Oxygen Carriers ("Blood Substitutes")—Raison d'Etre, Chemistry, and Some Physiology

Blut ist ein ganz besondrer Saft[1]

Jean G. Riess*,†

MRI Institute, University of California at San Diego, CA 92103

Received December 21, 2000

Contents

10.1021/cr970143c CCC: $36.00
Published on Web 09/12/2001

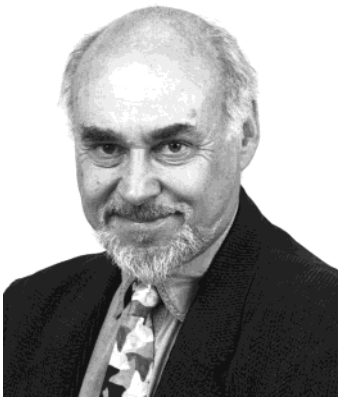
I. Introduction

After several decades of intensive efforts, safe injectable preparations capable of effectively delivering O_2 to tissues and removing CO_2, i.e., so-called blood substitutes, appear to be within reach of approval by the health authorities. "Artificial blood" has been on man's wish list for over a century; however, the undertaking turned out to be substantially more complex and arduous than expected. Numerous approaches have been explored. Major advances have alternated with setbacks and disappointments. Several promising products have met with ill fate. Two polymerized human hemoglobin (Hb) products, one polymerized bovine Hb and one perfluorocarbon (PFC) emulsion, are currently completing the last stages (Phase III) of clinical evaluation or have entered the regulatory review process for marketing approval. There is, therefore, hope that safe and effective commercial products will become available within the next few years.

The term "blood substitute" is clearly a misnomer, since the products under development only transport the respiratory gases, O_2 and CO_2, and for only a limited period of time at that. These products provide none of the complex and interrelated metabolic, regulatory, hemostatic, and host defense functions of blood. Oxygen carriers are nevertheless expected to play a pivotal role in easing the increasingly frequent blood shortages, solve some transfusion-related safety issues, and in the process, profoundly change patient care and the practice of transfusion medicine. Further, these carriers may find therapeutic value in situations where blood is no longer capable of adequately delivering O_2 to tissues, as in myocardial infarction or stroke. The present products are, therefore, more accurately described as temporary O_2 carriers, anti-hypoxic agents or, if need be, temporary red blood cell substitutes. In some cases, therapeutic value may rely on physiologic actions other than O_2 delivery.

Much of the research on blood substitutes was initiated and driven by the medical and life sciences research communities. While there are many thousands of papers on the subject, relatively few are published in the chemical literature. Key papers about the preparation of certain products that eventually went into clinical trials are sometimes found in *Surgery* or *Transfusion*. Reviewing this topic is also complicated by the fact that commercial, often venture capital-supported competition for a market that is estimated at several billions of dollars is intense. This means that all essential information is not available, especially where the chemistry of the systems under investigation is concerned. It has happened that extensive animal experimentation was reported on a product or formulation that was only identified by some undecipherable code name. It has also happened that untoward reactions observed during clinical trials (and their severity) were for a time only known through hearsay (or through the financial press; see, for example, refs 2 and 3) before there was mention of them in the scientific literature.

This review intends to summarize, with minimal bias, the different avenues that have been or are being explored for the purpose of developing injectable O_2 delivery systems, identify the challenges specific to each approach, analyze the obstacles that have been encountered en route, discuss the solutions that have been provided, along with their limitations, and offer some future perspectives. Numerous reviews have dealt with "blood substitutes"; most are limited to one type of product or emphasize one particular approach. Few integrate the chemistry that underlies the products' elaboration, characterization, and manufacturing. Because this area of research has been largely driven—and constrained—by medical, industrial, and marketing considerations, an attempt is made to outline the background of O_2 carrier development, thus alluding to questions concerning blood transfusion, the physiology of O_2 transport to tissues, clinical indications and methods of use, regulatory aspects, and economic and manufacturing challenges—the purpose of section II. Section III compares the principles that underlie the Hb and PFC approaches and their consequences and high-



Jean G. Riess received his chemical engineer and doctorat-ès-sciences degrees (the latter with Professor Guy Ourisson in 1963) from the University of Strasbourg. He then spent two years with John Van Wazer at Monsanto in Saint-Louis, MO, learning some phosphorus and transition-metal chemistry. In 1968 he became Professor at the University of Nice, France, where he founded, directed, and eventually became honorary director of the Unité de Chimie Moléculaire (associated with the Centre National de la Recherche Scientifique). His research successively involved phosphorus chemistry, transition-metal chemistry, organometallics, and eventually perfluorochemicals. His present interests are in fluorocarbons, fluorinated amphiphiles, and their colloid chemistry, including fluorocarbon emulsions for in vivo O_2 delivery, fluorinated self-assemblies, and drug delivery systems. Professor Riess has published about 360 papers and holds ca. 25 patents. He has served on numerous councils and committees and has chaired or co-chaired international conferences on phosphorus chemistry and blood substitutes. He has won awards from the French Academy of Sciences, French Chemical Society, Alexander von Humboldt Stifftung, City of Nice and Controlled Release Society, as well as Alliance's first Distinguished Contribution Award. He holds an honorary Research Associate position at the University of California at San Diego and sits on the Board of Directors of Alliance Pharmaceutical Corp.

* To whom correspondence should addressed. E-mail: jgr@allp.com. Address: Les Giaïnes F-06950 Falicon, France.
† The author is a member of the Board of Directors of Alliance Pharmaceutical Corporation in San Diego; however, the opinion expressed here are solely his and not necessarily those of the Corporation.

lights the difference. Sections IV and V focus on Hb and PFC products, respectively. While investigating Hb products is a "natural" thing to do, this author maintains the position that there are also grounds for believing that PFCs could provide a valuable solution to the blood substitute challenge. Finally, section VI sketches out some possible future directions.

One ambition of this review is to assemble or facilitate the access to a substantial part of the information a scientist attracted to the possibility of entering this field of research may want to have. Research on O_2 carriers entails crossing the traditional boundaries of our discipline and imposes constant dialogue with the medical community, factions of pharmaceutical development, and regulatory institutions. Literature coverage is provided through November 2000. The selection of references reflects that the emphasis of this review is on the chemistry of blood substitutes; the author is fully responsible for any oversights, omissions, and errors.

II. Blood, Blood Transfusion, Blood Substitutes

This section focuses on the challenges presently facing transfusion medicine with emphasis on alternatives and what is expected from blood substitutes.

A. Blood, an Organ Unlike Any Other

"The blood is the life" says the book of *Deuteronomy* (XII:11). Blood possesses stronger mystical, cultural, religious, and emotional connotations than any other organ. It is exchanged in rituals and has been offered to the gods as a sacrament. Blood symbolizes life, purity, identity, and kinship (blood brothers); strength, bravery, and violence (blood feuds); and death. It is the seat of the soul and of moral virtues. It provides vitality and ill fate. Among the ancient Greeks and throughout the Middle Ages, blood was considered one of the four fundamental humors responsible for health and illness. For 2000 years, bleeding out the "bad blood" has been used as a cure for all maladies.

Donating blood is an act of charity that can save a life. However, the perception of blood donation differs profoundly from one country to another and from one culture to another. In most countries blood donation is voluntary, unremunerated, and driven by altruism—the adequacy of our blood supply depends solely upon the generosity of a healthy public.

B. Current Issues in Blood Transfusion

Blood transfusion is surrounded by a complex history (see, for example, refs 4−9). Its practice was highly variable and somewhat suspect until Landsteiner's discovery of the ABO blood group system in 1900[10] provided the scientific basis for compatible and safe blood transfusion. Blood banking was made possible after it had been determined that addition of sodium citrate prevented blood from clotting.[11−13] However, the practice of transfusion developed slowly and its scope remained rather narrow until World War II. A "Centre de Recherche Hématologique et de Transfusion Sanguine" was established by Arnault Tzanck in Paris in 1923. The first blood banks were established in the late 1930s. Blood transfusion became of age when glucose was added to the citrate solution, extending the shelf life of red blood cells (RBC) to 21 days. The introduction of plastic storage bags improved blood safety dramatically by reducing the risk of bacterial contamination. More recently, use of a modified citrate−phosphate−dextrose anticoagulant solution supplemented with adenine allowed further extension of the permissible RBC storage period.[14] The number of transfusions in the United States increased steadily during the 1960s and 1970s, peaked in the mid-1980s, and has declined slowly but steadily thereafter.[15,16]

Today, an estimated 75−90 million units of blood are collected each year worldwide,[6,17,18] 80% of them in the developed countries (ca. 20% of the world's population). In the United States, blood collection amounted to 11.7 million in 1997,[15] 12.6 million when autologous donations are included.[19,20] Blood fractionation provides packed RBCs, platelet concentrates, plasma, and a range of important therapeutic proteins, including albumin, antithrombin, fibrinogen, factor VIII (or antihemophilic factor), factor IX, and immunoglobulins. In most cases, transfusion actually involves administration of packed RBCs or other components rather than whole blood. Further, recombinant factor VIII and serum albumin are now available and additional recombinant components can be anticipated.

After several decades of steady progress in improving the range of components available as well as their safety, blood banking and transfusion medicine suddenly came under scrutiny due to infectious safety issues triggered by the AIDS epidemic. It became more generally appreciated that AIDS was only one of several infectious diseases transmitted by blood transfusion and that transfusion was not devoid of risk. Transfusion-transmitted hepatitis is given to recipients far more commonly than AIDS and can progress into cirrhosis and/or liver cancer. Parasitic diseases take their toll in certain areas of the world. Other as yet unknown infectious agents are likely to emerge. Further issues concern the immunosuppressant effects of transfusions and the lack of immediate efficacy of RBCs stored more than a few days. New legal questions have also arisen concerning, in particular, the extent of testing.[21] As a consequence, the practice of transfusion medicine has undergone profound changes. This section will identify some of the issues that oxygen carrying blood substitutes could help relieve.

1. Transfused Blood: Safe or Not?

Trust in the safety of our blood supply and the blood banking community has been severely damaged by the AIDS tragedy. Acute anxiety about the safety of transfusion has developed.[22] Fortunately, the perceived risk is out of proportion with the real risk. More vigilant donor screening, new tests, handling practices, and tracking systems have drastically reduced the incidence of transfusion-related side effects, making the blood supply of developed countries safer than it ever has been.[16,23−33]

Blood transfusion nevertheless carries and will always carry a certain level of risk.[1] ("Blood products

should be considered potentially dangerous "drugs"";[25] "allogeneic blood remains an inherently defective raw material".[34]) Mild allergic reactions associated with fever, chills, pain and discomfort, and possibly urticaria can affect as many as 1 in 30 patients.[32] Delayed hemolytic transfusion reactions occur at an estimated frequency of 1 in 1000 patients, transfusion-related acute respiratory distress syndrome at a frequency of at least 1 in 5000, and fatal acute hemolytic transfusion reactions are observed for 1 in 250 000−1 000 000 transfusions.[16,32,35,36]

Infectious risks have declined abruptly. In the United States, the risk of acquiring hepatitis B is in the range of 1 in 30 000−250 000, hepatitis C of 1 in 30 000−100 000 (prior to 1965 the combined risk for hepatitis B and C was about 1 in 4); HIV is less than 1 in 500 000.[16,24,27,28,32] Transmission by transfusion of malaria, leishmania, and Chagas' disease (caused by *Trypanosoma cruzi*) raise serious concerns in certain geographic areas.[37] Septic shock from bacterial contamination, although rare, does occur in certain regions.[32,38,39] Whether and to which extent some forms of Creutzfeldt−Jakob disease (CJD) and other degenerative encephalopathies are transmissible by transfusion is being debated.[40−42] Bovine spongiform encephalopathy (BSE) is transmissible to other species, including mice, sheep, domestic cat, and macaque, by inoculation or ingestion of infected tissues.[43−45] The recent occurrence of a new variant of human Creutzfeldt−Jakob disease (nvCJD), distinct from previously known CJD and closely resembling BSE,[46−48] is a serious concern. Compelling evidence for transmission of BSE prions to humans has recently been inferred from experiments in which prions from bovines with BSE and from nvCJD were inoculated to transgenic mice expressing bovine prion protein (hence susceptible to foreign prions).[49] The nvCJD was readily transmitted to the mice; the incubation times for nvCJD and BSE were remarkably similar; clinical signs and neural damage were indistinguishable. Transmission of BSE by blood transfusion in sheep has recently been demonstrated.[45]

Finally, administrative errors (with an incidence of 1:12 000−30 000) remain one of the main causes of transfusion-related morbidity and mortality.[23,33,50,51]

Whatever the level of risk associated with a unit of blood, this risk is cumulative with the number of units transfused; conversely, each unit spared to a patient reduces the exposed risk.

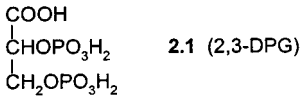
2. Transfusion-Mediated Immunosuppressive Effects

There is increasing evidence that allogeneic blood transfusion alters the host immune system and reduces the immune responsiveness (or immunocompetence) of the organism, thus predisposing a transfusion recipient to bacterial or viral infection.[38,52−65,65a] Transient blockade of the reticuloendothelial system (RES, also known as the mononuclear phagocyte system; in charge, among others, of clearing the circulation of foreign particulate matter) by transfused material may result in decreased clearance of bacteria by phagocytic cells. A significantly increased, dose-dependent incidence of infectious complications and septicemia was found in transfused versus nontransfused surgical patients.[66−68] A single unit of transfused allogeneic blood increased postoperative infections.[60] A recent prospective study in patients undergoing cardiac surgery demonstrated that intraoperative blood transfusion was associated with an enhanced inflammatory response, increased concentrations of inflammatory mediators, and increased postoperative morbidity.[62] Fewer infections were observed in patients receiving autologous rather than allogeneic blood,[54,69,70] and lower mortality was observed in cardiac surgery patients when leukocyte-depleted RBCs were used.[71] A retrospective study indicated that mortality in patients with sepsis is associated with the age of the RBCs transfused[72] (standard practice of blood banks is to release their oldest units first to avoid outdating). Blood transfusion has been identified as an independent consistent risk factor for postinjury multiple organ failure in trauma patients.[73] It has also been determined that packed RBC units contain substances that mediate inflammatory responses, including bactericidal permeability-increasing proteins (in amounts that increase with storage time), and that these substances are then transfused in the circulation along with RBCs.[62]

Various studies suggest that blood transfusions increase the risks of recurrence and spread of certain cancers.[55−57,59,67,74−78] Perioperative blood transfusions were found to reduce long-term survival following surgery for colorectal patients.[79] This risk may be lower when autologous is used.[80] On the other hand, transfusion-induced downregulation of the immune system has been used advantageously to improve the survival of kidney and heart transplants or to reduce the risk of recurrent spontaneous abortion.[55,57,81−84] A further safety issue with allogeneic blood is iron overload (requiring iron-chelating therapy), which can represent a serious complication for chronically transfused individuals such as sickle cell anemia and thalassemia patients.[85]

3. Efficacy of Stored Blood

Refrigeration and storage of RBCs results in so-called "storage lesions", which include changes in the affinity of Hb for O_2, a decrease in pH, hemolysis, changes in RBC deformability, formation of microaggregates, release of vasoactive substances, and denaturation of proteins.[25,86] Under normal circumstances, O_2 release and delivery to tissues is facilitated by the presence of an allosteric effector, 2,3-diphosphoglycerate (2,3-DPG, **2.1**) (section III). In



2.1 (2,3-DPG)

stored human RBCs, the concentration of 2,3-DPG decreases over time and little is left after 2 weeks.[87] This is why transfusion of stored blood is not immediately effective in delivering O_2 (Figure 1). It takes about 24 h for banked RBCs to restore their 2,3-DPG level to about one-half of normal.[87] During storage, RBCs also loose cellular adenosine triphos-

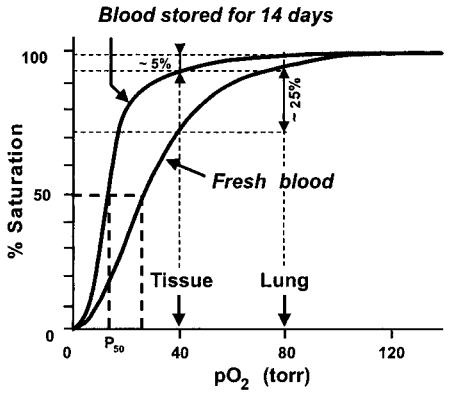


Figure 1. Capacity for banked blood to release O_2 diminishes over time as the O_2 binding isotherm of Hb shifts to the left and the affinity of Hb for O_2 increases due to loss of 2,3-DPG. After 2 weeks, release of O_2 between arterial blood and tissue drops from ~25% to ~5% of O_2 content at 37 °C. (Reprinted with permission from ref 1943. Copyright 1998 Karger Landes Systems.)

phate (ATP), resulting in loss of cell deformability,[88] which can impede on blood flow in the microcirculation.[89] One obvious use for an artificial O_2 carrier would be to serve, in case of emergency, as a bridge during the time required for the administered stored blood to reconstitute enough 2,3-DPG to deliver O_2 effectively.

An increasing number of studies have indicated that transfusion does not necessarily augment O_2 delivery and tissue oxygenation in critical care patients or improve outcome and that blood transfusions may be overused.[90−98] For example, one recent prospective, randomized clinical trial compared critically ill patients whose Hb levels were maintained either between 10 and 12 g/dL (liberal transfusion strategy) or between 7 and 9 g/dL (restrictive strategy). The results indicated that transfusion of fewer units of blood improved the patient's chances of survival.[98] Another study found no evidence that allogeneic transfusion improved survival in a cohort of elderly hip-fracture patients with Hb levels greater than 8 g/dL.[96] A low hematocrit (volume % of RBCs in blood, Hct) (<24%) at the conclusion of coronary artery bypass graft surgery was associated with a lesser incidence of myocardial infarction than a high Hct (≥34%).[97] No improvement in O_2 uptake was seen following transfusion of patients with sepsis having Hb levels around 9.0 g/dL; moreover, the patients who had received "old" blood (stored for over 2 weeks) developed evidence of gastrointestinal ischemia, possibly as a consequence of loss of RBC membrane deformability.[94] A carefully designed experimental study demonstrated that, in contrast to fresh RBCs, 28-day-old RBCs failed to improve tissue oxygenation in severely hemodiluted rats.[99] A new important awareness is thus emerging: banked blood (including predonated autologous blood) is not equivalent to *fresh* blood.

4. Supply Shortages and Cost Issues

Because of the intense efforts devoted to improving blood safety, the cost of blood collection, testing, storage, and distribution has escalated. In some countries, leukocyte (white blood cell) depletion has become mandatory. Leukodepletion removes about 99% of the leukocytes, which are considered responsible for certain transfusion reactions and immunosuppressive complications,[71] including a graft-versus-host response, cytomegalovirus infection,[23] and possibly the transmission of Creutzfeldt−Jakob encephalitis.[40−42] It is unlikely that this trend will reverse. Yet, achieving absolutely zero risk is illusory, whatever the expense. Past a certain point, additional testing and manipulation may actually increase the risk of errors.[23]

a. Blood Shortage: How Soon, How Serious? In developed countries, blood collection is declining[16] while its cost is increasing.[100] The demand for blood is likely to increase further with the aging of the populations, the development of aggressive new therapies, and the increase in the number of patients undergoing elective surgery. Baby boomers have now reached the age where the likelihood of their needing blood increases while their tendency to donate blood diminishes.[19,25,101] In the United States, for example, over 50% of transfusion recipients are over the age of 65, a segment of the population which will double in the next 30 years, while the prime donor age population will shrink in proportion. Seasonal shortages, especially of certain blood types, already occur, including the most frequently needed, universally compatible Group O blood. A recent survey indicated that the number of blood transfusions had increased by about 4% in the United States between 1994 and 1997 while collections decreased by 5.5% during the same period.[19,20] The margin between blood collections and transfusions has been shrinking consistently over the years, resulting in increased pressure on the blood banking system. Postponement of elective surgery due to blood shortages is increasingly frequent. A shortage of 4 million units has been projected by year 2030 in the United States.[102,103]

Blood availability and blood safety are interrelated. As our screening techniques become more sensitive, new agents, with yet unknown pathogenicity, are likely to be identified. Any further step to improve safety will invariably result in deferral of donors, hence directly impact blood availability. As an example, the discovery of the nvCJD, first described in 1996 in the United Kingdom, led the FDA to defer donors who have spend more than six months in Europe or 3 months in the United Kingdom since 1980. This policy could reduce blood collection by as much as 1.4 million units.[104] If safety increases at the expense of availability, people will eventually die from lack of blood. Finally, "the most unsafe unit of blood is the unit that is not there when you need it".[104] Here again, a blood substitute that does not rely on human blood collection could obviously play a considerable role in helping to relieve this blood supply shortage issue.

b. Developing versus Developed Countries. Transfusion recipients have very different profiles in developed relative to developing countries. Whereas in the former they tend to be patients undergoing surgery or older patients with malignancies, the vast

majority of the recipients in developing countries are women or children who require urgent transfusion.[105]

In the emerging countries, blood is chronically short due, among other things, to the limited number of suitable or/and willing donors.[106,107] Viral or parasitic infection with blood-borne diseases (HIV, hepatitis B, syphilis, malaria, and Chagas disease) can lead to the rejection of as much as 25% of the units collected.[106] In addition, the infrastructure and training required to collect, store, and deliver the available supply are sometimes lacking. Finally, if the present U.S. rate of transfusion was extended to the world's population, the shortfall would probably exceed 200 million units per year.[107] For developing countries, the availability of a blood substitute may be the best hope to meet future health care needs.

C. Trends and Alternatives to Transfusion

1. Transfusion Criteria

The infectious risks of blood transfusion have led to a drastic revision of transfusion practice. The trends are presently to transfuse only when there remains no other option, to lower transfusion "triggers", use autologous transfusion (one's own blood), and develop blood substitutes. In its clinical guidelines for RBC transfusion, the American College of Physicians clearly recommends to "regard elective transfusion with homologous blood (i.e., donor blood) as an outcome to be avoided".[108] Transfusion triggers or criteria (i.e., physiological or laboratory indicators of the need for transfusion) are being heavily debated.[97,109−121] While in the recent past patients would commonly receive blood as soon as their Hb levels fell below 10 g/dL, transfusion has now become uncommon above 8 or 9 g/dL. Unnecessary exposure to blood products may generate blame or even legal liability for any adverse outcomes.

Transfusion triggers are, unfortunately, far from being clear-cut physiological signals. They can be elusive and are highly patient- and indication-dependent, and there is no consensus on when RBC transfusions become necessary. Guidelines for transfusion have been issued,[108,109,117,122] but these guidelines all state that the ultimate decision to transfuse relies on the practitioner's experience and judgment on an individual patient basis. A practice parameter for the use of RBC transfusions has been developed with the view of assisting the physician in his decision.[123] While the general trend is to reduce the use of RBC transfusion, the need for preserving a sufficient safety margin and the risk of "undertransfusing" are recognized as well.[124−126] Transfusion indeed needs to be administered *before* a certain critical O_2 level is reached where O_2 consumption becomes supply dependent and an O_2 debt develops that can jeopardize vital tissues such as heart, brain, and guts. Such dilemma and risks could be significantly reduced, depletion of the O_2 reserve could be prevented, and an adequate safety margin could be preserved by recourse to an O_2 carrier other than RBCs. Such a carrier may possibly be used more liberally for comfort and safety, i.e., for preventing—rather than correcting—tissue hypoxia.

2. Autologous Blood and Blood Substitutes

Diverse alternatives to allogeneic (donor) blood transfusion are being used, and new ones are being actively developed. These alternatives include autologous blood predonation, acute normovolemic hemodilution (ANH, also called intraoperative autologous donation, IAD, especially by the cardiac surgeon community), intra- and postoperative blood salvage, use of hematopoietic growth factors such as erythropoietin (EPO, a glycoprotein that stimulates the production of RBCs), use of artificial O_2 carriers, and combinations of such techniques. Autologous transfusions eliminate the risk of infectious disease transmission, transfusion reactions, and alloimmunization, reduce the demand on allogeneic blood supply, and give the patients the psychological benefit of actively participating in their treatment. However, these alternatives have their own limitations,[126−132] and there is still today no autologous strategy available that is safe, effective, and widely applicable. Blood salvage is often not applicable, for example, in cancer and sepsis patients and is ineffective when only a limited amount of blood can be collected. It can also result in reinfusion of damaged RBCs and cell-free Hb, possible bacterial contamination, and infusion of microbubbles of air that can cause emboli. Use of predonated autologous blood, although it has had the favor of the public,[133,134] is logistically cumbersome, cannot be used in emergency procedures, and cannot be applied to small children, cancer patients, and other populations; certain associated risks, such as clerical error, may be comparable or superior to those of donor blood due to more complex handling and shipping.[32,135,136] Recent analysis even concluded that this procedure is inefficient[137,138] or not cost-effective.[139] Its practice is actually declining.[20] It should also be noted that predonated blood, by the time of surgery, is no longer fresh blood. Too frequent predonation or overhemodilution can cause anemia, which may not be appropriate for surgical patients with certain conditions, such as coronary artery disease.[126]

Acute normovolemic hemodilution[140−148] consists of withdrawing part of the blood from a patient (typically two units) shortly before undergoing surgery. This blood is replaced by an isoosmotic saline or isooncotic colloidal volume-expanding solution to maintain constant circulatory volume. The blood that is withdrawn is anticoagulated, set aside within the operating theater, and returned to the patient as needed during or after surgery. This procedure can be practiced on the day of surgery and is suitable for both emergency and elective surgery. Compared to other blood conservation strategies, ANH is logistically simpler and relatively inexpensive. Its main benefit lies in the reduction of RBC losses by decreasing the concentration of RBCs in the blood shed during surgery. An important advantage of ANH is that it also increases blood fluidity, which results in increased cardiac output, hence in increased systemic O_2 delivery. A further advantage is that the patient's room-temperature-stored fresh blood is immediately effective in O_2 delivery and conserves full functionality of fragile platelets and labile clotting factors.

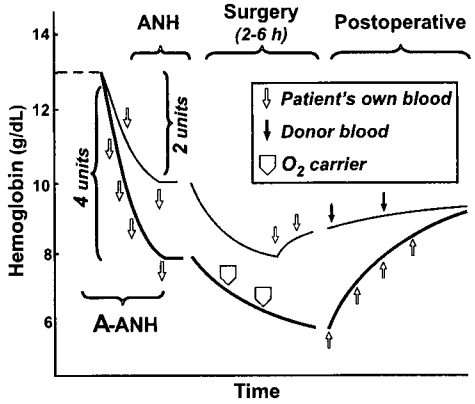


Figure 2. Schematic representation of the augmented acute normovolemic hemodilution (A-ANH) procedure as compared with standard ANH: 3−4 units of blood are withdrawn from the patient just before he/she undergoes surgery and are replaced by a volume-expanding saline or colloidal solution. When, during surgery, the need for transfusion is determined, an O_2 carrier is administered in lieu of blood, which prevents tissue hypoxia. When the Hb concentration reaches a level that is considered unsafe or at the end of surgery, the patient receives his/her own blood back. The A-ANH method is expected to avoid or reduce the patient's exposure to allogeneic blood; in addition, it should help relieve blood shortages. See also ref 166.

However, hemodilution needs to be relatively aggressive in order to provide clinically significant blood sparing,[135,149,150] which cannot always be practiced for fear of exposing the patient to ischemia.

This is one of the opportunities where O_2 carriers could be valuable. An "augmented" ANH procedure (A-ANH) that combines the use of an RBC substitute with ANH would allow more profound hemodilution, hence more effective reduction in allogeneic blood transfusion, while offsetting the risk of tissue hypoxia and cardiac ischemia during surgery.[151−155] In the A-ANH procedure (Figure 2) a larger portion of the patient's blood (typically four units) is collected than in the standard ANH procedure. When during the surgery the physician would normally administer a transfusion, he gives the O_2 carrier instead, thus delaying use of the precious fresh autologous blood while keeping the hemodiluted patient's tissue oxygenation in safe control. By operating at a lower Hb level, fewer precious RBCs are lost during surgical bleeding. If Hb reaches a level that is deemed too low or at the end of the procedure, the patient gets his own fresh, whole, and fully functional stored blood back. By allowing more profound hemodilution, the A-ANH procedure would result in reduced exposure to allogeneic blood transfusion. Mathematical modeling has indicated that substantial reduction in allogeneic blood exposure could indeed be achieved.[156,157] These expectations were recently confirmed by Phase III clinical trials, which demonstrated statistically significant avoidance or reduction in allogeneic blood transfusion in surgical patients when using the A-ANH procedure (see sections IV and V). Because the O_2 carrier enables the physician to perform ANH in a safer and more effective way, it should help ANH to become available to a broad population of surgical patients. This technique also has the potential to relieve banked blood shortages. How profound hemodilution can be has, however, not be determined and is expected to be highly dependent on patient condition.

D. Ideal and Real "Blood Substitutes"

For in vivo transport, O_2 can be chemically bound to the carrier (as in Hb) or dissolved in the carrier (as in PFCs). Use of a cell-free Hb solution as an artificial blood emerged over a century ago (see, for example ref 158). Between 1916 and 1978, at least 16 clinical trials of such solutions were reported; however, the results were disappointing.[159] Better understanding of the observed toxicities and significant modification of the Hb molecule led to improved preparations, several of which are now in Phase III clinical trials (section IV). Perfluorocarbon emulsions were proposed as an alternative approach to in vivo O_2 delivery in the late 1960s.[160] One such product, *Fluosol* (Green Cross Corp., Osaka, Japan), entered clinical trials a decade later and was licensed by the regulatory authorities of several countries, albeit for a rather limited indication, in 1989.[161] A similar product, *Perftoran* (Perftoran Co, Pushshino, Russia), has been approved in Russia.[162] A second-generation emulsion is presently in advanced clinical trials (section V). As noted earlier, these products are all very significantly different from blood, with advantages over RBCs in certain circumstances and limitations that may reduce the extent of their applications in others.

Injectable O_2 carriers need to be able to load O_2 rapidly during passage through the pulmonary capillary bed, reach the tissues and organs at risk of ischemia, and deliver their O_2 load rapidly and maximally to all organs. They should not increase O_2 demand and need to transport CO_2 as well. Blood substitutes should preferably be devoid of physiological activities other than O_2 delivery. They should not impair the cardiovascular compensatory mechanisms that normally prevent tissue hypoxia and should preserve the advantages of hemodilution. Ideally, they should have prolonged circulation lives. They must be free of bacteria, viruses, and endotoxins and should not promote the development of pathogens. They must be devoid of antigenic effects, and their side effects must be clinically minimal. They should not interfere with coagulation, immune system, and other organ functions. Being universal, i.e., free of blood type-specific antigens, these products would be useful in emergencies, e.g., on the site of an accident or disaster. It is essential that they be ready for use, easy to handle, and immediately effective. Shorter hospitalization and reduced cost of care are also desirable. Oxygen carriers must be manufacturable in any desired amount and stable enough to allow long-term storage in standard conditions. Finally, they need to be cost-effective and their price needs likely to remain in the neighborhood of that of blood.

The products under development meet several of these requirements. Devoid of blood group antigens, they require no compatibility testing, hence can be used without delay, and carry no risk of hemolytic reactions. They also eliminate the risk of clerical errors. They all consist in particles smaller than RBCs. Their ability to deliver O_2 to tissues in certain

circumstances has generally been established. The significance of the adverse effects observed with some of the products is, however, not yet clear. One limitation of all the present O_2 carriers is related to their short intravascular persistence (typical half-life of 6−24 h in the circulation) as compared to RBCs (several weeks). Therefore, these products can usually not be used as a direct replacement for blood. On the other hand, this limitation is clearly not an issue when coping with an acute transfusion need, as a bridge to transfusion, or for the duration of a surgical procedure. For these situations new strategies for use in combination with blood are being developed. The circulation half-life limitation does not apply to tissue or organ preservation. However, it excludes the use of such products when long-term O_2 delivery supplementation is needed, such as for the treatment of chronic anemia. Altogether, there is little doubt that "an effective, safe and stable oxygen carrier would provide a major advance in transfusion therapy".[163]

E. Principal Applications and Foreseeable Impact of an Oxygen Carrier

1. General Surgery

As indicated earlier, O_2 carriers may find major applications in the elective surgery setting where the carrier would, for example, be used in conjunction with autologous blood, as in the A-ANH procedure and other combination blood saving strategies. Three out of four transfusion recipients are surgical patients, and these patients receive about 60% of all blood products transfused.[32,103,109] ANH is increasingly advocated as being central to future blood-conservation strategies.[117,136,141−143,145,147−149,151,152,157,164−167] The availability of an O_2 carrier should allow ANH to be performed both more effectively and more safely and contribute to promoting the use of acute autologous transfusion practices in general. Strategies that combine the sustained action of EPO[168−171] with the immediate but transient action of an O_2 carrier should also be valuable.

Augmented-ANH is thus expected to reduce net blood loss during surgery, provide the patient with fresh, fully functional RBCs, preserve functional platelets and clotting factors that are his own, and eliminate clerical errors as well.

2. Cardiopulmonary Bypass Surgery—Neuroprotection

Coronary artery bypass graft surgery is being increasingly practiced and uses hemodilution routinely, often under hypothermic conditions. A saline solution is commonly used in order to prime the pump/oxygenator circuit. Oxygen carriers, combined with ANH (or IAD), should reduce transfusion needs in patients (more than 800 000 a year worldwide[172]) on cardiopulmonary bypass (CPB) circuits. One particular advantage of using O_2 carriers during cardiac surgery is that the harvested blood is protected from exposure to the bypass circuit and pump, which can activate white cells and is detrimental to platelet function and the clotting mechanism.[173]

Neurological dysfunction is a relatively common and potentially devastating complication of CPB surgery. Incidence of stroke and severe deterioration of intellectual function is around 6%.[172] In a recent study, the incidence of cognitive decline after coronary artery bypass surgery was 53% at discharge from the hospital and 42% at 5 years.[174] Such complications are a concern for the cardiac surgeon and entail a high societal cost. They appear to result, at least in part, from the formation of tiny air bubbles in the bypass circuit, which, once introduced in the circulation, may provoke air embolism. A product capable of simultaneously delivering O_2 and dissolving the air bubbles (primarily nitrogen) would, therefore, be extremely valuable.

3. Emergency—Trauma

A ready-for-use, immediately effective O_2 carrier should also be extremely valuable as part of the initial resuscitation stage of acute trauma patients, especially during the prehospital "golden hour" period, which largely determines the outcome for the patient. An O_2 carrier would provide a unique means of stabilizing the patient waiting for a transfusion, which becomes possible usually only after the victim has reached the hospital. Oxygen carriers should therefore find their place in any ambulance or rescue vehicle. By delaying the decision to transfuse, an O_2 carrier can also potentially reduce the number of one-unit RBC transfusions.

Storage-stable O_2 carriers would help respond to unforeseen needs, cope with a shortfall in available blood in case of disaster, seasonal or geographic temporary shortage, and bridge the gap until blood collection can be augmented. The military should be interested in O_2-carrying resuscitation fluids that would allow field use and bridging to transfusion. Hemorrhagic shock is the most serious and frequent complication of battlefield injury.[175] Artificial O_2 carriers should have advantages over the fragile and poorly stable "fresh" blood and RBCs and dispense with the need for their frequent and perpetual replacement. The products need, however, to fulfill specific requirements, such as long-term storage capabilities, low weight and volume, immediate availability, readiness for use, immediate effectiveness, universal compatibility, and sterility. The military tend to balk at the idea of using pure O_2 on the battlefield; this inconvenience no longer exists in the field hospital, where O_2 is available in the surgical/critical care units. Those patients who refuse blood transfusion on religious grounds should also eagerly accept O_2 carriers not derived from blood.

Further potential uses of O_2 carriers, often different from those of blood, as for treatment of ischemic states not related to anemia, such as stroke, myocardial infarction, coronary angioplasty, septic shock, cancer therapy, sickle cell anemia, and organ and tissue preservation, will be discussed in sections IV and V.

4. Impact on Blood Use and Blood Banking—Managing Our Own Blood

Speculating on the impact that O_2 carriers may have on blood banking[105] is a perilous exercise. One would expect blood bankers to easily accept new

products and procedures that could reduce the pressure on their inventory. Also, given the present intolerance of risk, even a relatively modest increase in safety margin, in particular during and after surgery, might provide a decisive market advantage for such products.[105] In developing countries lower cost and simpler logistics should prevail in decision making. The pace at which new products and procedures will penetrate the market is difficult to predict. In the current context of medical care expense control, the extent of use of O_2 carriers and of new allogeneic blood avoidance strategies in western countries will largely be determined by blood availability and cost considerations, second only to safety.

Together, O_2 carriers and novel strategies for blood utilization could provide a solution in the face of several weighty, interrelated challenges with which blood banking is presently confronted: demand from the public for ever increasing safety, need for high-quality blood, and blood shortages. Effective O_2 carriers and appropriate methods of use could further improve blood management and dramatically increase the donor pool by allowing many surgical patients to safely become their own donor. The objective is eventually to make the best possible use of that most precious source of blood, our own. There is also increasingly powerful evidence that advocates the use of *fresh* autologous blood. Among the new blood-saving/blood-providing strategies, A-ANH should be able to satisfy these needs best. It is also one of the easiest and least expensive to implement and one of the most universally applicable. Combination with use of recombinant EPO could further reduce the need for transfusion in the post-operative period.

How high the cost of a substitute can be is a matter of debate. A recent study indicates that in the United States the total cost of transfusing a unit of blood can reach $500, of which the product itself represents 20%, the rest of it being overhead and labor.[100] In this era of cost consciousness, it is unlikely that the cost of using an O_2 carrier can be significantly higher.

F. Regulatory Aspects

Blood substitutes present unique problems from a regulatory standpoint.[151,176] Regulatory agencies in charge of licensing new products include the European Medicines Evaluation Agency (EMEA) and the Food and Drug Administration (FDA) in the United States.[2] (At the FDA the unit that handles blood substitutes is the Center for Biologics Research and Review; Hb-based products are classified as biologics and PFC emulsions as drugs. Conducting clinical trials with a new product requires filing an Investigational New Drug Application (IND); marketing this product supposes the approval of a Product License Application (PLA) in the case of a biologic product or of a New Drug Application (NDA) in the case of a drug or a Marketing Authorization Application (MMA) in Europe.) These agencies are responsible for ensuring that biologics and drugs are safe and efficacious. Both safety and efficacy need, therefore, to be demonstrated prior to approval. In addition, the agencies inspect production facilities and verify process validation to ensure product quality and consistency.

One major handicap concerning the evaluation of injectable O_2 carriers comes from the absence of a standard with which to compare them. Red cells (or blood) have never been subjected to a controlled clinical trial for the purpose of demonstrating their efficacy and have never been formally approved by a regulatory agency.[177] The same holds for the autologous transfusion alternatives, including preoperative donation and normovolemic hemodilution. The opinion has been expressed that it is highly unlikely that clinical trials large enough to give a statistically valid comparison of outcomes of these techniques with allogeneic blood transfusion will ever be carried out.[129,173] Defining proper clinical endpoint(s), i.e., the clinical trial variable(s) that allow determining whether administration of O_2 carriers translates into clinical benefit for the patient, turns out to be difficult due, among others, to insufficient knowledge of O_2 transport physiology.

The first application for clinical investigation of a blood substitute was filed in 1970 and concerned a stroma-free Hb preparation.[176] The Phase I safety trials, however, uncovered major renal toxicity.[178] Several INDs have subsequently been granted for improved Hb products (section IV). So far the only O_2 carrier that has been licensed for use in humans is *Fluosol* in 1989, for use in conjunction with high-risk percutaneous transluminal coronary angioplasty.[179,180] This followed an initial rejection, in 1983, of the same product, when submitted as a treatment for chronic anemia, a logical decision in view of the product's intravascular persistence, too short for such an indication. A new PFC product is currently being evaluated for use in surgery (section V).

Unexpected adverse events with certain modified Hb products led the FDA to convene a panel of experts in 1990 to discuss these events and to issue a "Points to Consider" document on "the safety evaluation of Hb-based oxygen carriers".[181] Another "Points to Consider" was published in 1994, regarding the "efficacy criteria for Hb- and PFC-based O_2 carriers".[182] These documents are intended to offer guidance and suggestions to companies and clinical investigators on difficult product assessment issues. Clinical evaluation of O_2 carriers does indeed present unprecedented challenges. Concerns about product safety are important because of the unusually large doses of material that need to be administered (as compared with usual drugs), which amplifies the potential toxic effect of not only the carrier itself, but also of any trace contaminant it may contain. Disappointing results with certain products led to the requirement of Phase III populations larger than anticipated.

Demonstrating the product's efficacy is by no means easier. It is complicated by the existence of a significant O_2 reserve in man and of diverse compensatory mechanisms that set in place in case of anemia, including a rise in cardiac output and increased O_2 extraction by the tissues.[112,151,183,184] In addition, the actual clinical situations are extremely diverse, complex, and highly patient-dependent. Most importantly, it does not suffice to demonstrate physiological activity, as, for example, a significant in-

crease in mixed venous O_2 tension ($P\bar{v}O_2$) or in tissue O_2 tension, there must also be a clinical benefit such as a decrease in mortality or morbidity. Hence, the need for well controlled trials involving a significant number of patients as well as the interest in surrogate endpoints, i.e., measurable parameters other than direct clinical benefit. This supposes, however, that the relation between the surrogate endpoint and clinical benefit is firmly established. The endpoint(s) selected for clinical trials may depend on which indication is pursued. A consensus appears to have developed that demonstration of avoidance or reduction of exposure to transfusion of donor blood would be a valid surrogate endpoint for perioperative use (as in elective surgery).[182] Other logical endpoints are left ventricle function for angioplasty and tumor regression for tumor sensitization. No agreement on a realistic endpoint appears to have been found yet were resuscitation from hemorrhagic shock (as in trauma) is concerned.

Combination of perioperative hemodilution with use of an O_2 carrier may offer an effective model for demonstrating efficacy. It should be emphasized in this respect that each unit of transfused blood carries the same risk. Therefore, each unit spared to the patient is equally worth the effort. In other words, reduction of the number of units given is not a minor gain as compared to avoidance of transfusion. Reversal of transfusion triggers may also provide a valid surrogate clinical endpoint, as it indicates removal of risk of tissue hypoxia. Improvement of neurological outcome following CPB surgery, although it may prove more difficult to demonstrate, could also provide a valuable endpoint.

G. The Chemists' Role in Developing a "Blood Substitute"

Chemists are expected to design and provide the carrier molecule, to formulate it into a stable, injectable, scalable preparation, to properly characterize the product and its physical and chemical properties, and to contribute to the assessment and understanding of its in vivo behavior. Most importantly, chemists are expected to abide by the multiple requirements and constraints that govern the development of pharmaceuticals, as well as by countless manufacturing, regulatory, pharmaco-economic, and marketing considerations. This supposes a complex blend of synthetic chemistry, colloid chemistry, physical chemistry, biochemistry, and effective interactions with colleagues from the life sciences, process engineering, and medical worlds.[185] Finally, the importance of basic research in preparing the ground for new discoveries, as well as in the resolution of practical development issues, cannot be overstated.

The chemist in charge of developing an injectable O_2 carrier can call upon two fundamentally different principles depending on whether the O_2 molecule is covalently bound to the carrier or physically dissolved in it (section III). The first approach led to a large variety of products derived from modified and/or encapsulated Hbs from various sources (section IV). The second approach exploits the exceptional gas-dissolving capacity and biological inertness of PFCs, leading to the development of diverse injectable PFC emulsions (section V).

III. Fluorocarbon- versus Hemoglobin-Based Oxygen Carriers: Shifting the Paradigm of Oxygen Delivery

Cell-free Hb was logical to explore as a substitute for blood. Use of cell-free Hb solutions for this purpose has been disclosed for over a century (see, for example, ref 158). About a dozen human studies of such solutions were documented during the first half of the last century.[159,186] However, these studies uncovered a number of unexpected difficulties. Where efficacy was concerned, the increase in O_2 carrying capacity provided by cell-free Hb was short-lived and O_2 delivery was hindered by a right shift of the O_2 dissociation isotherm and further compromised by unexpected vasoconstrictive effects. Untoward reactions included kidney and liver toxicity, neurotoxicity, immune system activation, coagulation defects, peroxidative activity, and bacterial infection potentialization.[159,187−190] Extensive efforts were devoted to improving our understanding of these effects, and numerous avenues were explored in order to resolve or mitigate their consequences. This led to an array of products that involved substantial structural modification and/or re-encapsulation of the native protein.[191−198] Several such products are currently in advanced clinical trials (section IV).

Also based on the coordination of O_2 on a metal-centered complex is a range of synthetic iron chelates intended to mimic heme. This approach has inspired superb chemistry involving molecular "picket-fence", "basket-handle", and "capped" and "strapped" porphyrins,[199−204] polymer-bound chelates[205−207] and, more recently, lipid-embedded amphiphilic chelate self-assemblies[208,209] and semisynthetic heme−albumin conjugates.[210,211] Only the latter conjugates appear to have provided a workable solution to in vivo O_2 delivery and will be briefly reviewed in section IV.

Exploration of a fundamentally different approach to blood substitutes, which relies on the use of perfluorocarbons, i.e., synthetic inert O_2 *solvents* rather than on coordination complexes for in vivo O_2 transport, was initiated in the late 1960s.[212−214] The principal difficulties of this approach included the identification of appropriate, biocompatible, and readily excretable PFCs and emulsifiers, the engineering of stable biocompatible emulsions, and the understanding of PFC "physiology". The first product to have been licensed by the FDA for clinical use as an O_2 carrier was a PFC emulsion, *Fluosol* (Green Cross Corp., Osaka, Japan). *Fluosol*, however, did not achieve commercial success, largely because of the cumbersome reconstitution procedure required prior to administration, consequent to poor emulsion stability. A substantially improved PFC product is now in the final stages of clinical trials (section V).[155,215−220]

The purpose of this section is to outline and compare briefly the specific characteristics and key differences that exist between the Hb- and PFC-based approaches to in vivo delivery of O_2 (and

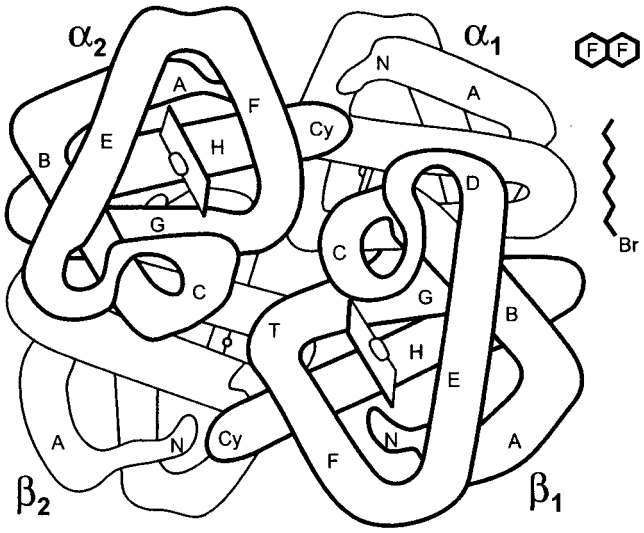


Figure 3. Schematic representation, on approximately the same scale, of the HbA tetramer with its two α and two *β* chains (Reprinted with permission from *Nature* (http://www.nature.com), ref 327. Copyright 1970 Macmillan Magazines Ltd.) and of two representative PFCs used for in vivo O_2 delivery, perfluorodecalin and perfluorooctyl bromide.

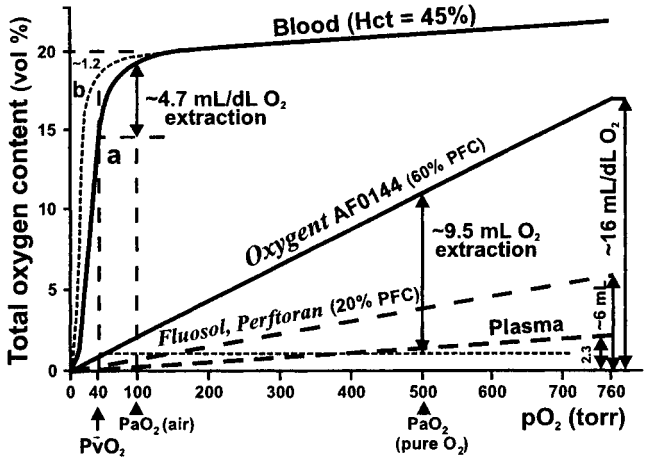


Figure 4. Total O_2 content of whole blood (Hb in the RBC) and cell-free Hb (i.e., in the absence of 2,3-DPG; dotted line) as compared to PFC emulsions of different concentrations, as a function of O_2 partial pressure.

uptake of CO_2), with reference, when appropriate, to blood and red blood cells. Comparison of different O_2 delivery systems is far from straightforward; it requires great care and the willingness to consider concepts that are not part of our traditional understanding of O_2 transport physiology. Any comparative assessment of PFC- and Hb-based products, whether from the standpoint of efficacy, safety, or manufacturing, must consider the paradigmatic differences that exist between these two types of carrier systems and their implications. It is essential also that the requirements and limitations of the products and the envisaged therapeutic indications and methods of use be taken into account. Failing to do so could result in erroneous conclusions.

A. Biomimetic or "Abiotic"?

The modified or encapsulated Hb approaches clearly follow nature's way of delivering O_2 to tissues in warm-blooded animals. They rely on the ability of certain metal-centered coordination complexes to reversibly bind the dioxygen ligand. The PFC approach differs from the above from every conceivable standpoint. Fluorocarbons cannot mimic Hb nor are they intended to. The two types of molecules could not be more dissimilar in terms of occurrence, structure (Figure 3), chemical reactivity, and biochemistry. The same can be said for the way they are formulated for injection (i.e., emulsions versus solutions or liposomes), their O_2 (and CO_2) uptake profiles (Figure 4) and mechanisms, the parameters that govern the delivery of O_2 to the tissues, their fate in vivo, and their possible interferences with normal physiology. Also radically different are raw material procurement, manufacturing procedures, and cost-effectiveness.

Hemoglobin is natural; fluorocarbons are synthetic. Is "natural" an advantage? However, does Hb, when stripped from its natural environment, still behave as the natural product? How far from natural is a modified (cross-linked, polymerized, conjugated, or genetically engineered) Hb product? These questions, as legitimate as they are, tend to be overlooked or the answers tend to be taken for granted. So far no PFC, or even CF_3 group, has been found in nature; they are so *un*natural that nature does not seem to recognize them (no receptors) or "know" how to process them (PFCs, including Teflon, can stay in living tissues for years without causing inflammation or rejection reaction.[221,222])

It should be kept in mind that the objective of this research is not to deliver Hb and mimic Hb's physiology but to deliver O_2 to tissues.

1. Different Molecular Entities

Normal adult human hemoglobin (HbA_0, Figure 3), which constitutes over 90% of the Hb present in the RBC, has a molecular weight (MW, atomic mass units) of about 64 500.[223,224] Its structure has been elucidated, primarily by Perutz and co-workers, using high-resolution X-ray crystallography.[225,226] It is made of four polypeptidic globin subunits (two α and two *β*, comprising 141 and 146 amino acid residues, respectively), arranged around a central water-filled cavity. The molecule has an almost spherical, slightly tetrahedron-like shape, about 5.5 nm in size, with a 2-fold axis of symmetry. Each subunit is centered on an iron(II) protoporphyrin IX (or heme) prosthetic group (Figure 5) that lies in a hydrophobic cleft between two helices within each globin polypeptide. The heme is held in place by several hydrogen bonds and numerous hydrophobic interactions with the globin and by coordination of its iron atom in axial position to the imidazole moiety of a histidine residue (His-87α or His-92*β*, depending on subunit) present in the hydrophobic cleft. The heme's nonpolar vinyl groups are buried deep into the hydrophobic interior of the cleft, while its propionic acid groups are located near the protein's more hydrophilic surface. The α and *β* subunits form tight α*β* dimers through numerous van der Waals and hydrogen-bonding interactions, while the α*β* dimers are more loosely bonded.

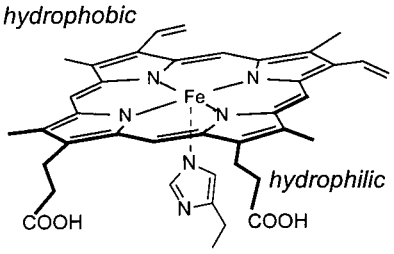


Figure 5. Iron(II) protoporphyrin IX (or heme) prosthetic group. The iron is coordinated to the His-87α or His-92β residues (depending on subunit) of the globin.

This structure is remarkably constant throughout the animal world. However, small variations in protein composition can have important physiological consequences. Sickle cell disease, for example, is caused by the substitution of one single amino acid (Glu-6β→Val).[223] The affinity of the hemes for O_2 and the cooperativity among the four hemes are regulated by subtle changes in the globin's structure.[227,228]

The molecular structure of PFCs has obviously little resemblance to any protein-embedded metal porphyrin. The PFCs used for O_2 transport are exceedingly simple small molecules with no specific structural requirements where O_2 transport and delivery are concerned. Examples of PFCs investigated in the course of the development of injectable O_2 carriers may be found in Chart 5. These PFCs are linear, branched, or cyclic and may contain heteroatoms, including oxygen, nitrogen, halogens other than fluorine, and hydrogen. The selection of PFC candidates for in vivo O_2 delivery eventually relied on excretion rate, emulsion stability, and ease of manufacture (section V.D).

Again, these man-made compounds have nothing in common with the complex, subtle, and environment-sensitive molecular machinery of Hb, which results from millions of years of evolutionary adaptation to the earth's atmosphere, and led to precisely defined primary, secondary, tertiary, and quaternary structural arrangements. However, it should also be clear that pyridoxalated, fumaroyl-cross-linked, glutaraldehyde-polymerized, dextran-conjugated, pegylated, or genetically cross-linked mutant Hbs (Figure 7) are no longer the natural molecule. Such modifications are intended to restore the O_2-delivery capacity and biocompatibility that are lost when the protein is extracted from the RBC. The physiological, immunological, or clinical consequences (including O_2 delivery) of these modifications obviously need to be determined. While the presently utilized PFCs are well defined pure molecular entities, this is never the case with the chemically modified Hb products.

2. Active and Labile versus Passive and Inert

Hemoglobin is a fragile biologic molecule. Any free Hb present in the circulation is normally disposed of rapidly. Oxidation and reduction of the protein take place continuously. Oxidation of Hb leads to methemoglobin (metHb, or ferrihemoglobin, the iron(III)-based oxidation product of Hb). MetHb levels normally do not exceed about 1−3% in the circulation due to effective reducing systems present in the RBC. Subsequent catabolism provides hemin, biliverdin, bilirubin, and eventually free iron. Hemoglobin and its breakdown products react with O_2, producing superoxide, hydrogen peroxide, and hydroxyl radicals which, in turn, can lead to a variety of peroxidation products. Is it possible to prevent Hb from being irreversibly oxidized to metHb (which is no longer able to bind O_2 reversibly) in the absence of a protective container membrane and appropriate reduction systems? Are the normal plasma antioxidant systems sufficient to protect large amounts of extracellular Hb from such oxidation? The reactivity of Hb is illustrated here by some aspects of its redox chemistry and by its reactions with nitric oxide, an ubiquitous molecule involved in various physiological processes.

a. The Redox Chemistry of Hemoglobin. Hemoglobin participates in complex oxidation and reduction processes that involve iron in oxidation states two, three, and four.[189,223,229−238] Autoxidation to metHb can occur through multiple pathways that can likely affect one or more subunits. One prominent mechanism involves the spontaneous dismutation of oxyHb to metHb and the superoxide radical $O_2^{\bullet-}$ (eq 3.1).

$$HbFe^{2+}O_2 \rightarrow HbFe^{3+} + O_2^{\bullet-} \qquad (3.1)$$

The latter can react with another oxyHb subunit to produce metHb and hydrogen peroxide, H_2O_2 (eq 3.2)

$$O_2^{\bullet-} + HbFe^{2+}O_2 + 2H^+ \rightarrow HbFe^{3+} + H_2O_2 + O_2 \qquad (3.2)$$

which in turn can react with an additional oxyHb subunit (in a Fenton-type reaction) to generate metHb and the highly reactive hydroxyl radical, $OH^{\bullet}$ (eq 3.3).

$$H_2O_2 + HbFe^{2+}O_2 \rightarrow HbFe^{3+} + OH^{\bullet} + OH^- + O_2 \qquad (3.3)$$

The hydroxyl radical can further react with oxyHb (Haber−Weiss reaction) to produce metHb and the hydroxide ion (eq 3.4).

$$OH^{\bullet} + HbFe^{2+}O_2 \rightarrow HbFe^{3+} + OH^- + O_2 \qquad (3.4)$$

The $O_2^{\bullet-}$ radical can also react with endogenous NO to generate peroxynitrite, $ONOO^-$ (eq 3.5)

$$O_2^{\bullet-} + NO \rightarrow ONOO^- \qquad (3.5)$$

which is a potent oxidant and powerful cytotoxic agent.[239,240] Peroxynitrite has a complex chemistry. It can rapidly oxidize free oxyHb to metHb and appears to reduce ferryl heme to ferric.[241]

Both ferrous and ferric Hb can be converted into reactive ferryl species by H_2O_2 (eq 3.6).[237,242−244]

$$HbFe^{3+} + H_2O_2 \rightarrow HbFe^{4+}{=}O + H_2O \qquad (3.6)$$

FerrylHb autoreduces back to ferric. Additional H_2O_2

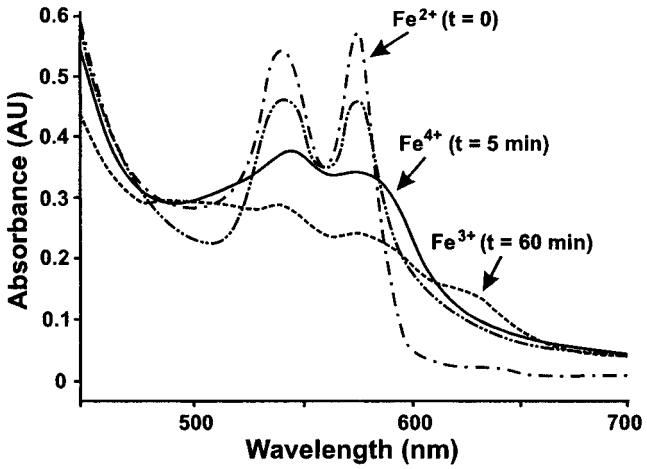


Figure 6. Oxidation of α,α-DBBF-cross-linked Hb following addition of H_2O_2 to cultured bovine aortic endothelial cells. When the culture supernatant was analyzed spectrophotometrically, the characteristic spectrum of ferrylHb became detectable after 5 min. Morphologic assessment of the cells after 6 h showed nuclear fragmentation indicative of apoptosis. (Reprinted with permission from ref 237. Copyright 2000 Elsevier.)

converts Hb^{3+} back to ferrylHb, completing a catalytic cycle.[238,245] H_2O_2 is produced endogenously as a byproduct of normal aerobic metabolism and is involved in the electron-transport function in the cell. It is also generated by activated macrophages and endothelial cells, especially during inflammatory processes, including reperfusion after ischemia.[230,236,246,247] The characteristic spectrum of ferrylHb has been detected following addition of H_2O_2 to cultured bovine aortic endothelial cells (Figure 6).[237] A globin-centered ferryl radical $^{\bullet}HbFe^{4+}{=}O$ has been detected in frozen normal human blood.[248] A ferryl species was formed when oxyHb was treated with $ONOO^-$ in the presence of CO_2.[249] FerrylHb reacts rapidly with oxyHb to produce metHb (eq 3.7).[242]

$$HbFe^{4+}{=}O + HbFe^{2+}O_2 \rightarrow 2HbFe^{3+} + {}^3/_2O_2 \quad (3.7)$$

These various active species, as well as Hb itself and its catabolic products, can damage proteins, carbohydrates, and nucleic acids, stimulate the peroxidation of unsaturated lipids, and promote the production of a range of toxic breakdown products.[189,230,236,247,250−253] MetHb is less stable than Hb and readily releases heme.[254] Because heme is hydrophobic, it can intercalate into endothelial membranes where it has the potential to catalyze the peroxidation of lipids. Cell damage has been observed upon exposure of endothelium to cell-free Hb products, especially when in their ferric and ferryl forms.[255−260] In an in vitro model of endothelial cells mimicking the biological responses to ischemia and reperfusion, the ferryl species was detected in a time frame that corresponded closely with Hb-mediated peroxide production and lipid peroxidation.[234] Damage to cultured cerebral endothelial cells by Hb has been reported.[261,262] Incubation of ferric HbA with liposomes resulted in lipid peroxidation, possibly involving a ferric/ferryl redox cycle, and increased ion permeability of the membrane.[263] Reaction of ferrylHb with H_2O_2 results in heme degradation.[264] Release of free iron from heme can trigger Fenton chemistry and the production of hydroxyl radical $OH^{\bullet}$.[251] Iron can catalyze oxidative injury to neuronal cell membranes and may participate in brain injury consequent to trauma.[265,266]

The rate of autoxidation of human cell-free Hb in a physiological salt solution reached 4% per hour at 37 °C.[267] It increased considerably with dilution as dissociation into more reactive dimers is favored[268] and depended on storage conditions.[269] Oxidation of unprotected free Hb to metHb during freeze-drying can reach 40%.[270] Within the RBC, metHb is reduced back to functional Hb by enzymatic pathways involving methemoglobin reductase and by nonenzymatic pathways involving ascorbic acid and glutathione. The $O_2^{\bullet-}$ radical and H_2O_2 generated during the autoxidation process are broken down by superoxide dismutase, glutathione peroxidase, and catalase, which are found in high concentrations in the RBC.

Excess free Hb can affect the normal balance between reactive oxygen and nitrogen species.[189,271−273] Free radicals and iron-derived reactive oxygen species are implicated in the pathogenesis of numerous diseases and vascular disorders, including atherosclerosis, arthritis, reperfusion injury, myocardial infarction, adult respiratory distress syndrome, and hemorrhagic shock and cancer, and may be a factor in post-traumatic damage to the central nervous system.[189,234,236,274] In particular, the balance between nitric oxide and superoxide generation appears to be a critical determinant in the ethiology of these diseases.[274]

Autoxidation of Hb decreases O_2 delivery for two reasons: direct loss of receptive ferrous hemes and higher affinity of the non-oxidized hemes caused by oxidation of other hemes within a given Hb tetramer.[275] It was noted that a random distribution of 20% ferric irons would imply that 58% of the tetramers would have at least one oxidized subunit, resulting in a leftward shift of the O_2 affinity curve of the remaining ferrous subunits. MetHb levels greater than 10% were estimated to significantly reduce the ability of a pegylated pyridoxalated Hb to oxygenate tissues in a rat exchange transfusion model.[276]

b. Ligand Coordination and Related Reactions. Hemoglobin has the capability of coordinating ligands other than O_2. Of particular relevance to the transport and delivery of the respiratory gases is the coordination of carbon monoxide and nitric monoxide. The toxicity of CO, because of the formation of highly stable carbonmonoxyHb, has been known for a long time. The physiological importance of NO as a chemical messenger has been realized more recently when it was identified as the endothelium-derived relaxing factor (EDRF) that regulates blood vessel tone and blood pressure, maintains the permeability barrier function of the endothelium, and controls a range of other physiological processes.[277−284] Nitric oxide has both protective and deleterious effects.[274,284,285] It is implicated in neurotransmission, angiogenesis in limiting leukocyte adhesion, prevent-

ing platelet adhesion, activation, and deposition, in the cytoprotection of mucosa,[286,287] the regulation of macrophage function, and immune response to bacterial and fungal infections,[281,286,288,289] and in gastrointestinal and hepatic physiology.[290] It has both pro-oxidant and antioxidant functions,[238,285,291] reducing, for example, heme-associated ferryl radicals (eq 3.9) and suppresses potent cytotoxic oxidants. It may also play a role in O_2 transfer from RBCs to tissues.[292] Nitric oxide is formed in tissues from L-arginine, and most of its functions are mediated through cyclic guanosine-3,5′-monophosphate (cGMP). Elevated NO levels are produced during certain pathological conditions, including septic shock and endotoxemia.[293,294] Microvascular dysfunction induced during reperfusion following ischemia appears to be attenuated by NO.[295] The formation of nitrosylHb was detected in animals exposed to NO[296] and in a rat endotoxin shock model.[297] Neurotransmitter functions similar to those of NO and which presumably operate according to similar mechanisms have subsequently been recognized for endogenously produced CO.[298−300]

$$HbFe^{2+}O_2 + NO \rightarrow HbFe^{3+} + NO^{3-} \quad (3.8)$$

$$HbFe^{4+} + NO \rightarrow HbFe^{3+} + NO_2^- \quad (3.9)$$

Hemoglobin is a potent inhibitor of the vasodilating effect of NO (or EDRF).[278,301−305] It can inactivate NO in a number of ways, the extent of each of which is still being debated (section IV.H). The NO ligand can react with both ferrous and ferric Hbs. It can bind to deoxyHb to form nitrosylHb or react with oxyHb in a reaction similar to its reaction with $O_2^{\bullet-}$. NO can also react with Cys-93β residues to form S-nitrosoHb. Displacement of O_2 from oxyHb to form nitroxylHb is not observed.

DeoxyHb has an extremely high affinity for NO (dissociation constant $\sim 4 \times 10^{-12}$ M).[306,307] However, it is essentially the rate of reaction, rather than the position of the equilibrium, that is relevant here. Coordination of NO to heme in deoxyHb is only diffusion limited, hence extremely rapid, as NO is one of the most diffusible molecules known. The half-life of NO in the presence of cell-free Hb, RBCs, and a buffer alone is on the order of picoseconds, milliseconds, and minutes, respectively.[308] (Direct reaction of NO with O_2 is slow and does not limit the diffusion of NO from the site of production to target molecules such as guanylate cyclase in myocytes and platelets.[309])

Substitution of O_2 by NO in oxyHb does not appear to occur to any significant extent. However, NO oxidizes oxyHb as rapidly (again in a diffusion-controlled reaction) as it binds with deoxyHb (eq 3.8). Unstable peroxynitrite $OONO^-$ is formed as an intermediate and eventually metHb and nitrate. Because oxyHb is the predominant Hb species in arterial blood, this reaction may actually be of greater significance than coordination of NO with heme iron.[307,308,310,311] The rate of reaction of NO with a variety of recombinant oxyHbs depended linearly on NO concentration and was independent of O_2 concentration.[307] The nitration of tyrosine residues on proteins,[239] including Hb,[241] has also been observed. Tyrosine nitration is a consequence of the interaction of NO or NO derivatives with reactive oxygen species and is associated with diverse pathological events.[311a] Peroxynitrite or $OONO^-$-derived species have been shown to play a critical role in tissue reperfusion injury and may be the true responsible agents for cellular injuries that were previously believed to be mediated by NO.[234,239] The superoxide and peroxynitrite anions appear to be involved in the interaction of NO with endogenous glutathione.[312] Oxygen free radicals appear to contribute to renal NO synthesis and renal blood flow regulation.[313]

The reaction of NO with ferric Hb is much slower, easily reversible, and may not play a significant part in the Hb-elicited hemodynamic effects.[305−307,314,315]

Nitric oxide can also react reversibly with the SH groups of the Cys-93β residues, yielding S-nitrosoHb, as part of a recently identified NO transport and blood flow control mechanism.[316,317] The binding of O_2 to heme irons appears to promote the binding of NO to Cys-93β while NO is released upon deoxygenation, thus contributing to regulation of blood vessel tone to tissue O_2 requirements. It was proposed that contrary to conventional views, the oxidation of NO to NO^{3-} by oxyHb may be of little significance under normal physiological conditions.[318] Instead, NO is believed by the authors to be bound to either vacant heme or cysteines. These reactions would help maintain NO in a bioactive state, i.e., *prevent* its rapid destruction by Hb. Other studies using mutant Hbs indicated, on the contrary, that reaction of NO with oxyHb and deoxyHb is the cause of the hemodynamic effects of cell-free Hb and that S-nitrosylation is not a key factor.[315] The same conclusion was reached following experiments on aortic rings with Hb derivatives having either or both Cys-93β and heme iron binding sites blocked using *N*-ethylmaleimide and cyanide, respectively.[305] A further study indicated that S-nitrosoHb and its metHb form also play a role in platelet aggregation regulation.[319] Certainly, the interplay of Hb and NO and other reactive endogenous species is more complex than anticipated and will require further clarification. Further complexity originates from the fact that any of the above reactions can take place in one or more subunits within the tetramer, can affect the properties of the other subunits, and can be affected by chemical or genetic modification of the protein.

c. Perfluorocarbons—Inertia par Excellence. Perfluorocarbons, as opposed to Hb, are among the most inert organic materials chemists have ever invented. Their initial industrial development was for handling the extremely corrosive uranium fluorides. PFCs have no coordination capability and cannot scavenge NO or bind CO. They are not subject to oxidation, and there is no indication that any sort of chemical modification occurs under the conditions of processing, storage, and use relevant to therapeutic O_2 delivery. Neat PFCs can typically be heated to 300 °C and higher for several days without detectable changes. Appropriately formulated PFC emulsions can be terminally heat-sterilized at the standard temperature of 121 °C.

B. Emulsion Droplets versus Hemoglobin Solutions or Encapsulated Hemoglobin and Red Blood Cells

1. The Red Blood Cell Has a Purpose

Does cell-free Hb behave like RBC-enclosed Hb? Assuredly not. The RBC allows very high blood concentrations of Hb in the circulation, thus permitting effective delivery of O_2 throughout large organisms. Such concentrations would be impossible with free Hb because of excessive oncotic pressure and viscosity, rapid dissociation, escape from circulation, and elimination. In the RBC, Hb is at an excess of 300 g/L concentration; simply diluting this Hb to a concentration where it is isoosmotic to human plasma (~60−80 g/L) results in dissociation of the tetramer into $\alpha\beta$ dimers which, in contrast to the tetramer, are rapidly filtered out by the kidney.[320]

Hemoglobin requires a particular environment, different from plasma, for optimal performance. Among the substances that accompany Hb in the RBC is the allosteric effector 2,3-diglycerophosphate **2.1**, an intermediate in glycolysis, whose role is reducing the affinity of Hb for O_2, hence facilitating its release to tissues.[321,322] Further O_2 affinity regulation is provided by CO_2 and H^+ and Cl^- ions. The RBC also contains large concentrations of various enzymes, such as carbonic anhydrase, methemoglobin reductase, superoxide dismutase, and catalase as well as glutathione, adenosine triphosphate (ATP), and phosphate ions, which participate in regulating O_2 and CO_2 transport and pH, controlling active oxygen species, limiting the level of metHb, and preventing the oxidation of the cysteines. In the absence of these cofactors, O_2 release to tissues is hindered and oxidation of Hb to metHb and to free-radical species is rapid. Further, the slightly higher pH of blood plasma (7.4) as compared to the internal fluid of the RBC (7.2), by reducing protonation of the C-terminal histidine residues of the β chains, reduces the aptitude of free Hb to release O_2, resulting in an allosteric augmentation of the affinity of the heme for O_2 (the alkaline Bohr effect).[323] Finally, the normal relationships between Hb, peroxide and superoxide species, and NO are also substantially altered when Hb is no longer confined within the RBC.[237,324] For example, the reaction of NO with oxyHb contained in RBCs, as it is limited by diffusion of NO into the cell, is about 650 times slower than with an equivalent concentration of cell-free oxyHb.[308]

The RBC, by sequestering the protein, plays a vital role in preserving Hb against degradation and the organism against Hb-induced toxicities. It prevents extravasation of the protein into the interstitial spaces. The cell's glycosylated surface ensures prolonged RBC circulation life, hence prolonging Hb life and functioning. Finally, it should be kept in mind that any cell-free Hb-based O_2 carrier will be used at doses that are several orders of magnitude larger than the amount of free Hb normally found in the plasma.

The optimal dimension of an artificial O_2 carrier is still a matter of debate. The Hb tetramers (about 6 nm in diameter) extravasate rather rapidly; particles of 20−50 nm may still diffuse through endothelial fenestra in the case of inflammation; particles around 150−250 nm (vesicles and small-size emulsions) do not appear to leak out; larger particles are taken up more readily by the RES; above ca. 5 μm capillary plugging becomes an issue.

2. A Diversity of Modifications and Formulations

Hemoglobin products have been derived from human, animal, or recombinant Hbs and have involved an impressive diversity of structural modifications and formulations (section IV). The modifications and combinations thereof can differ considerably depending on which physiological characteristics are believed most important, on raw material used, and on the indication pursued. The final products are eventually formulated as solutions or as suspensions of liposome or capsules. The Hb concentration ranges from 4 to 14 g/dL, with limitations due to colloidal osmotic pressure (COP, or oncotic pressure, the part of the osmotic pressure that is generated by colloids, normally proteins), viscosity, or encapsulation efficiency. Each product has its own unique O_2 delivery characteristics and physiological responses (section IV).

3. Fluorocarbon Emulsions

Due to their virtual insolubility in water, PFCs are formulated into emulsions for parenteral administration. The submicrometer size droplets of PFC are coated with a thin layer of a surfactant that serves as an emulsifier and an emulsion stabilizer. A range of emulsion concentrations—from 20% to 100% weight/volume (i.e., about 11−52 vol %)—has been investigated.[192,215,219,325] The present PFC emulsions use egg yolk phospholipids (EYP) as the emulsifier. Therefore, these emulsions have a definite similarity to the lipid emulsions used for parenteral nutrition. Emulsion osmolarity is independent of PFC concentration and is adjusted by the addition of salts or other tonicity agents. The PFC emulsion droplets do not normally filter out of the circulation.

C. Oxygen Coordination versus Oxygen Dissolution

The mechanisms by which O_2 is taken up, transported, and released by Hb products and by PFCs are different by essence and, consequently, so is the availability of O_2 to tissues.

1. Hemoglobin: An Exquisitely Well-Adapted Oxygen Coordination and Transport Machinery

Dioxygen coordination by Hb is accompanied by minute adjustments of the protein from the tensed (T-form) deoxyHb conformation to the relaxed (R-form) oxyHb conformation in which access to the heme pocket is more open. The deoxyHb form is stabilized by salt bridges within subunits, between subunits, and between subunits and 2,3-DPG (in man) and other anionic species, primarily ATP, inorganic phosphate and chloride ions, which serve as allosteric effectors.[223,326−329] Upon oxygenation of deoxyHb, these interactions are broken and 2,3-DPG,

Cl^-, and H^+ are released. Release of O_2 is accompanied by changes of Hb's conformation back from the R to the T form. The allosteric effectors, by stabilizing the protein's T conformation, reduce its affinity for O_2, facilitating O_2 off-loading.

The amount of bound O_2 in blood initially increases sharply when the partial pressure of O_2 increases and then levels off at about 150 Torr (the partial pressure of O_2 in air) when all four iron sites become essentially saturated (Figure 4). Above that pressure, the O_2 content of blood increases in a linear fashion with a very low slope corresponding to the dissolution of O_2 in the plasma. Since Hb is nearly saturated under atmospheric O_2 concentration and pressure, its O_2 content cannot be enhanced in any significant way by increasing O_2 availability.

The O_2 equilibrium characteristics of a given Hb product depend not only on the product's chemical constitution but also on environment and temperature. These equilibrium characteristics are primarily defined by O_2 affinity, cooperativity, and alkaline Bohr effect. The product's affinity for O_2, hence its tendency to retain or release the gas, is usually described by the O_2 partial pressure, P_{50}, at which 50% of the Hb is saturated with O_2. P_{50} is strongly dependent on the presence of an appropriate allosteric effector (primarily 2,3-DPG for man, dog, horse, rabbit, guinea pig, and rat,[330,331] chloride for bovines,[332] inositol tetraphosphate for ostrich,[333] bicarbonate for crocodiles,[334] etc.). At 37 °C, P_{50} for purified, cell-free human Hb (i.e., in the absence of 2,3-DPG) is about 14 Torr, as compared to about 26−28 Torr for Hb in the RBC. Only about 5% of the O_2 transported (instead of about 25%) is then released to tissues at the usual $P\bar{v}O_2$ (the mixed venous O_2 tension) of 40 Torr (cf. Figure 1).

P_{50} is also strongly dependent on temperature.[335,336] The capacity of Hb to release O_2 to tissues decreases rapidly when temperature decreases (i.e., the O_2 saturation curve of Figure 4 shifts leftward), thus reducing Hb's ability to oxygenate tissues in hypothermic conditions. For cell-free human Hb, P_{50} is 14 and 4 Torr at 37 and 15 °C, respectively.[337]

One remarkable property of Hb is its ability to bind O_2 in a cooperative fashion. Cooperativity among the four hemes allows the affinity of the protein for O_2 to increase as O_2 binds and vice-versa, enabling Hb to offload O_2 to the tissues at an O_2 tension lower than that at which it loads O_2 in the lungs.[228] Cooperativity is responsible for the sigmoid shape of the O_2 binding isotherm characteristic of Hb (Figure 4), as compared with the hyperbolic uptake curve of myoglobin, which has only one O_2 coordination site. Myoglobin provides a reserve of stored O_2 in red muscle tissue for release under conditions of O_2 deprivation but is unsuitable as a carrier because of too low P_{50} and lack of cooperativity. Restriction of cooperativity upon chemical modification is often assigned to a reduction in conformational mobility.

The change in the quaternary structure of Hb that occurs upon release of O_2 to tissues is accompanied by an increase in the pK of several functional groups. The protein is then capable of taking up several more protons, which it releases again during uptake of O_2 in the lungs, causing an increase in pH. When pH raises, the O_2 equilibrium curve shifts to the left. This alkaline Bohr effect also contributes to regulating the transport of CO_2 from the tissues to the lungs.[323,338] As CO_2 is released in the alveoli, the pH of blood increases, which increases O_2 affinity and facilitates O_2 uptake. Conversely, the CO_2 taken up by RBCs at the tissues level, after conversion into carbonic acid, leads to a decrease in pH within the RBCs, hence to a decrease in O_2 affinity, which facilitates O_2 offloading. In other words, CO_2 exchange facilitates O_2 exchange and vice-versa. As mentioned earlier, another synergistic effect was recently discovered between O_2 binding and NO uptake at the Cys-93β sites.[317]

Oxygen affinity, cooperativity, and the Bohr effect, stability and autoxidation rates, rates of reaction with NO and other factors present in the blood, as well as other functions of Hb, including CO_2 and NO transport and enzyme-like functions, can be substantially affected when the protein's structure is modified (section IV.H).

2. Fluorocarbons: Environment-Independent Passive Gas Solvents

Fluorocarbons are passive gas carriers. Physical dissolution of gases in PFCs obeys Henry's law, i.e., is directly proportional to the partial pressure of the gas (Figure 4). No chemical bonding is involved and there is no saturation. Oxygen solubility in a given PFC depends on the solubility coefficient of the PFC for that gas (section V.B). Uptake and release of O_2 by PFCs are essentially insensitive to the environment. Oxygen content can be adjusted by simply controlling pO_2. The principles that underlie O_2 transport by PFC emulsions are basically the same as those that operate for plasma. In both cases, dissolution is proportional to pO_2; simply, the solubility of O_2 in PFCs is typically 20 times larger than that for the plasma.[339]

Off-loading of O_2 from PFCs to tissues is not subordinated to any change in conformation and does not require the assistance of an allosteric effector. It is easy because the van der Waals interactions between O_2 and PFC molecules are an order of magnitude weaker than the covalent O_2−Fe(II) coordination bond in Hb, resulting in much higher extraction rates and ratios. The latter typically reach 90% with PFC emulsions, as compared to about 25% for Hb in normal conditions.[216,340] Oxygen release from PFCs is effective at any physiologically relevant partial pressure, rendering a cooperativity-like effect unnecessary. Likewise, O_2 release by PFCs is not dependent on pH and is not adversely affected by temperature. Since PFCs undergo no oxidation or other modification over time, their O_2 uptake and release characteristics are not affected by storage or during circulation.

Introducing a PFC emulsion into the circulation is akin to increasing the O_2 solubility of the plasma compartment of blood. When Hb and a PFC are present in the circulation simultaneously, the PFC will always release its O_2 load first, thus conserving the O_2 bound to the Hb. Hemoglobin is exquisitely

well adapted to supporting life in earth's atmosphere, but the conditions available in the operating room or critical care unit are different and adjustable. A valuable consequence of PFCs' following Henry's law is that the O_2 content of a PFC emulsion can be increased severalfold by just increasing the fraction of O_2 in the air inspired by the patient (FiO_2), which is a simple thing to do in a rescue vehicle or critical care or surgical setting. To achieve maximal benefit, the PFC-treated patient will generally inspire pure or close to pure O_2.

3. Carbon Dioxide Transport

Intravascular CO_2 transport relies on several mechanisms, including physical dissolution in plasma, carbonic anhydrase-induced transformation into bicarbonate (eq 3.10)

$$CO_2 + H_2O \overset{\text{carbonic anhydrase}}{\rightleftharpoons} H_2CO_3 \rightleftharpoons H^+ + HCO_3^- \quad (3.10)$$

and chemical binding consequent to reaction with the N-terminal amino groups of Hb to produce labile carbamino groups (eq 3.11).

$$Hb\}-NH_2 + CO_2 \rightleftharpoons Hb\}-NHCOO^- + H^+ \quad (3.11)$$

About 23% of total CO_2 transport occurs in the carbamino form.[338] This transport mode is depressed when the N-terminal NH_2 groups, which are among the most common targets of chemical modifications, are engaged in such modifications. Transport of CO_2 is also affected by an alteration of the Bohr effect since hydration of CO_2 and the formation of HCO_3^- depend on pH. The physiological consequences of reduced Bohr effect and CO_2 transport remain uncertain, however. It might affect tissue acid−base balance[341] and have clinical consequences in critically ill patients where tissue CO_2 build up can be high.[338]

As for any gas, PFCs transport CO_2 in the dissolved form. Solubilities for CO_2 are typically in the 120−250 vol % range, i.e., 3−5 times larger than for O_2.[339] There is no indication that transport of CO_2 by PFCs interferes with the CO_2 transport mechanisms by Hb and plasma.

4. Dissolution and Transport of Nitrogen and Other Gases

The capacity for PFCs to dissolve nitrogen (and air) may find applications in the treatment of decompression sickness[342−346] and for protection from neurologic damage caused by air microemboli during cardiopulmonary bypass surgery (section V.I). The solubility of xenon in PFCs can be exploited for magnetic resonance imaging (MRI).[347] Both Hb and PFCs transport NO but by different mechanisms, which changes the availability of the gas.

D. Oxygen Delivery to Tissues

Assessing the relative contributions of Hb and PFC products to O_2 delivery and consumption requires consideration of an unconventional situation. One commonly encountered error is to compare such products on the sole basis of their *static* O_2-binding or O_2-dissolving capacities on a gram-per-gram basis in *air*. Such an approach ignores the possibility of increasing O_2 dissolution in PFCs by a factor of almost five simply by giving the patient pure O_2 to breathe instead of air, i.e., ignores the actual conditions in which PFC emulsions are to be utilized. It also overlooks the ca. 4-fold higher tissue O_2 extraction ratio that is seen with PFCs under normal conditions. In addition, when normovolemic hemodilution is performed, there is normally a substantial increase in cardiac output (the volume of blood ejected from the heart in a given period of time) as a result of increased fluidity of the diluted blood.[141,145,348] This increase in cardiac output is preserved when PFC emulsions are administered, which further enhances their O_2 delivery capacity,[155,216,348] while Hb products classically display an unchanged or reduced cardiac output that can negate or even outweigh the benefit of increased fluidity.[304,349−360] Further unfounded conclusions have sometimes been drawn on the basis of inadequate protocol design and even on the implicit expectation that the O_2 carrier should still have been active when it was no longer present in the circulation (see, for example, ref 361 and the discussion that followed.[362,363]).

On the other hand, the notion that simply increasing the amount of Hb in the circulation will necessarily increase the amount of O_2 delivered to the tissues to the same extent as RBC-enclosed Hb would is overly simplistic and actually inaccurate (section IV). This is because O_2 release from cell-free Hb can be hindered by high O_2 affinity and/or vasoconstrictive effects. Over time, autoxidation can further reduce the O_2 transport capacity of Hb solutions.

1. Oxygen Content versus Delivery versus Consumption

In blood, O_2 is transported both in the coordinated and dissolved forms, coordinated to Hb and dissolved in the plasma compartment. Hemoglobin binds about 1.34 mL of O_2 per gram at full saturation,[216,364] and the normal Hb concentration in blood is about 14 g/dL. In room air, plasma dissolves only about 0.3 mL of O_2 per dL per 100 Torr and its contribution is negligible. One hundred milliliters of a 14 g/dL solution of Hb, therefore, carries about 19 mL of O_2. In room air, $P\bar{v}O_2$ is normally about 40 Torr and the O_2 binding (or saturation) isotherm (Figure 4) indicates that Hb remains 75% saturated with O_2, hence that blood normally gives up only an average 4.7 vol % of O_2. The rest constitutes an O_2 reserve.

The amount of O_2 dissolved in a given PFC emulsion is essentially the product of the PFC's O_2 solubility coefficient, emulsion concentration, and O_2 partial pressure (Figure 4). It amounts, for example, to about 3% in room air and 16 vol % under pure O_2 at 37 °C in a 60% w/v emulsion of *F*-octyl bromide. In the circulation, the arterial O_2 tension, PaO_2, can be increased from about 100 Torr, when the patient breathes room air, to 500 Torr when he breathes pure O_2. The emulsion will then carry around 10.5 vol % of O_2. Because O_2 is not covalently bound to the PFC, up to 90% of this O_2 can be released to tissues, which amounts to about 9.5 vol % O_2.

However, it is obviously not a carrier's O_2-dissolving capacity but the amount of O_2 that is delivered

to the tissues and, eventually, the contribution of that carrier to O_2 *consumption* that determines its effectiveness. Convective O_2 delivery, $\dot{D}O_2$ (the amount of O_2 offered to the tissues within a defined period of time), is the product of arterial blood O_2 content, CaO_2, and the cardiac output, CO (eq 3.12)

$$\dot{D}O_2 = (CaO_2) \times CO \qquad (3.12)$$

Oxygen delivery is indeed a dynamic notion that is dependent upon carrier flux. If an analogy to a shuttle service is used, it is the product of the number of seats available and the frequency of the shuttle's rotation. In blood, CaO_2 depends primarily on Hb concentration and O_2 saturation. When an O_2 carrier is used, the parameters that characterize that particular carrier are involved. These include, for a Hb product, its specific O_2 affinity, cooperativity, Bohr effect, profile and rate of removal from circulation, effect on cardiac output, etc. Cardiac output (shuttle frequency) normally increases when blood is diluted (as when hemodilution is practiced) by the administration of low-viscosity solutions or emulsions. Provided there are no contrary vasoactive effects, circulation is then facilitated, resulting in increased O_2 delivery. As PaO_2 is increased, proportionally more O_2 is delivered to the tissues from the plasma and PFC as compared to Hb, and $P\bar{v}O_2$ (which reflects global tissue oxygenation) can increase significantly.

Eventually, it is the amount of O_2 that is consumed by the tissues, and the prevention of hypoxia (inadequate O_2 supply), that is vital. Whole body O_2 consumption, $\dot{V}O_2$, is given by the Fick equation, eq 3.13

$$\dot{V}O_2 = (CaO_2 - C\bar{v}O_2) \times CO \qquad (3.13)$$

where the parenthesis represents the difference in O_2 content between arterial and mixed venous blood. When pure O_2 is inspired and PaO_2 is increased to around 500 Torr, the contribution of plasma is no longer negligible, raising to about 7% of $\dot{D}O_2$ and 32% of $\dot{V}O_2$, versus 1.6% and 4%, respectively, in room air.[216] Administration of a relatively small 2.7 g/kg b.w. dose of PFC can increase plasma solubility for O_2 by about 45%, which, because of high O_2 extraction, can translate into substantial contributions to O_2 delivery and consumption, although the amount of PFC-dissolved O_2 is smaller than the amount of Hb-dissolved O_2 (section V.G).

As noted, $P\bar{v}O_2$, i.e., the residual O_2 tension after the tissues have extracted the O_2 they needed, is often used as a global indicator of tissue oxygenation (although individual tissues may have different oxygenation status) and overall balance between O_2 supply and demand.[216,348,365−367] A substantial increase in $P\bar{v}O_2$ was consistently observed upon administration of PFC emulsions and was paralleled by an increase in tissue pO_2 (section V.G), which was often not the case with Hb preparations (section IV.H).

2. Oxygen Diffusion and Oxygen Diffusion Facilitation

Diffusion of O_2 from the RBCs into the tissues is driven by the pO_2 gradient that exists between the blood and these tissues. Thus, in an in vitro capillary system, the rates of both oxygenation and deoxygenation of an Hb solution were limited by diffusion and governed by the O_2 gradient between internal and external spaces.[335] Diffusive O_2 transport has been identified as a critical component of O_2 consumption.[368]

Diffusion should be facilitated when the RBC membrane is absent and when numerous small size (as compared to RBCs), highly mobile O_2 reservoirs are present in the circulation. Plasma gaps exist between red cells in the microcirculation (capillaries). These gaps are particularly large when the patient suffers from anemia or is hemodiluted. Cell-free Hb molecules, Hb-loaded liposomes, and PFC droplets, by filling these gaps in large numbers, increase O_2 content and potentially facilitate O_2 diffusion by providing numerous "stepping stones" or dynamic chains of particles over which O_2 can travel.[369−372] Much more numerous than RBCs, such particles also offer a several orders of magnitude larger area for gas exchange. In the larger vessels, a near-wall excess of the smaller particles is likely to develop as RBCs would tend to migrate nearer the lower shear central axis of the vessel. Effectiveness is expected (provided there is no vasoconstrictive activity) to be greatest in the capillary beds, at low RBC concentrations and, in the case of PFCs, when FiO_2 is high.

Several theoretical and experimental studies have addressed this question.[335,373−382] In an in vitro capillary model, an Hb solution took up and released O_2 2−5 times as rapidly as an equivalent RBC suspension.[374] Acellular Hb was shown to enter compressed capillaries more readily than RBCs.[383] The O_2 flux across a nonflowing layer of an Hb solution was increased as compared to N_2 and with non-Hb-containing solutions, but it decreased strongly when the viscosity of the medium increased.[381] Mathematical modeling and in vitro experiments with a capillary model have indicated that mixtures of cell-free Hb and RBCs should be more efficient than RBC suspensions in O_2 transport in small vessels and should facilitate O_2 diffusion.[382,384] Agreement between theory and experiment was significantly improved for O_2 uptake by RBC/cell-free Hb mixtures (but not for O_2 release) by using a shear-augmented diffusion coefficient. In vivo O_2 delivery has been demonstrated with Hb products (section IV.G), but diffusion facilitation could not be proved directly.[376,378]

Likewise, the large pO_2 gradients set in place at the high FiO_2 at which PFC emulsions are utilized provide a strong driving force for O_2 diffusion from the PFC droplets to the tissues. The small 0.1−0.2 μm-size droplets will circulate more easily in the capillary beds than the 30−70 times larger RBCs. The movement of emulsion microdroplets in the blood stream was proposed to create dynamic chains of particles; hence, channels which would help transfer O_2 from the RBCs to tissues.[372] The probability of formation of such channels increases sharply when the size of the particles decreases and their number increases. Numerous indications in the literature suggest that PFC emulsions can facilitate O_2 diffusion. Relatively small amounts of PFCs were fre-

quently observed to substantially increase arterial blood O_2 tension and tissue O_2 tension[385−388] (section V.G). However, in an experiment intended at providing direct evidence for O_2 diffusion facilitation, the observed improvement of O_2 uptake could be entirely accounted for by increased plasma O_2 solubility, without an added diffusion effect.[377]

3. Computer Modeling of Efficacy

The benefit that can be derived from use of a given Hb solution is difficult to predict as it depends on the multiple parameters that characterize that product, including P_{50}, cooperativity, constitution, hydrodynamic radius of components, formulation as solution or liposome, COP and viscosity of the preparation, rate of extravasation and clearance, rate of autoxidation, etc. Calculations can be rendered false when hemodynamic or other drug activity is present. Significant improvement of O_2 transport was predicted when cell-free Hb was present.[384] Possible vasoconstrictive activity in vivo was, however, not taken into account.

Computer simulation appears to be easier with PFC emulsions because of fewer product-related parameters. Simulation of O_2 transport has predicted that addition of a PFC emulsion to blood should increase O_2 flux at the blood vessel walls, decrease transport resistance of O_2 from RBCs to tissues in the capillaries, and be highly effective in increasing tissue O_2 tension at high FiO_2.[389−391] The primary contributor to this increase was identified as the augmented O_2 plasma solubility upon addition of the emulsion. The effect of a near wall excess of PFC droplets[392] was determined to be secondary.

A physiological model has been developed that allows calculation of the relative contributions of Hb, blood plasma, and O_2 carriers to O_2 delivery and consumption and prediction of $P\bar{v}O_2$ (i.e., global tissue oxygenation) with particular reference to the effects of hemodilution during surgery.[393] The input variables included Hb concentration, arterial O_2 tension, cardiac output and O_2 consumption, surgical blood, and O_2 carrier losses. Validation was achieved by comparing calculated $P\bar{v}O_2$ with $P\bar{v}O_2$ values measured during experimental and clinical investigations with a PFC emulsion. These simulations confirmed the benefits that can be derived from administration of PFCs during ANH (section V.I). The contribution of a dose of PFC to O_2 *consumption* was subsequently described in terms of an Hb equivalency value.

4. "Pressor Effect" and Efficacy

As indicated above, molecular solutions, liposome suspensions, and PFC emulsions were expected to circulate more freely than RBCs. However, contrary to hemodilution with conventional colloids,[141,145] hemodilution with Hb solutions usually produced no increase in cardiac output but a substantial increase (up to 40%) in arterial pressure due to increased vascular resistance, the so-called "pressor effect" (section IV.H). As a result, O_2 delivery often remained unchanged and was sometimes decreased, despite increased arterial O_2 content. For example, hemodilution of dogs with dextran or *Fluosol* led to significant elevation in cardiac output, while comparable hemodilution and reduction in blood viscosity with cell-free Hb did not.[352] Again, the mere presence of Hb in the circulation or an increase in arterial O_2 content do not guarantee that additional O_2 will be made available for consumption by the tissues. On the other hand, administration of a PFC emulsion, although it may transiently decrease blood O_2 content as a result of hemodilution, can result in a net increase in O_2 delivery.[386]

Animals close-to-totally exchange-perfused, whether with an Hb product[394] or a PFC emulsion,[222,395−397] usually survive. The question is whether such products are effective under clinically relevant conditions, i.e., when significant amounts of RBCs are present. Direct comparison of the O_2 delivery effectiveness of Hb and PFC products is difficult as the two systems are usually being evaluated using substantially different protocols. Eventually whether an O_2 carrier does or does not fulfill its objective will be determined by clinical data demonstrating safety and avoidance of tissue hypoxia. In surgical or trauma patients, this can be reflected by reversal of physiological indicators of acute anemia (transfusion triggers) and suppression or reduction of the need for allogeneic blood transfusion.

E. Pharmacologically Different

A few essential differences in in vivo behavior, characteristic of the two approaches (including clearance form circulation, metabolism, and excretion, effect on the RES, and immunogenic reactions), will be outlined here. The hemodynamic effects of Hb products as well as the effects linked to Hb's redox chemistry will be discussed in section IV, while the effects on the RES will be further discussed in both sections IV and V.

1. Intravascular Persistence

Extracellular Hb concentration in the plasma normally does not exceed 3−6 mg/L, and any such Hb is rapidly cleared from the circulation, primarily by the RES after binding with the plasma protein haptoglobin.[223] Excess Hb (once haptoglobin is depleted) is cleared in part by the kidneys (after dissociation to dimers) and in part directly by the RES, principally the liver.

Hemoglobin derivatives, Hb-loaded liposomes and capsules,[398−401] and PFC emulsion droplets,[222,402−406] like lipid emulsions,[407,408] other liposome preparations[409,410] and certain colloids, including gelatin and polysaccharides,[411] and senescent RBCs, being foreign particulate matter, are all handled by the RES. They are subject to opsonization, phagocytosis, and removal from the blood stream by macrophages. As a result of these and other mechanisms, both the present modified and/or encapsulated Hb products and PFC products have short, dose-dependent circulation half-lives ($t_{1/2}$), typically in the 8−24 h range in man, as compared to autologous RBCs (∼12 weeks). In the case of cell-free Hb products, $t_{1/2}$ depends on type of modification and MW. In the case of encapsulated Hb and PFC emulsions, vascular persistence depends primarily on size and, to some

extent, on surface characteristics, hence on membrane or film components.

Acellular Hb products have been observed to filter out of the circulation into the lymph, pleural, and peritoneal spaces and the intestine, resulting in a rapid initial fall in intravascular content.[188,412−418] Leakage into extravascular spaces is commonly believed to contribute to inactivation of endothelium-produced NO, hence in hemodynamic perturbations. Perfluorocarbon droplets, like RBCs or liposomes, are larger than the largest modified Hb molecules and do not leak out of normal vasculature.

2. Metabolism and Excretion

The in vivo distribution, metabolic pathways, and excretion routes of cell-free Hb and PFC products are widely different. Globin is catabolized in renal tubule cells to yield amino acids, while heme is converted into bilirubin, and the iron is stored in the form of ferritin for recycling.[223,419] If the kidney's Hb handling capacity is exceeded, hemoglobinurea will ensue. Infusion of large doses of cell-free Hb results in kidney damage.[178,186,187,420−422] One of the initial objectives of Hb modification was to suppress renal filtration by preventing the dissociation of the tetramer into dimers (section IV.D).

The liver is involved in the processing of Hb products.[423] Hemoglobin's degradation products, including hematin (the oxidized form of heme with water occupying the sixth coordination site of the ferric iron), hemin (the same with chloride instead of water), bilirubin (the product of hemin degradation by heme oxygenase), and eventually free iron, are all either toxic or susceptible of promoting toxic reactions.[189] Studies of the metabolism of modified Hb are potentially complicated by the extreme heterogeneity of most modified Hb products.

Fluorocarbons are not metabolized. The emulsion droplets are cleared from circulation by phagocytosis. The PFC is temporarily stored in the RES organs, slowly released back into the circulation via lipoproteins at a rate that depends on the PFC's MW and lipophilicity, and eventually excreted unchanged through the lungs with the expired air (section V.G). For these reasons and because PFCs are produced in a high state of purity, pharmacokinetic studies of PFCs are relatively simple.

3. Particle-Related Side Effects—Effects on the Reticuloendothelial System

Phagocytosis of foreign material can result in macrophage activation and the release of prostaglandins and pyrogenically active cytokines. These events can translate into fever, chills, and other flu-like symptoms. Such reactions have been described with early injectable lipid emulsions[424,425] and with non-Hb-loaded liposomes.[410,426] The same type of reactions were observed with Hb-loaded liposomes (section IV.F) and PFC emulsions (section V.H).

Uptake of large amounts of particulates can result in transient overload and depression of the RES and may limit the dose of O_2 carrier that can be administered.[208,399,403,404,407,408,427,428] Gelatin, starch, and dextran solutions (which are used as plasma expanders) are also known to depress the RES system.[411] Transient depression appears to occur with transfused RBCs as well[68] (section II). Dependence of circulation half-life on dose for both Hb and PFC products is likely to reflect loading and saturation of the RES.

4. Reactivity-Related Effects

Due to their reactivity with various endogenous factors, including O_2, NO, CO, and H_2O_2, and functions other than O_2 transport, Hb products and their degradation products can elicit diverse pharmacological activities. As indicated earlier, cell-free Hb and most of the modified Hb products elicit a "pressor effect" that can translate into an increase in vascular resistance and blood pressure and a reduction of cardiac output and tissue perfusion (section IV.H). This phenomenon is likely multifactorial with a major factor being vasoconstriction consequent to neutralization of NO.

PFCs have no capability to complex or neutralize NO or CO or react with H_2O_2. Clinical trials have shown no perturbation of hemodynamics or of O_2 consumption upon administration of the current emulsions, and the beneficial effect of hemodilution on cardiac output was fully preserved.

5. Immunogenic Reactions

Recrystallized horse Hb was shown long ago to be antigenic in rabbits.[429] Properly purified human Hb is considered to be a very weak antigen.[430,431] Repeated subcutaneous injections of polymerized bovine Hb into rabbits produced only weak antibody titer.[432] Transfusion of homologous pyridoxalated Hb or pyridoxalated and polymerized Hb in rats that had been immunized with the product by subcutaneous injection along with Freund's complete adjuvant did not elicit any immunological reaction, and no antibody titers were produced in the serum of the animals.[433] A single exposure to similarly modified human Hb products also provoked no adverse reactions. A single inoculation of liposome-encapsulated bovine Hb in mice produced only small immunologic changes.[434]

However, severe anaphylactic shock and death were observed when rats that had received immunizing doses of human Hb or of a pyridoxalated polymerized human Hb were subsequently transfused with these products.[433] Similar findings were made with o-raffinose-polymerized Hbs: the modified homologous Hb was not antigenic when administered to immunized rats while the heterologous product was antigenic.[435] The polymerized Hb was actually more antigenic than the nonmodified heterologous Hb. Small doses of dextran conjugates prepared from dog, rabbit, and sheep Hb were not immunogenic in homologous hosts; in heterologous species the anti-Hb response was comparable to that produced by the nonmodified Hb.[436] A pyridoxalated glutaraldehyde-polymerized human Hb was antigenic in dogs, and the antibody response was significantly greater in animals that were subjected to hemorrhagic shock (a condition which could impair the immune system) than in animals which had not been bled.[413] Liposome-encapsulated heterologous Hb minimally in-

creased antibody titers, and none was found with a pegylated heterologous Hb.[435] However, a single inoculation of liposomes containing bovine Hb in mice produced small immunologic changes (as measured by the acquisition of IgG antibody to bovine Hb), which were nevertheless deemed potentially important.[434] No immune response was reported during clinical trials of a cross-linked human Hb.[437] Although bovine Hb differs from human Hb by as much as 17 amino acids in the α chains and 24 amino acids in the β chains, only "low" antigenicity was mentioned in clinical studies with a glutaraldehyde-polymerized bovine Hb.[438]

Antigenic reactions with a neat pure PFC have not been reported. An anaphylactoid reaction elicited by *Fluosol* in some patients[439,440] was no longer seen when EYP was used as the emulsifier instead of *Pluronic* F68.[441] The recently developed PFC/EYP emulsions revealed no significant immunogenic or allergic reactions, complement activation, plasma contact system activation, or platelet activation.[442]

6. Potential for Promoting Bacterial Infection

Several studies have indicated that administration of Hb preparations can enhance the lethality associated with Gram-negative bacteria infection.[443−448] A sublethal dose of *Echerishia coli* can become lethal upon administration of a cell-free Hb preparation.[445−447] A pyridoxalated cross-linked Hb was more potent than the unmodified Hb in stimulating bacterial growth, possibly as a result of longer circulation half-life.[447] Both impure bovine Hb and highly purified Hb markedly increased the mortality rate of rats with *E. coli* peritonitis, while the effect of stromal elements was minimal.[449] Possible mechanisms for these effects include RES overload, potentiation of bacterial lipopolysaccharide activity by complexation with Hb,[450] and providing invading pathogens with an abundant supply of the iron essential for their replication and growth.[446,451,452] Phagocytic activity and the capacity of human granulocytes to kill bacteria were impaired when bovine Hb was added to a bacteria/granulocyte suspension.[453] On the other hand, Hb products were effective in restoring O_2 uptake and improving survival in septic rats[454,1251] and have been proposed as a treatment for septic shock.[455]

Synergistic toxicities from Hb and bacterial endotoxins have also been reported.[448,456−458] An α,α-cross-linked Hb increased the inflammatory response of hepatic macrophages to endotoxins.[458] Formation of stable complexes between Hb and diverse lipopolysaccharides[450] enhanced the latter's ability to activate coagulation.[459] A cross-linked tetrameric Hb strongly enhanced in vitro endothelial cell response to bacterial endotoxin, favoring coagulation and raising concerns about use of such material in patients at risk of endotoxemia.[460,461] Some potentiation of endotoxin induced lethality in mice was also seen with a recombinant human Hb.[462]

No microorganism is known to feed on PFCs, and no complexation of endotoxins by a PFC has been reported. *F*-Octyl bromide actually decreased the inflammatory response of cultured macrophages to endotoxin stimulation,[463] and partial ventilation of trauma patients with this PFC was seen to reduce alveolar inflammatory response.[464]

F. Commercial-Scale Production

Reliable access to raw materials, ease of manufacture, and cost-effectiveness will play critical roles in determining the degree of acceptance, breadth of application, and commercial success of an O_2 carrier. To have a valuable impact on medical practice, O_2 carriers must be able to replace a fair proportion of the blood and packed RBCs that are presently being transfused. A mere 10% of this amount represents over one million blood units in the United States alone and several million units worldwide. Only O_2 carriers that can be produced in that volume and can be sold for a price that remains in the range of that of packed RBCs will be able to potentially mitigate the current and projected blood shortages. Marketing of a blood substitute also requires involvement of a large organization, well established in the blood product industry.

1. Raw Material Procurement

a. Outdated Banked Human Blood. Access to raw material of appropriate quality and in sufficient quantity constitutes a major limitation in the development of Hb-based products. One reason for developing O_2 carriers is to ease blood collection difficulties and to cope with shortages. A further expectation is to provide developing countries with a cost-effective alternative to blood banking (section II). It may therefore sound paradoxical to rely on human blood as a raw material for manufacturing a "blood substitute". With improved blood banking practices, the proportion of donated blood that becomes outdated is steadily declining. It was only on the order of 4−7% in 1997 in the United States,[19,101] and not all of it is recoverable. The Internet is likely, by facilitating the movement of blood between blood banks, to help further reduce the proportion of units that become outdated. Since it takes at least two units of blood to manufacture one unit of a Hb-based substitute, the supply of human Hb from outdated RBCs can hardly be expected to support the demand for such a substitute to any commercially significant extent. Competition for this raw material may further reduce the share of blood available to each single product/company and is likely to increase its cost. Plans to extract and process Hb from placenta[465] have been abandoned, allegedly due to liability issues. Can one envisage the collection of blood from paid volunteers specifically for transformation into an O_2 carrier product for profit? And at which cost? These questions raise not only logistic, economic, and safety issues, but also ethical concerns.[466−468] In the developing countries, reliance on human blood as a raw material does not appear realistic. In any event, improved blood management and projected shortages are likely to challenge raw material procurement from human donors as raw material for a blood substitute.

b. Animal Blood. The assumption that animal Hb is abundant and cheap and could easily be collected in slaughterhouses must be tempered. Access to safe

bovine blood of constant quality probably requires raising dedicated, controlled herds of cows and harvesting the blood in accordance with expensive good manufacturing practices. The methods needed to do so on a large scale remain to be developed. Caution needs to be exercised as long as the absence of immunological effects has not been fully demonstrated. The risk of interspecies crossover of pathogens also remains to be assessed. Transmission of bovine spongiform encephalopathy (BSE) by blood transfusion has been demonstrated in sheep,[45] and there is also growing concern that BSE can pass from cattle to humans.[49] Other sources for animal Hb have been proposed for consideration, including from swine[469] and *Lumbricus terrestris*, the common earthworm,[470,471] but certainly require further evaluation.

c. Genetically Engineered Hemoglobin. Production of recombinant modified human Hb (rHb, section IV.E) is attractive and has resulted in superb scientific achievements.[315,472−477] Manufacture of rHb requires expression of α and β globins, the presence or addition of sufficient heme and its incorporation into the protein, proper folding, separation of the resulting Hb from other bacterial proteins, lipids, nucleic acids, and endotoxins, and thorough purification. Because of the large dose of protein that needs to be administered in order to provide clinical efficacy, the acceptable level of endotoxins and other contamination must be extremely low. Industrial development of rHb products thus faces unprecedented challenges related to the scale of the fermenters and separation and purification units that need to be built in order to satisfy a significant-size demand and the gigantic task of isolating the protein from the bacterial culture and of attaining, on a 100-ton scale, the mandatory level of purity. Pilot size production of an rHb from *E. coli* necessitated supplementation with heme extracted from bovine Hb. The large water consumption and the environmental impact of the effluents produced by large-scale rHb manufacture need to be taken into consideration. Production of human Hb in yeast[478] and in transgenic swine was initiated[479] and later abandoned. Recombinant drug protein synthesis is still an expensive avenue, which may not be suitable for the manufacture of a product that needs to be administered in 100-g-size doses while its price needs to stay in the vicinity of that of blood. The a priori lesser cost of protein production in plants as compared to bacteria[480] may be offset by the cost of meeting the special purity requirements for pharmaceutical-grade Hb.

d. Perfluorocarbons. Perfluorocarbons, being synthetic, have obvious advantages from the standpoint of raw material procurement, purity, safety, and cost-effectiveness. Pure PFCs can be manufactured within tight specifications, in any desired amount, using well-established procedures (section V.B). Perfluorooctyl bromide (also known as perflubron), for example, is directly derived from the production line that leads to poly(tetrafluoroethylene) (PTFE, i.e., Teflon) and to diverse large-tonnage industrial surfactants and can be obtained in better than 99.9% purity on a several hundred ton scale.

2. Manufacturing

Processing blood to extract pure Hb involves the lysis of the RBCs, removal of the immunogenic RBC membranes, thorough purification, and multiple virus inactivation steps. This is followed by chemical modification(s), separation of the modified product from excess reagents and byproducts, purification and chromatographic separation of undesirable unreacted Hb or modified Hbs having inappropriately high or low MW, and sterile filling. These operations result in losses of precious material. Processing also requires large amounts of highly purified water. As noted earlier, extraction of rHb from an *E. coli* culture and separation from bacterial endotoxins or separation of human from animal and chimerical Hb produced by transgenic animals is by no means simpler. Such manufacture also requires considerable capital expenditure.

Liposome technology has challenges of its own, some of which are again related to the large doses of Hb to be administered as compared to any of the liposomal preparations currently used for drug delivery, others to the capacity of Hb to promote lipid oxidation (section IV.F). Further development challenges include cost of components, encapsulation efficiency, particle size and metHb formation control, sterilization, and shelf stability. Liposome manufacturing is complex and has never been scaled up to meet the tonnage requirements of an O_2 carrier.

In comparison, the manufacture of a PFC emulsion is extremely simple, even though it requires specific know-how. Technology for producing injectable emulsions on a large scale is well established in the pharmaceutical industry, where emulsions for parenteral nutrition have been in use for over 30 years. The surfactant now utilized, egg phospholipids, also has a long history of use in pharmaceuticals, including for preparing injectable lipid emulsions and, more recently, liposomes. The formulation process being additive, its yield is essentially quantitative with respect to the raw materials utilized. The present products are terminally heat-sterilized at or above 121 °C. The investment in a given size production unit may be as much as an order of magnitude lower for PFC emulsions than for any Hb product.

Altogether, although use of Hb for developing an O_2 carrier appears to be a *natural* way to go and therefore attracted much interest and effort, it should be obvious that the PFC approach also has its advantages. Both types of products should hopefully become available in a not too distant future.

IV. Hemoglobin-Derived Oxygen Carriers

Numerous options have been explored during the century-long quest for an effective Hb-based blood substitute, from cell-free native Hb to extensively chemically and/or genetically modified and/or encapsulated Hb products.

A. Cell-Free Hemoglobin

Early experimentation with Hb solutions on animals has been reviewed by Sellards and Minot, who

also reported the first study involving intravenous injection of Hb to man for the purpose of investigating the protein's metabolism and excretion in normal and anemic patients.[481] During the 1930s and 1940s, Amberson's seminal work and review of the field accurately identified some of the prominent difficulties of the cell-free Hb approach to O_2 delivery.[186,482] These included the following: toxicity of stroma (debris of RBC membranes); rapid oxidation of Hb; rapid loss from the blood stream into the urine (resulting in kidney damage), lymph, and RES cells; and an elevation of blood pressure and reduction in heart rate (even by very low doses).

Nephrotoxicity, hemodynamic perturbations, coagulation abnormalities, and gastrointestinal and other disorders were documented in the over a dozen human clinical studies that were undertaken with cell-free Hb between 1916 and 1975 (see, for example, refs 186, 420, 421, 481, 483, 484, and 159). These untoward effects were first and in part rightfully blamed on the presence of residual stroma.[485−487] Careful separation of stroma is also essential because the RBC antigens that determine the blood group in the ABO and other systems are located on the cell membrane; once stroma is eliminated, Hb can be administered regardless of blood type. However, clinical trials of carefully purified Hb (from Warner-Lambert, Morris Plains, NJ) in low doses still provoked significant deterioration of renal function as well as hemodynamic perturbations, including hypertension and transient bradycardia and abdominal pain,[178] leading to the admittance that such solutions were not benign and indicating that stroma may not have been the only reason for Hb toxicity.

Improved purification alleviated some but not all of the side effects of Hb. Some residual nephrotoxicity was still seen.[187,422,488−492] Vasoactivity was eventually recognized as an intrinsic property of the Hb molecule.[188,301,305,493] Inflammatory responses,[494−498] coagulation disorders,[460,499−501] neurotoxicity,[265,502−505] and damage to the retina (an outgrowth of the central nervous system tissue sensitive to hypoxic and ischemic conditions)[506] have been observed. Release of heme from cell-free Hb can promote lipid peroxidation and induce endothelium toxicity.[257]

Unmodified cell-free Hb solutions were actually ineffective at delivering O_2 due to the left shift of the O_2 dissociation isotherm that follows extraction from the RBC and loss of 2,3-DPG. It was not until hematocrits reached 10% or less that O_2 began to be unloaded to a significant extent from Hb solutions at physiological O_2 tensions.[350,351,507,508] Oxygen delivery was further compromised by vasoconstrictive effects and decreased cardiac output.[304,376]

B. Challenges of the Hemoglobin Approach

After the 1978 trials,[178] the last documented human clinical investigation that was undertaken with unmodified Hb, it had become clear that native cell-free Hb, even when carefully purified, was unsafe.[159,187−190,234,422] A better understanding of the observed effects was mandatory. The native Hb molecule needed to be modified and/or encapsulated in order to restore functionality and reduce toxicity. It was necessary to prevent tetramer dissociation, reduce O_2 affinity, adjust oncotic pressure, increase intravascular persistence, control oxidation, limit vasoconstriction, and suppress or strongly reduce various other forms of toxicity or interference with physiologic processes. Further issues that needed consideration included raw material procurement, the difficulty of conducting site-specific chemistry on the Hb molecule, the cost and complexity of large-scale manufacturing of a sterile, pharmaceutical-grade biologic product, interferences with clinical analytical methods, and the presence of pharmacological activity other than O_2 delivery. The solutions that were designed in order to resolve these issues are discussed in subsequent sections (see also recent collections of articles[191,194−197,509,510]). Comparison of products prepared by different investigators is generally difficult. Different animal protocols may be one reason for divergent assessment of otherwise similar products.[511]

1. Avoiding Dissociation of the Tetramer–Renal Toxicity

As mentioned earlier, when cell-free Hb is released in the circulation and diluted in the plasma, the equilibrium between the $\alpha_2\beta_2$ tetramer and the $\alpha\beta$ dimers is shifted toward the latter, which are rapidly cleared by renal filtration, resulting in short intravascular half-life, tubule obstruction, and renal failure.[320,422,492] Furthermore, the dissociation of heme from $\alpha\beta$ dimers is much faster than from the tetramers.[512]

Successful strategies for preserving tetrameric Hb included chemical or genetically engineered cross-linking, cross-linking and polymerization, conjugation to a polymer, and encapsulation. These actions tend to shift the burden of metabolism from the kidney to the liver and RES.

2. Reducing Oxygen Affinity To Restore Oxygen-Delivery Capability

In the absence of 2,3-DPG, **2.1**, the O_2 dissociation curve of human Hb is shifted to the left (Figure 4), hindering the release of O_2 to tissues. The slightly higher pH of plasma as compared to that inside the RBC further contributes to increasing the affinity of Hb for O_2 because of the Bohr effect. As noted in section III.C, the amount of O_2 released by Hb to tissues at the usual pO_2 of 40 Torr decreases from about 25% (RBC-enclosed human Hb) to about 5% (cell-free Hb) of the amount of O_2 transported (Figure 1). The role of the Hb solution may then be essentially reduced to that of a plasma expander. The need for preserving an O_2 affinity, cooperativity, and Bohr effect similar to those of Hb in the RBC is usually considered important for Hb-derived products but is being periodically debated.[380,513] Emphasis has recently shifted to molecular dimension of the O_2 carrier and viscosity of its solution or suspension and their effects on endothelial function and O_2 delivery regulation at the microcirculatory level.[380,514−516]

Several types of chemical modifications were effective in moving the Hb affinity curve back to the right (section IV.D). Use of bovine Hb provided another way of circumventing this difficulty, since

offloading of O_2 by bovine Hb is primarily sensitive to chlorine rather than 2,3-DPG.[332] Genetic engineering offered further effective means of lowering Hb's affinity for O_2 (section IV.E).

3. Preserving Adequate Colloidal Osmotic Pressure

Maintaining proper colloidal osmotic pressure is vital since COP regulates the exchange of fluids between the intra- and extravascular spaces. The normal COP of blood is 27 Torr; a higher COP tends to draw fluids into the vascular space and upset fluid balance. To remain isooncotic with blood plasma, the concentration of a free tetrameric Hb in solution cannot exceed 6−8 g/dL, while normal Hb concentration in blood is around 14 g/dL. A 14 g/dL Hb solution would have a physiologically unacceptable COP of larger than 60 Torr. Only "anemic"[366] isooncotic solutions can therefore be prepared from tetrameric Hb. What the optimal COP of a blood substitute should be is, however, still being debated and may depend on patient condition.

Since COP depends critically on the number of colloidal particles present in the solution, one approach to reducing it was to "polymerize" Hb, the actual goal being to link together about 2−4 tetramers into one molecule. In many cases as, for example, in glutaraldehyde polymerization of human Hb, the procedure results, however, in further increase of the already too high O_2 affinity of cell-free Hb. Since pyridoxalation had been shown to correct such excessive O_2 affinity, combinations of pyridoxalation and polymerization have been explored.[190,517−526] Some cross-linkers, such as o-raffinose, allowed one to simultaneously polymerize Hb and reduce its affinity for O_2.[491,527,528] "Conjugation" of Hb to a polymer can reduce COP when several Hb molecules are linked to a same polymer molecule but not when several polymer chains are bound to the same Hb molecule. Conjugation to a hydrophilic polymer can actually increase COP due to extensive hydration.[529] Polymerization or conjugation to a polymer, however, tend to increase viscosity, which may impose another limit to the concentration of Hb in the solution. Finally, the COP issue can also be resolved by Hb reencapsulation.

4. Preventing Vasoconstrictive Activity—Nitric Oxide Scavenging

As mentioned earlier, an unanticipated rapid increase in blood pressure, indicative of blood vessel constriction, was consistently observed upon infusion of cell-free Hb products. There is compelling evidence that this effect is related, at least in part, to scavenging of the endothelial cell-produced nitric oxide by Hb products. Intervention of an autoregulatory process has also been demonstrated (section IV.H). The clinical implications of the pressor effect of Hb products are still being debated. How much vasoconstriction is acceptable and what beneficial use can be derived from this property is likely to depend on indication and patient condition.

Preventing Hb from interfering with blood flow regulation mechanisms and with NO's multiple functions is certainly one of the most serious challenges encountered in the development of O_2 carriers. The principal approaches being investigated for preventing such interference consist of hindering extravasation by enlarging or encapsulating Hb, in stimulating compensatory NO production, or in "teaching" Hb to express a preference for O_2 over NO, which represents a radical change with respect to Hb's normal behavior, a tour de force that has actually been partly achieved by genetic engineering.

5. Controlling Hemoglobin Redox Chemistry, Free Radical Formation, Free Iron Accumulation, and Related Toxicities

In order for Hb to remain functional, autoxidation to metHb needs to stay under control. Nonphysiological amounts of cell-free Hb can disrupt the delicate and critical balance that is normally achieved between various oxidant and antioxidant systems in living tissues, increase the production of harmful oxygen species, and create a catalytically active environment favorable to peroxidation of lipids, including from endothelial cell membranes (section III.A). The redox potentials, redox chemistry, and pseudo-enzymatic activity (including oxygenase and peroxidase functions) of Hb can be profoundly altered by chemical modifications.[189,233,242,271,530,530a] The catabolism of large amounts of Hb products results inevitably in nonphysiologically large amounts of free hemin, bilirubin, and ultimately free iron, known to catalyze various oxidative and peroxidative reactions.[189] Iron overload may become a complication in the case of chronic dosing.[85] The implications of such perturbations are not yet well understood. Normal oxidative processes may be critically altered in the case of pathological conditions, in which case patients may have diminished ability to control such processes. Reactive iron and free-radical species indeed underlie the pathophysiology of numerous clinical conditions.[189,234,236] Reactive oxygen metabolites are formed during ischemia and upon reperfusion of tissues following transfusion of RBCs to patients with hypovolemic shock and trauma. Free radicals and free iron released from Hb products may exacerbate reperfusion injury.[271,324,531−533]

Proper control of these complex and interrelated redox phenomena is difficult. The redox potential of Fe^{3+}/Fe^{2+} in Hb is such that O_2 will be reduced to superoxide $O_2^{\bullet-}$ before metHb will be reduced to ferrous Hb.[189] Strategies aimed at limiting the rate of autoxidation of Hb in vivo include co-administration of antioxidants, co-encapsulation of Hb with reducing enzymes in liposomes, conjugation of Hb with such enzymes, chemical modification of Hb that provides antioxidant properties, and genetic engineering of mutant proteins with lower autoxidation rates.

6. Achieving Site-Specific Chemistry on Hemoglobin

Achieving selective chemistry on Hb is not a trivial enterprise. Hemoglobin is a labile, multifunctional protein. Any reaction is potentially complicated due to the number of functional groups available for reactions. In addition, Hb itself is microheterogeneous. Most of the reagents utilized have limited or no site-specificity, thus yielding complex mixtures

that often also contain substantial amounts of unreacted Hb. Additionally, the outcome of most reactions can differ substantially depending on experimental conditions such as reagent/protein ratio, concentration, duration, pH, whether the reaction is conducted in the presence or absence of O_2, and of certain cofactors. The sensitivity of Hb to oxidation also needs to be taken into consideration. Hemoglobin has multiple physiological functions that can be significantly altered by chemical modification.[237] Finally, "minor modifications of Hb's molecular structure may greatly alter toxicities".[534]

The raw material procurement issues have been discussed in section III.F. Extensive fractionation and purification are generally needed after modification has been achieved in order to remove unreacted material, low- and/or high-MW derivatives, and byproducts. Despite this, Hb products always consist of highly heterogeneous mixtures.

7. Minimizing the Effects on the Immune System

There is abundant evidence that the antigenicity of human cell-free Hb is low. At this point, it is still unclear, however, whether repeated exposure to animal Hb is safe. There are indications that modification of the protein can enhance antigenic reactions (section III.E).

The question of whether chemically or genetically *modified* Hbs or products derived from animal Hb may under some circumstances, induce an immune response remains a legitimate one, and this question should be addressed for each new modification of the natural protein.

Clearance of large amounts of cell-free Hb involves the RES, also raising the question of possible blockade of this system.

8. Ensuring Sterility, Absence of Endotoxin

Absence of bacteria, viruses, and prions needs to be ensured. Native Hb does not withstand standard heat sterilization temperatures. Heat-denaturated Hb can increase human blood mononuclear cell procoagulant activity.[535] Terminal sterilization of Hb products is usually achieved by filtration. Some cross-linked materials, being more stable, have been pasteurized.[536−538] Complete removal of endotoxins from recombinant Hb produced in pathogenic bacteria is also a challenge.[539]

9. Avoiding the Promotion of Bacterial Growth and Enhancement of Endotoxin Potency

There exist numerous yet conflicting reports indicating that certain Hb products may be immunosuppressive and may enhance the virulence of bacteria (section III.E).

Concerns have therefore been expressed about the potential danger that the promotion of sepsis and other infections by Hb products could represent for trauma and surgical patients who are particularly susceptible to such infections.[189,446−448,458,540,541] The extent and mechanism(s) of such effects need, therefore, further clarification.

10. Sorting out Oxygen Carrier and Drug Activities

In addition to being O_2 carriers, most Hb products also have other important pharmacological actions, including substantial vasoactive properties and volume expanding capacity. Hemoglobin also contributes to CO_2 and NO transport, has a number of pseudo-enzymatic activities,[189,530,542,543] including as an antioxidant,[229,233,242,271] and can function as a receptor for drugs and peptides.[544] Different structural modifications are likely to affect these diverse functions differently.

This multifunctional role and carrier/drug ambivalence does not simplify the assessment and understanding of the O_2 delivery efficacy and side effects of Hb-derived products. Comparison of Hb solutions with Ringer solutions without oncotic activity may have little meaning. Differences in solution viscosity may explain differences in responses elicited by otherwise similar products. In some cases, the clinical benefit of products in development was actually not expected to originate from O_2 delivery as much as from pressor effect and NO scavenging ability.[294,455,545,546]

11. Interference with Clinical Laboratory Tests

The red color imparted to blood plasma by Hb preparations produces an appearance of hemolysis in the plasma and serum samples of patients having received such products. This color can affect blood chemistry analysis, cross matching, coagulation, and other tests and devices, in particular those that rely on or are controlled by optical measurements and use wavelengths that are absorbed by Hb. The extent of such interferences depends on assay, Hb product, analytical method, and instrumentation and can be a source of logistical problems.[547−551,1231]

Patient safety requires assessment of the extent to which O_2 carriers can affect routine clinical laboratory testing, the identification of analytical methods and instruments that show the least interference, and the development of new ones. The laboratory in charge of analyzing blood samples will need to know exactly which product a given patient has received. This issue supposes an ongoing effort since clinical laboratory tests and equipment are expected to evolve as analytical technology advances.

C. Doing Chemistry on Hemoglobin

The chemical modifications to which Hb has been subjected as part of O_2 carrier research are based on a limited number of basic reactions, including essentially the reductive alkylation and the acylation of primary amino groups, amidation of carboxylic functions, and S-alkylation of sulfhydryl groups. However, the number of reactive functions present on the protein, poor specificity of most reagents, and dependence of reaction product on experimental conditions complicate the issue. Chemical modification of Hb was also used for investigating the mechanisms by which the hemoprotein operates. Further modifications were directed at finding a treatment for sickle cell disease.

Reagents for Hb modification in view of therapeutic use should be nontoxic and nonimmunogenic. They must allow reactions to be carried out in aqueous media and should have minimal detergent activity to avoid protein denaturation. Other aspects demand-

ing attention include effective purification and deoxygenation of Hb prior to reaction, batch-to-batch reproducibility, and viral inactivation without protein denaturation.

This section briefly outlines the basic chemistry pertaining to Hb modification for use as O_2 carriers. Some of the reactions are discussed further in subsequent sections in relation to specific investigations, objectives, and products.

1. Hemoglobin Purification

Early Hb solutions relatively free of stromal substance were prepared using slow RBC lysis, centrifugation, filtration, and dialysis.[486,552] Purity assessment was, however, essentially based on the absence of coagulant activity; no chemical analysis for lipids or other impurities was performed. It should be noted that each percent of nonidentified material present in an O_2 carrier, because of the large doses utilized, translates into grams of this material being administered to a patient. Hemoglobin purification has since been improved using high-speed centrifugation, ultrafiltration, solvent extraction, dialysis, crystallization, gel permeation chromatography, anion and cation-exchange chromatography, affinity chromatography, and combinations of such techniques.[432,500,521,553−559] Purity was assessed using gel electrophoresis, isoelectric focusing, thin-layer chromatography, and high-pressure liquid chromatography. However, most papers provide little quantitative information on Hb purity and many of the preparations used appear to have still contained other proteins and peptides, lipids, and metHb.[178,560,561] As for all proteins, Hb is heat sensitive. It is denaturated when temperature exceeds about 55 °C.[562] Sterilization is usually achieved by filtration or pasteurization in the carbonmonoxyHb form. Denaturation was also observed when Hb solutions were agitated while exposed to an air−liquid interface.[563]

Long-term storage of Hb is made difficult due to autoxidation. MetHb formation has been slowed by reducing temperature, controlling pH (metHb forms less rapidly above pH 7.0), increasing concentration, and addition of ascorbic acid and deferoxamine. Reduced β-nicotinamide−adenine dinucleotide (NADH) and glutathione also provided good antioxidative protection.[564,565] The purified Hb solutions were often stored at −70 °C under nitrogen, sometimes in the form of carbonmonoxyHb. Lyophilized products were prepared usually in the presence of protective agents such as sugars, tris(hydroxymethyl)aminomethane (THAM), or amino acids.[270,566−569] In the absence of protectors, metHb formation during freeze-drying can reach 50%. Long-term storage of recombinant deoxyHb (rHb1.1) was achieved with sodium ascorbate at pH 7 after thorough deoxygenation of the solution.[570] Trace amounts of O_2 induced significant modifications of the protein, which was accompanied by increased P_{50} and decreased cooperativity.

Thorough purification, as by chromatography, increased Hb's susceptibility to autoxidation as it removed the enzymes that control its oxidation in the RBC.[571,572] It has therefore been recommended to use separation procedures that preserve these enzymes or to separate them only after modification has been completed.[526,573] Chemical modification or encapsulation has often been performed while the protein was in its carbonmonoxy form.

2. The Privileged Reaction Sites

Most of the Hb modification reactions are nucleophilic reactions. The strongest nucleophiles of Hb are sulfhydryl and amino groups. The rate of reaction depends on the nucleophilicity of the attacking group (lysine side-chain amino groups, for example) and on the ability of the leaving group to come off. Since protonation decreases nucleophilicity, the reaction rate is affected by pH. At a given pH, the groups with the lowest pK_a are usually the most reactive, and reactivity increases with increasing pH.

The reactivity of a given function depends also strongly on its accessibility and microenvironment, hence location in the three-dimensional structure of the protein and interactions with other functions and solvent molecules. Lysine ϵ-amino groups are the most abundant functions of Hb, and many of them are exposed on the protein's surface.

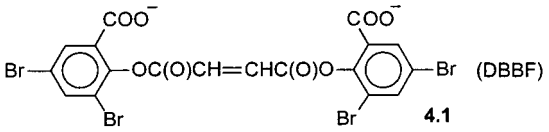
Since 2,3-DPG **2.1** plays an essential role in both O_2 affinity regulation and tetramer stability, the cavity that hosts 2,3-DPG (the so-called 2,3-DPG pocket), with its well exposed Val-1 and Lys-82 residues on both α and β chains, is a privileged target for site-directed Hb modification. Other popular target sites include the following: two Lys-99α residues; the two reactive Val-1α residues; two of the six sulfhydryl groups from cysteine residues, those located on the Cys-93β residues, which are easily accessible and readily oxidizable in oxyHb; and the reactive carboxylic acid termini (Arg-141α and His-146β) of the four globin chains. However, there are altogether 44 lysine residues with primary ϵ-amino groups on their side chains scattered across the molecule and 28 aspartic acid and 24 glutamic acid residues with β and γ side-chain carboxylic groups. In addition, some of the imidazole groups from histidines, guanidino groups from arginines, and phenolic groups from tyrosines can participate in various reactions.[574] The various minor glycosylated (or glycated) forms present in natural Hb[223,575,576] may participate in further reactions.

Some reagents are function-specific, i.e., allow derivatization of primary amines, carboxylic, sulfhydryl, guanidino, phenolic groups, or imidazole rings, with, in specific conditions, limited effect on other functions of proteins.[577−581] Other reagents have little specificity. Methyl bromide, for example, can react with cysteine, lysine, histidine, tyrosine, and aspartic acid residues simultaneously.[582] The reactivities of these different functions correlated with the pK_a and relative surface accessibility of the nucleophilic residues of Hb. Pyridoxalation, carboxymethylation, S-alkylation, and cross-linking reactions that utilize negatively charged reagents are usually categorized as site-specific, while intermolecular cross-linking (i.e., polymerization and conjugation to a polymer, as with glutaraldehyde, glycolaldehyde, and $NaIO_4$-oxidized polysaccharides occurs essentially at random

on multiple sites on the protein's surface. Specific is synonymous to preferential, not exclusive. The apparent specificity of a reagent is often simply a reflection of differences in reaction rates and can change with reagent/protein ratio or medium.

It should be born in mind that the same amino groups from the 2,3-DPG pocket, C-terminal carboxylic acid groups, and sulfhydryl groups from Cys-93β that are privileged target sites for chemical modification also play a decisive role in ensuring Hb tetramer stability, O_2 affinity regulation, CO_2 transport and pH regulation,[228,327,553,583] and NO transport.[316] For example, chemical derivatization of the terminal Val-1 residues, as by cyanate, inhibits CO_2 uptake and reduces the Bohr effect.[584] Intermolecular cross-linking, as with glutaraldehyde or o-raffinose, by restricting conformational changes, tends to suppress cooperativity. Alkylation of Cys-93β residues may interfere with NO transport, hence possibly with vascular tone regulation. Autoxidation and reactions with essential endogenous factors can be substantially accelerated.

a. Effect of Protein Conformation and Experimental Conditions on Reaction Outcome. Deoxy- and oxy- or carbonmonoxyHb have significantly different conformations, which can translate into different aptitudes of certain sites for reaction. Cys-93β thiols are available for reaction in oxyHb, not in deoxyHb. The amino group of valines and lysines can react differently according to accessibility, microenvironment, specific interactions, and presence of certain organic and inorganic ions. The amino groups of Lys-40α, Lys-127α, and Val-1α are involved in constraining salt bridges in deoxyHb but not in oxyHb.[323,327] This can result in different reactivity patterns and sometimes in drastically different product properties, such as, in particular, a right versus a left shift of the O_2 binding isotherm. For example, bis(3,5-dibromosalicyl) fumarate **4.1** cross-links Hb between α or between β chains depending on whether the reaction is conducted with deoxy or oxyHb, respectively, leading to opposite effects on O_2 affinity.



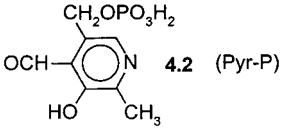
4.1

Bis(*N*-maleimidomethyl)ether cross-links horse oxyHb between the Cys-93β and His-97β residues, which immobilizes the protein's conformation and completely suppresses cooperativity and Bohr effect; with deoxyHb only one maleimide ring reacts, producing no cross-links and retaining some of the cooperativity and Bohr effect.[585] The reactivity of the NH_2-terminal amino groups of deoxyHb is modified by the presence of 2,3-DPG, Cl^- or CO_2.[586] The carboxylic end groups of Arg-141α and His-146β (which are implicated in the Bohr effect) are freely moving in oxyHb but restricted by salt bridges in deoxyHb.[583]

Multifunctional reagents are commonly used for cross-linking and conjugation of the protein to a polymer.[578,579,581,587] The distance between functions and the flexibility and bulkiness of the cross-linker can help select the residues that are to be cross-linked. Intramolecular cross-linking is usually achieved with reagents that demonstrate some site-specificity, which can be obtained by fitting the reagent with 2,3-DPG site-seeking negative charges. Intramolecular cross-linking can also be favored over intermolecular cross-linking by dilution techniques. However, a small proportion of the reagent will inevitably react through only one of its functional groups, while attachment of multiple cross-linkers on the same Hb molecule can also occur. Neutral reagents with two or more aldehyde functions tend to react at random and can affect any number of sites on the protein, no matter how the reaction is conducted.

b. Seeking the 2,3-Diphosphoglycerate Pocket—Site-Specific versus Random Reaction. 2,3-DPG forms salt bridges with the amino group of Val-1 and the imidazoles of His-2 and His-143 of both β chains and with the ϵ amino group of Lys-82 of one β chain.[588] The proton-rich 2,3-DPG pocket is strongly positively charged, a feature that has been extensively exploited for inducing site specificity by introducing negatively charged groups into the reagent. Carboxylate, phosphate, sulfate, and sulfonate groups have been used for this purpose. Two situations can be distinguished, depending on whether the negative charge is present in the moiety that is being grafted onto the protein or in a leaving group. In the former case, the charge on the protein is modified.

As mentioned, the 2,3-DPG pocket hosts four easily accessible reactive primary amino groups, those of the Val-1 and Lys-82 residues of both α and β chains, the ϵ-NH_2 of Lys-82β lying further outside the pocket than the α-NH_2 of Val-1β. These amino groups are readily available for reaction once the reagent is positioned at the 2,3-DPG site. For example, the reaction of deoxyHb with glucose 6-phosphate (in the absence of other organic phosphates) was 20 times faster than with glucose and occurred for 95% at the NH_2-termini of the β chains, indicating indeed that this reagent had behaved as an affinity label occupying the 2,3-DPG site.[589] Reagents carrying negative charges reacted preferentially with α amino groups of the β chains in the reductive alkylation of oxyHb, while the corresponding uncharged aldehydes reacted with both α and β chains.[590,591] Prominent examples of reagents that target the 2,3-DPG site include pyridoxal phosphate **4.2**, which has been used as an O_2 affinity modifier, and a wide range of negatively charged di- and multifunctional reagents that were utilized for cross-linking Hb and for conjugation with polymers (section IV.D).



4.2

3. Characterization of Modified Hemoglobin

A vast array of techniques is being used for analyzing, characterizing, and assessing modified or mutant Hbs.[223,557,592,593] Typically, the α and β chains can be separated by ion-exchange chromatography

by taking advantage of differences in surface charge and isoelectric points. The isolated chains can then be digested with enzymes that cleave the subunits into smaller peptides. The most popular of these enzymes, trypsin, cleaves the polypeptide specifically on the carboxyl side of lysine and arginine residues. The resulting peptides can be identified by a combination of electrophoresis and chromatography. They can also be separated by ion-exchange chromatography. Automated sequencing of individual isolated peptides allows determination of the protein's primary structure and identification of the residues that have been modified.

Detailed knowledge of the protein's three-dimensional structure has been acquired by X-ray crystallography on deoxyHb,[226] metHb, carbonmonoxyHb[594] and eventually oxyHb,[595] and carbaminoHb[586] as well as on an increasing number of natural or genetically engineered mutant Hbs and chemically modified Hbs. X-ray diffraction analysis requires that a single crystal be grown (sometimes from a complex mixture of molecules) that is representative of the species under investigation. Combined use of trypsin digestion and peptide mass mapping by matrix-assisted laser desorption/ionization mass spectrometry[596] and multidimensional NMR, in conjunction with isotopically labeled reagents, provided further tools for investigating chemical modification of Hb.[597,598]

4. Reductive Alkylation of Amino Groups (Schiff Base and Related Chemistry)

Many of the chemical modifications performed on Hb are based on reactions with aldehydes, resulting in the alkylation of some of the protein's amino groups. Carbonyl−amine reactions involving proteins and the formation of imines occur in a variety of enzymatic and nonenzymatic biological processes.[599] Reductive alkylation has been extensively used for the purpose of investigating protein structure and function.[600] The primary reaction involves a nucleophilic attack of an unprotonated amino nitrogen on the carbonyl group, followed by rapid proton transfer (eq 4.1).

$$\mathrm{Hb\}{-}NH_2 + RCH{=}O \rightleftharpoons Hb\}{-}N^{+}H_2CHRO^{-} \quad (4.1)$$

Dehydration of the hydroxymethyl intermediate **4.3** leads to the imine (or Schiff base) **4.4** (eq 4.2).

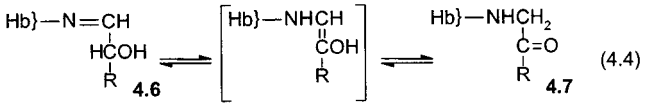
$$\rightleftharpoons \underset{\textbf{4.3}}{Hb\}{-}NHCHROH} \overset{-H_2O}{\underset{H^+}{\rightleftharpoons}} Hb\}{-}N^{+}H{=}CHR \overset{-H^+}{\rightleftharpoons} \underset{\textbf{4.4}}{Hb\}{-}N{=}CHR} \quad (4.2)$$

Such imines are susceptible to protonation and hydrolysis to regenerate the amine and carbonyl functions. Therefore, the imines are usually reduced into amines **4.5** with stable N−C bonds (eq 4.3).

$$Hb\}{-}N{=}CHR \xrightarrow{[H]} \underset{\textbf{4.5}}{Hb\}{-}NHCH_2R} \quad (4.3)$$

Both the initial nucleophilic attack and the subsequent dehydration reaction, which is acid-catalyzed, are pH-dependent and usually fastest at pH ca. 4−5.[600] Schiff base formation with Hb is expected to be fastest with the more basic primary α- and ϵ-amino groups. Aromatic aldehydes and ketones tend to react more slowly due to reduced conjugation in the first step of the reaction, but the resulting imines tend to be more stable than aliphatic analogues.

In the case of carbonyl functions having a neighboring hydroxyl group, as in glycolaldehyde, glyceraldehyde, glucose, and other reducing sugars, a rearrangement of the imine **4.6** into a 1-amino-1-deoxyketose **4.7** (the Amadori rearrangement) can take place that stabilizes the bond with respect to hydrolysis (eq 4.4).[590,600−603] As the Amadori rearrangement generates a new carbonyl function, the reaction can proceed further, leading to intra- and/or intertetrameric cross-linking.



(4.4)

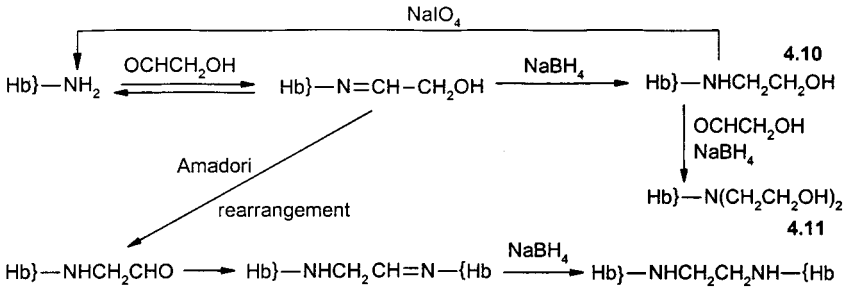
Schiff base chemistry has been used extensively to derivatize Hb and modify its O_2 affinity and other properties. A large variety of mono-, di-, or polyaldehydes has been utilized for this purpose. Negative charges were often introduced in such reagents in order to target the 2,3-DPG site. Multiple dialdehyde pairs were created on diverse mono-, oligo-, and polysaccharides through oxidation by $NaIO_4$ for the purpose of protein coupling. Reagents utilized for Hb polymerization include ring-opened, oxidized adenosine (o-adenosine), ATP (o-ATP) and raffinose (o-raffinose), and oxidized dextran, inulin, and modified starches (section IV.D).

The reductant, generally $NaBH_4$ or the milder $NaCNBH_3$, serves multiple functions: it terminates the reaction, reduces the imide in amine bonds (thus stabilizing the product), reduces any remaining free carbonyls (such as on aborted cross-links), and reduces any metHb that had formed during the reaction.

a. Formaldehyde, Acetaldehyde. Reductive methylation of Hb was performed using formaldehyde and [^{3}H]$NaBH_4$ for radiolabeling purposes.[604] The reaction took place primarily at the α-amino groups of the N-terminal valine residues and at ϵ-amino groups of lysine residues. Because of low steric hindrance it can, in this particular case, readily proceed to the *N,N*-dimethylamino derivatives. Addition of formaldehyde to sulfhydryl groups of cysteines and imidazole groups of histidines is usually rapid but reversible.[600]

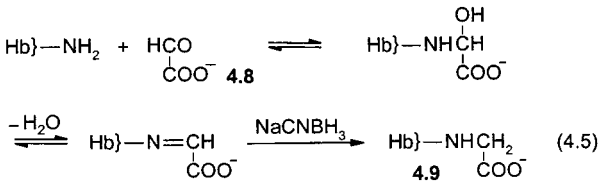
The reaction of ^{14}C-labeled acetaldehyde with HbA has been investigated under nonreductive conditions as part of a study of minor Hb derivatives that are present in above normal amounts in people abusing alcohol (acetaldehyde is a metabolite of ethanol).[605] Labeled adducts of valine, lysine, tyrosine, glucosylvaline, and glucosyllysine were identified after borohydride reduction and acid hydrolysis, indicating that acetaldehyde had reacted with amino groups of

Scheme 1



lysines and valines and with the phenolic ring of tyrosine residues. The secondary amines of glycosylated valines or lysines were also likely reaction sites. Additionally, acetaldehyde adducts were formed that, contrary to Schiff bases, were relatively stable to dialysis and were not susceptible to reduction by $NaBH_4$.[606] Up to four aldehyde residues could form stable bonds with the protein. The primary sites of attachment were the terminal valine residues. Evidence was provided for the formation of stable cyclic imidazolidinone derivatives as well, possibly from an initially formed Schiff base.

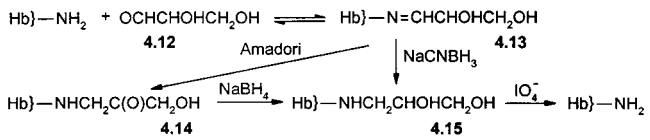
b. Glyoxylate−Carboxymethylation. Reductive *N*-carboxymethylation of amino groups of Hb using sodium glyoxylate **4.8** and cyanoborohydride yielded **4.9** (eq 4.5).[607−610] The objectives were to modify the



protein's surface charge by covalently attaching a small anion, reduce its O_2 affinity, and provide a stable analogue of carbaminoHb (Hb−NHCOOH, the CO_2 adduct of Hb) for further investigations.

The extent to which the reaction proceeded and the product distribution depended strongly on reaction conditions. Monosubstitution was favored by limiting the amount of reagent.[607,608] Carboxymethylation then occurred primarily on the N-terminal valines. Four fractions were separated by anion-exchange chromatography: unreacted Hb (9%), Hb with about two CH_2COO^- groups per Hb (26%), Hb with an average 3.5 substituents (i.e., carboxymethylated on essentially all four Val-1 residues, 28%), and a species with 5−6 mol of glyoxylate per Hb that was also carboxymethylated on some lysine residues.[607] Hybrid Hbs, specifically modified on the α or the *β* chains, were prepared by separating carboxymethylated α and *β* chains and recombining them with non-carboxymethylated chains. X-ray diffraction studies confirmed the modification of the N-terminal amino groups and the structural similarity of carboxymethyl deoxyHb with carbaminoHb.[586] The covalently bound carboxyl moieties replaced the inorganic anions that normally interact with the free terminal amino groups of deoxyHb. With higher concentrations of reagent, further lysine residues became carboxymethylated.[608] In excess of 90% of the lysine groups could eventually be modified. Any intermediate level

Scheme 2



of modification could be achieved, allowing adjustment of increased negative charge.

c. Glycolaldehyde−Hydroxyethylation. 2-Hydroxyaldehydes and ketones allow introducing in proteins 2-hydroxyalkyl moieties that could later be removed by treatment with $NaIO_4$ (Scheme 1).[611] With glycolaldehyde **4.10**, both mono- and dihydroxyethylation can occur, the latter providing the tertiary amine **4.11** that is resistant to attack by $NaIO_4$.

Reductive hydroxyethylation of HbA with glycolaldehyde was shown, using [^{3}H]$NaCNBH_3$ and tryptic peptide mapping, to take place selectively at all four α-NH_2 termini.[612] In the absence of reductant, the initially formed Schiff bases underwent an Amadori rearrangement, allowing formation of covalent cross-links (section IV.D).

d. Glyceraldehyde−Hydroxypropylation. Glycosylation (or glycation) of Hb has been achieved with a number of reducing sugars.[576] It is the free aldehyde forms of such sugars that undergo coupling to proteins. Glyceraldehyde **4.12**, an aldotriose, is more reactive than the common pentoses and hexoses because it lacks the cyclic hemiacetal form, which is prevalent in the latter sugars. The Shiff base adducts **4.13** initially formed in the reaction of **4.12** with HbS (the Hb variant responsible for sickle cell disease) underwent Amadori rearrangement into ketoamine adducts **4.14** (a reaction analogous to the nonenzymatic glucosylation of proteins), resulting in 2-oxo-3-hydroxypropylation (Scheme 2).[613]

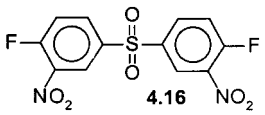
The reaction sites and final product distribution differed substantially depending on the presence or absence of a reducing agent.[590,614] This difference of behavior reflects the aptitude of the microenvironment around the glycation site (rather than the amino acid sequence) to facilitate the rearrangement of **4.13** into **4.14**.[615] The ratio of nonreductive to reductive modification was 13 between Val-1*β* and Val-1α and 600 between Lys-16*β* and Val-1α.[614] Reduction with $NaBH_4$ converted the 2-oxo-3-hydroxypropyl moieties to more stable 2,3-dihydroxypropyl groups, yielding **4.15**. Periodate oxidation of the latter regenerated the lysine residues.[610,614]

e. Glucose. The reaction of Hb with glucose has been extensively investigated, in particular because on the order of 5−7% of Hb in normal human RBCs

is covalently linked to glucose (which is evidence for Schiff base formation under physiological conditions). This percentage can be 2 times larger in patients with diabetes mellitus.[616] Glucose and metabolites of glucose, including glucose-6-phosphate, reacted with amino groups of Hb to form aldimine linkages, which subsequently rearranged into ketoamines.[576,589,617] A number of valine and lysine residues were affected, and there were significant differences between the sites of glycosylation in vivo and in vitro.[618] Glucose-6-phosphate readily reacted with the amino terminal Val-1β residues, while glucose did not.[589]

As noted, the rate of condensation of monosaccharides with amino groups depends strongly on the extent to which the sugar exists in the open (carbonyl) structure rather than in the ring (hemiacetal or hemiketal) structure. The fact that glucose was the least reactive of a series of aldohexoses was suggested to be one reason for which glucose emerged during the evolutionary process as the primary metabolic fluid, as the high stability of its ring structure limits potentially deleterious nonenzymatic glycosylation of proteins.[619]

f. Arylation using Halide Compounds. Aryl halides are usually considered as amino-group-specific. Fluorine is the most reactive leaving group. Aryl halides also react with other nucleophiles, including thiol, phenol, and imidazole, but the products of these reactions are unstable at alkaline pH. Arylation of Hb with ^{14}C-labeled 1-fluoro-2,4-dinitrobenzene took place primarily at Val-1 residues and led to increased O_2 affinity; cooperativity and Bohr effect were lost.[620] Reaction of carbonmonoxyHb with 4,4′-difluoro-3,3′-dinitrodiphenyl sulfone **4.16** resulted primarily in cross-linking between Val-1α residues; cross-links at other nonidentified positions were also seen as well as some intermolecular cross-linking.[621] Again, O_2 affinity was high and cooperativity was lost.

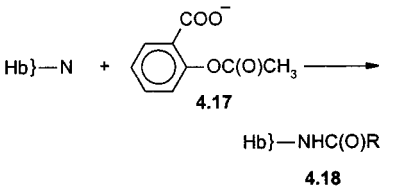


4.16

5. Acylation Reactions

Acylation involves nucleophilic attack at the carbonyl carbon of the reagent. Acylation of a primary amine of a protein to form an amide bond using carboxylic acid halides or anhydrides is difficult to control. Acetic anhydride, when used to determine the reactivity of amino groups of Hb as a function of their p*K* and environment, was found to react in each $\alpha\beta$ subunit with some 15 lysine residues on both chains, in addition to the terminal valines.[622] Milder agents were investigated, including activated carboxylic esters, mixed carboxylic/phosphoric anhydrides, isocyanates, isothiocyanates, and imidoesters. Anionic acylating agents display regiospecificity, again because they tend to position themselves at the 2,3-DPG site. Hydrolysis of the reagent is often an important side reaction.

a. Salicylic Acid Esters. Acylation of Hb with acetylsalicylic acid **4.17** (eq 4.6), yielding **4.18**, has initially been investigated as a potential cure for sickle cell disease.[623] Evidence was found for in vivo acetylation of Hb in patients on long-term, high-dose aspirin therapy.[624] The reaction was initially deter-

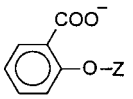
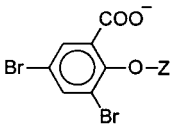


(4.6)

mined, using ^{14}C-labeled aspirin, to involve a number of sites on both α and β chains, primarily Lys-59β, Lys-144β, and Lys-90α.[624,625] Recent reanalysis (using ^{13}C-labeled aspirin, multidimensional NMR, blocking of the 2,3-DPG site, carbamylation of the Val-1 amino groups, spin-labeling at the Cys-93β site, and use of an Hb with a Lys-144$\beta\rightarrow$Arg mutation) supported the conclusion that many lysine residues were acetylated, the most rapidly and highly acetylated being Lys-82β rather than Lys-144β.[597] Partial acylation of Hb with glutarylsalicylamide caused a substantial decrease in O_2 affinity.[626]

Bis(salicyl) succinate **4.19** (Chart 1) was observed to acylate Hb more effectively than the monosalicy-

Chart 1

Z		
—C(O)CH$_3$	**4.17**	**4.20**
—C(O)(CH$_2$)$_n$C(O)—	**4.19** (n = 2)	**4.21a** (n = 2) **4.21b** (n = 8)
—C(O)CH=CHC(O)—		**4.1**
—C(O)CH=CHC(O)O$^-$		**4.22**
—C(O)(CH$_2$)$_3$C(O)O$^-$		**4.17b**

late compound, which was assigned to the presence of two negative charges rather than only one.[627] Subsequently, 2-acetyl-3,5-dibromosalicylic acid **4.20** ("dibromoaspirin") was found to be much more reactive than **4.17**, as the 3,5-dibromosalicylate leaving group facilitates the transfer of the acetyl moiety to Hb.[628,629] Reaction of bis(3,5-dibromosalicyl) succinate **4.21a** and fumarate **4.1** (commonly known as "DBBF") with intracellular Hb (i.e., directly with RBCs) was further enhanced by easier penetration of the erythrocyte membrane due to the lipophilic bromine atoms. Cross-linking of oxyHb occurred between the Lys-82β and led to increased O_2 affinity.[629,630] This was the main objective of the work: preventing RBCs from sickling by favoring the oxy form of HbS (it is the deoxy form that "polymerizes" into fibers that cause RBC sickling). Computer modeling of the reaction of **4.1** with oxyHb suggested that the carboxylate leaving groups not only help position the reagent, but also help deprotonate the ϵ-amino lysine group, thus producing a better nucleophile.[631] The additional flexibility of bis(3,5-dibromosalicyl)succinate and -glutarate, as compared to bis(3,5-dibromo-

salicyl)fumarate, resulted in reactions at more sites, yielding more heterogeneous products.[632] DBBF was utilized in the commercial development of an O_2 carrier (then renamed DCLHb, for diaspirin cross-linked Hb) that reached Phase III clinical trials (section IV.I).

Numerous other salicylic acid esters have been investigated for treating sickle cell disease.[595,630,633−636] Bis(salicyl)diesters of diacids were used profusely for intramolecular cross-linking of the tetramer. Mono-(3,5-dibromosalicyl)fumarate **4.22** produced a pseudo-cross-linked derivative specifically acylated at the Lys-82β residues[637] (section IV.D).

b. Mixed Carboxylic/Phosphoric Anhydrides. The electrophilic mixed anhydride methyl acetyl phosphate **4.23** was expected to react primarily with Hb's N-terminal amino and ϵ-amino groups (eq 4.7). Its negatively charged phosphate leaving group was intended for targeting the 2,3-DPG binding site.[598,610,638−640]

$$\text{Hb}\}\text{—NH}_2 + \text{CH}_3\text{C(O)OP(O)(OH)OCH}_3 \quad \mathbf{4.23}$$
$$\longrightarrow \text{Hb}\}\text{—NHC(O)CH}_3 + \text{(HO)}_2\text{P(O)OCH}_3 \qquad (4.7)$$

Treatment of intact human RBCs with ^{14}C-labeled **4.23** led to mixtures of Hb products derivatized at two, four, or six sites.[610,638,641] When only two acetyl groups were present, they were distributed among the Val-1, Lys-82, and Lys-144 residues of the β chains, within or near the 2,3-DPG-binding site. With bovine Hb, acetylation (which competes with Cl^- binding) occurred at Met-1β and Lys-81β for oxyHb and Val-1α and Lys-81β for deoxyHb.[641] Acetylation of bovine Hb with low reagent/protein ratios was used to identify the sites involved in Cl^- binding.[610,642] Peptide mapping indicated that, in the conditions used, acetylation had occurred at random throughout the protein. Fourteen of the 24 amino groups present in the $\alpha\beta$ dimer were acylated, although a given Hb tetramer had only 2−6 of its amino groups affected.

A recent ^{1}H−^{13}C NMR study of the reaction of Hb with [1-^{13}C]methyl acetyl phosphate revealed the formation of at least 10 adducts, confirming that the reaction was not as highly specific as initially thought.[598] The number of products formed could be reduced by blocking the 2,3-DPG site with inositol hexaphosphate (IHP). Methyl acetyl phosphate and aspirin were eventually concluded to roughly target the same sites, i.e., primarily Lys-82β in the 2,3-DPG pocket, but also Lys-59β and Lys-90α, which are outside the pocket.

Difunctional bis(methyl phosphate) dicarboxylates were investigated as intramolecular cross-linkers. For example, bis(methyl phosphate) fumarate **4.24** (Chart 2) reacted with Hb in the absence of 2,3-DPG to produce material cross-linked between Val-1 and Lys-82 within and between β chains and between Lys-99 residues of α chains; some unreacted Hb and traces of higher MW species were also found.[639] In the presence of 2,3-DPG, only α,α-cross-links appear to have been formed. The rigid cross-linkers **4.25** were also utilized. A disulfide cross-link was introduced into human deoxyHb using bis(*N*,*N*′-carbobenzyloxy)cystinyl bis(methyl phosphate) **4.26**.[643] The two major product constituents, one cross-linked and the other not, were modified at the Lys-82β residues; two minor products had modified α subunits.

c. Succinimidyl Esters. Succinimidyl esters (Chart 3) were used to graft poly(ethylene glycol) (PEG) strands on Hb. Monomethoxypoly(ethylene glycol) (mPEG) esters **4.27** (also known as PEG-succinimidyl oxyacetates) allowed grafting of PEG strands onto Hb or pyridoxalated Hb, forming amide bonds.[644,645] The *N*-hydroxysuccinimide diester of α-carboxymethyl, ω-carboxymethoxypoly(oxyethylenes) (also known as PEG-bis(succinimidyl oxyacetates) **4.28** were used when some degree of polymerization was sought.[525,646] Conjugation of pyridoxalated Hb was initially achieved with bis(succinimidyl succinate) esters of PEGs **4.30**.[647] The ester linkage between the PEG strand and the succinic residue had, however, limited stability under physiological conditions.[648]

The negatively charged monofunctional sulfosuccinimidyl acetate **4.33** and difunctional bis(sulfosuccinimidyl) suberate **4.34a** and sebacate **4.34b** were reported to selectively acylate and cross-link in the case of the difunctional esters (except in the case of

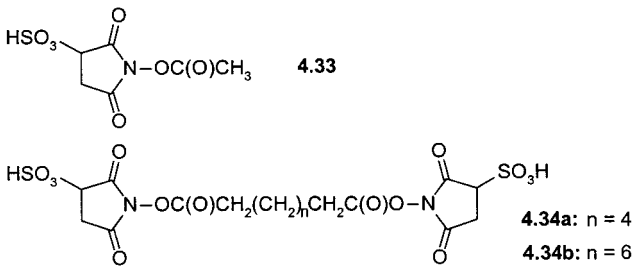
Chart 2

Cross-Linker	X = $OP(O)_2^-OCH_3$
X—(O)C—CH=CH—C(O)—X	**4.24**
X—(O)C—(1,4-C$_6$H$_4$)—C(O)—X	**4.25a**
X—(O)C—(1,2-C$_6$H$_4$)—C(O)—X	**4.25b**
X—(O)C—(1,3-C$_6$H$_4$)—CH=CH—(1,3-C$_6$H$_4$)—C(O)—X	**4.25c**
X—(O)CCHCH$_2$—S—S—CH$_2$CHC(O)—X; $C_6H_5CH_2OC(O)NH$; $NHC(O)OCH_2C_6H_5$	**4.26**

Chart 3

Activated PEG	Z: —C(O)CH$_2$—	—C(O)(CH$_2$)$_2$C(O)—	—C(O)—
NO—Z—(OCH$_2$CH$_2$)$_n$OCH$_3$	**4.27**	**4.29**	**4.31**
NO—Z—(OCH$_2$CH$_2$)$_n$O—Z—ON	**4.28**	**4.30**	**4.32**

the shorter tartaric diester) the β chains of Hb, the α chains being hardly affected.[591]

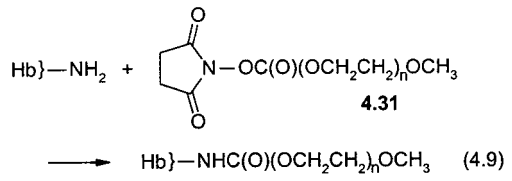


d. Cyanates, Isocyanates, and Isothiocyanates—Urea and Thiourea Bond Formation. Carbamylation by potassium or sodium cyanate to yield **4.35** (eq 4.8) has been used for establishing structure/function relations in Hb, including determination of the residues involved in the Bohr effect, the role of the terminal NH_2 groups in CO_2 binding, the mechanism of the CO_2-mediated reduction of O_2 affinity, and for increasing the O_2 affinity of HbS.[584,610,649−654]

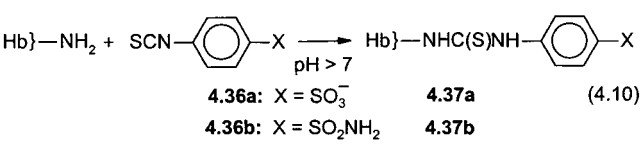
$$\text{Hb}\}-NH_2 + HN{=}C{=}O \rightarrow \text{Hb}\}-NHC(O)NH_2 \quad (4.8)$$
4.35

When performed on deoxyHb at a pH ca. 6, the reaction took place essentially at the Val-1 termini of both chains; the lysine residues were then minimally affected. Carbamylation of Val-1α was shown by X-ray diffraction and solution studies to block the two inorganic anion binding sites that are associated with this residue and eliminated the chloride-induced alkaline Bohr effect.[655]

Reaction of mPEG succinimidyl carbonates **4.31** and PEG bis(succinimidyl carbonates) **4.32** (Chart 3) with lysine residues allowed formation of rather stable urethane linkages (eq 4.9)[648,656] (section IV.D).

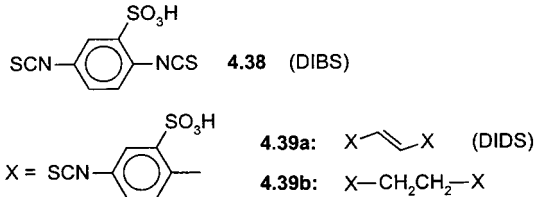


The reaction of aryl isothiocyanates with amino groups of proteins to form thiourea derivatives (the Edman reaction) has often been used for sequencing proteins. Provided the pH was maintained slightly above 7 (protonated amines do not react), the reaction of both oxy- and deoxyHb with 4-isothiocyanatobenzenesulfonic acid **4.36a** took place at the Val-1 residues of all four chain termini, yielding **4.37a** (eq 4.10).[657,658] Reaction at the α chain termini was favored by adding IHP or sodium tripolyphosphate. Alkyl isothiocyanates (but usually not aryl derivatives) also reacted with the Cys-93β sulfhydryl groups.



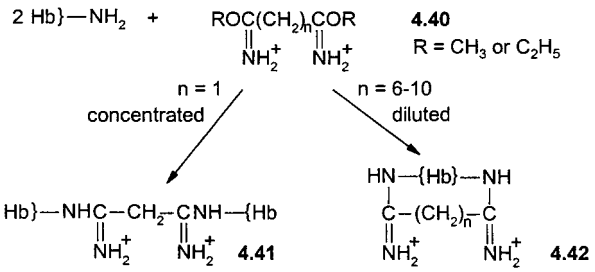
The negatively charged isothiocyanate **4.36a** provided Hb derivatives **4.37a** with markedly reduced O_2 affinity, while the neutral 4-isothiocyanatobenzenesulfonamide **4.36b** led to somewhat increased O_2 affinity.[659] The influence of these modifications on Bohr effect and cooperativity were discussed.[660]

Various diisothiocyanato compounds have been investigated as intramolecular cross-linkers, including 2,5-diisothiocyanatobenzene sulfonate **4.38** (DIBS) and 4,4′-diisothiocyanatostilbene-2,2′-disulfonate cross-linker **4.39a** (DIDS) (section IV.D).



e. Alkyl Imidates—Amidination. Alkyl imidates (imidoesters) show a high degree of selectivity for primary amino groups and produce amidines.[577,661] The bis(imidates) of malonic, adipic, suberic, and sebacic acids **4.40** (Scheme 3; n = 1, 4, 6, and 8,

Scheme 3



respectively) reacted primarily with lysine residues of Hb yielding diverse mixtures of monomers and oligomers, depending on conditions.

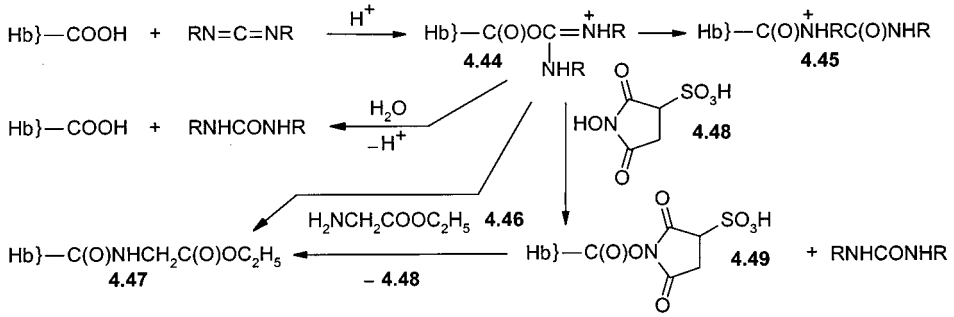
With diethyl malonimidate **4.40** (n = 1, R = C_2H_5), polymerized Hb **4.41** was obtained that had 30 out of the 44 lysine residues modified. Use of longer diacids, such as dimethylsuberimidate **4.40** (n = 6, R = CH_3), and dilute (1%) Hb solutions favored intramolecular cross-linking as in **4.42**.[661] Treatment of sickle RBCs by dimethyladipimidate **4.40** (n = 4, R = CH_3) prevented sickling.[662] Modification and cross-linking of HbS with this reagent led to increased O_2 affinity and reduced sickling.[663]

6. Amidation of Carboxylic Groups

Amidation was used to evaluate the functional role of Hb's carboxyl groups, identify the most basic ones, monitor conformational transitions, assess the effect of diverse mutations on such transitions, graft allosteric effectors on Hb, conjugate Hb with dextran or PEG derivatives, and achieve direct ("zero-link") intermolecular cross-linking between tetramers (section IV.D).

Water-soluble carbodiimides such as 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide (EDCI) **4.43** are effective reagents for protein amidation. They react with the protonated form of carboxylic groups, i.e.,

Scheme 4



at low pH, to provide the activated *O*-acylisourea intermediate **4.44** (Scheme 4).[664]

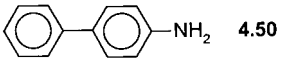
$CH_3CH_2N{=}C{=}N(CH_2)_3N(CH_3)_2$ (EDCI) **4.43**

Adduct **4.44** can hydrolyze to regenerate the carboxylic group, or rearrange into a stable *N*-acylurea adduct **4.45**, or react with a nucleophile. Nucleophilic attack by glycine ethyl ester **4.46** afforded the amidated Hb derivative **4.47**. The carboxylic function of the Glu-43β residue (located at an $\alpha\beta$ interface and implicated in the Bohr effect) was identified as the most basic carboxylic group of HbS.[665−667] Some amidation also occurred at Glu-22β and Asp-47β, very little on the heme carboxylic groups. The products were highly heterogeneous, presumably as a consequence of side reactions such as *N*-acylurea adduct formation. Rearrangement of **4.44** into **4.45** could be inhibited by trapping the activated carboxylic group with *N*-hydroxysulfosuccinimide **4.48**.[664] The sulfosuccinimidyl ester **4.49** that is formed is more stable than **4.45** but reacted with **4.46** to provide the Hb amide **4.47,** along with **4.48**. The reaction product consisted of unreacted Hb (60−65%), the amidated product disubstituted on the Glu-43β residues (nearly 25%), and a small amount of a product with four moles of glycine methyl ester per Hb. Preservation of cooperativity and sensitivity of the disubstituted product to 2,3-DPG suggested that the structure of Hb underwent little change on amidation of Glu-43β. Measurements of the rate of amidation indicated that the apparent pK_a of this residue increased when the protein changed from the oxy to the deoxy state, confirming the contribution of Glu-43β to the alkaline Bohr effect. Amidation of Glu-43β with glycine ethyl ester did not influence the reductive hydroxyethylation of HbS with glycolaldehyde, thus allowing the preparation of HbS with both modifications, resulting in additive effects on deoxyHbS solubility and decreased propensity to polymerize.[666]

Amidation of Hb has also been achieved with methylamine, glucosamine,[667] and galactosamine.[664]

7. S-Alkylation of Hemoglobin

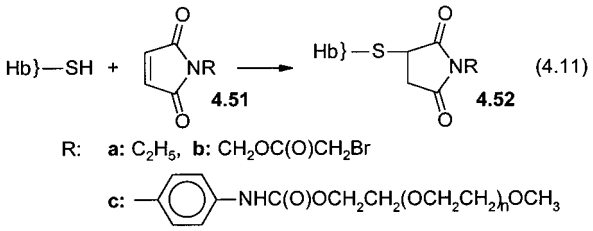
Since there are only two of them, the reactive sulfhydryl functions of the Cys-93β residues are obvious targets for controlled Hb chemistry. The SH groups are accessible and reactive in the oxyHb form only.[583,668,669] Haloacetate and maleimide reagents react fastest with thiols and are considered thiol-specific. The thiol group is a soft nucleophile, which can be titrated with iodoacetamide and various disulfides, including cystine, cystamine and dimercaptoethanol,[670] and 4,4′-dithiodipyridine. 4-Aminobiphenyl **4.50** (a carcinogen present in cigarette smoke) was bound to Hb at the Cys-93β sites (as determined by X-ray crystallography on the metHb form) when oxyHb was treated with *N*-hydroxy-4-aminobiphenyl.[671] S-Alkylation of Hb was also achieved using mercurials for analytical purposes.[672]



4.50

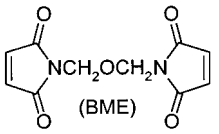
A wide range of thiol reagents, most of them disulfides, were investigated with regard to rate of reaction with Hb in solution and in intact RBCs, modification of functional properties, rate of autoxidation, and potential for antisickling and showed widely different behavior.[673] These differences appear to relate to differences in access to the Cys-93β residues, which is sterically restricted and shielded by negative charges from neighboring Asp-94β, Glu-90β, and C-terminal carboxylic residues.

In the case of maleimides **4.51**, the sulfur attacks the double bond in a Michael addition reaction (eq 4.11). There is no formal leaving group; a shift in



electrons stabilizes the product **4.52**. *N*-Ethylmaleimide **4.51a** was used to investigate the role of the SH groups of Hb and the dependence of their reactivity on degree of oxygenation,[668] to elucidate the origin of the Bohr effect,[323] to investigate their use as an antisickling agent,[673] to separate Hbs cross-linked at different sites,[674] and in studies of Hb interaction with NO.[305] N-Substituted maleimides reacted faster with the Cys-93β residues than with any other residue, the second most reactive sites being the Val-1α residues, followed in certain conditions by imidazole and phenol groups.[581,675,676] Horse Hb was S-alkylated with maleimide- and iodoacetamide-derivatized spin labels for the purpose of ESR[677] and NMR[597] studies.

Bis(*N*-maleimidomethyl)ether (BME) **4.53** was used for internal cross-linking in order to clarify the effects of conformational restraint on O_2 equilibrium and cooperativity of human Hb,[676,678] in Bunn's classical study of the renal handling of Hb,[320] and in Perutz's studies of conformational changes of Hb upon oxygenation and the cooperative effect.[327] BME reacted



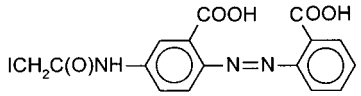
4.53

with horse oxyHb to cross-link the SH group of Cys-93β with the imidazole ring of His-97β.[585,679] With deoxyHb, alkylation of one of the maleimide rings occurred at Cys-93β while the other ring did not react and no covalent bridge was formed.

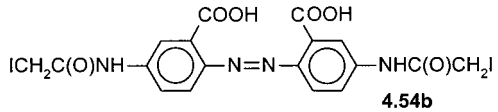
N-α(Bromoacetoxymethyl)maleimide **4.51b** alkylated the Cys-93β residues of human Hb; this was followed by rapid hydrolysis of the ester bond of the reagent.[680] With horse oxyHb, the same reagent first reacted with the Cys-93β residue of one β chain and then with Val-1β on the other β chain.[585,680] Spontaneous esterolysis of this bridge yielded a stable derivative doubly modified by S-alkylation of Cys-93β and by N-carboxymethylation of Val-1β. Only S-alkylation was observed with deoxyHb, as the rate of esterolysis of the reagent was faster than the rate of alkylation of Val-1β.

Maleimide-PEG reagents such as the maleimidophenyl compounds **4.51c** allowed grafting of PEG chains onto cysteine residues of proteins.[669,681,682] Bis(maleimide) derivatives with long PEG spacers (MW = 2000) resulted in intratetrameric cross-linking outside the central cavity of Hb rather than intermolecular binding. Shorter or longer spacers led to some oligomer formation. Bis(maleimide)-terminated polyamides were used to polymerize a DBBF-cross-linked Hb.[683]

α-Haloacetate groups (e.g., $ICH_2C(O)OR$) are regarded as SH-specific, as reactions with amino, imidazole, and phenol groups are much slower. Reaction of the monofunctional azo-dye reagent 4-iodoacetamido-2,3′-dicarboxyazobenzene **4.54a** with Hb resulted in the alkylation of both Cys-93β.[684] The difunctional analogue 4,4′-diiodoacetamido-2,2′-dicarboxyazobenzene **4.54b** achieved cross-linking primarily between Cys-93 and Lys-82 within the β chains. Some cross-linking between a Cys-93β and

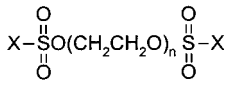


4.54a

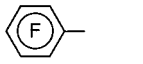


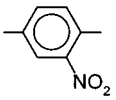
4.54b

the His-45α of the neighboring α chain was also observed. Oxygen affinity was increased, and cooperativity and Bohr effect were lost. The PEG-bis(benzene sulfonates) **4.55** were investigated as sulfhydryl-specific cross-linkers for Hb.[685] Substitution of the ring with electron-withdrawing groups was needed to activate the leaving group for nucleophilic displacement by the sulfhydryl groups. Primary amino groups were, however, also involved.

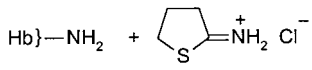


4.55

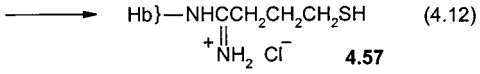
a: X = 

b: X = 

To increase the number of reactive SH functions on the protein, Hb was reacted with the cyclic imidothioester **4.56** (Traut's reagent),[686] which converted a number of NH_2 groups into SH groups according to eq 4.12, thus allowing maleimide-type chemistry to be pursued on **4.57**.[682] Cross-linking and polymerization with bis(maleimidohexane) yielded a 260 000 MW product with a very significantly reduced pressor effect.



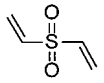
4.56



(4.12)

4.57

Divinyl sulfone **4.58** is primarily a sulfhydryl alkylating reagent that undergoes nucleophilic addition at its double bonds. Reaction of **4.58** with bovine Hb was highly dependent on experimental conditions.[687] Low Hb concentrations and reagent/Hb


4.58

ratios produced mixtures of non-cross-linked modified Hbs in anaerobic conditions and of intramolecularly cross-linked Hbs in aerobic conditions; larger concentrations and ratios produced polymerized species. The materials obtained in the absence of O_2 had low O_2 affinity. 1,2:3,4-Diepoxybutane **4.59** undergoes ring-opening reactions with nucleophiles, primarily thiols, but also amines and hydroxyl groups. It was used for Hb polymerization.[688]


4.59 (BUDE)

Disulfide cross-linking of cysteine residues between Hb molecules, yielding Hb microspheres, has been obtained in aqueous solution using high-intensity ultrasound.[689] The oxidant involved in the process was identified as superoxide, $O_2^{\bullet-}$, generated from water and O_2.

D. Chemical Modification of Hemoglobin for Effective Oxygen Transport

A large diversity of reagents, modifications, and products has been explored during the course of Hb-based O_2 carrier research (Figure 7). The patent literature is even more prolific. In many cases, more than one modification, for example, pyridoxalation

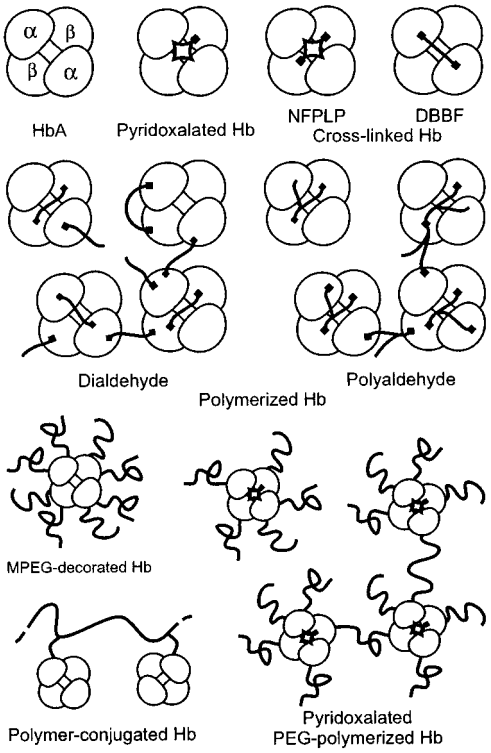
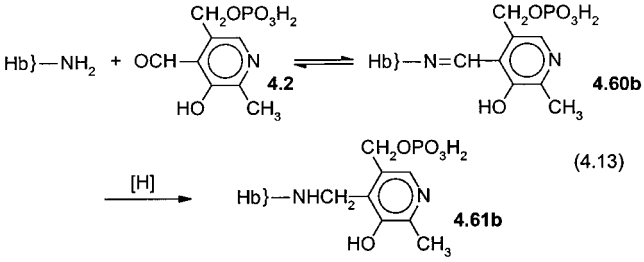


Figure 7. Schematic representation of some chemical modifications of Hb involved in O_2 carrier development.

and polymerization or intramolecular cross-linking and pegylation, was needed in order to meet the diverse challenges outlined in section IV.B. Successive modifications (Table 1) inevitably lead to increased product heterogeneity. In some cases, the various reagents compete for the same sites. Each modification can obviously have more than one consequence on the protein's properties and in vivo behavior. Different products can have substantially different characteristics (Table 2).

1. Oxygen Affinity Modification—Pyridoxalation

A first inappropriate characteristic of cell-free Hb to have been successfully corrected was its too high affinity for O_2 consequent, primarily, to the loss of 2,3-DPG. Attempts at restoring a functional P_{50} by simply adding 2,3-DPG to an HbA solution proved ineffective, as the allosteric effector was rapidly cleared from the circulation.[349,690] A persistent right shift of the O_2 dissociation curve (Figure 8) was achieved by allowing deoxyHb to react with pyridoxal phosphate **4.2**, a natural coenzyme related to vitamin B6 and a functional analogue of 2,3-DPG (eq 4.13). Permanent labeling with **4.2** was achieved by reducing the imines **4.60b** into amines **4.61b** with $NaBH_4$ or $NaCNBH_3$.[519,691−698]



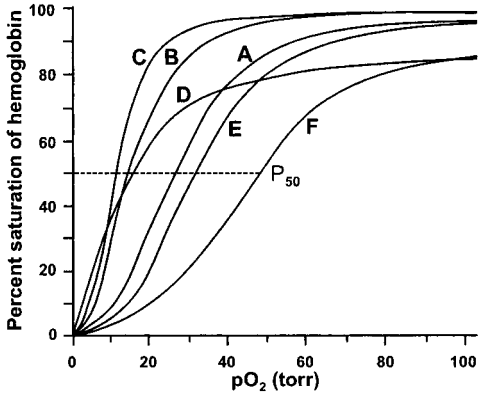


Figure 8. Oxygen binding isotherms for diverse human Hbs: (A) Hb in fresh whole blood, (B) unmodified cell-free Hb, (C) glutaraldehyde-polymerized Hb, (D) polymerized and pyridoxalated Hb, (E) α,α-DBBF-cross-linked Hb, and (F) NFPLP-cross-linked Hb. (Adapted with permission from ref 1944. Copyright 1992 Plenum Press.)

Imine adducts of proteins with pyridoxal phosphate have important biological functions. The affinity of this reagent for anion binding sites of proteins has led to its use as an affinity label for such sites.[699] With Hb, the two negative charges on the phosphate group helped position the reagent at the 2,3-DPG site during alkylation. The product's composition, the reaction sites, and P_{50} values depended on pyridoxal reagent and reductant utilized, conformation of the protein at the time of the reaction, and reagent/Hb ratio.[519,691,693,694,698,700−703] Pyridoxalation was initially reported to occur essentially at the N-terminal amino groups of the β chains when the reaction was performed on deoxyHb and at the N-termini of the α chains when performed on oxyHb. Binding to Val-1β and the formation of salt bridges with Lys-82β, His-143β, and His-2β residues led to an arrangement that mimics closely that found for 2,3-DPG.[704] A secondary reaction site was later identified as Lys-82β.[693] Subsequent studies indicated that P_{50} values and cooperativity were the same whether the reaction was conducted on oxyHb or deoxyHb and that the reaction could in both cases be limited to the β chains.[698]

Pyridoxal phosphate was sometimes used in the form of its Schiff base **4.62b** with THAM. Coupling of **4.62b** with Hb is then a transimination reaction (Scheme 5);[700,701] however, subsequent studies indicated no advantage in prior formation of the THAM imine.[698] When the reaction was performed on deoxyHb, a dipyridoxalated Hb with strongly decreased O_2 affinity and preserved cooperativity was separated from the reaction mixture, which also contained polypyridoxalated products.[701]

A typical pilot-scale preparation (20-L-size batch) of pyridoxalated Hb for in vivo experimentation involved deoxygenation of the stroma-free Hb solution using a blood oxygenator (but with N_2 in place of O_2), treatment with a 4-fold molar excess of **4.2**, reduction with $NaBH_4$, and the separation of excess reactants by ultrafiltration using an artificial kidney. The product was 68−75% pyridoxalated (up to 85% after passage through a Sephadex column).[519] This operation raised P_{50} from 12−14 Torr to 20−24 Torr;

Table 1. Examples of Hemoglobin Products with Two Successive Modifications of the Protein[a]

initial modification	subsequent modification(s)	ref
pyridoxalation	glutaraldehyde polymerization	521−524,770,792,797,798,1271
pyridoxalation	glycolaldehyde polymerization	776,803
pyridoxalation	conjugation to dextran	825
pyridoxalation	conjugation to inulin	837
pyridoxalation	copolymerization with albumin	849
pyridoxalation	mPEG decoration	644,645,836
pyridoxalation	PEG-decoration and polymerization	525,526,647,1284
pyridoxalation	S-pegylation	526
pyridoxalation	liposome encapsulation	1125
pyridoxalation	liposome encapsulation and liposome surface pegylation	836
carboxymethylation	glycolaldehyde polymerization	609,802
DBBF cross-linking	glutaraldehyde polymerization	789,836
DBBF cross-linking	glycolaldehyde polymerization	776,803
DBBF cross-linking	polyamide polymerization	683
DBBF cross-linking	conjugation to HES	836
DBBF cross-linking	PEG-polymerization	685,869,870
DBBF cross-linking	nitroxylation	198,1101,1101a
DBBF cross-linking	liposome encapsulation	1089
NFPLP cross-linking	glutaraldehyde polymerization	799
NFPLP cross-linking	glycolaldehyde polymerization	776,803
o-ATP cross-linking	o-adenine polymerization, glutathione binding	806
sebacoyl or adipoyl cross-linking	intermolecular zero-link amidation	811
sebacoyl cross-linking	succinic anhydride acylation	748
amidation	hydroxyethylation	666
amidation	conjugation to HES	835
thiolation (Traut's Reagent)	S-pegylation	682
heme−albumin conjugation	bis(maleimide) dimerization	853
glutaric acid cross-linking	alkylation with tetronic polymers	872
dextran conjugation	alkylation with oxidized inositol tetraphosphate	708,828
dextran conjugation	nitroxylation	1069
genetically engineered α,α-cross-linking	DBBF cross-linking	1942
genetically engineered α,α-cross-linking	glutaraldehyde polymerization	793
liposome encapsulation	PEG-PE decoration	1003
LEHb from diacyl chloride cross-linked Hb	glutaraldehyde polymerization	1009

[a] The reductive step following Schiff base formation, Amadori rearrangements and subsequent reactions, or reagent modifications are not listed as separate steps.

Table 2. Characteristics of a Series of Different Hb-Based O_2 Carriers Compared with RBC

parameter[a]	α,α-Hb	POE-PLP−Hb	Poly-α,α-Hb	HES-α,α-Hb	POE-LEHb	RBC
diameter (nm)	7 ± 2	22 ± 2	47 ± 17	68 ± 24	224 ± 76	8000
MW (number average)	72	186	510	431		
MW (weight average)	66	89	2154	2782		
monomeric Hb (%)	100	4.4	2.7	3.3	not detected	
Hb (g/dL)	5 10	5	5	5 10	5 10	12−17
MetHb (%)	2.2	4.5	8.0	4.5	2.5	<0.5
HES, POE, or lipid (g/dL)	0 0	4.5	0	3.4 6.8	2.8 5.8	
P_{50} (Torr)	32	14	20	22	18−32[b]	28
oxygen transport efficacy (%)[c]	32	17	22	27	20	28
COP (Torr)	15.8 36.4	70.2	2.5	9.5 32	20[d] 20[d]	ca. 25
viscosity (cP at 332 s^{-1})	1.0 1.3	6.1	1.5	2.2 7.8	3[d] 4[d]	3−4

[a] All formulations adjusted to pH 7.4 (37 °C) and 300 mOsm. [b] Depending on PLP/Hb ratio. [c] Oxygen transporting efficiency: the difference in O_2 saturation (%) between pO_2 of 40 Torr and 100 Torr. [d] After suspension in 5% human serum albumin. α,α-Hb = α,α-DBBF-*cross*-linked; PLP = pyridoxal phosphate; Poly = glutaraldehyde polymerized. From ref 836 with permission.

metHb levels were between 3% and 8%. Little was reported, however, on the product's actual composition.

Analysis of a similarly prepared pyridoxalated Hb by various chromatographic, electrophoretic, and isoelectric focusing techniques revealed some 21−25 Hb bands attesting to considerable heterogeneity.[518,705] Subsequent studies indicated that pyridoxalation was indeed much less selective than initially thought.[523,600,694−696,702,706] A critical reanalysis of the reaction of **4.2** with carefully purified HbA further confirmed that even this site-directed reagent led to extensive product heterogeneity (Figure 9).[698,703] Figure 9 also shows that the reductant can have a dramatic influence on product constitution. Reduction with $NaBH_4$ was deemed unnecessarily harsh, leading to pyridoxalation of both α and β chains and high metHb formation, while the unreduced and $NaCNBH_3$-reduced materials were modified only on the β chains and contained less metHb.

Reaction with the α globin chain amino terminals, resulting in increased O_2 affinity, was observed when the reagent had only one negative charge, as in pyridoxal 5-methylphosphonate **4.62c**, pyridoxal 5-phosphate monomethyl ester **4.62d**, or pyridoxal 5-sulfate **4.62e**.[700,701] The less polar, neutral 5-deoxy-

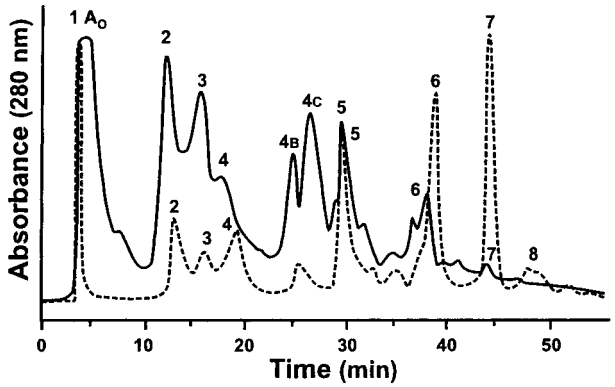
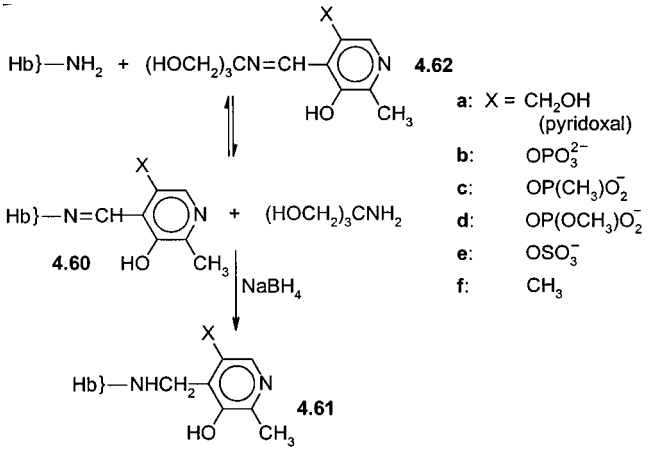


Figure 9. Chromatogram (anion-exchange column) of pyridoxalated HbA after reduction with $NaBH_4$ (—) or $NaCNBH_3$ (- - -). (Reprinted with permission from ref 703. Copyright 1990 Elsevier.)

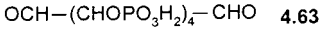
Scheme 5



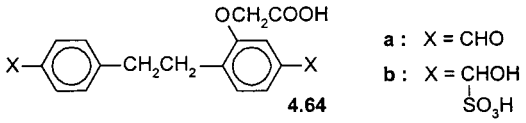
pyridoxal **4.62f** readily penetrated sickled RBCs and reacted with HbS's α chain termini, leading to increased O_2 affinity and reduced sickling.[707]

Significant reduction in O_2 affinity was also achieved by reductive carboxymethylation of the four N-terminal amino groups of deoxyHb using sodium glyoxylate and $NaCNBH_3$, yielding **4.9**.[607,610] An increasing excess of reagent affected an increasing number of lysine residues and led eventually to a left shift of the O_2 binding isotherm.[608]

Further effective means of reducing O_2 affinity included treatment of Hb or Hb conjugates with $NaIO_4$-oxidized inositol tetraphosphate **4.63**,[708,709] cross-linking with various di- and multifunctional agents,[527,710] or coupling with negatively charged benzene pentacarboxylate[711] or polycarboxylate dextran reagents.[712−715] Reaction with negatively charged



aromatic aldehydes such as **4.64a**, as part of a study of the 2,3-DPG site in various Hbs, generally also led to a right shift of the O_2 dissociation curve.[716,717] Other aromatic aldehydes were designed for producing high-affinity Hbs as a treatment for sickle cell disease.[718] However, despite careful molecular modeling, low-affinity Hbs were sometimes produced.[719] The structural features responsible for the direction in shift of the allosteric equilibrium could be determined, and the critical role of Lys-99α in obtaining low-affinity products was emphasized.[720]



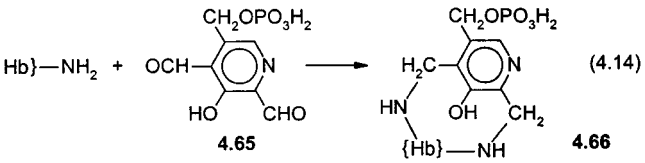
Treatment with monofunctional affinity modifiers did, however, not prevent dissociation of the Hb tetramer and rapid excretion nor did it remove the ca. 7 g/dL Hb concentration limit imposed by the need for physiological COP or suppress vasoconstrictive effects. Further modification of the Hb molecule was needed.

2. Intramolecular Cross-Linking

Intramolecular cross-linking of Hb was extensively practiced for the purpose of suppressing renal filtration by preventing tetramer dissociation, ideally reducing O_2 affinity by the same modification. Some site specificity was obtained by fitting the reagent with negative charges and by adjusting the distance between reactive sites and the flexibility and bulkiness of the cross-linker. However, multifunctional reagents have the potential for multiple intra- and intermolecular cross-linking combinations.

Negatively charged (i.e., site-directed) cross-linking agents fall into two categories, depending on whether the negative charges are part of the leaving groups or are born by the cross-link itself. In the former case, no extrinsic new negative charge is added to Hb as a consequence of cross-linking; in the latter case, the negative charge on the protein is increased. The most popular cross-linkers became bis(3,5-dibromosalicyl) fumarate **4.1** (DBBF, or "di-aspirin") and nor-2-formylpyridoxal 5-phosphate **4.65** (NFPLP). Intramolecular cross-linking was also achieved by genetic engineering (section V.E).

a. Negatively Charged Cross-Linkers. Reductive alkylation of deoxyHb by NFPLP **4.65** yielded **4.66** (eq 4.14) with internal cross-links between β chains, primarily between Val-1β and Lys-82β (Figure 10).[701,704,721−723] Significant perturbations in the



tertiary structure of one β chain was observed.[704] The increase in P_{50} was much larger than with pyridoxal phosphate, reaching 40−45 Torr.[701,721] The stability of the heme−globin linkage was increased, but the rate of autoxidation increased also.[724] Vascular retention was prolonged by a factor of 3 with respect to unmodified or pyridoxalated Hb, reaching a half-life of 3−7 h.[724,725] Elimination of Hb in the urine was almost completely prevented and accumulation in the kidneys greatly diminished.[415] Appearance in the lymph was, however, rapid and similar to that for unmodified Hb, indicating that cross-linking did not prevent Hb from entering the interstitial spaces.

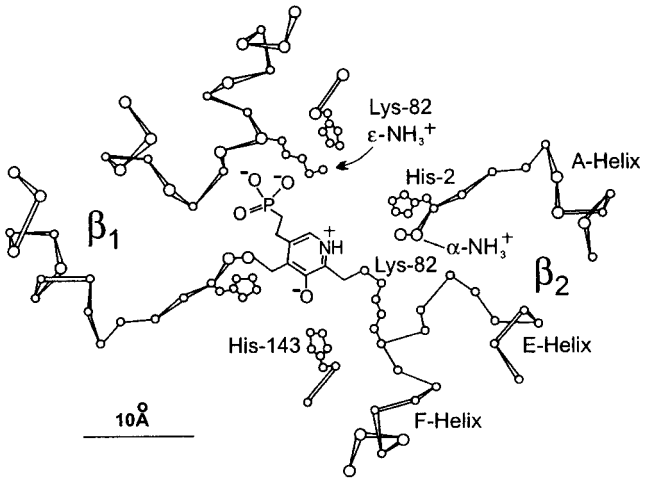
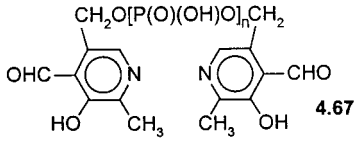


Figure 10. Sketch of the entrance of the central cavity between β chains in NFPLP-cross-linked Hb. The arrangement of NFPLP in the 2,3-DPG binding site closely mimics that found with the natural effector. (Adapted with permission from refs 701. Copyright 1981 Academic Press.)

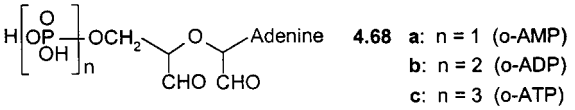
Accumulation of the ^{99m}Tc label was seen in the spleen and liver. Metabolic studies were initiated using ^{3}H-labeled NFPLP−Hb, but the metabolites were not identified.[726]

More flexible negatively charged polyphosphate chains flanked by two pyridoxal rings, such as **4.67**, provided cross-links between Val-1β of one β chain and a lysine (probably Lys-82β in human Hb and Lys-81β in bovine Hb) of the other β chain.[724,727,728]



4.67

Negatively charged dialdehydes were also derived from adenosine-5 mono-, di-, and triphosphate **4.68** and 5-phosphorylribose-1-phosphate by ring opening at the ribose 2,3-diol using $NaIO_4$.[412,729,730] Reaction of o-ATP with deoxyHb, followed by reduction with $NaBH_4$, produced complex mixtures of alkylated Hbs with substantially reduced O_2 affinity.[412]



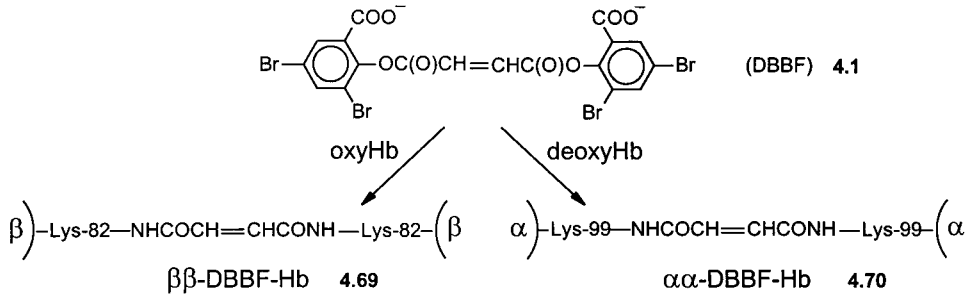
4.68 **a**: n = 1 (o-AMP)
b: n = 2 (o-ADP)
c: n = 3 (o-ATP)

Further negatively charged cross-linkers used with Hb include DIBS **4.38**,[731,732] DIDS **4.39a**,[592,733] its dihydrostilbene analogue **4.39b**,[734] and several series of carboxyl-group-bearing dialdehydes.[720] Reaction of human HbA with DIBS **4.38** yielded a predominant product cross-linked within the tetramer between the NH_2 termini of the α chains.[731] The reaction mixture was complex; P_{50} was slightly reduced, cooperativity was retained, and circulation half-life increased to around 3 h. The somewhat longer cross-linker **4.39a** (DIDS) reacted with CO-ligated Hb to produce an adduct having two DIDS molecules attached, one at each β chain termini, and high O_2 affinity. In the same conditions, deoxyHb provided a major product with one DIDS cross-linked between the β chain termini that had a greatly reduced O_2 affinity.[733]

b. Cross-Linkers with Negatively Charged Leaving Groups. The most thoroughly investigated of all neutral cross-linkers with negatively charged leaving groups was bis(3,5-dibromosalicyl) fumarate **4.1** (DBBF). When performed on oxyHb, acylation with **4.1** produced cross-links between β chains and O_2 affinity was augmented (**4.69**, Scheme 6).[629,630] The same reaction, when conducted on deoxyHb in the presence of tripolyphosphate to block competitive sites at the Lys-82β and Val-1β residues, led to **4.70**, which was cross-linked between the Lys-99α residues, the 2,3-DPG-binding site remaining accessible, and O_2 affinity was substantially reduced (Figure 8).[710,735−739] Removing chloride ions (which normally interact with Lys-99α) from the reaction mixture further facilitated acylation of this site. The reagent, being hydrolyzable, needed to be used rapidly. Recent improvements in processing allowed production of α,α-DBBF-cross-linked Hb with significantly higher purity (non-cross-linked Hb was virtually eliminated and metHb was reduced), increased yield (from 33% to 58%), and reduced cost.[738] Increased stability allowed pasteurization (76 °C for 90 min). However, production of a 20-L batch still required 30 working hours over a 5-day period. The product was sterile packaged and frozen at −80 °C. The thermal stability of metHb, carbonmonoxyHb, and cyanometHb also increased upon cross-linking with DBBF.[562] Both α,α- and β,β-cross-linked products prevented rapid renal excretion, the primary goal of the modification.[740]

The development by the Letterman Army Institute of Research in San Francisco of a consistent α,α-DBBF-cross-linked Hb product (also known as α,α-Hb), available to researchers, played a definite role in promoting basic research and understanding of O_2 delivery physiology by cell-free Hb. The O_2 carrying characteristics of this Hb product and the effect of allosteric modifiers were analyzed in detail.[341,741,742] Cooperativity between subunits was preserved, but

Scheme 6



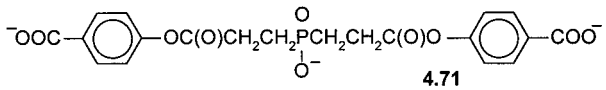
the Bohr effect and ability to bind CO_2 were reduced.[743] The rate constant for NO binding was essentially the same as that for unmodified cell-free Hb.[513] Intravascular persistence increased 2- to 3-fold.[416,737] Systemic hypertension and a decrease in heart rate were reported consistently (section IV.H). Diffusion across the endothelial walls into interstitial and lymphatic spaces was observed.[188,744] Pharmacokinetic studies in rats indicated bimodal clearance from the plasma, extravasation being an important mechanism. The ^{14}C-label was distributed throughout almost every tissue in the body, with the highest concentrations being found in the kidney and RES, and was eventually excreted in the urine (60% of the dose after 10 days) and feces (10%), the balance being not accounted for.[416] A series of studies to evaluate the clinical utility of α,α-DBBF-cross-linked Hb led the U.S. Army to abandon this product.[511]

Cross-linking of bovine deoxyHb using DBBF led to a heterogeneous product with modifications in both α and β chains, low O_2 affinity, and prolonged intravascular persistence.[745] An increase in metHb content in the circulation was noted.

Reaction of human deoxyHb with the bis(3,5-dibromosalicyl) diester of the 10-carbon-long sebacic acid, **4.21b,** resulted in cross-linking between either the Lys-82β of the two β subunits or simultaneously between both the Lys-82β of the two β subunits and between the Lys-99α of the two α subunits.[746] The cross-link between the two Lys-82β across the β cleft in the former species was determined by X-ray crystallography.[538] Non-cross-linked Hb was eliminated during pasteurization and by affinity chromatography. Oxygen affinity and cooperativity were close to those of blood. The same reagent, when reacted with oxyHbA, also produced cross-links between β chains and, surprisingly, a decrease in O_2 affinity.[747] Subsequent introduction of negative charges on a (3,5-dibromosalicyl) sebacate-cross-linked Hb by reaction with succinic anhydride failed to improve plasma half-life.[748]

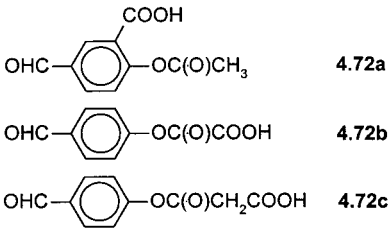
Chart 2 lists several rather rigid bis(methyl phosphate) dicarboxylate reagents[639] that were used as intramolecular cross-linkers and are reviewed in section IV. C.

c. Further Difunctional Cross-Linkers. The activated diester **4.71**, with negative charges on both leaving groups and cross-link, when allowed to react with human oxyHb, yielded a mixture of products cross-linked between β chains.[749] Bisimidoesters of

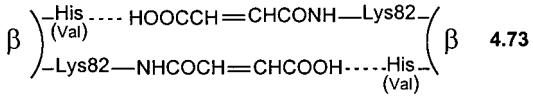


4.71

various lengths have been used to prepare Hb-based plasma expanders.[661] Bridging of Hb with **4.53** (BME) was used to reduce tetramer dissociation, prolong plasma retention, prevent glomerular filtration, determine structure/function relationships, and evaluate cross-linked Hb as a plasma expander.[320,327,585,678] Heterobifunctional cross-linkers that combine ester and aldehyde functions, including 5-formylaspirin **4.72a**, and related oxalyl **4.72b**, malonyl **4,72c**, and fumaryl monoaldehyde monoester derivatives were allowed to react with HbA and HbS.[750] The anionic reagents showed a preference for the β cleft region of Hb, and their reaction was inhibited by IHP, further confirming the role of the 2,3-DPG pocket in site selectivity.



d. Pseudo-Cross-Linked Hemoglobin. Stabilized "pseudo-cross-linked" tetramers were prepared by the reaction of mono(3,5-dibromosalicyl) fumarate **4.22** with human, bovine, or porcine oxyHb.[637,751,752] Two fumarate moieties were covalently bound by one end to the two Lys-82β residues, and electrostatic interactions were proposed to take place between the free carboxyl group of the fumarate moieties and positively charged residues of the other β chain as depicted in **4.73**. The absence of sensitivity of the P_{50}

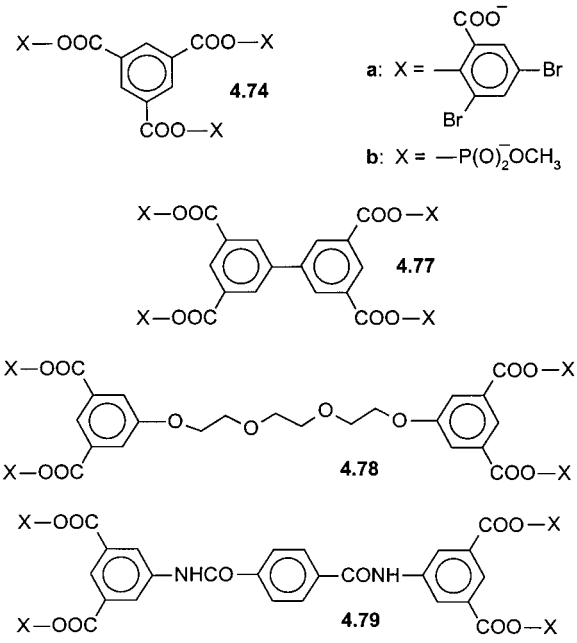


4.73

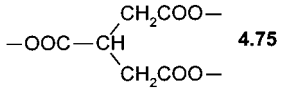
of the bovine product to Cl^- indicated that the fumaryl residue occupied the β cleft, where the free COO^- group could play the role of Cl^- in regulating O_2 affinity. The intravascular half-life of this product in rats was biphasic and increased about 5-fold (to 200 min) for the slower component.[751] Interestingly, the pseudo-cross-linked human Hb product was more resistant to oxidation by H_2O_2 than native HbA or DBBF-cross-linked Hb.[753] However, considerable amounts of Hb were rapidly excreted in the urine, indicating lesser stability in vivo than in vitro.[752]

e. Tri- and Tetrafunctional Acylating Agents—Cross-Linked Bis-Hemoglobin. Trimesoyltris(3,5-dibromosalicylate) **4.74a** (Chart 4) was designed as a site-directed trifunctional acylating agent.[754] However, reaction with deoxyHb or carbonmonoxyHb resulted primarily (~85%) in difunctional cross-linking between the Lys-82β residues of the two β chains. This specificity was attributed to the bulkiness of the reagent, which limits its chances for reaction once it has reached the 2,3-DPG site. The third salicylate ester group reacted much more slowly, principally undergoing hydrolysis, only forming a very small amount of triply linked trimesoyl Hb. Some α,α cross-linking was also detected. Cross-linking of human and bovine Hb with **4.74a** led to decreased O_2 affinity.[755] The less bulky trimesoyltris(methyl phosphate) **4.74b** reacted with deoxyHb to yield a principal product that was triply cross-linked between Val-1β, Lys-82 of one β chain, and Lys-82 of the other β chain.[756] The interchain Val-1β—Lys-82β cross-linked species was also formed in significant amounts, as well as a species bridged between Val-1β and Lys-82β within the same β chain and a number of minor derivatives. The triply cross-linked

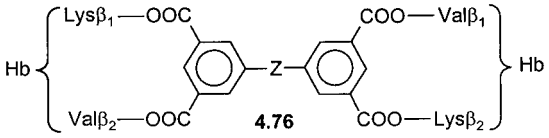
Chart 4



derivative and the Val-1β−Lys-82β species both displayed low O_2 affinities and high cooperativity but reduced Bohr effect. The functional properties of Hb cross-linked using **4.74a** and **4.74b** were substantially different.[757] The rate of O_2 dissociation was larger, the rate of CO association lower, the accessibility to the Cys-93β reduced, and the proximal His−Fe bond of the α chain disrupted when the latter reagent was used. These results were interpreted to mean that the quaternary structure of Hb had been shifted toward the R or T states by reaction with **4.74a** and **4.74b**, respectively.[757,758] Reaction of tris-(3,5-dibromosalicyl) tricarballylate **4.75** with oxyHb and deoxyHb yielded species with intersubunit cross-links.[759] Higher O_2 affinity, reduced cooperativity, and, in the case of the deoxyHb product, a higher autoxidation rate were observed. No evidence of triple cross-linking was found.



Tetrafunctional reagents with four 3,5-dibromosalicyl leaving groups were designed for the purpose of creating a specific connection within and between two Hb tetramers and producing a cross-linked bis-tetramer of type **4.76**.[760] Reagent **4.77a**, derived from



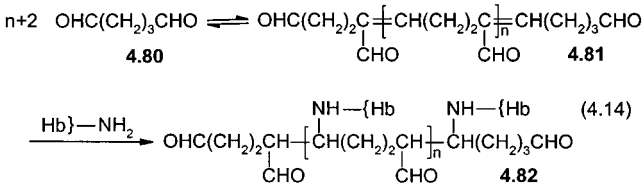
biphenyltetracarboxylic acid, reacted with Hb but only on two sites,[761] indicating that greater separation between functional sites was needed. The oligoether derivative **4.78a** reacted at all four sites but mainly within a single tetramer, suggesting that this reagent was too flexible to promote intermolecular connection.[762] Eventually, the products of the reaction of deoxyHb with **4.79a**, which cannot fold, included the desired species of type **4.76**.[760] Modifications of the Lys-82β and Val-1β residues were identified in the purified higher MW fraction. A low P_{50} value of 9 Torr was, however, measured for this fraction, and cooperativity was essentially lost.

3. Polymerization

Intermolecular cross-linking or "polymerization" (actually oligomerization) was used for the purpose of simultaneously reducing the protein's oncotic pressure and increasing its size so as to prevent rapid excretion and prolong plasma half-life. Molecules larger than the tetramer were also expected to extravasate less readily into the interstitial spaces and show lesser aptitude at NO inactivation. The patent literature covers the preparation of plasma and blood substitutes obtained by modification of various forms of Hb using virtually every possible bi- and multifunctional cross-linkers (see, for example, refs 763−765). Most difunctional agents achieve both intra- and intermolecular cross-linking simultaneously in proportions that depend on Hb ligation state, reactant ratio, dilution, other components present, reaction duration, etc. Dialdehydes such as glutaraldehyde or glycolaldehyde and reagents with multiple aldehyde functions, such as oxidized dextran, react essentially at random and can affect any number of sites. Polymerization involving Schiff base chemistry can be quenched at any moment by addition of excess lysine, glycine, glycylglycine, tris-(hydroxymethyl)methylamine, and other amines. Quenching with an amino acid can, however, modify the protein's surface charge by introducing new carboxylic residues. Limited polymerization leaves substantial amounts of unreacted tetramer; extended polymerization generates excessive viscosity and increased O_2 affinity. In both cases, fractionation is required, resulting in reduced overall yields and added cost. MetHb formation during polymerization and processing is often substantial. The polymerized Hb products reported so far were always highly heterogeneous and their chemical characterization generally poor. Polymerization alone was not always effective at restoring Hb functionality; combinations with other modifications were then explored (Table 1).

a. Glutaraldehyde. Glutaraldehyde is the reagent most frequently utilized for intermolecular cross-linking of Hb, pyridoxalated Hb, and other Hb derivatives (see, for example, refs 432, 521, 522, 569, 688, 732, 763, 766−777, 792 and 799). This highly reactive cross-linker has long been employed as a nonspecific tissue fixative.[778] While glutaraldehyde is commonly represented as a simple five-carbon dialdehyde **4.80**, its aqueous solutions actually consist of complex mixtures of components whose composition depends on experimental conditions, including pH and concentration. Free glutaraldehyde is then in equilibrium with various hydrates, tetrahydropyran derivatives, trioxane oligomers and higher polymers, α,β-unsaturated aldehydes, and hemiacetals resulting from aldol condensation.[581,779−783] It

has been presumed that it is an unsaturated polymer such as **4.81** that reacts with amino groups of proteins giving **4.82**, as speculatively illustrated in eq 4.14.[581,780,784]



A diversity of reactions can occur when glutaraldehyde reacts with amino groups of lysine and valine residues. In addition, glutaraldehyde can react with sulfhydryl groups of cysteines, imidazole rings of histidines, and phenolic rings of tyrosines.[574] The reaction of glutaraldehyde with proteins is therefore extremely complex, difficult to control, partially reversible, and still not well understood.[772,775,776,779,780,783,785−787] The β chains were extensively modified, but some reactions also occurred on α chains.[524] Reaction on oxyHb tended to produce larger size polymers than reaction on deoxyHb.[772] Electrostatic interactions between oppositely charged polymers, consequent to modification of surface charges, may generate "pseudopolymers".[775] Lysylhemoglobin products were present when the reaction was quenched with lysine.[697,775] Excess glutaraldehyde can, however, also be removed by dialysis.[521] Unless thorough reduction of the Schiff bases to amines is effected, depolymerization can occur and highly toxic[783] glutaraldehyde can be released into the solution.[788,789] Finally, glutaraldehyde-polymerized Hb products appear to be unstable on storage even after reduction.[524] Unstability has also been seen for certain products upon shaking.[522]

Chromatography, electrophoresis, gel isoelectric focusing, and other techniques have confirmed the polydispersity, heterogeneity, and dependence on experimental conditions of glutaraldehyde-polymerized Hb.[524,729,769,771,772,775,776,790,791] The reaction products comprised unreacted Hb and species that were intra- and intermolecularly cross-linked at various sites and to various degrees. MetHb formation was usually important but could be limited by addition of L-ascorbic acid to the reaction mixture.[521,791] Viscosity increased rapidly with Hb concentration, further reflecting lack of molecular uniformity.[569,774] There still exists considerable uncertainty about the reaction sites, number and identity of the groups involved in cross-linking, mechanisms of reaction, chemical constitution, and evolution of such products.

Glutaraldehyde polymerization usually resulted in a leftward shift of the O_2 saturation curve with respect to cell-free Hb, hence, a fortiori, with respect to RBC-enclosed Hb (Figure 8). The higher the degree of polymerization, the lower the P_{50}.[769,792] The presence of 2,3-DPG during glutaraldehyde treatment did not improve the O_2 binding properties.[769] Cooperativity was strongly reduced.[521,769,790] Interestingly, the changes in O_2 binding parameters and other biological characteristics,[769,772] including vasoconstrictive activity,[793] were similar for non-cross-linked (or "decorated") glutaraldehyde-modified tetramers and for glutaraldehyde-polymerized Hbs. Glutaraldehyde cross-linking stabilized Hb at high temperatures and destabilized it at physiological temperature.[772] The protein's redox potentials and autoxidation kinetics were significantly altered (section IV.H). The fate of the glutaraldehyde cross-link upon metabolic breakdown of polymerized Hb has not yet been clearly established.

One group of researchers produced a polymerized Hb that had reduced O_2 affinity (P_{50} ~25−30) by treating a diluted solution of human deoxyHb with glutaraldehyde.[794,795] The product consisted in 24% internally cross-linked Hb, 48% di- to tetra-Hb, and 28% polymers with MW > 500 000. A vascular half-life of 15 h was measured in the rat. There was no sign of renal toxicity but mean arterial pressure and pulmonary arterial pressure increased transiently in a hemorrhagic shock model in pigs. The elevations of blood pressure in the rat were similar with a monomeric, internally cross-linked fraction and a polymeric fraction (MW >124 000). Hemorrhagic disorders were seen in rats, rabbits, and monkeys that indicated endothelial cell damage. These disorders were attributed to the glutaraldehyde treatment on the basis of a comparison with nonglutaraldehyde-treated Hb solutions and on the fact that glutaraldehyde-polymerized albumin showed the same hemorrhagic disorders.[794,795]

Glutaraldehyde polymerization of human Hb solves the COP issue and significantly increased i.v. persistence but does usually not provide a functional product because of too high O_2 affinity. Since pyridoxalation and other treatments can successfully correct the P_{50} of Hb, combinations of such treatments and polymerization were explored, leading to the commercial development of a polymerized pyridoxalated human Hb (section IV.I). However, pyridoxal phosphate and glutaraldehyde compete for the same amino sites of Hb, and the combination of the two modifications adds further complexity to the product.[522,524,706,796−798] Combinations of NFPLP and glutaraldehyde treatment of Hb have also been investigated.[799] Finally, whether polymerization suffices to prevent vasoconstriction is highly controversial (section IV.H).

Since bovine Hb depends on Cl^- ions rather than 2,3-DPG for O_2 affinity modulation[332,800] and Cl^- concentration in human plasma is substantial, treatment with an affinity modifier is unnecessary. Stabilization of bovine Hb by glutaraldehyde polymerization resulted in a product that had a P_{50} of 20 together with a prolonged circulation half-life of about 10 h.[432,773] However, an immediate increase in systemic and pulmonary vascular resistance was elicited by a glutaraldehyde-polymerized bovine Hb in sheep.[801] MetHb also increased rapidly in the circulation, reaching 33% at 24 h and further limited the product's efficacy.[801] A commercial glutaraldehyde-polymerized bovine Hb is presently under development (section IV.I).

b. Glycolaldehyde Polymerization. Some authors have considered glutaraldehyde "unsuitable" because it is too reactive, it lacks specificity, and it

leads to uncontrolled intra- and intermolecular cross-linking.[776] Glycolaldehyde was tauted to be milder and more specific and to provide better control of polymerization and O_2 affinity. The initially formed Schiff base products were expected to undergo Amadori rearrangement, thereby generating the second aldehyde function that would allow cross-linking to occur (Scheme 1).[602,609,776] With deoxyHb, the reaction took place predominantly at the β chains, whereas both chains were extensively cross-linked under aerobic conditions. Polymerization was terminated with $NaBH_4$. A significant amount of non-cross-linked Hb was still present. The usual increase in O_2 affinity and decrease of cooperativity upon polymerization were observed. The reactions were slower than with glutaraldehyde and the products heterogeneous, and altogether, glycolaldehyde polymerization appears to offer little advantage over glutaraldehyde polymerization.

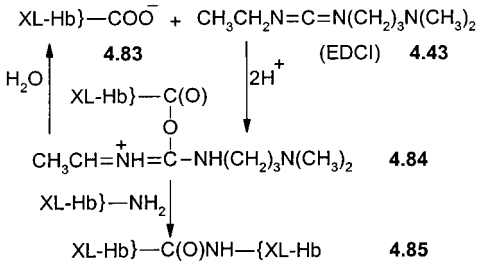
Glycolaldehyde polymerization has also been achieved on Hb that had first or subsequently been carboxymethylated,[609,802] pyridoxalated, NFPLP-cross-linked, or DBBF-cross-linked[776,803] as well as on bovine Hb.[804]

c. Further Intermolecular Cross-Linkers. Using an undisclosed "proprietary" cross-linking agent, different from glutaraldehyde, an internally cross-linked polymerized bovine Hb was developed in Texas, produced in Italy, and administered to children with sickle cell anemia in Zaire.[805] The patients' condition was reported to have improved, and no adverse reaction was reported. A Hb modification procedure was subsequently reported which comprised intramolecular cross-linking with ring-opened oxidized ATP (o-ATP) **4.68c**, intermolecular cross-linking with ring-opened oxidized adenine (o-adenosine), combination with reduced glutathione as an antioxidant, and optional enrichment with free-radical scavengers and other additives.[490,806] The product was stored at −90 °C. Little chemical characterization was provided. The increased negative surface charge due to the phosphate groups was postulated to reduce interactions with the RES and prevent extravasation and glomerular filtration. The vasodilatory activity of the adenine residues[807] was suggested to compensate for the usual vasoconstrictive effect of Hb products.[806] This Hb solution proved as effective or better than blood in resuscitating rats from severe hemorrhagic shock and did not exhibit the inflammatory responses seen with unmodified or glutaraldehyde-modified Hb.[808] The inflammatory response of human coronary artery endothelial cells to Hb was strongly diminished.[495] Neurotoxicity on human brain neuron cultures was also attenuated.[809]

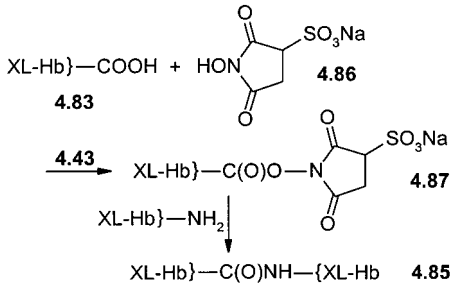
Polymerization using o-raffinose, a hexaldehyde that is obtained by oxidation of raffinose with $NaIO_4$, was essentially investigated as part of the development of *Hemolink* (section IV.I).

Polymeric Hbs with MW up to 600 000 and more were prepared as potential plasma expanders by treating Hb with short-chain diimidate esters, such as the diethyl malonimidate **4.40** (Scheme 3, $n = 1$, $R = C_2H_5$), too short to cross-link two lysines within the same tetramer, and relatively concentrated Hb solution.[661] Mixtures with high O_2 affinity and no cooperativity were obtained; circulation half-lives in rabbits were 4−12 times longer than for the native Hb.[577]

Scheme 7



Scheme 8



A range of water-soluble polyamides terminated at both ends by activated esters, aldehyde, or maleimide functions has recently been synthesized for the purpose of cross-linking proteins, producing amide or urea bonds, N-alkylation, or S- and N-alkylation, respectively.[810] Application to the polymerization of DCLHb indicated that degree of polymerization and O_2 carrying capacity could be modulated.[683]

d. Zero-Link Polymerization. "Zero-link polymerization" refers to direct intermolecular binding of Hb molecules without recourse to a cross-linker. Such polymerization was achieved using the carbodiimide EDCI **4.43** on human Hb internally cross-linked (XL-Hb, **4.83**) with sebacoyl residues and bovine Hb internally cross-linked with adipoyl residues.[811] The carbodiimide reacted with C-terminal and glutamic or aspartic side-chain carboxylic functions of **4.83**, forming *O*-acylisourea intermediates **4.84** that reacted with primary amine groups of lysines of another Hb molecule to form a peptide bond (Scheme 7). The yields in polymerized product **4.85** were, however, limited by the hydrolysis of intermediate **4.84**. Better yields were obtained by using a two-step approach in which **4.83** was first condensed with *N*-hydroxysulfosuccinimide **4.86** in the presence of EDCI to form the ester **4.87** (Scheme 8). The less water-sensitive activated ester **4.87** then reacted with amino, hydroxyl, and sulfhydryl groups of another Hb molecule, yielding **4.85**. Extensive polymerization was achieved by this means, yielding highly heterogeneous materials with MW ranging from about 1×10^5 to 2×10^6 for human Hb and up to 56×10^6 for bovine Hb. Fractionation by anion-exchange chromatography allowed determining that O_2 affinity and viscosity increased with MW, in line with observations made with other Hb polymers.[811]

e. "Hyperpolymeric" Hemoglobins. "Hyperpolymeric" Hbs with an average MW of 10^6 have been

produced by allowing a large molar excess of diverse difunctional cross-linkers, including DIBS **4.38**, DIDS **4.39a,** divinyl sulfone **4.58,** 1,2:3,4-diepoxybutane **4.59**, or glutaraldehyde **4.80**, to react with highly concentrated solutions of human or bovine[688,732] or porcine Hb.[469] Divinyl sulfone **4.58**, for example, when allowed to react directly with deoxygenated human RBCs (i.e., on a highly concentrated Hb solution), produced, after hemolysis, neutralization of excess reagent, fractionation, and concentration, highly polydisperse "hyper"polymerized Hb with high P_{50} values.[812] Viscosity and oncotic pressure increased exponentially when the concentration of Hb in solution reached around 70 g/L.

Comparison with intratetrameric cross-linked Hb products produced from diluted Hb solutions under otherwise similar conditions indicated that O_2 affinity had increased 2- to 5-fold upon polymerization and that there was considerable loss in cooperativity. Detailed studies have been published on the viscosimetry and structure in solution[732] and on MW determinations[813] for polymers obtained by reaction of glutaraldehyde or DIBS with RBCs.

This work was inspired by the existence of natural Hbs with MWs in the millions in certain lower phyla animal species, such as the common earth worm *Lumbricus terrestris*. The stable high MW (~3 500 000) Hb isolated from *L. terrestris* contains some 200 iron atoms and exhibits O_2 affinity and cooperativity similar to those of human Hb.[470,814] However, when this Hb was infused in mice, an unexplained in vivo interaction with mouse blood resulted in increased O_2 affinity.

f. Copolymerization with Enzymes. Bovine Hb has been polymerized with glutaraldehyde in the presence of small amounts of the enzymes superoxide dismutase and catalase.[815−817] The objective was to provide an O_2 carrier with antioxidant properties that could be useful in clinical situations involving ischemia and reperfusion, as it could protect both the tissues and the protein against the reactive oxygen species that are generated in vivo during reperfusion.

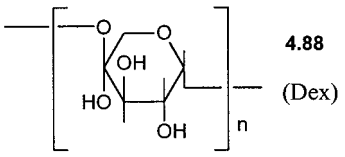
No chemical characterization of the reaction sites and polyhemoglobin-superoxide dismutase-catalase product was provided besides an HPLC analysis that showed the usual broad distribution of MWs. At the concentrations used, HPLC was not sensitive enough to detect the enzymes. However, metHb formation during polymerization was reduced (~7%) as compared to polymerization in the absence of enzymes (~20%).[818] The enzymes' circulation half-time was prolonged significantly, and the loss in their activity upon polymerization was minimal. Oxidation of Hb by superoxide and hydrogen peroxide and the formation of ferrylHb were prevented in vitro for certain enzyme ratios.[815,819] The increase in free-radical generation observed upon perfusion with a polymerized Hb solution in a rat ischemia-reperfusion model was markedly attenuated.[820]

4. Conjugation to Polysaccharides and Proteins

Covalent coupling of Hb to biocompatible, water-soluble polymers provided alternative means of increasing the protein's MW and size. Surface modification was a further objective for such coupling. Among the preferred polymers were those used in the clinic as plasma expanders, primarily natural or modified polysaccharides such as dextrans and starches.

Conjugation of Hb with polysaccharides requires prior activation of the latter. The activated polymers are complex mixtures since reaction can occur at multiple sites. They can then react with one or more Hb molecules, which can be conjugated through one or more bonds, diversely located at the protein's surface, to one or more polymers.

a. Dextrans. Dextrans are polydisperse biopolymers composed of α-D-glucopyranosyl units **4.88** with diverse degrees of branching and chain length. Some of them are used as plasma volume expanders. They are metabolized or excreted after temporary storage in the RES. The dextrans utilized for conjugation with Hb were generally low-MW (20 000−70 000) compounds, known to be less antigenic than those with higher MWs.[821] Conjugation to Hb was achieved by N-alkylation or acylation, amidation, and S-alkylation.

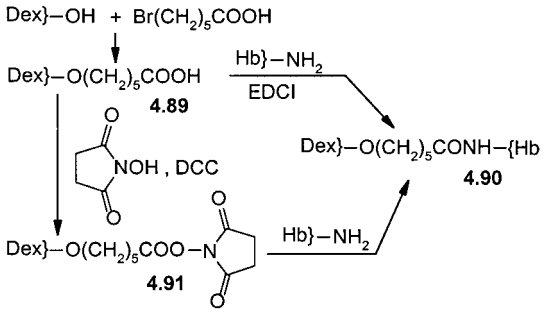


When N-alkylation of Hb was the objective, dextran was treated with $NaIO_4$, which produced a range of polyaldehydes in proportions depending on extent of oxidation.[822] The MW distribution and properties of the dextran-oxyHb conjugation products were strongly pH-dependent.[823] Stable bonds were formed at high pH, indicating that Amadori rearrangement of imine linkages into ketoamines had taken place. Affinity for O_2 was strongly augmented, while cooperativity and Bohr effect were decreased, indicating modification of amino groups involved in the salt bridges that stabilize the deoxyHb form. Dextran that had been either oxidized with $NaIO_4$ or derivatized by attachment of glutaraldehyde to an amino-substituted side chain reacted with Hb to produce, after $NaBH_4$ reduction, high O_2 affinity products.[824] These products were deemed unsuitable for use as blood substitutes on the basis of heart perfusion experiments.

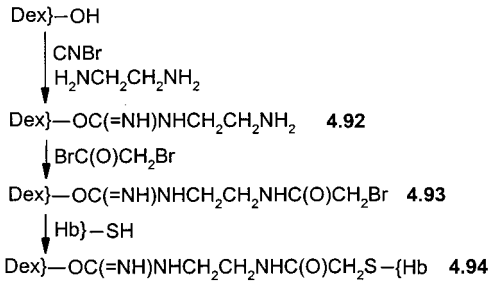
Amidation of oxy- and deoxyHb was achieved with activated carboxyl-derivatized dextrans.[825] Thus, dextran derivatized with 6-bromohexanoic acid, **4.89**, was allowed to react with Hb in the presence of EDCI, yielding the conjugate **4.90** (Scheme 9). Alternatively, the acid was treated with *N*-hydroxysuccinimide and dicyclohexylcarbodiimide (DCC) and the resulting dextran hydroxysuccinimide ester **4.91** was reacted with Hb. The conjugates **4.90** had very low P_{50} values that could not be corrected by pyridoxalation prior to conjugation, leading to the conclusion that these conjugates could not be used as O_2 carriers.

When the sulfhydryl functions were the targets of the modification, dextran was activated by treatment with cyanogen bromide, followed by diaminoethane, yielding **4.92**, which was acylated with bromoacetyl bromide into bromoacetylaminoethylaminodextran

Scheme 9



Scheme 10



4.93 (Scheme 10).[436,826−828] Conjugation of Hb with **4.93** led to mixtures of S-alkylated Hbs **4.94** with different stoichiometries and linking patterns. A sharp increase in viscosity upon storage indicated continued reaction of residual bromines on the dextran with Hb, which could be prevented by removing the unreacted bromines with sulfhydryl-containing compounds such as L-cysteine or β-mercaptopropionic acid.[708] High concentrations of reactants led to extensive three-dimensional networking, increased viscosity, and gelation.[827] Both carboxymethylation and conjugation to a dithiopyridyl group-activated dextran through a disulfide exchange reaction at the Cys-93β residues produced mixtures with less than 10% of Hb's reactive thiols remaining intact.[571]

The circulatory half-life of a 6% solution of a typical Hb-dextran conjugate reached 2 days in close-to-totally exchange-perfused dogs.[829] Conjugation with dextrans of MW larger than 20 000 did not further retard clearance from circulation.[830] Urinary excretion and impairment of kidney function were prevented (rat),[488] and the rate of extravasation of Hb into the peritoneal cavity was strongly reduced (rabbit).[708] The dextran−Hb conjugate was more resistant to acidic denaturation than free Hb and showed reduced affinity for haptoglobin.[831] The excessive O_2 affinity (P_{50} ~7−10 Torr) of the dextran−Hb conjugate was corrected by reductive alkylation with $NaIO_4$-oxidized inositol tetraphosphate **4.63** and dimethylamineborane.[708,709] Conjugates in which dextran was bound to either carboxyl groups or amino groups of Hb have also been investigated in the former Soviet Union.[767,832]

b. Hydroxyethylstarch and Inulin. Hydroxyethylstarch was derivatized with cyanogen bromide, ethylene diamide, and glutaraldehyde and oxidized with $NaIO_4$ in view of producing HES−Hb conjugates.[824,833] Significant cross-linking was observed, yielding viscous heterogeneous products with increased O_2 affinity.[834] In a further rather complicated seven-step approach, an aldehyde-derivatized HES obtained by treatment, first with cyanogen bromide, then with a di-, tri-, or tetraamine, then with glutaraldehyde, was further oxidized with $NaIO_4$; the resulting polyaldehyde mixture was eventually allowed to react with Hbs that had already been cross-linked intramolecularly with EDCI-activated glyoxylic, glutaric, or adipic acids, followed by $NaBH_4$ reduction.[835] More recently a Hb−HES conjugate was obtained from cyanogen bromide-activated HES in somewhat different conditions.[836] The Hb concentration of solutions of such conjugates was limited by viscosity, and mixtures with RBCs resulted in a kind of sludge that indicated strong interaction with RBCs.

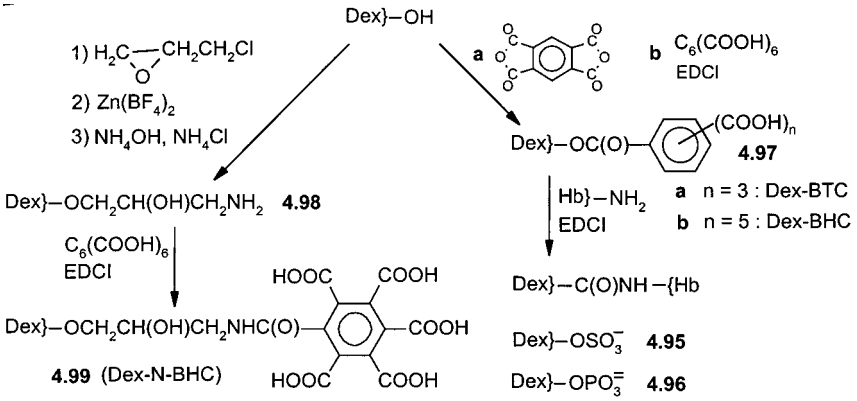
Inulin (a common polyfructan) was esterified with succinic anhydride, then activated with *N*-hydroxysuccinimide. A large excess of this reagent was allowed to react with pyridoxalated Hb.[837] Plasma half-life was significantly increased; the P_{50} values remained, however, on the low side.

c. Polyanionic Dextran Conjugates. Correction of the improper P_{50} of dextran/Hb conjugates was achieved by using polyanionic dextrans, including dextran sulfate **4.95**[838] and phosphate **4.96**,[714,839] and the benzene tetracarboxylate- or hexacarboxylate-derivatized dextrans **4.97a,b** (Scheme 11).[712−714,840−842] The latter derivatives provided particularly high negative charge densities.

$NaIO_4$-oxidized dextran phosphate[838] or sulfate[714,839] when allowed to react with deoxyHb yielded, after reduction with $NaBH_4$, anionic dextran−Hb conjugates with low O_2 affinity. Conjugation was facilitated as compared to noncharged analogues, resulting in larger MWs.

Dextran benzenehexacarboxylates were prepared by condensing benzene hexacarboxylate either onto a hydroxyl group of dextran, resulting in an ester function (**4.97b**, Dex-BHC), or onto an aminated dextran **4.98**, resulting in an amide link (**4.99**, Dex-N−BHC),[842,843] in both cases in the presence of EDCI **4.43**. Dextran−benzene tetracarboxylate **4.97a** (Dex-BTC) was obtained from dextran and benzene tetracarboxyl anhydride.[841] Some cross-linking between polymer chains occurred during condensation with Hb, and such cross-linking was more extensive when a flexible spacer was present, as in **4.99**.[840] Coupling of the benzene polycarboxylates with Hb using EDCI was proposed to take place preferentially at the Val-1β residues. Modulation of P_{50} (from 4.5 Torr for unmodified Hb to 28 Torr) was achieved by varying the density in negatively charged groups and the dextran polycarboxylate/deoxyHb ratio.[712] Oxygen affinity decreased with increasing BTC/Hb ratios and increasing EDCI concentration.[841] Subsequent addition of IHP did not increase P_{50} further, indicating that the allosteric sites of the conjugated Hb were occupied by BTC groups. When conjugation was effected on oxyHb in the presence of IHP, the yield was substantially decreased and the P_{50} of the conjugate, after IHP had been removed, was lowered, suggesting that IHP hindered the access of the 2,3-DPG site to the dextran-linked BTC groups. The Bohr effect was greatly reduced, indicating that the Val-

Scheme 11

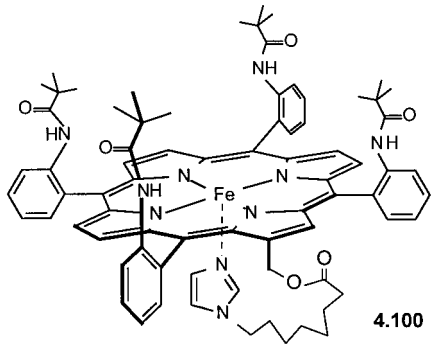


1a residues were among the sites of fixation of Dex-BTC. Whether other sites were involved and whether more than one of the three carboxylate functions available were implicated has not been determined. High COP, due to the presence of both unbound dextran and unbound Hb, could limit the Hb concentration of injectable solutions of such material. The amount of nonconjugated Hb could be decreased by using larger proportions of EDCI, but P_{50} then decreased also.[713] The O_2 affinity-reducing effect of the polyanionic polymers was powerful enough to allow the reactions to be conducted conveniently on oxyHb, which could be an advantage from a processing standpoint.[842] This advantage was lost, however, when $NaIO_4$-oxidized benzenehexacarboxyl dextran was used for conjugation through N-alkylation.[714] The circulation half-life of Dex-BTC−Hb was about 10 h in 50% exchange-transfused guinea pigs.[844] Some limited extravasation was observed as well as a gradual breakdown of the Hb−dextran conjugate.

Administration of Dex-BTC−Hb to rabbits in three situations, bolus injection, hemodilution, and resuscitation from hemorrhage, always resulted in increased mean arterial pressure and decreased heart rate.[845] In the hemodilution case, decreased abdominal aorta distension was also noted. Investigation of the effects of Dex-BTC−Hb solutions on RBC rheology indicated increased viscosity, especially at the low shear rates relevant to the microcirculation, and increased aggregation.[846,847]

d. Serum Albumin−Hemoglobin and Albumin−Synthetic Heme Conjugates. An early attempt at coupling Hb with albumin using a large excess of EDCI resulted in a product with a very low P_{50}.[848] Conjugates have been produced in which Hb was copolymerized with albumin using glutaraldehyde.[767,832] Copolymerization of pyridoxalated human Hb with serum albumin using glutaraldehyde yielded a very complex mixture.[849]

Although not a derivative of Hb, an O_2-carrying hybrid made of recombinant human serum albumin and the synthetic "picket-fence" tetraphenylporphinatoiron(II) complex **4.100** with a covalently bound proximal histidine must be mentioned.[210,211,850−852] Up to eight such synthetic hemes could be loaded into one albumin molecule. Exchange transfusion with the product restored arterial pressure and blood flow and demonstrated O_2 delivery in hemodiluted and hemorrhaged rats.[211] The in vivo half-life of the dioxygen complex was about 4 h. Slower autoxidation in vivo, as compared to Hb, may be due to the more hydrophobic environment provided by the albumin host molecule and to reduction of metHb by ascorbic acid provided by the RBCs. A lower COP was obtained by producing an albumin dimer using bis(maleiimide) cross-linking chemistry, thus allowing the preparation of a 10% Hb solution with the same COP as blood.[853]

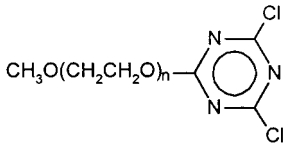


5. Pegylated Hemoglobins

Grafting poly(ethylene glycol) chains (pegylation) has become a popular means of altering and controlling the biodistribution, pharmacokinetics, and toxicity of drugs, proteins, and particulate matter.[854−862] Pegylation of Hb is an effective way of increasing the molecule's size. It was expected to hinder renal excretion, reduce toxicity, and prolong i.v. persistence by reducing the molecule's visibility to the RES. Very different products were obtained depending on whether only one or both ends of the PEG chain were functional. In the former case, the Hb molecule is simply "decorated" with PEG strands, which significantly increases its size and surface hydrophilicity, hence changes its diffusibility, but does not reduce its COP. The extensive hydration of the PEG chains that results from hydrogen bonding of water molecules to the ether oxygens can actually increase the colloid osmotic activity and viscosity of the solutions significantly,[529,836] which may limit Hb concentration. When both ends of the PEG chain are reactive, there are numerous additional possibilities of intra- and intermolecular cross-linking. Low MW PEG chains (5000 or less) appear to facilitate renal excretion and are better tolerated.

Conjugates of Hb with poly(vinylpyrrolidone) have been reported;[863] polyvinylpyrrrolidone solutions are, however, no longer used as plasma expanders.

a. Monofunctional Poly(ethylene glycol) Reagents. Cyanuryl chloride-activated monomethoxypoly(ethylene glycol) **4.101**, when allowed to react with Hb, provided adducts with unexpectedly high MW, indicating that intermolecular cross-linking was taking place.[864] Acylation of human Hb with an

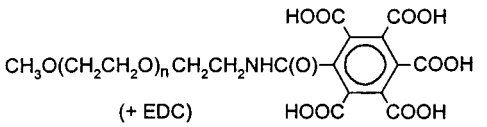


4.101

mPEG of MW around 5000 that had been converted into the succinimidyl succinate **4.29** or succinimidyl oxyacetate **4.27** (Chart 3) produced Hb conjugates with a MW around 190 000 and low P_{50}, whatever the ligand state and conditions.[644,864] Lesser O_2 affinity was obtained when condensation was achieved on deoxyHb in the presence of IHP. Conjugation of mPEG with pyridoxalated deoxyHb eventually provided low O_2 affinity, similar to that of the initial pyridoxalated Hb.[644] Lower MW mPEGs (MW 1900) were subsequently used, which limited the increase in viscosity and oncotic pressure.[645] The new conjugates were purified by ion-exchange chromatography and had about 13−14 PEG strands per Hb and a mean MW of 90 000. Their P_{50} was still rather low. The increase in arterial blood pressure was similar for the modified and unmodified Hbs in a rat model of hemorrhagic shock. A more recent mPEG-derivatized pyridoxalated Hb prepared from cyanuryl chloride mPEG was reported to cause RBC aggregation.[836]

Reaction of PEG-succinimidyl carbonate **4.31** with Hb yielded carbamate linkages that were less vulnerable to hydrolysis than ester bonds and resisted a variety of enzymes well.[648,656] When 7−8 PEG strands were attached, the P_{50} of 28 Torr of bovine Hb treated with **4.31** was essentially maintained and the half-life in the circulation was 10−13 h in 70% exchange-transfused rats. A larger number of PEG strands led to increased O_2 affinity and viscosity.

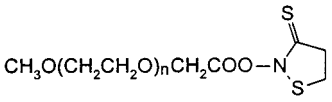
In another approach, an amino-mPEG was first coupled with benzene hexacarboxylate using EDCI, yielding **4.102**. Carboxylic groups of **4.102** were then



4.102

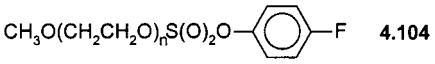
condensed with amino groups of oxyHb or deoxyHb, also with EDCI, resulting in polyanionic conjugates with an average MW of about 75 000.[712,865] Dramatic increases of P_{50} were obtained that were insensitive to IHP, indicating that coupling had taken place at the allosteric site.[865] Monosubstitution occurred primarily on the β chains (65% versus 15−20% on the α chains) at the Val-1β termini. In the case of oxyHb, an excess of EDCI led to lower P_{50}, which was suggested to indicate that some intramolecular cross-linking involving at least two carboxylate groups had occurred.

Further pegylation reagents used with Hb include the very stable PEG-thiazolidine-2-thione **4.103**, which conveniently provided amide bonds with bovine Hb.[866] Circulation half-life in rats was prolonged



4.103

4−5 times, but P_{50} was very significantly decreased as compared to native Hb. An attempt at modifying the surface of DCLHb specifically at the Cys-93β sites using the 4-fluorobenzenesulfonate-activated ester of mPEG **4.104** failed to give the expected methoxy-PEG grafted Hb.[685]



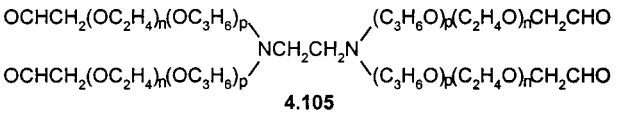
4.104

The relations between pegylation, increase in hydrodynamic volume, COP, and viscosity of the Hb product, and vasoactivity were investigated using mPEG chains of different length and different binding procedures. The latter included grafting of mPEG chains onto the Cys-93β residues by maleimide chemistry (eq 4.11) and onto the Val-1β sites by reductive alkylation chemistry and amidation of the Glu-43β residues of oxyHb with an amino-mPEG derivative using EDCI.[682] To increase the number of mPEG chains on the protein, several of Hb's NH_2 groups were derivatized into SH groups with Traut's reagent **4.56** prior to maleimide-mPEG derivative treatment (eq 4.12). The new pegylation sites were not identified. The size of the PEG chains (but not the chemistry involved in their binding) determined the hydrodynamic volume of the Hb product. Grafting of six mPEG chains onto Hb resulted in molecules with a large hydrodynamic radius of 15 nm (compared to about 3 nm for Hb), a low P_{50} of 6 Torr, and little cooperativity. The presence of six mPEG strands on the protein reduced its pressor effect more effectively than when only two PEG chains were bound.[682] No significant hemodynamic responses were seen during resuscitation of rat and swine following hemorrhage with a 4 g/dL solution that had a large COP of 50 Torr and a viscosity of 3 cP, comparable to that of blood.[867] Other data indicated some residual mean pulmonary artery pressure increase.[868] This technology was recently licensed to Sangart, Inc. (San Diego, CA).

b. Polyfunctional Poly(ethylene glycol) Reagents. A large part of the work done with Hb and difunctional PEG esters was directed at the development of a pyridoxalated Hb product that is being pegylated using the activated PEG diester **4.28** and is known as PHP (section IV.I). A highly polydisperse polymerized pyridoxalated Hb had initially been obtained using the PEG-bis(succinimidyl succinate) **4.30**.[647] Contrary to glutaraldehyde-polymerized pyridoxalated Hb, substantial cooperativity was retained. Polymerization and decoration of deoxy-DCLHb were achieved with the activated PEG-bis-

(benzenesulfonates) **4.55a** and somewhat less effectively with **4.55b**.[685] Pegylation occurred primarily at the Cys-93β residues but also at primary amino groups. P_{50} values and cooperativity remained in the commonly considered useful range. α,α-DBBF−Hb was also polymerized using a thiol-specific bis-(maleoylglycylamide)-PEG reagent[869] for the purpose of studying the impact of polymerization on Hb's redox and O_2 carrying and redox properties,[870] renal function, and pressor effect.[871] P_{50} diminished as usual with increasing extent of polymerization. Renal clearance was reduced and intravascular retention increased. Specific cross-linking of pyridoxalated Hb at the Cys-93β residues, also using a bifunctional maleimide PEG, has been briefly mentioned.[526]

Tetronic block polymers were treated with dimethyl sulfoxide and DCC, yielding tetraaldehydes of type **4.105**, which were then allowed to react with Hb that had first been cross-linked with glutaric acid, $HOOC(CH_2)_3COOH$.[872] Little detail was provided.



4.105

E. Genetic Engineering of Hemoglobin

Expression of the β globin chains of Hb in *E. coli* was achieved in the mid-1980s, allowing, by combination with native α chains and hence, the preparation of semi-recombinant tetramers.[472,873,874] Coexpression of both chains in *E. coli* was subsequently achieved.[875] Functional recombinant Hb (rHb) is obtained by assemblage of the genetically produced globins with heme endogenous to the expression system or exogenous. Heme is present in and common to numerous species, from bacteria to mammals, but not always in a sufficient amount. Heterologous gene expression in *E. coli* produced a rHb molecule that was constitutionally different from normal human Hb since it had additional N-terminal methionine residues on all four chains.[875,876] This mutant Hb (as well as the mutant with a Val-1β→Met substitution) was nevertheless structurally[877,878] and functionally[879] close to HbA, although it showed reduced cooperativity and Bohr effect and a loss of O_2 binding regulation by 2,3-DPG. Structural heterogeneity, resulting from misfolding or misassembly of the α and β subunits or initial misorientation of the heme, has been observed[880] as well as, in some cases, changes of native function over time.[881] Recombinant Hb with the exact sequence of human HbA was subsequently produced in *E. coli* through coexpression of the α and β globin genes and of a methionine aminopeptidase gene.[882,883] Incorrect heme insertion could be corrected by carrying out an oxidation−reduction cycle on the isolated rHb. The same expression system was used to produce diverse mutant Hbs.[477]

Normal and modified human Hb genes were expressed in bacteria (*E. coli*),[472,473,476,477,874−876,882,884−888] yeast (*Saccharomyces cerevisiae*),[478,889−891] mice,[892−895] pigs,[474,479,896] and plants,[897] in particular tobacco,[475] allowing the production of a large variety of normal and mutant rHbs. The availability of a precise three-dimensional structure of Hb[226] was instrumental for the design of these proteins. Purification of rHbs usually involved several successive chromatographic steps.[886,890]

Human rHb expressed in yeast had the correct N-terminal residues because yeast contains methionyl aminopeptidases that remove the initiating methionines.[478,889,890] However, part of the rHb molecules had higher O_2 affinity and lower cooperativity that were attributed to improper folding.[898] Subsequent studies indicated that the abnormal form was due to Hb incorporating a sulfheme (i.e., a structure with sulfur in the periphery of the protoheme IX group).[899] Human sickle rHbS has also been expressed in yeast.[895] Expression yields in yeast are, however, lower than in bacteria.

Site-directed mutagenesis has been used for the purpose of elucidating Hb and Mb structure/function relationships and determining the relations that govern them[307,473,879,891,900−905] or optimizing the functional properties of cell-free Hb.[473,477,885,887,889,906−908] Key objectives were to control O_2 affinity, rates of NO binding, autoxidation, and stability. Myoglobin has often been used as a prototype to develop methodologies and elucidate mechanisms.[909,910] Remarkable genetic engineering feats led to human rHbs that responded to chloride ions (as in bovine)[911] or bicarbonate ions (as in crocodile)[912] rather than 2,3-DPG for allosteric regulation, rHbs in which the positive charges at the 2,3-DPG sites are neutralized,[887,913] rHb with a carbonmonoxy form that is readily converted into a deoxy form by addition of IHP,[907] chimerical globins composed of the first half of the α chain and the last half of the β chain,[914] etc. Reengineering of the heme pocket, especially by replacing some amino acid residues by more bulky and less polar ones, produced variant Hbs with significantly lower rates of reaction with NO and lower vasoactivity but preserved O_2 delivery efficacy (i.e., capable, to some extent, of discriminating NO from O_2).[307,315,915,916] Reduced reactivity with NO translated into attenuated gastric dysmotility. Genetic engineering thus allowed one to independently manipulate P_{50} and the rate of NO uptake. However, these experiments, as in the case of chemical modifications, raise the question of which other characteristics of the Hb molecule might have been altered by the genetic mutation.[793] Random combinatorial mutagenesis techniques are also being developed.[908]

A variant of human Hb, rHb1.1, stabilized by a glycine bridge between the COOH and NH_2 termini of the α chains and bearing an Asn-108β→Lys mutation that provided reduced O_2 affinity, was expressed in *E. coli* and developed for commercialization. Human Hb was also expressed in transgenic pigs. Both enterprises are described in section IV.I.

Genetic engineering opens fascinating perspectives in terms of manipulation of the properties of Hb. It might ideally provide Hb mutants with (simultaneously) increased molecular stability, reduced O_2 affinity, reduced autoxidation rate, reduced rate of reaction with NO, and increased circulation half-life.[307,315,476,723,908,917]

F. Hemoglobin Encapsulation

Re-encapsulation of natural or modified Hb within an artificial barrier membrane is expected to achieve some of the features of RBCs (Figure 11), although with considerable differences in structure, shape, rheological behavior, and functioning and without the antigens present on the RBC surface. This approach has the potential to prevent Hb dissociation and clearance through the kidneys, solve the COP issue, reduce extravasation, vasoconstriction and organ toxicity, and increase intravascular persistence. Co-encapsulation of allosteric effectors can be used for correcting excessive O_2 affinity. Incorporation of reductants or reductases can limit metHb levels. Surface modifications may increase the particle's circulation half-life and reduce viscosity and aggregation. Large differences in formulation and processing methods used by different investigators led to widely different Hb liposome structures, characteristics, and in vivo behavior.

LEHb proved efficacious in diverse hemorrhagic shock[918] and exchange transfusion[919−921] models.

1. Early Artificial Red Blood Cells

Early attempts to encapsulate Hb relied on rather large semipermeable microcapsules made from materials such as nylon and collodion.[922,923] Hemoglobin-loaded biodegradable polyamide microcapsules, 0.5 μm in average diameter, were also reported.[924]

"Hemosomes" were obtained by sonicating concentrated Hb solutions with phospholipids and cholesterol; dicetyl phosphate or other negatively charged lipids were added to increase the surface charge and reduce aggregation.[925,926] A 35 vol % suspension of such vesicles allowed survival of rats after 92% blood exchange.[927] Co-incorporation of Hb and IHP in dipalmitoylphosphatidylcholine (DPPC)/cholesterol liposomes shifted the P_{50} from 17 to 50 Torr.[927a] Co-encapsulation of Hb and 2,3-DPG in liposomes made from egg yolk phosphatidylcholine, dipalmitoylphosphatidic acid, cholesterol, and α-tocopherol, using a reverse-phase evaporation and extrusion-based process yielded "neohemocytes".[928] The P_{50} was 24 Torr, most of the cooperativity was retained, and the half-life in the circulation was estimated as 5.8 h. However, a sufficiently fluid 25% suspension of these liposomes contained only about 3.8 g/dL of Hb. Some signs of liver toxicity were seen, and the need for limiting the increase in metHb was recognized.[928]

2. Specific Challenges

The most thoroughly investigated Hb confinement systems are lipid vesicles (or liposomes). Since their discovery,[929] liposomes have been extensively studied as drug delivery systems, the primary purpose being to reduce the toxicity of drugs and prolong their intravascular circulation. Commercial development of liposome formulations has been slow but was recently rewarded by the approval and marketing of several liposomal antifungal and anticancer preparations.[930,931]

However, the liposome-encapsulated Hb (LEHb) approach carries specific challenges which are related to the large doses of Hb that are to be administered, redox properties of the protein, need for high encapsulation efficiency, cost of ingredients, difficulties of large-scale manufacture, interference with analytical methods, metHb control, and shelf stability.[932] Matching the over 300 g/dL entrapment efficiency of the RBC is not a trivial task. High encapsulation efficiency is also required in order to not overload the patient with lipids. The tendency for liposomes to aggregate in plasma, which also appears to be Hb-specific because of the large doses administered, needs to be prevented.[933] Hydrophobic interactions between Hb and the liposomal membrane can result in the intercalation of the protein into the bilayer, which can facilitate peroxidation of lipids, unfolding of Hb, oxidation of heme iron, displacement of the heme from the globin, decoordination of the iron atom, and other alterations.[934] Peroxidation of membrane lipids by Hb and its redox byproducts is of particular concern, possibly limiting the use of oxidation-prone natural phospholipids (with unsaturated fatty acids). The latter may react with Hb products yielding metHb, fatty acid hydroperoxides, aldehydes, endoperoxides, malonic dialdehyde, aldol condensation products, etc.[935−937] Phospholipids and lipid vesicles appear to catalyze the free-radical-mediated oxidation of Hb and vice-versa.[938,939] Some LEHb also evoked undesirable hemodynamic, hematologic, and biochemical responses.[962]

LEHb constitute foreign particulates that are opsonized in the plasma, recognized and processed by the RES, mainly in the liver, spleen, and bone marrow.[398,400,401,928,932,940−942] Biodistribution was found independent from Hb type and source. There was no evidence of a deleterious effect on cells or accumulation of Hb in tissues.[943] Phagocytosis typically results in short plasma half-life and transient changes in serum enzymes. The rate of removal of liposomes from the blood stream decreases sharply with diminishing sizes and increasing doses. Positively charged liposomes are generally removed faster than negatively charged ones, which are removed faster than neutral ones. The intravascular half-life of LEHb also depends on the membrane surface. Removal of LEHb from circulation as well as phagocytosis by cultured monocytes are biphasic (Figure 12).[400,565,944,945] Up to one-half of the LEHb administered to rabbits was rapidly engulfed in the first two to 4 h, followed by a slower phase, possibly indicating RES saturation.

Transient blockage of the RES has been observed,[401] resulting in increased sensibility to *Listeria* infection.[946] RES uptake and possible overload by other liposomes has been discussed.[407,410,947−950] Lysophospholipids appear to enhance RES depression by liposomes.[410] A marked but transient increase in interleukin-6 level in the plasma of mice, indicative of macrophage activation, has been measured with LEHb that was larger than with empty liposomes.[434] Complement activation by LEHb in rat and pig has been documented.[951,952] The response of the RES to endotoxin stimulation was also affected by LEHb, as indicated by inhibition of tumor necrosis factor-α production (an indicator of inflammatory response) by mononuclear phagocytes in the presence of li-

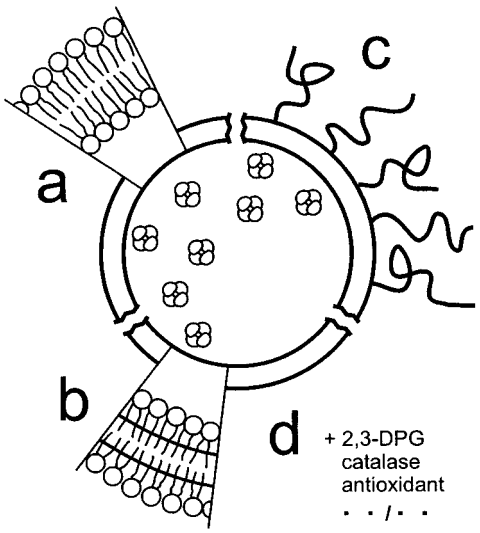


Figure 11. Schematic representation of liposome-encapsulated Hb products with (a) a plain phospholipid bilayer membrane, (b) a polymerized lipid membrane, (c) a poly(ethylene glycol) decorated membrane, and (d) allosteric effector, enzyme, antioxidant, and cryoprotector supplementation.

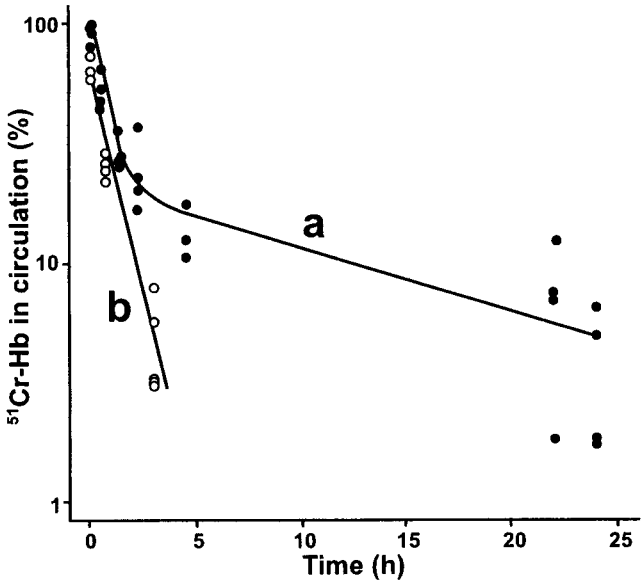


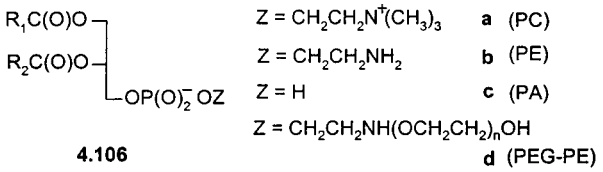
Figure 12. Clearance of (a) liposome-entrapped Hb as compared to (b) cell-free Hb from the blood of rats injected with a total of 17.2 mg of ^{51}Cr-labeled Hb in either form. The liposomes were made from hydrogenated egg phosphatidylcholine and cholesterol. (Reprinted with permission from ref 565. Copyright Elsevier 1994.)

popolysaccharides and LEHb in vitro[944a] and in vivo.[945] Modulation by LEHb of the inflammatory response of isolated splenocytes or cultured monocytes to endotoxins was also observed.[945] The binding of endotoxins on the surface of LEHb has been considered. Endotoxin-induced mortality in LEHb-treated rats depended strongly on the time interval between LEHb and endotoxin administration and on endotoxin level.[932] Transient thrombocytopenia, possibly due to transient sequestration of platelets in the liver and spleen, has been observed persistently with LEHb[932,953] and other liposomes.[954] Finally, accurate measurement of lipid-embedded endotoxin levels is difficult, possibly due to the particulate nature of LEHb or association of endotoxin with LEHb.[955]

Comparable challenges are encountered with nonliposomal capsules. The components of the containment system need to be biocompatible, inert, biodegradable, and rapidly excretable.

3. Phospholipids and Liposome Formulation

Phospholipids (e.g., **4.106a**−**d**) combine a hydrophilic polar moiety and a hydrophobic apolar moiety made of fatty acid chains. This amphiphilic character



is the driving force that causes phospholipids to form bilayers (as in liposomes) when dispersed in water and monolayers at certain interfaces (as in oil-in-water emulsions). Liposomes are primarily held together through hydrophobic forces. Phospholipids are the main constituents of cell membranes in all living organisms. Natural phospholipids consist of mixtures of phosphatidylcholine **4.106a** (PC), phosphatidylethanolamine **4.106b** (PE), phosphatidylinositol, phosphatidic acid **4.106c** (PA), other phospholipids, as well as a variety of other compounds, such as fatty acids, triglycerides, sterols, carbohydrates, and glycolipids. Phospholipids are extracted from vegetal tissue, such as soy bean, and animal tissues, primarily egg yolk, isolated by solvent extraction, and purified by recrystallization and/or chromatography.[956,957] Because of labile ester linkages and unsaturated fatty acids, natural phospholipids tend to undergo hydrolysis and oxidation over time. Acid/base-catalyzed hydrolysis yields free fatty acids and lysophospholipids, followed by glycerophospho compounds and eventually glycerophosphoric acid.[958,959] The rate of hydrolysis has a minimum around pH 6.5. Lysophospholipids can modify membrane performance and are known to have toxic effects when present in large amounts.[960] Their formation can, therefore, limit a product's shelf life. Oxidative degradation of EYP can occur in unsaturated acyl chains and cholesterol.

The physicochemical and biological properties of liposomes (including aggregation, intravascular persistence, and interaction with the RES) are largely determined by the structure and properties of the bilayer membrane that surrounds them and depend on phospholipid composition, particle size, surface charge, processing, and thermal history. The lipids used for preparing liposomes are similar to those used for manufacturing pharmaceutical emulsions for parenteral nutrition, drug delivery, and O_2 delivery with PFCs[219,961] (see also section V.D). Pure, synthetic saturated phospholipids, such as distearoylphosphatidylcholine (DSPC), are now available that are not oxidizable and provide improved biological properties,[953,962] but their use involves higher cost. Hydrogenated or partially hydrogenated natural lipids containing less oxidizable monounsaturated rather

than polyunsaturated fatty acids may provide a convenient, less costly raw material.[933,963−967] α-D-Tocopherol is often added as an antioxidant for protection of the lipids. However, α-tocopherol may destabilize liposomes with respect to particle size increase.[967]

Unless properly protected, liposome-encapsulated Hb rapidly converts into metHb. Extensive oxidation of Hb was observed during LEHb production and storage.[565] A liposomal bovine Hb preparation lost essentially all of its in vivo O_2 carrying capacity within 24 h, presumably due to metHb conversion, although about 40% of the initial Hb was still in circulation.[968] While Hb in liposomes solely made of egg PC converted into metHb within 2 days at 4 °C (i.e., much faster than Hb in solution), no acceleration in metHb formation was seen when a mixture of dimyristoylphosphatidylcholine (DMPC), cholesterol, and dicetyl phosphate was used.[935] Co-encapsulation of enzymatic reduction systems and other protectants appears indispensable. Glutathione,[564,944] D,L-homocysteine,[969−971] catalase,[966] methemoglobin reductase,[972] coenzyme NADH[564] (with glucose, adenine, and inositol as substrates),[933,972] or superoxide dismutase and catalase[817,969] were used for this purpose.

Cholesterol represents up to one-half of the lipids present in a liposomal formulation. Its purpose is to reduce membrane permeability, improve liposome resistance to fusion and lysis, hinder Hb oxidation and denaturation, and reduce lipid peroxidation. The rigid cholesterol molecule inhibits both hydrophobic- and ionic-type interactions of phospholipid membranes with proteins, probably through changes in membrane fluidity, in line with cholesterol's condensing/stabilizing effect in unsaturated bilayers.[934,973] It reduces intercalation of Hb into the lipidic bilayer and displacement of heme.[974] The presence of cholesterol in the formulation also prevents redistribution of cholesterol from RBC membranes to the liposome membrane, a process which renders RBCs osmotically fragile.[975]

Small amounts of negatively charged phospholipids, such as PA, PE, dicetyl phosphate, dimyristoylphosphatidylglycerol, phosphatidylserine, phosphatidyl inositol, and PEG-PE **4.106d**, have often been added to increase surface charge and inhibit liposome aggregation and fusion during storage.[964,966,976] Some of these additives can, however, enhance lipid peroxidation.[936] Further components were included in view of reconstituting an internal milieu closer to that found in the RBC. Such components include 2,3-DPG,[928] IHP,[565,977,978] or pyridoxal phosphate.[966,979] Co-encapsulation of Hb with **4.2** produced LEHb with an O_2 affinity comparable to or lower than that of fresh blood.[980] Uptake and release of O_2 and CO binding were substantially faster than for RBCs (indicating that the liposomal membrane did not constitute a significant barrier to O_2 diffusion) but nevertheless slower than for cell-free Hb.[964] Liposome suspensions tend to display non-Newtonian behavior, with increased viscosity at low shear rates.[964,979] Interaction between LEHb and albumin can cause a substantial increase in viscosity, especially at low shear rates.[981]

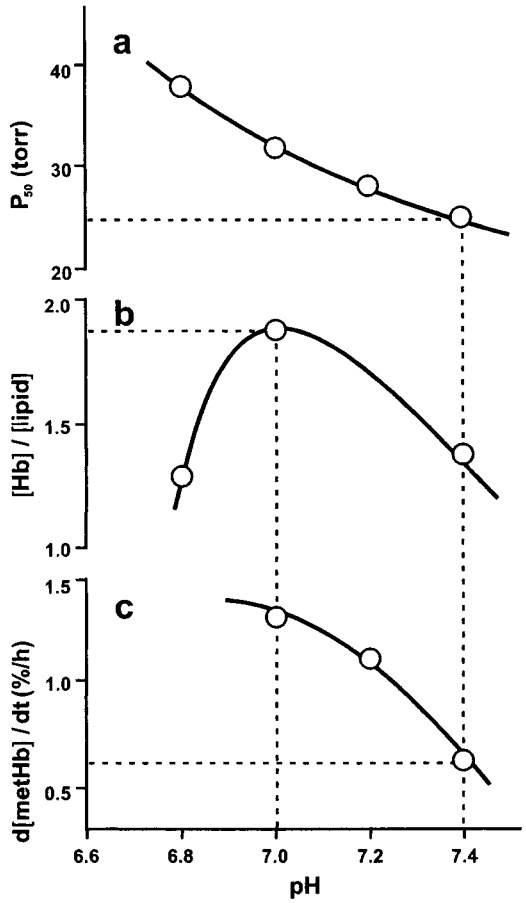


Figure 13. Effect of pH of the Hb solution during preparation of LEHb on (a) O_2 affinity, (b) encapsulation efficiency ([Hb]/[lipid]), and (c) rate of metHb formation of LEHb. (Reprinted with permission from ref 970. Copyright 1998 Elsevier.)

4. Processing

Large multilamellar vesicles (MLV) form spontaneously when lipid films are mixed with a Hb solution. These vesicles can be processed into small unilamellar vesicles (SUV) using sonication, extrusion, detergent dialysis, dehydration−rehydration, high-pressure shear (e.g., microfluidization), reverse-phase evaporation, double-emulsion techniques, and combinations thereof.[565,925,935,963,964,966,979,980,982−986] Sonication or the use of organic solvents and certain detergents can, however, be deleterious to the protein. Processing temperature was usually maintained below 50 °C, as irreversible denaturation of Hb was observed in the presence of phospholipid vesicles at higher temperatures.[565] Encapsulation efficiency, O_2 affinity, and the rate of metHb formation are rather sensitive to pH (Figure 13).[970] Encapsulation efficiencies differed widely with process but did usually not exceed 25−30% and could be as low as 7%. Thorough centrifugation, washing, dialysis, ultrafiltration, or size exclusion chromatography were used to remove nonencapsulated Hb.[933,964] Trace amounts of Hb that remained attached to or intercalated in the lipid bilayer[953] may account for differences in in vivo behavior of LEHb versus empty liposomes.[987] Some of the processing and concentration steps have often been achieved with Hb in its carbonmonoxy form in order to minimize oxidation during manufacture. As

terminal heat sterilization is prevented by the protein's fragility, LEHbs are usually sterilized by filtration and filled under sterile conditions.

The highest encapsulation efficiencies so far have been produced using dehydration−rehydration,[565] double-emulsion/dehydration/rehydration/high-shear,[966] and extrusion techniques.[970,979,981,985] The number of bilayers decreased when the surface potential of the phospholipid membrane became more negative and membrane fluidity was reduced, as by decreasing the temperature. The Hb concentration inside the vesicles increased when its zeta potential became positive, allowing higher Hb/lipid ratios to be attained.[985] The importance of pH control, as with CO_2, with respect to encapsulation efficiency, metHb formation, and O_2 affinity was emphasized.[988] Dehydration−rehydration encapsulation of human Hb along with glucose, fructose, and mannitol as cryoprotectants and IHP in vesicles made from equal amounts of hydrogenated egg phospholipids and cholesterol provided vesicles about 140−200 nm in diameter with a low lipid/Hb molar ratio and a high encapsulation efficiency of about 22%; metHb was, however, about 20%.[565] A rather complex double-emulsion, film dehydration/rehydration, high-shear procedure allowed incorporation of Hb within both the liposome and the liposomal membrane, thus improving "encapsulation" efficiency.[966]

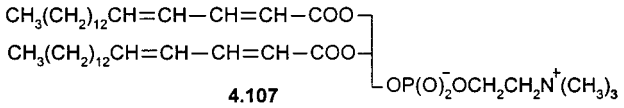
Vesicles about 250 nm in diameter that coencapsulated carbonmonoxyHb, pyridoxal phosphate, and D,L-homocysteine were prepared by extrusion from DPPC, dipalmitoylphosphatidylglycerol, cholesterol, and α-tocopherol.[979] The Hb concentration in the LEHb suspension was 10 g/dL (with only 6.2 g/dL of lipids) and the viscosity was 2.6 cP (230 s^{-1}); the P_{50} (after conversion of carbonmonoxyHb to Hb) was 32 Torr, and cooperativity was preserved. The aggregates that formed by interaction of the LEHb with plasma proteins readily dissociated at the normal shear rates found in the blood stream.

The shelf stability of aqueous LEHb suspensions was improved by lyophilization, polymerization, and surface modification. Lyophilization prolonged LEHb shelf life, provided cryoprotectants such as amino acids and carbohydrates, including glucose, sucrose, or trehalose, were added in order to minimize oxidation and loss of functionality during processing.[565,944,989−991] Trehalose is believed to bind to the membrane surface by multiple hydrogen bonding and maintain the lipid bilayer in a liquid-crystalline-like state in the absence of water. Saccharides may also contribute to hindering liposome membrane fusion and the leakage of content during the dehydration and rehydration procedures.[991,992] Lyophilization did not significantly alter the biological responses of LEHb. No hemodynamic perturbations were seen; only some differences in organ distribution and lesser changes in some serum enzyme levels were noted.[401,944,993] Large-scale end-process sterilization for LEHb production still awaits development.[932]

Manufacture of liposomes remains a complex and costly avenue, possibly not well suited to large-scale production of a relatively low-cost large-dose product.

5. Liposomes with Polymerized Membranes

The physical stability of liposomes was improved and the leakage of Hb was reduced by polymerization of the bilayer membrane. This was achieved by γ-ray irradiation of vesicles made of 1,2-bis(octadeca-2,4-dienoyl)-*sn*-glycero-3-phosphocholine **4.107**, octadeca-2,4-dienoic acid (or sodium palmitate), and cholesterol, while the Hb (human) was in the carbonmonoxy form, yielding vesicles about 250 nm in size.[991,994,995]

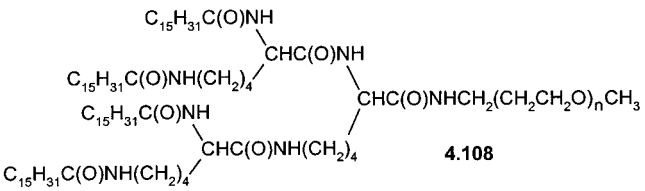


4.107

The polymerized vesicles resisted well to repeated freeze−thawing. Addition of saccharides further contributed to prevent Hb leakage and metHb formation during freeze-drying and storage.[991,996] After 1 year at 4 °C, P_{50} and cooperativity were well preserved and the metHb level was below 8% but increased rapidly in the presence of moisture or after the vesicles had been resuspended in water and CO had been substituted with O_2. The liposomal Hb had a high P_{50} of 40 Torr and low acute toxicity.[994] Its circulation half-life was 21 h in 40% exchange-transfused dogs. The metabolism of the polymerized lipids was, however, considered a problem.[971]

6. Surface-Modified Liposomes

Additives aimed a surface modification included, as for other liposomes,[860−862,930] gangliosides, PEG derivatives, phosphatidylinositol, and polysaccharide derivatives. Incorporation of monosialogangliosides (which are found on the surface of the RBC) into the bilayer membrane failed to prolong circulation persistence of LEHb.[997] Likewise for PEG-PE at clinically relevant doses.[998] Phosphatidylinositol and PE **4.106b** were reported to increase somewhat (to 15−20 h) the intravascular persistence of LEHb, 300−400 nm in diameter, containing pyridoxal phosphate and catalase.[966] Larger amounts of PEG increased the half-life of LEHb up to around 65 h.[999] Liposomes including PEG-PE appeared to have less adverse effects on the capacity of the RES to clear pathogens from the blood. Attachment of PEG strands on the LEHb's surface significantly reduced particle aggregation, viscosity, and hemodynamic side effects, indicating that albumin adsorption on the vesicles (which leads to substantially increased viscosity at low shear rates) was minimized.[981] A series of amphiphiles **4.108**, with mPEG polar heads and up to four palmitoyl hydrophobic chains connected by lysines, was recently synthesized for the purpose of improving the anchoring of PEG strands into a liposomal bilayer.[1000] Glycolipids with an oligosaccharide chain were successful in preventing vesicle



4.108

aggregation.[1001] Liposomes ca. 310 mm in mean diameter, coated with carboxymethylchitin for stabilization have also been reported.[1002]

Rapid spontaneous incorporation of PEG-PE compounds in the membrane of preformed vesicles has been reported.[1003] One advantage of this procedure is that the PEG chains are present on the outer layer of the membrane only, thus increasing the space available for Hb inside. Application to LEHb made of saturated phospholipids and cholesterol and containing pyridoxal phosphate and homocysteine led, after deoxygenation, to ready-for-use PEG-modified LEHb suspensions that were filtered through 0.45 μm filters and stable at 4 and 23 °C for 1 year (the nonmodified LEHb started to aggregate after 3 days).[971] These cellular Hb products were deemed advantageous, as compared to acellular intramolecularly cross-linked, pegylated, HES-conjugated, and glutaraldehyde-polymerized products (see Table 2), on the basis of absence of hemodynamic effects or RBC aggregation and easy O_2 affinity regulation.[836]

7. Further Hemoglobin Microcapsules and Red Cell Surrogates

Hemoglobin vesicles were prepared with 13−30% efficiency using primarily a PEG-cetyl ether, **4.109**, and cholesterol under high shear,[1004] according to the *Novasome technology*.[1005] A PEG-glyceryl monophos-

$$C_{16}H_{33}O(CH_2CH_2O)_nH \qquad \textbf{4.109}$$

phate, oleic acid, α-tocopherol, organic phosphates, and catalase were subsequently added to the formulation.[1006] No efficacy data were provided. Bovine Hb mixed with glutathione has been entrapped in bicontinuous cubic phases formed by glycerol monooleate and water.[1007] Encapsulation efficiency was high, but conversion to metHb and release of Hb from the cubic phase were rapid. Some Hb denaturation was observed. Enzyme-containing microcapsules were made from cellulose nitrate and other polymers.[1008] Hemoglobin microcapsules have been prepared by cross-linking Hb itself using various acyldichlorides.[1009] Large microcapsules, 5 μm in diameter, incorporating IHP and glucose with cross-linked Hb membranes were thus obtained using terephthaloyl chloride, followed by stabilization through glutaraldehyde. Smaller (about 0.5 μm) artificial red cells were prepared by surface treatment of pyridoxalated Hb-containing liquid membrane capsules using glutaraldehyde.[1010] Oxygen release was strongly reduced, which was blamed on glutaraldehyde polymerization. Aqueous suspensions of rather large (1−3 μm) hollow microspheres, with about 4−7 protein molecule-thick walls, have been generated from Hb using high-intensity ultrasound.[689] The Hb molecules were held together by disulfide cross-linking of cysteine residues, formed by reaction of superoxide generated during acoustic cavitation. Oxygen affinity was comparable to that of native Hb, cooperativity was preserved, and the system responded to the addition of IHP.[689] Oxygen-filled Hb microspheres of that type were suggested to possess many of the characteristics of a blood substitute; however, no in vivo experiments appear to have been reported.[1011]

Large biodegradable microcapsules (about 200 μm in size) made of poly(lactic acid) or ethylcellulose, containing human Hb, were prepared using a double-emulsion polymerization technique; encapsulation ratios attained 85% and 73%, respectively.[1012,1013] Hemoglobin was not altered by the process, but release from the capsules was rapid when diluted in a phosphate buffer. Such capsules were suggested to have potential in biotechnological rather than transfusional applications. Microcapsules (70−400 nm) made of polylactic acid or poly(isobutyl cyanoacrylate) containing bovine Hb and incorporating PEG-PE in their membrane have also been prepared.[1014] P_{50} and cooperativity were not affected by encapsulation. A Hb preparation was reported in which the protein was dissolved in a PEG/dextran "coacervate system" and the resulting mixture emulsified.[1015] No particle size, structural, or stability data were provided.

It has been suggested that a Hb/trehalose glass powder could be prepared by lyophilization that would be stable to oxidation, easily storable, and rapidly reconstituted.[1016] Trehalose is a nonreducing, biologically inert disaccharide that is abundant in living organisms adapted to survive conditions of extreme desiccation.[992,1017] Upon dehydration, aqueous solutions of this sugar tend to form glasses rather than crystallize. Chemical reactions within the highly viscous glass state are slowed considerably as the diffusion of reagents is slowed and the conformational changes required for Hb to bind ligands and react are frozen.[1016]

Red cell surrogates have been described that consisted of pyridoxalated Hb adsorbed to nanometer-size solid synthetic diamond, surface modified with cellobiose, and then coated with phospholipids, yielding particles 50−100 nm in size.[1018] Model RBCs have also been elaborated in which the role of the RBC's cytoskeleton was held by Sephadex ion-exchanger particles (reticulated dextran matrixes derivatized with ionic ligands).[1019] Hemoglobin was, however, completely released from the matrix at physiological pH.

G. Principal Therapeutic Applications of Hemoglobin-Based Oxygen Carriers

The applications of Hb products investigated in the clinic include resuscitation of trauma patients, avoidance or reduction of donor blood use in patients undergoing elective surgery (including cardiopulmonary bypass surgery), treatment of septic shock by neutralization of excess NO, and enhancement of tumor radio- and chemotherapy (section IV.I). Further potential uses include reduction of ischemia following myocardial infarction or stroke, treatment of sickle cell anemia, improvement of burns and wound healing, and perfusion of isolated organs destined for transplantation.

1. Trauma

The use of acellular Hb products in the treatment of hemorrhagic shock has received particular attention. Increased production of NO during severe hemorrhage can lead to vasodilation, hypotension, and cardiovascular collapse. Scavenging of excess NO

by an Hb product could, in principle, restore vascular tone and stabilize a patient's hemodynamics. This hypothesis was the basis for the clinical evaluation of *HemAssist* (section IV.I) in trauma patients. The efficacy of Hb products as a treatment of hemorrhagic shock is, however, controversial.[511,1020] Efficacy of DCLHb in animal models of hemorrhagic shock was demonstrated in terms of restoring arterial pressure, improving O_2 consumption, reversing ischemia and correcting an O_2 debt and base deficit.[1021−1033] For example, DCLHb was more effective than a starch solution (but less effective than blood) in resuscitating sheep from hemorrhagic shock.[1034] The same product was superior to albumin in reversing subendocardial ischemia and reducing mortality of severe hemorrhagic shock in pigs with critical coronary stenosis.[1033] Both the pressure activity and the O_2 carrying capacity were suggested to contribute to the treatment. On the other hand, no improvement in tissue O_2 delivery could be demonstrated in a swine resuscitation model with α,α-DBBF-cross-linked Hb.[304] Various reports concluded to lack of efficacy of this product because augmented vascular resistance, reduced cardiac output, and low peripheral perfusion partially offset the benefit of increased O_2 content.[188,304,356,357,359,1044,1257]

A pyridoxalated polymerized Hb was found effective in resuscitating rats from hemorrhagic shock,[1035] while another failed to improve survival in an ischemic intestinal shock model.[1036] An o-raffinose-polymerized Hb was equivalent to blood in terms of survival in a lethal hemorrhagic shock rat model.[1037] Successful resuscitation of dogs from hemorrhagic shock and recovery from an O_2 debt were also demonstrated with rHb1.1.[1038] Further investigations on these products are reviewed in section IV. I.

A high-O_2-affinity/high-viscosity/high-COP-pegylated bovine Hb and a low-O_2-affinity/low-viscosity/low-COP α,α-DBBF-cross-linked human Hb were compared with a non-O_2-carrying high-viscosity/high-COP starch solution in rats after 50% exchange transfusion followed by severe hemorrhage.[359] Arterial pressure, systemic vascular resistance, and lactic acid levels were seen to rise with the α,α-cross-linked material but not with the pegylated product. Two-hour survival was greatest in the PEG−Hb group, followed by the starch controls, and worst with the α,α-cross-linked product. Lactic acid levels (a measure of O_2 debt) correlated inversely to survival.

Trauma is, however, an extremely complex situation, usually involving multiple organs, poorly fit for controlled assessment of the benefit of an O_2 carrier. In addition, the window of time available for intervention is short.

2. Hemodilution

The effectiveness of Hb products in ANH is also being debated.[1020,1032] The efficiency of Hb products in allowing survival at extreme hemodilution (hematocrit below 5%) has been demonstrated in diverse animal models with various products.[167,355,365,829,1039−1043] On the other hand, isovolemic hemodilution experiments with DCLHb in hamsters led to an unfavorable reduction of tissue pO_2 and functional capillary density as compared to hemodilution with a dextran solution.[1044] Vasoconstriction due to NO scavenging or excessive arteriolar pO_2 values and reduced blood viscosity, resulting in reduced shear stress-induced production of endogenous endothelial vasodilator substances (NO, prostacyclin) in arteriolar vessels, were among the mechanisms invoked to explain impairment of tissue oxygenation. A 50% isovolemic exchange transfusion in rats led to heart rate reduction (despite lower viscosity) in DBBF-cross-linked Hb-treated animals, suggesting a direct myocardial depressant effect.[1045] Isovolemic exchange transfusion of cats with a β,β-cross-linked bovine Hb resulted in higher CaO_2 and lower levels of cerebral blood flow.[1046] DCLHb maintained myocardial perfusion and function at very low Hct values in hemodiluted pigs.[167] However, vasoconstriction eventually impaired peripheral tissue oxygenation despite augmented CaO_2. See also section IV. I.

3. Septic Shock

Sepsis is a systemic inflammatory response to major bacterial infection. Treatment of septic shock using cell-free Hb relies on the ability of the protein to scavenge the excess NO which is produced in that condition and causes vasodilation and hypotension. Cell-free Hb was indeed shown to scavenge the excess NO produced in endotoxin-treated isolated vessels in vitro.[1047] A polymerized bovine Hb restored cardiovascular and kidney function in an endotoxin-induced shock model in rats.[1048] Increased O_2 uptake was observed upon administration of DCLHb in a septic rat model.[454] A pyridoxalated−Hb−polyoxyethylene conjugate (PHP) is presently being evaluated in the clinic for treatment of septic shock (section IV.I).

4. Miscellaneous Applications

Hemoglobin products can potentially reduce ischemia consequent to myocardial infarction or stroke,[1049,1050] improve blood circulation during vaso-occlusive crisis in sickle cell patients,[805,1051] allow treatment of ischemia in case of autoimmune hemolytic anemias[1052] and anemia caused by malaria,[1053] improve burn and wound healing,[1054,1055] and allow improved preservation of isolated organs destined for transplantation.[1055a] DCLHb was investigated in ischemic stroke patients.[1050,1056] Efficacy of DCLHb (administered, however, along with nitroglycerine) in improving cardiac function during percutaneous transluminal coronary angioplasty (PTCA) was demonstrated.[1057] This product was also used to treat hypotensive episodes during hemodialysis, likely by scavenging excess NO.[1058] Oxygen carriers also have the potential to improve treatment of hypoxic tumors, which are resistant to radiation therapy and chemotherapy.[1059−1062] Enhancement of hematopoiesis has been observed with several products.[434,1063,1064] DCLHb enhanced wound healing as well as or better than blood in a rat model of surgical trauma.[1054] Prodrugs with peptides linked to recombinant Hb mutants have been designed.[1065] Hemoglobin can also be used as a nutrient for the cultivation of microorganisms.[1066]

Modified O_2 carriers with antioxidant properties could prove useful in clinical situations involving

ischemia and reperfusion, as they could help protect both the tissues and the protein against reactive oxygen species generated during reperfusion, stroke, myocardial infarction, and other forms of inflammation.[815−817,1067−1070]

H. Impact of Hemoglobin Modification on Physiologic Processes and Side Effects

Modification of Hb was intended for overcoming the changes of in vivo behavior of Hb when no longer confined in the RBC. A given modification can, however, have multiple consequences. For example, changes in oxygenation properties are not necessarily predictive of other functional changes.[870] The impact of Hb modification on toxicity has been reviewed periodically.[159,188,189,237,511,530a]

Cell-free Hb products appear to be extremely apt at defeating the redundancy in O_2 delivery regulation mechanisms that characterizes successful living species. Understanding the mechanism(s) of the hemodynamic perturbations observed with most Hb products, assessing their clinical consequences, and avoiding these effects has become a primary objective in the development of safe Hb-based products, even more so since the recent abandonment of two products in advanced clinical trials. Considerable progress has been made in such understanding in the recent years.

1. Protein Modification Has an Impact on Oxygen Delivery

a. Oxygen Transport Characteristics. Unmodified cell-free Hb does not appear to unload any significant amounts of O_2 when RBCs are present, unless these RBCs are deoxygenated to a high degree.[351] Depending on modification, the O_2 affinity of Hb was increased or decreased (Figure 8). Reactions with deoxyHb usually preserved low O_2 affinity better than reactions with oxyHb. Effective increase of P_{50}, resulting in enhanced O_2 extraction, was successfully achieved by pyridoxalation, carboxymethylation, diverse forms of intramolecular cross-linking, attachment to negatively charged polymers, and genetic engineering. Detailed O_2 binding studies suggested that α,α-cross-linking reduced O_2 affinity by affecting the intrinsic ligand binding properties of the heme rather than by changing allosteric contributions.[741] Modification of Cys-93β usually resulted in increased O_2 affinity.[320,668,673,678,683] Polymerization and conjugation with neutral polymers quasi-generally increased O_2 affinity.[524,577,732,769,790,811,823,828,870] Oxygen affinity increased consistently with degree of polymerization and more so when the reaction was performed on oxyHb rather than deoxyHb. Interestingly, O_2 affinity was also increased by simple, non-cross-linking "decoration" of Hb with glutaraldehyde, indicating that the chemical modification itself, rather than direct conformational freezing by polymerization, may be responsible for the effect.[769] However, functional O_2 affinity was reported to have been preserved in at least one case of glutaraldehyde treatment, where the extent of polymerization may have been relatively low.[794,795] Chemical modification can also result in reduced sensitivity to allosteric effectors.[769,865,879]

Cooperativity was preserved during intramolecular cross-linking with DBBF[710] and DIBS[731] but not with o-raffinose,[528] BME and other cysteine reagents[585,669] and sometimes by pegylation.[867] It was reduced or suppressed by polymerization.[521,528,569,577,769,790,823,870] Effects on cooperativity can occur in opposite directions at different steps of the oxygenation process.[538]

The Bohr effect was sometimes retained[769] but more often reduced or abolished[585,620,743,756,841,870,1071] by cross-linking or polymerization, likely reflecting the degree of participation in the modification reaction of the groups involved in the Bohr effect. Such reduction can compromise the buffering capacity of Hb.

Transport of CO_2 was depressed when the N-terminal amino groups were modified, as by pyridoxalation, carboxymethylation, DBBF-cross-linking glutaraldehyde polymerization, and other N-alkylation and acylation reactions. For example, DBBF-cross-linking reduced by one-half CO_2 binding as carbamino Hb.[743] This and the absence of carbonic anhydrase could affect the acid−base equilibrium in shock patients when large amounts of Hb products are infused.[338]

b. Intravascular Persistence. The circulation half-life of Hb products is generally described by a single number, although most products consist of an array of species with different pharmacokinetics and, possibly, different mechanisms contributing to clearance at different time points. Clearance from circulation is strongly species and dose-dependent.[476,737,994,1040,1071a,1072−1074] It did not always fit an exponential decay model and was sometimes clearly biphasic.[690,728,751,1071a] Many Hb products were found to diffuse out of the vasculature into the interstitial spaces, serous cavities, and gastrointestinal and respiratory tracts, which can translate into significant changes in intravascular volume. Initial rapid loss from circulation was seen with stroma-free Hb,[432] polymerized pyridoxalated Hb,[413] ATP-modified Hb,[414] α,α-DBBF-cross-linked Hb,[188,1075] pseudo-cross-linked Hb,[751] NFPLP-cross-linked Hb,[415] α,α-DBBF cross-linked Hb distributed rapidly in the skin, muscle, and skeleton.[744] Both DBBF-cross-linked Hb and a PEG−Hb were recently found to widen the endothelial gap junctions in the rat mesenteric microvasculature, leading to rapid extravasation.[418,1076] Phagocytosis of modified Hb by macrophages of the RES may also be involved in the clearance process.[1077] Removal of LEHb from the circulation is also bimodal: a rapid removal period (1−4 h) is followed by a slower period (Figure 12) that may be indicative of RES saturation.[428,565]

Intravascular half-life was usually prolonged 2- to 3-fold (typically to 3−4 h) upon intramolecular cross-linking[416,724,725,728,731,737,1078] and up to 10-fold by polymerization,[337,799,1040] conjugation to a polysaccharide,[829,844] or pegylation.[648,1079] For a given type of polymerized product, half-life increased with increasing MW.[799] The longest reported circulation persistence appear to have been obtained with pegylated,[1080] dextran-conjugated,[829] or PEG-coated LEHb

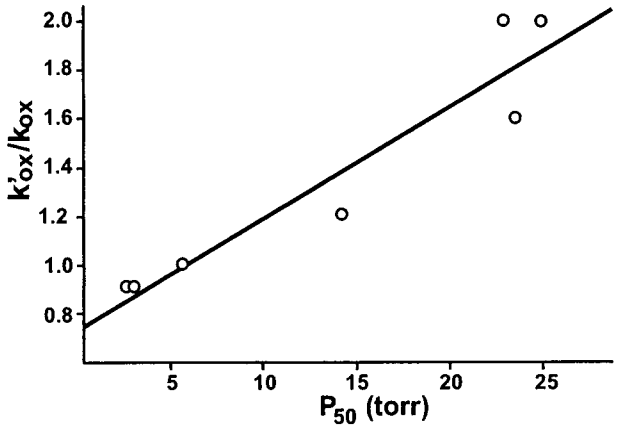


Figure 14. Rates of autoxidation of chemically modified Hbs relative to the rate of autoxidation of HbA (k'_{ox}/k_{ox}) as a function of O_2 affinity (P_{50}). The Hb products were, in increasing order of P_{50} o-raffinose-polymerized oxyHb, DBBF-cross-linked oxyHb, HbA ($k'_{ox}/k_{ox} = 1$), DBBF-cross-linked deoxyHb, Hb reacted with DIBS, o-raffinose-treated deoxyHb, and NFPLP-cross-linked Hb. (Reprinted with permission from ref 1085. Copyright 1995 Intercept.)

products.[999] However, surface modification only increased $t_{1/2}$ to a limited extent, coming nowhere near the intravascular persistence of RBCs.

The in vivo O_2 transport capacity of Hb products can be substantially reduced by autoxidation[275,276,801,968,1270] or, in some cases, by RBC aggregation.[1081] Vasoconstriction, reduced cardiac output, and reduced functional capillary density can further impact on O_2 delivery efficacy (see below). As indicated, autoxidation of Hb and myoglobin tends to increase when O_2 affinity decreases.[1082]

2. Impact of Hemoglobin Modification on Redox Behavior

Again, different modifications of Hb were found to alter Hb's redox chemistry differently. Autoxidation was usually more rapid with cross-linked or polymerized Hb than with native Hb,[259,724,747,759,772,801,870,1083,1084,1084a] although exceptions have been noted.[757] Autoxidation rates were generally inversely proportional to O_2 affinity (Figure 14),[235,245,275,772,870,1082,1085] again with exceptions.[757] The propensity of DBBF-cross-linked Hb for autoxidation is greater than for the native protein and was further enhanced by PEG-polymerization.[870] PEG-decorated bovine Hb was also prone to extensive oxidation.[1086] On the other hand, the rate of autoxidation of the PHP conjugate was similar to that of HbA.[1087] However, this product was found to still contain residual SOD and catalase from the RBC.[1070] Subtle changes in the polarity of and access to the heme pocket of myoglobin resulted in appreciable changes in autoxidation rate.[1082] Oxidation of Hb products with H_2O_2 also strongly depended on type of cross-linking.[753] Ferryl radical formation and stabilization were greatly increased by cross-linking Hb with DBBF between the Lys-99α residues as compared to unmodified HbA but not by cross-linking with the same agent between the Lys-82β residues.[245] The reactivity of ferric Hb with NO was substantially different for α,α-DBBF-cross-linked Hb and a Hb pseudo-cross-linked with fumaryl monodibromoaspirin.[1088] On the other hand, cross-linking of Hb had only minor effects on its peroxidase activity.[530] Glutaraldehyde polymerization enhanced the free-radical reactivity of bovine Hb, while intramolecular pseudo-cross-linking decreased it.[1084] The rate of autoxidation of α,α-DBBF-cross-linked Hb was increased by liposome encapsulation, even with α-tocopherol present in the formulation.[1089] Interactions between the Hb and the liposome membrane may be responsible for this effect.

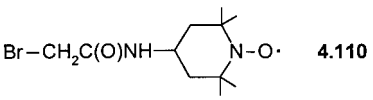
Evidence for oxyradical generation in the blood of dogs hemorrhaged then resuscitated with a stroma-free Hb solution was provided by the observation of greater concentrations of oxidation products of exogenously administered sodium salicylate.[532] Hepatic lesions, similar to those associated with lipid peroxidation syndromes, were seen in pigs exchange-transfused with α,α-DBBF-cross-linked Hb.[1090] Altered redox activity and the stabilization of long-lived ferrylHb species with high redox potential in α,α-DBBF-cross-linked Hb and in a PEG derivative of the former induced cytotoxicity in cultured bovine endothelial cells.[1091] A ferryl-α,α-DBBF-cross-linked Hb-enriched preparation induced morphologic changes and DNA fragmentation in the cells, indicative of apoptotic cell death. Incubation with DBBF-cross-linked Hb of endothelial cells subjected to hypoxia-reoxygenation (a condition that is likely to be encountered during trauma and surgery) caused oxidation of Hb to ferryl species and increased lipid peroxidation.[1092] A new radical was recently detected in the reaction of DBBF-cross-linked metHb with H_2O_2 that was not found with metHbA.[237] Interactions of cell-free Hb and DBBF-cross-linked Hb with H_2O_2 caused alterations in the normal endothelial cell death mechanisms.[273] Hemoglobin's neurotoxicity[188,189,265,266,504,505,1093,1094] could result from the particular sensitivity of cerebral tissue to oxidative damage. On the other hand, no increased free-radical formation was reported in a rabbit model of ischemia and reperfusion upon administration of DCLHb.[1095] The extent of oxidative damage that could be induced by ferrylHb species is still disputed.[189]

Glutaraldehyde polymerization considerably altered the redox potential and autoxidation kinetics of human Hb as well as its stability vis-à-vis heat and urea.[772] Redox potentials decreased with increasing polymerization. The autoxidation rate was 4 times faster than for native Hb. The decrease in redox potential and the increase in O_2 affinity and of autoxidation rates were tentatively explained by an opening of the heme pocket upon glutaraldehyde treatment.[772] Mössbauer and ESR studies of nitrosylHb indicated that polymerization had directly affected the environment of the iron atoms, including by charge transfer from the iron to O_2, changes in bond length, steric hindrance in the heme pocket, and access of this pocket.[1096] After 24 h, metHb accounted for as much as 33% of the plasma Hb in sheep exchange-perfused with a glutaraldehyde-polymerized bovine Hb.[801] Even limited polymerization caused bovine Hb to autooxidize faster and undergo more oxidative damage by H_2O_2 than the native protein.[1084] FerrylHb formation during enzymatic peroxidation was faster with the polymerized material. Differences

were also found in the rate of Hb-catalyzed NADPH oxidation and aniline hydroxylation.[243,1084] A 13-fold increase in hydroxyl radicals in the intestinal effluent was observed after intestinal reperfusion with a polymerized Hb product.[820] Generally speaking, polymerization did not appear to provide increased stability toward autoxidation and oxidative damage, on the contrary.

MetHb levels increased from 6% to about 30% after 24 h in rats exchange-transfused (>90%) with an NFPLP-polymerized Hb,[1097] but partial exchange-perfusion (40−70%) with fully oxidized Hb material showed a gradual decrease in metHb level providing evidence for the existence of an effective reducing system in the plasma. Likewise, DBBF-cross-linked metHb was partially reduced by a suspension of RBCs; addition of ascorbic acid increased the rate and extent of reduction.[1098] In vivo autoxidation of Dex-BTC−Hb was rapid in 50% exchange-transfused guinea pigs, with the metHb level reaching 30−40% before leveling off after about 12 h; partial reduction of >90% oxidized Dex-BTC−Hb was observed when the product was injected to the animals.[1099] Co-injection of ascorbic acid or riboflavin allowed maintaining metHb below 25%.

Concerns have been expressed that cell-free Hb may present a risk for patients with compromised vasculature and antioxidant status.[238] Control of Hb autoxidation was thoroughly investigated. β-Nicotinamide-adenine dinucleotide (NADH) and glutathione provided good antioxidative protection.[564,565] Co-administration of antioxidants (such as ascorbate and mannitol) and natural or synthetic iron chelators (such as deferoxamine, a chelating agent used clinically to treat iron overload) has been recommended.[189,252,531,1099] Ascorbic acid was highly effective in reducing metHb; however, it is not synthesized by humans. Incorporation of ascorbic acid,[570] carbohydrates,[565] homocysteine,[969,971] glutathione,[944] or enzymes[817,966,970,972] along with Hb into liposomes effectively prevented autoxidation. Site-directed mutagenesis provided further means of controlling the redox activity of cell-free Hb.[315,916] Other strategies involved cross-linking Hb with RBC enzymes[815,816,820] and modifying Hb in such a way that it acquires superoxide dismutase and catalase-like functions, as by derivatization with nitroxides.[198,1100] Such nitroxylation was achieved by reacting 4-(2-bromoacetamido)-2,2,6,6,-tetramethylpiperidine-1-oxyl **4.110** with an α,α-cross-linked Hb. About 16 nitroxide groups were distributed across both α and β chains. Stable nitroxides enhance the catalase-like activity of proteins, making them behave as antioxidants and antiinflammatory agents.[1068,1101,1101a] A dextran-conjugated polynitroxide product, free of components with MW around 64 000, with high O_2 affinity, no cooperativity, and no pressor activity was recently reported.[1069]



4.110

The stability of the heme−globin interaction is also affected by chemical modification. Loss of heme from metHb is biphasic.[1102] Both the fast and slow rate constants were 10−20-fold larger for glutaraldehyde- or glycolaldehyde-polymerized Hb than for intramolecularly cross-linked tetramers. The increase in release rate of heme from α,α-DBBF-cross-linked Hb as compared to HbA_0 paralleled the faster autoxidation of this product.[259,533] Incubation of DBBF-cross-linked Hb with liposomes also led to progressive heme degradation.[232] Destabilization of the heme−globin linkage was also observed with pegylated bovine Hb.[1103] Heme release and iron-mediated oxygen radical formation may be major contributors to the endothelial oxidative stress and cytotoxicity generated by this derivative. Elevated liver and pancreatic enzyme levels have been reported with various Hb products that may be indicative of free-radical-mediated tissue injury.[238,304,476,491,528,1056,1105−1108]

3. Impact of Hemoglobin Modification on Hemodynamics

As indicated, most Hb products elicit, even at very low doses, a "pressor effect", which includes increases in mean arterial pressure and systemic and pulmonary vascular resistance, reduction of heart rate and cardiac output, reduction in functional capillary density and tissue perfusion, and failure to increase cardiac output in response to hemodilution (Figure 15). These effects can offset the benefit of higher arterial O_2 content and even decrease O_2 delivery.

Several mechanisms appear to participate in causing the pressor effect, including scavenging of endothelial cell-produced NO and autoregulation processes triggered by excessive O_2 supply and/or reduced viscosity. A number of solutions have been devised in order to attenuate these effects. Whether molecular size increase alone, as by polymerization or pegylation, suffices to suppress Hb's pressor effect remains controversial.

a. The Pressor Effect. The pressor effect has been established in a number of in vitro and in vivo models to be intrinsic to cell-free Hb.[301,304,305,491,1109−1112] It has been observed to various extents upon infusion of virtually all types of Hb products, including diversely purified unmodified human[178,186,304,305,421,489,491,1113−1120] or animal Hb,[356,1120−1124] pyridoxalated Hb,[1114,1120,1125] intramolecularly α,α-cross-linked Hb[167,237,304,416,417,454,493,513,546,1021,1024,1026,1031,1034,1058,1116,1126−1146] or β,β-cross-linked Hb,[728,1118,1147] glutaraldehyde-polymerized Hb,[360,773,801,1041,1148−1155] dextran-conjugated Hb,[845,1143,1156] PEG-decorated Hb,[645,1060] α,α-cross-linked and polymerized Hb,[871] pyridoxalated and polymerized Hb,[1042,1123,1157,1158] o-raffinose-cross-linked and polymerized Hb,[491,513,1037,1074a,1143,1159−1161] carboxymethylated and glycolaldehyde-cross-linked Hb,[1060] pyridoxalated and pegylated Hb,[455,513,645,646,1081,1120,1141,1162−1166] recombinant Hbs,[307,315,462,476,1104,1106,1167,1168] or recombinant and chemically cross-linked or polymerized Hb.[793]

Failure to increase cardiac output upon hemodilution was observed consistently with Hb products,[304,349,352−356,489,511,1158,1169] thus impairing one of themost effective cardiovascular compensatory mechanisms that limits tissue hypoxia in hemodiluted or volume-resuscitated hemorrhagic patients. A drop in functional capillary density was observed upon he-

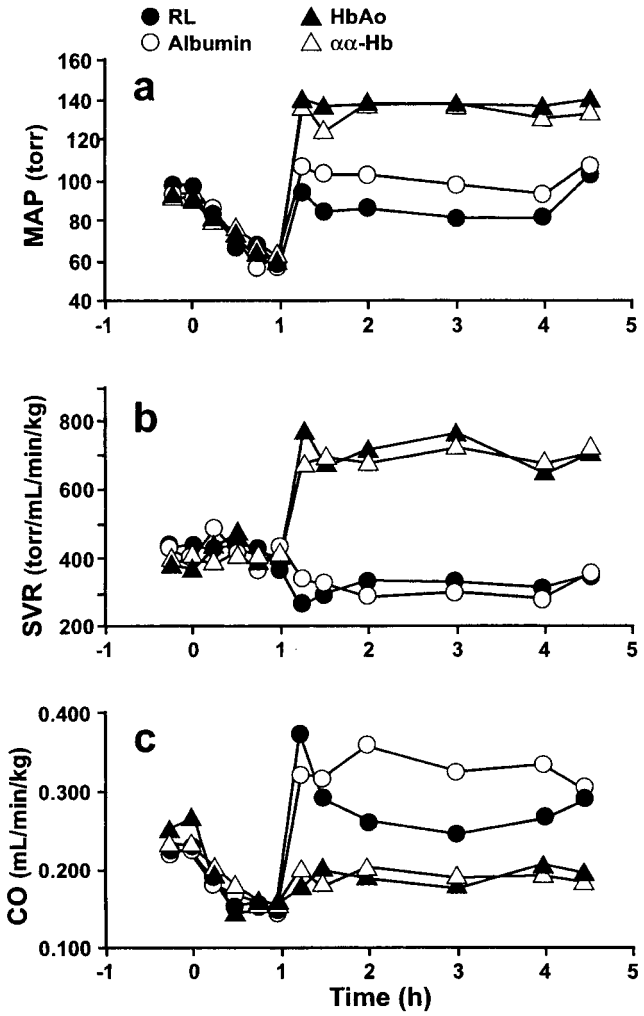


Figure 15. Pressor effect elicited by an α,α-DBBF-cross-linked Hb: (a) mean arterial pressure (MAP); (b) systemic vascular resistance (SVR); (c) cardiac output (CO); RL = Ringer's lactate solution. (Reprinted with permission from ref 511. Copyright Karger 2000.)

modilution with an α,α-DBBF-cross-linked Hb solution, whereas with a simple dextran solution, the number of active capillaries was maintained and dilution was compensated by higher flow rates.[1170] The O_2 partial pressure in the surrounding tissues was less with the O_2 carrying Hb solution than with the dextran solution. The changes of vascular tones can be different for different vascular beds.[1140]

Residual renal impairment, still seen in rats with DBBF-cross-linked Hb[740,1090,1126] and that was not suppressed by subsequent polymerization,[871] was attributed, at least in part, to interaction with NO. Further physiologic responses may be related to the pressor effect. A DBBF-cross-linked Hb affected O_2 delivery in dogs challenged with *E. coli* (possibly by neutralizing the NO normally produced in response to endotoxin challenge),[1171] enhanced platelet deposition in certain animal models, which could result in hypercoagulability and may have clinical implications,[1172] and caused myocardial lesions in various animal species.[1173] Gastrointestinal disturbances (attributed to defects in NO-mediated neuromuscular communication) with DCLHb[1138] and rHb1.1,[1104,1106,1174] and elevations of pancreatic plasma enzyme levels in preclinical and clinical studies with rHb1.1 were reported.[1038,1104,1106]

b. Mechanisms of the Pressor Effect. Several hypotheses of mechanisms are currently defended with compelling evidence and conviction. They differ depending on whether the primary role in the hemodynamic effects is thought to be held by direct scavenging of NO (within the vessel lumen or after crossing the endothelium) or by a largely viscosity-driven mechanism that autoregulates the diameter of precapillary arterioles or by impairment of NO transport as S-nitroxylated Hb. It is likely that these mechanisms are interdependent and all participate in the phenomena to an extent that depends on product and conditions.

The most widely accepted hypothesis is that NO is being neutralized by one or more reactions with Hb (section III.A), especially when the Hb product can leak out of the vascular space, thus gaining access to the basal site of the endothelium where NO is produced.[305,316,493,1028,1088,1111,1119−1121,1124,1128,1130,1136,1137,1146,1159,1175−1179] Binding of NO to Hb occurred at different rates for differently modified Hbs and was usually faster than for unmodified Hb.[271,314,315] Depletion of NO could also occur through reaction with $O_2^{\bullet-}$ resulting from Hb autoxidation.[241,1091]

Abundant data and arguments support Hb inactivation of NO as a primary mechanism in Hb-mediated vasoconstriction.[271] The vasoconstrictive potency of various types of Hb products, including unmodified human or bovine Hb, intramolecularly cross-linked Hb, recombinant cross-linked Hb (rHb1.1), pegylated Hb (PHP), pegylated LEHb, and membrane-polymerized LEHb, was assessed using arterial and venous rings isolated from diverse animal blood vessels.[301,1120,1146,1167,1175,1176,1178,1180] All the products caused the vessels to contract and mimicked the effects of a nitric oxide synthase inhibitor, e.g., *N*-nitro-L-arginine methyl ester (L-NAME). Scraping the endothelium also blocked the pressor effect. Nitric oxide precursors such as L-arginine and NO donors such as nitroglycerin[1128,1130,1181] or inhalation of NO attenuated the Hb-induced pressor effect.[1182,1183] Conversely, the potency of such agents was inhibited by Hb solutions and cGMP was reduced.[305,1124,1140,1175,1180,1184] Both free Hb and metHb were able to scavenge excess NO produced in endotoxin-treated isolated vessels in vitro.[1047] Highly purified ferrous Hbs, including oxyHb and carbonmonoxyHb (but not nitrosylated Hb), elicited dose-dependent contraction of rat aortic rings, while ferric derivatives, including metHb (whose reaction with NO is slower), did not.[305] Access to intact heme appeared to be required. Blocking the Cys-93β residues using *N*-ethylmaleimide did not notably attenuate Hb vasoactivity,[305] while blocking the heme with a cyano ligand did. These results were interpreted to mean that the primary mechanism was heme iron inactivation of endothelial NO and that non-heme interactions with NO did not play a significant role in the vessel model investigated. Many authors believe that extravasation is a prerequisite for Hb-induced vasoconstriction, hence that reduction of vasoactivity can be achieved by increasing the mo-

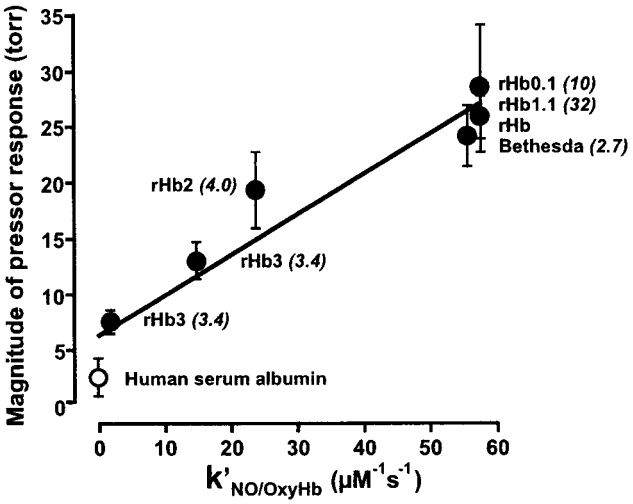


Figure 16. Correlation between pressor response (mean arterial pressure increase) and rate of reaction of NO with oxyHb for a series of identically formulated recombinant oxyHbs with different O_2 affinities (P_{50}). (Reprinted with permission from 315. Copyright 1998.)

lecular size of the product.[190,1178] However, based on the rate of reaction of NO with oxyHb, free Hb should have a 500-fold larger NO scavenging ability than the same amount of Hb contained within RBCs,[308] meaning that NO scavenging by cell-free Hb could be very rapid within the blood vessel lumen and that extravasation may not be a prerequisite to NO inactivation.

A linear correlation was found between the amplitude of the pressor effect (mean arterial pressure increase in rats) and the rate of reaction of NO with a series of identically formulated recombinant Hbs with heme pocket mutations, providing further compelling evidence for a role for NO scavenging by coordination to iron (Figure 16).[315] No correlation was found between pressor effect and O_2 affinity or autoxidation rates, the latter indicating that metHb production (and generation of superoxide) is unlikely to take a significant part in NO scavenging. An rHb mutant with decreased NO reactivity (rHb3011) elicited reduced gastric dysmotility.[915]

In another study, however, comparison of the blood pressure responses elicited in rats by cell-free HbA, DBBF- or trimesoyl tris(methyl phosphate)-crosslinked Hbs, o-raffinose-polymerized Hb, pyridoxalated PEG-conjugated Hb, and pegylated bovine Hb indicated an inverse correlation between pressure response and NO binding affinity and *no* correlation with NO reaction rates.[513] The Hb solutions that exhibited the tightest NO binding were actually those which elicited the lowest increases in blood pressure, leading to the unconventional conclusion that the hypertensive effect could not be the result of NO scavenging at the heme. An inverse correlation between vasopressor response and O_2 affinity was also found. However, the viscosity and other characteristics of the various preparations investigated were significantly different. An autoregulatory response to increased O_2 availability and lowered blood viscosity was invoked to explain these results.[379,516,1029,1170,1185−1187] Indeed, NO production and vascular reactivity depend, among others, on shear stress applied on the endothelial cells, hence on blood viscosity and flow.[514,847,1170,1188−1193] Elevated viscosity increases shear stress, which stimulates release of NO from endothelial cells, resulting in vascular smooth muscle relaxation. On the other hand, excess O_2 availability to the arterioles was demonstrated to produce an autoregulatory reflexive constriction of arterioles (possibly through inhibition of NO synthesis) and a decrease in functional capillary density, thus reducing O_2 availability to tissues.[1170,1194,1195] Vascular hindrance (the vascular resistance/blood viscosity ratio) in hemodiluted rabbits was lower with Hb preparations capable of maintaining blood viscosity.[515] Solutions of Dex-BTC−Hb, o-raffinose-polymerized Hb, and, to a lesser extent, α,α-DBBF-crosslinked Hb all induced an immediate small decrease in arteriolar diameter (mesentery) upon infusion in rats.[1196] These discoveries are important, although it seems unlikely that the observed complex hemodynamic effects can be reduced to solely a mechanical effect.

Further alternative theories to direct NO scavenging include stimulation by Hb of production of endothelin (an endothelium-derived vasoconstrictor),[1028,1124,1128,1197−1199] stimulation of adrenergic receptors,[1031,1131,1179,1200,1201] direct effect on peripheral nerves,[1201] interference with NO transport in the form of S-nitrosylated Hb,[316,317] implication of the platelet-activating factor,[1123] and, in the liver, scavenging of CO.[1202] Survival of hemorrhaged rats administered DCLHb was reduced when the pressor activity was inhibited by an endothelin receptor antagonist.[1028,1198] Differences in pressor activity among Hb derivatives and the relative contributions of these mechanisms some of which are interrelated (for example, NO inhibits the conversion of proendothelin to endothelin), likely depend on Hb modification, molecular size, accessibility of NO to the heme or Cys-93β residues, changes in heme affinity for NO or in thiol reactivity, aptitude to permeate endothelial walls, formulation and rheologic properties, experimental model, dose regime, O_2 demand, patient condition, and other factors. The type and mechanism of vasoactivity of a given product may even differ depending on dose.[1139,1203]

Clearly, despite considerable progress, the mechanism(s) and implications of Hb-induced vasoconstriction are not yet fully understood.[41,42]

c. Preventing Hemodynamic Perturbations by Cell-Free Hemoglobin. The principal strategies investigated for preventing vasoconstriction by Hb products have consisted in increasing the size of the Hb molecule by polymerization or conjugation to a polymer or in encapsulating the protein, so as to reduce extravasation, in reengineering the heme pocket genetically, so as to favor coordination of O_2 over NO, or in increasing solution viscosity, so as to stimulate NO production. Grafting of vasodilatory drugs onto the protein is also being explored.

Endothelial permeability of Hb products depends on molecular mass or size. Intramolecular crosslinking led to marginally decreased permeability; reduction was substantial with pegylation and even more so with liposome encapsulation.[1204] Acellular Hbs moved more rapidly through endothelial layers

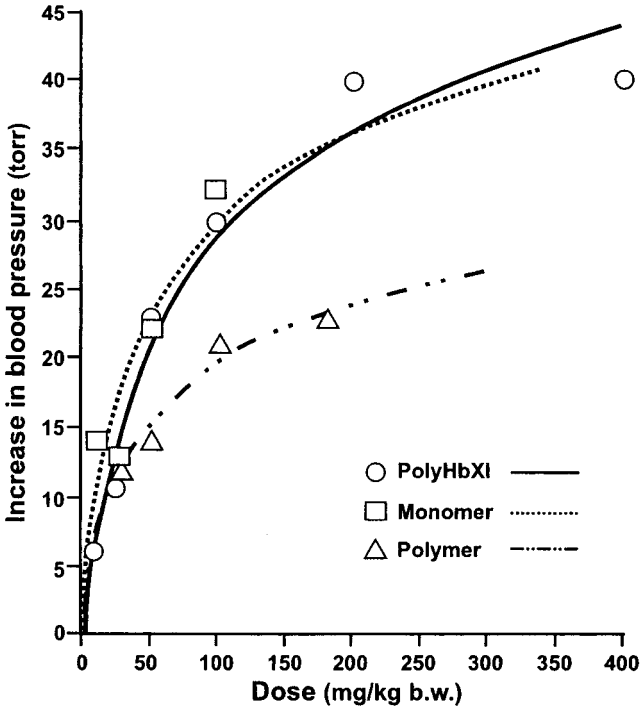


Figure 17. Glutaraldehyde polymerization of Hb reduces but does not suppress vasoactivity. (Reprinted with permission from ref 795. Copyright 1998 Elsevier.)

which had been rendered permeable by pretreatment with cytokine IL-6 in order to mimic a pathophysiological situation found in trauma patients; liposomes with a mean diameter of 200 nm appeared large enough to prevent extravasation even in these conditions.[1178]

Polymerization, by increasing molecular size, was expected to prevent Hb from leaking through the endothelial walls and reaching the NO production sites. The reports on this approach are mixed. On one hand, the glutaraldehyde-polymerized pyridoxalated human Hb *PolyHeme* (section IV.I), which is carefully freed from tetrameric species, was reported not to elicit any vasoconstrictive activity in trauma patients.[1205−1207] On the other hand, virtually all the data from other investigators indicate that polymerization (including another glutaraldehyde-polymerized pyridoxalated human Hb[795]), although it usually reduced vasoactivity, was unable to suppress it entirely (Figure 17).[795,1114] Definite evidence for vasoconstriction was found with polymerized bovine Hb in animal models[801,1208] and in the clinic.[360,1148,1153] o-Raffinose polymerization diminished but did not eliminate systemic vascular resistance and mean arterial pressure increases.[491,513,1143,1159,1161,1209] Likewise for dextran-conjugated Hb[845,1143] and a pyridoxalated PEG-decorated and polymerized Hb.[455,1178] Further purification of an o-raffinose-polymerized Hb, which reduced the proportion of tetrameric Hb species from 32% to about 1.8%, did not reduce systemic vascular resistance further.[491] In a comparative study, a Dex-BTC−Hb and an o-raffinose-polymerized Hb both elicited increases in mean arterial pressure and vascular resistance similar to those elicited by the smaller size tetrameric DCLHb, although with somewhat different rising trends.[1143] Despite the large molecular size of Dex-BTC−Hb, the presence of Hb was detected inside the aortic endothelial cells of guinea pigs exchange-transfused with this product, indicating that an endocytosis mechanism may help Hb cross the endothelial barrier.[1210] Also noteworthy is that the vascular resistance elicited by a nonpolymerized (i.e., "decorated") glutaraldehyde-modified rHb and by a glutaraldehyde-polymerized rHb were similar, although these effects were, in both cases, reduced relative to the unmodified rHb, suggesting that surface modification and/or intramolecular cross-linking rather than an increase in molecular size were responsible for the effect.[793]

In addition, various theoretical arguments have been put forward as to why eliminating the tetramer should not suffice to suppress the pressor effect of polymerized Hb. Why, for example, should bis-tetramers or linear oligomers not be able to cross the same size pores of the vascular epithelium as the tetramer since they have essentially the same cross-section?[1211] Transcapillary exchange of large molecules is only minimally affected by MW in the range from 60 000 to 300 000.[541] Furthermore, ischemia under hypovolemic conditions, as in hemorrhagic shock, and endotoxemia[1212] are known to increase endothelial permeability, which could allow leakage of large molecules. Substantial extravasation of a polymerized pyridoxalated Hb has been observed in a hemorrhagic shock model.[413] According to one hypothesis, the attenuation of the vasoconstrictive activity of Hb by conjugation to a polymer could, at least in part, be due to increased viscosity, which, by increasing shear stress, would increase NO production either directly or by increasing endothelin-1 production.[847]

The importance of the molecular dimension of the O_2 carrier in determining hemodynamic effects, as compared to direct interaction of NO with Hb, has become the latest hot debate in the field. The pressor effect was seen to diminish with increasing molecular size, the least vasoactive products being PEG-derivatized Hbs,[359] but the reason for the variation is not yet well established. Another study compared DBBF-cross-linked Hb, a pegylated pyridoxalated Hb, a HES-conjugated Hb, and a pegylated LEHb with diameters of 7, 22, 47, 68, and 224 nm, respectively, in the hamster skin microcirculation.[1213] The larger the size, the lesser the hypertensive activity and ability were to constrict the so-called "resistance" arterioles, the smallest changes being found for PEG-conjugated Hb vesicles (Figure 18).

The effects of increasing the size of Hb by pegylation are being actively investigated. Pegylation significantly attenuated the effects of bovine Hb on the GI tract.[1079,1214] A pegylated human Hb did not cause any hemodynamic response, although it had the same NO scavenging kinetics as the α,α-cross-linked Hb to which it was compared, which elicited a significant response; however, numerous other parameters were different between the two solutions.[1045] On the other hand, the increase in arterial blood pressure was similar for an mPEG-conjugated pyridoxalated Hb and for nonpegylated Hb in a rat model of hemorrhagic shock.[645] Vasoactivity of various acellular Hb

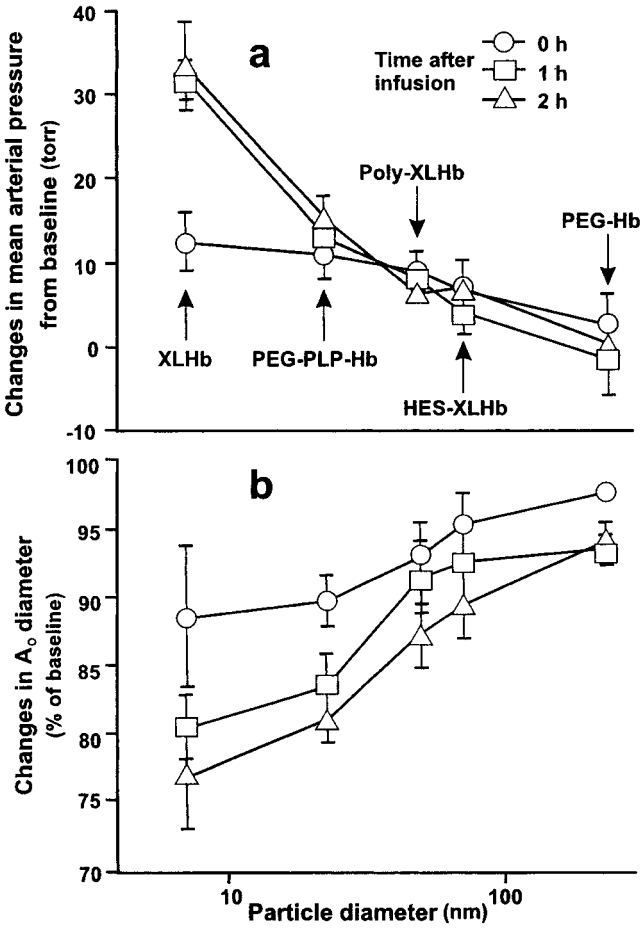


Figure 18. Influence of particle diameters on mean arterial pressure and on diameter of arterioles A_0 for diverse Hb products. (Reprinted with permission from ref 516. Copyright 2000 American Physiological Society.)

products, including a pyridoxalated Hb and a PEG-conjugated Hb, on rabbit aortic strips was almost independent from MW.[1120] Although it is hydrophobic, NO is extremely diffusible and it is unlikely that the hydrated outer layer of a pegylated Hb can slow its diffusion significantly. The rate of NO binding to the oxidized form of a PEG-polymerized, DBBF-cross-linked Hb with MW in the 320−645 000 range was essentially the same as that for the DBBF-cross-linked tetramer.[870] PEG polymerization did not suppress vasoactivity in a hypovolemic rat model, leading to the conclusion that polymerization of Hb may not be an effective strategy in overcoming vasoreactivity.[871] While pegylation is used by some researchers to attenuate vasoconstrictive effects, clinical trials currently investigate a pyridoxalated pegylated Hb product (PHP) for use of its NO scavenging potency in the treatment of NO-dependent shock (section IV.I).[455,1166]

The hypothesis that excessive O_2 delivery elicits an autoregulatory vasoconstrictive response led to the counterintuitive suggestion to use Hbs with higher O_2 affinity (i.e., that normally do not deliver O_2 substantially) and solutions with higher viscosity (i.e., which trigger shear stress-induced NO production but hinder cardiac output and may reduce O_2 diffusion facilitation by cell-free Hb).[380,514,1185] This hypothesis was challenged by the observation that Hb mutants with O_2 affinity so high that they cannot significantly contribute to O_2 delivery nevertheless induced a strong pressor effect.[315] Conversely, no autoregulatory response was reported with PFC-based O_2 carriers, which have little O_2 affinity and provide very high tissue pO_2. However, an mPEG-decorated DBBF-cross-linked Hb, yielding a slightly more viscous preparation, was superior to the non-pegylated product for resuscitating rats from hemorrhagic shock.[1215] The interrelations between size, viscosity, oncotic pressure, and vascular tone control are obviously complex.

Genetic engineering, including substitution in the distal heme pockets of Hb of Leu, Val, and Leu residues by larger, more hydrophobic aromatic Phe or Trp amino acids, allowed selective slowing down of the diffusion-controlled NO scavenging by cell-free Hb resulting in significantly reduced pressor effect.[307,315,908] Restricting access of NO to the heme pocket without also restricting the access of O_2 is quite a challenge as the two molecules have very similar sizes and electronic coating. It is noteworthy that structural differentiation of CO and O_2 binding using polar pocket effects had been demonstrated with model iron porphyrins.[1216]

Encapsulation of Hb was also effective in reducing hemodynamic effects. Encapsulation is expected to hinder diffusion of NO, as is the case with RBC-enclosed Hb.[308] A 30- to 100-fold reduced vasoconstrictive effect on rabbit arterial strips was measured.[1125,1217,1218] No significant hemodynamic perturbations were seen with a LEHb in the isolated rat liver,[1202,1219] or with a lyophilized LEHb in rats,[993] or in awake conscious cynomolgus monkeys following administration of clinically relevant doses of LE-Hb.[1220] Polymerized and pegylated liposome-encapsulated Hb products were significantly less vasoactive than acellular Hb products on rabbit aortic strips and in in vitro coronary perfusion experiments.[1120,1221] Are vesicles large enough to undergo hydrodynamic separation under vascular flow conditions, resulting in an Hb-free zone near the vessel wall in which NO's vasodilatory action would not be inhibited? Such a mechanism has been proposed to contribute to the reduced consumption of intraluminal NO by RBCs.[308,1222]

Further approaches to reducing vasoconstriction included cross-linking Hb with adenosine (which is known to have vasodilatory properties),[806,809] grafting nitroxides onto Hb (turning it into a vasodilator, antioxidant, and antiinflammatory agent),[1069,1101a] and cross-linking Hb with Trolox (a vitamin E analog) to provide antioxidant activity.[1223]

4. Impact on the Reticuloendothelial System

Clearance of excess cell-free Hb from circulation is primarily effected by the RES, principally the liver.[223] The extent and mechanism of removal of modified Hb is still largely unknown. The pharmacokinetics of Hb solutions are complex because most products consist of mixtures with each individual component having its own actions and rates of disappearance and because many components can leave the vascular space by several pathways and

diffuse into the interstitial and lymphatic spaces.[188,414,415,417,418]

Accumulation in the liver and spleen was seen with cross-linked Hb,[415,416] raising the possibility of depressed host defense mechanisms. No such depression was found in rats submitted to septic challenge 5 days after treatment with a pyridoxalated Hb.[423] On the other hand, the capacity for Hb to enhance bacterial growth has been repeatedly reported (section III.E). Perturbation of liver function was evoked, possibly as a consequence of the disposition of nonphysiological amounts of Hb and of vasoconstriction consequent to scavenging of carbon monoxide,[1202] a product of Hb degradation to biliverdin by heme oxygenase that appears to participate in liver cell function regulation.[1224]

Liposome-encapsulated Hb is captured by the RES, which may cause dose-dependent transient blockade of the RES, comparable to that seen with empty liposomes.[399,427,428,1225] Histopathologic studies in rats confirmed that liver and spleen are principally involved with LEHb removal, resulting in transient alterations in the weight and structure of these organs.[942] The presence of LEHb in vacuoles in fixed phagocytes was seen, which declined after 24 h, but still remained significantly above controls at 2 weeks. Lyophilized LEHb provoked further effects, including pulmonary infarcts, i.e., embolic injuries that may be associated with trapping of large particles or agglutinated LEHb. The highly species-dependent, complement activation-mediated side effects by LEHb, including impairment of cardiopulmonary function, and their clinical relevance have recently been discussed.[952] As for other particulates, the visibility of LEHb to the RES can be reduced by pegylation.

5. Miscellaneous Effects

Hemoglobin and certain Hb products have been associated with thrombocytopenia, platelet activation and deposition, and accelerated RBC aggregation.[836,1081,1172,1226−1232] A glutaraldehyde-polymerized Hb induced hemorrhagic lesions;[794] another caused RBC aggregation.[1230] Removal of NO (which inhibits platelet aggregation), generation of free radicals, and Hb-catalyzed metabolism of arachidonic acid were suggested as possible mechanisms for these effects. Incubation of white cells with purified cell-free Hb led to increased procoagulant activity in mononuclear cells[497] and to the release of inflammatory cytokines, interleukin-8, and tumor necrosis factor that caused leukocyte migration and adherence to umbilical vein endothelial cells.[498] Denaturation of Hb may cause white cell activation.[535] The capacity of Hb products to stimulate bacterial growth and amplify the response of macrophages to endotoxins has been reviewed in section III.E.

The effects of modification on Hb's neurotoxicity have not yet been fully assessed.[188,504,1233] The nervous system may be particularly at risk of peroxidation damage as it contains large proportions of polyunsaturated lipids. When mixed cultures of neurons and glial cells were exposed to purified cell-free Hb, the neurons were killed while the glial cells remained intact.[503] Low amounts of α,α-DBBF-cross-linked Hb were highly toxic to cortical cell cultures.[504] The effects were dose dependent and could be reversed by administration of an antioxidant or an iron chelator. Large infarcts were observed when Hb was injected in the hipocampus of gerbil brain, indicating deleterious effects in the central nervous system.[1233] Subarachnoid injection of autologous hemolysates resulted in DNA fragmentation and cell death.[505]

Myocardial lesions, characterized by cell degeneration and necrosis, have recently been reported to occur in certain animal species, primarily pig and rhesus monkey, upon infusion of DCLHb and other Hb or polyHb solutions.[1173] These lesions were also observed after administration of an NO synthase inhibitor and were decreased for rHbs having reduced rates of interaction with NO (rHb 3011), indicating a relation with NO scavenging.

A reduction in plasma volume was observed following administration of DBBF-cross-linked Hb,[1045] which could result in a dehydration effect, an outcome that is not desirable in hemorrhagic patients.[511]

I. Commercial Development of Hemoglobin Products

This section briefly summarizes the various Hb products that went into commercial development in the recent years. At this point, the cost of the different O_2 carriers remains difficult to project, as many issues, especially those concerning raw materials and large-scale manufacturing, are not yet fully resolved. The information available on the status of ongoing clinical trials has recently been reviewed.[1234]

1. Diaspirin Cross-Linked Human Hemoglobin—HemAssist

A DBBF-cross-linked Hb product was prepared from outdated banked human blood by Baxter Healthcare Corp. and became known as DCLHb (Diaspirin Cross-Linked Hb). The product, after formulation as a hyperoncotic (42 Torr), 10 g/dL solution, was trademarked *HemAssist*. The main difference between the Baxter and Letterman Army Institute products appears to be in the use of Ringer's lactate versus Ringer's acetate solutions for formulation, respectively. Since extensive preclinical and clinical investigation consistently documented a significant pressor effect, the possibility of using this feature as an advantage for treating critically ill hypotensive patients was investigated.[545,546] The combination of O_2 carrying capacity, volume expanding effect, and ability to restore blood pressure was expected to participate in hemorrhagic patient resuscitation.

Preparation and characterization of α,α-DBBF-cross-linked Hb[710,738,1235,1236] (and DCLHb)[739,789,1237−1239] is well documented. Tripolyphosphate was used in lieu of 2,3-DPG to maintain Hb in its deoxy form and block the Lys-82β site during the cross-linking reaction. Ultrafiltration was used at several stages of the process to remove viruses. Denaturation and precipitation of non-cross-linked Hb and other proteins was achieved by heating. Extent of cross-linking in the final preparation was superior to 98%; metHb content was 2−5%, and P_{50} reached 32 ± 3 Torr.[739] A heat treatment step for virus inactivation was included (60 °C for 10 h or 74 °C for 90 min).[536,537,738,1240] The

Table 3. Characteristics of *HemAssist* (DCLHb 10%); Reprinted with Permission from ref 739

parameter	typical value at batch release
total Hb concentration (g/dL)	9.5−10.5
metHb concentration (%)	2−5
pH at 37 °C	7.3−7.5
extent of cross-linking (%)	>98.0
total yield[a] (%)	55
P_{50} Torr (37 °C)	32 ± 3
osmolarity (mOsm)	285−310
colloidal osmotic pressure (Torr)[b]	42
endotoxin (EU/mL)	<0.06
sterility; rabbit pyrogen test	Pass

[a] From ref 511. [b] From ref 1033.

final product, DCLHb (Table 3), was filter-sterilized, filled into poly(ethylene-vinyl acetate) bags, and frozen at −20 °C for storage. It was to be thawed at user point.

DCLHb consisted of a family of proteins, first because Hb itself is microheterogeneous, then because DBBF, although it reacts predominantly with the Lys-99α residues, can additionally react at other sites on the protein (section IV.D). Multiple attachment of the reagent on a same Hb molecule can also occur, and some intermolecular cross-linking was seen.[739,1241] The product was also heterogeneous in terms of net charge. Autoxidation of α,α-DBBF-cross-linked Hb was more rapid than for the native Hb,[237] yielding 21% metHb after 5 h at 37 °C,[1083] and depended strongly on freezing/thawing conditions.[269] DCLHb was also more susceptible to oxidation by H_2O_2 than unmodified Hb, releasing iron, which could promote the formation of oxygen radicals.[753] Filtration of DCLHb into lymph was 2−3 times faster than for an albumin solution of similar COP, possibly due to pressor activity.[417]

Relatively small doses of DCLHb consistently produced a rapid and significant (20−30 Torr) increase in mean arterial pressure and systemic vascular resistance and a depression of cardiac output in essentially all animal species investigated, as well as the contraction of isolated blood vessels. Investigation of the pressor effect's mechanism indicated that this response is most likely multifactorial (section IV.H). Improved tissue oxygenation and reversal of base deficit by DCLHb solutions was demonstrated in a range of circumstances.[167,454,1050,1132,1198,1242−1247] Efficacy as a resuscitation fluid was reported in various hemorrhagic shock models.[294,739,1021−1024,1026,1030,1033,1034,1133,1135,1248−1250,1251a] Improved survival was reported in septic rats treated with DCLHb.[1251] Redistribution of blood flow to vital organs was observed.[1129,1247,1251,1251a] Attenuation of postischemic reperfusion injury in skin muscle has been reported.[1142] On the other hand, greater pulmonary contusion lesion size and stiffer lungs were observed after treatment with DCLHb, as compared to saline, in a porcine model of pulmonary contusion.[1251d] Clinical data indicated a reduction of allogeneic blood transfusion with *HemAssist* in cardiac surgery patients.[1145,1252]

The redox chemistry of DBBF-cross-linked Hb has recently been reviewed.[237] The modified Hb tended to be more cytotoxic to endothelial cells in vitro than native Hb, likely because of the generation of a long-lived ferryl species and suppression of protective pseudo-peroxidase activity.[1091] Alterations of redox behavior in vivo[237] and increased sensitivity to endotoxins[448,1253] were also discussed. Defective thromboregulation, with enhanced platelet deposition on injured blood vessel surface, was observed in a surgical rat model and determined to occur via NO scavenging.[1172] As mentioned earlier, myocardial lesions were seen consistently in certain animal species.[1173]

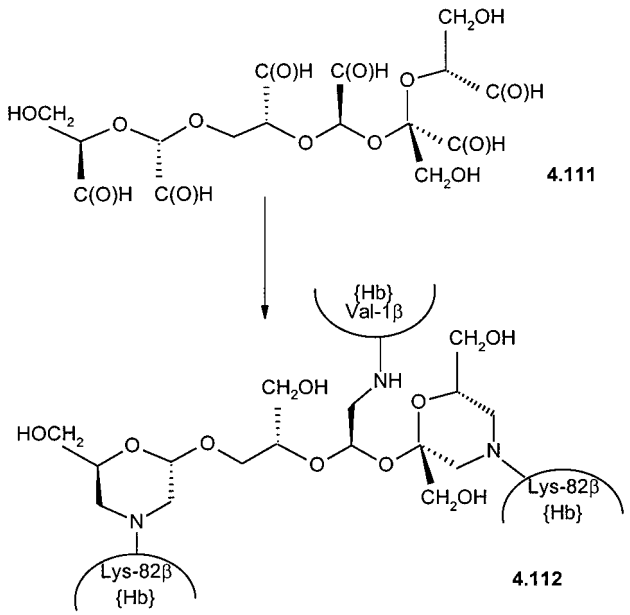
No significant adverse events or toxicities, except for the expected immediate increase in blood pressure and, in some patients, for transitory abdominal pain, were reported following a Phase I safety study of *HemAssist* in healthy volunteers[1138] or renal dialysis patients.[1028] A circulation half-life of 2−4 h was reported for 25−100 mg/kg b.w. doses. Subsequent safety and efficacy studies uncovered no significant side effects either.[546,1252,1254] No immunogenicity was found.[437] Several randomized multicenter clinical studies in various patient populations with doses up to ∼1.5 g Hb/kg b.w. of *HemAssist* confirmed the anticipated arterial pressure and systemic vascular resistance increases, and some blood saving was reported.[546,1145,1252,1255,1256] There were, however, also indications of depressed cardiac output and decreased O_2 delivery.[1257] The trials and development of *HemAssist* were terminated in early 1998 after drug-related serious adverse events including brain and pulmonary edema, pancreatic insufficiency, myocardial ischemia, and death were reported following use of DCLHb in patients with acute ischemic stroke,[1056,1211] and analysis of data from a Phase III trial enrolling 112 traumatic hemorrhagic shock patients in 17 trauma centers revealed serious adverse effects and higher mortality in the *HemAssist* patient group versus control group.[1144,1258]

A recent hindsight analysis concluded that the failure of HemAssist was predictable on the basis of preclinical testing but that other Hb-based products could still be successful provided the fundamental mechanisms of physiological effects such as the vasoactivity of Hb, interaction with bacterial infection, the endothelium, and the blood-brain barrier are better understood.[511]

2. o-Raffinose Cross-Linked and Polymerized Human Hemoglobin—Hemolink

Hemolink is produced by Hemosol Inc. (Toronto, Canada) from outdated human blood using o-raffinose **4.111** as an intra- and intermolecular cross-linker.[527,528,776,1259] The Hb resulting from cell lysis is converted to COHb, pasteurized, filtered, and chromatographed on both anionic and cationic ion-exchange columns. The reaction with o-raffinose is performed on deoxyHb in view of favoring the formation of a stabilized tetramer with low O_2 affinity. Excess o-raffinose is used, which also causes some intermolecular cross-linking to occur, leading to multiple Schiff bases, within and between individual Hb molecules. These Schiff bases are reduced with dimethylamine borane. The final product consists of

Scheme 12



about 33% cross-linked tetramers and 63% oligomers having MW comprised between 128 000 and about 600 000.[491,528] The primary sites involved were the Val-1β and Lys-82β residues of both β chains; modification of the α chains was observed to occur at later time points.[528,1074] Evidence for the formation of cyclic tertiary amines has been found, indicating further complexity. An example of a possible structure, **4.112**, with the o-raffinose residue in the tetramer's DPG-binding pocket is shown in Scheme 12.

Hemolink is formulated as a 10% w/v polymerized Hb solution in Ringer's lactate, has a P_{50} around 34 Torr, a COP of 24 Torr, and no cooperativity. Its shelf life was reported to be up to 1 year at 4 °C when in the deoxy form. A composite circulation half-life around 7.5 h was determined in the rat.[527,1074] Separation of fractions with diverse MW allowed measuring half-lives of 3.5 h for the tetramer, 9 h for a 128 000 fraction (Hb_2), and 12−15 h for a 192 000−576 000 fraction (Hb_{3-9}). Cross-linking effectively prevented renal filtration. The product became mainly concentrated in the liver, spleen, and kidneys and was eventually excreted in the urine.[527,1074]

Preclinical studies involving massive infusions of *Hemolink* to rats and dogs resulted in no death or adverse effects on renal function.[491,1259] Changes in blood and urine chemistry and in liver enzymes and creatinine clearance were all reversible. Little or no effect on the human immune or blood coagulation systems was seen.

Increases in mean arterial blood pressure and systemic vascular resistance were seen in several animal models.[1074,1161,1209,1260] These effects were comparable to those caused by unmodified Hb in the hemorrhaged hypotensive rat,[1159] while they were significantly less in 20% exchange-transfused rats.[491] A greater pressure rise was elicited in hypertensive rats but could be attenuated with anesthesia or treatment with antihypertensive agents.[1161] Effects on intestinal motor function were similar to those caused by an NO depleting agent and could be attenuated by administration of an NO donor.[1160]

A single-dose, blinded, randomized, placebo-controlled Phase I dose-escalation safety study conducted with healthy volunteers (up to 0.6 g/kg b.w.) demonstrated only a modest, dose-dependent increase in blood pressure and a decrease in heart rate.[528,1074] Small changes in liver and pancreas enzymes were noted. Average plasma half-life was around 5 h at 0.1 g/kg and 14 h at 0.5 g/kg (7.4 and 18.4 h for tetramers and oligomers, respectively). Renal functions were normal, and there were no blood coagulation anomalies or evidence for complement activation or immune response.

Adverse effects, none of which were considered life-threatening, included "moderate to severe" gastrointestinal pain, with dysphagia, dyspepsia, and abdominal cramps serious enough to interfere with normal daily activity, and mild to moderate genitourinary discomfort.[528,1074,1261] These effects were suggested to arise from smooth muscle spasm related to NO scavenging.

Multicenter, placebo-controlled Phase II trials demonstrated safety as direct blood replacement in orthopedic surgery. A further Phase II study in cardiac surgery involved patients undergoing coronary artery bypass grafting and intraoperative autologous donation (IAD).[1259] Increased blood pressure was again noted. An appearance of jaundice, probably related to high levels of bilirubin associated with metabolic clearance, was observed in a large proportion of patients. No significant coagulation disturbances or changes in hepatic enzyme levels were reported. The Company recently announced completion of a Phase III trial in patients undergoing coronary artery bypass surgery with ANH in Canada and the United Kingdom; avoidance or reduction of allogeneic blood transfusion was demonstrated.[1262] Phase III studies have begun in the United States.

3. Glutaraldehyde-Polymerized Bovine Hemoglobin—Hemopure

The glutaraldehyde-polymerized bovine Hb currently being developed by Biopure Corp. (Cambridge, MA) under the trade-name *Hemopure* is destined to serve as an O_2 carrier and plasma expander; its vasoconstrictive activity is also expected to contribute supporting blood pressure.[1108] The manufacturer specifies that it sources its raw material only from cattle from bovine spongiform encephalopathy-free regions of the world. Bovine Hb purification prior to modification includes ion-exchange HPLC. The polymerization procedure was not disclosed. The product, also known as HBOC-201, is "highly" polymerized (>95%), consists of a family of oligomers ranging predominantly in MW from 130 000 to 500 000, and is formulated as a 13 g/dL concentrated solution in a Ringer's lactate-type solution. Chemical characterization is limited to the mention of the expected modification of ϵ-amino groups of lysines by glutaraldehyde. There is no indication that the product is less heterogeneous than usually obtained by random reaction of this cross-linker with the protein. *Hemopure* has a very low O_2 affinity (P_{50} = 38 Torr) and a shelf life of 2 years. *Oxyglo$_2$bin*, a less refined version of this polymerized Hb (the yield with respect

to the starting material is 2.4 times larger than for *Hemopure*), is now commercially available for veterinary use. However, the product was reported to elicit a pronounced vasoconstrictive action and was ineffective at restoring O_2 delivery as compared to blood transfusion in a canine model of acute hypovolemia.[1263] A recent study confirms that glutaraldehyde polymerization of bovine Hb alters its allosteric mechanisms and increases its autoxidation rate.[1084a]

The O_2 delivery efficacy of *Hemopure* was demonstrated in diverse animal models,[1108] including hypotensive hemorrhagic shock,[1264,1265] profound isovolemic hemodilution,[1043] augmented preoperative autologous blood donation,[1042] near complete blood exchange,[1152] or hemodilution followed by artificial arterial stenosis.[1266] Its capacity for reducing the hypoxic regions of tumors (for the purpose of sensitizating tumors to radiation therapy or chemotherapy) depended on tumor model and antitumor agent.[1267,1268] Increased O_2 extraction and improved tissue oxygenation were generally attributed to the product's right-shifted O_2 dissociation curve.

Some papers reported significantly increased arterial pressure and systemic and pulmonary vascular resistance upon administration of *Hemopure*,[801,1150−1153,1155,1265,1266] others the absence of such effects.[355,1043,1264,1269] Alteration of the metabolism of the heart accompanied by increased O_2 consumption was noted in dogs.[1155] Conversion to metHb in vivo was rapid.[801]

Adequate oxygen transport and delivery were achieved with bovine Hb products in rats[1041] and sheep[355] in the near complete absence of RBCs. No unwanted side effects were seen on the microcirculation of striated skin muscle of the hamster.[1149] However, when used for isovolemic hemodilution of dogs in a range of clinically relevant hematocrit values down to 10%, the product, due to a significant decrease in cardiac output, failed to improve systemic O_2 delivery in comparison with a non-O_2-carrying HES solution.[1151] Actually, O_2 delivery was significantly lower in the treated animals until hematocrit reached 10%, indicating that the immediate increase in vascular resistance and marked decrease in cardiac output observed upon infusion outweighed the benefit of substantial additional O_2 content. Oxygen consumption was maintained because of a high extraction ratio.

A transient increase in total peripheral resistance and decrease in heart rate was observed during a dose-escalation Phase I safety study (doses up to ~0.6 g/kg b.w.) in healthy volunteers.[1150] A small clinical study of HBOC-201 in liver resection patients (0.4 g/kg b.w.) indicated increased arterial blood pressure and systemic vascular resistance and reduced cardiac index and O_2 delivery.[1153,1270] The product was otherwise well tolerated.

When subjected to a bicycle exercise experiment, healthy human volunteers phlebotomized of about 150 g of RBC Hb, who had received *Hemopure*, experienced greater O_2 uptake and CO_2 production and lower lactate levels as compared to controls who had received their own blood back.[1148] Since exercise capacity was maintained at a comparable level in both groups, the product was estimated to be about 3 times more efficient than RBCs on a gram-for-gram basis. A transient increase in peripheral vascular resistance and a decrease in heart rate and cardiac index were again noted. Phase II trials conducted in various populations of surgical patients revealed transient jaundice, elevation in liver enzyme levels, and gastrointestinal disturbances, but these side effects were not considered serious.[438,1108] A small study in sickle cell patients not in crisis uncovered no safety issues.[1051]

Contradictory reports were published on the product's efficacy in the clinic. A small study in aortic surgery patients resulted in avoidance of allogeneic blood transfusion for 27% of the patients, although it did not reduce total transfusion needs.[1154] On the other hand, no demonstrable benefit to the patients over hemodilution with a HES solution was found in patients undergoing preoperative hemodilution for abdominal aortic surgery.[360,912] Arterial O_2 content was maintained at levels higher in the HBOC-201-treated group of patients (0.9 or 1.2 g/kg b.w. doses),[360] but this advantage was offset by significant increases in systemic and pulmonary vascular resistance and a decrease in cardiac output, leading to decreased O_2 delivery.

Clinical experience with *Hemopure* had, by the end 1999, involved over 500 patients who were exposed to up to about 3.5 g/kg b.w. of the product over a period of 6 days. Reduced need for allogeneic blood was reported. Only some nonspecified "low" antigenicity was mentioned to have been observed during these trials, which was not considered to raise safety issues.[438] Further investigations seem to be in order to determine these antigenic responses exactly. A multicenter, single-blind Phase III study in orthopedic surgery patients has recently been completed, efficacy was demonstrated, but the safety results have not yet been announced. The use of bovine material in humans is still overshadowed by the possibility of interspecies transmission of diseases, including spongiform encephalitis,[43,44,49] whose agent could be transmitted by transfusion,[45] appears resistant to thermal treatment, and could stay dormant for many years.

4. Pyridoxalated Polymerized Human Hemoglobin—PolyHeme

The pyridoxalated and glutaraldehyde-polymerized human Hb-based product that is being developed by Northfield Laboratories Inc. (Evanston, IL) under the tradename *PolyHeme* is intended to serve as an alternative for blood in surgery and trauma.[190,1205] The suppression of Hb's vasoactive activity was listed among the desirable goals. Although details are scarce, the product's preparation is undoubtedly based on this group's extensive experience with Hb polymerization.[521,1271] The Hb obtained from outdated human banked blood is first pyridoxalated, then glutaraldehyde-polymerized. A further important step is the removal, presumably by chromatographic fractionation, of any nonpolymerized tetramer in view of preventing extravasation and reaction with

NO. In the absence of published information, one can only conjecture that the product consists of a highly heterogeneous mixture, in line with the standard published outcome of successive Hb pyridoxalation and glutaraldehyde polymerization reactions.[524,705,769] A unit of *PolyHeme* consists of 500 mL of a 10 g/dL solution of the modified Hb (MW 128 000−400 000; less than 1% residual tetramer) that was reported to have a COP of 20-25 Torr, a P_{50} of 28−30 Torr (the process improvements which allowed P_{50} to raise from 18 to 22 Torr[1272,1273] to 28−30 Torr[190,1274,1275] were not publicized) and metHb < 3%.[190,1275] *PolyHeme* can be stored for at least 1 year at 4 °C.

Preclinical efficacy studies in baboons at near zero hematocrit indicated that the product supported life better than nonpolymerized pyridoxalated Hb in such extreme conditions.[354,366,1276] No hemodynamic effects were noted.

No significant safety issues, vasoconstriction, or other adverse effects were reported following Phase I and Phase I/II clinical safety studies conducted in healthy volunteers and trauma or surgical patients.[190,1205,1277] Efficacy assessment was primarily based on measurement of Hb concentration. A small randomized trial in acute trauma and urgent surgery patients who received an average 4.4 units of *PolyHeme* (about 3 g/kg b.w.) indicated a reduction in use of allogeneic blood.[1205] Two Phase II trials in trauma and urgent surgery involved about 150 patients. A few patients with very low Hb levels received high doses of the product and demonstrated improvement of their condition, but massive transfusion of allogeneic blood could not be avoided.[1207] Randomized Phase III studies in elective aortic surgery with up to six units of *PolyHeme* are in progress.

5. Polyethylene Glycol "Decorated" Hemoglobin

A pegylated bovine Hb is being developed by Enzon Inc. (Piscataway, NJ) using a succinimidyl carbonate-PEG methodology[648] to graft mPEG chains at the surface of the protein. The sparsely documented 5-day/33% overall yield processing cycle[1278] yields a product in which each Hb molecule is derivatized at multiple sites with about 12 PEG strands of MW 5000 each, leading to a total MW of about 125 000, i.e., with about equal amounts of Hb and PEG.[1079] Extensive hydration of the POE chains resulted in greatly increased molecular size of Hb and very high COP.[529] Autoxidation was significant, leading to 53% metHb after 10 h at 37 °C.[1103] The product could, however, be stored for greater than 18 months at −20 °C without significant autoxidation.[276] This Hb, which is not cross-linked, neither intra- nor intermolecularly, was strongly destabilized with respect to heme transfer from Hb to serum albumin, especially when Hb concentration was low.[1103] P_{50} was strongly dependent on temperature and Hb concentration. It was suggested that PEG chains could access the central cavity of the bovine Hb tetramer through binding to Lys-81β and disrupt interdimer interactions. The pegylation procedure was also expected to reduce the immunogenicity of bovine Hb.[539,1103]

A 6 g/dL solution of this pegylated bovine Hb was evaluated in various animal models.[1079,1279−1281] A very significant increase in circulatory half-life with respect to unmodified bovine Hb was demonstrated, and some of the adverse effects, including vasoconstriction, were substantially reduced. The PEG−Hb product still inhibited the vasorelaxation effect of acetylcholine and NO donors in isolated aortic strips.[1120] Rapid extravasation from the intestinal mucosa capillaries, by a mechanism that includes widening of interendothelial gaps, and inflammation were observed in rats, potentially compromising the transport of nutrients and drugs between blood and tissue.[418,1076] The product allowed resuscitation of hemorrhaged dogs and piglets[539,1282] and improved survival of 80−85% exchange-transfused rats and piglets.[1279,1283] Increased tumor oxygenation and enhanced radiation sensitivity were demonstrated in rodents.[1060,1062] The product is currently undergoing clinical trials as a radiosensitizer for use in radiation treatment of hypoxic tumors.

6. Polyethylene Glycol-Cross-Linked Pyridoxalated Hemoglobin (PHP)

In a procedure initially utilized for pegylating albumin,[854] developed by Ajinamoto (Kawasaki, Japan) for Hb and now licensed to Apex Bioscience, Inc. (Durham, NC), a pyridoxalated Hb made from outdated human RBCs was decorated and cross-linked intra- and intermolecularly using a difunctional PEG ester, namely, the *N*-hydroxysuccinimide ester of an α-carboxymethyl, ω-carboxymethoxy-PEG **4.28** of MW 3600.[525,526,646,1284,1285] Acylation occurred on a number of amino groups on both α and β chains. The half-life in circulation of the conjugate increased with the number of PEGs attached, while P_{50} decreased. An average six PEGs per Hb was deemed an acceptable compromise. Adjustment of COP was achieved by controlling the degree of intermolecular cross-linking. In an early version the product consisted of about 83% monomers, 12% dimers, and 4% trimers, had an average six PEG strands per Hb, and was described as $Hb(PLP)_3(POE)_6$.[1286] The present pyridoxalated-hemoglobin−polyoxyethylene conjugate (PHP) is highly heterogeneous, has a weight average MW of about 187 000 with two main distributions around 106 000 (∼70%) and 300 000 (∼30%), and an average 3.3 pyridoxal phosphate groups and 5 PEG strands per Hb molecule.[526] It has a large hydrodynamic radii of approximately 7.2 nm. Some catalase, superoxide dismutase, and carbonic anhydrase from the original RBCs are maintained, which are also derivatized with PEG and sometimes bound to Hb through PEG cross-links. The product thus retains some antioxidant activity and resistance to oxidative modification.[1070] The injectable product is formulated as 6 and 8 g/dL Hb solutions that are stored frozen.

The O_2 transport efficacy of PHP has been established in various models.[1284,1286,1287] However, in a subsequent study of resuscitation after hemorrhagic shock in dogs, transient restoration of hemodynamic parameters with PHP solutions was followed by a large decrease in O_2 delivery, and massive use of PHP for resuscitation from hemorrhagic shock was not recommended.[1081] Aggregation of red cells and platelets and thrombocytopenia were observed in vitro.[1231]

Vasoconstrictive activity was demonstrated in animals and in heart perfusion experiments.[455,1162,1163,1178] The product restored cardiac index and small-bowel blood flow in a swine hemorrhagic shock model; however, O_2 delivery was decreased compared to blood and gastric mucosal pH remained low, indicating possible ischemia.[1141] No renal [1288] or hepatic[1289] toxicity were found.

As PHP strongly increased blood pressure, which was assumed to relate to NO scavenging, it was targeted for treatment of septic shock patients.[294,455] The systemic inflammatory response associated with septic shock leads to NO overproduction and subsequent loss of vascular tone. Because of its ability to neutralize NO, it was hypothesized that PHP could "mop up" this excess NO and restore blood pressure in NO-induced shock patients.[455,1070,1290] Reaching the NO production sites should be facilitated under some pathophysiological conditions when endothelial barrier functions are deteriorated.[1178] Continuous infusion of low doses (0.1 g/kg b.w.) of PHP restored mean arterial pressure in a septic sheep model.[455,1290] An increase of pulmonary arterial pressure and pulmonary vascular resistance but no impairment of organ or RES function were seen at these doses. However, treatment of endotoxemic swine with PHP, while improving mean arterial pressure, significantly exacerbated endotoxin-induced pulmonary hypertension and arterial hypoxemia.[1165]

Currently, PHP is in clinical trials for treatment of NO-dependent shock.[1070] The product showed no significant effect on blood pressure in a Phase I study in healthy volunteers at doses up to 0.1 g/kg. Similar doses of drug were reported to restore blood pressure, increase systemic vascular resistance, and decrease heart rate in septic shock patients. A Phase II continuous injection trial in such patients (up to 2.5 g/kg) concluded that PHP is indicated for the treatment of this type of shock. The vasopressor activity of PHP was deemed consistent with an NO-scavenging mechanism.

7. Recombinant Mutant Human Hemoglobin from E. coli—Optro

A variant of human Hb (rHb1.1, trademarked *Optro*), whose O_2-binding characteristics were close to those of Hb in human RBCs, has been successfully cloned, expressed, and purified by Somatogen, Inc. (now Baxter Hemoglobin Therapeutics, Boulder, CO).[473,476,888] In this variant, the two α globin chains were fused together by a glycine bridge located between the C-terminal arginine (Arg-141α) of one α chain and the N-terminal valine of the other for the purpose of preventing dissociation into dimers. rHb1.1 also featured an Asn-108β→Lys mutation in both β chains. This mutation, which is found in nature and known as Hb Presbyterian, increases P_{50} to around 30 Torr. The product contained methionine instead of valine at the N-terminus of the 3 chains (the fused di-α chain and the two β chains). An extra copy of the gene for ferrochelatase was subsequently introduced in order to improve the production of the enzyme that converts protoporphyrin IX into heme.[476] Accumulation of soluble rHb1.1 in *E. coli* implies proper protein folding, heme association, and subunit assembly. Endogenous heme was not present in sufficient amounts, requiring supplementation with heme from bovine origin.[888] Insoluble, misfolded rHb was also formed, especially in the absence of adequate amounts of heme.[888] Extraction, purification, and virus inactivation of rHb1.1 required a number of steps, including chromatography on a zinc-ion-chelate affinity capture resin and chromatography on an anion-exchange resin.[476]

Optro was formulated as an isotonic 8% solution of rHb1.1 in a phosphate-buffered saline and was stable to storage at 4−8 °C for over 1 year.[1107]

Preclinical investigation of rHb1.1 has recently been reviewed.[462] Efficacy of *Optro* in delivering O_2 was established in exchange-perfused rats whose brain and gut were monitored for ATP, phosphocreatine, and pH by ^{31}P NMR.[476] The close-to-totally exchange-transfused animals survived the 5 h observation period and demonstrated normal oxidative phosphorylation. Oxygen consumption was maintained in rHb-treated sheep undergoing CPB.[462] Resuscitation of dogs subjected to profound shock consecutive to severe hemorrhage and repayment of the O_2 debt were more effective with the rHb1.1 solution than with colloids and their own shed blood.[1038,1291] A rise in pancreatic enzymes was, however, observed.

The product was found to inhibit vasorelaxation by NO donors (such as arginine) or acetylcholine of vascular smooth muscle in vitro.[1167,1175] The effect was comparable to that elicited by purified cell-free Hb. Increased mean arterial pressure and systemic vascular resistance was observed in rats.[1168] Experimentation on opossums[1174,1292,1292a] and observations in human volunteers (0.11−0.15 g/kg b.w. dose)[1104] indicated significant impairment of the motor function of the esophagus in some subjects. These disorders were suggested to result primarily from NO scavenging and defects in the NO-dependent neuromuscular communication. rHb1.1 did not exacerbate bacterial growth in mice.[462]

A Phase I safety trial of *Optro* in healthy volunteers (25 g maximum total dose) provided no evidence for immunogenicity, coagulation changes, or renal toxicity.[476,1073] Plasma half-life of rHb1.1 increased with dose, being 2.8 and 12 h for plasma concentrations of 0.5 and 5 mg/mL, respectively. The most prevalent adverse reactions were those affecting the gastrointestinal tract (68% of patients), including nausea, vomiting, diarrhea, dysphagia, abdominal pain, and chest discomfort.

Arterial blood pressure increased in the rHb1.1-dosed patients and remained elevated until 6−8 h after infusion while heart rate decreased. "Flu-like" symptoms consisting of fever, chills, and headache, transitory increases in serum levels of the pancreatic enzymes amylase and lipase, and urticaria and pruritus were also noted. The gastrointestinal and hemodynamic symptoms indicated ischemic events possibly related, at least in part, with NO scavenging.[476,1073,1104,1107]

No clinically significant treatment-related adverse events were reported following Phase II safety/

efficacy studies in surgical patients (25−100 g total dose), with or without ANH, or enhanced ANH, including CPB surgery patients.[476,1107] Increased blood pressure was seen in two-thirds of the intraoperative blood replacement patient group as well as transient increases in lipase and amylase levels. Clinical trials with *Optro* were discontinued in 1998. Improved rHbs are now being developed by Baxter, including an octamer with both lower NO and lower O_2 binding capacity.[915,1258]

8. Recombinant Human Hemoglobin from Transgenic Pigs

Commercial development of transgenic pig-derived human rHb was attempted by DNX Biotherapeutics Inc. (Princeton, NJ). The choice of swine was dictated by relatively short gestation time, large litter size, and rapid growth.[479] Also, porcine Hb has some features in common with human Hb, including 2,3-DPG-dependent O_2 affinity. Transgenic pigs were produced by microinjection into the pronucleus of a fertilized single-cell pig egg of a genetic construct that contained the genes encoding human α and β globins along with a regulatory element, the β globin locus control region, which enhanced Hb expression and ensured that the gene will be expressed irrespective of its position in the chromosome.[474,896] The microinjected eggs were transferred into a mother pig for gestation. Of the order of 0.5−1% of the offspring may then carry the added gene (the transgene) and will be transgenic. The transgenic pigs can than be bred to a nontransgenic pig, and about one-half of their progeny will be transgenic, allowing a Hb-producing herd to be progressively generated.

An advantage of this expression system is that the Hb that is produced is confined and concentrated in the RBCs, which facilitates its recovery and purification. However, the actual level of expression of human Hb was low and the harvested human rHb needed to be separated (using ultrafiltration and preparative ion-exchange chromatography) from the highly preponderant and structurally very similar pig Hb, and from an even more closely related and hard to separate interspecies hybrid that comprised a human α chain and a pig β chain.[479] Finally, the human rHb thus produced still required modification in order to display functionality. Transgenic Hb was, for example, cross-linked, primarily between β chains, with bis(3,5-dibromosalicyl) sebacate **4.21b** under deoxy conditions.[479]

A number of challenging issues were listed that still needed resolution in order to obtain an exploitable system.[479] These issues include significant improvement of expression level of human Hb; improvement of hematopoiesis in pigs; development of new or more efficient technologies of breeding, blood collection, processing, separation, and control; demonstration of absence of immunological reaction due to pig or chimerical Hb; achievement of a functional, chemically or genetically cross-linked, or polymerized Hb; reduction in production cost; and acceptance of transgenic Hb by the regulatory agencies and, eventually, the public. The promoters of this source for Hb may not have foreseen the unprecedented legal, regulatory, and religious hurdles raised by the fate, processing, and disposition of animal carcasses that contain *human* Hb. The efforts to develop human Hb in transgenic pigs were discontinued in 1996.

9. Pegylated Liposome-Encapsulated Human Hemoglobin—Neo Red Cells

Neo Red Cells, which are being developed by Terumo (Kanagawa, Japan), consist of human Hb that is co-encapsulated in liposomes with an allosteric effector (IHP) and a metHb reduction system (the coenzyme NAD with glucose, adenine, and inositol as substrates).[933,972] The lipid formulation comprises hydrogenated soy phosphatidylcholines, cholesterol, myristic acid, and α-tocopherol. The liposomes are subsequently coated with PEG strands using PEG−phosphatidylethanolamine **4.106d**. The final liposome (180−220 nm) suspension contains 6 g/dL of Hb and has an encapsulation efficiency of 1.9 g of Hb per 1 g of lipid. These LEHb display a P_{50} of about 45 Torr and a circulation half-life of 21 h (1.5 g Hb/kg b.w. in rats). The rate of metHb formation was decreased 2- to 3-fold with respect to Hb liposomes not containing the reductant system.[933,972] Frozen storage was nevertheless deemed preferable.

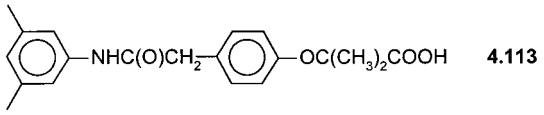
Blood pressure increased transiently during injection of this material to rats but returned rapidly to preinjection levels. Rabbits survived replacement of 85% of their blood by the preparation. Increased vascular resistance and decreased cardiac output were observed in dogs subjected to severe hemorrhagic shock, when treated with Neo Red Cells, despite the low viscosity of the preparation.[1293] Another study showed no such effects and concluded that normal aerobic metabolism could be maintained in severely hemodiluted animals.[1294] Heart preservation experiments were also reported.[1295] Clinical testing in humans does not seem to have yet begun.

10. Miscellaneous Development Efforts

Further documented efforts that were made to develop Hb-based O_2 carriers or that are in the early stages of development include a glutaraldehyde-polymerized human Hb (Dutch Red Cross, Amsterdam, Netherlands);[795] a recombinant human Hb expressed in yeast (Delta Biotechnology Ltd., Nottingham, U.K.);[478] conjugates of Hb from human placenta with dextran benzene tetracarboxylate or of pyridoxalated Hb with an mPEG (Pasteur-Mérieux, Marcy l'Etoile/University of Nancy, France);[1143,1297] an Hb encapsulated in polymerized lipid vesicles (NOF Corp., Tsukuba, Japan);[994,996,1298] an o-ATP-cross-linked, o-adenine-polymerized, and glutathione-decorated bovine Hb (Texas Tech University, Lubbock, TX);[806] *Hemospan*, a relatively viscous, high O_2 affinity pegylated human Hb preparation developed by Sangart, Inc.;[867] a hyperpolymerized porcine Hb product (SanguiBioTech AG, Witten, Germany);[469] and *HemoZym*, a dextran-polymerized polynitroxylated Hb with antioxidant and antiinflammatory properties (SynZyme Technologies, Irvine, CA).[1069]

Although not based on an O_2 carrier per se, another strategy for improving tissue oxygenation involves administration to the patient of an allosteric modifier that binds noncovalently to the central cavity of the

Hb tetramer, thus facilitating the release of O_2 to tissues.[1299] One such agent, RSR13 **4.113**, developed by Allos Therapeutics, Inc. (Denver, CO), is in clinical trials for improving tumor response to radiation therapy.[1300] Improvement of the outcome of moderate



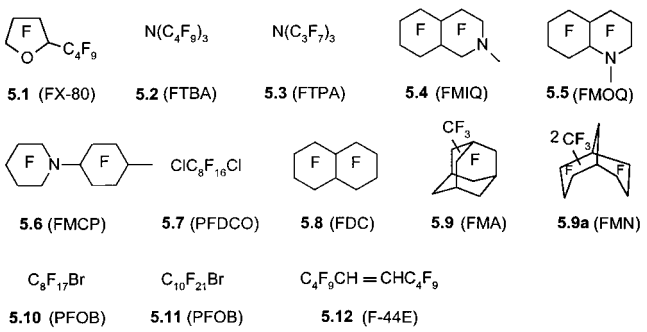
4.113

cerebral ischemia has been seen in the rat with this product.[1301] Introduction of IHP, a powerful allosteric effector, in a patient's RBC by continuous flow electroporation (EntreMed, Inc., Rockville, MD) follows a similar line.[1302] The latter technique also allowed exchanging native Hb with an exogeneous Hb with altered characteristics.[1302a]

V. Fluorocarbon-Based Oxygen Carriers

The basis for using PFCs for in vivo O_2 delivery is their unique combination of high gas-dissolving capacity (the highest known among liquids) and outstanding chemical and biological inertness. Chart 5 lists the PFCs that have been most investigated

Chart 5



5.1 (FX-80) **5.2** (FTBA) **5.3** (FTPA) **5.4** (FMIQ) **5.5** (FMOQ)

5.6 (FMCP) **5.7** (PFDCO) **5.8** (FDC) **5.9** (FMA) **5.9a** (FMN)

5.10 (PFOB) **5.11** (PFOB) **5.12** (F-44E)

for this purpose. Recent reviews on this topic include refs 153, 155, 185, 215, 218, 219, and 1303−1306.

A. The Challenges

In line with the paradigmatic differences that underlie the two approaches, the challenges encountered with PFC emulsions are very different in nature and resolution from those of the Hb-based products. Some issues concerned the PFC itself, others the emulsion; in many cases, the characteristics of the two cannot be dissociated. Many of the issues reflect our initial lack of knowledge about the in vivo behavior of PFCs and, consequently, of the most effective way to use them.

1. Identifying the "Right" Fluorocarbon

When PFCs were first investigated for O_2 delivery, there was no clue about which PFC—from among the hundreds that were a priori possible—would be appropriate. An essential task was to identify a PFC with high O_2 solubility that would be well tolerated, rapidly excreted, and amenable to producing a stable emulsion. The relationship between PFC structure and fate in vivo, particularly what determines excretion rate, needed to be established. Fluorocarbons have a number of distinctive characteristics in common, yet surprisingly few such compounds turned out to be appropriate for in vivo O_2 delivery. The selection process also needed to take into account the PFC's manufacturability and cost.

2. Engineering a Stable, Biocompatible Emulsion

Obtaining a stable, injectable, small-sized, narrowly distributed PFC emulsion was another absolute requirement that needed the chemist's skills. Related questions concerned the emulsion's formulation, concentration, fluidity, scale-up and sterilization, and user-friendliness. Manufacture of a sterile parenteral PFC emulsion, although it relied on existing technologies, required the development of specific know-how.

3. Understanding Fluorocarbon's "Physiology"

Understanding the mechanisms of O_2 delivery by PFCs and their implications in terms of product efficacy, assessing and understanding the PFC's absorption, distribution and excretion characteristics, as well as the emulsions' effects and side effects was mandatory. Such knowledge was also indispensable to identify the therapeutic indications and optimizing the methods of use that would maximize the benefits of PFC-based O_2 delivery for the patient.

4. Assessing Potential Effects on Host Defenses and Lung Function

Emulsion droplets being particulate matter are primarily handled by the RES. Phagocytosis limits the product's intravascular persistence. The potential side effects related to phagocytosis required investigation. Although no clinically significant side effects were seen at the currently investigated doses, temporary saturation of the RES may, like for other emulsions and particulates, constitute a dose-limiting factor for PFC emulsions or may preclude repeated dosing at intervals that are too close. Possible effects on the physiology of the lungs (the principal excretion route for PFCs) needed to be assessed. No antigens against PFCs have been reported to develop following infusion of PFC emulsions.

B. Some Fundamentals about Perfluorocarbons

1. A Sense of the Extremes

Liquid PFCs are uniquely characterized by exceptionally strong intramolecular (covalent) bonding and exceptionally weak intermolecular (van der Waals) interactions. These extremes are directly related to the position of the element fluorine in the extreme upper right corner of the Periodic Chart. The characteristics of fluorine are directly responsible for the specific properties of PFCs, including the lowest surface tensions (<20 mN m^{-1}), dielectric constants, and refractive indices of any liquid; high fluidity, density, compressibility, and gas solubilities; extreme hydrophobicity (as well as lipophobicity); and chemical inertness. Perfluorocarbons and perfluoroalkylated (*F*-alkylated) materials, including fluorosurfactants, have found multiple uses in the chemical,

electronic, nuclear, magnetic media, and aerospace industries, when extreme performance and/or resistance to highly corrosive environments are demanded.[1307−1309]

As noted above, the two essential features that are the foundation of the PFCs' major potential applications in medicine are their high gas-dissolving capacities and exceptional inertness. The first is a direct consequence of the weakness of the cohesive forces between molecules in liquid PFCs. This weakness facilitates the formation of "holes" that can accommodate gas molecules (which are also fluids with low cohesive forces) within the liquid. Because of weak intermolecular interactions, PFCs behave as nearly ideal liquids; their boiling points are close to those of the noble gases with comparable MW.

The PFCs' inertness, on the other hand, reflects the strength of the intramolecular chemical bonds and the low polarizability of the fluorine atoms. Several factors contribute to this inertness. The C−F bond is the strongest single bond encountered in organic chemistry (average 485 kJ mol^{-1}, compared to ∼413 kJ mol^{-1} for a standard C−H bond).[1310] Its strength is further increased when several fluorine atoms are present on the same carbon, reaching 530 kJ mol^{-1} in CF_3CF_3 as compared to 450 kJ mol^{-1} in CH_3CH_2F.[1311] The C−C backbone bonds are also reinforced due to the electroattracting character of the fluorines. The C−C bonds are, for example, stronger by 34 kJ mol^{-1} in poly(tetrafluoroethylene) compared to polyethylene.[1311] The larger size of fluorine atoms relative to hydrogens (estimated van der Waals radius of 147 versus 120 pm)[1312] and their high electron density result in a compact electron shield that ensures effective protection of the molecule's backbone. This dense electron sheath is also expected to have some sort of repellent "Scotchguard"-type effect against reagents at the molecular level. The bonds between *F*-alkyl chains (C_nF_{2n+1} = R_F) and oxygen, nitrogen, chlorine, or bromine are usually also strengthened and the potentially functional sites inactivated. When chlorine or bromine atoms are present in the *F*-alkyl chain, the enhanced electronegativity of the carbon to which they are bound tends to counterbalance their own electroattracting character, resulting in reduced bond polarity, polarizability, and reactivity. No enzymatic system is known to digest PFCs, and no bacterium or other microorganism is known to feed on them.

2. Hydrophobic and Lipophobic

Another direct consequence of low van der Waals forces—and a key characteristic of PFCs relevant to this discussion—is their extreme hydrophobicity. There are probably no two molecular liquids more different and more opposed in their structures and properties than PFCs and water. Fluorocarbons are significantly more hydrophobic than hydrocarbons. They are actually not only hydrophobic but lipophobic as well, repelling both water and lipids. The mixing of fluorocarbons and hydrocarbons is highly nonideal.[1313,1314] This explains why PFCs need to be emulsified for intravascular administration as well as why obtaining stable emulsions can be difficult.

Table 4. Gas Solubilities (vol %, 25 °C) of Representative Perfluorocarbons and Related Compounds

compound	O_2	CO_2	N_2	CO	H_2
H_2O	3.1	83	1.59	2.33	1.91
CCl_4	27.8	244	14.9	20.3	7.73
n-C_7H_{16}	15.2−16.7			26.3	10.4
n-C_7F_{16}	54.8	207	38.6	38.6	14.1
n-C_8H_{18}	28.6		18.0		9.4
n-C_8F_{18}	52.1				
C_6H_6	20.6−22.5	245	11.3	16.9	6.5
C_6F_6	46.8−48.8	426	34.8	41.1	
$C_8H_{17}Br$	8.4				
$C_8F_{17}Br$	50.0−52.7	210			

Hydro- and lipophobicity also contribute to the inertness (or passivity) of these compounds in vivo and largely determine their excretion rates. Extremely low solubility in water is the basis for the use of PFCs to osmotically stabilize injectable gas bubbles that can serve as contrast agents for use with ultrasound imaging.[1315] These characters of *F*-alkyl chains also explain the propensity of *F*-alkylated amphiphiles to self-assemble into a variety of stable colloidal and supramolecular systems.[1316,1317]

3. Oxygen-Dissolving Capacity

Fluorocarbons have the largest gas-dissolving capacities known for liquids.[339,1318] They behave like typical nonassociated, nonpolar liquids, with solubility for gases depending essentially on the molecule's shape,[1319] with small enthalpies and entropies of dissolution.[1320] At a given temperature, the solubility of a given gas in a PFC is directly proportional to the gas' partial pressure.

A formal two-step dissolution process, consisting first of opening a cavity in the solvent large enough to fit the gas molecule and then introducing the gas in this cavity, is often postulated for the purpose of discussing and predicting solubility data. The dissolution of O_2 or NO in diverse liquid PFCs has been investigated by NMR using the perturbation induced on the nuclear relaxation of the ^{13}C nuclei of the PFC by the paramagnetic species.[1321,1322] The results were rationalized on the basis of ease of formation of cavities within the liquid rather than the existence of any specific interaction and indicated that the cavities opened by these solutes in PFCs are significantly larger than in hydrocarbons. The compressibilities of PFCs were much higher and their internal pressures lower than those of hydrocarbon analogues, as expected for lower intermolecular interactions.[1323] The excess chemical potential, i.e., work needed to insert the ^{133}Xe radioisotope into a solvent, was much lower for PFCs than for hydrocarbons.[1324,1325] Likewise, the Gibbs free energy for opening a cavity in PFCs was much lower than in hydrocarbons.

The solubilities of O_2, CO_2, and N_2 in some typical PFCs and, for comparison, in a few other solvents are displayed in Table 4. Discrepancies found in the published solubility data may arise from differences in experimental methods and procedures and uncertainties about the purity of the PFC sample.[339] The methods utilized were manometric,[1326] volumetric,[1327] chromatographic,[219,1328,1329] or spectroscopic.[1321] The

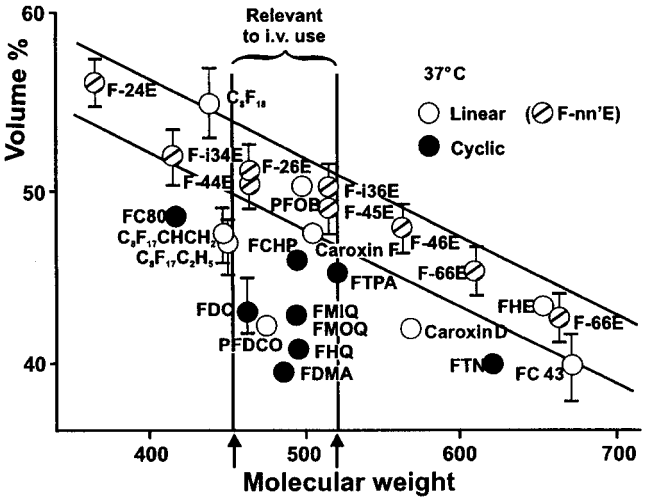


Figure 19. Solubility (vol %) of O_2 at 37 °C in various PFCs as a function of MW. The homologous series of bis-(*F*-alkyl)ethenes (striped dots) illustrates the decrease of solubility as MW increases. The window of MW relevant to intravascular use is highlighted. (Reprinted with permission from ref 1332. Copyright 1988 Ellis Horwood Ltd.)

O_2 solubilities of the PFCs pertinent to intravascular use range, under 1 atm, from 40 to 52 vol % and those for CO_2 from 140 to 240 vol %.[339,1328,1330] They are larger than in water by a factor of 20 or more if expressed in vol % or by a factor of 200 if expressed in molar fraction. Within a homologous series of PFCs, O_2 solubility increases with decreasing MW, as exemplified in Figure 19.[1331,1332] Solubilities of gases decrease in the order $CO_2 \gg O_2 > CO > N_2 > H_2 > He$, apparently following the decrease in molecular volume of the solute.[339]

Oxygen solubility depends somewhat on molecular structure. For similar MW, the differences in O_2 solubility can reach 20−25% (Figure 19), a factor that cannot be neglected when selecting a PFC for in vivo O_2 delivery. Linear PFCs, including those that have a double bond, an oxygen atom, or a terminal bromine atom, have an advantage over the cyclic and polycyclic PFCs.[1331,1332] Terminal alkyl chain resulted in slightly reduced O_2 solubility.[1333] Lesser O_2 solubility was also reported for α,ω-dichlorooctane.[406] The higher gas-dissolving capacities and compressibility of F-44E with respect to its saturated analogue was attributed to the "notch" introduced in the structure by the centrally located double bond, which was thought to facilitate the formation of the cavities that host the gas.[1321]

Predictions of gas solubilities in PFCs have been made using Hildebrand's solubility parameter δ (eq 5.1)

$$\delta = (\Delta E_v/V_m)^{1/2} \qquad (5.1)$$

where ΔE_v is the energy of vaporization and V_m the molar volume of the fluid.[1313] In order for two fluids to be mutually soluble, they need to have similar δ values. The δ values of PFCs are close to 6 hildebrands, as compared to 5.7 for O_2, 7−9 for hydrocarbons, and 23.4 for water. Semiempirical approaches to gas solubilities were derived from the scaled particle theory. These approaches express the free energy of solution as the sum of the free energies for formation of cavities in the solvent and that of the interaction between solute and solvent.[1320,1334] Correlations also exist between O_2 solubilities and the solvent's surface tension, compressibility,[1323] and viscosity,[1328] since these are all manifestations of the solvent's degree of cohesiveness. An empirical group additivity system was developed that provides estimates of the energy of vaporization and molar volume of PFCs from their structural formulas, thus allowing prediction of their vapor pressure and O_2 solubility.[1335]

Solubility of gases in PFC emulsions has been measured by chromatographic,[1336] polarographic,[1330] NMR,[1337] manometric, or radioisotopic[1338] methods, by redox titration after oxidation of ferrous thiocyanates by the O_2 present in solution,[1339] and by enzymatic determination of O_2 using glucose oxidase.[1340] The values obtained were close to those calculated by adding the gas solubilities of the PFC and aqueous phase, indicating that emulsification had no effect on the PFC's gas solubility. An NMR study of ^{19}F spin−lattice relaxation rates in *Fluosol* as a function of O_2 dissolution led to the same conclusion.[1337] The solubilities of xenon and hydrogen in water, blood plasma, *F*-tributylamine (FTBA), and *Oxypherol* (a 20% w/v FTBA emulsion from Green Cross) have been measured. Gas solubilities were about 9% less in plasma than in water, possibly reflecting a salting-out effect due to the Na^+ ions present in plasma.[1338] Increased solubility in *F*-decalin accelerated the elimination of xenon from tissues in dogs; elimination of tissue nitrogen should be even faster in view of the higher solubility in PFCs.[345] The O_2 content of *Fluosol* was not affected by the presence of blood.[1341,1342] It was not significantly affected by an increase in pH, while CO_2 solubility was reduced, as expected.[1342]

The gas transfer capacity of a liquid depends not only on gas solubility, but on the gas' aptitude to diffuse as well. The diffusion coefficients of O_2, N_2, and CO_2 in three 8- to 10-carbon fluorinated ethers were about twice as large as those in water,[1343] while in the heavier FTBA they did not exceed those in water.[1344] Kinetic measurements using stopped-flow spectrophotometry indicated that uptake and release of O_2 by an FTBA emulsion (half-time for uptake ∼2 ms) was about twice as fast as that by a cell-free Hb solution.[1345]

4. Synthesis of Fluorocarbons Relevant to in Vivo Use

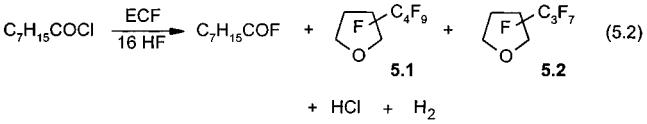
The candidate PFCs should be well defined and pure—which would seem rather obvious for compounds that are intended for injection in 100-gram-size doses. This requirement was not always met in the early days. Some of the materials investigated, including some PFCs that underwent significant development effort, consisted of complex, poorly defined mixtures with questionable batch-to-batch reproducibility.[1346−1348] This is because some perfluorination methods are not selective. No wonder these PFCs were sometimes toxic, and some of the published physicochemical data were subject to caution. This situation has changed: the number of PFCs of

interest has been reduced to a few, the manufacture of which (although the procedures used are not fully disclosed) appears to be under proper control, yielding material that reaches 99.9% purity.

Two major strategies are available for manufacturing PFCs: substituting fluorine atoms for hydrogen atoms in the parent hydrocarbon analogue of the desired PFC or combining smaller, already fluorinated, reactive building blocks.[1332] Electrochemical fluorination, fluorination by high-valency metal fluorides (usually cobalt trifluoride), and direct fluorination by elemental fluorine belong to the first category, while telomerization of tetrafluoroethylene or hexafluoropropene belong to the second. The preparation of cyclic, polycyclic, and branched compounds and of amines and α,ω-disubstituted compounds relies primarily on the substitution route. Telomerization readily provides well-defined pure linear PFCs but is limited in the number and diversity of products that can be prepared.[1349]

a. The Fluorine Substitution Processes. Replacing one hydrogen by a fluorine atom in a hydrocarbon releases about 75 kJ mol^{-1}; when 20 hydrogen atoms are to be replaced, the energy released can reach some 1500 kJ mol^{-1}. If the reaction is not properly controlled, this very large amount of energy easily causes C−C bond breakage, elimination of HF, isomerization, cyclization, and other structural rearrangements, while incompletely fluorinated compounds may still be present. Laborious purification/detoxification steps are then required.[1350,1351] The product's purity and consistency are highly dependent on individual molecular structures.

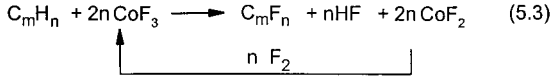
The first reported fluorocarbon, CF_4, was isolated during the preparation of beryllium by electrolysis of beryllium fluoride in an alkali fluoride in 1926.[1352] The first kilogram-size batches of higher MW PFCs were produced using Simons' electrochemical fluorination process shortly before World War II.[1353] Electrochemical fluorination (ECF) consists of the electrolysis of organic compounds dissolved in anhydrous liquid HF.[1353−1356] Fluorination takes place at the anode, probably through a radical cation mechanism,[1357] and hydrogen is liberated at the cathode. The anode potential needs to be greater than 3.5 V (versus the hydrogen reference electrode) in order for appreciable fluorination to occur but should remain less than 5.0 V in order to prevent extensive breakdown.[1356] The process requires that the substrate be somewhat soluble in HF and is, therefore, particularly effective for amines and acids. ECF usually results in complex mixtures of cleavage, coupling, rearrangement, and incomplete fluorination products. Several of the PFCs of Chart 5 were prepared by ECF. *F*-(2-*n*-Butyltetrahydrofurane) **5.1** (Chart 5), the major component of FX-80 (the 3M Company, St. Paul, MN) and one of the earliest PFCs investigated for medical use, results from cyclization during ECF of *F*-octanoyl chloride (eq 5.2).[1354,1358]



(5.2)

A sample of FX-80 used for in vivo distribution studies showed 6−8 peaks by GC.[1359] A sample of distilled FTBA **5.2** showed at least 15 components.[1348] After process optimization, a batch of electrochemically produced *F*-tripropylamine **5.3**, (FTPA, one of the constituents of *Fluosol*), contained actually 27% of the perfluorinated parent compound, along with numerous other materials.[1360] Electrochemical fluorination of *N*-methyldecahydroisoquinoline yielded FMIQ (cis and trans) **5.4** (selected by Green Cross for its second-generation emulsion[1361,1362]) as a mixture of the corresponding amines, plus diverse isomerized and fragmented products (Scheme 13).[1363] Lengthy purification was needed to remove products containing residual hydrogens or unsaturations and nitrogen fluorides. The similarly produced isomeric *F*-4-methyloctahydroquinolidizine (FMOQ) **5.5** was also given some consideration.[1364] *F*-Methylcyclohexylpiperidine **5.6** (FMCP, 3 isomers), present in *Perftoran* (Perftoran Co, Pushchino, Russia),[162] and numerous other *F*-amines investigated for O_2 delivery,[1365] as well as *F*-α,ω-dichlorooctane **5.7**, the PFC used in *Oxyfluor* (HemaGen-PFC, St Louis, Mo),[406] were all produced electrochemically.

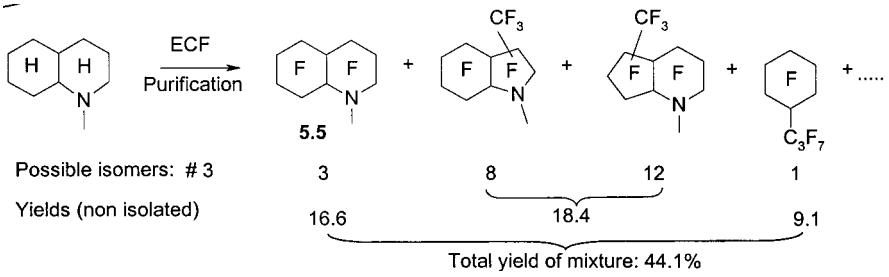
In the high-valency metal fluoride perfluorination process,[1366,1367] the material to be fluorinated is circulated in the vapor phase in a stream of nitrogen over a bed of CoF_3 at 200−400 °C. CoF_3 is regenerated in situ by passing F_2 over the bed of CoF_2 (eq 5.3).



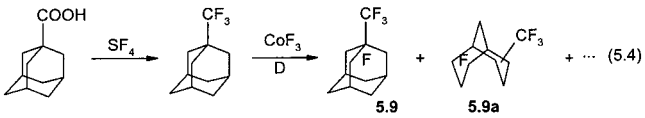
(5.3)

Although the reaction, for which a radical cation mechanism was suggested,[1368] involves less energy than direct fluorination, it generally yields complex mixtures and, because of the harsh reaction conditions, requires that the starting material be volatile and rather sturdy. Proper control of process and

Scheme 13



feedstocks may allow higher yields in the desired compound.[1369] Extensive purification is again needed to obtain a medical-grade product. This method applies well to fused ring aromatic compounds, which are then both fluorinated and saturated. Perfluorination of benzene yielded, however, 25−30 compounds.[1368] *F*-Decalin **5.8** (PFD, cis and trans), the primary PFC in *Fluosol*, is produced from naphthalene and available commercially in 94−98% purity for medical use. Perfluorination of 1,3-dimethyladamantane using CoF_3 gave only a small, 5−10% yield of the corresponding PFC **5.9**; ring opening was extensive.[1350] Better yields (60−65%) were obtained when the partially fluorinated substrates trifluoromethyladamantane (eq 5.4) and bis(trifluoromethyl)-adamantane were used.[1370] Despite a seven-step



5.9 **5.9a**

purification procedure, complex mixtures were obtained, with large proportions of derivatives of the *F*-bicyclononane **5.9a** present.[1350] Such PFCs were nevertheless used in *Addox*, an emulsion developed by Adamantech, Inc. (Marcus Hook, PA). A first emulsion used products derived from CoF_3 fluorination of dimethyladamantane,[1371] but the tissue residence half-life of more than 50 days was deemed unacceptable, and the PFCs derived from fluorination of monomethyladamantane were subsequently used.[1372]

Direct fluorination by molecular fluorine now also provides an effective industrial access to well defined PFCs.[1356,1373,1374] The difficulty was to control and dissipate the enormous amount of energy of the reaction (eq 5.5) in order to avoid C−C bond breaking and structural rearrangements[1373]

$$F_2 + (R)C{-}H \rightarrow (R)C{-}F + HF$$
$$\Delta G_{25} = -433 \text{ kJ mol}^{-1} \quad (5.5)$$

Such control was achieved by diluting F_2 and gradually increasing its rate of addition, controlling the rate of addition of the compound to be fluorinated and the temperature (the LaMar process).[1373,1375] Reaction conditions need indeed to be very mild at first and progressively more forcing. The process has been particularly successful with ethers and polyethers. It is currently widely applicable but does not seem to have yet been implemented on the 100-ton scale relevant to use as O_2 carriers. Liquid-phase photofluorination with elemental fluorine has been used for synthesizing diverse O_2 carrier candidates.[1376−1378] An aerosol direct fluorination process has also been developed.[1379]

b. The Telomerization Route. Telomerization involves the reaction of a telogen, such as C_2F_5I, with an olefin, such as tetrafluoroethylene, to form a series of longer PFC products, the telomers **5.13**, in high yields (Scheme 14, eq 5.6)[1380] The reaction, which follows a free-radical pathway, is initiated thermally, photochemically, or using a free-radical initiator. Stoichiometry and reaction conditions are used to maximize the yield in the desired telomer. Separation

Scheme 14

$$5\ CF_2{=}CF_2 + IF_5 + 2\ I_2 \longrightarrow 5\ C_2F_5I$$

$$C_2F_5I + n\ CF_2{=}CF_2 \longrightarrow C_2F_5(CF_2CF_2)_nI \quad \textbf{5.13} \quad (5.6)$$

$$\xrightarrow{1/2\ Br_2} C_2F_5(CF_2CF_2)_nBr \quad \textbf{5.10: n = 3} \quad (5.7)$$
$$\textbf{5.11: n = 4}$$

of individual PFCs by distillation is facilitated by the 100-mass-unit difference between successive homologous telomers. Higher yields can be achieved by recycling the lighter telomers.[1381] Production of a homologous series facilitates the investigation of structure/properties relationships.

The *F*-alkyl iodides **5.13** (about 2000 tons produced annually worldwide[1382]) are, for a large part, converted into fluorosurfactants for numerous industrial uses.[1308,1382,1383] The chemistry of *F*-alkyl iodides is notoriously different from that of alkyl iodides. This is due, among other things, to the inversion in carbon−iodine bond polarity and steric and electronic shielding against nucleophilic attack. Substitution of the halogen is extremely difficult. Direct bromination of *F*-octyl iodide produces *F*-octyl bromide (PFOB, also known as perflubron) **5.10** in high yields (eq 5.7). Purity of the distilled product is greater than 99.9%. For comparison, a sample of electrochemically produced PFOB was reported to consist of 82% *n*-C_8F_{17}-Br, 17% iso-C_8F_{17}Br, and 17 trace products.[1384] *F*-Octyl bromide **5.10** and *F*-decyl bromide **5.11** are the main components of *Oxygent* AF0144, the emulsion developed by Alliance Pharmaceutical Corp. (San Diego, CA).[219]

A series of linear and branched 1,2-bis(*F*-alkyl)-ethenes **5.12** (code-named F-*nn′*E, where *n* and *n′* are the number of carbons in the *F*-alkyl chains and E indicates the olefinic linkage) were obtained from *F*-alkyl iodides in a two-step process (eq 5.8) that can easily be scaled up for industrial production.[1329,1349,1385]

$$R_FI + CH_2{=}CHR'_F \rightarrow R_FCH_2{-}CHIR'_F \rightarrow R_FCH{=}CHR'_F \quad (5.8)$$
$$\textbf{5.12}$$

$$R_F = C_nF_{2n+1}$$

a: $n = n' = 4$

b: $n = n' = 6$

The materials isolated were essentially the trans isomers. Such compounds were also obtained by reaction of 1-bromo-1-(*F*-alkyl)ethylene with *F*-alkyl-copper compounds (eq 5.9); only the *vic*-disubstituted product **5.12** was formed.[1386]

$$R_FCBr{=}CH_2 + R'_FI \xrightarrow{Cu,\ DMF} R_FCH{=}CHR'_F \quad (5.9)$$

The internal double bond turned out to be very inert; F-44E **5.12a** and F-66E **4.12b**, for example, remained unaffected when heated at 130 °C with diethylamine or bromine for several weeks or treated by *m*-chloroperbenzoic acid or various biomimetic oxidation systems.[1329,1385] The availability of such homologous series contributed to determination of O_2 solubility (Figure 19) and organ retention half-times versus

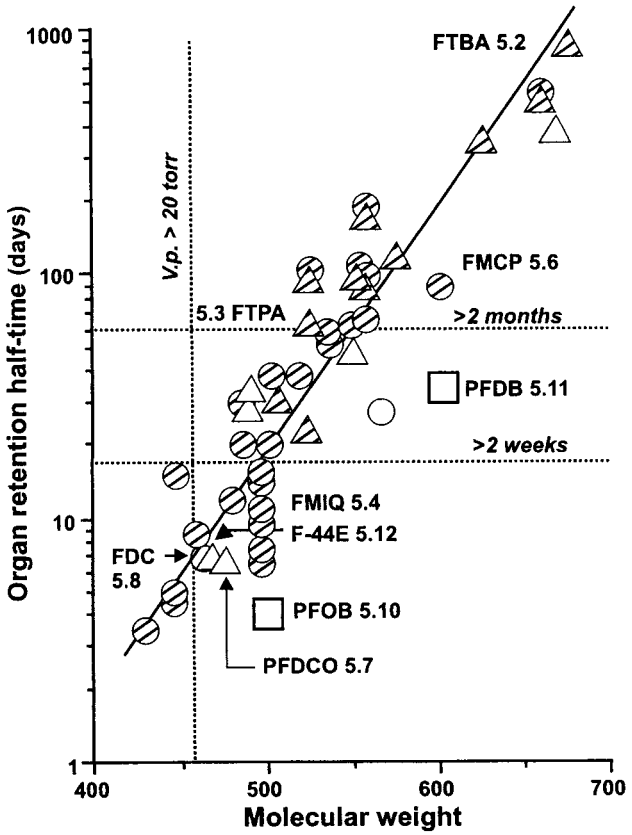
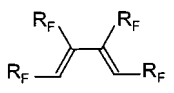


Figure 20. Semilogarithmic plot of organ retention half-times for PFCs versus MW. Triangles for linear and circles for cyclic compounds; striped signs indicate the presence of a heteroatom in the backbone; the lipophilic PFCs are indicated by squares. (Reprinted with permission from ref 219. Copyright 1998 Harwood Academic.)

MW (Figure 20).[1329,1385] No evidence of metabolic or enzymatic degradation was found when the PFCs extracted after several weeks from the liver of rats were chromatographically and spectroscopically analyzed. F-44E **5.12a** was selected by E. I. du Pont de Nemours, Inc. (Wilmington, DE) for preparing *Therox*, a concentrated PFC emulsion that was available in the 1980s and early 1990s for experimental use, and subsequently by Neuron Therapeutics Inc. (Malvern, PA) for the development of an emulsion for treatment of stroke. Copper-promoted coupling of $R_FCH{=}CIR'_F$ yielded the tetrakis(*F*-alkyl)butadienes **5.14**, which were conceived to provide bundles of covalently interconnected *F*-alkyl chains[1387] but proved too heavy for i.v. use.



5.14

F-Alkyl iodides also afforded easy access to fluorocarbon−hydrocarbon diblock compounds **5.15** and **5.16** by addition onto terminally unsaturated alkanes,[1383,1388] removal of HI, and optional hydrogenation (eq 5.10).

$$R_FI + RCH{=}CH_2 \rightarrow R_FCH_2CHIR \rightarrow R_FCH{=}CHR \rightarrow R_FCH_2CH_2R \quad (5.10)$$

5.15 **5.16**

Compound **5.17** was obtained by condensing an (*F*-alkyl)ethyl phosphonium iodide with an aldehyde (eq 5.11).[1389] A general synthesis of ^{14}C-labeled *F*-alkanes was developed from *F*-alkyl iodides and ethyl acetate.[1390]

$$R_FCH_2CH_2P^+(C_6H_5)_3I^- + RCHO \rightarrow R_FCH_2CH{=}CHR + (C_6H_5)_3PO + HI \quad (5.11)$$

5.17

C. The First Generation of Fluorocarbon Emulsions

1. Groundbreaking

The famous experiment of Clark and Gollan, in which they showed that a mouse could live while breathing an O_2-saturated liquid PFC, was published in 1966.[212] The fact that PFCs had the ability of dissolving larger amounts of gases than any other solvent was known.[1318,1326] What Clark and Gollan demonstrated in a spectacular fashion was that animals could withstand such treatment, i.e., that the PFC supported respiration *and* caused the animal no harm. Although PFCs had already been used in cellular microsurgery,[1391] in a blood oxygenator,[1392] and for isolating viruses,[1393] this experiment stimulated imagination and somewhat "officially" marked the entry of PFCs into the biomedical field. The same authors also reported that isolated rat hearts would continue to contract vigorously when perfused alternatively with an oxygenated liquid PFC and with diluted blood.[1394]

The first preparation of a physiologically adjusted PFC *emulsion* was originated by Sloviter and Kamimoto the following year,[213] making intravascular infusion of PFCs possible. The PFC was FX-80 (primarily compound **1**), and the surfactant was bovine serum albumin. This emulsion was as effective as if not superior to a suspension of RBCs in a buffered electrolyte solution for maintaining the spontaneous electrical activity and metabolic function of an isolated rat brain. Shortly thereafter, in 1968, Geyer et al. published the first blood substitution experiment in which a PFC assumed the O_2 transport function in live animals.[214,1395] The authors replaced virtually all of the RBCs of rats with a poloxamer-stabilized FTBA **5.2** emulsion and succeeded in keeping the animals alive for about 8 h while breathing pure O_2. During this time, while their Hb level was essentially zero, these rats continued to eat, drink, and display normal activity. The PFC-treated animals survived for a considerably longer time than untreated controls in carbon monoxide/air or oxygen mixtures.[1396,1397]

The first symposium on "Inert Organic Liquids for Biological Oxygen Transport" was held barely 3 years after Clark and Gollan's seminal paper.[160] By then, improved emulsions with lower particle sizes, complemented by blood serum, had allowed continuous survival of rats after better than 90% RBC replacement.[1398] Exchange-perfused rats thrived and reconstituted normal blood composition in 1−3 weeks. A greater than 3-fold increase in oxygen cathode current had been measured from polarographic elec-

Table 5. Composition of *Fluosol*[161]

composition (w/v%)		function
perfluorodecalin[a] **5.4**	14.0	oxygen carriers
perfluorotripropylamine[a] **5.3**	6.0	oxygen carriers
Pluronic F68[a] **5.18**	2.7	emulsifiers
egg yolk phospholipids[a]	0.4	emulsifiers
potassium oleate[a] **5.19**	0.03	emulsifiers
glycerol[b]	0.8	cryoprotector
NaCl[b]	0.60	ionic balance, pH and osmotic pressure control[d]
KCl[c]	0.034	ionic balance, pH and osmotic pressure control[d]
$MgCl_2$[b]	0.020	ionic balance, pH and osmotic pressure control[d]
$CaCl_2$[b]	0.028	ionic balance, pH and osmotic pressure control[d]
$NaHCO_3$[c]	0.21	ionic balance, pH and osmotic pressure control[d]
dextrose[b]	0.18	ionic balance, pH and osmotic pressure control[d]

[a] From stem emulsion. [b] From annex solution H. [c] From annex solution C. [d] The earlier *Fluosol*-DA formulation contained 3% HES and glucose instead of dextrose.

trodes implanted into the cortex of O_2-breathing cats perfused with an FC-43 (mainly FTBA)/poloxamer 188 emulsion, indicating a significant increase in cerebral O_2 tension.[1399] The possibility of preserving isolated heart, kidney, lung, and liver had been explored. For example, in an isolated rat liver perfusion experiment, an FX-80/albumin emulsion was shown to deliver O_2 to the organ in greater amounts than an RBC suspension.[1400] Neat PFCs had been used in liquid breathing experiments, and emulsified PFCs had already been injected intravenously in hamsters, rabbits, cats, dogs, chickens, mice, and frogs. As one would expect of such groundbreaking experiments, contradictory results, diverse side effects, and a host of unexplained observations were also reported. Hindsight tells us that the emulsions utilized were often too crude and the PFC, when FX-80 was used, too volatile to allow satisfactory results. In any case, reading the proceedings of this symposium played a decisive role in the present author's involvement in the then emerging field.[185]

2. A Capital Dilemma

A major problem with the PFCs used in the early experiments was that they were retained in the animals' tissues for an excessively long period of time. The half-life of FTBA in the rat was later estimated to be about 2.5 years.[1365] Such organ retention was not acceptable for a blood substitute, and the PFC approach would have been terminated in its embryonic stages had it not been for the discovery that *F*-decalin **5.8** was excreted within a few weeks of its administration (organ half-life of about 7 days).[1401,1402] This finding removed the first major roadblock from the development path, only to reveal another one, by no means less formidable: the difficulty of obtaining stable emulsions of such excretable PFCs. Indeed, in its attempt at developing an FDC-based commercial product, *Fluosol*-DC,[1403,1404] Green Cross was unable to produce a stable emulsion. A frustrating dilemma had surfaced, since those PFCs that gave stable emulsions were retained in the organism for an unacceptably long period of time, while those that were excreted rapidly did not produce an emulsion stable enough for practical use.[1331]

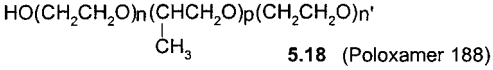
3. The First Commercial Emulsions—Fluosol-DA

Producing a stable, small-particle-size, physiologically compatible emulsion from excretable PFCs thus turned out to be a serious challenge. The emulsion also needed to be fluid, heat sterilizable, have large-scale industrial production feasibility, have long-term shelf stability in standard conditions, be easy to implement, and have minimal side effects. Progress in achieving these goals was discussed periodically by this author.[185,219,1306,1405−1409]

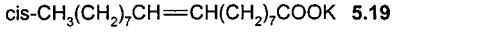
The early PFC emulsions were often crude and uncontrolled. Inappropriate (or insufficiently pure) components, particle sizes, osmotic pressure, sterilization conditions, etc., led to toxicities that had nothing to do with the PFC itself, although it was often blamed for them.[1410] For example, an endocrine toxicity elicited by a commercial FTBA emulsion was removed by dialysis and resin treatment.[1411] Likewise, the toxicity to human and animal cell lines of the surfactant used to prepare such emulsions, *Pluronic* F68, was substantially reduced by supercritical fluid fractionation.[1412] In general, these emulsions will not be reviewed here.

The first emulsion that was developed on a rational basis in a well-controlled industrial environment was *Fluosol*-DA. Having failed to produce a stable FDC emulsion, Green Cross added some *F*-tripropylamine (FTPA **5.3**, 30% of total PFC) to the formulation. By virtue of being, as FTBA **5.2**, a tertiary amine, FTPA was expected to provide stability.[1413−1415] The addition of FTPA resulted in some improvement in stability, at the cost, however, of a 65-day-long organ half-life. Yet, the product's stability was still not adequate: *Fluosol*-DA (later renamed *Fluosol*) had to be frozen for shipping and storage and reconstituted prior to use.[161,1413]

Fluosol's low PFC concentration (∼11 vol %) was probably dictated by the necessity to maintain proper emulsion fluidity, which is no longer preserved at higher concentrations when poloxamers are the surfactant. *Fluosol* actually utilized a three-component surfactant system consisting primarily of poloxamer-188 (*Pluronic* F68) **5.18** accompanied by small amounts of egg yolk phospholipids (EYP) and potassium oleate **5.19** (Table 5).[161,222,1414,1416] The poloxamer



5.18 (Poloxamer 188)



5.19

provided steric stabilization and also a hydrophilic

droplet coating. Potassium oleate introduced negative charges on the droplets, thus hindering flocculation (section V.E). The pH was adjusted to 7.3 and osmolarity to 410 mOsmol/L using mineral salts, glycerol, and dextrose. Glycerol also acted as a cryoprotectant. Hydroxyethylstarch was present in the early formulation to provide additional oncotic pressure. Weight average particle size was around 0.12 μm.

Fluosol underwent extensive preclinical evaluation and eventually human clinical trials, leading to approval in the United States for use in conjunction with an angioplasty procedure. A 35% w/v-concentrated version of *Fluosol* (*Fluosol*-DA 35%), with somewhat higher viscosity and possibly larger particles (the surfactants/PFC ratio remaining the same), was also available for laboratory experimentation.[222,386,1417−1419]

Another emulsion, similar to *Fluosol* in many respects (~11% PFC by volume; also based primarily on FDC, and incorporating a higher MW cyclic tertiary *F*-alkylamine, namely, FMCP **5.6**, also using a poloxamer as the emulsifier, was developed in Russia under the tradename *Perftoran* (Russian Academy of Sciences/Perftoran Company, Pushshino, Russia; this emulsion was initially known as *Ftorosan*).[162,372,1420,1421] An organ half-life of 90 days has been reported for FMCP.[1422] *Perftoran*, which is filter-sterilized, is stable for about 1 month at 4−8 °C but requires freezing for longer storage. The product was licensed by the Russian health authorities in 1997 for a wide range of indications. A further but very poorly documented emulsion that appears to have been similar to *Fluosol* in its formulation was investigated in China in the 1980s and was reported to have been administered to at least 340 patients, including war casualties.[1423,1424]

A highly stable 20% w/v FTBA/*Pluronic* F68 emulsion, *Fluosol*-43 (later renamed *Oxypherol*),[222] was commercially available for many years for experimental use. In view of the several-year-long organ-dwelling time of FTBA, use of this emulsion in humans can obviously not be considered.

4. What Was Wrong with Fluosol?

The first generation of PFC emulsions failed to meet several of the above outlined requirements. The shortcomings of *Fluosol* have been reviewed to satiety.[219,406,1331,1362,1406,1407,1425−1427] They include prolonged organ retention of FTPA, complement activation and hemodynamic effects attributed to *Pluronic*, excessive dilution, limited intravascular persistence, insufficient stability, and lack of user friendliness. Due to poor stability, the product actually came as three separate preparations: the frozen stem emulsion plus two annex salt solutions. The latter had to be admixed sequentially to the carefully thawed stem emulsion, and the reconstituted product had to be used within 8 h. This cumbersome procedure and the further need for administering a small test dose to patients prior to infusion in order to identify those patients who were sensitive to *Pluronic* contributed seriously to compromising the product's acceptance.

It should be noted that the reason *Fluosol* did not gain approval when first submitted to the FDA in 1983 was not related to side effects but to the fact that the proposed indication, anemia, was incompatible with the product's short circulation life. Stabilization of highly anemic surgical patients upon administration of the drug was demonstrated, but the benefit, being short-lived, did not change the outcome for these patients who, being Jehovah's Witnesses, refused blood transfusion.[361,1428,1429]

Fluosol was eventually licensed in 1989 for a more realistic indication: use in conjunction with PTCA.[179,180,1430−1432] In this procedure, a catheter fitted with a balloon is introduced into a blood vessel that is partially obstructed by an atheroma plaque. The balloon is inflated to crush the plaque and reopen the artery. During the inflation period, coronary blood flow is interrupted and the myocardium is deprived of oxygen, leading to transient myocardial ischemia and left ventricular contractile dysfunction. Such myocardial ischemia could be mitigated, both in preclinical[1433−1435] and clinical studies,[179,1430,1432,1436−1439] by infusion of oxygenated *Fluosol* distal to the balloon. Efficacy was demonstrated in patients based on reduced myocardial ischemia, maintenance of ventricular function, alleviation of angina, and decreased wall motion abnormalities. However, the unwieldy handling and testing requirements impeded the product's commercial success. The development of autoperfusion catheters (which allow blood to be perfused through the catheter during balloon inflation) eliminated the need for an O_2 carrier in this setting, leading Green Cross to halt manufacturing *Fluosol* in 1994.

5. In Search of Improved Emulsions

Research on *Fluosol* established firmly that PFC emulsions did carry and deliver the expected amount of O_2 to tissues. It also helped establish that large doses of such a product could be administered without significant side effects. The availability of *Fluosol* to researchers allowed collection of valuable information. Finally, the analysis of this preparation's shortcomings paved the way for the development of improved products. It must be emphasized indeed that the shortcomings of the *Fluosol*-type emulsions were, for the most part, related to the specific formulations, components and characteristics of these products, and not inherent to PFC emulsions in general. On the other hand, the failure of Green Cross to produce a practical emulsion and identify an application compatible with the product characteristics has created a negative image of the PFC approach, probably retarding the development efforts.

An essential lesson from the *Fluosol* experience was that the emulsion needed to be ready for use, hence significantly more stable. Frozen storage, complex reconstitution, and a short window of time for use were simply not acceptable. At least 1 year of shelf stability under standard refrigeration storage conditions was indispensable for the actual injectable preparation. It was also imperative that particles be small and remain small during heat sterilization and throughout shelf life. Small particle sizes (0.1−0.2 μm) translate into longer intravascular persistence

and reduced side effects, as these effects are related to phagocytosis and macrophage activation (section V.G). Although it is eventually the dose of PFC infused in the circulation that counts, rather than the volume of vehicle that is used, it was deemed necessary that the emulsion be relatively concentrated and yet fluid. Viscosity, convenience of use, marketing, and manufacturing considerations led eventually to the choice of a 60% w/v PFC concentration. The manufacturing process needed to be as "forgiving" as possible, i.e., insensitive to small changes in processing conditions, so bottle-to-bottle and lot-to-lot consistency could be easily achieved. Standard-temperature heat sterilization was also sought.

Intense efforts were devoted to the synthesis of new PFCs with cycles, branches, double bonds, and heteroatoms present within their structure and the experimentation of new formulations.[1349,1350,1371,1385,1440−1446] Green Cross was not the last in this race.[1362,1364,1365,1416] Its efforts were directed to improving emulsion stability by identifying both a new PFC and a better surfactant system. The new PFC, FMIQ **5.4**, was selected among a series of bicyclic tertiary amines, i.e., compounds that combined the structural features of FDC **5.8** and FTPA **5.3**. Its organ half-life was 10 days (4 g/kg b.w. dose in rats). The surfactant was EYP, with which no anaphylactic reaction was seen. The new emulsion was fluid, heat sterilizable, and ready for use and could be stored in normal refrigerated conditions; its concentration (25% w/v) remained low. Its development did not, however, survive the difficulties encountered by the company.

D. The Critical Components, Fluorocarbon and Surfactant

The question of the relationship between a PFC's molecular structure and its excretion characteristics and ability to provide stable emulsions remained open and central. Comparison of the published PFC and formulation evaluation data is not easy because of differences in protocols for measuring highly dose-, species-, and particle size-dependent excretion rates and often a subjective way of assessing emulsion stability. Only those data originating from the same team can usually be compared meaningfully. Particularly valuable in this respect were the papers by Green Cross, who reported data on no less than 53 different PFCs.[1364,1365,1447]

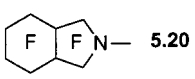
Proper selection of a biocompatible emulsifier or emulsifier system is also essential for successful PFC emulsion development. One of the emulsifier's roles is to reduce the large interfacial tension that opposes the dispersion of the very hydrophobic PFC into the aqueous phase; another is to stabilize the emulsion once it is formed. For intravascular use, the surfactant(s) must be devoid of significant toxicity. As it constitutes the external appearance of the PFC droplets, the surfactant may also play a part in particle recognition and clearance, hence influencing the particle's intravascular persistence and the PFC's organ distribution. The emulsifier should preferably be commercially available and, if possible, already used in pharmaceuticals.

Except for a range of new fluorinated surfactants, much less effort has been devoted to synthesizing and assessing surfactants suitable for emulsifying PFCs than to synthesizing and assessing new PFCs. This may arise not from lack of interest, but from the cost and delays involved in determining the pharmacology and toxicology of a new molecule and having it accepted by the regulatory authorities. Essentially two surfactants have so far been utilized in PFC emulsion development, namely, poloxamers and EYP.

1. Excretable Fluorocarbon and Stable Emulsion. Can One Have Both?

What structural features were responsible for organ retention and emulsion stability and whether the two characteristics could be dissociated were thoroughly investigated and intensely debated.[1331,1364,1365,1371] This research was, however, somewhat biased by preconceived ideas stemming from the early observations. Because FTBA contained a heteroatom, it was hypothesized that heteroatoms were responsible for increased organ retention[1401,1440] (and for emulsion stability as well). Likewise, because the first PFC shown to be rapidly eliminated from the body, FDC, was cyclic, the presence of cycles was deemed responsible for rapid excretion.[1350] The heteroatom-plus-cycle approach became the basis for the synthesis of FMIQ **5.4**, FMOQ **5.5**, and other nitrogen- or oxygen-containing cyclic compounds by Green Cross[1362,1363,1365] and of FMCP **5.6** by the Russian Academy of Sciences.[162,1448] All three compounds **5.4**−**5.6** are bicyclic (like FDC) and contain a nitrogen atom within their structure (like FTPA). Certain patents on PFC-based O_2 carriers voluntarily—but unnecessarily—teach away from noncyclic compounds.

Analysis of the available excretion data, including linear, branched, cyclic, and polycyclic alkanes, amines, ethers, and polyethers, mono- and dihalogenated PFCs, *F*-alkylated ethenes, etc., indicated that excretion rates were actually, within experimental error, an exponential function of the PFC's molecular weight (Figure 20).[1331] Neither the presence of cycles per se nor the presence of heteroatoms per se had any observable impact on excretion rate. These structural features appear to influence organ retention only inasmuch as their introduction changes the compound's MW. Thus, for example, FDC **5.8** ($C_{10}F_{18}$, MW = 462; bicyclic) has, within experimental error, the same excretion rate (6−7 days) as F-44E **5.12a** ($C_{10}F_{18}H_2$, MW = 464; noncyclic).[1385,1449] The addition of a nitrogen atom (and consequently of a fluorine atom), as in FMIQ **5.4** ($C_{10}F_{19}N$, MW = 495), increases organ half-life ($T_{1/2} \sim 11$ days), while the replacement of a carbon atom by a nitrogen, as in **5.20** ($C_9F_{17}N$, MW = 445) accelerates excretion ($T_{1/2} \sim 5$ days), in line with the respective increase or decrease in MW. For a given number of carbons,



5.20

excretion rate increased rapidly when the number of cycles increased,[1347,1371] reflecting the fact that each

cyclization implies the ejection of two fluorine atoms, hence 28 MW units.[1331] Another detailed analysis found that neither the connectivity (cyclization and ramifications) nor the presence of heteroatoms had significant effects on organ retention.[1365] Retention times were proposed to correlate best with a linear combination of the PFC's critical solution temperature (CST) in hexane and vapor pressure.[1365] Vapor pressure alone is a poor indicator of excretion potential. For the series of PFCs investigated in these studies, the CST in hexane actually correlated closely with MW.[1450]

The dependence of excretion rate on MW is consistent with the fact that excretion requires a certain degree of solubility of the PFC in lipids (section V.G), a characteristic that decreases rapidly with increasing MW. Likewise, the increase in emulsion stability with increasing MW is logical now that the mechanism of particle growth in PFC emulsions has been determined.

When MW diminishes, vapor pressure tends to increase, which can cause pulmonary complications.[222,1451−1454] Such reactions were observed with the volatile FX-80 **1** in diverse but not all animal species.[1451] These observations set an upper limit (around 20 Torr) at the acceptable vapor pressure, hence a lower limit (around 460) for the PFC's MW. The range of MWs acceptable for i.v. use was then established to be from ca. 460 to ca. 520,[1331] which leaves only a narrow window of possibilities, considering that the difference between these two limits equates to little more than one CF_2 group and less than a CF_3!

The above analysis also identified a "puzzling deviant point", a PFC for which excretion was faster than would have been predicted on the sole basis of its MW.[1331] This PFC was 1-n-*F*-octyl bromide (PFOB, **11**). Its half-life in rats has been determined by Long et al. to be 3.7 and 11 days for 1.5 and 5 g/kg b.w. doses, respectively,[1455] despite a MW of 499.

2. A Touch of Lipophilicity—Perfluorooctyl Bromide

The favorable impact of lipophilicity on PFC excretion was recognized rather early on,[1347,1361] and measuring the CST of a PFC in hexane (which reflects the PFC's lipophilicity) was proposed as a means of identifying excretable PFCs.[1371] However, the decrease of CST that was observed when, for a given number of carbon atoms, the number of cycles increased reinforced the authors' belief that it was the cycles that favored excretion.

The next significant breakthrough occurred rather serendipitously. Because they wanted to explore the possibility of using PFCs as contrast agents, Long, Mattrey, et al. became interested in brominated PFCs, the bromine atom providing the desired opacity to X-rays.[1456−1458] It turned out that one such compound, *F*-octyl bromide, **5.10**, had the benefit of a short organ-dwelling time and the capacity to be formulated into stable emulsions. The faster excretion of *F*-octyl bromide was subsequently attributed to the touch of lipophilicity induced by its well-exposed terminal bromine, facilitating uptake of the PFC by circulating lipids and transit through the organism.[1406,1459,1460] Enhanced lipophilicity is reflected by a significantly lower CST in hexane or octane as compared to regular PFCs with similar MWs (Figure 21, Table 6).

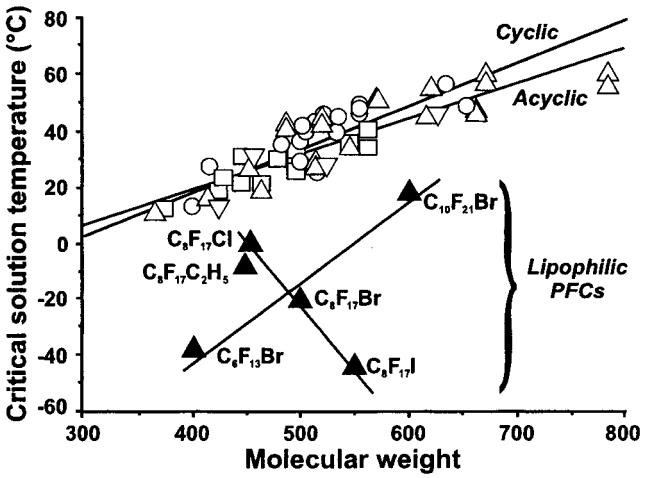


Figure 21. Critical solution temperatures (CST) in hexane of various regular PFCs (△, acyclic; ○, monocyclic; □, bicyclic; ▽, tricyclic) and lipophilic PFCs (▲). A lower CST indicates a higher solubility in lipids and correlates with faster excretion. (Reprinted with permission from ref 219. Copyright 1988 Harwood Academic.)

Table 6. Physical Properties of *F*-Octyl Bromide (PFOB, 1) and *F*-Decalin (FDC, 6)

property (units)	PFOB	FDC (cis + trans)
molecular formula	$C_8F_{17}Br$	$C_{10}F_{18}$
molecular weight (amu)	499	462
molar volume ($cm^3\ mol^{-1}$)	261	237
molecular volume ($Å^3$)	432	393
density ($g\ cm^{-3}$, 25 °C)	1.92	1.94
melting point (°C)	5	−10
boiling point (°C)	143	142
vapor pressure (Torr, 37 °C)	10.5	14
heat of vaporization ($kJ\ mol^{-1}$)	4.83	45.8
refractive index (25 °C)	1.30	1.313
kinematic viscosity (cS, 25 °C)	1.0	2.9
surface tension ($mN\ m^{-1}$)	18.0	15
interfacial tension vs saline ($mN\ m^{-1}$)	51.3	∼60
spreading coefficient ($mN\ m^{-1}$)	+2.7	−1.5
O_2 solubility (vol %, 25 °C)	50	40
CO_2 solubility (vol %, 25 °C)	∼210	∼140
critical solution temperature (*n*-hexane, °C)	−20	+22
solubility in water ($mol\ L^{-1}$)	5×10^{-9}	10×10^{-9}
solubility in olive oil ($mmol\ L^{-1}$)	37	4.6

PFOB **5.10** stands out as unique among candidate PFCs for therapeutic use. In addition to its exceptionally fast excretion rate (about 3 days in humans for a 2.7 g/kg dose), this PFC can easily be manufactured in very high purity using the telomerization route. It is just one step away from the industrially well developed *F*-alkyl iodides **5.13**, and a 100 ton/year production capacity is already in place that can be scaled higher without difficulty. PFOB also ranks among the PFCs that have the highest O_2 and CO_2 solubilities (50 and 210 vol %, respectively, at 37 °C); despite its bromine atom, it is inert in the conditions of processing, storage, and use relevant to applications for O_2 delivery; its emulsions show improved stability when phospholipids are the emulsifier; its

opacity to X-rays allows use as a contrast agent for radiography;[1457,1461,1462] its bromine atom also allows direct measurement of concentrations in tissues using X-ray computed tomography or neutron activation analysis;[1463] finally, its very low surface tension, low viscosity, and positive spreading coefficient could be advantageous in pulmonary applications.[1303,1464−1467] The principal physicochemical features of *F*-octyl bromide are collected in Table 6, where they are compared with those of *F*-decalin.[219] Table 6 shows that all PFCs are not equal, as indicated, for example, by differences in O_2 and CO_2 solubilities, lipophilicity, viscosity, surface properties, and solubility in water. PFOB is about 8 and 28 times more soluble in olive oil than FDC and FTPA, respectively.[1468] In view of this favorable combination of characteristics, *F*-octyl bromide was selected for developing a second-generation emulsion (section V.F).

The benefits of introducing lipophilic termini such as bromine or chlorine atoms or a short hydrocarbon segment into PFCs were confirmed with other compounds (Figures 20 and 21). Emulsions of various PFCs terminated by one or two Cl or Br atoms were investigated.[1459] The critical solution temperatures sharply decreased upon introduction of Cl or Br in the PFC, leading to the prediction of faster excretion rates. *F*-α,*ω*-Dichlorooctane **5.7** (MW 471) was chosen for development of *Oxyfluor*,[406] despite a somewhat longer organ half-life (~8 days for a 4 mL/kg b.w. dose in rats) and lesser O_2 solubility (43 vol % at 37 °C) as compared to PFOB. A group additivity scheme for predicting CST values has been proposed. The influence of one bromine atom on CST is essentially equivalent to that of two chlorine atoms and almost comparable to that of a C_2H_5 group.[1468]

3. Phospholipids

Egg yolk phospholipids have been selected as the emulsifier in the presently developed PFC emulsions for a number of reasons. Significantly better emulsion stability with EYP versus *Pluronic* was generally observed (see, for example, refs 1469−1471), although at least one paper reports the opposite.[1444] The stabilization effect is particularly remarkable with *F*-octyl bromide.[219,1472−1474] The low PFC/water interfacial tension achieved with phospholipids[1475] and the excellent match between the hydrophilic−lipophilic balance of EYP and PFOB were emphasized.[219,1460] An interfacial tension (σ^i) of 4.0 mN m^{-1} was measured with C12-diacyl phosphatidylcholine at the PFOB/water interface, and the σ^i value for EYP was estimated to be even lower.[1475] It is likely that the lower Ostwald ripening rates observed with emulsions stabilized with EYP as compared to poloxamers are a consequence of these low interfacial tensions.

Emulsions of PFCs prepared with EYP do not cause complement activation, as when poloxamers are used.[405,441,1476] Longer intravascular half-lives have been measured in rats for an EYP-based FDC emulsion (24.4 ± 0.9 h) as compared to a poloxamer-based FDC emulsion (13.5 ± 0.5 h).[1477] Egg yolk phospholipids also have the advantage of having a long history of use in pharmaceuticals. They have been in use for over 35 years for stabilizing intravenous injectable lipid emulsions for parenteral nutrition.[1478−1480] The rationale behind the design of lipid emulsions was that the small phospholipid-coated oil droplets should mimic chylomicrons, i.e., the natural blood fat particles produced by the mucosal cells of the small intestine.[1481,1482] Phospholipid's pharmacology is well documented,[956,1483] and there exist reliable commercial sources of pharmaceutical-grade EYP. Few side effects, without clinical consequences, were reported with such products, and these side-effects appear to relate to the particulate nature of the emulsion rather than to the EYP. Egg yolk phospholipids are, however, sensitive, oxidizable materials whose preparation, analysis, and handling require specific expertise.

Phospholipid composition, including minor components, such as phosphatidylinositol, phosphatidic acids, and lysophosphatidylethanolamine, and fatty acid composition and the level of fatty acid unsaturation can affect the oxidative stability, particle size stability, and viscosity of PFC[1484,1485] and lipid emulsions.[1486] Hydrolysis and oxidation of EYP can limit the shelf life of such emulsions. Generation of free fatty acids (FFA) in EYP-stabilized PFC emulsions is minimal at a pH arround 6.0.[1487] Increasing droplet growth rate with decreasing pH was reported, presumably due to a decrease in zeta potential as the pK_a of FFAs is approached. Phospholipid composition, degree of unsaturation, and the presence of trace metals can all have an effect on oxidation rate.[1488] Oxidation of EYP in PFC emulsions can be minimized by addition of minute amounts of metal chelators (e.g., EDTA) and antioxidants (e.g., α−D-tocopherol).

The strength of the surfactant film is related to the lateral hydrophobic interactions between the nonpolar moieties of the surfactant molecules. The stability of the emulsion also depends on cohesion at the PFC/water interface. The latter depends on the solubility of the surfactant's hydrophilic moiety in the aqueous phase and of its hydrophobic moiety in the PFC phase, which is expressed as the hydrophilic−lipophilic balance of this surfactant. The mixing of regular hydrocarbons and PFCs is highly nonideal. Because of PFOB's slight lipophilic character, cohesion between this particular PFC and the fatty acid chains of EYP is significantly enhanced, translating into higher emulsion stabilities than for standard PFCs. In other words, the hydrophilic−lipophilic balance between EYP and PFOB is, quite fortuitously, much closer than with other PFCs.[219,1460]

4. Poloxamers

Poloxamers are neutral block copolymers such as **5.18** consisting of two terminal hydrophilic polyoxyethylene (POE) blocks flanking a central hydrophobic polyoxypropylene block. When at an oil/water interface, the latter segment will tend to adhere onto the surface of the oil droplets, while the two POE chains will extend in a brush-like fashion into the aqueous phase and become substantially hydrated. Poloxamers are low-cost materials, produced in large tonnages

for countless applications in the food, cosmetic, and pharmaceutical industries.[1489−1492] Standard grade commercial poloxamers are, however, highly polydisperse and not intended for intravascular use. They may also contain traces of acetaldehyde, propionaldehyde, formic and acetic acids, unsaturated products, and an antioxidant that is added for preservation. Purification can be achieved by treatment with ion exchangers, charcoal or silica gel, dialysis, crystallization, and supercritical fluid fractionation.[1412,1493−1496]

Poloxamer 188 (e.g., *Pluronic* F68) has been the primary emulsifier in all *Fluosol*-type, first-generation PFC emulsions. In addition to providing a steric barrier against coalescence, the hydrophilic POE brush coating could also beneficially alter the opsonization and uptake of emulsion droplets by the RES. Poloxamer 188 is not metabolized, and its excretion occurs through the urine.[222] Its acute toxicity is low, with reported LD_{50} values of 8 and even 14−16 g/kg b.w. when given i.v. to rats.[372,1497]

However, the use of poloxamer 188 for emulsifying PFCs raised several problems. First, its surface activity is relatively poor. For example, the interfacial tension σ^i between FDC and water is only reduced from 56 to 20−30 mN m^{-1}, translating in lower stability for PFC emulsions made with poloxamers as compared to those prepared with EYP. Another difficulty arises from the fact that poloxamer 188 has a cloud point at about 110−115 °C (i.e., below the standard sterilization temperature of 121 °C), where dehydration of the POE chains suddenly occurs with consequent breakdown of the emulsion. The threshold temperature for destabilization of a PFC emulsion could, however, be raised to 128 °C by the addition of 2% soya oil.[1498] Poloxamers also tend to form gels at room temperature,[1490,1499,1500] which limits the PFC concentration in emulsion, as higher concentrations require larger amounts of surfactant while the volume of the continuous aqueous phase becomes smaller, resulting in increased viscosity.

Finally, *Pluronic* F68 has been found to be responsible for the unpredictable transient complement activation-mediated reaction observed in some patients in response to the injection of *Fluosol*.[439−441,1501,1502] Several papers indicate that dogs (a species particularly sensitive to complement activation) experienced an abrupt drop in arterial pressure when infused a FTBA/*Pluronic* F68 emulsion[1503] or *Fluosol*-DA.[222,1504−1506] Complement activation by *Fluosol* was also observed in the pig.[1507] There are a number of other, sometimes contradictory reports on physiological activities and side effects of *Pluronic* F68 or *Pluronic*-based emulsions.[1501,1508−1518] It is still uncertain, however, how much of these effects were due to the poloxamer itself and how much to some specific MW fraction(s) or impurity-(ies). Purified *Pluronic* F68 fractions showed markedly reduced toxicity.[1412,1493,1495,1496] In one study, *Pluronic* F68 alone did not activate complement in dogs, while the same material used in combination with FDC, FTPA, silicone oil, or mineral oil did; however, the same PFCs emulsified with phospholipids and glycerol did not.[1476] In *Perftoran*, a slightly different, somewhat more lipophilic, highly purified poloxamer was claimed not to cause complement activation.[162] Properly purified or modified poloxamers, poloxamines, or POE derivatives, as they are known to influence the distribution and disposition of injectable particles,[1491,1519−1521] may need to be reinvestigated for emulsion droplet surface engineering, intravascular persistence prolongation, and targeting purposes.

5. Fluorosurfactants

Fluorinated surfactants (or fluorosurfactants, i.e., surfactants with hydrophobic tails comprising a fluorocarbon moiety) are a logical choice for emulsifying PFCs as they can provide very low PFC/water interfacial tensions. Several *F*-alkylated monodisperse[1522] or polydisperse[1523−1527] POE or poloxamer derivatives, such as **5.21**−**5.23,** were synthesized with the aim of preparing PFC emulsions or microemulsions. Industrial-grade fluorinated surfactants, including the amine oxide derivative **5.24**, were also investigated.[1493,1528]

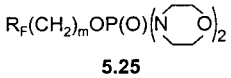
$R_F(CH_2)_mO(CH_2CH_2O)_nH$ **5.21**

$R_FC(O)NH(CH_2CH_2O)_mH$ **5.22**

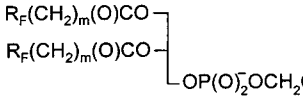
$R_FO(CH_2CH_2O)_n(CRHCH_2)_m(CH_2CH_2O)_nR_F$ **5.23**

$R_FC(O)NH(CH_2)_3N(CH_3)_2O$ **5.24**

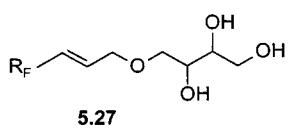
Commercial fluorosurfactants are usually complex mixtures, obviously not suited nor destined for biomedical uses.[1308] A range of fluorosurfactants with a modular structural design was synthesized for the purpose of providing well defined and pure material acceptable for pharmaceutical use.[1316,1529−1532] The polar heads included various types of polyols, anhydropolyols, mono- and disaccharides, amino acids, amine oxides, phosphoramides, phosphocholine, phosphatidylcholine, and other phosphatides and lipids as well as telomers with an adjustable number of hydrophilic THAM groups (see, for example, **5.25**−**5.30**). Tensions at the PFC/water interface were on



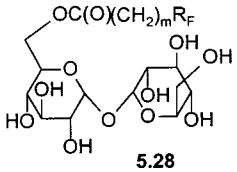
5.25



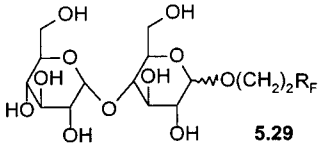
5.26



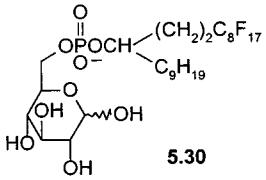
5.27



5.28



5.29



5.30

the order of 1−10 mN m^{-1}. Hemolytic activity,[1533,1534] impact on the growth and viability of cell cultures,[1535] and acute toxicity in mice[1536,1537] were determined. Hemolytic activity was surprisingly low and often undetectable, despite high surface activity. The self-aggregation behavior of the new amphiphiles was explored.[1317,1537] Fluorocarbon−hydrocarbon diblocks, such as **15** and **16**, also have amphiphilic character (fluorophilic/lipophilic) and can play a role as part of a surfactant system at a PFC/water or PFC/hydrocarbon interface.

E. The Emulsion Stability Issue and Its Resolution

Stability is a sine qua non condition for PFC emulsions to be of practical use. The mechanisms that can contribute to particle size increase over time and eventual breakdown of a PFC emulsion, namely, molecular diffusion, sedimentation or creaming, flocculation, and coalescence, have recently been discussed in detail.[219] The principal mechanism for irreversible droplet growth in such emulsions was determined to be molecular diffusion Coalescence may contribute to instability when mechanical stress is applied and at higher temperatures, as during heat sterilization. Sedimentation and flocculation are fully reversible and usually not considered as problematic. Several physicochemical responses to stability problems have been devised. It is essential that the biological implications of these solutions be taken into account.

1. Emulsion Degradation Mechanisms

a. Molecular Diffusion. Molecular diffusion (also known as Ostwald ripening or isothermal distillation) causes irreversible particle growth over time in submicronic PFC emulsions,[219,1331,1470,1538−1543] even in highly concentrated emulsions, where coalescence is a priori favored.[1544,1545] During Ostwald ripening, individual PFC molecules leave the smaller droplets, where the chemical potential is higher due to the Kelvin effect, diffuse through the continuous aqueous phase, and join larger particles. As a result, the larger particles enlarge further at the expense of the smaller ones. The smaller droplets have a higher curvature and capillary pressure, which results in greater local concentration of dispersed phase in their vicinity,[1546] as represented by eq 5.12

$$C(a) = C(\infty)\exp\frac{2\sigma^i V_m}{RTa} \approx C(\infty)\left(1 + \frac{2\sigma^i V_m}{RTa}\right) \quad (5.12)$$

where $C(a)$ is the solubility of the PFC in the vicinity of particle a, $C(\infty)$ its bulk phase solubility, σ^i is the interfacial tension between the two phases, V_m is the molar volume of the PFC, and T is the absolute temperature. The differences between $C(a)$ and $C(\infty)$ are significant only for submicronic droplets.

Particle growth by Ostwald ripening in crystalline dispersions is described by the Lifshitz−Slezov−Wagner theory,[1547,1548] which was subsequently adapted to emulsions[1549] and predicts a linear increase in number average particle radius $\bar{a}$ versus time (eq 5.13)

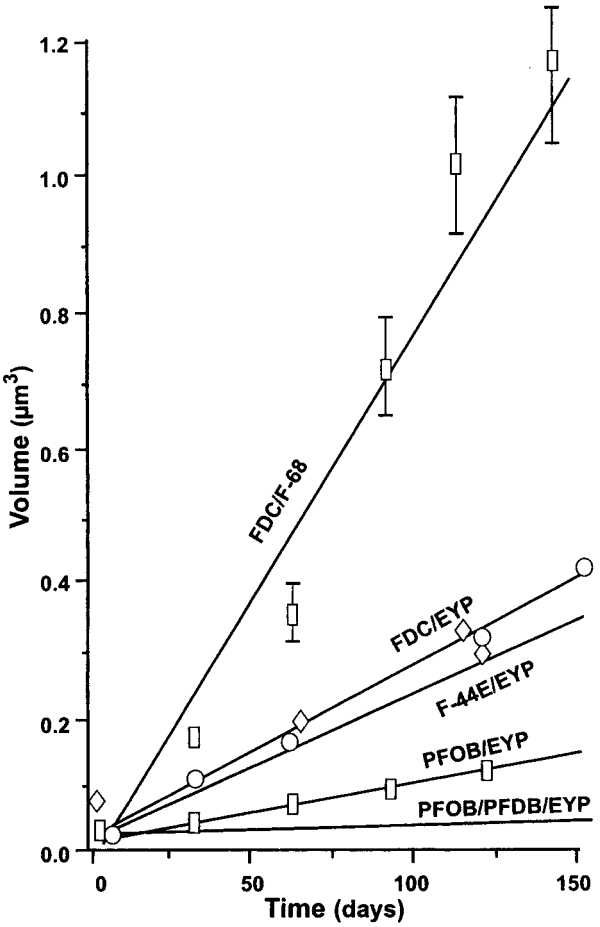


Figure 22. Increase of droplet volume over time for diverse PFC and surfactant combinations (F-68 is Pluronic F68). (Reprinted with permission from ref 1471. Copyright 1992 Dekker.)

$$\frac{d}{dt}(\bar{a}^3) = \omega = \frac{8\sigma^i DC(\infty)V_m}{9RT}\gamma(\phi) \quad (5.13)$$

where ω is the droplet's growth rate, D is the diffusion coefficient for the PFC in the aqueous phase, and $\gamma(\phi)$ reflects the dependence of ω on the volume fraction ϕ of PFC in the emulsion. Droplet volume was indeed observed to increase linearly with time[1470,1471,1474,1544,1545,1550,1551] and growth rate to increase nearly proportionally with PFC volume fraction in the 8−57% volume fraction range.[1552] It should be noted, however, that eq 5.13 does not take into account the presence of the surfactant film and supposes that the two phases are isotropic, which may not necessarily be the case, for example, in the vicinity of the interface for a multicomponent surfactant system.

The PFC has a dramatic influence on particle size growth with time (Figure 22).[339,1471v,1550,1553,1554] As noted earlier, highly stable emulsions can easily be produced with FTBA, while this was quasi-impossible with FDC. Of the parameters of eq 5.13, only $C(\infty)$, the solubility of the PFC in the aqueous phase, varies significantly among PFCs; hence, it is an effective predictor of emulsion stability.[1471,1542,1555,1556] Ostwald ripening is indeed slowed when the PFC is heavier,

hence generally less soluble in water. Measuring $C(\infty)$ is difficult because the values are often below the limits of sensibility of the usual analytical methods. The $C(\infty)$ values estimated from the growth rate of emulsions of 14 PFCs with 5−11 carbons using eq 5.13 were in the 10^{-6} to 10^{-10} mol L^{-1} range.[1555] As a rule of thumb, $C(\infty)$ decreased by a factor of 8 with each added CF_2 in a homologous series and by a factor of 2 when a tertiary amine nitrogen was added. The presence of cycles increased the solubility of PFCs in water, in line with reduced volume, hence the reduced surface area of the solvent cavity required to accommodate such structures. Thus, F-44 emulsions were more stable than FDC emulsions, although their MWs are similar.[1470] The single most important determinant of emulsion stability is eventually MW. However, as we have seen, organ retention increases exponentially with MW, an increase that can fortunately be mitigated by using lipophilic PFCs (e.g., *F*-octyl bromide).

b. Flocculation. Flocculation of a PFC emulsion is generally reversible. It can be limited by increasing the charge on the droplet, decreasing the concentration of electrolyte in the aqueous phase, or adding hydrophilic macromolecular components that provide a steric barrier between droplets. Minor components, such as negatively charged phospholipids and free fatty acids, can help prevent flocculation by increasing the zeta potential on the droplet's surface, which increases electrostatic repulsion between droplets. The small amount of potassium oleate present in *Fluosol* is likely to be related to preventing flocculation in this way. A significant zeta potential of around −24 mV was measured for phosphatidylcholine-stabilized FDC emulsions.[1444] Values close to zero were reported for a PFOB emulsion when a saturated phospholipid was used.[1557] Addition of negatively charged cholesteryl hemisuccinate prevented flocculation. Inducing a positive zeta potential was less effective, indicating that electrostatic forces alone cannot account for the prevention of flocculation. The role of hydration forces and steric effects was illustrated by the reduction of flocculation observed by addition of saccharides to the aqueous phase of emulsions.[1557,1558] The importance of droplet surface charge in improving the stability of injectable lipid emulsions during autoclaving was also recognized.[1559]

Emulsions stabilized by EYP usually have sufficient negative surface charge to prevent flocculation. Addition of cations can neutralize this charge, causing a decrease in zeta potential and flocculation when the repulsive electrostatic forces are no longer able to overcome the attractive van der Waals forces. Electrolytes, and in particular divalent cations such as Mg^{2+} and Ca^{2+}, tend to collapse the electrical double layer that forms around droplets, thus favoring flocculation. Such cations are known to complex phosphate headgroups.[1560] As the concentration of the cation increases, the surface charge can actually be reversed. The flocculation rate then diminishes as positive charge builds up.[1486]

Droplet flocculation can be promoted by free poloxamer. When distances between droplets become smaller than the surfactant micelle, the latter is excluded from the interparticle region, leading to an osmotic pressure difference that can cause droplets to flocculate.[1561] However, critical flocculation concentrations of poloxamer measured for *F*-decalin emulsions prepared with various poloxamers were found to be 2−4 times larger than the concentrations used in O_2 carrier formulations.[1541]

c. Coalescence. The stability of vesicles with respect to coalescence is controlled to a significant extent by the bending energy it takes to depart from the film's spontaneous curvature.[1562] The same principles hold for emulsion droplets. Monolayer elasticity and bending properties contribute to droplet coalescence by affecting the thermally activated rupture of the surfactant film at the oil−water interface.[1563,1564] The influence of monolayer curvature on emulsion stability is often overlooked because the surface of the droplets is virtually flat at the molecular level, even in submicronic emulsions. It was hypothesized that although the droplets are indeed not significantly curved, the monolayer at the edge of a nucleation hole, however, is.[1563] The strong dependence of the coalescence barrier on the film's curvature, especially when actual curvature is in the vicinity of spontaneous curvature, has been confirmed experimentally in the case of the ternary n-$C_{12}H_{25}(OCH_2$-$CH_2)_5OH$/*n*-octane/NaCl solution system.[1565] The data were in good agreement with the model, relating the rate of coalescence to the free energy penalty of hole nucleation in the emulsion film.

With EYP as the surfactant, the spontaneous curvature is nearly balanced at the PFC/water interface.[219] Fine adjustments of surfactant film characteristics can be made by changing the headgroups, length and degree of unsaturation of the fatty chains, and proportion of minor lipid components, including cholesterol and lysolecithins, electrolyte composition, FFA content, and PFC lipophilicity. Emulsion viscosity and stability to mechanical stress were, for example, affected by the degree of phospholipid chain unsaturation.[1484,1566] For a given phospholipid headgroup, increasing the length and degree of unsaturation of the acyl chains led to increased tail volume, hence to decreased spontaneous curvature and destabilization.[1484] Increased temperatures likewise increase tail volume by increasing the proportion of gauche conformers in the acyl chains, thus resulting in destabilization.

Poloxamers are believed to provide a hydrophilic steric barrier that can help prevent coalescence of emulsion droplets. A small angle neutron scattering study of the adsorption of various poloxamers to the surface of PFC emulsion droplets confirmed this view.[1567] The thickness of the poloxamer layer in the FTBA emulsion *Fluosol*-43 was estimated to about 12 nm.[222] The force required to achieve droplet coalescence of an FDC emulsion was measured for diverse poloxamers.[1568] Higher MW and longer POE chains, hence increased layer thickness, led to increased stability.

2. Counteracting Molecular Diffusion

a. Heavy Fluorocarbons as Stabilizers. Higushi and Misra demonstrated that molecular diffusion in

emulsions could be slowed by including in the dispersed phase a small amount of a component with lesser water solubility.[1549] An emulsion of hexane could be stabilized by adding a PFC[1538] and PFC emulsions by adding a secondary, higher MW ("heavier") PFC.[1443,1569] Molecular diffusion then modifies the composition of small versus large droplets over time. The smaller droplets become richer in the less soluble, heavier PFC, while the larger droplets are enriched in the more soluble primary PFC. Eventually the mass transfer stops when the differences in capillary pressures are compensated. Theoretical treatment of two-component dispersed phase emulsions showed that the rate of particle growth in such systems can be approximated by eq 5.14[1542,1570]

$$\omega_{mix} = (\phi_1/\omega_1 + \phi_2/\omega_2)^{-1} \qquad (5.14)$$

where subscripts 1 and 2 relate to the more and less water-soluble components, respectively. Experimental verification for emulsions made of mixtures of $C_8F_{17}Br$ and $C_{10}F_{21}Br$ was achieved using sedimentation field-flow fractionation to separate droplet populations according to size.[1545] The fractions, when analyzed by gas chromatography, showed the predicted component partitioning.

This emulsion stabilization principle had been used in *Fluosol*, where FTPA **5.3** is the heavy PFC, and in *Perftoran*, which utilizes FMCP **5.6** as the stabilizer. This does not go without a penalty, however, since these PFCs have organ half-lives of ~65 and ~90 days, respectively. One percent of the 16-carbon atom polycyclic PFC *F*-perhydrofluoranthene was sufficient to significantly stabilize FDC emulsions,[1443,1498] but the organ half-life of the additive is likely to be on the order of hundreds of days. *F*-Dimorpholinopropane (organ half-life 55 days) has also been proposed as a heavy PFC stabilizer.[1571]

The increased organ retention of the heavier PFC was mitigated by choosing a *lipophilic* PFC as the "heavy" additive.[1474,1572] The desired lipophilic touch, which improves PFC excretion, can be obtained by introducing polarizable halogen atoms, e.g., Cl or Br, or a short hydrocarbon segment in a terminal position in the PFC. *F*-Decyl bromide **5.11**, which has an RES half-life of only about 23 days, was thus selected as a stabilizing additive in *F*-octyl bromide emulsions (Figure 23).

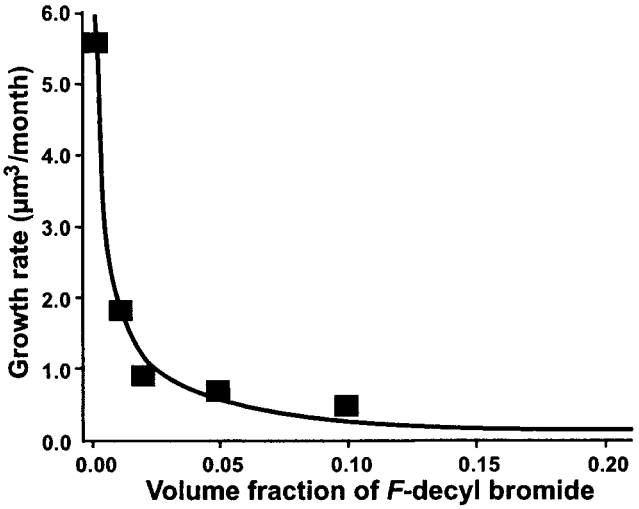


Figure 23. Increase in stability (decrease in growth rate) with increased proportion of *F*-decyl bromide incorporated in an *F*-octyl bromide emulsion. The fit to the experimental points is derived from eq 5.14. (Reprinted with permission from ref 1474. Copyright 1994 Dekker.)

b. Fluorosurfactants. The rationale for using fluorosurfactants for stabilizing PFC emulsions is that fluorosurfactants are capable of reducing PFC/water interfacial tensions σ^i, one of the terms of eq 5.13, to very low values. Various types of fluorosurfactants proved indeed to be highly effective in stabilizing PFC emulsions.[1316,1523−1525,1528,1573−1575] In some cases, small amounts of one single fluorosurfactant sufficed. In other cases, a second surfactant was needed. For example, the closely related *F*-alkylated derivatives of trehalose **5.28** and maltose **5.29** demonstrated dramatically different behaviors: the former gave highly stable emulsions, while the latter did not even allow obtaining an emulsion, probably reflecting differences in polar head conformation and hydration, hence in molecular shape.[1551,1576] Compound **5.29** (as well as the xylitol derivative **5.27**) provided, however, strong synergistic stabilization when used in association with a poloxamer, indicating hydrogen-bond interactions between the two species.[1577] Some such formulations showed no significant change in particle size distribution for the 6 years during which the emulsion was monitored.[219] Other surfactants, such as the anionic sugar derivatives **5.30**, were synergistic with phospholipids but not with poloxamers.[1551]

Fluorosurfactants attained their objective from the PFC emulsion stabilization perspective. However, the lack of knowledge about the pharmacology and possible toxicity of these compounds still hinders their development. The fact that the surfactant is not the active component, but only part of a delivery system, is a serious handicap to spending the time and money needed for their pharmacologic evaluation. The use of a fluorosurfactant as the stabilizer of an *F*-pentane emulsion-based contrast agent[1578] that has been licensed in Europe (which supposes that inocuity has been established for this fluorosurfactant) may break this reluctance.

c. Fluorocarbon−Hydrocarbon Diblocks. Another way of further stabilizing EYP-based PFC emulsions consists of supplementing standard phospholipids with mixed amphiphilic fluorocarbon-hydrocarbon (FC−HC) diblock compounds, such as **5.15** or **5.16**.[1579] It was hypothesized that the HC segments would tend to meddle with the EYP's fatty chains, while the FC segments would anchor themselves into the PFC droplet, so that the diblocks would behave as molecular "dowels" at this interface (Figure 24). As a consequence, the local concentration of the heavier diblocks at the interface would increase, which could reduce interfacial tension and dispersed phase solubility, hence slow down molecular diffusion. Highly stable emulsions were indeed obtained[1579,1580] that may provide the basis for a future generation of O_2 carriers. Such diblocks were more effective than a "heavy" PFC additive of similar MW,[219] and their presence had a definite impact on

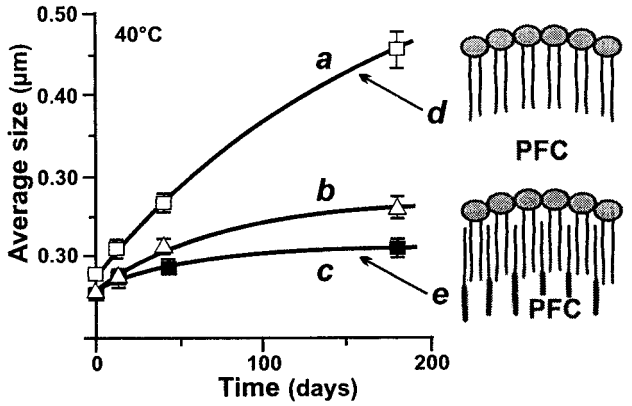


Figure 24. Particle size increase over time at 40 °C in a 90% concentrated *F*-octyl bromide/EYP emulsion prepared (a) with EYP alone and (c) with an equimolar mixture of EYP and $C_6F_{13}C_{10}H_{21}$ diblock; (e) hypothetical stabilizing "dowel effect" of the diblock compound at the PFC droplet/phospholipid film interface as compared to (d) a monolayer of EYP. (b) Stabilization with a heavier PFC of similar MW as the diblock. (Adapted with permission from ref 219. Copyright 1998 Harwood.)

the characteristics of the phospholipid film.[1581] The exact mechanism by which stabilization is achieved has, however, not yet been fully elucidated. Recent evidence confirms the aptitude of FC−HC diblocks to organize at the interface around a PFC droplet in water and reduce the interfacial tension.[1582] Partition coefficients of FnHm diblocks between PFCs and hexadecane indicated no marked preference nor phobicity for either phase, meaning that such diblocks could indeed improve the cohesiveness between the PFC molecules and fatty chains of the EYP.[1583] Because they are lipophilic, FnHm diblocks are excreted rather rapidly; $C_6F_{13}CH{=}CHC_{10}H_{21}$, for example, has an organ half-life of only 25 days at a dose of 3.6 g/kg b.w. in rats.[1584] No overt toxicity was seen at that dose, which is about 2 orders of magnitude larger than that required for emulsion stabilization. *Pluronic*/diblock combinations proved less effective, with the most stable FDC emulsions lasting only about 80 days.[1333] Use of fluorosurfactants or cosurfactants or FnHm diblock cosurfactants are probably the only way to effectively stabilize emulsions of volatile PFCs such as *F*-pentane.

d. Microemulsions. Microemulsions distinguish themselves from the usual emulsions (also called macroemulsions) in that they form spontaneously and are thermodynamically stable within a certain temperature-dependent composition domain. The characteristics of microemulsions are therefore independent of preparation conditions and highly reproducible. Since all particles are essentially identical in size, molecular diffusion is no longer effective as an emulsion coarsening mechanism. Obtaining microemulsions from PFCs usually requires at least one fluorosurfactant.

Microemulsions of PFCs with *F*-alkylated amine oxides and alcohols as surfactants and cosurfactants were first proposed for organ preservation,[1585] but no toxicity data were provided. A single neutral *F*-alkylated POE surfactant was subsequently shown to suffice for obtaining PFC microemulsions.[1586] Numerous PFC/fluorosurfactant/water phase diagrams have been investigated.[76,1586−1592] Mixtures of fluorinated and nonfluorinated surfactants allowed control over the temperature of microemulsion formation.[1593] Microemulsions were also produced from mixed FC−HC diblocks **5.17** using a pharmaceutical-grade nonfluorinated surfactant.[1594,1595]

Microemulsions could provide an ideal solution to long-term shelf stability if biocompatibility can be demonstrated. Possible problems could arise from the large amount of surfactant that is usually needed and from the very small size of the PFC particles, as such small particles could escape the vasculature through pores in the capillary endothelium. Also uncertain is the fate of the dispersion when, after mixing with blood, its composition falls outside of the often-narrow domain of existence of the microemulsion. Although the physicochemical aspects of this approach are rather well understood, further research is needed to evaluate it from a biological standpoint.

F. Formulation and Engineering of a Biocompatible Emulsion

Technology for producing injectable emulsions in compliance with pharmaceutically good manufacturing practices is well established. Small particles and narrow particle size distributions need to be achieved reproducibly. PFC particles of less than 0.2 μm should facilitate O_2 transport and evade phagocytosis more easily than larger ones, resulting in longer intravascular persistence and lesser side effects. Heat sterilization is a complex and challenging element of the process. Details on industrial PFC emulsion production are not disclosed.

1. Emulsification

The free energy of emulsion formation, ΔG_{form}, is given by eq 5.15

$$\Delta G_{form} = \Delta A\sigma^i - T\Delta S_{config} \qquad (5.15)$$

where ΔA is the variation in total interfacial area, σ^i the interfacial tension between the two liquids, and $T\Delta S_{config}$ a configurational entropic term that, for a macroemulsion, is negligible when compared to $\Delta A\sigma^i$.[1596] ΔG_{form} is positive and usually large, which means that energy needs to be supplied for the emulsion to form and that when it is formed this emulsion is thermodynamically unstable or metastable. As a consequence, the characteristics of PFC macroemulsions, including those that can have an impact on biological tolerance (e.g., particle size and size distribution, lysolecithin and free fatty acid content, viscosity, etc.) and their stability, depend not only on formulation but also on processing conditions as well.

Because deformation and disruption of droplets require an increase in their surface's curvature (local increase of the Laplace pressure, which is proportional to interfacial tension),[1597] the amount of energy that is involved is higher by several orders of magnitude than ΔG_{form}. The preparation of fine, narrowly dispersed PFC emulsions therefore requires emulsification procedures that provide a high-energy

density. Lipophilic PFCs such as PFOB or *F*-octylethane are easier to emulsify than standard PFCs, such as FDC, possibly because of lower interfacial tension. Particle sizes follow a typical normal logarithmic distribution.

For EYP-based PFC emulsions, the first step of the process involves the dispersion of the water-insoluble phospholipid in a saline solution. The PFC is then added to the saline phase, where it is broken down into fairly large droplets (average ~5 μm) using a high-shear rotor-stator-type homogenizer. This premix undergoes final emulsification by sonication, high-pressure homogenization, or microfluidization.[219] Minimal exposure to oxygen (through nitrogen sparging and blanketing), pyrogen-free water-for-injection, and a particulate-free environment are used throughout processing.

Sonication is often used in the research laboratory as it allows the preparation of very small, milliliter-size batches. This method, however, tends to yield rather wide particle size distributions, suffers from poor reproducibility, and has little large-scale feasibility. Microfluidization has been introduced for preparing parenteral emulsions relatively recently.[1598,1599] A flow of crude premix is divided into two separate streams funneled through precisely defined microchannels; these streams are forced to impinge on each other under high velocities in an interaction chamber. The device operates at pressures up to 20 000 psi and allows preparation of laboratory-size samples of ca. 50 mL as well as medium- and large-scale production. The Gaulin-type high-pressure homogenization process, which was invented in the 1890s for preparing dairy products, is the procedure of choice for industrial production of pharmaceutical emulsions.[1600] It is easy to control, gives narrow, consistent particle size distributions, and can be operated on very large scales. With EYP as the emulsifier, the emulsions prepared by microfluidization were consistently finer, more narrowly dispersed, resisted better to heat sterilization, and displayed higher shelf stability than those obtained by sonication.[1408] Concentrated (60% w/v) PFOB emulsions, with an average particle diameter of 0.16 $\pm$ 0.01 μm, are currently being produced in a commercial-scale facility using high-pressure homogenization.[219,1601]

Terminal heat sterilization is required for parenteral emulsions to be licensed. The present PFC emulsions are steam-sterilized in a rotary autoclave at or above 121 °C. In concentrated emulsions, it is difficult to achieve uniform heat penetration while maintaining emulsion integrity. Innovative sterilization procedures, providing the required probability of less than one nonsterile unit in 10^6 as well as a means of demonstrating sterility, were designed to meet this challenge.[1602] Such parameters as headspace in the bottles and cooling gradient after sterilization, which are usually overlooked in the research laboratory, were shown to influence the particle size profile and acceptability for an F-44E emulsion.[1603] Emulsions stabilized with *F*-decyl bromide had improved resistance to heat sterilization.[1572] In the laboratory, low-pressure room-temperature filtration on a 0.22 μm polyvinyldifluoride membrane provides an alternative to heat sterilization.[1604]

2. Process Optimization

Computer-assisted experimental design has been used for optimization of formulation, process, and storage parameters.[1552,1605] Understanding how certain parameters can influence emulsion properties is important. For example, the structure of the initial EYP dispersion has a definite impact on emulsion stability.[1606] Depending on the procedure used and energy applied, this dispersion can consist of poorly organized nonclosed "pre-liposomes", MLVs, or SUVs. A PFOB (90% w/v) emulsion prepared from "pre-liposomes" contained less PFC-free phospholipid vesicles than those obtained from the other two dispersions and was significantly more stable. The amount of EYP utilized largely determines the size of the emulsion droplets. It is difficult, however, to obtain droplet diameters lower than ca. 0.08 μm. The EYP efficiency (percentage of total EYP that is eventually present as a monolayer at the PFC/water interface) levels off when the EYP/PFC ratio increases. The percentage of phospholipids involved in PFC-free vesicles increases then sharply, and an excess of EYP can eventually impact negatively on emulsion stability.[1552,1607]

Excess energy (for example an excessive number of passes of the emulsion through the interaction chambers of a microfluidizer), by stripping the phospholipid layers from the PFC droplets, can result in increased particle sizes and lesser emulsion stability. The stripped lipids reorganize into liposomes whose presence is detrimental to emulsion stability.[219,1607] An optimal balance between number of passes and pressure is required in order to avoid such "overworking".[1552] Temperature, pressure, and number of passes can also affect the viscosity of the emulsions.[1608]

3. Second-Generation Formulations—Oxygent

Since the inception of *Fluosol*, PFC emulsion formulation has undergone major mutations, including selection of a PFC having some lipophilic character, replacement of the poloxamer by phospholipids as the emulsifier, a severalfold increase in PFC concentration, simplification of the overall formulation, a considerable increase in stability, and, consequently, a far superior user-friendliness.

All second-generation emulsions were 3−5 times more concentrated than *Fluosol*. The first highly concentrated PFC emulsion reported was a 100% w/v (52% v/v) PFOB emulsion.[1472] This very high concentration was dictated by the intended diagnostic application. It was subsequently shown that a slight decrease in concentration to 90% w/v (47% v/v) led to substantially improved fluidity and a rheological profile that was close to Newtonian.[1473] The physical characteristics of the new emulsion were also less sensitive to processing parameters; it was easier to sterilize and displayed better resistance to mechanical stress.

Further experimentation and optimization led to *Oxygent* AF0144, a ready-for-use, osmotically and

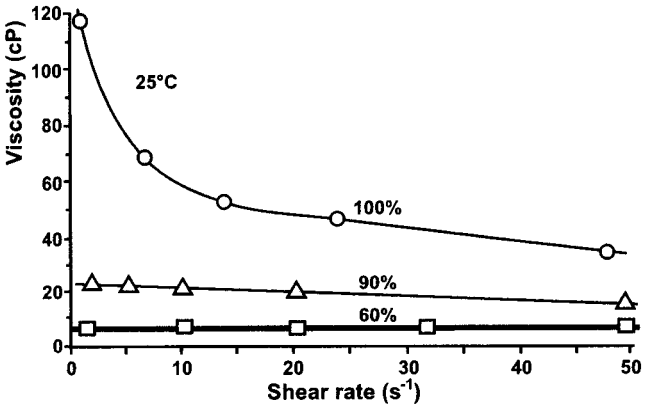


Figure 25. Viscosity at 25 °C of variously concentrated PFOB/EYP emulsions as a function of shear rate. (Adapted with permission from ref 1473. Copyright 1992 Dekker.)

pH-balanced emulsion of *F*-alkyl bromides. This formulation comprises 60% PFC by weight (about 32 vol %) consisting primarily of *F*-octyl bromide and a small percentage of *F*-decyl bromide as the stabilizing additive. The 60% w/v concentration was deemed best adapted to the clinical use in surgery. Egg yolk phospholipids are the emulsifier. Osmolarity is adjusted with NaCl and pH with a phosphate buffer. Minute amounts of α-D-tocopherol and EDTA are added to protect the phospholipids against oxidation. Average droplet size, after heat sterilization, is about 0.16 μm, and viscosity is around 5 cPs (Figure 25). This viscosity is close to that of blood and expected to contribute to the maintenance of normal hemodynamics. The product has a shelf life of at least 24 months when stored at 5−10 °C. It will be marketed by PFC Therapeutics, a joint company formed by Alliance and Baxter Healthcare Corp.

Several other post-*Fluosol* formulations have been reported. *Therox*,[1609] a concentrated emulsion of F-44E **5.12a** (48% v/v or 83% w/v) with a particle size of 0.25 μm, was developed by DuPont for research purposes. *Therox* and other F-44E emulsions were used in various studies.[1610−1613] Another F-44E emulsion is being investigated by Neuron Therapeutics Inc. for treatment of stroke. Adamantech locked onto a mixture of cyclic PFCs obtained by CoF_3 fluorination of methyladamantane (again in the belief that cyclization would help excretion) to produced *Addox*, a 40% w/v PFC emulsion.[1372,1614,1615] Both *Therox* and *Addox* were EYP-based and had a shelf life of 1 year at 4 °C. An FDC/poloxamer emulsion, stabilized by *F*-perhydrofluoranthene, was also investigated.[1443] An FDC emulsion with *F*-dibutylmethylamine instead of FTPA was reported[1616] but with no apparent advantage. A 35% v/v-concentrated *F*-perhydrophenanthrene/EYP emulsion destined to organ preservation was briefly mentioned.[1617]

A stable EYP-based emulsion of the radiopaque mixed FC−HC $C_6F_{13}CH{=}CIC_6H_{13}$, with an internal iodine atom, was reported.[1618,1619] Another radiopaque emulsion, made from PFOB, FMCP **6**, and a poloxamer, required freezing for storage.[1620] A highly stable PFOB/EYP formulation incorporating a FC−HC diblock[1579,1580] has been investigated for normothermic organ preservation.[1621−1623] Similarly stabilized emulsions, but with a poloxamer as the emulsifier, have been reported.[1333] Following a different approach, perfluorononane-filled microspheres have been prepared that were made of bovine serum albumin under ultrasonic conditions.[689]

HemaGen/PFC added some saffoil (essentially triglycerides) to its formulation for the purpose of improving emulsion stability.[406] An initial attempt to develop a concentrated FDC emulsion, using this principle, was, however, soon abandoned. The more lipophilic PFC α,ω-dichlorooctane **7** was eventually used for the development of *Oxyfluor*.[406] No convincing evidence was provided for a stabilizing effect of the added triglycerides. Attempts at reproducing such emulsions resulted actually in two populations of droplets, PFC droplets and saffoil droplets (Weers, 1998, unpublished results). On the other hand, addition of soya oil was reported to have some stabilizing effect on *Pluronic*-based FDC emulsions.[1498]

4. The Structure of Fluorocarbon/Phospholipid Emulsions

The phase equilibria of unsaturated phosphatidylcholines in water (e.g., dioleylphosphatidylcholine) are characterized by the presence of a lamellar liquid crystalline phase (L_α) in excess water.[1624] The addition of a PFC does not significantly alter this phase behavior, due to effective demixing between the phospholipid and the PFC.[1625] The ternary mixtures are thus characterized by a Winsor III equilibrium of an L_α phase in excess water and PFC. This behavior is similar to that found in soybean oil/water/EYP mixtures.[1626] Oil-in-water emulsions with large droplets, prepared within such three-phase regions, may exhibit enhanced stability due to the formation of a lamellar mesomorphic phase at the oil/water interface.[1627] In the case of submicron emulsions, however, the droplets are essentially coated by a single monolayer of phospholipid, with excess phospholipid forming SUVs.[1478,1572,1628−1633] Assuming a monolayer coverage, the area per molecule of EYP (60−65 Å^2) on the emulsion droplet's surface was independent of droplet size[1572] and in agreement with that reported for dipalmitoylphosphatidylcholine monolayers in the condensed state at an FTBA/water interface.[1634]

The coexistence of emulsion droplets and SUVs in PFC emulsions has been confirmed by a number of independent techniques. Figure 26 displays photomicrographs obtained by freeze-fracture transmission electron microscopy (TEM) for a 70% w/v dispersion of FDC in water, stabilized by 8% w/v EYP.[1630] The emulsion droplets are distinguishable from the SUVs by a characteristic granular appearance following cross-fracture. Because of large differences in density, the particles could be readily fractionated by centrifugation. The SUV-containing supernatant was shown by ^{19}F NMR to contain no PFC. Partitioning of the electron spin resonance label 2,2′,6,6′-tetramethylpiperidinenitrosyl (TEMPO) was also consistent with the presence of SUVs in the supernatant phase. Freeze-fracture TEM further confirmed the SUVs to be in the supernatant and the dense PFC-containing droplets (with their characteristic granular appearance) in the infranatant. Emulsion droplets and SUVs have also been separated using cross-flow field-

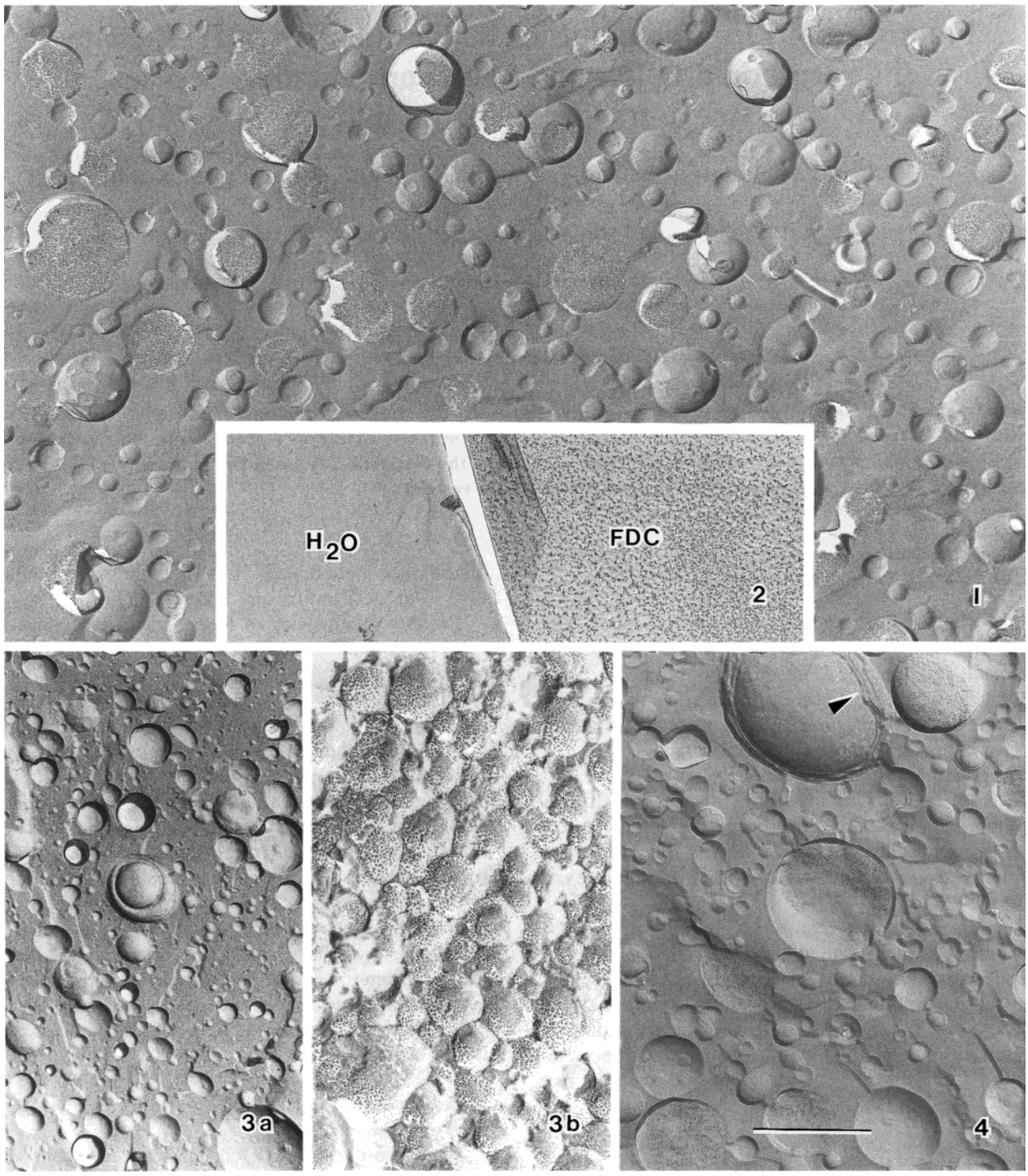


Figure 26. Freeze-fracture transmission electron micrographs of a 70% w/v concentrated *F*-decalin emulsion stabilized with 8% of EYP: (a) initial emulsion; (b) simultaneously frozen droplets of water (left) and PFC (on the right), showing the difference in aspect of the two phases after freeze-fracture; (c) supernatant taken after centrifugation of the emulsion; (d) infranatant taken after centrifugation of the emulsion; (e) micrograph showing rare multilamellar ordering on the droplets; the bar represents 0.2 mm. (Reprinted with permission from ref 1630. Copyright 1991 Elsevier.)

flow fractionation with multiangle laser light scattering detection. A fractogram obtained for *Oxygent* showed that the emulsion droplet size (root-mean-square radius) ranged from roughly 0.04 to 0.160 μm, with a peak at 0.060−0.065 μm, as compared to roughly 0.025−0.030 μm for the SUV population (Weers, unpublished results, cited in ref 219).

A description of PFC emulsions as vesicle-like aggregates or "asymmetric lamellar phospholipid aggregate systems"[1635] or "thermodynamically stable vesicles",[1636] possibly driven by the popularity of liposomes in cosmetics, is misleading: the number of lipid layers around a PFC droplet is uneven, as in any emulsion.

G. Some Elements of Fluorocarbon "Physiology"

Because of their extraneous nature and the presence of abundant fluorine probes, PFCs are easy to identify and quantify in blood, tissues, and expired air.[1463,1637−1644]

1. Pharmacokinetics

Numerous studies have focused on the intravascular persistence, in vivo distribution, and excretion of PFCs.[402,405,1361,1452,1455,1638,1639,1645−1650] In short, the PFC droplets infused in the vasculature are opsonized (i.e., made recognizable by the binding of specific plasma proteins, the opsonins), progressively phagocytized by circulating monocytes and fixed macrophages of the RES, the larger droplets being removed first. This mechanism is largely responsible for the limited intravascular half-life of the emulsions. The PFC is then temporarily stored in the RES organs, primarily the liver, spleen, and bone marrow. No evidence for metabolism has been reported for any of the PFCs investigated.

Removal of a PFOB emulsion from the blood stream over time was found to fit a double-exponential function.[1639] A rapid dose-dependent initial phase, following first-order kinetics, corresponds to loading of the RES, thereby slowing down the removal of the remaining emulsion droplets. The second much slower clearance phase also involves release of PFC from the RES back to the circulation and is no longer first order. The rate of RES uptake during the initial phase for EYP-coated emulsions was essentially determined by droplet size (Figure 27) and independent from identity of the PFC. The intravascular half-life ($t_{1/2}$) in rats was 14 h for a 2.7 g/kg PFC dose of an emulsion with average diameter of 0.09 μm. Surface modification using other surfactants than EYP generally led to shorter $t_{1/2}$. Clearance rates were also species-dependent.[1639]

When the PFC molecules diffuse back across cell membranes from the RES organs into the blood (the excretion rate determining step), they are taken up by circulating lipid carriers (lipoproteins and chylomicrons) and moved to the lungs for excretion. The rates of these steps depend critically on the PFC's MW and lipophilicity.[1347,1361,1365,1406,1407,1460,1651] The PFC is eventually excreted from the blood, through the lung alveoli, in the expired air. Some of the PFC is also deposited into adipose tissue, before being excreted by the same pathway described above. A small amount of PFC is found in the feces, the source of which is thought to be the lipid compartment reflected in bile. Interestingly, redistribution of the PFCs between organs and blood after successive administrations of different PFCs was shown to follow the Ostwald ripening pattern.[1642] Thus, when FDC or PFOB were injected in rats after FMCP had been administered and had accumulated in the organs, their elimination from circulation was accelerated while some of the FMCP was extracted from the organs back into the blood stream. Likewise, FDC was extracted from the organs back into circulation when FMCP was subsequently administered. The RES does not appear to be involved in these exchanges.

As indicated, the emulsion's circulatory half-life $t_{1/2}$ is particle size-, species-, and dose-dependent.[222,405,1452,1639,1652] For *Fluosol*, it was evaluated as 13, 25, and 29 h for rat, dog, and rabbit, respectively, for a 4 g/kg b.w. dose.[222] Dose response of *Fluosol* in rats is illustrated by initial half-lives of about 18 and 34 h for 4 and 14 g PFC/kg b.w., respectively.[1647] The FTPA fraction of the PFC that remained in the blood increased from the initial 30% to about 54% after 96 h (14 g PFC/kg dose). This is likely to reflect the faster removal of the larger droplets, which are being selectively enriched in FDC by Ostwald ripening, while the remaining droplets are smaller and richer in FTPA. Accelerated phagocytosis of *Fluosol*-43 in mice (as monitored by ^{19}F NMR of $t_{1/2}$) has been attributed to rapid elimination of the poloxamer, causing particle sizes to increase.[1638] Dose-dependence in humans is illustrated by $t_{1/2}$ values of 7.5 and 22 h for doses of 4 and 6 g of PFC/kg, respectively, for *Fluosol*-DA[1653] and 6.1 and 9.4 h for doses of 1.2 and 1.8 g/kg, respectively, for *Oxygent*.[220]

When *Fluosol* was administered to rats, the PFC content of liver, spleen, and lung increased rapidly and peaked after about 1 day whereas PFC in adipose tissues increased more gradually and peaked after ca. 2 weeks.[1361] PFC concentration was highest in the spleen and second highest in the liver, leading to temporary enlargement of these organs; lung PFC content was highly species- and PFC-dependent.[1654−1656] As a normal consequence of RES clearance, transient elevations in liver enzymes were observed when large amounts of *Fluosol* were administered.[1648] FDC was eliminated from the blood, liver, spleen, and lung more rapidly than FTPA, while the amount of FDC present in adipose tissues became relatively larger, confirming that the fate of individual PFCs depends on their affinity for lipids.[1361] Likewise, more FDC than FTPA was found in the milk of sucklings after the mother rat had received *Fluosol*. RES uptake of such emulsions decreased with increasing droplet surface hydrophilicity.[1657,1658]

Excretion of FTPA (administered to rats in the form of an FTPA/EYP emulsion) could be accelerated by subsequent daily intraperitoneal injections of lecithin.[1659] The $t_{1/2}$ was prolonged 3- to 4-fold, the amount of PFC temporarily retained in the liver decreased, and organ half-life reduced from ca. 65 to ca. 10 days, likely because of RES saturation by liposomes. Likewise, $t_{1/2}$ of an FDC/EYP emulsion and reduced accumulation in the liver were achieved by administration of EYP dispersions.[1477] The role of lipid carriers in PFC excretion was elegantly illustrated in a different type of experiment by demonstrating that the rate of excretion of FDC from rabbits could be substantially increased by intravascular injection of a fat emulsion after the PFC emulsion had been cleared from circulation.[1651] When the blood of rats that had been given an FMIQ/EYP emulsion was fractionated by HPLC 1 week later, most FMIQ was found in the high-density lipoprotein (HDL) fraction.[1361] This observation directly supports the view that PFCs move across cell membranes from the RES organs and return into the blood, taken up by lipoproteins. In line with this view, both. FDC and FTPA were shown by ^{19}F NMR studies to be partly soluble in the bilayer of phosphatidylcholine vesicles, while FTBA (whose organ half-life is several years) was not.[1660]

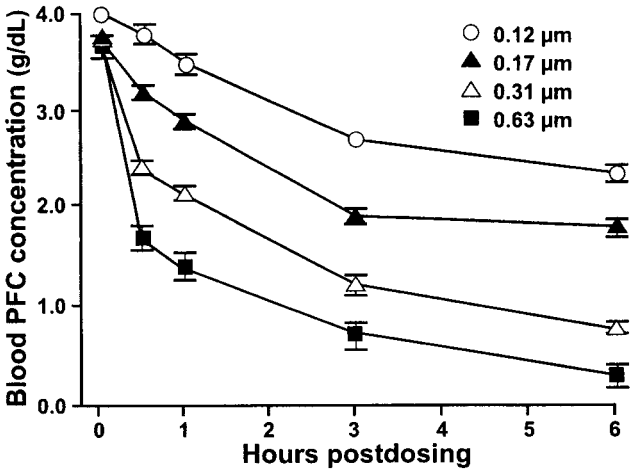


Figure 27. Blood *F*-octyl bromide levels in conscious rats following intravenous infusion (2.7 g/kg b.w. of PFC) of 90% w/v emulsions of various average sizes (obtained by varying the amount of emulsifier). (Reprinted with permission from ref 1725. Copyright 1994 Dekker.)

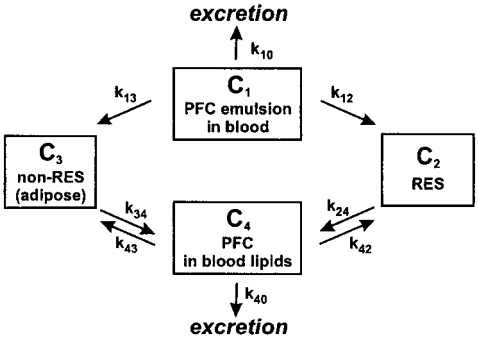


Figure 28. Pharmacokinetic compartment model for PFC emulsions. A first-order output from each compartment was assumed; k_{ij} represents the rate constant from compartment C_i to compartment C_j; k_{i0} represents the expiration rate constant from C_i to the air. (Reprinted with permission from ref 1650. Copyright 1996.)

A three-compartment pharmacokinetic model provided good correlation between observed and simulated blood and organ concentrations of FDC over time.[1361] It indicated that excretion of PFC as an emulsion and of PFC per se had to be distinguished and that the rate of excretion was lower for the emulsion form. No adipose tissue compartment was, however, taken into account, and the experimental data covered only a two-week period. Subsequently, a four-compartment model (PFC in blood, the RES, blood lipids, and adipose tissues) suggested that the rate-determining step in PFC excretion was the dissolution of the PFC into lipid carriers, further confirming that the excretion rate depended critically on PFC plasma lipid concentration.[1651] Another study evaluated six possible pharmacokinetic compartment models by nonlinear regression analysis with expiration data over a 28-day period from rats having received a 2.7 g/kg dose of a PFOB emulsion.[1650] One of these models with four compartments (PFC emulsion in blood, blood lipoproteins, RES tissues, and non-RES tissues; Figure 28) correlated the expiration data with tissue distribution data rather well.

2. Oxygen Delivery Efficacy

The principles of O_2 delivery by PFC emulsions have been outlined in section III and contrasted with those of Hb. Due to a linear increase in O_2 content with O_2 partial pressure, an immediate, large O_2 gradient is created between blood and tissues in the oxygen-breathing patient upon administration of a PFC emulsion. This provides a strong driving force for O_2 diffusion, which, because of the weak O_2/PFC interactions, results in high O_2 extraction rates and ratios by the tissues.

The efficacy of PFC emulsions in delivering O_2 to tissues has already been demonstrated with the first-generation emulsions. The aptitude of low doses of PFC to immediately increase tissue pO_2 and significantly contribute to O_2 consumption was further established with the current EYP-based concentrated emulsions.

a. Fluosol and Related Emulsions. Data from hemodilution or shock models have established that *Fluosol* could effectively increase O_2 consumption, prevent tissue hypoxia, and improve survival of the treated animals.[1661−1664] For example, O_2-breathing baboons, exchange-perfused with *Fluosol* to Hct 1%, all survived in the virtual absence of RBCs.[396] $P\bar{v}O_2$ increased significantly, and O_2 consumption was maintained despite decreased total arterial O_2 content and delivery. More importantly, a low PFC dose, despite a modest contribution to O_2 delivery (10%), substantially enhanced O_2 consumption (42 ± 5)%, even when almost one-half of the RBCs were still present (Hct 20%). When Hct was 1%, the PFC contributed about 55% to O_2 delivery and 60% to O_2 consumption. At Hct below 10%, delivery and consumption of O_2 were similar for *Fluosol* and a pyridoxalated human Hb solution in baboons.[1665] Hemodilution of dogs with *Fluosol*-DA 35% after predilution with HES resulted in substantial improvements in tissue pO_2 of heart, liver, kidney, and skeletal muscle.[385,1666] Resuscitation of dogs with *Fluosol* after hemorrhage led to significant increases in arterial pO_2 and $P\bar{v}O_2$, and O_2 consumption of the heart was restored.[1667] *Fluosol* was estimated to be about 4 times as efficient as blood Hb in tissue O_2 delivery in a canine adult respiratory distress syndrome model, despite lower arterial O_2 content.[1668] Administration of Fluosol 35% to cats with focal cerebral ischemia, although it transiently decreased blood O_2 content due to hemodilution, resulted in a net increase in O_2 delivery and cortical O_2 availability due to increased cerebral blood flow.[386]

Clinical trials in humans have established that *Fluosol* delivered the expected amount of O_2, had an immediate beneficial impact on the condition of anemic patients, and could contribute meaningfully to O_2 consumption in these patients; they also confirmed the short intravascular persistence, hence efficacy of the product.[361,1426,1429,1669,1670] When *Fluosol* (4 g of PFC/kg b.w.) was given to O_2-breathing severely anemic patients prior to surgery, $P\bar{v}O_2$ increased by ca. 60% and O_2 consumption by about 22%. The PFC provided (24 ± 7)% of the patients' O_2 consumption while mixed venous Hb saturation ($S\bar{v}O_2$) reached (90 ± 6)%, indicating that Hb re-

mained close to saturated.[1669] The amount of O_2 provided by *Fluosol* was deemed clinically important. However, the benefit was only temporary since the circulation half-life of the PFC was only about 20 h. An independent study with highly anemic surgical patients confirmed that *Fluosol* unloaded O_2 very effectively ((82 ± 5)%) and contributed at least as much to O_2 consumption ((28 ± 7)%) as the patients' own RBCs.[361] The outcome for these patients, who refused blood transfusion on religious grounds, however, could not be improved in view of the product's short intravascular residence. As a logical result, the FDA did not approve *Fluosol* when submitted as a treatment for anemia in the early 1980s.

The correct conclusion from these trials is not that *Fluosol* was ineffective as an O_2 delivery system, but that treatment of sustained anemia is not a valid indication for such a product. Subsequent approval of *Fluosol* for use during PTCA implies that efficacy (reduced ischemia of the myocardium[179,180]) had been demonstrated for this application. As indicated earlier, *Fluosol* has failed to reach commercial success, not for lack of O_2-delivering efficacy or because of side effects, but because of poor stability, lack of user friendliness, and inappropriate strategy of use.

b. Phospholipid-Based Emulsions. The early EYP-based concentrated PFC emulsions have demonstrated O_2 delivery efficacy as well. Intracoronary infusion of *Therox* in dogs was more effective in maintaining ventricular function than a non-O_2-carrying perfusion solution;[1609] intracranial hypertension and infarct volume following experimental stroke in cats were significantly reduced by perfusion of the brain (through the ventriculocisternal route) with an F-44E/EYP emulsion;[1612] substantially enhanced cardioplegic protection was obtained in an isolated rat heart model of cardioplegic arrest with *Addox*-type emulsions.[1614,1615]

The capacity of small doses of *Oxygent* to significantly increase O_2 consumption has been established in a number of models, in particular canine models that mimic hemodilution and surgical bleeding.[152,348,1671−1674] Infusion of the emulsion preserved the benefits of augmented cardiac input normally observed in response to hemodilution. Cardiac output was sometimes seen to increase even further upon infusion of the emulsion to the hemodiluted animals.[1675] The contribution to total O_2 delivery of a 3.3 g/kg b.w. dose of PFC, administered to O_2-breathing dogs, was only 8−10%, but due to high extraction, it accounted for 25−30% of total O_2 consumption, resulting in considerable sparing of O_2 extraction from Hb.[1672] This allowed O_2 consumption to be maintained while Hb levels were decreased to values as low as 2.0 g/dL (Figure 29). The treated dogs could lose almost two-thirds of their blood (70 mL per kg b.w.), compared to 10 mL/kg in controls, before $P\bar{v}O_2$ fell below the initial 100%-O_2-breathing baseline. Tissue pO_2 of skeletal muscle, gut, and brain was seen to increase substantially. Another study of this type concluded, on the basis of both indirect global indicators ($P\bar{v}O_2$, coronary venous pO_2) and direct measurements, using O_2-sensitive electrodes, of tissue pO_2 on the surface of liver and

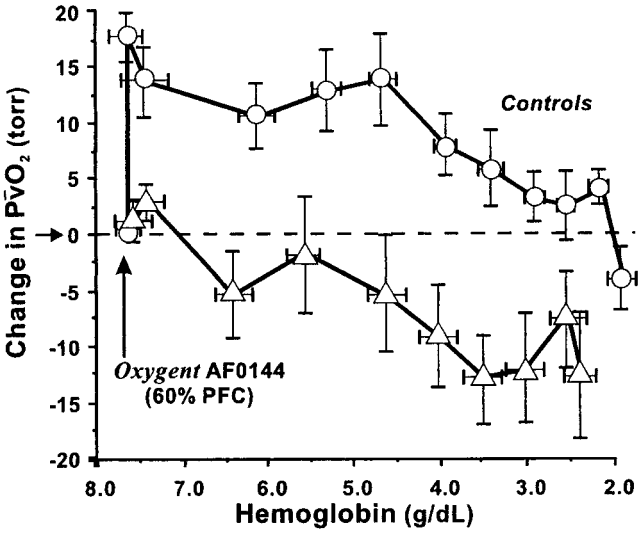


Figure 29. Efficacy of a PFC emulsion in a canine model mimicking surgical blood loss after acute normovolemic hemodilution. Both the treatment group and the control group animals are hemodiluted (from Hb about 14 to about 8 g/dL), breathe oxygen, and lose blood (hence Hb; *x*-axis) in a controlled manner. Mixed venous O_2 tension ($P\bar{v}O_2$, the oxygen tension in blood after tissues and organs have been irrigated; *y*-axis) reflects the adequacy of tissue oxygenation. The difference in tissue oxygenation in the two groups of animals is significant; the PFC-treated dogs (1.35 g of PFC per kg b.w. in this example) still have adequate tissue oxygenation at Hb levels one-fifth of normal. (Reprinted with permission from ref 1673. Copyright 1997 Birkhäuser.)

skeletal muscle, that the emulsion was as effective as fresh autologous RBC transfusion in preserving normal tissue oxygenation during profound ANH and could provide an additional margin of safety.[348] Cardiac output increased, and hemodilution of the dogs could be extended to a 3 g/dL Hb level without impairment of tissue oxygenation. A single infusion of 1.8 g/kg b.w. dose of PFC was as effective as transfusion of an amount of autologous RBC equivalent to about 3.8 g of Hb. There were no changes in systemic or pulmonary vascular resistance. Likewise, dogs that had been hemorrhaged until their $P\bar{v}O_2$ was equal or less than 25 Torr restored their $P\bar{v}O_2$ better when resuscitated with *Oxyfluor* than control animals treated with Ringer's solution.[1676] In a model of surgical hemodilution to a Hct of 20%, the PFC delivered 40% of the O_2 that was consumed.[1677]

Improved resuscitation and greater O_2 delivery were achieved by supplementing a standard crystalloid resuscitation solution with a PFOB emulsion in a swine model of near fatal hemorrhage.[1678] Infusion of the oxygenated emulsion into the aortic arch of dogs with ventricular fibrillation improved myocardial perfusion and restored spontaneous blood circulation.[1679] The following observations, using *Oxygent*, have further demonstrated that small doses of a PFC emulsion can contribute significantly to tissue oxygenation: increase in maximal O_2 consumption by an isolated working skeletal muscle in dogs in the presence of PFC,[377] effective preservation of cardiac muscle function in a canine model of low-flow coronary ischemia,[1680] prolonged sustainment of cardiac tissue oxygenation following arrest of coronary perfusion in the isolated rat heart,[1681] improved myo-

cardial O_2 delivery and aerobic metabolism during cold cardioplegic arrest of isolated rabbit heart with a PFC emulsion-enriched perfusate,[1682] protection of myocardium and oxygenation in a supply limited isolated perfused rat heart,[1683] graded increase in $P\bar{v}O_2$ and survival during CPB in dogs in response to increased doses of PFC emulsion,[371] improved tissue oxygenation and increased O_2 consumption in a porcine model of CPB,[1684] preservation of cerebral function following experimental brain stem ischemia in dogs,[1685] increase in retinal O_2 tension and oxygenation in cats,[388] improvement in primary visual cortex tissue pO_2 upon infusion of the PFC emulsion in cat,[1686] restoration of cerebral O_2 delivery with the emulsion in severely hemorrhaged rats,[1687] improvement of systolic function and reduction of myocardial edema and acidosis when standard blood cardioplegia was supplemented with PFC emulsion in dogs undergoing CPB and global myocardial ischemia followed by cold cardioplegic arrest and eventually normothermic reperfusion,[1688] sustained tissue perfusion and oxygenation of myocardium, intestinal mucosa, liver, lung, kidney, and skeletal muscle in heavily hemodiluted dogs treated with PFC emulsion,[1020] improved myocardial oxygenation and tolerance to low-flow ischemia (as found with certain surgical procedures) in isolated rabbit hearts,[1689] and enhanced tumor oxygenation leading to improved tumor-killer response to radio- and chemotherapy in animals and humans.[1690−1695]

Oxygen delivery by PFC emulsions was not counteracted by increased vascular resistance, and the emulsions did not trigger autoregulatory vasoconstriction despite substantial increases in $P\bar{v}O_2$ and tissue pO_2. Efficacy during clinical trials of *Oxygent* is discussed in section V. I.

Diverse observations suggest that in addition to bulk O_2 convection, PFC emulsions may also facilitate O_2 diffusion, although such facilitation has so far not been demonstrated directly. Unsickling of sickle cells in mice by administration of *Oxygent* indicates transfer of O_2 from the PFC droplets to Hb within the RBCs.[1696] Improvement in local tissue O_2 supply observed in *Fluosol*-perfused organs was greater than expected from the increased amounts of O_2 delivered by the emulsion, which was attributed to redistribution of microcirculatory blood flow.[1697] Significant increases in oxygenation were measured in the retina of O_2- or even air-breathing cats having received as little as 1 g/kg b.w. of PFC (Figure 30).[388] Several mechanisms could account for this improvement: contribution of PFC-dissolved O_2 to convective O_2 transport, the ability of the small emulsion droplets to reach microvessels that are usually only perfused by plasma, the high O_2 extraction ratio at the tissue level, characteristic of nonbonded O_2, and, possibly, facilitation of O_2 diffusion from RBCs to tissues by the numerous PFC droplets. Infusion of a PFC emulsion led to increased oxidation of cytochrome *a*, a_3 in tumors in rats, even when the animals breathed room air when convective O_2 transport is negligible.[1690] A near-wall excess of PFC droplets may explain the improvement of rheological properties and reduced hemolysis in blood exposed to mechanical stress when blood plasma was partially replaced by *Fluosol*.[392] The rate of RBC oxygenation/deoxygenation was seen to increase in vitro in the presence of small amounts of a poloxamer-based PFC emulsion.[1698,1699]

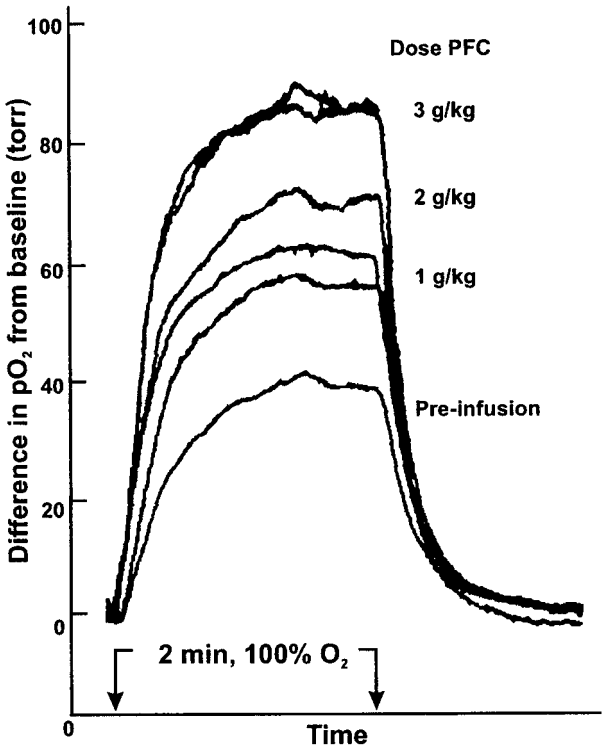


Figure 30. Effect of intravenous administration of a 100% w/v *F*-octyl bromide emulsion on tissue oxygenation of the cat retina. The difference in tissue pO_2 (as measured by an O_2-sensitive electrode) after three successive doses upon breathing pure O_2 is about twice that observed with 100% O_2 in the absence of PFC. (Reprinted with permission from ref 388. Copyright 1992 American Physiological Society.)

H. Product Safety and Mechanisms for Side Effects

Extensive efforts were devoted to establishing the safety profile of PFC emulsions and to identifying, understanding, and controlling their side effects.

1. Acute Toxicity

The intravenous LD_{50} of *Fluosol* was estimated at 26−29 and 35 g of PFC/kg b.w. in rats for 20% and 35% w/v concentrated emulsions, respectively, indicating that the volume injected (130−145 and 101 mL/kg, respectively) may have had a part in this toxicity.[222,1415] Similar figures were reported for *Perftoran*.[372] For PFOB, the LD_{50} reached 41 g/kg b.w. for a 100% w/v emulsion in rats.[1700]

When toxicity is observed with a particular PFC sample, this sample should be suspected of containing toxic byproducts or impurities. Distillation, washing with a KOH solution, and filtration over alumina usually suffice to remove such toxicity. Cell cultures provide a sensitive test for assessing impurity-related toxicity and monitoring purification.[1441,1535,1701] There is no sound evidence that the presence of isolated hydrogen, chlorine, bromine, secondary oxygen, or

tertiary nitrogen atoms or of a double bond in the PFC's structure induces any toxicity.

Fluosol elicited in some patients an anaphylactoid reaction related to complement activation (alternative pathway) that could include hemodynamic perturbations and was attributed to the surfactant.[440,1416,1702,1703] The approval by the FDA of *Fluosol* for use during PTCA in high-risk patients[161] implies that the product was nevertheless found safe for this indication. This untoward reaction was no longer seen with the EYP-based emulsions.[405,441,1609,1704,1705]

Contrary to *Fluosol*,[1706] *Oxygent* did not provoke endothelial cell activation.[1707] No leucocyte activation or substantial leucocyte adherence to vascular endothelium was found in vivo.[1708] Hypervolemic administration of up to 6 mL/kg of this emulsion had no adverse effects on the microcirculation in the hamster.[1709] Doses of 3 mL/kg induced no unwanted side effects on microvascular perfusion during profound ANH with the commonly used hemodiluents hydroxyethylstarch, albumin, and gelatin. On the other hand, use of a dextran solution in conjunction with the emulsion led to deterioration of microvascular perfusion, possibly related to activation and adhesion of leukocytes. Another EYP-based PFOB emulsion had no cytotoxic effect on cultured human vascular endothelium, the cells most exposed to the emulsion.[1622,1710] This PFC was actually shown to exert beneficial antiinflammatory effects and, in particular, to attenuate neutrophil adhesion to activated endothelial cells.[1711] Some of these antiinflammatory effects may be related to the lipophilic character of PFOB.[1712]

No carcinogenic, mutagenic, or teratogenic effects or immunological reactions have been reported for appropriately selected, purified, and formulated PFC emulsions.

2. Particle-Related Side Effects

a. Flu-Like Symptoms and Effect on Platelet Count. The clinical side-effect profile of the PFC/EYP emulsions is typical of macrophage activation related to RES uptake of particles. The side effects observed with the initial 90% and 100% w/v concentrated PFOB formulations consisted of early effects, including headache and occasional lower backache, which generally occurred during or shortly after infusion, and delayed effects (2−12 h) referred to as flu-like symptoms, e.g., fever, occasional chills and nausea.[405,1713−1715] These reactions, generally categorized as mild, were transient and fully reversible within 4−12 h.[406,1716] Transient increases in liver enzyme levels consequent to RES uptake of emulsions were also consistently noted. A temporary, moderate drop (about 15%) in platelet count was seen about 3 days after dosing. Similar effects have been reported with *Oxyfluor*,[406] and thrombocytopenia has been documented upon administration of parenteral fat emulsions,[1717] liposomes,[1718] including Hb-loaded liposomes,[987] and other injectable particulates.[1719−1721]

The mechanism of the flu-like side effects appears to be reasonably well understood. These effects are, for the most part, the natural consequence of RES clearance of particulate matter from the blood stream. Phagocytosis is accompanied by the release of products from the arachidonic acid cascade, including diverse prostaglandins and pyrogenically active cytokines.[405,1722] These responses could be effectively suppressed by prophylaxis with cyclooxygenase inhibitors or corticosteroids, which are known to interfere with the arachidonic acid cascade, and were not associated with the release of histamine, serotonin, bradykinin, or leukotriene D_4.[406,1722]

Acute toxicity and the magnitude and frequency of the above effects depend strongly on particle sizes.[405,1723−1725] Size minimization is also known to slow RES uptake of liposomes.[1726] Adjustments in the *F*-octyl bromide emulsion formulation and processing parameters and the addition of *F*-decyl bromide helped reduce particle sizes and narrow particle size distribution, resulting in significant attenuation of the side effects profile. During clinical safety studies in conscious volunteers with the optimized 60% w/v *Oxygent* formulation AF0144, fever frequency (∼15% of patients) and amplitude (seldom exceeding 1 °C) was not different from controls and platelet count, although temporarily depressed with respect to baseline, remained within normal range.[220,442,1727]

There were no effects on platelet function and coagulation parameters, no complement activation, immunogenic or allergic reactions, vasoconstriction or microcirculatory disturbances, abnormal changes in liver function, pulmonary or renal function, or clinically meaningful effects on blood chemistry at the doses administered.[442,1727] No emulsion-related adverse clinical events were reported in subsequent Phase II and III studies with *Oxygent* in surgical patients.[154,1728]

b. Potential Effects on Reticuloendothelial System Function. Extensive uptake of PFCs by macrophages results in enlargement of the liver and spleen.[404,1713,1729,1730] The effects of PFC emulsions on RES organ morphology, histology, and function have, therefore, been thoroughly investigated.[402,404,1505,1731,1732] Examination of liver, spleen, and bone marrow tissues of rats exchange-perfused with PFC emulsions indicated that the PFC-loaded cells had otherwise normal organelles and that the effect was fully reversible.

However, high doses of PFC can cause transient depression of RES clearance function. Magnetometric monitoring of ferromagnetic particles and ^{19}F NMR provided noninvasive methods for evaluating the influence of foreign particles on RES function.[1449] These effects were determined to be of the same nature for PFC emulsions as those of other particulates or colloids of similar size. Large particles are eliminated more rapidly and have greater potential for RES blockade.[222,1733] Clearance of colloidal carbon from circulation was transiently depressed in rats receiving high *Fluosol* doses.[1729,1733] Similar observations were made with colloidal iron chondroitin sulfate in surgical patients treated with that emulsion.[1731] Survival of human erythrocytes transfused to rats and primates was prolonged by infusing the recipients with *Fluosol* but was on the same order as that seen with a clinically used fat emulsion.[403,1734] Clearance by the liver of ^{51}Cr-labeled sheep RBCs

was slowed in rats injected with *Fluosol*-DA; overall RES function was, however, not significantly compromised.[1735] Tolerance of mice to endotoxins from *E. coli* was diminished by *Fluosol* in a dose-dependent manner; sensitivity to toxins was, however, significantly reduced by administration of hydrocortisone.[1736,1737] Interaction of PFCs with liver cytochrome P_{450} (as also evidenced by changes in clearance of various drugs) was PFC- and formulation-dependent.[1613,1738−1740] All structural changes resulting from the accumulation of PFCs in the tissues of rats having received very large 50 or 60 mL/kg doses of *Fluosol* were, however, fully reversible and considered nondetrimental.[1733,1741] The more recently developed PFC emulsions appear to have a lesser effect on the RES than the earlier ones.[1742,1743] Slight temporary overload and blockade of the RES is also known to occur upon administration of fat emulsions[408,1744,1745] and liposomes.[399,410,427,428,1745a] As noted earlier, Hb products as well as banked blood,[68] which contains nonviable RBCs, can also induce transient blockade of the RES.

c. Effect on Analytical Methods. The presence of a lipid emulsion in a blood sample can change its turbidity, which can interfere with measurements by CO-oximeters of oxyHb, carboxyHb, and metHb.[1746,1747] Interference was also noted for PFC emulsions with some instruments in the upper range of the intended clinical doses.[1748−1750] Use of a turbidity-correction algorithm that had been developed for an emulsion of the anesthetic *Propofol* (2,6-bis(1-methylethyl)-phenol) eliminated the effect of the emulsion on the CO-oximeter results.[1750] A PFDCO/saffoil/EYP emulsion led to overestimating platelet counts with certain automated cell counters,[1751] possibly due to the presence of lipid droplets or liposomes.[401] Such an interference was not found with emulsions with smaller particle sizes, such as *Oxygent*, even after 2 years of storage (0.18 μm).[1752]

3. Vapor-Pressure-Related Side Effects

The observation that the lungs of rats,[1398,1441] pigs,[1753] dogs, or rabbits[1453,1454,1754] given *Fluosol* or emulsions of certain PFCs, such as FX-80 or FDC, did not deflate normally at autopsy raised concerns about a possible toxic effect of PFCs in the lungs. This phenomenon was shown to relate to increased pulmonary residual volume (IPRV) due to retention of air in the alveoli.[1755,1756] It was found to be highly species-dependent and related to the PFC's vapor pressure and excretion rate. Sensitive species include rat, rabbit, swine, and macaque monkey, while the effect was not observed in mouse, dog, or man.[1757] The phenomenon was marked with FDC, unsignificant with PFOB, and absent PFDCO.[406] Biomechanical and O_2 transfer functions of lungs were essentially normal after administration of high doses (11 g/kg b.w.) of PFOB to dogs.[1758] Addition of a few percent of *F*-decyl bromide to *F*-octyl bromide reduced the vapor pressure of the PFC phase to less than 8 Torr, which sufficed to eliminate IPRV in most of the sensitive animal species.[405] No such effect was ever reported for the patients—several thousands—who had received *Fluosol*, although it contains FDC. Careful monitoring of patients receiving *Oxygent* revealed no effect either.[154,1759] Investigation of the mechanism of IPRV determined that the air bubbles that normally and continuously form in the alveoli are osmotically stabilized when water-insoluble PFC vapor is present.[1755] High transpulmonary pressures (which lead to bubble breakage), large airway dimensions, and low PFC vapor concentration in the lung (hence low capacity to stabilize the bubbles) ensure that the phenomenon is not operative in humans with the PFCs presently utilized.[1757] Interestingly, the phenomenon, once elucidated, was turned into a novel principle of osmotic stabilization of micrometer-sized air bubbles that was developed into a contrast agent for ultrasound imaging.[1315] The same principle, using microbubbles produced in vivo by injection of an *F*-pentane emulsion, has been proposed for O_2 delivery.[1760,1761] There is little doubt that microbubbles of O_2 can contribute to tissue oxygenation; circulation half-times have, however, not yet been reported for the *F*-pentane-stabilized bubbles, and withdrawal of a new drug application in the United States for use of this product as an ultrasound contrast agent may indicate that safety was not established.

I. Therapeutic Indications and Clinical Evaluation

Small doses of PFC emulsions are able to immediately and effectively deliver O_2 to tissues. The principal limitation of these products is, as for all the O_2 carriers presently under development, their short persistence in the circulation. While this limitation prevents their use as a treatment of chronic anemia, it does not restrict their temporary use, as for the duration of a surgical operation.[155,215−217,219,220,1762]

1. Avoidance and Reduction of Blood Use during Surgery

PFC emulsions could be important for avoiding or reducing exposure of surgical patients to donor blood. As indicated in section II.E (Figure 2), a novel strategy for reducing the need for donor blood transfusions has been defined that involves the use of an O_2 carrier in conjunction with ANH (or IAD) in the so-called "Augmented" ANH (or IAD) procedure. This strategy takes into account the emulsion's aptitude for delivering O_2, ability to preserve high cardiac output, and limited intravascular half-life. A computer model has been developed that allows prediction of $P\bar{v}O_2$ and other physiological responses to ANH and surgical blood loss when an O_2 carrier is administered to a certain patient.[393] This program was validated against a number of experimental and clinical studies. It indicated, for example, that at an FiO_2 of 1 and if the transfusion trigger is set at 7 g/dL, a single modest dose of 1.5 g/kg b.w. of PFC could allow a treated patient to lose 1700 mL more blood than a nontreated patient before $P\bar{v}O_2$ (reflecting global tissue oxygenation) falls below baseline.[216] The PFC should provide about 15% of total O_2 consumption when Hb levels decrease from 14 to 8 g/dL and cardiac output augments as a result of isovolemic hemodilution. $P\bar{v}O_2$ could be maintained at or above predosing levels as Hb concentration decreases from 8 to about 4 g/dL, corresponding to a

blood loss in the 3-L range for an average 70 kg individual.

Preclinical animal data and human clinical trials with *Oxygent* confirmed these expectations. Overall, the preclinical efficacy data indicated that the emulsion could allow for augmented ANH without impairment of tissue oxygenation, thus reducing the need for allogeneic blood transfusion.[152,1020] Even a low 0.9 g/kg dose of PFC, administered to patients after ANH, resulted in a measurable increase in $P\bar{v}O_2$ and $S\bar{v}O_2$.[1759] The $P\bar{v}O_2$ values remained at or above predosing levels, while Hb levels decreased substantially due to surgical blood loss. The contribution of a dose of PFC to O_2 consumption was subsequently described in terms of an Hb equivalency value. Using data from clinical trial patients the Hb equivalent of a 2.7 g/kg b.w. dose of *F*-octyl bromide was calculated to be about 4 g Hb (i.e., about 1.5 g Hb per g PFC) at an FiO_2 of 1.[1763]

Multicenter randomized Phase II trials involving patients undergoing elective surgery with ANH determined that a 1.8 g/kg dose of PFC was significantly more effective than *fresh* autologous blood (which is more effective than 2,3-DPG-depleted stored RBCs) at reversing physiological transfusion triggers.[154,155] Use of the O_2 carrier also substantially delayed the need for transfusion of the stored autologous blood. Similar results were obtained in patients undergoing genitourinary surgery.[1764]

Phase III clinical studies in surgical patients in Europe indicated that *Oxygent*, when administered according to the A-ANH procedure, substantially reduced the need for blood as compared to standard transfusion therapy. In the patient population (86% of the patients treated) for whom a transfusion is most likely needed (i.e., experiencing surgical blood loss from 10 to more than 80 mL/kg b.w.), *Oxygent* provided highly significant avoidance ($p = 002$) and reduction ($p < 0.001$) of blood transfusion.[1728]

2. Cardiopulmonary Bypass Surgery and Neuroprotection

Fluorocarbon emulsions may have value in CPB surgery.[371,1765,1766] They could potentially reduce the amount of blood needed for priming the extracorporeal circuit. In addition, because PFCs can dissolve N_2 as well as O_2, they could dissolve the tiny air bubbles that may be introduced in the CPB circuit and cause microemboli. They may thus provide a means of protecting the brain against postsurgical neurological damage and reduce the incidence and severity of the neurological deficits observed in a significant proportion of the patients who undergo cardiovascular surgery with CPB.[174,1767,1768] Experiments in which rats,[1769] rabbits,[1770] or dogs[1771] were injected air in an artery showed that animals that had received a PFC emulsion tolerated 3−5 times larger amounts of air than the untreated controls before irreversible brain damage or death occurred. The microvascular perfusion blockages that are observed on retinal angiography after a standard CPB procedure were reduced by 90% in dogs receiving a PFC-enriched priming solution.[1772] Both increased O_2 delivery and the capacity for PFCs to dissolve N_2 were invoked to explain these protective effects. No cerebral infarction was seen when *Oxyfluor* was added to the CPB priming solution of pigs that underwent a bypass procedure and subsequently received a massive bolus of air in the carotid artery.[1766] Cerebral blood flow was maintained, and electric activity was better preserved than in control animals that had received a saline solution. Improved tissue oxygenation was observed in an anemic canine model of hypothermic CPB with *Oxygent*; lower CPB flow rates than in control animals were allowed before a critical $P\bar{v}O_2$ had been reached.[371] There was also significantly lower mortality from cardiac failure on separation from CPB in the PFC-treated group. Reduction of the inflammatory response (as indicated by attenuated neutrophile activation) induced by an extracorporeal circuit was observed.[1773] Early clinical trials with this emulsion indicated an increase in cerebral blood flow in PFC-treated CPB patients.[1774]

3. Trauma—A Bridge to Transfusion

There are a number of instances where administration of a PFC emulsion could help temporarily correct an acute O_2 deficit. The emulsion could, for example, help bridge the time gap between a critical need for increased tissue oxygenation and transfusion of compatible blood or between the time of transfusion and when the transfused banked blood (including predonated autologous blood) becomes fully effective. Trauma is one such situation, in particular during the prehospital "golden hour" period when a patient's fate is decided. During this period, blood is usually not available and transfused stored blood is not effective yet. PFC emulsions could provide a means of stabilizing the patient waiting for an intervention and could therefore find their place in any ambulance or rescue vehicle.

4. Further Potential Indications and Uses of Fluorocarbon Emulsions

a. Cardiovascular. Potential cardiovascular uses for PFC emulsions, besides CPB, include treatment of acute myocardial infarction, cardioplegia, reperfusion, coronary angioplasty, and preservation of donor hearts for transplantation.[1775,1776] High O_2-delivering capacity, small particle sizes, and low viscosity are expected to improve tissue perfusion and oxygenation. Treatment of cardiac arrest is also being explored.[1679]

Reduction in myocardial ischemia and infarct size in dogs or pigs with coronary artery occlusions receiving PFC emulsions has been documented.[1434,1777−1779] Hemodilution with *Fluosol* of pigs with critical experimental coronary stenosis was better tolerated than with a non-O_2-carrying dextran solution.[1507] Because of small particle sizes, PFC emulsions may improve microvascular O_2 transport in tissues where RBC transport is impaired. Oxygen transport across a membrane-bound thrombus (blood clot) model suggested that the emulsions might carry O_2 across a thrombus and maintain tissue viability during acute ischemic events.[1780]

Addition of PFCs to cardioplegic solutions (used to protect the arrested heart during open-heart surgery) may be beneficial in case of prolonged myocardial

ischemia. In one study, cardioplegia with *Fluosol* was as effective as blood cardioplegia in allowing functional recovery of the heart after cold ischemic arrest[1781] but not in another study.[1782] Superior myocardial protection was provided by supplementation of blood cardioplegia with *Oxygent* in dogs; an endothelial-protecting effect was suggested.[1688]

Reperfusion of the myocardium after reconstructive artery surgery (angioplasty) can result in a reperfusion injury that limits the amount of salvageable myocardium.[180,1783] Several papers and reviews report the attenuation of such injury by PFC emulsions in animal models.[1784,1785] Oxygenated *Fluosol*, when infused in the coronary artery after the onset of reperfusion resulted, both in a canine model[1703,1786] and in a pilot clinical study,[180] in reduced infarct size and improved ventricular function. Accelerated thrombolysis by urokinase was observed in a dog model of acute myocardial ischemia.[1787] However, administration of *Fluosol* after treatment with the thrombolytic agent tissue plasminogen activator at the time of reperfusion of patients with acute myocardial infarction did not significantly reduce infarct size or improve cardiac function.[1788] On the other hand, dose-dependent protection against reperfusion injury was found with a more concentrated PFOB emulsion in a cardioplegic rat model, indicating that the emulsion could reduce the damage that can accompany cardiac surgery and organ transplantation.[1789] Mitigation of myocardial ischemia consequent to PTCA using oxygenated *Fluosol* resulted in the approval of this emulsion for high-risk procedures (section V.C).

b. Cerebrovascular. Stroke is one of the leading causes of death in the industrialized countries. The brain is extremely sensitive to O_2 deprivation, and irreversible damage occurs after brain ischemia greater than 5−10 min. In the case of vessel obstruction, the small size PFC droplets could improve perfusion and provide support to ischemic tissues by using smaller collateral vessels. Contrary to blood, the emulsions do not tend to have increased viscosity at low shear rate.

Experimentation with *Fluosol* for brain tissue rescue has met varying degrees of success.[1790−1795] *Fluosol* was reported to provide increased cortical O_2 delivery and some protection from cerebral ischemia and to reduce brain swelling, neuronal damage, and infarct size in cats with experimentally occluded middle cerebral artery.[386,1790,1791,1796] Other authors, although noting an improvement in brain tissue O_2 availability, found no improved outcome after 1 week.[1792] Using a similar stroke model, another group concluded that hemodilution with *Fluosol* did improve brain tissue oxygenation and reduced brain edema in the post-infarct period.[1794] These contradictions likely reflect the extreme complexity of the mechanisms implicated in neuronal death following stroke and the difficulty in defining proper strategies to combat them. A PFC/mannitol combination, when administered 0.5 h after onset of a 6 h period of cerebral artery occlusion in dogs, effectively prevented brain swelling[1797] and led to marked recovery of brain electrical activity,[1798] while PFC alone or mannitol alone was ineffective. Combination treatments involving PFCs, mannitol, vitamin E, and dexamethasone were deemed even superior.[1799] A *Fluosol*/mannitol combination was used in patients undergoing reconstructive vascular surgery to protect the brain against ischemic damage.[1800] An unspecified hyperosmolar saccharide-loaded FDC emulsion was reported to enhance brain pO_2 and reduce edema secondary to CPB in goats.[1801]

Intriguingly, it was shown that isolated brain slices could recover functionality when reoxygenated after up to 4 h in a nitrogen environment, leading to the hypothesis that microvascular dysfunction is the primary cause of brain damage and that neuronal changes and death are only secondary to these vascular effects.[1802] An original treatment of stroke was subsequently experimented that consisted of infusing an oxygenated, nutrient-enriched PFC emulsion directly into the cerebral ventricle (the cavity that contains the cerebrospinal fluid that bathes the brain), thus bypassing the clogged blood vessels.[1802,1803] Cats with experimental cerebral ischemia, when perfused in this fashion (ventriculocisternal perfusion) with an oxygenated F-44E emulsion within 3 h after onset of ischemia, demonstrated dramatically reduced infarct size and improved electrical brain activity.[1612] Other studies involving administration of a PFC into the cerebral ventricle were aimed at radiographic imaging of the central nervous system,[1804] at preventing brain tissue hypoxia,[1805] and at preventing paraplegia after aortic occlusion.[1806]

Prophylactic use of *Fluosol* improved hemodynamics and prevented paraplegia in dogs with spinal cord ischemia.[1807] These experiments are relevant to preventing irreversible damage to the spinal cord with resultant paraplegia in operations that require temporary clamping of the descending aorta. Likewise, cerebral functional protection during brain stem ischemia was demonstrated with *Oxygent* in dogs, suggesting that the emulsion could extend the allowable period of vascular occlusion during cerebrovascular repair surgery.[1685] Further studies indicated that small doses of *Oxygent* improved oxygenation of brain tissue[1687,1808] and of the retina (an outgrowth of the brain),[1686] sustaining the hope for treatment of cerebrovascular diseases, if proper strategies of use can be determined.

c. Oncology. Because of poor vascularization, solid tumors generally contain hypoxic cells. These cells are resistant to radiation therapy and to certain anticancer drugs because O_2 participates in the reactions that produce DNA damage. Numerous experimental studies have established that PFC emulsions could deliver O_2 deep into tumor regions that would otherwise be hypoxic, thereby improving the response of tumor cells to radio and chemotherapy.[1690,1691,1693−1695] Phase I/II clinical evaluation of *Fluosol* as an adjuvant to radiation in non-small-cell carcinoma in the lung demonstrated that large doses of PFC (∼10 g/kg in 5−7 weeks) could be administered without significant toxicity.[1809] Although these early clinical trials were considered encouraging,[1692,1809−1811] further studies were not initiated. Combination of a concentrated PFOB emulsion with hyperbaric (3 atm) O_2 selectively increased

the radiation sensibility of tumors relative to normal tissues.[1812] While repeated treatment of mammary tumors in the rat by irradiation or with cisplatin or cyclophosphamide was seen to increasingly reduce tumor oxygenation, administration of *Oxygent* was able to increase the O_2 content of the tumors, both in the absence of treatment and after each of the treatments, thus increasing their sensitivity to the treatment.[1813] Strategies combining several cytotoxic and sensitizing agents and improved PFC emulsions were advocated.[1695]

PFC emulsions were also investigated as adjuncts to photodynamic cancer therapy. This procedure depends on the use of tumor-localizing porphyrins that are activated by visible light in the presence of O_2 to induce tumor destruction.[1814] A PFC emulsion appeared to provide some protection to the skin against photodynamic therapy damage.[1815]

d. Preservation of Organ and Tissues for Transplantation. Use of PFC-enriched perfusates has the potential for increasing the availability and quality of organs suitable for transplantation. Kidney, heart, liver, lung, pancreas, testis, and multiple organ blocks have been preserved using such preparations. Early PFC-containing perfusates allowed preservation of rat liver[1816] and kidney[1817] and maintenance of beating rat hearts for over 10 h at 37 °C.[1818] *Fluosol*-DA protected ischemic transplanted hearts from reperfusion injury in a dog model.[1819] Improved preservation of isolated rat brains,[213,1820,1821] rat heart,[1822] canine heart,[1823] rabbit kidneys,[1824] rat, swine and canine livers,[1825−1827] pig lungs,[1828] rabbit heart and lung,[1122] and rabbit limbs[1829] has been achieved using Fluosol-43 or perfusion media containing this emulsion. The same emulsion was superior to hypothermia in inhibiting free-radical generation and preventing ischemia-reperfusion injury of skeletal muscle in rabbits.[1830] Reduced thromboxane release and skeletal muscle reperfusion injury were seen with *Fluosol* in dogs.[1831,1832] Increased synthesis of the high-energy phosphate phosphocreatine was seen during cold reperfusion of ischemic isolated pig hearts after cardioplegic arrest.[1833] Exchange perfusion of rats with Fluosol-43 to remove recipient humoral immune factors allowed prolonging the survival time of guinea pig heart xenografts.[1834] Perfusion of rat hearts using a concentrated PFOB emulsion produced increased contractile performance.[1835] Perfusion of rabbit hearts with a 35% v/v *F*-perhydrophenanthrene/EYP emulsion improved functional recovery after transplantation.[1836] *Oxygent* allowed effective preservation and transplantation of dog kidneys.[1837] Other PFOB emulsions allowed prolonged preservation of the intestine[1838] and improved warm preservation of the kidney[1839] and of multiple organ blocks.[1621] Traumatically severed fingers were preserved for 72 h using *Fluosol* prior to successful reimplantation.[1840]

Fluorocarbon emulsions have been used to improve cell cultures[1841] and preserve pancreatic islets.[1842] They helped enhance O_2 transfer and provided protection against damage by gas bubbles in hybridoma cell cultures[1843] and increased production of the antibiotic actinorhodin from *S. coelicolor* cultures.[1844]

e. Diagnostic and Tissue Oxygen Mapping. Various types of PFC-based contrast agents for diagnosis using X-ray computed tomography, MRI, and ultrasound imaging have been investigated.[1458] Differences in distribution of FMIQ **4** coupled with an anti-carcinoembryonic antigen antibody and of noncoupled FTPA **3** (administered as separate emulsions) allowed ^{19}F MRI of a human colon carcinoma implanted in nude mice.[1845] PFOB emulsions allowed in vivo delivery of hyperpolarized ^{129}Xe for MRI applications.[347]

Fluorocarbon emulsions were used for measuring tissue O_2 tension and mapping O_2 in tissues by exploiting the ability of the paramagnetic O_2 molecule to perturb the ^{19}F NMR signal.[1846−1851] ^{19}F NMR allowed measuring myocardial vascular volume,[1852] local cerebral volume,[1853] and tumor imaging[1854] and was used for PFC clearance studies.[1640]

f. Miscellaneous Applications of Perfluorocarbon Emulsions in the Biomedical Field. Incubation of sickled erythrocytes with an oxygenated FTBA emulsion reduced the percentage of sickled cells by one-half, increased their deformability, and significantly reduced the viscosity and resistance to flow of the cell suspension.[1855] *Oxygent* decreased peripheral resistance in a transgenic sickle cell mouse model by unsickling the sickled RBCs that partially obstructed vessels.[1696] PFCs may thus have potential for alleviating vaso-occlusive crisis and tissue infarction in sickle cell patients. PFC emulsions may also represent an alternative for patients with RBC incompatibilities.

Intraperitoneal perfusion with *Fluosol* resulted in significant increases in arterial pO_2 in an animal model, suggesting an alternative to pulmonary oxygenation during certain forms of respiratory failure.[1856] Intraluminal treatment with oxygenated FTBA emulsions had a protective effect against intestinal ischemia,[1857] leading to reduced mortality in rats.[1858] Tolerance to normally lethal doses of CO in rats exchange-perfused with a PFC emulsion has been demonstrated.[1396,1397] Accelerated displacement of CO from carboxyHb and improved survival were seen in PFC-treated dogs that had been exposed to CO.[1859] Three patients with CO intoxication were reported to have been treated with *Fluosol*.[1860]

Treatment of decompression sickness has been investigated.[342−346] In one experiment in hamsters 94% of the animals survived when given i.v. PFC emulsion as compared to 6% in the nontreated controls.[344] The incidence of severe decompression sickness was significantly reduced by treatment with *Oxygent* in a pig model, indicating potential for the rescue from ill-fated diving or underwater operations.[346]

Further potential applications for O_2-carrying PFC emulsions include total body washout for removal of toxins, viruses, drug overdoses, etc;[1861] treatment of acute pancreatitis;[1862] lavage of stomach to protect the gastric mucosa against damage provoked by hemorrhagic shock;[1863] use in an extracorporeal blood gas exchange system for treatment of respiratory failure;[1864] and as drug delivery systems.[1316,1317] *Perftoran* has even been used to sober up dead drunk

patients;[1865] accelerated oxidative metabolism of ethanol may be the basis for the cure! There are also indications that PFC emulsions might have antiinflammatory effects.[1866]

g. Perfluorocarbon Emulsions as a Research Tool. PFC emulsions can be used to stabilize and control animal models, organs and tissues destined for experimental studies.[1867,1868] "Bloodless" animals, obtained by near-total replacement of the blood of conscious animals, constitute a unique research tool.[1869−1871] They have been used for evaluation and screening of PFCs, surfactants, and emulsion formulations;[222,397,1441] investigating hematopoiesis, specific hormones, enzymes, and other proteins production and transport; prolonging the half-life of enzyme-sensitive compounds in the circulation;[1861] avoiding the optical interference caused by RBC Hb in studies on living tissues; investigating metabolic activities of tissues,[1872] the intramitochondrial respiratory process in vivo,[1873] pulmonary microvascular permeability;[1874] and brain physiology.[1875] A PFC emulsion was used for oxygenating immobilized biocatalysts[1876] and an FDC affinity emulsion for extracting an enzyme from yeast.[1877,1878]

5. Related Fluorocarbon-Based Products for Biomedical Applications

Further biomedical applications of PFC-based products, other than O_2 carrying PFC-in-water emulsions, will be briefly mentioned here.[1317,1409]

a. Neat Liquid Perfluorocarbons. Neat *F*-octyl bromide was evaluated for treatment of acute lung injury and acute respiratory distress syndrome by liquid ventilation therapy.[1464,1466,1467,1879,1880] The dense and fluid PFC was instilled into the patient's lungs, where it was expected to contribute to reopening collapsed alveoli, facilitate the exchange of the respiratory gases, and protect the lungs from some of the harmful side effects (barotrauma or volutrauma) of conventional mechanical ventilation. Phase I and II trials have indicated improvement of lung compliance and oxygenation status.[1464,1880] Mortality reduction in Phase II/III trials was, however, not better than with standard treatment using the latest, improved lung protection strategies. Antiinflammatory effects have been reported.[463,464,1466,1711,1880a]

F-Octyl bromide has gained approval in the United States for oral use as a bowel marker during MRI.[1882] The absence of protons, hence of signal, creates the desired contrast, allowing improved delineation of stomach and bowel walls, thus facilitating the identification of pathologic tissues. Aerosolized PFCs allowed analysis of lung structure and pulmonary oxygenation patterns using ^{19}F NMR.[1851] Externally applied PFC-filled pads are available commercially (*SatPad*, Alliance Pharmaceutical Corp.), which improve magnetic homogeneity, hence image quality, when fat saturation techniques are utilized during MRI.[1883] Gaseous and liquid PFCs and fluorocarbon−hydrocarbon diblocks are used as tools in vitroretinal surgery for treating complicated retinal detachments or managing the dislocated crystalline lens.[1884−1890]

Further applications investigated for neat PFCs include treatment of acute intestinal ischemia by intraluminal or peritoneal lavage,[1891] use in blood oxygenators,[1392,1892,1893] prevention of the bends by liquid breathing,[1894] as a foot bath therapy for treatment of ischemic ulcers,[1895] for preserving plant[1896] and animal[1897] semen, tissues, and transplants,[1898,1899] reducing the rejection of xenografts,[1900] growing nematodes,[1901] increasing growth rates of animal (including human) and plant cell cultures,[1844,1896,1902−1906] and removal of deleterious excess of O_2 photosynthesized by algae.[1907]A CO_2-gassed PFC was used to enhance the growth and multiplication of rose shoots.[1908] A further area of application of neat liquid PFCs relates to the delivery of drugs,[978,1909,1910] genes,[1911,1912] or immunoglobulins[1913] to the lung.

b. Injectable Gaseous Microbubbles. Diverse types of micrometer-size gas bubbles have been generated or stabilized using volatile PFCs, including *F*-propane, *F*-pentane, and *F*-hexane, to serve as contrast agents for diagnostic by ultrasound imaging.[1315,1578,1914−1917,1917a] When injected in the circulation, these microbubbles function as reflectors for ultrasound waves, provide clearer ultrasound images, and have the potential to facilitate assessment of cardiac structure and function, diagnosis of perfusion abnormalities, and detection of solid tumors in organs.[1917,1918] One such product, *Optison* (Molecular Biosystems, San Diego, CA), is now commercially available.[1919] Other products have been submitted to health authorities for approval. One of them, *Imavist* (Alliance Pharmaceutical Corp./Schering AG, Germany), exploits the extremely low solubility of PFCs in water in order to osmotically stabilize phospholipid-coated air bubbles.[1315,1920−1922] Another, *EchoGen* (Sonus Pharmaceuticals Inc., Bothell, WA), was a fluorosurfactant-stabilized emulsion of *F*-pentane which, once infused in the circulation, converted into micrometer size PFC gas bubbles.[1578,1915,1916] Such microbubbles, when used in conjunction with ultrasound, may have the potential for binding and breaking up blood clots that cause myocardial infarction or stroke.[1923,1924]

c. Further Fluorocarbon-Based Colloids. The availability of new fluorosurfactants[1530−1532,1537,1925] allowed the realization of a number of colloidal systems with a PFC phase, including stable reverse (i.e., water-in-PFC) emulsions and microemulsions,[1926,1927,1927a] hydrocarbon-in-fluorocarbon emulsions,[1928] and multiple emulsions (including combinations that involve three distinct nonmiscible phases, a PFC, a hydrocarbon, and water).[1929] Both lipophilic and hydrophilic drugs can be loaded in such multicompartment systems, which have potential for delivery through the pulmonary route. Various types of gels with continuous or dispersed PFC phases have been reported.[1930,1931]

Because of the extreme hydrophobic and definite lipophobic characters of their fluorinated chains, fluorinated amphiphiles tend to self-assemble into films, membranes, vesicles, tubules, and other supramolecular systems when dispersed in water and other solvents. Fluorinated and hydrogenated amphiphiles tend to phase-separate into distinct domains. *F*-Alkyl chains promote supramolecular or-

dering and provide exceptionally stable Langmuir films and bilayer membranes.[1316,1317,1932−1934] A variety of fluorinated vesicles (liposomes) have been reported. These are characterized by the presence of a well organized, hydro- and lipophobic (Teflon-like) fluorinated film within their bilayer membrane, thereby inducing enhanced stability, reduced membrane permeability, and reduced aptitude to fusion as compared to standard liposomes.[1317,1534,1537,1935,1936] Fluorosurfactants, including nonchiral ones, also formed stable tubular aggregates with diverse morphologies.[1937−1940] Fluorosurfactants were proposed as a means of stabilizing dispersions of micronized drugs in hydrofluoroalkanes (CFC substitutes) for delivery by meter-dose inhalers.[1941] Finally, fluorosurfactants may have therapeutic applications by themselves.[1866]

VI. Perspectives

Over time, the definition of blood substitutes has evolved. The initial intent of developing an all purpose "artificial blood" to be used instead of, and in the same circumstances as, blood has progressed into the more realistic goal of providing the physician with a novel means of temporarily alleviating tissue hypoxia and its consequences. This could be especially important for avoiding or reducing the surgical and critical care patient exposure to allogeneic blood and could be used in conjunction with, including global strategies for better using a patient's own fresh blood.

Blood transfusion has never been as safe as it is today. However, a new major risk has emerged: blood shortages. Blood shortages cause increasingly frequent postponement of elective surgeries and are predicted to increase further in the coming years. With the current shift of emphasis from blood safety to blood availability, the potential role of O_2 carriers as blood conservation agents has become even more critical. Furthermore, the safety and efficacy of allogeneic blood transfusion is increasingly questioned because of immunosuppressive effects and the realization that banked blood is not immediately effective and not as well tolerated as fresh blood. Therefore, the commercial availability of injectable O_2 carriers is likely to significantly impact blood transfusion practices.

Avoidance or reduction of the use of banked blood in surgery has become a primary goal of O_2 carrier development. Oxygen carriers may also become the first line of therapy in emergency situations, by providing a bridge to transfusion for trauma patients. Further likely applications include priming of CPB circuits, the treatment of myocardial and cerebral ischemia, use in cardioplegia, potentiation of radiotherapy and chemotherapy, and the treatment of sickle cell anemia. Some Hb products may provide a means of neutralizing excess NO produced during septic shock as well. Products with specific modifications, such as those integrating some antioxidant character, may find uses in specific settings. The ability of PFCs to also dissolve nitrogen could reduce the risk of cerebral air microembolism and consequent neurologic deficit following surgery involving CPB devices. Because their performances are preserved at low temperature, PFCs are additionally attractive for organ preservation and tissue culture.

The interference of O_2 carriers with physiology needs to be minimal. Preservation of normal hemodynamics and responses to hemodilution, nitric oxide functions, redox equilibria, and RES functions is necessary for most indications. Additionally, O_2 carriers should not be immunogenic, cause inflammation, elicit organ toxicity, or promote bacterial infection. The potential benefits for patients and society and whether these benefits outweigh the risks associated with the use of a given product need clear demonstration. Safety and efficacy alone, however, do not suffice. Successful products need to have long storage stability and large-scale product ion feasibility, implying the availability of safe and abundant raw material; they must also be user-friendly and gain widespread physician acceptance; finally, they must be cost-effective as well.

Reasons for the slow development of O_2 carriers include insufficient basic understanding of O_2 physiology and mode of action of modified Hb and PFC emulsion in vivo; an underestimation of the complexity of the enterprise; unexpected interferences with normal physiology, including Hb's many functions, especially in the ill patient; insufficient understanding of side effect mechanisms; lack of clear discernment of what the product characteristics, indications, and conditions of use should be; the difficulty of demonstrating the clinical benefit of such novel classes of drugs (even though O_2 delivery was usually established) in the absence of clear endpoints, validated protocols, and of a validated standard; increasingly stringent regulatory requirements; and the length of time and size of the investments required in order to develop them. Each product is unique and has its own set of chemical, physical, and biological characteristics, advantages, and side effects. Setbacks have been numerous. However, each setback has stimulated new research. Our knowledge of O_2 delivery to tissues, in vivo redox chemistry of Hb, in vivo behavior of PFCs, etc., has substantially advanced, largely due to the efforts and resources devoted to blood substitute research and to the diversity of the options that have been explored, resulting in considerable advances in product understanding, efficacy, and safety.

The Hb (biologic) and PFC (synthetic) approaches, while fundamentally different, are not exclusive from one another. Both types of products have demonstrated the capacity to carry and deliver O_2 to tissues in certain settings. The complexity encountered with Hb products derives from the fact that their functioning is highly dependent on environment; from their not only being O_2 carriers, but also having drug activity of their own and from their chemical reactivity and lability. As unwanted characteristics of the cell-free protein were understood and corrected, further layers of complexity were uncovered. Restoring adequate protein characteristics often required several successive modifications, resulting in increased heterogeneity, warranting investigation of any other clinical consequences of such modifications.

Understanding and alleviating the pressor effect elicited by most acellular Hb products is certainly one of the most trying challenges that the development of such products has faced. The mechanisms of vasoconstriction and blood flow redistribution remain controversial, although neutralization of endothelium-produced NO appears to be a prominent factor. The importance of molecular size and surface characteristics and of solution viscosity in controlling hemodynamics has recently been demonstrated. Exploitation of a potential benefit from the hypertensive activity of Hb products has become questionable in light of recent clinical experience. Because patients who may receive blood substitutes are particularly at risk for septic complications, the mechanisms and extent to which some O_2 carriers increase patient susceptibility to bacterial infection and endotoxemia also need to be further elucidated.

One interesting characteristic of PFCs is their chemical inertness and lack of sensitivity to environment. Due to physical dissolution, the O_2 transported by PFCs is readily and fully available, providing a simple, passive means of rapidly correcting an O_2 deficit. Furthermore, PFC emulsions do not elicit vasoconstrictive activity. Small doses of PFC emulsions can contribute significantly to O_2 consumption due to the high extraction ratio of O_2 by tissues, because of the possibility of increasing O_2 dissolution in PFCs by simply increasing the O_2 fraction in the air inspired by the patient, and because the increase in cardiac output that is normally observed in hemodiluted patients is preserved. Furthermore, PFC emulsions, because of simple processing and low capital expenditure and production cost, could play a part in allowing developing countries to benefit from an injectable O_2 carrier other than RBCs.

Cost has become an essential component of the equation for success, as managed healthcare has become a priority in all western countries. It is likely that the cost of blood substitutes will need to remain in the neighborhood of the cost for banked blood. Raw material procurement, difficulties with scale-up, processing costs, and limited yields may prevent some effective products from playing a significant part in this increasingly constraining pharmacoeconomic environment. Little relief of blood shortages can be expected from products derived from human blood. Bovine Hb may provide an abundant (but not inexpensive) supply of Hb, assuming that absence of immunologic effects and interspecies crossover of pathogens can be clearly established and accepted. Whether recombinant Hb can be produced cost-effectively remains to be determined. At this point, PFCs provide the only approach that does not depend on the collection of human or animal blood. Fluorocarbon emulsions can be manufactured by straightforward, cost-effective, high-yield additive procedures that are well established in the pharmaceutical industry.

The need for intensive basic research and close, constant, and continuously improved dialogue between those involved in basic research and pharmaceutical product development cannot be overemphasized. Drug development cannot proceed successfully without it. There will undoubdetly be future generations of both Hb- and PFC-based O_2 carriers. However, further basic research is needed to better understand the "pharmacology" of the drug *oxygen* itself, better define the desirable attributes of O_2 carriers, improve control of chemical modification of Hb and characterization of product composition, molecular structures, and evolution over time, better understand the influence of chemical or genetic modification or encapsulation on in vivo distribution, metabolism, and clearance and their effects on O_2 transport, vascular tone, hematopoiesis, and basic physiology in general. Further questions requiring attention include immunogenicity of modified Hb, inflammatory and microvasculature effects, autoxidation and the generation of reactive oxygen radicals, catalysis of lipid peroxidation, effect of saturation of clearance and catabolism capacity, effect of both Hb and PFC-based products on the RES and other defense mechanisms. Several promising new Hb preparations that address some of these concerns, including pegylated Hb products and liposome formulations, are undergoing preclinical evaluation. Future research on PFC emulsions will certainly aim at better understanding their interactions with the RES, further reducing residual side effects, and optimizing methods and conditions of use. Prolonging circulation persistence is another obvious goal. Surface engineering is being investigated for this purpose. Such modifications also have the potential of reducing any impact of large doses of product on the RES. However, the fundamental basis for developing O_2 carriers with a significantly prolonged circulation life, not to speak of one comparable to that of RBCs, is not yet available. Novel therapeutic applications will undoubtedly be explored, and certain O_2 carrier characteristics may be tuned to specific clinical applications. Clear endpoints and appropriate testing protocols will therefore be needed. Genetic engineering has reached a level of sophistication that allows precise manipulation of Hb's structure and properties, offering virtually endless possibilities. Recombinant Hbs that have simultaneously low O_2 affinities and low autoxidation rates and capable of discerning NO from O_2 while complying with the other requirements for a blood substitute, including cost-effectiveness may, at some point, provide a serious chance for O_2 carriers to overcome their present limitations. Each research program and each development effort brings us closer to viable commercial products. The market for effective and affordable O_2 carriers is very large and there should obviously be room for more than one "blood substitute", possibly for different applications. Each approach and product has its champions, without the dedication (and ambitions) of whom there would be no progress. Although the "natural" Hb route appears more intuitive a priori, there are also compelling reasons for developing PFC-based O_2 carriers. It is this author's opinion—and personal bias—that PFC-based products may have a good chance of meeting the requirements of a safe, effective, and affordable temporary O_2 carrier and of gaining widespread use in a near future. Clinical evaluation, regulatory decisions, and time will tell.

VII. Acknowledgments

I thank my former students, technicians, and coworkers of the Unité de Chimie Moléculaire in Nice and colleagues and friends at Alliance Pharmaceutical Corp. in San Diego and elsewhere, who over all these years helped to stay on line against all the odds. Grateful thanks also to those who reviewed and provided critical comments on various sections of the manuscript, including Abdu Alayash (Bethesda, MD), George Biro (Etobicoke, Canada), Alexis Caron (Nancy, France), Simon Faithfull (San Diego, CA), Steven Flaim (Santa Clara, CA), François Jeanneaux (Nice, France), Marie Pierre Krafft (Strasbourg, France), Maurice Lamy (Liége), Michael Marden (Paris, France), Viktor Obraztsov (San Diego, CA), Josée Pagnier (Paris France), Helen Ranney (San Diego, CA), Jolene Shorr (San Diego, CA), Claude Vigneron (Nancy, France), and Thomas Zuck (Cincinnati, OH), and Lora Barkley for her artful assistance with graphics, and Patricia Rusheen for help with documentation. I am also indebted for support provided over the years by numerous institutions and industries, particularly the Centre National de la Recherche Scientifique, AtoFina, and Alliance Pharmaceutical Corp.

VIII. Abbreviations

2,3-DPG	2,3-diphosphoglycerate
A-ANH	augmented acute normovolemic hemodilution
ANH	acute normovolemic hemodilution
ATP	adenosine triphosphate
b.w.	body weight
BME	bis(*N*-maleimidomethyl)ether
cGMP	cyclic guanosine-3,5′-monophosphate
CPB	cardiopulmonary bypass
DBBF	bis(3,5-dibromosalicyl) fumarate
DCC	dicyclohexylcarbodiimide
DCLHb	diaspirin-cross-linked hemoglobin
Dex-BHC	dextran-benzene hexacarboxylate
Dex-BTC	dextran-benzene tetracarboxylate
DIBS	2,5-diisothiocyanatobenzene sulfonate
DIDS	4,4′-diisothiocyanatostilbene-2,2′-disulfonate
DMPC	dimyristoyl-*sn*-glycero-3-phosphocholine
DPPC	dipalmitoyl-*sn*-glycero-3-phosphocholine
DSPC	distearoylphosphatidylcholine
ECF	electrochemical fluorination
EDCI	1-(3-dimethylaminopropyl)−3-ethylcarbodiimide
EDRF	endothelial-derived relaxing factor
ESR	electron spin resonance
EYP	egg yolk phospholipids
F-	perfluoro
F-44E	bis(perfluorobutyl)ethene
FDC	perfluorodecalin
FFA	free fatty acids
FiO_2	fraction of O_2 in the inspired air
FMA	perfluoromethyladamantane
FMCP	perfluoromethylcyclohexylpiperidine
FMIQ	perfluoro-*N*-methyldecahydroisoquinoline
FMOQ	perfluoro-4-methyloctahydroquinolidizine
FTBA	perfluorotributylamine
FTPA	perfluorotripropylamine
FX-80	perfluoro-2-butyltetrahydrofurane
Hb	hemoglobin
Hct	hematocrit
HES	hydroxyethylstarch
HPLC	high-performance liquid chromatography
i.v.	intravascular
IAD	intraoperative autologous donation
IHP	inositol hexaphosphate
IPRV	increased pulmonary residual volume
LEHb	liposome-encapsulated Hb
metHb	methemoglobin
MLV	multilamellar vesicle
mPEG	monomethoxypoly(ethylene glycol)
MRI	magnetic resonance imaging
MW	molecular weight
NADH	nicotinamide−adenine dinucleotide
NFPLP	nor-2-formylpyridoxal-5-phosphate
NMR	nuclear magnetic resonance
o-ATP	ring-opened oxidized ATP
o-Raffinose	ring-opened oxidized raffinose
PA	phosphatidic acid
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PEG	poly(ethylene glycol)
PFC	perfluorocarbon
PFDB	perfluorodecyl bromide
PFDCO	perfluoro-α,*ω*-dichlorooctane
PFOB	perfluorooctyl bromide
PHP	pyridoxylated hemoglobin poly(ethylene glycol)
PLP	pyridoxal-5-phosphate
POE	polyoxyethylene
PTCA	percutaneous transluminal coronary angioplasty
PTFE	poly(tetrafluoroethylene)
RES	reticuloendothelial system
SUV	small unilamellar vesicle
TEM	transmission electron microscopy
TEMPO	2,2′,6,6′-tetramethylpiperidinenitrosyl
THAM	tris(hydroxymethyl)aminomethane
R	C_nH_{2n+1}
R_F	C_nF_{2n+1}

IX. References

(1) von Goethe, J. W. *Faust I, First Part* **1808**. (Translation: Blood in a very special sap.)
(2) Siler, J. L.; Jereski, L.; Baker, S. *Bus. Week* **1990**, *May 21*, 42.
(3) Mitchell, J. *Wall St. J.* **1991**, *April 11*, p 34.
(4) Diamond, L. K. In *Blood, Pure and Eloquent*; Wintrobe, M. M., Ed.; McGraw-Hill: New York, 1980; p 659.
(5) *Blood, Pure and Eloquent*; Wintrobe, M. M., Ed.; McGraw-Hill: New York, 1980.
(6) Rossi, E. C.; Simon, T. L.; Moss, G. S.; Gould, S. A. *Principles of Transfusion Medicine*; Williams & Wilkins: Baltimore, 1996.
(7) Greenwalt, T. J. *Transfusion* **1997**, *37*, 550.
(8) Roux, J.-P. *Le Sang. Mythes, Symboles et Réalités*; Fayard: Paris, 1988.
(9) Starr, D. *Blood: an Epic History of Medicine and Commerce*; A. A. Knopf: New York, 1998.
(10) Landsteiner, K. *Wien Klin. Wochenschr.* **1901**, *14*, 1132.
(11) Hustin, A. S. *J. Méd. Bruxelles* **1914**, *12*, 436.
(12) Weil, R. *J. Am. Med Assoc.* **1915**, *64*, 425.
(13) Lewisohn, R. A. *Med. Rec.* **1915**, *87*, 141.
(14) Zuck, T. F.; Bensinger, T. A.; Peck, C. C.; Chillar, R. K.; Beutler, E.; Button, L. N.; McCurdy, P. R.; Josephson, A. M.; Greenwalt, T. J. *Transfusion* **1977**, *17*, 374.
(15) Wallace, E. L.; Churchill, W. H.; Surgenor, D. M.; Cho, G. S.; McGurk, S. *Transfusion* **1998**, *38*, 625.
(16) Goodnough, L. T.; Brecher, M. E.; Kanter, M. H.; AuBuchon, J. P. *N. Engl. J. Med.* **1999**, *340*, 438.
(17) *Blood Banking Data Base and Survey*; World Health Organisation: Geneva, 1993.
(18) Bassett, P. "Blood Substitutes, Selected Growth Factors, Stem Cells and Hemoglobin Modifiers. Products, Markets and Opportunities," International Business Communications, Inc. (citing 1995 World Health Organization sources), 1995.
(19) Sullivan, M. *Proceedings of the U.S. Public Health Service*; Advisory Committee on Blood Safety and Availability: Bethesda, MD, 1999; p 41.
(20) Sullivan, M. T.; Wallace, E. L.; Umana, W. O.; Schreiber, G. B. *Transfusion* **1999**, *39*, 1S.

(21) Zuck, T. F. *Arch. Pathol. Lab. Med.* **1990**, *114*, 309.
(22) Finucane, M. L.; Slovic, P.; Mertz, C. K. *Transfusion* **2000**, *40*, 1017.
(23) Sloand, E. M.; Pitt, E.; Klein, H. G. *J. Am. Med. Assoc.* **1995**, *274*, 1368.
(24) Dodd, R. Y. *Immunol. Invest.* **1995**, *24*, 25.
(25) Fakhry, S. M.; Sheldon, G. F. *Adv. Surg.* **1995**, *28*, 71.
(26) Klein, H. G. *Am. J. Surg.* **1995**, *170*, 21S.
(27) Lackritz, E. M.; Satten, G. A.; Aberle-Grasse, J.; Dodd, R. Y.; Raimondi, V. P.; Jansen, R. S.; Lewis, W. F.; Notari, E. P.; Petersen, L. R. *N. Engl. J. Med.* **1995**, *333*, 1721.
(28) Schreiber, G. B.; Busch, M. P.; Kleinman, S. H.; Korelitz, J. J. *N. Engl. J. Med.* **1996**, *334*, 1685.
(29) Holland, P. V. *N. Engl. J. Med.* **1996**, *334*, 1734.
(30) Schreiber, G. B.; Murphy, E. L.; Horton, J. A.; Wright, D. J.; Garfein, R.; Chien, H. C.; Nass, C. C. *J. Acquired Defic. Syndr. Hum. Retrovirol.* **1997**, *14*, 263.
(31) AuBuchon, J. P.; Birkmeyer, J. D.; Busch, M. P. *Ann. Intern. Med.* **1997**, *127*, 904.
(32) Fiebig, E. *Clin. Orthopaed. Relat. Res.* **1998**, *357*, 6.
(33) Williamson, L. M.; Lowe, S.; Lowe, E. M.; Cohen, H.; Soldan, K.; McClelland, D. B. L.; Skacel, P.; Barbara, J. A. J. *Br. Med. J.* **1999**, *319*, 16.
(34) Klein, H. G. cited by Keipert In *Blood Substitutes: Principles, Methods, Products and Clinical Trials*; Chang, T. M. S., Ed.; Karger Landes: New York, 1998; Vol. 2, p 127.
(35) Linden, J. V.; Tourault, M. A.; Scribner, C. L. *Transfusion* **1997**, *37*, 243.
(36) Silliman, C. C.; Paterson, A. J.; Dickey, W. O.; Stroncek, D. F.; Popovsky, M. A.; Caldwell, S. A.; Ambruso, D. R. *Transfusion* **1997**, *37*, 719.
(37) Dodd, R. Y. *Vox Sang.* **1998**, *74*, 161.
(38) Wagner, S. J.; Friedman, L. I.; Dodd, R. Y. *Clin. Microbiol. Rev.* **1994**, *7*, 290.
(39) Treakston, E. P.; Morris, A. J.; Streat, S. J.; Baker, B. W.; Woodfield, D. G. *Aust. N. Z. J. Med.* **1997**, *27*, 62.
(40) Ricketts, M. N.; Cashman, N. R.; Stratton, E. E.; ElSaadany, S. *Emerging Infect. Dis.* **1997**, *3*, 155.
(41) Dodd, R. Y.; Sullivan, M. T. *Transfusion* **1998**, *38*, 221.
(42) Mitka, M. *J. Am. Med. Assoc.* **1999**, *281*, 1157.
(43) Lasmezas, C. I.; Deslys, J. P.; Demalmay, R.; Adjou, K. T.; Lamoury, F.; Dormont, D. *Nature* **1996**, *381*, 743.
(44) Bruce, M. E.; Will, R. G.; Ironside, J. W.; McConnell, I.; Drummond, D.; Suttie, A.; McCardle, L.; Chree, A.; Hope, J.; Birkett, C.; Cousens, S.; Fraser, H.; Bostock, C. J. *Nature* **1997**, *389*, 498.
(45) Houston, F.; Foster, J. D.; Chong, A.; Hunter, N.; Bostock, C. J. *Lancet* **2000**, *356*, 999.
(46) Collinge, J.; Sidle, K. C. L.; Meads, J.; Ironsides, J.; Hill, A. F. *Nature* **1996**, *383*, 685.
(47) Hill, A. F.; Desbruslasi, M.; Joiner, S.; Sidle, K. C. L.; Gowland, I.; Collinge, J. *Nature* **1997**, *389*, 448.
(48) Andrews, N. J.; Farrington, C. P.; Cousens, S. N.; Smith, P. G.; Ward, H.; Knight, R. S. G.; Ironside, J. W.; Will, R. G. *Lancet* **2000**, *356*, 481.
(49) Scott, M. R.; Will, R.; Ironside, J.; Nguyen, H.-O. B.; Tremblay, P.; DeArmond, S. J.; Prusiner, S. B. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 15137.
(50) Linden, J. V.; Kaplan, H. S. *Transfus. Med. Rev.* **1994**, *8*, 169.
(51) Myhre, B. A.; McRuer, D. *Transfusion* **2000**, *40*, 879.
(52) George, C. D.; Morello, P. J. *Am. J. Surg.* **1986**, *152*, 329.
(53) Donnelly, P. K.; Proud, G.; Shenton, B. K.; Taylor, R. M. *Transfusion Med.* **1991**, *1*, 217.
(54) Murphy, P.; Heal, J. M.; Blumberg, N. *Transfusion* **1991**, *31*, 312.
(55) Mickler, T. A.; Longnecker, D. E. *J. Intensive Care Med.* **1992**, *7*, 176.
(56) MacLeod, A. M.; Roy-Chaudhury, P.; Catto, G. R. D. In *Blood, Blood Products and HIV*; Madhok, R., Forbes, C. D., Evatt, B. L., Eds.; Chapman & Hall: London, 1994; p 117.
(57) Bordin, J. O.; Blajchman, M. A. In *Principles of Transfusion Medicine*; Rossi, E. C., Simon, T. L., Moss, G. S., Gould, S. A., Eds.; Williams & Wilkins: Baltimore, 1996; Vol. 2, p 803.
(58) Jensen, L. S.; Kissmeyer-Nielsen, P.; Wolff, B.; Qvist, N. *Lancet* **1996**, *348*, 841.
(59) Landers, D. F.; Hill, G. E.; Wong, K. C.; Fox, I. J. *Anesth. Analg.* **1996**, *82*, 187.
(60) Vignali, A.; Braga, M.; Gianotti, L.; Radaelli, G.; Gentilini, O.; Russo, A.; Di Carlo, V. *Vox Sang.* **1996**, *71*, 170.
(61) Wheatley, T.; Veitch, P. S. *Br. J. Anaesth.* **1997**, *78*, 489.
(62) Fransen, E.; Maessen, J.; Dentener, M.; Senden, N.; Buurman, W. *Chest* **1999**, *116*, 1233.
(63) Corwin, H. L. *Chest* **1999**, *116*, 1149.
(64) Carson, J. L.; Altman, D. G.; Duff, A.; Noveck, H.; Weinstein, M. P.; Sonnenberg, F. A.; Hudson, J. I.; Provenzano, G. *Transfusion* **1999**, *39*, 694.
(65) Innerhofer, P.; Tilz, G.; Fuchs, D.; Luz, G.; Hobisch-Hagen, P.; Schobersberger, W.; Nussbaumer, W.; Lochs, A.; Irschick, E. *Transfusion* **2000**, *40*, 821.
(a) *Immunomodulatory Effects of Blood Transfusion*; Vamvakas, E. C., Blajman, M. A., Eds.; American Association of Blood Banks Press: Bethesda, MD, 1999.
(66) Duggan, J.; O'Connell, D.; Heller, R.; Ghosh, H. *Q. J. Med.* **1993**, *86*, 479.
(67) Tartter, P. I. *Immunol. Invest.* **1995**, *24*, 277.
(68) Houbiers, J. G.; van de Velde, C. J.; van de Watering, L. M.; Hermans, J.; Schreuder, S.; Bijnen, A. B.; Pahlplatz, P.; Schattenkerk, M. E.; Wobbes, T.; de Vries, J. E.; Klementschitsch, P.; van de Maas, A. H.; Brand, A. *Transfusion* **1997**, *37*, 126.
(69) Triulzi, D. J.; Blumberg, N.; Heal, J. M. *Crit. Rev. Clin. Lab. Sci.* **1990**, *28*, 95.
(70) Heiss, M. M.; Mempel, W.; Jauch, K.-W.; Delanoff, C.; Mayer, G.; Mempel, M.; Eissner, H.-J.; Schildberg, F.-W. *Lancet* **1993**, *342*, 1328.
(71) van de Watering, L. M. G.; Hermans, J.; Houbiers, J. G. A.; van den Broek, P. J.; Bouter, H.; Boer, F.; Harvey, M. S.; Huysmans, H. A.; Brand, A. *Circulation* **1998**, *97*, 562.
(72) Purdy, F. R.; Tweeddale, M. G.; Merrick, P. M. *Can. J. Anaesth.* **1997**, *44*, 1256.
(73) Moore, F. A.; Moore, E.; Sauaia, A. *Arch. Surg.* **1997**, *132*, 620.
(74) Francis, D. M. A. *Aust. N. Z. J. Surg.* **1990**, *60*, 743.
(75) Woolley, A.; Hogikyan, N. D.; Gates, G. A.; Haughey, B. H.; Schechtman, K. B.; Goldenberg, J. L. *Ann. Otol. Rhinol. Laryngol.* **1992**, *101*, 724.
(76) Busch, O. R.; Hop, W. C.; van Papendrecht, M. A.; Marquet, R. L.; Jeekel, J. *N. Engl. J. Med.* **1993**, *328*, 1372.
(77) Blumberg, N.; Heal, J. M. *Am. J. Med.* **1996**, *101*, 299.
(78) Vamvakas, E. C. *Transfusion* **1996**, *36*, 175.
(79) Edna, T.; Bjerkeset, T. *Dis. Colon Rectum* **1998**, *41*, 451.
(80) Heiss, M. M.; Mempel, W.; Delanoff, C.; Jauch, K.-W.; Gabka, C.; Mempel, M.; Dieterich, H.-J.; Eissner, H.-J.; Schildberg, F.-W. *J. Clin. Oncol.* **1994**, *12*, 1859.
(81) Claas, F. H. J.; de Koster, H. J.; Lagaaij, E. L.; van Rood, J. J. *Exp. Nephrol.* **1993**, *1*, 134.
(82) Shirwan, H.; Wang, H. K.; Barwari, L.; Makowka, L.; Cramer, D. V. *Transplantation* **1996**, *61*, 1382.
(83) Blajchman, M. A. *Transfusion* **1997**, *37*, 121.
(84) Opelz, G.; Vanrenterghem, Y.; Kirste, G.; Gray, D. W. R.; Horsburgh, T.; Lachance, J.-G.; Largiader, F.; Lange, H.; Vujaklija-Stipanovic, K.; Alvarez-Grande, J.; Schott, W.; Hoyer, J.; Schnuelle, P.; Descoeudres, C.; Ruder, H.; Wujciak, T.; Schwarz, V. *Transplantation* **1997**, *63*, 964.
(85) Collins, A. F.; Fassos, F. F.; Stobie, S.; Lewis, N.; Shaw, D.; Fry, M.; Templeton, D. M.; McClelland, R. A.; Koren, G.; Olivieri, N. F. *Blood* **1994**, *83*, 2329.
(86) Lovric, V. *Anaesth. Intensivther. Notfallmed.* **1984**, *12*, 246.
(87) Beutler, E.; Meul, A.; Wood, L. A. *Transfusion* **1969**, *9*, 109.
(88) Stuart, J.; Nash, G. B. *Blood Rev.* **1990**, *4*, 141.
(89) Simchon, S.; Jan, K.; Chien, S. *Am. J. Physiol.* **1987**, *253*, H898.
(90) Shah, D. M.; Gottlieb, M. E.; Rahm, R. L.; Stratton, H. H.; Barie, P. S.; Paloski, W. H.; Newell, J. C. *J. Trauma* **1982**, *22*, 741.
(91) Apstein, C. S.; Dennis, R. C.; Briggs, L.; Vogel, W. M.; Frazer, J.; Valeri, C. R. *Am. J. Physiol.* **1985**, *248*, H508.
(92) Maetani, S.; Nishikawa, T.; Tobe, T.; Hirakawa, A. *Ann. Surg.* **1986**, *203*, 275.
(93) Dietrich, K. A.; Conrad, S. A.; Hebert, C. A.; Levy, G. L.; Romero, M. D. *Crit. Care Med.* **1990**, *18*, 940.
(94) Marik, P. E. *J. Am. Med. Assoc.* **1993**, *269*, 3024.
(95) Heyland, D. K.; Cook, D. J.; King, D.; Kernermanm, P.; Brun-Buisson, C. *Crit. Care Med.* **1996**, *24*, 517.
(96) Carson, J. L.; Duff, A.; Berlin, J. A.; Lawrence, V. A.; Poses, R. M.; Huber, E. C.; O'Hara, D. A.; Noveck, H.; Strom, B. L. *J. Am. Med. Assoc.* **1998**, *279*, 199.
(97) Spiess, B. D.; Ley, C.; Body, S. C.; Siegel, L. C.; Stover, E. P.; Maddi, R.; D'Ambra, M.; Jain, U.; Liu, F.; Herskowitz, A.; Mangano, D. T.; Levin, J. *J. Thorac. Cardiovasc. Surg.* **1998**, *116*, 460.
(98) Hébert, P. C.; Wells, G.; Blajchman, M. A.; Marshall, J.; Martin, C.; Pagliarello, G.; Tweeddale, M.; Schweitzer, I.; Yetisir, E. *N. Engl. J. Med.* **1999**, *340*, 409; see also Hébert, P. C.; Blajchman, M. A.; Cook, D. J.; Yetisir, E.; Wells, G.; Marshall, J.; Schweitzer, I. *Chest* **2001**, *119*, 1850.
(99) Fitzgerald, R. D.; Martin, C. M.; Dietz, G. E.; Doig, G. S.; Potter, R. F.; Sibbald, W. J. *Crit. Care Med.* **1997**, *25*, 726.
(100) Crémieux, P.; Barrett, B.; Anderson, K.; Slavin, M. B. *J. Clin. Oncol.* **2000**, *18*, 2755.
(101) Vamvakas, E. C. *Transf. Med. Rev.* **1996**, *10*, 44.
(102) Scott, M. G.; Kucik, D. F.; Goodnough, L. T.; Monk, T. G. *Clin. Chem.* **1997**, *43*, 1724.
(103) Vamvakas, E. C.; Taswell, H. F. *Transfusion* **1994**, *34*, 464.
(104) Wilkinson, S. *Proceedings of the U.S. Public Health Service*; Advisory Committee on Blood Safety and Availability: Bethesda, MD, 1999; p 58.
(105) Stowell, C. P.; Tomasulo, P. In *Red Blood Cell Substitutes*; Rudolph, A. S., Rabinovici, R., Feuerstein, G. Z., Eds.; Dekker: New York, 1998; p 1.

(106) Bouskela, E.; Rocha e Silva, M.; Shorr, R. IBC Conference on Blood Substitutes and Oxygen Therapeutics, Washington, DC, 1998.
(107) Winslow, R. M. In *Blood Substitutes−Present and Future Perspectives*; Tsuchida, E., Ed.; Elsevier: Amsterdam, 1998; p 15.
(108) American College of Physicians. *Ann. Intern. Med.* **1992**, *116*, 403.
(109) Natl. Inst. of Health. *J. Am. Med. Assoc.* **1988**, *260*, 2700.
(110) Stehling, L.; Zauder, H. L. *Transfusion* **1990**, *30*, 1.
(111) Goodnough, L. T.; Verbrugge, D.; Vizmeg, K.; Riddell, J., IV *Transfusion* **1992**, *32*, 648.
(112) Stehling, L.; Simon, T. L. *Arch. Pathol. Lab. Med.* **1994**, *118*, 429.
(113) Hasley, P. B.; Lave, R.; Kapoor, W. N. *Transfusion* **1994**, *34*, 110.
(114) Greenburg, A. G. *Am. J. Surg.* **1995**, *170*, 44S.
(115) Frey, L.; Messmer, K. *Curr. Opin. Anaesthesiol.* **1996**, *9*, 183.
(116) Spence, R. K.; Swisher, S. N. In *Clinical Practice of Transfusion Medicine*; Petz, L. D., Swisher, S. N., Kelinman, S., Spence, R. K., Strauss, R. K., Churchill, R. G., Eds.; Livingstone: New York, 1996; p 177.
(117) American Society of Anesthesiologists Task Force on Blood Component Therapy. *Anesthesiology* **1996**, *84*, 732.
(118) Spence, R. K. *Semin. Hematol.* **1997**, *34*, 48.
(119) Weiskopf, R. B. *Transfusion* **1998**, *38*, 517.
(120) Winslow, R. M. In *Red Blood Cell Substitutes*; Rudolph, A. S., Rabinovici, R., Feuerstein, G. Z., Eds.; Dekker: New York, 1998; p 37.
(121) McFarland, J. G. *Chest* **1999**, *115*, 113S.
(122) Expert Working Group. *Can. Med. Assoc. J.* **1997**, *156*, S1.
(123) Simon, T. L.; Alverson, D. C.; AuBuchon, J.; Cooper, E. S.; DeChristopher, P. J.; Glenn, G. C.; Gould, S. A.; Harrison, C. R.; Milam, J. D.; Moise, K. J.; Rodwig, F. R.; Sherman, L. A.; Shulman, I. A.; Stehling, L. *Arch. Pathol. Lab. Med.* **1998**, *122*, 130.
(124) Lenfant, C. *Transfusion* **1992**, *32*, 873.
(125) Valeri, C. R.; Crowley, J. P.; Loscalzo, J. *Transfusion* **1998**, *38*, 602.
(126) Hogue, C. W.; Goodnough, L. T.; Monk, T. G. *Transfusion* **1998**, *38*, 924.
(127) National Institutes of Health Conference Report. *Transfusion* **1995**, *35*, 525.
(128) Reasoner, D. K.; Ryu, K. H.; Hindman, B. J.; Cutkomp, J.; Smith, T. *Anesth. Analg.* **1995**, *82*, 61.
(129) Thomas, M. J. G.; Desmond, M. J.; Gillon, J. *Transfusion* **1996**, *36*, 628.
(130) Faught, C.; Wells, P.; Fergusson, D.; Laupacis, A. *Transf. Med. Rev.* **1998**, *12*, 206.
(131) Manner, P. A.; Rubash, H. E.; Herndon, J. H. *Clin. Orthopaed. Relat. Res.* **1998**, *357*, 101.
(132) Napier, J. A. F.; Bruce, M.; Chapman, J.; Duguid, J. K. M.; Kelsey, P. R.; Knowles, S. M.; Murphy, M. F.; Williamson, L. M.; Wood, J. K. *Br. J. Anaesth.* **1998**, *4*, 768.
(133) Lee, S. J.; Liljas, B.; Churchill, W. H.; Popovsky, M. A.; Stowell, C. P.; Cannon, M. E.; Johannesson, M. *Tranfusion* **1998**, *38*, 757.
(134) Lee, S.; Liljas, B.; Neumann, P. J.; Weinstein, M. C.; Johannesson, M. *Med. Care* **1998**, *36*, 1162.
(135) Goodnough, L. T. *Br. J. Anaesth.* **1998**, *81*, 67.
(136) Goodnough, L. T.; Monk, T. G.; Brecher, M. E. *Transfusion* **1998**, *38*, 473.
(137) Kanter, M. H.; van Maanen, D.; Anders, K. H.; Castro, F.; Mya, W. W.; Clark, K. *J. Am. Med. Assoc.* **1996**, *276*, 798.
(138) Bierbaum, B. E.; Callaghan, J. J.; Galante, J. O.; Rubash, H. E.; Tooms, R. E.; Welch, R. B. *J. Bone Joint Surg.* **1999**, *81-A*, 2.
(139) Birkmeyer, J. D.; AuBuchon, J. P.; Littenberg, B.; Connor, G. T. O.; Nease, R. F.; Nugent, W. C.; Goodnough, L. T. *Ann. Thorac. Surg.* **1994**, *57*, 161.
(140) Messmer, K.; Sunder-Plassmann, L.; Klövekorn, W. P.; Holper, K. *Adv. Microcirc.* **1972**, *4*, 1.
(141) Stehling, L.; Zauder, H. L. *Transfusion* **1991**, *31*, 857.
(142) Ness, P. M.; Bourke, D. L.; Walsh, P. C. *Transfusion* **1992**, *32*, 226.
(143) Spahn, D. R.; Leone, B. J.; Reves, J. G.; Patsch, T. *Anesth. Analg.* **1994**, *78*, 1000.
(144) Freyburger, G.; Dubreuil, M.; Boisseau, M. R.; Janvier, G. *Br. J. Anaesth.* **1996**, *76*, 519.
(145) Kreimeier, U.; Messmer, K. *World J. Surg.* **1996**, *20*, 1208.
(146) Spahn, D. R.; Schmidt, E. R.; Seifert, B.; Pasch, T. *Anesth. Analg.* **1996**, *82*, 687.
(147) Monk, T. G.; Goodnough, L. T.; Brecher, M. E.; Pulley, D. D.; Colberg, J. W.; Andriole, G. L.; Catalona, W. J. *Anesth. Analg.* **1997**, *85*, 953.
(148) Rottman, G.; Ness, P. M. *Transfusion* **1998**, *38*, 477.
(149) Weiskopf, R. B. *Transfusion* **1995**, *35*, 37.
(150) Bryson, G. L.; Laupacis, A.; Wells, G. A. *Anesth. Analg.* **1998**, *86*, 9.
(151) Zuck, T. F.; Riess, J. G. *Crit. Rev. Clin. Lab. Sci.* **1994**, *31*, 295.
(152) Keipert, P. E.; Faithfull, N. S.; Roth, D. J.; Bradley, J. D.; Batra, S.; Jochelson, P.; Flaim, K. E. *Adv. Exp. Med. Biol.* **1996**, *388*, 603.
(153) Keipert, P. E. In *Blood Substitutes: Principles, Methods, Products and Clinical Trials*; Chang, T. M. S., Ed.; Karger Landes: New York, 1998; Vol. 2, p 127.
(154) Spahn, D. R.; van Brempt, R.; Theilmeier, G.; Reibold, J.-P.; Welte, M.; Heinzerling, H.; Birck, K. M.; Keipert, P. E.; Messmer, K. *Anesthesiology* **1999**, *91*, 1195.
(155) Spahn, D. R. *Adv. Drug Delivery Rev.* **2000**, *40*, 143.
(156) Faithfull, N. S.; Rhoades, G. E.; Keipert, P. E.; Ringle, A. S.; Trouwborst, A. *Adv. Exp. Med. Biol.* **1994**, *361*, 41.
(157) Brecher, M. E.; Goodnough, L. T.; Monk, T. *Transfusion* **1999**, *39*, 396.
(158) Deschiens. *Disclosure to the French Academy of Sciences* **1886**.
(159) Winslow, R. M. *Hemoglobin-Based Red Cell Substitutes*; The Johns Hopkins University Press: Baltimore, 1992.
(160) Proceedings of the Symposium on Inert Organic Liquids for Biological Oxygen Transport. In *Fed. Proc.*, Atlantic City, 1970; p 1696.
(161) Alpha Therapeutic Corporation. *FLUOSOL 20% intravascular perfluorochemical emulsion delivers oxygen to protect the heart during PTCA. Product Monograph*; Alpha Therapeutic Corp.: Los Angeles, 1990.
(162) Ivanitsky, G. R.; Vorobyev, S. I. *Perftoran Blood Substitute with Gas-Transporting Function. Product Monograph*; Perftoran Co.: Pushchino, Russia, 1997.
(163) Klein, H. G. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1994**, *22*, 123.
(164) Spence, R. K. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1995**, *23*, 367.
(165) Kochamba, G. S.; Pfeffer, T. A.; Sintek, C. F.; Khonsari, S. *Ann. Thorac. Surg.* **1996**, *61*, 900.
(166) Spahn, D. S. *Crit. Care* **1999**, *3*, R93.
(167) Meisner, F. G.; Kemming, G. I.; Habler, O. P.; Kleen, M. S.; Tillmanns, J. H.; Hutter, J. W.; Bottino, D. A.; Theim, E.; Meier, J. M.; Wojtczyk, C. J.; Pape, A.; Messmer, K. *Crit. Care Med.* **2001**, *29*, 829.
(168) Mercuriali, F.; Zanella, A.; Barosi, G.; Inghilleri, G.; Biffi, E.; A, V.; Colotti, M. T. *Transfusion* **1993**, *33*, 55.
(169) Biesma, D. H.; Marx, J. J.; Kraaijenhagen, R. J. *Lancet* **1993**, *344*, 367.
(170) Price, T. H.; Goodnough, L. T.; Vogler, W. R. *Am. J. Med.* **1996**, *101*, 22S.
(171) Monk, T. *Semin. Hematol.* **1997**, *33*, 48.
(172) Roach, G. W.; Kanchuger, M.; Mangano, C. M.; Newman, M.; Nussmeier, N.; Wolfman, R.; Aggarwal, A.; Marschall, K.; Graham, S. H.; Ley, C.; Ozanne, G.; Mangano, T. *N. Engl. J. Med.* **1996**, *335*, 1857.
(173) Gillon, J.; Thomas, M. J. G.; Desmond, M. J. *Transfusion* **1996**, *36*, 640.
(174) Newman, M. F.; Kirchner, J. L.; Phillips-Bute, B.; Gaver, V.; Grocott, H.; Jones, R. H.; Mark, D. B.; Reves, J. G.; Blumenthal, J. A. *N. Engl. J. Med.* **2001**, *344*, 395.
(175) Holmberg, J. A. In *Red Blood Cell Substitutes*; Rudolph, A. S., Rabinovici, R., Feuerstein, G. Z., Eds.; Dekker: New York, 1998; p 17.
(176) Fratantoni, J. C. In *Red Blood Cell Substitutes*; Rudolph, A. S., Rabinovici, R., Feuerstein, G. Z., Eds.; Dekker: New York, 1998; p 29.
(177) Fratantoni, J. C. In *Blood Substitutes−Present and Future Perspectives*; Tsuchida, E., Ed.; Elsevier: Amsterdam, 1998; p 33.
(178) Savitsky, J. P.; Doczi, J.; Black, J.; Arnold, J. D. *Clin. Pharmacol. Ther.* **1978**, *23*, 73.
(179) Kent, K. M.; Cleman, M. W.; Cowley, M. J.; Forman, M.; Jaffe, C. C.; Kaplan, M.; King, S. B.; Krucoff, M.; Lassar, T.; McAuley, B.; Smith, R.; Wisdom, C.; Wohlgelernter, D. *Am. J. Cardiol.* **1990**, *66*, 279.
(180) Forman, M. B.; Perry, J. M.; Wilson, B. H.; Verani, M. S.; Kaplan, P. R.; Shawl, F. A.; Friesinger, G. C. *J. Am. Coll. Cardiol.* **1991**, *18*, 911.
(181) FDA Center for Biologics Evaluation and Research. *Transfusion* **1991**, *31*, 369.
(182) FDA Center for Biologics Evaluation and Research. *Transfusion* **1994**, *34*, 712.
(183) Welch, H. G.; Meehan, K. R.; Goodnough, L. T. *Ann. Int. Med.* **1992**, *116*, 393.
(184) Crosby, E. T. *Can. J. Anaesth.* **1992**, *39*, 695.
(185) Riess, J. G. In *Fluorine at the Millenium*; Banks, R. E., Ed.; Elsevier: Amsterdam, 2000; p 385.
(186) Amberson, W. R.; Jennings, J. J.; Rhode, C. M. *J. Appl. Physiol.* **1949**, *1*, 469.
(187) Feola, M.; Simoni, J.; Tran, R.; Canizaro, P. C. *Biomater., Artif. Cells, Artif. Organs* **1990**, *18*, 233.
(188) Hess, J. R.; Reiss, R. F. *Transfusion Med. Rev.* **1996**, *10*, 276.
(189) Everse, J.; Hsia, N. *Free Radical Biol. Med.* **1997**, *22*, 1075.

(190) Gould, S. A.; Sehgal, L. R.; Sehgal, H. L.; Moss, G. S. In *Red Blood Cell Substitutes*; Rudolph, A. S., Rabinovici, R., Feuerstein, G. Z., Eds.; Dekker: New York, 1998; p 401.
(191) Hemoglobins, Part B. In *Methods in Enzymology*; Everse, J., Vandegriff, K. D., Winslow, R. M., Eds.; Academic Press: New York, 1994; Vol. 231.
(192) Blood Substitutes, General. Proceedings of the Vth International Symposium on Blood Substitutes, Vol. 1. In *Artificial Cells, Blood Substitutes, and Immobilization Biotechnology*; Chang, T. M. S., Riess, J. G., Winslow, R. M., Eds.; Dekker: New York, 1994; Vol. 22.
(193) *Advances in Blood Substitutes. Industrial Opportunities and Medical Challenges.*; Winslow, R. M., Vandegriff, K. D., Intaglietta, R., Eds.; Birkhäuser: Boston, 1997.
(194) *Blood Substitutes: Present and Future Perspectives*; Tsuchida, E., Ed.; Elsevier: Amsterdam, 1998.
(195) *Red Blood Cell Substitutes: Basic Principles and Clinical Applications*; Rudolph, A. S., Rabinovici, R., Feuerstein, G. Z., Eds.; Dekker: New York, 1998.
(196) *Blood Substitutes: Methods, Products and Clinical Trials.*; Chang, T. M. S., Ed.; Karger Landes: New York, 1998.
(197) *Tissue Oxygenation in Acute Medicine*; Sibbald, W. J., Messmer, K., Fink, M. P., Eds.; Springer-Verlag: Berlin, 1998.
(198) Hsia, C. J. C. IBC Conference on Blood Substitutes and Oxygen Therapeutics, Washington, DC, 1998.
(199) Collman, J. P. *Acc. Chem. Res.* **1977**, *10*, 265.
(200) Jones, R. D.; Summerville, D. A.; Basolo, F. *Chem. Rev.* **1979**, *79*, 139.
(201) Traylor, T. G.; Traylor, P. S. *Annu. Rev. Biophys. Bioeng.* **1982**, *11*, 105.
(202) Baldwin, J. E.; Perlmutter, P. In *Host−Guest Complex Chemistry III*; Vogte, F.; Weber, E., Eds.; Springer Verlag: Berlin, 1984, p 181.
(203) Momenteau, M.; Reed, C. A. *Chem. Rev.* **1994**, *94*, 659.
(204) Collman, J. P.; Zhang, X. *Compr. Supramol. Chem.* **1996**, *5*, 1.
(205) Fuhrhop, J. H.; Besecke, S.; Vogt, W.; Ernst, J.; Subramanian, J. *Makromol. Chem.* **1977**, *178*, 1621.
(206) Tsuchida, E. *J. Macromol. Sci. Chem.* **1979**, *A13*, 545.
(207) Tsuchida, E. *Memoirs Sch. Sci. Eng.* **1986**, *50*, 3.
(208) Kobayashi, K.; Tsuchida, E.; Nishide, H. In *Artificial Red Cells*; Tsuchida, E., Ed.; Wiley: New York, 1995; p 93.
(209) Komatsu, T.; Kuronuma, A.; Muramatsu, Y.; Nishide, H.; Tsuchida, E.; Kakizaki, T.; Kobayashi, K. *Artif. Organs Today* **1996**, *5*, 207.
(210) Komatsu, T.; Tsuchida, E.; Kobayashi, K. In *Blood Substitutes. Present and Future Perspectives*; Tsuchida, E., Ed.; Elsevier: Amsterdam, 1998; p 315.
(211) Tsuchida, E.; Komatsu, T.; Hamamatsu, K.; Matsukawa, Y.; Tajima, A.; Yoshizu, A.; Izumi, Y.; Kobayashi, K. *Bioconjugate Chem.* **2000**, *11*, 46.
(212) Clark, L. C.; Gollan, F. *Science* **1966**, *152*, 1755.
(213) Sloviter, H. A.; Kamimoto, T. *Nature* **1967**, *216*, 458.
(214) Geyer, R. P.; Monroe, R. G.; Taylor, K. In *Organ Perfusion and Preservation*; Norman, J. C., Ed.; Appleton-Century-Crofts: New York, 1968; p 85.
(215) Blood Substitutes, the Fluorocarbon Approach, Proceedings of the Vth International Symposium on Blood Substitutes, Vol 3. In *Artificial Cells, Blood Substitutes, and Immobilization Biotechnology*; Riess, J. G., Ed.; Dekker: New York, 1994; Vol. 22.
(216) Faithfull, N. S. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1994**, *22*, 181.
(217) Riess, J. G. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1994**, *22*, 215.
(218) Flaim, S. F. In *Red Blood Cell Substitutes*; Rudolph, A. S., Rabinovici, R., Feuerstein, G. Z., Eds.; Dekker: New York, 1998; p 79 and p 437.
(219) Krafft, M. P.; Riess, J. G.; Weers, J. G. In *Submicronic Emulsions in Drug Targeting and Delivery*; Benita, S., Ed.; Harwood Academic Publ.: Amsterdam, 1998; p 235.
(220) Riess, J. G.; Keipert, P. E. In *Blood Substitutes−Present and Future Perspectives*; Tsuchida, E., Ed.; Elsevier: Amsterdam, 1998; Chapter 7 p 91.
(221) Back, M. R.; White, R. A. *Biomaterials in Vascular Surgery*; Humana Press: Totowa, NJ, 1996.
(222) Naito, R.; Yokoyama, K. *Perfluorochemical Blood Substitutes. FC-43 Emulsion, Fluosol-DA, 20% and 35%*; Green Cross Corp.: Osaka, Japan, 1978, 1981.
(223) Bunn, H. F.; Forget, B. G. *Hemoglobin: Molecular, Genetic and Clinical Aspects*; Saunders: Philadelphia, 1986.
(224) Rifkind, J. M. *Prog. Inorg. Biochem.* **1988**, *7*, 155.
(225) Fermi, G. *J. Mol. Biol.* **1975**, *97*, 237.
(226) Fermi, G.; Perutz, M. F.; Shaanan, B.; Fourme, R. *J. Mol. Biol.* **1984**, *175*, 159.
(227) Perutz, M. F. *Annu. Rev. Biochem.* **1979**, *48*, 327.
(228) Perutz, M. F.; Wilkinson, A. J.; Paoli, M.; Dodson, G. G. *Annu. Rev. Biophys. Biomol. Struct.* **1998**, *27*, 1.
(229) Mal, A.; Nandi, A.; Chatterjee, I. B. *J. Biosci.* **1991**, *16*, 43.
(230) Grisham, M. B. *Reactive Metabolites of Oxygen and Nitrogen in Biology and Medicine*; Landes: Basel, 1992.
(231) Svistunenko, D.; Patel, R. P.; Wilson, M. T. *Free Radical Biol.* **1996**, *24*, 269.
(232) Alayash, A. I.; Brockner, B. A.; McLeod, L. L.; Goldman, D. W.; Cashon, R. E. In *Blood Substitutes: Principles, Methods, Products and Clinical Trials*; Chang, T. M. S., Ed.; Karger Landes: Basel, 1998; Vol. 2; p 157.
(233) Faivre, B.; Menu, P.; Labrude, P.; Vigneron, C. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1998**, *26*, 17.
(234) Alayash, A. I. In *Blood Substitutes. Present and Future Perspectives*; Tsuchida, E., Ed.; Elsevier: Amsterdam, 1998; p 201.
(235) Shikama, K. *Chem. Rev.* **1998**, *98*, 1357.
(236) Halliwell, B.; Gutteridge, J. M. C. *Free Radicals in Biology and Medicine*; Oxford Science Publ.: Oxford, 1999.
(237) D'Agnillo, F.; Alayash, A. I. *Adv. Drug Delivery Rev.* **2000**, *40*, 199.
(238) Alayash, A. I. *Free Radical Res.* **2000**, *33*, 341.
(239) Beckman, J. S.; Koppenol, W. H. *Am. J. Physiol.* **1996**, *271*, C1424.
(240) Wang, P.; Zweier, J. L. *J. Biol. Chem.* **1996**, *271*, 29223.
(241) Alayash, A. I.; Brockner Ryan, B. A.; Cashon, R. E. *Arch. Biochem. Biophys.* **1998**, *349*, 65.
(242) Giulivi, C.; Davies, K. J. A. *J. Biol. Chem.* **1990**, *265*, 19453.
(243) Alayash, A. I.; Fratantoni, J. C.; Bonaventura, C.; Bonaventura, J.; Bucci, E. *Arch. Biochem. Biophys.* **1992**, *298*, 114.
(244) Giulivi, C.; Davies, K. J. A. *Methods Enzymol.* **1994**, *231*, 490.
(245) Cashon, R. E.; Alayash, A. I. *Arch. Biochem. Biophys.* **1995**, *316*, 461.
(246) Panus, P. C.; Radi, R.; Chumley, P. H.; Lillard, R. H.; Freeman, B. A. *Free Radical Biol. Med.* **1993**, *14*, 217.
(247) McLeod, L. L.; Sevanian, A. *Free Radical Biol. Med.* **1997**, *23*, 680.
(248) Svistunenko, D. A.; Patel, R. P.; Voloshchenko, S. V.; Wilson, M. T. *J. Biol. Chem.* **1997**, *272*, 7114.
(249) Minetti, M.; Scorza, G.; Pietraforte, D. *Biochemistry* **1999**, *38*, 2078.
(250) Kanner, J.; Harel, S. *Arch. Biochem. Biophys.* **1985**, *237*, 314.
(251) Gutteridge, J. M. C. *FEBS Lett.* **1986**, *201*, 291.
(252) Faassen, A. E.; Sundby, S. R.; Panter, S. S.; Condie, R. M.; Hedlund, B. E. *Biomater., Artif. Cells., Artif. Organs* **1988**, *16*, 93.
(253) Simoni, J.; Feola, M.; Canizaro, P. C. *Biomater., Artif. Cells, Artif. Organs* **1990**, *18*, 189.
(254) Bunn, H. F.; Jandl, J. H. *J. Biol. Chem.* **1968**, *243*, 465.
(255) Balla, G.; Vercellotti, G. M.; Muller-Eberhard, U.; Eaton, J.; Jacob, H. S. *Lab. Invest.* **1991**, *64*, 648.
(256) Balla, J.; Jacob, H. S.; Balla, G.; Nath, K.; Eaton, J. W.; Vercellotti, G. M. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 9285.
(257) Vercellotti, G. M.; Balla, G.; Balla, J.; Nath, K.; Eaton, J. W.; Jacob, H. S. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1994**, *22*, 207.
(258) Balla, J.; Nath, K. A.; Balla, G.; Juckett, M. B.; Jacob, H. S.; Vercellotti, G. M. *Am. J. Physiol.* **1995**, *268*, L321.
(259) Motterlini, R.; Foresti, R.; Vandegriff, K.; Winslow, R. M. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1995**, *23*, 291.
(260) Simoni, J.; Simoni, G.; Lox, C. D.; Feola, M. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1994**, *22*, 777.
(261) Foley, P. L.; Takenaka, K.; Kassell, N. F.; Lee, K. S. *J. Neurosurg.* **1994**, *81*, 87.
(262) Machi, T.; Kassel, N. F.; Tanaka, Y.; Hudson, S.; Lehman, G. A.; Harrison, T. P. *Fukuoka Acta Med.* **1996**, *87*, 189.
(263) Sarti, P.; Hogg, N.; Darley-Usmar, V. W.; Sanna, M. T.; Wilson, M. T. *Biochim. Biophys. Acta* **1994**, *1208*, 38.
(264) Nagababu, E.; Rifkind, J. M. *Biochemistry* **2000**, *38*, 12503.
(265) Sadrzadeh, S. M. H.; Anderson, D. K.; Panter, S. S.; Hallaway, P. E.; Eaton, J. W. *J. Clin. Invest.* **1987**, *79*, 662.
(266) Sadrzadeh, S. M. H.; Eaton, J. W. *Am. Soc. Clin. Invest.* **1988**, *82*, 1510.
(267) Snyder, S. R.; Walder, J. A. *Biotechnology* **1991**, *19*, 101.
(268) Zhang, L.; Levy, A.; Rifkind, J. M. *J. Biol. Chem.* **1991**, *266*, 24698.
(269) Moore, G. L.; Zegna, A.; Ledford, M. E.; Huling, J. P.; Fishman, R. M. *Artif. Organs* **1992**, *16*, 513.
(270) Labrude, P.; Chaillot, B.; Vigneron, C. *J. Pharm. Pharmacol.* **1987**, *39*, 344.
(271) Alayash, A. I. *Nat. Biotechnol.* **1999**, *17*, 545.
(272) Kosaka, H. *Biochim. Biophys. Acta* **1999**, *1411*, 370.
(273) D'Agnillo, F.; Alayash, A. I. *Am. J. Physiol.* **2000**, *279*, H1880.
(274) Darley-Usmar, V.; Wiseman, H.; Halliwell, B. *FEBS Lett.* **1995**, *369*, 131.
(275) Marden, M. C.; Griffon, N.; Poyart, C. *Transfusion Clin. Biol.* **1995**, *6*, 473.
(276) Linberg, R.; Conover, C. D.; Shum, K. L.; Shorr, R. G. L. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1998**, *26*, 133.
(277) Ignarro, L. J.; Buga, G. M.; Wood, K. S.; Byrns, R. E.; Chaudhuri, G. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 9265.
(278) Palmer, R.; Ferrige, A. G.; Moncada, S. *Nature* **1987**, *327*, 524.

(279) Furchgott, R. F. In *Vasodilation*; Vanhoutte, P. M., Leusen, I., Eds.; Raven Press: New York, 1988; p 401.
(280) Kelm, M.; Scrader, J. *Circ. Res.* **1990**, *66*, 1561.
(281) Moncada, S.; Palmer, R. M. J.; Higgs, E. A. *Pharmacol. Rev.* **1991**, *43*, 109.
(282) Loscalzo, J.; Welch, G. *Prog. Cardiovasc. Dis.* **1995**, *38*, 87.
(283) Lincoln, J.; Hoyle, C. H. V.; Burnstock, G. *Nitric Oxide in Health and Disease*; Cambridge University Press: Cambridge, 1997.
(284) Wink, D. A.; Mitchell, J. B. *Free Radical Biol. Med.* **1998**, *25*, 434.
(285) Joshi, M. S.; Ponthier, J. L.; Lancaster, J. R. *Free Radical Biol. Med.* **1999**, *27*, 1357.
(286) Jones-Carson, J.; Vazquez-Torres, A.; van der Heyde, H. C.; Warner, T.; Wagner, R. D.; Balish, E. *Nat. Med.* **1995**, *1*, 552.
(287) Green, S. J. *Nat. Med.* **1995**, *1*, 515.
(288) Duncan, C.; Dougall, H.; Johnston, P.; Green, S.; Brogan, R.; Leifert, C.; Smith, L.; Golden, M.; Bengamin, N. *Nat. Med.* **1995**, *1*, 546.
(289) Pacelli, R.; Wink, D. A.; Cook, J. A.; Krishna, M. C.; DeGraff, W.; Friedman, N.; Tsokos, M.; Samuni, A.; Mitchell, J. B. *J. Exp. Med.* **1995**, *182*, 1469.
(290) Stark, M. E.; Szurszewski, J. H. *Gastroenterology* **1992**, *103*, 1928.
(291) Gorbunov, N. V.; Elsayed, N. M.; Kisin, E. R.; Kozlov, A. V.; Kagan, V. E. *Am. J. Physiol.* **1997**, *272*, L320.
(292) Kosaka, H.; Seiyama, A. *Biochem. Biophys. Res. Commun.* **1996**, *218*, 749.
(293) Bone, R. C. *Clin. Chest Med.* **1996**, *17*, 175.
(294) Fischer, S. R.; Bone, H. G.; Harada, M.; Jourdain, M.; Traber, D. L. *Sepsis* **1998**, *1*, 135.
(295) Kurose, I.; Wolf, R.; Grisham, M. B.; Granger, D. N. *Circ. Res.* **1994**, *74*, 376.
(296) Oda, H.; Kusumoto, S.; Nakajima, T. *Arch. Environ. Health* **1975**, *30*, 453.
(297) Westenberger, U.; Thanner, S.; Ruf, H. H.; Gersonde, K.; Sutter, G.; Trentz, O. *Free Radical Res. Commun.* **1990**, *11*, 167.
(298) Zhuo, M.; Small, S. A.; Kandel, E. R.; Hawkins, R. D. *Science* **1993**, *260*, 1946.
(299) Kharitonov, V. G.; Sharma, V. S.; Pilz, R. B.; Magde, D.; Koesling, D. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *90*, 2568.
(300) Snyder, S. H.; Jaffery, S. R.; Zakhary, R. *Brain Res. Rev.* **1998**, *26*, 167.
(301) Martin, W.; Villani, G. M.; Jothianandan, D.; Furchgott, R. F. *J. Pharmacol. Exp. Ther.* **1985**, *232*, 708.
(302) Fujiwara, S.; Kassel, N. F.; Sasaki, T.; Nakagomi, T.; Lehman, R. M. *J. Neurosurg.* **1986**, *64*, 445.
(303) McFarland, W.; Mvere, D.; Shandera, W.; Reingold, A. *Vox Sang.* **1997**, *72*, 85.
(304) Hess, J. R.; Macdonald, V. W.; Brinkley, W. W. *J. Appl. Physiol.* **1993**, *74*, 1769.
(305) Kim, H. W.; Greenburg, A. G. *J. Lab. Clin. Med.* **2000**, *135*, 180.
(306) Sharma, V. S.; Traylor, T. G.; Gardiner, R.; Mizukami, H. *Biochemistry* **1987**, *26*, 3837.
(307) Eich, R. F.; Li, T.; Lemon, D. D.; Doherty, D. H.; Curry, S. R.; Aitken, J.; Matthews, A. J.; Johnson, K. A.; Smith, R. D.; Phillips, G. N.; Olson, J. S. *Biochemistry* **1996**, *35*, 6976.
(308) Liu, X.; Miller, M. J. S.; Joshi, M. S.; Sadowska-Krowicka, H.; Clark, D. A.; Lancaster, J. R. *J. Biol. Chem.* **1998**, *273*, 18709.
(309) Kharitonov, V. G.; Sundquist, A. R.; Sharma, V. S. *J. Biol. Chem.* **1994**, *269*, 5881.
(310) Doyle, M. P.; Hoekstra, J. W. *J. Inorg. Biochem.* **1981**, *14*, 351.
(311) Huie, R. E.; Padmaja, S. *Free Radical Res. Commun.* **1993**, *18*, 195.
(a) Ischiropoulos, H. *Arch. Biochem. Biophys.* **1998**, *356*, 1.
(312) Foresti, R.; Clark, J. E.; Green, C. J.; Motterlini, R. *J. Biol. Chem.* **1997**, *272*, 18411.
(313) Myers, S. I.; Hernandez, R.; Castaneda, A. *Am. J. Surg.* **1995**, *169*, 604.
(314) Alayash, A. I.; Cashon, R. E. *Ann. N. Y. Acad. Sci.* **1994**, *738*, 378.
(315) Doherty, D. H.; Doyle, M. P.; Curry, S. R.; Vali, R. J.; Fattor, T. J.; Olson, J. S.; Lemon, D. D. *Nat. Biotechnol.* **1998**, *16*, 672.
(316) Jia, L.; Bonaventura, C.; Bonaventura, J.; Stamler, J. S. *Nature* **1996**, *380*, 221.
(317) Stamler, J. S.; Jia, L.; Eu, J. P.; McMahon, T. J.; Demchenko, I. T.; Bonaventura, J.; Gernert, K.; Piantadosi, C. A. *Science* **1997**, *276*, 2034.
(318) Gow, A. J.; Luchsinger, B. P.; Pawloski, J. R.; Singel, D. J.; Stamler, J. S. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 9027.
(319) Pawloski, J. R.; Swaminathan, R. V.; Stamler, J. S. *Circulation* **1998**, *97*, 263.
(320) Bunn, H. F.; Esham, W. T.; Bull, R. W. *J. Exp. Med.* **1969**, *129*, 909.
(321) Benesch, R.; Benesch, R. E. *Biochem. Biophys. Res. Commun.* **1967**, *26*, 162.
(322) Chanutin, A.; Curnish, R. R. *Arch. Biochem. Biophys.* **1967**, *121*, 96.
(323) Perutz, H. F.; Muirhead, H.; Mazzarella, L.; Crowther, R. A.; Greer, J.; Kilmartin, J. V. *Nature* **1969**, *222*, 1240.
(324) Alayash, A. I.; Cashon, R. E. *Mol. Med. Today* **1995**, *1*, 122.
(325) *Blood Substitutes*; Chang, T. M. S.; Geyer, R. P., Eds.; Marcel Dekker: New York, 1989.
(326) Monod, J.; Wyman, J.; Changeux, J.-P. *J. Mol. Biol.* **1965**, *12*, 88.
(327) Perutz, M. F. *Nature* **1970**, *228*, 726.
(328) Kilmartin, J. V.; Arnone, A.; Fogg, J. *Biochemistry* **1977**, *16*, 5393.
(329) Bonaventura, C.; Tesh, S.; Faulkner, K. M.; Kraiter, D.; Crumbliss, A. L. *Biochemistry* **1998**, *37*, 496.
(330) Benesch, R.; Benesch, R. E. *Nature* **1969**, *221*, 618.
(331) Bunn, H. F. *Science* **1971**, *172*, 1049.
(332) Fronticelli, C.; Bucci, E.; Orth, C. *J. Biol. Chem.* **1984**, *259*, 10841.
(333) Isaacks, R.; Harkness, D.; Sampsell, R.; Adler, J.; Roth, S.; Kim, C.; Goldman, P. *Eur. J. Biochem.* **1977**, *77*, 567.
(334) Komiyama, N. H.; Miyazaki, G.; Tame, J.; Nagai, K. *Nature* **1995**, *373*, 244.
(335) Lemon, D. D.; Nair, P. K.; Boland, E. J.; Olson, J. S.; Hellums, J. D. *J. Appl. Physiol.* **1987**, *62*, 798.
(336) Alayash, A. I.; Fratantoni, J. C. *Biomater., Artif. Cells, Immobilization Biotechnol.* **1992**, *20*, 259.
(337) Ilan, E.; Morton, P. G.; Chang, T. M. S. *Biomater., Artif. Cells, Immobilization Biotechnol.* **1992**, *20*, 263.
(338) Winslow, R. M. In *Blood Substitutes—New Challenges*; Winslow, R. M., Vandegriff, K. D., Intaglietta, M., Eds.; Birkhäuser: Boston, 1996; p 146.
(339) Riess, J. G.; Le Blanc, M. *Pure Appl. Chem.* **1982**, *54*, 2383.
(340) Lutz, J.; Decke, B.; Bauml, M.; Schultze, H. G. *Pflügers Arch.* **1978**, *376*, 1.
(341) Vandegriff, K. D.; Medina, F.; Marini, M. A.; Winslow, R. M. *J. Biol. Chem.* **1989**, *264*, 17824.
(342) Lutz, J.; Herrmann, G. *Pflügers Arch.* **1984**, *401*, 174.
(343) Spiess, B. D.; McCarthy, R. J.; Tuman, K. J.; Woronowicz, A. W.; Tool, K. A.; Ivankovich, A. D. *Undersea Biomed. Res.* **1988**, *15*, 31.
(344) Lynch, P. R.; Krasner, L. J.; Vinciquerra, T.; Shaffer, T. H. *Undersea Biomed. Res.* **1989**, *16*, 275.
(345) Novotny, J. A.; Bridgewater, B. J. M.; Himm, J. F.; Homer, L. D. *J. Appl. Physiol.* **1993**, *74*, 1356.
(346) Dromsky, D. M.; Fahlman, A.; Spiess, B. D. VIIIth International Symposium on Blood Substitutes, San Diego, 2000.
(347) Wolber, J.; Rowland, I. J.; Leach, M. O.; Bifone, A. *Magn. Reson. Med.* **1999**, *41*, 442.
(348) Habler, O. P.; Kleen, M. S.; Hutter, J. W.; Podtschaske, A. H.; Tiede, M.; Kemming, G. I.; Welte, M. V.; Corso, C. O.; Batra, S.; Keipert, P. E.; Faithfull, N. S.; Messmer, K. F. W. *Transfusion* **1998**, *38*, 145.
(349) Sunder-Plassman, L.; Dieterle, R.; Seifert, J.; Jesch, F.; Messmer, K. *Eur. J. Intensive Care Med.* **1975**, *1*, 37.
(350) Moss, G. S.; DeWoskin, R.; Rosen, A. L.; Levine, H.; Palani, C. K. *Surg. Gynecol. Obstet.* **1976**, *142*, 357.
(351) Messmer, K.; Jesch, F.; Endrich, B.; Hobbhahn, J.; Peters, W.; Schoenberg, M. *Eur. Surg. Res.* **1979**, *11*, 161.
(352) Biro, G. P. *Cardiovasc. Res.* **1982**, *16*, 194.
(353) Hobbhahn, J.; Vogel, H.; Kothe, N.; Brendel, W.; Peter, K.; Jesch, F. *Acta Anaesthesiol. Scand.* **1985**, *29*, 537.
(354) Gould, S. A.; Sehgal, L. R.; Rosen, A. L.; Sehgal, H. L.; Moss, G. S. *Ann. Surg.* **1990**, *211*, 394.
(355) Vlahakes, G. J.; Lee, R.; Jacobs, E. E.; LaRaia, P. J.; Austen, W. G. *J. Thorac. Cardiovasc. Surg.* **1990**, *100*, 379.
(356) Rooney, M. W.; Hirsch, L. J.; Mathru, M. *Anesthesiology* **1993**, *79*, 60.
(357) Poli de Figueiredo, L. F.; Mathru, M.; Solanki, D.; Macdonald, V. W.; Hess, J. R.; Kramer, G. C. *J. Trauma, Inj. Infect. Crit. Care* **1997**, *42*, 847.
(358) Nolte, D.; Steinhauser, P.; Pickelmann, S.; Berger, S.; Hartl, R.; Messmer, K. *J. Lab. Clin. Med.* **1997**, *130*, 328.
(359) Winslow, R. M.; Gonzales, A.; Gonzales, M. L.; Magde, M.; McCarthy, M.; Rohlfs, R. J.; Vandegriff, K. D. *J. Appl. Physiol.* **1998**, *85*, 993.
(360) Kasper, S.; Grune, F.; Walter, M.; Amr, N.; Erasmi, H.; Buzello, W. *Anesth. Analg.* **1998**, *87*, 284.
(361) Gould, S. A.; Rosen, A. L.; Sehgal, L. R.; Sehgal, H. L.; Langdale, L. A.; Krause, L. M.; Rice, C. L.; Chamberlain, W. H.; Moss, G. S. *N. Engl. J. Med.* **1986**, *314*, 1653.
(362) Correspondence. *N. Engl. J. Med.* **1987**, *315*, 1677.
(363) Sloviter, H. A. *Biomater., Artif. Cells, Artif. Organs* **1988**, *16*, 459.
(364) Prys-Roberts, C.; Foex, P.; Hahn, C. E. W. *Anesthesiology* **1971**, *34*, 581.
(365) Gould, S. A.; Rosen, A.; Sehgal, L.; Noud, G.; Sehgal, H.; DeWoskin, R.; Levine, H.; Kerstein, M.; Rice, C.; Moss, G. S. *J. Surg. Res.* **1980**, *28*, 246.
(366) Gould, S. A.; Rosen, A. L.; Sehgal, L. R.; Sehgal, H. L.; Moss, G. S. *J. Trauma* **1986**, *26*, 903.
(367) Snyder, J. V. In *Oxygen Transport in the Critically Ill*; Snyder, J. V.; Pinksy, M. R., Eds.; Year Book Medical Publ.: Chicago, 1987; p 179.

(368) Wagner, P. D. *Adv. Exp. Med. Biol.* **1988**, *227*, 245.
(369) Faithfull, N. S.; Cain, S. M. *J. Crit. Care* **1988**, *3*, 14.
(370) Faithfull, N. S. *Biomater., Artif. Cells, Immobilization Biotechnol.* **1992**, *20*, 797.
(371) Holman, W. L.; Spruell, R. D.; Ferguson, E. R.; Clymer, J. J.; Vicente, W. V. A.; Murrah, C. P.; Pacifico, A. D. *J. Thorac. Cardiovasc. Surg.* **1995**, *110*, 774.
(372) Ivanitskii, G. R.; Vorob'yev, S. I. *Biophysics* **1996**, *41*, 189.
(373) Wittenberg, J. B. *J. Biol. Chem.* **1966**, *241*, 104.
(a) Homer, L. D.; Weathersby, P. K.; Kiesow, L. A. *Microvasc. Res.* **1981**, *22*, 308.
(374) Boland, E. J.; Nair, P. K.; Lemon, D. D.; Olson, J. S.; Hellums, J. D. *J. Appl. Physiol.* **1987**, *62*, 791.
(375) Kreutzer, F.; Hoofd, L. J. C. *Respir. Physiol.* **1970**, *8*, 280.
(376) Biro, G. P.; Anderson, P. J.; Curtis, S. E.; Cain, S. M. *Can. J. Physiol. Pharmacol.* **1991**, *69*, 1656.
(377) Hogan, M. C.; Willford, D. C.; Keipert, P. E.; Faithfull, N. S.; Wagner, P. D. *J. Appl. Physiol.* **1992**, *73*, 2470.
(378) Hogan, M. C.; Kurdak, S. S.; Richardson, R. S.; Wagner, P. D. *Adv. Exp. Med. Biol.* **1994**, *361*, 375.
(379) Vandegriff, K. D.; Winslow, R. M. In *Blood Substitutes. Physiological Basis of Efficacy*; Winslow, R. M., Vandegriff, K. D., Intaglietta, M., Eds.; Birkhäuser: Boston, 1995; p 143.
(380) Winslow, R. M.; Vandegriff, K. D. In *Advances in Blood Substitutes*—Industrial Opportunities and Medical Challenges; Winslow, R. M., Vandegriff, K. D., Intaglietta, M., Eds.; Birkhäuser: Boston, 1997; p 167.
(381) Nishide, H.; Chen, X. S.; Tsuchida, E. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1997**, *25*, 335.
(382) Page, T. C.; Light, W. R.; McKay, C. B.; Hellums, J. D. *Microvasc. Res.* **1998**, *55*, 54.
(383) Conhaim, R. L.; Rodenkirch, L. A.; Wason, K. E.; Harms, B. A. *J. Appl. Physiol.* **2000**, *89*, 198.
(384) Page, T. C.; Light, W. R.; Hellums, J. D. *Microvasc. Res.* **1998**, *56*, 113.
(385) Kessler, M.; Höper, J.; Pohl, U. In *Oxygen Carrying Colloidal Blood Substitutes*; Frey, R., Beisbarth, H., Stosseck, K., Eds.; Zuckschwerdt Verlag: Munchen, 1982; p 99.
(386) Sutherland, G. R.; Farrar, J. K.; Peerless, S. J. *Stroke* **1984**, *15*, 829.
(387) Clark, L. C.; Spokane, R. B.; Hoffmann, R. E.; Sudan, R. *Adv. Exp. Med. Biol.* **1989**, *248*, 341.
(388) Braun, R. D.; Linsenmeier, R. A.; Goldstick, T. K. *J. Appl. Physiol.* **1992**, *75*, 1960.
(389) Patel, S.; Mehra, A. *Am. Soc. Artif. Intern. Organs J.* **1998**, *44*, 157.
(390) Patel, S.; Mehra, A. *Am. Soc. Artif. Intern. Organs J.* **1998**, *44*, 144.
(391) Eggleton, C. D.; Roy, T. K.; Popel, A. S. *Am. J. Physiol.* **1998**, *275*, H2250.
(392) Kameneva, M. V.; Borovetz, H. S.; Antaki, J. F.; Litwak, P.; Federspiel, W. J.; Kormos, R. L.; Griffith, B. P. *Adv. Exp. Med. Biol.* **1997**, *418*, 383.
(393) Faithfull, N. S.; Rhoades, G. E.; Keipert, P. E.; Ringle, A. S.; Trouwborst, A. *Adv. Exp. Med. Biol.* **1994**, *361*, 41.
(394) Gould, S. A.; Sehgal, L. R.; Rosen, A. L.; Sehgal, H. I.; Levine, H. D.; C, L., Rice; G, S., Moss. *J. Surg. Res.* **1982**, *33*, 189.
(395) Geyer, R. P.; Taylor, K.; Duffett, E. B.; Eccles, R. *Fed. Proc.* **1973**, *32*, 927.
(396) Gould, S. A.; Rosen, A. L.; Sehgal, L. R.; Sehgal, H. L.; Rice, C. L.; Moss, G. S. *Surg. Forum* **1981**, *23*, 299.
(397) Arlen, C.; Follana, R.; Le Blanc, M.; Long, C.; Long, D.; Riess, J. G.; Valla, A. *Biomater., Artif. Cells, Artif. Organs* **1988**, *16*, 455.
(398) Rabinovici, R.; Rudolph, A. S.; Ligier, R. S.; Yue, T.-L.; Feuerstein, G. *Circ. Shock* **1990**, *32*, 1.
(399) Beach, M. C.; Morley, J.; Spiryda, L.; Weinstock, S. B. *Biomater., Artif. Cells, Immobilization Biotechnol.* **1992**, *20*, 771.
(400) Rudolph, A. S.; Klipper, R. W.; Goins, B.; Phillips, W. T. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 10976.
(401) Rudolph, A. S.; Cliff, R. O.; Spargo, B. J.; Spielberg, H. *Biomaterials* **1994**, *15*, 796.
(402) Miller, M. L.; Wesseler, E. P.; Jones, S. C.; Clark, L. C. *J. Reticuloendothelial Soc.* **1976**, *20*, 385.
(403) Castro, O.; Nesbitt, A. E.; Lyles, D. *Am. J. Hematol.* **1984**, *16*, 15.
(404) Lutz, J. *Int. Anesthesiol. Clin.* **1985**, *23*, 63.
(405) Flaim, S. F. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1994**, *22*, 1043.
(406) Kaufman, R. J. In *Blood Substitutes: Physiological Basis of Efficacy*; Winslow, R. M., Vandegriff, K. D., Intaglietta, M., Eds.; Birkhäuser: Boston, 1995; p 53.
(407) Nugent, K. M. *J. Leukocyte Biol.* **1984**, *36*, 123.
(408) Davis, S. S.; Illum, L.; Washington, C.; Harper, G. *Int. J. Pharm.* **1992**, *82*, 99.
(409) Patel, H. M. *Crit. Rev. Ther. Drug Carrier Syst.* **1992**, *9*, 39.
(410) Lutz, J.; Augustin, A. J.; Jäger, L. J.; Bachmann, D.; Brandl, M. *Life Sci.* **1995**, *56*, 99.
(411) Lahnborg, G.; Berghem, L.; Jaarstrand, C. *Acta Chir. Scan.* **1979**, Suppl. 489, 271.
(412) Greenburg, A. G.; Maffuid, P. W. *Prog. Clin. Biol. Res.* **1983**, *122*, 9.
(413) Marks, D. H.; Brown, D. R.; Ottinger, W. E.; Atassi, M. Z. *Mil. Med.* **1987**, *152*, 473.
(414) Velky, T. S.; Lee, E. S.; Maffuid, P. W.; Robinson, G. T.; Yang, J. C.; Greenburg, A. G. *Arch. Surg.* **1987**, *122*, 355.
(415) Bleeker, W. K.; van der Plas, J.; Feitsma, R. I. J.; Ageterberg, J.; Rigter, G.; de Vries-van Rossen, A.; Pauwels, E. K. J.; Bakker, J. C. *J. Lab. Clin. Med.* **1989**, *113*, 151.
(416) Keipert, P. E.; Gomez, C. L.; Gonzales, A.; MacDonald, V. W.; Hess, J. R.; Winslow, R. M. *J. Lab. Clin. Med.* **1994**, *123*, 701.
(417) Conhaim, R. L.; Cooler, S. D.; McGrath, A. M.; DeAngeles, D. A.; Myers, G. A.; Harms, B. A. *Am. J. Respir. Crit. Care Med.* **1998**, *158*, 1204.
(418) Baldwin, A. L.; Wilson, L. M.; Valeski, J. E. *Am. J. Physiol.* **1998**, *275*, H615.
(419) Bunn, H. F.; Jandl, J. H. *J. Exp. Med.* **1969**, *129*, 925.
(420) Brandt, J. L.; Frank, N. R.; Lichtman, H. C. *Blood* **1951**, *6*, 1152.
(421) Miller, J. H.; McDonald, R. K. *J. Clin. Invest.* **1951**, *30*, 1033.
(422) Lieberthal, W. In *Red Blood Cell Substitutes*; Rudolph, A. S., Rabinovici, R., Feuerstein, G. Z., Eds.; Marcel Dekker: New York, 1998; p 189.
(423) Greenburg, A. G. *Prog. Clin. Biol. Res.* **1983**, *122*, 127.
(424) Wretlind, A. In *Fat Emulsions: Past—Present—Future. Intralipid Silver Jubilee Symposium*; Hagenfeldt, L., Ed.; Nutrition: Stockholm, 1987; Vol. 3; p 345.
(425) KabiVitrum Inc. *Intralipid 10%. A 10% i.v. Fat Emulsion* Product Monograph, 1988.
(426) Storm, G.; Oussoren, C.; Peeters, P.; Barenholz, Y. In *Liposome Technology*; Gregoriadis, G., Ed.; CRC Press: Boca Raton, 1993; Vol. 3; p 345.
(427) Rabinovici, R.; Neville, L.; Feuerstein, G.; Rudolph, A. S. In *Liposomes in Biomedical Applications*; Shek, P. N., Ed.; Harwood Academic Publ.: North York, Ontario, 1995; p 227.
(428) Rudolph, A. S. In *Liposomes in Biomedical Applications*; Shek, P. N., Ed.; Harwood Academic Publ.: North York, Ontario, 1995; p 217.
(429) Heidelberger, M.; Landsteiner, K. *J. Exp. Med.* **1928**, *38*, 561.
(430) Ovary, Z. *Immunochemistry* **1964**, *1*, 241.
(431) Reichlin, M.; Hay, M.; Levine, L. *Immunochemistry* **1964**, *1*, 21.
(432) Feola, M.; Gonzalez, H.; Canizaro, P. C. *Surg. Gynecol. Obstet.* **1983**, *157*, 399.
(433) Chang, T. M. S.; Varma, R. *Biomater., Artif. Cells, Artif. Organs* **1988**, *16*, 205.
(434) Rollwagen, F. M.; Gafney, W. C. M.; Pacheco, N. D.; Davis, T. A.; Hickey, T. M.; Nielsen, T. B.; Rudolph, A. S. *Exp. Hematol.* **1996**, *24*, 429.
(435) Chang, T. M. S.; Lister, C.; Nishiya, A.; Varma, R. *Biomater., Artif. Cells, Immobilization Biotechnol.* **1992**, *20*, 611.
(436) Cunnington, P. G.; Jenkins, S. N.; Tam, S. C.; Wong, J. T. F. *Biochem. J.* **1981**, *193*, 261.
(437) Patel, M. J.; Webb., E. J.; Shelbourn, T. E.; Mattia-Goldberg, C.; George, A. J. T.; Zhang, F.; Moore, E. G.; Nelson, D. J. *Blood* **1998**, *19*, 710.
(438) Jacobs, E. E. 52nd Annual Meeting of the American Association of Bood Banks—The Compendium, 1999; p 302.
(439) Vercellotti, G. M.; Hammerschmidt, D. E.; Craddock, P. R.; Jacob, H. S. *Blood* **1982**, *59*, 1299.
(440) Tremper, K. K.; Vercellotti, G. M.; Hammerschmidt, D. E. *Crit. Care Med.* **1984**, *12*, 428.
(441) Hammerschmidt, D. E.; Vercellotti, G. M. *Biomater., Artif. Cells, Artif. Organs* **1988**, *16*, 431.
(442) Noveck, R. J.; Shannon, E. J.; Leese, P. T.; Shorr, J. S.; Flaim, K. E.; Keipert, P. E.; Woods, C. M. *Anesth. Analg.* **2000**, *91*, 812.
(443) Litwin, M. S.; Walter, C. W.; Ejarque, P.; Reynolds, E. S. *Ann. Surg.* **1963**, *157*, 485.
(444) Lee, J. T.; Ahrenholz, D. H.; Nelson, R. D.; Simmons, R. L. *Surgery* **1979**, *86*, 41.
(445) Eaton, J. W.; Brandt, P.; Mahoney, J. R. *Science* **1982**, *215*, 691.
(446) Otto, B. R.; Verweij-van Vught, A. M. J. J.; MacLaren, D. M. *Nature* **1992**, *358*, 23.
(447) Griffiths, E.; Cortes, A.; Gilbert, N.; Stevenson, P.; MacDonald, S.; Pepper, D. *Lancet* **1995**, *345*, 158.
(448) Su, D.; Roth, R. I.; Yoshida, M.; Levin, J. *Infect. Immun.* **1997**, *65*, 1258.
(449) Dunn, D. L.; Nelson, R. D.; Condie, R. M.; Simmons, R. L. *Surgery* **1983**, *93*, 653.
(450) Kaca, W.; Roth, R. I.; Levin, J. *J. Biol. Chem.* **1994**, *269*, 25078.
(451) Bornside, G. H.; Bouis, P. J.; Cohn, I. *Surgery* **1970**, *68*, 350.
(452) Pruett, T. L.; Rotstein, O. D.; Wells, C. L.; Sorenson, J. J.; Simmons, R. L. *Surgery* **1985**, *98*, 371.
(453) Hau, T.; Simmons, R. I. *Surgery* **1980**, *87*, 588.
(454) Sielenkämper, A. W.; Chin-Yee, I. H.; Martin, C. M.; Sibbald, W. J. *Am. J. Respir. Crit. Care Med.* **1997**, *156*, 1066.
(455) Bone, H. G.; Schenarts, P. J.; Fischer, S. R.; McGuire, R.; Traber, L. D.; Traber, D. L. *J. Appl. Physiol.* **1998**, *84*, 1991.

(456) White, C. T.; Murray, A. J.; Smith, D. J.; Greene, J. R.; Bolin, R. B. *J. Lab. Clin. Med.* **1986**, *108*, 132.
(457) Yoshida, M.; Roth, R. I.; Levin, J. *J. Lab. Clin. Med.* **1995**, *126*, 151.
(458) Su, D.; Roth, R. I.; Levin, J. *Crit. Care Med.* **1999**, *27*, 771.
(459) Kaca, W.; Roth, R. I.; Ziolkowski, A.; Levin, J. *J. Endotoxin Res.* **1994**, *1*, 243.
(460) Roth, R. I. *Blood* **1994**, *83*, 2860.
(461) Roth, R. I.; Levin, J. *N. Engl. J. Med.* **2000**, *343*, 1273.
(462) Zuckerman, S. H.; Doyle, M. P.; Gorczynski, R.; Rosenthal, G. J. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1998**, *26*, 231.
(463) Thomassen, M. J.; Buhrow, L. T.; Wiedemann, H. P. *Crit. Care Med.* **1997**, *25*, 2045.
(464) Croce, M. A.; Fabian, T. C.; Patton, J. H.; Melton, S. M.; Moore, M.; Trenthem, L. L. *J. Trauma* **1998**, *45*, 273.
(465) Fasan, G.; Grandgeorge, M.; Vigneron, C.; Dellacherie, E. *J. Biochem. Biophys. Methods* **1991**, *23*, 53.
(466) Rodriguez del Pozo, P. *J. Med. Ethics* **1994**, *20*, 31.
(467) Domen, R. E. *Trans. Med. Rev.* **1995**, *9*, 53.
(468) Gustafsson, J.; Ljusberg-Wahren, H.; Almgren, M.; Larsson, K. *Langmuir* **1996**, *12*, 4611.
(469) Barnikol, W. K. R.; Burkhard, O.; Pötzschke, H.; Domack, U.; Dinkelmann, S.; Guth, S.; Özbek, M.; Fiedler, B.; Manz, B. International Society for Oxygen Transport to Tissue, 2000.
(470) Hirsch, R. E.; Jelicks, L. A.; Wittenberg, B. A.; Kaul, D. K.; Shear, H. L.; Harrington, J. P. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1997**, *25*, 429.
(471) Harrington, J. P.; Gonzalez, Y.; Hirsch, R. E. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **2000**, *28*, 477.
(472) Nagai, K.; Perutz, M. F.; Poyart, C. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 7252.
(473) Looker, D.; Abbott-Brown, D.; Cozart, P.; Durfee, S.; Hoffman, S.; Mathews, A. J.; Miller-Roehrich, J.; Shoemaker, S.; Trimble, S.; Fermi, G.; Komiyama, N. H.; Nagai, K.; Stetler, G. L. *Nature* **1992**, *356*, 258.
(474) Logan, J. S.; Martin, M. J. *Methods Enzymol.* **1994**, *231*, 435.
(475) Dieryck, W.; Pagnier, J.; Poyart, C.; Marden, M. C.; Gruber, V.; Bournat, P.; Baudino, S.; Mérot, B. *Nature* **1997**, *386*, 29.
(476) Freytag, J. W.; Caspari, R. F.; Gorczynski, R. J. In *Blood Substitutes. Present and Future Perspectives*; Tsuchida, E., Ed.; Elsevier: Amsterdam, 1998; p 55.
(477) Ho, C.; Sun, D. P.; Shen, T. J.; Ho, N. T.; Zou, M.; Hu, C. K.; Sun, Z. Y.; Lukin, J. A. In *Blood Substitutes. Present and Future Perspectives*; Tsuchida, E., Ed.; Elsevier: Amsterdam, 1998; p 281.
(478) Coghlan, D.; Jones, G.; Denton, K. A.; Wilson, M. T.; Chan, B.; Harris, R.; Woodrow, J. R.; Ogden, J. E. *Eur. J. Biochem.* **1992**, *207*, 931.
(479) Kumar, R.; Manjula, B. N. In *Red Blood Cell Substitutes*; Rudolph, A. S., Rabinovici, R., Feuerstein, G. Z., Eds.; Dekker: New York, 1998; p 309.
(480) Whitelam, G. C.; Cockburn, B.; Gandecha, A. R.; Owen, M. R. L. *Biotechnol. Genet. Eng. Rev.* **1993**, *11*, 1.
(481) Sellards, A. W.; Minot, G. R. *J. Med. Res.* **1916**, *34*, 469.
(482) Amberson, W. R. *Biol. Rev.* **1937**, *12*, 48.
(483) Gilligan, D. R.; Altschule, M. D.; Katersky, E. M. *J. Clin. Invest.* **1941**, *20*, 177.
(484) Goldberg, M. *J. Clin. Invest.* **1962**, *41*, 2112.
(485) Foix, C.; Salin, H. *Arch. Méd. Exp. Anat. Pathol.* **1913**, *25*, 104.
(486) Rabiner, S. F.; Helbert, J. R.; Lopas, H.; Friedman, L. H. *J. Exp. Med.* **1967**, *126*, 1127.
(487) Peskin, G. W.; O'Brien, K.; Rabiner, S. F. *Surgery* **1969**, *66*, 185.
(488) Tam, S.; Wong, J. *J. Lab. Clin. Med.* **1988**, *111*, 189.
(489) Amend, J.; Ou, C.; Ryan-MacFarlane, C.; Anderson, P. J.; Amend, N.; Biro, G. P. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1996**, *24*, 19.
(490) Simoni, J.; Simoni, G.; Hartsell, A.; Feola, M. *Am. Soc. Artif. Intern. Organs J.* **1997**, *43*, M714.
(491) Lieberthal, W.; Fuhro, R.; Freedman, J. E.; Toolan, G.; Loscalzo, J.; Valeri, C. R. *J. Pharmacol. Exp. Ther.* **1999**, *288*, 1278.
(492) Chan, W. L.; Tang, N. L. S.; Yim, C. C. W.; Lai, F. M.; Tam, M. S. C. *Toxicol. Pathol.* **2000**, *28*, 635.
(493) Muldoon, S. M.; Ledvina, M. A.; Hart, J. L.; Macdonald, V. W. *J. Lab. Clin. Med.* **1996**, *128*, 579.
(494) Simoni, J.; Feola, M.; Tran, R.; Buckner, M.; Canizaro, P. C. *Artif. Organs* **1990**, *14*, 98.
(495) Simoni, J.; Simoni, G.; Martinez-Zaguilan, R.; Wesson, D. E.; Lox, C. D.; Prien, S. D.; Kumar, R. V. *Am. Soc. Artif. Intern. Organs J.* **1998**, *44*, M356.
(496) Burhop, K. E.; Farrell, L.; Nigro, C.; Tan, D.; Estep, T. *Biomater., Artif. Cells, Immobilization. Biotechnol.* **1992**, *20*, 581.
(497) Smith, D. J.; Winslow, R. M. *J. Lab. Clin. Med.* **1992**, *119*, 176.
(498) McFaul, S. J.; Bowman, P. D.; Villa, V. M.; Gutierrez-Ibanez, M. J.; Johnson, M.; Smith, D. *Blood* **1994**, *84*, 3175.
(499) Rabiner, S. F.; Friedman, L. H. *Br. J. Haematol.* **1968**, *14*, 105.
(500) Moss, G. S.; DeWoskin, R.; Cochin, A. *Surgery* **1973**, *74*, 198.
(501) Kim, M. S.; Kim, H. W.; Sweeney, J. D.; Greenburg, A. G. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1997**, *25*, 289.
(502) Macdonald, R. L.; Weir, B. K. A. *Stroke* **1992**, *22*, 971.
(503) Regan, R. F.; Panter, S. S. *Neurosci. Lett.* **1993**, *153*, 219.
(504) Panter, S. S.; Vandegriff, K. D.; Yan, P. O.; Regan, R. F. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1994**, *22*, 399.
(505) Matz, P. G.; Fujimura, M.; Chan, P. H. *Brain Res.* **2000**, *858*, 312.
(506) Schuschereba, S. T.; Friedman, H. I.; DeVenuto, F.; Beatrice, E. S. *Lab. Invest.* **1983**, *48*, 339.
(507) Zuck, T. F.; DeVenuto, F.; Neville, J. R.; Friedman, H. I. In *Blood Substitutes and Plasma Expanders*; A. R. Liss: New York, 1978; p 111.
(508) DeVenuto, F.; Friedman, H. I.; Neville, J. R.; Peck, C. C. *Surg. Gynecol. Obstet.* **1979**, *149*, 417.
(509) Proceedings of the VIth International Symposium on Blood Substitutes; Chang, T. M. S., Greenburg, G., Tsuchida, E., Eds.; In *Artificial Cells, Blood Substitutes, Immobilization Biotechnology;* Dekker: New York, 1998; Vol. 25.
(510) Gulati, A. *Adv. Drug Delivery Rev.* **2000**, *40*, 129.
(511) Winslow, R. M. *Vox Sang.* **2000**, *79*, 1.
(512) Benesch, R. E.; Kwong, S. *J. Biol. Chem.* **1990**, *265*, 14881.
(513) Rohlfs, R. J.; Brune, E.; Chiu, A.; Gonzales, A.; Gonzales, M. L.; Magde, D.; Magde, M.; Vandegriff, K.; Winslow, R. M. *J. Biol. Chem.* **1998**, *273*, 12128.
(514) Tsai, A. G.; Friesenecker, B.; McCarthy, M.; Sakai, H.; Intaglietta, M. *Am. J. Physiol.* **1998**, *275*, H2170.
(515) Caron, A.; Menu, P.; Faivre-Fiorina, B.; Labrude, P.; Alayash, A.; Vigneron, C. *Am. J. Physiol.* **2000**, *278*, H1974.
(516) Sakai, H.; Hara, H.; Yuasa, M.; Tsai, A. G.; Takeoka, S.; Tsuchida, E.; Intaglietta, M. *Am. J. Physiol.* **2000**, *279*, H908.
(517) Jesch, F.; Bonhard, K.; Atippig, S.; Messmer, K. 5th European Congress on Anesthesiology, Paris, 1978.
(518) Pristoupil, T. I.; Kramlová, M.; Ulrych, S.; Fricová, V.; Kraml, J. *J. Chromatogr.* **1981**, *219*, 128.
(519) Sehgal, L. R.; Rosen, A. L.; Noud, G.; Sehgal, H. L.; Gould, S. A.; DeWoskin, R.; Rice, C. L.; Moss, G. S. *J. Surg. Res.* **1981**, *30*, 14.
(520) Lenz, G.; Junger, H.; Baur, K. F.; Schneider, M. In *Oxygen Carrying Colloidal Blood Substitutes.*; Frey, R., Beisbarth, H., Stosseck, K., Eds.; Zuckschwerdt Verlag: Munich, 1982; p 116.
(521) Sehgal, L. R.; Rosen, A. L.; Gould, S. A.; Sehgal, H. L.; Moss, G. S. *Transfusion* **1983**, *23*, 158.
(522) Keipert, P. E.; Chang, T. M. S. *Appl. Biochem. Biotechnol.* **1984**, *10*, 133.
(523) Hsia, C. J. C. *Prog. Clin. Biol. Res.* **1989**, *319*, 339.
(524) Marini, M. A.; Moore, G. L.; Christensen, S. M.; Fishman, R. M.; Jessee, R. G.; Medina, F.; Snell, S. M.; Zegna, A. I. *Biopolymers* **1990**, *29*, 871.
(525) Iwashita, Y. In *Artificial Red Cells*; Tsuchida, E., Ed.; John Wiley and Sons: New York, 1995; p 151.
(526) Talarico, T. L.; Guise, K. J.; Stacey, C. J. *Biochim. Biophys. Acta* **2000**, *1476*, 53.
(527) Hsia, J. C.; Song, D. L.; Er, S. S.; Wong, L. T. L.; Keipert, P. E.; Gomez, C. L.; Gonzales, A.; McDonald, V. W.; Hess, J. R.; Winslow, R. M. *Biomater., Artif. Cells., Immobilization Biotechnol.* **1992**, *20*, 587.
(528) Adamson, J. G.; Bonaventura, B. J.; Er, S. S.; Jones, R. T.; Langlois, S. F.; MacDonald, I. D.; Pilura, D. H.; Rydall, J. R.; Wicks, D. G.; Wiffen, D. E.; Wojciechowski, P. W.; Wong, L. T. In *Red Blood Cell Substitutes*; Rudolph, A. S., Rabinovici, R., Feuerstein, G. Z., Eds.; Dekker: New York, 1998; p 335.
(529) Vandegriff, K. D.; McCarthy, M.; Rohlfs, R. J.; Winslow, R. M. *Biophys. Chem.* **1997**, *69*, 23.
(530) Everse, J.; Johnson, M. C.; Marini, M. A. *Methods Enzymol.* **1994**, *231*, 547.
(a) Alayash, A. I.; Patel, R. P.; Cashon, R. E. *Antioxid. Redox Signaling* **2001**, *3*, 313.
(531) Paller, M. S. *Am. J. Physiol.* **1988**, *255*, F539.
(532) Biro, G. P.; Ou, C.; Ryan-Macfarlane, C.; Anderson, P. J. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1995**, *23*, 631.
(533) Motterlini, R.; Foresti, R.; Vandegriff, K.; Intaglietta, M.; Winslow, R. *Am. J. Physiol.* **1995**, *269*, H648.
(534) MacKenzie, C. F. IBC Conference on Blood Substitutes and Oxygen Therapeutics, Washington, DC, 1998.
(535) Kim, S. A.; Villa, V. O. M.; Hess, J. R. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1994**, *22*, 625.
(536) Estep, T. N.; Bechtel, M. K.; Miller, T. J.; Bagdasarian, A. *Biomater., Artif. Cells, Artif. Organs* **1988**, *16*, 129.
(537) Azari, M.; Ebeling, A.; Baker, R.; Burhop, K.; Camacho, T.; Estep, T.; Guzder, S.; Marshall, T.; Rohn, K.; Sarajari, R. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1998**, *26*, 577.
(538) Bucci, E.; Razynska, A.; Kwansa, H.; Gryczynski, Z.; Collins, J. H.; Fronticelli, C.; Unger, R.; Braxenthaler, M.; Moult, J.; Ji, X.; Gilliland, G. *Biochemistry* **1996**, *35*, 3418.

(539) Nho, K.; Glower, D.; Bredehoeft, S.; Shankar, H.; Shorr, R.; Abuchowski, A. *Biomater., Artif. Cells, Immobilization. Biotechnol.* **1992**, *20*, 511.
(540) Eaton, J. W. *J. Lab. Clin. Med.* **1996**, *127*, 416.
(541) Hess, J. R. *Semin. Hematol.* **1996**, *33*, 369.
(542) Zilletti, L.; Ciuffi, M.; Franchi-Micheli, S.; Fusi, F.; Gentilini, G.; Moneti, G.; Valoti, M.; Sgaragli, G. P. *Methods Enzymol.* **1994**, *231*, 562.
(543) Mieyal, J. J.; Starke, D. W. *Methods Enzymol.* **1994**, *231*, 573.
(544) Perutz, M. F.; Fermi, G.; Abraham, D. J.; Poyart, C.; Bursaux, E. *J. Am. Chem. Soc.* **1986**, *108*, 1064.
(545) Przybelski, R. J.; Daily, E. K.; Birnbaum, M. L. In *Advances in Blood Substitutes. Industrial Opportunities and Medical Challenges*; Winslow, R. M., Vandegriff, K. D., Intaglietta, M., Eds.; Birkhäuser: Boston, 1997; p 71.
(546) Reah, G.; Bodenham, A. R.; Mallick, A.; Daily, E. K.; Przybelski, R. J. *Crit. Care Med.* **1997**, *25*, 1480.
(547) Ma, Z.; Monk, T. G.; Goodnough, L. T.; McClellan, A.; Gawryl, M.; Clark, T.; Moreira, P.; Keipert, P. E.; Scott, M. G. *Clin. Chem.* **1997**, *43*, 1732.
(548) Wahr, J. A.; Anderson, M. M.; Giacherio, D. A.; Hallock, L.; Gawryl, M. S.; Lansden, C.; Tremper, K. K. *J. Cardiothorac. Vasc. Anesth.* **1997**, *11*, 10.
(549) Callas, D. D.; Clark, T. L.; Moreira, P. L.; Lansden, C.; Gawryl, M. S.; Kahn, S.; Bermes, E. W. *Clin. Chem.* **1997**, *43*, 1744.
(550) Chance, J. J.; Norris, E. J.; Kroll, M. H. *Clin. Chem.* **2000**, *46*, 1331.
(551) Ali, A. A.; Ali, G. S.; Steinke, J. M.; Shepherd, A. P. *Anesth. Analg.* **2001**, *92*, 863.
(552) Rabiner, S. F.; O'Brien, K.; Peskin, G.; Friedman, L. H. *Ann. Surg.* **1970**, *171*, 615.
(553) Kilmartin, J. V.; Rossi-Bernardi, L. *Biochem. J.* **1971**, *124*, 31.
(554) Bonhard, K. *Fed. Proc.* **1975**, *34*, 1466.
(555) Browdie, D.; Smith, H. *Am. J. Surg.* **1975**, *129*, 365.
(556) DeVenuto, F.; Zuck, T. F.; Zegna, A. I.; Moores, W. Y. *J. Lab. Clin. Med.* **1977**, *89*, 509.
(557) Riggs, A. *Methods Enzymol.* **1981**, *76*, 5.
(558) DeLoach, J. R.; Sheffield, C. L.; Spates, G. E. *Anal. Biochem.* **1986**, *157*, 191.
(559) Sheffield, C. L.; Spates, G. E.; Drolesky, R. E.; Green, R.; DeLoach, J. R. *Biotechnol. Appl. Biochem.* **1987**, *9*, 230.
(560) Christensen, S. M.; Medina, F.; Winslow, R. W.; Snell, S. M.; Zegna, A.; Marini, M. A. *J. Biochem. Biophys. Methods* **1988**, *17*, 143.
(561) Simoni, J.; Simoni, G.; Feola, M.; Canizaro, P. C. *Biomater., Artif. Cells, Immobilization Biotechnol.* **1991**, *19*, 488.
(562) Yang, T.; Olsen, K. W. *Arch. Biochem. Biophys.* **1988**, *261*, 283.
(563) Pristoupil, T. I.; Marik, T. *Biomater., Artif. Cells, Artif. Organs* **1990**, *18*, 183.
(564) Stratton, L. P.; Rudolph, A. S.; Knoll, J., W. K.; Bayne, S.; Farmer, M. C. *Hemoglobin* **1988**, *12*, 353.
(565) Brandl, M.; Gregoriadis, G. *Biochim. Biophys. Acta* **1994**, *1196*, 65.
(566) Labrude, P.; Vigneron, C.; Streiff, F. *J. Pharm. Belg.* **1976**, *31*, 191.
(567) DeVenuto, F.; Zegna, A. I.; Busse, K. R. *Surg. Gynecol. Obstet.* **1979**, *148*, 69.
(568) Thirion, C.; Larcher, D.; Chaillot, B.; Labrude, P.; Vigneron, C. *Biopolymers* **1983**, *22*, 2367.
(569) Keipert, P. E.; Chang, T. M. S. *Biomater. Artif. Cells, Artif. Organs* **1988**, *16*, 185.
(570) Kerwin, B. A.; Akers, M. J.; Apostol, I.; Moore-Einsel, C.; Etter, J. E.; Hess, E.; Lippincott, J.; Levine, J.; Mathews, A. J.; Revilla-Sharp, P.; Schubert, R.; Looker, D. L. *J. Pharm. Sci.* **1999**, *88*, 79.
(571) Hedlund, B.; Carlsson, J.; Condie, R.; Drayton, C. *Prog. Clin. Biol. Res.* **1983**, *122*, 71.
(572) Menu, P.; Labrude, P.; Grandgeorge, M.; Vigneron, C. *Int. J. Artif. Organs* **1991**, *14*, 672.
(573) Leal, O.; Anderson, D. L.; Bowman, R. G.; Basolo, F.; Burwell, R. L. *J. Am. Chem. Soc.* **1975**, *97*, 5125.
(574) Habeeb, A. F. S. A.; Hiramoto, R. *Arch. Biochem. Biophys.* **1968**, *126*, 16.
(575) Bunn, H. F.; Shapiro, R.; McManus, M.; Garrick, L.; McDonald, M. J.; Fallop, P. M.; Gabbay, K. H. *J. Biol. Chem.* **1979**, *254*, 3892.
(576) Cohen, M. P.; Wu, V.-Y. *Methods Enzymol.* **1994**, *231*, 65.
(577) Horowitz, B.; Mazur, A. *Prog. Clin. Biol. Res.* **1978**, *19*, 149.
(578) Ji, T. H. *Methods Enzymol.* **1983**, *91*, 580.
(579) Means, G. E.; Feeney, R. E. *Bioconjugate Chem.* **1990**, *1*, 2.
(580) Lundblad, R. L. *Chemical Reagents for Protein Modification*, 2nd ed.; CRC Press: Boca Raton, 1991.
(581) Wong, S. S. *Chemistry of Protein Conjugation and Cross-Linking*; CRC Press: Boca Raton, 1991.
(582) Scaloni, A.; Ferranti, P.; De Simone, G.; Mamone, G.; Sannolo, N.; Malorni, A. *FEBS Lett.* **1999**, *452*, 190.
(583) Perutz, M. F. *Nature* **1970**, *228*, 734.
(584) Kilmartin, J. V.; Rossi-Bernardi, L. *Nature* **1969**, *222*, 1243.
(585) Simon, S. R.; Arndt, D. J.; Konigsberg, W. H. *J. Mol. Biol.* **1971**, *58*, 69.
(586) Fantl, W. J.; Di Donato, A.; Manning, J. M.; Rogers, P. H.; Arnone, A. *J. Biol. Chem.* **1987**, *262*, 12700.
(587) Wold, F. *Methods Enzymol.* **1972**, *25*, 623.
(588) Arnone, A. *Nature* **1972**, *237*, 146.
(589) Haney, D. N.; Bunn, H. F. *Proc. Natl. Acad. Sci. U.S.A.* **1976**, *73*, 3534.
(590) Acharya, A. S.; Sussman, L. G.; Manning, J. M. *J. Biol. Chem.* **1983**, *258*, 2296.
(591) Manjula, B. N.; Roy, R. P.; Smith, P. K.; Acharya, A. S. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1994**, *22*, 747.
(592) Jones, R. T. *Methods Enzymol.* **1994**, *231*, 322.
(593) Hemoglobins, Part C. In *Methods in Enzymology*; Everse, J., Vandegriff, K. D., Winslow, R. M., Eds.; Academic Press: New York, 1994; Vol. 232.
(594) Baldwin, J. M. *J. Mol. Biol.* **1980**, *136*, 103.
(595) Manning, J. M. *Adv. Enzymol.* **1991**, *64*, 55.
(596) Yang, T.; Horejsh, D. R.; Mahan, K. J.; Zaluzac, E. J.; Watson, T. J.; Gage, D. A. *Anal. Biochem.* **1996**, *242*, 55.
(597) Xu, A. S. L.; Macdonald, J. M.; Labotka, R. J.; London, R. E. *Biochim. Biophys. Acta* **1999**, *1432*, 333.
(598) Xu, A. S. L.; Labotka, R. J.; London, R. E. *J. Biol. Chem.* **1999**, *274*, 26629.
(599) Feeney, R. E.; Blankenhorn, G.; Dixon, H. B. F. *Adv. Protein Chem.* **1975**, *29*, 135.
(600) Means, G. E.; Feeney, R. E. *Anal. Biochem.* **1995**, *224*, 1.
(601) Bunn, H. F.; Haney, D. N.; Gabbay, K. H.; Gallop, P. M. *Biochem. Biophys. Res. Commun.* **1975**, *67*, 103.
(602) Acharya, A. S.; Manning, J. M. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 3590.
(603) Flückiger, R.; Gallop, P. M. *Methods Enzymol.* **1984**, *106*, 77.
(604) Tack, B. F.; Dean, J.; Eilat, D.; Lorenz, P. E.; Schechter, A. N. *J. Biol. Chem.* **1980**, *255*, 8842.
(605) Stevens, V. J.; Fantl, W. J.; Newman, C. B.; Sims, R. V.; Cerami, A.; Peterson, C. M. *J. Clin. Invest.* **1981**, *67*, 361.
(606) San George, R. C.; Hoberman, H. D. *J. Biol. Chem.* **1986**, *261*, 6811.
(607) DiDonato, A.; Fantl, W. J.; Acharya, A. S.; Manning, J. M. *J. Biol. Chem.* **1983**, *258*, 11890.
(608) Saunders: S.; Hedlund, B. E. *Biochemistry* **1984**, *23*, 1457.
(609) ; Fantl, W. J.; Manning, L. R.; Ueno, H.; Di Donato, A.; Manning, J. M. *Biochemistry* **1987**, *26*, 5755.
(610) Manning, J. M. *Methods Enzymol.* **1994**, *231*, 225.
(611) Geoghegan, K. F.; Ybarra, D. M.; Feeney, R. E. *Biochemistry* **1979**, *18*, 5392.
(612) Acharya, A. S.; Sussman, L. G. *J. Biol. Chem.* **1983**, *258*, 13761.
(613) Acharya, A. S.; Manning, J. M. *J. Biol. Chem.* **1980**, *255*, 7218.
(614) Acharya, A. S.; Roy, R. P.; Dorai, B. *J. Protein Chem.* **1991**, *10*, 345.
(615) Nacharaju, P.; Acharya, A. S. *Biochemistry* **1992**, *31*, 12673.
(616) Trivelli, L. A.; Ranney, H. M.; Lai, H. T. *N. Engl. J. Med.* **1971**, *284*, 353.
(617) Bunn, H. F.; Gabbay, K. H.; Gallop, P. M. *Science* **1978**, *200*, 21.
(618) Shapiro, R.; McManus, M. J.; Zalut, C.; Bunn, H. F. *J. Biol. Chem.* **1980**, *255*, 3120.
(619) Bunn, H. F.; Higgins, P. J. *Science* **1981**, *213*, 222.
(620) Neer, E. H.; Konigsberg, W. *J. Biol. Chem.* **1968**, *243*, 1966.
(621) MacLeod, R. M.; Hill, R. J. *J. Biol. Chem.* **1970**, *245*, 4875.
(622) Bresciani, D. *Biochem. J.* **1977**, *163*, 393.
(623) Klotz, I. M.; Tam, J. W. O. *Proc. Natl. Acad. Sci. U.S.A.* **1973**, *70*, 1313.
(624) Bridges, K. R.; Schmidt, G. J.; Jensen, M.; Cerami, A.; Bunn, H. F. *J. Clin. Invest.* **1975**, *56*, 201.
(625) Shamsuddin, M.; Mason, R. G.; Ritchey, J. M.; Honig, G. R.; Klotz, I. M. *Proc. Natl. Acad. Sci. U.S.A.* **1974**, *71*, 4693.
(626) Tam, J. W. O. *Hemoglobin* **1978**, *2*, 101.
(627) Zaugg, R. H.; King, C.; Klotz, I. M. *Biochem. Biophys. Res. Commun.* **1975**, *64*, 1192.
(628) Walder, J. A.; Zaugg, R. H.; Iwaoka, R. S.; Watkin, W. G.; Klotz, I. M. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 5499.
(629) Walder, J. A.; Zaugg, R. H.; Walder, R. Y.; Steele, J. M.; Klotz, I. M. *Biochemistry* **1979**, *18*, 265.
(630) Walder, J. A.; Walder, R. Y.; Arnone, A. *J. Mol. Biol.* **1980**, *141*, 195.
(631) Pavlik, P. A.; Boyd, M. K.; Olsen, K. W. *Biopolymers* **1996**, *39*, 615.
(632) Huang, H.; Olsen, K. W. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1994**, *22*, 719.
(633) Dean, J.; Schlechter, A. N. *N. Engl. J. Med.* **1978**, *299*, Part I: 752; Part II: 804; Part III: 863.
(634) Zaugg, R. H.; Walder, J. A.; Walder, R. Y.; Steele, J. M.; Klotz, I. M. *J. Biol. Chem.* **1980**, *255*, 2816.
(635) Klotz, I. M.; Haney, D. N.; King, L. C. *Science* **1981**, *213*, 724.
(636) *Sickle Cell Disease and Thalassaemias: New Trends in Therapy*; Beuzard, Y., Lubin, B., Rosa, J., Eds.; INSERM/Libbey Eurotext Ltd.: Paris, 1995.

(637) Bucci, E.; Razynska, A.; Urbaitis, B.; Fronticelli, C. *J. Biol. Chem.* **1989**, *264*, 6191.
(638) Ueno, H.; Pospischil, M. A.; Manning, J. M.; Kluger, R. *Arch. Biochem. Biophys.* **1986**, *244*, 795.
(639) Kluger, R.; Grant, A. S.; Bearne, S. L.; Trachsel, M. R. *J. Org. Chem.* **1990**, *55*, 2864.
(640) Jones, R. T.; Head, C. G.; Fujita, T. S.; Shih, D.; Wodzinska, J.; Kluger, R. *Biochemistry* **1993**, *32*, 215.
(641) Ueno, H.; Pospischil, M. A.; Manning, J. M. *J. Biol. Chem.* **1989**, *264*, 12344.
(642) Ueno, H.; Manning, J. M. *J. Protein Chem.* **1992**, *11*, 177.
(643) Kluger, R.; Li, X. *Bioconjugate Chem.* **1997**, *8*, 921.
(644) Léonard, M.; Dellacherie, E. *Biochim. Biophys. Acta* **1984**, *791*, 219.
(645) Labrude, P.; Mouelle, P.; Menu, P.; Vigneron, C.; Dellacherie, E.; Leonard, M.; Tayot, J. L. *Int. J. Artif. Organs* **1988**, *11*, 393.
(646) Iwashita, Y.; Yabuki, A.; Yamaji, K.; Iwasaki, K.; Okami, T.; Hirata, C.; Kosaka, K. *Biomater., Artif. Cells, Artif. Organs* **1988**, *16*, 271.
(647) Iwasaki, K.; Iwashita, Y. *Artif. Organs* **1986**, *10*, 411.
(648) Nho, K.; Zalipsky, S.; Abuchowski, A.; Davis, F. F. In *Poly-(Ethylene Glycol) Chemistry: Biotechnical and Biomedical Applications*; Harris, J. M., Ed.; Plenum Press: New York, 1992; p 171.
(649) Cerami, A.; Manning, J. M. *Proc. Natl. Acad. Sci. U.S.A.* **1971**, *68*, 1180.
(650) Diederich, D. *Biochem. Biophys. Res. Commun.* **1972**, *46*, 1255.
(651) Kilmartin, J. V.; Fogg, J.; Luzzana, M.; Rossi-Bernardi, L. *J. Biol. Chem.* **1973**, *248*, 7039.
(652) Lee, C. K.; Manning, J. M. *J. Biol. Chem.* **1973**, *248*, 5861.
(653) Nigen, A. M.; Njikam, N.; Lee, C. K.; Manning, J. M. *J. Biol. Chem.* **1974**, *249*, 6611.
(654) Williams, R. C.; Chung, L. L.; Schuste, T. M. *Biochem. Biophys. Res. Commun.* **1975**, *62*, 118.
(655) O'Donnell, S.; Mandaro, R.; Schuster, T. M.; Arnone, A. *J. Biol. Chem.* **1979**, *254*, 12204.
(656) Zalipsky, S.; Seltzer, R.; Nho, K. *Polym. Prepr.* **1990**, *31*, 173.
(657) Currell, D. L.; Law, B.; Stevens, M.; Murata, P. *Biochem. Biophys. Res. Commun.* **1981**, *102*, 348.
(658) Currell, D. L. *Methods Enzymol.* **1994**, *231*, 281.
(659) Currell, D. L.; Nguyen, D. M.; Ng, S.; Hom, M. *Biochem. Biophys. Res. Commun.* **1982**, *106*, 1325.
(660) Bellelli, A.; Ippoliti, R.; Currell, D.; Condo, S. G.; Giardina, B.; Brunori, M. *Eur. J. Biochem.* **1986**, *161*, 329.
(661) Mok, W.; Chen, D.; Mazur, A. *Fed. Proc.* **1975**, *34*, 1458.
(662) Lubin, B. H.; Pena, V.; Mentzer, W. C.; Bymun, E.; Bradley, T. B.; Packer, L. *Proc. Natl. Acad. Sci. U.S.A.* **1975**, *72*, 43.
(663) Pennathur-Das, R.; Heath, R. H.; Mentzer, W. C.; Lubin, B. H. *Biochim. Biophys. Acta* **1982**, *704*, 389.
(664) Rao, M. J.; Acharya, A. S. *Methods Enzymol.* **1994**, *231*, 246.
(665) Seetharam, R.; Manning, J. M.; Acharya, A. S. *J. Biol. Chem.* **1983**, *258*, 14810.
(666) Acharya, A. S.; Sussman, L. G.; Seetharam, R. *J. Protein Chem.* **1985**, *4*, 215.
(667) Acharya, A. S.; Khandke, L. *J. Protein. Chem.* **1989**, *8*, 231.
(668) Riggs, A. *J. Biol. Sci.* **1961**, *236*, 1948.
(669) Acharya, A. S.; Upadhya, R.; Smith, P. K.; Manjula, B. N.; Friedman, J. M. VIIIth International Symposium on Blood Substitutes, San Diego, 2000.
(670) Taylor, J. F.; Antonini, E.; Brunori, M.; Wyman, J. *J. Biol. Chem.* **1966**, *241*, 241.
(671) Ringe, D.; Turesky, R. J.; Skipper, P. L.; Tannenbaum, S. R. *Chem. Res. Toxicol.* **1988**, *1*, 22.
(672) Bucci, E.; Fronticelli, C.; Chiancone, E.; Wyman, J.; Antonini, E.; Rossi-Fannelli, A. *J. Mol. Biol.* **1965**, *12*, 183.
(673) Garel, M. C.; Beuzard, Y.; Thillet, J.; Domenget, C.; Martin, J.; Galacteros, F.; Rosa, J. *Eur. J. Biochem.* **1982**, *123*, 513.
(674) Shibayama, N.; Imai, K.; Hirata, H.; Hiraiwa, H.; Morimoto, H.; Saigo, S. *Biochemistry* **1991**, *30*, 8158.
(675) Guidotti, G.; Königsberg, W. *J. Biol. Chem.* **1964**, *239*, 1474.
(676) Simon, S. R.; Konigsberg, W. H. *Proc. Natl. Acad. Sci. U.S.A.* **1966**, *56*, 749.
(677) Moffat, J. K. *J. Mol. Biol.* **1971**, *55*, 135.
(678) Moffat, J. K.; Simon, S. R.; Konigsberg, W. H. *J. Mol. Biol.* **1971**, *58*, 89.
(679) Arndt, D. J.; Simon, S. R.; Maita, T.; Konigsberg, W. *J. Biol. Chem.* **1971**, *246*, 2602.
(680) Arndt, D. J.; Konigsberg, W. *J. Biol. Chem.* **1971**, *246*, 2594.
(681) Zalipski, S.; Lee, C. In *Poly(Ethylene Glycol) Chemistry: Biotechnical and Biomedical Applications*; Harris, J. M., Ed.; Plenum Press: New York, 1992; p 347.
(682) Manjula, B. N.; Upadhya, R.; Nemkal, A.; Smith, P. K.; Vandegriff, K. D.; Winslow, R. M.; Friedman, J. M.; Acharya, A. S. VIIIth International Symposium on Blood Substitutes, San Diego, 2000.
(683) Hai, T. T.; Pereira, D. E.; Nelson, D. J.; Srnak, A.; Catarello, J. *Bioconjugates* **1999**, *10*, 1013.
(684) Fasold, H.; Meyer, C.; Steinkopff, G. *Eur. J. Biochem.* **1973**, *32*, 63.
(685) Hai, T. T.; Pereira, D. E.; Nelson, D. J.; Srnak, A. *Tetrahedron* **1999**, *55*, 2147.
(686) Jue, R.; Lambert, J. M.; Pierce, L. R.; Traut, R. R. *Biochemistry* **1978**, *17*, 5399.
(687) Ilan, E. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1994**, *22*, 677.
(688) Barnikol, W. K. R. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1994**, *22*, 725.
(689) Suslick, K. S.; Grinstaff, M. W.; Kolbeck, K. J.; Wong, M. *Ultrason. Sonochem.* **1994**, *1*, s65.
(690) Greenburg, A. G.; Schooley, M.; Peskin, G. W. *J. Trauma* **1977**, *17*, 501.
(691) Benesch, R. E.; Benesch, R.; Renthal, R. D.; Maeda, N. *Biochemistry* **1972**, *11*, 3576.
(692) Greenburg, A. G.; Schooley, M.; Ginsburg, K. A.; Peskin, G. W. *Surg. Forum* **1978**, *29*, 44.
(693) Benesch, R.; Benesch, R. E.; Kwong, S.; Acharya, A. S.; Manning, J. M. *J. Biol. Chem.* **1982**, *257*, 1320.
(694) DeVenuto, F.; Zegna, A. *J. Surg. Res.* **1983**, *34*, 205.
(695) McGarrity, M. J.; Er, S. S.; Hsia, J. C. *J. Chromatogr.* **1987**, *419*, 37.
(696) Menu, P.; Pauly, F.; Labrude, P.; Vigneron, C. *J. Chromatogr.* **1987**, *417*, 397.
(697) Marks, D. H.; Moore, G. L.; Medina, F.; Boswell, G.; Zieske, L. R.; Bolin, R. B.; Zegna, A. I. *Mil. Med.* **1988**, *153*, 44.
(698) Marini, M. A.; Moore, G. L.; Fishman, R. M.; Jessee, R.; Medina, F.; Snell, S. M.; Zegna, A. I. *Biopolymers* **1989**, *28*, 2071.
(699) Means, G. E.; Feeney, R. E. *J. Biol. Chem.* **1971**, *248*, 5532.
(700) Benesch, R.; Yung, S.; Suzuki, T.; Bauer, C.; Benesch, R. *Proc. Natl. Acad. Sci. U.S.A.* **1973**, *70*, 2595.
(701) Benesch, R.; Benesch, R. E. *Methods Enzymol.* **1981**, *76*, 147.
(702) McGarrity, M. J.; Er, S. S.; Nightingale, K. A.; Hsia, J. C. *J. Chromatogr.* **1987**, *413*, 53.
(703) Fishman, R. M.; Moore, G. L.; Zegna, A.; Marini, M. A. *J. Chromatogr.* **1990**, *532*, 55.
(704) Arnone, A.; Benesch, R. E.; Benesch, R. *J. Mol. Biol.* **1977**, *115*, 627.
(705) Pristoupil, T. I.; Kramlová, M.; Kraml, J.; Ulrych, S. *J. Chromatogr.* **1981**, *219*, 436.
(706) Pristoupil, T. I.; Kramlová, M.; Fortova, H.; Fricová, V.; Kadlecova, L. *J. Chromatogr.* **1984**, *288*, 469.
(707) Benesch, R.; Benesch, R. E.; Edalji, R.; Suzuki, T. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 1721.
(708) Wong, J. T. *Biomater., Artif. Cells, Artif. Organs* **1988**, *16*, 237.
(709) Xue, H.; Wu, X.; Wong, J. *Artif. Organs* **1992**, *16*, 427.
(710) Chatterjee, R.; Welty, E. V.; Walder, R. Y.; Pruitt, S. L.; Rogers, P. H.; Arnone, A.; Walder, J. A. *J. Biol. Chem.* **1986**, *261*, 9929.
(711) Brouwer, M.; Cashon, R.; Bonaventura, J. *Biomater., Artif. Cells, Immobilization Biotechnol.* **1992**, *20*, 323.
(712) Zygmunt, D.; Léonard, M.; Bonneaux, F.; Sacco, D.; Dellacherie, E. *Int. J. Biol. Macromol.* **1987**, *9*, 343.
(713) Dellacherie, E.; Grandgeorges, M.; Prouchayret, F.; Fasan, G. *Biomater., Artif. Cells, Immobilization Biotechnol.* **1992**, *20*, 309.
(714) Bonneaux, F.; Dellacherie, E.; Labrude, P.; Vigneron, C. *J. Protein Chem.* **1996**, *15*, 461.
(715) Caron, A.; Menu, P.; Faivre-Fiorina, B.; Labrude, P.; Vigneron, C. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1999**, *27*, 49.
(716) O'Kelly, T.; Brading, A.; Mortensen, N. *Gut* **1993**, *34*, 689.
(717) Beddell, C. R.; Goodford, P. J.; Stammers, D. K.; Wootton, R. *Br. J. Pharmacol.* **1979**, *65*, 535.
(718) Beddell, C. R.; Goodford, P. J.; Kneen, G.; White, R. D.; Wilkinson, S.; Wootton, R. *Br. J. Pharmacol.* **1984**, *82*, 397.
(719) Abraham, D. J.; Safo, M. D.; Boyiri, T.; Danso-Danquah, R. E.; Kister, J.; Poyart, C. *Biochemistry* **1995**, *34*, 15006.
(720) Boyiri, T.; Safo, M. K.; Danso-Danquah, R. E.; Kister, J.; Poyart, C.; Abraham, D. J. *Biochemistry* **1995**, *34*, 15021.
(721) Benesch, R.; Benesch, R. E.; Yung, S.; Edalji, R. *Biochem. Biophys. Res. Commun.* **1975**, *63*, 1123.
(722) Van der Plas, J.; de Vries-van Rossen, A.; Damm, J. B. L.; Bakker, J. C. *Transfusion* **1987**, *27*, 425.
(723) van der Plas, J.; Vries-van Rossen, A.; Koorevaar, J. J.; Buursma, A.; Sulstra, W. G.; Bakker, J. C. *Transfusion* **1988**, *28*, 525.
(724) Benesch, R. E. *Methods Enzymol.* **1994**, *231*, 267.
(725) Bleeker, W. K.; van der Plas, J.; Agterberg, J.; Rigter, G.; Bakker, J. C. *J. Lab. Clin. Med.* **1986**, *108*, 448.
(726) Keipert, P. E.; Triner, L. The Red Cell: Proceedings of the Seventh Ann Arbor Conference, Ann Arbor; Alan Liss: New York, 1989; p 383.
(727) Benesch, R. E.; Kwong, S. *Biochem. Biophys. Res. Commun.* **1988**, *156*, 9.
(728) Keipert, P. E.; Adeniran, A. J.; Kwong, S.; Benesch, R. E. *Transfusion* **1989**, *29*, 768.
(729) Scannon, P. J. *Crit. Care Med.* **1982**, *10*, 261.
(730) McGarrity, M. J.; Nightingale, K. A.; Hsia, J. C. *J. Chromatogr.* **1987**, *415*, 136.
(731) Manning, L. R.; Morgan, S.; Beavis, R. C.; Chait, B. T.; Manning, J. M.; Hess, J.; Cross, M.; Currell, D. L.; Marini, M. A.; Winslow, R. M. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 3329.

(732) Pötzschke, H.; Barnikol, K. R.; Kirste, R. G.; Rosenbaum, M. *Macromol. Chem. Phys.* **1996**, *197*, 1419.
(733) Kavanaugh, M. P.; Shih, D. T.-B.; Jones, R. T. *Biochemistry* **1988**, *27*, 7, 1804.
(734) Fuhrmann, G. F.; Kreutzfeldt, C.; Rudolphi, K.; Fasold, H. *Biochim. Biophys. Acta* **1988**, *846*, 25.
(735) Walder, J. A.; Chatterjee, R.; Arnone, A. *Fed. Proc.* **1982**, *41*, 651.
(736) Tye, R. W.; Medina, F.; Bolin, R. B.; Knopp, G. L.; Irion, G. S.; McLaughlin, S. K. *Prog. Clin. Biol. Res.* **1983**, *122*, 41.
(737) Snyder, S. R.; Welty, E. V.; Walder, R. Y.; Williams, L. A.; Walder, J. A. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 7280.
(738) Highsmith, F. A.; Driscoll, C. M.; Chung, B. C.; Chavez, M. D.; Macdonald, V. W.; Manning, J. M.; Lippert, L. E.; Berger, R. L.; Hess, J. R. *Biologicals* **1997**, *25*, 257.
(739) Nelson, D. In *Red Blood Cell Substitutes*; Rudolph, A. S., Rabinovici, R., Feuerstein, G. Z., Eds.; Dekker: New York, 1998; p 353.
(740) Urbaitis, B. K.; Razynska, A.; Corteza, Q.; Fronticelli, C.; Bucci, E. *J. Lab. Clin. Med.* **1991**, *117*, 115.
(741) Vandegriff, K. D.; Le Tellier, Y. C.; Winslow, R. M.; Rohlfs, R. J.; Olson, J. S. *J. Biol. Chem.* **1991**, *266*, 17049.
(742) MacDonald, V. W.; Winslow, R. M. *J. Appl. Physiol.* **1992**, *72*, 476.
(743) Vandegriff, K. D.; Benazzi, L.; Ripamont, M.; Perrella, M.; Le Tellier, Y. C.; Zegna, A.; Winslow, R. M. *J. Biol. Chem.* **1991**, *266*, 2697.
(744) Valeri, C. R.; Ichikura, T.; Pivacek, L. E.; Giorgio, A.; Prusty, S.; Dittmer, J. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **2000**, *28*, 451.
(745) Fronticelli, C.; Sato, T.; Orth, C.; Bucci, E. *Biochim. Biophys. Acta* **1986**, *874*, 76.
(746) Bucci, E.; Razynska, A.; Kwansa, H.; Matheson-Urbaitis, B.; O'Hearne, M.; Ulatowski, J. A.; Koehler, R. C. *J. Lab. Clin. Med.* **1996**, *128*, 146.
(747) Zhang, Q.; Olsen, K. W. *Biochem. Biophys. Res. Commun.* **1994**, *203*, 1463.
(748) Thomas, M.; Matheson-Urbaitis, B.; Kwansa, H.; Bucci, E.; Fronticelli, C. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1997**, *25*, 309.
(749) Peri, S. P.; Bhadti, V. S.; Hosmane, R. S.; Macdonald, V. W. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1997**, *25*, 511.
(750) Klotz, I. M.; Haney, D. N.; Wood, L. E. *J. Biol. Chem.* **1985**, *260*, 16215.
(751) Fronticelli, C.; Bucci, E.; Razynska, A.; Sznajder, J.; Urbaitis, B.; Gryczynski, Z. *Eur. J. Biochem.* **1990**, *193*, 331.
(752) Urbaitis, B.; Lu, Y. S.; Fronticelli, C.; Bucci, E. *Biochim. Biophys. Acta* **1992**, *1156*, 50.
(753) Osawa, Y.; Darbyshire, J. F.; Meyer, C. A.; Alayash, A. I. *Biochem. Pharmacol.* **1993**, *46*, 2299.
(754) Kluger, R.; Song, Y.; Wodzinska, J.; Head, C.; Fujita, T. S.; Jones, R. T. *J. Am. Chem. Soc.* **1992**, *114*, 9275.
(755) Bucci, E.; Fronticelli, C.; Razynska, A.; Militello, V.; Koehler, R.; Urbaitis, B. *Biomater., Artif. Cells, Immobilization Biotechnol.* **1992**, *20*, 243.
(756) Kluger, R.; Wodzinska, J.; Jones, R. T.; Head, C.; Fujita, T. S.; Shih, D. T. *Biochemistry* **1992**, *31*, 7551.
(757) Johnson, M. B.; Adamson, J. G.; Mauk, A. G. *Biophys. J.* **1998**, *75*, 3078.
(758) Schumacher, M. A.; Dixon, M. M.; Kluger, R.; Jones, R. T.; Brennan, R. G. *Nature* **1995**, *375*, 84.
(759) Zheng, Y.; Olsen, K. W. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1996**, *24*, 587.
(760) Kluger, R.; Lock-O'Brien, J.; Teytelboym, A. *J. Am. Chem. Soc.* **1999**, *121*, 6780.
(761) Paal, K.; Jones, R. T.; Kluger, R. *J. Am. Chem. Soc.* **1996**, *118*, 10380.
(762) Kluger, R.; Paal, K.; Adamson, J. G. *Can. J. Chem.* **1999**, *77*, 271.
(763) Bonsen, P.; Laver, M. B.; Morris, K. C.; Alza Corp. U.S. Pat. 4,001,401, 1977.
(764) Morris, K. C.; Bonsen, P.; Laver, M. B. U.S. Pat. 4,061,736, 1977.
(765) Hsia, J. C. U.S. Pat. 4,857,636, 1989.
(766) Payne, J. W. *Biochem. J.* **1973**, *135*, 867.
(767) Rozenberg, G. J. *Dokl. Akad. Nauk U.S.S.R.* **1978**, *243*, 1320.
(768) DeVenuto, F.; Zegna, A. I. *Transfusion* **1981**, *21*, 599.
(769) Guillochon, D.; Esclade, L.; Rémy, M. H.; Thomas, D. *Biochim. Biophys. Acta* **1981**, *670*, 332.
(770) Pristoupil, T. I.; Kramlová, M.; Fricová, V.; Ulrych, S.; Kraml, J.; Jojkova, K. *J. Chromatogr.* **1981**, *213*, 183.
(771) Kothe, N.; Eichentopf, B.; Bonhard, K. *Surg. Gynecol. Obstet.* **1985**, *161*, 563.
(772) Guillochon, D.; Esclade, L.; Thomas, D. *Biochem. Pharmacol.* **1986**, *35*, 317.
(773) Feola, M.; Simoni, J.; Canizaro, P. C.; Tran, R.; Raschbaum, G.; Behal, F. J. *Surg. Gynecol. Obstet.* **1988**, *166*, 211.
(774) Barnikol, W. K. R.; Burkhard, O. *Adv. Exp. Med. Biol.* **1989**, *248*, 335.
(775) Simoni, J.; Simoni, G.; Feola, M. *Anal. Chim. Acta* **1993**, *279*, 73.
(776) MacDonald, S. L.; Pepper, D. S. *Methods Enzymol.* **1994**, *231*, 287.
(777) Kuznetsova, N. P.; Gudkin, L. R.; Mishaeva, R. N.; Stragovich, L. M.; Bistrova, I. M.; Selivanov, E. A. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1998**, *26*, 213.
(778) Hopwood, D. *Histochem. J.* **1969**, *1*, 323.
(779) Korn, A. H.; Feairheller, S. H.; Filachione, E. M. *J. Mol. Biol.* **1972**, *65*, 525.
(780) Monsan, P.; Puzo, G.; Mazarguil, H. *Biochimie* **1975**, *57*, 1281.
(781) Peters, K.; Richards, F. M. *Annu. Rev. Biochem.* **1977**, *46*, 523.
(782) Tashima, T.; Imai, M.; Kuroda, Y.; Yagi, S.; Nakagawa, T. *J. Org. Chem.* **1991**, *56*, 694.
(783) Beauchamp, R. O.; St. Clair, M. B. G.; Fennell, T. R.; Clarke, D. O.; Morgan, K. T. *Crit. Rev. Toxicol.* **1992**, *22*, 143.
(784) Richards, F. M.; Knowles, J. R. *J. Mol. Biol.* **1968**, *37*, 231.
(785) Hardy, P. M.; Nicholls, A. C.; Rydon, H. N. *J. Chem. Soc., Perkin I Trans.* **1976**, 958.
(786) Lubig, R.; Kusch, P.; Röper, K.; Zahn, H. *Monatsh. Chem.* **1981**, *112*, 1313.
(787) Cheung, D. T.; Nimni, M. E. *Connect. Tissue Res.* **1982**, *10*, 187.
(788) Huang-Lee, L. L. H.; Cheung, D. T.; Nimni, M. E. *J. Biomed. Mater. Res.* **1990**, *24*, 1185.
(789) Nelson, D.; Hai, T.; Srnak, A.; Ebeling, A.; Kunas, G.; Catarello, J.; Burhop, K. *Biomater., Artif. Cells, Immobilization Biotechnol.* **1992**, *20*, 253.
(790) Fricová, V.; Pristoupil, I. I.; Kramlová, M.; Ulrych, S.; Marik, T. *Collect. Czech. Chem. Commun.* **1981**, *46*, 1990.
(791) Pietta, P.; Calatroni, A.; Palazzini, G.; Agostoni, A. *J. Chromatogr.* **1982**, *246*, 65.
(792) DeVenuto, F.; Zegna, A. *J. Surg. Res.* **1983**, *34*, 205.
(793) Doyle, M. P.; Apostol, I.; Kerwin, B. A. *J. Biol. Chem.* **1999**, *274*, 2583.
(794) Bleeker, W.; Agterberg, J.; La Hey, E.; Rigter, G.; Zappeij, L.; Bakker, E.; Bakker, J. In *Blood Substitutes—New Challenges*; Winslow, R. M., Vandegriff, K. D., Intaglietta, M., Eds.; Birkhäuser: Boston, 1996; p 112.
(795) Bakker, J. C.; Bleeker, W. K.; Hens, H. J. H.; Biessels, P. T. M.; van Iterson, M.; Trouwborst, A. In *Blood Substitutes. Present and Future Perspectives*; Tsuchida, E., Ed.; Elsevier: Amsterdam, 1998; p 225.
(796) DeVenuto, F.; Zegna, A. *Surg. Gynecol. Obstetr.* **1982**, *155*, 342.
(797) Fortova, H.; Suttnar, J.; Kramlova, M.; Pristoupil, T. I. *J. Chromatogr.* **1986**, *357*, 325.
(798) Clerc, Y.; Dubois, M.; Bihoreau, N.; Delamourd, L.; Brasseur, C.; Gond, B.; Goyffon, M.; Saint-Blanchard, J. *Appl. Biochem. Biotechnol.* **1987**, *14*, 241.
(799) Berbers, G. A. M.; Bleeker, W. K.; Stekkinger, P.; Agterberg, J.; Rigter, G.; Bakker, J. C. *J. Lab. Clin. Med.* **1991**, *117*, 157.
(800) Fronticelli, C.; Bucci, E. *Methods Enzymol.* **1994**, *231*, 150.
(801) Lee, R.; Neya, K.; Svizzero, T. A.; Vlahakes, G. J. *J. Appl. Physiol.* **1995**, *79*, 236.
(802) Manning, L. R.; Manning, J. M. *Biochemistry* **1988**, *27*, 6640.
(803) MacDonald, S. L.; Pepper, D. S. *Biomat., Artif. Cells., Immobilization Biotechnol.* **1991**, *19*, 424.
(804) Bu, F.; Wang, H.; Zhu, X. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **2000**, *28*, 493.
(805) Feola, M.; Simoni, J.; Angelillo, R.; Luhruma, Z.; Kabakele, M.; Manzombi, M.; Kaluila, M. *Surgery* **1992**, *174*, 379.
(806) Simoni, J.; Simoni, G.; Newman, G.; Feola, M. *Am. Soc. Artif. Intern. Organs J.* **1996**, *42*, M773.
(807) Haynes, J.; Obiako, B.; Thompson, W. J.; Downey, J. *Am. J. Physiol.* **1995**, *268*, H1862.
(808) Simoni, J.; Simoni, G.; Lox, C. D.; Prien, S. D.; Shires, G. T. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1997**, *25*, 193.
(809) Simoni, J.; Simoni, G.; Wesson, D. E.; Griswold, J. A.; Feola, M. VIIIth International Symposium on Blood Substitutes, San Diego, 2000.
(810) Hai, T. T.; Pereira, D. E.; Nelson, D. J. *Bioconjugate Chem.* **1998**, *9*, 645.
(811) Razynska, A.; Bucci, E. In *Blood Substitutes. Present and Future Perspectives*; Tsuchida, E., Ed.; Elsevier: Amsterdam, 1998; p 265.
(812) Pötzschke, H.; Guth, S.; Barnikol, W. K. R. *Adv. Exp. Med. Biol.* **1994**, *345*, 205.
(813) Pötzschke, H.; Barnikol, K. R.; Domack, U.; Kiste, R. G. *Macromol. Chem. Phys.* **1996**, *197*, 3229.
(814) Lamy, J. N.; Green, B. N.; Toulmond, A.; Wall, J. S.; Weber, R. E.; Vinogradov, S. N. *Chem. Rev.* **1996**, *96*, 3113.
(815) D'Agnillo, F.; Chang, T. M. S. *Biomater., Artif. Cells, Immobilization Biotechnol.* **1993**, *21*, 609.
(816) D'Agnillo, F.; Chang, T. M. S. *Nat. Biotechnol.* **1998**, *16*, 667.
(817) Chang, T. M. S.; D'Agnillo, F.; Yu, W. P.; Razack, S. *Adv. Drug Delivery Rev.* **2000**, *40*, 213.
(818) Quebec, E. A.; Chang, T. M. S. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1995**, *23*, 693.

(819) D'Agnillo, F.; Chang, T. M. S. *Free Radical Biol. Med.* **1998**, *24*, 906.
(820) Razack, S.; Agnillo, F. D.; Chang, T. M. S. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1997**, *25*, 181.
(821) Schneider, P. *Transfusion Sci.* **1992**, *13*, 357.
(822) Bonneaux, F.; Labrude, P.; Dellacherie, E. *Experientia* **1981**, *37*, 884.
(823) Dellacherie, E.; Bonneaux, F.; Labrude, P.; Vigneron, C. *Biochim. Biophys. Acta* **1983**, *749*, 106.
(824) Baldwin, J. E.; Gill, B.; Whitten, J. P.; Taegtmeyer, H. *Tetrahedron* **1984**, *39*, 1723.
(825) Pietta, P. G.; Pace, M.; Palazzini, G.; Agostini, A. *Prepr. Biochem.* **1984**, *14*, 313.
(826) Tam, S. C.; Blumenstein, J.; Wong, J. T. F. *Proc. Natl. Acad. Sci. U.S.A.* **1976**, *73*, 2128.
(827) Chang, J. E.; Wong, J. *Can. J. Biochem.* **1977**, *55*, 398.
(828) Xue, H.; Wong, J. T. *Methods Enzymol.* **1994**, *231*, 308.
(829) Tam, S.-C.; Blumenstein, J.; Wong, J. T.-F. *Can. J. Biochem.* **1978**, *56*, 981.
(830) Blumenstein, J.; Tam, S.-C.; Chang, J. E.; Wong, J. T.-F. *Prog. Clin. Biol. Res.* **1978**, *19*, 205.
(831) Tam, S.; Wong, J. *Can. J. Biochem.* **1980**, *58*, 732.
(832) Yarochkin, V. S.; Koziner, V. B.; Zeinalov, A. M.; Azihigirova, M. A. *Probl. Gematol. Pereliv. Krovi* **1980**, *25*, 29.
(833) Cerny, L. C.; Cerny, E. L.; Cerny, C. L.; Reath, M.; Liszczynskyj, M.; Gabel, S. *Clin. Hemorheol.* **1988**, *8*, 621.
(834) Cerny, L. C.; Stasiw, D. M.; Cerny, E. L.; Baldwin, J. E.; Gill, B. *Crit. Care Med.* **1983**, *11*, 739.
(835) Cerny, L. C.; Barnes, B.; Fisher, L.; Anibarro, M.; Ho, N.; Cerny, E. R. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1996**, *24*, 153.
(836) Sakai, H.; Yuasa, M.; Onuma, H.; Takeoka, S.; Tsuchida, E. *Bioconjugate Chem.* **2000**, *11*, 56.
(837) Iwasaki, K.; Ajisaka, K.; Iwashita, Y. *Biochem. Biophys. Res. Commun.* **1983**, *113*, 513.
(838) Sacco, D.; Klett-Zygmunt, D.; Vigneron, C.; Dellacherie, E. *Biochim. Biophys. Acta* **1990**, *1041*, 279.
(839) Sacco, S.; Bonneaux, F.; Dellacherie, E. *Int. J. Biol. Macromol.* **1988**, *10*, 305.
(840) Sacco, D.; Prouchayret, F.; Dellacherie, E. *Biomater., Artif. Cells, Immobilization Biotechnol.* **1992**, *20*, 331.
(841) Prouchayret, F.; Dellacherie, E. *Biopolymers* **1993**, *33*, 1803.
(842) Sacco, D.; Dellacherie, E.; Prouchayret, F. *J. Protein Chem.* **1994**, *13*, 1.
(843) Sacco, D.; Prouchayret, F.; Dellacherie, E. *Makromol. Chem.* **1989**, *190*, 1671.
(844) Menu, P.; Faivre, B.; Labrude, P.; Riffard, P.; Grandgeorge, M.; Vigneron, C. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1994**, *22*, 543.
(845) Caron, A.; Menu, P.; Labrude, P.; Vigneron, C. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1998**, *26*, 293.
(846) Menu, P.; Donner, M.; Faivre, B. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1995**, *23*, 319.
(847) Menu, P.; Longrois, D.; Faivre, B.; Donner, M.; Labrude, P.; Stolz, J.-F.; Vigneron, C. *Transfusion Sci.* **1999**, *20*, 5.
(848) Labrude, P.; Bonneaux, F.; Dellacherie, E.; Neel, J.; Vigneron, C. *Ann. Pharm. Fr.* **1979**, *37*, 291.
(849) Pristoupil, T. I.; Kramlová, M.; Fricová, V.; Kraml, J. *J. Chromatogr.* **1983**, *280*, 165.
(850) Tsuchida, E.; Ando, K.; Maejima, H.; Kawai, N.; Komatsu, T.; Takeoka, S.; Nishide, H. *Bioconjugate Chem.* **1997**, *8*, 534.
(851) Komatsu, T.; Tsuchida, E. *Polym. Prepr.* **1999**, *40*, 366.
(852) Wu, Y.; Komatsu, T.; Tsuchida, E. *Chem. Lett.* **2000**, 1194.
(853) Tsuchida, E. VIIIth International Symposium on Blood Substitutes, San Diego, 2000.
(854) Abuchowski, A.; van Es, T.; Palczuk, N. C.; Davis, F. F. *J. Biol. Chem.* **1977**, *252*, 3578.
(855) Nucci, M. L.; Shorr, R.; Abuchowski, A. *Adv. Drug Delivery Rev.* **1991**, *6*, 133.
(856) Papahadjopoulos, D.; Allen, T. M.; Garbizon, A.; Mayhew, E.; Matthay, K.; Huang, S. K.; Lee, K.-D.; Woodle, M. C.; Lasic, D. D.; Redemann, C.; Martin, F. J. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 11460.
(857) Katre, N. V. *Adv. Drug Delivery Rev.* **1993**, *10*, 91.
(858) Duncan, R.; Spreafico, F. *Clin. Pharmacokinet.* **1994**, *27*, 290.
(859) Zalipsky, S. *Adv. Drug Delivery Rev.* **1995**, *16*, 157. Zalipsky, S. *Bioconjugate Chem.* **1995**, *6*, 150.
(860) Lasic, D. D.; Needham, D. *Chem. Rev.* **1995**, *95*, 2601.
(861) *Long Circulating Liposomes*; Woodle, M. C., Storm, G., Eds.; Springer Verlag: Berlin, 1997; Torchilin, V. P. In *Advances in Blood Substitutes, Industrial Opportunities and Medical Challenges*; Winslow, R. M., Vandegriff, K. D., Intaglietta, M., Eds.; Birkhäuser: Boston, 1997; p 251.
(862) Allen, T. In *Liposomes, New Systems and New Trends in their Applications*; Puisieux, F., Couvreur, P., Delattre, J., Devissaguet, J.-P., Eds.; Editions de Santé: Paris, 1995; p 123.
(863) Schmidt, K. *Klin. Wochenschr.* **1979**, *57*, 1169.
(864) Léonard, M.; Néel, J.; Dellacherie, E. *Tetrahedron* **1984**, *40*, 1581.
(865) Dellacherie, E.; Léonard, M. *J. Protein Chem.* **1991**, *10*, 61.
(866) Greenwald, R. B.; Pendri, A.; Martinez, A.; Gilbert, C.; Bradley, P. *Bioconjugate Chem.* **1996**, *7*, 638.
(867) Vandegriff, K. D.; Lohman, J. M.; Tsai, A. G.; Intaglietta, M.; Drobin, D.; Kjellström, B. T.; Winslow, R. M. VIIIth International Symposium on Blood Substitutes, San Diego, 2000.
(868) Kjellström, B. T.; Drobin, D.; Intaglietta, M.; Lohman, J. M.; Vandegriff, K. D.; Winslow, R. M. VIIIth International Symposium on Blood Substitutes, San Diego, 2000.
(869) Hai, T. T.; Nelson, D.; Pereira, D.; Srnak, A. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1994**, *22*, 923.
(870) Rogers, M. S.; Ryan, B. B.; Cashon, R. E.; Alayash, A. I. *Biochim. Biophys. Acta* **1995**, *1248*, 135.
(871) Abassi, Z.; Kotob, S.; Pieruzzi, F.; Abouassali, M.; Keiser, H. R.; Fratantoni, J. C.; Alayash, A. I. *J. Lab. Clin. Med.* **1997**, *129*, 603.
(872) Cerny, L. C.; Cerny, E. L.; Liszczynskj, M.; Reath, M. *Biomater., Artif. Cells, Immobilization Biotechnol.* **1992**, *20*, 71.
(873) Nagai, K.; Thogersen, H. C. *Nature* **1984**, *309*, 810.
(874) Jessen, T. H.; Komiyama, H.; Tame, J.; Pagnier, J.; Shih, D.; Luisi, B.; Fermi, G.; Nagai, K. *Methods Enzymol.* **1994**, *231*, 347.
(875) Hoffman, S. J.; Looker, D. L.; Roehrich, J. M.; Dozart, P. E.; Durgee, S. L.; Tedesco, J. L.; Stetler, G. L. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 8521.
(876) Hernan, R. A.; Hui, H. L.; Andracki, M. E.; Noble, R. W.; Sligar, S. G.; Walder, J. A.; Walder, R. Y. *Biochemistry* **1992**, *31*, 8619.
(877) Kavanaugh, J. S.; Rogers, P. H.; Arnone, A. *Biochemistry* **1992**, *31*, 8640.
(878) Kroeger, K. S.; Kundrot, C. E. *Structure* **1997**, *5*, 227.
(879) Doyle, M. L.; Lew, G.; Young, A. D.; Kwiatkowski, L.; Wierzba, A.; Noble, R. W.; Ackers, G. K. *Biochemistry* **1992**, *31*, 8629.
(880) Hernan, R. A.; Sligar, S. G. *J. Biol. Chem.* **1995**, *270*, 24257.
(881) Sanders, K. E.; Ackers, G.; Sligar, S. *Curr. Opin. Struct. Biol.* **1996**, *6*, 534.
(882) Shen, T.; Ho, N. T.; Simplaceanu, V.; Green, B. N.; Tam, M. F.; Ho, C. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 8108.
(883) Shen, T.; Ho, N. T.; Zou, M.; Sun, D.; Cottam, P. F.; Simplaceanu, V.; Tam, M.; Bell, D. A.; Ho, C. *Protein Eng.* **1997**, *10*, 1085.
(884) Nagai, K.; Thogerson, H. C. *Methods Enzymol.* **1987**, *153*, 461.
(885) Baudin, V.; Pagnier, J.; Lacaze, N.; Bihoreau, M.; Kister, J.; Marden, M.; Kiger, L.; Poyart, C. *Biochim. Biophys. Acta* **1992**, *1159*, 223.
(886) Looker, D.; Mathews, A. J.; Neway, J. O.; Stetler, G. L. *Methods Enzymol.* **1994**, *231*, 364.
(887) Dumoulin, A.; Kiger, L.; Griffon, N.; Vasseur, C.; Kister, J.; Genin, P.; Marden, M. C.; Pagnier, J.; Poyart, C. *Protein Sci.* **1996**, *5*, 114.
(888) Weickert, M. J.; Curry, S. R. *Arch. Biochem. Biophys.* **1997**, *348*, 337.
(889) Wagenbach, M.; O'Rourke, K.; Vitez, L.; Wieczorek, A.; Hoffman, S.; Durfee, S.; Tedesco, J.; Stetler, G. *Bio/Technology* **1991**, *9*, 57.
(890) Ogden, J. E.; Harris, R.; Wilson, M. T. *Methods Enzymol.* **1994**, *231*, 374.
(891) Yanase, H.; Manning, L. R.; Vandegriff, K.; Winslow, R. M.; Manning, J. M. *Protein Sci.* **1995**, *4*, 21.
(892) Behringer, R. R.; Ryan, T. M.; Reilly, M. P.; Asakura, T.; Palmiter, R. D.; Brinster, R. L.; Townes, T. M. *Science* **1989**, *245*, 971.
(893) Hanscombe, O.; Vidal, M.; Kaeda, J.; Luzzatto, L.; Greaves, D. R.; Grosveld, F. *Genes Dev.* **1989**, *3*, 1572.
(894) Reilly, M. P.; McCune, S. L.; Ryan, T. M.; Townes, T. M.; Katsumata, M.; Asakura, T. *Methods Enzymol.* **1994**, *231*, 403.
(895) de Llano, J. J. M.; Schneewind, O.; Stetler, G.; Manning, J. M. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 918.
(896) Swanson, M. E.; Martin, M. J.; Donnell, J. K. O.; Hoover, K.; Lago, W.; Huntress, V.; Parsons, C. T.; Pinkert, C. A.; Pilder, S.; Logan, J. S. *Biotechnology* **1992**, *10*, 557.
(897) Dieryck, W.; Gruber, V.; Baudino, S.; Lenée, P.; Pagnier, J.; Mérot, B.; Poyart, C. *Transfusion Clin. Biol.* **1995**, *6*, 441.
(898) Adachi, K.; Konitzer, P.; Lai, C. H.; Kim, J.; Surrey, S. *Protein Eng.* **1992**, *5*, 807.
(899) Hofmann, O. M.; Mould, R. M.; Brittain, T. *Protein Eng.* **1994**, *7*, 281.
(900) Nagai, K.; Perutz, M. F.; Poyart, C. *Philos. Trans. R. Soc. London A* **1986**, *3*, 443.
(901) Fronticelli, C.; Gattoni, M.; Lu, A.-L.; Brinigar, W. S.; Bucci, J. L. G.; Chiancone, E. *Biophys. Chem.* **1994**, *51*, 53.
(902) Fronticelli, C.; Pechik, I.; Brinigar, W. S.; Kowalczyk, J.; Gilliland, G. L. *J. Biol. Chem.* **1994**, *269*, 23965.
(903) Olson, J. S. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1994**, *22*, 429.
(904) O'Donnell, J. K.; Birch, P.; Parsons, C. T.; White, S. P.; Okabe, J.; Martin, M. J.; Adams, C.; Sundarapandiyan, K.; Manjula, B. N.; Acharya, A. S.; Logan, J. S.; Kumar, R. *J. Biol. Chem.* **1994**, *269*, 27692.
(905) Kavanaugh, J. S.; Chafin, D. R.; Arnone, A.; Mozzarelli, A.; Rivetti, C.; Rossi, G. L.; Kwiatkowski, J. D.; Noble, R. W. *J. Mol. Biol.* **1995**, *248*, 136.

(906) Fronticelli, C.; Brinigar, W. S.; Olson, J. S.; Bucci, E.; Gryczynski, Z.; O'Donnell, J. K.; Kowalczyk, J. *Biochemistry* **1993**, *32*, 1235.
(907) Kim, H.; Shen, T.; Sun, D.; Ho, N.; Madrid, M.; Ho, C. *J. Mol. Biol.* **1995**, *248*, 867.
(908) Olson, J. S.; Eich, R. F.; Smith, L. P.; Warren, J. J.; Knowles, B. C. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1997**, *25*, 227.
(909) Springer, B. A.; Sligar, S. G.; Olson, J. S.; Phillips, G. N. *Chem. Rev.* **1994**, *94*, 699.
(910) Olson, J. S.; Phillips, G. N. *J. Biol. Chem.* **1996**, *271*, 17593.
(911) Baudin, V.; Kister, J.; Poyart, C.; Pagnier, J. *Transfusion Clin. Biol.* **1995**, *6*, 469.
(912) Kasper, S.; Walter, M.; Grune, F.; Bischoff, A.; Erasmi, H.; Buzello, W. *Anesth. Analg.* **1996**, *83*, 921.
(913) Weickert, M. J.; Pagratis, M.; Glascock, C. B.; Blackmore, R. *Appl. Environ. Microbiol.* **1999**, *65*, 640.
(914) Dumoulin, A.; Baudin, V.; Kiger, L.; Edelstein, S. F.; Marden, M.; Poyart, C.; Pagnier, J. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1994**, *22*, 733.
(915) Hartman, J. C.; Argoudelis, G.; Doherty, D.; Lemon, D.; Gorczynski, R. *Eur. J. Pharmacol.* **1998**, *363*, 175.
(916) Doyle, M. P.; Armstrong, A. M.; Brucker, E. A.; Fattor, T. J.; Lemon, D. D. VIIIth International Symposium on Blood Substitutes, San Diego, 2000.
(917) Vandegriff, K. D. In *Blood Substitutes. Physiological Basis of Efficacy*; Winslow, R. M., Vandegriff, K. D., Intaglietta, M., Eds.; Birkhäuser: Boston, 1995; p 105.
(918) Rabinovici, R.; Rudolph, A. S.; Vernick, J.; Feuerstein, G. *J. Trauma* **1993**, *35*, 121.
(919) Miller, I. F.; Mayoral, J.; Djordjevich, L.; Kashani, A. *Biomater., Artif. Cells, Artif. Organs* **1988**, *16*, 281.
(920) Rabinovici, R.; Rudolph, A. S.; Ligler, F. S.; Smith, E. F.; Feuerstein, G. *Circ. Shock* **1992**, *37*, 124.
(921) Phillips, W. T.; Lemen, L. D.; Goins, B.; Klipper, R.; Fresne, D.; Rudolph, A. S.; Martin, C.; Jerabek, A.; Emch, M. E.; Fox, P. T.; McMahan, C. A. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1996**, *24*, 403.
(922) Chang, T. M. S. *Science* **1964**, *146*, 524.
(923) Chang, T. M. S.; MacIntosh, F. C.; Mason, S. G. *Can. J. Physiol. Pharmacol.* **1966**, *44*, 115.
(924) Arakawa, M.; Kondo, T. *J. Pharm. Sci.* **1981**, *70*, 354.
(925) Djordjevich, L.; Miller, I. F. *Exp. Hematol.* **1980**, *8*, 584.
(926) Djordjevich, L.; Ivankovich, A. D. In *Liposomes as Drug Carriers*; Gregoriadis, G., Ed.; Wiley: New York, 1988; p 552.
(927) Djordjevich, L.; Mayoral, J.; Miller, I. F.; Ivankovich, A. D. *Crit. Care Med.* **1987**, *15*, 318.
(a) Doucet, D.; Soulard, C.; Vallez, M.-O.; Labrude, P.; Teisseire, B. *Artif. Organs* **1981**, *5(Suppl.)*, 392.
(928) Hunt, C. A.; Burnette, R. R.; MacGregor, R. D.; Strubbe, A. E.; Lau, D. T.; Taylor, N.; Kawada, H. *Science* **1985**, *230*, 1165.
(929) Bangham, A. D.; Standisch, M. M.; Watkins, J. C. *J. Mol. Biol.* **1965**, *13*, 238.
(930) *Medical Applications of Liposomes*; Lasic, D. D.; Papahadjopoulos, D., Eds.; Elsevier Science: Amsterdam, 1998; *Liposomes: Rational Design*; Janoff, A. S., Ed.; Dekker: New York, 1999.
(931) Storm, G.; Crommelin, D. J. A. *Pharm. Sci. Technol. Today* **1998**, *1*, 19; Barenholz, E. *Curr. Opin. Colloid Interface Sci.* **2001**, *6*, 66.
(932) Rabinovici, R.; Phillips, W. T.; Feuerstein, G. Z.; Rudolph, A. S. In *Red Blood Cell Substitutes*; Rudolph, A. S., Rabinovici, R., Feuerstein, G. Z., Eds.; Dekker: New York, 1998; p 263.
(933) Takahashi, A. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1995**, *23*, 347.
(934) Szebeni, J.; Hauser, H.; Eskelson, C. D.; Watson, R. R.; Winterhalter, K. H. *Biochemistry* **1988**, *27*, 6425.
(935) Gaber, B. P.; Chandrasekhar, I.; Pattiabiraman, N. *Biophys. J.* **1986**, *49*, 435a.
(936) Szebeni, J.; Toth, K. *Biochim. Biophys. Acta* **1986**, *857*, 139.
(937) Itabe, H.; Kobayashi, T.; Inoue, K. *Biochim. Biophys. Acta* **1988**, *961*, 13.
(938) LaBrake, C. C.; Fung, L. W.-M. *J. Biol. Chem.* **1992**, *267*, 16703.
(939) Yoshida, Y.; Kashiba, K.; Niki, E. *Biochim. Biophys. Acta* **1994**, *1201*, 165.
(940) Matsushita, Y.; Eshima, K.; Shindo, T.; Yamamoto, Y.; Hasegawa, E.; Nishide, H.; Tsuchida, E. *Biochim. Biophys. Acta* **1987**, *901*, 166.
(941) Phillips, W. T.; Rudolph, A. S.; Goins, B.; Klipper, R. *Biomater., Artif. Cells, Immobilization Biotechnol.* **1992**, *20*, 757.
(942) Rudolph, A. S.; Spielberg, H.; Spargo, B. J.; Kossovsky, N. *J. Biomed. Mater. Res.* **1995**, *29*, 189.
(943) Goins, B.; Klipper, R.; Sanders, J.; Rudolph, A. S.; Phillips, W. T. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1994**, *22*, 909.
(944) Rudolph, A. S.; Cliff, R. O.; Klipper, R.; Goins, B.; Phillips, W. T. *Crit. Care Med.* **1994**, *22*, 142.
(a) Langdale, L. A.; Maier, R. V.; Wilson, L.; Pohlman, T. H.; Williams, J. G.; Rice, C. L. *J. Leukocyte Biol.* **1992**, *52*, 679.
(945) Rudolph, A. S.; Cliff, R.; Kwasiborski, V.; Neville, L.; Abdullah, F.; Rabinovici, R. *Crit. Care Med.* **1997**, *25*, 460.
(946) Sherwood, R. L.; McCormick, D. L.; Zheng, S.; Beissinger, R. L. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1995**, *23*, 665.
(947) Poste, G. *Biol. Cell* **1983**, *47*, 19.
(948) Merion, R. M. *Transplantation* **1985**, *40*, 86.
(949) Allen, T. M.; Murray, L.; Alving, C. R.; Moe, J. *Can. J. Physiol.* **1987**, *65*, 185.
(950) Liu, D. *Adv. Drug Delivery Rev.* **1997**, *24*, 201.
(951) Szebeni, J.; Wassef, N. M.; Spielberg, H.; Rudolph, A. S.; Alving, C. R. *Biochem. Biophys. Res. Commun.* **1994**, *205*, 255.
(952) Szebeni, J.; Alving, C. R. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1999**, *27*, 23.
(953) Rabinovici, R.; Rudolph, A. S.; Yue, T.; Feuerstein, G. *Circ. Shock* **1990**, *31*, 431.
(954) Reinish, L. W.; Bally, M. B.; Loughrey, H. C.; Cullis, P. R. *Thromb. Haemost.* **1988**, *60*, 518.
(955) Cliff, R. O.; Kwasiborski, V.; Rudolph, A. S. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1995**, *23*, 331.
(956) Cevc, G. *Phospholipids Handbook*; M. Dekker: New York, 1993.
(957) Marsh, D. *CRC Handbook of Lipid Bilayers*; CRC Press: Boca Raton, 1987.
(958) Grit, M.; Zuidam, N. J.; Crommelin, D. J. A. In *Liposome Technology*, 2nd ed.; Gregoriadis, G., Ed.; CRC Press: Boca Raton, 1993; Vol. 1, p 455.
(959) Grit, M.; Underberg, W. J. M.; Crommelin, D. J. A. *J. Pharm. Sci.* **1993**, *82*, 362.
(960) Teelmann, K.; Schläppi, B.; Schüpbach, M.; Kistler, A. *Arzneim-Forsch./Drug Res.* **1984**, *34*, 1517.
(961) Nielloud, F.; Marti-Mestres, G. *Pharmaceutical Emulsions and Suspensions*; Marcel Dekker: New York, 2000.
(962) Rabinovici, R.; Rudolph, A. S.; Feuerstein, G. *Circ. Shock* **1990**, *30*, 207.
(963) Beissinger, R. L.; Farmer, M. C.; Gossage, J. L. *Trans. Am. Soc. Artif. Intern. Organs* **1986**, *32*, 58.
(964) Farmer, M. C.; Gaber, B. P. *Methods Enzymol.* **1987**, *149*, 184.
(965) Brandl, M.; Becker, D.; Bauer, K. H. *Drug Dev. Ind. Pharm.* **1989**, *15*, 655.
(966) Zheng, S.; Zheng, Y.; Beissinger, R. L.; Fresco, R. *Biochim. Biophys. Acta* **1994**, *1196*, 123.
(967) Huang, Y.-Y.; Chung, T.-W.; Wu, C.-I. *Int. J. Pharm.* **1998**, *172*, 161.
(968) Phillips, W. T.; Lemen, L.; Goins, B.; Rudolph, A. S.; Klipper, R.; Fresne, D.; Jerabek, P. A.; Emch, M. E.; Fox, P. T.; McMahan, C. A. *Am. J. Physiol.* **1997**, *272*, H2492.
(969) Takeoka, S.; Sakai, H.; Kose, T.; Mano, Y.; Seino, Y.; Nishide, H.; Tsuchida, E. *Bioconjugate Chem.* **1997**, *8*, 539.
(970) Takeoka, S.; Sakai, H.; Kobayashi, K.; Tsuchida, E. In *Blood Substitutes. Present and Future Perspectives*; Tsuchida, E., Ed.; Elsevier: Amsterdam, 1998; p 171.
(971) Sakai, H.; Tomiyama, K.; Sou, K.; Takeoka, S.; Tsuchida, E. *Bioconjugate Chem.* **2000**, *11*, 425.
(972) Ogata, Y.; Goto, H.; Kimura, T.; Fukui, H. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1997**, *25*, 417.
(973) Papahadjopoulos, D.; Cowden, M.; Kimelberg, H. *Biochim. Biophys. Acta* **1973**, *330*, 8.
(974) Szebeni, J.; Hauser, H.; Eskelson, C. D.; Winterhalter, K. H. *Biomater., Artif. Cells, Artif. Organs* **1988**, *16*, 301.
(975) Bruckdorfer, K. P.; Demel, R. A.; De Gier, J.; Van Deenen, L. L. M. *Biochim. Biophys. Acta* **1969**, *183*, 334.
(976) Yoshioka, H. *Biomaterials* **1991**, *12*, 861.
(977) Doucet, D.; Soulard, C.; Vallez, M.-O.; LaBrude, P.; Teisseire, B. *Artif. Organs* **1981**, *5* (*Suppl.*), 392.
(978) Cullen, A. B.; Cox, C. A.; Hipp, S. J.; Wolfson, M. R.; Shaffer, T. H. *Respir. Med.* **1999**, *93*, 770.
(979) Sakai, H.; Hamada, K.; Takeoka, S.; Nishide, H.; Tsuchida, E. *Biotechnol. Prog.* **1996**, *12*, 119.
(980) Farmer, M. C.; Rudolph, A. S.; Vandegriff, K. D.; Hayre, M. D.; Bayne, S. A.; Johnson, S. A. *Biomater., Artif. Cells, Artif. Organs* **1988**, *16*, 289.
(981) Sakai, H.; Takeoka, S.; Park, S.; Kose, T.; Nishide, H.; Izumi, Y.; Yoshizu, A.; Kobayashi, K.; Tsuchida, E. *Bioconjugate Chem.* **1997**, *8*, 23.
(982) Jopski, B.; Pirkl, V.; Jaroni, H.-W.; Schubert, R.; Schmidt, K.-H. *Biochim. Biophys. Acta* **1989**, *978*, 79.
(983) Mobed, M.; Nishiya, T.; Chang, T. M. S. *Biomater., Artif. Cells, Immobilization Biotechnol.* **1992**, *20*, 53.
(984) Deshpande, S. V.; Beissinger, R. L. *Biomater., Artif. Cells, Immobilization Biotechnol.* **1993**, *21*, 135.
(985) Takeoka, S.; Ohgushi, T.; Terase, K.; Ohmori, T.; Tsuchida, E. *Langmuir* **1996**, *12*, 1755.
(986) Illum, L.; Farrah, N.; Critchley, H.; Davis, S. S. *Int. J. Pharm.* **1988**, *46*, 251.
(987) Phillips, W. T.; Klipper, R.; Fresne, D.; Rudolph, A. S.; Javors, M.; Goins, B. *Exp. Hematol.* **1997**, *25*, 1347.
(988) Park, S.; Kose, T.; Hamasaki, M.; Takeoka, S.; Nishide, H.; Tsuchida, E. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1998**, *26*, 497.
(989) Rudolph, A. S. *Cryobiology* **1988**, *25*, 277.

(990) Cliff, R. O.; Ligler, F.; Goins, B.; Hoffmann, P. M.; Spielberg, H.; Rudolph, A. S. *Biomater., Artif. Cells, Immobilization Biotechnol.* **1992**, *20*, 619.
(991) Wang, L.; Takeoka, S.; Tsuchida, E.; Tokuyama, S.; Mashiko, T.; Satoh, T. *Polym. Adv. Technol.* **1992**, *3*, 17.
(992) Crowe, J. H.; Crowe, L. M.; Carpenter, J. F.; Rudolph, A. S.; Wistrom, C. A.; Spargo, B. J.; Anchordoguy, T. J. *Biochim. Biophys. Acta* **1988**, *947*, 367.
(993) Rabinovici, R.; Rudolph, A. S.; Vernick, J.; Feuerstein, G. *Crit. Care Med.* **1994**, *22*, 480.
(994) Satoh, T.; Kobayashi, K.; Sekiguchi, S.; Tsuchida, E. *Am. Soc. Artif. Intern. Organs J.* **1992**, *38*, M580.
(995) Hosoi, F.; Omichi, H.; Akama, K.; Awai, K.; Endo, S.; Nakano, Y. *Nucl. Instrum. Methods Phys. Res., Sect. B* **1997**, *131*, 329.
(996) Akama, K.; Gong, W.-L.; Wang, L.; Tokuyama, S.; Tsuchida, E. *Polym. Adv. Technol.* **1999**, *10*, 293.
(997) Goins, B.; Ligler, F. S.; Rudolph, A. S. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1994**, *22*, 9.
(998) Zheng, S.; Zheng, Y.; Beissinger, R. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1994**, *22*, 487.
(999) Phillips, W. T.; Klipper, R. W.; Awasthi, V. D.; Rudolph, A. S.; Cliff, R.; Kwasiborski, V.; Goins, B. A. *J. Pharmacol. Exp. Ther.* **1999**, *288*, 665.
(1000) Takeoka, S.; Mori, K.; Ohkawa, H.; Sou, K.; Eishun, T. *J. Am. Chem. Soc.* **2000**, *122*, 7927.
(1001) Takeoka, S.; Sakai, H.; Takisada, M.; Tsuchida, E. *Chem. Lett.* **1992**, 1877.
(1002) Kato, A.; Arakawa, M.; Kondo, T. *J. Microencapsulation* **1984**, *1*, 105; Kato, A.; Tanaka, I.; Arakawa, M.; Kondo, T. *Biomater., Med. Dev., Artif. Organs* **1985**, *13*, 61.
(1003) Sou, K.; Endo, T.; Takeoka, S.; Tsuchida, E. *Bioconjugate Chem.* **2000**, *11*, 372.
(1004) Vandegriff, K. D.; Wallach, D. F. H.; Winslow, R. M. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1994**, *22*, 849.
(1005) Wallach, D. F. H.; Philippot, J. R. In *Liposome Technology*, 2nd ed.; Gregoriadis, G., Ed.; CRC Press: Boca Raton, 1993; p 141.
(1006) Rohlfs, R. J.; Vandegriff, K. D. In *Blood Substitutes—New Challenges*; Winslow, R. M., Vandegriff, K. D., Intaglietta, M., Eds.; Birkhäuser: Boston, 1996; p 163.
(1007) Gibbs, W. W. *Sci. Am.* **1996**, *Sept.*, 44.
(1008) Chang, T. M. S. *Methods Enzymol.* **1985**, *112*, 195.
(1009) Lévy, M.-C.; Rambourg, P.; Lévy, J.; Potron, G. *J. Pharm. Sci.* **1982**, *71*, 759.
(1010) Davis, T. A.; Asher, W. J.; Wallace, H. W. *Trans. Am. Soc. Artif. Intern. Organs* **1982**, *28*, 404.
(1011) Wong, M.; Suslick, K. S. *Mater. Res. Soc. Symp. Proc.* **1995**, *372*, 89.
(1012) Cedrati, N.; Maincent, P.; Thomas, F.; Labrude, P.; Vigneron, C. *Artif. Cells, Blood. Substitutes, Immobilization Biotechnol.* **1994**, *22*, 867.
(1013) Cedrati, N.; Bonneaux, F.; Labrude, P.; Maincent, P. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1997**, *25*, 457.
(1014) Yu, W. P.; Chang, T. M. S. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1996**, *24*, 169.
(1015) Ecanow, J.; Ecanow, D.; Ecanow, B. *Biomater., Artif. Cells, Artif. Organs* **1990**, *18*, 359.
(1016) Hagen, S. J.; Hofrichter, H. J.; Bunn, H. F.; Eaton, W. A. *Transfusion Clin. Biol.* **1995**, *6*, 423.
(1017) Potts, M. *Microbiol. Rev.* **1994**, *58*, 755.
(1018) Kossovsky, N.; Gelman, A.; Sponsler, E. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1994**, *22*, 479.
(1019) Samain, D.; Leclerc, L.; Bonnafoux, M.; Moulai-Ali, S.; Kister, J.; Poyart, C. *Transfusion Clin. Biol.* **1995**, *6*, 449.
(1020) Habler, O. P.; Messmer, K. F. *Adv. Drug Delivery Rev.* **2000**, *40*, 171.
(1021) Przybelski, R. J.; Malcolm, D. S.; Burris, D. G.; Winslow, R. M. *J. Lab. Clin. Med.* **1991**, *117*, 143.
(1022) Malcolm, D.; Kissinger, D.; Garrioch, M. *Biomater., Artif. Cells, Immobilization Biotechnol.* **1992**, *20*, 495.
(1023) Schultz, S. C.; Hamilton, I. N.; Malcolm, D. S. *J. Trauma* **1993**, *35*, 619.
(1024) Schultz, S. C.; Powell, C. C.; Burris, D. G.; Nguyen, H.; Jaffin, J.; Malcolm, D. S. *J. Trauma.* **1994**, *37*, 408.
(1025) Powell, C. C.; Schultz, S. C.; Burris, D. G.; Drucker, W. R.; Malcolm, D. S. *Crit. Care Med.* **1995**, *23*, 867.
(1026) Frankel, H. L.; Nguyen, H. B.; Shea-Donohue, T.; Aiton, L. A.; Ratigan, J.; Malcolm, D. S. *J. Trauma* **1996**, *40*, 231.
(1027) Poli de Figueiredo, L. F.; Elgio, G. I.; Mathru, M.; Rocha e Silva, M.; Kramer, G. C. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1997**, *25*, 61.
(1028) Gulati, A.; Sen, A. P.; Sharma, A. C.; Singh, G. *Am. J. Physiol.* **1997**, *273*, H827.
(1029) Nolte, D.; Botzlar, A.; Pickelmann, S.; Bouskela, E.; Messmer, K. *J. Lab. Clin. Med.* **1997**, *130*, 314.
(1030) Gulati, A.; Sen, A. P. *Shock* **1998**, *9*, 65.
(1031) Gulati, A. In *Tissue Oxygenation in Acute Medicine*; Sibbald, W. J., Messmer, K., Fink, M. P., Eds.; Springer: Berlin, 1998; p 307.
(1032) Habler, O.; Messmer, K. In *Tissue Oxygenation in Acute Medicine*; Sibbald, W. J., Messmer, K., Fink, M. P., Eds.; Springer-Verlag: Berlin, 1998; p 291.
(1033) Habler, O.; Kleen, M.; Pape, A.; Meisner, F.; Kemming, G.; Messmer, K. *Crit. Care Med.* **2000**, *28*, 1889.
(1034) DeAngeles, D. A.; Scott, A. M.; McGrath, A. M.; Korent, V. A.; Rodenkirch, L. A.; Conhaim, R. L.; Harms, B. A. *J. Trauma* **1997**, *42*, 406.
(1035) Keipert, P. E.; Chang, T. M. S. *Biomater., Med. Dev., Artif. Organs* **1985**, *13*, 1.
(1036) Dawidson, I.; Drukker, S.; Hedlund, B.; Marks, D. H.; Reisch, J. *Crit. Care Med.* **1988**, *16*, 606.
(1037) Chang, T. M. S.; Varma, R. *Biomater., Artif. Cells, Immobilization Biotechnol.* **1992**, *20*, 503.
(1038) Siegel, J. H.; Fabian, M.; Smith, J. A.; Costantino, D. *J. Trauma: Injury, Infect., Crit. Care* **1997**, *42*, 199.
(1039) DeVenuto, F.; Moores, W. Y.; Zegna, A. I.; Zuck, T. F. *Transfusion* **1977**, *17*, 555.
(1040) Keipert, P. E.; Chang, T. M. S. *Vox Sang.* **1987**, *53*, 7.
(1041) Waschke, K.; Schrock, H.; Albrecht, D.; van Ackern, K. *Am. J. Physiol.* **1993**, *265*, H1243.
(1042) Slanetz, P. J.; Lee, R.; Page, R.; Jacobs, E. E.; LaRaia, P. J.; Vlahakes, G. J. *Surgery* **1994**, *115*, 246.
(1043) Standl, T.; Horn, P.; Wilhelm, S.; Greim, C.; Freitag, M.; Sputtek, A.; Jacobs, E.; Schulte am Esch, J. *Can. J. Anaesth.* **1996**, *43*, 714.
(1044) Tsai, A. G.; Kerger, H.; Intaglietta, M. In *Red Blood Cell Substitutes*; Rudolph, A. S., Rabinovici, R., Feuerstein, G. Z., Eds.; Dekker: New York, 1998; p 69.
(1045) Migita, R.; Gonzales, A.; Gonzales, M. L.; Vandegriff, K. D.; Winslow, R. M. *J. Appl. Physiol.* **1997**, *82*, 1995.
(1046) Ulatowski, J. A.; Bucci, E.; Nishikawa, T.; Razynska, A.; Williams, M. A.; Takeshima, R.; Traystman, R. J.; Koehler, R. C. *Heart Circ. Physiol.* **1996**, *39*, H466.
(1047) Kilbourne, R. G.; Joly, G.; Cashon, B.; DeAngelo, J.; Bonaventura, J. *Biochem. Biophys. Res. Commun.* **1994**, *199*, 155.
(1048) Heneka, M. T.; Löschmann, P. A.; Osswald, H. *J. Clin. Invest.* **1997**, *99*, 47.
(1049) Cole, D. J.; Drummond, J. C.; Patel, P. M.; Nary, J. C.; Applegate, R. L. *Anesth. Analg.* **1996**, *83*, 342.
(1050) Cole, D. J.; Nary, J. C.; Drummond, J. C.; Patel, P. M.; Jacobsen, W. K. *Artif. Cells, Blood Subst. Immobilization Biotechnol.* **1997**, *25*, 141.
(1051) Gonzalez, P.; Hackney, A. C.; Jones, S.; Strayhorn, D.; Hoffman, E. B.; Hughes, G.; Jacobs, E. E.; Orringer, E. P. *J. Invest. Med.* **1997**, *45*, 258.
(1052) Mullon, J.; Giacoppe, G.; Clagett, C.; McCune, D.; Dillard, T. *N. Engl. J. Med.* **2000**, *342*, 1638.
(1053) Freilich, D.; Branda, R.; Hacker, M.; Leach, L.; Barry, B.; Ferris, S.; Hebert, J. *Am. J. Trop. Med. Hyg.* **1999**, *60*, 322.
(1054) Xu, L.; Sun, L.; Rollwagen, F. M.; Li, Y.; Pacheco, N. D.; Pikoulis, E.; Leppäniemi, A.; Soltero, R.; Burris, D.; Malcolm, D.; Nielsen, T. B. *J. Trauma: Inj., Infect., Crit. Care* **1997**, *42*, 32.
(1055) Soltero, R. G.; Hansbrough, J. F. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1999**, *27*, 135.
(a) Willinger, C. C.; Schramck, H.; Pfaller, K.; Joannidis, M.; Deetjen, P.; Pfaller, W. *Renal Physiol. Biochem.* **1995**, *18*, 288.
(1056) Saxena, R.; Wijnhoud, A. D.; Carton, H.; Hacke, W.; Kaste, M.; Przybelski, R. J.; Stern, K. N.; Koudstaal, P. J. *Stroke* **1999**, *30*, 993.
(1057) McKenzie, J. E.; Cost, E. A.; Scandling, D. M.; Ahle, N. W.; Savage, R. W. *Cardiovasc. Res.* **1994**, *28*, 1188.
(1058) Swan, S. K.; Halstenson, C. E.; Collins, A. J.; Colburn, W. A.; Blue, J.; Przybelski, R. J. *Am. J. Kidney Dis.* **1995**, *26*, 918.
(1059) Robinson, M. F.; Dupuis, N. P.; Kusumoto, T.; Liu, F.; Menon, K.; Teicher, B. A. *Artif. Cells., Blood Substitutes, Immobilization Biotechnol.* **1995**, *23*, 431.
(1060) Nozue, M.; Lee, I.; Manning, J. M.; Manning, L. R.; Jain, R. K. *J. Surg. Oncol.* **1996**, *62*, 109.
(1061) Teicher, B. A.; Ara, G.; Herbst, R.; Takeuchi, H.; Keyes, S.; Northey, D. *In Vivo* **1997**, *11*, 301.
(1062) Linberg, R.; Conover, C. D.; Shum, K. L.; Shorr, R. G. L. *In Vivo* **1998**, *12*, 167.
(1063) Hughes, G. S.; Francom, S. F.; Antal, E. J.; Adams, W. J.; Locker, P. K.; Yancey, E. P.; Jacobs, E. E. *J. Lab. Clin. Med.* **1995**, *126*, 444.
(1064) Fowler, D. A.; Rosenthal, G. J.; Sommadossi, J. P. *Toxicol. Lett.* **1996**, *85*, 55.
(1065) Trimble, S. P.; Marquardt, D.; Anderson, D. C. *Bioconjugate Chem.* **1997**, *8*, 416.
(1066) Dive, D.; Piot, J. M.; Sannier, F.; Guillochon, D.; Charet, P.; Lutrat, S. *Enzyme Microb. Technol.* **1989**, *11*, 165.
(1067) Russell, J.; Okayama, N.; Alexander, J. S.; Granger, D.; Hsia, C. J. C. *Free Radical Biol. Med.* **1998**, *25*, 153.
(1068) Okayama, N.; Park, J. H.; Coe, L.; Granger, D. N.; Ma, L. I.; Hsia, C. J. C.; Alexander, J. S. *Free Radical Res.* **1999**, *31*, 53.
(1069) Hsia, C. J. C. VIIIth International Symposium on Blood Substitutes, San Diego, 2000.

(1070) Privalle, C.; Talarico, T.; Keng, T.; DeAngelo, J. *Free Radical Biol. Med.* **2000**, *28*, 1507.
(1071) Suzuki, T.; Benesch, R. E.; Benesch, R. *Biochim. Biophys. Acta* **1974**, *351*, 442.
(a) Hess, J. R.; Fadare, S. O.; Tolentino, L. S. L.; Bangal, N. R.; Winslow, R. M. In *The Red Cell: Seventh Ann Harbor Conference*; Brewer, G., Ed.; Alan Liss: New York, 1989; p 351.
(1072) Keipert, P. E.; Gomez, C. L.; Gonzales, A.; Macdonald, V. W.; Winslow, R. M. *Biomater., Artif. Cells, Immobilization Biotechnol.* **1992**, *20*, 737.
(1073) Viele, M. K.; Weiskopf, R. B.; Fisher, D. *Anesthesiology* **1997**, *86*, 848.
(1074) Adamson, J. G.; Moore, C. In *Blood Substitutes: Principles, Methods, Products and Clinical Trials*; Chang, T. M. S., Ed.; Karger Landes: Basel, 1998; Vol. 2, p 62.
(a) Carmichael, F. J. L.; Ali, A. C. Y.; Campbell, J. A.; Langlois, S. F.; Biro, G. P.; William, A. R.; Pierce, C. H.; Greenburg, A. G. *Crit. Care Med.* **2000**, *28*, 2283.
(1075) Hess, J. R.; Wade, C. E.; Winslow, R. M. *J. Appl. Physiol.* **1991**, *70*, 1639.
(1076) Baldwin, A. L. *Am. J. Physiol.* **1999**, *277*, H560.
(1077) Goldfischer, S.; Novikoff, A. B.; Albala, A.; Biempica, L. *J. Cell Biol.* **1970**, *44*, 513.
(1078) Keipert, P. E.; Verosky, M.; Triner, L. *Trans. Am. Soc. Artif. Intern. Organs* **1989**, *35*, 153.
(1079) Conover, C. D.; Gilbert, C. W.; Shum, K. L.; Shorr, R. G. L. *Artif. Organs* **1997**, *21*, 907.
(1080) Takahashi, T.; Iwasaki, K.; Malchesky, P. S.; Harasaki, H.; Emoto, H.; Goldcamp, J. B.; Matsushita, M.; Nosé, Y.; Rolin, H.; Hall, P. *Artif. Organs* **1991**, *15*, 462.
(1081) Sprung, J.; MacKenzie, C. F.; Barnas, G. M.; Williams, J. E.; Parr, M.; Christenson, R. H.; Hoff, B. H.; Sakamoto, R.; Kramer, A.; Lottes, M. *Crit. Care Med.* **1995**, *23*, 1540.
(1082) Brantley, R. E.; Smerdon, S. J.; Wilkinson, A. J.; Singleton, E. W.; Olson, J. S. *J. Biol. Chem.* **1993**, *268*, 6995.
(1083) Yang, T.; Olsen, K. W. *Biochem. Biophys. Res. Commun.* **1989**, *163*, 733.
(1084) Alayash, A. I. *Free Radical Biol. Med.* **1995**, *18*, 295.
(a) Alayash, A. I.; Summers, A. G.; Wood, F.; Jia, Y. *Arch. Biochem. Biophys.* **2001**, *391*, 225.
(1085) Vandegriff, K. D. *Biotechnol. Genet. Eng. Rev.* **1992**, *10*, 403.
(1086) Barnikol, W. K. R. *Adv. Exp. Med. Biol.* **1994**, *261*, 363.
(1087) Talarico, T.; Swank, A.; Privalle, C. *Biochem. Biophys. Res. Commun.* **1998**, *250*, 354.
(1088) Alayash, A. I.; Fratantoni, J. C.; Bonaventura, C.; Bonaventura, J.; Cashon, R. E. *Arch. Biochem. Biophys.* **1993**, *303*, 332.
(1089) Abugo, O. O.; Balagopalakrishna, C.; Rifkind, J. M.; Rudolph, A. S.; Hess, J. R.; Macdonald, V. W. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **2001**, *29*, 5.
(1090) Smith, C. D.; Schuschereba, S. T.; Hess, J. R.; McKinney, L.; Bunch, D.; Bowman, P. D. *Biomater., Artif. Cells, Artif. Organs* **1990**, *18*, 251.
(1091) Goldman, D. W.; Breyer, R. J.; Yeh, D.; Brockner-Ryan, B. A.; Alayash, A. I. *Am. J. Physiol.* **1998**, *275*, H1046.
(1092) McLeod, L. L.; Alayash, A. I. *Am. J. Physiol.* **1999**, *277*, H92.
(1093) Cole, D. J.; McKay, L.; Jacobsen, W. K.; Drummond, J. C.; Patel, P. M. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1997**, *25*, 95.
(1094) Panter, S. S.; Regan, R. F. In *Red Blood Cell Substitutes*; Rudolph, A. S., Rabinovici, R., Feuerstein, G. Z., Eds.; Marcel Dekker: New York, 1998; p 219.
(1095) Pincemail, J.; Detry, O.; Philippart, C.; Defraigne, J. O.; Franssen, C.; Burhop, K.; Deby, C.; Lamy, M. *Free Radical Biol. Med.* **1995**, *19*, 1.
(1096) Chevalier, A.; Guillochon, D.; Nedjar, N.; Piot, J. M.; Vijayalakshmi, M. W.; Thomas, D. *Biochem. Cell Biol.* **1990**, *68*, 813.
(1097) den Boer, P. J.; Bleeker, W. K.; Rigter, G.; Agterberg, J.; Stekkinger, P.; Kannegieter, L. M.; de Nijs, I. M. M.; Bakker, J. C. *Biomater., Artif. Cells, Immobilization Biotechnol.* **1992**, *20*, 647.
(1098) McGown, E. L.; Lyons, M. F.; Marini, M. A.; Zegna, A. *Biochim. Biophys. Acta* **1990**, *1036*, 202.
(1099) Faivre, B.; Menu, P.; Labrude, P.; Grandgeorge, M.; Vigneron, C. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1994**, *22*, 551.
(1100) Hsia, J. C. U.S. Pat. 5,591,710, 1997.
(1101) Krishna, M. C.; Samuni, A.; Taira, J.; Goldstein, S.; Mitchell, J. B.; Russo, A. *J. Biol. Chem.* **1996**, *271*, 26018.
(a) Buehler, P. W.; Mehendale, S.; Wang, H.; Xie, J.; Ma, L.; Trimble, C. R.; Hsia, C. J. C.; Gulati, A. *Free Radical Biol. Med.* **2000**, *29*, 764.
(1102) Vandegriff, K. D.; Le Tellier, Y. C. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1994**, *22*, 443.
(1103) Shorr, R. G. L.; Kwong, S.; Gilbert, C.; Benesch, R. E. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1999**, *27*, 185.
(1104) Murray, J. A.; Ledlow, A.; Launspach, J.; Evans, D.; Loveday, M.; Conklin, J. L. *Gastroenterology* **1995**, *109*, 1241.
(1105) Doyle, M. P.; Pickering, R. A.; DeWeert, T. M.; Hoekstra, J. W.; Pater, D. *J. Biol. Chem.* **1981**, *256*, 12393.
(1106) Herrington, T. M.; Midmore, B. R.; Sahi, S. S. In *Microemulsions and Emulsions in Food*; El-Nokaly, M., Cornell, D., Eds.; ACS Symposium Series 448; American Chemical Society: Washington, DC, 1991; p 82.
(1107) Freytag, J. W.; Templeton, D. In *Red Blood Cell Substitutes*; Rudolph, A. S., Rabinovici, R., Feuerstein, G. Z., Eds.; Dekker: New York, 1998; p 325.
(1108) Light, W. R.; Jacobs, E. E.; Rentko, V. T.; Gawryl, M. S.; Hughes, G. S. In *Red Blood Cell Substitutes*; Rudolph, A. S., Rabinovici, R., Feuerstein, G. Z., Eds.; Dekker: New York, 1998; p 421.
(1109) Beny, J. L.; Brunet, P. C.; Vanderbent, V. *Experientia* **1989**, *45*, 132.
(1110) Toda, N.; Kawakami, M.; Yoshida, K. *Am. J. Physiol.* **1991**, *260*, H420.
(1111) Collins, P.; Burman, J.; Chung, H.; Fox, K. *Circulation* **1993**, *87*, 80.
(1112) Macdonald, V. W.; Motterlini, R. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1994**, *22*, 565.
(1113) Hauser, C. J.; Kaufman, C.; Frantz, R.; Shippy, C.; Schwartz, S.; Shoemaker, W. C. *Arch. Surg.* **1982**, *117*, 782.
(1114) Vogel, W. M.; Dennis, R. C.; Cassidy, G.; Apstein, C. S.; Valeri, C. R. *Am. J. Physiol.* **1986**, *251*, H413.
(1115) Lieberthal, W.; Wolf, E. F.; Merrill, E. W.; Levinsky, N. G.; Valeri, C. R. *Life Sci.* **1987**, *41*, 2525.
(1116) Macdonald, V. W.; Winslow, R. M.; Marini, M. A.; Klinker, M. T. *Biomater., Artif. Cells, Artif. Organs* **1990**, *18*, 263.
(1117) Ning, J.; Peterson, L. M. N.; Anderson, P. J.; Biro, G. P. *Biomater., Artif. Cells, Immobilization Biotechnol.* **1992**, *20*, 723.
(1118) Ulatowski, J. A.; Koehler, R. C.; Nishikawa, T.; Traystman, R. J.; Razynska, A.; Kwansa, H.; Urbaitis, B.; Bucci, E. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1995**, *23*, 263.
(1119) Kim, H. W.; Greenburg, A. G. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1995**, *23*, 303.
(1120) Nakai, K.; Ohta, T.; Sakuma, I.; Akama, K.; Kobayashi, Y.; Tokuyama, S.; Kitabatake, A.; Nakazato, Y.; Takahashi, T. A.; Sadayoshi, S. *J. Cardiovasc. Pharmacol.* **1996**, *28*, 115.
(1121) Martin, W.; Smith, J. A.; White, D. G. *Br. J. Pharmacol.* **1986**, *89*, 563.
(1122) Kaplan, E.; Diehl, J. T.; Peterson, M. B.; Sommerville, K. H.; Daly, B. D. T.; Connolly, R. J.; Cooper, A. G.; Seiler, S. D.; Cleveland, R. J. *J. Thorac. Cardiovasc. Surg.* **1990**, *100*, 687.
(1123) Koch, T.; Duncker, H. P.; Heller, A.; Schaible, R.; van Ackern, K.; Neuhof, H. *Shock* **1994**, *1*, 146.
(1124) Heller, A.; Ragaller, M.; Schmeck, J.; Fluth, H.; Muller, M.; Albrecht, D.; Koch, T. *Shock* **1998**, *10*, 401.
(1125) Nakai, K.; Matsuda, N.; Amano, M.; Ohta, T.; Toluyama, S.; Akama, K.; Kawakami, Y.; Tsuchida, E.; Sekiguchi, S. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1994**, *22*, 559.
(1126) Keipert, P. E.; Gonzales, A.; Gomez, C. L.; Macdonald, V. W.; Hess, J. R.; Winslow, R. M. *Transfusion* **1993**, *33*, 701.
(1127) Motterlini, R.; MacDonald, V. W. *J. Appl. Physiol.* **1993**, *75*, 2224.
(1128) Schultz, S. C.; Grady, B.; Cole, F.; Hamilton, I.; Burhop, K.; Malcolm, D. S. *J. Lab. Clin. Med.* **1993**, *122*, 301.
(1129) Gulati, A.; Sharma, A. C.; Burhop, K. E. *Life Sci.* **1994**, *55*, 827.
(1130) Katsuyama, S. S.; Cole, D. J.; Drummond, K. B. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1994**, *22*, 1.
(1131) Malcolm, D. S.; Hamilton, I. N.; Schultz, S. C.; Cole, F.; Burhop, K. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1994**, *22*, 91.
(1132) Sharma, A. C.; Gulati, A. *J. Lab. Clin. Med.* **1994**, *123*, 299.
(1133) Dunlap, E.; Farrell, L.; Nigro, C.; Estep, T.; Marchand, G.; Burhop, K. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1995**, *23*, 39.
(1134) Hindman, B. J.; Dexter, F.; Cutkomp, J.; Smith, T. *Anesthesiology* **1995**, *83*, 1302.
(1135) Matheson-Urbaitis, B.; Lu, Y.; Fronticelli, C.; Bucci, E. *J. Lab. Clin. Med.* **1995**, *126*, 250.
(1136) Sharma, A. C.; Singh, G.; Gulati, A. *Am. J. Physiol.* **1995**, *269*, H1379.
(1137) Katusic', Z. S.; Lee, H. C.; Clambey, E. T. *Gen. Pharmacol.* **1996**, *27*, 239.
(1138) Przybelski, R. J.; Daily, E. K.; Kisicki, J. C.; Mattia-Goldberg, C.; Bounds, M. J.; Colburn, W. A. *Crit. Care Med.* **1996**, *24*, 1993.
(1139) Barve, A.; Sen, A. P.; Saxena, P. R.; Gulati, A. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1997**, *25*, 75.
(1140) Dietz, N. M.; Martin, C. M.; Beltran-del-Rio, A. G.; Joyner, M. J. *Anesth. Analg.* **1997**, *85*, 265.
(1141) Noone, R. B.; Mythen, M. G.; Vaslef, S. N. *J. Trauma: Inj., Infect., Crit. Care* **1998**, *45*, 457.
(1142) Pickelmann, S.; Nolte, D.; Leiderer, R.; Schutze, E.; Messmer, K. *Am. J. Physiol.* **1998**, *275*, H361.

(1143) Caron, A.; Menu, P.; Faivre-Fiorina, B.; Labrude, P.; Vigneron, C. *J. Appl. Physiol.* **1999**, *246*, 348.
(1144) Sloan, E. P.; Koenigsberg, M.; Gens, D.; Cipolle, M.; Runge, J.; Mallory, M. N.; Rodman, G. *J. Am. Med. Assoc.* **1999**, *282*, 1857.
(1145) Lamy, M. L.; Daily, E. K.; Brichant, J.-F.; Larbuisson, R. P.; Demeyere, R. H.; Vandermeersch, E. A.; Lehot, J.-J.; Parsloe, M. R.; Berridge, J. C.; Sinclair, C. J.; Baron, J.-F.; Przybelski, R. J. *Anesthesiology* **2000**, *92*, 646.
(1146) Vuylsteke, A.; Davidson, H. J.; Ho, W.-S. V.; Ritchie, A. J.; Callingham, B. A.; White, R.; Hiley, C. R. *J. Cardiovasc. Pharmacol.* **2001**, *37*, 394.
(1147) Ulatowski, J. A.; Nishikawa, T.; Matheson-Urbaitis, B.; Bucci, E.; Traystman, R. J.; Koehler, R. C. *Crit. Care Med.* **1996**, *24*, 558.
(1148) Hughes, G. S.; Yancey, E. P.; Albrecht, R.; Locker, P. K.; Francom, S. F.; Orringer, E. P.; Antal, E. J.; Jacobs, E. E. *Clin. Pharmacol. Ther.* **1995**, *58*, 434.
(1149) Botzlar, A.; Nolte, D.; Messmer, K. *Eur. J. Med. Res.* **1996**, *1*, 471.
(1150) Hughes, G. S.; Antal, E. J.; Locker, P. K.; Francom, S. F.; Adams, W. J.; Jacobs, E. E. *Crit. Care Med.* **1996**, *24*, 756.
(1151) Krieter, H.; Hagen, G.; Waschke, K. F.; Köhler, A.; Wenneis, B.; Brucker, U. B.; van Ackern, K. *J. Cardiothorac. Vasc. Anesth.* **1997**, *11*, 3.
(1152) Standl, T. G.; Reeker, W.; Redmann, G.; Kochs, E.; Werner, C.; Schulte am Esch, J. *Int. Care Med.* **1997**, *23*, 865.
(1153) Standl, T.; Wilhelm, S.; Horn, E. P.; Burmeister, M.; Gundlach, M.; Schulte am Esch, J. *Anaesthesist* **1997**, *46*, 763.
(1154) LaMuraglia, G. M.; O'Hara, P. J.; Baker, W. H.; Naslund, T. C.; Norris, E. J.; Li, J.; Vandermeersch, E. *J. Vasc. Surg.* **2000**, *31*, 299.
(1155) Loke, K. E.; Forfia, P. R.; Recchia, F. A.; Xu, X.; Osorio, J. C.; Ochoa, M.; Gawryl, M.; Hintze, T. H. *J. Cardiovasc. Pharmacol.* **2000**, *35*, 84.
(1156) Caron, A.; Menu, P.; Faivre, B.; Labrude, P.; Vigneron, C. *Transfusion Clin. Biol.* **1995**, *6*, 453.
(1157) Gilroy, D.; Shaw, C.; Parry, E.; Odling-Smee, W. *J. Biol. Chem.* **1988**, *28*, 1312.
(1158) Lenz, G.; Junger, H.; van den Ende, R.; Brotman, B.; Prince, A. M. *Biomater., Artif. Cells, Immobilization Biotechnol.* **1991**, *19*, 709.
(1159) Thompson, A.; McGarry, A. E.; Valeri, C. R.; Lieberthal, W. *J. Appl. Physiol.* **1994**, *77*, 2348.
(1160) Wong, L. T.; Er, S. S.; Ning, J.; Christoff, B.; Carmichael, F. J. L. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1998**, *26*, 529.
(1161) Ning, J.; Wong, L. T.; Christoff, B.; Carmichael, F. J. L.; Biro, G. P. *Transfusion Med.* **2000**, *10*, 13.
(1162) Kida, Y.; Iwata, S.; Gyoutoku, Y.; Aikou, A.; Yamakawa, T.; Nishi, K. *Artif. Organs* **1991**, *15*, 5.
(1163) Kida, Y.; Maeda, M.; Iwata, S.; Iwashita, Y.; Goto, K.; Nishi, K. *Artif. Organs* **1995**, *19*, 117.
(1164) Kida, Y.; Yamakawa, T.; Iwasaki, S.; Furusho, N.; Kadowaki, Y.; Iwata, S.; Iwashita, Y.; Nishi, K. *Artif. Organs.* **1995**, *19*, 511.
(1165) Aranow, J. S.; Wang, H.; Zhuang, J.; Fink, M. P. *Crit. Care Med.* **1996**, *24*, 807.
(1166) Bone, H. G.; Waurick, R.; Van Aken, H.; Booke, M.; Prien, T.; Meyer, J. *Intensive Care Med.* **1998**, *24*, 48.
(1167) Rioux, F.; Petitclerc, E.; Audet, R.; Drapeau, G.; Fielding, R. M.; Marceau, F. *J. Cardiovasc. Pharmacol.* **1994**, *24*, 229.
(1168) Loeb, A.; McIntosh, L. J.; Raj, N. R.; Longnecker, D. *J. Cardiovasc. Pharmacol.* **1997**, *30*, 703.
(1169) Rosen, A. L.; Gould, S.; Sehgal, L. R.; Noud, G.; Sehgal, H. L.; Rice, C. L.; Moss, G. S. *Crit. Care Med.* **1979**, *7*, 380.
(1170) Tsai, A. G.; Kerger, H.; Intaglietta, M. In *Blood Substitutes. Physiological Basis of Efficacy*; Winslow, R. M., Vandegriff, K. D., Intaglietta, M., Eds.; Birkhäuser: Boston, 1995; p 155.
(1171) Crowley, J. P.; Metzger, J.; Gray, A.; Pivacek, L. E.; Cassidy, G.; Valeri, C. R. *Circ. Shock* **1993**, *41*, 144.
(1172) Olsen, S. O.; Tang, D. B.; Jackson, M. R.; Gomez, E. R.; Ayala, B.; Alving, B. M. *Circulation* **1996**, *93*, 327.
(1173) Burhop, K. E.; Estep, T. E. VIIIth International Symposium on Blood Substitutes, San Diego, 2000.
(1174) Rattan, S.; Rosenthal, G. J.; Chakder, S. *J. Pharmacol. Exp. Therp.* **1995**, *272*, 1211.
(1175) Rioux, F.; Drapeau, G.; Marceau, F. *J. Cardiovasc. Pharmacol.* **1995**, *25*, 587.
(1176) Hart, J. L.; Ledvina, M. A.; Muldoon, S. M. *J. Lab. Clin. Med.* **1997**, *129*, 356.
(1177) Moisan, S.; Drapeau, G.; Burhop, K. E.; Rioux, F. *Can. J. Physiol. Pharmacol.* **1998**, *76*, 434.
(1178) Nakai, K.; Sakuma, I.; Kitabatake, A. In *Blood Substitutes. Present and Future Perspectives*; Tsuchida, E., Ed.; Elsevier: Amsterdam, 1998; p 251.
(1179) Palaparthy, R.; Wang, H.; Gulati, A. *Adv. Drug Delivery Res.* **2000**, *40*, 185.
(1180) Freas, W.; Llave, R.; Jing, M.; Hart, J.; McQuillan, P.; Muldoon, S. *J. Lab. Clin. Med.* **1995**, *125*, 762.
(1181) Bilello, K.; Schultz, S.; Powell, C.; Jaffin, J.; Cole, F.; Malcolm, D. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1994**, *22*, 819.
(1182) Poli de Figueiredo, L. F.; Mathru, M.; Solanki, D.; Kramer, G. C. *Crit. Care Med.* **1997**, *25*, A39.
(1183) Sefton, W.; Pudimat, P.; Bina, S.; Lojeski, E.; Mongan, P.; Muldoon, S. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1999**, *27*, 203.
(1184) Erhart, S. M.; Cole, D. J.; Patel, P. M.; Drummond, J. C.; Burhop, K. E. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **2000**, *28*, 385.
(1185) Intaglietta, M.; Johnson, P. C.; Winslow, R. M. *Cardiovasc. Res.* **1996**, *32*, 632.
(1186) Tsai, A. G.; Kerger, H.; Intaglietta, M. In *Blood Substitutes—New Challenges*; Winslow, R. M., Vandegriff, K. D., Intaglietta, M., Eds.; Birkhäuser: Boston, 1996; p 124.
(1187) Winslow, R. M. *Adv. Drug Delivery Rev.* **2000**, *40*, 131.
(1188) Pohl, U.; Herlan, K.; Huang, A.; Bassenge, E. *Am. J. Physiol.* **1991**, *261*, H2016.
(1189) Lamontagne, D.; Pohl, U.; Busse, P. *Circ. Res.* **1992**, *70*, 123.
(1190) Davies, P. F. *Physiol. Rev.* **1995**, *75*, 519.
(1191) Malek, A. M.; Izumo, S. *J. Biomech.* **1995**, *28*, 1515.
(1192) de Wit, C.; Schäfer, C.; von Bismarck, P.; Bolz, S.-S.; Pohl, U. *Pflügers Arch.—Eur. J. Physiol.* **1997**, *434*, 354.
(1193) Ballermann, B. J.; Dardik, A.; Eng, E.; Liu, A. *Kidney Int.* **1998**, *54*, S100.
(1194) Messina, E. J.; Sun, D.; Koller, A.; Wolin, M. S.; Kaley, G. *Microvasc. Res.* **1994**, *48*, 151.
(1195) Pries, A. R.; Heide, J.; Ley, K.; Klotz, K.-F.; Gaehtgens, P. *Microvasc. Res.* **1995**, *49*, 289.
(1196) Caron, A.; Malfatti, E.; Aguejouf, O.; Faivre-Fiorina, B.; Menu, P. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **2001**, *29*, 19.
(1197) Gulati, A.; Singh, G.; Rebello, S.; Sharma, A. C. *Life Sci.* **1995**, *56*, 1433.
(1198) Gulati, A.; Sharma, A. C.; Singh, G. *Crit. Care Med.* **1996**, *24*, 137.
(1199) Saxena, R.; Wijnhoud, A. D.; Veld, A.; van den Meiracker, A. H.; Boomsma, F.; Przybelski, R. J.; Koudstaal, P. J. *J. Hypertension* **1998**, *16*, 1459.
(1200) Gulati, A.; Rebello, S. *J. Lab. Clin. Med.* **1994**, *124*, 125.
(1201) Sharma, A.; Gulati, A. *Crit. Care Med.* **1995**, *23*, 874.
(1202) Suematsu, M.; Wakabayashi, Y.; Goda, N.; Takeoka, S.; Tsuchida, E.; Ishimura, Y. In *Blood Substitutes. Present and Future Perspectives*; Tsuchida, E., Ed.; Elsevier: Amsterdam, 1998; p 241.
(1203) Malcolm, D. S.; Hamilton, I. N.; Schultz, S. C.; Cole, F.; Burhop, K. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1994**, *22*, 91.
(1204) Nakai, K.; Sakuma, I.; Ohta, T.; Ando, J.; Kitabatake, A.; Nakazato, Y.; Takahashi, T. A. *J. Lab. Clin. Med.* **1998**, *132*, 313.
(1205) Gould, S. A.; Moore, E. E.; Hoyt, D. B.; Burch, J. M.; Haenel, J. B.; Garcia, J.; DeWoskin, R.; Moss, G. S. *J. Am. Coll. Surg.* **1998**, *187*, 113.
(1206) Johnson, J. L.; Moore, E. E.; Offner, P. J.; Haenel, J. B.; Hides, G. A.; Tamura, D. Y. *Am. J. Surg.* **1998**, *176*, 612.
(1207) Gould, S. A. Proceedings of the 52nd Annual Meeting of the American Association of Blood Banks, The Compendium, San Francisco, CA, 1999; p 297.
(1208) Xu, X.; Shen, W.; Hintze, T. H. *FASEB J.* **1994**, *8*, A883.
(1209) Sherman, I. A.; Dlugosz, J. A.; Perelman, V.; Hsia, C. J. C.; Wong, L. T.; Condie, R. M. *Biomater., Artif. Cells, Immobilization Biotechnol.* **1993**, *21*, 537.
(1210) Faivre-Fiorina, B.; Caron, A.; Fassot, C.; Fries, I.; Menu, P.; Labrude, P.; Vigneron, C. *Am. J. Physiol.* **1999**, *276*, 766.
(1211) Estep, T. N. IBC Conference on Blood Substitutes and Oxygen Therapeutics, Washington, DC, 1998.
(1212) Ishii, Y.; Shuyi, W.; Kitamura, S. *Life Sci.* **1995**, *56*, 2263.
(1213) Sakai, H.; Hara, H.; Yuasa, M.; Tsai, A. G.; Takeoka, S.; Tsuchida, E.; Intaglietta, M. *Am. J. Physiol.* **2000**, *279*, 908.
(1214) Conover, C. D.; Lejeune, L.; Shum, K.; Shorr, R. G. L. *Life Sci.* **1996**, *59*, 1861.
(1215) Leppäniemi, A.; Soltero, R.; Burris, D.; Pikoulis, E.; Ratigan, J.; Waasdorp, C.; Hufnagel, H.; Malcolm, D. *J. Trauma: Inj., Infect. Crit. Care* **1996**, *40*, 242.
(1216) Traylor, T. G.; Koga, N.; Deardurff, L. A. *J. Am. Chem. Soc.* **1985**, *107*, 6504.
(1217) Feuerstein, G.; Sulpizio, A.; Hieble, J. P.; Macdonald, V.; Chavez, M.; Rudolph, A. S. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1996**, *24*, 335.
(1218) Rudolph, A. S.; Sulpizio, A.; Hieble, P.; MacDonald, V.; Chavez, M.; Feuerstein, G. *J. Appl. Physiol.* **1997**, *82*, 1826.
(1219) Goda, N.; Suzuki, K.; Naito, M.; Takeoka, S.; Tsuchida, E.; Ishimura, Y.; Tamatani, T.; Suematsu, M. *J. Clin. Invest.* **1998**, *101*, 604.
(1220) Rudolph, A. S.; Sulpizio, T.; Kwasiborski, V.; Cliff, R. O.; Rabinovici, R.; Feuerstein, G. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1996**, *24*, 415.

(1221) Nakai, K.; Usuba, A.; Ohta, T.; Kuwabara, M.; Nakazato, Y.; Motoki, R.; Takahashi, T. A. *Artif. Organs* **1998**, *22*, 320.
(1222) Liao, J. C.; Hein, T. W.; Vaughn, M. W.; Huang, K.-T.; Kuo, L. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 8757.
(1223) Adamson, G.; McIntosh, G. A.; Stewart, J. B.; Wong., L. T. VIIIth International Symposium on Blood Substitutes, San Diego, 2000.
(1224) Verma, A.; Hirsch, D. J.; Glatt, C. E.; Ronnett, G. V.; Snyder, S. H. *Science* **1993**, *259*, 381.
(1225) Usuba, A.; Endoh, Y.; Motoki, R.; Ogata, Y.; Suzuki, K.; Kamitani, T. In *Liposomes in Biomedical Applications*; Shek, P. N., Ed.; Harwood Academic Publ.: North York, Ontario, 1995; p 265.
(1226) Bolin, R.; Smith, D.; Moore, G.; Boswell, G.; DeVenuto, F. *Prog. Clin. Biol. Res.* **1983**, *122*, 117.
(1227) White, C. T.; Murray, A. J.; Greene, J. R.; Smith, D. J.; Medina, F.; Makovec, G. T.; Martin, E. J.; Bolin, R. B. *J. Lab. Clin. Med.* **1986**, *108*, 121.
(1228) Iuliano, L.; Violi, F.; Pedersen, J. Z.; Pratico, D.; Rotilio, G.; Balsano, F. *Arch. Biochem. Biophys.* **1992**, *299*, 220.
(1229) Marcus, A. J.; Broekman, M. J. *Am. Heart Assoc.* **1996**, *93*, 208.
(1230) Tsai, S.-P.; Wong, J. T.-F. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1996**, *24*, 513.
(1231) Alonsozana, G. L. G.; Elfath, M. D.; Mackenzie, C.; Gregory, L. C.; Duh, S. H.; Trump, B.; Christenson, R. H. *J. Cardiothorac. Vasc. Anesth.* **1997**, *11*, 845.
(1232) Mondoro, T. H.; Alayash, A. I.; Ryan, B. A. B.; Terle, D. A.; Vostal, J. G. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1998**, *26*, 1.
(1233) Regan, R. F.; Panter, S. S. VIIIth International Symposium on Blood Substitutes, San Diego, 2000.
(1234) Vandegriff, K. D. *Exp. Opin. Invest. Drugs* **2000**, *9*, 1967.
(1235) Walder, R. Y.; Andracki, M. E.; Walder, J. A. *Methods Enzymol.* **1994**, *231*, 274.
(1236) Vandegriff, K. D.; Olson, J. S. *Biophys. J.* **1984**, *45*, 825.
(1237) Nelson, D.; Azari, M.; Brown, R.; Burhop, K.; Bush, S.; Catarello, J.; Chuang, H.; Downing, C.; Estep, T.; Loewen, A.; McClure, K.; McDaniel, A.; Michalek, E.; Mozier, N.; Rohn, K.; Spicuzza, J.; Zieske, P.; Zimmerman, G. *Biomater., Artif. Cells, Immobilization Biotechnol.* **1992**, *20*, 423.
(1238) Farmer, M.; Ebeling, A.; Marshall, T.; Hauck, W.; Sun, C. S.; White, E.; Long, Z. *Biomater., Artif. Cells, Immobilization Biotechnol.* **1992**, *20*, 429.
(1239) Azari, M.; Rohn, K.; Picken, J. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1994**, *22*, 701.
(1240) Azari, M.; Catarello, J.; Burhop, K.; Camacho, T.; Ebeling, A.; Estep, T.; Guzder, S.; Krause, K.; Marshall, T.; Rohn, K.; Sarajari, R. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1997**, *25*, 521.
(1241) Yu, Z.; Friso, G.; Miranda, J. J.; Patel, M. J.; Lo-Tseng, T.; Moore, E. G.; Burlingame, A. L. *Protein Sci.* **1997**, *6*, 2568.
(1242) Nolte, D.; Botzlar, A.; Hecht, R.; Csapo, C.; Menger, M. D.; Messmer, K. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1994**, *22*, 587.
(1243) Sharma, A. C.; Rebello, S.; Gulati, A. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1994**, *22*, 593.
(1244) Bowes, M. P.; Burhop, K. E.; Zivin, J. A. *Stroke* **1994**, *25*, 2243.
(1245) Schultz, S. C.; Powell, C. C.; Bernard, E.; Malcolm, D. S. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1995**, *23*, 647.
(1246) van Iterson, M.; Sinaasappel, M.; Burhop, K.; Trouwborst, A.; Ince, C. *J. Lab. Clin. Med.* **1998**, *132*, 421.
(1247) Chow, M. S. S.; Fan, C.; Tran, H.; Zhao, H.; Zhou, L. *J. Pharmacol. Exp. Ther.* **2001**, *297*, 224.
(1248) Cohn, S. M.; Farrell, T. J. *J. Trauma* **1995**, *29*, 210.
(1249) Powell, C. C.; Schultz, S. C.; Malcolm, D. S. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1996**, *24*, 197.
(1250) Kumar, A.; Sen, A. P.; Saxena, P. R.; Gulati, A. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1997**, *25*, 85.
(1251) Mourelatos, M. G.; Enzer, N.; Fergusson, J. L.; Rypins, E. B.; Burhop, K. E.; Law, W. R. *Shock* **1996**, *5*, 141.
(a) Shen, A. P.; Dong, Y.; Saxena, P. R.; Gulati, A. *Shock* **1998**, *9*, 223.
(b) Cohn, S. M.; Zieg, P. M.; Rosenfield, A. T.; Fischer, B. T. *Crit. Care Med.* **1997**, *25*, 484.
(1252) Baron, J.-F. *Br. J. Anaesth.* **1998**, *81*, 34.
(1253) Krishnamurti, C.; Carter, A. J.; Maglasang, P.; Hess, J. R.; Cutting, M. A.; Alving, B. M. *Crit. Care Med.* **1997**, *25*, 1874.
(1254) Sloan, H. P.; Koenigsberg, M. D.; Bickell, W. H.; Cohn, S. M.; Kruse, J.; Thompson, D. R.; Corne, L.; Micheels, J.; Mols, P. *Acad. Emerg. Med.* **1995**, *2*, 365.
(1255) Burhop, K. E.; Schmitz, T. H. In *Blood Substitutes. Present and Future Perspectives*; Tsuchida, E., Ed.; Elsevier: Amsterdam, 1998; p 75.
(1256) Remy, B.; Deby-Dupont, G.; Lamy, M. *Br. Med. Bull.* **1999**, *55*, 277.
(1257) Garrioch, M. A.; McClure, J. H.; Wildsmith, J. A. W. *Br. J. Anaesth.* **1999**, *83*, 702.
(1258) Estep, T. U.S. Public Health Service, Advisory Committee on Blood Safety and Availability, 1999.
(1259) Biro, G. P.; Carmichael, F. L. The Compendium−52nd Annual Meeting of the American Association of Blood Banks, San Francisco, 1999; p 294.
(1260) Ning, J.; Er, S. S.; Wong, L. T. In *Blood Substitutes. Present and Future Perspectives*; Tsuchida, E., Ed.; Elsevier: Amsterdam, 1998; p 211.
(1261) Biro, G. P.; Carmichael, F. J. L.; Lieberthal, W. IBC Conference on Blood Substitutes and Oxygen Therapeutics, Washington, DC, 1998.
(1262) Carmichael, F. J. L.; Biro, G. P.; Cheng, D. C. H. VIIIth International Symposium on Blood Substitutes, San Diego, 2000.
(1263) Driessen, B.; Jahr, J. S.; Lurie, F.; Gunther, R. A. *J. Vet. Pharmacol. Ther.* **2001**, *24*, 61.
(1264) Harringer, W.; Hodakowski, G. T.; Svizzero, T.; Jacobs, E. E.; Vlahakes, G. J. *Eur. J. Cardio-thorac. Surg.* **1992**, *6*, 649.
(1265) Hayward, R.; Lefer, A. M. *Methods Find. Exp. Clin. Pharmacol.* **1999**, *21*, 427.
(1266) Horn, E.; Standl, T.; Wilhelm, S.; Jacobs, E. E.; Freitag, U.; Freitag, U.; Schulte am Esch, J. *Surgery* **1997**, *121*, 411.
(1267) Teicher, B. A.; Holden, S. A.; Menon, K.; Hopkins, R. E.; Gawry, M. S. *Cancer Chemother. Pharmacol.* **1993**, *33*, 57.
(1268) Teicher, B. A.; Schwartz, G. N.; Sotomayor, E. A.; Robinson, M. F.; Dupuis, N. P. *J. Cancer Res. Clin. Oncol.* **1993**, *120*, 85.
(1269) Hodakowski, G. T.; Page, R. D.; Harringer, W.; Jacobs, E. E.; LaRaia, P. J.; Svizzero, T.; Guerrero, J. L.; Austen, W. G.; Vlahakes, G. J. *Biomater., Artif. Cells, Immobilization Biotechnol.* **1992**, *20*, 669.
(1270) Standl, T.; Burmeister, M.-A.; Horn, E.-P.; Wilhelm, S.; Knoefel, W. T.; Schulte am Esch, J. *Br. J. Anaesth.* **1998**, *80*, 189.
(1271) Sehgal, L. R.; Gould, S. A.; Rosen, A. L.; Sehgal, H. L.; Moss, G. S. *Surgery* **1984**, *95*, 433.
(1272) Sehgal, L. R.; Sehgal, H. L.; Rosen, A. L.; Gould, S. A.; DeWoskin, R.; Moss, G. S. *Biomater., Artif. Cells, Artif. Organs* **1988**, *16*, 173.
(1273) Gould, S. A.; Sehgal, L. R.; Sehgal, H. L.; Moss, G. S. *Crit. Care Clinics* **1992**, *8*, 293.
(1274) Gould, S. A.; Moss, G. S. *World J. Surg.* **1996**, *20*, 1200.
(1275) Gould, S. A.; Moore, E. E.; Moore, F. A.; Haenel, J. B.; Burch, J. M.; Sehgal, H.; Sehgal, L.; DeWoskin, R.; Moss, G. S. In *Blood Substitutes. Present and Future Perspectives*; Tsuchida, E., Ed.; Elsevier: Amsterdam, 1998; p 41.
(1276) Rosen, A. L.; Gould, S. A.; Sehgal, L. R.; Sehgal, H. L.; Levine, H. D.; DeWoskin, R. D.; Moss, G. S. *J. Appl. Physiol.* **1990**, *68*, 938.
(1277) Gould, S. A.; Moore, E. E.; Moore, F. A.; Haenel, J. B.; Burch, J. M.; Sehgal, H. L.; Sehgal, L. R.; DeWoskin, R. D.; Moss, G. S. *J. Trauma* **1997**, *43*, 325.
(1278) Bradley, R.; Sloshberg, S.; Nho, K.; Czuba, B.; Szesko, D.; Shorr, R. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1994**, *22*, 657.
(1279) Conover, C. D.; Malatesta, P.; Lejeune, L.; Chang, C.-L.; Shorr, R. G. L. *J. Invest. Med.* **1996**, *44*, 238.
(1280) Conover, C. D.; Linberg, R.; Gilbert, C. W.; Shum, K. L.; Shorr, R. G. L. *Artif. Organs* **1997**, *21*, 1066.
(1281) Conover, C. D.; Lejeune, L.; Shum, K. L.; Gilbert, C. W.; Shorr, R. G. L. *Artif. Organs* **1997**, *21*, 369.
(1282) Song, D.; Olano, M.; Wilson, D. F.; Pastuszko, A.; Tammela, O.; Nho, K.; Shorr, R. G. L. *Transfusion* **1995**, *35*, 552.
(1283) Conover, C. D.; Linberg, R.; Lejeune, L.; Gilbert, C. W.; Shum, K. L.; Shorr, R. G. L. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1998**, *26*, 199.
(1284) Malchesky, P. S.; Takahashi, T.; Iwasaki, K.; Harasaki, H.; Nose, Y. *Intl. J. Artif. Organs* **1990**, *13*, 442.
(1285) Talarico, T. L.; Guise, K. J.; Garg, V. K. *BioPharmacology* **1999**, *12*, 42.
(1286) Yabuki, A.; Yamaji, K.; Ohki, H.; Iwashita, Y. *Transfusion* **1990**, *30*, 516.
(1287) Matsushita, M.; Iwashita, Y.; Iwasaki, K.; Ohki, H.; Nasu, M.; Horiuchi, T.; Chen, J. F.; Goldcamp, J.; Murabayashi, S.; Harasaki, H.; Malchesky, P. S.; Nosé, Y. *Trans. Am. Soc. Artif. Intern. Organs* **1986**, *32*, 490.
(1288) Takahaski, T.; Iwasaki, K.; Malchesky, P. S.; Harasaki, H.; Matsushita, M.; Nosé, Y.; Rolin, H.; Hall, P. M. *Artif. Organs* **1993**, *17*, 153.
(1289) Eldridge, J.; Russell, R.; Christenson, R.; Sakamoto, R.; Williams, J.; Parr, M.; Trump, B.; Delaney, P.; Mackenzie, C. F. *Crit. Care Med.* **1996**, *24*, 663.
(1290) Bone, H. G.; Fischer, S. R.; Schenarts, P. J.; McGuire, R.; Traber, L. D.; Traber, D. L. *Shock* **1998**, *10*, 69.
(1291) Siegel, J. H.; Fabian, M.; Smith, J. A.; Constantino, D. In *Red Blood Cell Substitutes*; Rudolph, A. S., Rabinovici, R., Feuerstein, G. Z., Eds.; Dekker: New York, 1998; p 119.
(1292) Chakder, S.; Rosenthal, G. J.; Rattan, S. *Am. J. Physiol.* **1995**, *268*, G443.

(a) Conklin, J. L.; Murray, J.; Ledlow, A.; Clark, E.; Hayek, B.; Picken, H.; Rosenthal, G. *J. Pharmacol. Exp. Therap.* **1995**, *273*, 762.
(1293) Usuba, A.; Motoki, R. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1997**, *25*, 379.
(1294) Takaori, M.; Fukui, A. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1996**, *24*, 643.
(1295) Funakoshi, Y.; Fuchinoue, S.; Agishi, T.; Ota, K. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1997**, *25*, 407.
(1296) Waschke, K. F.; Albrecht, D. M.; van Ackern, K.; Kuschinsky, W. *Br. J. Anaesth.* **1994**, *73*, 522.
(1297) Labrude, P. *Ann. Pharm. Fr.* **1992**, *50*, 250.
(1298) Akama, K.; Morizawa, K.; Tokuyama, S.; Satoh, T.; Kobayashi, K.; Sekigushi, S.; Tsuchida, E. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1994**, *22*, 901.
(1299) Abraham, D. J.; Wireko, F. C.; Randad, R. S.; Poyart, C.; Kister, J.; Bohn, B.; Liard, J. F.; Kunert, M. P. *Biochemistry* **1992**, *31*, 9141.
(1300) Kleinberg, L.; Grossman, S. A.; Piantadosi, S.; Pearlman, J.; Engelhard, H.; Lesser, G.; Ruffer, J.; Gerber, M. *J. Clin. Oncol.* **1999**, *17*, 2593.
(1301) Grocott, H. P.; Bart, R. D.; Sheng, H.; Miura, Y.; Steffen, R.; Pearlstein, R. D.; Warner, D. S. *Stroke* **1998**, *29*, 1650.
(1302) Bruggemann, U.; Roux, E. C.; Hanning, J.; Nicolau, C. *Transfusion* **1995**, *35*, 478.
(a) Fischer, J. J.; Yabuki, H. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1998**, *26*, 377.
(1303) Faithfull, N. S. In *Yearbook of Intensive Care and Emergency Medicine*; Vincent, J. L., Ed.; Springer-Verlag: Brussels, 1994; p 237.
(1304) Lamy, M.; Mathy-Hartert, M.; Deby-Dupont, G. In *Tissue Oxygenation in Acute Medicine*; Sibbald, W. J., Messmer, K., Fink, M. P., Eds.; Springer-Verlag: Berlin, 1998; p 332.
(1305) Lowe, K. C. In *Blood Substitutes. Present and Future Perspectives*; Tsuchida, E., Ed.; Elsevier: Amsterdam, 1998; p 327.
(1306) Riess, J. G. In *Blood Substitutes: Methods, Products and Clinical Trials*; Chang, T. M. S., Ed.; Karger Landes: New York, 1998; p 101.
(1307) Banks, R. E.; Smart, B. E.; Tatlow, J. C. *Organofluorine Chemistry: Principles and Commercial Applications*; Plenum Press: New York, 1994.
(1308) Kissa, E. *Fluorinated Surfactants, Synthesis, Properties, Applications*; Marcel Dekker: New York, 1994.
(1309) Mallick, A.; Bodenham, A. R. *Br. J. Hosp. Med.* **1996**, *55*, 443.
(1310) Banks, R. E.; Tatlow, J. C. *J. Fluorine Chem.* **1986**, *33*, 227.
(1311) Smart, B. E. In *Organofluorine Chemistry: Principles and Commercial Applications*; Banks, R. E., Smart, B. E., Tatlow, J. C., Eds.; Plenum Press: New York, 1994; p 57.
(1312) Bondi, A. *J. Phys. Chem.* **1964**, *68*, 441.
(1313) Hildebrand, J. H.; Prausnitz, J. M.; Scott, R. L. *Regular and Related Solutions*; Van Nostrand Reinhold Co.: New York, 1970.
(1314) Mukerjee, P.; Yang, A. Y. S. *J. Phys. Chem.* **1976**, *80*, 1388.
(1315) Schutt, E. G.; Pelura, T. J.; Hopkins, R. M. *Acad. Radiol.* **1996**, *35*, S188.
(1316) Riess, J. G. *Colloids Surf. A* **1994**, *84*, 33.
(1317) Krafft, M. P.; Riess, J. G. *Biochimie* **1998**, *80*, 489.
(1318) Gjaldbaek, J.; Hildebrand, J. H. *J. Am. Chem. Soc.* **1949**, *71*, 3147.
(1319) Chandler, D. *Annu. Rev. Phys. Chem.* **1978**, *29*, 441.
(1320) Patrick, C. R. In *Preparation, Properties, and Applications of Organofluorine Compounds*; Banks, Ed.; Ellis Horwood: Chichester, 1982; p 323.
(1321) Hamza, M. A.; Serratrice, G.; Stébé, M. J.; Delpuech, J.-J. *J. Am. Chem. Soc.* **1981**, *103*, 3733.
(1322) Serratrice, G.; Stébé, M. J.; Delpuech, J.-J. *J. Chim. Phys.* **1985**, *82*, 579.
(1323) Serratrice, G.; Delpuech, J.-J. *Nouv. J. Chim.* **1982**, *6*, 489.
(1324) Kennan, R. P.; Pollack, G. L. *J. Chem. Phys.* **1988**, *89*, 517.
(1325) Pollack, G. L.; Kennan, R. P.; Himm, J. F.; Carr, P. W. *J. Chem. Phys.* **1989**, *90*, 6569.
(1326) Gjaldbaek, J. *Acta Chem. Scand.* **1952**, *6*, 623.
(1327) Zander, R. *Res. Exp. Med.* **1974**, *164*, 97.
(1328) Wesseler, E. P.; Iltis, R.; Clark, L. *J. Fluorine Chem.* **1977**, *9*, 137.
(1329) Arlen, C.; Y. Gauffreteau; F. Jeanneaux; M. Le Blanc; Riess, J. G. *Bull. Soc. Chim. Fr.* **1985**, 562.
(1330) Sharts, C. M.; Reese, H. R. *J. Fluorine Chem.* **1978**, *11*, 637.
(1331) Riess, J. G. *Artif. Organs* **1984**, *8*, 44.
(1332) Riess, J. G.; Le Blanc, M. In *Blood Substitutes: Preparation, Physiology, and Medical Applications*; Lowe, K. C., Ed.; Ellis Horwood Ltd.: Chichester, 1988; p 94.
(1333) Meinert, H.; Knoblich, A. *Biomater., Artif. Cells, Immobilization Biotechnol.* **1993**, *21*, 583.
(1334) Pierotti, R. A. *Chem. Rev.* **1976**, *76*, 717.
(1335) Lawson, D. D.; Moacanin, J.; Scherer, K. V.; Terranova, T. F.; Ingham, J. D. *J. Fluorine Chem.* **1978**, *12*, 221.
(1336) Navari, R. M.; Rosenblum, W. I.; Kontos, H. A.; Patterson, J. L., Jr. *Res. Exp. Med.* **1977**, *170*, 169.
(1337) Reid, R. S.; Koch, C. J.; Castro, M. E.; Lunt, J. A.; Treiber, E. O.; Boisvert, D. J. P.; Allen, P. S. *Phys. Med. Biol.* **1985**, *30*, 677.
(1338) Pollack, G. L.; Kennan, R. P.; Holm, G. T. *Biomater., Artif. Cells, Immobilization Biotechnol.* **1992**, *20*, 1101.
(1339) Watanabe, R.; Inahara, H.; Motoyama, Y. Proceedings of the Xth International Congress Nutrition−Symposium on Perfluorochemical Artificial Blood; Kyoto, 1975, Igakushobo Med. Publ.: Osaka, 1975, p 113.
(1340) Ghosh, A.; Janic, V.; Sloviter, H. A. *Anal. Biochem.* **1970**, *38*, 270.
(1341) Rosen, A. L.; Sehgal, L. R.; Gould, S. A.; Sehgal, H. L.; Dalton, L.; Rice, C. L.; Moss, G. S. *Crit. Care Med.* **1982**, *10*, 149.
(1342) Grote, J.; Steuer, K.; Müller, R.; Söntgerath, C.; Zimmer, K. *Adv. Exp. Biol. Med.* **1985**, *191*, 453.
(1343) Tham, M. K.; Walker, R. D.; Modell, J. H. *J. Chem. Eng. Data* **1973**, *18*, 411.
(1344) O'Brien, R. N.; Langlais, A. J.; Seufert, W. D. *Science* **1982**, *217*, 153.
(1345) Ohyanagi, H.; Itoh, T.; Sekita, M.; Okamoto, M.; Mitsuno, T. *Artif. Organs* **1978**, *2* (*Suppl.*), 90.
(1346) American Chemical Society Symposium on Organofluorine Compounds in Medicine and Biology, Las Vegas, 1982; abstracts.
(1347) Moore, R. E.; Clark, L. C. In *Oxygen Carrying Colloidal Blood Substitutes*; Frey, R., Beisbarth, H., Stosseck, K., Eds.; Zuckschwerdt Verlag: Mainz, 1982; p 50.
(1348) Le Blanc, M.; Riess, J. G. In *Preparation, Properties, and Industrial Applications of Organofluorine Compounds*; Banks, R. E., Ed.; Ellis Horwood Ltd: Chichester, 1982; Chapter 3; p 83.
(1349) Le Blanc, M.; Riess, J. G. In *Oxygen Carrying Colloidal Blood Substitutes*; Frey, R., Beisbarth, H., Stosseck, K., Eds.; Zuckschwerdt Verlag: Munchen, 1982; p 43.
(1350) Moore, R. E.; Clark, L. C.; Miller, M. L. In *Proceedings of the IVth International Symposium on Perfluorochemical Blood Substitutes*; Excerpta Medica: Amsterdam, 1978; p 69.
(1351) Moldavsky, D. D.; Furin, G. G. *J. Fluorine Chem.* **1998**, *87*, 111.
(1352) Lebeau, P.; Damien, A. *C. R. Acad. Sci. Fr.* **1926**, *182*, 1340.
(1353) Simons, J. H. *J. Electrochem. Soc.* **1949**, *95*, 47.
(1354) Abe, T.; Nagase, S. In *Preparation, Properties and Industrial Applications of Organofluorine Compounds*; Banks, R. E., Ed.; Ellis Horwood: Chichester, 1982; p 19.
(1355) Alsmeyer, Y. W.; Childs, W. V.; Flynn, R. M.; Moore, G. G. I.; Smeltzer, J. C. In *Organofluorine Chemistry. Principles and Commercial Applications*; Banks, R. E., Smart, B. E., Tatlow, J. C., Eds.; Plenum Press: New York, 1994; p 121.
(1356) Adcock, J. L. In *Chemistry of Organic Fluorine Compounds II. A Critical Review*; Hudlicky, M., Pavlath, A. E., Eds.; ACS Monograph 187; American Chemical Society: Washington, DC, 1995; p 97.
(1357) Gambaretto, G. P.; Napoli, M.; Conte, L.; Scipione, A.; Armelli, R. *J. Fluorine Chem.* **1985**, *27*, 149.
(1358) Drakesmith, F. G.; Hughes, D. A. *J. Appl. Electrochem.* **1979**, *9*, 685.
(1359) Holaday, D. C. *Fed. Proc.* **1970**, *29*, 1815.
(1360) Conte, L.; Napoli, M.; Gambaretto, G. P. *J. Fluorine Chem.* **1985**, *30*, 89.
(1361) Tsuda, Y.; Yamanouchi, K.; Yokoyama, K.; Suyama, T. *Biomater., Artif. Cells, Artif. Organs* **1988**, *16*, 473.
(1362) Ohyanagi, H.; Saitoh, Y.; Mitsuno, T.; Watanabe, M.; Yamaouchi, K.; Yokoyama, K. *Artif. Organs* **1990**, *14*, 199.
(1363) Naito, Y.; Inoue, Y.; Ono, T.; Arakawa, Y.; Fukaya, C.; Yokoyama, K.; Kobayashi, Y.; Yamanouchi, K. *J. Fluorine Chem.* **1984**, *26*, 485.
(1364) Yokoyama, K.; Suyama, T.; Okamoto, H.; Watanabe, M.; Ohyanagi, H.; Saitoh, Y. *Artif. Organs* **1984**, *8*, 34.
(1365) Yamanouchi, K.; Tanaka, M.; Tsuda, Y.; Yokoyama, K.; Awazu, S.; Kobayashi, Y. *Chem. Pharm. Bull.* **1985**, *33*, 1221.
(1366) Stacey, M.; Tatlow, J. C. *Adv. Fluorine Chem.* **1960**, *1*, 166.
(1367) Hudliky, M. In *Chemistry of Organic Fluorine Compounds II. A Critical Review*; Hudlicky, M., Pavlath, A. E., Eds.; ACS Monograph 187; American Chemical Society: Washington, DC, 1995; p 120.
(1368) Burdon, J.; Parson, I. W. *Tetrahedron* **1980**, *36*, 1423.
(1369) Green, S. W.; Slinn, D. S. L.; Simpson, R. N. F.; Woytek, A. J. In *Organofluorine Chemistry: Principles and Commercial Applications*; Banks, R. E., Smart, B. E., Tatlow, J. C., Eds.; Plenum Press: New York, 1994; p 89.
(1370) Moore, R. E.; Driscoll, G. L. *J. Org. Chem.* **1978**, *43*, 4978.
(1371) Moore, R. E.; Clark, L. C. *Int. Anesthesiol. Clin.* **1985**, *23*, 11.
(1372) Moore, R. E. *Biomater., Artif. Cells, Artif. Organs* **1988**, *16*, 443.
(1373) Lagow, R. J. In *Fluorine Chemistry: A Comprehensive Treatment*; Howe-Grant, M., Ed.; Wiley: New York, 1995; p 242.
(1374) Moldavskii, D. D.; Bispen, T. A.; Kaurova, G. I.; Furin, G. G. *J. Fluorine Chem.* **1999**, *94*, 157.
(1375) Lagow, R. J.; Margrave, J. L. *Prog. Inorg. Chem.* **1979**, *26*, 161.

(1376) Scherer, K. V.; Yamanouchi, K.; Ono, T. *J. Fluorine Chem.* **1990**, *50*, 47.
(1377) Ono, T.; Yamanouchi, K.; Scherer, K. V. *J. Fluorine Chem.* **1995**, *73*, 267.
(1378) Ono, T.; Yamanouchi, K.; Fernandez, R. E.; Scherer, K. V. *J. Fluorine Chem.* **1995**, *75*, 197.
(1379) Adcock, J. L.; Horita, K.; Renk, E. B. *J. Am. Chem. Soc.* **1981**, *103*, 6937.
(1380) Haszeldine, R. N. *J. Chem. Soc.* **1953**, 3761.
(1381) Zhang, L.; Zhang, J.; Yang, Z.; Wang, Y.; Fuss, W.; Weizbauer, S. *J. Fluorine Chem.* **1998**, *88*, 153.
(1382) Wakselman, C.; Lantz, A. In *Organofluorine Chemistry: Principles and Commercial Applications*; Banks, R. E., Smart, B. E., Tatlow, J. C., Eds.; Plenum Press: New York, 1994; p 177.
(1383) Brace, N. O. *J. Fluorine Chem.* **1999**, *93*, 1.
(1384) Sharts, C. M. *J. Fluorine Chem.* **1998**, *90*, 197.
(1385) Jeanneaux, F.; Le Blanc, M.; Riess, J. G.; Yokoyama, K. *Nouv. J. Chim.* **1984**, *8*, 251.
(1386) Santini, G.; Le Blanc, M.; Riess, J. G. *Tetrahedron* **1973**, *29*, 2411.
(1387) Jeanneaux, F.; Santini, G.; Le Blanc, M.; Cambon, A.; Riess, J. G. *Tetrahedron* **1974**, *30*, 4197.
(1388) Brace, N. O. *J. Org. Chem.* **1973**, *38*, 3167.
(1389) Cecutti, C.; Rico, I.; Lattes, A.; Novelli, A.; Rico, A.; Marion, G.; Gracia, A.; Lachaise, J. *Europ. J. Med. Chem.* **1989**, *24*, 485.
(1390) Haddach, M.; Sapienza, J.; Sharts, C. M. *J. Labelled Compd. Radiopharm.* **1999**, *42*, 227.
(1391) Kopac, M. J. *Trans. N. Y. Acad. Sci.* **1955**, *17*, 257.
(1392) Howlett, S.; Dundas, D.; Sabiston, D. C. *Arch. Surg.* **1965**, *91*, 643.
(1393) Gessler, A. E.; Bender, C. E.; Parkinson, M. C. *Trans. N. Y. Acad. Sci.* **1956**, *18*, 701.
(1394) Gollan, F.; Clark, L. C. *Physiologist* **1966**, *9*, 191.
(1395) Geyer, R. P.; Monroe, R. G.; Taylor, E. *Fed. Proc.* **1968**, *27*, 384.
(1396) Sloviter, H. A.; Petkovic, M.; Ogoshi, S.; Yamada, H. *J. Appl. Physiol.* **1969**, *27*, 453.
(1397) Geyer, R. P. Proceedings of the Xth International Congress for Nutrition: Symposium on Perfluorochemical Artificial Blood, Kyoto 1975; Igakushobo Med. Publ.: Osaka, 1975; p 3.
(1398) Geyer, R. P. *Fed. Proc.* **1970**, *29*, 1758.
(1399) Clark, L. C.; Kaplan, S.; Becattini, F.; Benzing, G. *Fed. Proc.* **1970**, *29*, 1764.
(1400) Triner, L.; Verosky, M.; Habif, D. V.; Nahas, G. G. *Fed. Proc.* **1970**, *29*, 1778.
(1401) Clark, L. C.; Becattini, F.; Kaplan, S.; Obrock, V.; Cohen, D.; Becker, C. *Science* **1973**, *181*, 680.
(1402) Okamoto, H.; Yamanouchi, K.; Imagawa, T.; Murashima, R.; Yokoyama, K.; Watanabe, R.; Naito, R. Proceedings of the IInd Intercompany Conference, Osaka, 1973.
(1403) Naito, R.; Yokoyama, K. Proceedings of the Xth International Congress Nutrition−Symposium on Perfluorochemical Artificial Blood, Kyoto 1975; Igakushobo Med. Publ.: Osaka, 1975; p 55.
(1404) Green Cross Corp. *Perfluorochemical emulsions as O_2−CO_2 carrier, Fluosol-43, Fluosol-DC*; Green Cross Corp.: Osaka, Japan, 1975.
(1405) Riess, J. G.; Le Blanc, M. *Angew. Chem., Int. Ed. Engl.* **1978**, *17*, 621.
(1406) Riess, J. G. *La Transfusinone del Sangue* **1987**, *32*, 316.
(1407) Riess, J. G. *Vox Sang.* **1991**, *61*, 225.
(1408) Riess, J. G.; Krafft, M. P. *Adv. Exp. Med. Biol.* **1992**, *317*, 465.
(1409) Riess, J. G. *New J. Chem.* **1995**, *19*, 891.
(1410) Riess, J. G. In *Blood Compatible Materials and Devices: Prospectives Towards the 21st Century*; Sharma, C. P., Szycher, M., Eds.; Technomic Publ. Co.: Lancaster, 1991; Chapter 14, p 237.
(1411) Chubb, C. *Biol. Reprod.* **1985**, *33*, 854.
(1412) Lane, T. A.; Krukonis, V. *Transfusion* **1988**, *28*, 375.
(1413) Naito, R.; Yokoyama, K. HS Symposium−Karolinska Institute Research Center, Research on Perfluorochemicals in Medicine and Biology, Huddlinge: Sweden, 1978; p 41.
(1414) Naito, R.; Yokoyama, K. *Blood Substitutes and Plasma Expanders*; Alan Liss: New York, 1978; p 81.
(1415) Yokoyama, K.; Suyama, T.; Naito, R. In *Biomedical Aspects of Fluorine Chemistry*; Filler, R., Kobayashi, Y., Eds.; Elsevier: Amsterdam, 1982; p 191.
(1416) Ohyanagi, H. In *Artificial Red Cells*; Tsuchida, E., Ed.; John Wiley and Sons: New York, 1995; p 199.
(1417) Lutz, J.; Bäuml, M.; Schulze, H. G. Proceedings of the IVth International Symposium on Perfluorochemical Blood Substitutes, Kyoto, 1978; Excerpta Medica: Amsterdam, 1979; p 123.
(1418) Ochi, S.; Sasaki, Y.; Sakabe, H.; Wada, Y.; Nakaji, S.; Hashimoto, I. *Clin. Ther.* **1982**, *4*, 465.
(1419) Gould, S. A.; Sehgal, L. R.; Rosen, A. L.; Langdale, L. A.; Sehgal, H. L.; Krause, L.; Moss, G. S. *J. Trauma* **1983**, *23*, 720.
(1420) *Perfusion and Nonperfusion Methods of Myocardium Perfusion with Perfluorocarbon Emulsion*, Beloyartsev, F. F.; Islamov, B. I.; Mayevsky, E. I. *Acad. Sci. U.S.S.R.*: Pushshino **1983**, 1.
(1421) Ivanitski, G. R. *Perfluorocarbon*; Acad. Sci. U.S.S.R.: Pushchino, Russia, 1994.
(1422) Perfluorocarbon Emulsion, Stabilized by Proxanol: Vorobiev, S. I.; Ivanitski, G. R. *Russ. Acad. Sci. Inst. Theor. Exp. Biophys.* Pushshino **1994**, 3.
(1423) Chen, H. S.; Yang, Z. H. *Biomater., Artif. Cells, Immobilization Biotechnol.* **1988**, *16*, 403.
(1424) Yang, C. M.; Chen, H. S.; Lu, T. X.; Wu, X. T. XIIth Congress of the International Society on Artificial. Cells, Blood Substitutes, and Immobilization Biotechnology, Beijing, China, Sept. 1997.
(1425) Motta, G.; Ratto, G. B.; Sacco, A.; Canepa, G. *Period. Biol.* **1986**, *88*, 261.
(1426) Mitsuno, T.; Ohyanagi, H.; Yokoyama, K.; Suyama, T. *Biomater., Artif. Cells, Artif. Organs* **1988**, *16*, 365.
(1427) Biro, G. P. *Transf. Med. Rev.* **1993**, *7*, 84.
(1428) Tremper, K. K.; Levine, E. M.; Waxman, K. *Int. Anesthesiol. Clin.* **1985**, *23*, 185.
(1429) Spence, R. K.; McCoy, S.; Costabile, J.; Norcross, E. D.; Pello, M. J.; Alexander, J. B.; Wisdom, C.; Camishion, R. C. *Crit. Care Med.* **1990**, *18*, 1127.
(1430) Anderson, H.; Leimgruber, P.; Roubin, G.; Nelson, D.; Gruentzig, A. *Am. Heart J.* **1985**, *110*, 720.
(1431) Young, L. H.; Jaffe, C. C.; Revkin, J. H.; McNutty, P. H.; Cleman, M. *Am. J. Cardiol.* **1990**, *65*, 986.
(1432) Kerins, D. M. *Am. J. Med. Sci.* **1994**, *307*, 218.
(1433) Roberts, C. S.; Anderson, H. V.; Carboni, A. A.; Justicz, A. G. S.; Leimgruber, P. P.; Kloner, R. A.; Gruentzig, A. R. *Am. J. Cardiol.* **1986**, *57*, 1202.
(1434) Tokioka, H.; Miyazaki, A.; Fung, P.; Rajagopalan, R. E.; Kar, S.; Meerbaum, S.; Corday, E.; Drury, J. K. *Circulation* **1987**, *75*, 473.
(1435) Virmani, R.; Kolodgie, F. D.; Osmialowski, A.; Zimmerman, P.; Mergner, W.; Forman, M. B. *Am. Heart J.* **1988**, *116*, 421.
(1436) Cleman, M.; Jaffee, C. C.; Wohlgelernter, D. *Circulation* **1986**, *74*, 555.
(1437) Jaffe, C. C.; Wohlgelernter, D.; Cabin, H.; Bowman, L.; Deckelbaum, L.; Remetz, M.; Cleman, M. *Am. Heart J.* **1988**, *115*, 1156.
(1438) Bell, M. R.; Nishimura, R. A.; Holmes, D. R.; Bailey, K. R.; Schwartz, R. S.; Vlietstra, R. E. *J. Am. Coll. Cardiol.* **1990**, *16*, 959.
(1439) Cowley, M. J.; Snow, F. R.; DiSciascio, G.; Kelly, K.; Guard, C.; Nixon, J. V. *Circulation* **1990**, *81*, IV27.
(1440) Clark, L. C.; Wesseler, E. P.; Miller, M. L.; Kaplan, S. *Microvasc. Res.* **1974**, *8*, 320.
(1441) Geyer, R. P. In *Proceedings of the IVth International Symposium on Perfluorochemical Blood Substitutes*; Excerpta Medica: Amsterdam, 1979; p 3.
(1442) Geyer, R. P. In *Oxygen Carrying Colloidal Blood Substitutes*; Frey, R., Beisbarth, H., Stosseck, K., Eds.; Zuckschwerdt Verlag: München, 1982; p 19.
(1443) Sharma, S. K.; Lowe, K. C.; Davis, S. S. *Biomater., Artif. Cells, Artif. Organs* **1988**, *16*, 447.
(1444) Johnson, O. L.; Washington, C.; Davis, S. S. *Int. J. Pharm.* **1990**, *63*, 65.
(1445) Gross, U.; Rüdiger, S.; Kolditz, L.; Reichelt, H. *Mittl. Chem. Ges.* **1990**, *37*, 1461.
(1446) Dimitrov, A.; Radeck, W.; Rüdiger, S. *J. Fluorine Chem.* **1991**, *52*, 317.
(1447) Yokoyama, K.; Naito, R.; Tsuda, Y.; Fukaya, C.; Watanabe, M.; Hanada, S.; Suyama, T. *Prog. Clin. Biol. Res.* **1983**, *122*, 189.
(1448) Beloyartsev, F. F.; Mayevsky, E. I.; Islamov, B. I. *Ftorosan−Oxygen Carrying Perfluorochemical Plasma Substitute*; Acad. Sci. U.S.S.R.: Pushchino, 1983.
(1449) Lutz, J.; Kettemann, M.; Racz, J.; Noth, U. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1995**, *23*, 407.
(1450) Grec, J.-J.; Riess, J. G.; Devallez, B. *Nouv. J. Chim.* **1985**, *9*, 637.
(1451) Sloviter, H. A.; Yamada, H.; Ogoshi, S. *Fed. Proc.* **1970**, *29*, 1755.
(1452) Okamoto, H.; Yamanouchi, K.; Yokoyama, K. *Chem. Pharm. Bull.* **1975**, *23*, 1452.
(1453) Clark, L. C.; Wesseler, E. P.; Kaplan, S.; Miller, M. L.; Becker, C.; Emory, C.; Stanley, L.; Becattini, R.; Obrock, V. *Fed. Proc.* **1975**, *34*, 1468.
(1454) Clark, L. C.; Hoffmann, R. E.; Davis, S. L. *Biomater., Artif. Cells, Immobilization Biotechnol.* **1992**, *20*, 1085.
(1455) Mitten, R. M.; Burgan, A. R.; Hamblin, A.; Yee, G.; Long, D. C.; Long, D. M.; Mattrey, R. F. *Biomater., Artif. Cells, Artif. Organs* **1988**, *16*, 683.
(1456) Long, D. M.; Liu, M. I.; Szanto, P. S.; Alrenga, P. *Rev. Surg.* **1972**, *29*, 71.
(1457) Long, D. M.; Higgins, C. B.; Mattrey, R. F.; Mitten, R. M.; Multer, F. K. In *Preparation, Properties, and Industrial Applications of Organofluorine Compounds*; R. E. Banks, Ed.; Ellis Horwood: New York, 1982; p 139.
(1458) Mattrey, R. F. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1994**, *22*, 295.

(1459) Kabalnov, A. S.; Makarov, K. N.; Shchukin, E. D. *Colloids Surf.* **1992**, *62*, 101.
(1460) Weers, J. G. *J. Fluorine Chem.* **1993**, *64*, 73.
(1461) Hirschl, R. B.; Pranikoff, T.; Wise, C.; Overbeck, M. C.; Gauger, P.; Schreiner, R. J.; Dechert, R.; Bartlett, R. H. *J. Am. Med. Assoc.* **1996**, *275*, 383.
(1462) Wolf, G. L.; Rogowska, J.; Gazelle, G. S.; Halpern, E. F. *Lymphology* (*Suppl.*) **1994**, *27*, 261.
(1463) Mattrey, R. F.; Nemcek, A. A.; Shelton, R.; André, M. P.; Mitten, R. M.; Peterson, T. *Invest. Radiol.* **1990**, *25*, 915.
(1464) Leach, C. L.; Greenspan, J. S.; Rubenstein, D.; Shaffer, T. H.; Wolfson, M. R.; Jackson, J. C.; DeLemos, R.; Fuhrman, B. P. *N. Engl. J. Med.* **1996**, *335*, 761.
(1465) Wiedemann, H. P. *Pulm. Perspect.* **1998**, *15*, 1.
(1466) Steinhorn, D. M.; Papo, M. C.; Rotta, A. T.; Aljada, A.; Fuhrman, B. P.; Dandora, P. *J. Crit. Care* **1999**, *14*, 20.
(1467) Fuhrman, B. P.; Hernan, L. J.; Rotta, A. T. *New Horiz.* **1999**, *7*, 433.
(1468) Le, T. D.; Arlauskas, R. A.; Weers, J. G. *J. Fluorine Chem.* **1996**, *78*, 155.
(1469) Mukherji, B.; Sloviter, H. A. *Transfusion* **1991**, *31*, 324.
(1470) Varescon, C.; Arlen, C.; Le Blanc, M.; Riess, J. G. *J. Chim. Phys.* **1989**, *86*, 2111.
(1471) Krafft, M. P.; Postel, M.; Riess, J. G.; Ni, Y.; Pelura, T. J.; Hanna, G. K.; Song, D. *Biomater., Artif. Cells, Immobilization Biotechnol.* **1992**, *20*, 865.
(1472) Long, D. C.; Long, D. M.; Riess, J. G.; Follana, R.; Burgan, A.; Mattrey, R. F. In *Blood Substitutes*; Chang, T. M. S., Geyer, R. P., Eds.; Dekker: New York, 1989; p 441.
(1473) Riess, J. G.; Dalfors, J. L.; Hanna, G. K.; Klein, D. H.; Krafft, M. P.; Pelura, T. J.; Schutt, E. G. *Biomater., Artif. Cells, Artif. Organs* **1992**, *20*, 839.
(1474) Weers, J. G.; Liu, J.; Fields, T.; Resch, P.; Cavin, J.; Arlauskas, R. A. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1994**, *22*, 1175.
(1475) Kabalnov, A.; Weers, J.; Arlauskas, R.; Tarara, T. *Langmuir* **1995**, *11*, 2966.
(1476) Ingram, D. A.; Forman, M. B.; Murray, J. J. *J. Cardiovasc. Pharmacol.* **1993**, *22*, 456.
(1477) Putyatina, T. K.; Aprosin, U. D.; Afonin, N. I. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1994**, *22*, 1281.
(1478) Rotenberg, M.; Rubin, M.; Bor, A.; Meyuhas, D.; Talmon, Y.; Lichtenberg, D. *Biochim. Biophys. Acta* **1991**, *1086*, 265.
(1479) Westesen, K.; Wehler, T. *Colloids Surf. A* **1993**, *78*, 115.
(1480) Hyltander, A.; Sandström, S.; Lundholm, K. In *Submicron Emulsions in Drug Targeting and Delivery*; Benita, S., Ed.; Harwood Academic Publ.: Amsterdam, 1998; p 7.
(1481) Wretlind, A. In *Current Concepts of Parenteral Nutrition*; Greep, J. M., Socters, P. B., Wisdrop, R. I. C., Phaf, C. W. R., Fisher, J. E., Eds.; Nartinus Hijhoff: The Hague, 1977; p 273.
(1482) Redgrave, T. G.; Maranhao, R. C. *Biochim. Biophys. Acta* **1985**, *835*, 104.
(1483) *Phospholipids: Characterization, Metabolism, and Novel Biological Applications*; Cevc, G., Paltauf, F., Eds.; American Oil Chemists Society: Champaign, 1995.
(1484) Pelura, T. J.; Johnson, C. S.; Tarara, T. E.; Weers, J. G. *Biomater., Artif. Cells, Immobilization Biotechnol.* **1992**, *20*, 845.
(1485) Yoon, J. K.; Burgess, D. J. *Pharm. Dev. Technol.* **1996**, *1*, 333.
(1486) Washington, C. *Adv. Drug Delivery Rev.* **1996**, *20*, 131.
(1487) Song, D.; Pelura, T. J.; Liu, J.; Ni, Y. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1994**, *22*, 1299.
(1488) Tarara, T. E.; Malinoff, S. H.; Pelura, T. J. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1994**, *22*, 1287.
(1489) Schmolka, I. R. *J. Am. Oil. Chem. Soc.* **1977**, *54*, 110.
(1490) Schmolka, I. R. *Ann. N. Y. Acad. Sci.* **1994**, *720*, 93.
(1491) Stolnik, S.; Illum, L.; Davis, S. S. *Adv. Drug Delivery Rev.* **1995**, *16*, 195.
(1492) Reeve, L. E. In *Handbook of Biodegradable Polymers*; Domd, A., Kost, J., Wiseman, D., Eds.; Harwood Academic Publ: Amsterdam, 1997; p 231.
(1493) Clark, L. C.; Kaplan, S.; Becattini, F. *J. Thorac. Cardiovasc. Surg.* **1970**, *60*, 757.
(1494) Geyer, R. P. *Intl. Anesthesiol. Clin.* **1985**, *23*, 25.
(1495) Bentley, P. K.; Davis, S. S.; Johnson, O. L.; Lowe, K. C.; Washington, C. *J. Pharm. Pharmacol.* **1989**, *41*, 661.
(1496) Edwards, C. M.; Gambaretto, G. P.; Conte, L.; Lowe, K. C. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1999**, *27*, 171.
(1497) Yokoyama, K.; Y., K.; Suyama, T. *Life Chem. Rep.* **1983**, *2*, 35.
(1498) Johnson, O. L.; Washington, C.; Davis, S. S. *Int. J. Pharmacol.* **1990**, *59*, 131.
(1499) Wanka, G.; Hoffmann, H.; Ulbricht, W. *Colloid Polym. Sci.* **1990**, *268*, 8, 101.
(1500) Nakashima, K.; Anzai, T.; Fujimoto, Y. *Langmuir* **1994**, *10*, 658.
(1501) Lane, T. A.; Lamkin, G. E. *Blood* **1986**, *68*, 351.
(1502) Hong, F.; Shastri, K. A.; Logue, G. L.; Spaulding, M. B. *Transfusion* **1991**, *31*, 642.
(1503) Clark, L. C.; Becattini, F.; Kaplan, S. *Alabama J. Med. Sci.* **1972**, *9*, 16.
(1504) Ohyanagi, H.; Mitsuno, T. Proceedings of the Xth International Congress for Nutrition−Symposium on Perfluorochemical Artificial Blood, Kyoto, Japan, 1975; Igakushobo Med. Publ.: Osaka, 1975; p 21.
(1505) Ohyanagi, H.; Sekita, M.; Yokoyama, K.; Itoh, T.; Toshima, K.; Okamoto, M.; Kawa, Y.; Mitsuno, T. Proceedings of the IVth International Symposium Perfluorochemical Blood Substitutes, Kyoto, 1978; Exerpta Medica: Amsterdam, 1979; p 373.
(1506) Hoke, J. F.; Ravis, W. R.; Hankes, G. H.; Spano, J. *Res. Commun. Chem. Pathol. Pharmacol.* **1991**, *73*, 315.
(1507) Biro, G. P.; White, F. C.; Guth, B. D. *J. Cardiovasc. Pathol.* **1986**, *1*, 99.
(1508) Colman, R. W.; Chang, L. K.; Mukherji, B.; Sloviter, H. A. *J. Lab. Clin. Med.* **1980**, *95*, 553.
(1509) Mitsuno, T.; Ohyanagi, H.; Naito, R. In *Oxygen Carrying Colloidal Blood Substitutes*; Frey, R., Beisbarth, H., Stosseck, K., Eds.; Zuckschwerdt Verlag: München, 1982; p 30.
(1510) Kitazawa, M.; Ohnishi, Y. *Virchow Arch.* **1982**, *398*, 1.
(1511) Shakir, K. M. M.; Williams, T. J. *Prostaglandins* **1982**, *23*, 919.
(1512) Bucala, R.; Kawakami, M.; Cerami, A. *Science* **1983**, *201*, 965.
(1513) Virmani, R.; Warren, D.; Rees, R.; Fink, L. M.; English, D. *Transfusion* **1983**, *23*, 512.
(1514) Lane, T. A.; Lamkin, G. E. *Blood* **1984**, *64*, 400.
(1515) Virmani, R.; Fink, L. M.; Gunter, K.; English, D. *Transfusion* **1984**, *24*, 343.
(1516) Janco, R. L.; Virmani, R.; Morris, P. J.; Gunter, K. *Transfusion* **1985**, *25*, 578.
(1517) Wake, E. J.; Studzinski, G. P.; Bhandal, A. *Transfusion* **1985**, *25*, 73.
(1518) Williams, J. H.; Chen, M.; Drew, J.; Panigan, E.; Hosseini, S. *Proc. Soc. Exp. Biol. Med.* **1988**, *188*, 461.
(1519) Porter, C. J. H.; Moghimi, S. M.; Illum, L.; Davis, S. S. *FEBS Lett.* **1992**, *305*, 62.
(1520) Moghimi, S. M.; Muir, I. S.; Illum, L.; Davis, S. S.; Kolb-Bachofen, V. *Biochim. Biophys. Acta* **1993**, *1179*, 157.
(1521) Davis, S. S.; Illum, L. In *Advances in System Constructs*; Gregoriadis, G., McCormack, B., Poste, G., Eds.; Plenum Press: New York, 1995; p 183.
(1522) Selve, C.; Castro, B.; Leempoel, P.; Mathis, G.; Gartiser, T.; Delpuech, J.-J. *Tetrahedron* **1983**, *39*, 1313.
(1523) Gangoda, M.; Fung, B. M.; O'Rear, E. A. *J. Colloid Interface Sci.* **1987**, *116*, 230.
(1524) Fung, B. M.; O'Rear, E. A.; Afzel, J.; Frech, C. B.; Mamrosh, D. L.; Gangoda, M. In *Blood Substitutes*; Chang, T. M. S., Geyer, R. P., Eds.; Dekker: New York, 1989; p 439.
(1525) Gross, U.; Herbst, M.; Szekrényesy, T. *Tenside Surf. Deterg.* **1991**, *28*, 250.
(1526) Meinert, H.; Reuter, P.; Mader, J. *Biomater., Artif. Cells, Immob. Biotech* **1992**, *20*, 115.
(1527) Edwards, C. M.; Lowe, K. C.; Heptinstall, S.; Lucas, P.; Trabelsi, H.; Cambon, A. *Adv. Exp. Med. Biol.* **1997**, *428*, 489.
(1528) Clark, L. C.; Clark, E. W.; Moore, R. E.; Kinnett, D. G.; Inscho, E. I. *Prog. Clin. Biol. Res.* **1983**, *122*, 169.
(1529) Riess, J. G.; Arlen, C.; Greiner, J.; Le Blanc, M.; Manfredi, A.; Pace, S.; Varescon, C.; Zarif, L. In *Blood Substitutes*; Chang, T. M. S., Geyer, R. P., Eds.; Dekker: New York, 1989; p 421.
(1530) Greiner, J.; Riess, J. G.; Vierling, P. In *Organofluorine Compounds in Medicinal Chemistry and Biomedical Applications*; Filler, R., Kobayashi, Y., Yagupolskii, L., Eds.; Elsevier: New York, 1993; p 339.
(1531) Riess, J. G.; Krafft, M. P. *Biomaterials* **1998**, *19*, 1529.
(1532) Riess, J. G.; Greiner, J. *Carbohydr. Res.* **2000**, *327*, 147.
(1533) Riess, J. G.; Pace, S.; Zarif, L. *Adv. Mater.* **1991**, *3*, 249.
(1534) Riess, J. G. *J. Drug Targeting* **1994**, *2*, 455.
(1535) Le Blanc, M.; Riess, J. G.; Poggi, D.; Follana, R. *Pharm. Res.* **1985**, 246.
(1536) Mahé, A. M.; Manoux, J.; Valla, A.; Follana, R.; Zarif, L.; Greiner, J.; Vierling, P.; Riess, J. G. *Biomater., Artif. Cells., Immobilization Biotechnol.* **1992**, *20*, 1025.
(1537) Riess, J. G.; Frézard, F.; Greiner, J.; Krafft, M. P.; Santaella, C.; Wierling, P.; Zarif, L. In *Handbook of Nonmedical Applications of Liposomes. From Design to Microreactors*; Barenholz, Y., Lasic, D. D., Eds.; CRC Press: Boca Raton, 1996; Vol. III.; p 97.
(1538) Davis, S. S.; Round, H. P.; Purewal, T. S. *J. Colloid Interface Sci.* **1981**, *80*, 508.
(1539) Kabalnov, A. S.; Pertsov, A. V.; Aprosin, Y., D.; Shchukin, E. D. *Kolloidn Zh.* **1985**, *47*, 1048.
(1540) Rüdiger, S. *J. Fluorine Chem.* **1989**, *42*, 403.
(1541) Amelina, E. A.; Kumacheva, E. Z.; Pertsov, A. V.; Shchukin, E. D. *Colloid J. U.S.S.R., Engl. Transl.* **1990**, *52*, 216.
(1542) Kabalnov, A. S.; Shchukin, E. D. *Adv. Colloid Interface Sci.* **1992**, *38*, 69.
(1543) Weers, J. G. In *Modern Aspects of Emulsion Science*; Binks, B. P., Ed., The Royal Society of Chemistry; Cambridge, U.K., 1998; p 292.

(1544) Trevino, L.; Solé-Violan, L.; Daumur, P.; Devallez, B.; Postel, M.; Riess, J. G. *New J. Chem.* **1993**, *17*, 275.
(1545) Weers, J. G.; Arlauskas, R. A. *Langmuir* **1995**, *11*, 474.
(1546) Thompson (Lord Kelvin), W. *Proc. R. Soc. Edinburgh* **1870**, *7*, 63.
(1547) Lifshitz, I. M.; Slezov, V. V. *Soviet Phys. Jept.* **1959**, *35*, 331.
(1548) Wagner, C. *Z. Elektroch.* **1961**, *65*, 581.
(1549) Higuchi, W. I.; Misra, J. *J. Pharm. Sci.* **1962**, *51*, 459.
(1550) Kabalnov, A. S.; Aprosin, Y. D.; Pavlova-Verevkina, O. B.; Pertsov, A. V.; Shchukin, E. D. *Kolloidn. Zh. Acad. Nauk. U.S.S.R.* **1986**, *58*, 27.
(1551) Riess, J. G.; Greiner, J.; Abouhilale, S.; Milius, A. *Prog. Colloid Polym. Sci.* **1992**, *88*, 123.
(1552) Ni, Y.; Pelura, T.; Sklenar, T. A.; Kinner, R. A.; Song, D. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1994**, *22*, 1307.
(1553) Geyer, R. P. *N. Engl. J. Med.* **1973**, *289*, 1077.
(1554) Clark, L. C.; Moore, R. E. In *Biomedicinal Aspects of Fluorine Chemistry*; Filler, R., Kobayashi, Y., Eds.; Elsevier: Amsterdam, 1982; p 213.
(1555) Kabalnov, A. S.; Makarov, K. N.; Shcherbakova, O. V. *J. Fluorine Chem.* **1990**, *50*, 271.
(1556) Postel, M.; Riess, J. G.; Weers, J. G. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1994**, *22*, 991.
(1557) Oleksiak, C. B.; Habif, S. S.; Rosano, H. L. *Colloids Surf.* **1994**, *84*, 71.
(1558) Washington, C.; Athersuch, A.; Kynoch, D. J. *Int. J. Pharmacol.* **1990**, *64*, 217.
(1559) Chansiri, G.; Lyons, R. T.; Patel, M. V.; Hem, S. L. *J. Pharm. Sci.* **1999**, *88*, 454.
(1560) Tatulian, S. A. In *Phospholipids Handbook*; Cevc, G., Ed.; Dekker: New York, 1993; p 511.
(1561) Aronson, M. P. *Langmuir* **1989**, *5*, 494.
(1562) Helfrich, W. *Z. Naturforsch.* **1973**, *28*, 693.
(1563) Kabalnov, A. S.; Wennerström, H. *Langmuir* **1996**, *12*, 276.
(1564) Kabalnov, A. S. In *Modern Aspects of Emulsion Science*; Binks, B. P., Ed.; The Royal Society of Chemistry: Cambridge, UK, 1998; p 205.
(1565) Kabalnov, A. S.; Weers, J. G. *Langmuir* **1996**, *12*, 1931.
(1566) Tarara, T. E.; Dellamary, L. A.; Kabalnov, A.; Trevino, L.; Weers, J. G. *Abstracts of Papers*, 210th National Meeting of the American Chemical Society, Chicago, IL, 1995; American Chemical Society: Washington, DC, 1995.
(1567) Washington, C.; King, S. M.; Heenan, R. K. *J. Phys. Chem.* **1996**, *100*, 7603.
(1568) Parfenova, A. M.; Amelina, E. A.; Vitvitskii, V. M.; Makarov, K. N.; Gervits, L. L.; Shchukin, E. D. *Colloid J. U.S.S.R. Eugl. Transl.* **1991**, *52*, 700.
(1569) Davis, S. S.; Purewal, T.; Round, H. P. *J. Pharm. Pharmacol.* **1979**, *31*, 4P.
(1570) Kabalnov, A. S.; Pertzov, A. V.; Shchukin, E. D. *Colloid Surf.* **1987**, *24*, 19.
(1571) Edwards, C. M.; Lowe, K. C.; Rohlke, W.; Geister, U.; Reuter, P.; Meinert, H. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1997**, *25*, 255.
(1572) Weers, J. G.; Ni, Y.; Tarara, T. E.; Pelura, T. J.; Arlauskas, R. A. *Colloids Surf. A* **1994**, *84*, 81.
(1573) Sharts, C. M.; Malik, A. A.; Easdon, J. C.; Khawli, L. A. *J. Fluorine Chem.* **1987**, *34*, 365.
(1574) Riess, J. G. *Curr. Surg.* **1988**, *45*, 365.
(1575) Edwards, C. M.; Lowe, K. C.; Trabelsi, H.; Lucas, P.; Cambon, A. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1997**, *25*, 327.
(1576) Milius, A.; Greiner, J.; Riess, J. G. *New J. Chem.* **1992**, *16*, 771.
(1577) Zarif, L.; Manfredi, A.; Varescon, C.; Le Blanc, M.; Riess, J. G. *J. Am. Oil Chem. Soc.* **1989**, *66*, 1515.
(1578) Worah, D. M.; Kessler, D. R.; Meuter, A. R.; Huang, M.; Correas, J.-M.; Quay, S. C. *Drug Future* **1997**, *22*, 378.
(1579) Riess, J. G.; Solé-Violan, L.; Postel, M. *J. Dispersion Sci. Technol.* **1992**, *13*, 349.
(1580) Riess, J. G.; Cornélus, C.; Follana, R.; Krafft, M. P.; Mahé, A. M.; Postel, M.; Zarif, L. *Adv. Exp. Med. Biol.* **1994**, *345*, 227.
(1581) Cornélus, C.; Krafft, M. P.; Riess, J. G. *J. Colloid Interface Sci.* **1994**, *163*, 391.
(1582) Thomas, J.-L.; Marie, P.; Krafft, M. P. Manuscript in preparation.
(1583) Solé-Violan, L.; Devallez, B.; Postel, M.; Riess, J. G. *New J. Chem.* **1993**, *17*, 581.
(1584) Zarif, L.; Postel, M.; Septe, B.; Trevino, L.; Riess, J. G.; Mahé, A.-M.; Follana, R. *Pharm. Res.* **1994**, *11*, 122.
(1585) Rosano, H. L.; Gerbacia, W. E. U.S Pat. 3,778,381, 1973.
(1586) Mathis, G.; Leempoel, P.; Ravey, J. C.; Selve, C.; Delpuech, J.-J. *J. Am. Chem. Soc.* **1984**, *106*, 6162.
(1587) Mukerjee, P.; Mysels, K. J. In *Colloidal Dispersions and Micellar Behavior*; Mittal, K. L., Ed.; ACS Symposium Series 9; American Chemical Society: Washington, DC, 1975; p 239.
(1588) Kunieda, H.; Shinoda, K. *J. Phys. Chem.* **1976**, *80*, 2468.
(1589) Oliveros, E.; Maurette, M.-T.; Braun, A. M. *Helv. Chim. Acta* **1983**, *66*, 1183.
(1590) Robert, A.; Tondre, C. *J. Colloid Interface Sci.* **1984**, *98*, 515.
(1591) Ravey, J. C.; Stébé, M. J. *Prog. Colloid Polym. Sci.* **1987**, *73*, 127.
(1592) Chittofrati, A.; Sanguinetti, A.; Visca, M.; Kallay, N. *Colloids Surf.* **1992**, *63*, 219.
(1593) Schubert, K.-V.; Kaler, E. W. *Colloids Surf.* **1994**, *84*, 97.
(1594) Cecutti, C.; Novelli, A.; Rico, I.; Lattes, A. *J. Dispersion Sci. Technol.* **1990**, *11*, 115.
(1595) Lattes, A.; Rico-Lattes, I. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1994**, *22*, 1007.
(1596) Tadros, T. F.; Vincent, B. In *Encyclopedia of Emulsion Technology*; Becher, P., Ed.; Marcel Dekker: New York, 1983; p 129.
(1597) Walstra, P.; Smulders, P. E. A. In *Modern Aspects of Emulsion Science*; Binks, B. P., Ed.; The Royal Society of Chemistry: Cambridge, UK, 1998; p 56.
(1598) Korstvedt, H.; Nikopoulos, G.; Chandounet, S.; Siciliano, A. *Am. Paint Coating J.* **1985**, *22*, 38.
(1599) Lidgate, D. M.; Fu, R. C.; Fleitman, J. S. *BioPharm.* **1989**, *Oct.*, 28.
(1600) Rees, L. H. *Chem. Eng. News* **1974**, *May*, 87.
(1601) Chapman, K. W.; Keipert, P. E.; Graham, H. A. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1996**, *24*, 318.
(1602) Dalfors, J. L.; Espinosa, C. A. *Biomater., Artif. Cells, Immobilization Biotechnol.* **1992**, *20*, 853.
(1603) Apte, S.; Turco, S. *J. Parenter. Sci. Technol.* **1992**, *46*, 12.
(1604) Cornélus, C.; Krafft, M. P.; Riess, J. G. Proceedings of the 1st World Congress on Emulsion, Paris, 1993, *1*, 12−207.
(1605) Hanna, G. K.; Ojeda, M. C.; Sklenar, T. A. *Biomater., Artif. Cell, Immobilization Biotechnol.* **1992**, *20*, 849.
(1606) Cornélus, C.; Giulieri, F.; Krafft, M. P.; Riess, J. G. *Colloid Surf.* **1993**, *70*, 233.
(1607) Krafft, M. P.; Rolland, J.-P.; Riess, J. G. *J. Phys. Chem.* **1991**, *95*, 5673.
(1608) Ni, Y.; Klein, D. H.; Pelura, T. J. *Biomater., Artif. Cells, Immobilization Biotechnol.* **1992**, *20*, 869.
(1609) Thoolen, M. J. M. C.; Rasbach, D. E.; Shaw, J. H.; Raynolds, S.; Timmermans, P. B. M. W. M. *Biomater., Artif. Cells, Immobilization Biotechnol.* **1993**, *21*, 53.
(1610) Teicher, B. A.; Herman, T. S.; Jones, S. M. *Int. J. Radiat. Oncol. Biol. Phys.* **1990**, *19*, 945.
(1611) Gross, U.; Rüdiger, S.; Reichelt, H. *J. Fluorine Chem.* **1991**, *53*, 155.
(1612) Bell, R. D.; Frazer, G. D.; Osterholm, J. L.; Duckett, S. W. *Stroke* **1991**, *22*, 80.
(1613) Lutz, J.; Krafft, M. P. *Adv. Exp. Med. Biol.* **1997**, *411*, 391.
(1614) Menasché, P.; Grousset, C.; Mousset, C.; Moore, R. E.; Piwnica, D. A. *Biomater., Artif. Cells, Artif. Organs* **1988**, *16*, 607.
(1615) Johnson, D. L.; Greene, P. S.; Gott, V. L.; Gardner, T. J. *Circulation, Suppl. III* **1988**, *78*, 153.
(1616) Gross, U.; Kolditz, L.; Papke, G.; Rüdiger, S. *J. Fluorine Chem.* **1991**, *53*, 163.
(1617) Segel, L. D.; Minten, J. M. O.; Schweighardt, F. K. *Am J. Physiol.* **1992**, *263*, H730.
(1618) Sanchez, V.; Zarif, L.; Greiner, J.; Riess, J. G.; Cippolini, S.; Bruneton, J. N. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1994**, *22*, 1421.
(1619) Sanchez, V.; Manfredi, A.; Greiner, J.; Riess, J. G. *Bull. Soc. Chim. Fr.* **1994**, *131*, 648.
(1620) Vorobyev, S. I.; Ivanitsky, G. R. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1996**, *24*, 454.
(1621) Voiglio, E. J.; Zarif, L.; Gorry, F. C.; Krafft, M. P.; Margonari, J.; Martin, X.; Riess, J.; Dubernard, J. M. *J. Surg. Res.* **1996**, *63*, 439.
(1622) Mathy-Hartert, M.; Deby, C.; Krafft, M. P.; Deby, G.; Bradfer, J. J.; DeRoover, A.; Lamy, M. *Transplantation*, in press.
(1623) Bouley, L.; Krafft, M. P.; Dutoit, P.; Bercik, P.; Riess, J. G.; Kucera, P. VII International Symposium on Blood Substitutes, Tokyo, 1997.
(1624) Sjolund, M.; Lindblom, G.; Rilfors, L.; Arvidson, G. *Biophys. J.* **1987**, *52*, 145.
(1625) Kabalnov, A.; Tarara, T.; Arlauskas, R.; Weers, J. *J. Colloid Interface Sci.* **1996**, *184*, 227.
(1626) Rydhag, L.; Wilton, I. *J. Am. Oil Chem. Soc.* **1981**, *58*, 830.
(1627) Friberg, S.; Jansson, P. O.; Cederberg, E. *J. Colloid Interface Sci.* **1976**, *55*, 614.
(1628) Groves, M. J.; Wineberg, M.; Brain, A. P. R. *J. Disp. Sci. Technol.* **1985**, *6*, 237.
(1629) Handa, T.; Saito, H.; Miyajima, K. *Biochemistry* **1990**, *29*, 2884.
(1630) Postel, M.; Chang, P.; Rolland, J. P.; Krafft, M. P.; Riess, J. G. *Biochim. Biophys. Acta* **1991**, *1086*, 95.
(1631) Habif, S. S.; Normand, P. E.; Oleksiak, C. B.; Rosano, H. L. *Biotechnol. Prog.* **1992**, *8*, 454.
(1632) Westesen, L.; Wehler, T. *Colloids Surf. A* **1993**, *78*, 125.
(1633) Meinert, H.; Reuter, P.; Rohlke, W.; Cambon, A.; Szonyi, S.; Gaysinski, M. *J. Fluorine Chem.* **1994**, *66*, 203.
(1634) Rosenberg, M. D. *Fed. Proc.* **1971**, *30*, 1623.
(1635) Gross, U.; Rüdiger, S. *J. Fluorine Chem.* **1994**, *69*, 31.
(1636) Gross, U.; Reichelt, H.; Draffehn, J. *Biomater., Artif. Cells, Immobilization Biotechnol.* **1992**, *20*, 831.

(1637) Yamanouchi, K.; Murashima, R.; Yokoyama, K. *Chem. Pharm. Bull.* **1975**, *23*, 1363.
(1638) Mallet-Martino, M. C.; Betbeder, D.; Lattes, A.; Lopez, A.; Martino, R.; François, G.; Cros, S. *J. Pharm. Pharmacol.* **1984**, *36*, 556.
(1639) Klein, D. H.; Jones, R. C.; Keipert, P. E.; Luena, G. A.; Otto, S.; Weers, J. G. *Colloid Surf. A* **1994**, *84*, 89.
(1640) Noth, U.; Jäger, L. J. E.; Lutz, J.; Haase, A. *Magn. Reson. Imaging* **1994**, *12*, 149.
(1641) Zarif, L.; Postel, M.; Trevino, L.; Riess, J. G.; Valla, A.; Follana, R. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1994**, *22*, 1193.
(1642) Sklifas, A. N.; Shekhtman, D. G.; Obraztsov, V. V.; Vorobiev, S. I.; Temnova, I. V. *Biophysics* **1998**, *43*, 149.
(1643) Audran, M.; Krafft, M. P.; De Ceaurriz, J.; Mathurin, J.-C.; Sicart, M.-T.; Marion, B.; Fabre, F.; Bressolle, F. *J. Chromatogr. B* **1999**, *734*, 267.
(1644) Audran, M.; Krafft, M. P.; De Ceaurriz, J.; Maturin, J.-C.; Sicart, M.-T.; Marion, B.; Bougard, G.; Bressole, F. *J. Chromatogr. B* **2000**, *745*, 333.
(1645) Yokoyama, K.; Yamanouchi, K.; Murashima, R. *Chem. Pharm. Bull.* **1975**, *23*, 1368.
(1646) Yokoyama, K.; Yamanouchi, K.; Ohyanagi, H.; Mitsuno, T. *Chem. Pharm. Bull.* **1978**, *26*, 956.
(1647) Lutz, J.; Stark, M. *Pflügers Arch.* **1987**, *410*, 181.
(1648) Ravis, W. R.; Hoke, J. F.; Parsons, D. L. *Drug Metabol. Rev.* **1991**, *23*, 375.
(1649) Obraztsov, V. V.; Kabalnov, A. S.; Sklifas, A. N.; Makarov, K. N. *Biofizika* **1992**, *37*, 379.
(1650) Ni, Y.; Klein, D. H.; Song, D. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1996**, *24*, 81.
(1651) Obraztsov, V. V.; Kabalnov, A. S.; Sklifas, A. N.; Makarov, K. N. *Biophysics* **1992**, *37*, 298.
(1652) Tsuda, Y.; Yamanouchi, K.; Okamoto, H.; Yokoyama, K.; Heldebrant, C. *J. Pharmacobio-Dyn.* **1990**, *13*, 165.
(1653) Yokoyama, K.; Watanabe, M.; Naito, R. In *Oxygen Carrying Colloidal Blood Substitutes (Mainz, March 1981)*; Frey, R., Beisbarth, H., Stosseck, K., Eds.; Zuckschwerdt Verlag: München, 1982; p 214.
(1654) Lutz, J.; Metzenauer, P.; Kunz, E.; Heine, W. D. In *Oxygen Carrying Colloidal Blood Substitutes*; Frey, R., Beisbarth, H., Stosseck, K., Eds.; Zuckschwerdt Verlag: München, 1982; p 73.
(1655) Pfannkuch, F.; Schnoy, N. *Prog. Clin. Biol.* **1983**, *122*, 209.
(1656) McGoron, A. J.; Pratt, R.; Zhang, J.; Shiferaw, Y.; Thomas, S.; Millard, R. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1994**, *22*, 1243.
(1657) Illum, L.; West, P.; Washington, C.; Davis, S. S. *Intl. J. Pharmaceutics* **1989**, *54*, 41.
(1658) Carstensen, H.; Müller, R. H.; Müller, B. W. *Clin. Nutr.* **1992**, *11*, 289.
(1659) Sloviter, H. A.; Mukherji, B. *Prog. Clin. Biol. Res.* **1983**, *122*, 181.
(1660) Kong, C. F.; Fung, B. M.; O'Rear, E. A. *J. Phys. Chem.* **1985**, *89*, 4386.
(1661) *Proceedings of the IVth International Symposium on Perfluorochemical Blood Substitutes*, Kyoto, 1978; Excerpta Medica: Amsterdam, 1979.
(1662) Hamilton, P. B.; Farr, L. E.; Hiller, A.; Van Slyke, D. *J. Exp. Med.* **1947**, *86*, 455.
(1663) *Advances in Blood Substitute Research*; Bolin, R. B., Geyer, R. P., Nemo, G. J., Eds.; A. R. Liss: New York, 1983; *Prog. Clin Biol. Med*. **1983**, *122*.
(1664) Perfluorochemical Oxygen Transport. In *International Anesthesiology Clinic*; Tremper, K. K., Ed.; Little, Brown and Co.: Boston, 1985; Vol. 23.
(1665) Gould, S. A.; Rosen, A. L.; Sehgal, L. R.; Sehgal, H. L.; Rice, C. L.; Moss, G. S. *J. Trauma* **1982**, *22*, 736.
(1666) Vogel, H.; Gunther, H.; Harrison, D. K.; Anderer, W.; Kessler, M.; Peter, K. *Adv. Exp. Med. Biol.* **1989**, *248*, 653.
(1667) Biro, G. P. *Int. Anesthesiol. Clin.* **1985**, *23*, 143.
(1668) Light, R. B.; Perez-Padilla, R.; Kryger, M. H. *Chest* **1987**, *91*, 444.
(1669) Tremper, K. K.; Friedman, A. E.; Levine, E. M.; Lapin, R.; Camarillo, D. *N. Engl. J. Med.* **1982**, *307*, 277.
(1670) Mitsuno, T.; Ohyanagi, H.; Naito, R. *Ann. Surg.* **1982**, *195*, 60.
(1671) Cernaianu, A. C.; Spence, R. K.; Vassilidze, T. V.; Gallucci, J. G.; Gaprindashvili, T.; Olah, A.; Weiss, R. L.; Cilley, J. H.; Keipert, P. E.; Faithfull, N. S.; DelRossi, A. J. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1994**, *22*, 965.
(1672) Keipert, P. E.; Faithfull, N. S.; Bradley, J. D.; Hazard, D. Y.; Hogan, J.; Levisetti, M. S.; Peters, R. M. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1994**, *22*, 1161.
(1673) Flaim, S. F. In *Blood Substitutes: New Frontiers*; Winslow, R. M., Vandegriff, K. D., Intaglietta, M., Eds.; Birkhäuser: Boston, 1997; p 91.
(1674) Habler, O. P.; Kleen, M. S.; Hutter, J. W.; Podtschaske, A. H.; Tiede, M.; Kemming, G. I.; Welte, M. V.; Corso, C. O.; Batra, S.; Keipert, P. E.; Faithfull, N. S.; Messmer, K. F. W. *Transfusion* **1998**, *38*, 135; *Res. Exp. Med.* **1998**, *197*, 301.
(1675) Keipert, P. E.; Faithfull, N. S.; Bradley, J. D.; Hazard, D. Y.; Hogan, J.; Levisetti, M. S.; Peters, R. M. *Adv. Exp. Med. Biol.* **1994**, *345*, 197.
(1676) Goodin, T. H.; Grossbard, E. B.; Kaufman, R. J.; Richard, T. J.; Kolata, R. J.; Allen, J. S.; Layton, T. E. *Crit. Care Med.* **1994**, *22*, 680.
(1677) Kaufman, R. J. In *Emulsions—A Fundamental and Practical Approach*; Sjoblom, J., Ed.; Kluwer Academic Publ.: Boston, 1992; p 207.
(1678) Stern, S. A.; Dronen, S. C.; McGoron, A. J.; Wang, X.; Chaffins, K.; Millard, R.; Keipert, P. E.; Faithfull, N. S. *Am. J. Emerg. Med.* **1995**, *13*, 269.
(1679) Manning, J. E.; Batson, D. N.; Payne, F. B.; Adam, N.; Murphy, C. A.; Perretta, S. G.; Norfleet, E. A. *Ann. Emerg. Med.* **1997**, *29*, 580.
(1680) Ogilby, J. D.; Noma, S.; DiLoretto, G.; Stets, G. *Biomater., Artif. Cells, Immobilization Biotechnol.* **1992**, *20*, 973.
(1681) Zweier, cited in ref 218, p 91.
(1682) del Balzo, U.; Harrel, R. A.; Duggan, M. T.; Flaim, S. F. *Ann. Thorac. Surg.* **1995**, *60*, 866.
(1683) del Balzo, U.; Strnat, C. A.; Harrell, R. A.; Flaim, S. F. *J. Invest. Med.* **1997**, in press.
(1684) Briceño, J. C.; Rincón, I. E.; Vélez, J. F.; Castro, I.; Marcos, M. I.; Velásquez, C. E. *Am. Soc. Artif. Int. Organs J.* **1999**, *45*, 322.
(1685) Guo, J.; White, J.; Batjer, H. *Neurosurgery* **1995**, *36*, 350.
(1686) Padnick, L. B.; Linsenmeier, R. A.; Goldstick, T. K. *Am. Physiol. Soc.* **1999**, *86*, 1497.
(1687) Waschke, K. F.; Riedel, M.; Albrecht, D. M.; van Ackern, K.; Kuschinsky, W. *Anesth. Analg.* **1994**, *79*, 874.
(1688) Mosca, R. S.; Rohs, T. J.; Waterford, R. R.; Childs, K. F.; Brunsting, L. A.; Bolling, S. F. *Surgery* **1996**, *120*, 197.
(1689) Symons, J. D.; Sun, X.; Flaim, S. F.; del Balzo, U. *J. Cardiovasc. Pharmacol.* **1999**, *34*, 108.
(1690) Sostman, H. D.; Rockwell, S.; Sylvia, A. L.; Madwed, D.; Cofer, G.; Charles, H. C.; Negro-Vilar, R.; Moore, D. *Magn. Reson. Med.* **1991**, *20*, 253.
(1691) Teicher, B. A.; Holden, S. A.; Ara, G.; Ha, C. S.; Herman, T. S.; Northey, D. *J. Cancer Res. Clin. Oncol.* **1992**, *118*, 509.
(1692) Evans, R. G.; Kimler, B. F.; Morantz, R. A.; Batnitzky, S. *Intl. J. Radiat. Oncol. Biol. Phys.* **1993**, *26*, 649.
(1693) Rockwell, S. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1994**, *22*, 1097.
(1694) Teicher, B. A. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1995**, *23*, 395.
(1695) Stern, S.; Guichard, M. *Radiother. Oncol.* **1996**, *41*, 143.
(1696) Fabry, M. E.; Does, M. D.; Suzuka, S. M.; Nagel, R. L. VIIIth International Symposium on Blood Substitutes, San Diego, 2000.
(1697) Harrison, D. K.; Günther, H.; Vogel, H.; Ellermann, R.; Brunner, M.; Höper, J.; Kessler, M. *Adv. Exp. Med. Biol.* **1985**, *191*, 445.
(1698) Zaritskii, A. R.; Kuznetsova, I. N.; Perevedentseva, E. V.; Fok, M. V. *Russian J. Phys. Chem.* **1993**, *67*, 533.
(1699) Perevedentseva, E. V.; Zaritskiy, A. R.; Fok, M. V.; Kutzetnova, I. N. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1998**, *26*, 223.
(1700) Burgan, A. R.; Herrick, W. C.; Long, D. M.; Long, D. C. *Biomater., Artif. Cells, Artif. Organs* **1988**, *16*, 681.
(1701) Northoff, H.; Haidmann, L.; Hansch, G.; Reuter, P.; Mader, J.; Meinert, H. *Infusionstherapie* **1992**, *19*, 115.
(1702) Vercellotti, G. M.; Hammerschmidt, D. E. *Int. Anesthesiol. Clin.* **1985**, *23*, 47.
(1703) Forman, M. B.; Puett, D. W.; Wilson, B. H.; Vaughn, W. K.; Friesinger, G. C.; Virmani, R. *J. Am. Coll. Cardiol.* **1987**, *9*, 1082.
(1704) Mattrey, R. F.; Hilpert, P. L.; Long, C. D.; Long, D. M.; Mitten, R. M.; Peterson, T. *Crit. Care Med.* **1989**, *17*, 652.
(1705) Rosoff, J. D.; Soltow, L. O.; Vocelka, C. R.; Schmer, G.; Chandler, W. L.; Cochran, R. P.; Kunzelman, K. S.; Spiess, B. D. *J. Cardiothorac. Vasc. Anesth.* **1998**, *12*, 397.
(1706) McCoy, L. E.; Becker, C. A.; Goodin, T. H.; Barnhart, M. I. *Scan. Electron Microsc.* **1984**, *1*, 311.
(1707) Lane, T.; Smith, D.; Wancewitz, E.; Funk, R. *Biomater., Artif. Cells, Immobilization Biotechnol.* **1992**, *21*, 1051.
(1708) Tsai, A. G.; Nolte, D.; Messmer, K.; Intaglietta, M. *Biomater., Artif. Cells, Immobilization Biotechnol.* **1992**, *20*, 959.
(1709) Nolte, D.; Pickelmann, S.; Lang, M.; Keipert, P.; Messmer, K. *Anesthesiology.* **2000**, *93*, 1261.
(1710) Mathy-Hartert, M.; Krafft, M. P.; Deby, C.; Deby-Dupont, G.; Meurisse, M.; Lamy, M.; Riess, J. G. *Artif. Cells, Blood. Subst., Immobilization Biotechnol.* **1997**, *25*, 563.
(1711) Woods, C. M.; Neslund, G.; Kornbrust, E.; Flaim, S. F. *Am. J. Physiol.* **2000**, *278*, L1008.
(1712) Obraztsov, V. V.; Neslund, G. G.; Kornbrust, E. S.; Flaim, S. F.; Woods, C. M. *Am. J. Physiol.* **2000**, *278*, L1018.

(1713) Bruneton, J. N.; Falewee, M. N.; François, E.; Cambon, P.; Riess, J. G.; Balu-Maestro, C.; Rogopoulos, A. *Radiology* **1989**, *170*, 179.
(1714) Behan, M.; O'Connel, D.; Mattrey, R. F.; Carney, D. N. *Am. J. Radiol.* **1993**, *160*, 399.
(1715) Cernaianu, A. C.; Spence, R. K.; Vassilidze, T. V.; DelRossi, A. J.; Carrig, T.; White, P. F.; Nathanson, M.; Okonkwo, N.; Wahr, J.; Faithfull, N. S.; Keipert, P. E.; Flaim, K. E. *Anesthesiology* **1994**, *81*, A397.
(1716) Flaim, S. F.; Hazard, D. R.; Hogan, J.; Peters, R. M. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1994**, *22*, 1511.
(1717) Dahl, P. E.; Østerud, B.; KjÆve, J. C. *Clin. Nutr.* **1992**, *11*, 269.
(1718) Loughrey, H. C.; Bally, M. B.; Reinish, L. W.; Cullis, P. R. *Thromb. Haemostasis* **1990**, *64*, 172.
(1719) Waddell, W. R.; Geyer, R. P.; Olsen, F. R.; Andrus, S. B.; Stare, F. J. *J. Lab. Clin. Med.* **1955**, *45*, 697.
(1720) Schuberth, O.; Wretlind, A. *Acta Chir. Scand.* **1961**, *278S*, 3.
(1721) Lucks, J. S. In *Pharmaceutical Emulsions and Suspensions*; Nielloud, F., Marti-Mestres, G., Eds.; Dekker: New York, 2000; p 229.
(1722) Flaim, S. F.; Hazard, D. R.; Hogan, J.; Peters, R. M. *Invest. Radiol.* **1991**, *26*, S122.
(1723) Fujita, T.; Sumaya, T.; Yokoyama, K. *Eur. Surg. Res.* **1973**, *3*, 436.
(1724) Yokoyama, K.; Yamanouchi, K. Y.; Watanabe, M.; Matsumoto, T.; Murashima, R.; Daimoto, T.; Hamano, T.; Okamoto, H.; Suyama, T.; Watanabe, R.; Naito, R. *Fed. Proc.* **1975**, *34*, 1478.
(1725) Keipert, P. E.; Otto, S.; Flaim, S. F.; Weers, J. G.; Schutt, E. A.; Pelura, T. J.; Klein, D. H.; Yaksh, T. L. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1994**, *22*, 1169.
(1726) Senior, J.; Crawley, J. C. W.; Gregoriadis, G. *Biochim. Biophys. Acta* **1985**, *839*, 1.
(1727) Leese, P. T.; Noveck, R. J.; Shorr, J. S.; Woods, C. M.; Flaim, K. E.; Keipert, P. E. *Anesth. Analg.* **2000**, *91*, 804.
(1728) Spahn, D. R. European Society of Anesthesiology Annual Meeting, Göteborg, Sweden, April 2001.
(1729) Lutz, J.; Metzenauer, P. *Pflügers Arch.* **1980**, *387*, 175.
(1730) Lutz, J.; Augustin, A. J.; Schwegler, J. S.; Milz, J. *Life Sci.* **1992**, *50*, 1503.
(1731) Fujita, T.; Suzuki, C.; Ogawa, R. *Prog. Clin. Biol. Res.* **1983**, *122*, 265.
(1732) Geyer, R. P. *Prog. Clin. Biol. Res.* **1983**, *122*, 157.
(1733) Mitsuno, T.; Ohyanagi, H.; Yokoyama, K. *Artif. Organs* **1984**, *8*, 25.
(1734) Castro, O.; Reindorf, C. A.; Socha, W. W.; Rowe, A. W. *Int. Arch. Allergy Appl. Immunol.* **1983**, *70*, 88.
(1735) Bottalico, L. A.; Betensky, H. T.; Min, Y. B.; Weinstock, S. B. *Hepatology* **1991**, *14*, 169.
(1736) Lutz, J.; Barthel, U.; Metzenauer, P. *Circ. Shock* **1982**, *9*, 99.
(1737) Mitsuno, T.; Tabuchi, Y.; Ohyanagi, H.; Sugiyama, T. *Prog. Clin. Biol. Res.* **1983**, *122*, 257.
(1738) Lutz, J.; Wagner, M. *Artif. Organs* **1984**, *8*, 41.
(1739) Mishin, V. M.; Obraztsov, V. V.; Grishanova, A. Y.; Gutkina, N. I.; Shekhtman, D. G.; Khatsenko, O. G.; Lyakhovich, V. V. *Chem-Biol. Interact.* **1989**, *72*, 143.
(1740) Obraztsov, V. V.; Kabalnov, A. S.; Makarov, K. N.; Gross, U.; Radeck, W.; Rüdiger, S. *J. Fluorine Chem.* **1993**, *63*, 101.
(1741) West, L.; McIntosh, N.; Gendler, S.; Seymour, C.; Wisdom, C. *Int. J. Radiat. Oncol. Biol. Phys.* **1986**, *12*, 1319.
(1742) Jäger, L. J. E.; Lutz, J. *Adv. Exp. Med. Biol.* **1994**, *345*, 221.
(1743) Koester, M.; Lutz, J. *Adv. Exp. Med. Biol.* **1994**, *345*, 215.
(1744) Sobrado, J.; Moldawer, L. L.; Pomposelli, J. J.; Mascioli, E. A.; Babyan, V. K.; Bistrian, B. R.; Blackburn, G. L. *Am. J. Clin. Nutr.* **1985**, *42*, 855.
(1745) Davis, S. S.; Illum, L.; Muller, R.; Landry, F.; Wright, F.; Harper, G. *Clin. Nutr.* **1990**, *9*, 260.
(a) Juliano, R. L. *Adv. Drug Delivery Res.* **1988**, *2*, 31.
(1746) Sehgal, L. R.; Sehgal, H. L.; Rosen, A. L.; Gould, S. A.; Moss, G. S. *Crit. Care Med.* **1984**, *12*, 907.
(1747) Rouillon, J. D.; Toubin, G.; Moussard, C.; Magnin, P. *Pathol. Biol.* **1985**, *33*, 57.
(1748) Sehgal, L. R.; Sehgal, H. L.; Rosen, A. L.; Gould, S. A.; Moss, G. S. *Arztl. Lab.* **1984**, *30*, 55.
(1749) Shepherd, A. P.; Steinke, J. M. *Clin. Chem.* **1998**, *44*, 2183.
(1750) Toffaletti, J. G.; Wildermann, R. F. *Clin. Chem.* **2000**, *46*, 136.
(1751) Cuignet, O. Y.; Wood, B. L.; Chandler, W. L.; Spiess, B. D. *Anesth. Analg.* **2000**, *90*, 517.
(1752) Obraztsov, V. V.; Woods, C. M. VIIIth International Symposium on Blood Substitutes, San Diego, 2000.
(1753) Hirlinger, W. K.; Grunert, A.; Herrmann, M.; Petutschnigk, D.; Langer, K. *Anaesthesist* **1982**, *31*, 660.
(1754) Clark, L. C.; Hoffmann, R. E.; Spokane, R. B.; Winston, P. E. *Mater. Res. Soc. Symp. Proc.* **1989**, *110*, 129.
(1755) Schutt, E.; Barber, P.; Fields, T.; Flaim, S.; Horodniak, J.; Keipert, P.; Kinner, R.; Kornbrust, L.; Leakakos, T.; Pelura, T.; Weers, J.; Houmes, R.; Lachmann, B. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1994**, *22*, 1205.
(1756) Obraztsov, V. V.; Tarakhovskii, Y. S.; Ponomarchuk, V. V.; Sklifas, S. I. *Biophysics* **1994**, *39*, 745.
(1757) Leakakos, T.; Schutt, E. G.; Cavin, J. C.; Smith, D.; Bradley, J. D.; Strnat, C. A.; Del Balzo, U.; Hazard, D. Y.; Otto, S.; Fields, T. K.; Keipert, P. E.; Klein, D. H.; Flaim, S. F. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1994**, *22*, 1199.
(1758) Millard, R. W.; McGoron, A. J. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1994**, *22*, 1251.
(1759) Wahr, J. A.; Trouwborst, A.; Spence, R. K.; Henny, C. P.; Cernaianu, A. C.; Graziano, G. P.; Tremper, K. K.; Flaim, K. E.; Keipert, P. E.; Faithfull, N. S.; Clymer, J. J. *Anesth. Analg.* **1996**, *82*, 103.
(1760) Burkard, M. E.; Van Liew, H. D. *Am. Physiol. Soc.* **1994**, *77*, 2874.
(1761) Liew, H. D. V.; Burkard, M. E. *Adv. Exp. Med. Biol.* **1997**, *411*, 395.
(1762) Flaim, S. F. In *Red Blood Cell Substitutes*; Rudolph, A. S., Rabinovici, R., Feuerstein, G. Z., Eds.; Dekker: New York, 1998; p 437.
(1763) Faithfull, N. S. *Adv. Exp. Med. Biol.* **2001**.
(1764) Monk, T.; Winston, R.; Wahr, J.; Frei, D.; Wang, J.; Keipert, P. *Anesth. Analg.* **1998**, *86*, S142.
(1765) Ménasché, P.; Pinard, E.; Desroches, A.-M.; Seylaz, J.; Laget, P.; Geyer, R. P.; Piwnica, A. *Ann. Thorac. Surg.* **1992**, *54*, 392.
(1766) Cochran, R. P.; Kunzelman, K. S.; Vocelka, C. R.; Akimoto, H.; Thomas, R.; Soltow, L. O.; Spiess, B. D. *Ann. Thorac. Surg.* **1997**, *63*, 1326.
(1767) Pugsley, W.; Klinger, L.; Paschalis, C.; Treasure, T.; Harrison, M.; Newman, S. *Stroke* **1994**, *25*, 1393.
(1768) Selnes, O. A.; Goldsborough, M. A.; Borowicz, L. M.; Mckhann, G. M. *Lancet* **1999**, *353*, 1601.
(1769) Ménasché, P.; Pinard, E.; Desroches, A.-M.; Seylaz, J.; Laget, P.; Geyer, R. P.; Piwnica, A. *Ann. Thorac. Surg.* **1985**, *40*, 494.
(1770) Spiess, B. D.; Braverman, B.; Woronowicz, A. W.; Ivankovich, A. D. *Stroke* **1986**, *17*, 1146.
(1771) Spiess, B. D.; McCarthy, R. J.; Tuman, K. J.; Ivankovich, A. D. *J. Cardiothorac. Anesth.* **1987**, *1*, 210.
(1772) Spiess, B. D.; Cochran, R. P. *J. Cardiothorac. Vasc. Surg.* **1996**, *10*, 83.
(1773) McDonagh, P. F.; Cerney, K.; Hokama, J.; Lai, G.; Gonzales, R. F.; Davis-Gorman, G.; Copeland, J. G. *J. Surg. Res.* **2001**, *99*, 7.
(1774) Amory, D. W.; Leone, B.; Croughwell, N. D.; White, W. D.; Richardson, K. M.; Baudet, B.; Osgood, C. F.; Gerstle, L.; Newman, M. F. *Anesth. Analg.* **1998**, *86*, SCA6.
(1775) Kloner, R. A.; Hale, S. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1994**, *22*, 1069.
(1776) Ogilby, J. D. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1994**, *22*, 1083.
(1777) Glogar, D. H.; Kloner, R. A.; Muller, J.; DeBoer, W. V.; Braunwald, E.; Clark, L. C. *Science* **1981**, *211*, 1439.
(1778) Kolodgie, F. D.; Dawson, A. K.; Roden, D. M.; Forman, M. B.; Virmani, R. *Am. Heart J.* **1986**, *112*, 1192.
(1779) Faithfull, N. S.; Fennema, M.; Erdmann, W. *Br. J. Anaesth.* **1988**, *60*, 773.
(1780) Nguyen, P. D.; O'Rear, E. A.; Fung, B. M. *Biomater., Artif. Cells, Artif. Organs* **1989**, *17*, 245.
(1781) Pearl, J. M.; Laks, H.; Drinkwater, D. C.; Meneshian, A.; Martin, S. M.; M.Curzan; Chang, P. A. *Ann. Thorac. Surg.* **1992**, *54*, 1144.
(1782) Acar, C.; Partington, M. T.; Buckberg, G. D. *J. Thorac. Cardiovasc. Surg.* **1991**, *101*, 284.
(1783) Braunwald, E.; Kloner, R. A. *J. Clin. Invest.* **1985**, *76*, 1713.
(1784) Forman, M. B.; Ingram, D. A.; Murray, J. J. *Clin. Hemorheol.* **1991**, *12*, 121.
(1785) Martin, S. M.; Laks, H.; Drinkwater, D. C.; Stein, D. G.; Capouya, E. R.; Pearl, J. M.; Barthel, S. W.; Chang, P.; Kaczer, E.; Bhuta, S. *Ann. Thorac. Surg.* **1993**, *55*, 954.
(1786) Bajaj, A. K.; Cobb, M. A.; Virmani, R.; Gay, J. C.; Light, R. T.; Forman, M. B. *Circulation* **1989**, *79*, 645.
(1787) Ohyanagi, H.; Saito, Y.; Uchida, T.; Watanabe, M.; Yamanouchi, K.; Yokoyama, K.; Mitsuno, T. *Biomater., Artif. Cells, Immobilization Biotechnol.* **1992**, *20*, 941.
(1788) Wall, T. C.; Califf, R. M.; Blakenship, J.; Talley, J. D.; Tannenbaum, M.; Schwaiger, M.; Gacioch, G.; Cohen, M. D.; Sanz, M.; Leimberger, J. D.; Topol, E. J.; Group, T. R. *Circulation* **1994**, *90*, 114.
(1789) Zhao, L.; Smith, J. R.; Eyer, C. L. *Proc. West. Pharmacol. Soc.* **1992**, *35*, 77.
(1790) Han, D. H.; Zervas, N. T.; Geyer, R. P.; Adams, J. F.; Ropper, A. H.; Kennedy, S. K.; Heros, R. C.; Varsos, V. G.; Borges, L.; Hedley-Whyte, E. T. In *Cardiovascular Disease*; Reivich, M., Hurtig, H. I., Eds.; Raven Press: New York, 1983; p 409.
(1791) Peerless, S. J.; Nakamura, R.; Rodriguez-Salazar, A.; Hunter, I. G. *Stroke* **1985**, *16*, 38.
(1792) Kolluri, S.; Heros, R. C.; Hedley-Whyte, E. T.; Vonsattel, J. P.; Miller, D.; Zervas, N. T. *Stroke* **1986**, *17*, 976.
(1793) Voynikov, T. I.; Nikolova, I. N.; Suzuki, A.; Higashino, H. *Neurosciences* **1990**, *16*, 591.

(1794) Kline, R. A.; Negendank, W.; McCoy, L.; Berguer, R. *Am. J. Surg.* **1991**, *162*, 193.
(1795) Sakas, D. E.; Stranjalis, G.; Wittaker, K.; Whitwell, H. L. *Cerebrovasc. Brain Metabol. Rev.* **1996**, *8*, 209.
(1796) Peerless, S. J.; Ishikawa, R.; Hunter, I. G.; Peerless, M. J. *Stroke* **1981**, *12*, 558.
(1797) Suzuki, J.; Tanaka, S.; Yoshimoto, T. *Acta Neurochir.* **1981**, *58*, 149.
(1798) Mizoi, K.; Yoshimoto, T.; Suzuki, J. *Acta Neurochir.* **1981**, *56*, 157.
(1799) Suzuki, J.; Fujimoto, S.; Mizoi, K.; Oba, M. *Stroke* **1984**, *15*, 672.
(1800) Suzuki, J.; Yoshimoto, T.; Kodama, N.; Sakurai, Y.; Ogawa, A. *Surg. Neurol.* **1982**, *17*, 325.
(1801) Runge, T. M.; MacGinity, J. W.; Frisbee, S. E.; Briceno, J. C.; Ottmers, S. E.; Calhoon, J. H.; Hantler, C. B.; Corvick, D. L.; Ybarra, J. R. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1997**, *25*, 297.
(1802) Osterholm, J.; Alderman, J. B.; Triolo, A. J.; D'Amore, B. R.; Williams, H. D.; Frazer, G. *Neurosurgery* **1983**, *13*, 381.
(1803) Bose, B.; Osterholm, J. L.; Payne, J. B.; Chambers, K. *Neurosurgery* **1986**, *18*, 270.
(1804) Dobben, G. D.; Long, D. M.; Szanto, P. S.; Mantegrano, V. C.; Liu, M.-S. *Neuroradiology* **1973**, *6*, 17.
(1805) Doss, L. L.; Kaufman, N.; Bicher, H. I. *Microvasc. Res.* **1977**, *13*, 253.
(1806) Maughan, R. E.; Mohan, C.; Nathan, I. M.; Ascer, E.; Damiani, P.; Jacobowitz, I. J.; Cunningham, J. N.; Marini, C. P. *Ann. Thorac. Surg.* **1992**, *54*, 818.
(1807) DelRossi, A. J.; Cernaianu, A. C.; Cilley, J. H.; Spence, R. K.; Camishion, R. C.; Yu, Y.; Costabile, J. P.; Vertrees, R. A. *J. Thorac. Cardiovasc. Surg.* **1990**, *99*, 665.
(1808) van Rossem, K.; Vermarien, H.; Faithfull, N. S.; Wouters, L.; Decuyper, K. *Adv. Exp. Radiat. Biol.* **1997**, *411*, 403.
(1809) Lustig, R.; Lowe, N.; Prosnitz, L.; Spaulding, M.; Cohen, M.; Stitt, J.; Brannon, R. *Intl. J. Radiat. Oncol. Biol. Phys.* **1990**, *19*, 97.
(1810) Lustig, R. A.; McIntosh-Lowe, N. L.; Rose, C. M.; Haas, J.; Krasnow, S.; Spaulding, M. B.; Prosnitz, L. *Intl. J. Radiat. Oncol. Biol. Phys.* **1989**, *16*, 1587.
(1811) Dowling, S.; Fischer, J. J.; Rockwell, S. *Biomater., Artif. Cells., Immobilization Biotechnol.* **1992**, *20*, 903.
(1812) Rockwell, S.; Irvin, C. G.; Kelley, M.; Hughes, C. S.; Yabuki, H.; Porter, E.; Fischer, J. J. *Int. J. Radiat. Oncol.* **1991**, *22*, 87.
(1813) Teicher, B. A.; Sotomayer, E. A.; Dupuis, N. P.; Kusumoto, T.; Menon, K. *J. Cancer Res. Clin. Oncol.* **1994**, *120*, 593.
(1814) Henderson, B. W.; Dougherty, T. J. *Photochem. Photobiol.* **1992**, *55*, 145.
(1815) Lowe, K. C.; Akande, S. L.; Bonnett, R.; White, R. D.; Berenbaum, M. C. *Biomater., Artif. Cells, Artif. Organs* **1992**, *20*, 925.
(1816) Novakova, V.; Birke, G.; Plantin, L.-O.; Wretlind, A. *Fed. Proc.* **1975**, *34*, 1488.
(1817) Berkowitz, H. D.; McCombs, P.; Sheety, S.; Miller, L. D.; Sloviter, H. *J. Surg. Res.* **1976**, *20*, 595.
(1818) Tomera, J. F.; Geyer, R. P. *J. Mol. Cell Cardiol.* **1982**, *14*, 573.
(1819) Ueda, K.; Genda, T.; Hirata, I.; Shimada, M.; Shibata, T.; Ueda, T.; Omoto, R. *J. Heart Lung Transplant.* **1992**, *11*, 646.
(1820) Dirks, B.; Kriegelstein, J.; Lind, H. H.; Rieger, H.; Schütz, H. *J. Pharmacol. Methods* **1980**, *4*, 95.
(1821) Höller, M.; Breuer, H.; Fleischhauer, K. *J. Pharmacol. Methods* **1983**, *9*, 19.
(1822) Segel, L. D.; Rendig, S. V. *Am. J. Physiol.* **1982**, *242*, H485.
(1823) Gohra, H.; Mori, F.; Esato, K. *Ann. Thorac. Surg.* **1989**, *48*, 96.
(1824) Kawamura, A.; Meguro, J.; Takahashi, M.; Ikeda, A.; Hirai, H.; Kukita, K.; Yonekawa, M.; Witmanowski, H.; Yokota, N.; Hayashi, T.; Ito, K. *Intl. J. Artif. Organs* **1994**, *17*, 053.
(1825) Kamada, N.; Calne, R. Y.; Wight, D. G. D.; Lines, J. G. *Transplantation* **1980**, *30*, 43.
(1826) Yamamoto, N.; Konishi, Y.; Wakashiro, S.; Takayasu, T.; Tatsumi, Y.; Shimahara, Y.; Tanaka, K.; Yamaoka, Y.; Ozawa, K. *J. Surg. Res.* **1991**, *51*, 288.
(1827) Ohya, T.; Ohwada, S.; Kawashima, Y. *J. Am. Coll. Surg.* **1995**, *182*, 219.
(1828) Kurki, T. S.; Harjula, A. L.; Heikkilä, L. J.; Lehtola, A. L.; Hämmäinen, P.; Taskinen, E.; Mattila, S. P. *J. Heart Transplant.* **1990**, *9*, 424.
(1829) Yabe, Y.; Ishiguro, N.; Shimizu, T.; Tamura, Y.; Wakabayashi, T.; Miura, T. *J. Reconstr. Microsurg.* **1994**, *10*, 185.
(1830) Yabe, Y.; Ishiguro, N.; Shimizu, T.; Kawasaki, S.; Sasaki, Y.; Iwata, H. *J. Surg. Res.* **1996**, *64*, 89.
(1831) Mohan, C.; Gennaro, M.; Marini, C.; Ascer, E. *Am. J. Surg.* **1992**, *164*, 194.
(1832) Gennaro, M.; Mohan, C.; Ascer, E. *Cardiovasc. Surg.* **1996**, *4*, 399.
(1833) Scheule, A. M.; Bohl, A.; Heinemann, M. K.; Ziemer, G.; Henze, E. *Eur. J. Cardiothor. Surg.* **1997**, *11*, 746.
(1834) Wada, S.; Murakami, H.; Sueda, T.; Kajihara, H.; Matsuura, Y. *Hiroshima J. Med. Sci.* **1994**, *43*, 153.
(1835) Biro, G. P.; Masika, M.; Korecky, B. *Adv. Exp. Med. Biol.* **1989**, *248*, 509.
(1836) Segel, L. D.; Follette, D. M.; Iguidbashian, J. P.; Contino, J. P.; Castellanos, L. M.; Berkoff, H. A.; Kaufman, R. J.; Schweighardt, F. K. *J. Heart Lung Transplant.* **1994**, *13*, 669.
(1837) Brasile, L.; Clarke, J.; Green, E.; Haisch, C. *Transplant. Proc.* **1996**, *28*, 349.
(1838) DeRoover, A.; Krafft, M. P.; Deby-Dupont, G.; Jacquet, N.; Lamy, M.; Meurisse, M.; D'Silva, M. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **2001**, *29*, 225.
(1839) Khalfoun, S.; Janin, P.; Machet, M. C.; Arbelle, B.; Lacord, M.; Locatelli, A.; Salmon, H.; Riess, J. G.; Gruel, Y.; Nivet, H.; Bardos, P.; Lebranchu, Y. *Transplant. Proc.* **1995**, *27*, 2210.
(1840) Smith, A. R.; Van Alphen, W.; Faithfull, N. S.; Fennema, M. *J. Plast. Reconstr. Surg.* **1985**, *75*, 227.
(1841) Keese, C. R.; Giaver, I. *Science* **1983**, *219*, 1448.
(1842) Zekorn, T.; Siebers, U.; Bretzel, R. G.; Heller, S.; Meder, U.; Ruttkay, H.; Zimmermann, U.; Federlin, K. *Hormone Metab. Res.* **1991**, *23*, 302.
(1843) Ju, L.-K.; Armiger, W. B. *BioTechniques* **1992**, *12*, 258.
(1844) Elibol, M.; Mavituna, F. *Appl. Microbiol. Biotechnol.* **1995**, *43*, 206.
(1845) Mishima, H.; Kobayashi, T.; Shimizu, M.; Tamaki, Y.; Baba, M.; Shimano, T.; Itoh, S.; Yamazaki, M.; Iriguchi, N.; Takahashi, M.; Mori, T. *J. Magn. Reson. Imaging* **1991**, *1*, 705.
(1846) Fishman, J. E.; Joseph, P. M.; Floyd, T. F.; Mukherji, B.; Sloviter, H. A. *Magn. Reson. Imaging* **1987**, *5*, 279.
(1847) Fishman, J. E.; Joseph, P. M.; Carvlin, M. J.; Saadi-Elmandjra, M.; Mukherji, B.; Sloviter, H. A. *Invest. Radiol.* **1989**, *24*, 65.
(1848) Mason, R. P. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1994**, *22*, 1141.
(1849) Thomas, S. R.; Millard, R. W.; Pratt, R. G.; Shiferaw, Y.; Samaratunga, R. C. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1994**, *22*, 1029.
(1850) Shukla, H. P.; Mason, R. P.; Bansal, N.; Antioch, P. P. *Magn. Reson. Med.* **1996**, *35*, 827.
(1851) Thomas, S. R.; Gradon, L.; Pratsinis, S. E.; Pratt, R. G.; Fotou, G. P.; McGoron, A. J.; Podgorski, A. L.; Millard, R. W. *Invest. Radiol.* **1997**, *32*, 29.
(1852) Judd, R. M.; Rottman, G. A.; Forder, J. R.; Yin, F. C. P.; Blackband, S. J. *Magn. Reson. Med.* **1992**, *28*, 129.
(1853) Lu, D.; Joseph, P. M.; Greenberg, J. H.; Lin, R.; Mukherji, B.; Sloviter, H. A. *Magn. Reson. Med.* **1993**, *29*, 179.
(1854) Mason, R. P.; Antioch, P. P.; Babcock, E. E.; Gerberich, J. L.; Nunnally, R. L. *Magn. Reson. Imaging* **1989**, *7*, 475.
(1855) Reindorf, C. A.; Kurantsin-Mills, J.; Allotey, J. B.; Castro, O. *Am. J. Hematol.* **1985**, *19*, 229.
(1856) Faithfull, N. S.; Salt, P. J.; Klein, J.; van der Zee, H.; Soini, H.; Erdmann, W. *Adv. Exp. Med. Biol.* **1985**, *191*, 463.
(1857) Oldham, K. T.; Guice, K. S.; Gore, D.; Gourley, W. K.; Lobe, T. E. *Am. J. Surg.* **1987**, *153*, 291.
(1858) Ricci, J. L.; Sloviter, H. A.; Ziegler, M. M. *Am. J. Surg.* **1985**, *149*, 84.
(1859) Yokoyama, K.; Yamanouchi, K.; Suyama, T.; Naito, R.; Ohyanagi, H.; Mitsuno, T. Proceedings of the IVth International Symposium on Perfluorochemical Blood Substitutes, Kyoto, 1978; Exerpta Medica: Amsterdam, 1979, p 273.
(1860) Matsuki, A.; Jin, T.; Fukushi, S.; Ishihara, H.; Satoh, Y.; Toyota, M.; Oyama, T. *Prog. Clin. Biol. Res.* **1983**, *122*, 445.
(1861) Geyer, R. P. *Fed. Proc.* **1975**, *34*, 1525.
(1862) Ohyanagi, H.; Ohashi, O.; Nakayama, S.; Yamamoto, M.; Okumura, S.; Saitoh, Y. *Biomater., Artif. Cells, Artif. Organs* **1988**, *16*, 585.
(1863) Matin, A. F. M.; Baba, S.; Choudhury, N. A. *Jpn. J. Surg.* **1991**, *21*, 661.
(1864) Mizuno, H.; Isobe, J.; Matsunobe, S.; Nakamura, T.; Shimizu, Y.; Hitomi, S. *Int. J. Artif. Organs* **1994**, *17*, 609.
(1865) Vaiciulis, K.; Dobrovolskiene, Z.; Petrauskiene, N.; Zukauskas, G. *Acta Med. Lituanica* **1995**, 3.
(1866) Lowe, K. C. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **2000**, *28*, 25.
(1867) Plauth, M.; Zimmermann, B.; Raible, A.; Vieillard-Baron, D.; Bauder-Gross, D.; Hartmann, F. *Res. Exp. Med.* **1991**, *191*, 339.
(1868) Pappenheimer, J. R.; Volpp, K. *Am. J. Physiol.* **1992**, *263*, C480.
(1869) Geyer, R. P. *Prog. Clin. Biol. Res.* **1975**, *6*, 565.
(1870) Lowe, K. C.; McNaughton, D. C.; Hardy, R. N. In *Oxygen Carrying Colloidal Blood Substitutes*; Frey, R., Beisbarth, H., Stosseck, K., Eds.; Zuckschwerdt Verlag: München, 1982; p 91.
(1871) Goodin, T. H.; Clarke, W. P.; Taylor, K.; Eccles, R.; Geyer, R. P.; McCoy, L. E. *Am. J. Physiol.* **1983**, *245*, H519.
(1872) Mayevsky, A.; Mizawa, I.; Sloviter, H. A. *Neurolog. Res.* **1981**, *3*, 307.
(1873) Sylvia, A. L.; Proctor, H. J.; Goldsmith, M. M.; Jobsis, F. F. *J. Trauma* **1982**, *22*, 815.
(1874) Schneeberger, E. E. *Biomater., Artif. Cells, Artif. Organs* **1988**, *16*, 565.

(1875) Harvey, S. A. K.; Trankina, M. L.; Olson, M. S.; Clark, J. B. *Biochim. Biophys. Acta* **1991**, *1073*, 486.
(1876) Adlercreutz, P.; Mattiasson, B. *Acta Chem. Scand.* **1982**, *B36*, 651.
(1877) McCreath, G. E.; Chase, H. A.; Lowe, C. R. *J. Chromatogr. A* **1994**, *659*, 275.
(1878) Chase, H. A.; Yang, Y. *Biotechnol. Appl. Biochem.* **1998**, *27*, 205.
(1879) Gauger, P. G.; Overbeck, M. C.; Chambers, S. D.; Cailipan, C. I.; Hirschl, R. B. *J. Appl. Physiol.* **1998**, *84*, 1566.
(1880) Greenspan, J. S.; Wolfson, M. R.; Shaffer, T. H. *Biomed. Instrum. Technol.* **1999**, *33*, 253.
(a) Koch, T.; Ragaller, M.; Haufe, D.; Hofer, A.; Grosser, M.; Albrecht, D. M.; Kotsch, M.; Lutter, T. *Anesthesiology* **2001**, *94*, 101.
(1881) Kinsella, J. P.; Parker, T. A.; Galan, H.; Sheridan, B. C.; Abman, S. H. *Am. J. Respir. Crit. Care Med.* **1999**, *159*, 1220.
(1882) Mattrey, R. F.; Trambert, M. A.; Brown, J. J.; Young, S. W.; Bruneton, J. N.; Wesbey, G. E.; Balsara, Z. N. *Radiology* **1994**, *191*, 841.
(1883) Eilenberg, S. S.; Tartar, V. M.; Mattrey, R. F. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1994**, *22*, 1477.
(1884) Chang, S.; Reppucci, V.; Zimmerman, N. J.; Heinemann, M.-H.; Coleman, J. *Ophthalmology* **1989**, *96*, 785.
(1885) Peyman, G. A.; Schulman, J. A.; Sullivan, B.; *Surv. Ophthalmol.* **1995**, *39*, 375.
(1886) Shapiro, M. J.; Resnick, K. I.; Kim, S. H.; Weinberg, A. *Am. J. Ophthalmol.* **1991**, *112*, 401.
(1887) Flores-Aguilar, M.; Munguia, D.; Loeb, E.; Crapotta, J. A.; Vuong, C.; Shakiba, S.; Bergeron-Lynn, G.; Weers, J. G.; Freeman, V. R. *Retina* **1995**, *15*, 3.
(1888) Langefeld, S.; Kirchhol, B.; Meinert, H.; Roy, T.; Aretz, A. *Graefes Arch. Clin. Exp. Ophthalmol.* **1999**, *237*, 201.
(1889) Colthurst, M. J.; Williams, R. L.; Hiscott, P. S.; Grierson, I. *Biomaterials* **2000**, *21*, 649.
(1890) Dinkelmann, S.; Geister, U.; Röhlke, W.; Meinert, H.; Northoff, H. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **2001**, *29*, 71.
(1891) Floyd, T. F.; Boroughs, A.; Garvey, C.; Dasher, J.; Ikeda, C. B.; Sloviter, H. A.; Ziegler, M. M. *J. Pediatric Surg.* **1987**, *22*, 1191.
(1892) Malchesky, P. S.; Nosé, Y. *Adv. Cardiol.* **1971**, *6*, 72.
(1893) Mori, Y. H.; Kaminaga, K.; Ando, T. *Ann. Biomed. Eng.* **1990**, *18*, 285.
(1894) Gollan, F.; Clark, L. C. *Trans. Assoc. Am. Physicians* **1967**, *80*, 102.
(1895) Iwai, T.; Sato, S.; Yamada, T.; Muraoka, Y.; Sakurazawa, K.; Inoue, Y.; Kimoshita, H.; Endo, M. *J. Cardiovasc. Surg.* **1989**, *30*, 490.
(1896) Anthony, P.; Lowe, K. C.; Power, J. B.; Davey, M. R. *Cryobiology* **1997**, *35*, 201.
(1897) Thurston, R. J.; Rogoff, M. S.; Scott, T. R.; Korn, N. *Poult. Sci.* **1993**, *72*, 598.
(1898) Kuroda, Y.; Morita, A.; Fujino, Y.; Tanioka, Y.; Ku, Y.; Saitoh, Y. *Transplantation* **1993**, *56*, 1087.
(1899) Tanioka, Y.; Sutherland, D. E. R.; Kuroda, Y.; Gilmore, T. R.; Asaheim, T. C.; Kronson, J. W.; Leone, J. P. *Surgery* **1997**, *122*, 435; Matsumoto, S.; Kandaswamy, R.; Sutherland, D. E. R.; Hassoun, A. A.; Hiraoka, K.; Sageshima, J.; Shibata, S.; Tanioka, Y.; Kuroda, Y. *Transplantation* **2000**, *70*, 771.
(1900) Sugawara, Y.; Matsuura, Y.; Sueda, T.; Wada, S.; Ochikubo, H.; Kajihara, H. *Tranplant. Proc.* **1998**, *30*, 3826.
(1901) Jewitt, N.; Anthony, P.; Lowe, K. C.; de Pomerai, D. I. *Enzyme Microb. Technol.* **1999**, *25*, 349.
(1902) Adlercreutz, P.; Mattiasson, B. *Eur. J. Appl. Microbiol. Biotechnol.* **1982**, *16*, 165.
(1903) Marcovich, H.; Riess, J. G. Congrès Génie Biologique et Médical, Toulouse, 1982.
(1904) Fischer, G. W.; Hunter, K. W.; Wilson, S. R. *Lancet* **1980**, *2*, 1300.
(1905) Anthony, P.; Lowe, K. C.; Davey, M. R.; Power, J. B. *Biotechnol. Tech.* **1995**, *9*, 777.
(1906) Lowe, K. C.; Anthony, P.; Wardrop, J.; Davey, M. R.; Power, J. B. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1997**, *25*, 261.
(1907) Wasanasathian, A.; Peng, C.-A. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **2001**, *29*, 47.
(1908) Wardrop, J.; Lowe, K. C.; Davey, M. R.; Marchant, R. *Plant Cell Rep.* **1997**, *17*, 17.
(1909) Wolfson, M. R.; Greenspan, J. S.; Shaffer, T. H. *Pediatrics* **1996**, *97*, 449.
(1910) Hazard, D.; Trevino, L.; Dellamary, L.; Kinner, R.; Meays, D.; Bradley, J.; Flaim, S. Ninth Annual Pediatric Critical Care Colloquium, Milwaukee, WI, 1996; p 55.
(1911) Lisby, D. A.; Ballard, P. L.; Fox, W. W.; Wolfson, M. R.; Shaffer, T. H.; Gonzales, L. W. *Hum. Gene Ther.* **1997**, *8*, 919.
(1912) Weiss, D. J.; Strandjord, T. P.; Jackson, J. C.; Clark, J. G.; Liggitt, D. *Exp. Lung Res.* **1999**, *25*, 317; Weiss, D. J.; Bonneau, L.; Liggitt, D. *Molec. Therapy* **2001**, *3*, 734.
(1913) Bot, A. I.; Tarara, T. E.; Smith, D. J.; Bot, S. R.; Woods, C. M.; Weers, J. G. *Pharm. Res.* **2000**, *17*, 275.
(1914) Fritz, T. A.; Unger, E. C.; Sutherland, G.; Sahn, D. *Invest. Radiol.* **1997**, *32*, 735.
(1915) Beppu, S.; Matsuda, H.; Shishido, T.; Matsumura, M.; Niyatake, K. *J. Am. Soc. Echocardiol.* **1997**, *10*, 11.
(1916) Grayburn, P. A.; Weiss, J.; Hack, T.; Klodas, E.; Raichlen, J.; Vannan, M.; Klein, A.; Kitzman, D.; Chrysant, S.; Cohen, J.; Abrahamson, D.; Foster, E.; Perez, J.; Aurigemma, G.; Panza, J.; Picard, M.; Byrd, B.; Segar, D.; Jacobson, S.; Sahn, D.; DeMaria, A. *J. Am. Coll. Cardiol.* **1998**, *32*, 230.
(1917) *Ultrasound Contrast Agents-Basic Principles and Clinical Applications*, 2nd ed.; Goldberg, B. B., Raichlen, J. S.; Forsberg, F., Eds.; Martin Dunite: London, 2001.
(a) Tiemann, K.; Lohmeier, S.; Kuntz, S.; Köster, J.; Pohl, C.; Burns, P.; Porter, T. R.; Nanda, N. C.; Lüderitz, B.; Becher, H. *Echoradiography* **1999**, *16*, 799.
(1918) Halpern, E. J.; Verkh, L.; Forsberg, F.; Gomella, L. G.; Mattrey, R. F.; Goldberg, B. B. *Am. J. Radiol.* **2000**, *174*, 1575.
(1919) Cohen, J. L.; Cheirif, J.; Segar, D. S.; Gillam, L. D.; Gottdiener, J. S.; Hausnerova, E.; Bruns, D. E. *J. Am. Coll. Cardiol.* **1998**, *32*, 746.
(1920) Taylor, G. A.; Ecklund, K.; Dunning, P. S. *Radiology* **1996**, *201*, 125.
(1921) Greenberg, R. S.; Taylor, G. A.; Stapleton, J. C.; Hillsley, C. A.; Spinak, D. *Radiology* **1996**, *201*, 119.
(1922) Mulvagh, S. L.; Foley, D. A.; Aeschbacher, B. C.; Klarich, K. K.; Seward, J. B. *J. Am. Coll. Cardiol.* **1996**, *27*, 1519.
(1923) Wu, Y.; Unger, E. C.; McCreery, T. P.; Sweitzer, R. H.; Shen, D.; Wu, G.; Vielhauer, M. D. *Invest. Radiol.* **1998**, *33*, 880.
(1924) Porter, T. R.; LeVeen, R. F.; Fox, R.; Kricsfeld, A.; Xie, F. *Am. Heart J.* **1999**, *132*, 964.
(1925) Riess, J. G.; Arlen, C.; Greiner, J.; Le Blanc, M.; Manfredi, A.; Pace, S.; Varescon, C.; Zarif, L. *Biomater., Artif. Cells, Artif. Organs* **1988**, *16*, 421.
(1926) Sadtler, V. M.; Krafft, M. P.; Riess, J. G. *Angew. Chem., Int. Ed. Engl.* **1996**, *35*, 1976.
(1927) Sadtler, V. M.; Krafft, M. P.; Riess, J. G. *Colloids Surf. A* **1999**, *147*, 309.
(a) Brubach, J.-B.; Mermet, A.; Filabozzi, A.; Gerschel, A.; Lairez, D.; Krafft, M. P.; Roy, P. *J. Phys. Chem. B* **2001**, *105*, 430.
(1928) Trevino, L.; Dellamare, L.; Tarara, T.; Krafft, M. P.; Riess, J. G.; U.S. Pat 5,733,526, 1997.
(1929) Krafft, M. P.; Sadtler, V. M.; Riess, J. G. International Symposium on Fluorine Chemistry, Vancouver, 1997.
(1930) Krafft, M. P.; Riess, J. G. *Angew. Chem., Int. Ed. Engl.* **1994**, *33*, 1100.
(1931) Krafft, M. P. In *Novel Cosmetic Delivery Systems*; Magdassi, S., Touitou, E., Eds.; Dekker: New York, 1998; Chapter 10; p 195.
(1932) Ringsdorf, H.; Schlarb, B.; Venzmer, J. *Angew. Chem., Int. Ed. Engl.* **1988**, *27*, 113.
(1933) Kunitake, T. *Angew. Chem., Int. Ed. Engl.* **1992**, *31*, 709.
(1934) Wang, S.; Lunn, R.; Krafft, M. P.; Leblanc, R. M. *Langmuir* **2000**, *16*, 2882.
(1935) Elbert, R.; Folda, T.; Ringsdorf, H. *J. Am. Chem. Soc.* **1984**, *106*, 7687.
(1936) Kunitake, T.; Higashi, N. *Makromol. Chem. Suppl.* **1985**, *14*, 81.
(1937) Giulieri, F.; Krafft, M. P.; Riess, J. G. *Angew. Chem., Int. Ed. Engl.* **1994**, *33*, 1514.
(1938) Giulieri, F.; Guillod, F.; Greiner, J.; Krafft, M. P.; Riess, J. G. *Chem. Eur. J.* **1996**, *2*, 1335.
(1939) Emmanouil, V.; El Ghoul, M.; André-Barrès, C.; Guidetti., B.; Rico-Lattes, I.; Lattes, A. *Langmuir* **1998**, *14*, 5389.
(1940) Imae, T.; Funayama, K.; Krafft, M. P.; Giulieri, F.; Tada, T.; Matsumoto, M. *J. Colloid Interface Sci.* **1999**, *212*, 330.
(1941) Li, L.; Schultz, R. D. Symposium on Respiratory Drug Delivery V, Phoenix, 1996; abstracts p 2.
(1942) Apostol, I. *Anal. Biochem.* **1999**, *272*, 8; Manjula, B. N.; Malavalli, A.; Prabhakaran, M.; Friedman, J. M.; Acharya, A. S. *Protein Eng.* **2001**, *14*, 359.
(1943) Nelson, D. J. In *Blood Substitutes: Principles, Methods, Products and Clinical Trials.*; Chang, T. M. S., Ed.; Karger Landes: Basel, 1998; Vol. 2, p 39.
(1944) Keipert, P. E. *Adv. Exp. Med. Biol.* **1992**, *317*, 453.

CR970143C