0031-6997/01/5302-283–318$3.00
PHARMACOLOGICAL REVIEWS
Vol. 53, No. 2
122/900547
Pharmacol Rev 53:283–318, 2001
Printed in U.S.A

Long-Circulating and Target-Specific Nanoparticles: Theory to Practice

S. MOEIN MOGHIMI,[1] A. CHRISTY HUNTER, AND J. CLIFFORD MURRAY

Molecular Targeting and Polymer Toxicology Group, School of Pharmacy and Biomolecular Sciences, University of Brighton, Brighton, United Kingdom (S.M.M., A.C.H.); and Laboratory of Molecular Oncology, CRC Department of Clinical Oncology, City Hospital, University of Nottingham, Nottingham, United Kingdom (J.C.M.)

[1] Address for correspondence: Dr. S. M. Moghimi, Molecular Targeting and Polymer Toxicology Group, School of Pharmacy and Biomolecular Sciences, University of Brighton, Brighton BN2 4GJ, UK. E-mail: s.m.moghimi@brighton.ac.uk

***Abstract*——The rapid recognition of intravenously injected colloidal carriers, such as liposomes and polymeric nanospheres from the blood by Kupffer cells, has initiated a surge of development for "Kupffer cell-evading" or long-circulating particles. Such carriers have applications in vascular drug delivery and release, site-specific targeting (passive as well as active targeting), as well as transfusion medicine. In this article we have critically reviewed and assessed the rational approaches in the design as well as the biological performance of such constructs. For engineering and design of long-circulating carriers, we have taken a lead from nature. Here, we have explored the surface mechanisms, which affords red blood cells long-circulatory lives and the ability of specific microorganisms to evade macrophage recognition. Our analysis is then centered where such strategies have been translated and fabricated to design a wide range of particulate carriers (e.g., nanospheres, liposomes, micelles, oil-in-water emulsions) with prolonged circulation and/or target specificity. With regard to the targeting issues, attention is particularly focused on the importance of physiological barriers and disease states.**

I. Introduction

Macrophages are widely distributed and strategically placed in many tissues of the body to recognize and clear altered and senescent cells, invading particulates, as well as macromolecular ligands via a multitude of specialized plasma membrane receptors (Gordon, 1995). This propensity of macrophages for endocytosis/phagocytosis of foreign particles in the past has provided an opportunity for the efficient delivery of therapeutic agents to these cells with the aid of colloidal drug delivery systems (usually in the form of liposomes, polymeric nanospheres, micelles, and oil-in-water emulsions), following parenteral administration (Poznansky and Juliano, 1984). For example, macrophages that are in contact with blood or lymph serve as sites of proliferation for certain microbes during some or all of the infection process. Numerous logical strategies for using colloidal drug carriers in the treatment of certain infectious diseases involving macrophages have been developed, based on analysis of the pathogenesis of microbial diseases (Alving, 1988; Agrawal and Gupta, 2000). For instance, the proposed lysosomotropic-parasitotropic process for delivery of liposome-encapsulated drugs to *Leishmania* within macrophages involves uptake of the phagocytosed liposome and delivery to a lysosome, fusion of lysosome with the parasitophorous vacuole, and delivery of the liposome to a lysosome within the parasite (Alving, 1988). Ambisome (Gilead, Boulder, CO), a liposomal formulation of amphotericin B, is an example of a world-wide marketed drug delivery product available for treatment of visceral leishmaniasis or confirmed infections caused by specific fungal species.

Some pathogens have also evolved ways of evading intracellular killing to survive within the cell; one example is by escaping the phagosome, which is followed by direct entry into cytoplasm (Provoda and Lee, 2000). To allow further cytoplasmic delivery, new strategies have been developed. A classical example is the use of a pH sensitive liposome (Horwitz et al., 1980). Such vesicles maintain stable phospholipid bilayers at neutral pH or above but destabilize and become fusion-competent at the acidic pH of endosomes and subsequently release their entrapped contents first into endosomes and then into cytoplasm. However, the efficiency of cytoplasmic delivery has further been enhanced by employing natural mechanisms that breach the endosomal membrane once the liposomal contents are delivered into the endosomal lumen. For example, Lee et al. (1996) have adopted a strategy used by a facultative intracellular pathogen (*Listeria monocytogenes*) that mediates bacterial passage from the phagosome into cytosol. After being internalized into phagosomes, *L. monocytogenes* permeabilizes phagosomal membranes with the aid of listeriolysin O to enter the cytosolic space of host cells. Indeed, coencapsulation of listeriolysin O along with protein-based antigens in liposomes resulted in efficient delivery of protein antigens to the cytosolic pathway of antigen processing and presentation in macrophages. This approach seems highly desirable in vaccine production to polysaccharide antigens that are poorly immunogenic. Similarly, breaching of the endosomal membrane is also critical for survival of DNA after delivery to macrophages.

Metabolic processes within the mature macrophage have also served as targets for drug carriers. These included degradation of accumulated macromolecules within lysosomes (storage diseases) and iron overloading (Poznansky and Juliano, 1984). Apart from therapeutic goals, colloidal carriers have proved to be useful for diagnostic purposes, for example, to assess macrophage phagocytic and clearance functions. Similarly, particulate colloids tagged with a suitable radiopharmaceutical or contrast agent were shown to be helpful for imaging certain pathologies (e.g., deep-seated tumors)

not via targeting but through macrophage loading (Seltzer, 1989; Tilcock, 1995; Kostarelos and Emfietzoglou, 1999). By this approach, it is the surrounding parenchyma, and not the pathology, that will change in intensity.

The rapid sequestration of intravenously injected colloidal particles from the blood by hepatic midzonal and periportal Kupffer cells is problematic for efficient targeting of drug carriers or diagnostic agents to a desired macrophage population (e.g., splenic red pulp macrophages) as well as to nonmacrophage sites. As a result, there has been a growing interest in the engineering of colloidal carrier systems that upon intravenous injection avoid rapid recognition by Kupffer cells and adequately remain in the blood. It is the aim of this article to critically discuss and evaluate the developments in the field of long-circulating and target-specific colloidal carrier systems for intravenous administration (and in selected cases with regard to subcutaneous injection). We have viewed the application of long-circulating particles in sustained-release of drugs within vasculature, transfusion medicine (artificial erythrocytes), and targeting. The latter is defined either as passive or active. With regard to passive targeting, this article identifies and discusses the opportunities afforded by certain physiological and pathological conditions that are amenable for treatment or diagnosis by using long-circulating colloidal carriers. In relation to active targeting, attention is focused toward identification of potential and accessible tissue-specific antigens and their ligands. For particle engineering, we have taken a lead from nature. That is, exploring the surface mechanisms that afford red blood cells long-circulatory lives and the ability of certain pathogenic microorganisms to evade macrophage recognition. Our analysis is centered where such strategies have been translated and fabricated to design a wide range of particulate carriers (e.g., nanospheres, liposomes, micelles, oil-in-water emulsions) with prolonged circulation and target specificity. Throughout our approach, we critically evaluate these engineered systems on the basis of biodistribution, target specificity, therapeutic end-points, biological activities, and toxicity.

II. Theoretical Applications of Long-Circulating Particulate Carriers in Experimental and Clinical Medicine

A. Circulating Drug Reservoir in the Blood Compartment

There are several reasons why the search for "macrophage-evading" or long-circulating particles is so extensive. One important reason is to provide a long-circulating drug reservoir from which the drug can be released into the vascular compartment in a continuous and controlled manner. Candidate drugs and therapeutic agents may be those with short elimination half-lives (e.g., ara-C, cytokines, growth factors). Therefore, the requirements in terms of drug release from a long-circulating carrier will depend on clearance kinetics of the system as well as a pharmacologically desired free drug profile.

B. Artificial Oxygen Delivery Systems

Long-circulating particulate carriers might also act as oxygen delivery systems and end the centuries-old search for "artificial or substitute blood". An artificial colloid-based blood substitute would be expected to be compatible with all blood types and have a better shelf life than blood itself, which is 6 weeks from the time of collection. Their use will reduce the risk of contracting viral or other transmittable diseases from the donated blood. Such blood substitutes are most likely to be used as part of blood conservation strategies, which in conjugation with blood pre-donation and preoperative hemodilution, are aimed at reducing the need for allogeneic blood transfusion. Another possible application is to temporarily augment oxygen delivery in patients at risk for acute tissue oxygen deficit due to either transient anemia or ischemia.

C. Blood-Pool Imaging

Similarly, one can also foresee long-circulating nanoparticles as carriers of radiopharmaceuticals or contrast agents for use in the imaging of vasculature. These may include blood-pool imaging, detection of vascular malformations, and gastrointestinal bleeding. Technetium-99m-labeled red blood cells are used routinely in nuclear medicine for blood-pool imaging, but technetium-99m is not strongly bound to the red cell and dissociates, resulting in the clearance of free technetium through the kidney and bladder (Phillips, 1998). This dissociation greatly interferes, for example, with the detection of sites of bleeding in the lower abdomen. This, together with the potential hazards associated with blood handling, justifies efforts for the development of synthetic long-circulatory particles in nuclear medicine.

D. Passive Targeting

The unique structural changes associated with a given vascular pathophysiology could also provide opportunities and insights for the use as well as engineering of long-circulating particulate carrier systems. For exam-

[2] Abbreviations: ara-C, 1-β-D-arabinofuranosylcytosine; DPPC, dipalmitoylphosphatidylcholine; DPPE, dipalmitoylphosphatidylethanolamine; DSPC, distearoylphosphatidylcholine; DSPE, distearoylphosphatidylethanolamine; E-selectin, endothelial leucocyte adhesion molecule-1; FcR, Fc-receptor, HIV, human immunodeficiency virus; IES, interendothelial cell slits; IL, interleukin; PE, phosphatidylethanolamine; PEG, poly(ethylene glycol); PI-3 kinase, phosphoinositide-3-OH kinase; PLGA, poly(lactide-co-glycolide); PLA, poly(lactic acid); POE, polyoxyethylene; POP, polyoxypropylene; PS, phosphatidylserine; RES, reticuloendothelial system; SIRPα, signal regulatory protein alpha; TNF-α, tumor necrosis factor-α .

ple, particle escape from the circulation is normally restricted to sites where the capillaries have open fenestrations as in the sinus endothelium of the liver (Roerdink et al., 1984) or when the integrity of the endothelial barrier is perturbed by inflammatory processes (e.g., rheumatoid arthritis, infarction, infections) (Turner and Wright, 1992) or by tumors, although such defects are not a consistent feature of all tumors (Jain, 1989; Hobbs et al., 1998). In the liver, the fenestrae-associated cytoskeleton controls the hepatic function of endothelial filtration, where the size of fenestrae can be as large as 150 nm (Braet et al., 1995). The size of fenestrae in certain inflammatory vessels as well as tumor capillaries can be up to 700 nm. Currently, there is evidence in support of liposome extravasation to hepatic parenchyma (Roerdink et al., 1984) as well as increased capillary permeability to liposomes and polymeric nanospheres (in the size range of 50–200 nm) during inflammation (Boerman et al., 1997a,b; Dams et al., 1998, 1999, 2000b) and in specific cancers (Poznansky and Juliano, 1984; Yuan et al., 1994; Hobbs et al., 1998; Drummond et al., 1999) based on observations in experimental animals and in humans. However, the efficiency and kinetics of particle delivery varies from one model to another. Particles of less than 10 nm can also leave the systemic circulation through the permeable vascular endothelium in lymph nodes (Wiessleder et al., 1994; Moghimi and Bonnemain, 1999). The sinus endothelium of bone marrow is also capable of removing small-sized particles from the systemic circulation. Here, the sinus endothelium can remove particles from the circulation by both transcellular and intracellular routes (Moghimi, 1995a). The transcellular route is through the diaphragmed fenestrae of endothelial walls, whereas the intercellular route is associated with the formation of bristle-coated pits containing matter on the luminal surface of the endothelium. However, regional dilation increased branching of sinuses, and even complete loss of bone marrow sinus endothelium can occur in certain pathological conditions thus resulting in particulate accumulation in affected areas. For instance, marked fragmentation of endothelial cytoplasm, as well as complete loss of the endothelium of bone marrow sinusoids, have been reported for dogs infected with a canine hepatitis virus (Linblad and Bjorkman, 1964).

Certainly one would expect this because of the prolonged residence in blood, long-circulating carriers with the appropriate sizes have a better chance of reaching the above-mentioned targets, resulting in improved treatment or diagnosis. Another topic that deserves investigation is the use of a tissue-specific pharmacological mediator that, by opening intercellular connections in selected vessels, may facilitate long-circulating colloidal entities to escape from the circulation and extravasate into the tissues of organs whose postcapillary endothelia contain receptors for the mediator (Rosenecker et al., 1996). In spite of the preceding evidence, unless the means are found to actively control local permeability and access, the issue of tissue selectivity is not properly addressed by the use of engineered nanoparticulate complexes, and significant therapeutic gain is unlikely.

Given the potency (and toxicity) of modern pharmacological agents, tissue selectivity is a major issue. In the delivery of chemotherapeutic agents to solid cancers, this is particularly critical, since the therapeutic window for these agents is often small and the dose-response curve is steep. Therefore, the idea of exploiting the well documented vascular abnormalities of tumors, restricting penetration into normal tissue interstitium while allowing freer access to that of the tumor, becomes particularly attractive. This has been the subject of considerable research; however, the data suggest that the solution to this problem will depend upon more than simply reducing mechanical barriers to *trans*-endothelial passage. Let us examine the idea of passive targeting more closely with regard to the process of extravasation of long-circulating particles from the blood to a solid tumor, a target that has received the most attention in the drug delivery field. A solid tumor comprises two major cellular components. These are the tumor parenchyma and the stroma, which incorporates the vasculature and other supporting cells. To meet the metabolic requirements of the expanding population of tumor cells, the pre-existing blood vessels become subject to intense angiogenic pressure (Folkman, 1995). Several factors produced by tumor cells are believed to signal the development of new capillaries from these vessels (angiogenesis). Scanning electron microscopy of microvascular corrosion casts has allowed visualization of the geometry of tumor architecture. From these studies, it has become apparent that tumor vessels are highly irregular and show gross architectural changes (e.g., the presence of interrupted endothelium and an incomplete basement membrane) with increased blood vessel tortuosity as well as abnormal and heterogeneous vessel density (Jain, 1988). As a consequence of these abnormalities, there may be profound physiological changes in blood flow within tumors and in transport properties of the tumor vessel. Due to the vascular heterogeneity of solid tumors, the blood flow, and consequently tissue oxygenation, tends to be nonuniform. Therefore, solid tumors usually contain well perfused, rapidly growing regions, and poorly perfused, often necrotic areas (Jain, 1988). As in normal tissues, diffusive and convective forces govern the movement of molecules into the interstitium of tumors. However, diffusion is believed to play a minor role in the movement of solutes across the endothelial barrier compared with bulk fluid flow. Examination of pressure gradients in experimental tumors has suggested that the movement of drugs and particulates out of tumor blood vessels into the extra-vascular compartment is remarkably limited (Jain, 1989). This has been attributed to a higher-than-expected interstitial pressure, in part due to a lack of functional lym-

phatic drainage, coupled with lower intravascular pressure. In addition, interstitial pressure tends to be higher at the center of solid tumors, diminishing toward the periphery, creating a mass flow movement of fluid away from the central region of the tumor.

These pathophysiological characteristics have serious implications for the systemic delivery of not only low-molecular weight drugs and macromolecules (e.g., antibodies, polymer conjugate therapeutics), but also particulate delivery vehicles (e.g., liposomes), and simply enhancing the plasma half-life of these agents will not necessarily lead to an increase in therapeutic effect. However, it is likely that long-circulating particles may have a better safety profile in terms of normal tissue toxicity than free anticancer agents, as well as anticancer drugs encapsulated in phagocyte-prone carriers. The utility of conventional colloidal carriers as vehicles for drug delivery in cancer treatment is inappropriate since the class of drug being used is able to induce apoptosis in macrophages of the RES (Daemen et al., 1995). In the liver, restoration of Kupffer cells may take up to periods of 2 weeks (Daemen et al., 1995). A potentially harmful effect is that bacteriemia may occur during the period of Kupffer cell deficiency (Daemen et al., 1995).

E. Active Targeting

The ability to target drugs and gene therapeutics to nonmacrophage cells within the vasculature has been one of the most sought after goals in clinical therapeutics. Therefore, attachment of specific (but not macrophage-recognizable) ligands onto the surface of macrophage-evading carriers will open the possibility of targeting specific cell types or subsets of cells within the vasculature and even elements of vascular emboli and thrombi. One example is the abnormal lymphocyte differentiation antigens on leukemia cells (Raso et al., 1982), which could serve as target molecules for ligand-directed targeting of macrophage-evading carriers containing anticancer agents. Another important target is vascular endothelial cells. The vascular endothelium is remarkably heterogeneous; endothelial cells from different tissues of the body differ in expression of surface antigens and receptors (Kumar et al., 1987; Belloni and Nicolson, 1988; Rajotte et al., 1998; Thorin and Shreeve, 1998). Endothelial cells also display phenotypic changes in response to various cytokines and growth factors (Pober and Cotran, 1990; Bevilacqua, 1993; Kraling et al., 1996; Murray, 1997). For example, ELAM-1 (E-selectin) is not detectable on the surface of quiescent endothelial cells of normal vessels, but is strongly up-regulated on the luminal surface by pro-inflammatory cytokines such as IL-1β and TNF-α (Lin et al., 1992). E-selectin induction on endothelial cells is clearly associated with inflammatory and immune processes (Pober and Cotran, 1990) as well as some noninflammatory angiogenic states (Kraling et al., 1996). E-selectin is known to be involved in the arrest of monocytes, neutrophils, and subsets of T-lymphocytes within the circulation. Therefore, it is not surprising that this molecule has become a potential target for several strategies designed to enhance delivery of therapeutic agents to, or to improve imaging of, particular vascular beds. For example, Wickham et al. (1997) have demonstrated that the enhanced expression of E-selectin may be used as a means to target entry of adenoviral vectors into endothelial cells. A bi-specific antibody, which reacts with both E-selectin and with a "FLAG" epitope genetically incorporated into one of the adenoviral coat proteins, was able to direct binding and entry of the adenovirus into endothelial cells pretreated with the TNF-α but not into quiescent nonstimulated cells. Another antibody against E-selectin, 1.2B6, has been used for radionuclide imaging of rheumatoid arthritis in humans (Jamar et al., 1997). In this study, the sensitivity of anti-E-selectin coupled to indium-111 was compared with a nonspecific antibody conjugated with technetium-99m, in terms of their ability to detect synovitis. This study showed that targeting with the E-selectin antibody was more intense and specific than with a nonspecific antibody (Jamar et al., 1997). Furthermore, the anti-E-selectin antibody was able to detect joint abnormalities in areas that were clinically silent.

Biochemical differences between the vasculature of the tumor and the normal tissues are also noteworthy. Indeed, various approaches have been taken to identify differences in luminal protein expression between the endothelial cells of tumor vessels and those of normal tissues. These include two-dimensional gel electrophoresis of cell surface proteins isolated from cultured endothelial cells (P. Hewett and J. C. Murray, manuscript submitted), screening of monoclonal antibodies on tissue sections (Hagemeier et al., 1986; Rettig et al., 1992), in vivo lectin labeling (Belloni and Nicolson, 1988), and the application of bacteriophage display technology in vivo (Rajotte et al., 1998) to study endothelial heterogeneity. These markers, which are usually up-regulated on the surface of tumor-associated endothelial cells, include proteins involved in cell-cell or cell-matrix interactions (e.g., E-selectin, vascular cell adhesion molecule-1, and the $\alpha_v\beta_3$ integrin complex) and growth factors receptors (Cheresh, 1987; Matthews et al., 1991; Kim et al., 1993; Brooks et al., 1994; Dumont et al., 1994; Sato et al., 1995; Max et al., 1997; Pasqualini et al., 1997; Charpin et al., 1998; Lauren et al., 1998; Lin et al., 1998; Maurer et al., 1998; Ellerby et al., 1999). The growth factor receptors of potential interest are the vascular endothelial growth factors-2 (KDR-2), which are up-regulated in a wide range of solid tumors (Matthews et al., 1991), and the Tie family of endothelial receptor tyrosine kinases (Tie-1 and Tie-2) (Lauren et al., 1998). Targeted disruption of gene function in murine embryos indicates that Tie-2 plays a pivotal role in both angiogenesis and vascular remodeling/stability, whereas Tie-1 is required for the maintenance of vascular integrity (Dumont et al.,

1994; Lin et al., 1998). The angiopoietin growth factors have been identified as the ligands for Tie-2, whereas Tie-1 remains an orphan receptor. Since the development of new blood vessels is essential for tumor growth (Griffioen and Molema, 2000), an attractive antitumor strategy is to exploit these biochemical differences and aim at the tumor vasculature for therapeutic intervention with ligand-bearing long-circulating particles. This strategy might overcome the physiological differences within tumors that have been described as heterogeneous and unpredictable. One must also be cautious with regard to heterogeneity in angiogenic stages in human tumor vasculature. This is because in the animal model, the majority of tumor vasculature is usually in a proangiogenic state, whereas in human tumors, the percentage of proangiogenic vessels is variable and usually low (Griffioen and Molema, 2000). Hence, such antiangiogenic approaches may only affect a minority of the tumor vasculature.

Even if engineered colloidal carriers can be targeted successfully to specific subsets of circulating blood cells or endothelia, subsets may differ significantly in their capacity to internalize particles bound to the cell surface. Therefore, in selected cases, it may be necessary to use particle-immobilized ligands that upon binding and signal transduction initiate local effects (e.g., apoptosis or cytokine production).

III. Rational Approaches in the Design of Long-Circulating Particles

If monocytes and macrophages in contact with blood are not the desired target, then how can we avoid their phagocytic "barrier"? To date, a classical approach has been to administer large doses of placebo carriers in an effort to impair the phagocytic capacity of macrophages in contact with the blood, thereby allowing subsequently administered particles containing active material to remain in systemic circulation for prolonged periods or to reach designated targets (Abra et al., 1980; Souhami et al., 1981; Moghimi and Davis, 1994). Another strategy is transient apoptotic destruction of liver and spleen macrophages by prior administration of gadolinium chloride (Hardonk et al., 1992) or liposomes with entrapped clodronate (Naito et al., 1996; Schmidt-Weber et al., 1996; van Rooijen et al., 1997). The latter approach has received some attention for the in vivo gene transfer protocols involving adenoviruses (Wolff et al., 1997). These strategies, although successful in experimental models, have little justification in clinical practice as they suppress the essential defense system of the body.

"If one way be better than another, you may be sure it is nature's way" [Aristotle]. It seems appropriate to design a long-circulating carrier based on nature's principles. For example, healthy erythrocytes evade the macrophages of the immune system and fulfill their function of transporting oxygen with a life span of 110 to 120 days. A multitude of physicochemical and physiological factors are believed to control the life span of red blood cells. These include surface characteristics (e.g., surface charge, membrane phospholipid composition, surface antigens) as well as bulk properties (e.g., shape and their extent of deformability) (Weiss and Tavassoli, 1970; Schnitzer et al., 1972; Chen and Weiss, 1973; Moghimi, 1995b; Oldenborg et al., 2000). For instance, red blood cells may avoid macrophage surveillance with the protection of a barrier of oligosaccharide groups. Furthermore, their deformable nature allows them to bypass the human splenic filtration process at the IES in the walls of venous sinuses. In the same context, two decades ago Densen and Mandell (1980) pointed out, "it is naive to assume that microbes passively accept their fate at the hands of phagocytes". In fact, for the successful growth, certain pathogens have deployed a clever array of surface strategies to avoid phagocytosis by macrophages (Ram et al., 1998; Alcami and Koszinowski, 2000). Perhaps we should also learn to translate feasible microbial surface strategies in the engineering of long-circulating or macrophage-evading nanoparticles.

A. The First Few Steps

1. Physicochemical Characteristics of Nanoparticles and Their Effect on Protein Adsorption and Opsonization. It has been repeatedly emphasized that the clearance behavior and tissue distribution of intravenously injected particulate drug carriers are greatly influenced by their size and surface characteristics (Poznansky and Juliano, 1984; Patel, 1992; Moghimi and Davis, 1994). These physicochemical parameters can control the degree of particle self-association (Ahl et al., 1997) in the blood as well as particle opsonization in biological fluids. The size of a particle may change substantially upon introduction into a protein-containing medium (e.g., plasma). Therefore, in the blood, particles and their aggregates should be small enough so that they are not removed from the circulation by simple filtration in the first capillary bed encountered (e.g., rat or mouse lung following tail vein injection). The opsonization process is the adsorption of protein entities capable of interacting with specific plasma membrane receptors on monocytes and various subsets of tissue macrophages, thus promoting particle recognition by these cells (Chonn et al., 1992; Moghimi and Davis, 1994; Gref et al., 1995; Moghimi and Patel, 1998; Moghimi and Hunter, 2000a). Classical examples of opsonic molecules include various subclasses of immunoglobulins, complement proteins like C1q and generated C3 fragments (C3b, iC3b), apolipoproteins, von Willebrand factor, thrombospondin, fibronectin, and mannose-binding protein (Absolom, 1986; Patel, 1992; Serra et al., 1992; Chonn et al., 1995; Moghimi and Patel, 1996; Szebeni, 1998). On exposure to blood, particles of differing surface characteristics, size, and morphology attract different arrays of opsonins as well as other plasma proteins, the content and con-

formation of which may account for the different pattern in the rate and site of particle clearance from the vasculature (Moghimi and Patel, 1998). Since opsonization plays a major role in particle clearance from the blood, then interindividual variations in blood opsonic activity and concentration must also be considered. Undoubtedly, a clear understanding of such events is the first rational step for the design of colloidal carriers that target not only a relevant macrophage population (see *Section III.A.2.*) but also for the engineering of long-circulating or macrophage-evading particles. It should also be emphasized that the interaction of particles with blood protein may have effects beyond opsonization. These may include interference with the blood-clotting cascade, a process that may lead to fibrin formation, and anaphylaxis because of complement activation.

From this discussion, it therefore appears that evasion of particulate binding to, or uptake by, macrophages could be achieved to a certain extent by interference with protein adsorption and classical mechanisms of opsonization (e.g., prevention of complement activation in relevant species). In support of this statement, an early study with liposomes (Senior and Gregoriadis, 1982) demonstrated that small neutral unilamellar vesicles (100 nm or below), made from equimolar amounts of saturated phospholipids and cholesterol, have a longer circulation time in rats (half-lives up to 20 h) than their anionic counterparts (half-lives less than 1 h). These observations are in agreement with the process of complement opsonization of liposomes; neutral vesicles are poor activators of the complement system when compared with anionic liposomes (Volanakis and Wirtz, 1979; Chonn et al., 1991; Devine and Bradley, 1998). Therefore, the small-sized neutral vesicles are not efficiently coated with the opsonizing complement proteins and as a result are poorly recognized by Kupffer cells. However, in the case of larger neutral or anionic liposomes, clearance rates increased progressively with increasing size (Senior et al., 1985). This indicates that surface curvature changes may affect the extent and/or type of protein or opsonin adsorption. Indeed, vesicle size has been shown to play a critical role in complement activation (Devine et al., 1994; Harashima et al., 1994). From static in vitro studies, it appears that at a fixed lipid concentration larger liposomes are more efficient at activating complement than smaller vesicles (Devine et al., 1994). This probably suggests the importance of geometric factors and surface dynamics on the initial assembly of proteins involved in complement activation. Therefore, for vesicles larger than 100 nm other strategies must be sought to prevent surface opsonization processes. Indeed, the simplicity of the above approaches have led to the development of DaunoXome. This is a regulatory approved (U.S. Federal Drug Administration) formulation of daunorubicin citrate entrapped in small neutral unilamellar liposomes, made of the high melting point phospholipid DSPC, for the treatment of Kaposi's sarcoma lesions.

2. Macrophage Heterogeneity, Physiological Status, and Species Differences. It is important to realize that not all macrophages and monocytes are identical; considerable heterogeneity with respect to phenotype and physiological properties (e.g., phagocytosis) exist between different types of phagocytes and even among phagocytic cells of the same tissue (Gordon et al., 1992; Naito, 1993; Rutherford et al., 1993). For example, rat Kupffer cells are heterogeneous with regard to phagocytosis and accessory functions such as cytokine production, tumoricidal capability, and antigen presentation to T cells (Sleyster and Knook, 1982; Hoedemakers et al., 1994). Kupffer cells in the periportal area are larger and have a higher lysosomal enzyme activity (on a per cell basis as compared with cells in other regions of the liver), which reflects their higher level of phagocytic and scavenging activities than those of central macrophages. On the other hand, macrophages of the pericentral region are more active in cytokine production and have a higher tumoricidal capability than the larger periportal Kupffer cells (Hoedemakers et al., 1994). With regard to phagocytic receptors, human Kupffer cells and splenic macrophages serve as good examples, Table 1 (Buckley et al., 1987; Tomita et al., 1994). For instance, in human liver the expression of the IgG-coated particle receptor (FcγRIIIA) is restricted only to Kupffer cells located in the central area of the liver lobule (Tomita et al., 1994). In murine, macrophages found within the marginal

TABLE 1
Expression of complement receptors (CR) by resident liver and spleen macrophages in selected species

Species	Liver Macrophages	Spleen Macrophages		
	Kupffer Cells	Red Pulp	Marginal Zone	White Pulp
Human	CR1 (++)[a] CR3 (++)[a] CR4 (++)[a]	CR1 (—)[b] CR3 (+)[b]	CR1 (—)[b] CR3 (++)[b]	CR1 (?)[a] CR3 (—)[b]
Murine	CR3 (—)[c]	CR3 (+)[d]	CR3 (++)[d]	CR3 (—)[d]
Rat	CR3 (++)[e] KCA-3[f]	CR3 (++)[e]	CR3 (+)[e]	CR3 (—)[e]

Tissue expression of the above receptors was analyzed by immunohistochemical techniques ([a]Hinglais et al., 1988; [b]Buckley et al., 1987; [c]Lee et al., 1986; [d]Gordon et al., 1992; [e]Robinson et al., 1986; [f]Maruiwa et al., 1993). (++) represents strong expression (25–50% of macrophages); (+) represents poor expression (less than 15% of macrophages); (—) represents no expression; and (?) represents unknown at present. CR1 recognizes particles bearing C3b as well as C4b; CR3 (CD11b/CD18) recognizes the iC3b region that contains the amino acid sequence Tyr-Arg-Gly-Asp-Gln; CR4 recognizes surface-bound iC3b. In human spleen, white pulp macrophages refers to T zone (interdigitating reticulum cells) and B zone (dendritic reticulum cells and tangible body macrophages). KCA-3 antigen is exclusive to periportal Kupffer cell surface facing the sinusoid rather than the endothelial lining.

zone, which surrounds the lymphoid regions of the spleen, differ in morphology, endocytosis, and receptor expression from those in the red pulp. For example, unlike other splenic macrophages, the marginal zone macrophages express a specific scavenger receptor (known as MARCO) that is distinct from type 1 and type 2 scavenger receptors (Ito et al., 1999). It is therefore conceivable that the particular populations of macrophages may respond differently to particulate delivery systems and, hence, employ one particular recognition mechanism. Therefore, understanding of macrophage properties, heterogeneity, and recognition mechanisms could provide new insights and opportunities for the design of long-circulating particles as well as carriers that can selectively deliver drugs and therapeutic materials to particular macrophage subpopulations (e.g., for optimizing delivery to recruited monocytes versus local resident macrophages, and selective and apoptotic ablation of macrophages serving as reservoirs of infectious agents, such as HIV and tuberculosis). Here, consideration must also be given toward the physiological status of macrophages of the RES. A responsive phagocyte can become primed and later selectively activated for one or more functions (Adams and Hamilton, 1992). Examples of such functions include enhanced phagocytosis, chemotaxis, antigen processing and presentation, and destruction of tumor cells. The capacity to accomplish a complex function is dependent, in turn, upon acquisition of the requisite capacities such as enhanced mobility of a given population of plasma membrane receptors for a given chemotactic agent or an immobilized opsonin, increase in the number or selective expression of a particular group of scavenging receptors (e.g., the expression of plasma membrane FcγRIA which is restricted to human Kupffer cells at the sites of inflammation and is apparently unrelated to the type of liver disease), and the amount of mediators secreted etc. There are strong indications that activated macrophages can recognize engineered long-circulating particles from the blood (Moghimi et al., 1993a; Moghimi and Murray, 1996; Schmidt-Weber et al., 1996; Moghimi and Gray, 1997) (discussed under *Section VII.*). Hence, such long-circulating particles may act as vehicles for delivery of therapeutic or diagnostic agents to such macrophage populations.

There are also species differences with regard to macrophage receptors involved in phagocytosis (Table 1). An intriguing example with regard to drug delivery and targeting is the hepatic Kupffer cell in mice. Unlike the splenic marginal zone macrophages and blood monocytes, murine Kupffer cells do not express complement receptors with scavenging functions (Lepay et al., 1985; Lee et al., 1986; Gordon et al., 1992). Based on immunohistochemical studies, the iC3b receptor (CR3) is hardly detectable in murine Kupffer cells. The inability for detection could be due to the absence of CR3 or to a blockade by a bound ligand. On the other hand, macrophages recruited to murine liver by infection express high levels of CR3 (Lepay et al., 1985; Lee et al., 1986). It is possible that CR3 plays a role in the adhesion of monocytes to liver sinusoids, which would be in agreement with the well documented role of CR3 as an adhesion molecule in cellular interactions. Despite this published immunological data, it is interesting to note that a group of investigators have emphasized that, in mice, some long-circulating particles avoid liver macrophages because they inhibit both classical and alternative pathways of complement activation; hence, they are not recognized by Kupffer cells via CR3! (Passirani et al., 1998).

3. Splenic Filtration. The size and the deformability of particles plays a critical role in their clearance by the sinusoidal spleens of humans and rats. Particles must be either small or deformable enough to avoid the splenic filtration process at the IES in the walls of venous sinuses (Chen and Weiss, 1973; Moghimi et al., 1991). The IES in sinusoidal spleens provides resistance to flow through the reticular meshwork. The endothelial cells of the sinus wall have two sets of cytoplasmic filaments: a set of loosely associated tonofilaments and a set of filaments tightly organized into dense bands in the basal cytoplasm containing actin and myosin, which can probably vary the tension in the endothelial cells and, hence, the size of IES (Drenckhahn and Wagner, 1986). However, the slit size rarely exceeds 200 to 500 nm in width, even with an erythrocyte in transit (Chen and Weiss, 1973). Hence, retention of blood cells and blood-borne particles at the IES depends on their bulk properties, such as size, sphericity, and deformability. These cell slits are the sites where erythrocytes containing rigid inclusions (e.g., Heinz bodies, malarial plasmodia) are believed to be "pitted" of their inclusions, which are eventually cleared by the red pulp macrophages (Weiss and Tavassoli, 1970; Schnitzer et al., 1972; Groom, 1987). Idealy, the size of an engineered long-circulatory particle should not exceed 200 nm. If larger, then the particle must be deformable enough to bypass IES filtration. Alternatively, long-circulating rigid particles of greater than 200 nm may act as splenotropic agents (Moghimi et al., 1991).

4. Confinement to Vasculature. Earlier we discussed the possibility of particle extravasation from the blood to selected sites in the body (*Section II.D.*). However, if we intend to keep long-circulating particles within the vasculature, as in certain cases (e.g., a circulating drug reservoir), then a lower size window in the design of long-circulating particles must also be considered. There is a clear-cut relationship between particle size (e.g., as in the case of liposomes) and the extent to which they reach the hepatic parenchyma. For instance, it has been demonstrated that the smaller the liposomes (usually those of below 100 nm in diameter), the larger the contribution of hepatocytes in total hepatic uptake (Roerdink et al., 1984). This is probably a reflection of the size of the fenestrations in the hepatic sinusoidal endothe-

lium, which are ~100 to 150 nm in diameter (Braet et al., 1995). Even highly deformable vesicles of up to 400 nm in diameter can also reach hepatocytes after intravenous injection by a process of "extrusion" through endothelial fenestrations (Romero et al., 1999). Therefore, as a rough approximation, the size of long-circulating particles, providing that they are rigid structures, should be in the range of 120 to 200 nm in diameter to substantially avoid particle trapping in space of Disse and hepatic parenchyma.

IV. Translation of Microbial and Related Mammalian Technologies to Nanoparticle Engineering

Considerable information has become available from pathogenic microorganisms that employ various surface strategies to avoid recognition by macrophages. For example, mucoid strains of *Pseudomonas aeruginosa* from patients with cystic fibrosis display a polyuronic acid polysaccharide that interferes with phagocytosis by virtue of its hydrophilicity (Cabral et al., 1987). The external envelope glycoproteins of HIV type-1 and its simian counterpart are heavily glycosylated. Recent studies have demonstrated that these carbohydrates form a barrier to help shield the virus from immune recognition and limit effective antibody responses to the virus (Reitter et al., 1998). A major constituent of various microbial envelope glycoproteins is sialic acid (Ram et al., 1998). Such sialyated proteins favourably bind factor H (Meri and Pangburn, 1994). The binding of factor H contributes to pathogenicity by inhibiting complement-mediated destruction; factor H acts as a cofactor for factor I-mediated cleavage of C3b and also inactivates alternative pathway convertase by dissociating Bb from the C3b, Bb complex (Fearon and Austen, 1977; Sim et al., 1981). Examples of factor H binding proteins include the streptococcal M6 protein (Fischetti et al., 1995), the Yad A protein *of Yersinia enterocolitica* (China et al., 1993), and also the envelope glycoproteins 120 and 41 of HIV-1 (Stoiber et al., 1996; Reitter et al., 1998). Microorganisms such as certain species of *Staphylococcus aureus* fix complement in a cryptic subcapsular location inaccessible for ligation by complement receptors. Eukaryotic infectious agents, such as *Candida albicans* and related species, subvert C3-mediated opsonization processes by expressing a surface protein that is antigenically, structurally, and functionally related to the mammalian integrin CR3 and p150,95 (Heidenreich and Dierich, 1985). Several viruses express complement regulatory proteins to reduce complement attack (Alcami and Koszinowski, 2000).

Can coating of synthetic nanoparticles with a hydrophilic microbial polysaccharide, factor H, or a factor H ligand fend off the body's defense system? Can such constructs release their entrapped materials in a controlled manner within the systemic circulation or at the desired pathological sites? And, finally, what are the toxicological variables inherent with these types of systems? One of the earliest attempts undertaken was the coating of liposomes with linear dextrans, which are polymers of α-D-glucose units (Pain et al., 1984). Linear dextrans are produced by the fermentation of sucrose with a strain of the bacterium *Leuconostoc mesenteroides* followed by hydrolysis and fractionation to give dextrans with different average molecular weights. Linear dextrans have frequently been used as plasma expanders in medicine; they remain in the systemic circulation for extended periods of time which are proportional to their molecular weights. The circulatory persistence of drugs (e.g., doxorubicin) and proteins such as carboxypeptidase G, superoxide dismutase, and arginase have all been increased following conjugation to dextran (Melton et al., 1987a,b; Wileman, 1991). Liposomes coated with dextran (molecular weight of 70,000) were also more efficient in retaining the entrapped radioactive markers within the circulation than uncoated liposomes (Pain et al., 1984). The rate of clearance of dextran-coated liposomes was dependent on the density of dextran molecules on the liposome surface. The steric brushes of the dextran macromolecules are believed to reduce protein adsorption, resulting in enhanced stability of liposomes in the blood (Pain et al., 1984). Perhaps, one of the most successful applications of dextran in nanoparticulate engineering is the development of ultrasmall superparamagnetic iron oxide particles, which are used in magnetic resonance imaging (Bengele et al., 1994; Guimaraes et al., 1994; Wiessleder et al., 1994; Moghimi and Bonnemain, 1999). These particles consist of hexagonally shaped iron cores of 4 to 5 nm in diameter surrounded by 20 to 30 hydrated "brush-like" structures composed of dextran molecules. By laser light scattering, such crystals exhibit a unimodal hydrodynamic radius of 20 to 25 nm. Because of their physical properties (small size and hydrophilic nature), iron oxide particles are poorly recognized by Kuppfer cells and splenic macrophages and, concomitantly, exhibit prolonged circulation in the blood with a half-life of 3 to 4 h (Moghimi and Bonnemain, 1999). The eventual macrophage recognition of such particles may be attributed to gradual opsonization with antidextran antibodies and complement activation. These partricles have found clinical applications in lymph node imaging (see *Section VIII.C.*).

Another example is the bacterial exopolysaccharide pullulan (an α-D-glucan). Cholesterol-pullulan conjugates with various degrees of substitution as well as monoalkyl(hexadecyl)-pullulan have all been shown to confer some degree of protection to the liposome surface and moderately reduce vesicle uptake by both the liver and spleen macrophages in vivo (Kang et al., 1997). The capsular polysaccharide of *N*-acetyl neuraminic acid (polysialic acids) from *Escherichia coli* K1 and K92, *Neisseria meningitidis* B and C as well as their shorter chain derivatives should also be considered for nanopar-

ticle surface engineering. Indeed, preliminary experiments have confirmed that long-chain polysialic acids exhibit prolonged circulation half-lives (Gregoriadis et al., 1993; Fernandes and Gregoriadis, 1997); due to their high hydrophilicity, little or no binding occurs between factor H and polysialic acids (Pangburn et al., 1991; Meri and Pangburn, 1994). Recently, successful attempts were made to increase the half-life of asparaginase following coupling to such polysialic acids (Fernandes and Gregoriadis, 1997). Theoretically, conjugation of liposomes or nanospheres to polysialic acids could be carried out by a variety of methods, depending on the reactive groups available on the interacting entities. However, caution is required for utilizing polysialic acids for human use. This is because coupling reactions could potentially alter or damage the tertiary structure of the long-chain polysialic acids and, hence, affect the clearance patterns of the engineered colloids. The bacterial polysialic acids C and K92 are immunogenic in humans whereas polysaccharide B and K1 are T-independent antigens and do not induce immunological memory (Moreno et al., 1985). However, it is possible that polysialic acids B and K1 could become T cell dependent antigens and induce memory when grafted to nanocarriers. Another concern is the possible antigenicity of polysialic acids; low levels of antibodies against polysialic acids exist in the circulation (Mandrell and Zollinger, 1982). Macrophages as well as other myeloid cells express several sialic acid binding receptors such as sialoadhesin, CD33, and siglec-5 (Crocker et al., 1991; Munday et al., 1999). The sialic acid-binding proteins are not phagocytic receptors (Munday et al., 1999). However, they could cooperate with phagocytic receptors to increase the efficiency of recognition and uptake, but they function primarily as ligands in cellular recognition events and signaling. Therefore, it will be of interest to determine whether interactions between sialic acid binding proteins and polysialic acids influence host defense functions.

Other successful approaches might be to tag drug carriers with complement regulatory proteins or specific complement inhibitors. Several novel complement inhibitors and chimeric or modified human complement regulatory proteins are currently available for pursuing such studies. Perhaps one of the easiest approaches is to enrich the surface of particulates with factor H ligands. For example, factor H has the ability to bind to polyanions such as heparin, and most sulfated glycosaminoglycans, to include dextran sulfate and chondroitin sulfate A (but not chondroitin sulfate C, keratan sulfate, hyaluronic acid, or polyaspartic acid) (Pangburn et al., 1991; Meri and Pangburn, 1994). It should be emphasized that the interaction between factor H and polyanions is specific and may depend upon the number, orientation, and polymeric arrangement of the anionic groups on nanoparticle surface. Recently, Passirani et al. (1998) covalently attached heparin to the surface of monodispersed poly(methyl methacrylate)-based nanoparticles with a mean diameter of 160 nm. Following intravenous injection into mice, heparin-coated nanoparticles exhibited an initial phase of elimination from the blood with a half-life of 5 h, the remaining heparin nanoparticles circulated for ~48 h. The prolonged circulation time of heparin nanoparticles was suggested to arise from the inhibition of complement activation (Passirani et al., 1998). Although, complement inhibition was only demonstrated in human serum, it is unlikely that the inhibition of complement activation in mice can explain the prolonged circulation time of nanoparticles and their poor hepatic localization. In the event of complement activation, resident murine Kupffer cells lack complement receptors with scavenging functions (Lepay et al., 1985; Lee et al., 1986; Gordon et al., 1992). The prolonged circulation time of nanoparticles in mice is most likely due to their high degree of surface hydrophilicity. It is also likely that the vascular endothelial cells may eventually play a critical role in the clearance of heparin-coated particles because these cells possess heparin receptors, although species variation must be considered (Patton et al., 1995).

Heparin-like polysaccharides can also be obtained from microorganisms. For example, *E. coli* serotype K5 synthesizes a form of a relatively low molecular mass (~50 kDa) desulfatoheparin (Vann et al., 1981). This molecule has repeating units of 4-β-D-glucuronosyl-1 and 4-α-*N*-acetyl-D-glucosamine, which is similar to *N*-acetylheparosan, a biosynthetic precursor of heparin. The bacterial product resembles type II glycosaminoglycuronan chains, which are synthesized in the Golgi complex of eukaryotes and then joined to core proteins. Unlike the eukaryotic products, the bacterial polymer lacks L-iduronosyl and sulfate residues. Because of its structural similarity to heparin and the abundance of the negatively charged glucronic acid, these heparin-like molecules may be worthy of particle-surface modification. A related and successful strategy has been the grafting of polyglucuronide to a liposome (100 nm) surface that conferred some degree of vesicle invisibility to macrophages of the RES of both mice and rats (Namba et al., 1992; Oku et al., 1992; Oku and Namba, 1994). For example, at 22 h after intravenous administration into rats, the ratios of the total amount of liposomal associated radioactive marker in the liver and the spleen to that in the blood were 2.4 and 6.3 for liposomes containing 20 mol% palmitylglucuronide and control formulation, respectively.

Sialic acid is an essential component of eukaryotic cell surfaces that plays an important role in preventing destruction of host tissue by constant low-grade activation of the alternative pathway. This is evident from desialyation of erthyrocyte membranes, which results in their conversion from nonactivators to activators of the alternative complement pathway due to reduction in factor H binding (Pangburn and Muller-Eberhard, 1978;

Kazatchkine et al., 1979). Surolia and Bachhawat (1977) conducted an elegant approach to rendering liposomes invisible to macrophages by mimicking the mammalian cell surface. Here, the liposome surface was enriched with sialic acid following the incorporation of cell derived glycolipids into the liposomal bilayer. This work initiated a major breakthrough in the liposome field; since then, various modifications of vesicles with gangliosides (Allen and Chonn, 1987; Gabizon and Papahadjopoulos, 1988; Allen et al., 1989; Chonn et al., 1992; Liu et al., 1995), ganglioside derivatives (Park and Huang, 1993; Yamauchi et al., 1993, 1995) and glycophorin (Yamauchi et al., 1993) have been made and investigated. Indeed, sialic acid containing gangliosides (e.g., ganglioside GM1), Fig. 1, when incorporated into liposomes, inhibit the alternative complement pathway as it promotes the binding of factor H to liposome-bound C3b (Michalek et al., 1988). In mice, DSPC or DPPC vesicles of 70 to 200 nm in size, containing high concentrations of cholesterol (>30 mol%) and GM1 at 5 to 7 mol%, behave as long-circulatory (Allen and Chonn, 1987; Gabizon and Papahadjopoulos, 1988; Allen, 1994a). Interestingly, in rats, GM1-containing vesicles are cleared rapidly from the circulation by hepatic Kupffer cells (Liu et al., 1995). The presence of naturally occurring anti-GM1 IgM in rat (but not in mouse) blood, through the activation of the classical pathway of the complement system, is thought to be responsible for the rapid detection of GM1-incorporated vesicles by Kupffer cells (Wassef et al., 1991; Alving and Wassef, 1992; Liu et al., 1995). What mechanisms are therefore responsible for prolonged circulation time of GM1 liposomes in mice? GM1 at 5 to 7 mol% is miscible with both DPPC and DSPC bilayers and forms the most stable vesicles in the presence of cholesterol (Bedu-Addo and Huang, 1996). The stability of the bilayer reduces vesicle susceptibility to lysis by mouse plasma components (e.g., lipoproteins) and to perturbation or penetration by plasma or cell surface proteins. Furthermore, due to the inhibition of complement activation, the assembly of the membrane attack complex at the vesicle bilayer is prevented. Gabizon and Papahadjopoulos (1988) postulated that the negative charge in GM1 is "shielded" by a bulky, neutral, hydrophilic sugar moiety that contributes to macrophage avoidance by decreasing or preventing protein adsorption or opsonization processes. This hypothesis is further supported by the fact that liposomal incorporation of other negatively charged glycolipids, where the negative charge is shielded by neutral sugars (e.g., phosphatidylinositol and sulfatides), also prolongs the blood retention time of the vesicle. Replacing phosphatidylinositol with phosphatidylinositol phosphate, in which an "exposed" charged phosphate is added to the inositol molecule, or replacing GM1 with G_{T1}, in which two silaic acid molecules with exposed carboxyl groups are present, enhances the hepatic clearance of liposomes (Gabizon and Papahadjopoulos, 1988, 1992). In spite of these experiments, the validity of this hypothesis is still questionable. Several studies have demonstrated that negatively charged glycolipids or phospholipids with exposed and unshielded carboxylic groups such as ganglioside GM3 (Fig. 1) (Yamauchi et al., 1993), [2-(2-palmitoylamido-1-

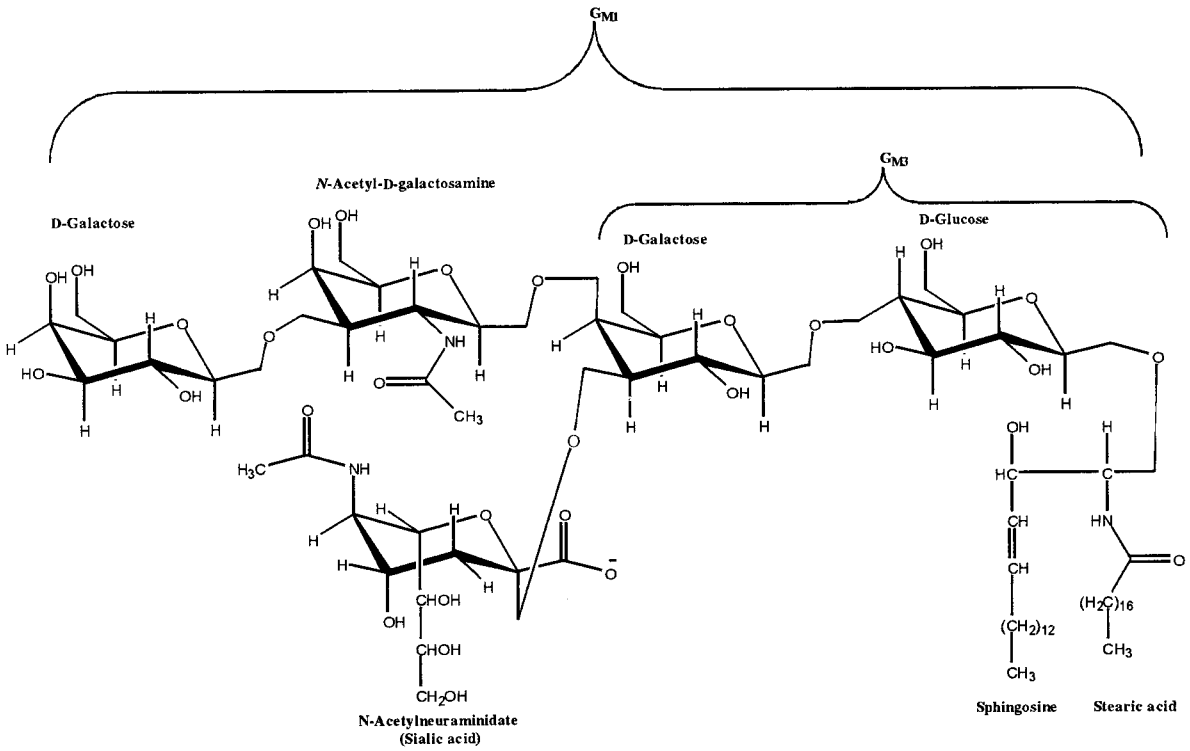


FIG. 1. Structure of gangliosides GM1 and GM3.

ethyl)-5-acetoamide-3,5-dideoxy-D-glycero-β-D-galacto-2-nonulipyranoside]onate (a synthetic derivative of sialic acid) (Yamauchi et al., 1995), *N*-glutaryldioleoylphosphatidylethanolamine, and *N*-adipyl dioleoylphosphatidyl-ethanolamine (Park et al., 1992)] show a considerable ability to prolong the circulation time of sub 200 nm liposomes in mice. Furthermore, derivatives of GM1 have also been synthesized such that their carboxyl group is either methylated or reduced (Park and Huang, 1993). These derivatives showed considerable ability in preventing hepatic sequestration of liposomes in mice. Reductive amination of a GM1 derivative containing a C7 analog of sialic acid in the presence of β-alanine adds to GM1 oligosaccharide an additional carboxyl group, which is not shielded by the neutral sugar residues, and yet the β-alaninyl GM1 had an activity very similar to native GM1 (Park and Huang, 1993). In contrast to GM1, ganglioside GM3 containing liposomes are long-circulatory in rats (Yamauchi et al., 1993). Therefore, it appears that particular stereo organization of the ganglioside sugar residues can control the extent of opsonization and the macrophage recognition of glycolipid-incorporated liposomes.

Studies by Moghimi and Patel (1989a,b, 1998) demonstrated that rat serum displays a dual role in Kupffer cell recognition of liposomes. In addition to stimulating liposome uptake by Kupffer cells via a noncomplement-mediated process, serum was shown to contain at least two heat-stable proteins that suppressed liposome recognition by Kupffer cells. Subsequently, it was suggested that a balance between the opsonic molecule and these suppressive proteins (dysopsonins) could regulate the quantity and the rate of clearance of liposomes from the blood by the hepatic macrophages (Moghimi and Patel, 1998). Dysopsonins could modulate the rate of liposome uptake by reducing the amount of liposome-bound opsonin and, hence, protect the phagocytic cells from being destroyed by excessive binding and ingestion of liposomes, particularly those vesicles which are more resistant toward lysosomal esterases. On the basis of this hypothesis, Park and Huang (1993) suggested that the prolonged circulation time of GM1-incorporated vesicles in mice may also be due to the binding of a putative dysopsonin onto the liposome surface leading to a reduced level of both opsonization and/or inactivation of bound opsonins. The dysopsonin hypothesis therefore suggests another possibility for enhancing the circulation time of liposomes (and perhaps other particulates). If dysopsonins play a significant role in vivo, then vesicles enriched with these proteins should be expected to display long circulation times. This approach warrants the identification of these blood dysopsonic molecules and their mode of action with regard to different types of colloidal carriers.

Attempts have also been made to introduce sialic acid groups onto the surface of polymeric nanospheres. One example was the noncovalent adsorption of glycoproteins rich in sialic acid such as orsomucoid onto the surface of poly(isobutylcyanoacrylate) nanoparticles (Olivier et al., 1996). This attempt was disappointing, since the half-life of these particulates was similar to those of uncoated systems. This was presumably due to insufficient density or altered conformation of the clustered glycans on the nanoparticle surface. Alternatively this could have been due to the desorption and exchange of sialic acid rich glycoproteins as soon as the particles were introduced into the systemic circulation.

Another potentially interesting mammalian system for disguising foreign particles as "self" is the integrin-associated protein CD47. This molecule has been suggested to function as a marker of self on murine red blood cells and lymphocytes since in the absence of CD47 the cells are cleared from the bloodstream by the splenic red pulp macrophages (Oldenborg et al., 2000). This marker protects the cells against elimination by binding to the SIRPα. CD47 analogues are also encoded by smallpox and vaccina viruses (Lindberg et al., 1994). It seems that these pathogens are taking advantage of SIRPα signaling to disable normal defenses. Therefore, CD47-SIRPα may represent a viable approach for the design of macrophage-evading colloidal carriers.

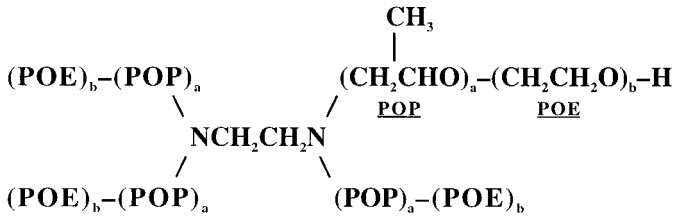
V. Synthetic Polymers in Colloid Engineering

A. Polymeric Nanospheres

From the preceding discussion it is now apparent that a relatively successful approach for prolonging the circulation times of colloidal particles in the blood is to create a steric surface barrier of sufficient density. Because of the possible immunological consequences associated with some bacterial polysaccharides and the high cost of recombinant complement regulators, tremendous efforts have been directed to design synthetic polymers that can fulfill these criteria. The design of long-circulating particles can be traced back to the late sixties. It has long been known that stabilization of emulsion systems may be achieved at the interface by the addition of an emulsifying agent or a surfactant. In a pioneering experiment Geyer (1967) demonstrated that intravenously injected lipid emulsions prepared with high molecular weight members of POE/POP copolymer nonionic surfactants (poloxamers and poloxamines) as emulsifiers remained in the blood for relatively long periods. This behavior was initially thought to arise from the interference of surfactants with the lipoprotein-lipase activity (Hart and Payne, 1971). Later, it was suggested that high molecular weight POP/POE surfactants in some way prevent lipid particles from sticking to the blood vessel endothelium as well as inhibiting recognition by macrophages (Jeppsson and Rossner, 1975).

Poloxamers consist of a central POP block that is flanked on both sides by two hydrophilic chains of POE (Fig. 2). A slightly different structure is exhibited by the

(a) Poloxamines:



poloxamine 908	a = 17	b = 119
poloxamine 1508	a = 31	b = 129

(b) Poloxamers:

$(POE)_b–(POP)_a–(POE)_b$

poloxamer 407	a = 67	b = 98
poloxamer 338	a = 54	b = 128

FIG. 2. The structure of selected poloxamines (a) and poloxamers (b).

poloxamines that are tetrafunctional block copolymers with four POE/POP blocks joined together by a central ethylene diamine bridge (Fig. 2). Numerous investigators have now demonstrated that such copolymers adsorb onto the surface of oil-in-water emulsions or any hydrophobic nanoparticulate systems [e.g., polystyrene, gold, PLGA, poly(phosphazene), poly(methyl methacrylate) and poly(butyl 2-cyanoacrylate)] via their hydrophobic POP center-block (Troster et al., 1990; Moghimi et al., 1993c; Stolnik et al., 1994; Storm et al., 1995; Vandorpe et al., 1997; Monfardini and Veronese, 1998). This mode of adsorption leaves the hydrophilic POE side-arms in a mobile state as they extend outward from the particle surface and provide stability to the particle suspension by a repulsion effect through a steric mechanism of stabilization involving both enthalpic and entropic contributions (Moghimi et al., 1993c). The strength of polymer adsorption and the resultant polymer conformation is dependent on the proportion and the size of both POP and POE segments as well as the physicochemical properties of the nanoparticle surface (Moghimi and Hunter, 2000b). With the help of field-flow fractionation, electron spin resonance and conventional labeling techniques, detailed analytical characterization of the adsorption complexes formed between poloxamers and polystyrene beads of different sizes have been accomplished. These studies have also pointed toward the importance of nanoparticle surface curvature on polymer chain mobility and conformation (Li et al., 1994). For a given triblock polymer, it was found that both surface concentrations and adlayer thicknesses are strongly related to the particle size, such that smaller particles (sizes below 100 nm) take up fewer polymer molecules per unit area than the larger ones. The reduced crowding around each POE chain results in thinner adlayers and higher chain mobilities. Therefore, the surface density decreases with decreasing particle size. For a particle of a given size, it is the size of the surfactant's hydrophobic center block (POP), rather than its flanking tails, that determines the surface concentration or density. Thus, triblocks of similar POP size showed comparable surface concentration, while the longer POE chains were associated with thicker adlayers as well as greater chain dynamics (Li et al., 1994). Therefore, by keeping the particle size constant, one can gain insight into the effect of POE chain lengths on plasma protein adsorption and phagocytosis. Indeed, among the various copolymer members, poloxamine-908, poloxamine-1508, poloxamer-238, and poloxamer-407 (Fig. 2) have proved to be among the most effective copolymer nonionic surfactants for prolonging the circulation time of hydrophobic nanoparticles of 15 to 150 nm in mice and rats. For example, reported half-lives of poloxamine-908-coated nanospheres in mice and rats vary from a few hours to 1 to 2 days, depending on both the particle size and its initial surface hydrophobicity (Storm et al., 1995; Monfardini and Veronese, 1998; Moghimi and Hunter, 2000b).

It has also been shown that surface modifications with poloxamers and poloxamines before intravenous injection is not really necessary for making nanoparticles long-circulatory. Intravenously injected uncoated 60 nm polystyrene nanoparticles (which are susceptible to phagocytosis by Kupffer cells) were converted to long-circulating entities in those rats that received a bolus intravenous dose of either poloxamer-407 or poloxamine-908, 1 to 3 h earlier (Moghimi, 1997, 1999). It can be argued that the altered biodistribution profile of nanoparticles is the result of cell-surface modification by the administered copolymers. For instance, block copolymers could adhere to cell membrane hydrophobic domains via their hydrophobic center block or act as an effective membrane-spanning entity (Watrous-Peltier et al., 1992). The extracellular steric constraints resulting from hydrophilic POE tails of copolymers will then prevent the interaction between an approaching particle and the cell. Interestingly, this was not the primary mechanism. Instead, nanoparticles were shown to acquire a coating of copolymer and/or copolymer-protein complexes in the blood (Moghimi, 1997); this event explains their phagocytic resistance.

Following intravenous injection to mice and rats, poloxamer- or poloxamine-coated sub-200 nm nanoparticles of albumin (Lin et al., 1994), poly(phosphazene) (Vandorpe et al., 1997), and PLGA (Stolnik et al., 1994) as well as liposomes (Woodle et al., 1992; Kostarelos et al., 1999) do not exhibit prolonged circulation times (reported half-lives are usually less than 2–3 h). Therefore,

alternative coating of grafting materials have been tailored. The majority of these materials are based on PEG and its derivatives (Dunn et al., 1994; Lin et al., 1994, 1997, 1999; Gref et al., 1994; Stolnik et al., 1994; Vandorpe et al., 1996, 1997; Lacasse et al., 1998; Peracchia et al., 1997a,c, 1998; Monfardini and Veronese 1998; De Jaeghere et al., 2000). PEG is a linear polyether diol that exhibits a low degree of immunogenicity and antigenicity (Abuchowski et al., 1977). The polymer backbone is essentially chemically inert, and the terminal primary hydroxyl groups are available for derivatization. Usually, the hydroxyl groups are first activated and then reacted with the chosen molecule; PEG activation and functionalization methods have been exhaustively reviewed elsewhere (Zalipsky, 1995; Monfardini and Veronese, 1998). Surface modification of nanoparticles with PEG and its derivatives can be performed by adsorption, incorporation during the production of nanoparticles, or by covalent attachment to the surface of particles. Examples of currently available PEG conjugates for nanoparticle surface engineering includes PEG-R type copolymers, where R is PLA (Stolnik et al., 1994; Bazile et al., 1995), PLGA (Gref et al., 1994), and poly-ϵ-caprolactone (Shin et al., 1998; Kim et al., 1998) with appropriate molecular weights. The molecular weight of the PEG segment varies between 2000 and 5000, which is necessary to suppress protein adsorption. Another related example is poly(phosphazene)-poly(ethyleneoxide)-5000 (Vandorpe et al., 1997). Despite these efforts, coating materials such as the two synthesized PLA-PEGs (PLA:PEG, 3:4, composed of methoxy-PEG-2000 and the weight average molecular weight of 5400 for the copolymer; and PLA:PEG, 2:5, composed of methoxy-PEG-5000 and the weight average molecular weight of 9500 for the copolymer) were found to be inferior to poloxamine-908 in keeping PLGA nanospheres in the rat blood (half-lives of less than 2 h in both cases of PLA-PEG) (Stolnik et al., 1994). On the other hand, biodistribution studies in rats have demonstrated longer retention time for poly(phosphazene) particles coated with poly(phosphazene)-poly(ethyleneoxide)-5000 (half-life of ~3 h) compared with poloxamine-908-coated counterparts (half-life of less than 30 min) (Vandorpe et al., 1997). A European Community Program in nanoparticle engineering (Brite/Euram, BE-3348-89) have further synthesized and used dextranox-methoxy-PEG (dextran-70,000 and methoxy-PEG-5000, total weight average molecular weight of 89,000) for cross-linking to preformed albumin nanospheres (Lin et al., 1994) as well as human serum albumin-poly(thioetheramido acid)-methoxy-PEG-2000 [albumin 49% w/w, PEG 38% w/w, and poly(thioetheramido acid) 13% w/w], albumin-poly(amidiamine)-PEG-2000 [albumin 50% w/w, PEG 40% w/w, and poly(thioetheramido acid) 10% w/w] and albumin-methoxy-PEG for incorporation during albumin nanosphere production (Lin et al., 1997, 1999). Physicochemical charaterization studies such as the measurement of in vitro protein adsorption, dynamic light scattering, turbidity ratio test, electrolyte and pH induced flocculation, and particulate electrophoretic mobility measurements have confirmed the presence of the steric barrier of PEG and dextran-PEG brush-like structures on the surface of albumin nanoparticles. Based on such observations it was proposed that these and structurally similar particles are expected to behave as long-circulatory entities (Lin et al., 1994, 1997, 1999). This, however, contradicts the earlier biodistribution data from the same group which demonstrated rapid localization of these particles in the rat liver (Dunn, 1995; Lin, 1996). Therefore, physicochemical characterization based on the described techniques can not predict the in vivo fate of nanoparticles. These assemblies presumably have an inadequate surface density of PEG which must be addressed.

Submicron sized PEG-ylated poly(isobutyl 2-cyanoacrylate) nanoparticles have also been produced by an emulsion/polymerization method (Peracchia et al., 1997c). Here, PEG was anchored to the nanoparticles through either only one end-group or through both end groups. The latter outcome surely limits the mobility of PEG chains and interaction with blood opsonins and, hence, particle retention time in the blood (Peracchia et al., 1997b). Efforts have also been made to form long-circulating nanospheres from diblock copolymers such as PLA-PEG (expressing a molecular weight of 20,000) in a one-step procedure, but again with rather disappointing results (Gref et al., 1994). Based on indium-111 labeling studies, the circulation half-life of PLA-PEG particles (90–150 nm) was less than 1 h in rats. Furthermore, the presented data indicated that at 90 min after injection, the circulatory pool of nanoparticles was less than 20% of the injected dose whereas the liver-associated activity was in the order of 30% of the initial dose (Gref et al., 1994). No explanation was provided for the missing 50% of the injected activity.

An interesting approach, however, has been the covalent attachment of semitelechelic poly[*N*-(2-hydroxylpropyl)methacrylamide]s of different molecular weights to nanospheres based on methyl methacrylate, maleic anhydride, and methacrylic acid (Kamei and Kopecek, 1995). One polymer preparation, with a weight average molecular weight of 18,800, was able to dramatically extend the circulation time of nanospheres (half-life of 12–15 h) in rats, whereas control preparations were cleared rapidly from the blood by the liver.

B. Micelles (Self-Assembly Constructs)

Multiblock copolymers such as POE-poly(L-lysine), POE-poly(β-benzyl-L-aspartate, POE-poly(ϵ-caprolactone) and poly(acrylic acid)-poly(methyl methacrylate) as well as those that have been used in particle coatings (e.g., poloxamers, poloxamines, PEG-PLA, PEG-PLGA) also self-disperse in water to form spherical polymeric micelles with diameters in the size range of 15 to 80 nm

(Yokoyama, 1992; Jones and Leroux, 1999). Some of these micellar structures have been suggested as promising long-circulating carriers of poorly water soluble and amphiphilic drugs, because of their small size and hydrophilic shell (Yokoyama et al., 1990, 1991; Yokoyama, 1992; Kwon et al., 1993, 1994; Hagan et al., 1996; Zhang et al., 1997a,b). Nonetheless, the effectiveness of such systems will depend on their critical micelle concentration. Upon intravenous injection, micelles are often diluted to less than their minimum micelle concentration and polymer molecules are known to behave in a dramatically different way. Furthermore, little information is known with regard to the stability of micellar systems in vasculature and their extent of interaction with blood and cellular components. Micellar stability may be enhanced by cross-linking procedures to produce a solid outer core (e.g., shell-cross-linked kendel-like particles, Thurmond et al., 1996; Bütün et al., 1999). However, such strategies may be rather premature for the design of a stable long-circulating micellar construct, as the reduced surface energy will probably result in rapid accumulation in liver macrophages.

C. Liposomes

For incorporation into the liposomal bilayer, numerous lipid derivatives of PEG have been made using lipids that, for example contain a primary amino group (particularly PE), an epoxy group or a diacylglycerol moiety (Blume and Cevc, 1990; Klibanov et al., 1990; Allen and Hansen, 1991; Allen et al., 1991a,b; Lasic et al., 1991; Mori et al., 1991; Maruyama et al., 1992; Parr et al., 1994; Kirpotin et al., 1996; Woodle, 1998). Alternatively, activated PEG can be anchored to reactive phospholipid groups of preformed liposomes (Senior et al., 1991). Another strategy has utilized the transfer of PEG-phospholipid conjugates from the micellar phase into the lipid bilayer of preformed vesicles (Uster et al., 1996). It was found by trial and error that PEG-grafted liposomes with extended circulation half-lives are in the size range of 70 to 200 nm and contain 3 to 7 mol% of methoxy-PEG-2000 grafted to DSPE or DPPE in addition to various amounts of phospholipids and cholesterol (Klibanov et al., 1990; Allen et al., 1991b; Woodle and Lasic, 1992; Woodle, 1998). The circulation half-lives of such vesicles is 15 to 24 h in rodents, and as high as 45 h in humans. To date, these are the best engineered long-circulating particles. Only recently, the biophysical basis of these observations was realized. It was shown that mixtures of PEG-phospholipid conjugates and phospholipids exist in primarily three physically distinct states (Bedu-Addo et al., 1996a). These included a lamellar phase with components exhibiting some miscibility, a lamellar phase with components phase separated, and mixed micelles. The relative proportion of the three states in a given mixture was dependent on PEG chain length, acyl chain length and the degree of unsaturation of the PEG-phospholipid conjugate, and the acyl chain composition of the phospholipid. For example, beyond 7 mol% short-chain PEG-DPPE conjugates (PEG molecular weight in the region of 1000–3000) show a strong tendency to form mixed micelles with DPPC. Long-chain PEG-DPPC conjugates (PEG molecular weight of either 5000 or 12,000) above 8 mol% formed phase separated lamellae with the phospholipid due to PEG chain-chain interactions; the PEG chain-chain interactions can be reduced by using PEG-dioleoylphosphatidylcholine due to the presence of the kink in the acyl chain. Changes in phospholipid composition also alters the miscibility of PEG-PE with phospholipids. Increasing phosphatidylcholine acyl chain length increases fatty acid chain-chain interaction and so the van der Waals cohesive force. This results in more tightly packed phosphatidylcholine and PEG-PE molecules and a greater tendency toward PEG chain-chain entanglement and micelle formation. The extent of demixing of PEG-PE in bilayers, therefore, decreases in the order of C18:0 > C16:0 > C14:0. These observations explain why long-circulatory PEG-ylated liposomes usually contain 3 to 7 mol% short-chain PEG-PE (PEG molecular weight in the range of 1000–3000). Large chain PEG-PE (PEG with a molecular weight of either 5000 or 12,000) seem unsuitable for preparing certain formulations of long-circulating liposomes; phase separation generates domains less enriched with PEG-PE and could lead to poor steric protection of the liposome surface and subsequent destabilization by lipoproteins and opsonic attack (Bedu-Addo et al., 1996a).

Inclusion of a high concentration of cholesterol (above 30 mol%) within the liposomal bilayer can further improve surface protection by PEG (molecular weight of 1000–3000)-PE (5–7 mol%). This is due to an increase in bilayer cohesive strength and, hence, a reduction in the formation of phase separated lamellae (Bedu-Addo et al., 1996b). Because of its relatively inflexible structure cholesterol thus acts as a spacer keeping lipid chains apart and reducing PEG chain-chain interaction. At higher concentrations of PEG-PE, solubilization of the bilayer occurs with preferential solubilization of cholesterol over phospholipid. For these reasons the most suitable formulations for a prolonged circulation time contains >30 mol% cholesterol and low concentrations (equal or less than 7 mol%) of short PEG-PE. Even in the presence of cholesterol the steric protection of long-chain PEG-PE is relatively poor. This is presumably due to the fact that reduction in the intramolecular expansion factor with increase in molecular weight could lead to coil shrinkage and, hence, reduced chain flexibility.

Apart from PEG lipid derivatives, other effective alternative materials for prolonging the circulation time of liposomes include phosphatidylpolyglycerols and phospholipid derivatives of amphiphilic poly(vinyl pyrrolidone), poly(vinyl alcohol), poly(2-methyl-2-oxazoline), poly(2-ethyl-2-oxazoline), poly(acryl amide), poly(acryloy morphine), and *N*-(2-hydroxylpropyl)methacrylamide (Torchilin et al., 1994, 1995; Maruyama et al., 1994;

Woodle et al., 1994; Takeuchi et al., 1996; Zalipsky et al., 1996; Shtilman et al., 1999; Whiteman et al., 1999). In contrast to these materials, incorporation of 5 to 7 mol% PEG stearates (Klibanov et al., 1990; Moghimi, 1996) and Tergitols (S. M. Moghimi, unpublished observations) have proved to be ineffective in prolonging liposome circulation times. The biophysical basis for these observations remains to be elucidated.

Coating of liposomes with chitin derivatives such as carboxymethylchitin and carboxymethyl/glycochitin has also been achieved (Mobed and Chang, 1998). *N*-Acetylglucosamine is the repeating unit of these chitin derivatives and is rather similar to *N*-acetyl and *N*-glyconeuraminic acid of the red blood cell outer membrane (Mobed and Chang, 1991). The adsorption of carboxymethychitin (molecular weight 101,000) onto the surface of neutral liposomes not only resulted in electrostatic stabilization of the vesicles but also provided an effective barrier to macromolecular plasma protein adsorption (Mobed and Chang, 1998). However, the retention time of such constructs in the systemic circulation remains to be evaluated.

D. Oil-in-Water Emulsions

Apart from poloxamers and poloxamines, PEG-PE and Tweens have all been used as emulsifiers for the production of long-circulatory oil-in-water emulsions. Again, the blood retention time of such emulsions was shown to be related to PEG chain length and its surface density (Liu and Liu, 1995; Lundberg, 1997).

VI. Essential Thoughts on Using Polymers in Nanoparticle Engineering

A. The Concept of Polymer Polydispersity: Does Size Matter?

An important consideration in clinical therapeutics is the ultimate fate of polymeric constituents used in nanoparticle engineering. To date this subject has received little attention. The fate of any polymer used will be determined by its method of synthesis, subsequent degree of purity and its behavior on administration into the individual biological system. Synthetic polymers exist as populations with a statistical distribution, i.e., they are polydisperse systems which vary in molecular weight (Fig. 3). The degree of polydispersity will tend to be greatest if the product has been synthesized by free radical polymerization, which is nonselective with chain termination occurring, by combination or diproportionation. On the other hand, starburst polyamidoamine dendrimers are a relatively new class of spherical macromolecules, which have been reported as monodisperse (Kukowska-Latallo et al., 1996). Ironically, the future usefulness of these monodisperse systems in medicine remains to be revealed, since in vivo studies so far have indicated their propensity to localize in the liver and spleen (Malik et al., 2000).

There have been some preliminary studies on the effects of specific molecular weight fractions present within a polydisperse system to discern individual biological activities in vivo on both short-term and long-term administration (Emanuele et al., 1997; Toth et al., 1997). Variation in polymer molecular weight fractions has been linked to tissue toxicity (Toth et al., 1997). For example, Flocor (CytRx, GA, USA), a well defined fraction of poloxamer-188 (molecular weight 8964, polydispersity 1.0280) only reduced nephrotoxicity by 68% in a recent clinical trial (Emanuele et al., 1997) when compared with the native poloxamer-188 (RheothRx) following intravenous administration. This suggests that the nephrotoxic constituent(s), which are below the renal threshold, had not been removed or that the polymer is inherently toxic in its own right or a joint effect of the two (see also *Section VI.C.*). Limited studies with polydispersed radiolabeled POP/POE copolymers have indicated that the primary route of polymer excretion is renal and the minor route is biliary (Wang and Stern, 1975; Willcox et al., 1978; Rodgers et al., 1984). In addition, radiolabeled copolymers have been found in all organs, particularly in the liver, lung and skeletal muscles, 24 h after intravenous administration into the dog (Willcox et al., 1978). Long-term effects of accumulated polymers in these organs still remain to be evaluated. Furthermore, no studies so far have determined to what extent these copolymers are excreted intact or in a modified form from the starting material. Poloxamers and poloxamines can also adsorb on to the surface of blood cells and endothelia but the long-term significance has not been thoroughly investigated (Smith et al., 1987). For example, adsorption on to endothelial cells may lead to activation of NF-κB or endothelial cells may respond with the expression of proteins stored in the cytoplasm.

Another classical example is the early work done with pyran copolymers. Here, the relation in variation between the polymer molecular weight and its LD_{50} was illustrated by Breslow (1976) which may be of relevance to polydisperesed systems used in nanoparticle engineering. For example, the LD_{50} for molecular weight fractions of 6900, 15,700, and 19,600 of pyran copolymer injected intravenously into mice were 127, 131, and 100 mg/kg, respectively. Molecular weight was found to affect the degree and type of physiological response. Below a molecular weight of ~15,000 stimulation of phagocytic activity of the RES was achieved, whereas with higher molecular weight polymers induced depression in phagocytic activity of the RES. Serum glutamate pyruvate transaminase was used as a marker of liver damage, higher levels were attained with increasing polymer molecular weight and greater polydispersity as was the inhibition of metabolism of the compounds aminopyrine and antipyrin. It is not clear from these studies to what extent the observed toxicities are related to the presence of impurities and/or oxidized contaminants in each polymer sample.

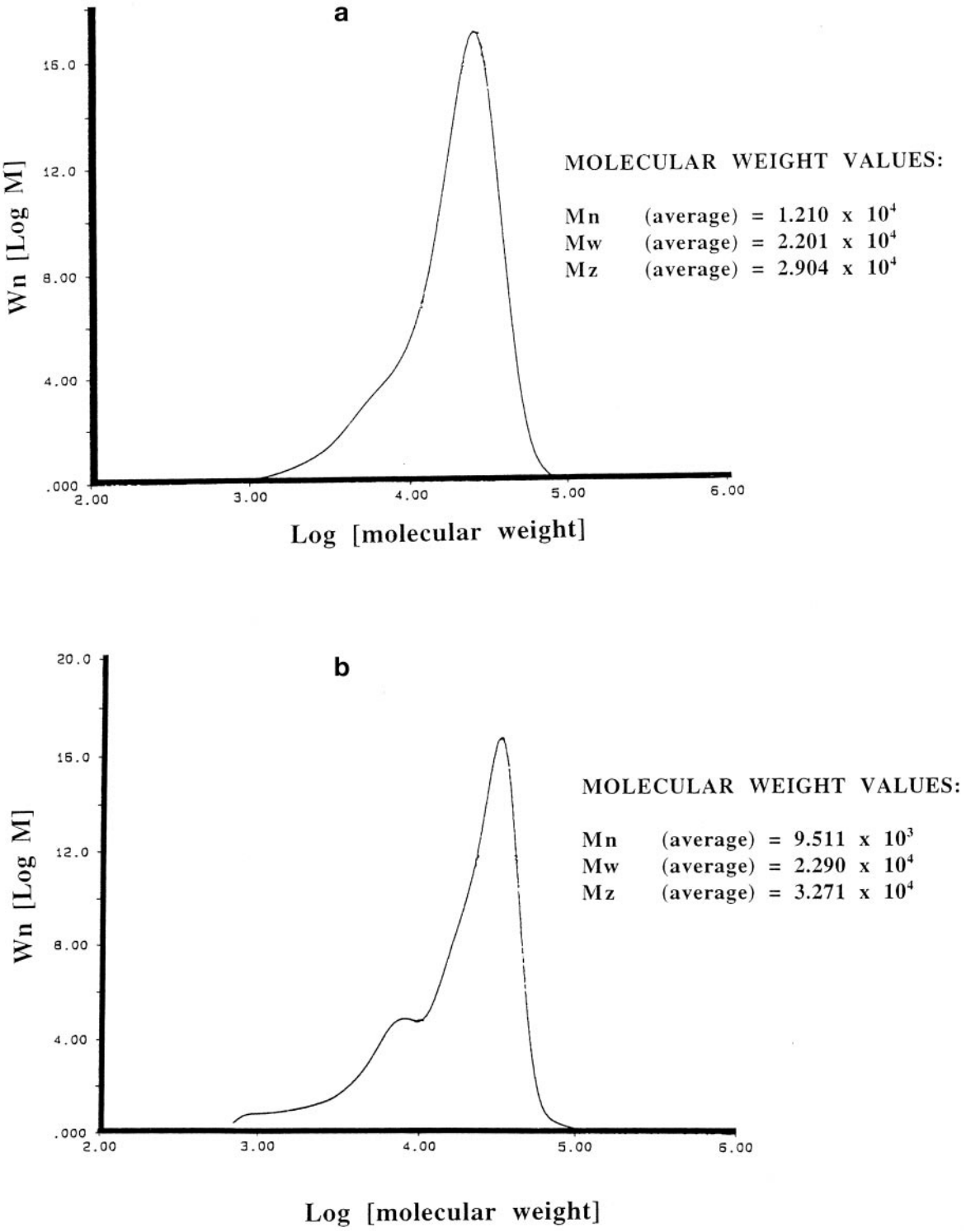


FIG. 3. A comparison of two batches of poloxamine-908 obtained from the same manufacturer and analyzed by gel permeation chromatography. Clear differences are seen in both log molecular weight range and weight fraction of the polymers. Wn[log M], weight fraction of polymer; Mn, number average molecular weight; Mw, weight average number weight; Mz, molecular weight average.

B. Sources of Chemical Contamination in Polymers

Most polymers are synthesized under relatively hostile conditions (elevated temperature and pressure) and are thus prone to processes of degradation during their formation. To counter the in-process environment processing modifiers (e.g., heat stabilisers and emulsifiers) are frequently used but readily separated from the polymer (Stevens, 1999). To ensure postprocessing polymer stability chemical property modifiers such as antioxidants and ultraviolet stabilisers are added. It must therefore be considered if these or other additives and/or low molecular weight degradative products or monomers

are capable of inducing immunological (e.g., IgE-independent hypersensitivity reactions) and/or pharmacological events following parenteral administration (see also part *C* below). There is relatively little information available regarding the biological fate of antioxidants or their break-down products. Unfortunately the concentration and types of additives and low molecular weight contaminants (e.g., monomers, oligomers, homopolymers) present in commercial products is proprietary information, which is not readily available. An investigation into the effect of removal of the antioxidant butylated hydroxy toluene, a known skin sensitizing agent, from poloxamer-188 has been attempted (Bentley et al., 1989). Unfortunately a complete lack of data supporting the presence of the poloxamer following column chromatography rendered the results of this study meaningless. Other techniques such as supercritical fluid fractionation (Edwards et al., 1999) have been used to purify polymers; however, in this case there was evidence for macromolecular cleavage.

Incomplete polymerization of diblock copolymers may result in homopolymer contamination in the product, which are exceptionally difficult to remove and may explain the presence of homopolymers in some commercial samples (Moghimi and Hunter, 2000b). It has not yet been determined whether these contaminants are essential for the useful activity of the polymer or they have a synergistic role in combination with the copolymer. A possible future method to circumvent the deleterious consequences of hostile synthetic processing conditions and negate the use of specific additives may be achieved through milder microbiological methods. To date commercially bacterially derived polymers have included polyhydroxyacids and polyesters (Kunioka and Doi, 1990; Gogolewski et al., 1993b).

C. Interspecies and Intraspecies Response(s) to Polymeric Systems

It should also be emphasized that different species may respond differently to the administered polymeric materials. For example an early study with polyelectrolyte poly(ethylene sulfonate) clearly demonstrated a broad spectrum of antitumor activity in mice following subcutaneous administration (Regelson and Holland, 1958). However, the antitumor activity of this polymer was found to be reduced in humans and it was also too toxic for clinical use (Regelson and Holland, 1958; Breslow, 1976). Variation in interspecies activity has also been observed with pyran copolymer. This material was able to induce interferon production in mice. A synergistic effect between pyran copolymer and the foot-and mouth-disease vaccine in mice (Campbell and Richmond, 1973) was observed. A 1.2 mg dose of pyran copolymer plus the vaccine protected 80% of the mice, with no antiviral activity observed for the copolymer alone. This activity was not carried over to cattle and pigs where dosages were too high and toxic side-effects were observed (Sellers et al., 1972; McVicar et al., 1973). These observations seem relevant to nanoparticle production and engineering with polymers and suggest caution in extrapolating animal model responses to humans. We may even experience variations among different individuals. For example, recently, we have demonstrated variation in complement activation in the sera of healthy individuals by various poloxamer and poloxamines (Szebeni et al., 2001). The surfactants failed to activate complement in the sera of some individuals, whereas in others complement activation did occur. Variation in the pathway of complement activation was also noticed among the positive population. We were not able to find any correlation between the molecular weight of the copolymers and the extent of complement activation among the positive population. Impurities in copolymers or derivatives of the copolymers generated in the manufacturing process have been proposed to be responsible for activation of the complement system (Ingram et al., 1993). Interestingly, removal of some impurities by dialysis or organic extraction resulted in further activation of complement system among some individuals. These treatments also activated complement in the sera of even some negative individuals (in those that no complement activation by the pretreated copolymers were observed). Complement activation may induce mild to severe hypersensitivity reactions. Therefore, future studies should explore the relationship between in vitro and/or in vivo complement activation by such copolymers and clinical signs and symptoms of hypersensitivity to poloxamer/poloxamine based nanoparticles. Similar to the above observations, variation in complement activation in serum of healthy individuals by PEG-bearing liposomes has also been observed (Szebeni et al., 2001).

D. Biodegradable Polymers

The situation regarding the fate and activity of polymers is further complicated by the current focus on the development of biodegradable/bioerodable polymeric drug delivery vehicles for parenteral use. At present there is no study available, which demonstrates these polymers (e.g., PLA, PLGA) will degrade in a uniform manner in vivo. Clearly the in vivo environment will vary depending on the site of parenteral administration, composition of tissue fluids and disease state which further complicates the uniformity of the degradation process. PLGA polymers have been used widely as biomaterials for medical applications over the last 30 years and are regarded as “biocompatible” and “nontoxic”. This has been due to the wide variety of materials achievable by varying the molar ratios of the lactic acid and glycolic acid moieties. For example, high molecular weight crystalline PLGA has been used effectively as surgical sutures and bone fixation nails and screws (Daniels et al., 1990; Pulapura and Kohn, 1992). Conversely low molecular weight amorphous PLGA has

been researched widely for controlled drug delivery applications (Asano et al., 1990; Wang and Wu, 1997). An example of a successful pharmaceutical product for the controlled delivery of lutenizing hormone in the form of injectable depot is Zoladex (Zeneca, UK). However, there is little information available regarding the rate of degradation as well as toxicological problems associated with PLGA and related biodegradable particulate drug delivery systems following parenteral administration. A 2-fold increase in the rate of degradation of PLA has been observed in plasma compared with buffer or water at 37°C (Mason et al., 1981). The specific plasma constituents responsible were not identified. The large surface area of the colloid, which is gradually presented to opsonic and other immunoregulatory proteins, may potentiate these effects. This surface area will be markedly increased once the degradation process starts. It will be essential to control the rate of degradation in the in vivo environment to ensure even dosage delivery assuming that the polymer and its by products are completely inert in vivo. Faster in vivo rates of degradation have been attributed, in part, to the foreign body response (Tokiwa and Suzuki, 1977; Williams and Mort, 1977; Ali et al., 1994). Investigation into the phagocytosis of both whole ($\leq$ 38 μm) and predegraded PLA particles following intraperitoneal administration into mice has been undertaken (Lam et al., 1993). Cells that had phagocytosed PLA particles showed increased signs of cell damage compared with the control which was nondegradable polytetrafluoroethylene particles of 4 to 9 μm. Predegradation of PLA particles induced cell damage (e.g., swollen mitochondria, widened endoplasmic reticulum, disappearance of membrane ruffling) on day 2 of the experiment with evidence of necrotic cells and cell debris on days 3 and 4. This clearly indicates that at least in the mouse model degradation products of the so called "bioinert" PLA can be cytotoxic to immune cells. Therefore, there is a clear need for a wide range of in vivo studies on biodegradable polymers to determine the nature and fate of the degradation product(s). The physicochemical nature of the particle (size, shape and porosity will also affect the degradation rate (Matlaga et al., 1976; Grizzi et al., 1995). The autocatalytic nature of some polymers will further accelerate degradation rates and this may be potentiated when the polymers are isolated or have restricted transport away from a region. For example, hydrolysis of the esteratic link of PLA will result in the formation of more acid groups and, hence, increased availability of protons to feed the esteratic hydrolysis. PLG polymers with the acid group capped (as an ester) have been demonstrated to have reduced rates of degradation compared with the free acid and that the length and type of capped moiety can be used to control the rate of degradation (Tracy et al., 1999). Perhaps the ideal drug delivery application for biodegradable polymer microparticulate systems is for the delivery of biologically active polymers. If controlled rates and site of degradation can be achieved it is an ideal opportunity for a sustained release "biologically active polymer-polymer therapeutic system".

The precise role of polymer stereochemistry on enzyme degradation and immunological response following parenteral administration has not yet been determined. Stereochemical preference for copolymer bonds has been demonstrated (Li et al., 2000), degradation of stereocopolymers (L-, DL- and meso-lactides) by proteinase K that showed preferential degradation of bonds with the stereochemical configuration of L-L, L-D and D-L bonds as opposed to D-D bonds. However enzymatic attack in the case of the meso form may have been facilitated by increase rates of water uptake. A comparison of the effect of poly-L- and D-lactides on inflammatory response was found to be reduced in the case of polymers containing the D-isomer (Gogolewski et al., 1993a). Whether different stereochemistry can modulate biological responses is still subject to speculation.

VII. Why Are Polymer-Coated Long-Circulating Particles Eventually Cleared by Macrophages?

All polymer-coated or -grafted long-circulating particles are at some point cleared from the blood of tested species by macrophages of the RES (Moghimi and Murray, 1996; Moghimi and Gray, 1997; Moghimi, 1998; Dams et al., 2000a). As discussed in the previous section, polymers are statistical macromolecules; any given sample usually contains several closely related species. In a particular polymer sample, the pattern as well as the strength of noncovalent binding of individual polymer macromolecules to a nanoparticle surface may vary from one to another. Therefore, it is possible that in the blood a fraction of the surface adsorbed polymers, depending on their physicochemical characteristics, is displaced by some plasma proteins (polymer dissociation by displacement). This partial displacement of the surface associated polymer decreases the steric shielding at particular surface sites and makes the nanoparticles prone to phagocytoses. In the case of long-circulating nanospheres with a polymeric texture, the processes of swelling, degradation and erosion of the polymer matrix may induce morphological and surface changes favorable for opsonization and/or phagocytosis. Similarly, with grafted systems (as in PEG liposomes), an alternative explanation is the chemical breakdown of the polymer-conjugate linkages (e.g., succinate, carbamate, amide) in the blood (Parr et al., 1994), rapid partitioning (e.g., PEG ceramides from the liposomes) (Webb et al., 1998) and complete transfer of the whole conjugate to vascular elements. These possibilities, at least, seem to be in agreement with two-phase partitioning of PEG-attached liposomes in the presence of plasma which resulted in gradual plasma protein build-up on the vesicle surface (Senior et al., 1991). The receivers of the coating materials may be the circulating lipoprotein particles or even

cells such as erythrocytes and vascular endothelial cells (Smith et al., 1987; Howerton et al., 1990).

The majority of surface coating polymers used to date are rich in hydroxyl groups, which may eventually trigger the activation of both the alternative and classical pathways of the complement system. This results in particle fixation by opsonizing complement fragments (e.g., C3 degradative products) and stimulation of particle recognition by activated complement receptors of phagocytic cells. For example, Cremophor EL, as well as poloxamer and poloxamine copolymers can activate the human complement system, causing conversion of C3 through both the alternative and classical pathways (Hunter and Bennett, 1984; Szebeni et al., 1998, 2000, 2001). As for the explanation of classical pathway activation of complement by such polymeric materials, we have speculated a role for C-reactive protein and antibodies such as anti-cholesterol and anti-phospholipid antibodies (Szebeni et al., 2001); these antibodies are abundant in most humans (Alving and Swartz, 1991). For instance, the binding of naturally occurring anticholesterol antibodies to the hydroxyl-rich surface of poloxamer- or poloxamine-coated nanoparticles is possible, since the epitope they recognize contains a hydroxyl group.

Complement activation has also been suggested to be responsible for the rapid clearance of radiolabeled methoxy-PEG-coated liposomes by macrophages of the RES in two patients (Dams et al., 2000b). An intriguing observation, however, is the poor clearance of such complement (C3b and iC3b)-opsonized PEG-bearing liposomes in certain human individuals (circulation half-lives of more than 45 h) (Szebeni et al., 2000). Several possibilities may account for these observations. First, the binding of vesicle-bound monomeric and/or polymeric iC3b to macrophage receptor CD11b/CD18 (CR3) may be sterically hindered by PEG. Second, competition between vesicle-bound and free iC3b for CR3 can not be ruled out. Thirdly, such vesicles may bind to erythrocytes as they express receptors for C3b (CR1). These cells outnumber circulating leukocytes and the vast majority of CR1 receptors present in circulation are located on the erythrocytes (Cornacoff et al., 1983). It is plausible that erythrocytes will eventually transfer bound liposomes to liver and spleen macrophages during their passage through these organs. Finally, generation of liposome-bound C3bn complexes requires detailed analysis as PEG may protect C3bn-containing complexes from inactivation. Indeed, these complexes are among the most potent nucleation sites for the alternative C3 convertases.

Another interesting possibility arises from the ability of stimulated or activated macrophages to rapidly recognize and ingest long-circulating particles. The majority of tissue macrophages are in the quiescent state but some macrophage populations in the same tissue may exist in primed or activated states. One study (Moghimi et al., 1993a) has shown the ability of zymosan-stimulated macrophages to recognize and ingest long-circulating particles by an opsonic-independent process. This effect of zymosan was attributed to changes in macrophage cell surface hydrophobicity as well as changes in mobility of certain plasmalemma receptors, leading to recognition of some structural determinants of long-circulating particles in vivo. Therefore, it seems that activated or stimulated macrophages can overcome the steric barrier of long-circulating particles and proceed with the phagocytic ingestion. This may be of particular relevance with regard to the intravenous administration of long-circulating carriers in clinical situations with enhanced regional or global macrophage activity. To date, the majority of studies in determining the pharmacokinetics and tissue distribution of long-circulating particles have relied on healthy animals or have used disease models with suppressed macrophage activity.

It is also likely that in certain cases the coating polymer triggers the clearance of long-circulating particles from the blood by modulating some functions of the host immune system. Two lines of investigations are in agreement with this suggestion. Moghimi and Gray (1997) were the first to report that a single intravenous injection of poloxamine-908 or poloxamer-407-coated polystyrene particles (60 nm) into rats could dramatically affect the half-life and body distribution of subsequent doses. The degree of this alteration was dependent on the interval between the doses. An interval of 2 days between the two injections had no effect on the pharmacokinetics of the second dose. Interestingly, when the interval between the two injections was 3 to 10 days, the second dose of particles were rapidly cleared from the blood by Kupffer cells and splenic red pulp macrophages. Again, intervals of longer time periods had no effect on half-life or tissue distribution of the second dose of polymer-coated particles as they behaved as long-circulatory; these observations were not associated with macrophage depletions or defects in phagocytosis. Subsequently, the coating polymer was found to trigger particle clearance from the blood by resident ED2 positive Kupffer cells and certain subpopulations of splenic red pulp macrophages. These coplymers can also activate the phagocytic activity of neutrophils (Ingram et al., 1992). It is also possible that activated neutrophils phagocytose polymer-coated particles and subsequently home in to other organs such as the spleen and lungs. The mechanism of mononuclear phagocyte stimulation by these copolymers is not clear but may be directed primarily through cytokine production by these cells. For example, a poloxamer member (CRL-1072) was shown to stimulate the production of IL-8, TNF-α and granulocyte-macrophage colony stimulating factor by macrophages in a dose-dependent manner (Jagannath et al., 1999).

In a similar line of investigation, Dams et al. (2000a) further demonstrated that a single intravenous injection of PEG-grafted long-circulating liposomes into rats or

monkeys significantly alters the pharmacokinetic profile of subsequently injected PEG liposomes. The outcome was rather similar to the earlier reported studies on repeated administration of POP/POE copolymer-coated particles (Moghimi and Gray, 1997; Moghimi, 1998) with the exception that a minimum time interval of 5 days between the two liposome injections was required to evoke macrophage clearance of the second dose by an opsonic dependent process. The presented data suggested a role for complement activation by an unknown heat labile serum factor. The above evidence probably explains why the circulatory life time of all engineered long-circulating particles never exceeds 3 to 5 days.

VIII. Experimental and Clinical Trials with Parenterally Administered Long-Circulating Particles: Achievements and Pitfalls

A. Circulating Drug Reservoir in the Blood Compartment

To date, long-circulating polymeric nanospheres have received little attention for their ability to act as a sustained-release system for continuous and controlled release of their entrapped drugs over a period of days, because the current technology is not sophisticated or smart enough. First, with the exception of one or two examples, all long-circulating nanoparticles are cleared from the systemic circulation of experimental animals within the first 8 to 10 h after intravenous administration. Repeated administration of a system based on the current technology could also lead to rapid accumulation in macrophages of the RES and induce toxicity. Second, the complex processes of gradual nanosphere degradation and erosion (e.g., random-chain and end scission) within the vasculature and the kinetic of drug release into the blood have been poorly addressed. Earlier work with classical "macrophage-prone" nanoparticles has shown that the molecular and bulk properties of the matrix polymer such as melting and glass transition temperatures, molecular weight distribution, crystallinity, degree of crosslinking, chemical structure etc., all control the efficiency of drug encapsulation as well as the extent of nanosphere degradation and drug release (Si-Nang et al., 1973; Ziff and McGrady, 1985; Kehlen et al., 1988; Shah et al., 1992; Grandfils et al., 1996; Batycky et al., 1997). Therefore, the release rate of drug from nanoparticles is expected to vary, among other factors, with the thickness and porosity as well as the size of the core drug particles, the dispersion state of the drug, drug solubility and molecular weight. The release profile of any nanoparticulate population is also a function of the drug payload and rate constants of the individual nanoparticles (Benita, 1984; Donbrow et al., 1986; Hoffman et al., 1986; Benita et al., 1988). The above issues must be considered for the development and vascular performance of long-circulating polymeric nanoparticles if they are going to act as a circulating reservoir for a particular drug. Hence, not only the clearance kinetics of the nanoparticle from the blood should be considered, but also the in vivo process of gradual nanosphere matrix degradation and the extent of drug release must be worked out in relation to a pharmacologically desired free drug profile. Batch-to-batch reproducibility, both in terms of nanoparticle production and drug release, is also of major concern since polymers are heterogeneous with regard to both molecular weight and macromolecular architecture (e.g., degree of branching and crosslinking).

In contrast to nanoparticles, liposomes may serve as a better example of circulating drug reservoir in humans, simply because of their rather longer blood residence and the presumed intrinsic safety of their matrix-forming components over polymeric nanospheres. In an experimental study, intravenously injected PEG liposomes were tested for their efficacy as a slow release system within the vasculature for the antineoplastic drug ara-C in the treatment of murine L1210/C2 leukemia (Allen et al., 1992). This drug is rapidly inactivated in vivo by cytidine deaminases with an initial half-life of 16 to 20 min in mice (Borsa et al., 1969). This is very close to the value found in humans (Baguley and Falkenhaug, 1971). The PEG-liposomal formulation of the drug was found to be superior to the 24 h free drug infusions given at the same total drug dose as well ara-C encapsulated in non-PEG containing liposomes, and prolonged mean survival times of the mice. Thus such constructs appear at least to protect rapidly degraded drugs from breakdown in vivo, with release of the drugs in a therapeutically active form over relatively long time periods. Although not investigated, the ability of PEG liposomes to localize in bone marrow, liver, and spleen may also be contributing to their therapeutic effect: indeed, high numbers of tumor cells can be found in the bone marrow and spleen within a few hours following intravenous injection of leukemia cells. Similarly, the increased reported therapeutic activity of doxorubicin-entrapped PEG liposomes in various models of solid tumors, when compared with free doxorubicin or doxorubicin-entrapped in macrophage-prone vesicles (Cabanes et al., 1999; Drummond et al., 1999), might be attributed to the increased circulation lifetimes of such vesicles; these systems could act as slow release or systemic infusion delivery vehicles in the blood compartment. Furthermore, PEG incorporation into the liposome bilayer significantly attenuates the toxic effects of doxorubicin to hepatic macrophages (Daemen et al., 1997).

B. Blood-Pool Imaging

Long-circulating PEG liposomes and micelles with entrapped radiopharmaceuticals or contrast agents have been successful in blood-pool imaging. For example, an iodine-containing amphiphilic block copolymer consisting of methoxy-PEG and poly[ϵ,*N*-(triiodobenzoyl)]-L-lysine was shown to form stable long-circulating micelles

of 80 nm in diameter with an iodine content of 35 to 40% wt (Torchilin et al., 1999). These micelles provided clear and informative images of the blood pool, using computer tomography. Similarly, PEG liposomes labeled with technetium-99m chelator hexamethylpropyleneamine, which are highly stable in vivo, have produced quality images of the heart and sites of gastrointestinal bleeding in experimental animals (Goins et al., 1996).

C. Passive Targeting

1. Pathologies with Leaky Vasculature: Solid Tumors. Numerous studies have confirmed passive accumulation of intravenously injected long-circulating particles in experimental and clinical cases of solid tumors (Papahadjopoulos et al., 1991; Huang et al., 1992; Yuan et al., 1994; Forssen et al., 1996; Northfelt et al., 1996; Parr et al., 1997; Drummond et al., 1999). The extent of particle extravasation, however, depends on the porosity of the angiogenic tumor vessels as well as tumor blood flow. Among the tested particles, PEG liposomes have received considerable attention and this subject has been examined by Drummond et al. (1999) in a recent issue of *Pharmacological Reviews*. From several experimental models as well as the Kaposi's sarcoma lesions in humans, it appears that the therapeutic activity of anticancer agents is substantially enhanced by entrapment in PEG liposomes (70–200 nm) when compared with the free drug and in some cases to drug-entrapped in unprotected liposomes (Gill et al., 1995; Harrison et al., 1995; Drummond et al., 1999). Undoubtedly, the encapsulation procedure reduces the free drug peak plasma concentration and drug exposure to susceptible tissue such as heart and macrophages of the liver. The question arises as to what extent passive accumulation of PEG liposomes in tumors accounts for the reported improvements in antitumor activity, particularly with respect to our earlier discussion (see *Section II.*). From the experimental tumor models it appears that the majority of localized liposomes do not interact with target cancer cells (Huang et al., 1992; Yuan et al., 1994). For example, in a human tumor xenograft implanted in severe combined immunodeficiency mice, the extravasated liposomes were found to be distributed heterogeneously where it formed perivascular clusters that did not move significantly and could be observed for up to 1 week (Yuan et al., 1994). Surely, for improved anti-tumor activity, the gradual process of vesicle extravasation must be followed by the efflux of drug from the liposomes, resulting in tumor cell exposure to drug molecules. The mechanisms responsible for drug leakage from extravasated liposomes in tumors has been poorly studied, primarily due to technical difficulties associated with monitoring drug release. However, several speculative mechanisms can induce drug release from extravasated vesicles. One possibility is the drug leakage induced by the interstitial fluid surrounding tumors. Such fluids usually contain oxidizing agents as well as lipases and other hydrolytic enzymes derived from dying tumor cells or the resident inflammatory cells (see next section). For example, in an in vitro study the leakage of doxorubicin from long-circulating liposomes was confirmed in the presence of fluid obtained from pleural malignant effusions (Gabizon, 1995).

Nonetheless, if drugs are released from the extravasated vesicles then it is very likely that long-circulating liposomes inherently overcome a certain degree of multidrug resistance by tumor cells, since such vesicles can deliver between 3 and 10 times more drug to solid lesions when compared with the administered drug in its free form (Krishna and Mayer, 1998). Indeed, the clinical formulation of doxorubicin in PEG-coated liposomes (Caelyx or Doxil) is able to deliver between 5 and 11 times more doxorubicin to Kaposi's sarcoma lesions than to normal skin, leading to an overall tumor response rate as high as 80% (Northfelt et al., 1996, 1997). Thus, if tumors exhibit a low resistance factor, then tumor regression can be expected. For tumors exhibiting higher resistance levels, as in a rat glioblastoma tumor model (Hu et al., 1995), PEG liposomes by themselves may be unable to circumvent multidrug resistance significantly. Therefore, an alternative approach is to use a temperature-sensitive long-circulating vesicle in conjugation with hyperthermia and indeed this has proved to be an effective strategy in many cases (Huang et al., 1994). This novel approach, however, has limited applicability in vivo given that the conditions for hyperthermia are unlikely to be useful for visceral or widespread malignancies. Other alternative approaches may elaborate on the use of biochemical triggers such as the pH sensitive lipid-anchored copolymers for liposome engineering (Meyer et al., 1998) and cleaveable PEG-PE (Kirpotin et al., 1996; Zalipsky et al., 1999) to generate fusion competent vesicles which has been reviewed by Drummond et al. (1999). Another possible strategy is the attachment of engineered protease-sensitive hemolysin to long-circulating vesicles (Panchal et al., 1996; Provoda and Lee, 2000). It is expected that, when such constructs reach the tumors, pore formation within the liposomal bilayer is activated by the proteases released from the cancer cells. The hemolysins may even begin to generate pores in the cell membrane, thereby enhancing their permeability to cytotoxic drugs. Studies with liposomes composed of certain acidic phospholipids such as PS have demonstrated that these phospholipids can increase the cytotoxicity of encapsulated anticancer drugs against multidrug resistance cells (Fan et al., 1990). Interestingly, inclusion of 5 to 7 mol% PS in the liposomal bilayer was shown to strongly antagonize the effect of PEG-PE and resulted in rapid localization of such liposomes to the liver macrophages (Allen et al., 1991b; Klibanov et al., 1991). One possible explanation is that liposomes undergo a phase separation in the presence of divalent cations and the domains enriched with PS-cation complex, but excluding PEG-PE, readily attract

plasma opsonins or directly bind to macrophage scavenger receptors (Allen et al., 1991b; Klibanov et al., 1991). Recently, it was demonstrated that the inclusion of 15 mol% DSPE-PEG 2000 can protect PS liposomes from binding to blood coagulation proteins and extend the circulation longevity of the vesicles (Chiu et al., 2001).

Attempts have also been made to encapsulate multidrug resistance modulators in liposomes. Unfortunately, the multidrug resistance modulators are either not suitable for liposomal encapsulation (Krishna and Mayer, 1998) or exhibit high leakage rates from liposomes (Webb et al., 1995; Ouyang et al., 1995) when exposed to the blood (e.g., verapamil and prochlorperzaine). Here, long-circulating polymeric nanospheres may be of use providing that they can efficiently release their entrapped agents following extravasation. On the other hand, a conventional method to circumvent multidrug resistance is to utilize coadministration of chemosensitizers and anticancer drugs. A potential problem with this approach is the exacerbated toxicity of the anticancer drug, usually due to its altered pharmacokinetics. Given the ability of long-circulating liposomes to accumulate in tumors, a promising approach will be to administer a potent multidrug resistance modulator in free form but in combination with an anticancer agent encapsulated in a long-circulatory carrier.

Human tumors are known to contain large numbers of macrophages. However, not much is known with regard to uptake of extravasated long-circulating carriers by tumor macrophages and their effect on macrophage function and viability. Such studies are necessary particularly with the view that some polymeric materials or their degradative products can affect macrophage accessory functions. For example, poloxamer-231 was reported to enhance the expression of macrophage major histocompatibility complex class II (Ia) molecules in mice following intraperitoneal administration in the absence of mature lymphocytes (Howerton et al., 1990). Poloxamer-231 induced macrophages were also primed for the secretion of superoxide anions, which can be stimulated by interferon-γ and lipopolysaccharide to lyse tumor cells (Howerton et al., 1990).

Numerous in vivo studies have shown that polymeric micelles are also able to improve the efficacy of anticancer agents against solid tumors (Yokoyama et al., 1990, 1991; Yokoyama, 1992; Zhang et al., 1997a,b). Comparisons between the activity of free drug to that of micelle-incorporated drug are rather difficult to make because these experiments have often been carried out at the maximum-tolerated dose, which may be different for the two formulations (Yokoyama et al., 1990, 1991). However, the improved reported efficacy with micellar formulation may be due to priming or activation of intratumor macrophages by the micellar polymeric components.

2. Pathologies with Leaky Vasculature: Inflammatory and Infectious Sites. Surprisingly, little attention has been paid with regard to passive accumulation of long-circulating particles at inflammatory sites and infectious foci. However, a preliminary experiment has confirmed passive accumulation of intravenously injected PEG-1900 grafted liposomes at arthritic sites in rats (Corvo et al., 1999); the processes of drug release and therapeutic monitoring were not addressed in this study. Long-circulating radiolabeled liposomes (e.g., indium-111 or technetium-99m-labeled PEG-1900-coated liposomes of 100 nm in size) have also proved to be valuable tools in detection of experimental and clinical infectious and inflammatory foci by gamma scintigraphy (Goins et al., 1993; Boerman et al., 1995, 1997a,b; Dams et al., 1998, 1999, 2000b). In terms of diagnostic accuracy, the scintigraphic results seem to be similar between radiolabeled liposomes and radiolabeled leukocytes (the established method for diagnostic imaging). Therefore, liposome-based radiopharmaceuticals may be an attractive alternative for labeled leukocytes, because they can be prepared easily and handling of human blood is not required. Such radiopharmaceutical vesicles also show advantages over radiolabeled polyclonal antibodies in the detection of infectious and inflammatory foci. For example, in rats with focal *S. aureus* infection the abscess-to-muscle ratios with indium-111-labeled PEG liposomes were approximately four times as those obtained with indium-111-labeled polylclonal IgG (Boerman et al., 1995). Current limitations associated with radiolabeled IgGs include the inability to image vascular lesions due to slow clearance of immunoglobulins from the blood, moderate sensitivity in particular patient groups, a relatively long time-span between injection and final diagnosis, a high radiation burden, and poor stability of the radiolabel (as in technetium-99m-labeled IgG) and accumulation in nontarget tissue (e.g., the kidneys) (Oyen et al., 1991).

At sites of inflammation or infection, activated phagocytes may phagocytose extravasated PEG-coated vesicles. The phagocytic process is probably enhanced as a result of the combined effect of an elevated concentration of phospholipase A2 at these sites, and an increase in phospholipase catalytic activity toward PEG-containing vesicles (Vermehren et al., 1998). From the therapeutic point of view, phagocytosis of liposome-encapsulated drugs could lead to phagocyte apoptosis (as in the case of inflammatory diseases) or efficient microbial destruction as macrophages may serve as sites of proliferation of microbes during some or all of the infection process.

3. Spleen. There are several clinical advantages for efficiently redirecting intravenously injected colloids to the spleen (Moghimi, 1995b). For example, the design of spleen-specific radiopharmaceuticals and contrast agents may enhance visualization of abnormalities of the spleen number or position, and detection of residual tissue after splenectomy, which is otherwise hampered by the left hepatic lobe. Similarly, encapsulation of

drugs such as antimicrobial, hemoxygenase inhibitors, vinca alkaloids, and cyclosporin in splenotropic carriers may offer an effective approach for the treatment of several splenic and hematological diseases and disorders (e.g., malaria, hairy-cell leukemia, idiopathic thrombocytopenic purpura and autoimmune hemolytic anemia) as well as for prolonging survival of allografts.

In *Section III.*, we discussed that rigid long-circulating carriers of larger than 200 nm may act as splenotropic particles, at least with regard to the sinusoidal spleen. Indeed, the splenic filtration of such engineered particles was first demonstrated by Moghimi et al. (1991) with poloxamer or poloxamine-coated polystyrene particles of 220 to 250 nm in rats. Prior administration of poloxamine-908 into the rat blood can also convert uncoated polystyrene beads to splenotropic particles (Moghimi, 1997). With both approaches, over 40% of the injected dose of particles localize to the spleen within a period of a few hours while the hepatic uptake is below 15% of the total administered dose. It was also found that the rat splenic pulp macrophages (Figs. 4 and 5), and to some extent marginal zone phagocytes, ingest such particles, despite their phagocyte resistant nature (Moghimi et al., 1993b). This raises the question as to what extent filtration at IES rather than direct recognition of particles by macrophages is responsible for splenic sequestration of these engineered particles. The filtration was found to be the key mechanism, based on two sets of experiments. First, there was no change in the amount of particle retention in the spleen between normal rats and those that received a prior injection of clodronate liposomes to destruct red pulp and marginal zone macrophages (S. M. Moghimi, unpublished observations, 1995b). Second, unlike the rat model, the sinusoidal rabbit spleen did not remove more than 5% of an intravenously administered dose of 220 nm poloxamine-coated polystyrene particles (Moghimi et al., 1991). This is due to the abundance of structural continuity between arterial capillaries and sinuses in the rabbit spleen (Snook, 1950), thus reducing filtration efficiency at the red pulp. Contrary to the rabbit model, over 95% of the blood travels through the open route of circulation (blood flows through the reticular meshwork of the marginal zone or red pulp to reach the venous vessels) in rat and human spleens (Chen, 1980; Groom, 1987). A gradual loss of the surface poloxamine in the splenic microenvironment (and hence the steric barrier) may explain their

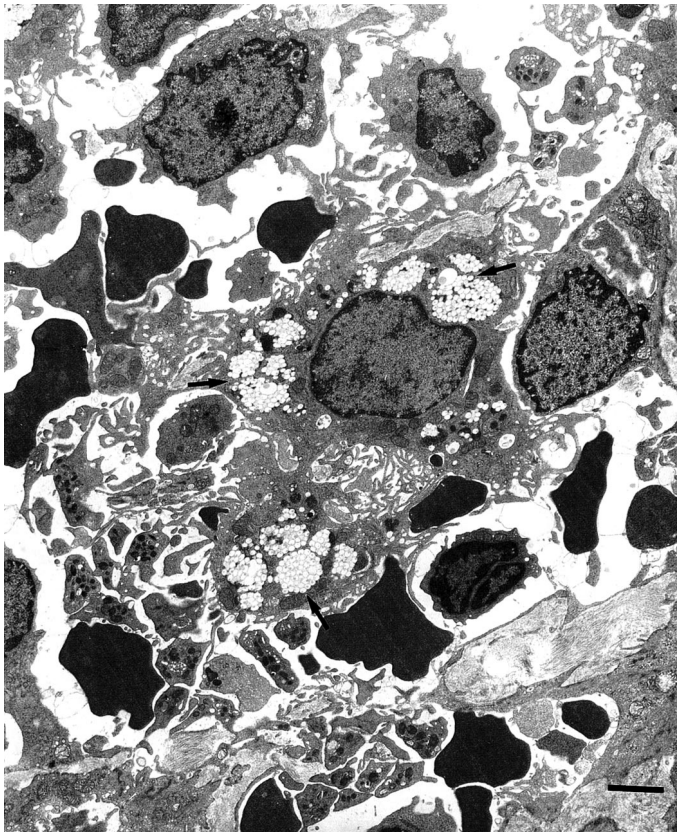


FIG. 4. Electron micrograph of a rat splenic red pulp macrophage showing massive accumulation of poloxamine-908-coated polystyrene nanospheres (220 nm) in lysosomal structures. Bar = 2 μm. Reproduced from Moghimi et al. (1993b) with permission of Elsevier Science (Oxford, UK).

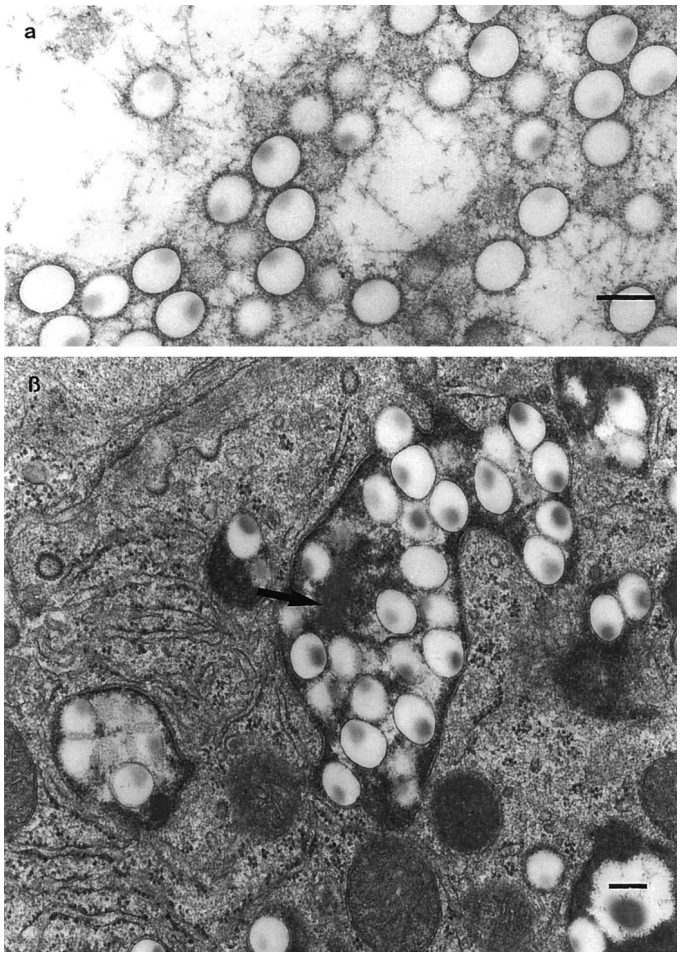


FIG. 5. Electron micrographs of poloxamine-908-coated polystyrene nanospheres (220 nm) incubated in the presence of rat serum (a) and a magnified view of a rat splenic red pulp macrophage lysosome with accumulated poloxamine-908-coated polystyrene particles (220 nm). Bar = 200 nm.

phagocytosis by macrophages. In support of this statement, it has been suggested that the adsorption of POP/POE copolymers on larger particles is relatively weaker than that on smaller particles (Lee et al., 1989). Studies with dually labeled constructs (radiolabeled beads and radiolabeled polymers) are still necessary to resolve this matter.

Another interesting observation is the efficient and again the size dependent clearance of GM1-incorporated liposomes as well as long-circulating polymer-coated particles by the mouse spleen (Klibanov et al., 1991; Liu et al., 1991), despite the nonsinusoidal structure of this organ (Schmidt et al., 1983; Moghimi, 1995b). In nonsinusoidal spleens, proximal venous vessels possess flat endothelium, a conventional basement membrane, and adventitial reticular cells where circulating blood cells enter the lumen of the pulp vein by passing through mural apertures that are large in size, offering little impedance to flow and, hence, filtration (Blue and Weiss, 1981). However, Weiss et al. (1986) have recognized a lineage of contractile fibroblastic cells, known as barrier-forming cells, which appear to fuse with one another in the filtration beds of the spleen to form syncytia. Barrier cells act in coordination with macrophages and other stromal cells, regulating splenic filtration, blood flow, cell homing and migration, and the clearance of particles. Although they occur in small numbers in the normal murine as well as human spleens, barrier cells are believed to provide normal basal filtration to marginal zone and red pulp regions (Weiss, 1991). However, barrier cells are present in large numbers in murine and human spleens under several pathological conditions or following administration of IL-1 (Weiss, 1991). Nonetheless, future experiments with clodronate-incorportaed liposomes are necessary to confirm the process of mechanical filtration as the main mechanism for particle retention in the murine spleen. Several other investigators later confirmed localization of various classes of sterically stabilized long-circulating particles in the spleen of mice and rats following intravenous injection (Gabizon and Papahadjopoulos, 1992; Litzinger and Huang, 1992; Maruyama et al., 1992; Li et al., 1993; Dams et al., 1999; Peracchia et al., 1999).

In the murine spleen, filtered polymer-coated nanoparticles and GM1-liposomes are primarily ingested by the marginal zone macrophages (Liu et al., 1991). The intrasplenic loss of the surface bound polymer could again explain the phagocytic clearance of the filtered particles. With regard to phagocytosis of GM1-liposomes it is possible that macrophage secretory products (e.g., a protease to expose a receptor for GM1) itself may be the regulator. The murine marginal zone macrophages also express a large number of silaic acid binding proteins (Crocker and Gordon, 1989; Kraal, 1992). Whether these receptors play a significant role in recognition and/or ingestion of GM1-liposomes remains to be studied. Some subpopulations of murine marginal zone macrophages exclusively express a scavenger receptor with collagenous structure (Ito et al., 1999). Apart from chemically modified low density lipoproteins not much is known regarding the ligand specificity of this receptor. It is likely that this receptor could play some role in the detection of such filtered particles.

Macrophage localization of particles in the spleen may have important consequences such as an immunological response or induction of tolerance. The polymeric coating materials and nanoparticles may have the ability to stimulate macrophages in their own right (see *Section VII.*). Macrophage activation could potentiate immune system responses to drugs coupled to or embedded in the carriers.

4. Lymph Nodes. The mammalian lymph nodes are aggregates of finely structured lympho-reticular tissues that arrest and eliminate antigenic materials, cells and microorganisms that have gained entry to the tissue fluids and have been conveyed to the nodes by their afferent lymph. In addition, direct passage of antigens or small foreign particles from the blood vessels to the lymphoid parenchyma occurs mainly through permeable vascular endothelium in lymph node vessels, although some species discrepancies do exist (Moghimi and Rajabi-Siahboomi, 1996). By taking advantage of these physiological processes colloidal carriers can be delivered to lymph nodes following intravenous, interstitial, and intramuscular injection. Experimental and clinical goals of delivering drugs and imaging agents to lymph node macrophages and other lymphatic elements are numerous and have been discussed in detail elsewhere (Moghimi and Rajabi-Siahboomi, 1996; Moghimi and Bonnemain, 1999). Here, we limit our discussion to the engineering aspects of long-circulating particles with regard to lymphatic targeting in two different scenarios of medical imaging to illustrate the role of the above physiological principles with regard to passive targeting and requirements in clinical medicine.

First, the availability of a contrast material that can reach lymph nodes after a single intravenous injection is highly desirable because of the large number of lymph nodes in the body and access being difficult to most of them. As a result of their very small size and prolonged circulation time, a significant fraction of intravenously injected dextran-coated iron oxide particles have been shown to appear in numerous lymph nodes in several animals as well as in humans (Bengele et al., 1994; Guimaraes et al., 1994; Wiessleder et al., 1994; Moghimi and Bonnemain, 1999). In lymph nodes, macrophages of medullary sinuses and paracortex participate in the clearance of iron oxide particles. Indeed, dextran-stabilized iron oxide crystals have helped to distinguish between normal and tumor-bearing nodes or reactive and metastatic nodes with magnetic resonance imaging (Moghimi and Bonnemain, 1999).

Second, the thin-walled and fenestrated lymphatic microvessel is easily penetrated by particulate agents after

injection into the extracellular space; although lymphatic localization of particles depends upon the potency of the lymphatic vessels as well as lymph node integrity (Moghimi and Rajabi-Siahboomi, 1996). Once inside the vessel, materials that are transported with the lymph become cleared by macrophages located in lymph nodes. With regard to medical imaging, an interstitially administered substance for lymphoscintigraphy or indirect lymphography should satisfy two characteristics. First, it should spread rapidly and efficiently from the injection site, and second, it should provide thorough uptake in regional nodes. Various kinds of particles and colloidal carriers have been examined in several animals and it has been demonstrated that particles less than 100 nm in size are preferable for such purposes (reviewed by Moghimi and Rajabi-Siahboomi, 1996; Moghimi and Bonnemain, 1999). However, with the majority of the particles, the drainage from the interstitial sites usually takes a time period of hours due to particle flocculation at the injection site as well as particle interaction with the interstitium ground substances. The slow transit could also make particles susceptible to phagocytosis at the injection site and decrease the efficiency of nodal delivery. To overcome these problems, attempts were made to sterically stabilize particles with hydrophilic polymers in a similar manner to long-circulating colloids (Moghimi et al., 1994). When long-circulating particles, such as poloxamer-407 or poloxamine-908-coated polystyrene particles (60 nm) were injected subcutaneously into rat footpads, more than 70% of the dose drained into initial lymphatics within 2 h compared with only 20% of uncoated particles (Fig. 6). As expected, the drained coated particles escaped clearance by macrophages of the regional nodes, and reached the systemic circulation where they remained in the blood for prolonged periods (Moghimi et al., 1994). Carriers based on this prototype can be used to visualize the lymphatic chain and for assessing the lymph. Indeed, this has been the case with polymeric micellar flow (Trubetskoy et al., 1996). On the other hand, for efficient and rapid delivery to lymph node macrophages the surface of interstitially injected nanoparticles must be covered with short PEG or POE chains (Fig. 6). The steric barrier imposed by such short hydrophilic coating materials (e.g., poloxamine-904) is not only sufficient in suppressing particle aggregation at the interstitial site but it still permits some degree of opsonin adsorption and macrophage recognition (Moghimi et al., 1994). The above experiments demonstrate that by simple engineering one can design particles for a specific clinical situation. Other examples in lymphatic delivery based on the described nanoengineering technique includes PEG-coated liposomes (Oussoren and Storm, 1997) and PLGA nanospheres coated with poloxamers, poloxamines and PLA-PEG (Hawley et al., 1997a,b)

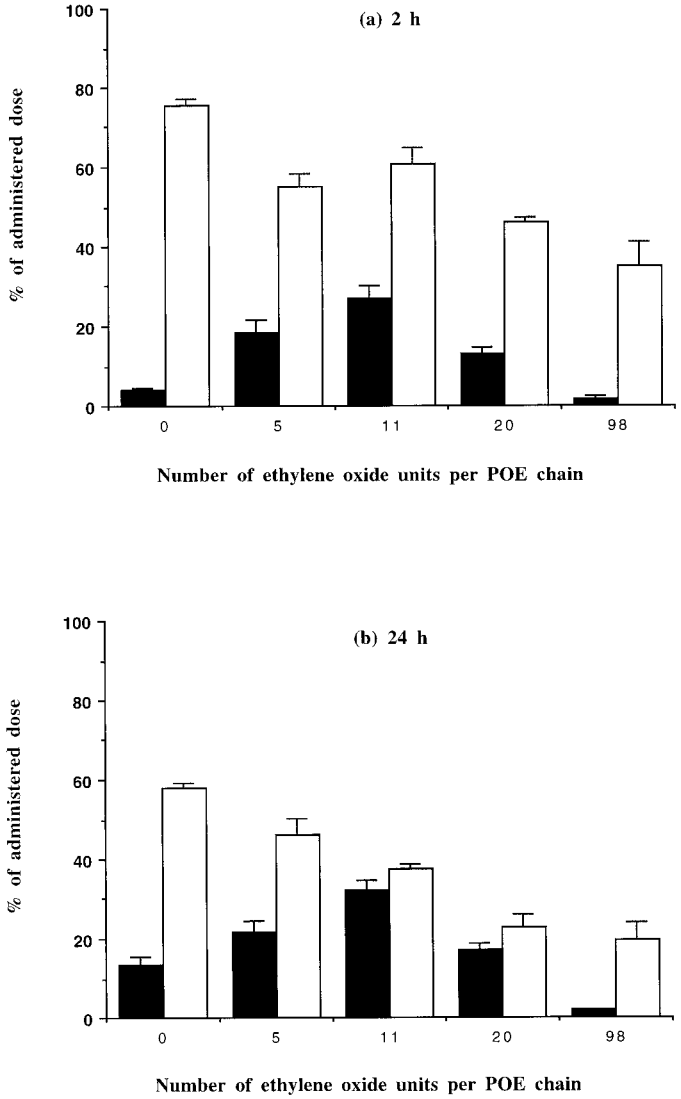


FIG. 6. Footpad and lymph node distribution of uncoated and POP/POE copolymer-coated polystyrene nanospheres (60 nm) following subcutaneous injection into rat footpads. Copolymers were poloxamer-401, -402, -403, and 407. These poloxamers all share a central POP segment consisting of 67 propylene oxide units. Closed columns represent regional lymph nodes (popliteal and iliac nodes), and open columns represent footpads. For experimental details see Moghimi et al. (1994).

D. Active Targeting

Active targeting increases the probability of redirecting long-circulating particles to a designated but accessible targets. Unlike passive targeting, in this case, ligands or homing devices that specifically bind to surface epitopes or receptors on the target sites, are coupled to the surface of the long-circulating carriers. Considerable attention has been focused on long-circulating liposomes for active or ligand-mediated targeting. For example, various endogenously occurring ligands and substances have been attached to PEG-grafted vesicles, which include oligosaccharides that mimic ligands for selectins (e.g., sialyl Lewis X) (Murohara et al., 1995; DeFrees et al., 1996), folic acid (Lee and Low, 1994; Lee and Huang, 1996; Goren et al., 2000), oligopeptides (Zalipsky et al.,

1995, 1997) plasminogen (Blume et al., 1993) and interestingly antibodies (Mori et al., 1991; Torchilin et al., 1992; Ahmad et al., 1993; Allen et al., 1995b; Bloemen et al., 1995; Spragg et al., 1997) and their fragments (Park et al., 1995; Kirpotin et al., 1997; Drummond et al., 1999). The biological performance of such constructs has only been tested in selected cases.

1. Ligand Coupling. The most important criteria in the coupling procedure is to control the surface density of the ligand. Among the polymers discussed so far, poloxamers and PEG have received most attention. Derivatization of the end group of poloxamer POE chains with pyridyl disulfide has be achieved (Li et al., 1996). The disulfide bridge is resistant to hydrolysis. Release of the pyridyl thioketone can only take place following disulfide exchange with a thiol-containing moiety on the peptide or protein to be attached to the surface. By mixing derivatized with native poloxamer before nanoparticle coating, it may be possible to control the density of the surface functional groups, since there is no preferential adsorption of either species.

Currently, three methods can be used to assemble ligand-PEG bearing liposomes. First, end group-functionalized PEG lipids are incorporated into liposomes and then conjugated to the desired ligand (Zalipsky, 1995; Kirpotin et al., 1996). However, this strategy could lead to a construct bearing some surface unreacted end groups. Furthermore, the reactive groups at the termini of the PEG chains on the inner monolayers could undergo side reactions with water or encapsulated drug molecules which may be of concern. Finally, conformational clouds of the PEG chains usually interfere with conjugation reactions between reactive groups on the surface of a vesicle and a ligand molecule, as well as the binding of surface attached ligands and their target receptors (Klibanov et al., 1991; Hansen et al., 1995). These problems are circumvented by the second approach where ligand-PEG-lipid conjugate is used directly during liposome preparation (DeFrees et al., 1996; Zalipsky et al., 1997). However, this approach suffers from the fact that a substantial portion of the ligand molecules face the inner aqueous compartment (Zalipsky et al., 1997). The final strategy is based on simple incubation of ligand-PEG-lipid conjugates with preformed vesicles. By this approach the three-component conjugate is exclusively positioned on the outer leaflet of the liposomal bilayer (Uster et al., 1996).

2. Demonstration of Longevity and Target Binding. Several experimental models have been explored for examining the target binding capability of ligand-bearing long-circulating particles, particularly the liposomes. In the majority of these models, only in vitro binding or internalization by target cells have been studied. When in vivo longevity and subsequent targeting has been demonstrated, the therapeutic endpoint has often been ignored. It seems that the majority of investigators are only interested in demonstrating the idea of active targeting with such constructs. Again, the majority of these efforts have peculiarly concentrated on cancer as the target site; opportunities offered within vasculature (e.g., blood clots) or by activated endothelium is often ignored. Furthermore, the possibility of an immune response against such constructs has received little attention. This is particularly important with regard to repeated administrations. To highlight some of these problems we will concentrate on antibody- and folate-mediated targeting approaches in experimental oncology.

3. Antibody-Mediated in Vivo Targeting (the Cart in Front of the Horse?). Long-circulating immunoliposomes are interesting but puzzling constructs. Surely, the presence of a surface attached monoclonal antibody can stimulate vesicle clearance by the nonspecific macrophage Fc receptor. However, the circulation time of PEG-grafted immunoliposomes is influenced by the density of the attached antibody (Maruyama et al., 1990; Allen, 1994b; Allen et al., 1995a). At densities below 50 μg antibody/μmol phospholipid, immunoliposomes are cleared at faster rates than those seen for antibody free long-circulating liposomes and demonstrate some target binding activity (Allen et al., 1995a). At densities above 100 μg antibody/μmole phospholipid, clearance rates are extremely rapid with massive immunoliposome localization within macrophages of the RES (Allen et al., 1995a). Indeed, the concept of in vivo target binding of long-circulating immunoliposomes have been demonstrated successfully by Maruyama et al. (1990) and Mori et al. (1993). These investigators have targeted the murine pulmonary endothelium using monoclonal antibodies against the lung endothelial protein thrombomodulin. Similarly, Ahmad et al. (1993) and Allen et al. (1995a) have shown antibody-targeted delivery of doxorubicin entrapped in PEG-grafted liposomes for the eradication of a lung metastatic cancer in a murine model, where the targeting was associated with a significant decrease in the tumor burden. However, it should be emphasized that lung targeting is easy since following tail vein injection the long-circulating immunoliposomes encounter lung capillary beds first, where a significant fraction of the dose will be retained.

Another major concern is the immunogenicity of long-circulating immunoliposomes (Harding et al., 1997; Harasym et al., 1998). For example, when injected intravenously into rats, PEG-grafted immunoliposomes (100 nm), with an average of 18 chimerized mouse IgG residues per vesicle, were relatively long-circulating (Harding et al., 1997). Subsequent injections of the immunoliposomes into the same animals resulted in rapid clearance, which was accompanied by a significant increase in anti-antibody specific titers. In contrast, repeated injections of the free antibody provided no evidence of an immune response. An alternative approach is the attachment of antibody fragments such as F(ab′) or F(ab′)$_2$ (Park et al., 1995; Kirpotin et al., 1997). Again, immunogenicity is also an important factor with regard

to antibody fragment-bearing liposomes and has been demonstrated by Phillips et al. (1994). This is not surprising, considering that damaged antibodies are removed rapidly from the circulation. Therefore, these approaches seem unsuitable for in vivo targeting of hematological tumors or vascular endothelial cells.

Surprisingly, the idea of active targeting of long-circulating immunoliposomes to solid tumors has been persued repeatedly. Here, several other difficulties can further be identified. First, within a solid tumor immunoliposomes tend to bind to cells at the periphery of the tumor mass. This process can further retard possible penetration of antibody-bearing vesicles into the tumor mass. In addition, binding to the targeted epitope may not lead to vesicle internalization and subsequent drug release within the cell interior, although this could be overcome with particles bearing antibody fragments. Second, an important consideration is the concept of tumor heterogeneity. Some populations of cancer cells in the target area may not express the epitope being targeted. Down regulation and/or shedding of the antigen from the cell surface further complicates the issue. A recent study has clearly demonstrated that despite optimal preparation of an immunoliposome with high in vitro affinity to tumor cells and reduced RES deposition, no advantage in enhancement of therapeutic efficacy could be achieved in comparison to plain long-circulating liposomes (Goren et al., 1996). This study further indicates that the rate limiting factor for accumulation in solid tumors is the process of vesicle extravasation.

In spite of the above arguments, selective manipulation of macrophage Fc receptors may be potentially useful in the application of antibody-bearing long-circulating particles. Such approaches require a detailed understanding of the mechanisms by which FcRs stimulate the polymerization of actin and induce formation of phagosomes. Recent studies have suggested a role for PI-3 kinase, the Rho family of GTPases and protein kinase C as downstream effectors of Fc receptor mediated phagocytosis (Honing et al., 2000). For instance, PI-3 kinase participates in the process of signaling cascade. Cross-linking of FcγRI and FcγRII increases the activity of PI-3 kinase but Fcγ*R*-mediated phagocytosis is prevented by PI 3-kinase inhibitors wortmannin and LY294002 (Araki et al., 1996). These agents prevent the formation of phagosomes. Such combined approaches are worthy of future experimentation.

4. Folate-Mediated Targeting. To overcome some of the hurdles obstructing the traditional antibody based approaches in cancer targeting, several investigators have turned to folic acid (Goren et al., 2000). In principle, folate offers many advantages as a targeting ligand over antibodies. It is presumed nonimmunogenic due to its small size, has good stability, and is highly specific for tumors (Sudimack and Lee, 2000). The elevated expression of the folate receptor has frequently been observed in various types of human cancers including ovarian, endometrial, colorectal, breast, lung, and renal cell carcinoma as well as brain metastases derived from epithelial cancers (Sudimack and Lee, 2000). The receptor is generally absent in most normal human tissues with the exception of placenta, choroid plexus, lung, thyroid, and kidney. Folic acid has been attached to liposomes via a long PEG spacer (molecular mass of 3350 Da) to overcome the steric hindrance encountered at the cell surface (Lee and Low, 1994). Such conjugates are rapidly internalized into a low pH endosomal compartment by folate receptor-bearing cancer cells via receptor-mediated endocytosis and proved successful in delivering of protein toxins, anticancer agents, genetic materials as well as diagnostic agents. Again, the incorporation of folate is unlikely to affect the extent of liposome extravasation from the blood into the tumor interstitum. Whether this strategy has any therapeutic advantage over nontargeted long-circulating particulate systems remains to be shown.

E. Passive or Active Targeting?

An interesting case of "accidental" targeting was reported with respect to long-circulating polystyrene nanospheres coated with poloxamer-407 (Porter et al., 1992a,b). Among three different commercially available samples of poloxamer-407, only one poloxamer sample was found to be capable of directing polystyrene particles (60–150 nm) to sinus endothelial cells of the rabbit bone marrow 12 to 24 h after intravenous injection (Fig. 7), although they all suppressed particle recognition by macrophages of the RES (Porter et al., 1992a). A similar process was also reported with a batch of poloxamer-338 (Illum and Davis, 1984). This homing process is apparently exclusive to the rabbit, as bone marrow targeting can not be demonstrated in other species such as mice and rats (Porter et al., 1992a). These observations are interesting as these particular polymers render nanospheres both anti-adhesive (by virtue of the extend POE configuration on the particle surface which acts as a steric barrier) and pro-adhesive (by virtue of recognition by bone marrow sinus endothelium). Therefore, the process is analogous to the anti-adhesive and pro-adhesive behavior of mucins. The mechanisms of recognition still remain to be elucidated.

An important question arises here, which must not be ignored, is the extent of reproducibility in biological targeting using polymeric materials. The main problem seems to be the exact composition of each polymer batch: the presence of a range of different molecular weight copolymers as well as homopolymers (Moghimi and Hunter, 2000b), which needs to be defined precisely. Nevertheless, hidden within this variability is the inference that exquisite levels of recognition and specificity are achievable with such polymers.

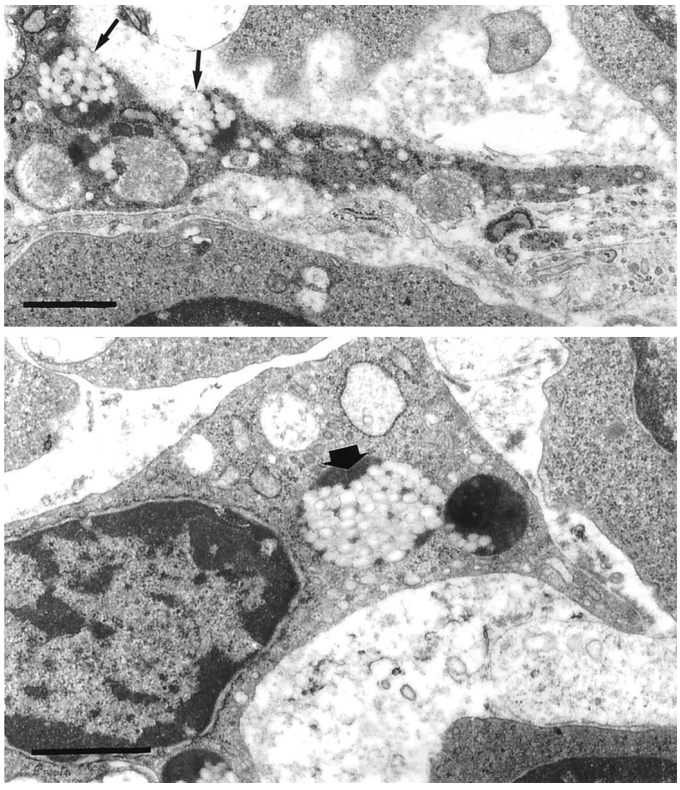


FIG. 7. Electron micrographs showing the localization of poloxamer-407-coated latex nanospheres (150 nm) in sections through sinusoidal endothelial cells of male New Zealand White rabbits. The poloxamer was supplied by Ugine Kuhlman Company (Manchester, UK) and used as received. Particles (4.0 mg) were injected intravenously via the marginal ear vein. Bone marrow was extracted from a femoral bone and fixed by an immersion technique (Porter et al., 1992b). Poloxamer-coated nanoparticles are present in large vesicles designated as dense bodies (arrowheads). The endothelium of other bone marrow vessels remained indifferent to the presence of poloxamer-407-coated beads. In animals that received uncoated polystyrene particles, the sinus endothelial cells in bone marrow rarely participated in particle uptake from the blood. Micrographs courtesy of Dr. S. M. Moghimi. Bar = 1 μm.

IX. Conclusions

In this article we have critically examined the wide range of approaches in the design and implementation of colloid based long-circulating drug carrier systems. The current focus of research in this area has generated a broad spectrum of carriers with the majority showing rather poor biological performance in terms of both circulation time and target specificity. Again, the concepts of drug incorporation and release from such particles has been poorly investigated. Also the ultimate fate of the constituent materials of the vehicles remain undefined. Our inadequate understanding of physicochemical interactions between a carrier and host immune system seems responsible for technical difficulties in constructing a multicomponent long-circulating system with optimal biological performance. However, the limited evidence suggests that the majority of polymers used in particle engineering can affect some macrophage accessory functions (e.g., cytokine release, major histocompatibility complex class II antigen expression). This may have important implications in vaccine design, particularly for subcutaneous and intramuscular protocols, using colloidal carriers. In designing long-circulating particles, attention must also be paid toward their application to a relevant pathology. Here, it is still unclear which approach to targeting is likely to yield the best results. Clearly much more needs to be learned about the physicochemical nature of interactions between polymers and the cell surface, in particular which components or combination of components on a given cell surface is responsible for these interactions. In terms of the endothelial cell surface, we have only begun to appreciate differences in the complement of molecules exposed on the surface.

We strongly believe that, understanding the nature of the scavenging properties of macrophages and more importantly the precise role of the different macrophage populations in these processes are fundamental to the rational design of long-circulating as well as target-specific colloidal entities. Although, the current limited evidence suggests that stimulated macrophages can recognize long-circulating carriers in vivo, the nature of the macrophage plasma membrane receptors involved in this process and their mode of action is still undetermined. It is interesting to note that in vivo a stimulated Kupffer cell will not phagocytose healthy erythrocytes. Is it therefore possible that an understanding of this mutual relationship between the stimulated phagocyte and the erythrocyte holds the key for the design of a truly synthetic long-circulating colloid. Perhaps, knowledge gained from research in this area could end the long search for an artificial red blood cell. This will require a greater understanding of erythrocyte membrane biochemistry and biophysics. For example, the unique biconcave disc shape in conjunction with the deformable nature of the erythrocyte may have an influential effect on the mode of interaction of red blood cells with other biological constituents.

At present the research focus tends to be on a single strategy rather than using a range of potentially complimentary tactics for engineering of long-circulatory particles. For example, inclusion of GM3 in liposomes has resulted in increased circulation time of the vesicles, whereas a combination of GM3 with complement regulatory proteins may further enhance vesicle longevity. Similarly, approaches with polymeric systems have rarely used a combination of coating polymers which may afford a higher energy steric barrier. The translation of microbial strategies to overcome macrophage recognition is another resource of elegant and evolutionary developed host uptake avoidance mechanism(s). However, caution must be exercised with respect to microbial surface expression which will vary depending on the exposed microenvironment (Cheung and Fischetti, 1988; Bayer et al., 1989).

To date synthetic polymers have been the materials of choice in the construction of long-circulating colloids. A deficiency in this field which have to be rectified is the

precise characterization of these macromolecules in terms of molecular weight and purity. The problem with molecular weight determination is due to technical difficulties associated with the techniques available at present. For instance, aqueous gel permeation chromatography, used for the separation and molecular weight determination of water-soluble polymers, is a relatively new technique, and its use is hindered by the fact that there are very few molecular weight standards that can be used to calibrate the system. Also, little work has been done in determining the nature of polymeric degradation products, their biological activity and ultimate fate following parenteral administration.

Finally, a major factor that has been ignored is genetics. Future considerations must be given toward the immunogenetic and pharmacogenetic differences and related polymorphisms.

REFERENCES

Abra RM, Bosworth ME and Hunt CA (1980) Liposome disposition in vivo: effects of pre-dosing with liposomes. *Res Commun Chem Pathol Pharmacol* **29:**349–360.

Absolom D (1986) Opsonins and dysopsonins: an overview. *Methods Enzymol* **132:** 281–318.

Abuchowski A, van Es T, Palczuk NC and Davis FF (1977) Alteration of immunological properties of bovine serum albumin by covalent attachment of polyethylene glycol. *J Biol Chem* **252:**3578–3581.

Adams DO and Hamilton TA (1992) Molecular basis of macrophage activation: diversity and its origin, in *The Macrophage* (Lewis CE and McGee JOD eds) pp 75–114, IRL Press, Oxford, UK.

Agrawal AK and Gupta CM (2000) Tuftsin-bearing liposomes in treatment of macrophage-based infections. *Adv Drug Delivery Rev* **41:**135–146.

Ahl PL, Bhatia SK, Meers P, Roberts P, Stevens R, Dause R, Perkins WR and Janoff AS (1997) Enhancement of the in vivo circulation lifetime of L-distearoylphosphatidylcholine liposomes: importance of liposomal aggregation versus complement activation. *Biochim Biophys Acta* **1329:**370–382.

Ahmad I, Longenecker M, Samuel J and Allen TM (1993) Antibody-targeted delivery of doxorubicin entrapped in sterically stabilized liposomes can eradicate lung cancer in mice. *Cancer Res* **53:**1484–1488.

Alcami A and Koszinowski UH (2000) Viral mechanisms of immune evasion. *Immunol Today* **21:**447–455.

Ali SAM, Doherty PJ and Williams DF (1994) Molecular biointeractions of biomedical polymers with extracellular exudates and inflammatory cells and their effects on the biocompatability, in vivo. *Biomaterials* **15:**779–785.

Allen TM (1994a) The use of glycolipids and hydrophilic polymers in avoiding rapid uptake of liposomes by the mononuclear phagocyte system. *Adv Drug Delivery Rev* **13:**285–309.

Allen TM (1994b) Long-circulating (sterically stabilized) liposomes for targeted drug delivery. *Trend Pharmacol Sci* **15:**215–220.

Allen TM, Ahmad I, Lopes de Menezes DI and Moase EH (1995a) Immunoliposome-mediated targeting of anticancer drugs in vivo. *Biochem Soc Trans* **23:**1073–1079.

Allen TM, Austin GA, Chonn A, Lin L and Lee KC (1991a) Uptake of liposomes by cultured mouse bone marrow macrophages: influence of liposome composition and size. *Biochim Biophys Acta* **1061:**56–64.

Allen TM, Brandeis E, Hansen CB, Kao GY and Zalipsky S (1995b) A new strategy for attachment of antibodies to sterically stabilized liposomes resulting in efficient targeting to cancer cells. *Biochim Biophys Acta* **1237:**99–108.

Allen TM and Chonn A (1987) Large unilamellar liposomes with low uptake by the reticuloendothelial system. *FEBS Lett* **223:**42–46.

Allen TM and Hansen C (1991) Pharmacokinetics of Stealth verses conventional liposomes: effect of dose. *Biochim Biophys Acta* **1068:**133–141.

Allen TM, Hansen C, Martin F, Redemann C and Yau-Young A (1991b) Liposomes containing synthetic lipid derivatives of poly(ethylene glycol) show prolonged circulation half-lives in vivo. *Biochim Biophys Acta* **1066:**29–36.

Allen TM, Hansen C and Rutledge J (1989) Liposomes with prolonged circulation times: factors affecting uptake by reticuloendothelial and other tissues. *Biochim Biophys Acta* **981:**27–35.

Allen TM, Mehra T, Hansen C and Chin YC (1992) Stealth liposomes: an improved sustained release system for 1-β-D-arabinofuranosylcytosine. *Cancer Res* **52:**2431–2439.

Alving CR (1988) Macrophages as targets for delivery of liposome-encapsulated antimicrobial agents. *Adv Drug Delivery Rev* **2:**107–128.

Alving CR and Swartz GM Jr (1991) Antibodies to cholesterol, cholesterol conjugates and liposomes: implications for atherosclerosis and autoimmunity. *Crit Rev Immunol* **10:**441–453.

Alving CR and Wassef NM (1992) Complement-dependent phagocytosis of liposomes: suppresion of "stealth" lipids. *J Liposome Res* **2:**383–395.

Araki N, Johnson MT and Swanson JA (1996) A role for phosphoinositide 3-kinase in the completion of macropinocytosis and phagocytosis by macrophages. *J Cell Biol* **135:**1246–1260.

Asano M, Fukuzaki H, Yoshida M, Kumakura M, Mashimo T, Yuasa H, Imai K, Yamanaka H, Kawaharada U and Suzuki K (1990) In vivo controlled release of a luteinizing-hormone-releasing hormone agonist from poly(DL-lactic acid) formulations of varying degradation pattern. *Int J Pharm* **67:**67–77.

Baguley BC and Falkenhaug EM (1971) Plasma half-life of cytosine arabinoside (NSC-63878) in patients treated for acute myeloblastic leukaemia. *Cancer Chemother Rep* **55:**291–298.

Batycky RP, Hans J, Langer R and Edwards DA (1997) A theoretical model of erosion and macromolecular drug release from biodegradable microspheres. *J Pharm Sci* **86:**1464–1477.

Bayer AS, Eftekhar F, Tu J, Nast CC and Spreet DP (1989) Oxygen-dependent up-regulation of mucoid exopolysaccharide (alginate) production in *Pseudomanos aeruginosa. Infect Immun* **58:**1344–1349.

Bazile D, Prud'homme C, Bassoullet MT, Marlard M, Spenlehauer G and Veillard M (1995) Stealth MePEG-PLA nanoparticles avoid uptake by the mononuclear phagocyte system. *J Pharm Sci* **84:**493–498.

Bedu-Addo FK and Huang L (1996) Effect of matrix lipid chain length on liposomes containing cholesterol and ganglioside G_{M1}: implications in drug delivery. *J Pharm Sci* **85:**714–719.

Bedu-Addo FK, Tang P, Xu Y and Huang L (1996a) Effects of polyethyleneglycol chain length and phospholipid acyl chain composition on the interaction of polyethyleneglycol-phospholipid conjugates with phospholipid: implications in liposomal drug delivery. *Pharm Res (N Y)* **13:**710–717.

Bedu-Addo FK, Tang P, Xu Y and Huang L (1996b) Interaction of polyethyleneglycol-phospholipid conjugates with cholesterol-phosphatidylcholine mixtures: sterically stabilized liposome formulations. *Pharm Res (N Y)* **13:**718–724.

Belloni PN and Nicolson GL (1988) Differential expression of cell surface glycoproteins on various organ derived microvascular endothelia and endothelial cell cultures. *J Cell Physiol* **136:**398–410.

Bengele HH, Plamacci S, Rogers J, Jung CW, Crenshaw J and Josphson L (1994) Biodistribution of an ultrasmall superparamagnetic iron oxide colloid, BMS 180549, by different routes of administration. *Magn Reson Imaging* **12:**433–442.

Benita S (1984) Kinetic model identification of drug relaese from microcapsules using the nonlinear regression search procedure. *Appl Biochem Biotechnol* **10:**255–258.

Benita S, Babay D, Hoffman A and Donbrow M (1988) Relation between individual and ensemble release kinetics of indomethacin from microspheres. *Pharm Res (N Y)* **5:**178–182.

Bentley PK, Davis SS, Johnson OL, Lowe KC and Washington C (1989) Purification of pleuronic F-68 for perfluorochemical emulsification. *J Pharm Pharmacol* **41:** 661–663.

Bevilacqua MP (1993) Endothelial-leukocyte adhesion molecules. *Annu Rev Immunol* **11:**767–804.

Bloemen PGM, Henricks PAJ, van Bloois L, van den Tweel, Bloem AC, Nijkamp FP, Crommelin DJA and Storm G (1995) Adhesion molecules: a new target for immunoliposome-mediated drug delivery. *FEBS Lett* **357:**140–144.

Blue J and Weiss L (1981) Species variation in the structure and function of the marginal zone: an electron microscope study of cat spleen. *Am J Anat* **161:**169–187.

Blume G and Cevc G (1990) Liposomes for sustained drug release in vivo. *Biochim Biophys Acta* **1029:**91–97.

Blume G, Cevc G, Crommelin MDJA, Bakker-Woudenberg IAJM, Kluft C and Storm G (1993) Specific targeting with poly(ethylene glycol)-modified liposomes: coupling of homing devices to the ends of the polymeric chains combines effective target binding with long circulation times. *Biochim Biophys Acta* **1149:**180–184.

Boerman OC, Oyen WJG, Storm G, Corvo ML, van Bloois L, van der Meer JWM and Corstens FHM (1997a) Technetium-99m labelled liposomes to image experimental arthritis. *Ann Rheum Dis* **56:**369–373.

Boerman OC, Oyen WJG, van Bloois L, Koenders EB, van der Meer JWM, Corstens FHM and Storm G (1997b) Optimization of technetium-99m-labelled PEG liposomes to image focal infection: effects of particle size and circulation time. *J Nucl Med* **38:**489–493.

Boerman OC, Storm G, Oyen WJG, van Bloois L, van der Meer JWM, Claessens RAMJ, Crommelin DJA and Corstens FHM (1995) Sterically stabilized liposomes labeled with In-111 to image focal infection. *J Nucl Med* **36:**1639–1644.

Borsa J, Whitmore GR, Valeriote FA, Collins D and Bruce WR (1969) Studies on the persistence of methotrexate, cytosine arabinoside, and leucovorin in the serum of mice. *J Natl Cancer Inst* **42:**235–242.

Braet F, Dezanger R, Baekeland M, Crabbe E, van der Smissen P and Wisse E (1995) Structure and dynamics of the fenestrae-associated cytoskeleton of rat-liver sinusoidal endothelial cells. *Hepatology* **21:**180–189.

Breslow DS (1976) Biologically active synthetic polymers. *Pure Appl Chem* **46:**103–113.

Brooks PC, Clark RAF and Cheresh DA (1994) Requirement of vascular integrin $\alpha_v\beta_3$ for angiogenesis. *Science (Wash DC)* **264:**569–571.

Buckley PJ, Smith MR, Braverman MF and Dickson S (1987) Human spleen contains phenotypic subset of macrophages and dendritic cells that occupy discrete microanatomic locations. *Am J Pathol* **128:**505–520.

Bütün V, Lowe AB, Billingham NC and Armes SP (1999) Synthesis of zwitterionic shell cross-linked micelles. *J Am Chem Soc* **121:**4288–4289.

Cabanes A, Even Chen S, Zimberoff J, Barenholz Y, Kedar E and Gabizon A (1999) Enhancement of antitumor activity of polyethylene glycol-coated liposomal doxorubicin with soluble and liposomal interleukin 2. *Clin Cancer Res* **5:**687–693.

Cabral DA, Loh BA and Speert DP (1987) Mucoid *Pseudomonas aeruginosa* resist nonopsonic phagocytosis by human neutrophils and macrophages. *Pediatr Res* **22:**429–431.

Campbell CH and Richmond JY (1973) Enhancement, by two carboxylic acid interferon inducers, of resistance stimulated in mice by foot-and-mouth disease vaccine. *Infect Immun* **7:**199–204.

Charpin C, Bergeret D, Garcia S, Andrac L, Martini F, Horschowski N, Choux R and Lavaut MN (1998) ELAM selectin expression in breast carcinomas detected by

automated and quantitative immunohistochemical assays. *Int J Oncol* **12:**1041–1048.
Chen LT (1980) Intrasplenic microcirculation in rats with acute hemolytic anemia. *Blood* **56:**737–740.
Chen LT and Weiss L (1973) The role of the sinus wall in the passage of erythrocytes through the spleen. *Blood* **41:**529–537.
Cheresh DA (1987) Human endothelial cells synthesize and express an Arg-Gly-Asp-directed adhesion receptor involved in attachment to fibrinogen and von Willebrand factor. *Proc Natl Acad Sci USA* **84:**6471–6475.
Cheung AL and Fischetti VA (1988) Variation in the expression of cell-wall proteins of *Staphylococcus-aureus* grown on solid and liquid-media. *Infect Immun* **56:**1061–1065.
China B, Sory BT, N'Guyen, De Bruyere M and Cornelis GR (1993) Role of the YadA protein in prevention of opsonization of *Yersinia enterocolitica* by C3b molecules. *Infect Immun* **61:**3129–3136.
Chiu GNC, Bally MB and Mayer LD (2001) Selective protein interactions with phosphatidylserine containing liposomes alter the steric stabilization properties of poly(ethylene glycol). *Biochim Biophys Acta* **1510:**56–69.
Chonn A, Cullis PR and Devine DV (1991) The role of surface charge in the activation of the classical and alternative pathways of complement by liposomes. *J. Immunol* **146:**4234–4241.
Chonn A, Semple SC and Cullis PR (1992) Association of blood proteins with large unilamellar liposomes in vivo. Relation to circulation lifetimes. *J Biol Chem* **267:**18759–18765.
Chonn A, Semple SC and Cullis PR (1995) Beta 2 glycoprotein I is a major protein associated with very rapidly cleared liposomes in vivo, suggesting a significant role in the immune clearance of "non-self" particles. *J Biol Chem* **270:**25845–25849.
Cornacoff JB, Hebert LA, Smead WL and VanAman ME, Birmingham DJ and Waxman FJ (1983) Primate erythrocyte-immune complex-bearing mechanism. *J Clin Invest* **71:**236–247.
Corvo ML, Boerman OC, Oyen WJG, Van Bloois L, Cruz MEM, Crommelin DJA and Storm G (1999) Intravenous administration of superoxide dismutase entrapped in long circulating liposomes. *Biochim Biophys Acta* **1419:**325–334.
Crocker PR and Gordon S (1989) Mouse macrophage hemagglutinin (sheep erythrocyte receptor) with specificity for sialylated glycoconjugates characterized by a monoclonal antibody. *J Exp Med* **169:**1333–1346.
Crocker PR, Kelm S, Dubois C, Martin B, McWilliam AS, Shotton DM, Paulson JC and Gordon S (1991) Purification and properties of sialoadhesin, a sialic acid-binding receptor of murine tissue macrophages. *EMBO J* **10:**1661–1669.
Daemen T, Hofstede G, Kate MTT, Bakker-Woudenberg IAJM and Scherphof G (1995) Liposomal doxorubicin-induced toxicity: depletion and impairment of phagocytic activity of liver macrophages. *Int J Cancer* **61:**716–721.
Daemen T, Regts J, Meesters M, Ten Kate MT, Bakker-Woudenberg IAJM and Scherphof GL (1997) Toxicity of doxorubicin entrapped within long-circulating liposomes. *J Control Release* **44:**1–9.
Dams ETM, Becker MJ, Oyen WJG, Boerman OC, Storm G, Laverman P, de Marie S, van der Meer JWM, Bakker-Woudenberg INJM and Corstens FHM (1999) Scintigraphic imaging of bacterial and fungal infection in granulocytopenic rats. *J Nucl Med* **40:**2066–2072.
Dams ETM, Laverman P, Oyen WJG, Storm G, Scherphof GL, van der Meer JWM, Corstens FH and Boerman OC (2000a) Accelerated blood clearance and altered biodistribution of repeated injections of sterically stabilized liposomes. *J Pharmacol Exp Ther* **292:**1071–1079.
Dams ETM, Oyen WJG, Boerman OC, Storm G, Laverman P, Koenders EB, van der Meer JWM and Corstens FHM (1998) Technetium-99m-labeled liposomes to image experimental colitis in rabbits: comparison with technetium-99m-HMPAO-granulocytes and technetium-99m-HYNIC-IgG. *J Nucl Med* **39:**2172–2178.
Dams ETM, Oyen WJG, Boerman OC, Storm G, Laverman P, Kok PJM, Buijs WCAM, Bakker H, van der Meer JWM and Corstens FHM (2000b) Tc-99M-PEG liposomes for the scintigraphic detection of infection and inflammation: clinical evaluation. *J Nucl Med* **41:**622–630.
Daniels AU, Chang MKO and Andriano KP (1990) Mechanical properties of biodegradable polymers and composits proposed for internal fixation of bone. *J Appl* **1:**57–78.
DeFrees SA, Phillips L, Guo L and Zalipsky (1996) Sialyl Lewis X liposomes as a multivalent ligand and inhibitor of E-selectin mediated cellular adhesion. *J Am Chem Soc* **118:**6101–6104.
De Jaeghere F, Allemann E, Feijen J, Kissel T, Doelker E and Gurny R (2000) Cellular uptake of PEO surface-modified nanoparticles: evaluation of nanoparticles made of PLA:PEO diblock and triblock copolymers. *J Drug Target* **8:**143–153.
Densen P and Mandell GL (1980) Phagocyte strategy vs. microbial tactics. *Rev Infect Dis* **2:**817–838.
Devine DV and Bradley AJ (1998) The complement system in liposome clearance: can complement deposition be inhibited? *Adv Drug Delivery Rev* **32:**19–39.
Devine DV, Wong K, Serrano K, Chonn A and Cullis PR (1994) Liposome-complement interactions in rat serum: implications for liposome survival studies. *Biochim Biophys Acta* **1191:**43–51.
Donbrow M, Hoffman A and Benita S (1986) Variation of population release kinetics in polydisperse multiparticulate systems (microcapsules, microspheres, droplets, cells) with heterogeneity of one, two or three parameters in the population of individuals. *Int J Pharm* **29:**195–211.
Drenckhahn D and Wagner J (1986) Stress fibers in the splenic sinus endothelium in situ: molecular structure, relationship to the extracellular matrix and contradictibility. *J Cell Biol* **102:**1738–1747.
Drummond DC, Meyer O, Hong K, Kirpotin DB and Papahadjopoulos D (1999) Optimizing liposomes for delivery of chemotherapeutic agents to solid tumors. *Pharmacol Rev* **51:**691–743.
Dumont DJ, Gradwohl G, Fong GH, Puri MC, Gertsenstein M, Auerbach A and Breitman ML (1994) Dominant-negative and targeted null mutations in the endothelial receptor tyrosine kinase, Tek, reveal a critical role in vasculogenesis of the embryo. *Genes Dev* **8:**1897–1909.
Dunn SE (1995) *Biodegradable nanosphere systems for drug targeting with emphasis on systems for bone marrow targeting*. PhD Thesis, University of Nottingham, UK.
Dunn SE, Brindley A, Davis SS, Davies MC and Illum L (1994) Polystyrene-poly(ethylene glycol) (PS-PEG2000) particles as model systems for site specific drug delivery. 2. The effect of PEG surface density on the in vitro cell interaction and in vivo biodistribution. *Pharm Res (N Y)* **11:**1016–1022.
Edwards CM, Gambaretto GP, Conte L and Lowe KC (1999) Evaluation of commercial and purified Pluronic F-68 in a human blood neutrophil bioassay. *Artif Cells Blood Substit Immobil Biotechnol* **27:**171–177.
Ellerby HM, Arap W, Ellerby LM, Kain R, Andrusiak R, Rio GD, Krajewski S, Lombardo CR, Rao R, Ruoslahti E, Bredesen DE and Pasqualini R (1999) Anticancer activity of targeted pro-apoptotic peptides. *Nat Med* **5:**1031–1038.
Emanuele R, Martin R, Hunter RL, Robert L, Culbreth PH and Paula H (1999) inventors, CytRx Corporation assignee. Polyoxypropylene/polyoxyethylene copolymers with improved biological activity. US Patent 5,990,241. 1999 Nov 23.
Fan D, Bucana CD, O'Brian CA, Zwelling LA, Seid C and Fidler IJ (1990) Enhancement of murine tumor cell sensitivity to adriamycin by presentation of the drug in phosphatidylcholine-phosphatidylserine liposomes. *Cancer Res* **50:**3619–3626.
Fearon DT and Austen KF (1977) Activation of the alternative complement pathway due to resistance of zymosan-bound amplification convertase to endogenous regulatory mechanisms. *Proc Natl Acad Sci USA* **74:**1683–1687.
Fernandes A and Gregoriadis G (1997) Polysialyated asparaginase: preparation, activity and pharmacokinetics. *Biochim Biophys Acta* **1341:**26–34.
Fischetti VA, Horstmann RD and Pancholi V (1995) Location of the complement factor H binding site on streptococcal M6 protein. *Infect Immun* **63:**149–153.
Folkman J (1995) Tumor angiogenesis, in *The Molecular Basis of Cancer* (Mendelson J, Howley PM, Israel MA and Liotta LA eds) pp 206–232, WB Saunders Company, Philadelphia.
Forssen EA, Malé-Brune R, Adler-Moore JP, Lee MJA, Schmidt PG, Krasieva TB, Shimizu S and Tromberg BJ (1996) Fluorescence imaging studies for the disposition of daunorubicin liposomes (DaunoXome) within tumor tissue. *Cancer Res* **56:**2066–2075.
Gabizon AA (1995) Liposome circulation time and tumor targeting: implications for cancer chemotherapy. *Adv Drug Delivery Rev* **16:**285–294.
Gabizon A and Papahadjopoulos D (1988) Liposome formulations with prolonged circulation time in blood and enhanced uptake in tumors. *Proc Natl Acad Sci USA* **85:**6949–6953.
Gabizon A and Papahadjopoulos D (1992) The role of surface charge and hydrophilic groups on liposome clearance in vivo. *Biochim Biophys Acta* **1103:**94–100.
Geyer RP (1967) Studies on the mechanism of intravenous fat emulsions. *Fette Med* **6:**59–61.
Gill PS, Espina BM, Muggia F, Cabriales S, Tupule A and Esplin JA (1995) Phase I/II clinical and pharmacokinetic evaluation of liposomal danuorubicin. *J Clin Oncol* **13:**996–1003.
Gogolewski S, Jovanovic M, Perren SM, Dillon JG and Hughes MK (1993a) Tissue response and in vivo degradation of selected polyhydroxyacids: polylactides (PLA), poly(3-hydroxybutyrate) (PHB) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHB/VA). *J Biomed Mater Res* **27:**1135–1148.
Gogolewski S, Jovanovic M, Perren SM, Dillon JG and Hughes MK (1993b) The effect of melt processing on degradation of selected poly(hydroxyacids): polylactides, polyhydroxybutyrates and polyhydroxybutyrate-co-valerates. *Polymer Degrad Stability* **40:**313–322.
Goins B, Klipper R, Cliff RO, Blumhardt R and Phillips WT (1993) Biodistribution and imaging studies of technetium-99m-labeled liposomes in rats with focal infection. *J Nucl Med* **34:**2160–2168.
Goins B, Phillips WT and Klipper R (1996) Blood-pool imaging using technetium-99m-labeled liposomes. *J Nucl Med* **37:**1374–1379.
Gordon S (1995) The macrophage. *Bioessays* **17:**977–986.
Gordon S, Lawson L, Rabinowitz S, Crocker PR, Morris L and Perry VH (1992) Antigen markers of macrophage differentiation in murine tissues. *Curr Top Microbiol Immunol* **181:**1–37.
Goren D, Horowitz AT, Tzemach D, Tarshish M, Zalipsky S and Gabizon A (2000) Nuclear delivery of doxorubicin via folate-targeted liposomes with bypass of multidrug-resistance efflux pump. *Clin Cancer Res* **6:**1949–1957.
Goren D, Horowitz AT, Zalipsky S, Woodle MC, Yarden Y and Gabizon A (1996) Trageting of Stealth liposomes to erbB2 (Her/2) receptor: in vitro and in vivo studies. *Br J Cancer* **74:**1749–1756.
Grandfils C, Flandroy P and Jerome R (1996) Control of the biodegradation rate of poly(D, L-lactide) microparticles intended as chemoembolization materials. *J Control Release* **38:**109–122.
Gref R, Domb A, Quellec P, Blunk T, Müller RH, Verbavatz JM and Langer R (1995) The controlled intravenous administration of drugs using PEG-coated sterically stabilized nanospheres. *Adv Drug Delivery Rev* **16:**215–233.
Gref R, Minamitake Y, Peracchia MT, Trubetskoy V, Torchilin V and Langer R (1994) Biodegradable long-circulating polymeric nanospheres. *Science (Wash DC)* **263:**1600–1603.
Gregoriadis G, McCormack B, Wang Z and Lifely R (1993) Polysialic acids: potential in drug delivery. *FEBS Lett* **315:**271–276.
Griffioen AW and Molema G (2000) Angiogenesis potentials for pharmacologic intervention in the treatment of cancer, cardiovascular diseases, and chronic inflammation. *Pharmacol Rev* **52:**237–268.
Grizzi I, Garreau H, Li S and Vert M (1995) Hydrolytic degradation of devices based on poly(D, L-lactic acid) size dependence. *Biomaterials* **16:**305–311.
Groom AC (1987) Microcirculation of the spleen: new concepts. *Microvasc Res* **34:** 269–289.
Guimaraes R, Clement O, Bitton J, Carnot F and Frija G (1994) MR lymphography with superparamagnetic iron nanoparticles in rats: pathological basis for contrast enhancement. *Am J Roentgenol Radium Ther Nucl Med* **162:**201–207.
Hagan SA, Coombes AGA, Garnett MC, Dunn SE, Davies MC, Illum L, Davis SS, Harding SE, Purkiss S and Gellert PR (1996) Polylactide-poly(ethylene glycol)

copolymers as drug delivery systems. I. Characterization of water dispersible micelle-forming systems. *Langmuir* **12:**2153–2161.
Hagemeier HH, Vollmer E, Goerdt S, Schulzeosthoff K and Sorg C (1986) A monoclonal antibody reacting with endothelial cells of budding vessels in tumors and inflammatory tissues, and non-reactive normal adult tissues. *Int J Cancer* **38:**481–488.
Hansen CB, Kao GY, Moase EH, Zalipsky S and Allen TM (1995) Attachment of antibodies to sterically stabilized liposomes: evaluation, comparison and optimization of coupling procedures. *Biochim Biophys Acta* **1239:**133–144.
Harashima H, Sakata K, Funato K and Kiwada H (1994) Enhanced hepatic uptake of liposomes through complement activation depending on the size of liposomes. *Pharm Res (N Y)* **11:**402–406.
Harasym TO, Bally MB and Tardi P (1998) Clearance properties of liposomes involving conjugated proteins for targeting. *Adv Drug Delivery Rev* **32:**99–118.
Harding JA, Engbers CM, Newman MS, Goldstein NI and Zalipsky S (1997) Immunogenicity and pharmacokinetic attributes of poly(ethylene glycol)-grafted immunoliposomes. *Biochim Biophys Acta* **1327:**181–192.
Hardonk MJ, Dijkhuis FWJ, Hulstaert CE and Koudstaal J (1992) Heterogeneity of rat-liver and spleen macrophages in gadolinium chloride-induced elimination and repopulation. *J Leukoc Biol* **52:**296–302.
Harrison M, Tomlinson D and Stewart S (1995) Liposomal-entrapped doxorubicin: an active agent in AIDS-related Kaposi's sarcoma. *J Clin Oncol* **13:**914–920.
Hart PD and Payne SN (1971) Effects of non-ionic surfactants that modify experimental tuberculosis on lipase activity of macrophages. *Br J Phamacol* **43:**190–196.
Hawley AE, Illum L and Davis SS (1997a) Lymph node localization of biodegradable nanospheres surface modified with poloxamer and poloxamine block copolymers. *FEBS Lett* **400:**319–323.
Hawley AE, Illum L and Davis SS (1997b) Preparation of biodegradable, surface engineered PLGA nanospheres with enhanced lymphatic drainage and lymph node uptake. *Pharm Res (N Y)* **14:**657–661.
Heidenreich F and Dierich MP (1985) *Candida albicans* and *Candida stellatoidea*, in contrast to other Candida species, bind iC3b and C3d but not C3b. *Infect Immun* **50:**598–600.
Hinglais N, Kazatchkine M, Mandet C, Appay MD and Bariety J (1988) Human liver Kupffer cells express CR1, CR3 and CR4 complement receptors. *Lab Invest* **82:** 640–651.
Hobbs SK, Monsky WL, Yuan F, Roberts WG, Griffith L, Torchilin VP and Jain RK (1998) Regulation of transport pathways in tumor vessels: role of tumor type and microenvironment. *Proc Natl Acad Sci USA* **95:**4607–4612.
Hoedemakers RMJ, Morselt HWM, Scherphof GL and Daemen T (1994) Secretion pattern of the rat-liver macrophage population following activation with liposomal muramyl dipeptide in-vivo and in-vitro. *J Immunother* **15:**265–272.
Hoffman A, Donbrow M and Benita S (1986) Direct measurements on individual microcapsule dissolution as a tool for determination of mechanism. *J Pharm Pharmacol* **38:**764–766.
Honing H, van Rooijen N and van der Berg TK (2000) Manipulation of macrophage activities using liposomes. *J Liposome Res* **10:**359–389.
Horwitz BA, Shintizky M, Kreutz W and Yatvin MB (1980) pH-sensitive liposomes: possible clinical implications. *Science (Wash DC)* **210:**1253–1255.
Howerton DA, Hunter RL, Ziegler HK and Check IJ (1990) Induction of macrophage Ia expression in vivo by a synthetic block copolymer, L81. *J Immunol* **144:** 15781584.
Hu YP, Henry-Tolume N and Robert J (1995) Failure of liposome encapsulation of doxorubicin to circumvent multidrug resistance in an in vitro model of rat glioblastoma. *Eur J Cancer* **31A:**389–394.
Huang SK, Lee KD, Hong K, Friend DS and Papahadjopoulos D (1992) Microscopic localization of sterically stabilized liposomes in colon carcinoma-bearing mice. *Cancer Res* **52:**5135–5143.
Huang SK, Stauffer PR, Hong K, Guo JWH, Phillips TL, Huang A and Paphadjopoulos D (1994) Liposomes and hyperthermia in mice: increased tumor uptake and therapeutic efficacy of doxorubicin in sterically stabilized liposomes. *Cancer Res* **54:**2186–2191.
Hunter RL and Bennett B (1984) The adjuvant activity of nonionic block polymer surfactants. II. Antibody formation and inflammation related to the structure of triblock and octablock copolymers. *J Immunol* **133:**3167–3175.
Illum L and Davis SS (1984) The organ uptake of intravenously administered colloidal particles can be altered using a non-ionic surfactant (Poloxamer 338). *FEBS Lett* **167:**79–82.
Ingram DA, Forman MB and Murray JJ (1992) Phagocytic activation of human neutrophils by the detergent component of fluosol. *Am J Pathol* **140:**1081–1087.
Ingram DA, Forman MB and Murray JJ (1993) Activation of complement by Fluosol attributable to the Pluronic detergent micelle structure. *J Cardiovasc Pharmacol* **22:**456–461.
Ito S, Naito M, Kobayashi Y, Takatsuka H, Jiang S, Usuda H, Umezu H, Hasegawa G, Arakawa M, Shultz LD and Elomaa O (1999) Roles of a macrophage receptor with collagenous structure (MARCO) in host defence and heterogeneity of splenic marginal zone macrophages. *Arch Histol Cytol* **62:**83–95.
Jagannath C, Pai S, Actor JK and Hunter RL (1999) CRL-1072 enhances antimycobacterial activity of human macrophages through interleukin-8. *J Interferon Cytokine Res* **19:**67–76.
Jain RK (1988) Determinants of tumor blood flow: a review. *Cancer Res* **48:**2641–2658.
Jain RK (1989) Delivery of novel therapeutic agents in tumors: physiological barriers and strategies. *J Natl Cancer Inst* **81:**570–576.
Jamar F, Chapman PT, Manicourt DH, Glass DM, Haskard DO and Peters AM (1997) A comparison between 111In-anti-E-selectin mAb and 99Tcm-labelled human non-specific immunoglobulin in radionuclide imaging of rheumatoid arthritis. *Brit J Radiol* **70:**473–481.
Jeppsson R and Rossner S (1975) The influence of emulsifying agents and of lipid soluble drugs on the fractional removal rate of lipid emulsions from the blood stream of rabbit. *Acta Pharmacol Toxicol* **37:**134–144.
Jones MC and Leroux JC (1999) Polymeric micelles –a new generation of colloidal drug carriers. *Eur J Pharm Biopharm* **48:**101–111.
Kamei S and Kopecek J (1995) Prolonged blood circulation in rats of nanospheres surface-modified with semitelechlic poly[*N*-2(hydroxypropyl)methacrylamide]. *Pharm Res (N Y)* **12:**663–668.
Kang EC, Akiyoshi K and Sunamoto J (1997) Surface coating of liposomes with hydrophobized polysaccharides. *J Biact Compat Polym* **12:**14–26.
Kazatchkine MD, Fearon DT and Austen KF (1979) Human alternative complement pathway: membrane-associated sialic acid regulates the competition between B and beta1 H for cell-bound C3b. *J Immunol* **122:**75–81.
Kehlen H, Rätzsch MT and Bergmann J (1988) Continuous kinetics of first order degradation reactions in polydisperse mixtures. *Chem Eng Sci* **43:**609–616.
Kim KJ, Li B, Winer J, Armanini M, Gillett N, Phillips HS and Ferrara N (1993) Inhibition of vascular endothelial growth factor induced angiogenesis suppresses tumour growth in vivo. *Nature (Lond)* **362:**841–844.
Kim SY, Shin IG, Lee YM, Cho CS and Sung YK (1998) Methoxy poly(ethylene glycol) and ϵ-caprolactone amphiphilic block copolymeric micelle containing indomethacin. II. Micelle formation and drug release behaviours. *J Control Release* **51:**13–22.
Kirpotin D, Hong KL, Mullah N, Papahadopoulos D and Zalipsky S (1996) Liposomes with detachable polymer coating. Destabilization and fusion of dioleoylphosphatidylethanolamine vesicles triggered by cleavage of surface-grafted poly(ethylene glycol). *FEBS Lett* **388:**115–118.
Kirpotin D, Park JW, Hong K, Zalipsky S, Li WL, Carter P, Benz CC and Papahadjopoulos D (1997) Sterically stabilized anti-HER2 immunoliposomes: design and targeting to human breast cancer cells in vitro. *Biochemistry* **36:**66–75.
Klibanov AL, Maruyama K, Beckerleg AM, Torchilin VP and Huang L (1991) Activity of amphipathic poly(ethylene glycol) 5000 to prolong the circulation time of liposome depends on the liposome size and is unfavourable for immunoliposome binding to target. *Biochim Biophys Acta* **1062:**142–148.
Klibanov AL, Maruyama K, Torchilin VP and Huang L (1990) Amphipathic polyethyleneglycol effectively prolong the circulation time of liposomes. *FEBS Lett* **268:** 235–237.
Kostarelos K and Emfietzoglou D (1999) Liposomes as carriers of radionuclides: from imaging to therapy. *J Liposome Res* **9:**429–460.
Kostarelos K, Tadros TF and Luckham PF (1999) Physical conjugation of (tri-) block copolymers to liposomes toward the construction of sterically stabilized vesicle systems. *Langmuir* **15:**369–376.
Kraal G (1992) Cells in the marginal zone of the spleen. *Int Rev Cytol* **132:**31–74.
Kraling BM, Razon MJ, Boon LM, Zurakowski D, Seachord C, Darveau RP, Mulliken JB, Corless CL and Bischoff J (1996) E-selectin is present in proliferating endothelial cells in human hemangiomas. *Am J Pathol* **148:**1181–1191.
Krishna R and Mayer LD (1998) Applications of liposome technology to overcome multidrug resistance in solid tumors, in *Targeting of Drugs 6: Strategies for Stealth Therapeutic Systems* (Gregoriadis G and McCormack B eds) pp 95–108, NATO ASI Series, Plenum Press, New York.
Kukowska-Latallo JF, Bielinska AU, Johnson J, Spindler R, Tomalia DA and Baker Jr (1996) Efficient transfer of genetic material into mammalian cells using Starburst polyamidoamine dendrimers. *Proc Natl Acad Sci USA* **93:**4897–4902.
Kumar SD, West DC and Ager M (1987) Heterogeneity in endothelial cells from large vessels and microvessels. *Differentiation* **36:**57–70.
Kunioka M and Doi Y (1990) Thermal degradation of microbial copolyesters: poly(3-hydroxybutyrate-co-3-hydroxyvalerate) and poly(3-hydroxybutyrate-co-4-hydroxybutyrate). *Macromolecules* **23:**1933–1936.
Kwon G, Suwa S, Yokoyama M, Okano T, Sakurai Y and Kataoka K (1993) Biodistrinution of micelles forming polymer-drug conjugates. *Pharm Res (N Y)* **10:**970–974.
Kwon G, Suwa S, Yokoyama M, Okano T, Sakurai Y and Kataoka K (1994) Enhanced tumor accumulation and prolonged circulation times of micelle-forming poly(ethylene oxide-aspartate) block copolymer-adriamycin conjugates. *J Control Release* **29:**17–23.
Lacasse FX, Filion MC, Phillips NC, Escher E, McMullen JN and Hildgen P (1998) Influence of surface properties at biodegradable microsphere surfaces: effects on plasma protein adsorption and phagocytosis. *Pharm Res (N Y)* **15:**312–317.
Lam KH, Schakenraad JM, Esselbrugge H, Feijen and Nieuwenhuis (1993) The effect of phagocytosis of poly(L-lactic acid) fragments on cellular morphology and viability. *J Biomed Mat Res* **27:**1569–1577.
Lasic DD, Martin FJ, Gabizon A, Huang SK and Papahadjopoulos D (1991) Sterically-stabilized liposomes: a hypothesis on the molecular origin of the extended circulation time. *Biochim Biophys Acta* **1070:**187–192.
Lauren J, Gunji Y and Alitalo K (1998) Is angiopoietin-2 necessary for the initiation of tumour angiogenesis? *Am J Pathol* **153:**1333–1339.
Lee J, Martic PA and Tan JS (1989) Protein adsorption on Pluronic co-polymer coated polystyrene particles. *J Colloid Interface Sci* **131:**252–266.
Lee KD, Oh YK, Portnoy DA and Swanson JA (1996) Delivery of macromolecules into cytosol using liposome containing hemolysin from Listeria monocytogenes. *J Biol Chem* **271:**7249–7252.
Lee RJ and Huang L (1996) Folate-targeted, anionic liposome-entrapped polylysine-condensed DNA for tumor cell-specific gene transfer. *J Biol Chem* **271:**8481–8487.
Lee RJ and Low PS (1994) Delivery of liposomes into cultured KB cells via folate receptor-mediated endocytosis. *J Biol Chem* **269:**3198–3204.
Lee SH, Crocker P and Gordon S (1986) Macrophage plasma membrane and secretory properties in murine malaria. Effects of *Plasmodium yoelii* blood-stage infection on macrophages in liver, spleen, and blood. *J Exp Med* **163:**54–74.
Lepay DA, Steinman RM, Nathan CF, Murray HW and Cohen ZA (1985) Liver macrophages in murine listeriosis. Cell-mediated immunity is correlated with an influx of macrophages capable of generating reactive oxygen intermediates. *J Exp Med* **161:**1503–1512.
Li C, Yang DJ, Kuang LR and Wallace S (1993) Polyamino acid microspheres: preparation, characterization and distribution after intravenous injection in rats. *Int J Pharm* **94:**143–152.

Li JT, Caldwell KD and Rapoport N (1994) Surface properties of Pluronic-coated polymeric colloids. *Langmuir* **10:**4475–4482.
Li JT, Carlsson J, Lin JN and Caldwell KD (1996) Chemical modification of surface active poly(ethylene oxide)-poly(propylene oxide) triblock copolymers. *Bioconjugate Chem* **7:**592–599.
Li SM, Tenon M, Garreau H, Braud C and Vert M (2000) Enzymatic degradation of stereocopolymers derived from L-, DL- and meso-lactides. *Polymer Degrad Stability* **67:**85–90.
Lin P, Buxton JA, Acheson A, Radjiejewski C, Maisonpierre PC, Yancopoulos GD, Channon KM, Hale LP, Dewhirst MW, George SE and Peters K (1998) Antiangiogeneic gene therapy targeting the endothelium specific receptor tyrosine kinase Tie2. *Proc Natl Acad Sci USA* **95:**8829–8834.
Lin PS, Ho KC, Sung SJ and Gladding J (1992) Effect of tumor necrosis factor, heat, and radiation on the viability and microfilament organization on cultured endothelial cells. *Int J Hyperther* **8:**667–677.
Lin W (1996) *The preparation and characterization of surface modified albumin nanoparticles for site specific drug delivery*. PhD Thesis, University of Nottingham, UK.
Lin W, Coombes AGA, Garnett MC, Davies MC, Schacht E, Davis SS and Illum L (1994) Preparation of sterically stabilized human serum albumin nanospheres using a novel dextranox-MPEG crosslinking agent. *Pharm Res (N Y)* **11:**1588–1592.
Lin W, Garnett MC, Davies MC, Bignotti F, Ferruti P, Davis SS and Illum L (1997) Preparation of surface-modified albumin nanospheres. *Biomaterials* **18:**559–565.
Lin W, Garnett MC, Schacht E, Davis SS and Illum L (1999) Preparation and in vitro characterization of HSA-mPEG nanoparticles. *Int J Pharm* **189:**161–170.
Linblad G and Bjorkman N (1964) Ultrastructural alterations in sinusoidal endothelium of liver and bone marrow in dogs with experimental hepatitis contagiosa canis. *Acta Pathol Microbiol Scand* **62:**155–161.
Lindberg FP, Lublin DM, Telen MJ, Veile RA, Miller YE, Doniskeller H and Brown EJ (1994) RH-related antigen CD47 is the signal-transducer integrin-associated protein. *J Biol Chem* **269:**1567–1570.
Litzinger DC and Huang L (1992) Amphipathic poly(ethylene glycol) 5000-stabilized dioleoylphosphatidylethanolamine liposomes accumulate in spleen. *Biochim Biophys Acta* **1127:**249–254.
Liu D, Mori A and Huang L (1991) Large liposomes containing ganglioside GM1 accumulate effectively in spleen. *Biochim Biophys Acta* **1066:**159–165.
Liu D, Song YK and Liu F (1995) Antibody dependent, complement mediated liver uptake of liposome containing GM_1. *Pharm Res (N Y)* **12:**1775–1780.
Liu F and Liu D (1995) Long-circulating emulsions (oil-in-water) as carriers for lipophilic drugs. *Pharm Res (N Y)* **12:**1060–1064.
Lundberg B (1997) A submicron lipid emulsion coated with amphipathic polyethylene glycol for parental administration of Paclitaxel (Taxol). *J Pharm Pharmacol* **49:**16–21.
Malik N, Wiwattanapatapee R, Klopsch R, Lorenz K, Frey H, Weener JW, Meijer EW, Paulus W and Duncan R (2000) Dendrimers relationship between structure and biocompatibility in vitro, and preliminary studies on the biodistribution of 125I-labelled polyamidoamine dendrimers in vivo. *J Control Release* **65:**133–148.
Mandrell RE and Zollinger WD (1982) Measurement of antibodies to meningococcal group B polysaccharide: low avidity binding and equilibrium binding constants. *J Immunol* **129:**2172–2178.
Maruiwa M, Mizoguchi A, Russell GJ, Narula N, Stronska M, Mizoguchi E, Rabb H, Arnaout MA and Bhan AK (1993) Anti-KCA-3, a monoclonal antibody reactive with a rat complement C3 receptor, distinguishes Kupffer cells from other macrophages. *J Immunol* **150:**4019–4030.
Maruyama K, Kennel SJ and Humah L (1990) Lipid composition is important for highly efficient target binding and retention of immunoliposomes. *Proc Natl Acad Sci USA* **87:**5744–5748.
Maruyama K, Okuizumi S, Ishida O, Yamauchi H, Kikuchi H and Iwatsuru M (1994) Phosphatidylpolyglycerols prolong liposome circulation in vivo. *Int J Pharm* **111:**103–107.
Maruyama K, Yuda T, Okamoto A, Kojima S, Suginaka A and Iwatsuru M (1992) Prolonged circulation time in vivo of large unilamellar liposomes composed of distearoylphosphatidylcholine and cholesterol containing amphipathic poly(ethylene glycol). *Biochim Biophys Acta* **1128:**44–49.
Mason NS, Miles CS and Sparks RE (1981) Hydrolytic degradation of poly D, L-(lactide), in *Biomedical and Dental Applications of Polymers* (Gebelein CG and Koblitz FF, eds) pp 279–291, Plenum Press, New York.
Matlaga BF, Yasenchak LP and Salthouse TN (1976) Tissue response to implanted polymers: the significance of sample shape. *J Biomed Mat Res* **10:**391–397.
Matthews W, Jordan GT, Gavin M, Jenkins N, Copeland NG and Lemischka IR (1991) A receptor tyrosine kinase cDNA isolated from a population of enriched primitive hematopoietic cells and exhibiting close genetic linkage to c-kit. *Proc Natl Acad Sci USA* **88:**9026–9030.
Maurer CA, Friess H, Kretschman B, Wildi S, Muller C, Graber H, Schilling M and Buchler M (1998) Over-expression of ICAM-1, VCAM-1 and ELAM-1 might influence tumor progression in colorectal cancer. *Int J Cancer* **79:**76–81.
Max R, Gerristen RPCM, Nooijen PTGA, Goodman SL, Sutter A, Keilholz U, Ruiter DJ and De Waal Rmw (1997) Immunohistochemical analysis of integrin $\alpha_v\beta_3$ expression on tumor-associated vessels of human carcinomas. *Int J Cancer* **71:**320–324.
McVicar JW, Richmond JY, Campbell CH and Hamilton LD (1973) Observations of cattle, goats and pigs after administration of synthetic interferon inducers and subsequent exposure to foot and mouth disease virus. *Can J Comp Med* **37:**362–368.
Melton RG, Wiblin CN, Foster RL and Sherwood RF (1987a) Covalent linkage of carboxypeptidase G_2 to soluble dextrans–I. Properties of conjugates and effects on plasma persistance in mice. *Biochem Pharmacol* **36:**105–112.
Melton RG, Wiblin CN, Baskerville A, Foster RL and Sherwood RF (1987b) Covalent linkage of carboxypeptidase G_2 to soluble dextrans–II. In vivo distribution and fate of conjugates. *Biochem Pharmacol* **36:**113–121.
Meri S and Pangburn MK (1994) Regulation of alternative pathway complement activation by glycosaminoglycans: specificity of the polyanion binding site on factor H. *Biochem Biophys Res Commun* **198:**52–59.
Meyer O, Papahadjopoulos D and Leroux J-C (1998) Copolymers of *N*-isopropylacrylamide can trigger pH sensitivity to stable liposomes. *FEBS Lett* **421:**61–64.
Michalek MT, Bremer EG and Mold C (1988) Effect of gangliosides on activation of the alternative pathway of human complement. *J Immunol* **140:**1581–1587.
Mobed M and Chang TMS (1991) Preparation and surface characterization of carboxymethylchitin-incorporated submicron bilayer-lipid membrane artificial cells (liposomes) encapsulating liposomes. *Biomater Artif Cell Immobil Biotechnol* **19:**731–744.
Mobed M and Chang TMS (1998) Comparison of polymerically stabilized PEG-grafted liposomes and physically adsorbed carboxymethylchitin and carboxymethyl/glycolchitin liposomes for biological applications. *Biomaterials* **19:**1167–1177.
Moghimi SM (1995a) Exploiting bone marrow microvascular structure for drug delivery and future therapies. *Adv Drug Delivery Rev* **17:**61–73.
Moghimi SM (1995b) Mechanisms of splenic clearance of blood cells and particles: towards development of new splenotropic agents. *Adv Drug Delivery Rev* **17:**103–115.
Moghimi SM (1996) Apoptotic cell death in activated monocytes following incorporation of clodronate-liposomes. *J Leukoc Biol* **61:**643–644.
Moghimi SM (1997) Prolonging the circulation time and modifying the body distribution of intravenously injected polystyrene nanospheres by prior intravenous administration of poloxamine-908: a "hepatic-blockade" event or manipulation of nanosphere surface in vivo? *Biochim Biophys Acta* **1336:**1–6.
Moghimi SM (1998) Humoral-mediated recognition of "phagocyte-resistant" beads by lymph node macrophages of poloxamine-treated rats. *Clin Sci* **95:**389–391.
Moghimi SM (1999) Re-establishing the long-circulatory behaviour of poloxamine-coated particles after repeated intravenous administration: applications in cancer drug delivery and imaging. *Biochim Biophys Acta* **1472:**399–403.
Moghimi SM and Bonnemain (1999) Subcutaneous and intravenous delivery of diagnostic agents to the lymphatic system: applications in lymphoscintigraphy and indirect lymphography. *Adv Drug Delivery Rev* **37:**295–312.
Moghimi SM and Davis SS (1994) Innovations in avoiding particles clearance from blood by Kupffer cells: cause for reflection. *Crit Rev Ther Drug Carrier Syst* **11:**31–59.
Moghimi SM and Gray T (1997) A single dose of intravenously injected poloxamine-coated long-circulating particles triggers macrophage clearance of subsequent doses in rats. *Clin Sci* **93:**371–379.
Moghimi SM, Hawley AE, Christy NM, Gray T, Illum L and Davis SS (1994) Surface engineered nanospheres with enhanced drainage into lymphatics and uptake by macrophages of the regional lymph nodes. *FEBS Lett* **344:**25–30.
Moghimi SM, Hedeman H, Christy NM, Illum L and Davis SS (1993a) Enhanced hepatic clearance of intravenously administered sterically stabilized microspheres in zymosan-stimulated rats. *J Leukoc Biol* **54:**513–517.
Moghimi SM, Hedeman H, Muir IS, Illum L and Davis SS (1993b) An investigation of the filtration capacity and the fate of large filtered sterically-stabilized microspheres in rat spleen. *Biochim Biophys Acta* **1157:**233–240.
Moghimi SM and Hunter AC (2000a) Recognition by macrophages and liver cells of opsonized phospholipid vesicles and phospholipid headgroups. *Pharm Res (N Y)* **18:**1–8.
Moghimi SM and Hunter AC (2000b) Poloxamers and poloxamines in nanoparticle engineering and experimental medicine. *Trends Biotechnol* **18:**412–420.
Moghimi SM, Muir IS, Illum L, Davis SS and Kolb-Bachofen V (1993c) Coating particles with a block copolymer (poloxamine-908) suppresses opsonization but permits the activity of dysopsonins in the serum. *Biochim Biophys Acta* **1179:**157–165.
Moghimi SM and Murray JC (1996) Poloxamer-188 revisited: a potentially valuable immune modulator? *J Natl Cancer Inst* **88:**766–768.
Moghimi SM and Patel HM (1989a) Differential properties of organ-specific serum opsonins for liver and spleen macrophages. *Biochim Biophys Acta* **984:**379–383.
Moghimi SM and Patel HM (1989b) Serum opsonins and phagocytosis of saturated and unsaturated phospholipid liposomes. *Biochim Biophys Acta* **984:**384–387.
Moghimi SM and Patel HM (1996) Altered tissue-specific opsonic activities and opsonophagocytosis of liposomes in tumour-bearing rats. *Biochim Biophys Acta* **1285:**56–64.
Moghimi SM and Patel HM (1998) Serum-mediated recognition of liposomes by phagocytic cells of the reticuloendothelial system. The concept of tissue specificity. *Adv Drug Delivery Rev* **32:**45–60.
Moghimi SM, Porter CJH, Muir IS, Illum L and Davis SS (1991) Non-phagocytic uptake of intravenously injected microspheres in rat spleen: influence of particle size and hydrophilic coating. *Biochem Biophys Res Commun* **177:**861–866.
Moghimi SM and Rajabi-Siahboomi AR (1996) Advanced colloid-based systems for efficient delivery of drugs and diagnostic agents to the lymphatic tissues. *Prog Biophys Mol Biol* **65:**221–249.
Monfardini C and Veronese FM (1998) Stabilization of substances in circulation. *Bioconjugate Chem* **9:**418–450.
Moreno C, Lifely MR and Esdaile J (1985) Immunity and protection of mice against *Neisseria meningitidis* group B by vaccination using polysaccharide complexed with outer membrane proteins: a comparison with purified B polysaccharide. *Infect Immun* **47:**527–533.
Mori A, Kennel SJ and Huang L (1993) Immunotargeting of liposomes containing lipophilic antitumor prodrugs. *Pharm Res (N Y)* **10:**507–514.
Mori A, Klibanov AL, Torchilin VP and Huang L (1991) Influence of the steric barrier activity of amphipathic poly(ethylene glycol) and ganglioside GM1 on the circulation time of liposomes and on the target binding of immunoliposomes in vivo. *FEBS Lett* **284:**263–266.
Munday J, Floyd H and Crocker PR (1999) Sialic acid binding receptors (siglecs) expressed by macrophages. *J Leukoc Biol* **66:**705–711.
Murohara T, Margiotta J, Phillips JM, Paulson JC, DeFrees S, Zalipsky S, Guo LLS

and Lefer AM (1995) Cardioprotection by liposome-conjugated sialyl Lewis(x)-oligosaccharide in myocardial and reperfusion injury. *Cardiovasc Res* **30:**965–974.
Murray JC (1997) Endothelial gene products, cytokines and radiation, in *Radiation Biology of the Endothelial cell* (Rubin D ed) pp 147–160, CRC Press, Boca Raton, FL.
Naito M (1993) Macrophage heterogeneity in development and differentiation. *Arch Histol Cytol* **56:**331–351.
Naito M, Nagai H, Kawano S, Umezu H, Zhu H, Moriyama H, Yamamoto T, Takatsuka H and Takei Y (1996) Liposome-encapsulated dichloromethylene diphosphonate induces macrophage apoptosis in vivo and in vitro. *J Leukoc Biol* **60:**337–344.
Namba Y, Oku N, Ito F, Sakakibara T and Okada S (1992) Liposomal modification with uronate, which endows liposomes with long circulation in vivo, reduces the uptake of liposomes by J774 cells *in vitro*. *Life Sci* **50:**1773–1779.
Northfelt DW, Dezube BJ, Thommes JA, Levine R, Von Roem JH, Dosik GM, Rios A, Krown SE, DuMond C and Mamelok RD (1997) Efficacy of pegylated-liposomal doxorubicin in the treatment of AIDS-related Kaposi's sarcoma after failure of standard chemotherapy. *J Clin Oncol* **15:**653–659.
Northfelt DW, Martin FJ, Working P, Volberding PA, Russell J, Newman M, Amantea MA and Kaplan LD (1996) Doxorubicin encapsulated in liposomes containing surface-bound polyethylene glycol: pharmacokinetics, tumour localization, and safety in patients with AIDS-related Kaposi's sarcoma. *J Clin Pharmacol* **36:**55–63.
Oku N and Namba Y (1994) Long-circulating liposomes. *Crit Rev Ther Drug Carrier Syst* **11:**231–270.
Oku N, Namba Y and Okada S (1992) Tumor accumulation of novel RES-avoiding liposomes. *Biochim Biophys Acta* **1126:**255–260.
Oldenborg PA, Zheleznyak A, Fang YF, Lagenaur CF, Gresham HD and Lindberg FP (2000) Role of CD47 as a marker of self on red blood cells. *Science (Wash DC)* **288:**2051–2054.
Olivier JC, Vauthier C, Tarverna M, Puisieux F, Ferrier D and Couvreur P (1996) Stability of orosomucoid-coated polyisobutylcyanoacrylate nanoparticles in the presence of serum. *J Control Release* **40:**157–168.
Oussoren C and Storm G (1997) Lymphatic uptake and biodistribution of liposomes after subcutaneous injection. III. Influence of surface modification with poly(ethylene glycol). *Pharm Res (N Y)* **14:**1479–1484.
Ouyang C, Choice E, Holland J, Meloche M and Madden TM (1995) Liposomal cyclosporin. Characterization of drug incorporation and interbilayer exchange. *Transplantaion (Baltim)* **60:**999–1006.
Oyen WJG, Claessens RAMJ, van der Meer Jwm and Corstens Fhm (1991) Detection of subacute infectious foci with indium-111-labeled autologous leukocytes and indium-111-labeled human nonspecific immunoglobulin-G: a prospective comparative study. *J Nucl Med* **32:**1854–1860.
Pain D, Das PK, Ghosh PC and Bachhawat BK (1984) Increased circulatory half-life of liposomes after conjugation with dextran. *J Biosci* **6:**811–816.
Panchal RG, Cusack E, Cheley S and Bayley H (1996) Tumor protease-activated, pore-forming toxins from a combinatorial library. *Nat Biotechnol* **14:**852–856.
Pangburn MK, Atkinson MA and Meri S (1991) Localization of the heparin-binding site on complement factor H. *J Biol Chem* **266:**16847–16853.
Pangburn MK and Muller-Eberhard HJ (1978) Complement C3 convertase: cell surface restriction of β1H control and generation of restriction on neuraminidase-treated cells. *Proc Natl Acad Sci USA* **75:**2416–2420.
Papahadjopoulos D, Allen TM, Gabizon A, Mayhew E, Matthay K, Huang SK, Lee K-D, Woodle MC, Lasic DD, Redemann C and Martin FJ (1991) Sterically stabilized liposomes: improvements in pharmacokinetics and antitumor therapeutic efficacy. *Proc Natl Acad Sci USA* **88:**11460–11464.
Park JW, Hong K, Carter P, Asgari H, Guo LY, Keller GA, Wirth C, Shalaby R, Kotts C, Wood WI, Papahadopoulos D and Benz CC (1995) Development of anti-p185(HER2) immunoliposomes for cancer-therapy. *Proc Natl Acad Sci USA* **92:**1327–1331.
Park YS and Huang L (1993) Effect of chemically modified GM1 and neoglycolipid analogs of GM1 on liposome circulation time: evidence supportring the dysopsonic hypothesis. *Biochim Biophys Acta* **1166:**105–114.
Park YS, Maruyama K and Huang L (1992) Some negatively charged phospholipid derivatives prolong the liposome circulation in vivo. *Biochim Biophys Acta* **1108:**257–260.
Parr MJ, Ansell SM, Choi LS and Cullis PR (1994) Factors influencing the retention and chemical-stability of poly(ethylene glycol)-lipid conjugates incorporated into large unilamellar vesicles. *Biochim Biophys Acta* **1195:**21–30.
Parr MJ, Masin D, Cullis PR and Bally MB (1997) Accumulation of liposomal lipid and encapsulated doxorubicin in murine Lewis lung carcinoma: the lack of beneficial effects of coating liposomes with poly(ethylene glycol). *J Pharmacol Exp Ther* **280:**1319–1327.
Pasqualini R, Koivunen E and Ruoslahti E (1997) Alpha v integrins as receptors for tumour targeting by circulating ligands. *Nat Biotechnol* **15:**542–546.
Passirani C, Barratt G, Devissaguet JP and Labarre D (1998) Long-circulating nanoparticles bearing heparin or dextran covalently bound to poly(methyl methacrylate). *Pharm Res (N Y)* **15:**1046–1050.
Patel HM (1992) Serum opsonins and liposomes: their interaction and opsonophagocytosis. *Crit Rev Ther Drug Carrier Syst* **9:**39–90.
Patton WA, Granzow CA, Getts LA, Thomas SC, Zotter LM, Gunzel KA and Lowe-Krentz LJ (1995) Identification of a heparin-binding protein using monoclonal antibodies that block heparin to porcin aortic endothelial cells. *Biochem J* **311:**461–469.
Peracchia MT, Desmaële D, Couvreur P and d'Angelo J (1997a) Synthesis of a novel poly(MePEG cyanoacrylate-co-alkyl cyanoacrylate) amphiphilic copolymer for nanoparticle technology. *Macromolecules* **30:**846–851.
Peracchia MT, Fattal E, Desmaele D, Besnard M, Noel JP, Gomis JM, Appel M, d'Angelo J and Couvreur P (1999) Stealth PEGylated polycyanoacrylate nanoparticles for intravenous administration and splenic targeting. *J Control Release* **60:**121–128.
Peracchia MT, Vauthier C, Desmaële D, Gulik A, Dedieu JC, Demoy M, d'Angelo J and Couvreur P (1998) Pegylated nanoparticles from a novel methoxypolyethylene glycol cyanoacrylate-hexadecyl cyanoacrylate amphiphilic copolymer. *Pharm Res (N Y)* **15:**550–556.
Peracchia MT, Vauthier C, Passirani C, Couvreur P and Labarre D (1997b) Complement consumption by poly(ethylene glycol) in different conformations chemically coupled to poly(isobutyl 2-cyanoacrylate) nanoparticles. *Life Sci* **61:**749–761.
Peracchia MT, Vauthier C, Puisieux F and Couvreur P (1997c) Development of sterically stabilized poly(isobutyl 2-cyanoacrylate) nanoparticles by chemical coupling of poly(ethylene glycol). *J Biomed Mat Res* **34:**317–326.
Phillips NC, Gagne L, Tsoukas C and Dahman J (1994) Immunoliposome targeting to murine $CD4^+$ leucocytes is dependent on immune status. *J Immunol* **152:**3168–3174.
Phillips WT (1998) Use of radiolabeled liposomes for PEG-liposome-based drug targeting and diagnostic imaging applications, in *Targeting of Drugs 6: Strategies for Stealth Therapeutic Systems* (Gregoriadis G and McCormack B eds) pp109–120, Plenum Press, New York.
Pober JS and Cotran RS (1990) Cytokines and endothelial cell biology. *Physiol Rev* **70:**427–451.
Porter CJH, Moghimi SM, Davies MC, Illum L and Davis SS (1992a) Differences in the molecular weight profile of poloxamer 407 affect its ability to redirect intravenously administered colloids to the bone marrow. *Int J Pharm* **83:**273–276.
Porter CJH, Moghimi SM, Illum L and Davis SS (1992b) The polyoxyethylene/polyoxypropylene block co-polymer poloxamer-404 selectively redirects intravenously injected microspheres to sinusoidal endothelial cells of rabbit bone marrow. *FEBS Lett* **305:**62–66.
Poznansky M and Juliano RL (1984) Biological approaches to the controlled delivery of drugs: a critical review. *Pharmacol Rev* **36:**277–336.
Provoda CJ and Lee K-D (2000) Bacterial pore-forming hemolysins and their use in the cytosolic delivery of macromolecules. *Adv Drug Delivery Rev* **41:**209–221.
Pulapura S and Kohn J (1992) Trends in the development of bioresobable polymers for medical applications. *J Biomater Appls* **6:**216–250.
Rajotte D, Arap W, Hagedorn M, Koivunen E, Pasqualini R and Ruoslahti E (1998) Molecular heterogeneity of the vascular endothelium revealed by in vivo phage display. *J Clin Invest* **102:**430–437.
Ram S, Sharma AK, Simpson SD, Gulati S, McQuillen DP, Pangburn MK and Rice PA (1998) A novel sialic acid binding site on factor H mediates serum resistance of sialylated Neisseria gonorrhoeae. *J Exp Med* **187:**743–752.
Raso V, Ritz J, Basala M and Schlossman ST (1982) Monoclonal antibody-ricin A chain conjugate selectively cytotoxic for cells bearing the common acute lymphoblastic leukemia antigen. *Cancer Res* **42:**457–464.
Regelson W and Holland JF (1958) The anionic polyelectrolyte, polyethylene sulphonate, as a new anti-neoplastic agent. *Nature (Lond)* **181:**46–47.
Reitter JN, Means RE and Desrosiers RC (1998) A role for carbohydrates in immune evasion in AIDS. *Nat Med* **4:**679–684.
Rettig WJ, Garin-Chesa P, Healey JH, Su SL, Jaffe EA and Old LJ (1992) Identification of endosialin, a cell-surface glycoprotein of vascular endothelial-cells in human cancer. *Proc Natl Acad Sci USA* **89:**10832–10836.
Robinson AP, White TM and Mason DW (1986) Macrophage heterogeneity in the rat as delineated by two monoclonal antibodies MRC-OX41 and MRC OX-42, the latter recognizes complement receptor type 3. *Immunology* **57:**239–247.
Rodgers JB, Friday S and Bochenek WJ (1984) Absorption and excretion of the hydrophobic surfactant, C-14 poloxalene 2930, in the rat. *Drug Metab Dispos* **12:**631–634.
Roerdink FH, Regts J, Van Leeuwen B and Scherphof GL (1984) Intrahepatic uptake and processing of intravenously injected small unilamellar vesicles in rats. *Biochim Biophys Acta* **770:**195–202.
Romero EL, Morilla MJ, Regts J, Koning GA and Scherphof GL (1999) On the mechanism of hepatic transendothelial passage of large liposomes. *FEBS Lett* **448:**193–196.
Rosenecker J, Zhang W, Hong K, Lausier J, Geppetti P, Yoshihara S, Papahadjopoulos D and Nabel JA (1996) Increased liposome extravasation in selected tissues: effect of substance-P. *Proc Natl Acad Sci USA* **93:**7236–7241.
Rutherford MS, Witsell A and Schook LB (1993) Mechanisms generating functionally heterogeneous macrophages: chaos revisited. *J Leukoc Biol* **53:**602–618.
Sato TN, Tozawa y, Deutsch U, Wolburg Buchholz K, Fujiwara Y, Gendron Maguire M, Gridley T, Wolburg H, Risau W and Qin Y (1995) Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. *Nature (Lond)* **376:**70–74.
Schmidt EE, MacDonald IC and Groom AC (1983) Microcirculation in mouse spleen (nonsinusal) studied by means of corrosion casts. *J Morphol* **186:**17–29.
Schmidt-Weber CB, Rittig M, Buchner E, Hauser I, Schmidt I, Palombo-Kinne E, Emmrich F and Kinne RW (1996) Apoptotic cell death in activated monocytes following incorporation of clodronate-liposomes. *J Leukoc Biol* **60:**230–244.
Schnitzer B, Sodeman T, Mead MJ and Contacos PG (1972) Pitting function of the spleen in malaria: ultrastructural observations. *Science (Wash DC)* **177:**175–177.
Sellers RF, Herniman AJ and Hawkins CW (1972) The effect of a synthetic anionic polymer (pyran) on the development of foot-and-mouth disease in guinea-pigs, cattle and pigs. *Res Vet Sci* **13:**339–341.
Seltzer SE (1989) The role of liposomes in diagnostic imaging. *Radiology* **171:**19–21.
Senior J, Crawley JCW and Gregoriadis G (1985) Tissue distribution of liposomes exhibiting long half-lives in the circulation after intravenous injection. *Biochim Biophys Acta* **839:**1–8.
Senior J, Delgado C, Fisher D, Tilcock C and Gregoriadis G (1991) Influence of surface hydrophilicity of liposomes on their interaction with plasma protein and clearance from the circulation: studies with poly(ethylene glycol)-coated vesicles. *Biochim Biophys Acta* **1062:**77–82.
Senior J and Gregoriadis G (1982) Is half-life of circulating small unilamellar liposomes determined by changes in their permeability? *FEBS Lett* **145:**109–114.
Serra MV, Mannu F, Matera A, Turrini F and Arese P (1992) Enhanced IgG- and

complement-independent phagocytosis of sulfatide-enriched human erythrocytes by human monocytes. *FEBS Lett* **311:**67–70.
Shah SS, Cha Y and Pitt CG (1992) Poly(glycolic acid-co-DL-lactic acid): diffusion or degrdation controlled drug delivery? *J Control Release* **28:**261–270.
Shin IL, Kim SY, Lee YM, Cho CS and Sung YK (1998) Methoxy poly(ethylene glycol)/ε-caprolactone amphiphilic block copolymeric micelle containing indomethacin. I. Preparation and characterization. *J Control Release* **51:**1–11.
Shtilman MI, Torchilin VP, Tsatsakis AM, Mihailova EV, Rizos A, Yaroslavov AA and Shashkova IM (1999) Amphiphilic polymers of N-niylpyrrolidone as potential modificators for liposome membranes. *Proc Int Symp Control Rel Bioact Mater* **26:**921–922.
Sim E, Wood AB, Hsiung LM and Sim RB (1981) Pattern of degradation of human complement fragment, C3b. *FEBS Lett* **132:**55–60.
Si-Nang L, Carlier PF, Delort P, Gazzola J and Lafont D (1973) Determination of coating thickness of microcapsules and influence upon diffusion. *J Pharm Sci* **62:**452–454.
Sleyster EC and Knook DL (1982) Relation between localization and function of rat liver Kupffer cells. *Lab Invest* **47:**484–490.
Smith CM II, Hebbel RP, Tukey DP, Clawson CC, White JG and Vercellotti GM (1987) Pluronic F-68 reduces the endothelial adherence and improves the rheology of liganded sickle erythrocytes. *Blood* **69:**1631–1636.
Snook T (1950) A comparative study of vascular arrangements in mammalian spleen. *Am J Anat* **87:**31–37.
Souhami RL, Patel HM and Ryman BE (1981) The effect of reticuloendothelial blockade on the blood clearance and tissue distribution of liposomes. *Biochim Biophys Acta* **674:**354–371.
Spragg DD, Alford DR, Greferath R, Larsen CE, Lee KD, Gurtner GC, Cybulsky MI, Tosi PF, Nicolau and Gimbrone MA (1997) Immunotargeting of liposomes to activated vascular endothelial cells: a strategy for site-specific delivery in the cardiovascular system. *Proc Natl Acad Sci USA* **94:**8795–8800.
Stevens MP (1999) Polymer Chemistry 3rd Ed. Oxford University Press.
Stoiber H, Pinter C, Siccardi AG, Clivio A and Dierich MP (1996) Efficient destruction of human immunodeficiency virus in human serum by inhibiting the protective action of complement factor H and decay accelerating factor (DAF, CD55). *J Exp Med* **183:**307–310.
Stolnik S, Dunn SE, Garnett MC, Davies MC, Coombes AGA, Taylor DC, Irving MP, Purkiss SC, Tadros TF, Davis SS and Illum L (1994) Surface modification of poly(lactide-co-glycolide) nanospheres by biodegradable poly(lactide)-poly(ethyleneglycol) copolymers. *Pharm Res (N Y)* **11:**1800–1808.
Storm G, Belliot SO, Daemen T and Lasic DD (1995) Surface modification of nanoparticles to oppose uptake by the mononuclear phagocyte system. *Adv Drug Delivery Rev* **17:**31–48.
Sudimack J and Lee RJ (2000) Targeted drug delivery via the folate receptor. *Adv Drug Delivery Rev* **41:**147–162.
Surolia A and Bachhawat BK (1977) Monosialoganglioside liposome-entrapped enzyme uptake by hepatic cells. *Biochim Biophys Acta* **497:**760–765.
Szebeni J (1998) The interaction of liposomes with the complement system. *Crit Rev Ther Drug Carrier Syst* **15:**57–89.
Szebeni J, Baranyi L, Savay S, Lutz H, Jelezarova E, Bunger R and Alving CR (2000) The role of complement activation in hypersensitivity to Pegylated liposomal doxorubicin (Doxil). *J Liposome Res* **10:**467–481.
Szebeni J, Muggia FM and Alving CR (1998) Complement activation by Cremophor EL as a possible contributor to hypersensitivity to paclitaxel: an in vitro study. *J Natl Cancer Inst* **90:**300–306.
Szebeni J, Savay S, Alving CR, Hunter AC and Moghimi SM (2001) Variation of complement activation in human serum by poloxamer 188 (Pluronic-F68). *Proc Int Symp Control Rel Bioact Mater,* in press.
Takeuchi H, Toyoda T, Toyobuku H, Yamamoto H, Hino T and Kawashima Y (1996) Improved stability of doxorubicin-loaded liposomes by polymer coating. *Proc Int Symp Control Rel Bioact Mater* **23:**409–410.
Thorin E and Shreeve SM (1998) Heterogeneity of vascular endothelial cells in normal and diseased states. *Pharmacol Therap* **78:**155–166.
Thurmond KB II, Kowalewski T and Wooley KL (1996) Water-soluble knedel-like structures: the preparation of shell-cross-linked small particles. *J Am Chem Soc* **118:**7239–7240.
Tilcock C (1995) Imaging tools: liposomal agents for nuclear medicine, computed tomography, magnetic resonance, and ultrasound, in *Liposomes as Tools in Basic Research and Industry* (Philippot JD and Schubar F eds) pp 225–240, CRC Press, Boca Raton, FL.
Tokiwa Y and Suzuki T (1977) Hydrolysis of polyesters by lipases. *Nature (Lond)* **270:**76–78.
Tomita M, Yamamoto K, Kobashi H, Ohmoto M and Tsuji T (1994) Immunohistochemical phenotyping of liver macrophages in normal and diseased human liver. *Hepatology* **20:**317–325.
Torchilin VP, Frank-Kamenetsky MD and Wolff GL (1999) CT visualization of blood pool in rats using long-circulating, iodine-containing micelles. *Acad Radiol* **6:**61–65.
Torchilin VP, Klibanov AL, Huang L, O'Donnell S, Nossiff ND and Khaw BA (1992) Targeted accumulation of polyethylene gylcol-coated immunoliposomes in infarcted rabbit myocardium. *FASEB J* **6:**2716–2719.
Torchilin VP, Shtilman MI, Trubeyskoy VS, Whiteman K and Milstein AM (1994) Amphiphilic vinyl polymers effectively prolong liposome circulation time in vivo. *Biochim Biophys Acta* **1195:**181–184.
Torchilin VP, Trubetskoy VS, Whiteman KR, Caliceti P, Ferruti P and Veronese FM (1995) New synthetic amphiphilic polymers for steric protection of liposomes in vivo. *J Pharm Sci* **84:**1049–1053.
Toth K, Bogar L, Juricskay I, Keltai M, Yusuf S, Haywood LJ and Meiselman HJ (1997) The effect of RheothRx injection on the hemorheological parameters in patients with acute myocardial infarction. *Clin Hemorheol Microcirc* **17:**117–125.
Tracy MA, Ward KL, Firouzabadian L, Wang Y, Dong N, Qian R and Zhang Y (1999) Factors affecting the degradation rate of poly(lactide-co-glycolide) microspheres in vivo and in vitro. *Biomaterials* **20:**1057–1062.
Troster SD, Müller U and Kreuter J (1990) Modification of the body distribution of poly(methyl methacrylate) nanoparticles in rats by coating with surfactants. *Int J Pharm* **61:**85–100.
Trubetskoy VS, Frankkamenetsky MD, Whiteman KR, Wolf GL and Torchilin VP (1996) Stable polymeric micelles: lymphoangiographic contrast media for gamma scintigraphy and magnetic resonance imaging. *Acad Radiol* **3:**232–238.
Turner NC and Wright NA (1992) The response to injury, in *Oxford Textbook of Pathology* (McGee JOD, Isaacson PG and Wright NA eds) pp 351–390, Oxford University Press, Oxford, UK.
Uster PS, Allen TM, Daniel DB, Mendez CJ, Newman MS and Zhu GZ (1996) Insertion of poly(ethylene glycol) derivatized phospholipid into pre-formed liposomes results in prolonged in vivo circulation time. *FEBS Lett* **386:**243–246.
Vandorpe J, Schacht E, Dunn S, Hawley A, Stolnik S, Davis SS, Garnet MC, Davies MC and Illum L (1997) Long circulating biodegradable poly(phosphazene) nanoparticles surface modified with poly(phosphazene)-poly(ethyleneoxide) copolymer. *Biomaterials* **18:**1147–1152.
Vandorpe J, Schacht E, Stolnik S, Garnet MC, Davies MC, Illum L and Davis SS (1996) Poly(organo phosphazene) nanoparticles surface modified with poly(ethylene oxide). *Biotechnol Bioeng* **52:**89–95.
Vann WF, Schmidt MA, Jann B and Jann K (1981) The structure of the capsular polysaccharide (K5 antigen) of urinary-tract-infective Escherichia coli 010:K5:H4. A polymer similar to desulfo-heparin. *Eur J Biochem* **116:**359–364.
van Rooijen N, Bakker J and Sanders A (1997) Transient suppression of macrophage functions by liposome-encapsulated drugs. *Trends Biotechnol* **15:**178–185.
Vermehren C, Kiebler T, Hylander I, Callisen TH and Jørgensen K (1998) Increase in phospholipase A_2 activity towards lipopolymer-containing liposomes. *Biochim Biophys Acta* **1373:**27–36.
Volanakis JE and Wirtz Kwa (1979) Interaction of C-reactive protein with artificial phosphatidylcholine bilayers. *Nature (Lond)* **281:**155–157.
Wang N and Wu XS (1997) Synthesis, characterization, biodegradation and drug delivery application of biodegradable lactic/glycolic acid oligomers. Part II. Biodegradation and drug delivery application. *J Biomater Sci Polym Ed* **9:**75–87.
Wang ZJ and Stern IJ (1975) Disposition in rats of a polyoxypropylene-polyoxyethylene copolymer used in plasma fractionation. *Drug Metab Dipos* **3:**536–542.
Wassef NM, Matyas GR and Alving CR (1991) Complement-dependent phagocytosis of liposomes by macrophages. Suppresive effects of stealth lipids. *Biochem Biophys Res Commun* **176:**866–874.
Watrous-Peltier N, Uhl J, Steel V, Borphy L and Merisko-Liversidge E (1992) Direct suppression of phagocytosis by amphipathic polymeric surfactants. *Pharm Res (N Y)* **9:**1177–1183.
Webb MS, Saxon D, Wong FMP, Lim HJ, Wang Z, Bally MB, Choi LSL, Cullis PR and Mayer LD (1998) Comparison of different hydrophobic anchors conjugated to poly(ethylene glycol): effects on the pharmacokinetics of liposomal vincristine. *Biochim Biophys Acta* **1372:**272–282.
Webb MS, Wheeler IJ, Bally MB and Mayer LD (1995) The cationic lipid stearylamine reduces the permeability of the cationic drugs verpamil and prochlorperazine to lipid bilayers: implications for drug delivery. *Biochim Biophys Acta* **1238:**147–155.
Weiss L (1991) Barrier cells. *Immunol Today* **12:**24–29.
Weiss L, Powell R and Schiffman FJ (1986) Mechanisms of splenic control of murine malaria: reticular cell activation and the development of a blood-spleen barrier. *Am J Anat* **176:**251–285.
Weiss L and Tavassoli M (1970) Anatomical hazards to the passage of erythrocytes through the spleen. *Semin Hematol* **7:**372–380.
Whiteman KR, Subr V, Ulbrich K and Torchilin VP (1999) Attachment of HPMA derivatives to the liposome surface makes them long-circulating. *Proc Int Symp Control Rel Bioact Mater* **26:**1064–1065.
Wickham TJ, Haskard D, Segal D and Kovesdi I (1997) Targeting endothelium for gene therapy via receptors up-regulated during angiogenesis and inflammation. *Cancer Immunol Immunother* **45:**149–151.
Wiessleder R, Heautot JF, Schaffer BK, Nossiff N, Papisov MI, Bogdanov AA and Brady TJ (1994) MR lymphography: study of a high lymphotropic agent. *Radiology* **191:**225–230.
Wileman TE (1991) Properties of asparaginase-dextran conjugates. *Adv Drug Delivery Rev* **6:**167–180.
Willcox ML, Newman MM and Paton BC (1978) A study of labeled Pluronic F-68 after intravenous injection into the dog. *J Surg Res* **25:**349–356.
Williams DF and Mort E (1977) Enzyme-accelerated hydrolysis of polyglycolic acid. *J Bioeng* **1:**231–238.
Wolff G, Worgall S, van Rooijen N and Crystal RG (1997) Enhancement of in vivo adenovirus-mediated gene transfer and expression by prior depletion of tissue macrophages in the target organ. *J Virol* **71:**624–629.
Woodle MC (1998) Controlling liposome blood clearance by surface-grafted polymers. *Adv Drug Delivery Rev* **32:**139–152.
Woodle MC, Engbers CM and Zalipsky S (1994) New amphipathic polymer-lipid conjugates forming long-circulating reticuloendothelial system-evading liposomes. *Bioconjugate Chem* **5:**493–496.
Woodle MC and Lasic DD (1992) Sterically stabilized liposomes. *Biochim Biophys Acta* **1113:**171–199.
Woodle MC, Newman MS and Martin FJ (1992) Liposome leakage and blood circulation: comparison of adsorbed copolymers with covalent attachment of PEG. *Int J Pharm* **88:**327–334.
Yamauchi H, Kikuchi K, Yachi K, Sawada M, Tomikawa M and Hirota S (1993) Effects of glycophorin and ganglioside GM_3 on the blood circulation and tissue distribution of liposomes in rats. *Int J Pharm* **90:**73–79.
Yamauchi H, Yano T, Kato T, Tanaka I, Nakabayashi S, Higashi K, Miyoshi S and Yamada H (1995) Effects of sialic acid derivative on long circulation time and tumor concentration of liposomes. *Int J Pharm* **113:**141–148.

Yokoyama M (1992) Block copolymers as drug carriers. *Crit Rev Ther Drug Carrier Syst* **9:**213–248.
Yokoyama M, Miyauchi M, Yamada N, Okano T, Sakurai Y, Kataoka K and Inoue S (1990) Characterization and anticancer activity of the micelle-forming polymeric anticancer drug adryamicin-conjugated poly(ethylene glycol)-poly(aspartic acid) block copolymer. *Cancer Res* **50:**1700–1703.
Yokoyama M, Okano T, Sakurai Y, Ekimoto H, Shibazaki C and Kataoka K (1991) Toxicity and antitumor activity against solid tumors of micelle-forming polymeric anticancer drug and its extremely long circulation in blood. *Cancer Res* **51:**3229–3236.
Yuan F, Lwunig M, Huang SK, Berk DA, Papahadjopoulos D and Jain RK (1994) Microvascular permeability and interstitial penetration of sterically stabilized (stealth) liposomes in a human tumour xenograft. *Cancer Res* **54:**3352–3356.
Zalipsky S (1995) Functionalized poly(ethylene glycol)s for preparation of biologically relevant conjugates. *Bioconjugate Chem* **6:**150–165.
Zalipsky S, Hansen CB, Oaks JM and Allen TM (1996) Evaluation of blood clearance rates and biodistribution of poly(2-oxazoline)-grafted liposomes. *J Pharm Sci* **85:**133–137.
Zalipsky S, Mullah N, Harding JA, Gittelman J, Guo L and DeFrees SA (1997) Poly(ethylene glycol)-grafted liposomes with oligosaccharide or oligopeptide ligands appended to the termini of the polymer chains. *Bioconjugate Chem* **8:**111–118.
Zalipsky S, Puntambekar B, Bolikas P, Engbers CM and Woodle MC (1995) Peptide attachment to extremities of liposomal surface grafted PEG chains: preparation of the long-circulating form of laminin pentapeptide, YIGSR. *Bioconjugate Chem* **6:**705–708.
Zalipsky S, Qazen M, Walker JA, Mullah N, Quinn YP and Huang SK (1999) New detachable poly(ethylene glycol) conjugates: cysteine-cleavable lipopolymers regenerating natural phospholipid, diacylphosphatidylethanolamine. *Bioconjugate Chem* **10:**703–707.
Zhang X, Burt HM, Mangold G, Dexter D, Von Hoff D, Mayer L and Hunter WL (1997a) Anti-tumor efficacy and biodistribution of intravenous polymeric micellar paclitaxal. *Anti-Cancer Drugs* **8:**686–701.
Zhang X, Burt HM, Von Hoff D, Dexter D, Mangold G, Degen D, Oktaba AM and Hunter WL (1997b) An investigation of the antitumour activity and biodistribution of polymeric micellar paclitaxel. *Cancer Chemother Pharmacol* **40:**81–86.
Ziff RM and McGrady ED (1985) Kinetics of polymer degradation. *Macromolecules* **19:**2513–2516.