ELSEVIER

Journal of Chromatography B, 715 (1998) 65–80

JOURNAL OF CHROMATOGRAPHY B

Review

Application of bioaffinity technology in therapeutic extracorporeal plasmapheresis and large-scale fractionation of human plasma

T. Burnouf[a,*], H. Goubran[b], M. Radosevich[a]

[a]*Haemonetics Plasma Product Services (HPPS), 88 rue Nationale, 59800 Lille, France*
[b]*Faculty of Medicine, Cairo University, Cairo, Egypt*

Abstract

This paper describes the increasingly unique and powerful role that affinity chromatography is occupying both as a tool for the treatment of extracorporeal plasma exchange (to discard biological compounds with noxious metabolic or immunologic effects in patients) and as a purification tool in the production of therapeutic plasma protein derivatives. Management of both applications requires careful monitoring of the parameters applied to the plasma material, to avoid immunological stimulation or activation of the coagulation cascade. Examples of direct current applications of affinity ligands in therapeutic removal and industrial production of plasma compounds are presented.

Keywords: Reviews; Plasmapheresis; Plasma fractionation; Proteins

Contents

*Corresponding author.

0378-4347/98/$19.00
PII: S0378-4347(98)00052-8

1. Introduction

The on-going refinement in the chemistry of adsorption materials and, in particular, the ability to prepare binding media with high selectivity and limited toxicity, has opened up the potential applications of bioaffinity technology to plasma-processing fields. Applications include direct in vivo therapeutic extracorporeal plasmapheresis, to purify plasma from patients and remove compounds that cause disease or a particular symptom [1]. Another area is the large-scale production of therapeutic protein drugs through the fractionation of human (or animal) plasma [2,3]. Clearly, the availability of refined bioaffinity media has played a major role in this biopharmaceutical field and has added new tools to the separation portfolio of transfusion technologists and plasma fractionators.

The requirements behind the development of affinity binding material for extracorporeal adsorption and plasma fractionation have led to similar development strategies. In both cases, the selected media (chromatographic support or membrane) must have a high capacity and selectivity for the targeted compounds, which is generally a protein, and it must be biocompatible. The packing material must not activate the coagulation cascade or the complement system and it must not contribute to the generation of a proteolytic activity of enzymes of the inflammatory and hemostatic systems; this is essential to avoid side-effects from the adsorbed plasma being returned to the patient as well as from the infusion of the purified protein drugs. Similarly, in the case of plasma fractionation, washing and elution buffer steps should not induce changes in the protein's conformation, in order to avoid the possible formation of neo-antigens. In addition, the adsorption system should demonstrate a high stability, to avoid uncontrollable leaching of the ligand or toxic degradation of the support, a phenomenon which can be especially critical when performing plasmapheresis in patients with an unbalanced hemostatic system. Finally, for both types of applications, the affinity material should resist standard, or sometimes harsh, cleaning and sanitization procedures (e.g. to allow potential reuse in the same patient during plasmapheresis and re-use of the chromatographic material during successive purification cycles at the industrial scale). Hence, although the principles of bioaffinity are very attractive and simple, development of these principles in these two areas has had to face several issues dealing with ligand specificity, toxicity, leakage and/or cost. Designing a bioaffinity extraction strategy that maximizes purity, yield and economy is important.

In principle, the ligands used can be selected from small molecules such as amino acids, sugars, nucleotides, or from macromolecules such as proteins, polysaccharides or nucleic acids. In practice, however, insufficient knowledge of the complex structure of plasma proteins and their fragile physiological properties has made it difficult to find and/or design ideal ligands that are specific for individual plasma proteins, such as coagulation factors or protease inhibitors, or specific plasma contaminants, such as cholesterol or fatty acids. In addition, a number of potentially attractive protein ligands are not readily available or even recognised. Therefore, many of the ligands used have relatively general binding properties; this is the case for protein A, heparin, gelatin, chelated metals, lectins and dyes. More specific ligands include monoclonal antibodies and competitive enzyme inhibitors. The mechanisms pertaining to the interactions between protein and ligand sites may involve single or combined phenomenon, e.g. ionic, hydrophobic, hydrogen bonding or van der Waals interactions.

This paper reviews the clinical application of

bioaffinity technology in the extracorporeal adsorption of patients' plasma during therapeutic plasmapheresis procedures and as a purification tool that is suitable for the large-scale fractionation of human plasma.

2. Plasma: a delicate biological equilibrium to be preserved

Plasma is an amazingly complex biological fluid where a delicate balance is maintained between pro-enzymes and protease inhibitors of the hemostatic, fibrinolytic and complement systems, under normal physiological conditions [4]. However, pro-enzymes are prone to powerful cascade activation mechanisms when the physiological equilibrium is altered, as may occur, in vivo, under certain pathological circumstances or during the complex cascade reactions that lead to blood coagulation or, in vitro, when plasma is in contact with activating surfaces, such as silica [5]. Indeed, silica, and any surface with negative reactive groups, has the potential to activate the proteins of the blood contact phase system, such as prekallikrein, high-molecular-mass kininogen and factor XII (FXII). These may, in turn, activate enzymes of the coagulation system, potentially leading to the formation of thrombin and the activation of fibrinogen into fibrin. Therefore, a short summary of the complex systems involved is important to gain an understanding of the requirements of any bioaffinity adsorption system that is in contact with whole plasma or plasma fractions. The risk of imbalance of the hemostatic system is particularly high in complex plasma fractions, where all biological compounds of the hemostatic system are present.

2.1. The coagulation system

Following vascular injury, coagulation may be activated through the extrinsic pathway. Tissue factor is first involved in the activation of factor VII (FII), leading to FIX and FX activations, or it may activate FX directly [6]. Another route of activation is the intrinsic pathway, which requires exposure of a surface (vascular endothelium or a foreign surface) and the components of the contact system (prekallikrein, high-molecular-mass kininogen and FXII) [7]. The corresponding reactions lead to the consecutive activation of FXI and, then, FIX and FX consecutively. Here, the activation of FX is mediated by FVIIIa. The common pathway is initiated once sufficient FXa has been generated to convert prothrombin into thrombin in the presence of FVa, calcium and phospholipids. The thrombin then activates fibrinogen, which is polymerized into fibrin in the presence of FXIIIa.

Endotoxins, generated during a bacterial infection (or present in poorly sanitized chromatographic or adsorption material), may also stimulate monocytes to produce tissue factor and induce blood coagulation [8]. Cardiologists are well aware of the fact that artificial surfaces, such as the valves or grafts that are used in surgery, may stimulate thrombosis [9]. These materials are usually negatively charged and, on contact, the components of the contact system may become activated. FXI is particularly sensitive to activation by surface-bound FXIIa. This risk must then be particularly well controlled during therapeutic plasmapheresis. Similarly, due to a poorly sanitized purification process, the presence of endotoxins in therapeutic concentrates could create serious side-effects in treated patients. Additionally, materials used in the production of blood/plasma derivatives (e.g. chromatographic supports, filters, containers, etc.) may induce activation of coagulation factors if their surfaces are not properly coated to mask hidden negatively charged groups. In addition to activation, which involves proteolytic degradation, proteins may also adsorb to different surfaces during purification (particularly when they are highly purified) and, thus, affect the overall yield of a production process or modify a given protease/anti-protease balance.

2.2. The fibrinolytic system

Once coagulation has been achieved, fibrin deposits are cleared from the circulation by the fibrinolytic system. In-vivo fibrinolysis is initiated by endothelial plasminogen activator or by activated Hageman factor (FXII), which, in both cases, through a series of reactions, will convert plasminogen into plasmin through a series of reactions [10]. This protease is capable of degrading fibrinogen, fibrin and coagulation factors (FV, FVIII, FIX, FXI), among other plasma proteins. Thus, if the

delicate balance between the procoagulant and the fibrinolytic systems is disrupted, hemorrhage or thrombosis may develop in the patient.

2.3. The complement system

The complement system may play an important role in many thrombohaemorrhagic disorders since its activation causes vascular permeability, leading to hypotension and shock, as is commonly observed in disseminated intravascular coagulation [11]. Hageman factor, as in the case of the contact and fibrinolytic systems, may be responsible for complement activation inducing osmotic lysis of red cells and platelets. These lysed cells release procoagulant factors that will accelerate the coagulation process. Plasmin may also directly activate the complement components C1 or C3 through two independent pathways. The plasmin-mediated activation of the complement system may cause serious clinical complications.

2.4. Inhibitor systems

The intrinsic coagulation pathway is mainly inhibited by antithrombin III (AT III), protein C (Pc) and protein S, while the extrinsic pathway is regulated by the tissue factor pathway inhibitor. C1 inhibitor is known to inhibit activated FXI as well as the C1 complement component. The fibrinolytic system is inhibited by either plasma antiproteases (α-2-antiplasmin and α-2-macroglobulin) or cellular inhibitors (plasminogen activator inhibitors types 1 and 2). Generally, plasma inhibitors are less susceptible to negatively charged surfaces but they are sensitive to oxidation [12,13], which may occur, under certain conditions, during a production process.

3. Bioaffinity in therapeutic extracorporeal plasma adsorption

In the past, apheresis devices were solely used for blood component collection. Within the last 30 years, therapeutic plasmapheresis procedures have been employed to remove plasma with its protein-bound solutes, such as circulating protein-bound toxins, autoantibodies, immune complexes or other offending molecules, for the treatment of a large number of diseases, namely collagen vascular disorders, neurological diseases, familial hyperlipidaemias or for the purpose of removing circulating toxins [1].

The concept of selectively binding and discarding a large variety of macromolecules that may have a noxious metabolic or immunological effect, as the patient's plasma passes through an apheresis system, was extremely appealing and eliminated the need for infusion of foreign plasma that was potentially incompatible and the concomitant risk of viral transmission. The fragile equilibrium of plasma's biochemical complexity implies, however, very careful management of any system where plasma is returned to the patient, in order to avoid immunological stimulation or activation of the coagulation cascade.

On-line membrane plasma filtration–fractionation techniques have been developed and applied to plasma therapy and include cascade double filtration, initially used for the selective removal of low density lipoproteins (LDLs) from the plasma of patients with familial hypercholesterolaemia, cryofiltration and thermofiltration [14–16]. On-line filtration was rapidly surpassed and replaced by on-line sorption plasma fractionation, which offered another alternative to the selective removal of pathological molecules using bioaffinity concepts [16,17]. This technique entails an initial separation of plasma from cellular components using an apheresis machine, followed by perfusion through a sorbent column. Table 1 illustrates the various affinity sorbents and their sorbing potentials [1].

Nonspecific adsorption on activated charcoal was introduced a few decades ago to offer patients with advanced hepatic encephalopathy the chance of recovery, by removing the offending toxins and conjugated bilirubin as the plasma flowed through an apheresis system [18].

Columns containing polyanion-exchange resins (Plasorba BR 350 & 500 series, from Asahi Medical, Tokyo, Japan) were later introduced to adsorb bilirubin and other anionic protein-bound substances and these offered a more selective approach to hepatic patients [19–21].

A substantial subgroup of patients with familial hypercholesterolaemia respond inadequately to stan-

Table 1
Ligand or adsorption material used in therapeutic extracorporeal plasmapheresis (modified from ref. [1])

Ligand or material of adsorption	Biological entity adsorbed
Polylysine methylated albumin	T4 phage DNA
Anion exchange/semi-affinity resin	
• Polyanion	• Bilirubin
• Dextran sulfate	• LDL-anti-dsDNA
Heparin/heparin–agarose	LDL
Tryptophan IM-TR	Anti-acetylcholine receptor antibodies IC RF IgG anti-GQ1b ganglioside antibody
Phenylalanine IM-PH	Anti-MBP antibodies, IC, RF
Modified PVA gel	RF, IC, anti-DNA, anti-RNP anti-SM antibodies
Oligosaccharide	Anti-blood type antibodies
Charcoal absorbent	Bilirubin, creatinine urea and potassium
DNA	Anti-DNA antibodies
Monoclonal or polyclonal antibodies to given antigens:	
• insulin	• anti-insulin antibody
• FVIII	• anti-FVIII antibodies
• FIX	• anti-FIX antibodies
• anti-LDL antibodies	• LDL
• anti-α-fetoprotein antibodies	• α-fetoprotein
• anti-IgE antibodies	• IgE
C1q	IC
Protein A	IC, IgG 1,2,43 and, C1
α-NCI Type IV collagen	Anti-α NCI; type IV collagen antibodies

PVA=polyvinyl alcohol; RF=rheumatoid factor; IC=immune complex; RNP=ribonuclear protein; SM=smooth muscles; C1q=subfraction q of the complement component 1.

dard diet and drug therapy and are, therefore, at high risk for premature development and progression of coronary disease. Initially, apheresis with double filtration was attempted with some clinical success. Dextran sulfate cellulose (DSC) (Liposorber La-40, Kanegafuchi Chemical Industry, Osaka, Japan), used for the adsorption of LDL cholesterol offered an excellent therapeutic option for these patients, who were doomed to the complications of advanced atherosclerosis. Homma et al. [22] compared double filtration to DSC column apheresis and documented that DSC columns remove atherogenic lipoproteins more effectively than double filtration plasmapheresis.

The DSC columns are formed from porous cellulose beads, which are covalently linked to DS (M_r 5000). In contrast to polyanions with carboxyl groups showing low LDL affinity, the charge density and molecular mass of DSC are suitable for LDL. The DSC–LDL affinity rapidly increases as the charge density is increased and then it reaches a constant value. There is little affinity with columns containing either DS monomer or DS with a molecular mass that is higher than 10 000 Da, whereas DS with a molecular mass in the mid-range showed strong LDL affinity.

Gordon et al. [23] evaluated LDL and the removal of lipoprotein (a) [Lp(a)] using a Liposorber LA-15 system. The controlled trial comprised patients with familial hypercholesterolaemia who had not respond-

ed adequately to diet and maximal drug therapy. Mean acute reduction in LDL–cholesterol was 76% in heterozygous patients and 81% in homozygous ones, with a substantial unprecedented reduction in Lp(a) by 65% in heterozygous and by 68% in homozygous patients. LDL–bioavid Liposorber LA-15 represents an important therapeutic option in hypercholesterolaemic patients who are unresponsive to diet or drug therapy [23].

LDL adsorption columns packed with an LDL adsorbent and a polysulfonate hollow-fiber plasma separator (Sulflux) were developed as an efficient LDL apheresis system [24]. DS-based systems, however, seem to bind some coagulation factors, such as FVIII, von Willebrand factor (vWF; the physiological protein carrier of FVIII), FIX and FXI as avidly as LDL and LP(a). The reduction in fibrinogen is also pronounced, ranging from 20 to 30%, but may also contribute to the therapeutic advantages of the DS system, which represents a model of the bioaffinity apheresis system [25]. An apheresis system based on heparin precipitation of LDL at low pH (HELP) [HELP Plasmat-secura system, Braun Melsungen, Melsugen, Germany] has been developed. It seems to be well tolerated, with low side-effects [26].

Suzuki et al. [27] studied the kinetics of anti-DNA antibody following selective removal by adsorption using DSC columns in fourteen patients with systemic lupus erythematosus. They compared the one- and two-compartment model of DSC columns and demonstrated, by mathematical analysis, that the one-compartment model is suitable. In addition, the generation- and catabolic rates of anti-dsDNA could be obtained using this model [27].

Immunoadsorbent columns were developed initially to remove the rheumatoid factor for patients with rheumatoid arthritis or the circulating antibodies (hence, the terminology of ‘‘immunoadsorbent’’), such as antiacetylcholine antibodies, of myasthenic patients. Immunoadsorption was also implicated in infective polyneuropathy and demyelinating neuropathies, with some efficacy in antibody clearance [28,29]. Immunoadsorption columns are based on a matrix of a tryptophan- or phenylalanine-immobilized polyvinyl alcohol gel (IM-TR or IM-PH). IM-TR gel adsorbs a larger amount of the IgG than the IM-PH gel and seems to be more suitable for patients with Fisher’s syndrome, a pathologic situation characterized by polybeuroradiculitis with ophthalmoplegia, ataxia and areflexia, due to anti-GQ1b ganglioside antibodies [30]. IM-PH has been used successfully with patients with rheumatoid arthritis, systemic lupus erythematosus (SLE) and multiple sclerosis [31].

Immunoadsorption using monoclonal or polyclonal antibodies has also been tested for LDL, SLE and a large group of disorders; it has also been used to determine the reduction in inhibitors against coagulation factor inhibitors [32] and whenever the offending molecules could be fully characterized.

Anti-alpha 3 (IV) collagen auto-antibodies have been implicated in the pathogenesis of Goodpasture syndrome, an autoimmune disorder that causes glomerulonephritis and pulmonary haemorrhages. Affinity chromatographic columns containing the alpha 3 NCI domain of bovine type IV collagen or the recombinant alpha 3 NCI domain of human type IV collagen, immobilized to agarose beads, are capable of binding the antibodies and may offer a simple therapeutic alternative to patients [33].

Protein A produced by *Staphylococcus aureus* has a very high affinity for IgG subclasses 1, 2 and 4 in their free form or even after the formation of immune complexes. Apheresis with protein A columns (Prosorba–DuPont) has beneficial effects in the treatment of idiopathic thrombocytopenic purpura and a larger variety of clinical disorders, including anti-coagulation FVIII and FIX [32] and von Willebrand factor [34] antibodies, and anti-HLA antibodies [35]. By virtue of its IgG binding properties, the total immunoglobulin levels of the processed plasma are significantly altered [36]. Continuous immunotherapy columns are based on two parallel recyclable immunoadsorbent protein A columns with selective IgG affinity that allow the recirculation of plasma in the column until saturation occurs [1]. Staphylococcal protein A is immunogenic and any leakage from the column is capable of inducing immunological stimulation, together with hypertension. In practice, however, the leakage seems to be negligible. Protein A-based columns have been approved by the Food and Drug Administration (FDA) for use in patients with immune idiopathic thrombocytopenic purpura [ITP] [37,38]. Published clinical data support their use in a multitude of other clinical

situations, such as Kaposi's sarcoma [39] and chemotherapy-induced haemolytic uraemic syndrome–thrombotic thrombocytopenic purpura [HUS–TTP] [40] and for grade 3–4 bone marrow transplant associated thrombotic microangiopathy [41].

4. Bioaffinity in plasma fractionation

4.1. General

Industrial plasma fractionation processes were developed in the 1940s [42]. The technology used, based on successive ethanol precipitation steps, is still the way the vast majority of industrial plasma fractionator conduct the fractionation process on human plasma to produce albumin and immunoglobulins, the two major human plasma products [43,44]. As both products obtained by ethanol fractionation (a) have been licensed for many years by regulatory agencies, (b) are obtained with satisfactory yield and (c) overall have satisfactory safety and efficacy records, there has not been pressure from the medical and regulatory community on plasma fractionators to develop significantly different purification strategies. Therefore, the increased use of chromatographic techniques over recent years by plasma fractionators has mostly been for the extraction of new or improved therapeutic plasma products, particularly of coagulation factors, anticoagulants and protease inhibitors [2,3,45,46]. The medical and economic rationale behind these essential trends is summarized in Table 2 [45].

One requirement of plasma fractionation, in comparison to other biopharmaceutical areas where only one protein is to be extracted from the starting mixture, is (due to the cost of human plasma and its scarcity) to design fractionation schemes that allow the simultaneous co-extraction of between four and fifteen therapeutic proteins from the starting plasma [2,3,44]. This implies that any purification process developed for new products should not have a negative impact on the licensed production methods of established products, especially albumin and IgG [45]. Therefore, the human plasma fractionation process must use complex, multi-step procedures to extract individual therapeutic proteins in a compatible fashion. This has led plasma fractionators to implement chromatographic purification procedures from side-fractions or precipitates (''pastes'') that are generated as by-products of the current production process for albumin and IgG. Examples of side-fractions include the cryoprecipitate (a cold-insoluble plasma fraction obtained by thawing plasma at 2–4°C, which is used as a starting material for the purification of FVIII, vWF and fibrinogen) and fraction IV1 (a fraction generated during albumin fractionation following precipitation of IgG). Further details on the traditional ethanol plasma fractionation process and plasma fraction nomenclature can be found in reviews [47,48].

Table 2
Rationale for the implementation of chromatographic techniques in plasma fractionation

- Medical reasons: Need for products with improved purity and safety through elimination of harmful contaminants, including:
  - ◇ Thrombogenic components in FIX concentrates
  - ◇ Anti-A, anti-B blood group hemolytic antibodies in FVIII and vWF concentrates
  - ◇ Prekallikrein activator in albumin preparations
  - ◇ Vasoactive substances in IgG preparations
  - ◇ Endotoxins in albumin; IgG; FVIII concentrates
  - ◇ Viruses (validated on a case-by-case basis) in all plasma products
- Biochemical reasons: Need for mild purification conditions to ensure:
  - ◇ The use of relatively non-denaturing conditions
  - ◇ The maintenance of protein biological activity
  - ◇ A low risk of neoantigen formation
- Economical reasons: Need to implement more efficient fractionation process:
  - ◇ More products can be made from plasma
  - ◇ The yield can be improved
- Technical reasons: Better control of critical process steps
  - ◇ Relatively easy scaling up
  - ◇ Standardisation and recording of the separation process

4.2. Most currently used ligands

Although the first use of chromatography in plasma product purification was initiated in the 1960s, it was only in the mid/late 1980s that applications of column chromatography were developed [2,3]. The first attempt to implement chromatography in plasma fractionation was based on ion-exchange chromatography, a principle that is still

widely used nowadays [45]. With the development of improved affinity media, its application in plasma fractionation has increased, allowing the purification of new plasma protein drugs and improving product purity and safety. Because of limitations due to the instability of plasma proteins under extreme conditions of pH, ionic strength or pressure, and their sensitivity to most organic solvents, affinity chromatography has become a useful tool in the production of several plasma proteins. It was introduced because the need to make increasingly pure and safer plasma protein products available has required more refined chromatographic techniques and the combination of several purification principles. Nevertheless, lack of knowledge of plasma protein structure has made it difficult to find and/or design ideal, non-toxic, ligands that specifically recognize target plasma proteins, such as coagulation factors or protease inhibitors.

As human plasma proteins are often used in therapeutics to ensure long term substitutive treatment of congenital or acquired deficiency states (e.g. coagulation factor deficiency, as seen in hemophilia), this implies that patients will be exposed to plasma protein products for a long period of time, typically during their whole life, to restore physiologically active circulating levels of the lacking factor. Alternatively, in the treatment of acute blood loss or following injuries, patients may receive huge amounts (typically several liters) of albumin over a period of only a few days, to ensure fluid replacement. Exposure of patients to large quantities of plasma protein products requires that issues dealing with the absence of leachables, stability and/or the absence of short-term and long-term toxicity (particularly of immunological potential) of the ligands used during protein purification are extremely well controlled and validated.

Textile dyes have turned out to be useful pseudo-specific ligands in biochemical research and biotechnological processes [49]. For example, synthetic textile dyes, in particular those with a chlorotriazine group, have been found to be useful for the purification of blood proteins at a research level. A good example is Cibacron Blue 3GA, a blue anthraquinone dye, containing a monochlorotriazine reactive group. The trypsin-like family of enzymes forms one of the largest groups of enzymes requiring cationic substrates and includes enzymes that are involved in blood clotting (kallikrein, thrombin, FXa). These enzymes bind the side chains of lysine or arginine in a binding pocket that is proximal to the reactive serine (Ser-195) [50]. Development of dye affinity chromatography has been hampered because those ligands that were available initially were obtained under conditions which met the requirements of the textile industry but not those of the biopharmaceutical industry; they were heterogeneous and, due to their low purity, contained a high proportion of contaminants [51]. Binding of plasma proteins seems to be governed mainly by nonspecific mixed ionic/hydrophobic interactions, which explains why many plasma proteins are bound to immobilized Cibacron Blue or Procion Red (for example) at low ionic strength and neutral pH. However, dyes do not behave as simple ion exchangers [52]. Immobilized metal-affinity chromatography (IMAC) purifies biological entities by exploiting the affinities of surface functional groups for metal ions, like Ni^{2+}, Zn^{2+}, Cu^{2+} and Fe^{3+}. IMAC has now become a helpful and more widely used tool in preparative protein separation, but it is only recently that the first therapeutic plasma protein drug, purified by a process involving an IMAC processing step, was licensed [53]. Specific monoclonal antibodies designed for large-scale immunopurification processes of coagulation factors or anticoagulants have been developed. Table 3 summarizes the various ligands used or described in plasma product manufacture. Experience shows that immobilized heparin and monoclonal antibodies are currently the most used ligands in industrial plasma fractionation.

4.3. Examples of industrial application

Affinity chromatography may be used in plasma fractionation, either upstream, to capture a protein from a complex plasma fraction, or downstream, as a polishing step. When used upstream on a complex plasma fraction that serves for the preparation of several plasma products, a major concern is the absence of any negative influences of the ligand or the support on the specifications of all finished products derived from this common purification step. So far, the more commonly used approach has been to focus the application of bioaffinity chromatog-

Table 3
Examples of immobilized ligands used in the affinity or semi-affinity purification of therapeutic plasma derivatives (references are indicated in brackets)

Protein	Ligand						
	Heparin	Gelatin	Monoclonal Ab	Thiol	Cu (IMAC)	Dextran sulfate	Cibacron Blue
FVIII	+ [46]		+ [64,65]			+ [63]	
FIX	+ [69,71,72]		+ [73]		+ [53]	+ [68]	
vWF		+ [75]					
Fibronectin	+ [79]	+ [76–78]					
FXI	+ [80]						
ATIII	+ [80–82]						
AAT				+[a] [88]			
Protein C	+ [90]		+ [91,92]				
Albumin							+[a] [58,59]

[a]Pilot-scale only; no current therapeutic product.

raphy to the downstream purification part of plasma protein drug manufacture, often as a polishing step to improve product purity. Examples of industrial application are presented below.

4.3.1. Albumin

Essentially all current therapeutic albumin preparations are still prepared by fractionation of plasma by ethanol precipitation [42,43]. This process consists of several cryoprecipitate–supernatant incubation steps with 8 to 40% cold ethanol, carried out under defined conditions of pH and osmolarity [47,48]. The precipitates generated during this process are separated by centrifugation or filtration. Recovery of albumin from plasma can reach 75–85% and a purity of up to 96–98%. Some processes combine ethanol fractionation with ion-exchange chromatography, which generally improves product purity to 99%, sometimes at the expense of the yield [48,54].

Alternative approaches based on affinity chromatography have been described. Ligands evaluated include immobilized bilirubin, fatty acids and dyes. Immobilized bilirubin [55] showed poor specificity, low capacity and decomposed in light. Chromatography on immobilized alkyl derivatives of succinic acid yielded albumin [56] with a relatively low recovery of 60% upon elution with octanoate. Both ligands have been found to be inappropriate for industrial applications. Triazine dye affinity chromatography using immobilized Cibacron Blue F3GA has been used on a pilot-scale to purify albumin that was obtained by a complete chromatographic procedure starting from cryo-poor plasma [57]. The yield of albumin was over 80% and the purity was more than 99%. However, dye leakage needed to be properly controlled. Cibacron Blue F3GA–Sepharose (or Procion Blue Sepharose CL6B) has also been described for the recovery of albumin entrapped in a Cohn fraction IV recovered by centrifugation [58,59]. These processes have been scaled up to a 40-l adsorbent column that could bind 1.1 kg of albumin. Elution was achieved with 20 m*M* sodium octanoate or 3 *M* NaCl. Octanoate elution yields an albumin fraction with higher purity (about 98%). Recovery from fraction IV was >98%. The final product met pharmacopoeia-quality requirements, but was not developed further for therapeutic application at that time due to regulatory constraints (with safety and toxicity studies and clearance of Cibacron Blue). Another concern relates to the fact that Cibacron Blue 3GA was shown to have the capacity to bind more than 99.5% of hepatitis B particles from plasma protein solution, suggesting the risk of co-elution of viruses with the albumin fraction [60].

Considering that albumin is the major plasma protein and that the backbone of plasma fractionation technology is highly dependent on the albumin purification process used, any change in the upstream purification process of albumin would likely require revalidation of the purification techniques used for most other plasma products. Since ethanol fractionation (sometimes combined with terminal chromatographic adsorption used as a polishing step) is

considered to provide albumin preparations with good recovery from plasma, and quality profiles meeting current therapeutic applications, it is unlikely that affinity-based purification processes will be widely implemented on an industrial scale. Nevertheless, some alternative uses of albumin, e.g. as a stabilizer of recombinant products or as a culture medium substitute, may prompt the development of refined, affinity-based polishing purification techniques that yield ultra-pure albumin from Cohn supernatant IV or precipitate V.

4.3.2. Immunoglobulin G

Like albumin, most industrial-scale plasma fractionation processes used to extract IgG (for either intramuscular or intravenous infusion) are based on the ethanol fractionation method. However, ion-exchange chromatography is increasingly combined with ethanol fractionation. These additional chromatographic steps yield products with improved quality profiles, although they have often been implemented primarily to remove chemicals (such as tri-*n*-butyl phosphate, Tween 80 or Triton X-100) or enzymes (pepsin or papain) that are used during viral inactivation treatments or chemical modifications of IgG preparations, respectively [61]. Affinity or pseudoaffinity chromatography, similar to laboratory-scale approaches using immobilized histidine [62], have not been applied industrially.

As monoclonal IgG became available for therapeutic applications, affinity chromatography using immobilized protein A and native or modified protein G has been developed. However, such an approach has not been used in the large-scale purification of IgG from plasma, probably because products obtained by traditional approaches have established clinical profiles and because those affinity media are costly and would require a careful regulatory approach that addresses the risk of residual protein A or protein G ligands in the final product.

4.3.3. Coagulation factors

If production techniques for albumin and IgG have not been dramatically modified in the last 40 years, due to a conservative approach in favor of established processes, the technologies used to extract all coagulation factors have evolved considerably during the last ten years, allowing the development of products with improved safety profiles.

4.3.3.1. *Factor VIII*. FVIII concentrates are used for the substitution therapy of hemophilia A. All plasma-derived licensed concentrates are produced from cryoprecipitate. ''Intermediate purity'' preparations (specific activity of about 1 IU/mg) are obtained by precipitation from cryoprecipitate. Higher purity preparations (specific activity$>$50 IU/mg) are obtained using either anion-exchange-, size-exclusion- or affinity chromatography [46].

Most FVIII products are obtained by processes comprising the following steps: Solubilization of cryoprecipitate, adsorption with aluminium hydroxide or polyethylene glycol, to remove residual vitamin K-dependent proteins or proteolytic enzymes, centrifugation or filtration, viral inactivation treatment, by solvent–detergent or pasteurization, chromatographic purification, formulation, concentration, aseptic filling and freeze-drying.

The following affinity ligands have been used for large-scale chromatographic purification of FVIII: Heparin and murine monoclonal antibodies that specifically bind human FVIII coagulant proteins or vWF (in vivo, FVIII binds to vWF, its physiological plasma carrier). Matrix-bound dextran sulfate has also been found to be useful for the purification of the complex FVIII–vWF [63], however, there has been no industrial application.

In the process using immunoaffinity on immobilized anti-FVIII [64] or anti-vWF antibodies [65], the virally inactivated cryoprecipitate extract is run through the column under conditions where FVIII, or the FVIII–vWF complex, are specifically bound. The columns are then subjected to extensive washing, to remove slightly bound cryoprecipitate proteins as well as the virus-inactivating agents. FVIII is subsequently eluted, e.g. using 40% ethylene glycol [64] or calcium, when dissociation from bound vWF is required [65]. Subsequent chromatography on either aminohexyl agarose or on a QAE column is performed to bind FVIII and eliminate murine monoclonal antibodies, or other possible trace murine components leaching from the immunoadsorption column, in the breakthrough fraction. This step also helps to remove chemical agents or residual plasma protein contaminants that were used during viral

inactivation or immunoaffinity chromatography steps. To limit FVIII loss during production, albumin must be added during both processes, thus decreasing the specific activity from ca. 2000–4000 to 3–10 FVIII IU/mg.

A process based on direct adsorption of FVIII from plasma by chromatography through an affinity matrix, dimethylamino–propylcarbamylpentyl–Sepharose CL-4B, has been evaluated experimentally [66]. However, the approach has been abandoned, in part due to the difficulty in recovering other plasma fractions. Guanidinium immobilized on Sepharose 4B prepared by isourea modification of amino–Sepharose 4B has been shown, at the laboratory scale, to bind 36 units of FVIII/vWf per ml of matrix from cryoprecipitate [67].

4.3.3.2. *Factor IX*. FIX concentrates are used for hemophilia B substitution therapy. The prothrombin complex concentrate (PPSB or PCC), a complex mixture of vitamin K-dependent coagulation factors in which FIX has a specific activity of about 1 IU/mg (corresponding to only 1 to 2% of the total protein) has long been the traditional substitutive therapeutic product in FIX deficiency. The prothrombin complex is obtained by DEAE–Sephadex or DEAE–cellulose adsorption of cryo-poor plasma (obtained after the removal of cryoprecipitate), a step that co-extracts many components sharing broad biochemical properties. Those proteins include vitamin K-dependent proteins that share structural analogies, such as the presence of γ-carboxyglutamic acid residues [4].

FIX products with increased purity have been obtained by chromatography of the prothrombin complex using a combination of one or two anion-exchange chromatographic steps with various affinity, or semi-affinity, media, such as sulfated dextran, heparin, metal chelate or murine anti-human FIX antibodies [68–74].

Sulfate dextran and heparin are negatively charged polymers that have the potential to bind several plasma proteins, including FIX, by a combination of electrostatic and affinity interactions. The DEAE–Sephadex eluate is directly chromatographed on sulfated dextran, providing isolated fractions [FII, FX, FIX, and protein C (Pc)] in a single step [68]. The specific activity of FIX is increased from 1 to about 10 IU/mg. Other processes [69,70] combine DEAE–Sephadex, DEAE–Sepharose and heparin–Sepharose; implementation of the anion-exchange step on DEAE–Sepharose, compared to a process using only DEAE–Sephadex as the initial purification step [70], improves the purification capacity of the heparin step and permits the FIX purity to be increased to about 150 IU/mg. FIX can also be extracted from cryo-poor plasma by successively running the cryo-poor extract through DEAE–Sepharose, heparin–Sepharose and a cation exchanger [72].

An alternative large-scale production process uses immobilized murine anti-FIX monoclonal antibodies [73]. The purification scheme involves adsorption of the cryo-poor plasma on DEAE–Sephadex, followed by immunoaffinity and aminohexyl–Sepharose chromatography; the last step aims to decrease the level of contamination by murine antibodies. The process yields a FIX concentrate with a specific activity above 150 IU/mg. Another approach has been described where cryo-poor plasma is pre-purified on DEAE–Spherodex, to obtain a PCC fraction from which FIX is isolated by immunoaffinity chromatography; FIX is eluted under mild conditions using a buffer containing EDTA [74]. A subsequent step on DEAE–Sepharose removes leached anti-FIX antibodies.

Another FIX concentrate is produced by IMAC [53]. A DEAE–Sepharose eluate at high ionic strength is loaded onto a column of chelating Sepharose that has been charged with copper ions. Under those conditions, prothrombin and thrombin do not bind to the Cu^{2+} IMAC gel. FX is eluted with a buffer at low ionic strength and low pH, whereas Pc and FIX are subsequently washed out by glycine-containing buffers.

4.3.3.3. *von Willebrand factor*. Most concentrates used to treat von Willebrand disease are low-purity FVIII preparations that also contain active vWF. A high-purity preparation specific for the treatment of this disease has recently been developed [75]. Obtained from cryoprecipitate, it is first purified by two anion-exchange chromatographic steps on DEAE–Toyopearl 650 M; this is followed by affinity chromatography on immobilized gelatin. Under the

applied conditions, the partially purified vWF fraction obtained after the two ion-exchange steps is still contaminated by fibronectin. The protein fraction is then run through a gelatin–Sepharose column that has been equilibrated in a citrate buffer containing 0.15 *M* NaCl at neutral pH, to selectively bind fibronectin [76]. vWF does not bind to the gel and is recovered in the breakthrough fraction and concentrated by ultrafiltration.

4.3.3.4. *Fibronectin*. Although there are no licensed fibronectin concentrates, some preparations have been used for clinical evaluation. Purification of fibronectin on immobilized gelatin is the usual, rapid, standard pilot-scale purification process used [77,78]. An ultra-pure product with good biological activity has been purified directly from plasma by means of a two-step bio-affinity chromatographic process that combines immobilized gelatin and immobilized heparin adsorption [79].

4.3.3.5. *Factor XI*. A FXI concentrate for clinical use has been obtained by a chromatographic process in which cryo-poor plasma is first adsorbed on DEAE–cellulose and then on heparin–Sepharose. This process is similar to the one described for the production of an ATIII concentrate [80]. After washing the heparin column with 0.35 *M* NaCl, FXI elution is achieved with 2 *M* NaCl. The eluate is then desalted on Sephadex G-25 prior to the final formulation steps [3].

4.3.4. Protease inhibitors and anticoagulants

4.3.4.1. *Antithrombin III*. ATIII concentrate, developed over twenty years ago, was the first plasma product obtained by affinity chromatography [81]. The most common method of preparing therapeutic ATIII concentrate is by affinity chromatography on immobilized heparin [82]. In vivo, heparin binds to ATIII, its natural cofactor. Although heparin also binds several other plasma proteins, such as histidine-rich glycoprotein, fibronectin, fibrinogen, FIX and various plasma growth factors, the processes used on a large-scale make it possible to obtain concentrates with high specific activity, due to the high binding capacity for ATIII. Commercial immobilized heparin gels have an ATIII binding capacity of about 10 U/ml or higher.

ATIII production processes usually include cryoprecipitation, ion-exchange chromatography on DEAE–Sephadex or DEAE–cellulose (under conditions where ATIII is eluted in the breakthrough fraction) followed by immobilized heparin adsorption [80–82]. The gel is usually equilibrated in a phosphate buffer at neutral pH, to bind ATIII, while albumin and IgG are recovered in the unbound fraction. Loosely bound proteins are selectively washed by a buffer containing 0.25 to 0.35 *M* NaCl. ATIII is then eluted with 0.45 *M* NaCl or with a higher salt concentration [80,82].

Viral inactivation of the purified fraction of ATIII is most often carried out by pasteurization (a heat treatment at 60°C for 10 h in the presence of a stabilizer, usually sodium citrate or a combination of sucrose and glycine). As this heat treatment step may induce some protein denaturation, a second adsorption step on a column of immobilized heparin allows selective elimination of the non-heparin-binding ATIII molecules, which do not bind to the gel [83].

Alternative production methods start from the fraction IV-1 generated during ethanol fractionation of albumin. Final purification of ATIII is also achieved using immobilized heparin [45].

4.3.4.2. *α1-Antitrypsin*. One difficulty encountered with α1-antitrypsin (AAT) purification, as with several other proteins from the alpha-fraction, is the separation from albumin. This is because of similarities in physicochemical properties, in particular, the size and isoelectric point. Several small-scale purification techniques have been based on affinity chromatography using various ligands, such as immobilized concanavalin [84] to bind AAT, or Blue-Cibacron or Red Procion [85,86], to remove albumin and other protein contaminants. Anti-albumin antibodies have also been described [87]. Another interesting approach is based on thiol–disulfide exchange chromatography of plasma treated with 0.02 *M* 2-mercaptoethanol, to induce a mild reduction of disulfide bridges. AAT, a glycoprotein with reactive thiol groups, is then adsorbed, through SH–SS interchange, to a matrix bound with activated thiol compounds [88]. However, risks of ligand

leakage, incompatibility with the recovery of other important plasma fractions or economical considerations have impaired its application at an industrial scale. Current AAT concentrates are therefore produced using a combination of ion-exchange- and size-exclusion chromatography [89].

4.3.4.3. *Protein C.* Pc is the precursor of activated Pc, which is an important naturally occurring anticoagulant. Highly purified human Pc concentrate can be prepared by a four-step chromatographic procedure that includes a DEAE–Sephadex A50 adsorption step, DEAE–Sepharose FF to separate FII, FVII and FIX, and further purification by an on-line chromatographic system that combines DMAE–Fractogel EMD and heparin–Sepharose. This chromatographic design greatly reduces the contact time of free Pc in the acidic buffer solution used during DMAE–Fractogel EMD and heparin–Sepharose chromatography, thus preserving the biological activity, reducing the risk of microbial contamination and decreasing the overall processing time. Yields were consistently higher than 70% for the DMAE–EMD Fractogel/heparin–Sepharose purification steps [90].

A preparation based on immunoadsorption using murine anti-human Pc antibodies is produced industrially [91]. This Pc concentrate is processed by two ion-exchange- and one immunoaffinity steps using murine anti-Pc antibodies. Another experimental-scale procedure has been described based on a process using PCC as the starting material, and subsequently purification by immunoaffinity chromatography [92].

4.3.5. Other proteins

Purification of transthyretin (or prealbumin) can be achieved by affinity chromatography on immobilized Remazol Yellow GGL [93]. An experimental procedure that was considered to be suitable for large-scale production has been adapted from plasma fraction II+III; it yields an 80% pure transthyretin, using a side fraction of a chromatographic albumin purification process as the starting material [94]. An immobilized lectin, jacalin, has been found to bind plasma proteins bearing *O*-linked oligosaccharides, including IgA, C1 inhibitor and plasminogen [95]. There have been no industrial applications of such a purification approach for therapeutic products. Plasminogen can be purified industrially by affinity chromatography on immobilized lysine.

5. Future trends and conclusion

Bioaffinity adsorption fulfils an important role in the processing of plasma. Bioaffinity plasmapheresis is developing as a helpful therapeutic procedure in a limited number of therapeutic applications for which there is often no other efficient alternative that allows specific elimination of unwanted biological substances from a patient's plasma to be achieved. This technique is likely to develop further, provided that ligands with increased specificity and capacity can be found, and less costly adsorption systems are made available for clinical evaluation.

In plasma fractionation, bioaffinity chromatography has brought significant advantages due to its discriminating ability to bind selected proteins and allow dramatic single-step increases in purity. The role of bioaffinity chromatography in plasma fractionation is likely to increase in the future, especially for the isolation of new plasma protein products or if affinity media with increased selectivity, specificity, improved rigidity, low toxicity and reasonable cost continue to be made available. Because of its current cost and limitation (e.g. restricted resistance to harsh cleaning procedures), affinity chromatography, as it is known and used today, is likely to continue to be implemented more as a polishing purification step than as a mainstream fractionation process. Indeed, considering current regulatory implications, whether or not bioaffinity chromatography would substitute for the dominant plasma fractionation approaches used to purify albumin and IgG and other currently licensed products remains doubtful, unless new processes bring about marked advantages in recovery, productivity or safety. This is explained in part by a general consensus from the medical and scientific community that, at current levels of knowledge, most available therapeutic plasma products, unlike those of earlier generations, have reached a convenient level of purity to properly satisfy therapeutic needs, and because the presence of residual,

contaminating plasma proteins, because of their human origin, do not generally induce therapeutic problems or side-effects in most patients. In addition, established fractionators may be reluctant to implement new purification processes that would require potentially lengthy validation procedures to establish the quality, and safety and efficacy of a new preparation (e.g. viral safety, absence of leakage products, stability, etc.). One may therefore speculate that the economies of emerging countries starting plasma fractionation programs may be more inclined to turn to innovative plasma fractionation technologies that provide enhanced recovery of proteins in high demand in their market territory, thus allowing a more efficient use of a limited plasma supply. One example of the technologies that is in line with a higher recovery of plasma proteins is the chromatographic capture of FVIII directly from plasma (the cryoprecipitation step) to improve overall yield [96].

Because of current safety concerns (potential risk of contamination with prions; immunologic potential of high-molecular-mass ligands leaching in the product), due to the use of ligands from animal sources (e.g. monoclonal murine antibodies, heparin from porcine or equine origin, or gelatin from bovine sources), as well as economic reasons, future trends in bioaffinity technology may be in the use of synthetic ligands that allow one to custom-design optimal, rapid purification schemes and that ensure a better discriminating power of impurities. As an example, structured peptide ligands (also referred to as ''constrained peptides'') [97], obtained by phage display technology, may now be specifically engineered and screened for their capacity to bind and release a protein target that is present in a complex biological fluid, under predefined purification conditions [98]. This approach may allow improved yield and specificity in the purification of the proteins of interest, and may permit better maintenance of their integrity. Such engineered peptide ligands appear to be good candidates for applications in the plasma fractionation industry, for instance, to replace the use of murine monoclonal antibodies that may exhibit non-specific binding properties of impurities and, as any large protein entities, are unstable, sensitive to harsh sanitization procedures and are potentially immunogenic.

Further progress in therapeutic plasmapheresis and in the plasma fractionation industry requires the continuous implementation of carefully selected extraction technologies that do not alter the physiological functions and clinical potential of plasma proteins. Success in this field needs sustained research and development projects to design bioaffinity ligands with enhanced specificity for plasma proteins. Developments in such areas could also be helpful in the design of purification strategies for recombinant plasma proteins obtained from engineered cells, as well as from transgenic animals or plants, where very high levels of purity of the final product are an absolute requirement.

6. List of abbreviations

AAT	α1-antitrypsin
ATIII	antithrombin III
C1, C3	complement system components 1 and 3
DS	dextran sulfate
DSC	dextran sulfate cellulose
F	factor
IgG	immunoglobulin G
IC	immune complex
IMAC	immobilized metal-affinity chromatography
IM-PH	immobilized phenylalanine
IM-TR	immobilized tryptophan
IU	international unit
LDL	low density lipoprotein
LP	lipoprotein
Pc	Protein C
PVA	polyvinyl alcohol
PPSB/PCC	prothrombin complex concentrate
Ps	Protein S
RF	rheumatoid factor
RNP	ribonuclear protein
SLE	systemic lupus erythrematosus
SM	smooth muscles
vWF	von Willebrand factor

References

[1] K. Sawada, P.S. Malchesky, Y. Nosé, Curr. Stud. Hematol. Blood Transfus. 57 (1990) 341–383.

[2] T. Burnouf, Bioseparation, 1 (1991) 383–396.
[3] P. Feldman, L. Winkelman, in J.R. Harris (Editor), Blood Separation and Plasma Fractionation, Wiley–Liss, New York, 1991, pp. 341–383.
[4] F.W. Putnam. in F.W. Putnam (Editor), The Plasma Proteins, Structure, Function, and Genetic Control, Vol. IV, Academic Press, Orlando, FL, 1984, pp. 45–166.
[5] D.A. Pankowski, N.P. Ziats, N.S. Topham, O.D. Ratnoff, J.M. Anderson, J. Vasc. Surg. 11 (1990) 599–606.
[6] G.J. Broze Jr., Semin. Hematol. 29 (1992) 159–169.
[7] M. Verstraete, in E. Haber, E. Braunwald (Editors), Thrombolysis. Basic Contributions and Clinical Progress. Mosby-Year Book, St Louis, MO, 1991, pp. 3–16.
[8] R.I. Handin, J. Loscalzo, in E. Braunwald (Editor), Heart Disease: A Textbook of Cardiovascular Medicine, W.B. Saunders, Philadelphia, 4th ed., 1992, pp. 1767–1789.
[9] R.L. Bick, in R. Pifarré (Editor), Anticoagulation, Hemostasis, and Blood Preservation in Cardiovascular Surgery, Hanley and Belfus, Philadelphia, 1993, pp. 23–55.
[10] F.J. Castellino, Semin. Thromb. Hemost. 10 (1984) 18.
[11] R.L. Bick, in Disorders of Hemostasis and Thrombosis: Principles of Clinical Practice, Thieme, New York, 1985, pp. 1–25.
[12] T.W. Stief, A. Aab, N. Heimburger, Thromb. Res. 49 (1988) 581–589.
[13] W. Vogt, Free Radic. Biol. Med. 18 (1995) 93–105.
[14] P.S. Malchesky, Y. Nosé, in Control Aspects of Biomedical Engineering. International Federation of Automatic Control, Pergamon Press, New York, 1987, pp. 111–122.
[15] P.S. Malchesky, Y. Asanuma, D.E. Hammerschmidt, Y. Nose, Trans. Am. Soc. Artif. Internal Organs 26 (1980) 541–545.
[16] P.S. Malchesky, J. Wojcicki, T. Horiuchi, J.M. Lee, Y. Nosé, in Y. Nosé, P.S. Malchesky, J.W. Smith (Editors), Plasmapheresis: New Trends in Therapeutic Applications, ISAO Press, Cleveland, 1983, pp. 51–67.
[17] Y. Nosé, P.S. Malchesky, Y. Asanuma, J.W. Smith, W.D. Carey, D.R. Ferguson, R.E. Hermann, in S. Sideman, T.M.S. Chang (Editors), Hemoperfusion, Kidney and Liver Support and Detoxification, McGraw–Hill, New York, 1980, pp. 265–272.
[18] H. Mandani, A.W. Siemsen, G. Dunea, Clin. Toxicol. 17 (1980) 534–536.
[19] S. Sideman, L. Mor, L.S. Fishler, L.M. Thaler, J.M. Brandes, Bilirubin Removal by Sorbent Hemoperfusion from Jaundiced Blood; Artificial Liver Support, Springer, Berlin, 1981, pp. 103–105.
[20] Z. Yamazaki, N. Inoue, T. Wada, T. Oda, K. Atsumi, K. Kataoka, Y. Fujisaki, Trans. Am. Soc. Artif. Internal Organs 15 (1979) 480–486.
[21] T. Kanto, T. Yashima, in Therapeutic Plasmapheresis, Vol. VII, Schattauer, Stuttgart, 1988, pp. 93–97.
[22] Y. Homma, Y. Mikani, H. Tamachi, N. Nakaya, H. Nakamura, G. Araki, Y. Goto, Atherosclerosis 60 (1986) 23–27.
[23] B.R. Gordon, S.F. Kelsey, D.W. Bilheimer, D.C. Brown, P.C. Dau, A.M. Gotto Jr, D.R. Illingworth, P.H. Jone, S.F. Leitman, J.S. Prihoda, E.A. Stein, T.N. Stern, J.H. Zovaral, R.J. Zweiner, Am. J. Cardiol. 70 (1992) 1010–1016.
[24] N. Tani, Artif. Organs 20 (1996) 922–929.
[25] I.S. Jovin, Y. Taborski, K.M. Heideinger, Lancet 346 (1995) 117–118.
[26] R.S. Lees, N.N. Holmes, R.W. Stadler, S.F. Ibrahim and, A.M. Lees, J. Clin. Apheresis 11 (1996) 132–137.
[27] K. Suzuki, Y. Matsuki, T. Hidaka, T. Ishizuka, M. Kawakami, S. Takata, H. Kutsuki, H. Nakamura, J. Clin. Apheresis 11 (1996) 16–22.
[28] N. Shibuya, S. Shirabe, K. Nagasato, in Oda (Editor), Therapeutic Plasmapheresis, Vol. IV, Schattauer, Stuttgart, 1988, pp. 165–169.
[29] N. Shibuya, K.K. Nagasato, T. Kobayashi, K. Yoshida, in S. Shiokawa, N. Inoue (Editors), Current Practice in Therapeutic Plasmapheresis, Excerpta Medica, Amsterdam, 1985, pp. 166–172.
[30] N. Yuki, Neurology 46 (1996) 1644–1651.
[31] Z. Yamazaki, Y. Idezuki, N. Inoue, H. Yoshizawa, N. Yamawaki, K. Inagaki, N. Tsuda, Biomater. Artif. Cells Artif. Organs 17 (1989) 117–124.
[32] I.M. Nillson, C. Freiburghauss, Adv. Exp. Med. Biol. 386 (1995) 175–184.
[33] A.A. Boutaud, R. Kalluri, T.Z. Kahsai, M.E. Noelken, B.G. Hudson, Exp. Nephrol. 4 (1996) 205–212.
[34] J. Uehlinger, G.R. Button, J. McCarthy, A. Forster, R. Watt, L.M. Aledort, Transfusion 31 (1991) 265–269.
[35] F. Kriaa, Y. Laurian, C. Hiesse, G. Tchernia, B. Charpentier, Nephrol. Dial. Transplant. 10 (1995) 108–110.
[36] H.W. Snyder, D.E. Reed and, F.R. Jones, Semin. Haematol. 16 (1989) 31–41.
[37] T.R. Guthrie, A. Oral, Semin. Haematol 26(suppl I) (1989) 3–9.
[38] K. Muroi, R. Sasaki, Y. Miura, Semin. Haematol. 26(suppl I) (1989) 10–14.
[39] H.W. Snyder, D.E. Reed, F.R. Jones, Semin. Haematol. 26(suppl I) (1989) 25–30.
[40] A. Mittelman, J. Bertram, Semin. Haematol. 26(suppl I) (1989) 15–18.
[41] Z.R. Zeigler, R.K. Shadduck, R. Nath, D.D.R. Andrews, Bone Marrow Transplant. 17 (1996) 81–86.
[42] E.J. Cohn, L.E. Strong, W.L. Hughes Jr., D.J. Mulford, J.N. Ashworth, M. Melin, H.L. Taylor, J. Am. Chem. Soc. 68 (1946) 459–475.
[43] P. Kistler, H.S. Nitschmann, Vox Sang. 7 (1962) 414–424.
[44] P.R. Foster, R.V. McIntosh, in C.V. Prowse (Editor), Plasma and Recombinant Blood Products in Medical Therapy, Molecular Medical Science Series, Wiley, Chichester, 1992, pp. 19–45.
[45] T. Burnouf, in C.V. Prowse (Editor), Plasma and Recombinant Blood Products in Medical Therapy, Wiley, Chichester, 1992, pp. 67–87.
[46] T. Burnouf, J. Chromatogr. B 664 (1995) 3–15.
[47] P. Kistler, H. Friedli, in J.M. Curling (Editor), Methods of Plasma Protein Fractionation, Academic Press, London, 1980, pp. 77–91.
[48] J.E. More, M.J. Harvey, in J.R. Harris (Editor), Blood Separation and Plasma Fractionation, Wiley–Liss, New York, 1991, pp. 261–306.
[49] S. Subramanian, Crit. Rev. Biochem. 16 (1984) 169–205.

[50] J.A. Thomas, C.R. Lowe, Colloque INSERM 227 (1993) 25–29.
[51] S.J. Burton, Colloque INSERM 227 (1993) 19–24.
[52] G. Birkenmeier, G. Kopperschlaeger, J. Chromatogr. 235 (1982) 237–248.
[53] P.A. Feldman, L. Harris, D.R. Evans, H.E. Evans, Colloque INSERM 227 (1993) 63–68.
[54] J.M. Curling. in J.M. Curling (Editor), Methods of Plasma Protein Fractionation, Academic Press, London, 1980, pp. 77–91.
[55] M. Hierowski, R. Brodersen, Biochem. Biophys. Acta 354 (1974) 121–129.
[56] A. Wickman, L.O. Andersson, Biochem. Biophys. Acta 372 (1974) 218–224.
[57] J. Saint-Blancard, J. Fourcart, F. Limonne, P. Girot, E. Boschetti, in T.C.J. Gribnau, J. Visser, J.F. Nivard (Editors), Affinity Chromatography and Related Techniques, Elsevier, Amsterdam, 1982, pp. 305–312.
[58] M.J. Harvey, R.A. Brown, J. Rott, D. Lloyd, R.S. Lane, in J.M. Curling (Editor), Separation of Plasma Proteins, Pharmacia Fine Chemicals, Uppsala, 1983, pp. 79–88.
[59] J.E. More, A.G. Hitchcock, N. Thompson, J.L. Young, Colloque INSERM 175 (1989) 177–183.
[60] R.A. Brown, B.S. Combridge, J. Virol. Methods 14 (1986) 267–274.
[61] M. Burnouf-Radosevich, TCB 3 (1995) 167–179.
[62] S. Kanoun, L. Amourache, S. Krishnan, M.A. Vijayalaksmi, Colloque Inserm 227 (1993) 189–195.
[63] P. Harrison, R.H. Saundry, G.F. Savidge, Throm. Res. 50 (1988) pp. 295–304.
[64] S. Liu, J. Addiego, E. Gomperts, C. Kessler, L. Garanchon, G. Neslund, V. Foster, R. Berkebile, S. Courter, M. Lee, H. Kingdon, M. Griffith, Colloque INSERM 175 (1989) 263–270.
[65] M.E. Hrinda, C. Tarr, W. Curry, J. Newman, A.B. Schreiber and, R. D'Alisa, Colloque INSERM 175 (1989) 413–418.
[66] M.P. te Booy, A. Faber, E. de Jonge, E.P. Wolterink, W. Riethorst, T. Beugeling, A. Bantjes, J. Over, B.W. Konig, J. Chromatogr. 501 (1990) 103–114.
[67] R.H. Saundry, G.F. Savidge, Bioseparation 2 (1991) 177–186.
[68] D. Ménaché, H.E. Behre, C.L. Orthner, H. Nunez, H.D. Anderson, D.C. Triantaphyllopoulos, D.P. Kosow, Blood 64 (1984) 1220–1227.
[69] T. Burnouf, C. Michalski, M. Goudemand, J.J. Huart, Vox Sang. 57 (1989) 225–232.
[70] C. Michalski, F. Bal, T. Burnouf, M. Goudemand, Vox Sang. 55 (1988) 202–210.
[71] A.H.L. Koenderman, H.G.J. ter Hart, C.T. Hakkennes, E.J. Muller, L. Brands, N. van Duren, H. Hiemstra, J. Over, Colloque INSERM 227 (1993) 81–86.
[72] A.L. Löf, E. Berntorp, B. Eriksson, C. Mattson, L. Svinhufvud, S. Winge, A. Östlin, Colloque INSERM 227 (1993) 69–74.
[73] H.C. Kim, C.W. Mc Millan, G.C. White, G.E. Bergman, P. Saidi, Semin. Hematol. 27 (1990) 30–35.
[74] C. Lutsch, P. Gattel, B. Fanget, J.-L. Véron, K. Smith, J. Armand, M. Grandgeorge, Colloque INSERM 227 (1993) 75–80.
[75] M. Burnouf-Radosevich, T. Burnouf, Vox Sang. 62 (1992) 1–11.
[76] M. Burnouf-Radosevich, J. Tissue Culture Methods 16 (1994) 223–226.
[77] V. Regnault, C. Rivat, C. Geschier, J.F. Stoltz, Rev. Fr. Transfus. Hemobiol. 33 (1990) 391–405.
[78] M.-F. Rouleau, E. Boschetti, T. Burnouf, J.-M. Kirzin, J. Saint-Blancard, in T.C.J. Gribnau, J. Visser, J.F. Nivard (Editors), Affinity Chromatography and Related Techniques, Elsevier, Amsterdam, 1982, pp. 323–331.
[79] M. Allary, E. Boschetti, D. Lafargue, C. Sene, J. Saint-Blancard, Colloque INSERM 175 (1989) 61–66.
[80] J.K. Smith, L. Winkelman, D.R. Evans, M.E. Haddon, G. Sims, Vox Sang. 48 (1985) 325–332.
[81] M. Miller-Andersson, H. Borg, L.-O. Andersson, Thromb. Res. 5 (1974) 439–452.
[82] M. Wickerhauser, C. Williams, Vox Sang. 47 (1984) 397–405.
[83] Y. Camacho, G. Navio, J. Acosta, E. Rocha, Thromb. Haemost. 54 (1985) 90.
[84] R. Murthy, A. Hercz, Febs Lett. 32 (1973) 243–246.
[85] G. Gunzer, N. Hennrich, J. Chromatogr. 296 (1984) 221–229.
[86] G. Birkenmeier, G. Kopperschlaeger, Colloque INSERM 175 (1989) 217–228.
[87] R. Myerowitz, Z. Handzel, J. Robbins, Clin. Chim. Acta 39 (1972) 307–317.
[88] C.B. Laurell, I. Dahlqvist, U. Persson, J. Chromatogr. 278 (1983) 53–61.
[89] T. Burnouf, Colloque INSERM 175 (1989) 53–60.
[90] F.L. Zhou, T. Burnouf, J.J. Huart, M. Radosevich, Vox Sang., (Submitted).
[91] E. Johann, P. Ludwig, S. Hans-Peter, European patent EP 0533210A, 1991.
[92] V. Regnault, C. Geschier, M.-E. Bricquel, C. Rivat, P. Alexandre, J.-F. Stolz, Colloque INSERM 227 (1993) 97–102.
[93] S. Copping, P.G.H. Byfield, Biochem. Soc. Trans. 10 (1982) 104–105.
[94] V. Regnault, L. Vallar, C. Geschier, C. Rivat, J.-F. Stoltz, Colloque Inserm 227 (1993) 301–306.
[95] G.L. Hortin, B.L. Trimpe, Anal. Biochem. 188 (1990) 271–277.
[96] P. Kaersgaard, K.A. Barington, C. Baerentzen, J. Kiye, Thromb. Haemostas. 65 (1991) 999.
[97] R.C. Ladner, Tibtech 13 (1995) 426–430.
[98] J. Maclennan, Biotechnology 13 (1995) 1180–1183.