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Review

Advanced sorbents for preparative protein separation purposes

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ABSTRACT

Concepts of the preparative liquid chromatography of proteins are well documented and provide the necessary information to design purification schemes. The success of these concepts depends, however, on the intrinsic characteristics of the packing material. Specific suppliers have proposed chromatographic sorbents based on different polymeric matrices, the intrinsic characteristics of which are responsible for the final results. A review of existing base matrix properties permits an optimized selection for a particular separation purpose. Selectivity, efficiency, capacity and productivity are described with respect to specific packings. Additionally, a section is devoted to sorbent deterioration and cleaning aspects, both of which have a large impact on the choice criteria for preparative purposes. Chemical treatments necessary to clean the sorbent may have a deleterious effect, consequently reducing their loadability or their long-term usage.

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1. INTRODUCTION

The large-scale purification of recombinant and natural proteins and increases in purity standards and competitive cost requirements are stimulating, much more than in the past, improvements in the productivity of bioprocesses. Most downstream processes include some chromatographic steps among other conventional separation methods, because they permit very pure products to be obtained in compliance with regulatory recommendations. This compulsory choice however, is generally sophisticated and expensive, which explains the development of more effective approaches to improve the productivity of chromatographic technologies. Continuous *versus* discontinuous approaches have been investigated, computerized optimization strategies have been proposed, various column technologies have been studied and automated multi-task approaches have been proposed. However, one of the most important aspects of liquid chromatography is the sorbent. During 25 years of development, packing materials for preparative protein separations have been continuously improved, mostly with respect to performance.

Extremely soft gels based on dextran, diluted polyacrylamide or non-cross-linked agarose have been progressively replaced by more and more rigid materials. The initial driving force of this approach was the great technological difficulty of running a separation cycle on columns longer than a few dozen centimetres on the laboratory scale. However, more recently, the driving force has changed towards the possibility of decreasing the cycle time by increasing the speed without sorbent shrinkage.

Improvements in resolution have been an important field of investigation; here the approaches chosen were the improvement of the separation efficiency or the enhancement of the selectivity (or both), which were mostly applied for analytical purposes or for preparative separations, respectively. Resolution capabilities for preparative applications, however, were much more closely linked to the intense development of special mechanisms of action such as affinity interactions and hydrophobic associations, both based on better selectivity. Attempts have also been made to improve the resolution of gel filtration media by acting on the slope of the selectivity curves (better selectivity) and on the particle size (better efficiency). The former was achieved by the introduction of new packing materials and the latter became possible with mechanically stronger matrices.

As far as the chemical resistance of chromatographic sorbents is concerned, improvements are linked with the need for cleaning after each cycle. Regeneration and cleaning are now routine operations dictated not only by the final purity of the product but also the need to increase the life of the sorbent and consequently to limit the cost of the separation process.

Sorption capacity has also been improved over the years, but its progression was not really spectacular. At the beginning of the develop-

ment of ion exchangers, the sorption capacity for biological macromolecules was already impressive at about 80 mg/ml [1]. At present, even with recently introduced ion exchangers (see below), the sorption capacity is sometimes even lower (a few dozen mg/ml). This appears to conflict with the need for productivity on a large scale when considering that the first dextran-based ion exchangers, despite their great softness, showed sorption capacities as high as 200–300 mg/ml. This situation could be explained by the development of the concept of "productivity", which is the result of the association of two parameters, capacity and speed. This means that productivity improvements have essentially been accomplished by increasing the flow-rate. As shown in the following sections however, this approach is also reaching its limits.

In the last decade, new concepts have been introduced with the development of packing materials specific for protein separations on a preparative scale. Homogeneous cross-linked polysaccharides (*e.g.*, agarose) have been successfully developed for large-scale use [2]. These gels show interesting chromatographic performances such as good sorption capacity and acceptable pressure resistance at least for moderate column lengths. Their ability to withstand extreme alkaline washings is also important and explains their easy adoption for preparative use.

Macroporous materials based on synthetic polymers with a large number of hydroxyl groups and designed to have large pores were then developed in order to overcome some of the limitations of homogeneous networks [3–5]. Elimination of sieving effects, stability in strongly acidic media, increased physical stability towards pH and ionic strength changes, non-biodegradability and resistance to medium pressure are the major advantages. In some instances, the macroporosity concept was pushed beyond certain limits where the separation of molecules is defined as "perfusive chromatography" [6]. Here the main advantage is the very high speed permitted by rapid mass transfer.

The kinetics of adsorption–desorption and the availability of interacting chemical ligands (such as ion-exchange groups) have been enhanced by the introduction of so-called "tentacular sorbents" [7]. Active groups are attached to linear flexible polymeric chains that increase the mobility of interacting groups and the chances of rapid interactions with the macromolecules to be separated.

More recently, another concept has been introduced that combines the very good sorption properties of soft gels with the rigidity of composite materials [8]. The "soft gel in a rigid shell" concept defining these sorbents results in a particularly high starting binding capacity which is maintained at high levels, even at flow-rates as high as 7–10 m/h.

All of these approaches for making sorbents were developed in order to achieve better economics for preparative applications. Today, all the parameters of the chromatographic cycle have been analysed in detail and sorbents have been adapted to improve the level of productivity. Although the packing material plays a decisive role in the performance of a preparative separation, technological expertise contributes substantially to adapting sorbents, even when some intrinsic characteristics are not perfect. Semi-rigid sorbents can be packed in large, wide columns to avoid problems of back-pressure; highly porous mechanically stable materials can be used at very high flow-rates to compensate for their low level of capacity per cycle; and aqueous–organic or acidic washing can replace alkaline washings with base-sensitive materials.

In this review, we examine the different aspects of preparative liquid chromatography for protein separations, with particular reference to the main sorbents currently commercially available.

2. MAIN PRINCIPLES OF LIQUID CHROMATOGRAPHY AND THEIR IMPACT ON SORBENT DESIGN

Column liquid chromatography is a separation method based on the differential migration speeds of sample components through a solid phase. The main mechanism on which chromatography is based is the adsorption of sample components as a result of complex physico-chemical interactions. Column separation of proteins with no adsorption is restricted to gel

filtration, which is simply based on the differential diffusion rate of proteins into the pores of the matrix according to their hydrodynamic volume. Interactions used in the adsorption chromatography of proteins include ionic interactions, hydrophobic associations, biorecognition and covalent linkages. The distribution of components between the liquid and solid phases depends on a number of parameters, some of which directly linked to the nature and structure of the solid phase. To predict the behaviour of molecules in preparative chromatography, mathematical models have been developed.

Equilibrium models are based on the distribution of the investigated molecules between the two phases. As this distribution depends on temperature, isotherms are obtained that are characterized by thermodynamic equilibrium constants. Under linear conditions (low protein concentration), the selectivity factor characterizing two components is given by the ratio of the individual equilibrium constants. The composition at any point in the column can be calculated, based on the behaviour of a single component, by solving differential equations of material balance. The equilibrium rate is not reached immediately, however, and depends on mass transfer resistance.

The film diffusion rate, pore diffusion rate and surface adsorption rate control the mass transfer, which is in any case finite, indicating that equilibrium between the mobile and solid phases is never reached. In addition, isotherms are not linear in preparative applications: several distribution models exist [9], the best known being the Langmuir isotherm. All these contingencies make the mathematical equations extremely complex. They explain at least intuitively the importance of the size of the chromatographic sorbent beads and their pores and the influence of any modified parameter.

Statistical models [10] introduced the number of theoretical plates constituting the column and described the distribution residence time of a given molecule that probably corresponds to a Gaussian-type function. Again, this model does not consider interactions between different molecules of the sample and mass-transfer resistance is also neglected.

Complex examples of protein mixtures have not yet been resolved with the use of models. Practically nothing is known about the behaviour of a simple protein when it is in a complex mixture where protein–protein interactions considerably affect the adsorption isotherms of single components.

Complex mathematical models, which are mostly inaccessible to the biochemist–chromatographer, usually fail because of the lack of data on protein behaviour with respect to the sorbent. The separation of a mixture of components is a consequence of a very complex interdependence of kinetics, mass transfer and thermodynamic phenomena where the chromatographic sorbent is the most important factor.

More practically, resolution and sorption capacity are systematically considered when selecting a packing material for preparative protein applications. Both depend on two categories of parameters, one associated with the experimental conditions defined by the chromatographer and the other essentially with the properties of the polymeric network defined by the manufacturer.

2.1. Relationship between resolution and the nature of the sorbent

Resolution is defined simply by the distance between two peaks (retention time or elution volume) and their respective width. Extensive information is available in the literature [11,12]. Measurement of resolution is a common operation in protein separations by liquid chromatography and is used as a method to optimize the column geometry and the separation procedure.

Most of the parameters linked to resolution are easily accessible to the user; they can additionally be modified by optimizing some operational parameters as indicated in Table 1.

Specific characteristics of the packing material, however, have a significant impact on the resolution; the most important are those described in Fig. 1. Small particles provide a large number of plates, increasing dramatically the column efficiency. The back-pressure generated when decreasing the particle size can be easily over-

TABLE 1

SELECTED OPERATIONAL FACTORS THAT MODIFY RESOLUTION IN LIQUID CHROMATOGRAPHY

Parameters governing the resolution	Main operational conditions that modify resolution
Selectivity factors	Mobile phase composition: pH, ionic strength, nature of buffer, temperature Elution gradient slope and shape Column geometry
Efficiency	Flow-rate Temperature Column length Loading volume and solute concentration
Capacity factors	Mobile phase composition Elution conditions Temperature

come by using rigid materials. Manufacturers of solid phases essentially modify several parameters as described in Fig. 1, most of them, however, acting on the selectivity such as the chemical structure of the sorption sites, the accessibility of the pores, the sorption capacity and the flexibility of the polymer.

2.2. Impact of packing material on sorption capacity

Sorption capacity is defined as the amount of protein reversibly adsorbed on a given volume of matrix. This phenomenon is governed by surface processes, but the available surface of most

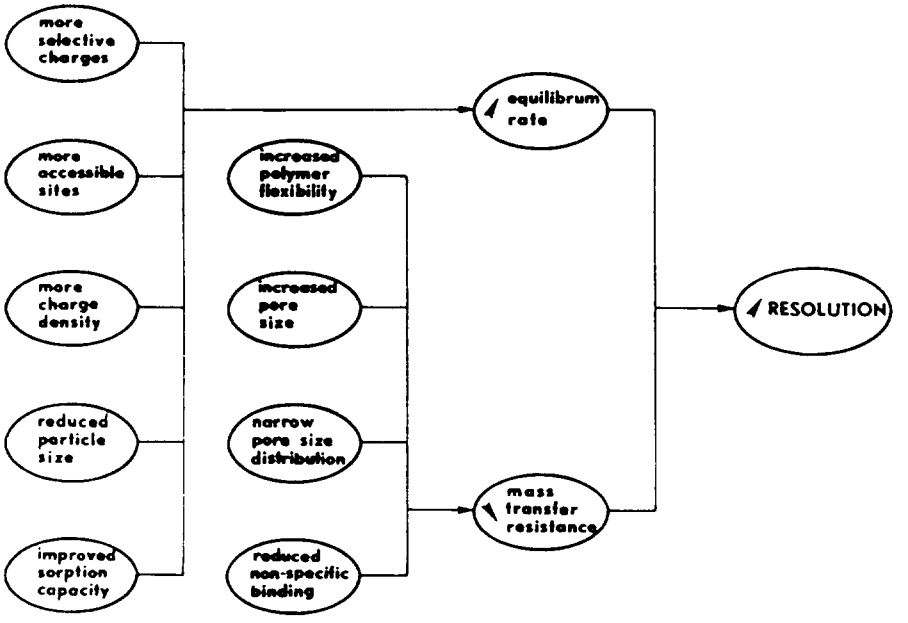

Fig. 1. Selected factors acting on resolution in liquid chromatography. All these factors are directly linked to the sorbent design only.

sorbents is found inside the polymer network. Independently of the accessibility, sorption capacity is proportional to the number of active sites. This is true for very low-molecular-mass solutes, but for proteins the accessibility is much lower because of their large molecular size and the statistical distribution of pores in the polymeric network [13]. Sorption capacity decreases rapidly when the size of large pores is close to the hydrodynamic diameter of the proteins of interest.

As the polymeric pores are not all identical, proteins behave differently when interacting with surface sites or with sites located in pores with dimensions similar to the size of the protein in question. In this instance, the interaction kinetics depend on the diffusion time of the protein.

To avoid slowing down diffusion and to keep the chromatographic separation velocity high, it is obvious that the site accessibility must be kept very high when designing sorbents. Attempts are being made by manufacturers to produce more and more accessible surfaces with extremely porous solid material defined as perfusive [6] or with special porous flexible networks [8]. The former sacrifices to a certain extent the intrinsic sorption capacity while increasing the surface availability, and the latter takes advantage of the much larger surface with facilitated diffusion properties for high sorption capacities.

The capacity varies as a function of a number of parameters, some of which are dependent on the experimental conditions, but many others are associated with the packing properties (see Fig. 2).

For high throughputs, mostly considered on a preparative scale, the dynamic binding capacity frequently represents the discriminating factor. Phenomena on which the dynamic binding capacity is dependent have been extensively described [14,15]; the nature and composition of the network, pore diameter, pore size distribution and particle diameter are the major factors related to the sorbent itself. Other dependences are known, such as solute characteristics and concentration, flow-rate, pH, ionic strength and nature of the counter ion, all of which depend on experimental conditions easily accessible to the chromatographer.

It is clear that significant variations in dynamic binding capacity between different sorbents exist, as reported [16] and described later in this

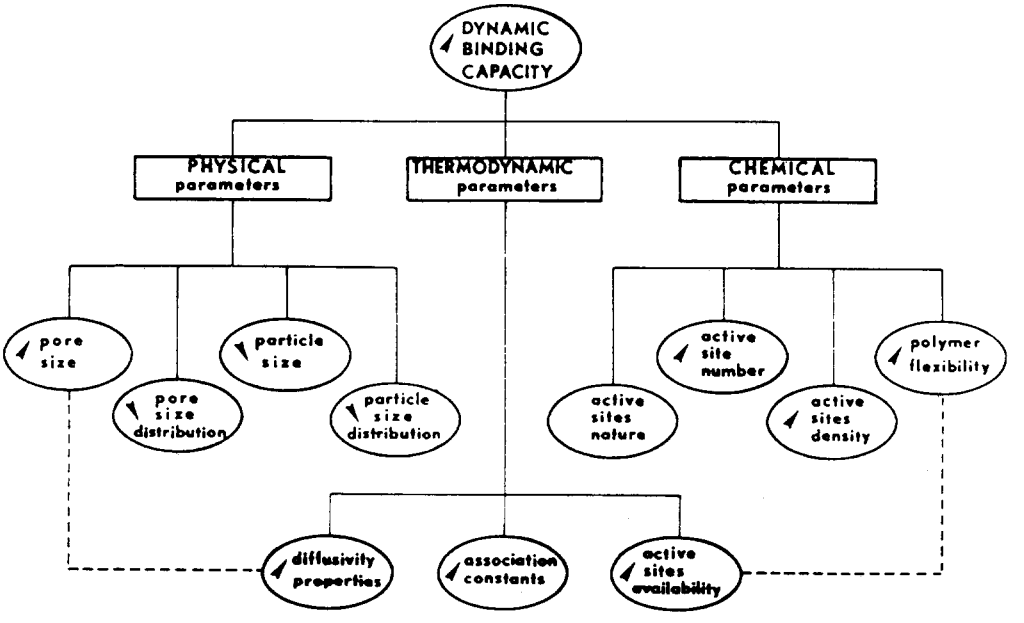

Fig. 2. Selected factors related to dynamic binding capacity in liquid chromatography. All these factors are connected with the sorbent design only.

review for ion exchangers. The available surface may also contain non-specific adsorption sites. Variation in the number of these sites is at the origin of the performance difference between a given group of adsorbents with similar pore and particle sizes.

2.3. Importance of particle size of chromatographic sorbent

As indicated above, particle size plays a role in both resolution and variation of the dynamic binding capacity *versus* flow-rate. On the preparative scale, however, choosing a small particle size induces back-pressures that are unacceptable for soft and semi-rigid materials and additionally influences the cost of the process. Small particles (<20 μm) are generally expensive and represent the largest capital investment affecting the separation costs. Large particles (>80 μm) cost much less but give lower performance.

General economic models have been studied [17] to calculate typical productivities of beads of different diameter, assuming that the overall sorbent life is between 100 and 300 separation cycles. For medium-scale columns designed for preparative purposes, current economic data based on productivity and cost *versus* particle size show that 20–40-μm particles have a definitive advantage over smaller particles (2–10 μm) with respect to cost considerations. When compared with particles of about 80–100 μm, the advantage is essentially based on the time/performance ratio.

3. REVIEW ON BASE MATRIX PROPERTIES

To define a base matrix, a number of classifications have been proposed based on chemical composition, applications and specific properties, but the one that is always applicable is the distinction between xerogels and aerogels.

The xerogel category includes sorbents with shrinking and swelling properties as a function of the amount of solvent (aqueous solutions in the case of a hydrophilic matrix) present inside the network. Examples of xerogels are homogeneous cross-linked dextran and polyacrylamide.

Aerogels are very rigid matrices that are not affected in their volume by the amount of solvent and its nature. Examples of aerogels are porous silica, porous glass, macroreticular rigid polystyrene (in aqueous systems) and to a certain extent polymethacrylates.

In spite of this rigid classification, there are examples of xerogel–aerogel hybrids with limited properties of shrinking and swelling such as macroreticular polyacrylamide derivatives, polyvinyl derivatives and cross-linked agarose gels.

3.1. Polysaccharide materials

Dextran polymers cross-linked with epichlorhydrin [18] or other bisepoxiranes have been used to make gel filtration media and ion exchangers. They are characterized by poor mechanical properties, a high degree of hydrophilicity and a decreased exclusion limit.

Agarose gels are typical natural and neutral macroporous polysaccharidic networks used as such, or after cross-linking [2,19] to improve their mechanical properties and to render them insensitive to dissociating agents such as urea. They are inert, hydrophilic and easy to derivatize, explaining their wide use in bioseparations. Many derivatives have been made for gel filtration, ion exchange, affinity chromatography and related techniques.

Cellulose is another polysaccharide used for gel filtration and ion exchange after appropriate modifications [1]. It has long been very popular in macrofibres for ion exchange; more recently, beaded cellulose has been proposed [20] for all kinds of liquid biochromatography. Cellulose gels are not totally inert, and in fibrous shape they generate back-pressure problems when used in long columns.

3.2. Synthetic organic polymers

Polyacrylamide cross-linked with methylenebisacrylamide is well known [21]. It is essentially used for gel filtration. Its low exclusion limit does not permit its use to be extended to ion exchange and other applications by just decreasing the polymer concentration in order to obtain

a higher exclusion limit, because the resulting softness of the gel is not compatible with column usages.

Polyacrylamide is sensitive to alkaline treatments (above pH 10); it is, however, stable in acidic media and in the presence of any dissociating agents. Supports based on poly(hydroxymethyl methacrylate) (HEMA) were originally proposed in the 1970s. HEMA sorbents are based on copolymers of ethylene dimethacrylate and hydroxyethyl methacrylate, the biocompatibility of which has been demonstrated by their widespread use in soft contact lenses. The original patented technique of suspension polymerization of water-soluble monomers enables spherical particles to be prepared with size and porosity controlled over a wide range. These copolymers have strong resistance to hydrolysis, high mechanical strength and a high surface hydroxyl group content. The subsequently hydrolysed poly-HEMA sorbents exhibit pressure resistance up to 250 p.s.i. (1 p.s.i. = 6894.76 Pa), long-term stability over the pH range 2–12 and compatibility with most organic solvents.

During the past 20 years, derivatives of HEMA sorbents suitable for size-exclusion ion-exchange, hydrophobic interaction and affinity chromatography have been developed. Recent developments include HEMA-based reversed-phase sorbents, phenyl modification for hydrophobic interaction chromatography, immobilized protein A for affinity chromatography of immunoglobulins and iminodiacetic acid modification for immobilized metal affinity chromatography. HEMA polymers are used for the preparation of size-exclusion media with calibration graphs that are nearly linear from the exclusion limit to low molecular masses. According to manufacturers' information, the resolution is usually lower than that of soft gels as a result of a lower selectivity.

HEMA ion-exchange media have been extensively described [5,22–24]. They are based on macroreticular structures where ionic groups are chemically attached via various chemical reactions. Finally, derivatives for affinity applications, hydrophobic chromatography and reversed-phase materials exist, as shown in Tables 3, 6, 7 and 11.

Owing to their solvent compatibility, HEMA polymers can be used either in aqueous systems or in aqueous–organic mixtures.

Other hydrophilic synthetic sorbents are well known under the trade name Fractogel, but their chemical composition has not been revealed. They have reasonably good hydrophilic properties and, depending on their substituted groups, they can be used as gel filtration media and ion exchangers [25] and as affinity sorbents. More recently, a new group of ion exchangers have been obtained by grafting linear polymer chains on the surface of Fractogel. These chains or "tentacles" possess active ionic sites and they are stated to reduce the contact between the solute and the matrix, diminishing the level of non-specific binding. Moreover, they confer a much higher polymer flexibility to improve the selectivity when compared to classical sorbents [7,26]. Tentacle-type sorbents have first been restricted to ion exchangers and then extended to affinity media.

Styrene–divinylbenzene copolymers have long been used in liquid chromatography [27] but their use for protein separations has only recently been developed as a result of the modifications of their strongly hydrophobic surface. In aqueous media they are used mostly as ion exchangers after the introduction of a hydrophilic material providing cationic or anionic groups.

Microreticular and macroreticular hydrophilic polystyrene matrices are now available. The most recently available packing in this category is Poros; it has a special structure with extremely large pores permitting a relatively constant dynamic binding capacity over a large range of flow-rates up to a superficial linear flow of several thousand cm/h [6,28]. Adsorption–desorption mechanisms occur on the surface of the large channels inside the microbeads, improving the mass transfer of large, slowly diffusing proteins. As a surface-based process the sorption capacity of these ion exchangers is lower than that of the classical gels.

Another acrylamide derivatives is Trisacryl, which results from the polymerization of a derivative of trishydroxymethylaminomethane [29,30]. The base polymer is used for preparing ion exchangers, gel filtration media desalting and affinity sorbents. They are characterized by their high hydrophilicity due to the presence of three

hydroxyl groups per repeating unit, their mechanical resistance to 30–45 p.s.i. as a result of their macroporous structure and their chemical stability in the presence of dissociating agents, strong acids and solvents. The stability of some of them in strongly alkaline media is limited, but other such as Trisacryl Plus can be repeatedly washed with sodium hydroxide with no effect on their behaviour [31,32].

3.3. Inorganic materials

The main feature of this sorbent series is their incompressibility in any medium. The most common inorganic material is porous silica, which is obtained by polycondensation of sodium silicate under mild acidic conditions [33]. The first stage of the synthesis, the formation of an aqueous silica gel, is followed by a number of physicochemical operations including wet and dry heat ageing to create the appropriate surface area, porous volume and pore size [34]. Silica is well known in reversed-phase HPLC [35] and as a base material to make hydrophilic composite material (see below).

The major problems encountered with naked porous silica are the low stability in alkaline conditions and the non-specific binding, both due to the presence of acidic surface silanol groups. These drawbacks can be considerably reduced by several approaches: diminution of the surface area decreases enormously the alkali sensitivity and the non-specific binding; high-temperature heating contributes to the condensation of silanol groups; and layering of special chemicals such as zirconium oxide [36], aluminium [37] or polyethyleneimine [38] increase considerably the pH stability of porous silica. Hydroxyapatite is another inorganic sorbent long used in the separation of biological materials [39]. It is a special crystalline form of calcium phosphate with the property of reversibly adsorbing proteins by a complex ionic mechanism [40].

The ease with which it is possible to obtain crystals is counterbalanced by the greater difficulty of being able to maintain stable column flows. Crystals are highly fragile, generating "fines" that preclude any long-term usage of columns. However, alternative solutions have been found, consisting in trapping microcrystals in a macroreticular agarose network readily used in packed columns (see HA-Ultrogel, Section 8). When faced with difficult protein separations using classical ion-exchange supports, hydroxyapatite has been demonstrated to be a successful alternative even on a large scale.

Porous glass is another inorganic support for the separation of proteins. As with porous silica, the presence of surface silanols generates non-specific binding for proteins [33] that can be suppressed by a passivation operation with special polymers or by covalent grafting.

3.4. Composite materials

Composite materials are mixed networks constituted of at least two components with independent roles. Most generally a component acts as a rigid skeleton, the other being responsible for the interaction with the proteins to be separated.

Polyacrylamide–agarose gels for molecular sieving [41] were the first composite polymers to be introduced, under the trade-name Ultrogel AcA. This association was justified by the good separation properties of polyacrylamide, which it is impossible to exploit directly because of their great softness in diluted forms. Hard agarose gels compensate for the mechanical failure of low-density polyacrylamide with no interference with the gel filtration effect. The concentrations of agarose and polyacrylamide are between 2 and 4% and 2 and 20%, respectively.

Other composite gel filtration media are obtained by combining dextran derivatives and bisacrylamide polymers (see Sephacryl below) [42]. This combination also allows a wide variety of sorbents to be obtained with different exclusion limits as a function of their chemical composition. They are considered more copolymer like than real composite materials because of the covalent links between the two moieties.

Another well know composite material, Spherodex, associates porous silica and dextran derivatives. These products are used essentially as ion exchangers for large-scale use. They associate the good interacting properties of ioniz-

able dextran derivatives with the highly rigid silica matrix [43,44].

Layering dextran derivatives on the silica surface additionally permits the total suppression of the non-specific binding of acidic silanols (they are no longer accessible to proteins) and limits the alkaline sensitivity of the silica moiety. This composite material, owing to the presence of silica, is extremely stable to pressure and it does not shrink or swell in any solvent.

Most recently, the group of composite sorbents has been enriched with a new material consisting of a macroporous organic composite, the pores of which are filled with a soft flexible synthetic gel. The result of this association is to enhance the accessibility of the biological macromolecules to the active sites by rapid diffusivity mechanisms while maintaining at high levels the dynamic capacity, flow-rate and resolution. All the components of the matrix have been specially selected for their stability towards strong acidic, alkaline and oxidizing agents. These composites have so far been designed for protein ion exchange and are known under the trade-name HyperD [8].

Many other composites have been described in the literature but are not necessarily commercially available. It is nevertheless interesting to note that alginate gels alone [45] or in association with polyacrylamide [46] have been described for the separation of macromolecules. Others examples are copolymers of agarose and acrolein [47] for protein immobilization and affinity chromatography. Reticulated gelatin has been proposed for gel filtration and affinity chromatography [48]; chitosan gels have also been described for the preparation of ion exchangers [49].

4. SORBENTS FOR GEL FILTRATION

Commercially available gel filtration media for preparative applications are generally composed of natural cross-linked polysaccharides or synthetic three-dimensional polymers, or are composite material.

Independently of their chemical composition, gel filtration media for proteins are characterized by their exclusion limit and their selectivity range [50,51] (see also the theory and practice of gel filtration in ref. 52). Both are directly dependent on the size of the gel pores and on the pore size distribution. Gel filtration is generally a limited resolution technique where the selectivity between peaks is increased when the pore size distribution is very narrow. On the other hand, on decreasing the pore size distribution the selectivity range becomes smaller. This situation explains why, for a given medium, several gels exist to cover, by zone overlapping, the total range of protein molecular masses.

Regularity in polymer synthesis is the key to success in increasing the selectivity and the lot-to-lot consistency. Macroporous polymerization does not provide access to high-performance gel filtration media. Such gels are generally soft or semi-rigid, especially when designed for the separation of medium and large proteins.

As shown in Tables 2–4, natural and synthetic polymers are used to manufacture these gels covering an M_r fractionation range from several hundred to millions. To cover the entire range, sequential combinations of gel filtration media are necessary [53].

As a general rule, unsubstituted agarose-based gels are applied to the separation of very large macromolecules; dextran- and polyacrylamide-based materials are used for medium-sized proteins; some low-porosity gels such as Sephadex G-25, Biogel P-30, Ultrogel AcA-202 and Trisacryl GF 05 are used for desalting.

It is interesting that some sorbents present a larger fractionation range than others; this means that numerous proteins must be placed in the limited space between the total column volume and the void volume. For a similar column efficiency (essentially similar particle size) the resolution is substantially lower in this instance. A larger fractionation range must consequently be associated with a small particle size to increase the separation efficiency in order to compensate for the poor selectivity factor. Selectivity curves are therefore good "identity cards" of the general characteristics of the gel filtration media: when the slope is shallow, the selectivity performance is low. Selectivity curves are dependent on the nature of molecules used for their determination. For a similar molecular mass the

TABLE 2

PREPARATIVE POLYSACCHARIDIC-BASED GEL FILTRATION MEDIA FOR BIOLOGICAL MACROMOLECULES

Trade name and type	Supplier	Nature of polymer	Particle diameter (d_p) (μm)[a]	Fractionation range ($M_r \times 10^{-3}$)[b]
Bio-Gel				
A-0.5M	Bio-Rad	Agarose	40–80; 80–150; 150–300	10–500
A-1.5M			40–80; 80–150; 150–300	10–1500
A-5M			40–80; 80–150; 150–300	10–5000
A-15M			40–80; 80–150; 150–300	40–15 000
A-50M			40–80; 80–150; 150–300	100–50 000
A-150M			40–80; 80–150; 150–300	1000–150 000
Cellufine				
GCL-25	Amicon	Cellulose	84–45; 45–105	0.1–4
GCL-90			84–45; 45–105	0.5–40
GCL-300			84–45; 45–105	1–100
GCL-700			84–45; 45–105	20–300
GCL-1000			84–45; 45–105	2–600
GCL-2000			84–45; 45–105	2–3000
Sephadex				
G-10	Pharmacia	Dextran	40–120	0–0.7
G-15			40–120	0–1.7
G-25			10–40; 20–80; 50–150	1–5
G-50			10–40; 20–80; 50–150	1.5–30
G-75			10–40; 40–120	3–70
G-100			10–40; 40–120	4–150
G-150			10–40; 40–120	5–400
G-200			10–40; 40–120	5–800
Sepharose				
6B	Pharmacia	Agarose	45–165	10–4000
4B			60–140	60–20 000
2B			60–200	70–40 000
CL-6B		Cross-linked agarose	45–165	10–4000
CL-4B			60–140	60–20 000
CL-2B			60–200	70–40 000
Ultrogel				
A-6	Sepracor	Agarose	60–140	25–2400
A-4			60–140	55–9000
A-2			60–140	120–23 000

[a] Spherical particles.
[b] Globular macromolecules.

exclusion limit is lower when the molecule is linear.

To achieve high levels of resolution or to determine the best compromise between the resolution and the flow-rate, different particles sizes are generally proposed from about 30 to 200–300 μm diameter.

The mechanical strength of gel filtration media is dependent on the chemical nature of the matrix, the cross-linking agent and its ratio and the amount of solid polymer. Most gel filtration media are deformable when subjected to pressure; when compressed by high flow-rates, an empty space is formed at the top of the column that could have an adverse effect on the overall separation efficiency.

TABLE 3

PREPARATIVE SYNTHETIC-BASED GEL FILTRATION MEDIA FOR BIOLOGICAL MACROMOLECULES

Trade name and type	Supplier	Nature of polymer	Particle diameter (d_p)(μm)[a]	Fractionation range ($M_r \times 10^{-3}$)[b]
Bio-Gel				
P-2	Bio-Rad	Polyacrylamide	40–80	0.1–1.8
P-4			40–80; 80–150	0.5–4
P-6			40–80; 80–150	1–6
P-10			40–80; 80–150	1.5–20
P-30			80–150; 150–300	2.5–40
P-60			80–150; 150–300	3–60
P-100			80–150; 150–300	5–100
P-150			80–150; 150–300	15–150
P-200			80–150; 150–300	30–200
P-300			80–150; 150–300	60–400
Fractogel				
HW-40	Merck	Vinyl polymer[c]	25–40; 32–63	0.1–10
HW-50			25–40; 32–63	0.5–200
HW-55			25–40; 32–63	1–1000
HW-65			25–40; 32–63	50–5000
HW-75			25–40; 32–63	500–50 000
Separon				
Hema-Bio 40	Tessek	Poly-HEMA[d]	60 ± 20	40–80
Hema-Bio 100			60 ± 20	80–250
Hema-Bio 300			60 ± 20	250–800
Hema-Bio 1000			60 ± 20	800–2000
Toyopearl				
HW-40	TosoHaas	Vinyl polymer[c]	25–40; 32–63	0.1–10
HW-50			25–40; 32–63	0.5–200
HW-55			25–40; 32–63	1–1000
HW-65			25–40; 32–63	50–5000
HW-75			25–40; 32–63	500–50 000
Trisacryl				
GF-05	Sepracor	Poly-THMMA[e]	40–80; 80–150	0.2–2.5
GF-2000			40–80; 80–150	10–15 000

[a] Spherical shape.
[b] Globular molecules.
[c] No other information available from supplier.
[d] Hydroxymethyl methacrylate.
[e] N-Tris(hydroxymethyl)methylacrylamide.

Generally, gel filtration media do not show parasitic interactions with solutes. However, several polymers show non-specific adsorption under particular conditions of ionic strength and pH. They may exhibit weak electrostatic interactions that could be eliminated by increasing the buffer salinity; they may exhibit weak hydrophobic characteristics that can be cancelled by decreasing the ionic strength or by adding small amounts of chaotropic substances.

5. SORBENTS FOR ION EXCHANGE

Within the groups of sorbents for protein separation, ion exchangers are the most commonly used and valuable for large-scale applica-

TABLE 4

COMPOSITE-BASED GEL FILTRATION MEDIA FOR BIOLOGICAL MACROMOLECULES

Trade name and type	Supplier	Nature of polymer	Particle diameter (d_p)(μm)[a]	Fractionation range ($M_r \times 10^{-3}$)[b]
Sephacryl				
S-100	Pharmacia	Allyldextran–MBA[c]	20–75; 40–105	1–100
S-200			20–75; 40–105	5–250
S-300			20–75; 40–105	10–1500
S-400			20–75; 40–105	20–8000
S-500			20–75; 40–105	40–2000
S-1000			20–75; 40–105	500–100 000
Superdex				
75	Pharmacia	Agarose–dextran	22–44	3–70
200			22–44	10–600
Ultrogel				
AcA-202	Sepracor	Polyacrylamide–agarose	60–140	1–15
AcA-54			60–140	5–70
AcA-44			60–140	10–130
AcA-34			60–140	20–350
AcA-22			60–140	100–1200

[a] Spherical particles.
[b] Globular macromolecules $M_r < 1.5 \cdot 10^6$; polysaccharides $M_r > 5 \cdot 10^6$.
[c] MBA = methylene-bis-acrylamide.

tions. They are composed of a polymeric porous structure (as described earlier) where electrically charged chemical groups are covalently attached. Mechanisms of ion exchange have been extensively studied [54] and are easily applicable to protein separations as a protein in a polyelectrolyte with a dominant net charge modulated by the environmental pH.

It is not the purpose of this paper to explain the laws governing the fundamental mechanisms of ion-exchange interactions but rather to discuss the influence of the ion-exchange sorbent on the separation performance. Different aspects are explained in the following sections.

5.1. Nature of charged groups

A wide variety of charged groups attached to a polymeric matrix are commercially available and are classified into two categories: anionic and cationic. Anionic groups include strong acids such as sulphonates, sulphates and phosphates and weak acids such as carboxylates. Cationic groups are essentially represented by tertiary amino groups (weak) and quaternary amino groups (strong). All these chemical groups are attached to a polymeric backbone through a short, medium or long hydrocarbon chain by means of ether, alkylamine or amido bonds.

Table 5 summarizes the different possible configurations of ionic groups and their mode of anchorage to the matrix.

Sulphates are generally attached to polysaccharidic networks; these derivatives can be used in any applications of ion exchange of pH <7. In several cases, as they mimic some natural polysaccharidic structures such as heparin, they have been used successfully to separate coagulation factors under better conditions than with classical sulphonates [55]. Sulphonate groups are more common and are attached either directly to the polymer or via a hydrocarbon chain. Alternatively, they can be attached by direct copolymerization of sulphonated monomers [7] or by chemical reaction with sulphonation reagents [56].

Carboxylates, generally obtained by alkaline

TABLE 5

ION-EXCHANGE GROUPS USED IN CHROMATOGRAPHY OF PROTEINS

Group	Structure	Attachment to matrix	p*K*	Example of sorbents
Sulphate	$-OSO_3H$	Ether linkage	<2	SP-Spherodex
Sulphonate	$-(CH_2)_nSO_3H$	Ether linkage	<2	SP-Toyopearl
Phosphate	$-OPO_3H_2$	Ether linkage	<2 and 6	Phosphocellulose
Carboxylate	$-(CH_2)_nCOOH$	Ether linkage Amido linkage	3.5–4.2	CM-Sepharose
Tertiary amine	$-(CH_2)_n-\overset{\overset{C_2H_5}{\vert}}{\underset{\underset{C_2H_5}{\vert}}{N^+}}-(CH_2)_2-\overset{\overset{C_2H_5}{\vert}}{\underset{\underset{C_2H_5}{\vert}}{N}}$	Ether linkage	8.5–9.5	DEAE-Trisacryl
Quaternary amine	$-(CH_2)_n-N^+\equiv(R)_3$	Ether linkage Amido linkage	>9	Q-HyperD

reactions of chloroacetic acid on hydroxyl-containing polymers [1], are widely used as cation exchangers but in a more pH restricted range than sulphonated resins.

Diethylaminoethyl (DEAE) groups, well known in protein separations, are complex structures resulting from reaction between diethylaminoethyl chloride and a non-ionic sorbent under alkaline conditions that induce secondary reactions on the monomer itself, generating oligo-DEAE chains. These complex structures with different p*K* values are evidenced easily by titration curves and do not modify the ion-exchange mechanism with proteins. Their stability is a concern, however, and is mentioned in Section 9.4.

Quaternary amino groups are well known structures; owing to their high p*K* values they can be used in a wider pH range than DEAE materials with in most instances a higher selectivity in separating anionic proteins.

5.2. *Major ion exchangers used for preparative protein separation*

Ion-exchange sorbents for protein separation are characterized essentially by their anionic or cationic nature and their ability to adsorb reversibly a protein model (generally BSA or haemoglobin) under well defined conditions. In contrast to gel filtration media, the matrix does not have a defined pore size. Here the pores are larger to avoid any possible molecular sieving effects during separation. Available ion exchangers have various mechanical resistances and are based on natural, synthetic, mineral and composite materials.

A general overview of commercial ion exchangers is given in Table 6. However, to give in greater detail the individual characteristics of these sorbents, a specific technical analysis is given.

5.3. *Comparative physico-chemical and dynamic performances*

For preparative applications, productivity is the most important aspect to consider. In a first approach, productivity is the result of the combination of sorption capacity and flow-rate. Sorption capacity in fact varies in a non-linear fashion with the flow-rate and the extent of this variation depends considerably on the sorbent structure.

Information on all parameters having a direct impact on the productivity are not easily available from producers and only limited data can be obtained. To understand the simple influence of flow-rate on dynamic sorption capacity as has been clearly defined [57], some date are shown in Fig. 3.

It can be observed that some sorbents undergo a low dynamic sorption capacity decrease with increasing flow-rate, such as Poros and Fractogel EMD; others, *e.g.*, Sepharose Fast Flow, show a

TABLE 6

LARGE-SCALE ION-EXCHANGE MEDIA FOR SEPARATION OF BIOLOGICAL MACROMOLECULES

Trade name and type	Supplier	Nature of polymer	Ion-exchange groups[a]
Accell	Waters	Silica–acrylic polymer	QMA, CM
Bio-Gel A	Bio-Rad	Agarose	DEAE, CM
Cellufine-Matrex	Amicon	Beaded cellulose	DEAE, CM, sulphate
Cellulose	Whatman	Fibrous cellulose	DE, CM, SE, phospho
Fractogel EMD	Merck	Vinylic grafted polymer	TMAE, DEAE, DMAE, SP, CM
Separon HEMA-Bio	Tessek	Acrylic polymer	Q, DEAE, CM, SB
HyperD	Sepracor	Composite shell + synthetic gel	Q, S
Macroprep-50	Bio-Rad	Synthetic polymer	Q, S, CM
Poros	Perseptive	Coated polystyrene	Q, S
Sephacel	Pharmacia–LKB	Beaded cellulose	DEAE
Sephadex	Pharmacia–LKB	Dextran	QAE, DEAE, CM, SP
Sepharose	Pharmacia–LKB	Agarose	DEAE, CM
Sepharose Fast Flow	Pharmacia–LKB	Cross-linked agarose	Q, DEAE, CM, S
Silica-PAE	Amicon	Silica–polyethyleneimine	Various amino groups
Spherodex	Sepracor	Silica–dextran	DEAE, SP
Spherosil	Sepracor	Grafted silica	QMA, DEA, C, S
Toyopearl	TosoHaas	Vinylic polymer	QAE, DEAE, CM, SP
Trisacryl	Sepracor	Macorporous synthetic polymer	QA, DEA, CM, SP
Trisacryl Plus	Sepracor	Base-resistant polymer	DEAE, SP
Ultrogel	Sepracor	Cross-linked agarose	Phospho
Zephyr	Sepracor	Silica–dextran	D, S

[a] DEAE and D = diethylaminoethyl; CM = carboxymethyl; Q = quaternary amino groups; S = sulphonate groups; SP = sulphopropyl groups; QMA = quaternary aminomethyl groups; SE = sulphoethyl groups; phospho = phosphoester groups; TMAE = tetramethylaminoethyl groups; DMAE = dimenthylaminoethyl groups; SB = sulphobutyl groups.

rapid decrease in dynamic binding capacity with increasing flow-rate. Sorbents such as Sepharose HP, SuperQ and HyperD show a moderate decrease in dynamic capacity and their differentiation with respect to productivity performance is dependent on the absolute level of the dynamic capacity. In this experimental context and independently of any possible lot-to-lot variations that can affect all sorbents, HyperD seems to behave substantially better than the others.

The loadibility of an ion exchanger at a specified flow-rate is a major feature with regard to productivity. Variations with sample input depend on a number of factors linked to the structure of the ion exchanger. The number of active ionic sites must be high enough to cover all the polymer surface area; additionally, the availability of these sites must be maximized. However, between pellicular-based sorbents with a very high availability of ionic sites but low sorption capacity and dense polymeric networks with much higher surface area covered with more ionic sites but with low diffusivity, a compromise must be found.

The analysis of the curves obtained by plotting dynamic sorption capacity *versus* flow-rate are of major importance for the user to identify the best productivity level for a specific fractionation.

High productivity unfortunately does not necessarily mean high throughput (maximum amount of protein purified per unit time at a desired level of purity). Resolution variation *versus* flow-rate is obviously of importance when calculating the productivity of an ion exchanger. With respect of what was described earlier, it can be considered here that highly selective ion-exchange phases (generally those having quaternary ammonium and sulphonate groups), combined with a high static capacity and large pores in a three-dimensional network, constitute the best choice for large-scale preparative applica-

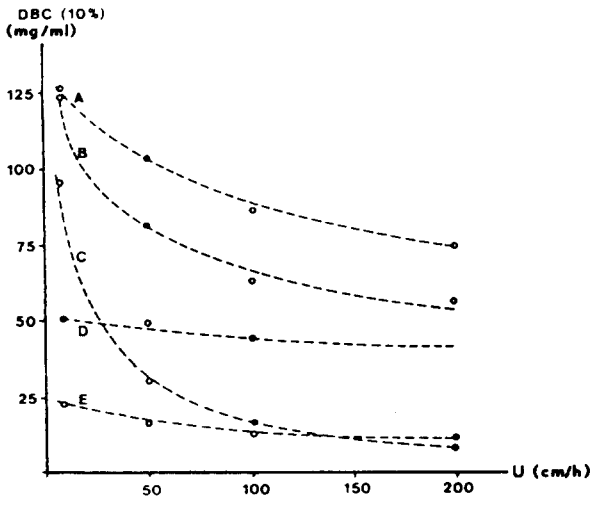

Fig. 3. Variation of dynamic binding capacity (*DBC*) as a function of the flow-rate (*U*) for ion exchangers with quaternary ammonium groups. Determinations of *DBC* were performed using breakthrough curves (10%) with a column of 1 cm I.D. containing 4 ml of sorbent. Protein solution was bovine serum albumin at 5 mg/ml in 50 m*M* Tris–HCl buffer (pH 8.6). (A) Q-HyperD F; (B) Super Q; (C) Q-Sepharose Fast Flow; (D) Q-Fractogel EMD; (E) Q-Poros.

tions. At this point, however, the sorbent must not be limited in flow characteristics. In addition to the productivity of a separation cycle, it is also fundamental on a preparative scale to analyse the impact of the sorbent behaviour in the context of a multi-cycle operation process.

Re-equilibration factors, regeneration, recovery levels and lifetime are other important parameters to be taken into account. The re-equilibration factor contributes also to the productivity because it influences the length of a separation cycle; it is defined as the minimum amount of buffer (volumes per column volume) needed to restore the correct ionic strength and pH in order to start another run. This parameter is not linked to the number of ionizable groups, but rather to the pH drift generated when passing from a concentrated to a diluted buffer (for details see Fig. 4).

Table 7 shows that sorbents with a relatively small number of ionizable groups, such as DEAE Bio-Gel A, possess a relatively high re-equilibration factor; conversely, some other sorbents with a high level of ionic groups need a small buffer volume for complete re-equilibration (*e.g.*, DEAE-Trisacryl). The sorbent efficiency therefore gives the level of accessibility of ionic groups (steric hindrance, sorbent porosity) for macromolecules such as bovine serum albumin.

6. SORBENTS FOR AFFINITY CHROMATOGRAPHY OF PROTEINS

Affinity separation takes advantage of selective and reversible complexation between the

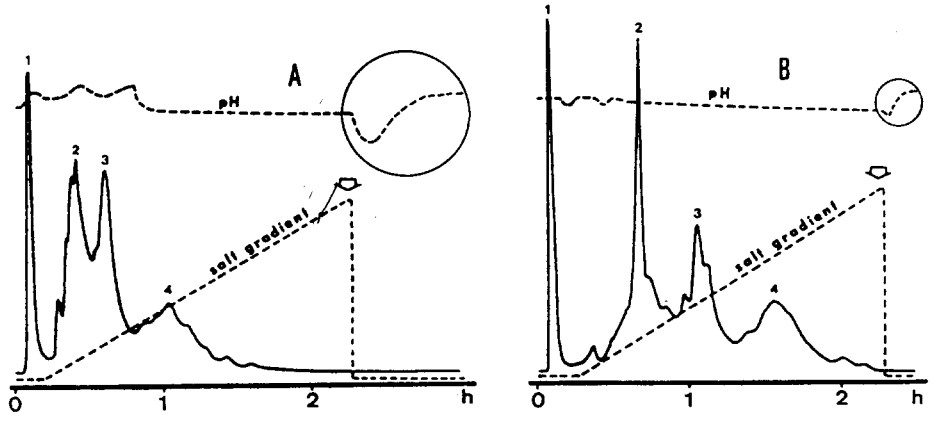

Fig. 4. Chromatographic separations of a protein mixture consisting of (1) cytochrome *c*, (2) haemoglobin, (3) ovablumin and (4) β-lactoglobulin on (A) DEAE-Spherodex and (B) Q-HyperD. The particle size of both sorbents was between 25 and 55 μm. Columns, 9 cm × 1 cm I.D.; buffer, 50 m*M* Tris–HCl (pH 8.6); elution, salt gradient up to 0.4 *M* in the same buffer; flow-rate, 50 cm/h. At the end of the elution gradient, re-equilibration of the column was effected with the initial buffer (arrows). pH drift was recorded on-line to determine the amount of buffer needed to restore the initial pH (see circled areas).

TABLE 7

GENERAL CHARACTERISTICS OF MAIN ION EXCHANGERS FOR PREPARATIVE PROTEIN SEPARATIONS

Ion exchanger	Amount of ionic groups (μequiv./ml)[a]	BSA sorption capacity (mg/ml)[b]	Sorption efficiency (mg/μequiv.)	Re-equilibration volume (volume of buffer per column volume)[c]
QMA Accell	104	111	1.06	1.8
DEAE Bio-Gel A	16	38	2.37	2.5
DEAE Cellufine	186	113	0.61	5.3
DEAE Cellulose	373	131	0.35	2.9
DEAE Fractogel 650	132	32	0.24	1.1
DEAE-Separon Hema-Bio 1000	320	70	0.21	Un[d]
DEAE Sephacel	116	131	1.13	2.3
DEAE Sepharose CL 6B	161	164	1.02	4.8
DEAE Sepharose Fast Flow	132	88	0.66	3.8
DEAE Spherodex	104	98	0.94	3.6
DEAE Trisacryl	340	86	0.25	1.5
PAE Matrex	244	42	0.17	4.5
Q Fractogel EMD	35	55	1.57	1.2
Q Poros II	375	40	0.10	5.9
Q Sepharose Fast Flow	179	115	0.64	1.5
Q Super	198	127	0.64	1.7
Q HyperD	160	125	0.83	1.2

[a] Determined experimentally by breakthrough curve; titration with 50 m*M* HCl on alkaline-regenerated sorbent.
[b] Determined for BSA in 50 m*M* Tris–HCl buffer (pH 8.6).
[c] Calculated on the passage from a 50 m*M* Tris–HCl buffer containing 1 *M* NaCl (pH 8.6) to 50 m*M* Tris–HCl (pH 8.6).
[d] Un = unknown. All data for DEAE-Separon Hema-Bio 1000 were from Tessek and related to 1 g of dry material; transformation to ml was calculated assuming that the swelling volume of Hema-Bio beads is 2.5 ml/g.

protein to be purified and another complementary molecule called the ligand. Among several existing affinity techniques [58], affinity adsorption involves a solid-phase adsorbent on which the ligands are chemically attached. The term affinity chromatography is generally used to cover a wide range of adsorption chromatographic approaches in which the biospecific recognition between the two molecules is the result of complex multivalent interactions.

The association of a protein with an immobilized ligand on a porous macromolecular matrix is dependent on individual or combined physicochemical interactions: ion-exchange effects; hydrogen bonding; hydrophobic associations [59]; π electron interactions [60]; metal chelation interaction; covalent bonding.

Briefly, an affinity sorbent is prepared by the attachment of an identified ligand on an inert porous matrix by means of a chemical reaction. This concept and the design of affinity chromatographic media involves numerous considerations to guide choices at different levels. The selection of specific ligands, the identification of the most appropriate immobilization chemistry and the choice of the matrix are obviously parameters of importance.

Affinity mechanisms of separation are highly selective; they are frequently protein specific or can be used for group separation. Affinity separation is an on–off method consisting of three steps, adsorption, elution and regeneration; as all the steps can be accomplished rapidly the choice of the matrix is determined by its high level of porosity, its high rigidity and by the possibility of developing defined surface chemistries for ligand immobilization.

6.1. Selection of basic media for affinity applications

The selection of the appropriate solid matrix is a consequence of a rational approach to keep all the affinity molecular mechanisms free from any

side-effects. The pore size must obviously be large enough to help the free diffusion of macromolecules for a better interaction with the ligand active sites. Inertness of the matrix is also important to maintain the solute–ligand interaction as "pure" as possible without any additional non-specific binding, which would decrease the level of selectivity.

The matrix is also selected in order to have available chemical groups where ligands can be chemically attached after treatment with activating agents. These groups are most frequently hydroxyl groups, which are at the same time very hydrophilic, devoid of any non-specific binding and chemically derivatizable. In special cases, the solid matrix may contain amido, carboxyl or amino groups to adapt the immobilization chemistry.

Cross-linked agarose and macromolecular hydrophilic polymers are well known as basic matrices for making affinity sorbents.

6.2. *Affinity ligands*

Ligands are molecules of different nature and origin that have the property of forming a specific reversible complex with the protein to be separated. They can be of small size (*e.g.*, amino acids, nucleotides, haptens, sugars, chelators or dyes) or of large size [*e.g.*, proteins (antibodies, lectins, receptors), polysaccharides (*e.g.*, heparin) and nucleic acids].

Very specific ligands bind one type of protein whereas general ligands can interact with whole classes of proteins less specifically. The extent of the affinity between a ligand and the adsorbed proteins is defined by the affinity constant. The normal range of affinity constants is between 10^4 and 10^8 l/mol. Above this value desorption requires drastic denaturing conditions; below 10^4 l/mol the interaction does not result in a real adsorption but rather in a delay of the interacting protein in crossing the chromatographic column.

Depending on their nature, ligands for affinity chromatography can be fragile. This is particularly evident with proteinaceous ligands such as antibodies, receptors and lectins. Repeated association–dissociation steps, extended contact with crude material, treatment with drastic washing solutions and the presence of protease traces in the feeds modify or destroy these ligands. Partial losses of ligands from affinity sorbents related to leakage mechanisms reduce the sorption capacity over the separation cycles and limit the life span of these materials.

Most generally, the smaller the ligand size, the lower is its sensitivity to external aggression. The success of an affinity sorbent synthesis depends on several factors such as matrix effects, chemical linkage effects and ligand choice.

6.3. *Immobilization chemistries*

Ligand immobilization consists of two distinct operations called matrix activation and ligand immobilization. The design of an affinity sorbent implies a number of choices to obtain the most suitable solution. The decision process obviously starts with the nature of protein to be purified and the impurities to be eliminated, which allows the appropriate ligand to be chosen.

Depending on the respective molecular sizes of the ligand and of the protein, a matrix with a medium or large pore structure will be adopted. The activation reaction should also be considered as a function of the nature of the ligand and the chemical composition of the matrix.

Activation reactions which consist in placing in contact a chosen matrix and an activating reagent is not a trivial operation. Inadequate control of the reaction can result in micro- or macroheterogeneity, hence the activating agent concentration, temperature, mode of agitation, reaction time and solvent choice are important considerations. Various activating agents are not soluble in aqueous systems and must be used in organic solvents, which may not be compatible with the hydrophilic nature of the matrix. Cross-linking agents are also used as activating materials and under certain conditions can decrease the size of the matrix pores, with obvious consequences on the diffusivity of the macromolecules. A list of the most common activating agents is given in Table 8.

Ligand coupling reactions most frequently involve nucleophilic attack, meaning that any contact of the activated matrix with nucleophiles

TABLE 8

LIST OF MAIN ACTIVATING AGENTS USED WITH HYDROXYL-CONTAINING MATRICES

Chemical activating agent	Activation reaction medium	Linkage produced on the matrix
Acyl chlorides	Acidic	Ester
Benzoquinone	Slightly alkaline	O-Quinone derivative
Bisepoxirane	Alkaline	Ether
Carbodiimide (water soluble)	Slightly acidic	Isourea ester[a]
Carbonyldiimidazole	Anhydrous	Carbonate
Cyanogen bromide	Alkaline	Imidocarbonate
Cyanodimethylaminopyridinium	Alkaline	Cyanate
Divinyl sulphone	Alkaline	Ether
Ethoxycarboxylethoxydihydroquinoline	Slightly acidic in ethanol	Mixed anhydride[a]
Epichlorohydrin	Alkaline	Ether
Fluoromethylphyridine	Anhydrous	O-Methylpyridine
Glutaraldehyde	Neutral or acidic	Acetal or Michael adducts
Nitrophenyl chloroformate	Anhydrous	O-Active ester
Periodates	Neutral	Shiff bases[b]
Tosyl chloride	Alkaline	Tosylate
Tresyl chloride	Alkaline	Tresylate
Trichlorotriazine	Aqueous–organic	O-Triazine chloride

[a] On carboxy-containing sorbents.
[b] After immobilization of amino-containing ligand.

except the ligand must be avoided. During coupling reactions in aqueous buffers, the ligand competes with water molecules which hydrolyse activated sites. This undesirable reaction, which occurs with premature exposure of the activated matrix in aqueous solutions, decreases considerably the potential coupling capacity of the support and may introduce non-specific binding sites on the matrix [61]. Depending on the nature of the activating agent, aqueous hydrolysis can produce chemical derivatives at the surface of the matrix that constitute possible sites for non specific binding; however, with certain activating agents, aqueous hydrolysis restores the initial structure of the matrix. This is the case, for instance, with nitrophenyl chloroformate, 2-fluoro-1-methylpyridinium toluene-4-sulphonate, carbonyldiimidazole and tosyl chloride.

Activation of the desired ligand which can react with the matrix can be a useful alternative to the above-mentioned approach. The coupling reaction should be chosen so that biological inactivation of the ligand is avoided or minimized; pH, temperature, time and protecting agents (*e.g.*, sugars when immobilizing lectins) should be selected accordingly.

After ligand coupling, unreacted groups have to be capped with small hydrophilic molecules without damaging the immobilized active ligand.

6.4. *Ready-to-use activated sorbents*

To help the user in the complex procedure of obtaining activated sorbents, a number of specialized companies provide ready-to-use solutions. As the combination possibilities are enormous (type of matrix, activating agent, degree of activation, etc.) ready-to-use affinity sorbents probably represent the largest list among chromatographic materials. To simplify the presentation, the main preactivated sorbents and group-specific sorbents are listed in Tables 9 and 10.

In affinity chromatographic separations, it is common to distinguish several main subgroups such as dye-ligand affinity chromatography, metal chelate affinity chromatography, covalent

TABLE 9

MAIN PREACTIVATED AFFINITY PACKINGS (SORBENTS WITH SPACERS ARE NOT CONSIDERED)

Nature of matrix	Activating agent	Active group for coupling	Ligand specificity	Trade name	Supplier
Agarose	Cyanogen bromide	Imidocarbonate	NH_2	CNBr-activated Sepharose 4B	Pharmacia–LKB
Agarose	Bisepoxirane	Epoxy	NH_2, OH, SH	Epoxy-activated Sepharose 6B	Pharmacia–LKB
Agarose	N-Hydroxysuccinimide	N-Hydroxy-succinimide ester	NH_2	Activated CH-Sepharose 4B	Pharmacia–LKB
Agarose	N-Hydroxysuccinimide	N-Hydroxy-succimimide ester	NH_2	Affi-Gel 10 and 15	Bio-Rad
Agarose	Carbonyldiimidazole	Imidazolecarbamate	NH_2	Reactigel	Pierce
Agarose	Unknown	Thiol	SH, heavy metals, double bonds	Activated-thiol Sepharose 4B	Pharmacia–LKB
Agarose	Unknown	Thiol	SH, heavy metals, double bonds	Thiopropyl-Sepharose 6B	Pharmacia–LKB
Agarose	Tresyl chloride	Tosylate	NH_2, SH	Tresyl-activated Sepharose 6B	Pharmacia–LKB
Agarose	Fluoromethylpyridinium	O-Methylpyridinium	NH_2, SH	Avid-Gel	Bio-Probe
Cellulose	Glutaraldehyde	Aldehyde	NH_2	Formyl-Cellufine	Amicon
Acrylic and vinyl polymers	Glutaraldehyde	Aldehyde	NH_2	Glut-Act-Trisacryl	Sepracor
Acrylic and vinyl polymers	*p*-Nitrophenyl-chloroformiate	O-Active ester	NH_2	NPC-Trisacryl	Sepracor
Acrylic and vinyl polymers	Tresyl chloride	Tosylate	NH_2, SH	Toyopearl AF Tresyl 650 M	TosoHaas
Acrylic and vinyl polymers	Bisepoxirane	Epoxy	NH_2, OH, SH	Toyopearl AF Epoxy 650 M	TosoHaas
Acrylic and vinyl polymers	Epichlorohydrin	Epoxy	NH_2, SH, OH	Epoxy-Fractogel	Merck
Acrylic and vinyl polymers	Carbonyldiimidazole	Imidazolecarbamate	NH_2	CDI-Fractogel	Merck
Polyacrylamide–agarose	Glutaraldehyde	Aldehyde	NH_2	Act-Ultrogel AcA-22	Sepracor

TABLE 10

GROUP-SPECIFIC SORBENTS FOR AFFINITY CHROMATOGRAPHY

Class of immobilized ligands	Class of proteins to be separated	Suppliers[a]
Lectins	Glycoproteins and glycoconjugates	1,2,5,7
Dyes	Various proteins and NAD-dependent enzymes	1,2,3,4,5,6,7
Heparin	Coagulation-related factors and growth factors	1,2,3,4,5,7
Protein A	Antibodies of IgG class	1,2,3,4,5,6,7,8
Boronates	Glycosylated proteins	5,6,7
Metal chelators	Metal-interacting proteins, histidine-rich structures	1,5,6,7,8
Lipopolysaccharide-binding ligands	Endotoxins	2,5,7

[a] 1 = Pharmacia–LKB; 2 = Sepracor; 3 = Merck; 4 = TosoHaas; 5 = Bio-Rad; 6 = Amicon; 7 = Pierce; 8 = Perseptive Biosystems.

chromatography and immunoaffinity chromatography.

6.5. Dye-ligand sorbents

Reactive textile dyes covalently immobilized on a porous hydrophilic matrix constitute a large class of affinity adsorbents, some of which are ready to be used and others are accessible by a simple and rapid immobilization [62].

A single rective dye permits an affinity column useful for the purification of a number of biochemicals to be prepared. It can simultaneously adsorb many different proteins from a crude extract or only a few according to the starting conditions. Its selectivity can also be exploited by using competitive displacers in the mobile phase, permitting the specific elution of the desired protein.

Typically, NAD- and NADP-dependent enzymes are easily separated; DNA-related enzymes are also adsorbed on dyes non-specifically. A few examples exist, however, where the specificity of a dye for a given molecule is sufficiently high that the desired target molecule can be isolated in a single step [63].

Dye adsorbents are inexpensive for large-scale applications, where they can be used repeatedly after regeneration involving acidic and alkaline solvents and chaotropic solutions.

6.6. Chelating sorbents

Transition metals can form complexes with electron-rich compounds (aromatic molecules and olefins) and may coordinate molecules containing O, N and S by ion–dipole interactions [64]. These special metal interactions can be utilized in metal chelate protein chromatography.

In practice, the metals have to be trapped on a chromatographic support without destroying their capacity to form a complex with the solute. Nevertheless, to immobilize the metal, it is necessary to couple on the solid matrix a compound that is able to ligate metals. The ligands used to chelate the metals, are, *e.g.*, aminosalicylic acid, 8-hydroxyquinoline, carboxymethylated amino acids, iminoacetic acid and EDTA. Iminoacetic acid is the most widely used ligand, forming different multi-coordinated metal complexes according to the spatial situation. Metals chelated with iminoacetic acid show reversible interactions with certain amino acids such as cysteine and histidine. Other amino acids such as trytophan, tyrosine and phenylalanine are affected by Cu-, Ni- or Zn-chelated gels.

6.7. Sorbents for covalent chromatography

In covalent chromatography, the purification of the product is carried out through the formation of a reversible covalent bond with the immobilized ligand. This affinity sorbent is synthesized by the immobilization of a ligand having either thiol or mercury groups. The former system initiates the adsorption of thiol-containing molecules by a thiol disulphur exchange with

the formation of a mixed disulphur bond. In the latter system, the adsorption is concomitant with the formation of Hg–S bonds. The covalently adsorbed product can be eluted by reduction of the S–S bonds or by competition with other thiol molecules. Only few thiol-containing ligands are used for the synthesis of covalent-based affinity sorbents such as cysteine, N-acetylhomocysteine thiolactone and glutathione; some thiol-containing sorbents are commercially available through the main suppliers listed in Section 12.

6.8. Sorbents for immunoaffinity chromatography

In immunoaffinity sorbents the ligand is a specific antibody. The selectivity of such sorbents is obviously extremely high and consequently they are only usable on a case by case basis. The matrices on which these macromolecular ligands must be chemically attached are very macroporous and the immobilization technique must preserve the active sites against denauturation and steric inaccessibility. Immobilization via the Fc fragment side is generally preferred and, to favour this orientation, the coupling method frequently involves the glycosidic moiety of the antibody.

In spite of their high cost, a few examples of large-scale immunoaffinity chromatography are known; their main weaknesses are the hydrolysis of the protein ligand in the presence of proteases and the risk of leakage.

7. SORBENTS FOR HYDROPHOBIC INTERACTION CHROMATOGRAPHY

Hydrophobic interaction chromatography (HIC) is a special type of affinity chromatography involving the association of lipophilic regions of certain amino acids of the protein and hydrophobic ligands immobilized on hydrophilic gels [65]. The hydrocarbon regions which are responsible for the hydrophobic character are either aliphatic or aromatic or both, and possess the common properties of excluding water and forming a hydrophobic association, resulting in the rearrangement of water molecules.

Hydrophobic interactions are relatively strong and their strength is dependent on the nature of the molecule and can be influenced by salt concentration and by temperature.

Experimentally, a support for hydrophobic chromatography can be synthesized by coupling hydrophobic hydrocarbon chains on an inert activated matrix. The adsorption of a protein by hydrophobic interaction is generally performed at high ionic strength. Elution of the adsorbed material is achieved by a decreasing ionic strength gradient, induced by addition of detergents, urea, ethylene glycol or chaotropic agents such as potassium isothiocyanate or sodium perchlorate.

A number of hydrophobic ligands have been described and coupled to an insoluble matrix for the separation of proteins and enzymes; butyl, octyl and phenyl groups are the most popular. Pentyl, hexyl, dodecyl, palmityl, naphthoyl and trityl groups have also been reported for a number of applications. In contrast to reversed-phase sorbents where there is a need to cover all the available surface of a sorbent with hydrocarbon chains, in HIC the degree of substitution is lower and the balance between hydrophilic and hydrophobic groups gives the appropriate binding capacity and specificity.

Basic sorbents for hydrocarbon chain grafting are generally based on both agarose and hydrophilic synthetic polymers where hydrophobic ligands are chemically attached via non-ionic linkages. The most common way to immobilize these ligands is to use an oxirane-activated matrix yielding ether bonds. More recently, however, a thioether linkage obtained by reacting oxirane-activated supports with thiols [66] has been described and used successfully for specific applications.

Phenyl-substituted sorbents are more often used with uncharacterized proteins. Their degree of hydrophobicity is intermediate and the interaction occurs with aromatic residues of amino acids of proteins. Whereas phenyl groups are used for strongly hydrophobic proteins, aliphatic chains are used for weakly hydrophobic structures.

Sorbents for HIC are commercially available and the best known materials are reported on the Table 11.

TABLE 11

MAIN SORBENTS AVAILABLE FOR PREPRATATIVE HYDROPHOBIC INTERACTION CHROMATOGRAPHY

Hydrophobic Interacting group	Chemical nature of matrix	Amount of hydrophobic ligands (μmol/ml)	Sorption capacity (mg/ml)[a]	Trade name	Supplier
Butyl	Vinyl polymer	1000	40	Butyl Fractogel	Merck
	Cellulose	Unknown	5–15	Butyl Cellufine	Amicon
	Vinyl polymer	Unknown	Unknown	Butyl Toyopearl	TosoHaas
Octyl	Agarose	50	30–50	Octyl-Sepharose[b]	Pharmacia–LKB
	Cellulose	Unknown	5–15	Octyl-Cellufine	Amicon
Phenyl	Polyacrylate	600	Unknown	Phenyl-Separon	Tessek
	Agarose	50	30–50	Phenyl-Sepharose[b]	Pharmacia–LKB
	Cellulose	Unknown	3–10	Phenyl-Cellufine	Amicon
	Vinyl polymer	Unknown	Unknown	Phenyl-Toyopearl	TosoHaas
Oligoethylene oxide	Vinyl polymer	Unknown	Unknown	Ether-Toyopearl	TosoHaas

[a] Values given by the manufacturers according to the proteins and working conditions.
[b] A Fast Flow grade is also available.

8. HYDROXYAPATITE SORBENTS FOR PROTEIN SEPARATION

The use of hydroxyapatite for protein separations was first described by Tiselius *et al.* [67]. This inorganic sorbent consists of a mosaic of crystals obtained through a combination of phosphate and calcium salts after a number of steps of wet alkaline treatment and heat ageing. During the maturation process several forms of complex calcium phosphate are formed that display various properties towards protein adsorption.

The principles of adsorption and desorption described for ion-exchange chromatography are not applicable to hydroxyapatite. Here the interaction mechanisms are more complex and have been demonstrated after specific modifications of protein structure. Carboxyl groups have been modified in addition to amino groups along with inversion of protein charge to understand the mechanism of protein–hydroxyapatite interactions [68]. The hydroxyapatite surface is essentially electronegative when equilibrated with phosphate buffers at neutral pH owing to neutralization of positive calcium sites by phosphate ions. Amino groups of proteins thus interact electrostatically with the hydroxyapatite surface; the lower the pH of the equilibrating buffer, the higher is the molarity of the buffer necessary for elution. Basic proteins are eluted by displacement with phosphate or chloride gradients or calcium salts. Free carboxyl groups of proteins interact with calcium sites by complexation; the displacement of acidic proteins is possible by ions that form stronger complexes with calcium-like phosphates and fluorides. For the carboxyl–calcium interaction, an increase in buffer ionic strength, *e.g.*, using sodium chloride, is ineffective in desorbing proteins [69]. The use of calcium ions is also ineffective in this instance as a result of the formation of more calcium loci with an enhanced number of carboxy–calcium interactions.

Hydroxyapatite has been ignored for a long time in medium- and large-scale applications. It is known that the crystals are very fragile; "fines" are present in most of the preparations, leading to column clogging.

Only a few chromatographic suppliers offer preparative hydroxyapatite adsorbents (see Table 12). Most of these sorbents are in pow-

TABLE 12

MAIN HYDROXYAPATITE SORBENTS AVAILABLE

Trade name	Physical shape and nature	Sorption capacity (mg/ml)	Supplier
HA-Ultrogel	Spherical; microcrystals trapped in agarose beads	10[a]	Sepracor
Bio-Gel-HTP	Crystals	10[b]	Bio-Rad
Hydroxyapatite	Spheroidal particles	Unknown	Merck

[a] Calculated for cytochrome *c* in mg/ml swollen sorbent at pH 6.8.
[b] Determine for BSA in mg/ml dry material (data from manufacturer).

dered or crystalline form, and some others are aggregated crystals or spheroids or are spherically shaped (microcrystals are trapped in the macroporous structures of cross-linked agarose).

9. SORBENT DETERIORATION ASPECTS AND LEAKAGE

The long-term performance of chromatographic packings is dependent on two distinct phenomena: deterioration due to irreversible deposition of contaminants that occupy adsorption active sites, therefore reducing their accessibility, and chemical damage resulting from harsh washings to clean or sterilize contaminated columns. In this section, only the latter aspect will be considered. It should be mentioned, however, that activation steps prior to ligand immobilization can also damage the matrix.

9.1. Damage by chemical agents

Both chemical and physical factors are responsible for damaging sorbents by polymer degradation, but their action depends on a number of factors, the first of which is the chemical nature of the sorbent.

Although many synthetic structures tolerate extreme conditions of washing, polyamides and polysaccharides are sensitive to acidic and alkaline pH conditions. Treatment of polyacrylamide gels in strongly alkaline conditions partially hydrolyses the side-chains, creating carboxylic residues. The backbone is not damaged, however, preserving the integrity of the three-dimensional structure. Substituted polyacrylamides and particularly polymethacrylamides are much more resistant [31] to extended exposure to sodium hydroxide with no loss of ligand or of their sorption capacity. Native or substituted polyacrylamide gels are very resistant to acidic treatments.

Alkaline treatment of polysaccharides generates complex reaction processes giving rise to a number of compounds; moreover, these reactions are catalysed by the presence of divalent cations and oxygen. The most important consequence of this complex reactive pathway is the depolymerization of the polysaccharide and the introduction of acidic sites into the remaining degraded sorbent [70]. Degradation, which is temperature and time dependent, is limited with cross-linked polysaccharides, however.

Acidic treatments are particularly destructive for polysaccharides; even in cross-linked form, they can be completely dissolved when treated for a few hours at $pH < 3$. Whereas alkaline treatment introduces non specific acidic adsorption sites, acidic attack progressively destroys the polymer backbone.

Silica is also known for its tendency to solubilize when exposed to strongly alkaline media. Several means have been tried to reduce this susceptibility of the silica material in order to render it compatible with biochromatographic requirements. Methods to increase the pH stability of porous silica have been reported. Decreasing the surface area is a very effective

means of decreasing the dissolution of silica in alkali; it has been found, for instance, that the rate at which silica dissolves was decreased at least by a factor of 3 on decreasing the surface area from 50 to 5 m^2/g [71].

Aluminium-mediated stabilization in alkaline media has also been suggested [37]; complexation of free silanols with aluminium ions was demonstrated to provide effective protection against the formation of sodium silicate. Silanol groups derivatized by incubation with zirconium oxides [36] and protection with polymer layers [38] have also been reported as means of increasing the stability of porous silica.

Strong oxidizing chemicals used also as cleaning and sterilizing agents can be destructive for polysaccharidic structures whereas some synthetic polymers are resistant in the presence of dilute hypochlorites and peracetates. Polysaccharides are very succeptible to oxidation, which has been described as a means of creating aldehyde reactive groups for immobilization of affinity ligands [72].

9.2. Damage caused by physical treatments

Most chromatographic sorbents are generally stable up to 120–130°C. Instability can be due to the destruction of the hydrogen bonding that stabilizes non-cross-linked agarose-based matrices; temperature above 35–36°C may damage substituted ligands for affinity chromatography (especially proteins) or some quaternary ammonium groups, particularly when they are in the hydroxide form.

Radiation damage of synthetic resins is non-existent or very limited, except when they contain substituted amines. Radiolytic action is more effective with polysaccharide-based supports, particularly in dry form [73].

9.3. Mechanically related damage

Compression and attrition damage chromatographic beads. They do not modify the interaction properties of the sorbent but rather physical properties such as flow-rate. Grinding of hydroxyapatite crystals was reported as a major factor impeding the large-scale utilization of this sorbent (see above) as a result of the progressive decrease in column flow-rates.

Attrition is a problem with inorganic materials and mineral composite matrices; the hardness of the surface promotes self-grinding with possible generation of fines when agitated. They are consequently not recommended for use in mechanically stirred vessels.

With soft or semi-rigid material, compression may also cause damage problems when cracks are formed, modifying the column performance.

9.4. Ligand leakage aspects

Although ligand leakage has been particularly described for affinity sorbents, it also occurs for any kind of packing material where adsorption sites are available. Thus ion-exchange sites and hydrocarbon chains [74] (HIC and RPC) can be the source of ligand deterioration and leakage.

Mechanisms resulting in the release of the ligand into the mobile phase during any step of the chromatographic separation cycle are classified as a function of the exact zone where they occur. Breakage of the attachment point, leaching of the ligand subsequent to partial matrix hydrolysis, release of physically entrapped material into the matrix network, dissociation of adsorbed ligand (non-covalently bound), subunit dissociation of ligands and chemical hydrolysis of the ligand itself are the major reasons. Fig. 5 illustrates chemically these mechanisms.

Cleavage of covalent bonds can be the result of redox reactions (*e.g.*, disulphite bonds are sensitive to reductive cleavage; osidic linkages are destroyed in the presence of oxidative conditions) or nucleophilic attack. This last reason has been particularly investigated in affinity sorbents once the ligand has been attacked after cyanogen bromide activation [75,76], glutaraldehyde activation [77], or N-hydroxysuccinimide or carbodiimide treatments [78]. Fewer studies have been carried out on ligands immobilized with other activation methods; however, activation procedures with divinyl sulphone, carbonyldiimidazole, chloroformate, sulphonyl chloride or triazine are likely to release at least under extreme conditions original or modified forms of attached ligands [79–81]. Progressive

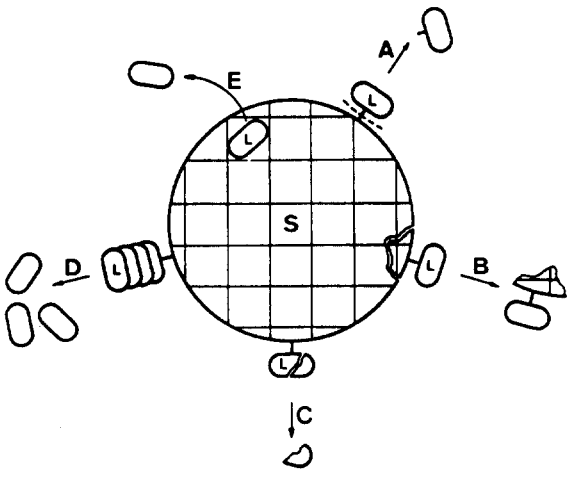

Fig. 5. Schematic representation of the major mechanisms inducing leakage from chromatographic sorbents (S). Ligand (L) can be released as a consequence of linkage hydrolysis (A); B represents a possible ligand leakage after partial hydrolysis of the sorbent matrix; partial ligand hydrolysis is shown in C; D represents a leakage of ligand molecules which are not chemically attached but rather adsorbed on the immobilized ligand; physically trapped ligand can also be released (E).

release of entrapped material is more specifically related to large molecules and to sorbents with a high degree of swelling and shrinking. Ligand immobilized in organic or aqueous–organic conditions and with a tendency to form aggregates in aqueous systems can also be easily trapped. As they are not necessarily covalently attached they can be expelled as a result of relaxation of polymeric chains. Release of physically entrapped material is also a consequence of molecular denaturation or partial hydrolysis with the production of small "pieces" that may be released.

Partial hydrolysis of the gel matrix for reasons explained above is equally at the origin of ligand leakage which is released in association with short polymer chains [74,81].

Immobilization of dyes is a good example of ligand leakage from the dissociation of non-covalently adsorbed molecules. Owing to their complex physico-chemical characteristics and some planar regions, free dyes can adsorb face to face (stacking effects) to chemically immobilized dyes with subsequent progressive release in the presence of low salt solutions of solvents or of chaotropic agents.

Partial hydrolysis or subunit dissociation of ligands has been evidenced with macromolecular ligands such as proteins. Dissociation of concanavalin A into individual subunits in the presence of chaotropic agents [82] can result in the loss of partial structures that may contaminate the purified protein. All multimeric ligands involving subsparts in their structure that may be dissociated reversibly (*e.g.*, LDH, antibodies) represent a potential danger of leakage. Additionally, the presence of traces of proteases in the feedstock can have a deleterious action on any immobilized proteinaceous ligand (immobilized lectins for glycoconjugate separation, protein A for immunoglobulin G separation, antibodies for immunopurification). Large macromolecular ligands, however, take advantage of multi-point attachment, which dramatically stabilizes the immobilization even when using an incompletely stable chemical bond with the matrix [83].

With regard to ion-exchange chemical groups, it should be noted that a certain level of instability is known for substituted amines used in cationic sorbents. For instance, diethylaminoethyl ion-exchange groups attached to polysaccharides are sensitive to strong alkali treatment [84]. Decomposition resulting in diethylaminoethyl alcohol and piperazinium ions is temperature dependent and increases with increasing pH.

10. CLEANING OF PACKING MATERIALS

Clean conditions in the production of pure biological materials require procedures to remove contaminating material to be established. Sterilization, which is one of the main aspects of cleaning, is the inactivation of living organisms present within the chromatographic packing such as bacteria, yeasts and viruses. A second aspect of cleaning implies the removal of any undesirable adsorbed material from any source, including pyrogens. Sodium hydroxide has been suggested for sterilizing stable chromatographic media [85]; however, recent studies demonstrated the limitation of this treatment for non-sporulated material [86]. Sodium hydroxide remains, however, a good agent for removing alkali-soluble impurities, some fatty acids and macromolecular aggregates. When associated

with ethanol or detergents or with urea, the effect of sodium hydroxide on cleaning is enhanced [87,88].

Decontamination approaches using sequential washing methods or alkaline solutions of ethanol are more effective for spore destruction. Removal of endotoxins can also be accomplished by alternative washings: acidic treatments release the so-called lipid A by hydrolysis of the ketosidic linkage with the core polysaccharide [89] and alkaline treatments contribute to detoxification by the saponification of fatty acids associated with lipid A [90].

All these approaches are obviously applicable only with acid- and alkali-stable sorbents. For the treatment of labile sorbents such as those used in affinity chromatography, more suitable solutions must be designed. Examples are the use of acidic ethanol solution [26] or hibitane digluconate associated with benzyl alcohol [91]. As a general rule for sorbent cleaning, acidic washings must be avoided with polysaccharidic-based material, ethanol-containing solutions are appropriate only for unshrinkable sorbents and strongly alkaline solutions must be avoided with some chromatographic polymers [31]. Today the tendency is for sorbent manufactuers to develop materials that are stable to any washing solutions including those that contain oxidizing agents.

11. FUTURE TRENDS

With the advent of new polymer chemistries, of new concepts in composite materials and surface chemistry development, improvements in the characteristics of chromatographic sorbents are to be expected. As far as a solid surface is involved in the separation of biochemical macromolecules, the main question is how to put in the smallest volume the largest surface area without sacrificing the accessibility of the active sites located on this surface.

Complex purification schemes involving numerous steps to obtain a pure protein should be simplified dramatically, taking into consideration the selectivity of the interacting surface and the efficiency of the separation. In contrast to some more or less accepted historical rules, very selective particles (such as affinity sorbents) should be used in the early stages of the separation as a capture-like step. Lower selectivity but higher efficiency is expected in the following steps with a high capacity of treatment of purer feedstocks issuing from a preliminary very selective capture phase.

In this configuration, specific sorbents must be manufactured and their characteristics should correspond to the requirements of the step for which they have been designed. Higher selectivity means greater specificity and consequently case-by-case design, implying the development of tailor-made manufacture of packings for preparative usages. For generic selective packings, selectivity should be intended either for group separation or related more to the elution conditions than to the adsorption phase.

Rational chemical design of the surface, grafting selected peptidic sequences, and also using so-called imprinting techniques should be rapidly developed consistent with the emerging selectivity needs. The adoption of, *e.g.*, "expanded beds" or "moving beds" in bioprocessing will necessitate specially designed particles where attention will be devoted to their density, robustness and ability to support very rapid exchange kinetics.

The barriers that still exist between HPLC and regular low-pressure liquid chromatography should also be overcome with the advent of medium-sized and medium-pressure packings but with high separating performance.

Finally, techniques involving or not surface interactions are also to be expected. Undoubtedly they will lead to the use of other new separating tools challenging the established technologies. However, only a slow development from the laboratory scale to production scale is to be expected owing to the difficulties of modifying already defined biotechnological processes unless they will permit unsolved problems to be overcome.

12. LIST OF SUPPLIERS AND TRADE NAMES

12.1. Suppliers

Amicon, 17 Cherry Hill Drive, Danvers, MA 01923, USA.

Bio-Probe International, 14272 Walmut Avenue, Tustin, CA 92680, USA.

Bio-Rad Laboratories, 2200 Wright Avenue, Richmond, CA 94804, USA.
E. Merck, Frankfurter Strasse 250, 6100 Darmstadt, Germany.
Perseptive Biosystems, University Park, 38 Sidney Street, Cambridge, MA 01213 USA.
Pharmacia–LKB Biotechnology, S-75182 Uppsala, Sweden.
Pierce Chemicals, P.O. Box 117, Rockford, IL 61105, USA.
Sepracor, 33 Locke Drive, Marlborough, MA 01752, USA.
Tessek, Krizovnicka 3, 11000 Prague 1, Czech Republic.
TosoHaas, Zettachring 6, 7000 Stuttgart 80, Germany.
Toyo Soda Manufacturing, 1-7-7 Akasaka, Minato-ku, Tokyo, Japan.
Waters Chromatography, 34 Maple Street, Milford, MA 01757, USA.
Whatman Chemical Separation, Springfield Mill, Maidstone, Kent ME12 2LE, UK.

12.2. Trade names of chromatographic sorbents

Accell: trade name of Waters Chromatography Division of Millipore.
Affi-Gel, Bio-Gel, Macroprep: trade names of Bio-Rad Laboratories.
Cellufine and Matrex: trade names of Amicon.
Avid-Gel: trade name of Bio-Probe international.
Fractogel and Super Q: trade names of TosoHaas.
Separon: trade name of Tessek.
HyperD, Spherodex, Spherosil, Trisacryl, Ultrogel and Zephyr: trade names of Sepracor.
Poros: trade name of Perseptive Biosystems.
Reacti-Gel: trade name of Pierce Chemicals.
Sephadex, Sephacel, Sephacryl, Sepharose and Superdex: trade names of Pharmacia–LKB Biotechnology.
Toyo-Pearl: trade name of TosoHaas.

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