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Journal of Chromatography A, 1065 (2005) 3–12
JOURNAL OF CHROMATOGRAPHY A
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Review

Chromatographic media for bioseparation

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Abstract

Bioseparation processes are dominated by chromatographic steps. Even primary recovery is sometimes accomplished by chromatographic separation, using a fluidized bed instead of a fixed bed. In this review, the action principles, features of chromatography media regarding physical and chemical properties will be described. An attempt will be made to establish categories of different media. Characteristics for bioseparation are the large pores and particle sizes. To achieve sufficient capacity for ultralarge molecules, such as plasmids or nanoparticles, such as viruses monoliths are the media of choice. In these media, the mass transport is accomplished by convection, and thus, the low diffusivity can be overcome. Common to all modern chromatography media is the fast operation. There are examples where a residence time of less then 3 min, is sufficient to reach the full potential of the adsorbent.

Keywords: Proteins; Plasmids; Pore; Diffusivity; Monolith; Ion exchange chromatography; Affinity; Hydrophobic interaction

Contents

1. Introduction

Bioseparation of high value products is mainly based on packed beds. An ideal process is composed of three steps: the capture, the purification and the polishing step. In contrast to analytical chromatography and separation of chiral compounds, biochromatography is characterized by low efficiency but high selectivity columns. The biomolecule is captured unbound material is washed out and then elution is effected by change of mobile phase composition known as step gradient elution. For high-resolution applications, linear gradient elution is also applied. Selection of a stationary phase is still accomplished in an empirical way. If not, a generic stationary phase is available, such as in the case of antibody purification with staphylococcal Protein A media.

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doi:10.1016/j.chroma.2004.08.162

Table 1
Action principles in protein chromatography

Name	Action principle	Separation by
Adsorption chromatography	Surface binding	Molecular structure
Ion-exchange chromatography	Ionic binding	Surface charge
Size-exclusion chromatography (molecular sieve chromatography[a], gel filtration[b])	Size exclusion	Molecular size and shape
Affinity chromatography	Biospecific adsorption/desorption	Molecular structure
Hydrophobic (interaction) chromatography	Hydrophobic complex formation	Hydrophobicity and hydrophobic patches
(Metal-) chelate chromatography	Coordination complex	Complex formation with transition metals
Normal-phase chromatography	Hydrophobic complex formation	Hydrophobicity
Reversed-phase chromatography	Hydrophobic complex formation	Hydrophobicity

[a] Used in context with polymer chemistry.
[b] Name as originally used by the inventors of size-exclusion chromatography.

Usually, the biomolecules are separated under such conditions that biological activity is maintained. Normally, buffer with pH around 7 and moderate salt concentrations are used. For elution, some times chaotropic salts or extreme pH values are applied. Resistance of media to NaOH is requested for industrial applications, since alkaline solutions are excellently suited for sanitization due to fast degradation of proteins, lipids and other biopolymers. The removal of viruses is also achieved by application of above-mentioned treatment of chromatography materials. This review focuses on the physical properties of the chromatography beds and the chemistry of the surface. We also try to categorize the different media designed for bioseparation. In the past 30 years, the properties of chromatographic media have been constantly improved. This concerns flow properties, binding capacities and kinetics of adsorption.

2. The action principles

Currently, eight different action principles are employed for chromatography of biomolecules (Table 1). These action principles are adsorption chromatography or mixed mode chromatography, ion-exchange chromatography (IEX), size-exclusion chromatography (SEC) also called gel filtration, affinity chromatography (AFC), hydrophobic interaction chromatography (HIC), normal phase chromatography (NPC), and reversed phase chromatography (RPC).

The nature of the stationary phase also determines the nature of the mobile phase. The optimization of both stationary and mobile phase is required to get optimal performance, because they are interrelated. In addition, possibilities to optimize protein chromatography are cut down by the stability of proteins. At extreme pH, salt conditions or in certain organic solvents they are not stable. In NPC the stationary phase is polar and the eluent consists of a mixture of water and organic solvent. A certain variant of NPC is also used for peptide and protein separation, the so-called hydrophilic interaction chromatography (HILIC) [1]. A polar ligand, such as diol or cyanogen is immobilized and elution is effected by a mixture of water and organic solvent. HILIC has been used for separation of peptides [2,3] or for instance for separation of proteins, such as histones [4] or quantitative analysis and process monitoring of site-specific glycosilation microheterogeneity in recombinant human interferon-gamma [5].

When the first nomenclature of chromatography media was established reversed phase chromatography was considered as an exotic technique, and therefore named in contrast to NPC reversed phase chromatography. Retention in RPC is achieved through discrete interactions between these nonpolar ligands and hydrophobic patches accessible on the surface of a protein or peptide. In RPC, the stationary phase is less polar than the NPC. Reversed phase chromatography was originally introduced in 1950 by Howard and Martin [6] and for some time widely used in paper chromatography [7]. It resurrected with the development of modern stationary phases for liquid chromatography. HIC is related to RPC but as eluent aqueous salt solutions are used as mobile phase. In both cases, the proteins are bound at conditions of high surface tension and eluted by lowering it.

IEX is indispensable for separation of proteins; in analytical as well as preparative and industrial scale. Ion-exchange can also be performed in the presence of high concentrations of urea or in the presence of non-charged detergents. Under such conditions, proteins are solubilized and aggregates are dissociated. Affinity chromatography has been specifically developed for protein chromatography. The beginning of the technique can be related to the activities of Porath and co-workers [8,9] and of Cuatrecasas et al. [10]. In affinity chromatography, the high resolution is obtained through biorecognition: the ability of proteins to specifically interact with another molecule (ligand). The formed complex can be split under relative mild conditions, which is necessary to desorb the protein from the column without damaging its native structure.

Mixed mode separations have been also successfully applied for separation of proteins [11]. As already indicated by the name, several physical principles are responsible for the interaction. It can be simultaneous cation and ion-exchange, or ion-exchange together with hydrophobic interaction, or ion-exchange with a kind of biorecognition. A typical example for mixed mode adsorption is hydroxyapatite [12,13]. Another example of a mixed mode column is the ABx-column [14].

SEC in the past also named gel filtration [9], or gel permeation chromatography, is also widely used for proteins separation, mainly as the polishing step. The method is limited by low productivity. For high-resolution application, the feed volume must be lower than 5% of the column volume [15]. The method is excellently suited for separation and determination of di- and oligomers of proteins [16–20]. With the novel stationary phases, a run can be finished within less than 1 h. The method has been also performed in continuous mode as continuous annular chromatography [21] or simulated moving bed [22,23]. Size exclusion is also used for refolding of proteins [24–26]. Refolding with SEC has been also performed in a continuous mode [27–29].

3. Requirements for media for protein chromatography

The ideal features of a medium suited for protein chromatography are summarized in Table 2. It is often not possible to reconcile all features, and therefore compromises have to be searched. The chemical nature of proteins determine the surface properties of the media, while the large size of proteins determine its physical properties. First of all, a large surface is requested to get a high binding capacity.

A large surface can be obtained with a highly porous material, containing numerous small pores, but small pores would prevent diffusion into the pores. Thus, a compromise must be found between surface area and pore size. Media for protein chromatography have a pore size of 30 nm. Hindered transport can be neglected when the pore diameter (d_P) is 10 times the protein diameter. Most proteins have a diameter below 3 nm [30]. In order to allow a reversible adsorption, the chromatography material must be very hydrophilic. Natural polymers, such as cellulose, agarose and dextran have been frequently used. Their high substitution with hydroxyl groups make them hydrophilic and enough groups are available to introduce ligands to specifically modify the surface according to its intended use. For protein chromatography, a ligand density below 100 μM is usually sufficient. A drawback of the hydrophilic natural polymer is their soft structure. Upon increasing flow, the medium will compress and pressure drop will increase [31]. Above the critical velocity, the column is clogged. In order to improve pressure stability media have been crosslinked. Introduction of crosslinkers leads to a more hydrophobic medium and generally unspecific adsorption increases. The chromatography media with the lowest unspecific adsorption are cellulose media, such as DE52 from Whatman or simply crosslinked Agarose or Sephadex media. Ideal features of a medium for protein chromatography are: (i) high selectivity, (ii) high binding capacity, (iii) high mass transfer, (iv) low unspecific adsorption, (v) incompressibility, (vi) chemically stable and stable immobilization of ligands, (vii) non-toxic leachables, (viii) high number of cycles (reusability), (ix) sanitation by alkaline conditions (only necessary for production of therapeutic proteins), and (x) inexpensive (cost effective).

Table 2
Features of media suited for protein chromatography

Feature	Dimension
Surface area (m^2/cm^3)	10–400
Functional group ($\mu mol/cm^3$)	1–100
Porosity (ε_P)	
Non-porous	0
Porous	0.25–0.75
Pore size (nm)	
Conventional	10–100
Monolith	1000–5000

Any chemical modification of the surface bears the risk of introduction of charged or hydrophobic moieties, which will unspecifically interact with the protein. Sanitation by NaOH is almost mandatory for media suited for industrial applications in the biopharmaceutical industry [32]. Proteins are rapidly degradated by alkaline hydrolysis and lipid are efficiently dissolved in NaOH. Up to 1 M NaOH is used for cleaning and sanitization of chromatography columns. If has to be noted that NaOH does not sterilize a packed bed. It reduces the germ count but does not eliminate all viable germ [33]. For chemical sanitization an oxidizing agent, such as peracetic acid would be very useful [32]. The compound efficiently destroys bacteria and breaks down into acetic acid and water. Interestingly, NaOCl (a compound with the same oxidizing capacity as peracetic acid) is less efficient in sterilizing bacteria. It has been assumed that peracetic acid can more easily penetrate into bacteria than NaOCl. In addition, NaOCl forms dioxins upon reaction with organic material containing cyclic hydrocarbons. A lot of chromatography materials are not resistant to oxidizing chemicals, and thus, the method is out of question. Chemical sterilization also requires a certain design of a chromatography column. If it is necessary to sterilize columns, it can be done by heat sterilization at 100 °C or above. To our knowledge, in situ sterilization of packed beds is not done, but packed columns are put, as a whole, into an autoclave. It is assumed that conditions applied for sanitization of media contaminated with bacteria will also inactivate viruses.

The chemical modifications, as well as the immobilized ligand, should result in a chemically stable matrix. The ligand should not be released during decomposition. An absolute stability is not possible and also not desirable. The controlled leakage of ligand has been accepted. For instance, leaked Protein A in the range of 4 ppm and above can be detected in IgG-eluates of Protein A columns. It is important that the leaked material is not toxic. This requirement excludes a variety of chemistries for manufacturing of media for production of therapeutic proteins. The ligand itself should also not be toxic. Dye-ligands have been suspected of being toxic [34], but recent studies showed that they are relatively safe. Chemistry of these ligands have been improved and currently a big variety of such ligands meeting

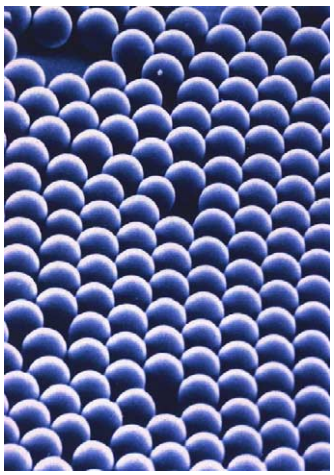

Fig. 1. SEM of monobeds produced by Amersham Biosciences. Reproduced by courtesy of Amersham Biosciences.

the requirements of manufacturing of therapeutic proteins are available.

3.1. Particle size and particle size distribution

The particle size for protein chromatography ranges from 2 to 300 μm. The size distribution varies from monodispers media (Fig. 1) up to 30% standard deviation of the size distribution. The feature depends on application. Media characterized by small particles and narrow particle size distributions are used for analytical applications.

In these applications high resolution (R_s) is obtained by high efficiency. Efficiency is measured as number of plates (N):

$$R_s = \frac{\sqrt{N}}{2}\frac{\alpha - 1}{\alpha + 1}\frac{k}{k + 1} \quad (1)$$

where k is the average relative retention of two solutes, and α the selectivity defined as k_2/k_1. For such applications, narrow small particles are required, since efficiency is directly related to the particle diameter.

$$\mathrm{HETP} = \frac{2\varepsilon_b D_L}{u} + \frac{2u}{1 - \varepsilon_b}\left[\frac{k'}{1 + k'}\right]^2\left[\frac{r_P}{3k_f} + \frac{r_P^2}{15\varepsilon_P D_P}\right] \quad (2)$$

where ε_b is the bed porosity, k' the relative retention factor, r_P the particle radius, ε_P the porosity of the particle, k_f the film mass transfer coefficient, D_P the particle diffusivity and D_L the axial dispersion coefficient. An alternative comprises monoliths, because they exhibit a high mass transfer property [35].

For preparative applications, resolution is often obtained by selectivity and chromatography is performed in an on/off mode. Conditions are searched where the protein of interest is captured, while the impurities are passing the column. After washing out the unbound material from the interstitial space the protein of interest is eluted. For such application, large particle diameter (>50 μm) are advantageous, since the pressure drop is lower, they are easier to pack and they are easier to manufacture, and therefore, less expensive than media with small particle size and narrow size distribution. The pressure drop (ΔP) for beds packed with rigid media is given by the Kozeny–Karman equation:

$$\frac{\Delta P}{L} = \frac{\mu u}{d_P^2}\frac{(1 - \varepsilon)^2}{\varepsilon^3} \times 150 \quad (3)$$

where L is the column length, d_P the particle diameter, u the chromatographic velocity and μ the dynamic viscosity, and ε the porosity. Several manufacturers offer chromatography media with the same surface chemistry, similar pore structure but different particle size. This provides more freedom to optimize a chromatographic separation process. A lot of chromatographic media are almost spherical. Spherical media produce a lower back pressure in a column than non-spherical ones, but manufacturing of spherical media is complicated and expensive. For preparative purposes with large particles, the increase of pressure drop due to deviation from spherical form is negligible (Fig. 2). Modern chromatography media do not contain fries or debris. These small particles may be washed down and clog the column over a certain time of operation. Fine material can also cause problems during packing. When a column is often repacked such fine material may be generated.

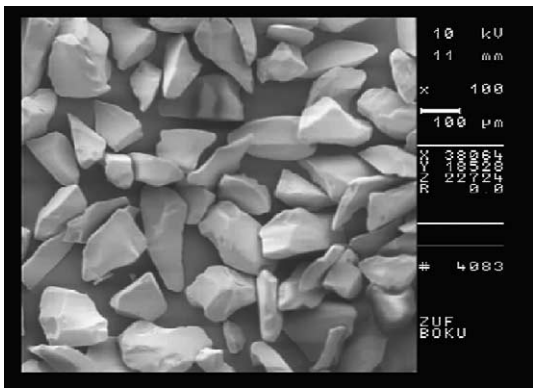

Fig. 2. SEM of a chromatography packing made of glass used (Prosep from Millipore) for industrial affinity chromatography.

The latest generation of chromatography media, the monoliths are stationary phases, which do not require packing of a bed. Either a continuous stationary phase is cast, which is intersected with channels [36,37] or a chromatography material is packed and then the bed is compressed [38–40]. In case of capillary LC, the wall acts as stationary phase. Both stationary phases, monoliths and capillaries are characterized by high mass transfer efficiency [35].

3.2. Pore size and pore size distribution

Media for protein chromatography are characterized by large pore size and compressibility. Thus, the pore size de-

termination is not possible by conventional methods used in classical adsorption science. A classical adsorbent, such as activated carbon contains micro meso and macro pores. For protein chromatography micropores are not of interest, because they are not available for proteins. The surface area of a rigid medium can be determined by nitrogen adsorption (BET-isotherm) or mercury intrusion. The soft natural and synthetic polymers must be dewatered before analysis and then the structure will noticeably change. The material will shrink and the obtained data are meaningless. Chromatography media from polymethacrylate, such as Toyopearls, CIM monoliths, or silica beads and glass can be characterized by above-mentioned methods.

Recently, the pore size of soft chromatography media has been measured by inverse SEC [41]. Dextran molecules with defined size serve as reference material. These molecules are inert enough and do not interact with the chromatography surface. The molecular size of the dextrans is given by the viscosity radius:

$$R = \left(\frac{3[\eta]M}{10\pi N_A}\right)^{1/3} \quad (4)$$

where $[\eta]$ is the intrinsic viscosity in cm^3/g, M is the molecular mass and N_A is Avagadro's number. The viscosity radius has been proposed as a universal calibration parameter for SEC as it captures the dependence of SEC elution volume on both the molecular mass and the molecular shape, as reflected in the intrinsic viscosity. This size description has proven useful in SEC as it permits comparison and calibration between macromolecules of very different shapes (e.g., proteins, polysaccharides, nucleic acids and viral particles) and is independent on pore geometry and mobile phase flow rate [41].

A mixture of dextran molecules with defined size is injected and they are eluted according to their size (Fig. 3). From the distribution coefficient of the retained dextran molecules (K_D) the fractional pore volume can be calculated

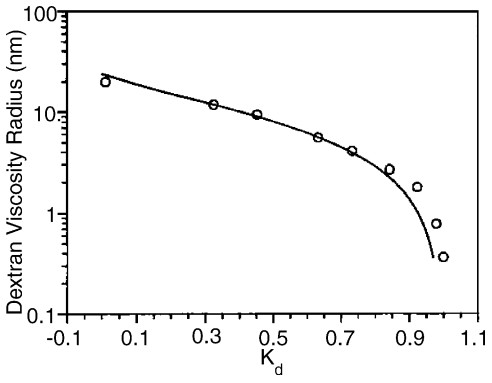

Fig. 3. Relationship between radius of gyration and distribution coefficient of dextran samples according to DePhillips et al. [41].

Table 3
Comparison of pore size of a selection of chromatography media determined by inverse size-exclusion chromatography and data from manufacturers

Stationary phase	Mean pore diameter (nm)	
	ISEC calculated	As given by supplier
Amersham Biosciences		
SP Sepharose FF	49.4	–
CM Sepharose FF	54.6	–
TosoH Biosep		
HW 65 F	132.2	100
SP 650 M	153.2	100
CM 650 M	147.8	100
HW 55 F	39.2	30
SP 550 C	17.6	30
EM Industries		
EMD SO_3^- M	33.0	100
EMD SO_3^- M (1 M NaCl)	59.3	100
EMD COO^- M	161.0	100
BioSepra		
Silica	136.0	100
SP Spherodex M	68.6	100
SP Spherodex M (1 M NaCl)	43.2	100
CM Spherodex M	20.8	100

Data from DePhillips et al. [41].

and a fairly good pore size distribution is obtained:

$$K_D = \frac{V_R - V_0}{V_T - V_0} \quad (5)$$

where V_R is the elution volume of the respective dextran molecule, V_0 the void volume, and V_T the total column volume. Because of the finite radius r_m of the probe molecule, the part of the pore immediately adjacent to the wall is inaccessible to the center of the probe, so the fractional accessible volume is $(1 - (r_m/r))^2$. Thus, for a given probe size:

$$K_D = \frac{\int_{r_m}^{\infty} f(r)(1 - (r_m/r)^2)\,dr}{\int_0^{\infty} f(r)\,dr} \quad (6)$$

where the denominator serves for normalization. This relation shows K_D to be calculated as the ratio of the pore volume accessible to a probe of radius r_m to the total pore volume, consistent with the experimental definition. The method has been also compared with the classical methods for measuring pore size distribution and good agreement has been observed for some media, for others a much smaller pore diameter has been found (Table 3).

Interestingly, enough has been observed for the grafted media, such as Fractogel [42]. Under certain conditions, such as low ionic strength, the grafted layer may behave as hydrogel and large uncharged molecules cannot penetrate into the pores. When salt is added then the hydrogel shrinks and pores are opened.

For separation of plasmids and viruses, an extreme pore geometry is required. This may lead to a very soft bead. Another way to design a medium with a high capacity for plasmid DNA is the utilization of plasmid partitioning into

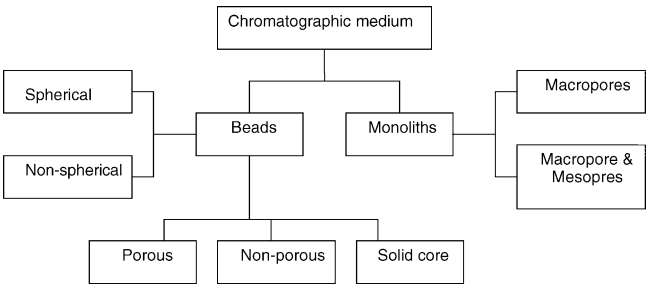

Fig. 4. Classification of chromatography media according to the shape of the bead.

a hydrogel. Chromatography beads particularly designed for plasmid separation are not yet commercially available.

4. Categories of media

4.1. General classification

It is not easy to classify media. They can be categorized according to the basic material, according to the transport mechanisms or according to manufacturing processes. Classification according to the transport mechanism is not reasonable, since upon chromatography conditions and composition of feed, the transport mechanism may change as demonstrated by experiments with laser confocal microscopy [43].

Here, we try to classify the media according to basic material and shape of the bed (Fig. 4). Chromatography material not suited for protein chromatography will not be considered here.

We can distinguish between packed beds by particles and continuous stationary phases the so-called monoliths. The beads can be porous or non-porous, but a monolith has to be porous, otherwise it would be useless as chromatography medium. Chromatography material with a solid core (Fig. 5) surrounded by a shell of sintered small particles are also available [44]. The monoliths are either solely composed of macropores, such as the CIM disks [36,37] or both macro and mesopores, such as the chromolith [45–48]. Initially, the chromolith has not been designed for protein chromatography. They have been also successfully applied for peptide separation and test have been made with larger proteins.

Classification according to basic material is straight forward: we have natural polymers, such as agarose, dextran, cellulose, chitoson, synthetic polymers, such as polymethacrylate, polyacrylamide, trisacryl and polystyrene, inorganic material, such as silica, zirconium oxide, glass, hydroxyapatite and composite materials where a gel has been filled into beads with gigapores [49,50].

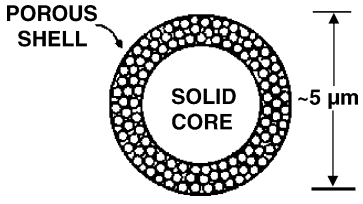

Fig. 5. Schematic drawing of a chromatography bead with a porous shell and a solid core. According to Kirkland et al. [44].

The basic beads are often further modified in order to coat the surface (silica, polystyrene) or to graft an additional layer onto the surface (Fractogel, Sepharose XL). Classifying media into preparative and analytical ones, makes a priori no sense. The only difference between analytical and preparative separation is the result. In analytical chromatography, we are interested in the information, while in preparative separation interest lies in purified product. For industrial scale operation, material with a particle diameter >30 μm is commonly used.

4.2. Beads

4.2.1. Natural polymers

The first application of a natural polymer was reported by Peterson and Sober in 1956 [51]. They derivatized cellulose to cellulose beads with ion-exchange functionalities. Three years later, the first dextran-based media Sephadex G-25 and G-50 were commercially available. The first paper on gelfiltration describing Sephadex was published by Porath and Flodin [52]. Common to all natural polymers, such as cellulose, agarose, dextran and chitosan used for protein chromatography is their low unspecific adsorption. These polymers are extremely hydrophilic and proteins do not adhere. This is explained by the high degree of hydroxy groups in the polymer chain. It seems that chitosan is the most hydrophobic one. An overview of most common media made out of natural polymers can be found in Table 4.

The big success stories in protein chromatography are dextran- and agarose-based chromatography beads, Sephadex and Sepharose, respectively [53]. The success is closely linked to the company Pharmacia now General Electric Health Care. Without these materials, the big advances in protein separation would not be possible. In 1959, Sephadex G-25 and G-50 were introduced, and 1960 and 1962 saw the introduction of ion-exchangers based on Sephadex block polymerizates.

Advantages of dextran and agarose beads over cellulose are better flow properties. Fibrous cellulose is extremely hydrophilic but also difficult to pack. Bed height is usually below 20 cm. Cellulose beads are also commercially available, such as Cellufine from Millipore or the cellulose based ion exchangers from Whatman [54]. To enforce the soft structure, agarose has been crosslinked. This material is known as Sepharose CL 2B (4B, 6B). Crosslinked agarose beads have been further modified by covalent binding of dextran to it. The medium has been commercialized as Superdex.

To exploit their excellent binding properties, these soft chromatography media are often used in batch contactors. This operation principle is very popular in blood plasma fractionation. The medium in the form of a dry powder or swollen in an appropriate buffer is mixed with the plasma in a tank

Table 4
Examples of chromatography media made from natural polymers

Basic material	Physical shape	Example	Manufacturer
Cellulose	Fibrous	DE 32	Whatman
	Micropellicular	DE 52	Whatman
	Pellicular	Express-Ion D	Whatman
		Express-Ion Q	
	Pellicular	Sephacel	Amersham Bioscienes
	Pellicular	Cellufine	Millipore
Dextran	Pellicular	Sephadex G	Amersham Biosciences
		Sephadex G	
		DEAE	
Agarose	Pellicular	DEAE-Sepharose 4B	Amersham Biosciences
	Pellicular crosslinked	DEAE-Sepharose FF	
Agarose-dextran composite	Pellicular	Superdex 30	Amersham Biosciences
		Superdex 200	
		DEAE-Sepharose XL	

then the medium swells and adsorbs proteins. After that, the loaded material is removed by a nutsch filter and the proteins are desorbed. In such processes, the chromatography material can only be used one time. Another way to handle these soft materials is via suspended bed chromatography introduced by Levison [55]. This is a hybrid technology between chromatography and batch contactor. The equilibrated chromatography medium is suspended together with the protein solution as a slurry and then pumped in the chromatography column where the residual steps are performed. The time consuming loading phase is cut short by this technology.

4.2.2. Synthetic polymers

Synthetic polymers are also frequently used as basic material for protein chromatography. Three polymers are of importance: hydrophobic vinyl polymers, polyacrylamide polymers and polyvinylstyrene. Polyacrylamide polymers have been introduced by Hjerthen and polymethacrylate by the group of Kalal in Prague, CZ [54]. Later material is known as Spheron, but it never really penetrated the market. Another synthetic polymer is polystyrene, which can be used without further modification of the surface for RPC. For other modes of chromatography, the surface must be coated with a hydrophilic polymer. A derivative of polyacrylamide is Trisacryl [56]. An overview of common polymeric media for protein chromatography can be found in Table 5.

Common to all synthetic polymers is their relative hydrophobicity. Therefore, for a lot of applications they must be coated into order to prevent low recovery. A plethora of other materials have been described, but they are not commercially available, and therefore of less interest for industrial applications. An advantage of the synthetic polymer-based media is their resistance to extreme chemical conditions, such as pH or an oxidizing environment presumably the coating is stable. Some of these media can be also sterilized in an autoclave. In situ sterilization is not possible due to lack of appropriate columns.

4.2.3. Inorganic media

The first chromatography material was $CaCO_3$ used by Tswett. This material is not suitable for protein chromatography. Arne Tiselius introduced hydroxyapatite for protein

Table 5
Example of basic material for protein chromatography

Basic material[a]	Trade name	Manufacturer
Polyacrylamide derivate	Trisacryl	BioSepra
	Hyper D	BioSepra, hydrogel filled into the porous shell
Hydrophilic crosslinked vinyl polymer	Toyopearl	TosoH Biosep
Polyacrylamide	Bio-Gel	Bio-Rad
Styrene divenyl benzene copolymers	Aberlit, Leweht	Merck, not optimal suited for protein chromatography
Polystyrene divenyl benzene	Source, Resource	Amersham Biosciences
Polystyrene divenyl benzene		Polymer labs, coated highly porous material available
Polystyrene divenyl benzene	Porous	Erseptive Biosystems. Similar material as polymer labs
Polymethacrylate	CIM	BIA separations, available as monolithic column

The surface has been further modified to functionalize it for IEX, AFC, HIC, RPC or SEC.
[a] Exact chemical composition is not available and often not disclosed by the manufacturer.

separation. The original microcrystalline hydroxyapatite had extremely poor flow properties, although selectivity was excellent. A breakthrough was accomplished by the ceramic hydroxyapatite [57]. The small hydroxyapatite particles are sintered to a spherical particle with large pores. The material has excellent flow properties, good selectivity and high binding capacity. It is used for large industrial processes, such as recombinant antibody production. The prime inorganic support material is silica [58–60]. The OH group can interact with proteins especially at high pH. Therefore, it is a necessity to react the residual Si-OH groups in order to inactivate them. Such a medium is called bonded silica. Peaks with good symmetry are only obtained in RPC with fully capped supports. On the other hand, with partially capped supports, the peak symmetry can be greatly improved by the addition of amines or quaternary ammonium compounds to the mobile phase. This is also known under reversed phase ion-pair chromatography. Silica has been also grafted with dextran [61] or polyvinylpyrrolidone [62] by interaction of a copolymer of vinylmethyldiethoxysilane and vinylpyrrolidone with Lichrospher Si 300 and Lichrospher Si 500. The coating procedure retained the wide-pore structure and the material showed good selectivity for proteins in the HIC mode. Silicas have been also coated with hydrophilic layers [63], cellulose [64], polystyrol [65], dextran [66,67], agarose [67] and poly(alkylaspart-amide) [1]. Another approach to stabilize silica supports was obtained by a zirconium salt treatment and then covalently bonded with a hydrophilic organo-silone (Zorbax Bioseries). The zirconium treated surface presents no unusual restraints in operating conditions and even permits short-term use of buffered eluents at pH 9.0 [68]. Full zirconium beads are used for small molecules but not for proteins [69]. It would be of interest, since zirconium oxide is stabile in alkaline solutions.

Glass has been also used a support. The most commonly used one is the controlled porous glass (CPG). It exhibits excellent flow properties, although the material is not spherically shaped (Fig. 2). The surface has reactive hydroxy groups which can be used for further modification. A revival of glass has been made by the Protein A adsorbent ProSep A (Millipore). This adsorbent is successfully applied for large scale therapeutic antibody purification. A proprietary chemistry is used to attach the staphylococcus Protein A ligand. The material has both excellent flow properties (back pressure) and excellent mass transfer properties [70].

4.2.4. Composite materials

To some extent the coated silica could be also regarded as a composite material, but in this review media made an inorganic and organic phase are considered. The HyperD material is a typical representative of such a composite material [49,50]. The manufacturers used a soft hydrogel similar to Trisacryl with a low degree of crosslinking and a high degree of functional groups. This leads to an extremely high binding capacity for proteins. But the material as such is not suited for chromatography in a packed bed. The material is too soft and could be only operated under extreme flow rates. Therefore, the gel has been polymerized into a porous shell originally made of silica and later of zirconium. The medium is also called gel in a shell. It can be operated at extremely high velocity without loosing binding capacity. The sorption mechanism was found to be dominated by solid diffusion. This was also confirmed by experiments where a gel was polymerized into a slab and the mixing protein front was observed in a microscope. The solid diffusion model was able to describe the moving concentration profile best. The high capacity of the composite medium allows direct capture of proteins out of a culture supernatant without prior conditioning [71].

4.3. Monoliths

Monoliths are a relatively new class of stationary phases, completely different when compared to conventional stationary phases. The material is cast into a chromatography column as a continuous block interlaced with channels [72]. The ramified channels do not have dead ends. Owing to this structure, the transport of the solute to the surface is solely by convection instead of diffusion as observed in conventional media [35,73]. Monolithic media are characterized by excellent mass transfer properties and a low-pressure drop. The large channel diameter make monoliths as excellent stationary phase for protein chromatography. The first monoliths were developed by Hjerthen et al. [38–40] and Tennikova et al. [74]. Hjerthen et al. [38–40] compressed polyacrylamide gels and observed excellent resolution. The polymethacrylate monoliths the most widely used for protein chromatography are produced as follows. When the polymer chain is growing the solubility is decreasing and at a certain polymer length defined particles precipitate and agglomerate to a homogenous porous network (Fig. 6). Silica monoliths grown as a single block by the gel–sol process have been developed for separation of small molecules [45–47]. These types of monoliths also contain mesopores. Numerous concepts of monoliths have been published and it seems that these configurations of a chromatographic bed comprises a great future for bioseparation. They are already widely used for analytical purposes. Scale up was difficult in the past, but

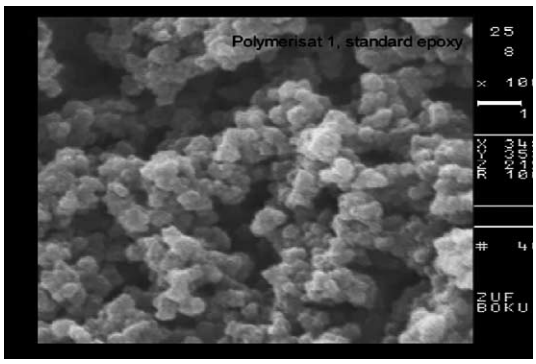

Fig. 6. SEM of a monolith made of polymethacrylate (CIM disks).

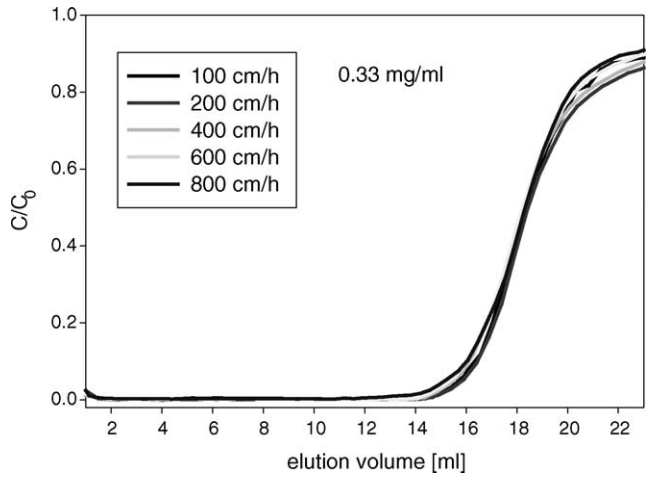

Fig. 7. Breakthrough curves of IgG on CIM SO_3 monoliths.

recently was successfully performed [75]. Polymethacrylate monoliths were scaled to 8000 ml. The conventional axial flow was replaced by a radial flow. The high mass transfer efficiency is demonstrated by the shape of the breakthrough curves (Fig. 7). Breakthrough curves have been performed at various velocities and superimposed. Breakthrough does not change with velocity and feed concentration. This is an indication that the adsorption is not mass transfer limited.

Monoliths have been also used as a support for solid phase synthesis [76]. An interesting application is the direct synthesis of peptides onto monolithic columns. Since the synthesis has been performed on polymethacrylate monoliths, the directly grown peptide can be used as an affinity ligand without any further treatment [77–79]. This strategy provides an excellent screening platform for affinity ligands. The screening of the ligand can be performed in a microtiter format and the same resin can be used for screening and large-scale separation. Monoliths are also interesting supports for enzyme reactors. The link to protein chromatography is in fusion proteins. After purification of the fusion protein, the fusion partner must be cleaved off. This can be either done in solution or in an enzyme reactor with immobilized enzymes. In packed bed reactors the efficiency is often limited by pore diffusion. The high porosity of monoliths ensures that the enzymatic process is not mass transfer limited [80].

Monolithic media are the best solution for separation of plasmids and viruses. The diffusivity of plasmids into pores of conventional media is extremely small. In monoliths, the large plasmid or virus is transported by convection. Recent studies have shown that the high efficiency of monoliths for this separation task [81].

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