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Review

Monoliths as stationary phases for separation of proteins and polynucleotides and enzymatic conversion

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Abstract

Monoliths are considered as a novel generation of stationary phases. They were applied for capillary electrochromatography and liquid chromatography exploiting every action principle such as ion-exchange, affinity recognition, reversed-phase, and hydrophobic interaction. The fast separation was explained by convective transport of the solutes through the bed. The contribution of this mode of transport is similarly explained as done for the beds packed with particles with gigapores. For monolithic beds, the concept of an ultrashort bed was frequently used. This mode of operation allows very short separation time. In many cases a gradient elution is necessary to achieve separation. Examples of applications for protein and polynucleotide separation performed on monoliths are given. Enzymatic conversion was described showing the examples of several immobilzed enzymes.

Keywords: Monoliths; Enzymatic conversion; Proteins; Polynucleotides

Contents

1. Introduction

Monoliths also called continuous beds, are cast as homogenous columns or disks [1–3]. They can be made of synthetic organic material [3–28], natural

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polymers [29,30] or inorganic material [31–37]. Monoliths differ to conventional columns in respect of hydrodynamic properties especially as they seem to have a lower mass transfer resistance [38] and pressure drop [39]. Both properties play a crucial role in chromatography performance, speed and scale up. Meanwhile, monoliths have been widely used for preparative and analytical separation of biopolymers. The material has been either casted in the form of disks, rods or tubes.

2. Pore structure of monoliths

The pore structure of monoliths is made responsible for excellent resolution at high flow-rates. Similar effects have been observed with particles interlaced with big pores. These types of media are described as having a flow-independent resolution. The band spreading does not increase with velocity when a high intraparticle Peclet number (*Pe*) is observed. The intraparticle Peclet number is defined as the ratio of flow around the particle to the intraparticle diffusion as shown in Eq. (1).

$$Pe = \frac{ud_p}{D_e} \tag{1}$$

Above a *Pe* number of 50 substantial flow is predicted. This is only observed when a sufficiently high particle porosity is present. General results on intraparticle convection for transport for spherical particles have been reported by Carta et al. [40] and Carta and Rodrigues [41].

The function of $1/f(Pe_{intra})$ represents the convective enhancement of intraparticle mass transfer. A Pe_{intra} of approximately 50 is needed to double the rate (Fig. 1). The intraparticle convection positively effects the performance. With an intraparticle flow of 1% the reduced HETP does not change over a broad range of velocities (see Fig. 2). The effect on dynamic capacity has been demonstrated by Heeter and Liapis [42] and Carta [43]. The breakthrough curve is substantially steeper when a sufficiently high intraparticle convective flow is assumed (Fig. 3).

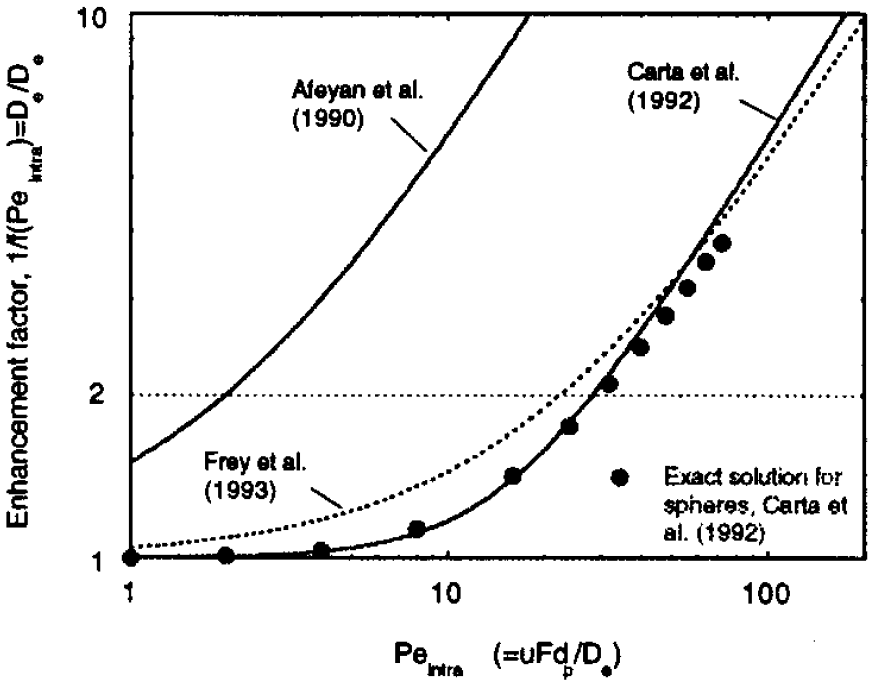

Fig. 1. Effect of intraparticle convection on transport. Reproduced with the kind permission of G. Carta, Workshop on Mass Transfer in Liquid Chromatography, PREP 2000, International Symposium, Exhibit and Workshop on Preparative Chromatography, Ion Exchange, Adsorption/Desorption Processes and Related Separation Techniques, Washington DC, 2000.

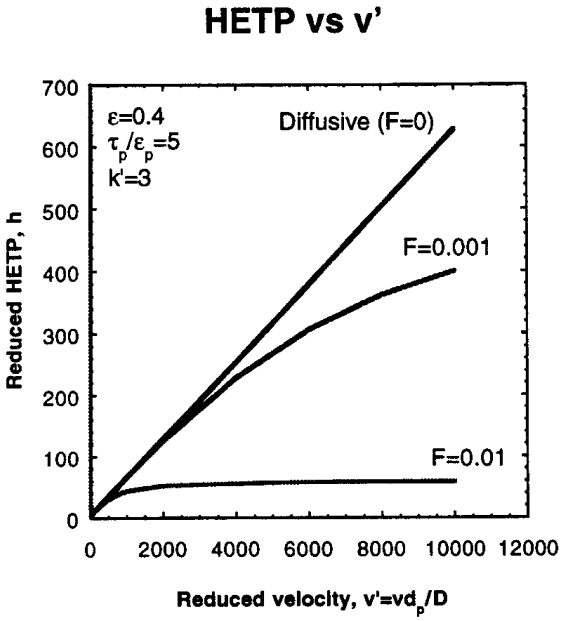

Fig. 2. Effect of intraparticle convection on performance, HETP versus velocity. Lines were calculated with the theory of Carta and Rodrigues. Reproduced with the kind permission of G. Carta, Workshop on Mass Transfer in Liquid Chromatography, PREP 2000, International Symposium, Exhibit and Workshop on Preparative Chromatography, Ion Exchange, Adsorption/Desorption Processes and Related Separation Techniques, Washington DC, 2000.

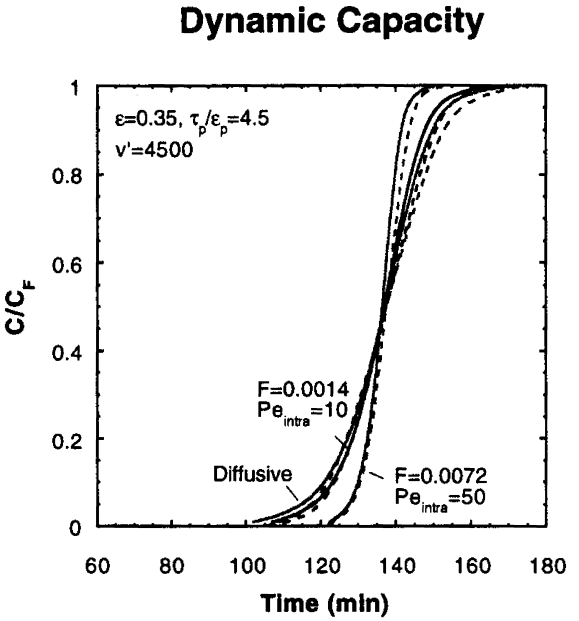

Fig. 3. Effect of intraparticle convection on dynamic capacity. Solid lines: Heeter and Liapis [42], numerical solution and dashed lines Carta [43] analytical approximation.

A logical consequence would be the extension of the whole flow through the pores. This occurs to a high extent in monolithic beds.

With these chromatography beds, the positive effects of intraparticle convection on performance should be even higher compared to beds packed with particles allowing intraparticle convection. This has been experimentally confirmed by Iberer et al. [2] for monoliths based on polymethacrylate block polymerisates and in-column polymerized beds of the copolymer of acrylic acid and *N,N′*-methylenebisacrylamide.

Meyers and Liapis [44] have proposed a theoretical model to describe the pore structure of monoliths and the mass transport of a solute either by convection or diffusion (Fig. 4). This theory is based on a model which has been previously described, the so-called cubic lattice network. The pore network model constructed by Meyers and Liapis [44] can be also used in an a priori manner to predict the convective velocity and pore diffusivity of a solute in monoliths. The representation of how the network was modeled is shown in Fig. 4.

The electron micrograph of such a monolith produced by copolymerisation of glycidyl methacrylate and ethylene dimethacrylate does not allow the extraction of information, except that small particles are agglomerated and channels are formed. So far, methods to quantitatively describe the connectivity are not available. Instead of a particle diameter they have introduced a diffusional length. Filling up of the pores (interstitium) with a material having a different diffraction than the monolith could be the basis measuring the connectivity by electron microscopy. Hahn and Jungbauer, have conceptualized the problem of diffusion into the agglomerates [39]. For a detailed study the value of connectivity is needed.

The effect of porous structure of a 2-mm thick diethylamine functionalized monolithic polymethacrylate disk on chromatographic performance was studied by Tennikov et al. [45]. It was shown that manufacturing conditions influence the pore structure; the fraction of glycidyl methacrylate

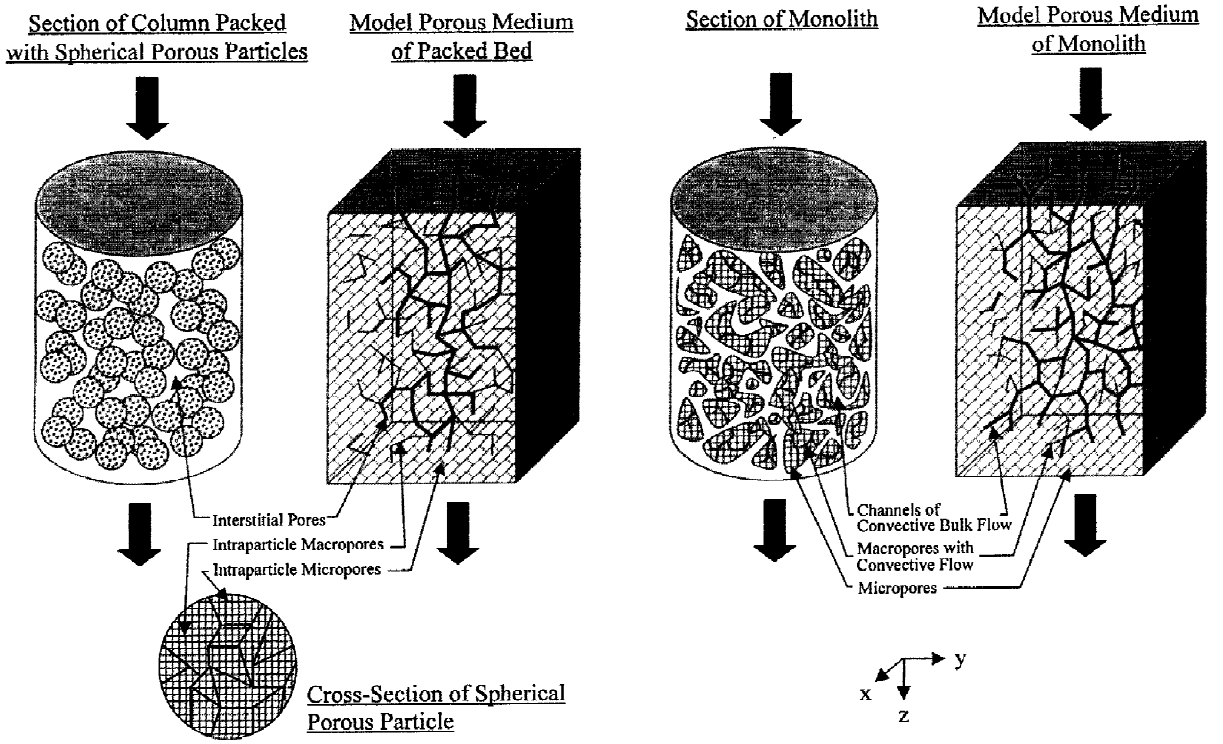

Fig. 4. Schematic representation of through pore network model of a finite small section of a packed bed and of a monolith (continuous bed). Reproduced by kind permission of authors [44].

(GMA) in the polymerization mixture and polymerization temperature determines the pore size, which is positively correlated; the higher the temperature, the lower is the pore size obtained. High GMA content in the polymerization mixture is responsible for large pores. In Fig. 5 differential pore size distribution profiles for such poly(glycidyl methacrylate-co-ethylene dimethacrylate) monoliths are shown. Monoliths characterized with pores larger than 1000 nm did not provide good separations because the residence time (contact time) of the protein was too short. Optimal pore size was about 700 nm. Sufficient resolution was achieved by these monoliths even at very steep gradients and high flow-rates. The separation time could be substantially shortened.

3. Mass transfer properties

The better performance of monoliths compared to conventional columns is usually explained by enhanced mass transport. The solute reaches the pores by convection not by diffusion. In this flow regime the separation process is much faster [2,38].

Several authors have laid down a theoretical concept to describe the enhanced mass transport of intraparticle convection. Iberer et al. [2] have shown the enhanced mass transport of the monoliths based on polymethacrylates.

The pores are connected in a way that the diffusional path is extremely short. The film resistance may be different. It can be assumed that the film thickness is smaller and the area is higher. Both effects contribute to an enhanced mass transport. Actual measurements must be performed to validate this hypothesis [39].

Due to the narrow channels in monoliths the Eddy dispersion is significantly reduced. The performance is similar to HPLC-type packed beds although monoliths exhibit a much lower back pressure. The specific permeability of polymethacrylate-based monoliths is such as CIM-disks (convective interaction media) is in the range equivalent to 45-μm

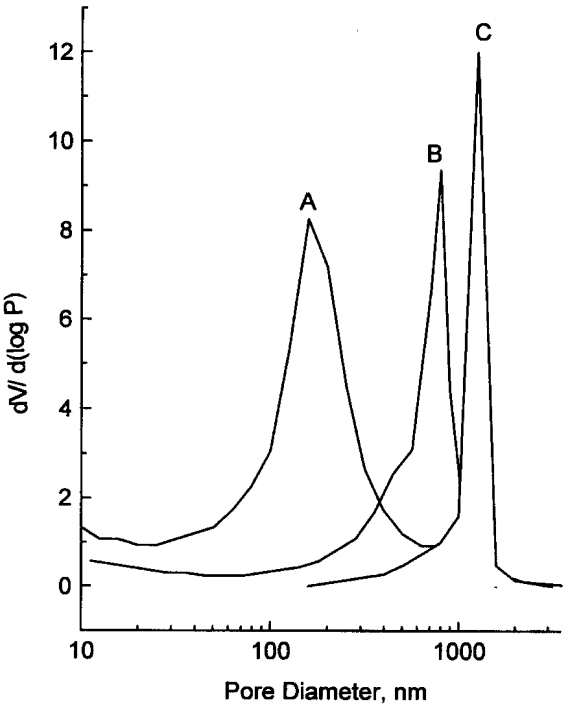

Fig. 5. Differential pore size distribution profiles for polyglycidyl methacrylate-co-ethylene disks measured by mercury intrusion porosimetry according to Tennikov et al., [45] reproduced by kind permission of authors.

particles. From SEM it can be clearly seen that such a monolith is composed of much smaller particles. Due to the ordered structure, Eddy vortices, made responsible for energy loss in a packed bed, may be less frequent [39].

Kinetics of adsorption might not differ with monoliths except the material composition is different from conventional chromatography media. Polymethacrylate based CIM-disks exhibit high binding capacity for proteins at high salt concentration in the mobile phase. They have a high ligand density in the mmol range such as the soft agarose and dextrane based ion exchangers. This property allows the use of CIM-disks for capture of proteins out of crude solutions [46].

Kinetics of adsorption do not differ from conventional chromatography and might be the limiting process for very fast separations. So far affinity chromatography was not often used in combination with monoliths [47–51]. Amatschek et al. [50] immobilized peptides directed against the very large blood coagulation factor VIII, on monoliths. They argued that slow adsorption kinetics may be the limiting factor for separation efficiency at high speed. Schuster et al. [51] immobilized monoclonal antibodies onto polymethacrylate monoliths. They could show that the recovery and resolution of various model proteins expressed in yeast were independent on a wide range of velocities. Unfortunately kinetic data are not available for that system. For rough calculation, if the affinity ligands are fast enough, the following assumptions are made. Assuming a ration with a simple equilibrium

$$A + B \overset{k}{\leftrightarrow} AB$$

the rate equation is

$$\frac{dc}{dt} = -(k_f - k_r)(C - C^*) \quad (2)$$

where C is the free solute, t the time, k_f the forward, k_r the reverse reaction constant and C^* the equilibrium concentration.

The time required (t_{50}) to bind 50% of the initial solute is given by

$$t_{50} = \frac{\ln 2}{k_f + k_r}$$

For an average antibody $k_f + k_r$ is in the range of $10^{-3} - 10^{-6}$. Even for a small disk with a height of 2 mm and a very high flow (i.e. 35 cm/min = 0.58 cm/s) enough time for binding must be available in such a system.

To what extent solid diffusion plays a role in adsorption of proteins in monoliths cannot be answered at the moment. Interestingly a high binding capacity can be observed at high salt concentration. This may be either explained that a substantial fraction of pores cannot be reached by the protein but by salt ions or that solid diffusion is likely. In the later case the model lets the protein move along the polymer fibers which are the base material of the sorbent.

4. The concept of disks

The fact that only a short layer of a chromatography column is able to separate proteins has been experimentally demonstrated by Tennikova et al. [52]. Later the theoretical concept of small disks for

separation of macromolecules without loosing resolution was laid down [53]. This approach allows us to work at extremely low pressure drops and owing to the geometrical shape of the ''column'', sufficient amount of sample can be loaded. By doing so, the problem of insufficient detector sensitivity is overcome; especially observed in narrow pore columns and columns packed with non-porous particles.

The concept of disks is based on the fact that at a certain position of the column the solute migrates with the almost same velocity as the eluent [54,55].

The position in the column (X_0) where migration velocity of the solute is identical to the migration velocity of the eluent can be empirically described by the following equation

$$X_0 = \frac{\lambda u}{SB} \tag{3}$$

where λ was called an auxiliary parameter depending on the ratio of inner pore volume of the sorbent to the intra particle volume and a few more empirical parameters, u is the linear velocity of the eluent, S a dimensionless protein adsorption parameter and B the steepness of the linear gradient. If the column is short and a shallow linear gradient is applied than the solute-velocity will never reach the eluent velocity. Therefore the full resolution of a sorbent is not used. In this case an increase of column length would enhance the resolution. Consequently when columns with a disk-shaped geometry are used then a very steep gradient must be used to obtained high resolution. This implies that the column header must be designed in a proper way that the sample is homogeneously distributed over the surface of the column.

A plethora of experimental evidences were reported in the past showing that the concept of disk-chromatography works for macromolecules as well as for small molecules [3,7,9,11,18,23,45,46,49,56–58].

The concept of ultrashort columns was also discussed by Coffman et al. [59]. When the column height is reduced to a size equivalent to one stage, separation can be only achieved when the solutes exhibit a large difference in affinity to the stationary phase.

They defined the separation factor as the ratio velocities of the solutes when they have reached equilibrium. This corresponds also to the selectivity. Furthermore they have defined a cut time τ_{cut}

$$\tau_{cut} = \frac{tQ}{\varepsilon \cdot V_{bed}}$$

with Q the flow-rate, ε the void fraction and V_{bed} (the bed volume) as the dimensionless separation time. Fig. 6 demonstrates that for a column with one stage an extremely short separation time is needed. When comparing the data of Tennikov et al. [45] and Sykora et al. [27] the shortcomings of an ultrashort

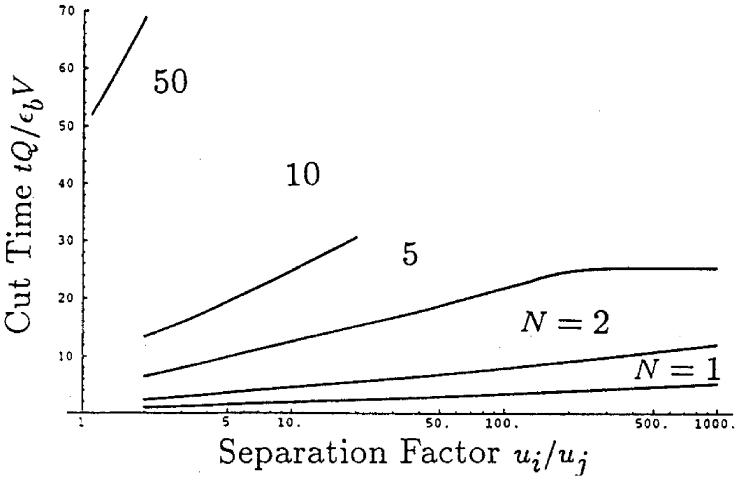

Fig. 6. Separation time or cut time for an N-plate column operated isocratically. Shown is the cut time τ_{cut} as a function of separation factor *vi*/*vj*. Reproduced from Coffmann et al. [59] by kind permission of authors.

column become clear. A monolith with >1000 nm pore size did not produce sufficient resolution with a height of 2 mm, when increased to 50 mm, sufficient resolution could be obtained.

5. Scale up of monoliths

Scale up of monoliths can be carried out either by the concept of similarity or the concept of residence time.

The simplified van Deemter Equation [60] is given as

$$H = A + \frac{B}{u} + Cu \tag{4}$$

where H is the plate height and A, B and C are parameters and u the chromatographic velocity. A counts for the Eddy dispersion, B for the molecular diffusion and C for the mass transfer resistances. When dealing with macromolecules then B can be neglected.

For situations with linear isotherms, the following approach can be used for scaling up a column

$$N = \frac{L}{H} \tag{5}$$

Or rewritten as

$$N = \frac{1}{\frac{A}{L} + \frac{C}{T_0}} \tag{6}$$

with t_0 the residence time

$$t_0 = \frac{L}{V} \tag{7}$$

of a solute in the column.

This equation can be used as a rule of thumb for scale-up of chromatographic separations and shows that especially for monoliths, the late number depends mainly on the residence time t_0.

The A term is very small for monoliths. All parameters such as loading volume, gradient volume, column size and flow-rate must be scaled according to the same residence time.

Another concept for scaling up is radial chromatography. This was described by Strancar et al. [61]. Such a system is shown in Fig. 7. A general rate model was described by Gu et al. [62] for that purpose. The shortcoming of their approach is that the concentration gradients in the axial direction are negligible. This means that the male-distribution of radial flow is ignored. Nevertheless they could predict the peak profiles of different scales.

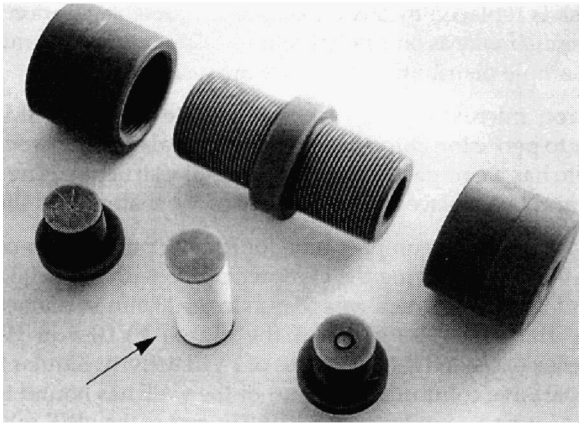

Fig. 7. Construction of the separation unit, which contains a compact porous tube (see arrow). The mobile phase or the sample is pumped from the inner side of the cylinder, flows through the wall and is collected on the other side of the unit. The separation process takes place during flow through the porous wall of the tube, as in the case of a radial column. Reproduced by the kind permission of the authors [61].

6. Applications for protein separation

The majority of applications of monoliths are in the field of capillary electrochromatography [63], chromatography with microcolumns [64] and capillaries [65]. The concept of the ultra-short bed has been applied to a great extent for separation of biopolymers such as proteins and polynucleotides. In Table 1 commercially available monoliths suited for purification of proteins and polynucleotides are listed.

A few representative examples were selected to demonstrate the benefits of monoliths. They were used for analytical as well as preparative separation systems.

Factor IX (FIX), a vitamin K-dependent multidomain plasma glycoprotein with an apparent molecular mass of 65 kD in SDS–PAGE was monitored

Table 1
Commercially available monoliths

Monolith	Company	Material	Pore size	Groups	Stability	Products
CIM	BIASeparations	Methacrylate	1500 nm	DEAE, QA, S, CM, EDA, C4, C2, epoxy, affinity	pH 1–13, NaOH (up to 1 *M*), urea (up to 8 *M*) and Guanidine HCl (up to 6 *M*), organic solvents	0.34 ml disk, 8-ml, 80-ml tube
UNO	Bio-Rad	Acrylate	Estimated from the picture 1000 nm	Q, S	pH range 2 to 12	Columns 0.2, 1, 6, 12 ml
Chromolith	Merck	Silica	Macropore: 2000 nm Mesopore: 13 nm	C_{18}	pH 2–7.5, organic solvents	Columns 50×4.6 mm
Seprasorb	Sepragen	Cellulose	(50–300 micron)	DEAE, QA, CM, S	NaOH (up to 1 *M*), urea (up to 8 *M*) and guanidine HCl (up to 6 *M*), 1 *M* HCl, pH 1–14	Cartridges (10 ml, 2.5×2.0 cm)
Conchrom	Conchrom	Silica	Mesopores: 30 nm Micropores: 5 nm	RP 18, RP 8, etc., ion-exchange	Organic solvents; pH 1–8	Different shapes

and purified by CIM-disks and tubes. Branovic et al. [66] describe the application of monolithic columns for down-stream processing of different clotting factor IX concentrates. An example of separation of FIX from other contaminants is shown in Fig. 8. The dynamic binding capacity of FIX on QA and DEAE disk was determined to be approximately 2000 and 1700 IU/ml support (this corresponds to about 1 and 0.85 mg protein per ml), respectively. A regeneration procedure using 1 *M* NaCl and 0.5 *M* NaOH was

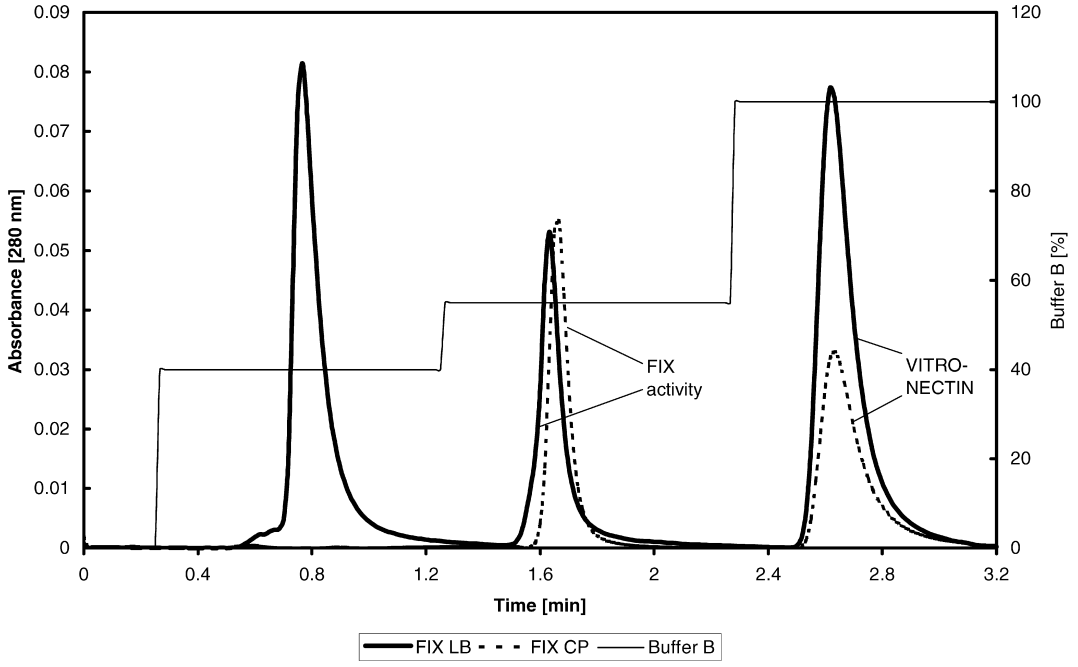

Fig. 8. Optimized chromatographic conditions for the separation of FIX from accompanying plasma proteins in FIX commercial concentrate (FIX CP) and a FIX laboratory batch (FIX LB) [66].

proven to be possible, since the capacity was maintained over five cycles. This feature is very important when sorbents are intended for production of biopharmaceuticals. Silica rods, when sufficiently coated might be resistant enough against alkaline treatment. The dynamic binding capacity of FIX on the upscaled tubes (8 ml monolithic matrix) was 20% lower than on disk. The radial flow profile of the tube from the outside to the inner hole was made responsible for that decrease. The application of a step gradient led to a good separation of FIX and plasma protein impurities as well on the disks as on the tubes. The separated peaks eluted from the tube were analyzed on DEAE disk again in a very short time (3 min) showing that these disks are also a very useful tool for fast in-process control for FIX production. The time scale could be shortened, if another chromatography system was used. CIM disks have also been applied for fast in process control during manufacturing of FIX in large scale [18]. A QA disk with 10-mm diameter and 3-mm thickness was operated at 5 ml/min. Contaminating plasma proteins like transferrin, human serum albumin and IgG could be separated from FIX in less then 1 min. Strancar et al. [23] demonstrated with model protein mixtures that the separation can be accomplished even with a time span less than 15 s (Fig. 9).

Presuming appropriate equipment would be available, separation could be achieved even in less than 1 s. With a flow-rate corresponding to 120 column volumes per min, no loss of resolution was observed. Data on sensitivity accuracy and robustness are not given for the FIX in process control, but they should not differ from normal HPLC experiments.

Another example is the separation of the large complex of clotting factor VIII–von Willebrand factor (FVIII–vWF). This FVIII is a very important therapeutical protein used for treatment of Hemophilia A. The complex has a molecular mass of up 2×10^7 kD depending on the various sizes of the multimers of vWF. The FVIII molecule is sensitive to degradation and tends to unspecifically bind to various kinds of surfaces. Further separation of FVIII–vWF complex was successfully achieved with

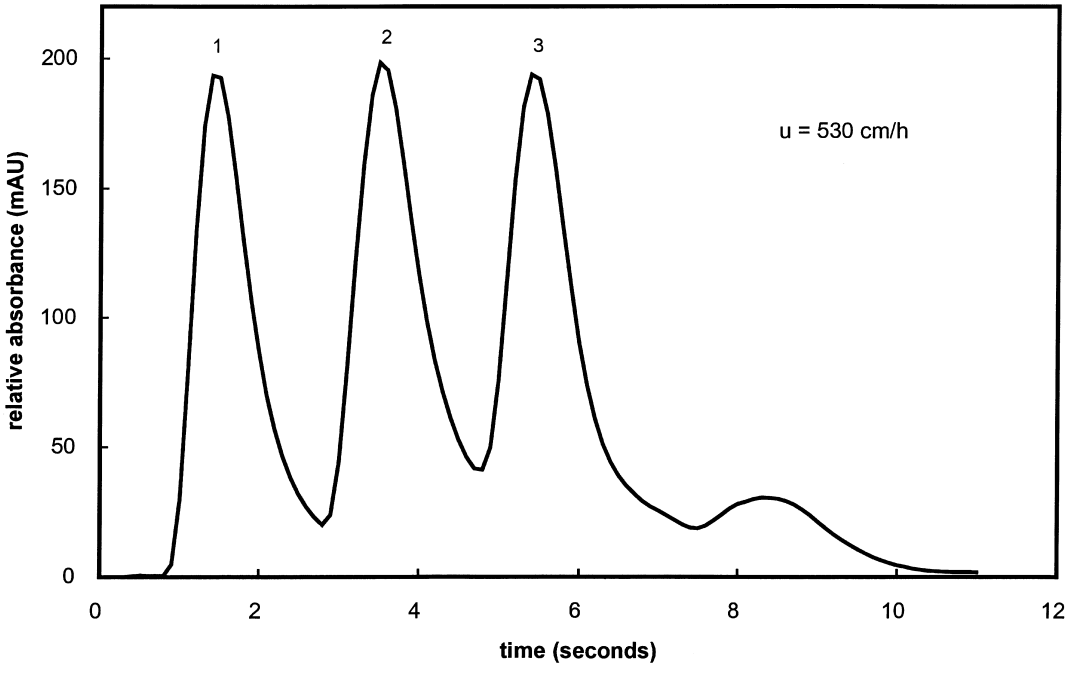

Fig. 9. Separation of a mixture of four standard proteins using a QA-CIM disk from BIA-Separations. (1) Myoglobin, (2) conalbumin, (3) soybean trypsin inhibitor and (4) solvent peak. A segmented gradient was used for this separation. Reproduced by kind permission of authors [23].

QA-tubes [61]. Owing to the nature of FVIII–vWF complex, great attention must be paid on (a) loss of biological activity, caused by adverse interaction with the support material or by decomposition during the purification process; (b) blockage of the separation unit through non-specific interaction with components of the mixture. In the case described by Strancar et al. [61] adverse interaction with vWF, a very sticky glycoprotein with a high molecular mass, is extremely critical. The protein often causes the clogging of the separation unit, leading to the loss of valuable sample. In this application, factor VIII bound to vWF could be separated from vWF which did not form a complex with FVIII by performing a step gradient with a FVIII recovery of more than 80%.

Peptides directed to FVIII [67] were also immobilized to CIM disks by the expoxy-chemistry [50]. This peptide column was used for affinity purification of recombinant and plasma-derived FVIII. Some problems concerning preservation of activity and clogging were also observed as described by Strancar et al. [61].

To obtain high yield and to reduce blocking all running buffers including the feed must be supplemented with a non-ionic detergent. We used frequently Tween 20 in a final concentration of 0.1%.

Hahn et al. [68] demonstrated for a model system, that CIM disks optimally present small affinity peptides. Ligand utilization was highest among all tested supports, either based on natural polymers such as agarose, cellulose or synthetic polymers such as polymethacrylate (Table 2). The authors speculated that the glycidylmethacrylate chains act as spacers. A similar observation was made by Amatschek et al. [50]. In these experiments, regarding FVIII separation, the highest capacity was obtained with CIM-disks.

Fast capture of antibodies and antigens by immunoaffinity chromatography and bacterial immunoglobulin binding proteins were described by various authors [11,47,49]. Tennikova packed disks with various affinity ligands into one cartridge. By doing so they could capture antisera directed to different antigens in one step. After loading and washing, the cartridges are disassembled and each disk is eluted in a single unit. Monospecific polyclonal antibodies were obtained. They conclude that quantitative affinity chromatography with monoliths can become a method of choice for the fine fractionation of pools of polyclonal antibodies from blood serum.

Immunoaffinity chromatography with immobilized monoclonal antibodies on CIM-disks was also used for purification of recombinant proteins by Schuster et al. [51]. They were able to enrich the recombinant proteins in one step to homogeneity. The same antibody immobilized to glass beads with gigapores resulted in a higher capacity, but CIM-disk showed flow independent dynamic capacity as expected for monoliths.

Three annexins CB 33, CBP 35 and CBP 65/67 derived from the plasma membranes of Morris heptoma 7777 were separated on DEAE disk applying a linear gradient as well as a step gradient [11]. In this paper also, the enrichment of immunoglobulins from rabbit serum is shown by loading the serum on disks with immobilized protein A or G.

Table 2
Summary of binding capacity and ligand density of all investigated sorbents

Sorbent	Ligand density mg peptide per ml gel	Binding capacity mg lysozyme per ml gel
Sepharose CL 6B epibromohydrin	9.2	6.0
Sepharose CL 6B diglycidylether	9.1	$\ll 1$
CIM epoxy	4.0	5.0
Fractogel azlacton	8.0	$\ll 1$
Fractogel epoxy	8.0	$\ll 1$
	18.5	4.2
Fractogel β-alanin	8.5	$\ll 1$
Fractogel EDA-SA	9.0	1.0
Fractogel DADPA-SA	9.0	12.5

The eluted immunoglobulin pool is further purified into monospecific polyclonal anti-annexin antibodies on disks with coupled annexins, each annexin on a separate disk.

Recombinant human basic fibroblast growth factor was purified by Garke et al. [69] using polyacrylamide based monoliths the so-called UNO columns. The dynamics of rh-b FGF aggregation and reaggregation in the crude feedstock was monitored by fast gradient elution chromatography.

Besides ion-exchange and affinity adsorption, hydrophobic interaction (HI) and reversed-phase (RP) interaction were described in connection with monoliths and protein separation. Limited applications are available for these modes.

A recombinant human tumor necrosis factor α (TNF-α) was purified by HI chromatography on C4 disks (25×3 mm) after isolation from an *E. coli* extract by an anionic-exchange step [70]. The separation efficiency and recovery of TNF-α was comparable to those on a Phenyl-Sepharose column. However, the separation time was reduced considerably. The active form of the protein is a trimer. HIC caused a dissociation of the trimer into monomers, a partial loss of activity being the consequence. The authors assume that monomers are formed due to the interaction of the sample with the hydrophobic ligand.

Xie et al. [21] reported hydrophobic interaction chromatography of proteins on monolithic columns containing butyl methacrylate. The authors claim that the hydrophobicity of the interacting surface can be easily controlled by the percentage of butyl methacrylate in the polymerization mixture. A protein mixture of cytochrome C, ribonuclease and lysozme was separated within 4 min.

Tennikova et al. [71] showed the separation of a mixture of proteins (myoglobin, ovalbumin, lysozyme and chymotrypsinogen) on monolithic ''membranes'' (20×1 mm) with C4 or C8 ligands. A separation of 5 mg of protein was achieved in 30 min.

Microcolumns filled with a monolithic bed C_{18}-derivatized were used for the separation of proteins in the reversed-phase mode [16]. Standard proteins were separated within 100 s using a linear acetonitrile gradient.

Tennikova and Svec [38] investigated the effect of gradient volume, gradient time, gradient profile and flow-rate on resolution performing ion-exchange, hydrophobic interaction and reversed-phase chromatography. The experiments confirmed that separation on monoliths obeys the rules of typical column chromatography. According to their calculations, the protein diffusivity enhanced by the convective flow through the monolith is about four orders of magnitude higher than the free diffusivity of the protein in the stagnant mobile phase located in the pores of a standard separation medium.

7. Purification of oligonucleotides and DNA

The full potential of monoliths was shown by Podgornik et al. [57] separating oligonucleotides from 8 to 14 mers in isocratic mode on a very thin (0.75 mm) DEAE disk. This application confirms the utility of the concept of a ultra short column to speed up separation. Although separation significantly improved with increased column length (0.75–12 mm) the optimization of the mobile phase led to a very good separation of all four oligomers on a 0.75-mm disks. The convective mass transport through the flow-through pores presumably causes an increase in the number of adsorption/desorption steps. HETP was calculated to be 18 μm. Separation behavior was very similar to HPLC filled with conventional porous particles. The increase of salt concentration in the mobile phase decreased the retention time. Higher retention times caused broadening of peaks. The maximum peak height was inversely proportional to the retention time.

A similar work was published almost at the same time by Sykora et al. [27], but not exploiting the concept of the ultra short bed. They were able to separate oligonucleotides from 12 to 24 mers at different flow-rates with a resolution of at least one. They applied a linear gradient. We make the nature of the stationary phase responsible for the better performance of the system reported by Podgornik et al. [57]. Unfortunately the molecular mass of the oligonucleotides in the samples of the different authors were not identical. Sykora et al. [27] used oligos with a higher molecular mass meaning that the number of interacting charges with the sorbent increase. It would be interesting to see if the samples

separated by Sykora et al. [27] could be also separated to the same resolution by the system of Podgornik et al. [57].

Plasmid DNA separation was described by Giovannini et al. [56]. Under optimized conditions a plasmid DNA preparation could be separated in 3 peaks presumably corresponding to supercoiled, nicked and open circular plasmid DNA. Applying linear gradients a much higher salt concentration can be used in the starting buffer. Isocratic separation was also possible using QA disks in contrast to protein chromatography. Higher flow-rates led to a decrease in resolution although the operation with these units is normally flow independent to a certain limit. The authors assume that at flow-rates higher than 1 ml/min the residence time is too low for adsorption. The applicability was demonstrated but further work will be necessary to optimize the separation for analytical as well as preparative purposes.

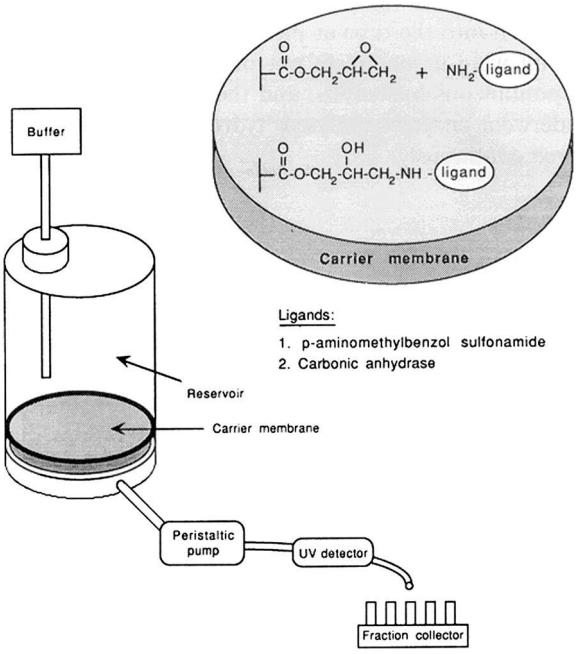

Fig. 10. Structure of the carrier membranes and its carrying device. Membranes consisted of a polymer matrix with an epoxy-activated surface. Ligands containing amino groups could be coupled by amine bonds. The membrane was tightly fixed in a vial. Buffer was loaded on the membrane and drawn by means of a peristaltic pump through the membrane and the UV detector towards the fraction collector [7].

8. Immobilization of enzymes and fast conversion of substrates with low or high molecular masses

As early as 1991, shortly after the first use of monolithic supports made of poly-glycidyl-methacrylate [52], Abou-Rebyeh et al. [7] have carried out the first conversion of a substrate with the immobilized enzyme carbonic anhydrase in flow-through. At first, this enzyme was isolated from the hemolysate of human erythrocytes, using affinity chromatography with the inhibitor p-aminomethylbenzene sulphonamide, which was immobilized to a monolith. The purified, active carbonic anhydrase was subsequently immobilized to an epoxy-activated disk. The disk was fixed at the bottom of a container. In this way a kind of flow-through reactor was created, with an enzyme immobilized to a monolith (cf. Fig. 10). Immobilization of carbonic anhydrase provided an opportunity to carry out kinetic experiments under dynamic conditions. The substrates used were 4-nitrophenyl acetate and 2-chloro-4-nitrophenyl acetate. Although in these experiments rather low flow-rates of only up to 1.2 ml/min were used, it was shown that a higher flow-rate led to an increase in enzymatic activity (cf. Fig. 11). This result was at first surprising, but agreed with the findings of Unarska et al. [72] that diffusion in monolithic supports is much faster and no longer a limiting factor for enzyme-substrate interaction. The following investigations have shown that in reactors like this a transport based on diffusion hardly occurs at all (see above).

Subsequent experiments have shown that other enzymes such as trypsin can be successfully immobilized and used for the conversion of substrates of both high [23] and low [19] molecular masses. In experiments similar to those in Fig. 11, Petro et al. [17] have shown within the framework of a comparative study that trypsin immobilized on to both macroporous beads and a monolithic support not only results in higher catalytic activity of trypsin bound to a monolith, but also in a much higher throughput. This is achieved with a monolith because of its efficient mass transfer, which is superior to that

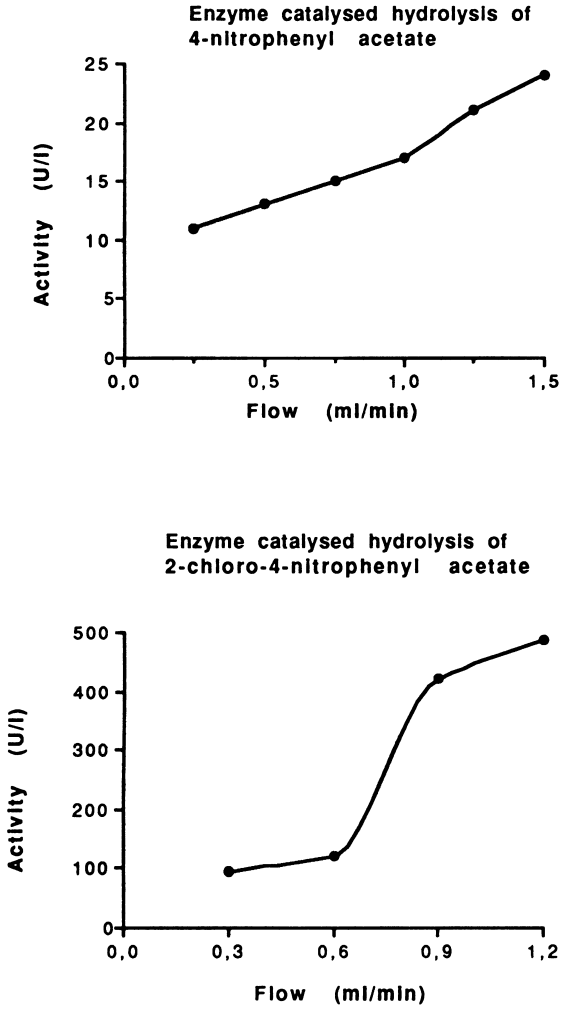

Fig. 11. Relationship between flow and activity of carbonic anhydrase immobilized on a carrier membrane. 4-Nitrophenyl acetate and 2-chloro-4-nitrophenyl acetate were used as substrates. Substrate solution was pumped through the membrane at different flow-rates. The enzymatic activity for both substrates increased at greater flow-rates [7].

of a compact porous support. If enzymes such as trypsin, immobilized to monoliths, are used for conversion of substrates with high molecular masses, the flow-rate may become a limiting factor [23]. In order to cause digestion of protein samples by trypsin immobilized to a monolith, the polypeptide chain has to be unfolded by adding SDS. Hydrolysis of such substrates is more successful at lower flow-rates. In order to investigate further the enzymatic conversion of substrates with high molecular masses under such conditions, more detailed experiments have to be carried out.

Other enzymes that have been used for immobilization on monoliths, are invertase [23] and glucose oxidase [23,73]. Recently, research has been concentrating on the optimization of enzyme immobilization, e.g., the introduction of spacers and the improvement of support chemistry. Besides, the options for miniaturization on the one hand and for scaling-up on the other hand have been tested [23,73,74]. The most frequently used model enzymes are trypsin [17,74] and glucose oxidase [23]. Vodopivec et al. [73] have recently used the monolithic, so-called convective interaction media (CIM) for immobilization of glucose oxidase. A so-called CIM-disk with immobilized enzyme, 12 mm in diameter and 3 mm thick, was used for on-line monitoring of glucose during cultivation of *Saccharomyces cerevisiae*. These disks successfully replaced conventional packed-bed columns.

The application described above shows the options for the use of such reactors on an analytical scale. However, scaling-up experiments with enzymes immobilized to monoliths are not found very often. Although it is technically possible even now to produce reactors like this in sizes of up to 100 ml and more [75], the experiments published so far deal with entities of only a few ml [23,74]. The production of the larger reactors will require the solution of problems in connection with the design of such units. When used for continuous conversion of complex substrate solutions, fouling will be one of the problems, and a continuous cleaning of the surface of the reactor has to be provided [76].

Despite such obstacles, the experiments carried out so far indicate that there are many options for the use of enzymes immobilized to monolithic supports, analytical scale as well as large scale.

9. Conclusion

Monoliths were successfully used for fast separation and in-process control of proteins and polynucleotides. The special feature of all reported examples was the fast separation and enzymatic conver-

sion. Even extremely big molecules such as the FVIII–VWF complex with a molecular mass higher than a relative molecular mass of 1 000 000 could be accomplished. Scale up was achieved by casting monolithic tubes. The changing flow profile with radius did not effect the resolution, since the mass transfer is not highly effected by the velocity. Monoliths may become an alternative separation medium for analytical and preparative applications in biotechnology.

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