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Modern Advances in Chromatography





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Modern Advances in Chromatography

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Preface

Due to their versatility and resolution, chromatographic separations of complex mixtures of biologicals are used for many purposes in academia and industry. If anything, recent developments in the life sciences have increased the interest and need for chromatography be it for quality control, proteomics or the downstream processing of the high value products of modern biotechnology. However, the many "challenges" of present day chromatography and especially of the HPLC of biomacromolecules such as proteins, are also present in the mind of any practitioner. In fact, some of these latter were such hindrances that much research was necessary in order to overcome and circumvent them. This book introduces the reader to some of the recently proposed solutions. Capillary electrochromatography (CEC), for example, the latest and most promising branch of analytical chromatography, is still hindered from finding broader application by difficulties related to something as simple as the packing of a suitable column. The latest solutions for this but also the state of art of CEC in general are discussed in the chapter written by Frantisek Svec. The difficulty of combining speed, resolution and capacity when using the classical porous bead type stationary phases has even been called the "dilemma of protein chromatography". Much progress has been made in this area by the advent of monolithic and related continuous stationary phases. The complex nature of many of the samples to be analyzed and separated in biochromatography often requires the use of some highly specific ("affinity") ligands. Since they can be raised in a specific manner to many bioproducts, protein ligands such as antibodies have allowed some very selective solutions in the past. However, they also are known to have some disadvantages, including the immunogenicity (toxicity) of ligands contaminating the final products, or the low stability of such ligands, which prevents repeated usage of the expensive columns. This challenge may be overcome by "molecular imprinting", a techniques, which uses purely chemical means to create the "affinity" interaction. Finally we were most happy to have two authors from industry join us to report on their experience with chromatography as a continuous preparative process. Readers from various fields thus will find new ideas and approaches to typical separation problems in this volume. Finally, I would like to thank all the authors for their contributions and their cooperation throughout the last year.

Lausanne, April 2002

Ruth Freitag

Capillary Electrochromatography: A Rapidly Emerging Separation Method

Frantisek Svec

F. Svec, Department of Chemistry, University of California, Berkeley, CA 94720-1460, USA. *E-mail: svec@uclink4.berkeley.edu*

This overview concerns the new chromatographic method – capillary electrochromatography (CEC) – that is recently receiving remarkable attention. The principles of this method based on a combination of electroosmotic flow and analyte-stationary phase interactions, CEC instrumentation, capillary column technology, separation conditions, and examples of a variety of applications are discussed in detail.

Keywords. Capillary electrochromatography, Theory, Electroosmotic flow, Separation, Instrumentation, Column technology, Stationary phase, Conditions, Applications

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1 Introduction

The recently decoded human genome is believed to be a massive source of information that will lead to improved diagnostics of diseases, earlier detection of genetic predispositions to diseases, gene therapy, rational drug design, and pharmacogenomic "custom drugs". The upcoming "post-genome" era will then target the gene expression network and the changes induced by effects such as disease, environment, or drug treatment. In other words, the knowledge of the exact composition of proteins within a living body and its changes reflecting both healthy and sick states will help to study the pharmacological action of potential drugs at the same speed as the candidates will be created using the methods of combinatorial chemistry and high throughput screening. This approach is assumed to simplify and accelerate the currently used lengthy and labor-intensive experiments with living biological objects. To achieve this goal, new advanced very efficient and selective multidimensional separation methods and materials must be developed for "high-throughput" proteomics [1, 2]. The limited speed and extensive manual manipulation required by today's two-dimensional gel electrophoresis introduced by O'Farrell 25 years ago [3] is unlikely to match the future needs of rapid screening techniques due to the slow speed and complex handling of the separations, and the limited options available for exact quantification [4]. Therefore, new approaches to these separations must be studied [5]. Microscale HPLC and electrochromatography are the top candidates for this mission since they can be included in multidimensional separation schemes while also providing better compatibility with mass spectrometry, currently one of the best and most sensitive detection methods [6].

After several decades of use, HPLC technology has been optimized to a very high degree. For example, new columns possessing specific selectivities, drastically reduced non-specific interaction, and improved longevity continue to be developed. However, increases in the plate counts per column - the measure of column efficiency – have resulted almost exclusively from the single strategy of decreasing the particle size of the stationary phase. These improvements were made possible by the rapid development of technologies that produced well-defined beads with an ever-smaller size. Today, shorter 30-50 mm long column packed with 3 µm diameter beads are becoming the industry standard while 150–300 mm long columns packed with 10-µm particles were the standard just a few years ago [7]. Although further decreases in bead size are technically possible, the lowered permeability of columns packed with these smaller particles leads to a rapid increase in flow resistance and a larger pressure drop across the column. Accordingly, only very short columns may be used with current instrumentation and the overall improvement, as measured by the efficiency per column, is not very large. In addition, the effective packing of such small beads presents a serious technical problem. Therefore, the use of submicrometer-sized packings in "classical" HPLC columns is not practical today and new strategies for increasing column efficiency must be developed.

Another current trend in HPLC development is the use of mini- and microbore columns with small diameters, as well as packed capillaries that require much smaller volumes of both stationary and mobile phases. This miniaturization has been driven by environmental concerns, the steadily increasing costs of solvent disposal, and, perhaps most importantly, by the often limited amounts of samples originating from studies in such areas as proteomics. The trade-off between particle size and back pressure is even more pronounced in these miniaturized columns. For example, Jorgenson had to use specifically designed hardware that enabled operating pressures as high as 500 MPa in order to achieve an excellent HPLC separation of a tryptic digest in a 25 cm long capillary column packed with 1-µm silica beads [8].

In contrast to mechanical pumping, electroendoosmotic flow (EOF) is generated by applying an electrostatic potential across the entire length of a device, such as a capillary or a flat profile cell. While Strain was the first to report the use of an electric field in the separation of dyes on a column packed with alumina [9], the first well documented example of the use of EOF in separation was the "electrokinetic filtration" of polysaccharides published in 1952 [10]. In 1974, Pretorius et al. realized the advantage of the flat flow profile generated by EOF in both thinlayer and column chromatography [11]. Although their report did not demonstrate an actual column separation, it is frequently cited as being the foundation of real electrochromatography. It should be noted however that the term electrochromatography itself had already been coined by Berraz in 1943 in a barely known Argentine journal [12].

The real potential of electrochromatography in packed capillary columns (CEC) was demonstrated in the early 1980s [13-15]. However, serious technical difficulties have slowed the further development of this promising separation method [16, 17]. A search for new microseparation methods with vastly enhanced efficiencies, peak capacities, and selectivities in the mid 1990s revived the interest in CEC. Consequently, research activity in this field has expanded rapidly and the number of published papers has grown exponentially. In recent years, general aspects of CEC has been reviewed several times [18–24]. Special issues of Journal of Chromatography Volume 887, 2000 and Trends in Analytical Chemistry Volume 19(11), 2000 were entirely devoted to CEC and a primer on CEC [25] as well as the first monograph [26] has recently also been published.

2 Concept of Capillary Electrochromatography

Capillary electrochromatography is a high-performance liquid phase separation technique that utilizes flow driven by electroosmosis to achieve significantly improved performance compared to HPLC. The frequently published definition that classifies CEC as a hybrid of capillary electrophoresis (CE) and HPLC is actually not correct. In fact, electroosmotic flow is not the major feature of CE and HPLC packings do not need to be ionizable. The recent findings by Liapis and Grimes indicate that, in addition to driving the mobile phase, the electric field also affects the partitioning of solutes and their retention [27–29].

Although capillary columns packed with typical modified silica beads have been known for more then 20 years [30, 31], it is only now that both the chro-

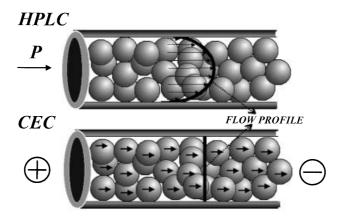
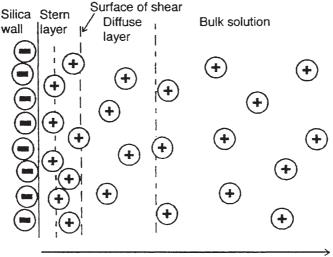


Fig. 1. Flow profiles of pressure and electroosmotically driven flow in a packed capillary

matographic industry and users are starting to pay real attention to them. This is because working with systems involving standard size columns was more convenient and little commercial equipment was available for the microseparations. This has changed during the last year or two with the introduction of dedicated microsystems by the industry leaders such as CapLC (Waters), UltiMate (LC Packings), and 1100 Series Capillary LC System (Agilent) that answered the need for a separation tool for splitless coupling with high resolution mass spectrometric detectors. Capillary μ HPLC is currently the simplest quick and easy way to clean up, separate, and transfer samples to a mass spectrometer, the feature valued most by researchers in the life sciences. However, the peak broadening of the μ HPLC separations is considerably affected by the parabolic profile shown in Fig. 1 typical of pressure driven flow in a tube [32]. To avoid this weakness, a different driving force – electroosmotic flow – is employed in CEC.

2.1 Electroosmotic Flow

Robson et al. [21] in their excellent review mention that Wiedemann has noted the effect of electroosmosis more than 150 years ago. Cikalo at al. defines electroosmosis as the movement of liquid relative to a stationary charged surface under an applied electric field [24]. According to this definition, ionizable functionalities that are in contact with the liquid phase are required to achieve the electroosmotic flow. Obviously, this condition is easily met within fused silica capillaries the surface of which is lined with a number of ionizable silanol groups. These functionalities dissociate to negatively charged Si–O⁻ anions attached to the wall surface and protons H⁺ that are mobile. The layer of negatively charged functionalities repels from their close proximity anions present in the surrounding liquid while it attracts cations to maintain the balance of charges. This leads to a formation of a layered structure close to the solid surface rich in



Distance from capillary wall

Fig. 2. Scheme of double-layer structure at a fused silica capillary wall. (Reprinted with permission from [24]. Copyright 1998 Royal Chemical Society)

cations. This structure consists of a fixed Stern layer adjacent to the surface covered by the diffuse layer. A plane of shear is established between these two layers. The electrostatic potential at this boundary is called ζ potential. The doublelayer has a thickness δ that represent the distance from the wall at which the potential decreases by e^{-1} . The double-layer structure is schematically shown in Fig. 2. Table 1 exemplifies actual thickness of the double-layer in buffer solutions with varying ionic strength [33].

After applying voltage at the ends of a capillary, the cations in the diffuse layer migrate to the cathode. While moving, these ions drag along molecules of solvating liquid (most often water) thus producing a net flow of liquid. This phenomenon is called electroosmotic flow. Since the ionized surface functionalities are located along the entire surface and each of them contributes to the flow, the overall flow profile should be flat (Fig. 1). Indeed, this has been demonstrated in several studies [32, 34] and is demonstrated in Fig. 3. Unlike HPLC, this plug-like flow profile results in reduced peak broadening and much higher column efficiencies can be achieved.

c, mol/l	δ, nm	
0.1	1.0	
0.01	3.1	
0.001	10.0	

Table 1. Effect of buffer concentration c on thicknessof the electrical double layer δ [33]

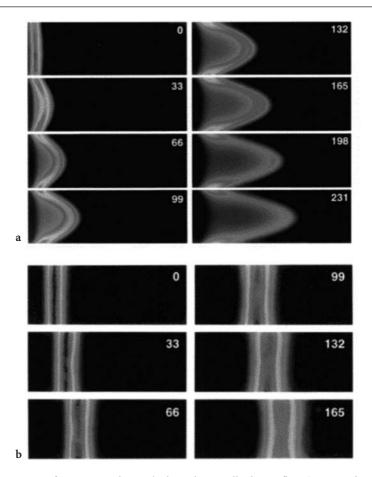


Fig. 3 a, b. Images of: **a** pressure-driven; **b** electrokinetically driven flow. (Reprinted with permission from [32]. Copyright 1998 American Chemical Society). Conditions: (**a**) flow through an open 100 μ m i.d. fused-silica capillary using a caged fluorescein dextran dye and pressure differential of 5 cm of H₂O per 60 cm of column length; viewed region 100 by 200 μ m; (**b**) flow through an open 75 μ m i.d. fused-silica capillary using a caged rhodamine dye; applied field 200 V/cm, viewed region 75 by 188 μ m. The frames are numbered in milliseconds as measured from the uncaging event

The plug flow profile would only be distorted in very narrow bore capillaries with a diameter smaller than the thickness of two double-layers that then overlap. To achieve an undisturbed flow, Knox suggested that the diameter should be 10-40 times larger than δ [15]. This can easily be achieved in open capillaries. However, once the capillary is packed with a stationary phase, typically small modified silica beads that carry on their own charged functionalities, the distance between adjacent double-layers is only a fraction of the capillary diameter. However, several studies demonstrated that beads with a submicrometer size can be used safely as packings for CEC columns run in dilute buffer solutions [15, 35].

	Pressurized flow		Electroosmotic flow	
Packing size, µm	3	1.5	3	1.5
Column length, cm	66	18	35	11
Elution time, min	33	n.a.	18	6
Pressure, MPa	40	120 ^b	0	0

 Table 2. Comparison of parameters for capillary columns operated in pressurized and electrically driven flow^a [37]

^a Column lengths, elution times, and back pressures are given for a capillary column affording 50,000 plates at a mobile phase velocity of 2 mm/s.

^b The back pressure exceeds capabilities of commercial instrumentation (typically 40 MPa).

In columns with thin double layers typical of dilute buffer solution, the electroosmotic flow, u_{eo} , can be expressed by the following relationship based on the von Smoluchowski equation [36]:

$$u_{eo} = \varepsilon_r \varepsilon_o \zeta E/\eta \tag{1}$$

where ε_r is the dielectric constant of the medium, ε_o is the permittivity of the vacuum, ζ is the potential at the capillary inner wall, *E* is the electric field strength defined as *V/L* where *V* is the voltage and *L* is the total length of the capillary column, and η is the viscosity of the bulk solution. The flow velocity for pressure driven flow *u* is described by Eq. (2):

$$u = d_p^2 \,\Delta P / \phi \,\eta L \tag{2}$$

where d_p is the particle diameter, ΔP is the pressure drop within the column, and ϕ is the column resistance factor that is a function of the column porosity (typically $\phi = 0.4$). In contrast to this, Eq. (1) does not include a term involving the particle size of the packing. Therefore, the lower limit of bead size in packed CEC columns is restricted only by the requirement of avoidance of the double-layer

d _p , μm ^a	Pressurized flow, HPLC		Electroosmotic flow, CEC		
	L, cm ^b	Plates/column	L, cm	Plates/column	
5	50	45,000	50	90,000	
3	30 c	50,000	50	150,000	
1.5	15 c	33,000	50	210,000	

Table 3. Comparison of efficiencies for capillary columns packed with silica particles operatedusing pressurized and electrically driven flow [37]

^a Particle diameter.

^b Column lengths.

^c Column length is dictated by the pressure limit of commercial instrumentation (typically 40 MPa).

overlap. However, a more important implication of this difference is the absence of back pressure in devices with electrically driven flow. Table 2 demonstrates these effects on conditions that have to be met to achieve an equal efficiency of 50,000 plates in columns packed with identical size beads run in both HPLC and CEC modes. Obviously, CEC requires much shorter column length and the separation is faster. Table 3 shows that the decrease in particle size leads to an increase in the column efficiency per unit length for both HPLC and CEC. However, the actual efficiency per column in HPLC decreases as a result of the shorter column length that must be used to meet the pressure limits of the instrumentation. In contrast, the use of the CEC mode is not limited by pressure, the columns remain equally long for beads of all sizes in the range of $1.5 - 5 \mu m$, and the column efficiency rapidly increases [37].

3 CEC Instrumentation

The simplest CEC equipment must include the following components: a highvoltage power supply, solvent and sample vials at the inlet and a vial to collect waste at the outlet of the capillary column, a column that simultaneously generates EOF and separates the analytes, and a detector that monitors the component peaks as they leave the column. Figure 4 shows a scheme of an instrument that

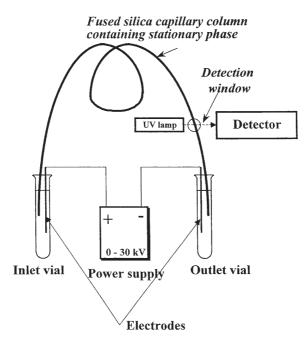


Fig. 4. A simplified schematic diagram of CEC equipment

in addition to the basic building blocks also includes a module that enables pressurization of the vials to avoid bubble formation within the column. The column itself is then placed in a temperature-controlled compartment that helps to dissipate the Joule heat created by the electric field. All these elements are built in more sophisticated commercial instruments such as the Capillary Electrophoresis System (Agilent Technologies).

Pressurization of the vials at both the inlet and the outlet ends of the CEC capillary column packed with particles to about 1.2 MPa is required to prevent formation of bubbles that lead to a noisy baseline. Typically, equal pressure of an inert gas such as nitrogen is applied to both vials to avoid flow that would otherwise occur resulting from the pressure difference. Hydraulic pressure applied only at the inlet end of the capillary column is occasionally used in pressure-assisted electrochromatography [38, 39].

The number of dedicated commercial instruments for CEC is very limited. Large manufacturers such as Agilent Technologies (Wallbron, Germany) and Beckmann/Coulter (Fullerton, CA, USA) implemented relatively minor adjust-

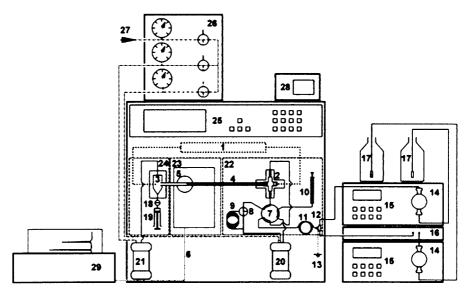


Fig. 5. Capillary electrochromatograph with gradient elution capability. (Reprinted with permission from [153]. Copyright 1997 American Chemical Society): 1, high-voltage power supply; 2, inlet reservoir with electrode; 3, outlet reservoir with electrode; 4, packed capillary column; 5, on-line sensing unit (UV detector); 6, detector output, 0-1 V; 7, sample injection valve; 8, purge valve; 9, restrictor; 10, syringe for introduction of sample or buffer; 11, capillary resistor; 12, static mixing tee; 13, grounding; 14, pumps; 15, pump control panels and readouts; 16, manometer; 17, eluent reservoirs; 18, switching valve; 19, syringe for buffer introduction; 20, waste reservoir at the inlet; 21, waste reservoir at the outlet; 22, thermostated inlet compartment; 23, detector compartment; 24, outlet compartment; 25, CEC instrument control panel; 26, gas pressure control; 27, gas inlet, 1.4 MPa nitrogen; 28, temperature control; 29, data acquisition. Line symbols: ..., electric wiring; –, liquid lines; –·–, gas lines; –––, separating lines between instrument compartments

ments to their well-established instrumentation for capillary electrophoresis. Smaller companies such as Microtech Scientific, Inc. (Sunnyvale, CA, USA) and Unimicro Technologies, Inc. (Pleasanton, CA, USA) have developed instruments that can be used for μ HPLC, CE, CEC, and pressurized CEC. Although this type of equipment addresses some of the weaknesses of the adapted CE instrumentation, the current market still lacks a reliable instrument for CEC that enables gradient elution, electrical fields higher than 1 kV/cm, or that includes a column compartment with well-controlled heating and accommodates even short capillaries. Current instrumentation is also not compatible with 96 or 384 well plate formats for direct sampling [40].

Since the commercial instrumentation does not satisfy the needs of specific CEC research, a number of groups described their home-built equipment. For example, Dittmann et al. developed an additional module that, once attached to HP

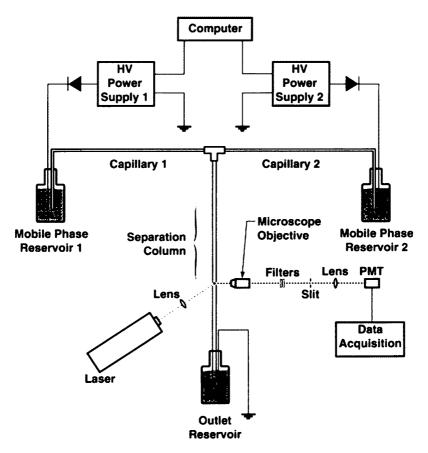


Fig.6. Schematic of the solvent gradient elution CEC apparatus with ramping voltage accessory. (Reprinted with permission from [204] Copyright 1996 American Chemical Society)

^{3D}CE instrument, allows operation in a gradient mode [41]. Horváth's group developed equipment for gradient CEC shown in Fig. 5 that allowed for combination of several chromatographic modes. These two and some other groups used a standard gradient HPLC system for the preparation of a mobile phase gradient that is delivered to the inlet of the capillary column through an interface. In contrast, Zare's group used electroosmotic pumping from two eluent reservoirs (Fig. 6). The gradient was obtained by ramping the voltage between these two reservoirs. A detailed description of CEC instrumentation has been published recently by Steiner and Scherer [39].

4 Column Technologies for CEC

CEC is often inappropriately presented as a hybrid method that combines the capillary column format and electroosmotic flow employed in high-performance capillary electrophoresis with the use of a solid stationary phase and a separation mechanism, based on specific interactions of solutes with the stationary phase, characteristic of HPLC. Therefore CEC is most commonly implemented by means typical of both HPLC (packed columns) and CE (use of electrophoretic instrumentation). To date, both columns and instrumentation developed specifically for CEC remain scarce.

Although numerous groups around the world prepare CEC columns using a variety of approaches, the vast majority of these efforts mimic in one way or another standard HPLC column technology. However, aspects of this technology have proven difficult to implement on the capillary scale. Additionally, the stationary phases packed in CEC capillaries are often standard commercial HPLCgrade beads. Since these media are tailored for regular HPLC modes and their surface chemistries are optimized accordingly, their use incorrectly treats CEC as a subset of HPLC. Truly optimized, CEC packings should play a dual role: in addition to providing sites for the required interactions as in HPLC, they must also be involved in electroosmotic flow. As a result, packings that are excellent for HPLC may offer limited performance in the CEC mode. This realization of the basic differences between HPLC and CEC [33] has stimulated the development of both specific particulate packings having properties tuned for the needs of CEC as well as alternative column technologies. Generally, column technology remains currently one of the "hottest" issues in CEC and the progress in this area has been summarized in several recent review articles [42-46].

4.1 Packed Columns

The influence of HPLC on the development of separation media for CEC is rather obvious. For example, HPLC-like "hardware", such as frits and packed columns, are employed. A number of various packing technologies have been reported that enable packing particles into narrow bore capillary columns. The solvent slurry packing appears to be the most popular technique that has been transferred di-

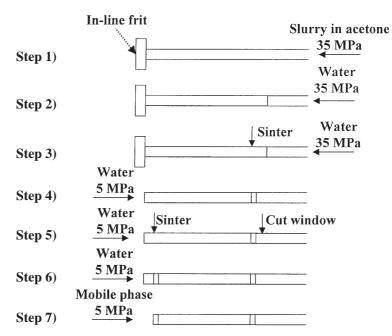


Fig.7. Scheme of a typical process used for packing CEC columns with beads

rectly from the HPLC. In contrast to relatively simple procedures widely used in HPLC, slurry packing of columns for CEC is more complex. The scheme in Fig. 7 shows as an example the individual steps required to fabricate an efficient column [47]. These include:

- 1. Attaching an in-line end-frit and packing the column by pumping a slurry of beads and solvent into the capillary under high pressure. Sonication is recommended to achieve better quality.
- 2. Flushing the packed column with water at high pressure to replace the solvent.
- 3. Preparing the outlet end-frit at the desired distance from the column end by sintering the silica beads using heating to a temperature of over 550 °C.
- 4. Removing the in-line end-frit and flushing out the extra-column packing materials using reversed flow direction.
- 5. Sintering of the packing materials to create the inlet end-frit at a distance representing the desired packed segment length followed by the removal of the polyimide coating from the detection window close to the outlet frit.
- 6. Cutting off the excess capillary close to the inlet frit.
- 7. Washing the packed capillary with the desired mobile phase

Since the general concept in CEC is to use packing materials with a beads size as small as possible, the viscosity of the liquid used for slurring the beads is critical. Equation (2) rearranged to

$$\Delta P = u \phi \eta L/d_p^2 \tag{3}$$

Packing method	Analyte	k'	<i>N</i> , plates/m
Pressurized slurry	Thiourea Amylbenzene	- 2.4	86,600 104,100
Supercritical CO ₂	Thiourea Amylbenzene	- 2.1	143,200 179,400
Centripetal force	Thiourea Amylbenzene	- 2.2	181,800 181,800
Electrokinetic	Thiourea Amylbenzene	2.3	98,800 136,700

Table 4. Retention factors *k'* and column efficiencies *N* for an unretained thiourea and retained compound amylbenzene in columns packed by different methods [53]

clearly shows that the pressure required to push a liquid through the packed capillary exponentially increases with the decrease in bead diameter. Although use of slower flow velocity could be the solution to this problem, it would lead to excessively long packing times and the uncontrolled sedimentation of particles would reduce the homogeneity of the bed, thus negatively affecting the efficiency. Therefore, the use of liquids with lower viscosity is more convenient and enables packing columns at reasonable pressures. Several groups have reported the use of supercritical CO_2 , a liquid that has very low viscosity and is easy to handle, in slurry packing of CEC columns [48, 49].

Yan developed a method that employs electrokinetic migration of charged silica beads [50]. The capillary is attached to a reservoir filled with slurry and the electric field is applied. The beads then move towards the anode in a stagnant liquid phase thus substituting the typical pumping of a liquid through the capillary. This remarkably simple method requires beads of very narrow size distribution since their surface area and consequently their net charge and migration rate increase with the decreasing bead diameter. If a polydisperse mixture of particles is used, the smaller beads migrate faster and this leads to the formation of inhomogeneous beds.

Colón and Maloney demonstrated another packing method that also avoids pumping the slurry through the column [51]. They used centripetal force to drive beads, which have a higher density than the liquid contained in solvent slurry, through the capillary. Their packing equipment enables a rotation speed of up to 3000 rpm at which the packing time is only 5–15 min.

Since the packing process always includes several steps, it requires specific skills to prepare highly efficient capillaries reproducibly. Obviously, the procedures described above are not trivial and the results obtained with each of them may differ substantially [52]. Table 4 compares data obtained for capillaries packed using four different methods [53]. The major challenge appears to be the in situ fabrication of retention frits. Tapered ends of the capillary columns introduced recently may help to solve this serious problem [54, 55]. The other problem is rearrangement of beads in the bed affected by their electromigration.

4.1.1 Packing Materials

The correct choice of the packing material, typically functionalized silica beads, is extremely important to achieve the best performance in CEC. Since specialized CEC packings are emerging only slowly [7], typical HPLC separation media are being frequently used to pack CEC columns. Figure 8 demonstrates the effect of the stationary phase in the separation of polyaromatic hydrocarbons (PAHs) [56]. The results are simple to interpret: the base deactivated BDS-ODS-Hypersil contains the lowest surface coverage with silanol groups that are the driving force for flow. Therefore, the separation requires a long time. The magnitude of electroosmotic flow produced by the packings largely depends on the extent of endcapping of residual silanol groups that is required to avoid peak tailing in HPLC. In contrast, the specifically developed CEC Hypersil C18 affords both good flow and fair selectivity. Table 5 summarizes properties and electroosmotic mobilities for a selected group of commercial packings [57].

In order to increase the electroosmotic flow, a number of studies used beads with specifically designed surface chemistries that involved strong ion-exchange functionalities. The famous yet irreproducible separations of basic compounds with an efficiency of several millions of plates has been achieved with silica based

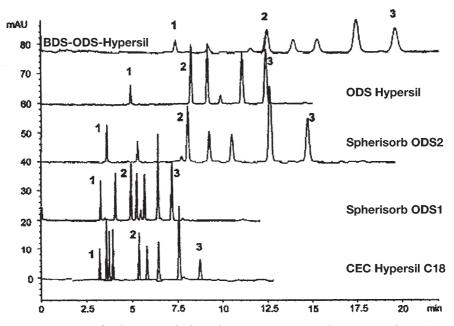


Fig. 8. Separation of polyaromatic hydrocarbons using commercial stationary phases. (Reprinted with permission from [56]. Copyright 1997 VCH-Wiley). Conditions: voltage 20 kV, capillary column 100 μ m i. d., total length 33.5 cm, active length 25 cm, isocratic separation using 80:20 acetonitrile-50 mmol/l TRIS buffer pH = 8. Peaks thiourea (1), naphthalene (2), and fluoranthrene (3)

Stationary phase	End-capping	Carbon content %	Surface area ^a m²/g	$\frac{{{\mu _{EO}}^b}}{{10^4} \cdot {cm^2}{V^{ - 1}}{s^{ - 1}}}$
Nucleosil 5 C18	Fully capped	13.6	350	1.56
LiChrospher RP-18	Uncapped	21.7	450	1.45
Spherisorb Diol	Uncapped	1.9	220	0.80
Spherisorb S5 ODS2	Fully capped	14.5	350	0.68
Zorbax BD-ODS	Fully capped	10.8	220	0.50
Hypersil ODS	Fully capped	11.0	170	0.14
Partisil 5 ODS3	Fully capped	10.9	350	< 0.01
Purospher RP-18	Chem. treated	17.8	500	< 0.01

Table 5. Properties of commercial stationary phases used in CEC [53]

^a Values published by manufacturers.

^b Electroosmotic mobility.

strong cation exchanger [58]. El Rassi and Zhang developed "layered" chemistries with sulfonic acid ion exchange functionalities attached to the silica surface forming a sub-layer covered with a top layer of C_{18} alkyl chains [59, 60]. These materials afford much higher electroosmotic flow than their non-sulfonated counterparts and exhibit an interesting selectivity in the separation of nucleosides and other families of compounds.

The majority of CEC-studies in the early 1990s have been carried out with columns packed with the then state-of-the-art 5-µm octadecyl silica (ODS) beads [15, 61, 62]. Later in the decade, 3-µm beads became the HPLC industry standard and found their way rapidly to CEC. Their use enabled easy separation of hydrocarbons in the CEC mode with an efficiency of up to 400,000 plates/m [48,58]. Even better results were obtained with experimental particles having a diameter of 1.5 µm [63-67]. Unger's group prepared and used even smaller beads with diameters in the submicrometer range [35, 68, 69]. Indeed, they achieved a further increase in efficiency to over 650,000 plates/m at a flow velocity of 3 mm/s. However, this was three time less than the value predicted by theory. This was explained by the effect of axial diffusion that does not depend on the particle size and becomes the dominating contribution to the peak broadening under these conditions, especially at the typical flow rates. Since an increase in the flow velocity of the magnitude required to minimize the effect of axial diffusion is difficult to achieve with the current instrumentation, the submicron sized packings do not offer any considerable advantage over the more common somewhat larger beads that are also easier to pack.

The effect of pore size on CEC separation was also studied in detail [70–75]. Figure 9 shows the van Deemter plots for a series of 7- μ m ODS particles with pore size ranging from 10 to 400 nm. The best efficiency achieved with the large pore packing led to a conclusion that intraparticle flow contributes to the mass transfer in a way similar to that of perfusion chromatography and considerably improves column efficiency. The effect of pore size is also involved in the CEC separations of synthetic polymers in size-exclusion mode [76].

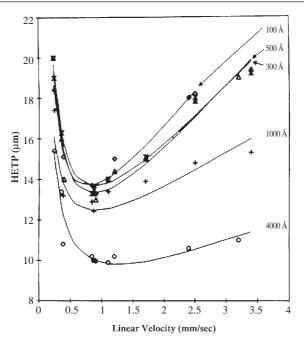
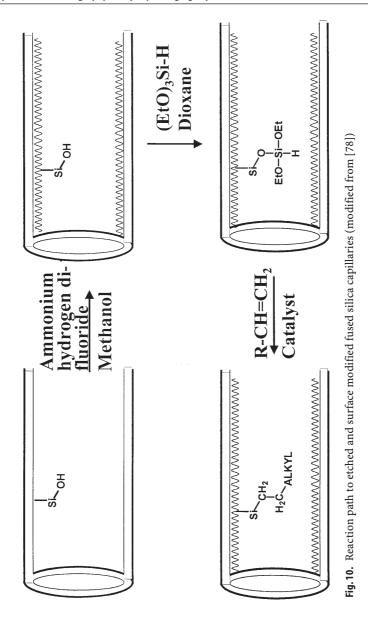


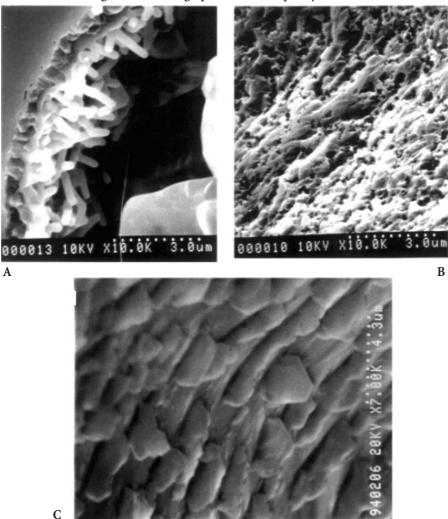
Fig. 9. Effect of pore size on the efficiency of CEC columns. (Reprinted with permission from [70]. Copyright 1997 VCH-Wiley). Conditions: field strength 100-500 V/cm, capillary column 75 µm i. d., total length 30 cm, active length 25 cm, isocratic separation using 20:80 acetonitrile-100 mmol/l phosphate buffer pH=6.9, marker acetone

4.2 Open-Tubular Geometry

In order to avoid tedious procedures required to prepare packed CEC columns, some groups are studying the use of empty capillaries. Since solute-stationary phase interactions are key to the CEC process, appropriate moieties must be bound to the capillary wall. However, the wall surface available for reaction is severely limited. For example, a 100 μ m i.d. capillary only has a surface area of 3×10^{-4} m² per meter of length, with a density of functional sites of approximately 3.1×10^{18} sites/m², which equals 0.5 μ mol sites/m². Moreover, surface modification cannot involve all of the accessible silanol groups, since some must remain to support the EOF. As a result, the use of bare capillaries in CEC has been less successful.

In contrast, chemical etching of the inner wall of the fused-silica capillaries was used to increase the surface area. This enables achievement of a higher phase ratio since more alkyl functionalities can be attached to the surface, thus improving both the separation process and loadability of the column. The surface morphology of the etched capillary depends on the time the methanol solution of ammonium hydrogen difluoride is left in contact with the capillary and temperature at which the reaction is carried out (Fig. 10) [77]. The surface features have been described by Pesek to range "from spikes of silica material extending





Scanning Electron Micrographs of Etched Capillary Surfaces

Fig. 11A–C. Scanning electron micrographs of fused silica capillary surfaces etched with methanolic ammonium hydrogen difluoride solution. (Reprinted with permission from [78]. Copyright 2000 Elsevier). Etching process was carried out for: A 3 h at 300 °C; B, 2 h at 300 °C and 2 h at 400 °C; C 2 h at 300 °C and 1 h at 400 °C

 $3-5 \,\mu$ m from the surface (Fig. 11 A), to a series of hills or sand dunes (Fig. 11 B), to large uniform boulder-like pieces of silica on the surface (Fig. 11 C)" [78]. Each of these structures easily survives conditions typical of the CEC separations. This group also used their silanization/hydrosilation process to attach the alkyl moieties shown in Fig. 10. First, the surface is treated with a triethoxysilane to afford hydride functionalities. The desired alkyl is then attached by a catalyzed hy-

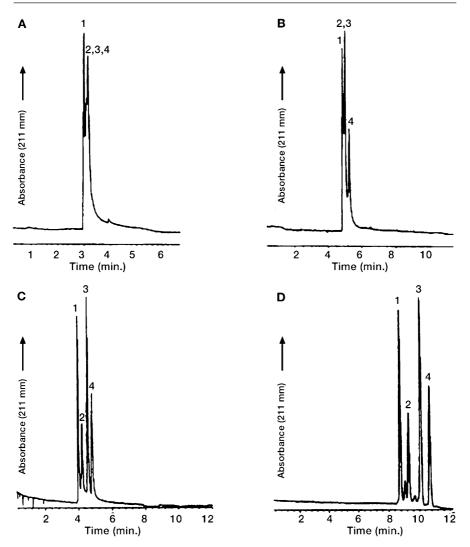


Fig. 12 A–D. Separation of a mixture of cyctochromes C from various sources in 20 µm i.d. capillary columns. (Reprinted with permission from [78]. Copyright 2000 Elsevier). Conditions: A bare capillary; **B** unetched C18 modified capillary; **C**, **D** etched C18 modified capillary, total column length 50 cm, active length 25 cm, voltage 30 kV (**A**, **B**, **C**) and 15 kV (**D**), mobile phase 60 mmol/l a-alanine and 60 mmol/l lactic acid pH 3.7, detection at 211 nm, pressurized injection for 2 s using vacuum

drosilylation reaction. The bonded phase was characterized using a number of analytical methods such as diffuse reflectance infrared Fourier transform (DRIFT), solid-state cross-polarization magic-angle spinning (CP-MAS) NMR, photoelectron spectroscopy (ESCA) and optical methods such as UV-visible and fluorescence spectroscopy. Figure 12 demonstrates the significant effect of the surface treatment on the CEC separation of very similar proteins [79].

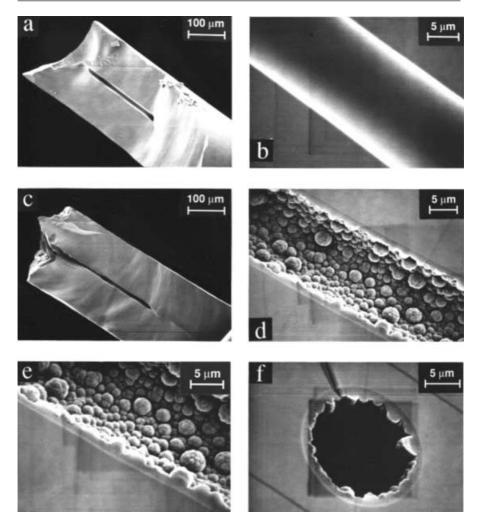


Fig.13a-f. Scanning electron micrographs of the raw fused-silica capillary and a PLOT column. (Reprinted with permission from [183]. Copyright 1999 Elsevier): **a** fractured end of raw 20 μm i.d. fused-silica capillary; **b** enlarged lumen of the raw fused-silica capillary shown in (a); **c** fractured end of a PLOT column; **d** the rugulose porous layer in the capillary column shown in (**c**); **e** the rugulose porous layer at higher magnification than in (**d**); **f** cross-section of PLOT column

Another approach is similar to that used in for the preparation of polymerlayer open tubular GC columns (PLOT). Horváth's group prepared capillaries with a porous polymer layer as shown in Fig. 13 by in situ polymerization of vinylbenzylchloride and divinylbenzene [183]. The reaction of the *N*,*N*-dimethyldodecylamine with chloromethyl groups at the surface simultaneously afforded strong positively charged quaternary ammonium functionalities and attachment of C_{12} alkyl chains to the surface. The unreacted chloromethyl groups

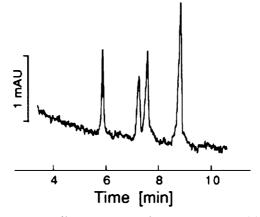


Fig. 14. Electrochromatogram of basic proteins α -chymotrypsinogen A (1), ribonuclease A (2), lysozyme (3), and cytochrome c (4) obtained under isocratic elution conditions by using a PLOT column. (Reprinted with permission from [183]. Copyright 1999 Elsevier). Conditions: fused-silica capillary column, length 47 cm (effective 40 cm), i. d. 20 µm, with a ca. 2 µm thick polymer layer having dodecyltrimethylammonium functionalities at the surface as the stationary phase; mobile phase, 20% acetonitrile in 20 mmol/l aqueous sodium phosphate pH 2.5, voltage – 30 kV

were hydrolyzed under basic conditions to hydroxymethyl groups, thus increasing the compatibility of the surface with the aqueous mobile phase. The CEC separation of four basic proteins using this PLOT column with the positively charged stationary phase and dodecylated chromatographic surface at pH 2.5 is shown in Fig. 14. The column featured very high efficiencies of up to 45,000 theoretical plates for proteins in isocratic elution. The order of elution does not follow the order of hydrophobicity, which indicates that both chromatographic retention and electrophoretic migration contribute to the protein separation.

Yet another approach to PLOT-like CEC columns was reported by Colón and Rodriguez [80, 81]. They used a mixture of tetraethoxysilane (TEOS) and octyltriethoxysilane (C8-TEOS) for the preparation of a thin layer of an organic-inorganic hybrid glass composite by the sol-gel process. This composite was used as the stationary phase for CEC separations. Figure 15 demonstrates the critical effect of the longer alkyl-containing component on the separation of aromatic hydrocarbons. A similar method was also proposed by Freitag and Constantin [82].

Another way to improve the performance of open-tubular columns was suggested by Sawada and Jinno [83]. They first vinylized the inner surface of a 25 µm i.d. capillary and then performed in situ copolymerization of *t*-butylacrylamide and 2-acrylamido-2-methyl-1-propanesulfonic acid (AMPS) to create a layer of polymeric stationary phase. This process does not currently allow good control over the homogeneity of the layer and the column efficiencies achieved in CEC separations of hydrocarbons were relatively low. These authors also recently thoroughly reviewed all the aspects of the open tubular CEC technologies [84].

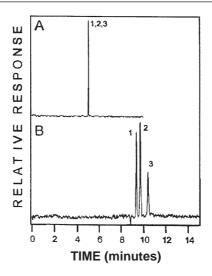


Fig.15. Electrochromatograms obtained in columns coated with sol-gel composites: (A) TEOS and (B) C8-TEOS/TEOS. (Reprinted with permission from [80]. Copyright 1999 American Chemical Society). Separation conditions: fused silica capillary, $12 \mu m i. d.$, 60 cm total length, 40 cm active length, mobile phase 60/40 methanol/1 mmol/l phosphate buffer, voltage 30 kV, electrokinetic injection 5 s at 6 kV, UV detection at 214 nm. Peaks: toluene (1), naphthalene (2), and biphenyl (3)

4.3 Replaceable Separation Media

Several research groups used another interesting column technology as an alternative to the modification of the capillary surface. This method is inherited from the field of electrophoresis of nucleic acids and involves capillaries filled with solutions of linear polymers. In contrast to the monolithic columns that will be discussed later in this review, the preparation of these pseudostationary phases need not be performed within the confines of the capillary. These materials, typically specifically designed copolymers [85–88] and modified dendrimers [89], exist as physically entangled polymer chains that effectively resemble highly swollen, chemically crosslinked gels.

In contrast to the polyacrylamide homopolymers typical of CE, Fujimoto et al. incorporated charged functionalities into the neutral polyacrylamide chains to accelerate the migration of neutral compounds through a capillary column [90]. Despite this improvement, nearly 100 min were required to effect the separation of acetone and acetophenone, making this approach impractical even with the use of high voltage. Alternatively, Tanaka et al. [86] alkylated commercial polyallylamine with C_8-C_{16} alkyl bromides, followed by a Michael reaction with methyl acrylate and subsequent hydrolysis of the methyl ester to obtain free carboxyl functionalities. This polymer effected the efficient separations of ketones and aromatic hydrocarbons shown in Fig. 16 in about 20 min at 400 V/cm. Similarly, Kenndler's group [87] has demonstrated the separation of

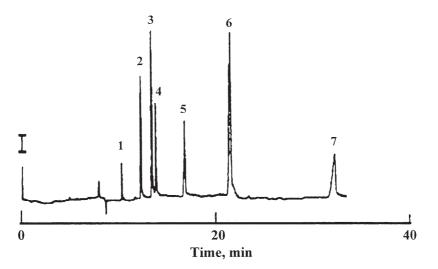


Fig. 16. CEC separation of naphthalene (1), fluorene (2), phenanthrene (3), anthracene (4), pyrene (5), triphenylene (6), and benzo(a)pyrene (7) using capillary filled with C10 alkyl substituted polyallylamine. (Reprinted with permission from [86]. Copyright 1997 Elsevier). Conditions: capillary 50 µm i. d., 48 cm total length, 33 cm active length, field strength 400 V/cm, carrier concentration 20 mg/ml, mobile phase 60:40 methanol-20 mmol/l borate buffer pH=9.3

phenols using a partially hydrolyzed polyacrylamide solution. Schure et al. [88] published an excellent study employing a pseudostationary phase of methacrylic acid, ethyl acrylate, and dodecyl methacrylate Increasing the concentration of the linear polymer solution increased the number of interacting moieties, thereby improving the efficiency to a maximum of 293,000 plates/m in a 3.72% polymer solution. Rheological measurements indicated that the dissolved pseudostationary phase afforded the best separation for concentrations at which the viscosity of the solution was the highest, and the polymer chains were most entangled.

Columns filled with polymer solutions are extremely simple to prepare, and the "packing" can easily be replaced as often as desired. These characteristics make the pseudostationary phases excellent candidates for use in routine CEC separations such as quality control applications where analysis and sample profiles do not change much. However, several limitations constrain their widespread use. For example, the sample capacity is typically very low, pushing typical detection methods close to their sensitivity limits. Additionally, the migration of the pseudostationary phase itself may represent a serious problem, e.g., for separations utilizing mass spectrometric detection. The resolution improves with the concentration of the pseudostationary phase. However, the relatively low solubility of current amphiphilic polymers does not enable finding the ultimate resolution limits of these separation media [88].

4.4 Polymer Gels

CEC capillary columns filled with hydrophilic polymer gels mimic those used for capillary gel electrophoresis [91]. Typically, the capillary is filled with an aqueous polymerization mixture that contains monovinyl and divinyl (crosslinking) acrylamide-based monomers as well as a redox free radical initiating system, such as ammonium peroxodisulfate and tetramethylethylenediamine (TEMED). Since initiation of the polymerization process begins immediately upon mixing all of the components at room temperature, the reaction mixture must be used immediately. It should be noted, that these gels are very loose, highly swollen materials that usually contain no more than 5% solid polymer.

For example, Fujimoto et al. [90] polymerized an aqueous solution of acrylamide, methylenebisacrylamide (5%), and AMPS within the confines of a capillary. Despite the lack of chemical attachment to the inner wall of the capillary, these crosslinked gels showed fair physical stability. However, retention times on these columns were prohibitively long. Column efficiencies of up to 150,000 plates/m were observed for the slightly retained acetophenone. The good correlation of the migration times of acetone and acetophenone with the expected "pore size" characteristics of the gel and the lack of explicit hydrophobically interacting moieties led Fujimoto to the conclusion that the prevailing mechanism of the separation was sieving [85].

Replacement of the hydrophilic acrylamide by the more hydrophobic *N*-isopropylacrylamide, in combination with the pre-functionalization of the capillary with (3-methacryloyloxypropyl) trimethoxysilane, afforded a monolithic gel covalently attached to the capillary wall. A substantial improvement in the separations of aromatic ketones and steroids was observed using these "fritless" hydrogel columns, as seen by the column efficiencies of 160,000 found for hydrocortisone and testosterone [92]. The separations exhibited many of the attributes typical of reversed-phase chromatography and led to the conclusion that, in contrast to the original polyacrylamide-based gels, size-exclusion mechanism was no longer the primary mechanism of separation.

4.5

Monolithic Columns

One of the most important competing column technologies spurred by the technical difficulties associated with packed columns are monolithic media. This technology was adopted from a concept originally developed for much larger diameter HPLC columns [93–100]. As a result of their unique properties, the monolithic materials have recently attracted considerable attention from a number of different research groups resulting in a multiplicity of materials and approaches used for the preparation of monolithic CEC columns. Silica and synthetic organic polymers are two major families of materials that have been utilized together with one of two different technologies: (i) packing with beads followed by their fixation to form a monolithic structure and (ii) the preparation of the monolith from low molecular weight compounds in situ. All these monolithic columns are also referred to as fritless CEC columns or continuous beds.

4.5.1 "Monolithized" Packed Columns

The first approach to monolithic columns formed from beads can be assigned to Knox and Grant [15] who prepared a particle-embedded continuous-bed CEC column. They packed beads into a Pyrex glass tube of 1-2 mm i.d. and then drew the packed column to create a capillary. The particles were partly incorporated in the glass wall and the column was stable unless the column-to-particle diameter exceeded a value of 10. The success of this procedure was very sensitive to the presence of water in the original packing material.

Dittmann at al. later developed a very simple method for preparing such stationary phases [41]. They packed a capillary with 3-µm ODS beads and then drew a heated wire along the capillary to achieve sintering of the beads. Since changes in the drawing speed directly affected both EOF and retention, they inferred that the heat treatment led to detachment of a part of the C18 ligands from the silica beads.

Horváth et al. sintered the contents of a capillary column packed with 6 μ m octadecylsilica by heating to 360 °C in the presence of a sodium bicarbonate solution [101]. These conditions also strip the alkyl ligands from the silica support, thus significantly deteriorating the chromatographic properties. However, the performance was partly recovered after resilanization of the monolithic material with dimethyloctadecylchlorosilane allowing the separation of aromatic hydrocarbons and protected aminoacids with an efficiency of up to 160,000 plates/m.

Several groups used sol-gel transition to immobilize the beads packed in a capillary. For example, Dulay et al. [102] packed a slurry of ODS beads in tetraethylorthosilicate solution and heated it to 100 °C to achieve the sol-gel transition and create the monolithic structure shown in Fig. 17. This technology is extremely sensitive and even a small deviation from the optimal conditions leads to cracks in the monoliths and a rapid deterioration in the column performance. However, even the best efficiency of 80,000 plates/m achieved with these column was relatively low. Henry et al. modified the original procedure and increased the efficiencies to well over 100,000 plates/m [103, 104].

Chirica and Remcho first created the outlet frit, packed the column with ODS beads, and then fabricated the inlet frit. The column was filled with aqueous solution of a silicate (Kasil) and the entrapment achieved by heating the column to 160 °C [105, 106]. The monolithic column afforded considerably reduced retention times compared to the packed-only counterpart most likely due to a partial blocking of the pores with the silicate solution. This approach was recently extended to the immobilization of silica beads in a porous organic polymer matrix [107].

Tang et al. used columns packed with a slurry of beads suspended in supercritical CO_2 . This packed column was filled with a dilute sol solution prepared by hydrolysis and polycondensation of tetramethoxysilane and ethyltrimethoxysilane precursors. The column was dried using supercritical CO_2 and heated first to 120 °C for 5 h followed by another 5 h at a temperature of 250 °C [108–110]. Column efficiencies of 127,000 and 410,000 plates/m were reported

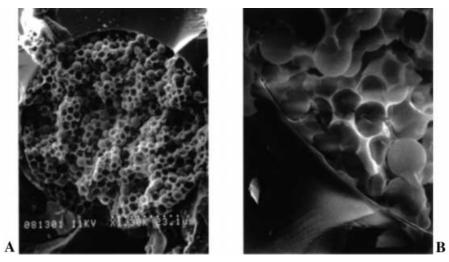


Fig. 17 A, B. Scanning electron micrographs of a 75 μm sol-gel/3 μm ODS filled capillary column. Cross-sectional view at a magnification of: A 1300_; B 4900_. (Reprinted with permission from [102]. Copyright 1998 American Chemical Society)

for system consisting of 5- μ m, 90-Å and 3- μ m, 1500-Å ODS beads, respectively [49].

All these methods are solving the problem of column stability since the fused beads cannot move. However, these approaches often do not avoid the in situ fabrication of frits, one of the critical operations in the preparation of CEC columns.

4.5.2 In Situ Prepared Monoliths

In contrast to the above technologies that involve packing beads, the most appealing aspect of the monolithic materials discussed in this section is their ease of preparation in a single step from low molecular weight compounds. In situ created monoliths can be prepared from both silica and organic polymers.

4.5.2.1 Silica Sol-Gel Transition

Although Fields already mentioned the possible preparation of monolithic silicabased CEC columns, the lack of experimental data leads to the assumption that this option has not been tested [111]. In fact, it was Tanaka et al. who demonstrated the preparation of monolithic capillary columns using a sol-gel transition within an open capillary tube [99, 112]. The trick was in the starting mixture that in addition to tetramethoxysilane and acetic acid also includes poly(ethylene oxide). The gel formed at room temperature was carefully washed with a variety of solvents and heated to 330 °C. The surface was then modified with octadecyltrichlorosilane or octadecyldimethyl-*N*,*N*-dimethylaminosilane to attach the hy-

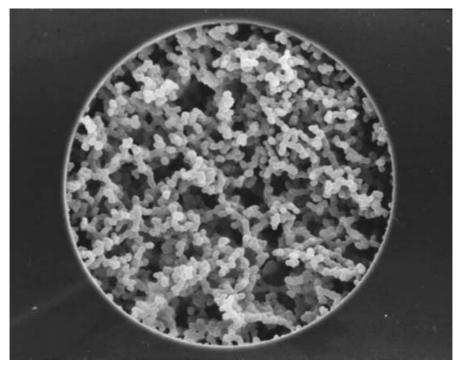


Fig. 18. Scanning electron micrograph of monolithic silica-based capillary column. (Reprinted with permission from [205]. Copyright 2000 American Chemical Society)

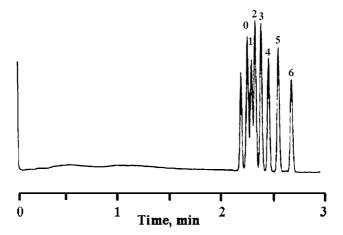


Fig. 19. Separation of alkylbenzenes $C_6H_5C_nH_{2n+1}$ (n=0-6) on an in situ prepared monolithic silica column. (Reprinted with permission from [99]. Copyright 2000 VCH-Wiley). Conditions: voltage 900 V/cm, capillary column 100 μ m i.d., total length 33.5 cm, active length 25 cm, isocratic separation using 90:10 acetonitrile-50 mmol/l TRIS buffer pH=8, column efficiency 58,000 plates/m

drophobic ligands required for the desired reversed-phase separations. The structure of the monolith was very regular (Fig. 18). These columns afford efficiencies of almost 240,000 plates/m as demonstrated on the separation of alkyl-benzenes shown in Fig. 19 [99]. A similar approach was also used by Fujimoto [113].

While only a few reports concern the in situ preparation of monolithic CEC columns from silica, much more has been done with porous polymer monoliths and a wide variety of approaches differing in both the chemistry of the monomers and the preparation technique is currently available. Obviously, free radical polymerization is easier to handle than the sol-gel transition accompanied by a large decrease in volume.

4.5.2.2

Acrylamide Polymerized in Aqueous Solutions

An approach towards continuous CEC beds involving highly crosslinked acrylamide polymers was reported by Hjertén et al. in 1995 [114]. The original technique was complex, requiring multiple steps [115]. In order to simplify the tedious preparation method, they later developed a simpler procedure [116]. The same group recently described another method for the preparation of monolithic capillary columns that was used for CEC separation of proteins in a mobile phase gradient [117]. The first step involved a polymerization initiated by the ammonium persulfate/TEMED system in a reaction mixture consisting of an aqueous phase, namely a solution of acrylamide and piperazine diacrylamide in a mixture of a buffer and dimethylformamide, and highly hydrophobic, immiscible octadecyl methacrylate. Continuous sonication was applied in order to emulsify this monomer. Finally, two new monomers, dimethyldiallylammonium chloride and piperazine diacrylamide, were added and the resulting dispersion was then forced into a methacryloylsilylated capillary in which the polymerization process was completed.

Hoegger and Freitag modified the Hjertén's procedure and prepared a variety of monolithic acrylamide-based CEC columns [118]. Their approach allowed them to adjust both rigidity and porous properties of the monoliths and to achieve excellent separations of model compounds as well as selected pharmaceuticals.

Despite the undeniable success, the use of purely aqueous-based polymerization systems for the preparation of monolithic capillaries for CEC also has some limitations. Perhaps the greatest limitation is that the typical non-polar monomers that are required to achieve the necessary hydrophobicity for a reversed-phase CEC are insoluble in water. In contrast to the "fixed" solubilizing properties of water, the wealth of organic solvents possessing polarities ranging from highly nonpolar to extremely polar enables the formulation of mixtures with solvating capabilities that may be tailored over a very broad range. An additional feature of organic solvents is their intrinsic ability to control the porous properties of the monoliths.

Novotny and Palm simplified the incorporation of highly hydrophobic ligands into acrylamide-based matrices by using mixtures of aqueous buffer and N-

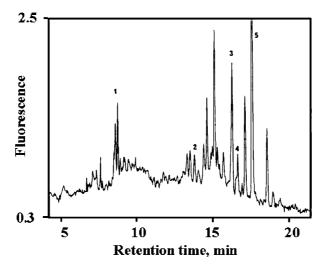


Fig. 20. Gradient electrochromatogram of derivatized urinary neutral steroids extracted from pregnancy urine. (Reprinted with permission from [120]. Copyright 2000 Elsevier). Conditions: monolithic capillary column 100 μ m i.d., total length 35 cm, active length 25 cm, voltage 600 V/cm, gradient of 35–65% acetonitrile in water and 5% 240 mmol/l ammonium formate buffer (pH 3). Peaks: labeling reagent (1), 11-hydroxyandrosterone (2), dehydroisoandrosterone (3), estrone (4), and spiked androsterone (5)

methylformamide to prepare homogeneous polymerization solutions of acrylamide, methylenebisacrylamide, acrylic acid, and C_4 , C_6 , or C_{12} alkyl acrylate [119]. Columns with high efficiencies were only obtained when the polymerization was performed in the presence of poly(ethylene oxide) dissolved in the polymerization mixture. In contrast to the typical model hydrophobic aromatic hydrocarbons often used, Novotny and coworkers extended the range of potential analytes to include carbohydrates [119], steroids [120], and bile acids [121]. The potential of the method developed by Novotny's group is demonstrated on the separation of steroids extracted from a "real world" sample of pregnant human urine (Fig. 20). Using retention times, spiking, and mass spectroscopy, several of the peaks could be safely assigned to specific compounds [120].

4.5.2.3 Imprinted Monolithic Columns

Molecular imprinting has recently attracted considerable attention as an approach to the preparation of polymers containing recognition sites with predetermined selectivity. The history and specifics of the imprinting technique pioneered by Wulff in the 1970s have been detailed in several excellent review articles [122–124]. Imprinted monoliths have also received attention as stationary phases for capillary electrochromatography.

The imprinting process shown schematically in Fig. 21 involves the preorganization of functional monomer molecules such as methacrylic acid and

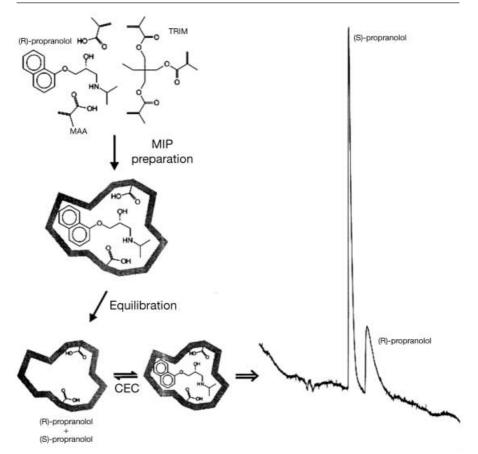


Fig. 21. Molecular imprinting of (*R*)-propranolol using methacrylic acid (MAA) as the functional monomer and trimethylolpropane trimethacrylate (TRIM) as the crosslinking monomer. (Reprinted with permission from [126]. Copyright 1998 Elsevier). The enantiose-lectivity of a given polymer is predetermined by the configuration of the ligand, *R*-propranolol present during its preparation. Since the imprinted enantiomer possesses a higher affinity for the polymer, the separation is obtained with a predictable elution order of the enantiomers

vinylpyridine around a template molecule and subsequent copolymerization of this complex with a large amount of a crosslinking monomer (ethylene dimethacrylate-EDMA, trimethylolpropane trimethacrylate-TRIM) [125]. Under ideal conditions, imprints possessing both a defined shape and a specific arrangement of chemically interactive functional groups that reflect those of the templated molecule remain in the polymer after extraction of the template.

The monolithic technology was used for CEC by Nilson et al. who introduced "superporous" imprinted monolithic capillaries in 1997 [125–127]. Isooctane was used as a porogen in order to produce a macroporous structure with large pores without interfering with the imprinting process. These imprinted monoliths were

successfully used for the separation of the enantiomers of propanolol, metoprolol, and ropivacaine. Using a similar process, Lin et al. developed imprinted monolithic columns for the CEC separation of racemic amino acids [128-131].

4.5.2.4 Polystyrene-Based Monolithic Columns

Horváth's group has reported the preparation of porous rigid monolithic capillary columns for CEC by polymerizing mixtures of chloromethylstyrene, divinylbenzene and azobisisobutyronitrile in the presence of porogenic solvents [132]. The reactive chloromethyl moieties incorporated into the monolith served as sites for the introduction of quaternary ammonium functionalities (see above). These capillary columns possessing positively charged surface functionalities were used for the reversed-phase separations of basic and acidic peptides such as angiotensins and insulin with plate numbers as high as 200,000 plates/m at pH=3. Good separation of chemically similar tripeptides (Gly-Gly-Phe and Phe-Gly-Gly) was also observed in a pH 7 buffer using this type of functionalized poly(styrene-*co*-divinylbenzene) monolithic column. However, the addition of acetonitrile to the mobile phase significantly decreases the mobility of the analytes thus making this approach less attractive [132].

Zhang developed a monolithic poly(styrene-*co*-divinylbenzene) CEC column in which the EOF is supported by carboxyl groups of polymerized methacrylic acid [133]. Using benzene as a probe, column efficiencies of 90,000 – 150,000 were observed within a flow velocity range of 1 - 10 cm/min (0.2 - 1.7 mm/s). Different families of compounds such as phenols, anilines, chlorobenzenes, phenylendiamines, and alkylbenzenes were well separated typically in less than 5 min using 20 cm long columns.

4.5.2.5

Methacrylate Ester-Based Monolithic Columns

In contrast to the reported investigations of acrylamide and styrene-based monoliths that have largely been limited to evaluation of their chromatographic performances, our group has performed extensive materials development and optimization for monolithic CEC capillaries prepared from methacrylate ester monomers [134-136]. Production of these monolithic capillary columns is amazingly simple (Fig. 22). Either a bare or a surface treated capillary is filled with a homogeneous polymerization mixture, and radical polymerization is initiated only when desired using either heat (thermostated bath) or UV irradiation [137] to afford a rigid monolithic porous polymer shown in Fig. 23. Once the polymerization is complete, unreacted components such as the porogenic solvents are removed from the monolith using a syringe pump or electroosmotic flow. This simple method for preparing monolithic capillary columns has numerous advantages. For example, the final polymerization mixture contains free radical initiators such as benzoyl peroxide or azobisisobutyronitrile, ensuring its stability and easy handling for several hours at room temperature or for days in the refrigerator without risking the onset of polymerization. The methacrylate-

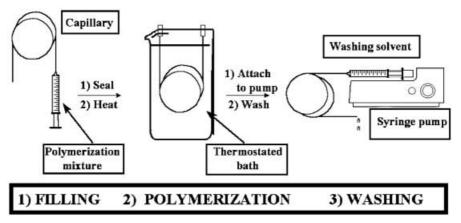


Fig. 22. Schematics for the preparation of monolithic capillary columns

based polymers are stable even under extreme pH conditions such as pH 2 or 12 [144]. The sulfonic acid functionalities of the monolithic polymer remain dissociated over this pH range creating a flow velocity sufficient to achieve the desired separations in a short period of time. In contrast to the stationary phase, the analytes are uncharged, yielding symmetrical peaks. It should be noted that such extreme pH conditions especially in the alkaline range cannot be tolerated by typical silica-based packings.

This technology was extended to the preparation of chiral capillary columns [138–141]. For example, enantioselective columns were prepared using a simple copolymerization of mixtures of *O*-[2-(methacryloyloxy)ethylcarbamoyl]-10,11-dihydroquinidine, ethylene dimethacrylate, and 2-hydroxyethyl methacrylate in the presence of mixture of cyclohexanol and 1-dodecanol as porogenic solvents. The porous properties of the monolithic columns can easily be controlled through changes in the composition of this binary solvent. Very high column efficiencies of 250,000 plates/m and good selectivities were achieved for the separations of numerous enantiomers [140].

5 Separation Conditions

Since the separation process in CEC has a number of attributes similar to those of HPLC, the most important variables affecting the separation are the same for both of these techniques. However, in HPLC mobile phase, flow and separation are independent variables. Therefore, the most important operational variables are the analyte-sorbent interactions that can be modulated by the chemistry of the packing, composition of the mobile phase, and temperature. In contrast, the CEC column has a dual role as it serves as both (i) a flow driving device and (ii) separation unit at the same time. Although the set of variables typical of HPLC is also effective in CEC, their changes may affect in one way or another both column functions. Therefore, optimization of the separation process in CEC is more complex than in HPLC.

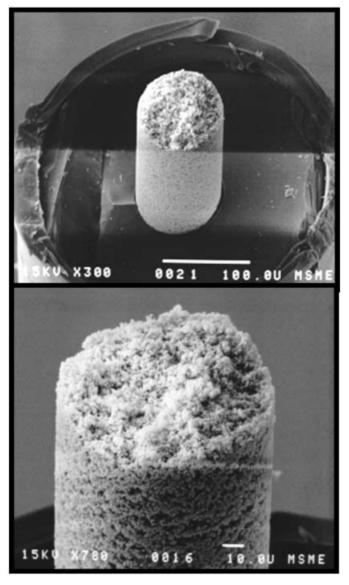


Fig. 23. Scanning electron micrograph of monolithic capillary column prepared according to [134]

5.1 Mobile Phase

Reversed-phase separations currently dominate in CEC. As a result, the vast majority of the mobile phases are mixtures of water and an organic solvent, typically acetonitrile or methanol. In addition to the modulation of the retention, the mobile phase in CEC also conducts electricity and must contain mobile ions. This is achieved by using aqueous mixtures of salts instead of pure water. The discussion in Sect. 2 of this chapter indicated that the electroosmotic flow is created by ionized functionalities. The extent of ionization of these functionalities that directly affects the flow rate depends on the pH value of the mobile phase. Therefore, the mobile phase must be buffered to a pH that is desired to achieve the optimal flow velocity. Obviously there are at least three parameters of the mobile phase that have to be controlled: (i) percentage of the organic solvent, (ii) the ionic strength of the aqueous component, and (iii) its pH value.

5.1.1

Percentage of Organic Solvent

The effect of the organic solvent on a CEC separation is very similar to that in HPLC. For example, Dorsey demonstrated this effect on the separation of a mixture of aromatic hydrocarbons using mobile phases containing 90 and 60% acetonitrile (Fig. 24) [142]. As expected, the retention in a mobile phase rich in the organic solvent is significantly shorter but the selectivity is reduced under these conditions. This problem has been solved using a gradient elution. Figure 24c shows an excellent baseline separation of all components in about 16 min, a run time only 5 min longer than that of the isocratic separation in the acetonitrile rich mobile phase. Although this effect could be predicted based on the knowledge of separation mechanism in reversed-phase HPLC, the situation is more complex in CEC.

Thus, an increase in the strength of the mobile phase may not be the best choice to achieve acceleration of the CEC separations. Although the retention rapidly decreases with the increasing percentage of the organic modifier, the organic solvents also affect the electroosmotic flow. As shown in Eq. (1), the flow velocity is directly proportional to the ε/η ratio. This in turn depends on the composition of the organic solvent/water mixture and typically passes through a minimum at 50-80% of the modifier. Figure 25 shows that the overall effect depends on the type of the organic solvent. Clearly, the electroosmotic mobility follows the changes in ε/η values for methanol. In contrast, the velocity continuously increases for acetonitrile [56]. This indicates that the solvent may also affect the zeta potential ζ by changing the surface charge density. Dorsey's pioneering work also demonstrated that even non-buffered non-aqueous media such as pure acetonitrile, methanol, and dimethylformamide could support an electroosmotic flow in CEC [143]. The use of polar non-aqueous mobile phases also proved useful in a variety of CEC separations [144–146]. For example, Lämmerhofer used mixtures of methanol and acetonitrile buffered with acetic acid and triethylamine to achieve very efficient (N=250,000 plates/m) enantioselective separations [140].

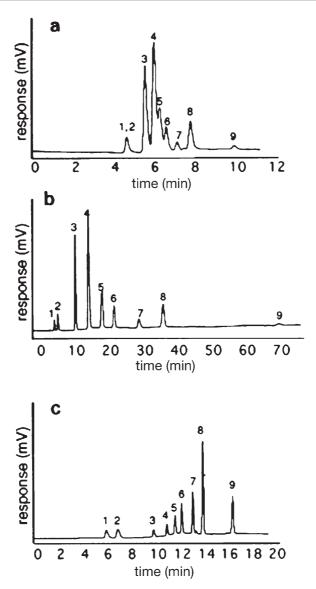


Fig. 24a–c. Comparison of isocratic and gradient separation of a model mixture. (Reprinted with permission from [142]. Copyright 1998 Elsevier). Conditions: capillary column 75 μ m i.d., total length 50 cm, packed length 20 cm, packing 5 μ m ODS Hypersil, voltage 15 kV, isocratic separation using: a 90:10 acetonitrile-water; b 60:40 acetonitrile-water; c a gradient elution using a gradient from 60 to 90% acetonitrile in water in 5 min. Peaks: acetone (1), phenol (2), benzene (3), toluene (4), naphthalene (5), acenaphthylene (6), fluorene (7), anthracene (8), 1,2-benzanthracene (9)

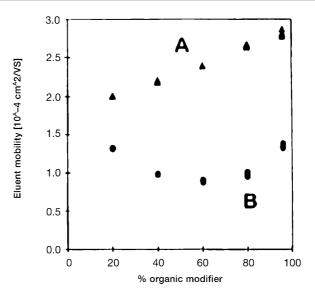


Fig. 25. Effect of percentage of acetonitrile (A) and methanol (B) on electroosmotic mobility in a packed column. (Reprinted with permission from [56]. Copyright 1997 Elsevier). Conditions: capillary column 100 μ m i. d., total length 33.5 cm, active length 25 cm packed with 3 μ m CEC Hypersil C18, mobile phase organic modifier-water +4% 25 mmol/l TRIS pH = 8, voltage 30 kV, temperature 20 °C, marker thiourea

5.1.2 Concentration and pH of Buffer Solution

The electroosmotic velocity as defined in Eq. (1) is directly proportional to the ζ potential at the surface of shear defined as

$$\zeta = \sigma \, \delta / \varepsilon_o \, \varepsilon_r \tag{4}$$

where σ is the charge density at the surface of shear and δ is the thickness of the double layer, with

$$\delta = [\varepsilon_o \varepsilon_r RT/2 F^2 c]^{0.5} \tag{5}$$

where *R* is the gas constant, *T* is the temperature, *F* is the Faraday constant, and *c* is the concentration of the electrolyte. Combination of Eqs. (1), (4), and (5) gives [147]

$$u_{\infty} = \frac{\sigma \left(\frac{\varepsilon_o \varepsilon_r RT}{2 F^2 c}\right)^{0.5}}{\eta} E.$$
 (6)

Equation (6) confirms that the electroosmotic velocity decreases with the square root of the salt concentration in the buffer. This trend is demonstrated in Fig. 26 [110]. However, the increase in concentration of the electrolyte also increases the conductivity of the mobile phase and leads to a rapid increase in current. High

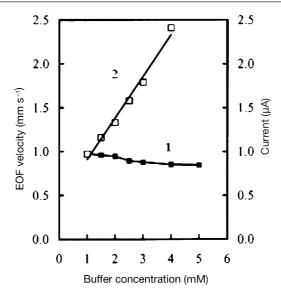


Fig. 26. Effect buffer concentration in the mobile phase on EOF velocity (1) and current (2). (Reprinted with permission from [110]. Copyright 2000 Elsevier). Conditions: monolithic capillary column 75 μ m i. d., total length 30 cm, active length 25 cm, containing sol-gel bonded 3 μ m ODS/SCX with 80 Å pores, mobile phase 70:30 acetonitrile/phosphate buffer pH 3.0, electric field strength 442 V/cm (voltage 15 kV)

currents generate more Joule heat, thereby increasing the temperature within the column. Unless dissipated through the walls, the heat results in a radial temperature gradient that is deleterious for the separations. Although according to Eq. (6) very low buffer concentrations should afford high electroosmotic flow and prevent Joule heating, their buffering capacity may quickly be depleted. Therefore buffer solutions with a compromise concentration in the range 5-50 mmol/l are suggested to achieve good CEC separations.

The effect of the pH is complex. First, it affects the ionization of the chargeable groups at the surface of the stationary phase. This is particularly important for stationary phases in which the weakly acidic silanol groups are the only driving force for the EOF. Figure 27 clearly shows that the separation of neutral compounds is considerably accelerated in a buffer with a pH value of 8 compared to 2.5 at which the acidic silanol groups are no longer completely ionized [148]. The situation is different for separation media with strong ion-exchange functionalities. For example, a pH changes in the range of 2-10 indeed does not affect notably the overall ionic strength of the mobile phase in such cases, since the electric current through the monolithic capillary column that includes strongly acidic sulfonic acid functionalities remains almost constant (Fig. 28). However, a simple calculation reveals that, in order to achieve pH values higher than 12, solutions with a rather high concentration of NaOH have to be used. For example, a 10 mmol/l NaOH solution exhibits a pH of 12, while a 100 mmol/l solution is necessary to produce a pH value of 13. Obviously, these concentrations consid-

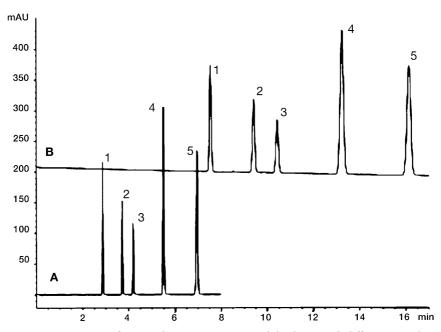


Fig. 27. CEC separation of a neutral test mixture at in mobile phases with different pH values. (Reprinted with permission from [148]. Copyright 2000 Elsevier). Conditions: capillary column 100 μ m i.d., total length 33.5 cm, active length 25 cm packed with 3 μ m Waters Spherisorb ODS I, mobile phase (A) 4:1 acetonitrile-25 mmol/l TRIS pH=8; (B) 4:1 acetonitrile-25 mmol/l phosphate, 0.2 % hexylamine pH=2.5, voltage 25 kV, temperature 20 °C. Peaks: thiourea (1), dimethylphthalate (2), diethylphthalate (3), biphenyl (4), o-terphenyl (5)

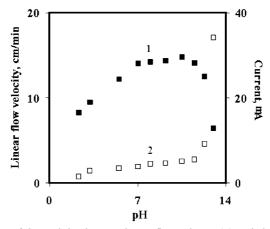


Fig. 28. Effect of pH of the mobile phase on linear flow velocity (1) and electrical current (2) in the monolithic capillary column. (Reprinted with permission from [149]. Copyright 1998 American Chemical Society). Conditions: monolithic capillary column 100 μ m i.d._30 cm, mobile phase 80:20 acetonitrile/5 mmol/l phosphate buffer, pH adjusted by addition of concentrated NaOH, flow marker thiourea 2 mg/ml, UV detection at 215 nm, voltage 25 kV, pressure in vials 0.2 MPa, injection, 5 kV for 3 s

erably exceed those of the original buffer solution (5 mmol/l). As a result of this increase in ionic strength, the conductivity of the mobile phase increases, and much higher currents are observed. Since the electroosmotic flow is reciprocally proportional to the concentration of ions in the mobile phase the flow velocity decreases dramatically at high pH values [149].

The pH value also affects the ionization of acidic and basic analytes and their electromigration. Since this migration can be opposite to that of the electroosmotic flow, it may both improve and impair the separation. This effect is particularly important in the separation of peptides and proteins that bear a number of ionizable functionalities. Hjertén and Ericson used monolithic columns with two different levels of sulfonic acid functionalities to control the proportion of EOF and electromigration. Under each specific set of conditions, the injection and detection points had to be adjusted to achieve and monitor the separation [117]. Another option consists of total suppression of the ionization. For example, an excellent separation of acidic drugs has been achieved in the ion-suppressed mode at a pH value of 1.5 [150].

5.2 Temperature

Temperature is an important variable in all modes of chromatography since it affects the mobile phase viscosity, as well as solute partitioning, solute diffusivity, the degree of ionization of buffers, the buffer pH, and the phase transitions of ligands in the reversed-phase stationary phases [151, 152]. The viscosity of liquids is generally reduced at higher temperatures. Since the flow velocity in CEC increases with decreasing viscosity (Eq. 1), elution should be faster while working at elevated temperatures. Indeed, Fig. 29 demonstrates this effect on the separation of amino acid derivatives [153]. The flow velocity increases from 1.2 to 1.7 mm/s and, compared to room temperature, the separation is completed in about one-third of the time at 53 °C.

However, the temperature also affects the solute partitioning between the mobile and stationary phase and therefore also the chromatographic retention. The distribution of the solute between the mobile and stationary phases is a function of (i) its solubility in the liquid phase and (ii) its adsorption on the solid phase. This is characterized by the distribution ratio *K* defined as the ratio of the concentration of the solute in the stationary phase to its concentration in the mobile phase. The higher this ratio, the longer the retention. According to the Van't Hoff equation

$$\ln K = -\Delta H/RT + \Delta S/R \tag{7}$$

where $-\Delta H$ is the enthalpy associated with the transfer of the solute to the stationary phase, and ΔS is the corresponding change in the entropy.

The effect is better expressed in the form of the ratio of the distribution factors K_{T1} and K_{T2} for two different temperatures:

$$K_{T1}/K_{T2} = \exp\left[-\Delta H (T_2 - T_1)/R T_2 T_1\right].$$
(8)

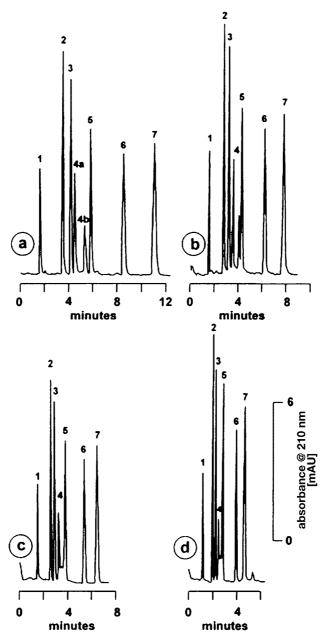


Fig. 29 a – d. Effect of temperature on the separation of PTH-amino acids. (Reprinted with permission from [153]. Copyright 1997 American Chemical Society). Conditions: capillary column 50 μm i. d., total length 20.7 cm, active length 12.7 cm packed with 3.5 μm Zorbax ODS particles having a mean pore size of 80 Å, mobile phase 30:70 acetonitrile/5 mmol/l phosphate buffer pH 7.55, voltage 10 kV, current 1 μA, temperature: **a** 25 °C; **b** 35 °C; **c** 45 °C; **d** 53 °C, UV detection at 210 nm, electrokinetic injection 0.5 s at 1 kV. Peaks: formamide (1), PTH-asparagine (2), PTH-glutamine (3), PTH-threonine (4), PTH-glycine (5), PTH-alanine (6), PTH-tyrosine (7)

Obviously, the magnitude of the temperature effect on retention depends on the difference in the enthalpy of the solute in either phase, and is specific for each solute. Therefore, it also changes the column selectivity. There is no retention and no temperature effect for ΔH =0.

Since the column temperature controls both the overall flow rate and the retentions of the individual compounds, a programmed temperature gradient can be used to shorten the CEC run times and optimize the selectivity [151].

5.3 Field Strength

According to Eq. (1), the electroosmotic velocity is directly proportional to the field strength *E* that is defined as the ratio of the voltage applied at the ends of the capillary and the length of the capillary. Typically, the u_{eo} vs *E* plots are linear over a broad range of voltages. As an example, Fig. 30 shows the effect of voltage on flow velocities in mobile phases with different percentages of acetonitrile [35]. In theory, very high values of *E* might be used to achieve high flow rates and consequently decrease the time required for a separation. Unfortunately, this does not apply completely. First, the maximum field strength is instrument dependent and typically does not exceed 30 kV. Arching, which may occur at higher voltages,

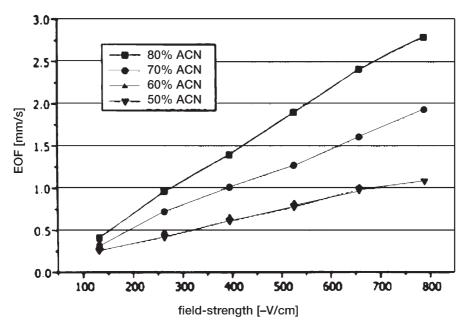


Fig. 30. Effect of field strength and percentage of acetonitrile in the mobile phase on electroosmotic flow in a packed capillary column. (Reprinted with permission from [35]. Copyright 2000 Elsevier). Conditions: capillary column 100 μ m i.d., total length 38 cm, active length 8.5 cm packed with 0.5- μ m C8 silica beads, mobile phase acetonitrile/25 mmol/l TRIS-HCl buffer pH = 8, temperature 20 °C, marker thiourea

is deleterious for both the instrument and the separation. Second, while managing the flow rate, the well-known effect of flow rate on column efficiency most often demonstrated by the van Deemter plot also has to be considered. Although the mass transfer term plays a much smaller role in CEC, the flow rate used for the separation in a specific column packed with porous beads should match that at the minimum of the curve. The situation is different for very small non-porous particles for which the A and C terms of the van Deemter equation can be neglected and only the axial diffusion effectively affects the column efficiency. Since the value of the B term decreases with the increasing flow rate, higher flow rates are desirable for achieving very efficient CEC separations [35].

6 Conclusions and Future Outlook

During the evolution of CEC in the past 10 years, the attention slowly shifted from the separations of mixtures of well-selected model compounds, typically aromatic hydrocarbons due to their good "visibility" in a UV detector, to more "real life" samples. These range from inorganic ions [154-157], low molecular weight compounds such as polyaromatic hydrocarbons (PAHs) [105, 158], ketones [34, 125, 159-165], drugs and their precursors, antibiotics [163, 166-168], saccharides [119, 169], fatty acids and their triglycerides [170, 171], steroids [34, 120, 172-174], amino acids [129, 175-178], pesticides and herbicides [179, 180], to biopolymers like peptides and proteins [117, 132, 147, 181-186], nucleic acids and their constituents [184, 187, 188], to organic polymers [76, 189, 190]. Several separations of these mixtures were discussed in this chapter. Similarly, the spectrum of separation mechanisms was extended from the most popular reversed-phase to the other chromatographic modes such as normal-phase [191-193], ion-exchange [58, 185, 194-196], immunoaffinity [186], and size-exclusion [135].

Despite its infancy, CEC made a substantial progress to become a "full member" of the family of chromatographic methods. As of today, UV detection is mostly used to monitor the separations. Unfortunately, on-column detection is required in CEC to avoid excessive peak broadening and loss of the high intrinsic efficiency of this method. Therefore, the capillary diameter is the only light pass length available for detection. This situation dramatically impairs the sensitivity of the UV detection. Several attempts to address this weakness such as the high sensitivity Z-cell developed by Agilent Technologies did not meet with a broad acceptance. The very sensitive laser induced fluorescence is another option; however, only a small fraction of molecules fluoresce while a tedious functionalization must precede the detection of others. Mass spectrometry (MS) is likely to be the ideal choice for detection in CEC [165, 197–201]. The typical flow rates in CEC are in the range of 20-1000 nl/min and correspond well with those easily handled by nanoelectrospray ionization interfaces. Since MS is very sensitive, affords additional information about the molecular weight of separated compounds, and in MSⁿ implementation allows even their identification, this truly orthogonal detection method appears most promising for the future. The currently high price of these detectors is the major obstacle to their broad application in both HPLC and CEC.

Knox, one of the pioneers of electrochromatography, recently called CEC "the liquid chromatography equivalent of gas chromatography (GC)" [201]. Indeed, efficiencies of hundreds of thousands of theoretical plates per column enable resolutions on a par with those of GC. Further improvements in performance of CEC are expected from the use of instrumentation that will allow higher voltages or short capillaries and consequently enable higher flow rates. This in turn will reduce the deleterious effects of axial diffusion that currently obliterates the use of non-porous submicrometer packing materials.

Packed capillaries with a larger inner diameter may be useful in "preparative" separations. These will find an application in proteome research as a part of multidimensional separation systems that will replace 2-D gel electrophoresis. The preparative CEC will require solving of the problems related to heat dissipation since the radial temperature gradient negatively affects the separations, and sample injection. The fabrication of sintered frits in larger bore capillaries is also very difficult. However, in situ polymerized monolithic frits can be fabricated in capillaries of virtually any diameter [190].

Also very promising are the monolithic separation media prepared directly in situ within the confines of the capillary by a free-radical polymerization of liquid mixtures [44]. They are easy to prepare and completely eliminate packing of beads which, for the very small beads, might require new technical solutions. In addition, the in situ prepared monoliths appear to be the material of choice for the fabrication of miniaturized microfluidic devices that represent the new generation of separation devices for the twenty-first century [202, 203].

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Short Monolithic Columns as Stationary Phases for Biochromatography

Ales Strancar¹ · Ales Podgornik¹ · Milos Barut¹ · Roman Necina²

¹ BIA Separations d.o.o., Teslova 30, 1000 Ljubljana, Slovenia

² Boehringer Ingelheim Austria GmbH, Dr. Boehringer Gasse 5–11, 1121 Vienna, Austria

Monolithic supports represent a novel type of stationary phases for liquid and gas chromatography, for capillary electrochromatography, and as supports for bioconversion and solid phase synthesis. As opposed to individual particles packed into chromatographic columns, monolithic supports are cast as continuous homogeneous phases. They represent an approach that provides high rates of mass transfer at lower pressure drops as well as high efficiencies even at elevated flow rates. Therefore, much faster separations are possible and the productivity of chromatographic processes can be increased by at least one order of magnitude as compared to traditional chromatographic columns packed with porous particles. Besides the speed, the nature of the pores allows easy access even in the case of large molecules, which make monolithic supports a method of choice for the separation of nanoparticles like pDNA and viruses. Finally, for the optimal purification of larger biomolecules, the chromatographic column needs to be short. This enhances the speed of the separation process and reduces backpressure, unspecific binding, product degradation and minor changes in the structure of the biomolecule, without sacrificing resolution. Short Monolithic Columns (SMC) were engineered to combine both features and have the potential of becoming the method of choice for the purification of larger biomolecules and nanoparticles on the semi-preparative scale.

Keywords. Short monolithic columns, Monoliths, Chromatography, Separation, Purification, Proteins, DNA, Bioconversion, Solid-phase synthesis

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1 Introduction

The developments in molecular and cell biology in the last quarter of the 20th century led to new technologies for the production of complex biomolecules which have the potential to assist in human health care in the areas of diagnostics, prevention and treatment of diseases. One of the most important and at the same time most expensive step in their production is the isolation and cleaning (down-stream processing) of the target biomolecule(s). It represents more than 50% of the total costs of the production process. Precipitation, ultrafiltration and liquid chromatography are most widely used for these purposes, but usually only liquid chromatography can purify the product to the level recognized as safe for therapeutic use. Usually, more than one liquid chromatographic step is necessary to purify the bioproduct to the desired level. A speeding-up and reduction of the chromatographic steps within any given purification process would result in increased cost effectiveness. For example, just by shortening the time required for the purification of the highly unstable protein component P of the Euphausia pacifica luciferase system from 3-4 days to 5 h a 50-100 fold increase in the specific activity could be achieved [1]. Therefore, only the concomitant development of molecular biology and more effective bioseparation techniques can lead to the production of complex biomolecules for therapeutic use.

Apart from reduced yield, down-stream processing can cause minor or even bigger modifications in the structure of the biomolecule. Often, these modifications do not affect the activity of the product, but may change its antigenicity. Along with virus safety, the reduction of such risks is a main objective in the down-stream processing of such biomolecules. Chromatographic purification, especially the introduction of ion exchange chromatography (IEC) and affinity chromatography (AC) has allowed the production of highly purified biomolecules. Improved column designs, especially the introduction of radial columns, have considerably reduced the time required for separation. However, even with these methods the risk of unwelcome changes in structure or loss of activity during purification cannot be excluded and has to be prevented in each case by careful investigation of the production process.

Liquid chromatography is, as a rule, a rather slow process. It often causes significant product degradation and requires expensive separation media and large volumes of solvents. Diffusional constrains in particular limit the speed of the separation, as they cause a rapid reduction in resolution with increasing eluent velocity in the case of conventionally columns packed with porous particles [2]. On the other hand, the efficient isolation of labile, valuable biomolecules requires a fast, reliable and affordable separation process under mild conditions. Also, the product should be rigorously characterized and the process thoroughly documented, making in-process control a necessary prerequisite [3]. Besides all these constrains, the cost of the production process is an important factor, which consequently forces the separation scientist to be effective with regard to the scaleup of the separation processes and to use reliable, fast means of controlling them. Thus liquid chromatographic supports should meet the following requirements [2, 4-6]:

- fast and efficient separation in order to decrease losses due to biomolecule degradation
- high, flow unaffected capacity
- good, flow unaffected resolution
- low backpressure
- easy and fast scale-up and scale-down potential
- meet all safety regulations, especially regarding leachables and sanitation
- the same material may be used for analytical and preparative purposes
- easy to handle and operate
- stable even if harsh conditions are applied during sanitation
- high batch-to-batch reproducibility

Chromatographic columns packed with conventional porous particles meet these requirements only to a limited extent. Slow diffusion of large molecules limits the speed of the separation due to the low rate of intraparticle mass transfer [7]. New approaches have been introduced to overcome this problem including:

- improved conventional chromatographic media
- microporous membranes
- monolithic continuous bed supports

1.1 Improved Conventional Chromatographic Media

To improve the efficiency of separation based on conventional porous particle technology, new particles and techniques have been introduced, mainly focusing on the improvement (acceleration) of the exchange of the solute between the mo-

bile and stationary phases by introducing micropellicular [4,7-9], superporous [10], superficially porous [11] and gigaporous supports such as the "perfusion" [12] and the "gel in a shell" [13] supports. While micropellicular stationary phases due to their very low capacity are mostly used for analytical purposes, superporous and gigaporous particles exhibit a much higher capacity and can be used on the preparative scale as well. Optimizing the length and the structure/chemistry of the interactive groups (ligands) on the support was another approach to improve the efficiency of the separation media. For these purposes many new ligands have been introduced, ranging from "tentacles" [14] and thiophilic groups [15] to amino acids, peptides, metal chelates, affinity and many other ligands, which offer high selectivity for particular groups of biomolecules. Extended reviews on different ligands used for separation of biopolymers have been presented by Boschetti [13] and Narayanan [16]. Finally, yet another approach was directed towards a new design of the chromatographic hardware (column) and process. Radial chromatography [17] and expanded bed chromatography [18] are two useful examples of this approach.

1.2 Microporous Membranes

Membrane technology in bioseparation reflects technological advances in both membrane filtration (ultrafiltration) and fixed-bed liquid chromatography. Ultrafiltration membranes (filters) are employed mainly as "cut off" devices used to separate biomolecules whose sizes differ by at least one order of magnitude. They are mainly used in down-stream processing to remove cell debris, colloidal or suspended solids and viruses, and to separate large biomolecules from small ones, for example in desalting processes. When affinity, ion exchange, hydrophobic interaction or reversed phase ligands are coupled to such membranes (filters), an increase in selectivity can occur. Comprehensive reviews of membrane technologies have, e.g., been presented by Heath and Belfort [19], Zeng and Ruckenstein [20], and by Klein [21].

The chromatographic interactions in the membranes are usually assumed to be similar to those in the porous particulate material. The main difference between the two "column" types is hydrodynamic. Membrane-based chromatographic supports can generally be distinguished from porous particle-based ones by the fact that the interaction between a solute (for example a protein) and the matrix (immobilized ligand) does not take place in the dead-end pores of a porous particle, but mainly in the through-pores of the membrane. While the mass transport in dead-ended pores necessarily takes place by diffusion, the liquid moves through the pores of the membrane by convective flow, drastically reducing the time required for mass transfer from the liquid to the stationary phase and back. As a consequence, membrane separation processes are generally very fast, in fact one order of magnitude or more faster when compared with columns packed with corresponding porous particles [6]. Since membranes are very short (usually a stack of few mm in height of several thin membranes is used for chromatographic purposes) compared to conventional chromatographic columns packed with porous particles, reduced pressure drops are found along the chromatographic unit, allowing increased flow rates and consequently higher productivity.

Many membrane separations are performed by using a conventional filtration apparatus; others are configured for compatibility with existing chromatography pumps and detectors. Regardless of the configuration of the apparatus and the type of matrix, the problem of uniform flow distribution from a relatively thin pipe to a large area has to be solved, as well as the problem of recollecting the eluate at the other end of the device with minimal back-mixing and distortion of zones, to assure the resolution power of the membrane column.

Membrane devices can be classified into four main types:

- porous sheets loaded with specific binding particles [22, 23] (these do not belong to membrane chromatography systems in the strictest sense, as the binding process does not take place at the pore wall itself, but at the very small particles that are embedded in the outer porous matrix [6])
- hollow fiber membranes [24, 25]
- single membrane or stack of sheet membranes [5, 26]
- radial flow membranes [27]

While hollow fiber units are potentially quite useful for "bind-release" separations, their use for conventional chromatography operations have met with minimal success to date [26]. The main problem lies in the relatively large dead volume of the units resulting in large band spreading. Stacked flat-sheet membranes, on the other hand, have been successfully used for complex chromatography separations on both the analytical and the preparative scale [2, 5, 26]. Their design was mainly derived from filtration modules and such columns exist in a variety of configurations, all representing short, wide chromatographic columns, in which the adsorptive material consists of one or more microporous membranes in series, each derivatized with the desired interactive moieties. They are very effective when a low concentration of the target molecule has to be isolated (captured) from large quantities of raw solution [19].

1.3 Monoliths – Continuous Beds

Apart from their predominantly diffusive means of mass transport, the problem of particulate separation media is their inability to completely fill the space within the chromatographic column. This also contributes to peak broadening and decreased column efficiency. By introducing separation media with a higher degree of continuity consisting of a monolith or continuous bed, i. e. typically a very large cylindrical particle of rigid, highly porous polymer, the void volume can be decreased to a minimum [28, 29]. The most important feature of such media is that the mobile phase is forced to flow through the large pores of the medium. As a consequence, mass transport is enhanced by convection and has a positive effect on the separation. For a more detailed theoretical discussion of the mass transport in monolithic supports, the reader can refer to the work of Meyers and Liapis [30, 31]. It is now over a decade since chromatographic supports based on such monolithic structures have been introduced. There were three main groups active in this field, each developing the support based on different material and having different characteristics. The first two groups, Nakanishi and co-workers [32] and Hjerten [33] were trying to replace the standard (long) chromatographic column with the new type of continuous support allowing convective mass transport. The idea of the third group (Tennikova, Svec and co-workers) was to combine the advantages of continuous bed supports with the advantages of short column length (i. e. that of "membrane" chromatography) [34]. As a result the theory of short column layer was developed, which is based on the fact that in gradient chromatography of proteins and other biomacromolecules, a critical distance X_0 exists at which the separation of zones is at a maximum and band spreading is at a minimum. With step gradients and high elution velocity the column length may be reduced to the level of membrane thickness [35] as will be shown later in this chapter.

1.3.1 Silica Based Monoliths

The silica-based monolithic beds were first introduced by Nakanishi and coworkers [32, 36], then further developed by Tanaka and co-workers [37] and Cabrera and co-workers [38] and now are commercially available from E. Merck (Darmstadt, Germany) under the trade name of "Chromolith". These columns are continuous rods of silica monolith, formerly named SilicaROD and are prepared by a sol-gel process, which is based on the hydrolysis and polycondensation of alkoxysilanes in the presence of water-soluble polymers. The method leads to "rods" made of single piece of porous silica with a defined bimodal pore structure having macro (of about 2 µm) and mesopores (of about 0.013 µm) when smaller rods intended for analytical purposes are prepared. The main feature of these columns is a porosity of about 80%, which is 15% more than columns packed with standard particulate packing. As a result, the pressure drop along the column is one-third to one-fifth of that on columns packed with 3- or 5-µm beads. The resulting pressure drop is therefore much lower, allowing operation at higher flow rates while the optimized separation efficiencies are comparable to silica columns packed with 4 µm particles [39]. Scaled up units intended for semi-preparative purposes were developed as well and were first reported by Schulte et al. [40]. These columns which are suitable for laboratory and semi industrial scale purification have macropores of about 4 µm and mesopores of about 0.014 µm and this allows even higher flow rates to be used then in case of the analytical ones. The authors reported that the achievable optimum separation efficiencies are comparable to those of columns packed with 10 µm particles, while the pressure drop is several times lower. All silica rods can be modified using the same derivatization chemistries that are used for regular HPLC packings, creating for example, C18 bonded phases suitable for reversed phase chromatography.

Another group working on silica monoliths is the one of A. and M. Kuehn [41]. Their Continuous-Bed-Silica (CB-Silica) is a highly porous monolith having meso- and micropores. The structure of the CB-Silica is very porous and con-

sists of pores with different sizes. The mesopores, which pass through the silica like channels allow favorable flow conditions of the mobile phase and keep the backpressure to a minimum. Due to the high amount of meso- and micropores per volume there is a large inner surface of about $450-550 \text{ m}^2/\text{g}$. The porosity of the monolith is about 70% and hence inferior to that of the Chromolith column, which typically has 80% porosity.

1.3.2 Soft Organic Gel-Based Monoliths

Continuous beds made of swollen polyacrylamide gel compressed in the shape of columns were first introduced by Hjerten and his group [33, 42]. Their technology relies on the polymerization of suitable monomers and ionomers directly in the chromatographic column. In the presence of salt, the polymer chains formed aggregate into large bundles by hydrophobic interaction, creating voids between the bundles (irregularly shaped channels) large enough to permit a high hydrodynamic flow at low backpressure. Following the polymerization, the bed is compressed by connecting it to an HPLC pump adjusted to a flow rate equal or higher than that to be used in the subsequent separations. The bed obtained can be regarded as a rod or plug permeated by channels through which the eluent can pass upon application of pressure. The polymer chains form a dense, homogeneous network of nodules consisting of microparticles with an average diameter of 2 µm. The channels between the nodules are large enough to permit a high hydrodynamic flow. Due to the high cross-linking of the polymer matrix, the nodule themselves can be considered as nonporous. These supports are now commercially available from Bio-Rad (Hercules, USA) under the trade name "UNO".

1.3.3

Rigid Organic Gel-Based Monoliths

In the adsorptive modes of protein chromatography the slope of the capacity factor k', defined as the molar ratio of the separated compound in the stationary phase and the mobile phase, plot versus composition of the mobile phase is very steep. Up to a certain composition of the mobile phase, k' is so high that the protein can be considered bound to the stationary phase and not capable of moving along the column. Reaching a defined point, a small change of the mobile phase composition causes a rapid decrease in k' to a value near zero. At this point, the protein dissolves in the mobile phase and passes through the column practically without any retention. In other words, the protein remains adsorbed at the top of the column until the eluting power of the mobile phase reaches the point at which a small change in the composition of the mobile phase causes the movement of the protein without any retention. One can also speak about selective elution of the compound. As a result of this process, even very short columns can provide very good separations and very good recovery (for details refer to Sect. 2), while longer columns might cause problems due to unspecific binding, product degradation and minor changes in the structure of the protein, which increase with the

length of the column. On the other hand, short beds are very difficult to pack with particles and tend to form channels, which spoil the resolution power of the column.

Monolithic supports offer an ideal solution to avoid such packing and channeling problems, which led Tennikova, Belenkii and Svec to develop 1-mm thick "membranes" made of rigid macroporous methacrylate polymer for that purpose [34]. They have proved that with such structures very efficient separations can be achieved and have originally suggested the name "High Performance Membrane Chromatography" (HPMC) for the technique using short chromatographic layers with high-resolution power. However, to avoid confusion of these types of support with the real membrane separation units discussed in Sect. 1.2, the name High Performance Monolith Chromatography (HPMC) is now preferred [43]. In addition, the structures themselves are nowadays generally referred to as "disks" rather than "membranes".

This newly developed type of chromatographic support (Short Monolithic Columns, SMC) was first produced and commercialized by Knauer Saeulentechnik (Berlin, Germany) under the trade name of "QuickDisk" [44, 45]. The group of developers in the company headed by Josic and Reusch had solved the problem of proper sample distribution by introducing a disk holder optimized for short chromatographic beds [45]. The product did not reach wide acceptance on the market due to problems with batch-to-batch reproducibility and bypassing. Also the scale-up strategy based on producing disks with larger diameters was not fully successful. A major breakthrough in the scale up of SMC was subsequently achieved by Strancar et al. [46], who introduced tube-shaped monolithic units, which resolved the problem of scale-up while retaining the idea of short chromatographic separation distances. Podgornik et al. [47] then resolved the problem of the preparation of larger homogeneous monolithic units by introducing the "tube in a tube" approach to column synthesis (for details refer to Sect. 3). In addition, the polymerization of the monoliths has been optimized resulting in much better batch-to-batch reproducibility and homogeneity. For smaller units, the problem of bypassing has been resolved by introducing a novel disk holder design [48]. Newly developed monolithic units were put on the market by BIA Separations d.o.o. (Ljubljana, Slovenia) under the trade name "CIM" (Convective Interaction Media) in 1998.

Another approach for increasing the capacity (scaling-up) on rigid organic gels was introduced by Svec and Fréchet [49]. These authors reported the preparation of a rod-shaped monolithic column consisting of a single "molded" piece of macroporous polymer, practically the same as the one introduced by Tennikova et al. [34]. The continuous rod of porous polymer was prepared by an insitu polymerization of a suitable monomer mixture within the confines of the tube of a chromatographic column. The chromatographic tube sealed at one end was filled with the polymerization mixture, sealed at the other end, and than heated in the water bath. Once the polymerization was complete, the seals were removed, the column was provided with fittings, attached to the HPLC system and washed. To provide different functionalities, the reagent was pumped at a slow rate through the column, which was kept at the desired reaction temperature for a certain time. After the reagents were washed out of the column, the col-

Table 1. Main c	Table 1. Main characteristics of different monolithic supports	olithic supports						
Name of the Material product	Material	Pore diameter	Total porosity	pH range	Regeneration	Main area of application	Producer	Introduced to the market
Chromolith TM silica	silica	2 and 0.013 µm 4 and 0.014 µm	80%			Small molecules	Merck	2000
SB-Silica	silica	0.03 and 0.005 μm	70%	1 - 8	avtoclavable		Conchrom	2000
UNO TM	polyacrylamide	about 1 µm		2 - 12		Biomolecules	Bio-Rad	1997
Seprasorb	cross-linked, regenerated cellulose	50 to 300 µm		2 - 14	0.5 M HCl 0.5 M NaOH	Biomolecules	Sepragen	
CIM®	polyglycidylmethacrylate-co- 1.5 and 0.030 µm ethyleneglycoldimethacrylate	1.5 and 0.030 µm	60%	1-13	avtoclavable, stable in 1 M NaOH	Biomolecules	BIA Separations	1990 ^a 1998
CIM® RP	styrene-divinylbenzene	1.5 and 0.030 µm	60%	1-13	avtoclavable, stable in 1 M NaOH	Biomolecules	BIA Separations	2000
"Rod"	styrene-divinylbenzene					Small molecules, Biomolecules	ISCO Inc	Not yet available
^a A very simila Disk.	^a A very similar product has already been introduced on the market in 1990 by Knauer Saeulentechnik, Berlin, Germany under the trade name Quick-Disk.	roduced on the marke	et in 1990 by	/ Knauer	Saeulentechnik,	Berlin, Germany	under the trac	le name Quick-

umn was ready for use. Monoliths of this type were successfully applied in the field of capillary electrochromatography as well. Details of this application can be found elsewhere in this book.

The main characteristics of all these types of monolithic supports are summarized in the Table 1.

2 Theoretical Background

From the point of view of fast and efficient chromatographic separations of large biomolecules such as proteins, the SMC are characterized by some very interesting features such as low flow resistance, the absence of a gradient in the mobile phase composition between the inlet and the outlet of the column as well as improved mass transfer characteristics. Let us briefly go through some of these features and discover what were the basic postulates and ideas that have led to a successful implementation of extremely short continuous beds for the chromatography of biomolecules. As already mentioned and as shown by many different authors (e.g., Moore and Walters [50], Vanecek and Regnier [51] and Tennikova and Svec [28]) the column length does not usually influence the resolution of the separation of proteins in a linear gradient to a great extent. This served as a rationale for developing the so-called high performance membrane chromatography (HPMC) in the late 1980s and early 1990s by Tennikova et al. [34, 52]. Although the exact influence of the column length on the resolution was not as straightforward as in the case of isocratic separations of small molecules, results have shown that it is possible to use extremely short columns for efficient separations of proteins [35, 45, 53-55], even though the reason for this was not well-understood [56]. Only recently, were the models developed that enable a better understanding of the phenomena involved [43, 55, 57]. Historically, HPMC was hence used exclusively for the separation of proteins by gradient elution.

2.1

Early Results Concerning the Influence of Column Length on Resolution

Snyder and co-workers [56, 58, 59] have developed a general model for the prediction of the peak position and bandwidth in a linear gradient for both small and large molecules using the so-called linear-solvent-strength (LSS) theory. As far as large biomolecules are concerned, the most important information retrieved from this approach is that of the role of the mobile phase composition during the desorption of large biomolecules from the matrix in the gradient elution. The LSS model for large molecules was based entirely on conventional chromatographic theory as developed for small molecules, taking only into account some special properties of large molecules. For small molecules in isocratic and gradient elution, the resolution is proportional to the square of the column length. Therefore, in order to increase the resolution two times, a four times longer column should be used (all other parameters being the same). On the other hand, with large biomolecules, the picture is somewhat different. First of all, with few exceptions, large biomolecules are separated using mostly gradient and not isocratic elution. Additionally, the effect of column length on the resolution is not completely understood and it is much more complicated than that for small molecules. Nevertheless, Snyder et al. [58] have presented the following equation for the reversed-phase gradient elution of macromolecules valid for the values of a average capacity factor between 1 and 10 and for flow rates higher than 1 ml/min:

$$PC \propto R_s \propto t_G^{05} F^0 L^0 d_p^{-1}.$$
 (1)

According to Eq. (1), resolution increases with the gradient time t_G and is not effected by the flow rate F or the column length L. This was found to be approximately correct for many reversed phase peptide and protein separations [56]. One such example can also be found in the paper by Moore and Walters [50] showing the gradient separation of ribonuclease A, cytochrome C and ovalbumin on columns of lengths ranging from 4.5 cm to 0.16 cm. In that case, an almost 30fold change in column length had little apparent effect on the separation quality. This phenomenon was interpreted as being the result of two opposing effects: (1) a decrease in the average value of the capacity factor, k', with increasing column length versus (2) an increase in N with increase in column length. These two factors cancel each other in the range 1 < k' < 10. However, the authors also claimed that, when gradient conditions were optimized, an increase in column length Lshould lead to increased peak capacity and resolution.

Yamamoto et al. [60] presented similar results regarding the influence of the column length on the separation of proteins in ion-exchange chromatography. These authors have applied a quasi-steady state model based on the continuous-flow plate theory. Briefly, their approach can be summarized as follows. In equilibrium theory, in which zone spreading effects are ignored, the moving rate of the protein zone in the column is expressed by the following equation:

$$v = \frac{u}{1+k'}.$$

In Eq. (2), v is the protein zone velocity, u is the mobile phase linear velocity and k' is the momentary value of the capacity factor. In a linear gradient experiment, the value of k' is large at the beginning of the elution owing to the low ionic strength and therefore v is low. Since the linear increase in the ionic strength is continuously applied to the column, the value of k' decreases. Therefore, the protein zone moves slowly at first and accelerates gradually with time. However, v approaches its maximum value a short distance from the top of the column owing to a drastic decrease in k' with the ionic strength, see, e.g., Snyder et al. [56]. The protein zone then moves until it reaches the outlet of the column with the velocity close to the maximum velocity attainable under non-binding conditions. At a fixed slope of the gradient and flow rate the resolution increases with column length until a certain length and then becomes constant. As the slope of the gradient becomes steeper, the length above which resolution becomes constant decreases. Therefore, the whole separation takes place in the relatively short part of the chromatography column and the rest of the column is not used to improve

the resolution further. To summarize, for steep gradients, the resolution soon becomes independent of the column length, while for shallow gradients the resolution gradually increases with the column length.

2.2 The Concept of SMC

The first study that made it possible to estimate the critical length of a column in gradient HPLC of proteins was presented by Belenkii and co-workers in 1993 [53]. Their approach was based on the concept of critical chromatography of synthetic polymers. They introduced the concept of a critical distance, X_0 , after which the protein zone travels with the same velocity as the mobile phase (similarly to what has been shown previously by Yamamoto et al. [60]). The equation for the critical distance at which the zone velocity v(x) becomes virtually identical to the displacer velocity, u, is defined as:

$$X_0 = \frac{\lambda \cdot u}{S \cdot B}.$$
(3)

Here, λ is the parameter characterizing the precision of the fulfillment of the equality $v(X_0) = u$, while u is the linear velocity, S is a dimensionless protein adsorption parameter (in reversed phase chromatography) and B is the steepness of the linear gradient. According to the authors, in the precritical region ($L \ll X_0$) the protein zone is significantly broadened as it is eluted at high values of k'. At the point $L = X_0$ the variance of the zone is at minimum. At $L > X_0$ there is no gradient compression and the variance becomes proportional to the square root of the column length. Furthermore, with steep gradients and small elution velocities, the column length may be reduced to the level of membrane thickness, i.e. about one millimeter or less, therefore, the concept of the critical distance X_0 is of fundamental importance to short continuous bed chromatography.

Similar to Yamamoto et al. [60], Tennikova and co-workers [55], used the socalled quasi-steady state approach to predict SMC chromatography. The basic equation used in their modeling was the dependence of the zone migration on the composition of the mobile phase and on the gradient function, which in its differential form is given by:

$$\frac{\mathrm{d}x}{\mathrm{d}t} = u \cdot f\left(c \cdot \left(t - \frac{x}{u'}\right)\right). \tag{4}$$

Where *u* is the elution velocity and *u*' is the displacer velocity.

The general solution of this equation was obtained and applied for a special case where f(c) is defined according to the stoichiometric model of protein retention as:

$$f(c) = \frac{1}{1 + K \cdot c^{-z}}$$
(5)

and the linear gradient is described by:

$$c = c_0 + B \cdot t \,. \tag{6}$$

For this special case, the following expression for the critical distance, X_0 , at which the quasi-steady state is obtained:

$$X_0 = \frac{\lambda \cdot u \cdot C_c}{Z \cdot B}.$$
(7)

In Eq. (7), λ is an auxiliary parameter, *u* is the linear velocity of the mobile phase, C_c is critical concentration of the displacing salt, *Z* is the effective charge on the solute ion divided by the charge on the mobile phase ion and *B* is the gradient steepness.

The results obtained by Tennikova et al. [55] for ion-exchange chromatography, together with the results previously presented by Belenkii et al. [53] for reversed phase chromatography, form a theoretical basis for HPMC, which is now more conveniently called chromatography on short monolithic columns (SMC). According to the authors, the quasi-steady state is realized at an X_0 less than L, thus, it might occur at steep gradients or on long columns. As the aim of any separation is to obtain good resolution, it is not important in which state the separation is performed. The main conclusion was that it is possible to use ultra-short columns for the separation of proteins and other molecules that traditionally have been separated using steep gradients, with appropriate resolution and at low gradient times. Furthermore, because of the short separation layer length, the pressure drop on short continuous beds is much lower (typically less than 5 MPa) and extremely high flow rates can be applied. This, together with the improved mass transfer characteristics, may lead to the possibility of performing very fast separations, which are extremely important for large and labile therapeutic macromolecules.

Very similar results were presented by Coffman et al. [2]. They introduced a yet different approach to the prediction of the efficiency of large molecule (proteins) separations on very short columns. Their approach is based on the fact that since short columns yield non-Gaussian effluent distributions, measuring the degree of binary separation using conventional chromatographic resolution is inadequate. Instead, they proposed the fractional purification P_i of component *i*, defined as:

$$P_i = \frac{Y_i}{Y_i + Y_i}.$$
(8)

Where Y_i represents the fractional mass yield of *i* in the *i*-rich product.

The purity of the final product in a single-stage (ultra-short) column is defined through the separation factor calculated as:

$$\frac{u_i}{u_j} = \frac{\ln\left[1 - P_i\right]}{\ln P_i}.$$
(9)

As further shown by the authors, for e.g., a fifty-plate column, a separation factor of about 1.5 is needed to achieve a 99% purity. This value of the separation factor is in the range of many practical protein separations. Therefore, the use of a fifty-plate column can achieve high purity indicating that the tens of thousands of plates found in many conventional chromatography columns are not necessary for most protein separations. Short columns can be efficiently used for the separation of proteins and probably for other large molecules as well, especially in reversed phase chromatography, where extremely large values of separation factors are not uncommon for some protein separations.

2.3 Resolution and Efficiency in SMC-Chromatography

Finally, let us briefly speculate about the efficiency of SMC to separate different (bio)molecules. The usual measure for the efficiency of conventional HPLC column is the so-called height equivalent of a theoretical plate (HETP) [56], which is simply the ratio between the column length, L, and the number of theoretical plates, N. Normally, the lower the HETP value, the more efficient the column is (larger N at the same column length). Columns with a large efficiency have HETP values in the range from 8 µm to 14 µm. For the typical column lengths (e.g. 10-25 cm) this HETP value translates into approximately 12000 to 30000 theoretical plates, N, per column. In ion-exchange chromatography of large molecules like proteins and DNA the HETP are much larger and can even reach 100-200 µm depending on the column type. These HETP values still translate into relatively high number of theoretical plates, N, ranging from 1000 to 2500. In the case of SMC with a "length" of only a few mm, the number of theoretical plates is much lower, ranging only from 10 to 50. As has been shown [57], this number is still high enough to allow an isocratic separation of different oligonucleotides. This was explained by a large difference in the Z factor between individual nucleotides owing to the increased chain length and, consequently, to the increased charge density. If the difference in Z factors of two molecules is large enough, the resolution achievable even on a short column can be satisfactory. This is especially true for large (bio)molecules, which differ significantly in the Z factor [59]. The reason lies in their high molecular mass and, consequently, a high heterogeneity of charged groups on the molecule surface. Due to these pronounced differences, the binding characteristics between individual molecules are quite different and an efficient separation can be achieved by selective elution using a linear gradient. A more detailed and comprehensive description of the processes governing differential elution of large biomolecules on the SMC can be found in a recent review paper published by Tennikova and Freitag [43].

3 Preparation of SMC and Scale-Up Strategies

3.1 Synthesis of the SMC

Most of the SMC described in this chapter are prepared by free radical polymerization of a mixture of glycidyl methacrylate (providing functional groups), ethylene dimethacrylate (as a cross-linking reagent), 2,2'-azobisisobutyronitrile (as an initiator) and a porogenic solvent (cyclohexanol and dodecanol) in barrels of polypropylene syringes, as published elsewhere [61, 62], yielding glycidyl methacrylate-*co*-ethylene dimethacrylate (GMA-EDMA) monoliths. Another method uses a free radical polymerization of the mixture of styrene and divinylbenzene (the latter as cross-linking reagent) using 2,2'-azobisisobutyronitrile as an initiator and a porogenic solvent (dodecanol and toluene) to ensure adequate porosity. After the polymerization, the block of polymer formed in disk or tube shape, is mounted in a specially designed housing allowing good sample distribution and low dead volume. Then the disk or tube is washed with methanol, a methanol-water mixture (50:50) and distilled water to remove porogenes and residual monomers from the porous polymer. After this the monolithic bed is ready for further derivatization or ligand immobilization if desired. GMA-EDMA monoliths have active epoxide groups which can easily be further modified using various chemistries, e.g. diethyl amine, propane sulfone for ion exchange chromatography, butyl for hydrophobic interaction chromatography or any desired protein ligand for affinity chromatography.

The preparation of monolithic columns is in many cases considered an easy and straightforward process, especially when compared to the tedious and timeconsuming preparation of monosized spherical particles and subsequent packing of conventional columns. In principle, this is true for the in-situ preparation of methacrylate and styrene-divinylbenzene based HPLC columns, or capillary micro HPLC and CEC columns [63]. According to various authors, the procedure consists of simply filling the column or capillary with a liquid monomer mixture (that also contains an organic solvent and an initiator), sealing the column/capillary at both ends and triggering the polymerization procedure by placing the column in an appropriate water bath. This procedure is very easily done and it removes the need for tedious slurry column packing. However, according to our own experience, this method of monolithic column preparation only works in the case of micro or small-scale (up to a few ml in volume) monolithic columns. The preparation of large volume monolithic columns with a well-defined and homogeneous structure still represents a considerable challenge to manufacturers. In contrast to the scale up of particle columns, containing particles that range in size from a few micrometers up to 100 micrometers, which is obtained by packing these very small particles in larger columns, large-scale monolithic columns are obtained by producing a large block of a polymer cast in a proper cartridge (monolith holder). The main problems that occur during this process are connected to the heat release and heat dissipation (gel-effect) during polymerization.

3.2 Preparation of Large Scale SMC

The production of conventional stationary phases in the form of porous polymer particle is based on suspension polymerization. Namely, the polymerization is allowed to proceed in a solvent under vigorous stirring that assures obtaining particles of the desired diameter. Since the particle size is typically in the range of a few micrometers, no problems with heat transfer are encountered. In contrast, the preparation of monoliths requires a so-called 'bulk' polymerization. A polymer mixture consisting of monomers and porogenic solvent is mixed with an initiator. As the temperature is increased, the initiator decomposes and oligomer nuclei start to form. The solubility of the polymers in the reaction mixture decreases during growth and at some point they start to precipitate. Thermodynamically speaking, the monomers are better solvents for the polymer than the porogenes. Consequently, the precipitated nuclei are swollen with the monomers. Since the monomer concentration is higher than in the surrounding solution, the polymerization in the nuclei is kinetically preferred. In the absence of mixing and due to their higher density, insoluble nuclei sediment and accumulate at the bottom of the mould. Initially, they form a very loose structure, which is highly porous. During the course of the polymerization, nuclei continue to grow and crosslink until the final structure is achieved. As it can be deduced from the above description, the pore size distribution of the polymer depends on the chemical composition, but also the polymerization temperature. In particular, the temperature defines the degradation rate of the initiator and, therefore, also the number of nuclei formed in a given time. Since the amount of the monomers is constant, the lower number of nuclei formed at lower temperatures within a defined volume corresponds to a larger size and thus, to larger pores between the clusters of growing nuclei. In contrast, at higher polymerization temperatures, where the initiator decomposition is much faster, the number of growing nuclei is much larger. Therefore, the pores formed will be smaller. A dramatic effect of the polymerization temperature is demonstrated in Fig. 1. As can be seen, a change of only 8 °C shifts the average pore radius from 400 to 850 nm and completely changes the flow characteristics of such a monolith. Therefore, the polymerization temperature is a powerful tool for the control of pore formation.

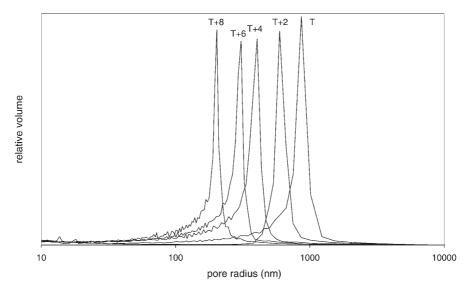


Fig. 1. Effect of the polymerization temperature on the pore size distribution. At the highest temperature (T+8) the average pore radius is 400 nm while at the lowest T the pores are much larger with an average pore radius of 850 nm

The polymerization of a methacrylate-based monolith is an exothermic process. Therefore, during the course of the reaction heat is released. If no mixing takes place and if the size of the mould is in a range of a centimeter or more, the released heat cannot be dissipated fast enough. As a consequence, an increase of the temperature inside the reaction mixture occurs as shown in Fig. 2. The temperature increase during polymerization is over 77 °C in a mould of 5 cm diameter. The effect of this temperature increase during the polymerization was carefully studied by Peters et al. [64]. They initially performed experiments in a 26 mm mould using AIBN as an initiator. The extremely fast reaction led to a monolith with a badly scarred structure due to the nitrogen released during the reaction. In subsequent experiments, benzoyl peroxide was used instead as an initiator. During the polymerization in a 26-mm mould an increase of temperature of only 7 °C across the radius of the column was recorded and no influence on the pore size distribution across the radius as well as along the height was found. On the other hand, when a mould of 50 mm was used, a temperature increase of 113 °C was observed and a 25 °C temperature differential was recorded across the radius of the column. Pore size distribution measurements revealed that the pores in the middle of the polymer were larger than on the outer part resulting in pore size distribution inhomogeneity. Obviously, the preparation of large volume monoliths is limited by the exothermic nature of the polymerization and the fact that the temperature exerts a pronounced influence on the pore

size distribution. To avoid these problems, Peters et al. [64] suggested perform-

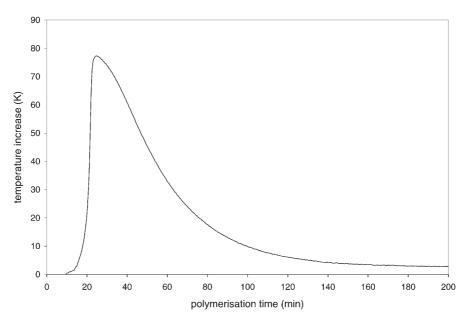


Fig. 2. Temperature profile in the middle of a 5 cm cylindrical mould during the polymerization of a GMA-EDMA monolith. The increase of the temperature by 77 °C significantly influences the structure of the GMA-EDMA monolith

ing the polymerization at a slow reaction rate. This is accomplished by gradually adding the reaction mixture to the mould. To investigate this, the authors fed the reaction mixture into the mould at a rate of 20 ml/h for 12 h to ensure slow polymerization. They found only a slight temperature increase of 10 °C and a much more uniform pore size distribution. The problem, which might arise with such a procedure, is the conditioning of the reaction mixture. The initiator is either continuously added and dissolved into a thermostatted reaction mixture or the reaction mixture must be significantly colder to prevent polymerization over such a long period of time. That the conditions in a gradual addition of the polymerization mixture were not the same as in the previously presented experiments can be concluded from the comparison of the pores of the monolith prepared in a conventional way (batch mode) in a 26 mm mould and with a gradual feeding (fed-batch mode) in a 50 mm mould. Although the temperature increase was similar in both cases, a pore diameter of the highest peak of the pore size distribution ranges from 1.50 to 1.56 µm in the former case and 1.66 to 1.76 µm in the latter case (in the upper part of the later monolith, the pores were even larger, i.e. up to $2 \mu m$).

Due to the problems with scale up by increasing the diameter, an alternative approach has been proposed, which consists of preparing long monolithic rods with small diameter. Indeed, there are several publications describing GMA-EDMA monolithic columns with a length of up to 300 mm [29]. As with conventional columns, an increased length results in increased backpressure. Since the monolithic structure is the most advantageous for fast separation of large molecules, which is predominantly based on a gradient elution, the column length should not improve the resolution significantly. In fact, as was discussed in Sect. 2.2, longer columns might even result in additional band spreading, thus lowering resolution. Therefore, to take advantage of the monolithic structure on a large scale, the most suitable design seems to be a tube shaped monolith. The first such monolith used in a radial chromatography mode was designed by Strancar et al. [46] in 1997. It was a 22 ml tubular monolithic GMA-EDMA column used for the purification of plasma proteins. The backpressure was significantly lower than the rod-shaped monolithic columns of similar volume.

Still the production of larger volume tubular shaped monoliths was hampered by the problem of providing the required uniform pore size distribution. To overcome this problem, a new approach has recently been proposed by Podgornik et al. [47]. Instead of gradually adding the polymerized mixture to form a single large volume monolith, this approach is based on the preparation of monoliths of a precisely defined shape. This process avoids temperature increases during polymerization, thus preventing uneven pore size distribution. To accomplish this in practice, a mathematical model based on the heat balance during the polymerization process was developed. To simplify the model, the authors assumed that the heat released per unit volume (*S*) is constant during the polymerization and uniformly released over the entire volume. Furthermore, they assumed that the thermal conductivity λ is constant and that the system is in thermal equilibrium. These assumptions were justified since only the determination of the maximal temperature increase is of interest and all other parameters can be considered to be in steady state during this process. For a more precise mathematical model predicting the temperature profile during the course of a polymerization and analysis of the reaction rate is required. Recently, the experiment has been conducted that indicates that this polymerization system follows a first order reaction kinetics [65]. However, using the previously described assumptions, the following equation for determination of the maximal temperature increase inside the reaction mixture can be derived:

$$T_{\max} = T_0 + \frac{S}{4\lambda} \cdot \left[r_1^2 + \frac{r_1^2 - r_0^2}{2\ln\frac{r_1}{r_0}} \cdot \left(\ln\left(\frac{1 - \frac{r_0}{r_1}}{2\ln\frac{r_1}{r_0}}\right)^2 - 1 \right) \right].$$
(10)

Based on this equation one can predict the temperature increase to be expected for a defined annulus thickness as shown in Fig. 3. With the above-described approach one can in addition construct a monolithic annulus of a desired radius but limited thickness. By preparing a series of annuluses where the outer diameter of the smaller monolith is equal to the inner diameter of a larger one, a large volume monolithic unit can be constructed by forming a so called "tube in a tube" system, as shown in Fig. 4. In this way, a monolithic unit of the required volume and uniform pore size distribution can be prepared. Furthermore, the voids between the annuluses can be filled with the reaction mixture and polymerization is allowed to proceed for a second time. Since the voids are very thin, no increase in temperature during the course of the reaction is expected.

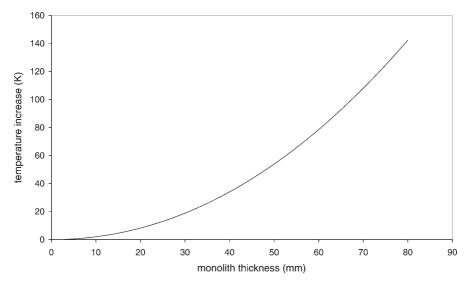


Fig. 3. Effect of the annulus thickness on the maximal temperature increase during the polymerization of a GMA-EDMA monolith. Inner annulus radius is 10 mm; calculation is based on Eq. (10). (Reprinted with permission from Podgornik A, Barut M, Strancar A, Josic D, Koloini T (2000) Anal Chem 72:5693)

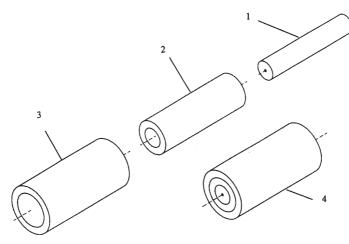


Fig. 4. Construction of a large volume GMA-EDMA monolithic unit. The monolithic unit (4) consists of three monolithic annuluses (1, 2 and 3). Total thickness of the unit 4 is a sum of the thickness of the monolithic annuluses 1, 2 and 3. (Reprinted with permission from Podgornik A, Barut M, Strancar A, Josic D, Koloini T (2000) Anal Chem 72:5693)

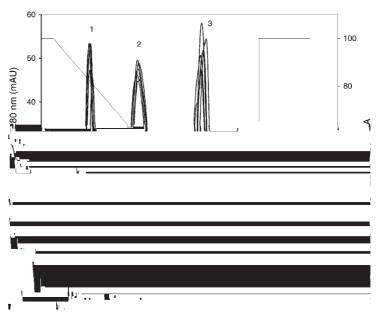


Fig. 5. Effect of the flow rate on the separation efficiency. Separation of a protein mixture at six different flow rates (40, 80, 120, 160, 200 and 240 ml/min) normalized to the elution volume. Conditions: Column: 80 ml CIM® DEAE Tube Monolithic Column; Mobile phase: buffer A: 20 mM Tris-HCl buffer, pH 7.4; buffer B: 20 mM Tris-HCl buffer + 1 M NaCl, pH 7.4; Gradient: 0–100% buffer B in 200 ml; Sample: 2 mg/ml of myoglobin (peak 1), 6 mg/ml of conalbumin (peak 2) and 8 mg/ml of soybean trypsin inhibitor (peak 3) dissolved in buffer A; Injection volume: 1 ml; Detection: UV at 280 nm. (Reprinted with permission from Podgornik A, Barut M, Strancar A, Josic D, Koloini T (2000) Anal Chem 72:5693)

This approach was verified by the construction of an 80 ml tubular monolithic column. The monolithic column was characterized by low backpressures even at high flow rates (below 2.5 MPa at the flow rate of 250 ml/min). One interesting feature, which should be highlighted at this point, is that, in contrast to conventional radial columns of large diameter and small bed thickness, the bed in this case had an outer diameter that was 35 mm while the inner diameter was only 1.5 mm. Because of that, the linear velocity of the mobile phase increases more then 23 times from the outer to the inner surface of the column. In the case of conventional porous particle supports, such changes in the linear velocity would generally result in a pronounced deterioration of the column efficiency. However, the characteristics of the monoliths were found to be flow independent, therefore the change in linear velocity should not have any influence either on the resolution or on the binding capacity. This was proved by the separation of a protein mixture as well as by measuring the dynamic binding capacity determined at different flow-rates. As shown in Fig. 5, the curves obtained overlap nicely at different flow rates. As the authors calculated, this unit can purify around 15 g of protein per hour.

4 Characteristics and Application of SMC in the Liquid Chromatography of Biomolecules

4.1 Characteristics of the SMC

Most of the SMC presented in this chapter are highly cross-linked porous rigid monolithic polyglycidylmethacrylate-*co*-ethyleneglycoldimethacrylate or styrene-divinilybenzene polymers produced by BIA Separations under the trade name of Convective Interaction Media (CIM). Following the idea of a short chromatographic layer, the smaller units are produced in the form of disks (see Fig. 6) and the larger units in the form of tubes (see Fig. 4).

Both units are engineered to ensure well-defined, narrow pore-size distributions, excellent separation power and exceptional chemical stability and flow characteristics. To ensure scalability, the smaller and bigger units are of the same



Fig. 6. Some of the smaller commercially available SMC – the CIM® Disk Monolithic Columns from BIA Separations d. o. o., Ljubljana, Slovenia

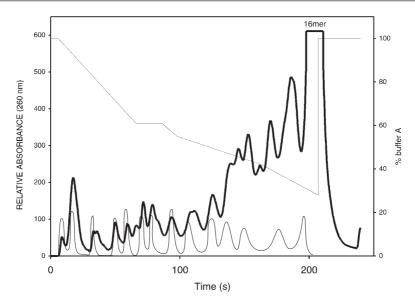


Fig. 7. Semi-Preparative Anion Exchange Purification of a 16-mer Oligodeoxynucleotide on a CIM[®] DEAE Disk Monolithic Column. Conditions: Column: 0.34 ml CIM[®] DEAE Disk (3×12 mm ID); Instrumentation: Gradient HPLC system with extra low dead volume mixing chamber; Sample: 16mer oligodeoxynucleotide from the reaction mixture – bold line, standards of 1, 2, 3, 4, 5, 6, 7, 9, 10, 11, 12, 14, 15, 16mer – thin line; Injection Volume: 20 µL; Mobile Phase: Buffer A: 20 mM Tris-HCl, pH 8.5; Buffer B: Buffer A+ 1 M NaCl; Gradient: as shown in the Figure; Flow Rate: 4 ml/min; Detection: UV at 260 nm

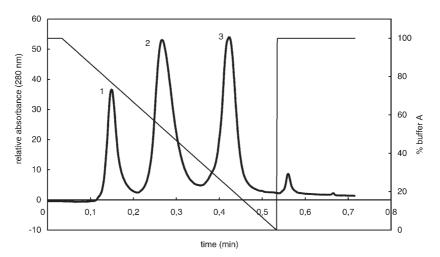


Fig. 8. Fast semi-industrial scale separation of a protein mixture using an 80 ml CIM® DEAE Tubular Monolithic Column. Conditions: Column: 80 ml CIM® DEAE Tubular Monolithic Column; Mobile phase: Buffer A: 20 mM Tris-HCl buffer, pH 7.4; Buffer B: 20 mM Tris-HCl buffer +1 M NaCl, pH 7.4; Gradient: 0–100% Buffer B in 30 s; Sample: 2 mg/ml of myoglobin (peak 1), 6 mg/ml of conalbumin (peak 2) and 8 mg/ml of soybean trypsin inhibitor (peak 3) dissolved in buffer A; Flow Rate: 400 ml/min; Injection volume: 1 ml; Detection: UV at 280 nm

monolithic structure and ligand density. The most important characteristics of SMC when compared to the conventional particle based materials are:

- flow independent resolution and binding capacity [48]
- capability of achieving very fast separations with good resolution both at small and large scale [47, 54, 66] as presented in Fig. 7 and Fig. 8 respectively
- simple handling, eliminating time-consuming column packing and repacking
- reduced biomolecule inactivation due to short contact times with the chromatographic matrix
- high binding capacity for very large biomolecules (see Sect. 5.1.2)
- air bubbles are not entrapped in the monolith, they are just washed out with the mobile phase
- concomitantly, the target molecule can be eluted in a very concentrated form by pushing a small volume of the elution buffer by the means of compressed air through the monolith
- multidimensional, so-called Conjoint Liquid Chromatography (CLC), see below.

Besides fast separation and purification of different biomolecules, the SMC technology allows the combination of different chromatographic modes in a single run by inserting disks of different chemistries in the same housing. This operational mode has been named Conjoint Liquid Chromatography (CLC) and enables, by proper buffer selection, single-step separations using different chromatographic modes with a very low dead volume. An example of CLC is demonstrated in Fig. 9 where the separation of proteins and the isolation of monoclonal antibody IgG from mouse ascites in one step was obtained within 2 minutes. In this case, the chromatographic column has been constructed by stacking one CIM QA ion exchange and one CIM Protein A affinity disk in the CIM holder (all from BIA Separations) and connecting the resulting column to the HPLC system in such a way that the mobile phase first passes the QA disk and afterwards the Protein A disk. At first this column was equilibrated with a buffer (20 mM Tris-HCl, pH 7.4), which allows binding in both the anion exchange and the affinity mode. After the injection of the sample most of the proteins were bound to the QA disk while the IgG's were bound by the Protein A disk. Afterwards the column was eluted using a linear gradient of strong salt (20 mM Tris-HCl, 1 M NaCl; pH 7.4). By this procedure the proteins were separated on the anion exchange disk and finally the IgG's were eluted from the Protein A disk by lowering the pH using a third buffer (0.1 M acetic acid). Similarly, the separation of proteins from human plasma and the concomitant isolation of IgG antibody molecules using a combination of CIM DEAE (weak anion exchanger) and CIM Protein G (affinity) disks has been described [48]. By using a series of four different SMC disks, each carrying different affinity ligand, the fractionation of the mixture of four different antibodies in a single step directly from biological mixture was possible [67].

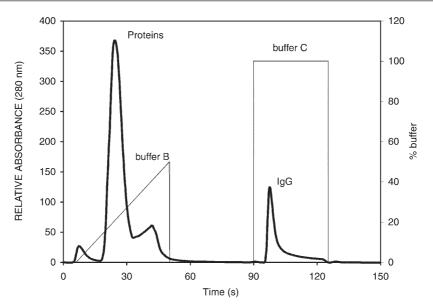


Fig. 9. Conjoint Liquid Chromatography (CLC). Separation of proteins from mouse ascites and isolation of monoclonal antibody IgG in one step obtained by a combination of CIM[®] QA and CIM[®] Protein A Disks. Conditions: Separation mode: CLC (first disk CIM[®] QA, 12×3 mm ID, 0.34 ml; second disk – CIM[®] Protein A, 12×3 mm ID, 0.34 ml, inserted in monolithic column housing); Instrumentation: Gradient HPLC system with extra low dead volume mixing chamber; Sample: Mouse ascites; Injection volume: 20 µL; Mobile Phase: Buffer A: 20 mM Tris-HCl, pH 7.4; Buffer B: Buffer A + 1 M NaCl; Buffer C: 0.1 M Acetic acid; Conditions: Gradient: 0–50% B in 50 s, 100% A for 40 s, 100% C for 30 s; Flow Rate: 4 ml/min; Detection: UV at 280 nm

4.2 Application of SMC for Liquid Chromatography of Biomolecules

The purpose of this section is twofold. The first is to give the reader an overview of the various applications which have already been developed using SMC. Several detailed reviews have already been published in this context [43, 68, 69, 70] and we will just summarize these works in Table 2. For more detailed information, the reader is invited to consult the original literature or in the corresponding review articles. The second aim of this section is to give a more detail description of the production process in order to discuss the steps needed to produce a biomolecule of sufficient quality for therapeutic use. Plasmid DNA (pDNA) has been selected for this purpose because gene therapy is a very promising concept in the treatment of many diseases and has generated a lot of interest in the development of efficient and reliable processes for its active principle. In addition, the size of pDNA is ideal for demonstrating the potential of SMC for the purification of large biomolecules.

Target molecule	Application	Mode	Reference
Proteins	Theoretical aspects	Ion Exchange, Hydrophobic Interaction, Reversed-Phase	[28]
Proteins	Theoretical aspects	Hydrophobic Interaction	[34]
Rat serum and plasma membrane proteins	Design of the monolith cartridge, purification, comparison with conventional columns	Anion Exchange, Affinity (Heparin)	[45]
Factor VIII from human plasma	In-process control, semi- preparative purification	Anion Exchange disks and tubes	[46]
Mixture of proteins (Myoglo- bin, Ovalbumin, Lysozyme and Chymotrypsinogen)	Purification	Hydrophobic Interaction	[52]
α ₁ -antitrypsin from human plasma, clotting Factor IX, from human plasma	In-process control	Ion Exchange disks	[54]
Proteins	Theoretical aspects – SMC theory	Ion Exchange	[55]
Proteins, Peptides, Oligonucleotides	Separation, Purification	Reversed Phase disks	[66]
Fractionation of the mixture of four different antibodies in a single step directly from biological mixture	Purification	CLC using four different affinity disks (different immunogens)	[67]
Antithrombin III and Fac- tor IX from human plasma	In-process control	Affinity (Heparin)	[71]
Recombinant human Tumor Necrosis Factor	Purification, comparison with conventional columns	Ion Exchange disks, Hydro- phobic Interaction disks	[72]
Annexins from liver plasma membranes, monospecific polyclonal antibodies	Purification, comparison with cellulose fiber modules	Anion Exchange disks, Affinity (Protein A, Protein G), Affinity (annexins)	[73]
Recombinant Protein G from cell lysates of <i>E. coli</i>	Semi-preparative puri- fication	Affinity (human immunoglobulin G) disks	[74]
Effect of porous structure of the SMC on resolution in chromatography of proteins	Theoretical aspects	Anion Exchange disks	[75]
Glucose oxidase, glyco- proteins from plasma membranes of rat liver	Analytical and semi pre- parative separations	Lectin Affinity (Concanavalin A) disks	[76]
IgG and other proteins from mouse ascites fluid	Purification	CLC (combination of Anion Exchange and Affinity (Protein A) disks	[76]
IgG from precipitated blood fraction and crude blood se- rum of immunized animals	Analytical and semi pre- parative separation, compa- rison with ELISA method	Affinity (different peptides, e.g. Bradykinin) disks	[77]

Table 2. Application of SMC for the separation of biomolecules

Target molecule	Application	Mode	Reference
Oligonucleotides	Analytical and semi preparative separations, Theoretical aspects	Anion Exchange disks	[78]
Oligonucleotides, Peptides, Steroids	Analytical separations	Ion Exchange, Reversed Phase disks	[79]
Lignin peroxidases enzymes	In-process control, semi- preparative purification, comparison with conven- tional columns	Anion Exchange disks	[80]
Polyclonal bovine IgG, recombinant human antibody (type IgG-κ)	Separation, comparison of the properties of Protein A, Protein G and Protein L SMC (CIM®) disks	Affinity (Protein A, Protein G, Protein L) disks	[81]
GTP gamma S binding proteins from membranes of porcine brain	Purification, comparison with conventional column	Anion Exchange and Affinity (Melittin) disks	[82]
Peak broadening in protein chromatography	Theoretical aspects	Anion Exchange disks	[83]
Factor IX from human plasma	In-process control, Purification	Anion Exchange disks and tubes	[84]
Model system for testing utilization of immobilized affinity peptides – SMC (CIM®) optimally present small affinity ligands	Ligand utilization, com- parison with agarose, cellulose and synthetic particle based polymers	Affinity (peptides) disks	[85]
Factor VIII from human plasma	Purification, comparison with porous particle materials	Affinity (peptides) disks	[86]
Various model proteins expressed in yeast	Purification	Affinity (monoclonal antibodies) disks	[87]
Control method for integrity of monolithic beds	Theoretical aspects, quality control	All phases	[88]
Viruses, pDNA	Purification, Concentration	Anion Exchange disks	[89]
Cell-bound Xylanases from Butxrivibrio sp. Mz5	Isolation	Anion Exchange tube	[90]
7.2 kb pDNA (supercoiled, nicked and open circular)	Analytical and semi-prepara- tive separation, comparison with conventional column and soft monolithic column	Anion Exchange disks	[91]
Model proteins	Comparison of the separa- tion on SMC (CIM®), UNO, Mono Q and Sartobind columns	Anion Exchange disks	[92]
Bovine Serum Albumin (BSA)	Theoretical study of dynamic binding capacity under different conditions	Anion Exchange disks	[93]

Table 2 (continued)

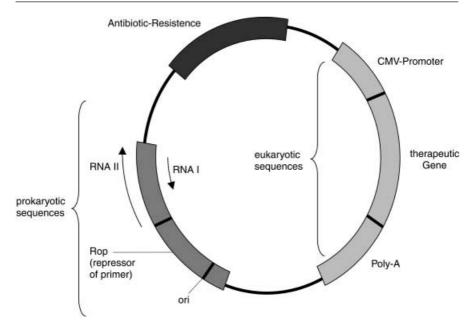


Fig. 10. Typical building blocks of a therapeutic plasmid

4.2.1 Plasmid DNA Purification

DNA plasmid-based treatment ("gene therapy") is considered an alternative to the one based on classical chemical drugs or proteins recovered from recombinant cells. Treatment of acquired and inherent genetic diseases as well as the use of DNA for the purpose of vaccination are potential applications of plasmid DNA (pDNA). The plasmid carries information that allows protein expression in the targeted human cells as well as eukaryotic regulatory elements and specific prokaryotic sequences that control replication in the host cell, see Fig. 10. Formulation is required for ex- or in-vivo administration. Selected systems for gene expression can be viral or non-viral.

Due to the increasing amounts of pDNA required for preclinical and clinical trials, production of pDNA needs to be performed on a large scale. These production processes must fulfill FDA regulatory requirements and be economical feasible. A typical production process is schematically presented in Fig. 11.

Plasmids are produced in prokaryotic cells (*E. coli* currently being the most popular host organism) with coding regions for proper replication in the bacteria. A high copy number per cell and stable maintenance during the fermentation is crucial for a robust process with high yield. Fermentation is performed in batch or fed-batch mode. Since fed batch processes reach higher cell densities (OD >100) they are considered as superior for large-scale production. After fermentation the cell broth is harvested by centrifugation, aliquoted, and frozen. For downstream processing of pDNA cells are thawed and broken up ("lysis"). A combination of column chromatography and/or precipitation steps is utilized for

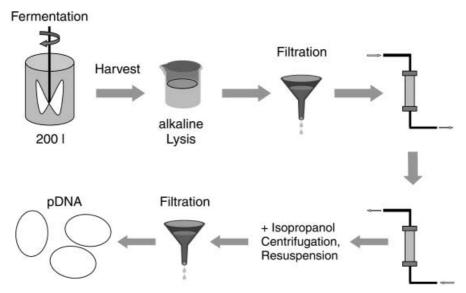


Fig.11. Flow chart of a typical pDNA production process. After fermentation cells are harvested and lysed by addition of alkaline solution. Clarification by filtration is followed by a series of chromatographic steps. After a final 0.22 μ m filtration step the purified plasmid is aliquoted and stored

the purification of the pDNA. After a final sterile filtration, the pDNA bulk is aliquoted and stored under proper conditions.

Manufacturing of pDNA is different from manufacturing of recombinant proteins since the two macromolecules differ significantly in their physico-chemical properties. Plasmids are negatively charged over a wide pH range. They are large molecules and have a long, thin shape. A typical plasmid contains between 5 and 20 kilo base pairs (kb) which corresponds to a mass between 3×10^6 and 13×10^6 Da and several thousand Å in length. The molecule is hence very sensitive to mechanical stress. There are several different forms of pDNA. The supercoiled or covalently closed circular (ccc) form is the most stable. The degree of supercoiling is dependent on the environmental conditions, such as the temperature and the pH. The open circular (oc) or nicked form is produced by breaking a single strand. Breakage of both strands can be caused by chemical and physical stress and produces the linear form. The final product obtained from the production process should contain more than 90% ccc pDNA. Critical points in large-scale production of pDNA are cell lysis, subsequent clarification, chromatography and final filtration.

Plasmid DNA, due to its size and shape, is very sensitive to shear forces. Therefore, lysis of the cells cannot be performed using high-pressure techniques such as homogenization. Chemical and enzymatic (Lysozyme) methods, on the other hand, cause minimal mechanical stress and minimal irreversible changes of the plasmid. During cell lysis under alkaline conditions, cells are subjected to NaOH and SDS. Subsequent neutralization to pH 5.5 causes flocculation of cell debris, proteins, and genomic DNA (gDNA). Very often RNAse is added to digest RNA into small pieces to prevent this molecule from interfering during the downstream process. Today, RNAse is used in large-scale production and is considered as a rate-limiting step. After addition of NaOH and SDS, the solution becomes highly viscous. Mixing without destroying the plasmid is difficult. Usually glass bottles containing the viscous solution are mixed very gently by hand. Some processes use optimized tanks and stirrers or a combination of different mixers in order to overcome these problems. Addition of enzymes obtained from animal sources is considered as critical and will be restricted by regulations. Lysozyme for cell lysis or RNAse for reduction of RNA should hence be avoided if possible. Cell debris and other particles need to be separated by filtration or centrifugation. Due to the high viscosity of the mixture, conventional dead-end filters clog very fast and clarification success is rather poor.

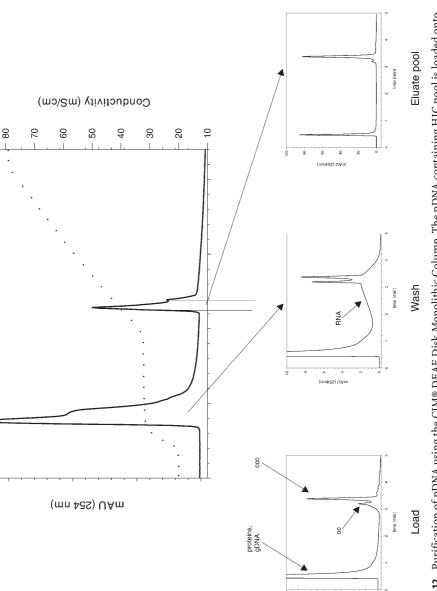
Conventional chromatographic resins are designed for binding and elution of proteins or small chemical substances. Chromatographic beads exhibit only a small percentage of their functional ligands on the outer surface. The majority are located within the pores. Molecules need to diffuse into these pores in order to interact with these ligands. Do to their size, plasmids have only restricted access to the ligands located within the pores. Therefore, binding of pDNA is limited to the surface of the beads and this is why conventional chromatographic supports have very low binding capacity for pDNA, usually less than 0.5 mg pDNA/ml packed chromatographic resin.

Plasmid DNA separation using SMC was first described by Giovannini et al [91]. In this pioneering work, the authors reported that, by using optimized conditions, a pDNA can be separated on an SMC into 3 peaks which presumably correspond to supercoiled, nicked and open circular pDNA. Separations under gradient and isocratic conditions were studied and it was shown that, in contrast to protein chromatography, different forms of pDNA could be separated on a strong anion exchange unit by using isocratic conditions. The experiments on SMC were compared to similar experiments performed using a conventional column packed with 10 µm porous particles and a monolithic column based on soft gel (UNO, Bio-Rad). The results demonstrated the superior performance of the SMC with regard to speed and capacity. The potential of SMC for the separation of nanoparticles (pDNA and measles virus) has also been investigated by Branovic et al. [89]. The authors have successfully separated pDNA from cellular RNA. Further, they were able to use the DEAE disk for even larger "molecules", i.e. for the purification and concentration of measles virus.

We have recently started to use the SMC for the purification of pDNA for therapeutic use. For this purpose we have selected the SMC (CIM) produced by BIA Separations. In comparison to conventional chromatographic resins, the CIM showed several advantages for the isolation of pDNA. The CIM stationary phase is highly porous and exhibits most of its ligands on the surface of the flow through pores. With a size of around 1500 nm the pores are large enough to be accessed by the plasmids. Consequently, the CIM media have an extremely high capacity for pDNA (>10 mg pDNA/ml in the case of the weak anion exchange DEAE-disk) and can be operated under high flow rates with low backpressures.

Resin	Yield ^a	Recovery ^a	Capacity ^b µg pDNA/ml	gDNA ^c ng/µg Plasmid	RNA ^d ng/µg Plasmid	LAL ^e EU/ml	CCC ^a purity	OD 260/280
Toyopearl 650 M DEAE column (35×10 mm ID)	78%	100%	240	n.d.	43	<0,3	94%	2,09
CIM® DEAE disk (3×12 mm ID)	75%	100%	2900	80	28	0,3-3	93%	1,94
Sepharose DEAE column (50×10 mm ID)	77%	93%	270	0.25	44	0,3-3	92%	2,01
Qiagen column (30×10 mm ID)	8%	55%	34	32	217	<0,3	83%	1,85
 ^a HPLC method using TosoHaas DANN NPR column (7.5 cm × 4.6 mm). ^b binding capacity was determined by overloading the columns and quantification of the eluted material. ^c fluorescence measurement with Picogreen (Novagene); Range: 1 ng-1 µg dsDNA/ml. ^d fluorescence measurement with Ribogreen (Novagene); Range: 25 pg-1 µg RNA/ml. ^e according to the current USP; Sensitivity: 0.03 EU/ml. 	soHaas DA termined ant with Pi ent with R USP; Sen	oHaas DANN NPR column (ermined by overloading the nt with Picogreen (Novagene nt with Ribogreen (Novagen USP: Sensitivity: 0.03 EU/ml	nn (7.5 cm × 4.6 mn (the columns and q gene); Range: 1 ng- agene); Range: 25 p; J/ml.	n). uantification of the ε 1 μg dsDNA/ml. g-1 μg RNA/ml.	luted material.			

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For the isolation of pDNA from a crude cell lysate obtained by alkaline lysis, different strategies can be applied. Impurities such as RNA and gDNA having similar properties to pDNA are difficult to remove by chromatography, in addition, the binding capacity of a given stationary phase for pDNA can be reduced significantly by competition from RNA and gDNA. A combination of different chromatographic principles is necessary for obtaining a highly purified plasmid, which meets all specifications. In our experiments, we were using different weak anion exchange columns as an intermediate step, while the capture step was performed by hydrophobic interaction column. The pDNA containing pool from the capture step was loaded onto the different columns and bound material was eluted by increasing the salt gradient. Results are summarized in the Table 3. Figure 12 shows the UV profile from the washing and elution steps using CIM DEAE supports.

As it can be seen from the figure during the washing step mainly the impurities (RNA and proteins) were eluted. The main peak contains the highly purified pDNA (ccc >95%). By adding a final polishing step, in this case size exclusion chromatography, high quality pDNA can be obtained without using enzymes, organic solvents or detergents. In comparison to other chromatographic material, CIM DEAE disks have a much higher binding capacity (see Table 3). Thus, efficient separation of gDNA, RNA and proteins from pDNA is possible. In addition, the separation of oc from ccc pDNA can be achieved on a preparative scale. Due to the properties of the CIM monolithic units, high flow rates at low backpressures are possible. Therefore, short process and cycle time enable high productivity. Losses due to product instability are minimized by the fast processing of the raw solutions and the CIM's resistance against chemical and thermal stress enables sanitation under harsh conditions.

5 Other Applications of SMC

5.1

Use of SMC as Biosensors and for Fast Bioconversion

In 1991, Abou-Rebyeh et al. [44] were the first to carry out a conversion of a substrate in flow-through enzyme reactor consisting of a polyglycidylmethacrylate*co*-ethyleneglycoldimethacrylate disk to which a suitable enzyme (carbonic anhydrase) had been immobilized. Immobilization of the enzyme provided an opportunity to carry out kinetic experiments under dynamic conditions. The authors have shown that a higher flow-rate led to an increase in enzymatic activity. According to them the mass transfer in monolithic supports is much faster and no longer a limiting factor for enzyme-substrate interaction.

In addition, it has been shown that other enzymes such as trypsin can be successfully immobilized and used for the conversion of substrates with higher molecular masses [76]. Petro et al. [94] compared the activity of trypsin immobilized on macroporous beads and on monolithic supports. They were able to show that the catalytic activity of trypsin bound to a monolith was much higher and resulted in a much higher throughput. Other enzymes such as invertase [76] and

glucose oxidase [76, 95] have also been immobilized on SMC. Monolithic disks with immobilized glucose oxidase used as enzyme reactors gave linear and reproducible results with short response times of several minutes, which is comparable to the response time of the commercially available packed-bed enzyme reactors. Hagedorn et al. [96] have investigated the potential of SMC with immobilized antibodies to be used for fast analysis of Protein G from cell lysate of recombinant E. coli using flow injection analysis (FIA). According to these authors, a reliable analysis can be performed at a much higher flow rate (shorter analysis time) than with conventional cartridges. Another application of SMB was described by Platonova et al. [97] successfully using polynucleotide phosphorylase immobilized on polyglycidylmethacrylate-co-ethyleneglycoldimethacrylate disk placed in a flow type bioreactor to synthesize polyriboadenylate from ADP and to carry out its reverse phosphorolysis. Very recently, Lim et al. [98] used elastase immobilized on monolithic disks for a rapid preparative cleavage of Inter- α -inhibitor complex proteins. They have shown that such units are stable and still active after repeated runs and that by varying the flow rate one can achieve partial or complete digestion. All these works indicate the potential of SMC for bioconversion purposes.

5.2 SMC for Solid Phase Extraction or Fast Sample Clean Up

When using SMC under isocratic conditions one cannot expect good resolution power due to the very short chromatographic layer and the low number of interactions (theoretical plates) of the molecules with the stationary phase's active sites. Still, such beds can be used for solid phase extraction and partial purification purposes. Strancar et al. [99] have demonstrated that monolithic disks can be used for on-column solid phase extraction (SPE) of Triton X-100 from human plasma before applying it to the analytical HPLC column by means of a column switching technique. Luksa et al. [100] have successfully used reversed phase disks for the fast clean up of drug samples before application to an MS/MS instrument. The same idea was used by LeThanh and Lendl [101], who used CIM QA disk for solid phase extraction and sample enrichment together with a sequential injection FTIR instrument for the rapid analysis of organic acid samples. However, a lot still needs to be done especially in the area of miniaturization and optimization of proper disk cartridges to satisfy the prerequisite of narrow peaks necessary for accurate MS studies.

5.3 Use of SMC for Solid Phase Synthesis

Other fields using short monolithic units are solid phase synthesis and combinatorial chemistry. Hird et al. [102] predicted that having a single polymer particulate block of porous polymer or monolith would allow the optimization of automation in the field of solid phase (combinatorial) synthesis. They have developed a method for the preparation of monolithic rods, which were then cut into discs of 1.0 to 2.5 mm thickness and used for solid phase synthesis. They have demonstrated that disks of an individual mass of up to 0.25 g were capable of yielding up to 0.5 mmole of a single compound in a solid phase synthesis. Korol'kov et al. [103] have demonstrated that SMC in the shape of disks can be used for conventional solid phase synthesis of bradykinin. Later Pflegerl et al. [104] were using 12 mm wide and 3 mm thick methacrylate ethylenediamine activated CIM monoliths corresponding to a volume of 0.34 ml with a ligand substitution of 1.7 mmol per ml CIM for a directed synthesis of peptides against human blood coagulation factor VIII. The disks were mounted in a cartridge designed for chromatography and were washed by injection of N,N-dimethylformamide prior to synthesis. Peptide synthesis was performed by injecting 0.3 M solutions of amino acid pentafluorophenyl esters with N-terminal Fmoc-protection in N-methylpyrrolidone. Side chain protection groups were trityl- for Cys, tert-butylfor Glu, Ser, Thr, and Tyr, and butoxycarbonyl- for Lys. Two consecutive 0.4 ml volumes of the respective amino acid ester solution were injected into the CIM monolithic column and allowed to react with the free amino group for 15 min each. After three consecutive DMF washes the Fmoc-group was cleaved with 20% piperidine in DMF, leaving a free amino group for reaction with the next Fmocprotected amino acid ester. Side-chain deprotection was performed with 90% trifluoroacetic acid in dichloromethane with 1% phenol, 3% triisobutylsilane, and 2% deionized water. After intensive washing with DMF and methanol, the disks were transferred to the aqueous running buffer system for chromatography or stored in 20% methanol at 4 °C. The authors reported that first of all the amino acids sequence was correct as demonstrated by a corresponding analysis, indicating that the solid phase synthesis could be carried out on such a support, that furthermore the synthesis could be performed without additives for swelling of the support, and that finally a pure FVIII-vWF molecule was able to be recovered, indicating that the directly synthesized peptide can function as an affinity chromatography ligand.

With the development of appropriate cartridges and proper chemistry that would allow the synthesis of the molecule on the disk and than the use of the same disk directly for affinity chromatography by placing it in a chromatographic cartridge, it is reasonable to expect that SMC will become a very efficient tool in the solid phase synthesis of peptides, oligonucleotides, and similar molecules.

6 Conclusions and Perspectives

Over the last decade many, attempts have been made to develop an optimal chromatographic support for the separation of (large) biomolecules taking into account their relatively unstable nature and the complicated samples they derive from. It has become clear that only supports which allow very fast separation processes using low back pressure offer good separation power, capacity and stability during the sanitation processes can satisfy the requests of the modern biotech industry. The experiments carried out so far clearly demonstrate that monolithic supports have the potential to satisfy the most stringent demands related to the separation and purification processes, especially when they are prepared as Short Monolithic Columns (combining the advantages of chromatographic columns with regard to separation power and those of membrane technology with regard to the speed of separation process). This is especially true when very large molecules or nanoparticles need to be analyzed or purified.

Still, a lot needs to be done to develop large monolithic units, which would handle kilogram and larger production scales and guarantee to the process managers the stability of the support and its presence on the market over several decades. However it is realistic to expect that SMC will attract widespread use within a decade in a variety of applications, from chromatography to bioconversions, solid phase extractions and solid phase synthesis.

7

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Porous Polymer Monoliths: An Alternative to Classical Beads

Shaofeng Xie¹ · Robert W. Allington¹ · Jean M.J. Fréchet² · Frantisek Svec²

¹ ISCO Inc., 4700 Superior Street, Lincoln, NE 608504-1328, USA. E-mail: shaofengx@isco.com

² Department of Chemistry, University of California, Berkeley, CA 94720 – 1460, USA.

E-mail: svec@uclink4.berkeley.edu

Porous polymer monoliths are a new category of materials developed during the last decade. These materials are prepared using a simple molding process carried out within the confines of a closed mold. Polymerization of a mixture that typically contains monomers, free-radical initiator, and porogenic solvent affords macroporous materials with large through-pores that enable flow-through applications. The versatility of the preparation technique is demonstrated by its use with hydrophobic, hydrophilic, ionizable, and zwitterionic monomers. The porous properties of the monolith can be controlled over a broad range. These, in turn, determine the hydrodynamic properties of the devices that contain the molded media. Since all the mobile phase must flow through the monolith, the mass transport within the molded material is dominated very much by convection, and the monolithic devices perform well even at very high flow rates. The applications of monolithic materials are demonstrated on the chromatographic separation of biological compounds and synthetic polymers, electrochromatography, gas chromatography, enzyme immobilization, molecular recognition, and in advanced detection systems. Grafting of the pore walls with selected polymers leads to materials with completely changed surface chemistries.

Keywords: MonolithPorous polymer, Separation, HPLC, Capillary electrochromatography, Enzyme immobilization, Modification

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1 Introduction

Particulate sorbents are available almost exclusively in the shape of micrometersized beads. These beads are packed in columns and represent currently the most common stationary phases for high-performance liquid chromatography (HPLC). Despite their immense popularity, slow diffusional mass transfer of macromolecular solutes into the stagnant pool of the mobile phase present in the pores of the separation medium and the large void volume between the packed particles are considered to be major problems in the HPLC of macromolecules, frequently impairing their rapid and efficient separation [1].

The attention of chromatographers has hitherto focused mainly on the first problem. For example, considerably improved mass transfer properties were observed for the perfused beads that have been introduced in the early 1990s [2, 3]. These beads have some pores that are large enough to allow a small portion of the mobile phase to flow through them. Separation media consisting of a rigid porous silica matrix with pores filled with a soft hydrogel also exhibit very good mass transfer characteristics [4, 5]. The use of non-porous beads made from both silica [6, 7] and synthetic polymers [8, 9] is the ultimate solution to the problem of diffusion into pores of a separation medium. Non-porous media are becoming very popular for the separation of proteins, oligonucleotides, and DNA fragments. However, only relatively short column lengths can be used to avoid unreasonably high backpressures in the columns packed with very small $2-5-\mu m$ non-porous beads.

The lowest theoretical interparticular volume of perfectly packed uniformly sized spherical beads is calculated to be about 26% of the total available volume. In practice, even the best packed columns still contain about 30-40% void volume in addition to the internal porosity of the beads. The problem of interparticular volume does not exist in systems in which a membrane is used as the separation medium. Both theoretical calculations and experimental results clearly document that membrane systems can be operated in a "dead-end" filtration

mode, at much higher flow rates than packed beds because all substrate solution flows through the support and the mass transfer is much faster as a result of this convective flow. This is particularly true for separations in which macromolecular analytes are involved. However, membranes tend to have a lower binding capacity per unit volume than particles. Therefore, an extremely large membrane unit would be required to achieve a capacity equivalent to that of a packed column [10, 11]. Therefore, a reasonable compromise between the membranes with their fast mass transfer and the beads with their high capacity had to be found. Early on, stacked thin membranes based on modified cellulose [12-14], cellulose acetate [15], spun poly(ether-urethane-urea), or Nylon [16] were used. Similarly, porous sheets in which beads of a separation medium are embedded into a web of polymer such as poly(vinyl chloride) [17] or poly(tetrafluoroethylene) [18], and then placed in a cartridge were used to simulate the function of a column with almost no voids. Rolled cellulose sheets [19] and woven matrices [20, 21] placed in the tube of a chromatographic column are other examples of separation media that exhibit almost no interstitial porosity. A number of these approaches have been described in detail in an excellent review by Roper and Lightfoot [22].

The separation in a medium that is essentially a single particle and does not contain interparticular voids, which normally contribute to peak broadening, has been treated theoretically [23, 24] but experimental work remained scarce as a result of the lack of suitable materials. The first attempt to make "single-piece" separation media dates back to the late 1960s and early 1970s. For example, highly swollen monolithic polymer gels were prepared by free-radical polymerization of an aqueous solution of 2-hydroxyethyl methacrylate in the presence of 0.2% ethylene dimethacrylate (crosslinking monomer) [25]. The gel was prepared in a glass tube, and this column was used for size-exclusion chromato graphy. The authors claimed that the effectiveness of the separation was rather low as a result of a pronounced longitudinal diffusion resulting from the very slow flow rate of only 4 ml/h. In contrast, the permeability of the monolithic open-pore polyurethane foams used by other groups was excellent [26-29]. However, excessive swelling in some solvents and softness were deleterious characteristics that prevented their successful use in both liquid and gas chromatography.

Macroporous discs [30-32] and compressed soft polyacrylamide gels [33] placed in a cartridge or column also represent examples of media that exhibit no interstitial porosity. These elegant approaches have recently been described in detail in a series of excellent review articles [34-36] and are also dealt with elsewhere in this issue. In the early 1990s, yet another category of *rigid* macroporous monoliths formed by a very simple "molding" process in which a mixture of monomers and solvent is polymerized and immediately brought to reaction *within* a closed tube or other container under carefully controlled conditions has been developed [37]. Since porous inorganic materials are very popular supports widely used in catalysis and chromatography [1], monolithic columns prepared from silica were developed almost simultaneously with the ones based on the organic polymers [38, 39]. A detailed account of these materials has been published recently [40-42].

Since a comprehensive description of all monolithic materials would exceed the scope of this chapter and a number of other monolithic materials are also described elsewhere in this volume, this contribution will be restricted mainly to monoliths for chromatographic purposes and prepared by polymerization of monomer mixtures in non-aqueous solvents. Monolithic capillary columns for CEC are treated in another chapter and will not be presented in detail here.

2 Macroporous Polymers

Macroporous polymers emerged in the late 1950s as a result of the search for polymeric matrices suitable for the manufacture of ion-exchange resins with better osmotic shock resistance and faster kinetics. The history of these inventions has been reviewed recently [43]. In contrast to the polymers that require solvent swelling to become porous, macroporous polymers are characterized by a permanent porous structure formed during their preparation that persists even in the dry state. Their internal structure consists of numerous interconnected cavities (pores) of different sizes, and their structural rigidity is secured through extensive crosslinking. These polymers are typically produced as spherical beads by a suspension polymerization process that was invented in Germany in the early 1910s [44, 45]. To achieve the desired porosity, the polymerization mixture should contain both a crosslinking monomer and an inert agent, the porogen [46–49]. Solvating or non-solvating solvents for the polymer that is formed, but also other soluble non-crosslinked polymers, or even mixtures of such polymers and solvents can serve as porogens.

Macroporous polymers are finding numerous applications as both commodity and specialty materials. While the former category includes ion-exchangers and adsorbents, supports for solid phase synthesis, polymeric reagents, polymersupported catalysts, and chromatographic packings fit well into the latter [50]. Although the vast majority of current macroporous beads are based on styrene-divinylbenzene copolymers, other monomers including acrylates, methacrylates, vinylpyridines, vinylpyrrolidone, and vinyl acetate have also been utilized [50].

While the suspension polymerization that affords macroporous polymers has been analyzed in the literature in detail [46–49], little could be found until recently [37, 51, 52] on how to prepare macroporous polymers by bulk polymerization within a mold.

2.1

Preparation of Rigid Polymer Monoliths

The preparation of rigid macroporous organic polymers produced by a straightforward "molding" process is simple and straightforward (Fig. 1). The mold, typically a tube, is sealed at one end, filled with the polymerization mixture, and then sealed at the other end. The polymerization is then triggered, most often by heating in a bath at a temperature of 55-80 °C or by UV light. The seals are then removed, the tube is provided with fittings, attached to a pump, and a solvent is pumped through the monolith to remove the porogens and any other soluble

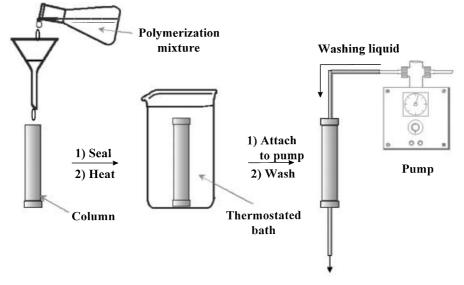
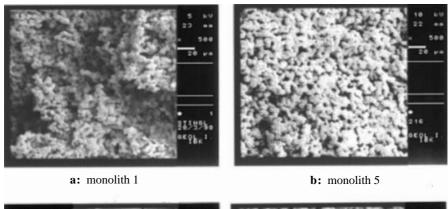


Fig. 1. Preparation of macroporous monolith by a "molding" process

compounds that remained in the polymer after the polymerization was completed. A broad variety of tube sizes and materials, such as stainless steel, poly(ether-ether-ketone) (PEEK), fused silica, and glass tubes, have been used as molds for the preparation of monoliths [53-56].

While the preparation of cylindrical monoliths with a homogeneous porous structure in capillaries and tubes up to a diameter of about 10-25 mm is readily achieved in a single polymerization step, larger size monoliths are somewhat more difficult to prepare. Dissipation of the heat of polymerization is frequently slow and the heat production may be sufficient to increase substantially the reaction temperature, accelerate the polymerization dramatically, and cause a rapid decomposition of the initiator. If this process is not controlled, monoliths with unpredictable radial and axial gradients of porosity are obtained. However, the slow and gradual addition of the polymerization mixture to the reaction vessel in which the polymerization proceeds minimizes the exotherm and allows the preparation of very large diameter monoliths with homogeneous porous structures [55]. An elegant method that helps to solve the problem of heat dissipation has been demonstrated recently [57]. Using analysis of the heat release during the polymerization, Podgornik at al. derived a mathematical model for the prediction of the maximum thickness of the monolith that can be prepared in a single step process without affecting the radial homogeneity of the material. To obtain large cylindrical objects, these authors prepared a few annular monoliths with various well-defined outer and inner diameters that inserted one into another to form a monolith with the desired large volume.

Sinner and Buchmeiser developed a less typical approach to monolithic columns [58, 59]. They used ring-opening metathesis copolymerization of norborn-2-ene and 1,4,4a,5,8,8a-hexahydro-1,4,5,8-exo,endo-dimethanonaphtha-



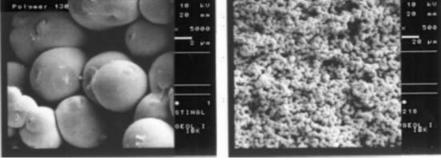






Fig.2a-d. Scanning electron micrographs of the inner part of the norborn-2-ene monolith prepared by ring-opening metathesis copolymerization (Reprinted with permission from [58]. Copyright 2000 American Chemical Society)

lene within borosilicate columns in the presence of porogenic solvents such as toluene, methylene chloride, methanol, and 2-propanol to obtain functionalized monolithic materials. A ruthenium catalyst was used to prepare monolithic separation media with a morphology shown in Fig. 2. By variation of the polymerization conditions such as the ratio of monomers, the porogenic solvents, and the temperature, the porous properties could be varied within a broad range of $2-30 \ \mu m$ affording materials with specific surface areas in the range of $60-210 \ m^2/g$.

2.2 Control of Porous Properties and Morphology

Many applications of porous materials such as for catalysis, adsorption, ion exchange, chromatography, solid phase synthesis, etc. rely on the intimate contact with a surface that supports the active sites. In order to obtain a large surface area, a large number of smaller pores should be incorporated into the polymer. The most substantial contributions to the overall surface area comes from micropores with diameters smaller than 2 nm, followed by the mesopores ranging from 2 to 50 nm. Large macropores make only an insignificant contribution to the overall surface area. However, these pores are essential to allow liquid to flow through the material at reasonably low pressure. This pressure, in turn, depends on the overall porous properties of the material. Therefore, the pore size distribution of the monolith should be adjusted properly to fit each type of application.

The pore size distributions of the molded monoliths are quite different from those observed for "classical" macroporous beads. An example of pore size distribution curves is shown in Fig. 3. An extensive study of the types of pores obtained during polymerization both in suspension and in an unstirred mold has revealed that, in contrast to common wisdom, there are some important differences between the suspension polymerization used for the preparation of beads and the bulk-like polymerization process utilized for the preparation of molded monoliths. In the case of polymerization in an unstirred mold the most important differences are the lack of interfacial tension between the aqueous and organic phases, and the absence of dynamic forces that are typical of stirred dispersions [60].

The porosity and flow characteristics of macroporous polymer monoliths intended for use as separation media for chromatography, flow-through reactors, catalysts, or supports for solid phase chemistry have to be adjusted during their preparation. Key variables such as temperature, composition of the pore-forming solvent mixture, and content of crosslinking monomer allow the tuning of the average pore size within a broad range spanning at least two orders of magnitude from tens to thousands of nanometers.

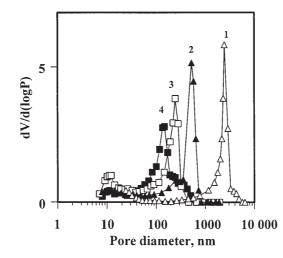


Fig.3. Effect of dodecanol in the porogenic solvent on the differential pore size distribution of molded poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) monoliths (Reprinted with permission from [62]. Copyright 1996 American Chemical Society). Conditions: polymerization time 24 h, temperature 70 °C, polymerization mixture: glycidyl methacrylate 24%, ethylene dimethacrylate 16%, cyclohexanol and dodecanol contents in mixtures 60/0 (curve 1), 57/3 (curve 2), 54/6 (curve 3), and 45/15 vol.% (4)

The polymerization temperature, through its effects on the kinetics of polymerization, is a particularly effective means of control, allowing the preparation of macroporous polymers with different pore size distributions from a single composition of the polymerization mixture. The effect of the temperature can be readily explained in terms of the nucleation rates, and the shift in pore size distribution induced by changes in the polymerization temperature can be accounted for by the difference in the *number of nuclei* that result from these changes [61, 62]. For example, while the sharp maximum of the pore size distribution profile for monoliths prepared at a temperature of 70 °C is close to 1000 nm, a very broad pore size distribution curve spanning from 10 to 1000 nm with no distinct maximum is typical for monolith prepared from the same mixture at 130 °C [63].

The choice of pore-forming solvent is another tool that may be used for the control of porous properties without changing the chemical composition of the final polymer. In general, larger pores are obtained in a poorer solvent due to an earlier onset of phase separation. The porogenic solvent controls the porous properties of the monolith through the *solvation of the polymer chains* in the reaction medium during the early stages of the polymerization [52, 62]. Supercritical carbon dioxide is the most recent contribution to the broad family of porogenic solvents [64]. This type of porogen is attractive since it is nontoxic, non-flammable, and inexpensive. In addition, the properties of this "solvent" can be tuned by varying the pressure. Once the polymerization is completed, the porogen is simply evaporated with no need for washing and no residual solvent traces in the monolith. Using ethylene dimethacrylate and trimethylolpropane trimethacrylate as monomers, a broad range of materials with typical macroporous structures and pore sizes in a range of 20–8000 nm were prepared.

In contrast to temperature, increasing the proportion of the crosslinking agent present in the monomer mixture affects not only the porous properties but also the chemical composition of the final monoliths. It decreases their average pore size as a result of an earlier formation of highly crosslinked globules with a reduced tendency to coalesce. This approach is useful for the preparation of monoliths with very large surface areas [52]. The experimental results imply that, in this case, the pore size distribution is controlled by limitations in *swelling of crosslinked nuclei* [62].

The morphology of the monoliths is closely related to their porous properties, and is also a direct consequence of the quality of the porogenic solvent as well as the percentage of crosslinking monomer and the ratio between the monomer and porogen phases. The presence of synergistic effects of these reaction conditions was verified using multivariate analysis [65].

In general, the morphology of macroporous materials is rather complex. The scanning electron micrograph shown earlier in Fig. 2 reveals the details of the globular internal structure of a molded monolith prepared by ring-opening metathesis copolymerization of norborn-2-ene [58]. Although this morphology featuring individual microglobules and their irregular clusters is similar to that found for beads [66], the size of both the clusters and the irregular voids between clusters are much larger.

2.3 Hydrodynamic Properties

For practical reasons, the pressure needed to drive the liquid through any system should be as low as possible. Because all of the mobile phase must flow through the monoliths, the first concern is their permeability to liquids, which depends fully on the size of their pores. A monolith with pores only of the size found in typical macroporous beads would be physically damaged by the extremely high pressures required for flow under such circumstances. Obviously, lower flow resistance can be achieved with materials that have a large number of broad channels. However, many applications also require a large surface area in order to achieve a high loading capacity. This high surface area is generally a characteristic of porous material that contains smaller pores. Therefore, a balance must be found between the requirements of low flow resistance and high surface area, and an ideal monolith should contain both large pores for convection and a connected network of shorter and smaller pores for high capacity [62].

Figure 4 shows the back pressure as a function of the flow rate. Typically, the pressure needed to sustain even a very modest flow rate is quite high for materials that have a mean pore diameter of less than about 500 nm, while high flow rates can be achieved at low pressures with materials that have pores larger than 1000 nm. Although the shape of the pores within the monoliths is very different from those of a tube, the Hagen-Poiseuille equation essentially holds also for the flow through the molded porous poly(glycidyl methacrylate-*co*-ethylene

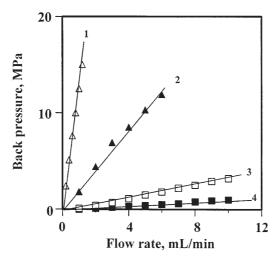


Fig. 4. Effect of the flow velocity on the back pressure in a molded poly(glycidyl methacrylate*co*-ethylene dimethacrylate) 100 mm × 8 mm monolithic column (Reprinted with permission from [62]. Copyright 1996 American Chemical Society). Conditions: mobile phase tetrahydrofuran; polymerization mixture: glycidyl methacrylate 24%, ethylene dimethacrylate 16%, cyclohexanol and dodecanol contents in mixtures 54/6%, temperature 80 °C (line 1), 54/6, 70 °C (line 2), 54/6, 55 °C (line 3), and 57/3, 55 °C (line 4)

dimethacrylate) and poly(styrene-*co*-divinylbenzene) monoliths because flow does not depend on the chemistry of the material [62].

2.4 Surface Chemistries

Obviously, the monolithic material may serve its purpose only if provided with a suitable surface chemistry, which depends on the desired application. For example, hydrophobic moieties are required for reversed phase chromatography, ionizable groups must be present for separation in the ion-exchange mode, and chiral functionalities are the prerequisite for enantioselective separations. Several methods can be used to prepare monolithic columns with a wide variety of surface chemistries.

2.4.1 Preparation from Functional Monomers

The number of monomers that may be used in the preparation of polymer monoliths is much larger than those used for classical suspension polymeriza-

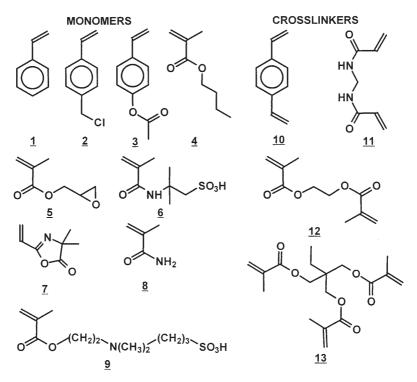


Fig. 5. Examples of monomers used for the preparation of porous monoliths

tion because there is only one phase in the mold. Therefore, almost any monomer, including water-soluble hydrophilic monomers, which are not suitable for standard polymerization in aqueous suspensions, may be used to form a monolith. This greatly increases the variety of surface chemistries that can be obtained directly. However, the polymerization conditions optimized for one system cannot be transferred immediately to another without further experimentation, and the use of new monomer mixtures always requires optimization of polymerization conditions in order to achieve sufficient permeability of the resulting monolith [67]. A few examples of monomers (1-9) and crosslinking agents (10-13) that have been used for the preparation of porous rigid monoliths are shown in Fig. 5. The list of monomers includes a broad variety of chemistries varying from very hydrophilic (acrylamide 8, 2-acrylamido-2-methyl-1-propanesulfonic acid 6) through reactive (glycidyl methacrylate 5, chloromethylstyrene 2, 2-vinyl-4,4-dimethylazlactone 7), to protected (4-acetoxystyrene 3), hydrophobic (styrene 1, butyl methacrylate 4) and even zwitterionic 9 functionalities, and chiral monomers [67-73].

2.4.2 Modification of Reactive Monoliths

Chemical modification is another route that increases the number of available chemistries, allowing the preparation of monoliths with functionalities for which monomer precursors are not readily available. These reactions are easily performed using monoliths prepared from monomers containing reactive group such as 2 and 5. For example, Fig. 6 shows the reaction of glycidyl methacrylate-based monolith with diethylamine, which leads to an useful ion-exchanger [37]. The reaction of poly(chloromethylstyrene-*co*-divinylbenzene) with ethylenediamine and then with γ -gluconolactone completely changes the surface polarity from hydrophobic to highly hydrophilic [74].

The living character of the ring opening metathesis polymerization described earlier in this review enables a simple preparation of functionalized norbornenebased monoliths. Adding one more in situ derivatization step that involves functional norborn-2-ene and 7-oxanorborn-2-ene monomers that react with the surface-bound initiator, the pores were provided with a number of typical functional groups such as carboxylic acid, tertiary amine, and cyclodextrin [58, 59].

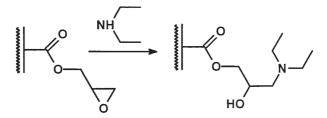


Fig. 6. Reaction of poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) monolith with diethylamine

2.4.3 Grafting

The preparation of functionalized monoliths by copolymerization of functional monovinyl and divinyl monomers requires optimization of the polymerization conditions for each new set of functional monomers and crosslinkers in order to obtain monoliths with the desired properties. Since the functional monomer constitutes both the bulk and the active surface of the monolith, a substantial percentage of the functional units remains buried within the highly crosslinked polymer matrix and is inaccessible for the desired interactions. A better utilization of a rare functional monomer might involve its graft polymerization within large pores of a "generic" monolith. Using the simple modification processes, only a single functionality is obtained from the reaction of each functional site of the surface. In contrast, the attachment of chains of reactive polymer to the reactive site at the surface of the pores would provide multiple functionalities emanating from each individual surface site, and thus dramatically increase the surface group density. Such materials, which possess higher binding capacities, are attractive for use in chromatography, ion exchange, and adsorption. Müller has demonstrated that the cerium(IV)-initiated grafting of polymer chains onto the internal surface of porous beads affords an excellent separation medium for biopolymers [75]. A similar reaction was used to graft poly(acrylamidomethylpropanesulfonic acid) 6 onto the internal surface of hydrolyzed poly(glycidyl methacrylate-co-ethylene dimethacrylate) monoliths [69].

Grafting can also provide the monolithic polymers with rather unexpected properties. For example, the two-step grafting procedure summarized in Fig. 7, which involves the vinylization of the pore surface by reaction of the epoxide moiety with allyl amine, and a subsequent in situ radical polymerization of *N*-isopropylacrylamide (NIPAAm) initiated by azobisisobutyronitrile within these pores leads to a composite that changes its properties in response to external temperature [76].

Living free-radical polymerization has recently attracted considerable attention since it enables the preparation of polymers with well-controlled composition and molecular architecture previously the exclusive domain of ionic polymerizations, using very robust conditions akin to those of a simple radical polymerization [77–86]. In one of the implementations, the grafting is achieved by employing the terminal nitroxide moieties of a monolith prepared in the presence of a stable free radical such as 2,2,5,5-tetramethyl-1-pyperidinyloxy (TEMPO). In this way, the monolith is prepared first and its dormant free-

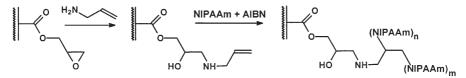


Fig.7. Grafting of a poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) monolith with *N*-isopropylacrylamide

radical ends can be used in a subsequent step involving growth of the functional polymer within the pores of the previously formed monolith. This makes the stable free-radical assisted two-step polymerization process a versatile tool, since it allows the preparation of functionalized porous materials with a large variety of surface chemistries that originate from only a single type of parent monolith.

TEMPO mediated crosslinking polymerization can also be used for the preparation of macroporous monoliths [63]. The latent TEMPO capped free radicals have a great potential for the preparation of a variety of macroporous materials with different chemistries and enhanced capacities using grafting. However, the polymerization conditions have to be modified to obtain monoliths with suitable porous properties. In general, this type of polymerization leads to products with a less permeable porous structure as a result of the rather high reaction temperature of 130 °C required to obtain high conversions using TEMPO as a stable free radical. In contrast, the use of "low" temperature mediators such as 2,2,5trimethyl-3-(1-phenylethoxy)-4-phenyl-3-azahexane [87] in the preparation of porous monoliths substantially simplifies the control of porous properties and polymers with a pore size of 50-1100 nm can be prepared [88]. Viklund et al. utilized yet different stable free radicals to prepare macroporous poly(styrene-co-divinylbenzene) monoliths. They used 3-carboxy-2,2,5,5-tetramethylpyrrolidinyl-1-oxy (carboxy-PROXYL) or 4-carboxy-2,2,6,6-tetramethylpiperidinyloxy (carboxy-TEMPO) as mediators and a binary porogenic solvent consisting of poly(ethylene glycol) and 1-decanol [89]. These polymerizations were found to be faster and led to higher degrees of monomer conversions in a shorter period of time compared to corresponding TEMPO mediated reactions. The use of mediators with carboxylic functionality simultaneously accelerated the reaction kinetics and improved the permeability of the prepared monoliths. Modification of the composition of porogenic mixture enabled control of the porous properties of the monolithic polymers over a wide range.

Grafting of these preformed monoliths with "dormant" radicals is achieved by filling the pores with a monomer solution and heating to the desired temperature to activate the capped radicals. For example, a functionalization of poly(styrene-divinylbenzene) monolith with chloromethylstyrene and vinylpyridine to obtain material with up to 3.6 mmol/g of functionalities has been demonstrated [88].

3 Application of Rigid Polymer Monoliths

Although the history of rigid monolithic polymers is relatively short, a number of applications have already been explored. These applications cover a rather broad range of fields from heterogeneous catalysis and solid-phase extraction, to polymer-supported chemistry and a variety of separation processes.

3.1 High Throughput Enzyme Reactors

Because the monoliths allow total convection of the mobile phase through their pores, the overall mass transfer is dramatically accelerated compared to conventional porous structures. Based on the morphology and porous properties of the molded monoliths, which allow fast flow of substrate solutions, it can be safely anticipated that they would also provide outstanding supports for immobilization of biocatalysts, thus extending the original concept of monolithic materials to the area of catalysis.

The immobilization of enzymes onto solid supports is beneficial because it allows for the repetitive use of the (expensive) biocatalysts, and also facilitates work-up and product isolation once an enzyme-mediated reaction has been carried out. However, a recurring problem is that the apparent activity of an immobilized enzyme is generally lower than that of its soluble counterpart. This is because the rate-determining step is the slow diffusion of the (large) substrate molecules to the active sites. With the highly porous monoliths, the faster mass transfer should thus translate into a higher activity. Comparative studies with trypsin immobilized onto both macroporous beads and fully permeated poly(glycidyl methacrylate-co-ethylene dimethacrylate) [90] and poly(2-vinyl-4,4-dimethylazlactone-co-methylene bisacrylamide) [71] monolithic supports revealed that the enzymatic activity of trypsin immobilized on the monoliths is always higher than that of the enzyme immobilized on beads even when small (11 µm) beads were used to minimize the effect of diffusion on the reaction rate (Fig. 8). The higher activity of the monoliths does not vary much even at high flow rates, and reaches up to 240 µmol/min when recalculated for 1 ml of the support. The backpressure in the molded poly(glycidyl methacrylate-co-ethylene

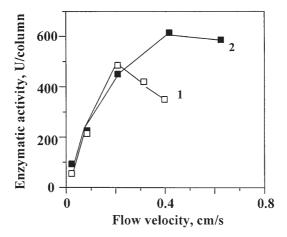


Fig. 8. Effect of linear flow velocity of an L-benzoyl arginine ethylester solution (0.2 mol/l) on the enzymatic activity of trypsin immobilized on poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) beads (curve 1) and monolith (curve 2) (Reprinted with permission from [90]. Copyright 1996 Wiley-VCH). Reactor 50 mm × 8 mm i.d., temperature 25 °C

Monolith	А	В	С	D
VAL/AA ^b Porogenic solvent	16/4 Tetradecanol	12/8 Tetradecanol	8/12 Tetradecanol	4/16 Decanol + oleyl alcohol 1 : 1
D _{p, med} , µm ^c V _p , ml/g ^d A, µmol/min/ml ^e	2.92 1.52 103	2.72 1.51 203	2.57 1.44 221	2.65 1.48 136

 Table 1. Porous properties and enzymatic activities of monolithic poly(2-vinyl-4,4-dimethyl-azlactone-co-acrylamide-co-ethylene dimethacrylate) reactors^a

^a Conditions: polymerization mixture: 20 wt% ethylene dimethacrylate, 20% vinyl azlactone+acrylamide, 60% porogenic solvent, and azobisisobutyronitrile (1% with respect to monomers), temperature 65 °C; polymerization time 24 h.

^b Percentage of vinyl azlactone (VAL) and acrylamide (AA) in polymerization mixture.

^c Median of the pore size distribution profile.

^d Total pore volume.

^e Activity of immobilized trypsin at a flow rate of 127 cm/min and a BAEE concentration of 5 mmol/l.

dimethacrylate) monolith reactor is a linear function of the flow velocity, and remains very low. In fact, the flow through this system is not limited by the hydrodynamics of the polymer monolith, but rather by the maximum flow rate of the pump used [90]. In contrast, the range of available flow rates for the packed column is limited by the rapid increase in backpressure, which must not exceed the upper limits of the equipment. The enzyme bound to the monolith thus not only has a higher activity, but a much higher throughput can also be achieved because of the efficient mass transfer even at high flow rates.

In contrast to glycidyl methacrylate-based matrices, 2-vinyl-4,4-dimethylazlactone/acrylamide supports are more hydrophilic and, therefore, more "enzyme friendly". Table 1 shows the effect of the percentage of vinylazlactone in the polymerization mixture on the overall activity of the immobilized enzyme. The highest activity of 221 μ mol/min per 1 ml of support is obtained with the support containing 20% of azlactone and 30% of acrylamide. Although this activity for the low molecular weight substrate is not higher than that of the enzyme immobilized on the glycidyl methacrylate-based monoliths, the vinylazlactone monoliths provide much simpler access to the conjugate because the attachment of the enzyme to the azlactone moieties of the monolith may be achieved in a single step [71].

The positive effect of convection of the substrate solution on mass transfer can be observed even better with macromolecular substrates that undergo processes such as protein digestion. For example, Fig. 9 compares reversed-phase chromatograms of cytochrome c digests obtained by cleavage with trypsin immobilized in both packed and molded column reactors, and clearly demonstrates the much higher activity of the monolithic device under otherwise similar circumstances [90].

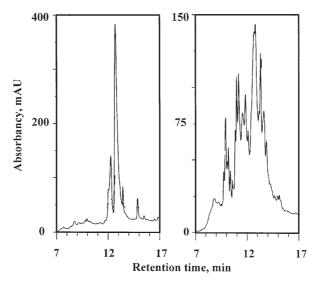


Fig. 9. Reversed-phase separations of cytochrome c digests obtained with trypsin-modified beads (*left*) and trypsin-modified monolithic reactor (*right*) in a tandem with a chromatographic column (Reprinted with permission from [90]. Copyright 1996 Wiley-VCH). Conditions: digestion: (*left curve*) trypsin-modified beads: reactor, 50 mm × 8 mm i.d., 0.2 mg of cytochrome c, digestion buffer, flow rate 0.2 ml/min, 25 °C, residence time, 15 min; (*right curve*) trypsin immobilized onto molded monolith: other conditions the same as with trypsin-modified beads. Reversed-phase chromatography column, Nova-Pak C18, 150 mm × 3.9 mm i.d., mobile phase gradient 0–70% acetonitrile in 0.1% aqueous trifluoroacetic acid in 15 min, flow rate, 1 ml/min, injection volume 20 µl, UV detection at 254 nm

3.2 Solid Phase Detection

Peroxyoxalate chemiluminescence is one of the most efficient methods for the direct detection of hydrogen peroxide [91]. This approach can be further extended to the indirect detection of some other compounds. The experimental setup consists typically of a reactor packed with a solid particulate support with a bound fluorophore such as 3-aminofluoranthene. Irgum used a bulk polymerization in a glass mold initiated by UV light for the preparation of his solid phase macroporous poly(glycidyl methacrylate-*co*-trimethylolpropane trimethacrylate) monolithic reactor [92]. The 3-aminofluoranthene immobilized onto the monolithic supports exhibited a light generation efficiency twice of that of reactors packed with modified 50-µm beads when evaluated in a flow system based on 1,1'-oxalyldiimidazolyl peroxyoxalate chemiluminescence detection of hydrogen peroxide. The results were correlated with the physical characteristics of the materials, and the efficiency was found to correlate with the amount of accessible reactive groups. As a result of "inner filtering" a lower functionalization density leads to an increase in the sensitivity for hydrogen peroxide in the flow system.

3.3 Solid Phase Extraction

Currently, sorption materials in bead shape are most frequently used in solidphase extraction (SPE) as a consequence of their wide commercial availability. The recovery of highly polar organic compounds from most of the typical C18 silica-based devices is less then ideal, although some newly developed silica adsorbents containing hydrophilic moieties enable significantly improved recoveries. Therefore polymer beads with an increased polarity have been developed for this purpose, for example, those based on poly(styrene-divinylbenzenevinylpyrrolidone) commercialized by Waters Corp. under the trade name Oasis. However, the inherent problem of all particulate separation media is their inability to fill the available space completely. This may be less critical for applications in column-like tubular formats, where the length of the packed bed partly compensates for the effect of the irregular interparticular voids. However, it is very difficult to avoid channeling between particles packed in a thin layer that has the low aspect ratio typical of applications such as disc SPE. This has led to the development of formats that include discs with embedded sorbent particles or HPLC-type beads tightly retained between two screens.

In contrast, monolithic materials are easily amenable to any format. This has been demonstrated by using short monolithic rods prepared by copolymerization of divinylbenzene and 2-hydroxyethyl methacrylate in the presence of specifically selected porogens [93]. Table 2 compares recoveries of substituted phenols from both the copolymer and poly(divinylbenzene) cartridges and clearly confirms the positive effect of the polar comonomer.

Compound	Recovery %		
	(DVB)	(HEMA-DVB)	
Phenol	58	92	
4-Nitrophenol	77	90	
2-Chlorophenol	82	97	
2-Nitropĥenol	88	96	
2,4-Dinitrophenol	76	91	
2,4-Dimethylphenol	85	95	
4-Chloro-3-methylphenol	88	99	
2,4-Dichlorophenol	79	97	
4,6-Dinitro-2-methylphenol	80	94	
2,4,6-Trichlorophenol	82	96	
Pentachlorophenol	91	97	
Average	80	95	

 Table 2. Recovery of phenols from porous poly(divinylbenzene) (DVB) and poly(2-hydroxylethyl methacrylate-co-divinylbenzene) (HEMA-DVB) monoliths [93]

3.4 Polymer Supports and Reagents

Although still very new to these applications that concern solution phase combinatorial chemistry, monolithic objects in various shapes are expected to offer new opportunities in this area [93-96]. Chemical reactivity and high capacity of accessible functionalities are the basic requirements for solid-phase chemistry. Obviously, the flow-through application of monolithic objects characterized by convective flow that considerably increases the mass transfer rate compared to diffusion through the pores of classical beads allows for decreasing the contact times and speeds up the procedures. Here again, grafting of functional monomers to the internal pore surface appears to be best suited for the preparation of monoliths with all of the functional groups exposed for interactions. For example, using a specifically designed reaction path, a monolith of poly-(chloromethylstyrene-DVB) was first modified with 4,4'-azobis(4-cyanovaleric acid) and then the bound initiation sites used to graft 2-vinyl-4,4-dimethylazlactone finally affording a monolith with 1.6 mmol/g of reactive functionalities. The product was then cut into discs and used as a scavenger for the rapid removal of excess amine from reaction mixtures [95].

3.5 Molecular Recognition

Materials with an enhanced selectivity towards specific substrate molecules can be produced using the technique of molecular imprinting in which interacting monomer(s) and a crosslinker are polymerized in the presence of template molecules. The template is then extracted from the polymer, leaving behind an imprint containing functional groups capable of chemical interaction. The shape of the imprint and the arrangement of the functional groups are complementary to the structure of the template. The current literature contains numerous examples of potential applications of imprinted polymers, such as chromatographic resolution of racemates, artificial antibodies, chemosensors, selective catalysts, and models of enzymes [97–100]. However, until recently, all of them relied on the use of particles that very often have an irregular shape and poor flow characteristics when packed into a column.

In contrast to these particulate materials, the molding technique has some advantages when used to the preparation of molecularly imprinted monoliths. Matsui et al. was the first to prepare an imprinted monolith [101]. Using acrylic acid and ethylene dimethacrylate, he demonstrated the capabilities of these materials for molecular recognition in a series of separations of positional isomers of diaminonaphthalene and phenylalanine anilide enantiomers. Sellergren later duplicated these experiments with phenylalanine anilide, and also mimicked earlier work with the preparation of imprinted monoliths with selectivities toward pentamidine, tri-O-acetyladenosine, and atrazine [102]. In addition, a similar porous polymer monolith has been prepared within a fused-silica capillary and used successfully for the selective electrophoretic separations of pentamidine and benzamidine [103]. Capillary columns containing "megaporous" imprinted monoliths found recently a specific application in the area of enantioselective capillary electrochromatography [104–109].

3.6 Gas Chromatography

Several approaches towards monolithic GC columns based on open pore foams prepared in large diameter glass tubes were reported in the early 1970s [26, 27, 110]. However, these columns had poor efficiencies, and the foams possessed only limited sample capacities in the gas-solid GC mode. Subsequent experiments with polymerized polymer layer open tubular (PLOT) columns where the capillary had completely been filled with the polymer were assumed to be failures since the resulting stationary phase did not allow the gaseous mobile phase to flow [111].

A preliminary study with the new generation of capillary columns with specifically designed monolithic poly(divinylbenzene) stationary phase shown in Fig. 10 has recently demonstrated that the application range of the rigid porous polymer monoliths can be extended to include gas-solid chromatography [112]. TGA measurement indicates that the porous monolith does not undergo any significant thermal degradation until a temperature of 380 °C is reached. This excellent thermal stability enables the monolith to operate routinely at temperatures up to 300 °C, and up to 350 °C for short periods of time, without observing

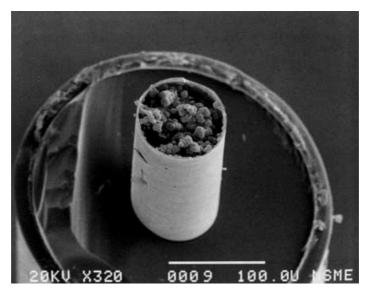


Fig. 10. Scanning electron micrographs of monolithic poly(divinylbenzene) capillary column. Note that the porous monolith is surrounded by an impervious tubular outer polymer layer resulting from copolymerization of the monomer with the acryloyl moieties bound to the capillary wall. This layer minimizes any direct contact of the analytes with the surface of the fused-silica capillary

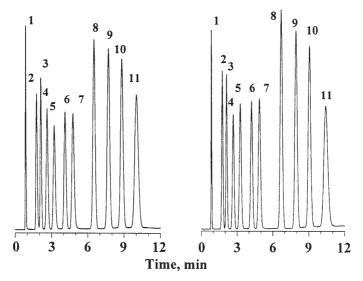


Fig. 11. Separation of a mixture of organic solvents using 50 cm long 100 (*left*) and 320 μm i.d. (*right*) monolithic capillary columns (Reprinted with permission from [112]. Copyright 2000 Wiley-VCH). Conditions: temperature gradient 120–300 °C, 20 °C/min, inlet pressure 0.55 MPa, split injection. Peaks: methanol (1), ethanol (2), acetonitrile (3), acetone (4), 1-propanol (5), methyl ethyl ketone (6), 1-butanol (7), toluene (8), ethylbenzene (9), propylbenzene (10), butylbenzene (11)

any deterioration of its properties. Figure 11 shows the separation of 11 model compounds. Major advantages of these monolithic columns are the simplicity of their single step in situ preparation method and the fact that, unlike in the case of conventional packed-bed GC columns, the length of the column may be adjusted easily by cutting. In addition, the ability to vary readily the surface chemistry of these materials by using different monomers should enable the fine control of both the polarity as well as the selectivity of such separation media. However, an improvement in column efficiency is required to match those of their coated open-tubular counterparts.

3.7 High-Performance Liquid Chromatography

Despite a growing number of applications in various areas, separations in the HPLC and CEC modes remain the focus of almost all groups working with the monoliths. Since the use of monolithic media in CEC has been summarized recently in several review articles [42, 107, 113, 114], we will focus in this chapter only on the HPLC separations.

3.7.1

Reversed-Phase Chromatography of Small Molecules

Recent chromatographic data indicate that the interactions between the hydrophobic surface of a molded poly(styrene-*co*-divinylbenzene) monolith and solutes such as alkylbenzenes do not differ from those observed with beads under similar chromatographic conditions [67]. The average retention increase, which reflects the contribution of one methylene group to the overall retention of a particular solute, has a value of 1.42. This value is close to that published in the literature for typical polystyrene-based beads [115]. However, the efficiency of the monolithic polymer column is only about 13,000 plates/m for the isocratic separation of three alkylbenzenes. This value is much lower than the efficiencies of typical columns packed with small beads.

The efficiency of the polymer-based monolithic columns is also rather low compared to efficiencies of up to 96,000 plates/m that were found for C18 modified silica-based monoliths reported by Tanaka's group [39]. The penalty paid for their more regular internal structure and higher efficiency is the more complicated method used for the preparation of these monolithic columns. In order to simplify the preparation, Fields prepared monolithic silica columns directly within the capillary [38]. Although his process works in capillaries, it may not be well suited for the preparation of larger size columns. The morphology of this monolith is quite different from that shown by Minakuchi et al. [116] for the silica and by Viklund et al. [65] for the organic polymer-based monoliths. Efficiencies achieved with these in situ prepared monolithic silica capillary were also only 5000–13,000 plates/m, i.e., in the range of those observed for polymer monoliths. This indicates that the efficiency is directly related to the morphology and not to the chemistry of the monolith.

The effect that the quality of the bed structure has on the chromatographic properties of columns packed with particles has been well known for a long time [1]. Similarly, the efficiency of capillary electrophoretic separations reaches its maximum for a specific capillary diameter, and then decreases steeply for both larger and smaller size [117]. Therefore, any improvement in the efficiency of the polymeric monolithic columns for the isocratic separations of small molecules is likely to be achieved through the optimization of their porous structure rather than their chemistry.

In contrast, it is already known that very high column efficiencies can be achieved in capillary electrochromatography. For example, Yu et al. demonstrated separations of benzene derivatives in revered-phase mode with an efficiency of over 200,000 plates/m [118] while Lämmerhofer et al. achieved efficiencies of 250,000 plates/m for the separations of enantiomers of functionalized amino acids [119].

3.7.2 Separation of Oligomers

Examples of the separation of styrene oligomers by HPLC on reversed-phase octadecylsilica columns in a gradient of the mobile phase follow the expected tendency for reversed-phase chromatography of small molecules [120]. Their retention depends both on the composition of the mobile phase and on the number of the repeat units in the oligomer. Larger polystyrene oligomers, being the more hydrophobic, exhibit longer retention times. This means that the elution order is opposite to that of size-exclusion chromatography where the larger molecules elute first. Figure 12 shows the separation of a commercial sample of styrene oligomers with a number average molecular weight of 630 in a short molded column that uses the gradient HPLC mode, and compares it with the separation achieved in the size-exclusion chromatography mode [121, 122]. The chromatograms are mirror images, and exhibit a number of peaks that can be assigned to the individual styrene oligomers. The resolution achieved with the molded rod column is very good, with the left chromatogram in Fig. 12 even indicating the presence of an undecamer [122].

In general, an increase in the resolution of an SEC system can only be achieved with better column packing or a longer column. In contrast, gradient elution provides additional options for improving the separation. If variables such as the range of mobile phase composition remain constant for a specific column and a specific set of solutes, the average retention factor in the gradient elution will only depend on the gradient time and the flow rate. Because the product of these variables is the gradient volume, equal separations independent of flow rate and gradient steepness should be achieved within the same gradient volume [31]. Figure 13 shows separations of styrene oligomers obtained with gradient times of 200 and 20 min and flow rates of 1 and 10 ml/min, respectively. The gradient volume is 200 ml in both cases and, indeed, no significant differences can be seen

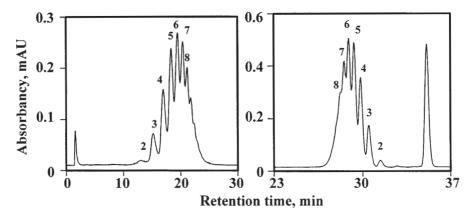


Fig. 12. Separation of styrene oligomers by reversed-phase (*left*) and size-exclusion chromatography (*right*) (Reprinted with permission from [121]. Copyright 1996 American Chemical Society). Conditions: (*left*) column, molded poly(styrene-*co*-divinylbenzene) monolith, 50 mm × 8 mm i.d., mobile phase, linear gradient from 60 to 30% water in tetrahydrofuran within 20 min, flow rate 1 ml/min, injection volume 20 μ l; UV detection, 254 nm; (*right*) series of four 300 mm × 7.5 mm i.d. PL Gel columns (100 Å, 500 Å, 105 Å, and Mixed C), mobile phase tetrahydrofuran, flow rate, 1 ml/min; injection volume 100 μ l, toluene added as a flow marker, UV detection, 254 nm; temperature 25 °C, *peak numbers* correspond to the number of styrene units in the oligomers

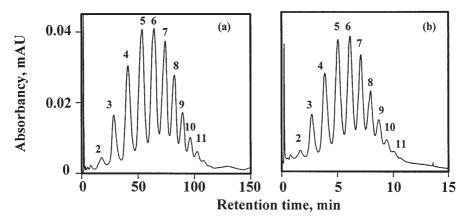


Fig. 13. Effect of flow rate and gradient time on the separation of styrene oligomers in a molded poly(styrene-*co*-divinylbenzene) monolithic column (Reprinted with permission from [121]. Copyright 1996 American Chemical Society). Conditions: column, 50 mm × 8 mm i.d.; (*left*) mobile phase, linear gradient from 60 to 30% water in tetrahydrofuran within 200 min, flow rate, 1 ml/min; (*right*) mobile phase, linear gradient from 60 to 30% water in tetrahydrofuran within 20 min, flow rate, 10 ml/min, analyte, 15 mg/ml in tetrahydrofuran, injection volume 20 μ l, UV detection, 254 nm, *peak numbers* correspond to the number of styrene units in the oligomer

between the two chromatograms. In contrast to SEC, these results indicate that the additional tools of flow rate and gradient time are available for the optimization of separation in gradient elution chromatography [122]. Molded rod columns allow the use of very high flow rates at reasonable backpressures, thus making very fast chromatographic runs possible. In addition, they also permit much higher sample loads than reversed-phase columns packed with typical C18-bonded silica particles.

3.7.3

Precipitation-Redissolution Separation of Synthetic Polymers

In this technique originally developed for packed columns [123], the polymer solution is injected into a stream of the mobile phase in which the polymer is not soluble. Therefore, the macromolecules precipitate and form a separate gel phase, which adsorbs onto the surface of the separation medium and does not move along the column. The solvating power of the mobile phase is then increased gradually until it reaches a point at which some of the macromolecules start to redissolve again and travel with the stream. Since the medium contains pores smaller than the size of the polymer molecules, the mobile phase can penetrate these small pores while the dissolved molecules move only with the stream through the larger channels. As a result, the polymer solution moves forward faster than the solvent gradient, and therefore the polymer eventually precipitates. The newly formed precipitated gel phase will then redissolve only when the solvent strength is again sufficient. A multitude of such precipitation-redissolution steps is repeated until the macromolecule finally leaves the column. The solubility of each polymer molecule in the mobile phase depends on both its molecular weight and its composition. As a result, separation of species differing in these properties is achieved.

Although higher molecular weight synthetic polymers such as polystyrene behave differently from small and midsize molecules in reversed-phase chromatographic separations, the general elution pattern from a monolithic column remains unchanged, as the more soluble species with lower molecular weights elute prior to those with higher molecular weights. For example, very good separations of a mixture of eight low dispersity polystyrene standards with molecular weights ranging from 519 to 2,950,000 g/mol was achieved using a very short length (5 cm) monolithic column at different flow rates in a gradient of methanol in tetrahydrofuran. The separation could be carried out at a higher flow rate in a much shorter period of time. For example, 16 min are needed for the separation at a flow rate of 2 ml/min, while only 4 min are sufficient for the same separation at 8 ml/min without decreasing the quality of the separation. The gradient volume required for the elution of a specific peak remains constant at both flow rates. In addition, the position of the peaks in the chromatogram can be adjusted by a simple change of the gradient profile. Similar results were also obtained using mobile phases in which acetonitrile and water were used as precipitants [122].

Generally, gradient separations can be performed faster by using higher flow rates and steeper gradients [31]. This also applies to the precipitation-redissolution chromatography of synthetic polymers. Figure 14 shows the separation of three polystyrene standards that was carried out using steep gradients and a flow rate of 20 ml/min. The separation is excellent at a gradient time of 1 min, and three baseline resolved peaks are obtained within 16 s.

Although successful, the separations described above required a high flow rate of 20 ml/min and consumed large volumes of the mobile phase, thus limiting a broader use of this technique. Subsequent studies improved the applicability of monolithic columns for the rapid determination of molecular parameters of synthetic polymers since the separation could be then carried out at much lower flow rates. This was achieved by (i) using columns with a smaller diameter and (ii) optimization of the mobile phase gradients. In addition to polystyrenes, separations of poly(methyl methacrylates), poly(vinyl acetates), and polybutadienes have also been demonstrated on such molded rod columns and compared with those obtained using size exclusion chromatography [124]. For example, the separation of nine polystyrene standards using a gradient that has been optimized to obtain a linear calibration curve was achieved in less than 2 min at a flow rate of only 1 ml/min. This method also enables the rapid determination of molecular parameters of commercial polymers with a broad molecular weight distribution affording results fully comparable with those obtained by the much slower size exclusion chromatography. The speed of this method proved to be a definite advantage in the characterization of large libraries of synthetic polymers prepared using methods of combinatorial chemistry [125].

It is worth noting that the commercial (ISCO, Inc.) monolithic column utilized in these evaluations is very stable. Over a period of about 7 months, approxi-

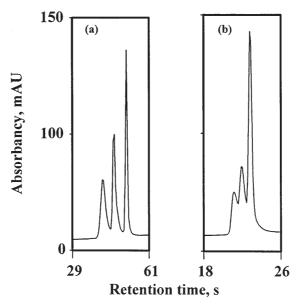


Fig. 14 a, b. Effect of gradient steepness on the very fast separation of polystyrene standards in a molded monolithic poly(styrene-*co*-divinylbenzene) column (Reprinted with permission from [121]. Copyright 1996 Elsevier). Conditions: column, 50 mm × 8 mm i.d., mobile phase, linear gradient from 100% methanol to 100% tetrahydrofuran within: **a** 1 min; **b** 12 s, flow rate, 20 ml/min, peaks represent polystyrene standards with molecular weights of 9200, 34,000 and 980,000 (order of elution), 3 mg/ml of each standard in tetrahydrofuran, injection volume 20 µl, UV detection, 254 nm

mately 3000 chromatographic measurements were carried out using a single 50×4.6 mm poly(styrene-co-divinylbenzene) monolithic column. In each gradient run one component of the mobile phase was a good swelling agent for the material of the column while the other was a precipitant. Although the high level of crosslinking does not allow extensive swelling of the monolithic material, even small volumetric changes of the matrix constitute a periodic stress for the column. However, this repeated stress had no effect on long-term column performance. During the course of this study the flow rate, one of the most critical variables, was changed quite often, routinely reaching values of up to 8 ml/min. The superior chemical stability of the column was demonstrated by the fact that several different solvents such as THF, dichloromethane, methanol, hexane, and water with repeated changes in gradient composition could be used without any adverse effect on the performance. An occasional low flow rate flushing with THF was the only "maintenance" carried out on the column. During the entire period of study, no change in back pressure, flow, and separation characteristics were observed for the monolithic column. Figure 15 shows two HPLC separations of a mixture of 8 polystyrene standards that were carried out more than 2 months and about 400 injections apart. The very small difference that can be observed between these two runs lies within the experimental error of chromatographic measurements.

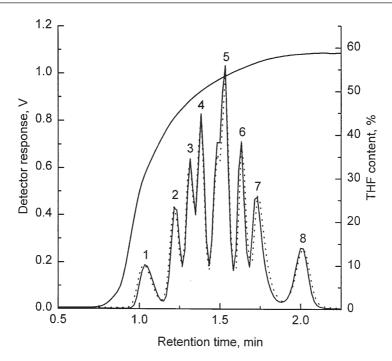


Fig. 15. Rapid separation of a mixture of eight polystyrene standards using a monolithic poly(styrene-*co*-divinylbenzene) column and the corresponding gradient profile monitored by the UV detector (Reprinted with permission from [124]. Copyright 2000 Wiley-VCH). Separation conditions: 1.25 min gradient of THF in methanol consisting of 0-35% THF in methanol in 0.12 min, 35-50% in 0.38 min, 50-55% in 0.25 min, 55-59% in 0.25 min, and 59-60% in 0.25 min, overall sample concentration 16 mg/ml (2 mg/ml of each standard) in THF, ELSD detection. Molecular weights of polystyrene standards: 3000 (1), 7,000 (2), 12,900 (3), 20,650 (4), 50,400 (5), 96,000 (6), 214,500 (7), and 980,000 (8). *Dotted line* shows the same separation recorded more than two months and about 400 injections later

3.7.4 Chromatography of Midsize Peptides

Short peptide molecules are a very important family of compounds produced by the pharmaceutical industry using both biotechnology and synthetic processes. HPLC is a valuable tool for both monitoring their preparation and achieving their purification. Because of their higher molecular weights, the slower mass transport (diffusion) of the analytes within the pores of typical poly(styrene-*co*-divinylbenzene) beads in a packed column negatively effects the quality of the separation. In contrast, the separation in a molded column with the same styrenic chemistry can be considerably faster, owing to the much better mass transport. For example, the isocratic separation of the peptides bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) and (D-Phe7)-bradykinin, which differ only in their seventh amino acid residue (L-proline and D-phenylalanine, respectively), can be achieved in only 3 min. The efficiency of the molded column for peptides with a molecular weight of about 1000 as determined with bradykinin in 50% aqueous acetonitrile is very good, amounting to 7900 plates/m [67]. Even faster separations of five peptides in about 1 min were achieved with a commercially available monolithic reversed phase column [127]. These monolithic columns manufactured by ISCO Inc. feature smaller pores and exhibit resolution similar to that of typical HPLC columns packed with 5-µm C8 silica beads.

Excellent performance for the elution of another peptide, insulin (molecular weight 5800 g/mol), was also observed using silica-based monoliths. The efficiency of the monolithic column was much better than that of a column packed with beads, and did not change much even at high flow rates.

3.7.5 Gradient Elution of Proteins

Gradient elution is a very popular method for the separation of natural macromolecules because the retention of different components of a complex biological mixture may vary considerably. In contrast to isocratic separations, the use of a gradient of mobile phase accelerates the elution, allowing separation of the sample components to be completed within a reasonable period of time. The mechanism of gradient elution is similar for many of the retentive HPLC modes such as reversed-phase, ion-exchange, and hydrophobic interaction chromatography [126]. Typically, the first step is the adsorption of the sample in the separation medium close to the top of a column, followed by successive dissolution of individual components as the composition of the mobile phase is changed. The nature of the components selected for the mobile phase is dictated by the separation mode used.

For example, mixtures of water or a dilute buffer solution and organic solvent such as acetonitrile are typically used for elutions from a highly hydrophobic separation medium in the *reversed-phase chromatographic mode*. The monolithic media tolerate fast flow rates, thus easily enabling high throughput separations. Figure 16 shows the reversed-phase separation of three proteins in a molded poly(styrene-co-divinylbenzene) rod column at two different flow rates using a constant gradient volume. The individual proteins are baseline separated into sharp and narrow peaks. No significant differences can be seen between separations done over the broad flow rate range of 5–25 ml/min. As expected, the quality of the separation does not change for runs that use the same gradient volume [31]. This, together with the low back pressure observed for the monolithic columns even at very high flow rate, enables rapid separations to be achieved simply with an increase in the flow rate or by using an even steeper gradient [53]. Figure 17 shows the rapid separation of five proteins in less than 17 s using a commercial column [127]. Norbornene-based monoliths were also used for the separation of model proteins using reversed phase chromatography [58]. These monoliths easily tolerate high flow rates of up to 10 ml/min and the good separations achieved even at this high flow rate confirm the existence of fast mass transfer.

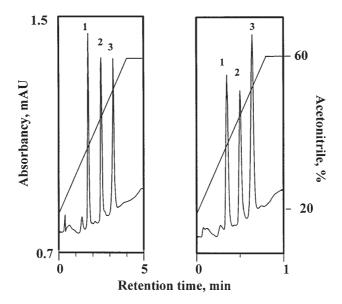


Fig. 16. Separation of cytochrome c (1), myoglobin (2), and chicken egg albumin (3) by reversed-phase chromatography on a monolithic poly(styrene-*co*-divinylbenzene) column at flow rates of: **a** 5 ml/min; **b** 25 ml/min. (Reprinted with permission from [53]. Copyright 1996 American Chemical Society). Conditions: column 50 mm \times 8 mm i.d., mobile phase: linear gradient from 20 to 60% acetonitrile in water

The ability to prepare monoliths within a mold of any shape was used by Lee et al. [128] who prepared monolithic ST-DVB microbeads within pulled fused silica needles and used them for the reversed-phase separation and on-line electrospray ionization mass spectrometry (ESI-MS) detection of proteins and peptides. As illustrated by Fig. 18, these monolithic microcolumns separated proteins far better than capillaries packed with commercial C18 silica or polymeric beads.

Huber's group recently prepared poly(styrene-*co*-divinylbenzene) monolithic columns in the capillary format using tetrahydrofuran/decanol mixtures as porogen. These columns were tested for the HPLC separation of protein digests followed by ESI MS detection enabling protein identification [129]. This technique represents an important contribution to the currently emerging techniques for studying of proteomes as it is more convenient and accurate to use than the classical 2-D gel electrophoresis.

In contrast to reversed-phase chromatography, the separation in *ion exchange mode* occurs under mild condition using an entirely aqueous mobile phase. The elution from an ion exchange monolith, which must contain charged ion exchange functionalities, as for any other ion exchange column is achieved using a gradient of increasing salt concentration in the mobile phase. The epoxide groups of a molded poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) monolith can be readily modified to form ion exchangers [130–132]. For example, the reaction with diethylamine leads to an analog of the diethylaminoethyl (DEAE)

chemistry (Fig. 6), which is well suited even for large scale separations. Figure 19 shows the ion exchange separation of 20 mg of a protein mixture including myoglobin, conalbumin, and soybean trypsin inhibitor using a relatively large molded 60×16 mm i.d. diethylamine modified monolith [133]. The proteins are baseline separated and the symmetry of the peaks is very good. Using commercial monolithic columns with a similar chemistry recently developed by ISCO for fast ionexchange chromatography, four proteins were separated in 3 min with an excellent resolution. These columns are also very stable and no changes in recovery

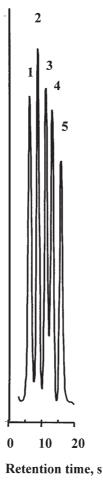


Fig. 17. Rapid reversed-phase separation of proteins at a flow-rate of 10 ml/min (Reprinted with permission from [127]. Copyright 1999 Elsevier). Conditions: Column, 50×4.6 mm i.d. poly(styrene-*co*-divinylbenzene) monolith, mobile phase gradient: 42% to 90% acetonitrile in water with 0.15% trifluoroacetic acid in 0.35 min, UV detection at 280 nm. Peaks: ribonucle-ase (1), cytochrome c (2), bovine serum albumin (3), carbonic anhydrase (4), chicken egg albumin (5)

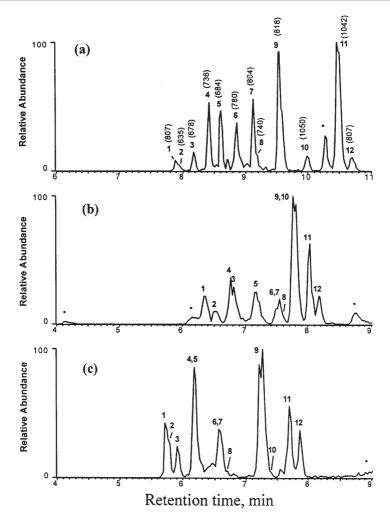


Fig. 18a-c. Base peak chromatograms for the LC/MS analyses of a cytochrome c Lys-C digest (0.7 pmol injected) on: **a** a poly(styrene-*co*-divinylbenzene) monolith-filled needle; **b** Vydac C18-packed needle; **c** Poros R2-packed needle. (Reprinted with permission from [128]. Copyright 1998 American Chemical Society)

have been found even after several hundreds of runs. This makes them well suited for high throughput separation of biomacromolecules.

In addition to the monolithic DEAE weak anion exchanger, ISCO has also developed several other monolithic columns including a strong anion exchanger as well as weak and strong cation exchangers. It has been demonstrated that, using these monolithic ion exchangers, resolution similar to that of conventional HPLC columns can be achieved. Specific attention has also been paid to the long-term stability of repeatedly used columns. Figure 20 shows the separations of three proteins achieved over a long period of time and a large number of injections.

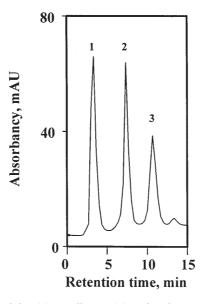


Fig. 19. Separation of myoglobin (1), conalbumin (2), and soybean trypsin inhibitor (3) by ionexchange chromatography on a diethylamine modified molded poly(glycidyl methacrylate-*co*ethylene dimethacrylate) monolithic column. (Reprinted with permission from [133]. Copyright 1995 Wiley-VCH). Conditions: column, monolith 60 mm×16 mm i.d., mobile phase gradient from 0.01 mol/l TRIS-HCl buffer pH 7.6 to 1 mol/l NaCl in the buffer in 30 min; flow rate 2ml/min; total protein loading 20 mg, UV detection at 280 nm

High performance is in liquid chromatography often synonymous with high pressure since small size particles are packed in the column. Columns packed with larger beads that can be run at medium pressure typically afford relatively poor performance because of the mass transfer resistance within the "long" pores of these large diameter particles. In contrast, monolithic media enable the high performance characteristic of HPLC to be achieved at a medium or even low pressure thus offering the separations in a unique mode that is called "high performance medium pressure liquid chromatography" (HPMPLC).

The breakthrough curves measured for the monolithic columns with different proteins are very sharp and confirm again the fast mass transport kinetics of the monoliths [133, 134]. The frontal analysis used for the determination of the breakthrough profile can also be used for calculation of the dynamic capacity of the column. For example, the capacity for the 60×16 mm i.d. monolith at 1% breakthrough is 324 mg of ovalbumin and represents the specific capacity of 40.0 mg/g of separation medium or 21.6 mg/ml of column volume.

The ultimate goal in the development of any separation medium, i.e., its use for the separation of "real-life" samples, has also been demonstrated by the separation of baker's yeast (*Saccharomyces cerevisiae*) extract. This separation compares favorably to those obtained with commercial packed ion-exchange

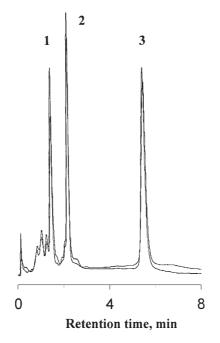


Fig. 20. Test of stability of weak cation exchange monolithic column (ISCO). Conditions: column, 50×4.6 mm i.d., mobile phase gradient of sodium chloride in 0.01 mol/l sodium phosphate buffer (pH 7.6) from 0.1 to 0.5 mol/l in 4.5 min and to 1 mol/l in 6.5 min, overall gradient time 11 min, flow rate 10 ml/min. Peaks: Ribonuclease (1), cytochrome c (2), lysozyme (3). The two separations shown in this figure were achieved 503 runs apart

columns, and even to the recently introduced compressed polyacrylamide-based monolithic media (UNO-columns by Bio-Rad) [133].

In order to accelerate further the ion-exchange separations, the rigid porous monoliths were provided with short chains of poly(2-acrylamido-2-methyl-1-propanesulfonic acid) grafted to the pore surface using a cerium(IV)-based redox initiating system [69]. In contrast to the typical chemical modification that occurs within the bulk of the matrix where each epoxide group is transformed into a single charged moiety, the grafting procedure provides chains in which each repeat units bears the required functionality. This increases the local concentration of ion-exchange groups and improves the separation properties of the matrix. Due to the low resistance to flow, elution can be carried out at a flow rate of 7 ml/min, and the separation of proteins is achieved in a linear gradient of the mobile phase within 2.5 min. Although this separation is rather quick, removing the dead volume between the peaks of chymotrypsinogen and lysozyme using nonlinear stepwise gradients accelerates it even more. This simple change in the gradient shape reduces the separation time by more than 30%, and the proteins are baseline separated within only 1.5 min.

A slightly different mechanism of proteins separation results from the use of porous polymeric monoliths containing zwitterionic sulfobetaine groups [68].

The approach developed by Irgum's group involves photoinitiated copolymerization of *N*,*N*-dimethyl-*N*-methacryloxyethyl-*N*-(3-sulfopropyl) ammonium betaine and ethylene dimethacrylate. Alternatively, the internal surface of porous poly(trimethylolpropane trimethacrylate) monoliths were grafted with zwitterionic "combs" by thermally initiated polymerization of the zwitterionic monomer within the pores. While the flow resistance of grafted monoliths was strongly affected by the type of electrolyte, no changes were observed upon variations in the ionic strength of the mobile phase. Since these monoliths interact reversibly with proteins in aqueous solutions they can also be used for bioseparations.

Another gentle method designed for the separation of proteins is *hydrophobic interaction chromatography* (HIC). The concept of HIC is based on the interactions of surface hydrophobic patches of proteins with hydrophobic ligands interspersed in the hydrophilic surface of the separation medium. The interaction occurs in an environment, such as an aqueous salt solution, that promotes these interactions. The column-bound ligands are typically short alkyl chains or phenyl groups. The strength of the interaction depends on many factors, including the intrinsic hydrophobicity of the protein, the type of ligands, their density, the separation temperature, and the salt concentration. In contrast to ion-exchange chromatography, the separation is achieved by decreasing the salt concentration in the mobile phase, causing the less hydrophobic molecules to elute first.

Since the hydrophobicity of styrene- or alkyl methacrylate-based monolithic matrices is too high to make them useful for hydrophobic interaction chromatography, porous monoliths based on highly hydrophilic copolymers of acrylamide and methylenebisacrylamide were developed [70, 135]. The hydrophobicity of the matrix required for the successful separations of proteins is controlled by the addition of butyl methacrylate to the polymerization mixture. The suitability of this rigid hydrophilic monolith for the separation of protein mixtures is demonstrated in Fig. 21, which shows the rapid separation of five proteins in less than 3 min using a steeply decreasing concentration gradient of ammonium sulfate.

Typically, proteins are eluted consecutively in hydrophobic interaction chromatography by applying a decreasing gradient of salt concentration. However in order to operate satisfactorily, a typical HIC column must be re-equilibrated in the initial mobile phase prior to the next run. This decreases the number of runs that can be performed within a given amount of time, and thus represents a serious limitation for high throughput processes. Therefore, a new concept of hydrophobic interaction chromatography has been developed which employs thermally induced change in the surface polarity of the grafted composites to achieve the HIC separation of proteins in a simple *isocratic* mode [76].

The preparation of monoliths with polyNIPAAm chains grafted to the internal pore surface was discussed previously. The extended solvated polyNIPAAmchains that are present below the lower critical solution temperature of this particular polymer are more hydrophilic, while the collapsed chains that prevail above the lower critical solution temperature are more hydrophobic. In contrast to isothermal separations in which the surface polarity remains constant throughout the run [136], HIC separation of proteins can be achieved at *constant salt concentrations* (isocratically) while utilizing the hydrophobic-hydrophilic

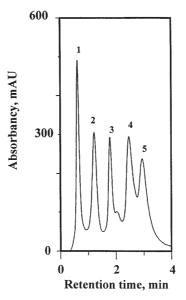


Fig. 21. Separation of cytochrome (peak 1), ribonuclease, (peak 2), carbonic anhydrase (peak 3), lysozyme (peak 4), and chymotrypsinogen (peak 5) by hydrophobic interaction chromatography on a molded poly(acrylamide-*co*-butylmethacrylate-*co*-*N*,*N*'-methylenebisacrylamide) monolithic column. (Reprinted with permission from [135]. Copyright 1998 Elsevier). Conditions: column, 50 × 8 mm i.d., 10% butyl methacrylate, mobile phase gradient from 1.5 to 0.1 mol/l ammonium sulfate in 0.01 mol/l sodium phosphate buffer (pH 7) in 3 min, gradient time 3.3 min, flow rate 3 ml/min

transition of the grafted chains of polyNIPAAm, which occurs in response to *changes in temperature*. For example, carbonic anhydrase and soybean trypsin inhibitor were easily separated. First, the grafted monolith is heated to 40 °C, and a mixture of the two proteins is injected. The more hydrophilic carbonic anhydrase is not retained under these conditions, and elutes from the column. In contrast, the more hydrophobic trypsin inhibitor does not elute even after 10 min. However, the elution occurs almost immediately once the temperature of the column is lowered to 25 °C [76].

3.7.6 Separation of Nucleic Acids

To prepare a suitable medium for the ion-exchange chromatography of nucleic acids poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) monolithic columns were modified to different extents by reaction with diethylamine to afford 1-*N*,*N*-diethylamino-2-hydroxypropyl functionalities. The performance of the resulting stationary phases was demonstrated in the separation of a homologous series of oligodeoxyadenylic (pd(A)₁₂₋₁₈) and oligothymidylic acids (d(pT)₁₂₋₂₄) at different flow rates. Very good separations of the oligonucleotides were achieved even at the high flow rate of 4 ml/min [137].

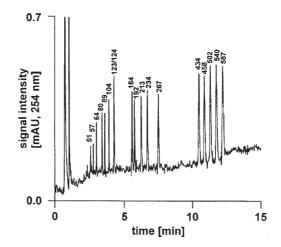


Fig. 22. High-resolution capillary ion-pair reversed-phase high-performance liquid chromatography separation of a mixture of double-stranded DNA fragments in a 60×0.20 mm i.d. monolithic poly(styrene-*co*-divinylbenzene) capillary column (Reprinted with permission from [138]. Copyright 2000 American Chemical Society). Mobile phase (A) 100 mmol/l triethylammonium acetate, pH 7.0, (B) 20% acetonitrile in 100 mmol/l triethylammonium acetate, pH 7.0, linear gradient 35–75% B in 3.0 min, 75–95% B in 12.0 min, flow-rate, 2.2 µl/min, temperature 50 °C, UV detection at 254 nm, sample pBR322 DNA-Hae III digest, 1.81 fmol of each fragment

Huber at al. prepared monolithic capillary columns by copolymerization of styrene and divinylbenzene inside a 200 µm i.d. fused silica capillary using a mixture of tetrahydrofuran and decanol as porogen. With gradients of acetonitrile in 100 mmol/l triethylammonium acetate, these monolithic columns allowed the rapid and highly efficient separation of single-stranded oligodeoxynucleotides and double-stranded DNA fragments by ion-pair reversed-phase high-performance liquid chromatography (IP-RP-HPLC) [138, 139]. These authors also compared the performance of monolithic columns with that of micropellicular, octadecylated poly(styrene/divinylbenzene) beads and found a considerably better performance for the monolithic columns. The use of this type of column enabled the analysis of an 18-mer oligodeoxynucleotide with an efficiency of more than 190,000 plates/m. An ESI MS was on-line-coupled to the chromatographic system to achieve detection of femtomole amounts of 3-mer to 80-mer oligodeoxynucleotides. Similarly, double-stranded DNA fragments ranging in size from 51 to 587 base pairs could also be separated as demonstrated in Fig. 22. This method also allowed the sequencing of short oligodeoxynucleotides.

4 Conclusion

Although much remains to be done in the study of macroporous monoliths, recent achievements open new vistas for the preparation of supports and separation media with exactly tailored properties. The experimental work done so far and the commercial availability of some types of monolithic columns confirms the great potential of these new molded continuous materials since the rigid macroporous polymer monoliths possess a number of unique properties compared to their more traditional macroporous beads counterparts. Although these materials are unlikely to replace particulate supports completely, they can complement the beads in a variety of applications. In addition to the number of their documented uses in reversed-phase, hydrophobic interaction, ion-exchange, precipitation chromatography, and capillary electrochromatography, these materials also show promise as potential flow-through supports in heterogeneous catalysis, as polymeric scavengers, as reagents for combinatorial chemistry, and as novel stationary phases in a variety of less common formats such as membranes, capillaries, and media for microfluidic devices.

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Molecularly Imprinted Materials – Receptors More Durable than Nature Can Provide

Oliver Brüggemann

Technische Universität Berlin, Institut für Chemie, TC 8, Strasse d. 17. Juni 124, 10623 Berlin, Germany. *E-mail: brueggemann@chem.tu-berlin.de*

The chapter describes the concept of molecular imprinting. This technology allows the fabrication of artificial polymeric receptors applicable in many areas of biotechnology. Polymers imprinted with selected template molecules can be used as specific recognition elements in sensors or as selective stationary phases in affinity chromatography or in capillary electrochromatography. However, also in solid phase extraction or immunoassays these polymers (MIP) are able to compete with traditional materials such as biological antibodies. Furthermore, polymers molecularly imprinted with so-called transition state analogue templates can be applied as catalysts. In other words, these kind of polymers may be used as artificial antibodies (plastibodies) or biomimicking enzymes (plastizymes). Compared to their biological counterparts, MIP offer different advantages such as simplicity in manufacturing and durability. Thus, the author expects MIP to have a major impact on the whole area of biotechnology.

Keywords: Catalysis, Capillary electrochromatography, MIP, Polymer, Sensor, Solid phase extraction

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List of Abbreviations

CE	Capillary Electrophoresis
CP	Control Polymer
DVB	Divinylbenzene
EGDMA	Ethyleneglycol dimethacrylate
FET	Field-Effect Transistor
GC	Gas Chromatography
HPCE	High Performance Capillary Electrophoresis
HPLC	High Performance Liquid Chromatography
LC	Liquid Chromatography
MAA	Methacrylic Acid
MI	Molecular Imprinting/Molecularly Imprinted
MIP	Molecularly Imprinted Polymer
MI-SPE	Solid Phase Extraction Based on Molecularly Imprinted Phase
PAH	Polycyclic Aromatic Hydrocarbon
QCM	Quartz Crystal Microbalance
RFGD	Radio-Frequency Glow-Discharge Plasma Deposition
SPE	Solid Phase Extraction
TFMAA	Trifluoromethacrylic Acid
TSA	Transition State Analogue

1 Introduction

In chromatography the demand for highly selective stationary phases is greater than ever, due to the high number of analytes to be separated from complex matrices such as urine, blood or other biological fluids. Bio-receptors, such as antibodies, are popular ligands to be immobilized on stationary phases in order to recognize selectively their specific counterparts (antigens). However, such biological molecules are quite sensitive to harsh conditions which are commonly used in chromatographic approaches, e.g., during elution. These problems are based on the fact that enzymes and antibodies are proteins which denature easily, e.g., under acidic conditions or at elevated temperatures or as the result of proteolytic digestion. In this sense plastics and inorganic materials are the exact opposite of the biological materials. Thus, the idea arose to transfer the recognition mechanisms of biological systems such as antibody/antigen or enzyme/substrate to polymeric networks by a technique called "Molecular Imprinting", the principle of which is shown in Fig. 1 [1–5].

In Molecular Imprinting (MI) a template acting as a substrate or antigen analogue is associated with a number of so-called "functional monomers" in a solvent ("porogen") prior to the addition of a cross-linker and a polymerization initiator. After polymerization the template is extracted from the three-dimensional polymer network leaving an imprint bearing a steric arrangement of interactive groups defined by the template structure. This allows later specific recognition and hence selective separation of analytes, which resemble the template. Columns

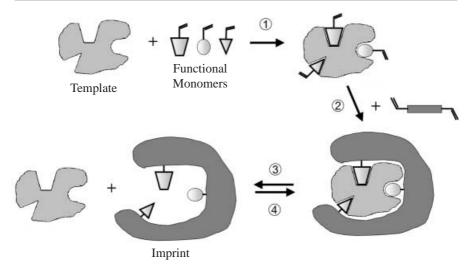


Fig.1. Concept of molecular imprinting – the non-covalent approach. 1. Self-assembly of template with functional monomers. 2. Polymerization in the presence of a cross-linker. 3. Extraction of the template from the imprinted polymer network. 4. Selective recognition of the template molecule

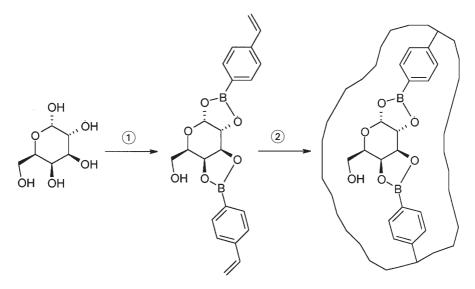


Fig.2. Concept of molecular imprinting – the covalent approach. 1. Derivatization of sugar template with *p*-vinylphenyl boronate. 2. Polymerization in the presence of a cross-linker

Application	Class of imprinted compound	Template	Refer- ences
Liquid chromatography	Herbicides, pesticides	Atrazine Bentazone Prometryn Triazine	[9-12] [13] [14] [9,15]
	Food components	Amino acids/peptides Carbohydrates Cholesterol Phenylalanine Proteins Nucleotide bases	[16, 17] [2, 18, 19] [20, 21] [22] [23-25] [26]
	Pharmaceuticals	Erythromycin A Oleandomycin Tylosin Chloramphenicol Penicillin V Oxacillin Hexestrol Cortisol Estradiol	[27] [27] [28] [29] [29, 30] [31] [32] [33]
Capillary electrophoresis	Herbicides, pesticides Food components Pharmaceuticals	2-Phenylpropionic acid L-Phenylalanine anilide S-Propranolol Pentamidine S-Ropivacaine R-Propranolol	[34] [35] [36] [11] [37] [38]
Solid phase extraction	Herbicides, pesticides	Atrazine Bentazone Yerbuthylazine Triazine	[39-41] [13] [42] [15]
	Pharmaceuticals	Bupivacaine Clenbuterol Theophylline Nicotine	[43] [44] [45] [46]
Sensors	Herbicides, pesticides	Atrazine Triazine 2,4-Dichlorophenoxy- acetic acid	[47, 48] [49] [50, 51]
	Food components	Cholesterol Flavonol Methyl-β-glucose Glucose Dansyl-L-phenylalanine	[52] [53] [54] [55] [8,56]
	Food additives Pharmaceuticals	Caffeine S-Propranolol Phenacetin	[57] [58] [59]
	Toxins	PAHs	[60, 61]

 Table 1. Examples of templates in molecular imprinting

Application	Class of imprinted compound	Template	Refer- ences
Catalysis	Transition state analogues	N-Isopropyl-N- nitrobenzylamine N-Benzyl-N-	[62] [63,64]
		isopropylamine Indol	[65]
Assays	Herbicides, pesticides	Atrazine 2,4-D-Phenoxyacetic acid Triazine	[9, 10, 66] [67, 68] [9]
	Food components	Amino acids/peptides Carbohydrates Cholesterol	[16] [2,18] [21,69,70]
	Food additives	Caffeine Menthol	[71] [72,73]
	Food contaminants	Listeria monocytogenes Staphylococcus aureus	[74] [74]
	Pharmaceuticals	Ampicillin Epinephrine Estradiol Ethynylestradiol	[75] [76] [77] [78]
Screening	Pharmaceuticals	11-α-Hydroxyprogesterone 11-Deoxycortisol	[79] [80]

Table 1 (continued)

and cartridges packed with these biomimetic stationary phases can be used hundreds of times without loss of performance [6]. Beside that, as polymeric materials such MI-stationary phases are characterized by an exceptional durability in organic solvents as well as at extreme temperatures, pH, or pressures [7, 8]. Hence, molecularly imprinted polymers (MIP) become more and more important for application in liquid chromatography (HPLC), capillary electrophoresis (CE), and solid phase extraction (SPE). Table 1 shows examples of templates used in imprinting procedures. At present the MIPs are predominantly used for analytical purposes, but also in areas such as catalysis or purification based on selective extraction.

2 Manufacturing of MIP

2.1 Preparation Techniques

Two different techniques have been developed for MIP production, namely the covalent and the non-covalent approaches. The covalent way is based on the chemical derivatization of the template with molecules containing polymerizable groups using reversible covalent bonds. Different chemical reactions can be ap-

plied for binding the template such as esterification with hydroxy or carboxy monomers, or the generation of amides or Schiff bases with amino monomers. Table 2 shows a few examples of compounds for the covalent approach.

The use of a *p*-vinylphenyl boronate as functional monomer to be covalently linked with a diol-template [2] is demonstrated in Fig. 2. Following polymerization in the presence of a cross-linker, the template has to be extracted from the polymer network. This requires breaking the covalent bond. During the application of covalently imprinted materials, the target molecules have to reform such bonds in order to be retained. Both making and breaking the bonds is at best a time-consuming process.

Imprinting technique	Monomer	Structure
Covalent	<i>p</i> -Vinylphenylboronic acid	B OH
	<i>p</i> -Vinylbenzylamine	NH ₂
	<i>p</i> -Vinylphenyl carbonyl	► C
Covalent/non-covalent	<i>p</i> -Vinylbenzoic acid	ОН
Non-covalent	Methacrylic acid	HOO
	4(5)-Vinylimidazole	N N
	4-Vinylpyridine	
	Itaconic acid	O OH OH
	2-Acrylamido-2-methyl-1- propane-sulfonic acid	H-CH ₂ -SO ₃ H

Table 2. Examples of functional monomers

Name	Structure
Ethyleneglycol dimethacrylate (EGDMA)	
<i>p</i> -Divinylbenzene (p-DVB)	
Trimethylolpropane trimethacrylate (TRIM)	
N,N´-Methylendiacrylamide	
Pentaerythritol tetraacrylate	

A much faster imprinting technique is the non-covalent strategy where the template simply self-assembles with functional monomers [1]. The types of noncovalent interactions of the monomers with the template used in this approach are manifold and include simple van der Waals, hydrophobic, or electrostatic interactions, but also hydrogen or $\pi - \pi$ bonds. This indicates the possibility of choosing from a broad range of monomers, e.g., acidic or basic substances like the often used methacrylic acid (MAA) or 4-vinylpyridine. Table 2 presents some typical examples of monomers applicable to non-covalent molecular imprinting. After mixing template and functional monomers with porogen and initiator, the following polymerization is done in the presence of relatively high amounts of

Table 3. Examples of cross-linkers

cross-linkers. The cross-linker establishes a rigid network around the template. Furthermore, the high percentage of cross-linker compared to the total amount of monomers used in the imprinting procedure prevents the dissolution of the final MI-polymer in most solvents. Cross-linkers typically used in non-covalent imprinting are listed in Table 3.

Ethyleneglycol dimethacrylate (EGDMA), but also the more hydrophobic divinylbenzene (DVB), have been chosen by many researchers for that purpose. Both components contain two vinyl groups and therefore can serve as crosslinkers. However, corresponding molecules with more than two polymerizable groups have also been suggested as cross-linkers and even seem to lead to more specific imprints. An example is trimethylolpropane trimethacrylate, also known as TRIM, which appears to result in well-performing MI-phases. Whereas the cross-linker ensures imprint stability and thus geometric recognition of the template, the functionalities incorporated by the functional or interactive monomer act as anchors recognizing chemical elements of the template. Both principles provide the imprints with the desired high specificity towards the template molecule. Figure 3 demonstrates the use of acidic groups as anchors for fixing the template molecule - in this example the antibiotic penicillin V. The methacrylic acid interacts with the template via hydrogen and ionic bonds involving both the amino as well as with the carboxy and the carbonyl functions of the β -lactam structure [29].

In addition to these two fundamental imprinting techniques, a hybrid of the two mechanisms has been suggested. In this case the polymerization is performed in the presence of a template covalently linked with the functional monomer, followed by a basic cleavage of the template via decarboxylation leaving imprints able to interact non-covalently with the template [21]. Furthermore, a procedure has been developed resulting in molecularly imprinted ligand-exchange adsorbents. Achiral functional monomers linked with a Cu²⁺ ion were used to chelate amino acid templates. The following cross-linking led to enantioselective MIPs to be used for chiral separations in LC [22]. However, the semi-covalent approach as well as the technique of imprinting metal-chelating complexes has not yet found broad applicability.

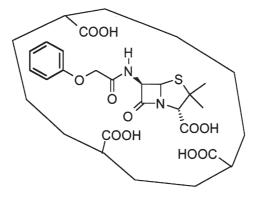


Fig. 3. Non-covalent imprinting of penicillin V

2.2 Different Formats of MIP

MIP are often generated as simple bulk polymers to be ground into fine particles, which are subsequently sieved and sedimented – admittedly a time-consuming process, which requires large amounts of solvents. The loss of fine polymer particles in the sedimentation procedure is also not negligible. The result usually is a polymer powder with particle sizes of a relative broad size distribution. After the template has been extracted, this material can be packed into LC-columns [17, 29, 30], CE-capillaries, or be used directly in the batch mode.

Due to the drawbacks of bulk polymer preparation, alternative techniques have been developed for the production of MIP. When particles are required, researchers try more and more to establish procedures resulting directly in (monosized) beads or microspheres. These processes are often dispersion or suspension polymerization, using, e. g., perfluorocarbon liquids where dispersed monomertemplate droplets with a narrow size distribution are automatically formed [81, 82]. After a final extraction step, the polymerized droplets can be applied immediately as MIP; no further processing is required. However, the result is again a polymer powder. A fundamentally different approach is establishing the MIP in a membrane format. In this case either a membrane base material such as fiberglass or cellulose is coated with an MIP [47, 83, 84] or membranes are fabricated directly from molecularly imprinted materials by a casting process [85–88]. The latter approach has been known to result in much higher capacities. Such membranes have been used successfully for separation processes; however, they are not yet competitive to columns packed with MIP.

MI-coatings and thin films are also produced for different applications such as capillary electrochromatography or sensor technologies [8, 34]. MIP have been immobilized on the tips of optic fibers or on electrodes and were shown to be able to engender a signal (fluorescence or conductivity) as the result of selective analyte binding. It should also be mentioned that molecular imprinting is not limited to the area of organic polymers, as the technique has been extended to proteins and silica as well.

3 Applications

3.1 Chromatographic Applications

3.1.1 *HPLC*

Liquid chromatography and especially HPLC is at present a most versatile tool in analytical chemistry. The separation is generally mediated by two phases, a solid one packed into the column and a mobile one, which percolates through this column. Substances are separated due to differences in the affinity towards the two phases. Nowadays different kinds of stationary phases are available for a broad range of analytical problems, including, e.g., reversed phase and ion exchange columns. However, all of these typically interact with groups of potential analytes. They are not specific for any one compound. This is advantageous in cases where HPLC-columns are required in routine applications for different kinds of analytical problems. Whenever a specific compound has to be separated from a host of chemically similar ones, they may not be the best choice. A typical example is the analysis of a protein in a biological fluid or a cell culture supernatant. In such cases biospecific affinity ligands such as antibodies are typically used to produce chromatographic columns of the required specificity. However, their use is beset with a host of technical and ethical problems. MIP present an interesting alternative in this context.

Although the first applications of MIP were in batch assays, a little later the biomimetic plastic powders also were packed into LC-columns in order to separate a given analyte (used as template) from other sample components. The idea was to use the MI-stationary phases similar to the conventional phases derivatized with chemical compounds or even biological (affinity) ligands. The first academic approaches concentrated on the generation of phases imprinted with an enantiomer in order to achieve chiral separation of the template from its optical counterpart. This demonstrated already the high potential of the MI-technique since extreme selectivities are required for such separations, while concomitantly commercially available phases for chiral separations tend to be rather expensive. Figure 4 shows the separation of the dipeptide Z-L-Ala-L-Ala from its enantiomer Z-D-Ala-D-Ala applying a phase imprinted with the L-L-peptide [17]. Although the isocratic elution (Fig. 4, right) only gave a fairly broad peak for the L-L-peptide compared to the sharp signal caused by the less retarded D-D-enantiomer, the separation was nearly quantitative. A change to gradient elution (Fig. 4, left) resulted in a baseline separation with two sharp peaks.

Not only chiral separations have been achieved with MI-stationary phases. It has also been demonstrated that the MIP could distinguish between ortho- and para-isomers of carbohydrate derivatives. For example, a polymer imprinted with *o*-aminophenyl tetraacetyl β -D-galactoside was used to analyze a mixture of *p*- and *o*-aminophenyl tetraacetyl β -D-galactoside. As expected, the imprinted ortho analyte eluted after the non-imprinted para component; see Fig. 5. Although baseline separation was not obtained, a separation factor of α =1.51 was observed [19].

An interesting example of MIP-LC analytics was presented in a paper, which focused on the separation of antibiotics of similar structures. Columns are (commercially) available to separate penicillins (β -lactams) from other antibiotics. However, if the quantification of each of the β -lactam compounds is required, a more selective stationary phase has to be found. Molecular imprinting allows the fabrication of phases specifically for each β -lactam. If for instance the concentration of the β -lactam oxacillin in a food sample has to be selectively determined, a polymer imprinted with oxacillin is the right choice. Compared to a standard stationary phase, which only allowed the separation of the entire group of β -lactams from other non- β -lactam analytes (e.g., bacitracin), the MIP enables the separation of the imprinted species from the pair of non-imprinted β -lactams penicillin V and penicillin G; see Fig. 6 [29, 30].

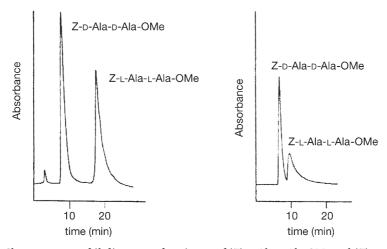


Fig. 4. Chromatogram of (*left*) 100 μg of a mixture of (*Z*)-L-Ala-L-Ala-OMe and (*Z*)-D-Ala-D-Ala-OMe on a (*Z*)-L-Ala-L-Ala-OMe-imprinted MIP-phase, gradient elution; (*right*) 1 mg of a mixture of (*Z*)-L-Ala-L-Ala-OMe and (*Z*)-D-Ala-D-Ala-OMe on a (*Z*)-L-Ala-L-Ala-OMe-imprinted MIP-phase, isocratic elution. Reprinted with permission from: Kempe M, Mosbach K (1995) Tetrahedr Lett 36:3563. Copyright 1995 Elsevier Science

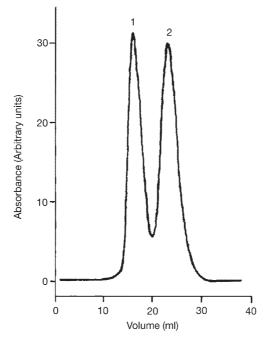


Fig.5. Separation of (1) para- and (2) ortho-aminophenyl tetraacetyl β-D-galactoside, using a polymer imprinted with *o*-aminophenyl tetraacetyl β-galactoside. Reprinted with permission from: Nilsson KGI, Sakguchi K, Gemeiner P, Mosbach, K (1995) J Chromatogr 707:199. Copyright 1995 Elsevier Science

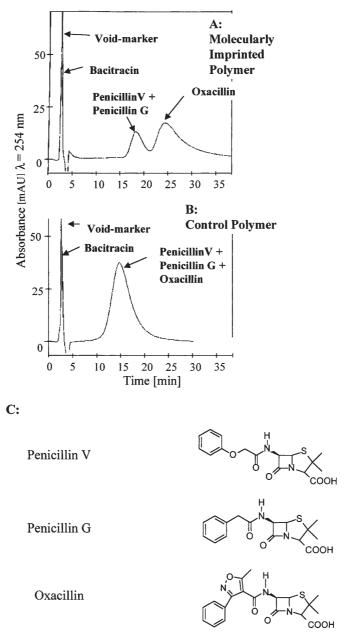


Fig. 6. A Chromatogram of a mixture containing the print molecule (oxacillin), two other β -lactam-antibiotics (penicillin G and penicillin V) and a non- β -lactam-antibiotic (bacitracin) on an oxacillin imprinted MIP containing 4-vinylpyridine residues, cross-linked with TRIM. The analysis was performed in organic mobile phase (ACN/AcOH, 99:1). **B** Same conditions but using the respective non-imprinted control polymer. **C** Structures of penicillin V, penicillin G, and oxacillin. Reprinted with permission from: Skudar K, Brüggemann O, Wittelsberger A, Ramström O (1999) Anal Commun 36:327. Copyright 1999 The Royal Society of Chemistry

These examples demonstrate the versatility of MIP in LC. Although broad peaks caused by the heterogeneity of imprint qualities are often observed for the imprinted species, MIP most certainly present an interesting alternative to traditional stationary phases in LC.

Beside the use of MIPs in conventional HPLC, MI-polymers may also be established in supercritical fluid chromatography, which is characterized by faster equilibration times combined with the use of the environmental friendly CO_2 as mobile phase. Although preliminary results show relatively broad peaks, chiral separation could be performed based on polymers imprinted with an enantiomer. However, the long-term stability of the photochemically generated polymers seems to be a problem [89].

3.1.2 Capillary Electrophoresis

Capillary electrophoresis stands for a group of separation techniques, which allow the separation of most analyte classes based on their mobility in an electrical field. In its simplest form, capillary zone electrophoresis, a fused silica capillary filled with a buffer is dipped into buffer reservoirs, which are connected to a high voltage power supply. After injection of a small volume of sample into the capillary the electric field is applied, which leads to the establishment of an electroosmotic flow forcing the whole sample plug past the detector to the cathode. Ionic analytes are in addition attracted by their respective electrodes, and thus are separated electrophoretically on the basis of their different polarities and mass-to-charge-ratios. Other separation principles used in capillary electrophoresis are isoelectric focusing based on a separation due to differences in the isoelectric points of the analytes, or gel electrophoresis, where the molecules are sieved in a gel matrix and hence are separated according to size. However, the separation of neutral compounds in such a system requires the addition of a second phase. An example is Micellar Electrokinetic Chromatography, where surfactants are added to the buffer in concentrations above the critical micelle concentration. The analytes interact with these micelles according to their hydrophobicity and hence travel either at the speed of the electroosmotic flow in the bulk buffer or at the (differing) speed of the micelles.

The principle is carried even further in capillary electrochromatography, where a true chromatographic stationary phase is introduced into the capillary. The stationary phases for these applications may be produced outside the capillary and subsequently packed into it, in a manner similar to packing HPLC columns. Alternatively, it has been shown that it is possible to generate the stationary phase in situ, i.e., within the capillary. To date reverse phase type stationary phases have mostly been used in capillary electrochromatography, although other interaction modes have been tried occasionally. For certain applications at least, the method would obviously profit from the ability to introduce a specific interaction between the stationary phase and a given analyte. However, it is problematic to rely on biological receptors to provide this selective interaction in capillary electrochromatography. MI-type stationary phases may once more have considerable advantages in terms of stability and reliability.

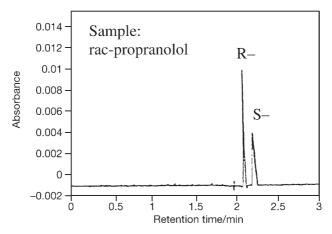


Fig. 7. Separation of *R*- and *S*-propranolol using MIP particles as chiral additive in the background electrolyte, MIP prepared using *S*-propranolol as template. Reprinted with permission from: Walshe M, Garcia E, Howarth J, Smyth MR, Kelly MT (1997) Anal Commun 34:119. Copyright 1997 The Royal Society of Chemistry

eral different techniques for producing such MIP for capillary electrochromatography have already been described in the literature. Particles molecularly imprinted with *S*-propranolol have been used as a model to evaluate the imprinting approach in a subsequent separation from its R-enantiomer; the result is shown in Fig. 7. Clearly, baseline separation could be achieved [36].

Capillaries coated with MIP have also been used successfully for chiral separation [34]. Schweitz et al. also synthesized highly porous, monolithic MI-polymers inside such capillaries for the separation of *S*- and *R*-propranolol [38, 90]. Figure 8 A shows a baseline separation of *S*-propranolol from the *R*-enantiomer in such capillaries, the latter of which was used as template. The difference in retention times for the two enantiomers was verified by analyzing the two chiral compounds also individually; see Fig. 8 B, C.

In a different approach, Lin et al. have used particles derived from a ground MI-bulk polymer and mixed with a polyacryl amide gel for chiral separation. Using a polymer imprinted with L-phenylalanine, D-phenylalanine could be separated from the template with a separation factor of 1.45 [35]. Although the combination of MIP with capillary electrochromatography is still not widely used, the ability to separate enantiomers in nanoliter samples promises interesting developments for the future.

3.1.3 Solid Phase Extraction, SPE

Currently, perhaps the most promising approach based on the use of molecularly imprinted polymers is solid phase extraction (SPE). Traditionally, when complex samples require a clean-up/enrichment step prior to high-resolution analysis,

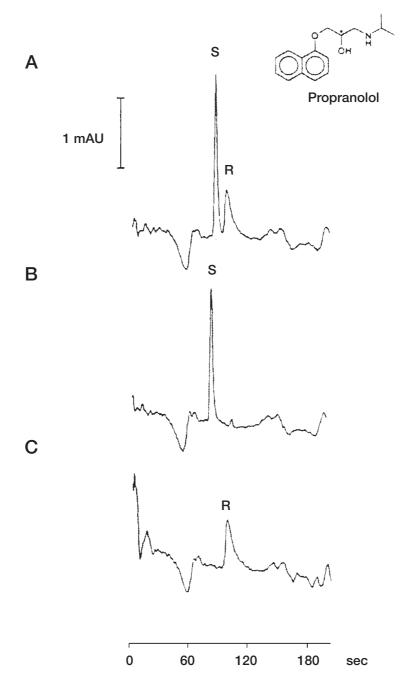


Fig.8A–C. Electropherograms of: A a racemic mixture of propranolol; **B** (*S*)-propranolol; **C** (*R*)-propranolol (using in all three experiments the MI-capillary prepared by using (*R*)-propranolol as template). Reprinted with permission from: Schweitz L, Andersson LI, Nilsson S. (1997) Anal Chem 69:1179. Copyright 1997 American Chemical Society

techniques are applied which lack specificity, e.g., liquid-liquid-extractions or flush chromatography with naked silica phases. In principle, solid phase extraction is also based on differences in interaction of the analytes with a solid phase. Other than LC, SPE is a one-stage procedure relying on a single adsorption/desorption event for separation. High selectivity is thus an advantage and biospecific ligands have been suggested for SPE as well. However, the difficulties of using such ligands with crude and complex samples are non-negligible and once more MI presents a cheap and powerful alternative. MI-phases are capable of extracting only the compound, which has been used as the template from the complex mixture, thus preparing it for further analysis, e.g., by LC, CE, or GC. Especially the analysis of urine or blood samples benefits from such a specific extraction step due to the large number of substances present in these sample matrices.

In addition to their high specificity, MI solid phases offer the option of oneway use, because their starting materials and production processes are inexpensive. For example, polymers imprinted with the triazine herbicide atrazine were to be used for the pre-concentration of simazine, a related triazine herbicide, from water. Using a typical recipe based on MAA as functional monomer and EGDMA as cross-linker, the polymerization was performed in aqueous suspension. The obtained beads were chromatographically tested with respect to their affinity towards the template atrazine, resulting in atrazine capacity factors of 31.2 for the MIP and 2.5 for the control polymer (CP). For simazine, values of 25.4 and 2.3 were measured for the MIP and CP respectively, demonstrating the high selectivity of the MIP. Subsequently, the MIP beads were packed into a column for the selective extraction of simazine from aqueous samples, containing simazine and other structurally non-related herbicides. After sample loading, washing, and eluting of the adsorbed substances, the analysis of the extract showed pure simazine with a recovery rate of 91% after a third elution step [39]. The separation performance for these samples could be improved by the use of polymers imprinted with dibutylamine. Atrazine was recovered with a yield of 96.8% [41]. MIP imprinted with terbutylazine have been shown to improve the analytical determination of pesticides in natural waters or sediments. Figure 9 shows the LC chromatograms of groundwater samples spiked with six chlorotriazines at concentrations of 1 μ g l⁻¹ after SPE either with an unspecific polymer (Fig. 9A) or with a MIP-phase (Fig. 9B). Only the chlorotriazine analytes were selectively bound to the latter and could be recovered with a yield of about 80% [42].

When environmental water was analyzed with respect to a possible contamination with 4-nitrophenol, it could be shown that by using an MI-SPE polymer selective for 4-nitrophenol the following LC-analysis was much facilitated due to the cleaner matrix and the reduction of interference caused by humic acids [91].

The direct comparison of a standard liquid-liquid extraction with a liquid-liquid extraction followed by MI-SPE demonstrated the enhancing character of the MI-SPE technique. In GC chromatograms, peak heights of sameridine extracted from spiked human plasma were increased by a factor of 5 after introducing an SPE step; see Fig. 10. Besides that, the number of impurities was noticeably reduced by this selective extraction step [92–94]. Interestingly, in this case it was possible to use as template a structure which was a little different from that of the analyte (R_2 = methyl for the template and R_2 = ethyl for the analyte sameridine).

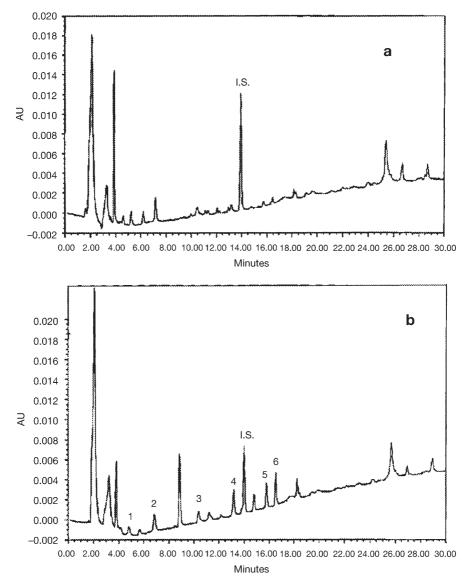


Fig. 9a, b. Chromatograms obtained after pre-concentration of a 100 ml groundwater sample spiked at 1 μ g l⁻¹ through: **a** a CP-cartridge; **b** a cartridge filled with a polymer imprinted with terbuthylazine. Peaks: 1=deisopropylatrazine, 2=deethylatrazine, 3=simazine, 4=atrazine, 5=propazine, 6=terbuthylazine, I.S.=internal standard (diuron). Reprinted with permission from: Ferrer I, Lanza F, Tolokan A, Horvath V, Sellergren B, Horvai G, Barcelo D (2000) Anal Chem 72:3934. Copyright 2000 American Chemical Society

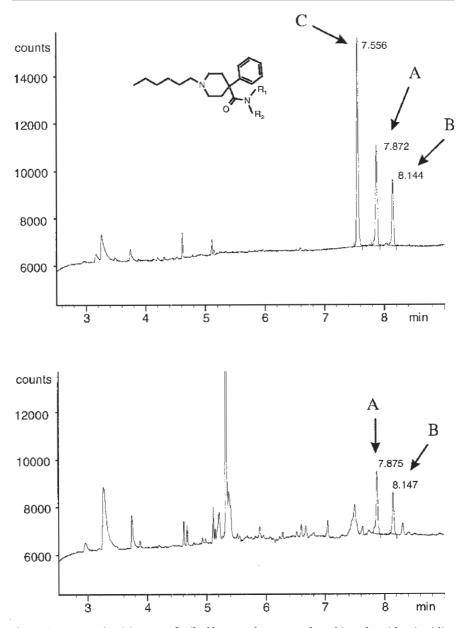


Fig. 10. Representative GC-traces of spiked human plasma samples subjected to either (*top*) liquid-liquid extraction followed by MI-SPE or (*bottom*) standard liquid-liquid extraction only. Human plasma was spiked with 66.8 nmol/l of sameridine and 50.2 nmol/l of internal standard. A: sameridine (R₁=methyl, R₂=ethyl), B: internal standard, C: imprint species (R₁=methyl, R₂=methyl). MIP was composed of MAA and EGDMA. Reprinted with permission from: Andersson LI (2000) J Chromatogr 739:163. Copyright 2000 Elsevier Science

Pre-concentration of bupivacaine from human plasma was performed with an MIP, followed by elution, and analysis via gas chromatography also demonstrated the high specificity of the SPE based on a MIP compared to a CP; see Fig. 11. Compared to the MI-SPE method, SPE on a C18-column led to an extraction of not only the desired bupivacaine but also of many other ingredients of the plasma sample, thus complicating the subsequent analysis [43].

In another example, urine samples were extracted with MIP phases imprinted with clenbuterol in order to determine the concentration of this β -agonist, which is known to be misused in animal breeding and thus is occasionally found as a food contaminant. Recovery rates of up to 75% were observed for spiked samples when extracting the clenbuterol. However, in subsequent control experiments clenbuterol was detected also in non-spiked blank urine samples, and further experiments lead to the conclusion that the clenbuterol used as template permanently bled from the MI-polymer. Consequently, the authors decided to use in the future a structural analogue as template instead of clenbuterol in order to avoid this problem [44].

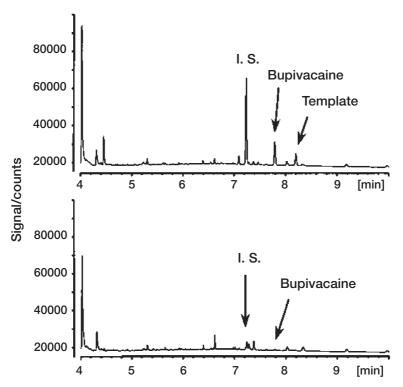


Fig. 11. Representative GC-traces of spiked human plasma subjected to (*top*) MI-SPE and (*bot-tom*) SPE with a non-imprinted control polymer. Plasma was spiked with 160 nmol/l bupivacaine. MIP was composed of MAA and EGDMA and imprinted with pentycaine as a structural analogue to bupivacaine. Reprinted with permission from: Andersson LI (2000) Analyst 125:1515. Copyright 2000 The Royal Society of Chemistry

In a further application of MI-SPE, theophylline could be separated from the structurally related caffeine by combining the specific extraction with pulsed elution, resulting in sharp baseline-separated peaks, which on the other hand was not possible when a theophylline imprinted polymer was used as stationary phase for HPLC. A detection limit of 120 ng ml⁻¹ was obtained, corresponding to a mass detection limit of only 2.4 ng [45]. This combination of techniques was also used for the determination of nicotine in tobacco. Nicotine is the main alkaloid in tobacco and is the focus of intensive HPLC or GC analyses due to its health risk to active and passive consumers. However, HPLC- and GC-techniques are time-consuming as well as expensive, due to the necessary pre-purification steps required because the sample matrices typically contain many other organic compounds besides nicotine. However, a simple pre-concentration step based on MI-SPE did allow faster determination of nicotine in tobacco samples. Mullett et al. obtained a detection limit of 1.8 μ g ml⁻¹ and a mass detection limit of 8.45 ng [95]. All these examples demonstrate the high potential of MI-SPE to become a broadly applicable sample pre-purification tool.

3.2 Non-Chromatographic Applications

3.2.1 Sensors

Today the ability to determine fast and reliably concentrations of the main components as well as of certain key trace components or toxic agents in biotechnological, biochemical, pharmaceutical, or food samples represents a major challenge to analytical chemistry. Specific sensors based on transducers such as microchip or fiber optics have been developed, which in combination with a more or less specific recognition element allow the detection of nearly all target molecules even in complex sample matrices. The main components of such sensor systems are first of all an element capable of recognizing as specifically as possible the analyte of interest and in addition a component, which is capable of transducing the signal generated by the recognition event into a measurable signal. Not surprisingly, MIP are more and more frequently suggested as specific and robust recognition elements in sensor technology. They are mostly bonded in the form of thin MIP-coatings to various transducing elements. Many types of signals and hence transduction principles have already been used in connection with MIP-sensing including optical signals such as luminescence, colorimetry or fluorescence, electrochemical principles like conductometry, capacitance or amperometry, but also mass-sensitive systems like quartz crystal microbalances (QCM), or surface acoustic waves (SAW). An overview of these techniques is given in Table 4.

Quite a number of recent papers have concentrated on techniques which allow the gravimetric detection of only a few femtograms of an analyte [105]. In one of these, PAHs could be measured in concentration of down to a few ng ml⁻¹ in degraded oil when a piezoelectric quartz crystal coated with MI-polymers imprinted either with a fresh or with a degraded lubricant was immersed into the

Transducer	References	
Ellipsometry	[96]	
Potentiometry	[97]	
Amperometry	[98]	
Conductometry	[47, 48, 83, 99, 100]	
Capacitance (field-effect)	[51, 101]	
Luminescence	[102]	
Fluorescence	[8, 53, 103]	
Quartz crystal microbalance	[55-61, 104]	

	Table 4.	Transducers f	for MIP-based	sensors
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oil sample. Since the degradation of oil causes a change in its viscosity, while an MIP-sensor concomitantly prefers the type of oil used as template, the resonance frequency shift of the quartz (due to recognition) showed explicit dependence on the degradation grade of the oil [60]. In this way PAH concentrations could even be determined in aqueous samples [61].

In another application, a thin MIP layer imprinted with dansyl-L-phenylalanine was immobilized on a QCM electrode and placed in a liquid medium for enantiomer analysis. The frequency shift caused by the L-enantiomer (template) was more pronounced than the effect caused by the adsorption of the dansyl-Dphenylalanine [56]. Such an enantio-discrimination via QCM was also established for S- and R-propranolol. In this case the gold electrode of a quartz crystal was coated with an S-propranolol-imprinted MIP. Afterwards, S-propranolol could be detected down to concentrations of 50 µmol l⁻¹ [58]. A glucose QCM sensor was developed by producing MIP-films on conducting electrodes via electropolymerization. Poly(o-phenylenediamine) was chosen as matrix to be imprinted with glucose and one of the platinum electrodes of the quartz crystal was used as working electrode during polymerization [55]. A low detection limit of 200 µg l⁻¹ for odorants such as geosmin in the gas phase was obtained with a similar QCM device combined with a polymer film imprinted with 2-methylisoborneol [104]. Other similar sensor systems were used for determination of caffeine with a detection limit of 5×10^{-9} moll⁻¹ [57] or for detecting phenacetin in urine and human serum showing similar detection limits. Figure 12 demonstrates the high selectivity of an MIP-QCM sensor towards the analgesic phenacetin compared to non-imprinted structural analogues like paracetamol, antifebrin, and phenetole [59].

In a different approach, optical fibers were dip coated with MIP-films imprinted, e.g., with dansyl-L-phenylalanine for the detection of this compound in a variety of fluids. The compound adsorbed selectively on the MIP-film, emitting a fluorescence signal when excited by light coming from the fiber. The generated signal was transported back by fiber and could be used for quantification of the concentration of the adsorbed dansyl-L-phenylalanine [8]. This technique has been further developed for the detection of pesticides and insecticides in water by incorporating luminescent europium into the MI-polymer working as signal transducer. When choosing glyphosate as template the resulting MIP provided a

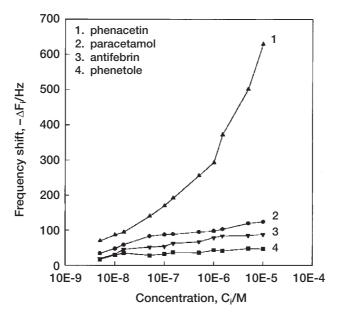


Fig. 12. Response of the MIP sensor to phenacetin (curve 1), paracetamol (curve 2), antifebrin (curve 3), phenetol (curve 4). The same electrode was used for all detections in a 10-ml sample volume (aqueous system). MIP was composed of MAA and EGDMA and imprinted with phenacetin. Reprinted with permission from: Tan Y, Peng H, Liang C, Yao S (2001) Sensors Actuators B 73:179. Copyright 2001 Elsevier Science

detection limit of 9 ppt. The imprinting of diazinon as representative of the organothiophosphates resulted in an even lower detection limit of 7 ppt [102].

Other workers have concentrated on fluorescence as transduced signal in connection with MIP, e.g., for quantification of 3-hydroxyflavon in a flow-through opto-sensing device [53], or for the fluorescence detection of cAMP with a fluorescent dye as an integral part of the recognition site, based on a fluorescence quenching effect in the presence of the imprinted molecule [103]. In other publications membranes have been used as conductometric sensors when being imprinted with L-phenylalanine or sialic acid. Detection was possible at analyte concentrations of 1-50 µmol l⁻¹ in solution [83]. MIP membranes have also been used for the conductometric determination of atrazine in aqueous matrices. The imprinted analyte could be detected at concentrations down to 5 nmol l⁻¹ within a response time of 6 – 15 min [48]. Furthermore, cholesterol-specific MIP films on gold electrodes were used to detect cholesterol in a range of $15-60 \text{ }\mu\text{mol} \text{ }l^{-1}$ within an analysis time of 5 min [52]. An interesting approach was presented recently which consisted of a combination of a field-effect transistor (FET) and molecular imprinting. In this case, it was not a synthetic polymer but TiO₂ which was imprinted with herbicides such as 4-chlorophenoxyacetic acid or 2,4dichlorophenoxyacetic acid (2,4-D). The ion-sensitive FET was applied for the determination of both substances as sodium salts, and resulted for 2,4-D in a detection limit of 1×10^{-5} mol l⁻¹ [51]. The examples presented here should serve

to give an impression for the versatility of the MIP-sensor approach. For more information a recent review on MIP and their use in biomimetic sensors is recommended [106].

3.2.2 Catalysis

Although MIP are mostly generated to be utilized as binding or capturing agents, they can also be applied as "artificial enzymes". Enzymes are known to interact via a specific binding site with a respective substrate or better transition state molecule. The strong binding is responsible for lowering the activation energy of the transition state during conversion of the substrate towards the product. However, as typical proteins, enzymes tend to denaturate at extreme pH, elevated temperatures, or under "non-physiological conditions" in general. Thus, for many catalytic bioconversion processes it would be advantageous to use catalysts of higher durability than enzymes. Consequently, molecular imprinting has been suggested as tool for the generation of biomimetic catalysts. Other than for imprinting capturing devices, however, the template should be different from the substrate but

		•		
Type of reaction	Substrate	TSA	Relative catalytic effect of the MIP	Refer- ence
Hydrolysis	Nitrophenyl ester	Pyridin-derivatives of N-Boc-aminoacids	5	[108]
	<i>p</i> -Nitrophenol acetate	<i>p</i> -Nitrophenylmethyl phosphonate	1.6	[109]
	Aminoacid ester	Phosphonate	3 2.54	[110] [111]
Dehydro- fluorination	4-Fluoro-4- (<i>p</i> -nitrophenyl)- 2-butanone	N-Benzyl-N- isopropylamine	2.4	[63]
		Benzylmalonic acid	3.2	[112]
		N-Methyl-N-(4-nitro- benzyl)-δ-aminovaleric acid	3.3	[113]
		<i>N</i> -Isopropyl- <i>N</i> - <i>p</i> -nitro- benzylamine	3.27	[62]
		N-Benzyl-N-isopropylamine	5.97	[64]
Diels-Alder reaction	Tetrachlorothiophen- dioxide + maleic anhydride	Chlorendic anhydrid		[114]
Aldol con- densation	Acetophenone and benzaldehyde	Dibenzoylmethane (DBM) + Co ²⁺	2	[115]
Isomerization	Benzisoxazol	Indol	7.2	[65]

Table 5. Examples of reactions catalyzed by MIPs

also from the product of the reaction of interest and resemble more the transition state. In fact, both substrate and product when used as templates would most likely inhibit the catalytic process due to strong and even irreversible binding to their respective MIP. However, due to the fact that the transition state itself is usually non-stable, such molecules are also not available as templates and therefore stable analogues of the transition state have to be found or synthesized. Such a use of transition state analogues (TSA) as template molecules has been proven to be efficient for developing catalytic (proteinous) antibodies [107]. For every reaction a specific template must be chosen. This requires profound knowledge of the mechanisms of the enzyme reaction and the structure of the transition state. Only a few reactions have to date been described sufficiently well to allow catalyzation by MIP. This small set of examples includes mainly hydrolyses of esters and dehydrofluorinations; see also Table 5.

The saponification of the ester is a process where the trigonal carbon of the substrate passes through a transition state characterized by a tetrahedral structure to be transformed into a product, which again has a trigonal configuration. The challenge is to find a stable TSA with four tetrahedrally ordered ligands including two with oxygen groups, which is typical for this reaction. The problem could be solved by choosing a phosphonic ester with phosphor having four ligands with two of them oxygen, one with a double bond. MIP imprinted with such phosphonates showed clear catalytic effects in the hydrolyses of structurally related carbon esters, although catalytic effects did not exceed a value of 3 when comparing MIP and CP [109–111]. Using pyridine derivatives of *N*-Boc-amino acids instead, an acceleration of the reaction with a factor of 5 was obtained [108].

Comparable effects were observed in the catalysis of the dehydrofluorination of 4-fluoro-4-(*p*-nitrophenyl)-2-butanone using MI-polymers or even MI-proteins (see later) imprinted with suitable TSA templates. Focusing on the two sp³ hybridized carbons (C2 and C3) of the butanone which are converted to sp² carbons as a result of dehydrofluorination, the search for the right TSA led to *N*-benzyl-*N*-isopropylamine (Fig. 13) either containing a nitro-group [62] at the aromatic ring or not [63, 64].

Reaction to be catalysed:

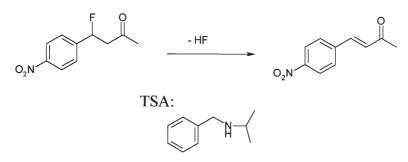


Fig.13. Dehydrofluorination reaction catalyzed by a MIP imprinted with the TSA *N*-benzyl-*N*-isopropylamine

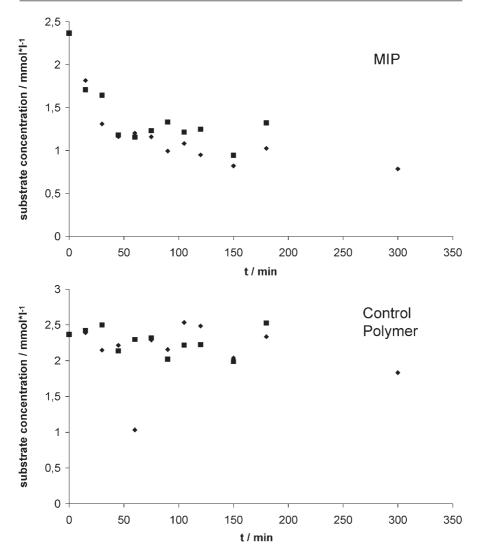


Fig. 14. Application of an *N*-benzylisopropylamine imprinted MAA/EGDMA copolymer as catalyst for the dehydrofluorination of 4-fluoro-4-(*p*-nitrophenyl)-2-butanone in a batch reactor. Given is the substrate concentration versus time. The reaction was carried out at 50° C in 10 ml of a mixture of water and acetonitrile 1:1 (v/v), containing 5 mg of the substrate 4-fluoro-4-(*p*-nitrophenyl)-2-butanone (i.e., a final concentration of 2.4 mmol/l) and 500 mg MIP or non-imprinted control polymer (CP). *Top*: use of MIP, 1. experiment (\blacklozenge), 2. experiment (\blacksquare). *Bottom*: use of CP, 1. experiment (\blacklozenge), 2. experiment (\blacksquare). Reprinted with permission from: Brüggemann O (2001) Anal Chim Acta 435:197. Copyright 2001 Elsevier Science

The early results did not lead to increases of the catalytic effects by more than a factor of 3.3 compared to the CP, observed when applying imprinted materials either generated by executing the imprinting procedure as usual with monomers or by using proteins such as BSA as imprinting matrices. However, one recent publication, which focused on the optimization of catalysis from a chemical reaction engineering point of view, reported on an acceleration of the dehydrofluorination by a factor of 5.97 when an EGDMA/MAA-based MIP imprinted with *N*-benzyl-*N*-isopropylamine was used. This was achieved in a batch reactor by systematically varying parameters such as the temperature, the substrate concentration, or the amount of MI-catalyst used. The highest catalytic effect was observed at a temperature of 50 °C when catalyzing the dehydrofluorination of 5 mg of 4-fluoro-4-(p-nitrophenyl)-2-butanone with 500 mg MIP in 10 ml water/acetonitrile 1:1 (v/v). Figure 14 (top) shows the degradation of the substrate in the presence of the MI-catalyst within 40 min, followed by a drastic slowdown. The average rate constant calculated from the two experiments was $k_{MIP} = 2.96 \times 10^{-3}$ min⁻¹. For the control experiment the MI-catalyst was replaced by 500 mg of a CP, Fig. 14 (bottom). Only a marginal decrease of the substrate concentration was measured with a corresponding average rate constant of only $\overline{k_{CP}} = 4.96 \times 10^{-4} \text{ min}^{-1} [64].$

One publication discussing the use of a MIP as catalyst for the isomerization of benzisoxazol presents an even higher relative effect with an acceleration of 7.2 compared to the CP [65].

The transfer hydrogenation of aromatic ketones, which is typically catalyzed by ruthenium half-sandwich complexes using, e.g., formic acid as hydrogen source, was chosen as another model system. After applying an appropriate TSA molecule as template, i.e., a ruthenium phosphinato complex, the resulting MIP catalyzed the hydrogenation of benzophenone approximately twice as effectively as the CP [116].

Enzymes are known to show high enantio-selectivity, which is a parameter one wishes to install in the MIP as well. That this is possible was demonstrated in a recent paper on enantio-selective ester hydrolysis catalyzed by MIP. The MIP imprinted with the D-enantiomer preferentially hydrolyzed the D-ester with rate enhancements of up to three compared to the CP [117]. Although these findings may be far from outstanding, they represent remarkable results on the route towards the generation of competitive biomimetic catalysts.

3.2.3 Biomimetic Assays

The enzyme linked immunosorbent assay (ELISA) is known as a well performing, highly specific immunoassay. This binding test is based on antibodies immobilized in excess on suitable surfaces, which specifically capture their particular antigens from complex solutions. In a second step a second type of antibody (detection antibody) labeled with an enzyme is bound to another epitope of the antigen. The more antigen is attached to the immobilized capturing antibody, the more enzyme linked antibodies will be bound. After a washing step, the addition of a substrate solution to the linked enzymes, followed by an incubation step, results in the degradation of the substrate into a (usually colored) product. The intensity of the color gives direct information about the amount of bound enzyme and, thus, an indirect indication of the antigen concentration in the sample. This type of assay and a host of related immunoassays are used world-wide in medical and biochemical analyses and represent one of the most sensitive types of quantitative analytical tools. However, both the antibodies and the enzyme used are sensitive (proteinaceous) materials and therefore the use of stable MIP both for capturing and as detection "enzyme" was just a matter of time.

Monodisperse microspheres imprinted with theophylline or 17β -estradiol were used in competitive radioimmunoassays showing the MIP's high selectivity for the template molecule. In this case the assay is based on the competition of the target molecule with its radioactively labeled analogue for a limited number of "antibody" binding sites [77, 118]. Figure 15 demonstrates that displacing the radioactively marked theophylline from the imprinted polymer was only possible with theophylline as competitor. Structurally related molecules showed effects solely at elevated concentrations [77].

Magnetic (iron oxide core) microspheres have been imprinted with *S*-propranolol. The magnetism allowed the facile separation of the imprinted beads from the liquid matrix. The particles exhibited the expected affinity towards the template molecule. This technique was also proposed as a putative tool for cell sorting [81].

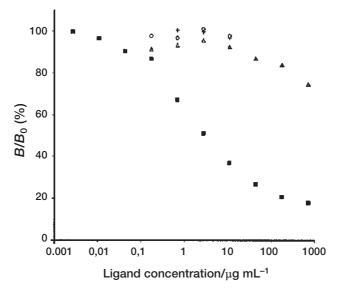


Fig. 15. Displacement of radio-labeled analyte analog binding to MI spheres under equilibrium condition. B/B_0 is the ratio of the amount of radio-labeled ligand bound in the presence of the displacing ligand (analyte), B_0 . Displacement of $[8-^3H]$ theophylline binding to a polymer imprinted with theophylline. Displacing ligands: theophylline (\blacksquare); theobromine (+); xanthine (\bigcirc); caffeine (Δ). Reprinted with permission from: Ye L, Cormack PAG, Mosbach K (1999) Anal Commun 36:35. Copyright 1999 The Royal Society of Chemistry

The use of an enzyme tag but also of radioactivity is avoided when applying a non-related fluorescent probe as competitor which itself binds to the imprinted polymer. In this manner, the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) could be detected in a binding assay with a detection limit of 100 nmol l⁻¹ in both aqueous and organic media when using a 2,4-D imprinted polymer and 7-carboxymethoxy-4-methylcoumarin as fluorescent probe [67]. A chemiluminescence imaging ELISA was presented based on an MIP as recognition element and an analyte labeled with an enzyme. The herbicide 2,4-D was once more chosen as template. Later a competitive assay was developed for 2,4-D using 2,4-D labeled with tobacco peroxidase as competitor for the binding sites. Thereby, the concentration of 2,4-D in the sample could be quantified down to a concentration of 0.01 µg ml⁻¹ [68].

3.2.4 Screening of Combinatorial Libraries

Molecularly imprinted polymers have been used in screening procedures in two different ways. Either a library of MIP was created in a combinatorial fashion to find the best performing artificial receptor for a selected ligand, or MIP were used for screening libraries of various structurally similar and hence difficult to separate compounds. One of the earliest papers in that field described the automated in-situ generation of an array of differently composed MIP as bottom coatings in glass vials. The MI-polymers imprinted either with atrazine or ametryn were examined in binding assays using both templates as ligands. It was found that the use of MAA as an interactive monomer in the imprinting process resulted in the most efficient atrazine receptors while TF MAA was the best choice for producing ametryn receptors. Not only the best interactive monomer was determined in each case, but also its right concentration and molar ratio within the monomer/cross-linker-composition [119]. A similar approach was described for screening large groups of MIP imprinted with terbutylazine using different functional monomers [120]. MI-generated combinatorial libraries were utilized as mixed stationary phase in an LC column for the simultaneous separation of different derivatives of amino acids and peptides. By using only pure enantiomers as templates it was possible to separate several racemates in a single chromatographic run, although the chromatograms showed the typical broad peak shapes [121].

A single biomimetic receptor was also used for screening an entire phage display hexapeptide library. The MI-polymer imprinted with yohimbine was used as α_2 -adrenoreceptor-analogue in this case. However, the measured affinity of the phages for the yohimbine MIP was similar to that of the CP, respectively a polymer imprinted with corynanthine, a compound that is structurally related to yohimbine. It was pointed out that presumably the phages had problems to access the imprints, while the heterogeneity of the binding site population led to difficulties unknown in the case of true bio-receptors with their consistent properties [122]. In another publication combinatorial libraries of steroids were screened using MI-polymers imprinted with either 11- α -hydroxyprogesterone or corticosterone. The results obtained in this work clearly demonstrated the util-

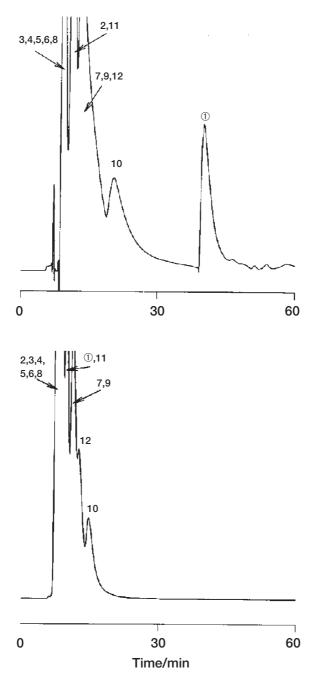


Fig. 16. Screening of a steroid library; using (*top*) MIP prepared against $11-\alpha$ -hydroxyprogesterone (1), gradient elution; using (*bottom*) non-imprinted control polymer, isocratic elution. Reprinted with permission from: Ramström O, Ye L, Krook M, Mosbach K (1998) Anal Commun 35:9. Copyright 1998 The Royal Society of Chemistry

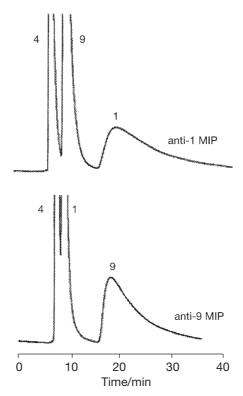


Fig. 17. Chromatographic confirmation of the imprinting effect. Applying (*top*) polymer ("anti-1 MIP") imprinted with 11- α -hydroxyprogesterone ("1"), and (*bottom*) polymer ("anti-9 MIP") imprinted with 11-deoxycortisol ("9"). Component "4": progesterone. Reprinted with permission from: Ye L, Yu Y, Mosbach K (2001) Analyst 126 (Advance Article). Copyright 2001 The Royal Society of Chemistry

ity of MIP for selective separation of the imprinted compound from other analytes in libraries, and in addition that MIP can discriminate between analytes with only small structural differences. Figure 16 shows the screening of a steroid library based on an MI-polymer imprinted with $11-\alpha$ -hydroxyprogesterone combined with a gradient elution HPLC. The last eluted peak (1) corresponds to the $11-\alpha$ -hydroxyprogesterone (Fig. 16, top), which could not be separated from the other compounds when the CP column was used instead (Fig. 16, bottom) [79].

Comparable results were presented when the number of MI-polymers was extended to other templates of similar steroid libraries. The use of a polymer imprinted with 11-deoxycortisol in a separation of 11- α -hydroxyprogesterone, progesterone and 11-deoxycortisol resulted in a chromatogram where the 11-deoxycortisol eluted last (Fig. 17, bottom). When an 11- α -hydroxyprogesterone imprinted polymer was used, on the other hand, the 11- α -hydroxyprogesterone was found to elute last (Fig. 17, top) [80].

3.2.5 Non-Polymeric Matrices for Molecular Imprinting

Molecular imprinting is not limited to organic polymer matrices, but can also be applied to silica-based materials and even proteins. Proteins freeze-dried in the presence of a transition state analogue as template have been used successfully as catalysts, e.g., for the dehydrofluorination of a fluorobutanone. For instance, lyophilized β -lactoglobulin imprinted in this manner with *N*-isopropyl-*N*-ni-trobenzyl-amine could accelerate the dehydrofluorination by a factor of 3.27 compared to the non-imprinted protein; see Table 5 [62]. In a similar procedure, BSA was imprinted with *N*-methyl-*N*-(4-nitrobenzyl)- δ -aminovaleric acid and showed an enhancement of the catalytic effect by a factor of 3.3 compared to the control protein for the same reaction; see Table 5 [113].

The use of silica as matrix has been developed even further. In the beginning, research was focused on generating affinity phases. Sol-gel glasses imprinted with uranyl ions were found to show significantly higher affinities towards the template. Interestingly, the concept of adsorption of the uranyl ion was explained by a cation-exchange equilibrium with a strong pH-dependence [123]. However, the long preparation period of four weeks for the glasses has to be regarded as a disadvantage, when comparing to the much shorter polymerization procedures for standard MIP, which seldom surpass 12 h. Catalytically active sol-gel matrices were also developed in the form of shape selective MI-glasses imprinted with aromatic rings carrying three 3-aminopropyltriethoxysilane groups. Using these MI-glasses as catalysts, the Knoevenagel condensation of malononitrile with isophthalaldehyde was clearly accelerated [124].

Silica particles surface-imprinted with a TSA of α -chymotrypsin were applied for the enantio-selective hydrolyzation of amides. Surprisingly, the particles showed reverse enantio-selectivity, i. e., the sol-gel imprinted with the L-isomer of the enzyme's TSA showed a higher selectivity for the D-isomer of the substrate [125]. Also TiO₂ gels have been imprinted, e.g., with 4-(4-propyloxyphenylazo)benzoic acid. QCM coated with ultrathin films of this gel were prepared by an immersion process and showed selective binding of the template [126]. These examples demonstrate once more the broad applicability of the concept of molecular imprinting.

4 Future Outlook

Beside the many advantages of MI, e.g., in regard to the versatility of the approach in terms of the numerous application and matrices, the simplicity of generating MI-surfaces, their durability and low costs, imprinted polymers admittedly also show some disadvantages. First of all, the heterogeneity of the imprint population leads to broad peaks or non-satisfying resolutions of the analytes. Second, it is very difficult to perform non-covalent imprinting in aqueous media, since water acts as a strong competitor in the non-covalent interactions. However, such aqueous media would in principle be the preferred medium for the imprinting of many biomolecules including native proteins. Beside that, the typical MIP is made with a high amount of cross-linker. This leads to rather rigid polymers, which do not allow for an "induced-fit", as is typical for many biological enzymes.

While a variety of small molecules have been used as template for MI, the most important question for the immediate future clearly is whether molecular imprinting will also be applicable for large biomolecules such as proteins, DNA, or even complete cells. It has already been shown that polymeric receptors for individual DNA-bases can be generated via MIP [26, 127]. However, the imprinting of complete DNA strands or at least of oligomers has not been achieved yet. The use of proteins as templates has been published a few times. In one pertinent example, glass surfaces have been imprinted with complete proteins via radiofrequency glow-discharge plasma deposition (RFGD) [128]. The proteins were first immobilized on mica and then coated with sugar molecules to be covered with a plasma film. After attaching the plasma film on the glass, mica and template were removed leaving just the polysaccharide cavities; see Fig. 18. The surfaces created by this process were subsequently able to specifically recognize the chosen large template molecules like BSA or fibrinogen.

Rather than using the protein molecule as a whole, the imprinting of selected protein epitopes may present a more practical approach. Imprints of such "patches" may then act as receptors for these parts of the protein. It could be shown that an MIP imprinted with a tetrapeptide was able to recognize not only the template but also a protein bearing the same 3-amino acid terminus as the peptide template [129]. If this approach proves to be successful in other cases as well, MI-based recognition will no longer be limited to small molecules. The result will be even more "antibody like" biomimetic polymers.

The improvement of "enzyme like" MIP is currently another area of intense research. Beside the use of the MIP themselves as catalysts, they may also be applied as enhancer of product yield in bio-transformation processes. In an exemplary condensation of *Z*-L-aspartic acid with L-phenylalanine methyl ester to *Z*-aspartame, the enzyme thermolysin was used as catalyst. In order to shift the equilibrium towards product formation, a product imprinted MIP was added. By adsorbing specifically the freshly generated product from the reaction mixture, the MIP helped to increase product formation by 40% [130]. MIP can also be used to support a physical process. Copolymers of 6-methacrylamidohexanoic acid and DVB generated in the presence of calcite were investigated with respect to promotion of the nucleation of calcite. Figure 19 (left) shows the polymer surface with imprints from the calcite crystals. When employing these polymers in an aqueous solution of Ca²⁺ and CO₃²⁻ the enhanced formation of rhombohedral calcite crystals was observed; see Fig. 19 (right) [131].

As most other MIP, current imprinted enzyme mimics are based on highly cross-linked polymers. However, such "artificial enzymes" lack solubility in most solvents and do not permit an "induced fit" when interacting with the substrate. In a rather interesting recent publication, a cross-linked microgel was instead chosen as imprinting matrix to allow formation of a specific – yet flexible – cavity as well as solubility in solvents such as dimethylformamide. First results showed that specific recognition was possible with these MIP, but usability in homogeneous catalysis still has to be proven [132]. When focusing on enzyme-

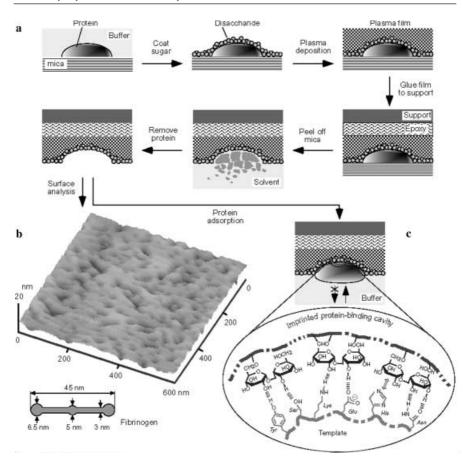


Fig. 18a-c. Protocol for imprinting of proteins: a template protein adsorbed onto a freshly cleaved mica surface in citrated phosphate-buffered saline, pH 7.4. A 1 to 10 mmol/l solution of disaccharide was spin-cast to form a 10-50 Å sugar overlay. The sample was put into the inglow region of a 13.56 MHz RFGD reactor. Plasma deposition of C_3F_6 was conducted at 150 mtorr and 20 W for 3-6 min, forming a 10-30-nm fluoropolymer thin film. The resulting plasma film was fixed to a glass cover slip using epoxy resin and oven-cured. The Mica was peeled off and the sample was soaked in a NaOH/NaClO (0.5/1.0%) solution for 0.5-2 h for dissolution and extraction of the protein. A nanopit with a shape complementary to the protein was created on the imprint surface; b a tapping mode AFM image of the surface of a fibrinogen imprint, together with a drawing of fibrinogen; c mechanisms for the specific protein recognition of template-imprinted surfaces. A nanocavity-bound template protein is prevented from exchange with other protein molecules in the solution because of steric hindrance and an overall strong interaction; the latter is due to many cooperative weak interactions, involving hydrogen bonds, van der Waals forces and hydrophobic interactions for example. Reprinted with permission from: Shi H, Tsai W-B, Garrison MD, Ferrari S, Ratner BD (1999) Nature 398:593. Copyright 1999 Macmillan Magazines Ltd

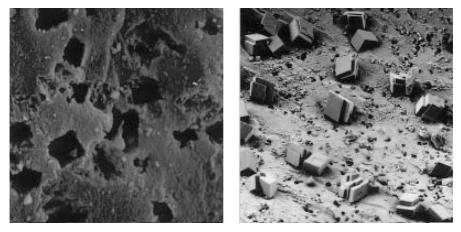


Fig. 19. SEM: *left*: a calcite-imprinted polymer surface after HCl/MeOH wash; *right*: nucleation of calcite at the imprinted polymer surface in the presence of CaCl₂ (1.0 mmol/l), Na₂CO₃ (0.8 mmol/l). Reprinted with permission from: D'Souza SM, Alexander C, Carr SW, Waller AM, Whitcombe MJ, Vulfson EN (1999) Nature 398:312. Copyright 1999 Macmillan Magazines Ltd

like polymers with higher flexibility, other efforts concentrated on the use of liquid crystalline networks in order to lower the cross-linker content of the MIP, thus reducing its stiffness [133].

Various novel imprinting techniques have also been presented recently. For instance, latex particles surfaces were imprinted with a cholesterol derivative in a core-shell emulsion polymerization. This was performed in a two-step procedure starting with polymerizing DVB over a polystyrene core followed by a second polymerization with a vinyl surfactant and a surfactant/cholesterol-hybrid molecule as monomer and template, respectively. The submicrometer particles did bind cholesterol in a mixture of 2-propanol (60%) and water [134]. Also new is a technique for the orientated immobilization of templates on silica surfaces [135]. Molecular imprinting was performed in this case by generating a polymer covering the silica as well as templates. This step was followed by the dissolution of the silica support with hydrofluoric acid. Theophylline selective MIP were obtained.

In conclusion, molecularly imprinted polymers and related materials have every potential to become popular tools in analytical chemistry, catalysis, and sensor technology. Obviously this will require further research, especially in the "problem areas" of MI mentioned above. Nevertheless, the author of this contribution fully expects that in the near future MIP will become real competitors for biological enzymes or antibodies, and thus will have a major impact on the whole area of biotechnology.

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Chromatographic Reactors Based on Biological Activity

Ales Podgornik¹ · Tatiana B. Tennikova²

- ¹ BIA Separations d. o. o., Ljubljana, Slovenia. *E-mail: Ales.Podgornik@guest.arnes.si*
- ² Institute of Macromolecular Compounds, Russian Academy of Sciences, St. Petersburg, Russia. E-mail: tennikova@mail.rcom.ru

In the last decade there were many papers published on the study of enzyme catalyzed reactions performed in so-called chromatographic reactors. The attractive feature of such systems is that during the course of the reaction the compounds are already separated, which can drive the reaction beyond the thermodynamic equilibrium as well as remove putative inhibitors. In this chapter, an overview of such chromatographic bioreactor systems is given. Besides, some immobilization techniques to improve enzyme activity are discussed together with modern chromatographic supports with improved hydrodynamic characteristics to be used in this context.

Keywords: Chromatographic reactor, Chromatographic bioreactor, Immobilization, Chromatographic supports

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List of Abbreviations and Symbols

ε	overall bed void fraction
θ	fraction of the surface covered by molecules
Φ	Thiele modulus
η	effectiveness factor
6	stationary phase mass density
a _m	external surface area per unit mass of catalyst
С	concentration
С	feed concentration of acetic acid
c_l	concentration of reactant in the bulk liquid
c_s	concentration on a surface

c_s^*	maximal concentration on a surface
\tilde{D}_e	effective diffusivity
k _a	adsorption constant
K_a, K_b, K_c	distribution coefficients of compounds A, B, and C
k _d	desorption constant
k_m	mass-transfer coefficient
K_s	saturation constant
M	molar mass of acetic acid
m_j	flow rate ratio
Ń	number of columns
p	pressure of the gas
PR	productivity
Q_j	volumetric flow rates through j-th section
r	reaction rate
r_a	rate of adsorption
r _d	rate of desorption
r _e	transport rate through external film
r _{max}	maximal reaction rate
r_p	reaction rate for the whole particle
R_s	particle radius
r _s	reaction rate on the surface
t	switching time
V	column volume

1 Introduction

Almost one hundred years have passed since first documented publication of a chromatography method. Today, chromatography is one of the most commonly used analytical methods in a number of different disciplines. There are several reasons for this wide acceptance of chromatography. The method is relatively simple to use, it is robust and highly reproducible and it can be applied for the analysis and purification of almost any existing stable chemical compound. The working range of chromatography varies between ng amounts, e.g., in trace analysis, and the purification of tons of product in preparative applications.

Being an excellent tool for purification and analysis, the combination of chromatography with a chemical reaction offers additional benefits. Most obviously, since the separation is performed already during the reaction step, no additional purification is required afterwards. Therefore, there is no need for an additional separation operation, which reduces production costs. Moreover, for reversible reactions, the removal of a product from the reaction zone results in higher conversions than under equilibrium conditions. These benefits were recognized already in the early 1960s when the first chromatographic reactor was designed and applied mainly in heterogeneous gas-solid catalytic reactions. More recently, in the 1980s, a type of chromatographic reactor was applied in enzyme catalysis. These early works stimulated several fundamental and application-orientated studies in the last decade, the results of which are collected in this review. Besides the application of a chromatographic reactor in enzyme catalyzed reactions, a significant part of the chapter is dedicated to chromatographic supports in terms of their hydrodynamic properties, which influence the apparent enzyme activity as well as different immobilization techniques, which are also crucial for reproducible and stable enzyme functioning.

2 Common Features of Processes Performed in Flow-Through Dynamic Systems

2.1

Chromatographic Principles

Liquid chromatography (LC) and, in particular, high performance liquid chromatography (HPLC), is at present the most popular and widely used separation procedure based on a "quasi-equilibrium"-type of molecular distribution between two phases. Officially, LC is defined as "a physical method ... in which the components to be separated are distributed between two phases, one of which is stationary (stationary phase) while the other (the mobile phase) moves in a definite direction" [1]. In other words, all chromatographic methods have one thing in common and that is the dynamic separation of a substance mixture in a flow system. Since the interphase molecular distribution of the respective substances is the main condition of the separation layer functionality in this method, chromatography can be considered as an excellent model of other methods based on similar distributions and carried out at dynamic conditions.

Being a complex dynamic process, separation by high performance chromatography based on positive (adsorption) or negative (exclusion) interactions between a substance and a stationary phase surface represents a unique tool to control the transport properties of specially designed solid supports. Comparatively weak interactions (such as ionic or hydrophobic) allow evaluation of not only the morphology but also the topography of the inner surface of an investigated stationary phase. In other words, the model of dynamic interphase mass distribution developed for chromatography can be applied to other processes based, for example, on biological complementary interactions. Moreover, it is necessary to remember that all chromatographic modes play an important role in the modern bioseparation.

It is known that most biological interactions taking place in vivo are based on the formation of specific complexes of the involved biomolecules. Pairs like enzymes and their substrates, antigens and their antibodies, or receptors and their complements can be listed as very well known examples. Such biospecific interactions are also used successfully in analytical biology, biotechnology, immunochemistry, medicine, and related fields [2]. In this range of techniques, the affinity chromatography plays a very important role. Affinity chromatography is based on the natural affinity of a component to be separated to its natural biological complement – the ligand – which is immobilized on the surface of the stationary phase. Thus, a highly specific separation may be based on the formation of the related biospecific affinity. Regardless of the type of interaction used, the efficiency of a chromatographic process depends significantly on the molecular transport from the bulk liquid to the stationary phase surface and back, as well as on the flow regime. All involved effects are commonly expressed in terms of a single parameter, namely the HETP (Height Equivalent of a Theoretical Plate). This parameter depends on the structure of the matrix as well as on the type of molecules to be separated [3–5]. The phenomena determining the overall characteristics of the system are similar to those in heterogeneous catalytic reaction systems. In this case also, a chemical reaction takes place besides several transport phenomena. All together determine the behavior of the chromatographic reactor. To enable a better understanding, the factors which mainly influence the behavior of such systems are briefly discussed in the following section.

2.2 Chemical Reactions on Solid Surfaces

Chemical reactions on solid surfaces can be realized in gas-solid and liquid-solid systems. In both cases the reaction takes place on the surface of the solid matrix, and therefore the molecules to be reacted need to get in contact with the reactive surface. Several transport regimes and interaction mechanisms define the mass transfer efficiency. They can be summarized as follows [6]:

- Transport of the reactants from the bulk fluid to the external surface of the catalyst particle (external resistance)
- Penetration of the reactants into the pores of the catalyst particle (intraparticle transport)
- Adsorption of the reactants at the interior sites of the catalyst particle
- Chemical reaction of the adsorbed reactants to adsorbed products (surface reaction – the intrinsic chemical step)
- Desorption of the adsorbed products
- Transport of the products from the interior sites to the outer surface of the catalyst particle (internal resistance)
- Transport of the products from the external surface into the bulk fluid system (external resistance)

At steady-state conditions the velocities of all the individual steps are identical. Therefore, the slowest transport process will determine for the overall reaction rate.

2.2.1 External Resistance

External transport resistance describes the rate of transport of the reactants from a bulk liquid phase through the stagnant liquid layer around the particles, which has to be overcome to reach the surface. The transport rate is commonly described by the equation

$$r_e = k_m \cdot a_m \cdot (c_l - c_s) \tag{1}$$

where c_l represents the concentration of the reactant in the liquid, c_s is the surface concentration, a_m is the external surface area per mass unit of catalyst, while k_m represents the mass transfer coefficient. k_m depends on hydrodynamic conditions as well as on liquid characteristics. k_m value for many particular systems may be found in the pertinent literature [7].

2.2.2 Rates of Adsorption and Desorption Processes

By far the most commonly used expression for the rate of adsorption is the one derived by Langmuir as follows:

$$r_a = k_a \cdot p \cdot (1 - \theta) \tag{2}$$

where k_a represents the adsorption rate constant, p is the gas pressure, and θ is the fraction of the surface already occupied by the adsorbed molecules.

Concomitantly, the rate of desorption can be expressed as

$$r_d = k_d \cdot \theta \tag{3}$$

where k_d is the desorption rate constant.

At steady-state conditions, the two rates must be equal and the well-known Langmuir isotherm is obtained:

$$\Theta = \frac{p}{\frac{k_d}{k_a} + p}.$$
(4)

Although this concept was originally derived for gas-solid adsorption, it can also be successfully applied to describe a liquid-solid interaction process when using the equations

$$r_a = k_a \cdot c_l \cdot (c_s^* - c_s) \tag{5}$$

for the adsorption and

$$r_d = k_d \cdot c_s \tag{6}$$

for the desorption.

It can be seen from Eq. (5) that the maximum possible concentration on the surface, c_s^* , influences significantly the transport rate. This parameter is a function of the available surface area as well as of the density of the reactive sites. Because of that, the matrix structure plays a very important role in such adsorption/desorption processes. In the case of biological reactions, where the chemical conversion is performed by immobilized enzymes, the immobilization also plays an important role in order to achieve an optimal enzyme density on the reactive surface.

2.2.3 Internal Resistance

Internal resistance relates to the diffusion of the molecules from the external surface of the catalyst into the pore volume where the major part of the catalyst's surface is found. To determine the diffusion coefficients inside a porous space is not an easy task since they depend not only on the molecules' diffusivity but also on the pore shape. In addition, surface diffusion should be taken into account. Data on protein migration obtained by confocal microscopy [8] definitely demonstrate that surface migration of the molecules is possible, even though the mechanism is not yet well understood. All the above-mentioned effects are combined in a definition of the so-called effective diffusivity [7].

2.2.4 Effect of Intraparticle Resistance on Reaction Rate

To evaluate the effect of intraparticle resistance on the overall reaction rate, an approach based on the introduction of effectiveness factor η is usually proposed:

$$\eta = \frac{\text{actual rate for the whole particle}}{\text{rate evaluated at outer surface conditions}} = \frac{r_p}{r_s}$$
(7)

where r_p is the average reaction rate for the whole particle, and r_s is the reaction rate on the particle surface.

The rate of enzyme reaction is often described by the Michaelis-Menten equation:

$$r = \frac{r_{\max} \cdot c}{K_s + c} \tag{8}$$

where r_{max} is the maximal reaction rate, *c* is the substrate concentration, and K_s is the saturation constant.

There is no analytical solution for the effectiveness factor in the case of Michaelis-Menten kinetics. However, at very low substrate concentrations, the kinetics are known to become first-order. For this particular case, an analytical solution for η can be found:

$$\eta = \frac{1}{\Phi} \cdot \left(\frac{1}{\tanh 3\Phi} - \frac{1}{3\Phi} \right)$$
(9)

where Φ is a dimensionless group called the Thiele modulus for spherical particles defined as

$$\Phi = \frac{R_s}{3} \cdot \sqrt{\frac{r_{\max} \cdot \varrho}{K_s \cdot D_e}}$$
(10)

where R_s is the particle radius, r_{max}/K_s is the reaction constant, ϱ is the particle density, and D_e is the effective diffusivity of the molecule under consideration.

Although this expression is valid only in a certain range of substrate concentrations, it yields information about some very important parameters, which in turn determine the overall reaction rate. The effectiveness factor η is close to 1 when the Thiele module is close to 0. Therefore, it is beneficial to have particles of a small diameter. However, as we shall see in the following section, such small particles lead to a more pronounced pressure drop over a given column length, which soon will become a limiting parameter. The second parameter, which greatly influences the value of Φ , is the effective diffusion. This value may vary over orders of magnitude between small and large molecules. Since many biological reactions involve large molecules, this may become the limiting factor of the entire conversion process.

The expression for the effectiveness factor η in the case of zero-order kinetics, described by the Michaelis-Menten equation (Eq. 8) at high substrate concentration, can also be analytically solved. Two solutions were combined by Kobayashi et al. to give an approximate empirical expression for the effectiveness factor η [9]. A more detailed discussion on the effects of internal and external mass transfer resistance on the enzyme kinetics of a Michaelis-Menten type can be found elsewhere [10, 11].

As can be concluded from this short description of the factors influencing the overall reaction rate in liquid-solid or gas-solid reactions, the structure of the stationary phase is of significant importance. In order to minimize the transport limitations, different types of supports were developed, which will be discussed in the next section. In addition, the amount of enzyme (operative ligand on the surface of solid phase) as well as its activity determine the reaction rate of an enzyme-catalyzed process. Thus, in the following sections we shall briefly describe different types of chromatographic supports, suited to provide both the high surface area required for high enzyme capacity and the lowest possible internal and external mass transfer resistances.

3 Modern Chromatographic Stationary Phases

Based on the previous analysis of the different transport phenomena, which determine the overall mass transport rate, the structure of the solid phase matrix is of extreme importance. In the case of any chromatographic process, the different diffusion restrictions increase the time required for separation, since any increase of the flow rate of the mobile phase leads to an increase of the peak broadening [12]. Thus, the improvement of the existing chromatographic separation media (column packing of porous particles) and hence the speed of the separation should enable the following tasks:

- True on-line analysis of produced biologicals [13]
- Decrease of production costs [12]
- Decrease product loss caused by degradation [12]

Taking into account the numerous other requirements of modern separation processes (such as high capacity, high volumetric throughput, biocompatibility, mechanical, chemical, and biological stability, low operative back pressure, etc.), a great number of new and allegedly improved chromatographic stationary phases were developed over the last decade [13–26].

3.1 Dispersed Particles

Preparative and analytical chromatographic columns packed with conventional porous particles meet only part of the requirements discussed above. To achieve the required surface area necessary for high binding capacity, dispersed particles should be highly porous. The pores are typically of the "dead-end"-type and thus the liquid inside is stagnant. The molecules can only penetrate into the pores by molecular diffusion resulting in a significant intraparticle mass transfer resistance. Columns packed with such particles typically require analysis times of several minutes to several hours [27]. As already discussed, this is especially true for large and hence slowly diffusing biomolecules, such as proteins and DNA [23]. To improve this behavior, the new types of dispersed sorbents were suggested over the past ten years. Among these are nonporous [21, 22], micropellicular [13, 19, 23-25], or the gigaporous structures found in the so-called "perfusion" [14–17] and the "gel in a shell" [20] sorbent beads, but also a number of beads with improved accessibility of the active ligands [18, 26], as well as some new and original designs of the separation modules and methods [28].

3.1.1 Non-Porous Particles

To overcome the limitations of intraparticle resistance in the case of particulate stationary phases, non-porous particles were suggested. Due to a total absence of pores, mass transfer resistance is to be expected on the particle surface only, resulting in a very fast exchange of the molecules between the bulk liquid and the adsorptive surface [29]. To overcome the problem of low specific surface and to obtain high chromatographic efficiency, such particles typically are very small, with a diameter of $1.5-3.0 \ \mu m$ [13]. Similar to the non-porous are the so-called micropellicular beads. These are have no internal pores, even though their surface is covered by a thin layer of porous material in order to increase the adsorptive surface area. However, due to the extremely high-pressure drop caused by such small particles and the still relatively low capacity, such non-porous and micropellicular beads are generally not recommended for preparative purposes.

3.1.2 Gigaporous or Perfusion Particles

Perfusion particles were introduced at the beginning of the 1990s [14]. In contrast to the conventional porous particles discussed above, perfusion particles contain a network of large throughpores accessible by convection (perfusion pores) in addition to the usual diffusion pores accessible only by molecular diffusion. The intraparticle mass transport is significantly facilitated by the throughpores, since molecules are transported into and through them by the convective mobile phase flow [14]. This results in faster mass exchange between the mobile and the stationary phase and hence considerably less dependency of the chromatographic separation (resolution) on the flow rate [30]. Gigaporous particles were developed to increase the capacity of such stationary phases, which decreases when the pores become too large. Such particles consist of a rigid skeleton containing the large pores, which are filled with the soft gel. In this case, the capacity is significantly increased while the flow rate dependency of the chromatographic separation lies between that of the perfusion and the conventional porous particles [30]. Despite a significant improvement of the mass transfer characteristics inside the particles, the fact that particle are used at all, automatically results in the formation of voids between the particles when packed in a column. Hence most of the mobile phase still flows around rather than through the particles.

3.2 Membrane Adsorbers

Membrane absorbers are continuous chromatographic supports, which circumvent some of the above-mentioned problems of particulate stationary phases. They were originally derived from membrane (filtration) technology. The immobilization of interactive (ionic, hydrophobic, or biospecific) groups on the surface of microfiltration membranes was found to increase the selectivity of certain separation procedure. Ideally such activated membranes, or membrane adsorbers, allow the selective adsorption of certain substances and substance classes, which may subsequently be eluted by means of a stepwise change of the mobile phase (elution buffer). More complete information on the various types of modern membrane technology can be found in some recent reviews [e.g., 31-33].

Membrane adsorbers in various configurations have been inserted into chromatographic systems and were found to act as ultra short separation layers otherwise analogous to conventional columns. The molecules to be separated interact with such structures very much as in the packed columns and especially gradient elution protocols have been very successful in such cases [34-41]. The main difference between membrane adsorbers and conventional columns (packed beds) consists in hydrodynamics of the intraporous space. Inside the pores of the conventional chromatographic stationary phase particles, the interaction between the molecules to be adsorbed and the surface takes place under conditions where there is no flow of mobile phase. In fact, the stagnant intraporous liquid can be considered to be part of the stationary phase and as already mentioned the molecules reach the actual site of adsorption (interaction) only by molecular diffusion. In contrast, the same interaction inside a chromatographic membrane takes place in a flow through channel and thus under conditions of convective flow in the bulk mobile phase. In addition, such thin membranes allow the use of very high volumetric flow rates at comparatively low operative backpressures. This fact together with the almost flow rate independent performance of the stationary phase increases significantly the productivity of the separation process. Method scale up is also realized very easily in membrane technology, e.g., by increasing the cross section of a membrane module at constant thickness of membrane used. A wide set of membrane units with different configurations (stacks, radial flow layers, hollow fibers, etc.) may be used in membrane chromatography, as summarized elsewhere [42].

3.3 Continuous Separation Layers (Macroporous Monoliths)

As already noted, besides the diffusion restrictions, there exists one more disadvantage in the conventional columns. The column volume is not filled completely by the beads of stationary phase. This fact leads to additional peak broadening and decrease of separation efficiency. It is obvious that the interparticle volume can be decreased by using continuous macroporous media. However, the chromatographic membranes are usually extremely thin and thus many separated layers are stacked together to provide the required adsorption capacity. Macroporous monolithic stationary phases, which resemble the membranes to some extent can, in contrast, be produced in different shapes like flat disks [43–56], rods [57–69], or even tubes [70, 71]. The latter has been introduced as a way to scale up these stationary phases. In the case of monoliths the column can be regarded as a single highly porous particle. All pores are highly interconnected. Small pores provide the specific surface required for a high binding capacity while large throughpores facilitate the flow of the liquid through the column at comparatively low back pressure.

The most important feature of monolithic media is that the mobile phase flows exclusively through the separation unit. In contrast, there is no flow inside the conventional porous chromatographic particles and only a partial flow through the perfusion beads. Just as with the membrane adsorbers, monolith stationary phases may be operated with a minimum in mass transfer resistance with the concomitant advantages in terms of speed and throughput.

3.4

Stationary Phases for Affinity Chromatography and Enzyme Immobilization

The supports for affinity chromatographic separations can be produced using as a base the commercially available chromatographic sorbents of different morphology and design discussed in the previous sections. These include the conventional porous (or partially porous) particles as well as the membranes, fibers, and monoliths. The development of affinity chromatography gave rise to the design of a variety of new stationary phases as well as new approaches to the immobilization chemistry for such ligands. At present, an extensive range of dispersed sorbents are used [72], including natural polymers (agarose, dextrane, cellulose), synthetic polymers (polyacrylamide, polyhydroxymethyl methacrylate and other polymethacrylates, different latexes), inorganic porous materials (silica, macroporous glass, porous titanium), and finally, the composites (silica covered by polysaccharides, polyacrylamide-agarose). Recently, perfusion sorbents have been proposed especially for high-speed affinity chromatography [73]. A new type of a stationary phase for HyperDiffusion affinity chromatography was presented by Sepracor Inc. and called HyperD [74]. This support is based on gigaporous particles in which the porous space is filled with a homogeneous soft hydrophilic polymer gel. In this case, the high adsorption capacity is most likely reached not so much by an increase in surface, but rather by an increase in the volume. The advantages

of using macroporous monoliths for affinity separations is discussed elsewhere [46, 53, 75–83].

The principles of enzyme immobilization on solid phases and the requirements for such solid phases are in general the same as those applied for sorbents and protein ligands used in affinity chromatography. Some time ago the main requirements for the "ideal" matrix were formulated as follows [84]:

- Insolubility
- High permeability and high specific surface
- Defined shape of the particles
- Absence of non-specific adsorption
- High chemical reactivity and capacity for the ligand (enzyme) to be immobilized
- High chemical stability at the conditions required for immobilization reaction and ligand (enzyme) regeneration
- High biological (microbial) resistance
- High hydrophilicity (biocompatibility)

It is obvious that a reasonable combination of productivity and biocompatibility must be taken into account during the development of new stationary phases for affinity techniques. The final goal of such developments should be a porous sorbent with optimized morphology and topography of the inner surface allowing for maximum accessibility of the immobilized ligands. This will guarantee a high-speed process with a minimum of degradation of the valuable product.

Once developed, such biocompatible high throughput stationary (solid) phases are used not only in affinity chromatography but also in heterogeneous biocatalysis, where immobilized biological molecules (enzymes) bound covalently to the surface of an inert support (sorbent) also play a key role. Just as in chromatography, such supports require the immobilization of active biological ligands at high density. The ligands should be well accessible to other biologicals (substrates) dissolved in a mobile phase and concomitantly the entire process should be geared towards high throughput without loss in performance. However, in this case it is not a simple adsorption, but a more complicated bioconversion process, which takes place. The first step is the formation of specific (affinity) complex between the immobilized enzyme and soluble substrate. The characteristics (affinity) of the complexation between the immobilized enzyme and the soluble substrate should approach those of the same pair formed in free solution. The solid support chosen for immobilization is of obvious importance in this context. Most interesting both from a scientific and a practical perspective are in this context the flow through units resembling chromatographic columns. Such systems are widely used both at the analytical and the preparative scale, i.e., from on-line heterogeneous FIA-systems or biosensors to high productive bioconverters [85, 86]. For the construction of such units, a proper immobilization technique is of utmost importance.

4 Immobilization Techniques

Immobilization represents an important parameter in the preparation of bioactive supports. Another important factor is the structure of the support, since this determines accessibility (mass transfer). Different types of supports were discussed in the previous section and here we are going to focus on various immobilization techniques. There are several types of immobilization, which can be categorized in the following groups [87]:

- Cross-linking (for example, with glutaraldehyde)
- Adsorption to a solid matrix
- Adsorption to a solid matrix with subsequent cross-linking
- Covalent binding to a solid carrier material
- Techniques like entrapment or compartmentation
- Enzyme crystallization, with or without cross-linking

In general, these groups can be divided into two main approaches: non-covalent and covalent immobilization. Non-covalent immobilization is performed via adsorption, entrapment, or crystallization. The immobilization via adsorption is easily achieved and it is very attractive since it commonly causes only insignificant changes in the structure and hence the activity of the protein. The main drawback of the method is that the protein is only weakly bound to the matrix and already small variations in the mobile phase composition or temperature can cause its desorption from the surface, resulting in a rapid loss of biological activity. Entrapment immobilization stabilizes the protein within the pores of a matrix formed around it during the process. In order to prevent the protein from escaping, the pores of the matrix should be small. Typical matrices used for this type of immobilization are alginate and polyacrylamide gels as well as different mycelia. As in the case of adsorption, there is no significant influence on the protein structure and, consequently, on its biological functionality. The main problem of this type of bioactive matrix may be mass transfer limitations due to density of the matrix necessary to stabilize the protein as well as the ensuing limitations in regard to the molecular size of the substrates that can be used. The method is therefore mostly suitable for low molecular mass substrates and products. Similar problems can occur in the case of immobilization by cross-linking, which results in the formation of enzyme aggregates. An extensive discussion of various methods can be found in the literature [88].

4.1 Covalent Immobilization

Covalent immobilization is performed through a chemical reaction between the protein molecule and the solid support (matrix). While the reactive moieties on the support can be chosen relatively freely, chemical modification of the protein tends to result in a decrease of its biological activity. Therefore, immobilization via reactive residues of the amino acids is preferred. To design suitable immobilization methods some guidelines should be followed.

Amino acids differ by their hydrophobicity and hydrophilicity. In most cases, the more hydrophobic amino acids like glycine, alanine, valine, leucine, isoleucine, methionine, proline, phenylalanine, and tryptophane are found in the interior of a compact globular protein and their residues are not accessible for chemical reaction with surface functional groups. In addition, it is desirable that the reactive groups used to immobilize the protein are located far away from the protein's functional site, since a coupling near or at the site might result in a significant loss of biological activity. Unfortunately, precise information on the protein structure is not always available and thus it is difficult to predict the effect of immobilization for each particular case. Among the 20 protein forming amino acids, only few were found to be reactive. These are the guanidyl group of arginine, the y- and β -carboxyl groups of glutamic and aspartic acids, the sulfhydryl group of cysteine, the imidazolyl group of histidine, the ε-amino group of lysine, the thioether moiety of methionine, the indolyl group of tryptophan, and finally the phenolic group of tyrosine [89]. Besides the polypeptide chain, many proteins also contain prosthetic groups. By far the most important of these groups for the chemical attachment of proteins to solid supports are the carbohydrates. They are chemically reactive, especially in oxidized form. As such they are very suitable for immobilization, especially because they are not commonly involved in biological activity [90].

In the last 30 years many different methods for the covalent immobilization of proteins were developed. Several facts should be taken into consideration regarding the linkage between the protein and the matrix. Since the biological activity should not be affected by the immobilization, the reaction should proceed under mild conditions. The formed covalent bond has to be stable in a wide range of experimental conditions and it shouldn't introduce any nonspecific interactions. Putative unreacted active groups should be easily deactivated to prevent any subsequent unspecific reactions. Among the most commonly used methods are (1) activation of a matrix by CNBr forming cyanate ester groups [91], (2) formation of N-hydroxysuccinimide ester groups [92] by N-hydroxysuccinimide (NHS), (3) formation of imidazolylcarbamate groups using 1,1'-carbonyldiimidazole (CDI) [93], (4) formation of aldehyde groups by periodate oxidation [94] commonly applied for oxidation of saccharides residues on protein or by using glutaraldehyde, (5) formation of hydrazide groups using adipic dihydrazide [95] and some others, which introduce amino groups, epoxy groups, etc. Detailed protocols for the listed immobilization strategies together with their characteristics can be found elsewhere [32, 88, 89, 96, 97].

Despite the fact that all the above-mentioned procedures form stable covalent bonds, some leakage of the ligand (bleeding) from the solid support is always taking place. Although the amount of the released protein is normally very small, it results in a slow decrease of biological activity during prolonged usage. What is more important, it might cause allergic reactions when the products thus prepared are intended for a therapeutic usage [98]. Due to the low level of ligand release, the ligand leakage is commonly determined using immunoassays with labeled agents. Using such an assay Hermanson et al. [96] compared different immobilization chemistries and determined that the most stable coupling method is periodate/reductive amination. In this case the leakage during 28 days of constant use was only 0.02%. Periodate/reductive amination was followed by immobilization via CNBr with 0.03% leakage and the CDI method with 0.04% leakage. The supports activated with tresyl and NHS chemistries gave much higher leakage (0.22% and 0.43%, respectively). Even more detailed investigation of the three most stable methods can be found in the study by Riedstra and coworkers [99], which investigated the effect of the storage conditions on the leakage. They found the same trends as Hermanson et al., namely the most stable immobilization was again found to be with aldehyde groups using reductive amination, followed by CNBr and CDI immobilization. Besides the immobilization chemistry, the media in which the activated supports are stored, was found to significantly influence the amount of a leakage. A storage in 0.1 mol/l glycine-NaOH buffer, pH 10.5 at room temperature caused a 5–20 times higher leakage than the storage of the same support in 25% EtOH at 4 °C. This is a clear indication that each system should be studied individually for evaluation of such factors.

The stability is certainly an extremely important issue when long-term usage is considered. Along with the stable ligand immobilization, a high biological activity is of utmost importance. In the following paragraphs we will focus on different approaches to increase the biological activity of the immobilized proteins such as the orientation of the immobilized protein, the introduction of a spacer, and the possibility of multilayer immobilization.

4.2 Oriented Immobilization

As already discussed, a covalent immobilization can be performed via different chemical moieties on the protein surface. Because of that, protein molecules are immobilized in random orientation with at least one, but often several, covalent bonds to the matrix. As a result, the active site might be oriented toward the matrix surface and its accessibility to the substrate molecule hence significantly reduced. This results in a decrease of biological activity and consequently in lower binding capacity or decrease of reaction rate in the case of enzymes.

The most extensively studied proteins in this regard are the immunoglobulins (antibodies) which are routinely used for affinity purification. There are at least three different approaches for oriented immobilization of IgG [100, 101]: (1) via Protein A or Protein G, (2) via the carbohydrate moiety, and (3) via sulfhydryl groups. Immobilization via Protein A or Protein G takes the advantage of high specificity of these ligands for the Fc domain of many IgG subclasses. Because of that, after the immobilization the antibody molecules are uniformly oriented in a way, which leaves the antigen-specific binding sites free. Although the bond is stable toward the changes in ionic strength of the mobile phase, lowering of pH values releases the coupled IgG from the Protein A/G ligands. To avoid this problem, the affinity bond can be further stabilized by the formation of an additional covalent linkage between the Protein A and the bound immunoglobulin using a bifunctional reagent [102].

Immunoglobulins contain carbohydrate moiety linked mainly to the C_{H2} domain of the Fc fragment, i.e., again far from the antigen binding sites. These sugar

molecules contain hydroxyl groups, which can be oxidized with periodate to form hydrazone bonds. O'Shannessy and Hoffman [95] found that the antigen-binding capacity was three times higher when the antibodies were immobilized by this approach compared to the random immobilization via ϵ -amino groups. The binding efficiency was found to vary from 0.6 to 1.35 (mole Ag bound/mole Ab coupled), depending on the size of the antigen. Prisyazhnoy and co-workers [103] found even higher efficiency factors (up to 1.6) using a similar immobilization method while Domen et al. [104] reached the theoretical efficiency maximum of two molecules of antigen retained per molecule of antibody. A similar improvement of the efficiency using this immobilization protocol was also reported by other groups [105].

Oriented immobilization of antibodies can also be performed via the sulfhydryl groups of the Fab' fragment. The disulfide bonds can be reduced with 2-mercaptoethanol to form two half-immunoglobulin molecules each containing a single antigen binding site [96]. Before disulfide reduction a pepsin digestion may be performed to obtain just the F(ab')2 fragments, which can also be immobilized in an orientated manner after disulfide reduction. The immobilization appears to be the most effective if iodoacetyl groups are present on the matrix [96]. In this way, an about three times higher capacity was obtained as compared with random immobilization [106]. A similar approach has also been used for oriented immobilization of antibody molecules on a gold surface [107].

To achieve oriented immobilization of proteins in general, their chemical modification represents another option. We already discussed that IgG linkage through the sugar residues leads to oriented immobilization. If proteins do not have such sugar residues originally, they can be introduced chemically. One interesting approach is a glycosylation of proteins such as galactosylation [108]. Another approach to achieve, in this case, reversible immobilization was proposed by Loetscher et al. [109]. They modified the glycosylic part of a monoclonal antibody with a chelating peptide and immobilized the molecule on a nickel affinity resin. Another very important approach to chemical modifications is biotinilation. In this way, proteins can be immobilized in a defined manner by interaction with streptavidin while forming an extremely stable affinity complex with the highest known affinity dissociation constant of 10⁻¹⁵ M [110].

Guanaranta and Wilson [111] compared the different methods for immobilization of acetylcholinesterase by direct immobilization on the matrix, introduction of 1,6 diaminohexane as a spacer, and two methods of oriented immobilization via antibody and avidin-biotin linkage. They found that the latter gave the best efficiency, i. e., tenfold higher than direct immobilization on the matrix (which was the lowest) followed by immobilization on the antibody and immobilization using a spacer. Details about the biotin-avidin technology were published by Wilchek and Bayer [112].

A very interesting approach was presented recently by Niemeyer et al. [113]. They prepared covalent DNA-streptavidin conjugates to which biotinylated alkaline phosphatase, beta-galactosidase, and horseradish peroxidase, as well as biotinylated anti-mouse and anti-rabbit immunoglobulins, were coupled. Immobilization of DNA-streptavidin conjugates was performed by hybridization with the complementary oligonucleotides, bound to the surface. It was demonstrated that such a procedure gives higher immobilization efficiencies than the direct coupling of biotinylated proteins to streptavidin-coated surfaces which was attributed to the formation of a rigid, double stranded DNA spacer. As the authors concluded, this method seems to be very promising for reversible simultaneous immobilization of different compounds using microstructured oligonucleotide arrays as immobilization matrices, which proceeds with site selectivity due to the unique specificity of the Watson-Crick base pairing.

Some recent approaches to orientated immobilization exploited methods of genetic engineering to introduce suitable binding site like a cysteine residue [101]. Mansfeld and Ulbrich-Hofmann introduced cysteine on a thermolysine-like neutral protease from *Bacillus stearothermophilus* [114]. This approach is especially interesting since it allows the introduction of cystein residues in different positions of the protein molecule and therefore enables one to study the effects of molecule orientation on its biological activity [115]. More information about the oriented immobilization can be found in recent reviews of Turkova [101, 110].

4.3 Spacers

Certain fairly small linear molecules are commonly used as spacer "arms" between the matrix and the affinity ligand. A spacer molecule should contain two reactive groups, one to be attached to the matrix and the second to be bound to an immobilized ligand. As a result, the ligand is separated from the matrix by a certain distance defined by the spacer length. The access to the active site of the immobilized protein is facilitated; as a consequence, the biological activity seems to be higher. In addition, a spacer molecule also gives to the protein greater flexibility in comparison with a direct coupling to the matrix, which might also contribute to the improved biological activity. Zhuang and Butterfield [116] immobilized the proteolytic enzyme papain directly on the matrix and via a six-atom spacer. They found that the K_s value was lower in the case of immobilization with spacer and closer to the value of the free enzyme. Therefore, spatial hindrances appears to have been significantly reduced.

It is important to emphasize that the introduction of a spacer should never affect the binding characteristics of the support. The selected spacer must not introduce any charges and should not be sufficiently hydrophobic to cause any kind of non-specific interaction. Several molecules can fulfill this demand but only a few of them are regularly used. Most of these contain terminal amino or carboxylic groups. In particular these are diaminodipropyl amine, 6-aminocapronic acid, 1,6-diaminohexane, ethylenediamine, 1,3-diamino-2-propanol, succinic acid, 1,4-butanediol diglycidyl ether, and others [96]. Extensive description of other bifunctional reagents can be found in the book of Wong [89].

Different types of spacers can be introduced on a matrix by a variety of grafting procedures [32, 117]. Normally, the length of a spacer is not more that ten atoms. Longer spacers can cause problems due to their folding. Hou and co-workers [118] reported this phenomenon for spacers longer than 12 atoms, which were found to cause a dramatic reduction in the binding capacity of the affinity support. However, some commercial supports with longer spacers like Affi-gel 15 from Bio-Rad, CA are available on the market. Especially protein ligands sometimes do require the use of a larger spacer. Recently the use of inert dextrans as long hydrophilic spacers for protein immobilization was reported. Immobilization of rennin on such a spacer resulted in 15-fold higher caseinolytic activity in comparison to the direct immobilization on the matrix while an immobilized via Protein A (as "spacer") was able to bind up to the maximal theoretical value of two molecules [119].

4.4 Multilayer Immobilization

One of the most recent approaches to increase the biological activity is so-called multilayer immobilization. The basic idea is to immobilize enzymes consecutively in several layers connected with a suitable linker, which is able to form strong affinity binding. For this purpose, three types of linkers were suggested: (1) avidin, (2) antibodies, and (3) concanavalin A. Multilayer immobilization with avidin linkage is based on the strong affinity bond between avidin and biotin. To perform this type of immobilization, the enzyme to be immobilized should be biotinilated. Rao et al. [120] prepared a support with horseradish peroxidase immobilized in multilayer using this technology as shown in Fig. 1.

The immobilization proceeded as follows: horseradish peroxidase (HRP) was biotinilated with biotinamidocaproate *N*-hydroxysuccinimide ester to obtain biotinylated HRP with two biotin molecules per enzyme molecule. Avidin was immobilized on polystyrene support beads using the carbodiimide method. This procedure was followed by an attachment of the disubstituted biotinylated HRP

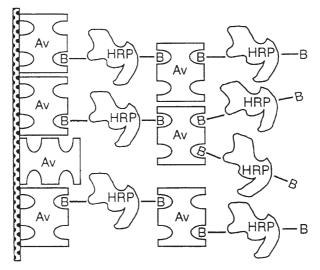


Fig.1. Schematic presentation of the principle of multilayer immobilization. Biotinilated horseradish peroxidase (HRP) is linked via avidin. (Reprinted with permission from [120])

via one of the two biotin moieties through avidin-biotin affinity binding. Another layer of avidin was attached to the second biotin on the biotinylated horseradish peroxidase, and so on, leading to layer-by-layer protein assembly of the enzyme. The biological activity increased approximately three times for a two-layer structure when compared to conventional immobilization. Similar immobilization approaches were reported for the construction of enzyme electrodes [121–123] where high biological activity is of utmost importance to assure a low detection limit.

A first report of successful multilayer immobilization using antibodies as a linker was also reported for the construction of an enzyme electrode. In this particular case glucose oxidase was the immobilized enzyme [124]. This approach was further developed by Farooqi and co-workers [125]. A polyclonal antibody preparation was generated in rabbits against *Aspergillus niger* glucose oxidase and horseradish peroxidase. Antibodies and glucose oxidase were alternately immobilized on the matrix resulting in multilayer immobilization. The effectiveness of multilayer immobilization is shown in Fig. 2. As can be seen, the activity increases linearly with the number of immobilized layers. In this way, final activities higher than by any other type of immobilization can be obtained.

A similar approach for the immobilization of glucose oxidase was reported by the same group using concanavalin A as a linker for the multilayer formation [126]. From the examples described so far it is obvious that the immobilization method significantly influences the biological activity of the immobilized biological (enzyme) resulting in higher or lower binding capacity or in higher or lower enzyme reaction rate. The support and the immobilization strategy should hence be selected carefully to obtain optimal performance.

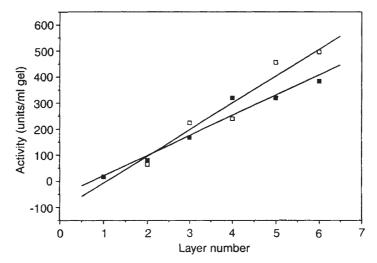


Fig. 2. Effect of the number of immobilized enzyme layers on the enzyme activity. Purified *(filled square)* and non-purified *(open square)* anti-(glucose oxidase) Ig was used as a linker for immobilization of enzyme glucose oxidase, the activity of which was measured. (Reprinted with permission from [125])

5 Chromatographic Reactors

5.1 Short Description

The idea of a chromatographic reactor was presented at the beginning of the 1960s almost simultaneously by several researchers [127–129]. They recognized the benefits of a simultaneous removal of products during a reaction, especially in the cases of reversible reactions where the conversion can be pushed beyond the thermodynamic equilibrium under these circumstances. In particular, a discontinuous chromatographic reactor was defined by Langer and Patton [130] as "a chromatographic column in which a solute or several solutes are intentionally converted, either partially or totally, to products during their resistance in the column. The solute reactant or reactant mixture is injected into chromatographic reactor as a pulse. Both conversion to products and separation take place in the course of passage through the column; the device is truly both a reactor and a chromatograph". A schematic presentation of such a batch chromatographic reactor is shown in Fig. 3.

In such an apparatus, a chemical reaction takes place with a conversion of compound A into the products B and C. Typically, a sharp pulse of component A is fed into the column. During the passage through the column, compound A is converted into the products B and C and the amount of component A decreases. Because of their different retention times, the products B and C are concomitantly separated from each other and component A. Due to the removal of the products from the reaction zone, chemical equilibrium is never reached and the reaction will ideally proceed until the total conversion of the compound A. The reaction may take place in the stationary and/or the mobile phase. Heterogeneous reactions may be either catalyzed by the packed adsorbent or by an additional catalyst, which is mixed with the adsorbent.

The products leave the chromatographic reactor already separated and thus, no further purification steps are required. Therefore, two operations, namely reaction and separation, are combined in a single unit, which significantly reduces the costs of the whole process [131].

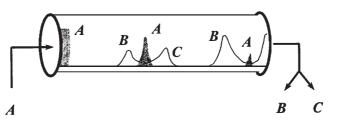


Fig. 3. Schematic presentation of the operating principle of a batch chromatographic reactor. A pulse of compound A is injected into the reactor. As the substance travels through the reactor it is converted into compounds B and C, which are continuously separated. (Reprinted with permission from [134])

5.2 Reaction Types

The full advantage of the chromatographic reactor (simultaneous reaction and separation) is fully realized only for selected types of reactions, which are briefly summarized below [132].

5.2.1 Reversible Reaction $A \leftrightarrow B + C$

In this case the application of a chromatographic reactor leads to significantly higher conversion when B and C are eluted on either side of A. It means that the capacity factors should be as follows: $K_b > K_a > K_c$ or alternatively $K_c > K_a > K_b$. In the case of B and C eluting on the same side of A, even if they are separated, the improvement to be expected is much smaller [133].

5.2.2 Reversible Reaction: $A + B \leftrightarrow C + D$

To obtain the best performance in this particular case, the products C and D should be separated in order to slow down the reverse reaction while good contact between the reactants A and B should be preserved. Three main possibilities can be distinguished:

- The retention times of A and B are different: a benefit of the separation can be obtained with a suitable feeding mode in a way that the pulse of the faster reactant travels through that of the slower reactant during their passage through the reactor.
- A and B have the same retention time: in this case the problem is similar to the case of a reaction of type A ↔ B + C with the difference that the forward reaction is of second order and very sensitive to the injected concentrations.
- B is used as a carrier fluid: since B is in large excess, the problem is reduced to a reaction of type $A \leftrightarrow C + D$.

5.2.3 Consecutive Competing Irreversible Reactions: $A + B \rightarrow R$, $R + B \rightarrow S$

Let R be the desired product. In this case, we have a competition between the rate of the second reaction and the rate of separation of R from B. Under these circumstances, the feeding mode determines the efficiency of the conversion. However, so far there are no published experimental results that would demonstrate the possibility of increasing yield of R by using a chromatographic reactor with simultaneous separation.

5.2.4 Removal of Inhibitors

There are several reactions, especially in the case of enzymes, where either the substrate(s) or the product(s) inhibit partially or even totally the reaction. In such a case the removal of the inhibitor from the reaction zone results in a higher yield [133, 134].

5.3 Reactor Types

Increased conversion and product purity are not the only benefits of simultaneous separation during the reaction. The chromatographic reactor was also found to be a very suitable tool for studying kinetics and mechanisms of chemical and biochemical reactions. Some recent publications describe the results on investigation of autocatalytic reactions [135], first-order reversible reactions [136], and estimation of enantioselectivity [137, 138]. It is beyond the scope of this chapter to discuss the details, but the interested reader is referred to an overview published by Jeng and Langer [139].

Most publications dealing with chromatographic reactors focus on theoretical issues of this very complex system. Models of different complexity were derived and used to predict the behavior of chromatographic reactors. Such models typically take into consideration different types of mass transfer, adsorption isotherms, flow profiles, and reactions. A general scheme of these models, not including the reaction, is presented in Fig. 4. There are also several review papers

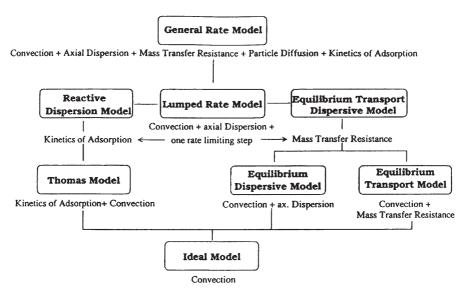


Fig. 4. Different types of models used for describing chromatographic processes. (Reprinted with permission from [131])

which describe the results of mathematical simulations based on these models assuming different reaction rates. On the other hand, there is a smaller but significant number of publications with experimental data on the performance of chromatographic reactors. In most cases, gas-solid systems were studied although some experiments with liquid-solid systems were reported as well. An overview can be found in the book of Ganestos and Barker [140].

A chromatographic reactor can be realized with different configurations from a single fixed-bed reactor to multireactor arrangement enabling continuous operation. Here, a short description of the basic types together with some recent results are presented.

5.3.1 Batch Chromatographic Reactors

5.3.1.1 Fixed-Bed Chromatographic Reactor

Chromatographic fixed-bed reactors consists of a single chromatographic column containing a solid phase on which adsorption and reaction take place. Normally a pulse of reactant is injected into the reactor and, while traveling through the reactor, simultaneous conversion and separation take place (Fig. 3). Since an extensive overview of the models and applications of this type of reactor was presented by Sardin et al. [132], only a few recent results will be discussed here. Most of the practical applications have been based on gas-liquid systems, which are not applicable for the enzyme reactions, but a few reactions were also reported in the liquid phase. One of these studies, performed by Mazzotti and co-workers [141], analyzed the esterification of acetic acid into ethyl acetate according to the reaction:

ethanol + acetic acid \leftrightarrow ethyl acetate + water.

The reaction is reversible and therefore the products should be removed from the reaction zone to improve conversion. The process was catalyzed by a commercially available poly(styrene-divinyl benzene) support, which played the dual role of catalyst and selective sorbent. The affinity of this resin was the highest for water, followed by ethanol, acetic acid, and finally ethyl acetate. The mathematical analysis was based on an equilibrium dispersive model where mass transfer resistances were neglected. Although many experiments were performed at different fed compositions, we will focus here on the one exhibiting the most complex behavior; see Fig. 5.

Prior to injection, the reactor was saturated with ethanol. At time 0, feeding of a mixture of ethanol and acetic acid in the ratio of 70:30 into the reactor was started. The concentrations of the different compounds were measured at the reactor outlet.

As the reactants entered the column, they were adsorbed and the reaction started. Water was strongly retained by the resin while the ethyl acetate was readily desorbed and carried by the mobile phase. Since the product was removed from the reaction zone, esterification proceeded until the full consumption of the

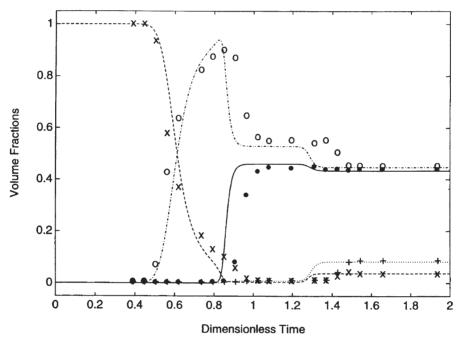


Fig. 5. Complex behavior of a batch chromatographic reactor system. After an inlet step, three steady states were detected at the reactor outlet. Experimental data for acetic acid (*filled circle*), ethanol (×), water (+) and ethyl acetate (*open circle*) were successfully fitted by a mathematical model (*solid and dashed lines*). (Reprinted with permission from [159])

limiting reactant. The process continued until the entire resin was saturated with water. The concentration profiles at the outlet of the reactor exhibited interesting dynamics. After approximately 0.5 dimensionless units of time, a sudden decrease of ethanol and a simultaneous increase of ethyl acetate was observed. This was the consequence of the weak retention of ethyl acetate. After approximately 0.8 units of time, a steady state characterized by a high ethyl acetate concentration was achieved. This increase was the result of the selective role of the resin. Water was adsorbed and the reaction was allowed to proceed to completion, i.e., far beyond the thermodynamic equilibrium. The second steady state was observed between 0.9 and 1.3 time units. This plateau was the result of the acetic acid excess in the feed. As the reaction was not complete, an equilibrium mixture enriched in ethyl acetate and acetic acid together with small amounts of water and ethanol was formed. A final steady state occurred after 1.2 units of time. At that point the resin became saturated with water, and thus no further retention of any component took place. As a result all concentrations corresponded to the thermodynamic equilibrium. A detailed analysis with a description of all the phenomena supported by the pertinent simulations was presented in the work. However, even this short summary should serve to indicate the very complex behavior of a chromatographic reactor, which depends on the composition of the feed, as well as on the adsorption characteristics of the matrix.

A similar reaction, namely the hydrolysis of methyl acetate,

methyl acetate + water \leftrightarrow acetic acid + methanol

was recently studied by Sircar and Rao [142]. In this case, two different supports were used, namely an ion exchanger as a catalysts and activated carbon as selective adsorbent for the acetic acid. The authors found that at a temperature of 35 °C a conversion of 31.4% could be achieved in the chromatographic reactor in comparison with 21.0%, which could be obtained with a fixed-bed reactor containing only the catalysts. Similar to the previous case, after saturation of the resin, a regeneration step was needed and a process scheme including this procedure to perform continuous process is presented in the paper.

As already discussed, the enhanced conversion is due to the separation of the products from the reaction zone. This can be realized via different distribution coefficients of the compounds (and consequently, a separation of the components) or via (selective) adsorption on a support. Since in the first case the compound travels through the reactor with different speeds, a continuous feed would cause repeated mixing of the separated compounds. Therefore, no improvement can be expected. In the second case, a regeneration of the adsorbent is needed after a certain operative period. This is an inherent drawback of the discontinuous operation of the fixed-bed chromatographic reactor.

For optimal performance, the feeding strategy for the chromatographic reactor should be carefully designed. In addition, the following criteria should be fulfilled [132]:

- The reaction rates should be as high as possible (under these circumstances reversible reactions are close to equilibrium).
- At least two chromatographically separable products must be formed.
- The reactants are introduced sequentially according to their retention properties.
- The adsorbent (and possibly, the mobile phase if a liquid) is chosen such as to obtain elution as specified by the stoichiometric ratios.

Recently, Falk and Seidel-Morgenstern [143] performed a detailed comparison between fixed-bed reactors and fixed-bed chromatographic reactors. The reaction studied was an equilibrium limited hydrolysis of methyl formate into formic acid and methanol using an ion-exchange resin as both the catalyst and the adsorbent. The analysis was based on a mathematical model, which was experimentally verified. The comparison was based on the following four assumptions:

- The same amount of feed was introduced into both reactors; i.e., pulses of higher concentration were injected into chromatographic reactor but due to the periods without feeding, the average concentration was equal to the concentration continuously feed into the fixed-bed reactor.
- The volumetric flows were the same.
- The properties of the solid phase were the same.
- The reactor dimensions and operating temperatures were the same.

The criterion used for this comparison was the achievable conversion. It was demonstrated that, taking into account the periodic nature of the batch chro-

matographic reactor, a conventional fixed-bed reactor might lead to a higher overall conversion. Although this conclusion was drawn for a particular chemical reaction, the acknowledged drawback of a discontinuous operation may only be overcome by an alternative design of the chromatographic reactor.

5.3.2 Continuous Chromatographic Reactors

Three types of continuous chromatographic reactors can be generally distinguished: (1) the continuous annular chromatographic reactor, (2) the countercurrent moving bed chromatographic reactor, and (3) the simulated moving bed chromatographic reactor [144]. These types differ significantly in the design as well as in their performance. Each type will be described separately.

5.3.2.1

Continuous Annular Chromatographic Reactor

In the continuous annular chromatographic reactor the stationary phase is realized in the shape of an annulus, which is slowly rotating around its axis, while a continuous feed stream enters from a stationary inlet. A carrier liquid is distributed uniformly from above the annulus. The chemical reaction occurs in the bed and the reactant(s) and products are separated along the column's axis by the carrier. Due to the rotation of the stationary phase, the components elute at different outlet angles according to the strength of their interaction with the stationary phase. The system is schematically presented in Fig. 6. To obtain optimum performance, the reaction rate should be fast enough for the reaction to occur primarily on top of the bed, while the bottom serves largely as a separator [144].

Reactions in which a single reactant gives more than one product are the most suitable for this type of chromatographic reactor. Therefore the reaction should be of type $A \leftrightarrow B + C$ and the adsorption of A, B, and C should differ significantly. As in the case of the discontinuous reactors discussed above, the most satisfactory operation is obtained if the distribution coefficient of A is between the values for B and C [144]. Few examples of real applications of this type of continuous chromatographic reactor exist in the literature. Cho et al. [145, 146] studied a liquid-phase hydrolysis of methyl formate. A gas-solid phase catalytic dehydrogenation of cyclohexane into benzene using a Pt catalyst was studied by the same group [147]. In addition, there are two reports on the application of this type of reactor to perform biochemical reaction using enzymes, both by Sarmidi and Barker [148, 149], which will be described in detail later on.

Recently, a theoretical analysis of the productivity of a heterogeneous catalytic reaction of the type

 $A + eluent \leftrightarrow C + D$

performed in a continuous annular chromatographic reactor was presented [150]. The annular chromatographic reactor was connected in two ways. In the first case the system consisted of a fixed-bed reactor followed by an annular chromatographic reactor and in the second of a fixed-bed reactor followed by a sim-

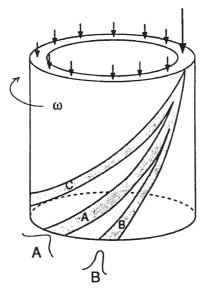


Fig. 6. Schematic presentation of a continuous annular chromatographic reactor. The sample (*big arrow*) and the mobile phase (*small arrows*) are continuously introduced from the top of the column, which rotates with the constant angular velocity ω . Passing through the column, compound A is converted into the compounds B and C. Due to their different retention they split in three streams and exit at different positions from the column. (Reprinted with permission from [144])

ple annular chromatographic separator. The efficiency of the particular systems depended on the differences in distribution coefficients of C and D, the reaction equilibrium constant, and whether the reaction is instantaneous or not. In addition, the productivity of the annular chromatographic reactor was shown to be highly dependent on the rotation rate. We can probably expect more experimental studies of this attractive type of chromatographic reactor in the future.

5.3.2.2 Countercurrent Moving Bed Chromatographic Reactor

Another approach to continuous reaction chromatography is the countercurrent moving-bed chromatographic reactor (CMCR). In this type of reactor the stationary (solid) phase travels in the opposite direction to the liquid phase. In practice this is performed by introducing the stationary phase from the top of the reactor. The stationary phase flows downwards under the influence of gravity while the liquid phase is pumped upwards from the bottom. A schematic presentation of such a system is shown in Fig. 7. Depending on the adsorption characteristics of the different components, they can travel in the direction of the liquid or the solid phase resulting in their separation.

The results of many, albeit mainly theoretical studies of the behavior of this type of reactor based on different reaction types and adsorption isotherms have

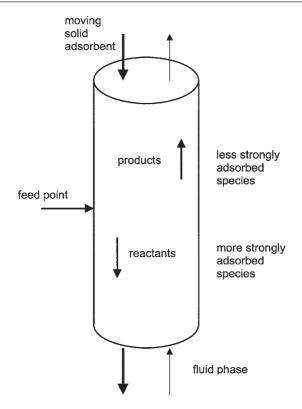


Fig.7. Schematic presentation of a true countercurrent moving bed chromatographic reactor. (Reprinted with permission from [151])

been published recently [144, 151]. However, there are very few examples of experimental results obtained with this type of chromatographic reactor. Takeuchi and Uraguchi [152] studied the oxidation of CO using aluminum oxide as a catalyst. Another experimental study was the catalytic hydrogenation of 1,3,5trimethylbenzene into 1,3,5-trimethylcyclohexane [153]. In this case, a catalyst (Pt on aluminum particles) was continuously introduced into the reactor from the top. A higher purity of the product and a conversion much higher than the equilibrium one were obtained. The results were supported by theory [154]. According to the authors' best knowledge no application of this type of reactors in the area of enzyme (or other biological) reactions has been published so far.

Although the countercurrent moving bed chromatographic reactor represents an interesting model for theoretical studies, the very few experimental examples already indicate the difficulties in realizing such a process in practice. One of the main difficulties is handling the solid phase. Its movement inevitable causes back mixing thereby reducing the efficiency of the process. Abrasion of the particles is another problem [131, 151]. To avoid the above-mentioned problems the simulated moving bed reactors discussed in the next section were developed.

5.3.2.3 Simulated Moving Bed Reactors (SMBR)

In a simulated moving bed reactor, as its name already indicates, the movement of the solid phase is simulated. This is achieved by using a set of fixed-bed reactors (columns) connected in series and periodically switching the feed and withdrawal points from one column to the other. A schematic presentation of a simulated moving bed chromatographic reactor is shown in Fig. 8.

The process can be divided into four different sections. Section I is located between the desorbent and extraction node. The flow rate is higher than in all the other sections, which is necessary to remove the more strongly adsorbed product (here component B) from the adsorbent. Section II is located between the extract and the feed node. In this section the components B and C are formed. The less strongly adsorbed product (here component C) is desorbed and transported upstream together with the solvent, whereas B is still held on the adsorbent and transported to the extract port. The extract stream therefore contains the more strongly adsorbed product B. In Section III the conversion of component A takes place. Component B is retained and, thus, component C can be collected at the raffinate port. In Section IV component C is adsorbed and transported back to Section III together with the adsorbent, while the fluid phase is cleaned and recycled.

There are a number of papers published recently dealing with the modeling of the SMBR. Several researchers explored the effects of different parameters on

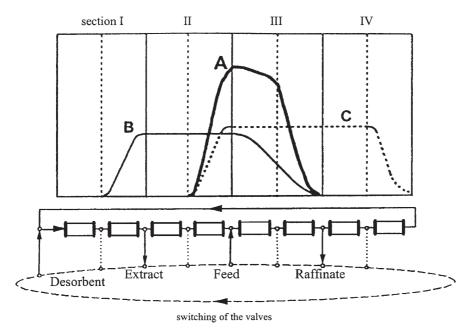


Fig. 8. Schematic presentation of a simulated moving bed chromatographic reactor together with profiles inside the columns. (Reprinted with permission from [131])

the process efficiency. Fricke et al. [155] studied the effects of different factors, like the distribution coefficients, the separation factors, and the reaction kinetics, in terms of reaction rate and reaction equilibrium on taking the reversible reaction $A \leftrightarrow B + C$ as example. Based on this analysis, the following parameters limit the use of the process:

- For adsorption limited by mass transfer kinetics, the educt should be significantly less retained than the most strongly adsorbed product.
- The reaction rate should be higher than 10^{-2} s⁻¹.
- The reaction equilibrium constant should be greater than 0.01 mole.

The best performance can be achieved under the following conditions:

- The mass transfer resistance should be small to avoid band spreading.
- K_a should lie between K_b and K_c .
- The separation factor of the products should be high.
- K_a should lie closer to K_b in the case of adsorption limited by mass transfer kinetics.
- The reaction rate should be high in order to minimize the reaction zone.

The same group investigated the effect of reactor design for this type of reaction [156]. By simulation, they compared the behavior of a chromatographic reactor with two different ways of arranging adsorber and catalyst. In one case, the two were mixed together to form a homogeneous bed. In the second case, adsorbent and catalyst were arranged into alternating segments of equal size. They found that conversion increases significantly with the number of segments from approximately 47% for one segment to more then 80% for two segments while the homogeneous packing gave the best conversion of about 92%. They also concluded that an increase of the number of columns from 2 to 3 in the segment, where the reaction took place, had beneficial effects.

The main goal of any investigation of the effect of different parameters on the characteristics of an SMBR is to optimize the performance. However, this represents a challenging task due to the complexity of the process and the many degrees of freedom. Because of the cyclic port switching, providing a detailed mathematical model becomes extremely complex. One approach is to model the SMBR as a simple countercurrent moving bed reactor. It was shown by Storti et al. [157] that the steady state solution of a detailed countercurrent moving bed model describes the solution of a simulated moving bed model reasonably well in the case of three or more columns per zone and linear adsorption isotherm without a reaction. Therefore, two approaches to optimizing SMB were proposed [131]: (1) to develop a short cut design methodology based on the model of the equivalent countercurrent moving bed reactor process or (2) a heuristic strategy combined with experiments and dynamic simulation of the SMB.

Currently, the most successful methodology for the optimization of an SMB's performance is the so-called triangle theory, which was recently also applied to the SMBR [158]. The analysis was based on a mathematical model describing the esterification of acetic acid and ethanol into ethyl acetate and water in a fixed-bed chromatographic reactor [159]. A mixture of ethanol and acetic acid is intro-

duced into the system between Sections II and III (see Fig. 8). Ethanol is used as an eluent and introduced at the bottom of Section I. The two products, ethyl acetate and water, are collected in the raffinate and extract, respectively, both diluted in ethanol. The aim of this work was to find the experimental conditions where complete conversion of acetic acid and complete separation of the products is achieved. The applied procedure was based on the triangle-shaped region of complete separation, which characterizes non-reactive SMB. The flow rate ratios (m_i) are defined by the equation

$$m_i = \frac{(Q_j \cdot t - V \cdot \varepsilon)}{V \cdot (1 - \varepsilon)} \qquad j = 1, 2, 3, 4 \tag{11}$$

where Q_j represents the volumetric flow rates through the different sections (see Fig. 8), *t* is switching time, *V* is the column volume, and ε is overall bed void fraction.

Values of m_2 and m_3 change with the variation of flow rates Q_2 and Q_3 (the flow rates through Sections II and III where the reaction takes place). This procedure was repeated for different feed compositions.

As shown in Fig. 9, the size of the region within which a complete conversion and separation occurs depends on the feed ratio. However, in all cases the existence of such a region was confirmed. A feed ratio of 0/100 (acetic acid/ethanol) actually represents a limiting case where no reaction occurs (identical to a nonreactive SMB). Since obviously there are many operating conditions where complete conversion and separation is achieved, other criteria should be introduced

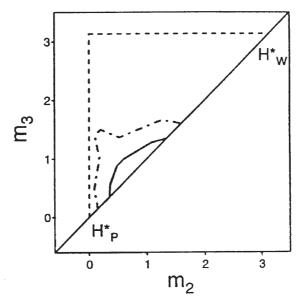


Fig. 9. Regions with complete conversion/separation for different feed compositions in an SMBR: Acetic acid to ethanol ratio: (---) 0/100; (-.-) 40/60; (---) 100/0. (Reprinted with permission from [158])

to determine the optimum ones. One such criterion can be the productivity per unit mass of resin (PR) defined by Eq. (12):

$$PR = \frac{(m_3 - m_2) \cdot C \cdot M}{t \cdot \varrho \cdot N} \tag{12}$$

where ρ is a stationary phase mass density, *N* is the number of columns, *C* is the feed concentration of acetic acid, and *M* is the molar mass of acetic acid.

For different feed compositions, a maximum value of PR was found for a feed ratio of 40/60. This demonstrates the existence of an optimum. The results obtained in this study were compared with the simulation of an SMB bioreactor for the inversion of sucrose to fructose and glucose and found to be consistent [160].

A different approach was proposed very recently by Dünnebier et al. [131], who introduced a novel optimization and design strategy for SMBR based on mathematical optimization and a complex dynamic process modeling strategy. As optimization criteria they used a detailed cost function and explicitly considered the product quality requirements. The strategy is based on the simulation of the SMBR till steady state is achieved. This steady state is then evaluated in terms of certain optimization criteria. If the required optimum is reached, the optimization is completed; otherwise, by using a gradient optimization method, a new cycle of simulation with different input values is started. The computer calculation takes 1-2 days on a modern PC. The approach was tested taking the inversion of sucrose into fructose and glucose [134, 148] and the production of β -phenethyl acetate from acetic acid and β -phenethyl alcohol [161] as examples. The authors concluded that a potential saving in operating cost of up to 20% and reduction of desorbent consumption of up to 60% can be achieved.

In the last decade, several applications of the SMBR were developed. A Japanese group reported on a reversible esterification of acetic acid and β -phenethyl alcohol (B) into β -phenethyl acetate and water [161]. The equilibrium conversion under experimental conditions without adsorption was 63%. An ion-exchange resin was used both as catalyst and as adsorbent for acetic acid. Applying a mathematical model originally proposed by Hashimoto et al. [162], a good agreement with experimental data was found. Almost 100% conversion was achieved. The same group also investigated the production of bisphenol A (2,2,-bis(4-hydroxyphenyl) propane) and water, as a side product, from acetone and phenol (also used as solvent) on an ion-exchange resin [163]. The reaction was of the $A+2B\leftrightarrow C+D$ -type. The authors found that the water adsorption decreases the reaction rate. Applying an SMBR system, supported by a mathematical model, conditions could be found where the adsorbed water was continuously removed, resulting in a stable long-term operation without any reaction inhibition.

The esterification of acetic acid with ethanol using sulfonic ion-exchange resins as catalyst/selective sorbent was studied by Mazzotti et al. [164]. The authors developed a detailed mathematical model, which was able to predict correctly the system's behavior. They succeeded in obtaining 100% conversion of acetic acid in addition to a complete separation. Several other studies involving enzymatic reactions were also carried out and will be presented in more detail in the next section. Recently there were also some reports of the application of SMBR for gas-solid systems. Kruglov et al. [165] studied the oxidative coupling of methane. This is a rather complex process consisting of four reactions leading to ethane and ethylene as desired products considered together as C_2 , as well as to CO and CO_2 as by-products. Using a conventional reactor design it was not possible to obtain a yield of C_2 of more than 20-25%. A significant increase in yield due to the use of an SMBR was firstly shown by Tonkovich et al. [166, 167], who achieved a methane conversion of 65% and a C_2 yield of more than 50%. In this work further improvement was obtained by the selection of a proper catalyst and final yields of 55% were achieved for C_2 together with a methane conversion of 75%.

Another study was performed on a catalytic hydrogenation of 1,3,5-trimethylbenzene to 1,3,4-trimethylcyclohexane, which is a typical first-order reversible reaction [168]. By optimizing various operating conditions it was possible to achieve a product purity of 96% and a reactant conversion of 0.83 compared to a thermodynamic equilibrium conversion of only 0.4. The results were successfully described with a mathematical model derived by the same authors [169]. Comparison to a real countercurrent moving bed chromatographic reactor yielded very similar results for both types [170].

5.4 Chromatographic Bioreactors

In this chapter chromatographic bioreactors are considered as chromatographic reactors where the reaction is catalyzed by an enzyme or enzyme system, which can be present in pure form or as a cell component. The enzyme can be immobilized on the matrix or it can be dissolved in a liquid phase. Therefore, the reaction can take place in either phase. Several different bioreactions were performed in chromatographic reactors of different types. In the following part some pertinent examples are presented according to their type of reaction.

5.4.1 Dextran Biosynthesis

Several types of enzymatic reactions were studied during the last decade for the biosynthesis of dextran. Reports on the application of chromatographic reactors for an enzyme catalyzed reaction were first demonstrated in the late 1980s [171, 172].

The reaction proceeds according to the following scheme:

sucrose $\xrightarrow{\text{dextransucrase}}$ dextran + fructose.

The formation of the biopolymer dextran is a complex process, where the fructose is known to inhibit the polymer chain growth. Consequently, the separation of the fructose from the reaction zone results in a higher molecular mass of the dextran even at high initial sucrose concentration [173]. In a first investigation, a fixed-bed chromatographic reactor was filled with calcium charged polystyrene. Fructose was retarded on this matrix while the dextran was prevented from entering the pores (size-exclusion) and migrated with the mobile phase. The sucrose migrated at an intermediate rate and was gradually converted. In this way, at a feed concentration of 20% w/v of sucrose over 77% of dextran with a molecular mass of more than 150,000 Da were obtained. When a column of larger diameter was used (5.4 rather than 1 cm) a similar conversion was obtained indicating the possibility of process scale up. It should be emphasized that the enzyme was not immobilized on the support but was continuously added to the inlet stream. This is important since polymeric dextran causes a high viscosity, which results in mass transfer limitations, decreasing the efficiency of the process.

The same group also performed experiments with a continuous chromatographic reactor of the SMBR type [133, 174]. The system used consisted of 12 columns with inner diameters of 5.4 cm and a length of 75 cm. Approximately 12 cycles were necessary for stabilization of the system. Complete inversion of sucrose even at feed concentrations of up to 55% w/v was achieved, while the product purity was over 90%. After prolonged usage, the system efficiency decreases due to the loss of the calcium ions from the matrix and a consequent decreased selectivity of the resin. However, this problem could be overcome by regeneration of the resin with calcium nitrate.

5.4.2 Inversion of Sucrose into Fructose and Glucose

Several papers investigating the inversion of sucrose into glucose and fructose appeared a decade ago [133, 174, 175]. The reaction is performed using the enzyme invertase according to the following reaction:

sucrose $\xrightarrow{\text{invertase}}$ glucose + fructose.

Although the reaction is not reversible, the use of a chromatographic reactor was shown to be beneficial since substrate inhibition occurs at sucrose concentrations of more than 10% w/v [174]. An SMBR system, similar to the one used for dextran synthesis, consisted of 12 columns filled with calcium charged polystyrene. Since all three compounds have different distribution coefficients, they could be separated on the resin. Again, the enzyme was not immobilized and the reaction consequently occurred in the liquid phase. By employing the combined chromatographic bioreactor/separator-principle, the simultaneous reaction and separation reduced the on-column sucrose concentration and minimized the substrate inhibition-related problems. Complete conversion was obtained even at initial sucrose concentrations as high as 55% w/v and the final fructose purity was up to 94%. Furthermore, the enzyme consumption was only 34% of the amount required to invert the same quantity of sucrose under the same conditions over the same period of time in a traditional fermenter. This particular process was analyzed theoretically by Ching and Lu [160]. They developed a mathematical model and estimated the effects of the feed flow rate, the eluent flow rate, and the column switching time on the final purity and recovery. One of their conclusions was that not the entire bed was used in the process. To improve

the purity and recovery one can reduce the feed flow rate. This, however, leads to lower productivity and more diluted products. An alternative is to use an adsorbent with better separation performance for glucose and fructose. In both cases purity and recovery for glucose and fructose would be higher than 95%. The change of the system configuration is another option.

The configuration proposed by Ching and Lu [160] consisting of four sections and a recycle unit was theoretically analyzed by Meurer et al. [134]. They concluded from their simulations that with a proper adjustment of the experimental conditions almost 100% product purity could be achievable. Furthermore, they compared the SMBR configuration with two other set-ups, one consisting of

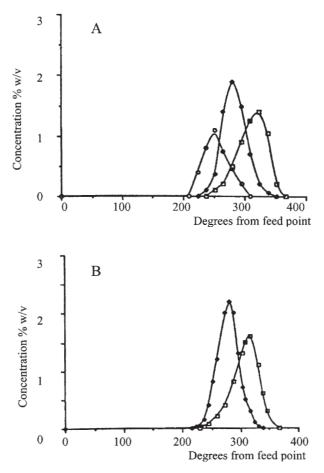


Fig. 10A, **B**. Outlet profiles of a continuous annular chromatographic bioreactor. Sucrose (*open circle*) was converted into glucose (*filled circle*) and fructose (*open square*) and separated due to different retention on the stationary phase: A only partial conversion was obtained and a sucrose peak can clearly be seen; **B** a larger amount of enzyme was used and complete conversion was obtained. (Reprinted with permission from [148])

an inversion reactor and a chromatographic batch process to separate glucose and fructose, and a second one consisting of the same inversion reactor and an SMB separation unit. The comparison was made for a given productivity. It was shown that, to achieve a high purity product, the SMBR configuration is the most effective, followed by a configuration consisting of the reactor and an SMB separation unit.

Conversion of sucrose into glucose and fructose was also performed on a rotating annular chromatography reactor [148] using similar operating conditions as described for the separation on the SMBR [133]. The effects of the flow rate and the enzyme activity were studied experimentally and a mathematical model was developed. A typical chromatograph is shown in Fig. 10. Complete conversion of sucrose was possible up to a feed concentration of 50% w/v. Although no data about the enzyme efficiency were given, the advantage of such a system might be in the ability to separate more than two compounds simultaneously.

5.4.3

Isomerization of Glucose and Fructose

Isomerization of glucose and fructose is a reversible reaction with an equilibrium constant of 1.0. It was, e.g., studied by Hashimoto et al. [162, 176]. A high content of fructose is desired since this increases sweetness and water solubility. Thus a shift of the equilibrium is required. To perform this task, an SMBR system was developed, which consisted of 23 columns. In this case the enzyme glucose isomerase was immobilized on a quaternary pyridine matrix in the form of a microorganism, namely Streptomyces phaeochromogenes. Seven of the columns were filled with the immobilized "enzyme". The other columns were adsorptive columns containing Y zeolite for the selective adsorption of fructose. Although the reaction and the adsorption were performed in separated columns, the behavior of the system should bear some similarity to one where both matrixes are mixed together due to the high number of columns [156]. A feed containing glucose and fructose in equimolar amounts was introduced into the system. A mathematical model was developed for predicting the system's behavior, which correlated well with the experimental values. Experiments confirmed that it is possible to obtain fructose conversion of 55%, which was at the desired level. Subsequently the developed system was compared with two others, one consisting of an enzyme reactor and a conventional fixed-bed adsorber column and the other of an enzyme reactor and an SMB adsorber. In both cases there was a recycle. The authors demonstrated that, for a given fructose content of 55%, the SMBR system required the lowest amount of desorption buffer. This system was also analyzed theoretically by Ching and Lu [160]. It was found that the efficiency of the process was rather low. Significant improvement can be achieved with the proper adjustment of the desorbent flow-rate and the dilution ratio. However, the most significant improvement could be expected with a higher reaction rate, demonstrating the importance of the enzyme support preparation.

5.4.4 Conversion of Maltose into Glucose

Another interesting application of the chromatographic reactor was proposed by Hashimoto et al. [176]. In this case the chromatographic reactor was not used to increase a conversion or to prevent an inhibition but to overcome the difficulties of enzyme immobilization. In their paper the authors proposed a system for the continuous conversion of a substrate to a product by recirculation of a non-immobilized enzyme. The separation of the enzyme from the substrate and the product is performed by a gel filtration based on the much higher molecular mass of the enzyme. To investigate the performance of such a system a continuous hydrolysis of maltose into glucose using the enzyme glucoamilase was tested. The system was very simple SMBR consisting of only two columns filled with size exclusion support. The enzyme was passing directly through the column while both maltose and glucose were retained, having approximately the same size and hence the same distribution coefficients. Although the performance of the system was not very robust, a conversion of 90% was reached in the beginning and only a small leakage of the enzyme was detected. Based on the calculations, it should be possible to achieve 99% conversions while avoiding totally all leakage of the enzyme.

5.4.5 Conversion of Starch into Maltose

Another system, which was intensively studied in the context of a possible chromatographic reactor, is the saccharification of starch into maltose and dextrin using the enzyme maltogenase [149, 177]. This enzyme is able to hydrolyze 1,4- α glucosidic linkages to produce maltose as well as maltotriose, which in turn may form maltose and glucose. The enzyme was not immobilized but instead was continuously added to the eluent. Two types of reactors were investigated: a continuous rotating annular chromatograph (CRAC) [149] and semi-continuous counter-current chromatographic reactor-separator SCCR-S (or SMBR) unit consisting of 12 columns [177]. In the latter case, the system was stabilized after approximately six cycles and a typical concentration profile along the columns is shown in Fig. 11.

Both reactors were filled with a calcium charged polystyrene resin as the adsorber, which retains only maltose. In the case of the CRAC, conversions of up to 79% at feed flow rates of up to 400 cm³/h and substrate concentrations of 15.5% (w/v) were achieved. An even better performance was obtained with the SCCR-S, which required only 34.6-47.3% of the enzyme required by CRAC. This was assigned to the longer contact time between the substrate and the enzyme. The advantage of the CRAC might be the possibility to separate also maltose and glucose, which cannot be done with the SCCR-S. In their paper the authors speculated that further improvement was possible because of a decrease of substrate or product inhibition as shown in the batch experiments [149]. By changing parameters like the column switch time, the eluent flow-rate, the feed concentration, and the enzyme activity, purity close to 100% was achieved.

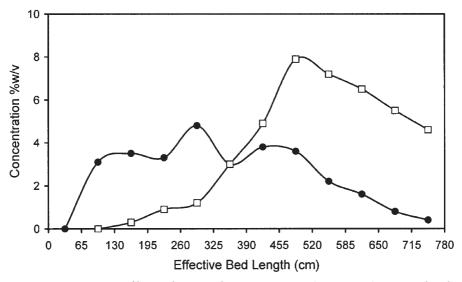


Fig. 11. Concentration profiles inside an SMB bioreactor. Dextrin (*open square*) was introduced in the reactor and enzymatically converted into maltose (*filled circle*), which was separated due to differences in the retention on the stationary phase. (Reprinted with permission from [177])

5.4.6 Reactions with Lipases

A few publications dealing with enzymatic conversion using lipases in a chromatographic reactor appeared in the recently literature. Mensah et al. [178] studied the enzymatic esterification of propionic acid and isoamyl alcohol (dissolved in hexane) to produce isoamyl propionate according to the following scheme:

isoamil alcohol + propionic acid $\xrightarrow{\text{lipase}}$ isoamil propionate + water.

In this context the lipase was immobilized on a support which also adsorbed water and propionic acid. During the reaction, the water caused a decrease of the reaction rate. While the water adsorption on the catalyst results in a reversible decrease of the enzyme activity, an excessive accumulation of water in the bulk mobile phase resulted in rapid irreversible deactivation of the enzyme.

The dynamics of the system were studied using a batch chromatographic reactor. The reactor was saturated with hexane prior to feeding with the mixture of 1 mol/l isoamyl alcohol and propionic acid dissolved in hexane. The concentration profiles recorded at the column outlet are shown in Fig. 12.

Both adsorption and reaction plays an important role. Since the adsorption isotherm is favorable for the adsorption of propionic acid, the ester is formed at the beginning of the reactor thus ester and unreacted alcohol move ahead of the propionic acid front. Water is retained on the catalyst and stays behind the front. Thus no further reaction occurs. When the propionic acid front reaches the reactor outlet, the reaction takes place over the entire reactor volume, thus assur-

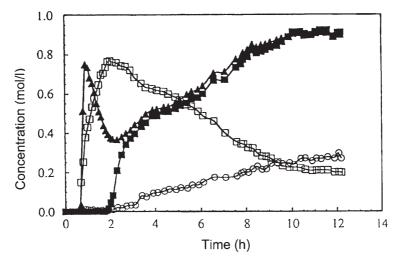


Fig. 12. Outlet concentration profiles from a batch chromatographic bioreactor for enzyme catalyzed esterification. Water, which when in the liquid phase irreversibly inhibits the reaction, is adsorbed. The profiles of water (*open circle*), propionic acid (*filled square*), isoamyl alcohol (*filled triangle*) and isoamyl propionate (*open square*) at the reactor outlet are presented. (Reprinted with permission from [178])

ing that the highest conversion is obtained. The water, however, accumulates on the biocatalyst, decreasing its activity. Consequently, the conversion decreases with time. In addition, once the matrix is saturated, water starts to accumulate in the liquid phase, causing further irreversible deactivation of the enzyme. To prevent enzyme degradation, the reaction should be stopped at this point and the resin should be regenerated. Such a procedure was performed several times without significant changes in performance with a conversion, which was close to 80%. To increase further the efficiency of the process an ion-exchange resin was added to improve the water adsorption. Experiments performed under otherwise identical conditions resulted in a longer period of operation at high conversion. Continuous operation of such a system, including continuous regeneration, was shown to be possible by the same authors [179]. They constructed a periodic counter-current adsorptive reactor (or simulated moving bed reactor) for the regeneration of the adsorber and described it with a mathematical model, which was further used for process optimization. It was shown that with a configuration consisting of just two beds in series, a 50% greater productivity in comparison to the conventional fixed-bed reactor could be achieved.

The second reaction studied using lipase as catalyst was the reversible regioselective esterification of propionic acid and 2-ethyl-1,3-hexanediol [180]. While the previously described reaction was almost irreversible, this reaction is equilibrium limited with an apparent equilibrium constant of 0.6 ± 0.1 . In addition, the accumulated water inhibits the enzyme. Therefore, only the removal of the water from the reaction zone assures high enzymatic activity as well as drives the reaction beyond thermodynamic equilibrium. Experiments with two

fixed-bed chromatographic reactors were performed: the first contained only the enzyme catalysts while in the second an ion-exchange resin was added. In both cases the reactors were saturated with hexane and fed with a mixture of 1 mol/l propionic acid and 2-ethyl-1,3-hexanediol dissolved in hexane. The behavior of the systems was very similar to the one reported by Mensah et al. [178]. As the reactants enter into reactor they are converted into monoester and water. The propionic acid and the water were adsorbed on the catalyst while the non-retained diol and the ester are moved ahead. As the propionic acid front moves across the reactor the reaction take place in a larger and larger volume, resulting in an increased monoester concentration. However, concomitantly, the water accumulates on the catalyst. Once the resin is saturated the concentration of water increases until the thermodynamically defined steady state is reached. The fact that an adsorber was added in the second reactor resulted in a much higher conversion (64% in the chromatographic reactor compared to 44% in the simple reactor) due to the separation of the water from the reaction mixture and the ensuing higher biocatalyst activity. In addition, due to higher water uptake rate on the ion-exchanger, a longer period with enhanced conversion was achieved. By changing the inlet composition, conversions of up to 80% during the transition period became possible. For repeated operation a regeneration step is required.

5.4.7 Chiral Hydrolysis

Another interesting approach was presented by den Hollander et al. [181]. They performed a selective enzymatic hydrolysis of the L-enantiomer of a racemic mixture of N-acetyl-methionine to produce L-methionine and acetic acid using *N*-acylamino acid amidohydrolase as catalyst. In this reaction only the L-acetylmethionine is hydrolyzed while the D-form remains untouched. The reaction is reversible, and therefore the separation of the products is necessary to shift the equilibrium toward higher conversion. The chromatographic reactor was in this case based on the principle of centrifugal partition chromatography (CPC). This liquid-liquid chromatographic system consisted of an aqueous two-phase system. The reaction occurred in the stationary phase and the separation is determined by the partition coefficients of the involved substances in the two-phase system. The system behavior was successfully predicted by a mathematical model. A more detailed study was presented from the same group [182]. With the adjustment of the operating conditions almost complete conversion was obtained. However, closer investigation showed that the enhanced conversion was not due to the separation effect but rather to the slower mass transfer.

5.4.8 Penicillin Hydrolysis

Wu et al. [183] studied the reversible hydrolysis of penicillin G into 6-aminopenicillanic acid (6-APA) and phenylacetic acid (PAA) in a chromatographic reactor. *E. coli* cells containing penicillin acylase (the catalyst) were immobilized by entrapment into gelatine and further cross-linking with glutaraldehyde. The ad-

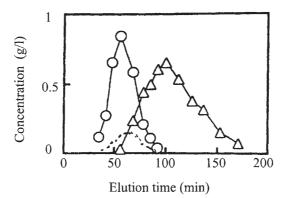


Fig.13. Outlet concentration profiles from a batch chromatographic bioreactor for enzyme catalyzed hydrolysis. Both products, i. e., 6-aminopenicillanic acid (*open triangle*) and phenylacetic acid (*open circle*), are separated resulting in a very high conversion. The penicillin G profile is presented as a *dashed line* (Reprinted with permission from [183])

sorbent was macroporous cross-linked polystyrene. The reactor was filled with equal volumes of both particles and used as a fixed-bed chromatographic reactor. As can be seen in Fig. 13, good separation of product and substrate was obtained. Penicillin G is indicated by a dotted line since its concentration was too low to be detected. Conversions of up to 98% were achieved.

6 Conclusions and Further Perspectives

In recent years we have seen many efforts to understand and describe properly the behavior of a chromatographic reactor. Although the proper mathematical description in principle has already been known for decades, the solutions for the more complex but also the more realistic cases can be effected only numerically. This has recently become possible thanks to the constantly increasing power of modern computers now capable of handling even complex systems of differential equations within a reasonable amount of time. Along with the hardware, the development of suitable software resulted in faster and more efficient procedures for numerical solving of differential equations. A second significant development occurred in the material sciences, where the production of many new chromatographic supports with increased chemical stability, binding capacity, and excellent hydrodynamics characteristics was achieved. From the field of biotechnology, the rapid development of different genetic and biochemistry techniques enabling the isolation or even the creation and characterization of thousands of biologic compounds should be mentioned. Their purification and selective conversion is a challenging task, which requires sophisticated solutions and efficient process instrumentation.

So far, all applications of the chromatographic enzyme reactor were limited to conversions of small molecules serving mainly as "case studies". In the near future these studies will hopefully be extended to larger substrate molecules and

enzymes immobilized on supports like monoliths, which enable fast mass transfer between the liquid and the solid phase and consequently able of preserving high enzyme activity. The knowledge obtained from batch chromatographic bioreactors on the laboratory level can nowadays be successfully used to predict the behavior of complex continuous reactor systems like the SMBR. Already a huge amount of knowledge has been collected and we can expect that it will find its application in real industrial applications.

7

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Simulated Moving Bed Chromatography (SMB) for Application in Bioseparation

Sabine Imamoglu

Aventis Pharma Deutschland GmbH, Industriepark Hoechst, 65926 Frankfurt, Germany. *E-mail: sabine.imamoglu@aventis.com*

Simulated Moving Bed (SMB) technology is of rising interest in the field of bioseparation. This is particularly due to its advantages such as reduction of solvent consumption, high productivity and final purities as well as low investment costs in comparison to eluent chromatography. SMB units can operate under high productivity overloaded conditions. This leads to non-linear competitive adsorption behavior, which has to be accounted for when designing and optimizing new SMB separations. The so called "Triangle Theory", which is briefly reviewed in this chapter, provides explicit criteria for the choice of the operating conditions of SMB units to achieve the prescribed separation of a mixture characterized by Langmuir, modified Langmuir and bi-Langmuir isotherms.

The application of the SMB-technique to the downstream processing of biotechnological products requires some specific changes to meet the special demands of bioproduct isolation. Some exemplary applications are given including separations of sugars, proteins, monoclonal antibodies, ionic molecules and optical isomers and for desalting.

Keywords: Preparative chromatography, Simulated moving bed chromatography, Continuous separation technique, Triangle theory, Bioseparation

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Abbreviations

- *c* Fluid phase weight concentration
- H Henry constant
- K Adsorption equilibrium constant
- m_i Mass flow ratio in section *j*, defined by Eq. (6)
- n Adsorbed phase weight concentration
- Q Volumetric flow rate
- *t** Switch time in a SMB unit
- *V* Volume of the column

Greek letters

- ε Void fraction of the bed
- $\epsilon_{\rm p}$ Intraparticle void fraction
- $\varepsilon^{\frac{1}{2}}$ Overall void fraction, $\varepsilon^* = \varepsilon + \varepsilon_p (1 \varepsilon)$
- ω Equilibrium Theory parameter defined by Eq. (20)

Subscripts and superscripts

- *A* More firmly retained component in the feed
- B Less firmly retained component in the feed

1 Introduction

At present, the purification by chromatographic processes is the most powerful high-resolution bioseparation technique for many different products from the laboratory to the industrial scale. In this context, continuous simulated moving bed (SMB) systems are of increasing interest for the purification of pharmaceuticals or specialty chemicals (racemic mixtures, proteins, organic acids, etc.). This is particularly due to the typical advantages of SMB-systems, such as reduction of solvent consumption, increase in productivity and purity obtained as well as in investment costs in comparison to conventional batch elution chromatography [1].

An SMB is a multi-column continuous chromatographic separator, based on the counter-current movement of a liquid and a "stationary" phase packed in the columns. The SMB technology was introduced 40 years ago [2] and has to date mainly been applied to very large-scale production/purification processes, e.g., in the petrochemical and sugar industries [3]. Although from the start, the SMB was recognized as a very efficient technology, it was for a long time more or less ignored in the field of fine chemistry and pharmaceuticals [4]. To some extent this was due to the patent situation and the complexity of the concept. In the 1970s, however, High Performance Liquid Chromatography (HPLC) was developed for the preparative separation of fine chemicals. This technique was shown to be very efficient but also expensive to use, with pronounced product dilution quickly recognized as a major drawback. Starting in the 1980s and continuing until today, there has been a drastic increase in the demand for technologies allowing the quick and efficient preparation of pure pharmaceutical and food products, because regulations concerning the purity and consistency of such substances became more and more strict [5]. Finally, in the 1990s, the arrival of "biotechnology" as a full blown industry resulted in an ever increasing number of sensitive, high-value products such as peptides, recombinant proteins and antibodies, which required a yet unheard of level of final purity.

In this context the integration of HPLC in the SMB concept has shown a tremendous potential for the development of separation process which are efficient and versatile as well as economically sound. The first separations of pharmaceutical compounds using HPLC-SMB technology were performed in the early 1990s [6–8]. Other areas of application, e.g., the fine chemicals, cosmetics and perfume industries have since followed suit [9]. Most importantly and as a reaction to the needs of these new areas of application, SMB systems smaller than the huge SMB-plants adapted to the needs of the petrochemical industry, are now commercially available.

There are two disadvantages to using SMB-chromatography in the field of biomolecule separation. With SMB chromatography it is only possible to divide the feed mixture into two product streams and not into a multitude of fractions. However, each of the product streams can consist of more than one compound. A more serious limitation for SMB-biochromatography is the restriction to an isocratic elution mode. To solve this problem, several attempts have been made to modify the adsorption strength by influencing different parameters, e.g. the system pressure or the pH. Even today, the SMB-technology is not used extensively in the biotechnology and biopharmaceutical industry, but the potential is there and given the current state-of-the-art in both instrumentation and process development tools (e.g., simulation software) very attractive applications can be expected for the near future.

2 The principle of SMB

In preparative chromatography, selectivity and efficiency no longer have the same importance they do in analytical chromatography. A certain selectivity is required in preparative chromatography as everywhere else in order to achieve the separation, but other parameters are at least as important if not more so. These include the loading capacity of the stationary phase and the maximum speed (throughput) of the process. The three main economic criteria for a large scale separation process are

- *Productivity*, i.e. the amount of product produced per unit of time and stationary phase respectively column volume

- *Eluent consumption*, since this determines the cost for mobile phase in terms of preparation and handling (tanks, water preparing systems, pumps)
- *Product dilution*, since this determines the cost for further product processing (e.g., concentration, polishing) [10].

To optimize a given preparative chromatographic process (highest productivity, lowest mobile phase consumption, and product dilution) the separation has to be performed with the highest product concentrations still compatible with the system. One immediate consequence of this is that each column has to be operated in the non-linear range of the adsorption isotherm.

A counter current movement of the mobile phase and the sorbent has some unique advantages when designing separation processes for maximum economy. The efficiency requirement for the sorbent is lower compared to other chromatographic modes, since no individual column has to achieve full resolution. Instead only the pure fractions of the zones obtained are withdrawn from the system. The time-space yield in terms of productivity is enhanced considerably by the improved utilization of the sorbent capacity. The product dilution is lower, pure fractions are withdrawn with high yield and it is not necessary to consider fractions of less then the desired purity. Early on it was re-

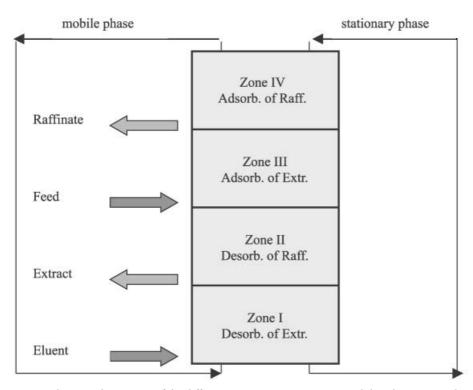


Fig.1. Schematic description of the different zones in an SMB system and the adsorption and desorption processes in these zones

cognized however, that it is extremely difficult to operate a true moving bed (TMB) system because it would involve the circulation of a solid adsorbent, which would lead to significant mechanical stress for the solid phase. It was soon shown, that all the theoretical advantages of TMB chromatography could also be achieved by the SMB-approach, which uses several fixed-bed columns in series and an appropriate shift of the injection and collection points to simulate the movement of the solid phase. SMB is a continuous process and hence can be perfectly implemented into all continuous production processes. SMB is also much more suited to large-scale production than conventional (batch) chromatography. Another distinct advantage of the SMB-approach is that it generally requires significantly less eluent than other chromatographic separation modes

The classical moving bed consists of four different zones, in which different constraints must be met, Fig. 1:

- Zone I (between the eluent and the extract): the more firmly retained product (A, extract) must be completely desorbed.
- Zone II (between the extract and the feed): the less firmly retained product (B, raffinate) must be completely desorbed.

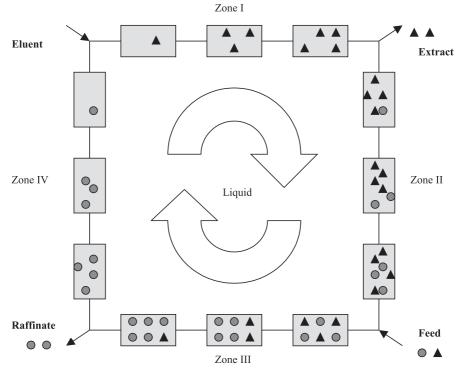


Fig. 2. Principle of the SMB

- Zone III (between the feed and the raffinate): the more firmly retained product (A, extract) must be completely adsorbed.
- Zone IV (between the raffinate and the eluent): the less firmly retained product (B, raffinate) must be completely adsorbed.

Under these circumstances, all the internal flow rates (volumetric flow rates, *Q*) are related to the inlet/outlet flow rates by simple mass balances:

$$Q_{\rm II} = Q_{\rm I} - Q_{\rm Ext} \tag{1}$$

$$Q_{\rm III} = Q_{\rm II} + Q_{\rm Feed} \tag{2}$$

$$Q_{\rm IV} = Q_{\rm III} - Q_{\rm Raff} \tag{3}$$

$$Q_{\rm I} = Q_{\rm IV} + Q_{\rm El} \tag{4}$$

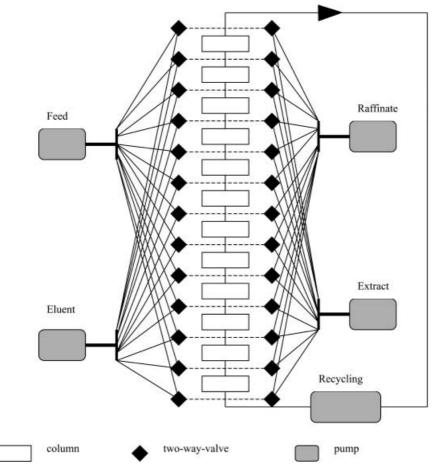


Fig. 3. General set-up of a SMB separation system

The inlet/outlet flow rates are related by:

$$Q_{\rm Ext} + Q_{\rm Raff} = Q_{\rm Feed} + Q_{\rm El} \tag{5}$$

Between Zone II and III, the feed mixture is introduced into the system and transported with the mobile phase into Zone III, Fig. 2.

In Zone III the compounds which have higher affinity to the sorbent are adsorbed and transported with the stationary phase to Zone I. There they are desorbed by a mixture of fresh eluent introduced between Zones I and IV and the recycled eluent from Zone IV. The less adsorbed compounds in Zone III are moved with the mobile phase to Zone IV. There they adsorb and are transported in that form together with the stationary phase (column) to Zone II, where they finally become desorbed.

The different adsorption and desorption events are controlled via the flow rates adjusted by the means of 3 or 5 external pumps and the column switch times, Fig. 3. The key element for success is the proper selection of the respective flow rates, which must be chosen in such a way that the extract front between zones I and II and the raffinate front between zones III and IV are stabilized, while the separation between zones II and III is assured. A simple trial-anderror approach to such an optimization of the system parameters is unlikely to be successful. Instead, the chromatographic behavior of all compounds has to be modeled and simulated.

In the first step, the adsorption isotherms of the compounds should be determined under non-linear chromatographic conditions, which can be done in several ways [11]. Afterwards, models should be implemented and used to simulate the chromatographic behavior and to find the optimum system parameters for a given separation problem. Different approaches for finding the optimum parameter are described in the literature [12–16] mainly for adsorption and ion exchange chromatography.

2.1

Technical Aspects of SMB Implementation

A classical Simulated Moving Bed system consists of 4 to 24 columns distributed between 4 zones, in addition to 3 to 5 pumps and valves which connect the different streams between the columns. In general a 4 column SMB should be sufficient to test and optimize the conditions for any given separation problem. The optimal number of columns per zone must be determined in the simulation of the SMB process. The rule is more columns per zone result in a better separation, while too many columns per zone make the system too complex. If an infinite number of columns per zone are used the SMB approaches a TMB.

There are different ways to connect the columns to build a SMB system. An important aspect is always the position of the recycling pump. The recycling pump ensures the internal flow of the mobile phase. Most often the recycling pump is placed between the last and the first column, i.e. columns 12 and 1 in Fig. 2. Once the recycling pump is fixed with respect to the columns, it moves with respect to the zones and is alternatively located in zones IV, III, II, and I. The flow rates required in the different zones are different and so the pump flow rates vary from

zone to zone. With small variations, most large-scale SMB-units show this basic design. Process control is comparatively easy under these circumstances, since the design of the system is relatively simple. For small SMB-systems the volume of the recycling pump can lead to an asymmetry, which in turn can result in a decrease in final purity. Possible solutions to that problem are the use of a shorter column (near the recycling pump) or an asynchronous shift of the inlets and outlets [17].

Another option for the recycling pump is to fix it with respect to the zones rather than the columns. In this case the pump is always located between zone IV and I where only the eluent is present. However, since the columns rotate, additional valves are needed for this design, which makes the system more complex. A third possibility is to use an eluent pump instead of a recycling pump. Again this variant requires more valves than, e.g., the first option and has the disadvantage of requiring the connection of one outlet to the eluent reservoir. Independent of the location of the recycling pump, there are different options to control the outlet flow rates. In particular this can be done by pumps, by analogous valves, by flow meters, or by pressure control.

Summing up, a robust and easy to handle SMB-design uses 4 zones, a recycling pump fixed in respect to the columns and two pumps for the control of the outlet flow rates. Extremely high precision of all technical components of the SMB is needed. All pumps and valves have to be exactly synchronized. The flow rates should not vary by more than1% from the preset value. All connections between the different parts of the system must be carefully optimized in order to minimize the dead volume. All columns should be stable and nearly identical in performance. If the SMB-technology is to be used in Biotechnology, GMP issues (cleaning, process and software validation) also have to be considered. In addition and as with any continuous process in that particular area, the definition of a batch could be a problem.

2.2 Operating Conditions

The important step in designing an SMB is to find the operating conditions suited to processing a given amount of feed per day or week or month [18]. The procedure, which is illustrated here, is based on the modeling of non-linear chromatography. Since the SMB is to be used as a preparative separation technique, which is supposed to operate under high product overload conditions, i. e. in the non-linear part of the adsorption isotherm, the linear part of the adsorption isotherm is normally of little importance. SMBs can also be operated under linear conditions but this somewhat academic case will only be considered marginally here. The determination of the competitive non-linear adsorption isotherms of both compounds of interest is, on the other hand, generally required. It is usually possible to get all relevant data defining suitable operating conditions from measuring the feed mixture directly. The use of the pure components is usually not necessary [18].

As mentioned previously, the design of an SMB-separation requires the correct choice of the different flow rates of the recycle stream, the feed stream, the eluent stream, the extract stream, the raffinate stream and the shift period for the columns/zones, which corresponds to the simulated "solid stream". Other important parameters for the operating conditions are:

- The feed concentrations
- The number of columns per zone
- The column length
- The column diameter and
- The particle size.

All these parameters can be determined and optimized by data measuring on the laboratory scale.

3 Theoretical Background

3.1 The "Triangle Theory"

For a deeper understanding of SMB behavior, a more synthetic view of the process is required. This is, e.g., possible by applying the Equilibrium Theory Model, i.e. a model where mass transfer resistance and axial dispersion are neglected (columns of infinite efficiency). The application of this highly idealized model to SMB units under the concomitant assumption of Langmuir-type adsorption isotherms forms the basis of the so-called "Triangle Theory" proposed by Morbidelli and his group [19]. The Triangle theory facilitates the determination of optimal and robust operating conditions of SMBs suitable for achieving the desired separation [20-27]. A major feature of this approach consists of the fact that the typical overloaded operating conditions of the SMB can be taken into account, i.e. the highly non-linear and competitive adsorption behavior. This makes this approach superior to particularly when compared with others, which are based on empirical extrapolations of the linear adsorption isotherms to design the non-linear SMB operations [28].

Let us assume a standard four-zone SMB unit, in which the complete separation of a binary mixture, constituted of the more retained component A and the less retained component B is to be achieved. In the framework of the Equilibrium Theory, the key operating parameters through which the performance of the SMB can be controlled are the flow rate ratios, m_j , j=1,...,4, in the four sections of the SMB unit, according to:

$$m_j = \frac{Q_j t^* - V \varepsilon^*}{V(1 - \varepsilon^*)} \tag{6}$$

where *V* is the column volume, *t*^{*} is the column switch time, i. e., the time between two successive switches of the inlet and outlet ports, $\varepsilon^* = \varepsilon + (1 - \varepsilon) \varepsilon_p$ is the overall void fraction of the column, with ε and ε_p , being the bed void fraction and the macroporosity of the stationary phase particles, and Q_j is the volumetric flow rate in the *j*th section of the SMB unit.

Constraints on the values of the flow rate ratios can thus be determined, which depend solely on parameters characterizing the adsorption equilibrium of the species to be separated. In the most general case, these can be derived from a bi-Langmuir multicomponent adsorption isotherm [27] or, as previously suggested, from the Langmuir and the modified Langmuir isotherm [20,25]. For the sake of simplicity the binary Langmuir isotherm will be used from hereon, as defined by:

$$n_i = \frac{\mathrm{H}_i c_i}{1 + \mathrm{K}_A c_A + \mathrm{K}_B c_B}, \quad i = A, B \tag{7}$$

where n_i and c_i are the adsorbed and mobile phase concentration, H_i is the Henry constant of the *i*th component, i. e. the slope of the single component adsorption isotherm at infinite dilution, K_i is the equilibrium constant of the *i*th component, which accounts for the competitive and overload effects.

Subsequently, the condition of complete separation has to be coupled with the material balances derived for the nodes of the SMB unit and implemented in the Equilibrium Theory Model for Langmuir-type systems. That leads to the set of mathematical conditions given below, which the flow rate ratios have to fulfil in order to achieve complete separation, in particular:

$$\mathbf{H}_A < m_1 < \infty \tag{8}$$

$$m_{2,cr}(m_2, m_3) < m_2 < m_3 < m_{3,cr}(m_2, m_3)$$
 (9)

$$\frac{-\varepsilon_{\rm p}}{1-\varepsilon_{\rm p}} < m_4 < m_{4,\,cr}(m_2,\,m_3) \tag{10}$$

$$= \frac{1}{2} \left\{ H_B + m_3 + K_B c_B^F (m_3 - m_2) - \sqrt{[H_B + m_3 + K_B c_B^F (m_3 - m_2)^2 - 4 H_B m_3]} \right\}$$

where the superscript *F* indicates the feed conditions.

The constraints on m_1 and m_4 are explicit. The lower limit of m_1 , however, does not depend on the other flow rate ratios, whereas the upper limit of m_4 is an explicit function of the flow rate ratios m_2 and m_3 and of the feed composition respectively [25]. The constraints on m_2 and m_3 are implicit (see Eq. 4), but they do not depend on m_1 and m_4 . Therefore, they define a unique region of complete separation in the (m_2, m_3) plane, which is the triangle-shaped region abw in Fig. 4. The boundaries of this region can be calculated explicitly in terms of the adsorption equilibrium parameters and the feed composition as follows [25]:

Straight line wf:

$$(\mathbf{H}_{A} - \boldsymbol{\omega}_{G}(1 + \mathbf{K}_{A} c_{A}^{F})) \ m_{2} + \mathbf{K}_{A} c_{A}^{F} \boldsymbol{\omega}_{G} \ m_{3} = \boldsymbol{\omega}_{G}(\mathbf{H}_{A} - \boldsymbol{\omega}_{G}).$$
(11)

- Straight line wb:

$$(\mathbf{H}_{A} - \mathbf{H}_{B}(1 + \mathbf{K}_{A} c_{A}^{F})) \ m_{2} + \mathbf{K}_{A} c_{A}^{F} \mathbf{H}_{B} \ m_{3} = \mathbf{H}_{B}(\mathbf{H}_{A} - \mathbf{H}_{B}).$$
(12)

- Curve ra:

$$m_3 = m_2 + \frac{(\sqrt{H_A} - \sqrt{m_2})^2}{K_a c_A^F}.$$
 (13)

- Straight line ab:

$$m_3 = m_2$$
. (14)

The co-ordinates of the intersection points are given by:

point
$$a(H_A, H_A)$$
 (15)

point
$$b(H_B, H_B)$$
 (16)

$$\operatorname{point} f(\omega_G, \omega_G) \tag{17}$$

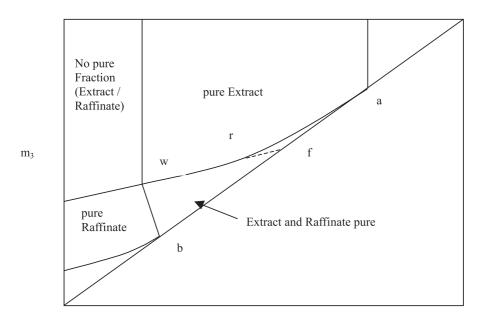
$$\operatorname{point} r\left(\frac{\omega_G^2}{H_A}, \frac{\omega_G[\omega_F(H_A - \omega_G)(H_A - H_B) + H_B\omega_G(H_A - \omega_F)]}{H_A H_B(H_A - \omega_F)}\right)$$
(18)

and point w
$$\left(\frac{H_B\omega_G}{H_A}, \frac{\omega_G[\omega_F(H_A - H_B) + H_B(H_B - \omega_F)]}{H_B(H_A - \omega_F)}\right)$$
 (19)

In the above equation ω_F and ω_G depend on the feed composition. They are the roots of the following quadratic equation, with $\omega_G > \omega_F > 0$:

$$(1 + K_A c_A^F + K_B c_B^F) \omega^2 - [H_A (1 + K_B c_B^F) + H_B (1 + K_A c_A^F)] \omega + H_A H_B = 0.$$
(20)

As illustrated in Fig. 4, the region of complete separation is surrounded by three regions corresponding to three different operating regimes. In the region



 m_2

Fig. 4. Separation of a two component mixture using a non-adsorbable desorbent/eluent

of pure raffinate, as the name indicates, the raffinate stream is pure but the extract is polluted by component *B*. In the region of pure extract, the extract is pure, but the raffinate is polluted by component *A*. In the third region (no pure fraction), both components *A* and *B* are found in both the extract and the raffinate streams. The information from the geometrical representation of the separation regions in the (m_2, m_3) plane in Fig. 4 is only correct if the relevant constraints on m_1 and m_4 are fulfilled, in particular inequalities (8) and (10).

The vertex *w* of the region of complete separation in the (m_2, m_3) plane represents the optimal operating conditions in terms of solvent consumption and productivity per unit mass of stationary phase. However, under such circumstances, even the slightest disturbance in the process conditions or the smallest error in the evaluation of the adsorption equilibrium parameters will result in a slight deviation of the operating point from the optimal location into a region, where complete separation is no longer possible. Since the optimal operating conditions are not very robust, the operating point under realistic conditions is chosen somewhere inside the complete separation triangle and not at its vertex. That is a compromise between separation performance (productivity and solvent requirement) and robustness of the performance.

The multicomponent Langmuir adsorption isotherm given in Eq. (7) is the simplest model for the description of non-linear, multicomponent, adsorption equilibrium. At high concentration, the model predicts "saturation" of the stationary phase and overload of the chromatographic column. At low concentration (high dilution) the behavior can be correctly described by the non-competitive linear adsorption isotherm:

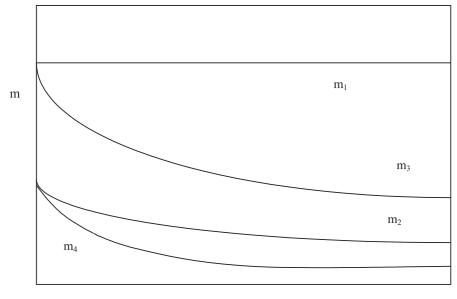
$$n_i = \mathcal{H}_i c_i (i = A, B). \tag{21}$$

When translated to the SMB conditions, these features imply that increasing feed concentration lead to an increasing degree of non-linearity due to the fact that the adsorption columns increasingly are operated under overload conditions. This effect is predicted by the approach summarized in the previous section, in particular by Eqs. (8) to (19), which allow the calculation of the constraints on m_1 and m_4 and the boundaries of the complete separation region in the (m_2, m_3) plane as a function of feed composition [19].

The non-linearity effect can easily be demonstrated by the following theoretical separation of a binary mixture. Let us assume that the concentrations of A and B are the same and correspond each to half of the overall feed concentration. The feed concentration is in addition assumed to be the only parameter necessary to characterize the feed composition. The mass flow ratio in section 1 (constrained by Eq. (8)) does not depend on the feed composition. On the contrary, the upper limit on the flow rate ratio m_4 given by Eq. (10) is a function of the feed composition. Both dependencies are illustrated in Fig. 5.

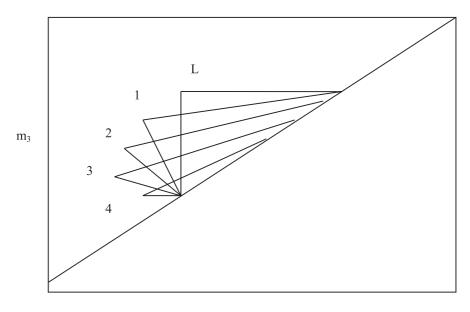
When the constraint on m_4 is not fulfilled, some of the weakly adsorbed component B is carried over by the recycled mobile phase and starts to pollute the extract.

Figure 6, on the other hand, illustrates the differences between operating an SMB under linear and non-linear conditions. In particular, this figure illustrates the effect of the overall concentration on the region of complete separation re-



c [g/l]

Fig. 5. Optimal values of the flow rate ratios as a function of the overall feed concentration



 m_2

Fig. 6. Effect of the overall concentration of the feed mixture on the region of complete separation

gion in the (m_2, m_3) plane, under conditions where all other system parameters, such as the values of the adsorption equilibrium parameters, are kept constant. Five complete separation regions plotted using Eqs. (11) to (19) are shown. Region *L* corresponds to the limiting situation of a feed mixture constituted of *A* and *B* infinitely diluted in the solvent. Regions 1 to 4 correspond to higher and higher feed concentrations.

As the feed concentration increases the basis of the triangle and the position of the vertex shifts downwards to the left. The complete separation region becomes narrower and concomitantly also less robust. This implies that when the concentration of the feed is increased, the flow rate ratios in Sects. 2 and 3, as well as the difference $(m_3 - m_2)$ decrease in consequence (see also Fig. 5). Material balances show that the maximum productivity increases with the feed concentration increases, productivity improves, but robustness becomes poorer. So the optimum value for the feed concentration of an SMB tends to be defined by a compromise between the opposite needs of productivity and robustness [25, 27].

When the feed mixture is infinitely diluted, the competitive Langmuir isotherms of the two component approach the respective non-competitive, linear, single-component isotherms (21) and the constraints on the m_j parameters of the SMB unit reduce to the following set of decoupled inequalities:

$$\mathbf{H}_A < m_1 < \infty \tag{22}$$

$$\mathbf{H}_{B} < m_{2} < \mathbf{H}_{A} \tag{23}$$

$$\mathbf{H}_{B} < m_{1} < \mathbf{H}_{A} \tag{24}$$

$$\frac{-\varepsilon_{\rm p}}{1-\varepsilon_{\rm p}} < m_4 < {\rm H}_B \tag{25}$$

These are the classical constraints for SMB separation [29, 30].

3.2

Choice of Process Operating Conditions

When a specific feed composition is given, the constraints on m_1 and m_4 as well as the complete separation region in the (m_2, m_3) plane can be determined, since these depend only on the parameters of the adsorption equilibrium isotherms and the feed composition itself. Based on these values an operating point can be selected, i. e. a set of four values of $m_j = 1, ..., 4$ fulfilling the complete separation requirements. Since the flow rate ratios are dimensionless groups combining column volumes, flow rates and switching intervals, the constraints on the flow rate ratios are independent of the size and productivity of the SMB unit.

Once the four flow rate ratios are selected, two additional constraints are necessary to determine the values of the six design and process parameters required for operating an SMB. In particular, V, t^* and Q_j , j = 1, ..., 4 are wanted for the set up of the separation. The volume of the columns is normally a given one, especially if the plant is already in use, or it is selected based on productivity requirements. A second process parameter, the switching time or the upper flow rate limit of the unit, is often also determined, e.g., by the pressure limit of the unit and the flow rate dependency of the column efficiency (theoretical plate height) [22, 30, 31]. Based on this, the other process parameters can be calculated from the selected values of the flow rate ratios. If the volume of the column and the switching time are known, the flow rates in the sections of the unit are calculated by applying the definition of m_i (1) through the equation:

$$Q_j = \frac{V[m_j(1-\varepsilon^*)+\varepsilon^*}{t^*}.$$
(26)

3.3 Simulation of SMB

For the simulation of SMB-separations efficient software packages, based on the Triangle-Theory, are commercially available. The number of columns, the column dimensions, the theoretical number of plates in the columns, the feed concentration, the bi-Langmuir adsorption isotherm parameters and the number of cycles need to be defined by the user. Then the separation is simulated and values for the flow rate ratios, the flow rates, the switching time and the quality of the separation, purity and yield, are calculated. Based on these values an actual separation can be performed. However, some optimization/further development is usually necessary, since the simulations are based on an ideal model and the derived parameters and results therefore can only be taken as indications for the test runs.

4 Applications

SMB have been used for many bioseparation problems. These problems are reviewed in the next sections and include:

- the separation of sugars,
- desalting steps,
- the purification of proteins,
- the purification of monoclonal antibodies,
- the separation of ionic molecules,
- the separation in organic solvents and
- the separation of optical isomers.

4.1 Separation of Sugars

The separation of the two sugars fructose and glucose, is currently perhaps the industrial separation of biomolecules performed on the largest scale. Since it is a typical two-component separation, the advantages of utilizing an SMB for this purpose are obvious and glucose/fructose separations by SMB are well estab-

lished in industry. Since the pioneering work of Barker [32], this particular separation has been investigated by scientists and process engineers [33-35]. The separation is preferably performed on an ion-exchange resin (typically a polystyrene-based cation exchange resin in the calcium form), using warm water as eluent. The fructose forms a complex with the calcium ions and is retained on the column, while glucose and other oligosaccharides are eluted with the eluent. For improving the productivity, some work has been done with zeolites (calcium form) as alternative stationary phase [36]. The so-called Sarex process [37] has been developed for the continuous separation of inverted carbohydrate syrup containing 42% fructose. The process yields 90-94% pure fructose at a recovery yield of over 90%. The glucose-rich fraction is about 80% pure. This separation can be implemented on columns of a few meters internal diameter.

SMB packed with cationic resins in the calcium form have also been used for the production of other monosaccharides such as xylose or arabinose [3]. The separation of mono- from disaccharides or of different disaccharides is another interesting application. For instance, the separation of palatinose and trehalulose has been studied by Kishihara [38] and the separation of fructose and trehalulose by researchers at NOVASEP [4]. The SMB technology has also been used for the fractionation of dextran (polyglucoside mainly used as a blood plasma volume expander) by size exclusion chromatography using columns packed with Spherosil XOB075 (200 to 400 µm porous silica beads) [39]. The technology has proved itself as being efficient allowing one to obtain, according to the flow rates, different dextran fractions from 10,000 to 125,000 Dalton.

4.2 Desalting

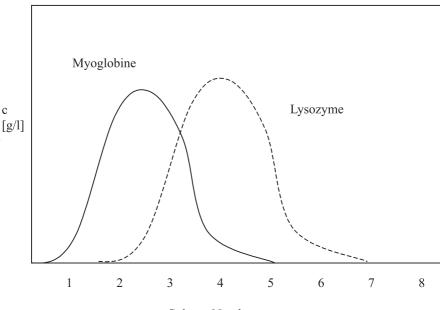
Desalting is another simple and interesting application of the SMB in biotechnology [4]. Different mechanisms can be used for this purpose, including ion-exclusion, hydrophobic interaction, size exclusion or ion exchange effects [36]. Glucose and NaCl have, for instance, been separated from feed mixtures containing the same amount of Glucose and salt using a Retardion 11 A-8 [40] resin. A very high purity was obtained for both products. NaCl and Glycerol have been separated using an Amberlite HFS-471X (8% DVB) phase, which is a strongly acidic cation-exchange resin in the sodium form. The mechanism of the separation is ion exclusion, i. e. based on the fact that the glycerol can enter the internal pores of the resin whereas the salt ions are excluded. The adsorption isotherm of glycerol was found to be linear (as to be expected for a substance with no interaction with the resin), whereas the adsorption isotherm of the salt was anti-Langmuir (as expected for an ion exclusion process).

Instead of ion-exclusion, size exclusion has been used in the separation of NH_4SO_4 from a protein [41]. In that case, the adsorption isotherms were found to be simply linear. A hydrophobic interaction separation has been used for desalting in the case of phenylalanine and NaCl [41]. NaCl shows almost no interaction with the packing and consequently has a linear adsorption isotherm. The phenylalanine, on the other hand, showed a classical Langmuir-type adsorption isotherm.

4.3 Purification of Proteins

Proteins have, to date, only rarely been purified by SMB. The first attempt was made by Huang et al. in 1986 [42]. They isolated trypsin from porcine pancreas extracts using an SMB made of only six columns. In addition, this example also demonstrates that SMB systems with a very limited number of columns can be efficient. Another example for a successful protein-separation by SMB is the purification of human serum albumin (HSA) using two SMB-systems connected in series [43]. The first SMB was used for removing the less strongly retained components and the second one for removing the more strongly retained components of the sample matrix.

A separation of myoglobin and lysozyme has been presented by Nicoud [44]. This purification was performed on SMB containing 8 columns using ACA 54 (Biosepra, France) as support. Very pure extracts (>98%) and raffinates (>98%) were obtained from a 50–50 mixture. An internal profile is given in Fig. 7. Another recently presented example is the separation of cyclosporine A from cyclic oligopeptide and other impurities in the reversed phase mode or by adsorption on silica gel presented by Schulte et al. [10].



Column Number

Fig. 7. Separation of myoglobin/lysozyme: internal profile on an 8 column SMB

4.4 Purification of Monoclonal Antibodies

The purification of a monoclonal antibody has been realized by SMB chromatography on an affinity chromatography stationary phase [45]. The recovery possible with the two-section SMB system depended on the desired extract purity. By adding two purge steps to the set-up, the monoclonal antibody could be isolated directly from cell culture supernatant with a yield of \geq 90%. The product purity was >99% based on SDS-Page. In this case the SMB chromatography offered not only the advantage of a continuous process but also a better exploitation of the adsorption capacity of the solid phase and, therefore, a smaller dilution of the product compared to conventional column chromatography.

4.5 Separation of Ionic Molecules

The SMB technology has also been used for the purification of different ionic molecules, such as amino acids [4], one pertinent example is the large-scale production of lysine [46]. Pure betaine has been isolated from molasses by a two step chromatographic process involving first ion exclusion chromatography, during which a mixture containing betaine and glycerol is separated from the rest of the feed, whereas in the second ion exclusion step pure betaine is separated from the glycerol [47].

Another related example concerns L-Glutathione, which is produced by yeast fermentation. L-Glutathione is a tripeptide used as a therapeutic in certain liver diseases. L-Glutathione of a purity of at least 99% purity is required in the final crystallization step of the pharmaceutical production process. Obtaining an Lglutathione of such purity is difficult especially with regard to the amino-acid impurities present in the original fermentation broth. One of the most challenging molecules in this regard is glutamic acid. For the separation of glutathione and glutamic acid a cation-exchange resin (Amberlite IR200C, 350–590 µm, Rohm & Haas) was used. The separation was implemented in a 16 column-SMB. The glutathione was obtained in the raffinate stream at 99% purity with 99% yield [48].

4.6 Separation in Organic Solvents

Many separations of organic molecules in organic solvents have been performed with an SMB, but only two of them concern the area of biotechnology/bioseparation. Much work has been done with regard to the separation of fatty acids and their derivatives since Szepy published the first results [49] on the separation of C_{16} to C_{22} methyl esters. The other relevant case is the separation of stereoisomers of phytol by SMB [9] using a classical silica phase with heptane-ethyl acetate as eluent and the Licosep 8–200 SMB system from NOVASEP.

4.7 Separation of Optical Isomers

One of the most challenging, but also very typical two-component separations encountered in the pharmaceutical industry is the separation of optical isomers. Since the two optical isomers are chemically equivalent, most chemical syntheses result in racemic mixtures. On the other hand, because the biological activity of the two isomers may differ dramatically, an efficient (chromatographic) separation is called for. The SMB technology seems to be predestined for the separation of optical isomers on a large-scale. The two first separations were done in 1992 by Negawa and Shoji for phenyl-ethyl alcohol [53] and by Fuchs et al. for threonine [54] isomers. By now a comparatively large number of application protocols exist [44,50] and process development is typically characterized by very short development times and extremely high probabilities of success. Attractively low purification costs can usually be achieved.

Particular examples for the separation of optical isomers in the (pharmaceutical) industry include prazinquatel [51], β -blockers [52], chiral epoxide [6], thiadiazin EMD5398 [18] and hetrazipine [7]. The Belgian company UCB Pharma uses a large-scale SMB from NOVASEP to perform optical isomer separation at a scale of several tons per year. Almost all of these separations are performed on cellulose-based stationary phases using organic eluents [4].

5 Conclusions

Simulated Moving Bed Chromatography has been used successfully for almost 30 years on a very large scale in the petrochemical industry. More recently, the high potential of the SMB-approach has also been recognized by the fine chemistry and pharmaceutical industries. Applications in the biotechnology field are increasing, where an SMB can be used putatively for many different products and applications including, for example, proteins (enzymes), isomers, or in desalt-ing/polishing steps. Originally designed for large-scale production processes (100,000 tons/year) SMB-systems can nowadays also be operated on a comparatively small scale, e.g., for production processes involving less than 1 kg per run, since such small-scale units have become commercially available, e.g., from NOVASEP.

The SMB separates binary mixtures into the components or multi-component mixtures into two fractions. The latter option is especially attractive if chromatographic conditions can be defined under which the target molecule is the first or the last to elute in a multi-component mixture. Under such circumstances the SMB can be used without technical modifications. In other situations, two SMB systems have to be used in series.

One important aspect concerns the difference between SMB and Batch Chromatography. In a continuous system the solvent processing is easier and the continuous nature of SMB may also have advantages in situations where bacterial growth is possible [4]. For example, bacterial growth in dilute solution of sugars may lead to plugging of a chromatographic system. In a continuous system like the SMB stagnant zones are easier to prevent and thus growth becomes less likely. Concomitantly, however, the continuous approach may require the redefinition of certain established concepts, such as that of a "batch", which is fundamental to many quality control schemes. In summing up, the SMB-approach has been found to have three main advantages over batch chromatography.

- In the SMB a significant amount of eluent can be saved. This is generally the case for binary separations, but can be less simple for multi-component systems.
- The SMB maximizes the productivity of chromatography. This becomes especially interesting for separations characterized by low selectivity or low efficiency.
- The continuous SMB process simplifies the operation and the connection with associated equipment (e.g., evaporation).

On the other hand, SMB requires strict process control and is less versatile than normal elution chromatography. In that sense, SMB should be viewed predominately as a very powerful tool for production plants, while batch chromatography with its higher flexibility is equally well suited for development purposes. The fact that efficient simulation software is needed to set up an SMB, while an "empirical" approach is often sufficient for success in batch chromatography points in the same direction.

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Continuous Annular Chromatography

Jürgen Wolfgang · Adalbert Prior

Prior Separation Technology GmbH, Vorarlberger Wirtschaftspark, 6840 Götzis, Austria. *E-mail: juergen.wolfgang@priorsep.com*

In recent years the demand for process scale chromatography systems in the industrial downstream process has been increasing steadily. Chromatography seems to be the method of choice when biological active compounds must be recovered from a mixture containing dozens of side products and contaminants as it is for example the case when processing fermentation broths. Since chromatography can solve almost any separation problem under mild operating conditions, a continuous chromatography system represents an extremely attractive and powerful option for such large-scale applications. The increasing number of biotechnological products forces system suppliers of the downstream processing side to develop new and improved high throughput purification technologies.

Continuous Annular Chromatography (CAC) has been shown to be the only continuous chromatography technique to fulfill the high demands raised by modern biotechnological productions. In recent years Prior Separation Technology has transferred the principle of Continuous annular chromatography from the research laboratories to the fully developed industrial downstream process scale. The technology is now called Preparative Continuous Annular Chromatography – P-CAC. It can be placed at any stage in the downstream line starting at the very early stages where capturing and concentration of the desired product is required down to the polishing steps, which assure a sufficient final purity of the end product.

Keywords: Continuous chromatography, Preparative chromatography, P-CAC, Downstream processing, Continuous process chromatography

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List of Abbreviations and Symbols

ε	void fraction
С	liquid-phase solute concentration [g/l]
C_F	feed solute concentration [g/l]
	average liquid-phase solute concentration [g/l]
$\overline{C^*}$	equilibrium solute concentration [g/l]
D_	angular dispersion coefficient [cm ² /s]
D_z	axial Dispersion coefficient [cm ² /s]
Κ	equilibrium distribution coefficient
k _o a	global mass transfer coefficient
LF	loading factor
N_{th}	number of theoretical plates
9	solid-phase average solute concentration [mmol/g dry resin]
	feed flow rate [cm ³ /s]
Q_T	total flow rate [cm ³ /s]
R_0	mean bed radius [cm]
	time [s]
ť	transformed time [s]
t'_F	length of time corresponding to the feed arc [s]
$t_F \hat{t}_{\max} \hat{t}$	peak chromatographic elution time [s]
Î	chromatographic time [s]
и	superficial velocity [cm/s]
	width of solute band at half the maximum concentration [deg]
W_0	initial feed bandwidth [deg]
Z	bed axial position [cm]
Δ	time interval at half of peak maximum concentration [s]
θ	displacement from feed point [deg]
$ heta_F$	feed arc [deg]
$egin{array}{c} heta_E \ ar{ heta} \end{array}$	elution arc [deg]
θ	angular displacement of the maximum solute concentration at the chro-
	matograph exit [deg]

 ω rotation rate [deg/h]

1 Introduction

Continuous annular chromatography is the only chromatography-based technology, which allows the continuous separation of a multicomponent mixture. Other than in SMB (Simulated Moving Bed) [1] based purifications where only two fractions – raffinate and extract – can be separated per unit, continuous annular chromatography allows the recovery of more than two fractions (up to five different peaks could be resolved [2]). Furthermore all commonly used chromatographic techniques like step-elution, gradient-elution and even displacement-elution can be performed continuously on a continuous annular chromatograph. With the CAC technology it becomes for the first time possible to run an Ion-Exchange purification continuously.

Figure 1 shows a schematic drawing of a CAC apparatus. The apparatus consists of two concentric cylinders standing one inside the other, forming an annulus into which the stationary phase is packed. This annular bed is slowly rotating about its vertical axis. Under isocratic elution conditions the feed mixture to be separated is introduced continuously at the top of the bed at a space that remains fixed in space while the rest of the annulus is flooded with elution buffer. As time progresses, helical component bands develop from the feed point, with

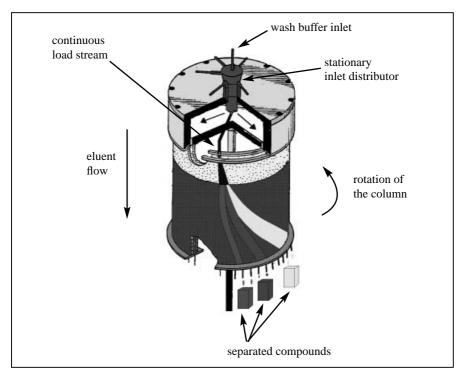


Fig. 1. Principle of a continuous annular chromatograph

slopes dependent upon elution velocity, rotational speed, and the distribution coefficient of the component between the fluid and adsorbent phase. At steady state the component bands form regular helices between the feed sector at the top of the bed and the individual fixed exit points at the bottom of the annular bed, where the separated components can be continuously recovered. As long as conditions remain constant, the angular displacement of each component band from the feed point will also remain constant.

Under non-isocratic conditions as in ion exchange chromatography, any portion of the annular bed which is not receiving feed at a given time is either receiving wash buffer, step elution buffer(s), regeneration buffer, or equilibration buffer. Currently the P-CAC units allow the usage of up to seven different feed and buffer solutions. Thus, the P-CAC is a truly continuous, steady-state process, which retains the very attractive characteristics of being able to assure effective multicomponent separations with a flexibility typical for chromatography in general.

Giddings was the first to demonstrate that theoretically the rotating annular column can be superior to a fixed column of the same volume for process scale applications [3]. He recognized that many industrial scale packed columns exhibit non-uniformities in flow at large diameters, resulting in an increased plate height and loss of resolution. By using a rotating column with the same total cross-sectional area and bed height as a fixed column, but with an annulus size small enough so that flow non-uniformities do not occur, a process can be scaled up without loss of resolution. In other words, all things being equal, the geometry of a rotating in better resolution. He also pointed out that process control might be easier in continuous operation because it results from the column geometry, and not from careful timing of feed injection and product withdrawal as in a simulated continuous operation. Due to its truly continuous character the CAC technology features a very high throughput compared to traditional batch chromatographic separations.

2 General Overview

2.1 Brief Historical Survey

The idea of annular chromatography was first mentioned by A.J.P. Martin [4] in 1949 where he summarized a discussion with his colleagues Prof. Tiselius and Dr. Synge. In his summary Martin writes:

"An idea rising from a discussion with Prof. Tiselius and Dr. Synge during this discussion can also enable chromatography to be a continuous process, provided that the developing solvent returns the adsorbent to its initial state within a reasonable period. Imaging the chromatogram to be packed within a narrow annular space between two concentric cylinders. The upper surface of the chromatogram is flooded with solvent and at one point the solution to be separated

is fed on slowly. The annular chromatogram is slowly and uniformly rotated with the result that different zones will form helices of characteristic angle which can be collected at various fixed points around the bottom of the chromatogram".

Martin further mentioned that the scheme he described has already been tried out by Dr. Wadman of the University of Bristol. Wadman, however, never published any results of his very first work on annular chromatography. Martin and Synge won a joint Nobel price in chemistry for their work in partition chromatography, Tiselius won a Nobel price in chemistry for his work in electrophoreses.

Between the time annular chromatography was first mentioned and the time when the first practical work was published, several years passed by. Between 1970 and 1990 most of the work on annular chromatography was performed at Oak Ridge National Laboratories (ORNL, Oak Ridge TN, USA). As we can find in the literature, most of the work done at ORNL focused on separation of metals [5-9], purification of sugars [10, 11] and the purification of standard model proteins such as hemoglobin, Bovine Serum Albumin [12] and amino acids [13]. In the 1990s some publications on annular chromatography from a Japanese group can be found. The work performed there mainly concentrates on the non-isocratic elution of proteins and amino acids [14-19].

2.2 Current Research and Development Status

In 1994 the work on annular chromatography at the Oak Ridge National Laboratory (ORNL) ended. The Japanese group mentioned above has not published anything related to annular chromatography since 1997. In 1994 Prior Technology GmbH (Götzis Austria), which was already in contact with C. Byers from the ORNL, started to use the annular chromatography technology to develop a system for the continuous separation of the precious metals rhodium, palladium, platinum, and iridium [2]. These studies carried out as Ph.D. work were performed in close cooperation between Prior Technology GmbH and the University of Technology of Graz, Austria, the Technion in Haifa Israel, and the Oak Ridge National Laboratories.

During the first four years (1994–1998) several papers were published by that group describing the separation of fructose, mannitol, and sorbitol [20, 21], the desalting of BSA [22], the recovery of a rhodium-based homogeneous catalyst [23], the separation of a steroid mixture [24], and the simultaneous separation of platinum group metals and iron [25]. Recently a study on the removal of ashes from a lactose concentrate was performed [26]. All work mentioned here was done on an annular chromatograph STD-100 E which was at that time sold by IsoPro Int. (Knoxville, TN, USA). In 1996 engineers at Prior Technology GmbH started to redesign and develop a new annular chromatograph; see Fig. 2.

In 1999 a new type of annular chromatograph was presented by Prior Separation Technology GmbH, a spin-off company of Prior Technology GmbH. At the same time the selling of the machine under the brand name of P-CAC started. The most important changes in the design compared to the previous system include the usage of only FDA approved materials such as pharmagrade stainless steel or polyetheretherketone – PEEK and the implementation of glide ring sealings as interface between the rotating and the stationary parts of the column. Other changes concern the possibility to use the inner cylinder of the annular column as a heat exchanger, thus allowing a precise temperature control of the separation column. In 2000 Prior Separation Technology added a UV detector to the P-CAC system, allowing UV active substances to be monitored as they exit the annular column. Currently wavelengths of 280 nm and 260 nm can be used for the detection. As can be seen in Fig. 2 the P-CAC system itself currently consists of the annular column and the drive as well as a series of peripheral equipment such as pumps and a thermostat.

Using the new P-CAC systems basic research is currently performed at four different academic institutions. The first group at the center of Biotechnology of the Swiss Federal Institute of Technology in Lausanne, Switzerland, studies the usage of the P-CAC for the isolation of biologically active substances such as IgG [27] using affinity chromatography with r-Protein A Sepharose (Amersham Pharmacia Biotech, Uppsala Sweden) as the stationary phase as well as the continuous purification of plasmid DNA. The second group at the Institute of Applied Microbiology at the University of Agricultural Sciences in Vienna, Austria, studies the usage of the P-CAC for the separation and isolation of biomolecules in general [28, 29]. This group also examines the usage



Fig. 2. Picture of a commercially available P-CAC system from Prior Separation Technology GmbH

of two different stationary phases in one P-CAC column (ion-exchange resin on top of a size-exclusion resin) for the recovery of green fluorescent protein [29].

The third group at the department of Chemical Engineering at the University of Kaiserslautern, Germany uses the P-CAC as a chromatographic reactor. In this case chemical reactions coupled to the concomitant separation of the reactants in the P-CAC are studied. For these investigations the upper part of the annular column is used as the reaction zone, while the lower part of the column separates the products as well as the reactants. With the P-CAC as a continuous chromatographic reactor it is possible to shift the chemical equilibrium to the product side due to the fact that the products are always removed from the chemical reaction in the separation zone. As a second focal point the group in Kaiserslautern studies the heat transfer in a scaled-up P-CAC version. The fourth group at the department of Process Engineering at the Swiss Federal Institute of Technology in Zurich, Switzerland also uses the P-CAC as a chromatographic reactor and studies the esterification of glycerol and the recovery of the three different glycerides. This group started their work only in November 2000, and therefore no reference can be found on these studies.

Another group independently published the continuous purification of porcine lipase on a size-exclusion resin in an annular chromatograph [30]. The annular chromatograph used in this study was designed and fabricated at the department of Chemical Engineering at the University of New Hampshire (Durham). In 2001 studies on the refolding of recombinant proteins on a P-CAC will be started at the department of Chemical Engineering at the University of Cambridge, United Kingdom. In addition this group will also start to investigate the separation of peptides according to their chain-length.

3 Technical Description of the P-CAC System

Compared to the continuous annular chromatography systems sold by Isopro International and used throughout the ORNL studies, the P-CAC units developed by Prior Separation Technology feature several design modifications which will be presented hereafter. As can be seen from Fig. 3, the P-CAC system consist of three major parts: the P-CAC head, the annular column, and the drive including the control panel. Figure 3 represents a schema of the laboratory sized P-CAC used as a Research and Development tool.

3.1 P-CAC Head

At the P-CAC head seven different inlet ports can be used to supply the column with the feed and with different eluent solutions, while the ORNL units were equipped with only four ports. One inlet port at the P-CAC is reserved for the indication of pressure and one for the pressure relief valve. Another inlet port is designed as an inlet for the main eluent, which floods the entire annulus.

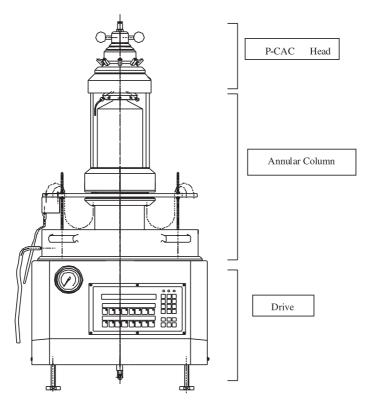


Fig. 3. Schematic drawing of a Lab P-CAC

The remaining inlets can be used for process adaptation and optimization, e.g.:

- As multiple feed-inlet ports
- For the implementation of techniques such as step-elution, gradient-elution, displacement-elution, as well as for the wash and sanitation steps needed for ion exchange chromatography and continuous downstream processing in general.

The head is made of polypropylene and polyetheretherketone-based materials and is designed to run at an operating pressure of maximal 10 bar. All feed and eluent solutions are pumped directly into the column through tubes, to minimize dead zones and prevent fouling.

3.2 Annular Column

The annular column consists of an outer and an inner cylinder standing one inside the other and held together by the ground plate. Depending on the material, the outer cylinder withstands an operating pressure of maximal 3 bar in the case of the glass cylinder and maximal 10 bar in the case of the stain-

	Type 1	Type 2	Type 3
Total gel volume	1000 ml	2000 ml	3000 ml
Maximum bed height	20 cm	40 cm	60 cm
Inner diameter of the outer cylinder	15 cm	15 cm	15 cm
Outer diameter of the inner cylinder	13 cm	13 cm	13 cm
Annular bed cross sectional area	44 cm ²	44 cm^2	44 cm ²

Table 1. Physical characteristics of the laboratory scale P-CAC units (columns)

less steel cylinder. A pressure relieve valve installed in the P-CAC head prevents exceeding of the pressure maximum. The columns of the CAC units used in the ORNL studies were ordinary Plexiglas tubes and the inner cylinders were made of polypropylene. The inner cylinder of the P-CAC unit is made of pharmagrade stainless steel and is designed to withstand a pressure of up to 10 bar. At the same time, the inner cylinder serves as a heat exchanger and is able to keep the temperature of the annular column within a range from 4 to 80 °C.

The space between inner and outer cylinders forms the annulus. The column bottom plate is made of stainless steel and typically contains 90 exit holes below the annulus. The holes are covered by a filter plate to keep the stationary phase in place. Three different column sizes are available for the laboratory P-CAC unit; the physical characteristics of the different annular columns are summarized in Table 1. The collection of the different fractions at the lower end of the annular column is regulated by a fixed glide ring system. Each chamber in the fixed glide-ring corresponds to an exit holes in the bottom plate of the column. The number of exit holes equals the number of chambers. The fixed glide ring system allows the continuous and controlled recovery of the separated fractions at the end of the column. Thus cross contamination is avoided and precise fraction collection is ensured. The whole process of collecting the fractions is conducted in a closed system. Unused eluent can be easily recycled.

3.3 Drive

The drive of the P-CAC units consists of a high precision stepping motor, a control panel, and a software package, which allows the column to be run in various different operation modes.

In the production mode the rotation rate of the P-CAC can be varied between 0°/h and 5000°/h. By comparison, the drivers used in the CAC units throughout the ORNL studies were only able to rotate the column between 2°/h and 1000°/h. The housing of the drive is made of stainless steel coated with polyethylene and protects the drive as well as the electronic parts against environmental influences.

In addition to the regular rotation, the drive can also be used in the (fast rotating) "packing mode". In particular a P-CAC column may be packed with the resin automatically in the way that the resin slurry is pumped to the annular col-

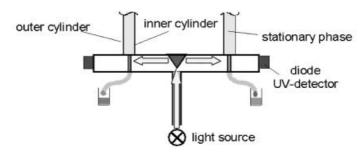


Fig. 4. Schematic drawing of the on-line UV detector in the P-CAC

umn while the column is rotated in a fast rotation mode (up to ten revolutions per minute). This guarantees a plane surface throughout the entire annulus.

3.4 UV-Detector

For monitoring the output of the annular column an online UV detector was developed [31] by Prior Separation Technology. In this case the P-CAC system is equipped with a separate measuring plate located directly under the slip-ring (see Fig. 4). The measuring plate contains 98 quartz capillaries, one for each of the outlets of the P-CAC and 8 reference channels. The online UV detection unit contains an external light source (UV lamp) and the light emitted (260 nm or 280 nm) by this light source is transferred through a quartz fiber to the center of the measuring plate. There the light beam is reflected on a conical mirror and is evenly distributed throughout the inner circumference of the measuring plate. The refracted light travels through the light path and hits the quartz capillary. Light is absorbed by the fluid stream in the capillary depending on the concentration of the molecules dissolved in the liquid stream according to the Lambert Beer Equation. The light portion being transmitted through one of the capillaries hits a diode creating a voltage signal. Corresponding to the 98 quartz capillaries there are 98 diodes wired in series and linked to a computer. On the computer a data-acquisition and monitoring software allows one to measure the absorbance and the elution position of the species which were separated in the annular column. The implementation of the on-line UV detector allows a continuous monitoring of all the products eluted from the P-CAC during the separation.

4 Mathematical Background – Theory

4.1 Analogy Between Fixed Bed and CAC

In fixed bed columns (batch columns), the fluid and solid phase concentrations are functions of both position and time. Considering a conventional, idealized,

stationary bed with void fraction ε , a one-dimensional steady state, material balance for a solute with concentration *C* may be written as

$$\varepsilon D_z \frac{\partial^2 C}{\partial z^2} = \varepsilon \frac{\partial C}{\partial t} + (1 - \varepsilon) \frac{\partial q}{\partial t} + u \frac{\partial C}{\partial z}, \qquad (1)$$

where D_z is the axial dispersion coefficient, u is the superficial velocity, and C and q are the liquid and solid phase concentrations, respectively. Using a simple fluid film model to describe fluid-particle mass transfer, the following rate equation may be written to relate the fluid and solid phase concentrations [32]:

$$(1-\varepsilon)\frac{\partial q}{\partial t} = k_0 a (C - C^*), \qquad (2)$$

where $k_o a$ is an overall mass transfer coefficient and C^* is the liquid phase concentration in equilibrium with the solid phase.

Continuity and rate equations can also be written in a cylindrical coordinate system for the two-dimensional annular chromatography. Assuming steady state and neglecting velocity and concentration variations in the radial direction, the above-mentioned equations may then be written as

$$\varepsilon D_z \frac{\partial^2 C}{\partial z^2} + \frac{\varepsilon D_\theta}{R_0^2} \frac{\partial^2 C}{\partial \theta^2} = \omega \varepsilon \frac{\partial C}{\partial \theta} + \omega (1 - \varepsilon) \frac{\partial q}{\partial \theta} + u \frac{\partial C}{\partial z}$$
(3)

and

$$\omega(1-\varepsilon)\frac{\partial q}{\partial \theta} = k_0 a \left(C - C^*\right),\tag{4}$$

where D_z and D_{θ} are the axial and the angular dispersion coefficients, R_o is the mean radius of the annular bed, ω is the rate of rotation, and z and θ are the axial and angular coordinates respectively. If angular dispersion is negligible, then the one-dimensional, unsteady-state, fixed bed equations (Eqs. 1 and 2) can be transformed into the corresponding steady-state, two-dimensional, continuous equations (Eqs. 3 and 4) with the change of variable:

$$\theta = \omega t'. \tag{5}$$

where ω is the rotation rate, t' is a transformed time, and θ is the angle [33, 34]. Equations (3) and (4) then become

$$\varepsilon D_z \ \frac{\partial^2 C}{\partial z^2} = \varepsilon \ \frac{\partial C}{\partial t'} + (1 - \varepsilon) \ \frac{\partial q}{\partial t'} + u \ \frac{\partial C}{\partial z} , \qquad (6)$$

and

$$(1-\varepsilon)\frac{\partial q}{\partial t'} = k_0 a(C-C^*).$$
⁽⁷⁾

These equations can be solved with the appropriate boundary conditions. For isocratic operation, for example, the boundary conditions may be written as

$$t' = 0, \quad \text{all } z: \quad q = c = 0$$

$$z = 0, \quad 0 < t \le t'_F \quad C - \frac{\varepsilon D_z}{u} \quad \frac{fC}{fz} = C_F$$

$$t' > t'_F \quad C - \frac{\varepsilon D_z}{u} \quad \frac{fC}{fz} = 0$$

$$z = Z, \quad \text{all } t' \quad \frac{fC}{fz} = 0$$

where C_F is the feed concentration and t'_F is the length of time corresponding to the feed arc

$$\theta_F = \omega t'_F.$$

Because of this analogy, the mathematical treatment of the steady-state performance of the CAC is no more complicated than the corresponding mathematical treatment of the analogous transient conventional chromatographic operation. Thus, solutions that are available to describe the latter can be used very simply to describe the former, making use of Eq. (5). This of course holds only if angular dispersion is negligible, which however is the case in most typical preparative or production-scale liquid chromatographic operations as shown by Howard and coworkers [10, 35].

4.2 Analytical Solution for the CAC Steady State Equation

An analytical solution of these mass-transfer equations for linear equilibrium was found by Thomas [36] for fixed bed operations. The Thomas solution can be further simplified if one assumes an infinitely small feed pulse (or feed arc in case of annular chromatography), and if the number of transfer units $(n=k_0az/u)$ is greater then five. The resulting approximate expression (Sherwood et al. [37]) is

$$C(z, \hat{t}) = \frac{LF}{2\pi^{0.5}} \left\{ \frac{(k_0 a)^2}{u^3 z \,\hat{t} \, [(1-\varepsilon)K]^3} \right\}^{0.25} \exp\left\{ -\left[\sqrt{\frac{k_0 a z}{u}} - \sqrt{\frac{k_0 a \hat{t}}{K(1-\varepsilon)}} \right]^2 \right\},\tag{8}$$

where

$$\hat{t} = \frac{\theta}{\omega} - \frac{\varepsilon z}{u} \tag{9}$$

The amount of solute introduced per unit cross-sectional area of the sorbent bed, *LF* (Loading Factor), can be calculated from

$$LF = \frac{C_F u Q_F}{Q_T} \frac{360^\circ}{\omega} , \qquad (10)$$

where Q_F is the feed flow rate and Q_T is the total flow rate.

Equation (8) is especially useful in determining equilibrium and mass transfer parameters from experimental chromatographic concentration profiles. As shown by Sherwood et al. [37] the peak maximum will occur when

$$\sqrt{\frac{k_0 a z}{u}} - \sqrt{\frac{k_0 a \hat{t}}{K (1 - \varepsilon)}} = 0 , \qquad (11)$$

or

$$\hat{t}_{\max} = \frac{K(1-\varepsilon) z}{u}$$
(12)

Thus the equilibrium distribution coefficient for a CAC experiment is given by

$$K = \left(\frac{\theta_{\max}}{\omega} - \frac{\varepsilon z}{u}\right) \frac{u}{z(1-\varepsilon)}.$$
(13)

The overall mass transfer coefficient, $k_0 a$, can be found from the width of the experimental peak at one-half of the maximum peak height, Δ . As shown by Sherwood et al. [37], from Eq. (8) one finds that

$$k_0 a = \frac{u}{z} \ 16(\ln 2) \left(\frac{\hat{t}_{\max}}{\Delta}\right)^2. \tag{14}$$

At the high feed loadings typically used in preparative-scale applications, the assumption of an infinitely small feed arc no longer applies and Eq. (8) can no longer be used. Carta [38] developed an exact analytical solution for the general case of finite-width, periodic feed applications while retaining the assumptions of a linear equilibrium and negligible axial dispersion. Carta's solution, originally obtained to describe the behavior of a fixed bed, can be transformed with the use of Eq. (5) to give the two-dimensional, steady state solution for the CAC under high feed loading conditions. The resulting expression

$$\frac{C(z,\theta)}{C_F} = \frac{\theta_F}{\theta_F + \theta_E} + \frac{2}{\pi} \sum_{j=1}^{\infty} \left\{ \frac{1}{j} \exp\left[-\frac{j^2 k_0 a z}{(j^2 + r^2) u}\right] x \sin\left[\frac{j \pi \theta_F}{\theta_F + \theta_E}\right] \\ x \cos\left[-\frac{j \pi \theta_F}{\theta_F + \theta_E} + \frac{2 j \pi \theta}{\theta_F + \theta_E} - \frac{2 j z \omega \varepsilon}{u(\theta_F + \theta_E)} - \frac{j r k_0 a z}{(j^2 + r^2) u}\right] \right\}$$
(15)

can then be used to compute concentration profiles where θ_F and θ_E are the feed and the elution arcs, and *r* is given by

$$r = \frac{k_0 a(\theta_F + \theta_E)}{2\pi (1 - \varepsilon) K \omega}$$
(16)

This equation applies for both large and small feed sectors, or when multiple, evenly spaced feeds are introduced into the same column.

The average concentration, *C*, between any two angles, θ_1 and θ_2 , can be calculated by integrating Eq. (16) resulting in

$$\frac{\hat{C}(z)}{C_F} = \frac{\theta_F}{\theta_F + \theta_E} + \frac{2(\theta_F + \theta_E)}{\pi(\theta_2 - \theta_1)} \sum_{j=1}^{\infty} \left\{ \frac{1}{j^2} \exp\left[-\frac{j^2 k_0 a z}{(j^2 + r^2) u}\right] x \sin\left[\frac{j \pi \theta_F}{\theta_F + \theta_E}\right] x \sin\left[\frac{j \pi (\theta_2 - \theta_1)}{\theta_F + \theta_E}\right] \right\} \\
x \cos\left[-\frac{j \pi \theta_F}{\theta_F + \theta_E} + \frac{j \pi (\theta_1 + \theta_2)}{\theta_F + \theta_E} - \frac{2j z \omega \varepsilon}{u(\theta_F + \theta_E)} - \frac{j r k_0 a z}{(j^2 + r^2) u}\right] \right\}$$
(17)

The average concentration can, e.g., be used to calculate the purity of the product fraction collected between any two angles θ_1 and θ_2 .

In case an analytical solution of Eqs. (6) and (7) is not available, which is normally the case for non-linear isotherms, a solution for the equations with the proper boundary conditions can nevertheless be obtained numerically by the method of orthogonal collocation [38, 39].

4.3 Theoretical Plate Concept for the CAC System

The continuous annular chromatograph can be described mathematically by a theoretical plate approach similar to the one developed by Martin and Synge [40] and exemplified by Said [41] for stationary columns [5]. The mathematical description results in algebraic expressions for the elution position of each solute relative to the feed point and for the "bandwidth" of the eluting zone as a function of the elution position or other system parameters. However, a series of simplifications have to be made in order to describe the CAC with the theoretical plate concept:

- The annulus consists of a series of equally sized segments arranged circumferentially.
- Each of the annular segments is made up of a series of theoretical plates, progressing from the top of the resin layer to the bottom. All segments have identical heights.
- As a solute leaves the theoretical plate its concentration is at equilibrium with the average concentration of the solute sorbed in the stationary phase.
- There is no lateral mass transfer of the solute or solvent to adjoining annular segments.
- No radial variation exists in either the fluid or the sorbent phase.
- The superficial velocity of the eluent is constant throughout the annulus.
- A single annular segment as it rotates represents one reference point for the mathematical description and the feed point will be the other.
- All of the solute is assumed to be in the first theoretical plate at the end of the introduction period.

Assuming that all these assumptions are fulfilled the number of theoretical plates in the vertical section of the CAC can be calculated as follows [5]:

$$N_{th} = \frac{8 \ln 2\bar{\theta}}{W^2 - W_0^2},$$
(18)

where N_{th} is the number of theoretical plates, $\bar{\theta}$ is the angular displacement of the maximum solute concentration at the CAC exit, and W and W_0 are the width of solute band at half the maximum solute concentration and the initial feed band-width respectively.

5 Scale-Up of the CAC System

The most critical scale-up issue in the CAC technology is the effect of increased annulus thickness. While most of the experiments conducted to date used systems with a thin annulus, at least some of experiments performed at the ORNL used packed annuli ranging from 1% to about 96% of the available cross section of the outer shell. A summary of the conditions used for these studies are given in Table 2. Different annular chromatographs with outer diameters ranging from 9 cm to 45 cm were used. The annulus width in the different CAC units ranged from about 0.60 up to 12.4 cm. In particular, the different CAC models were used to study the separation of mixtures of copper, nickel, and cobalt on a Dowex 50W-X8 ion-exchange resin [9]. The results show that neither the annulus thickness nor the size of the CAC appeared to affect the resolution under appropriately scaled conditions. While the resolution remained constant, the throughput increased with the annulus thickness. Mechanical scale up issues for a CAC unit with an outer diameter of 100 cm and a possible throughput of up to 200 l/h of feed are discussed in [42].

Designation	Annulus width, Δr	Inner diameter outer cylinder	$\Delta r/r_0$	Annular bed cross sectional area (cm ²)	Available area ^a
CAC-ME	0.64 cm	9.0. cm	0.14	16.5 cm ²	26.5%
CAC-ME-2	1.30 cm	9.0 cm	0.29	30.4 cm ²	48.9%
CAC-ME-4	3.20 cm	9.0 cm	0.71	57.0 cm ²	91.6%
CAC-II-2	5.10 cm	28.0 cm	0.36	364.8 cm ²	59.5%
CAC-II-3	12,4 cm	28.0 cm	0.82	592.0 cm ²	96.7%
CAC-III	3.20 cm	44.5 cm	0.14	4117 cm ²	26.5%

Table 2. Physical characteristics of the CAC units used at ORNL

^a The available area corresponds to the cross-sectional area of the annular bed divided by the maximum possible area based on r_0 .

6 Industrial Applications

Since the commercial introduction of the P-CAC in 1999, several industrial applications have been shown to be transferable to the system. Moreover, users in the biopharmaceutical and foodstuff industry have seen their productivity increasing dramatically as a result of using the P-CAC technology. Furthermore, a P-CAC has been shown capable of continuously separating stereoisomers when using chiral stationary phases even when there is more than one chiral center in the desired molecule. Below some of the applications are described in more details. Others are proprietary and hence cannot be disclosed.

6.1 Continuous Annular Size Exclusion Chromatography

Buchacher et al. [43] discussed the continuous separation of protein polymers from monomers by continuous annular size exclusion chromatography. The P-CAC used for the experiments was a laboratory P-CAC type 3 as described in Table 1. The results were compared to conventional batch column chromatography in regard to resolution, recovery, fouling, and productivity. The protein used in the studies was an IgG preparation rich in aggregates. Under the conditions used, the polymers could be separated from the monomers, although no baseline separation could be achieved in either the continuous or the batch mode. The

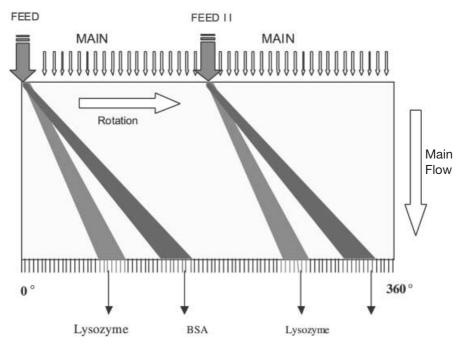


Fig. 5. Using two feed inlets to double the throughput on a low capacity resin

productivity of the P-CAC system, however, was twice as high as that of the conventional batch column. At the same time the buffer consumption was halved. At high protein concentrations (25 g/l), fouling of the resin occurred at the upper part of the annular column. The high protein concentration in the feed as well as the sticky nature of the proteins was responsible for the accelerated fouling, which also occurred in batch chromatography. Continuous regeneration of the annular column (using an NaOH solution) could not be accomplished without harming the protein zones. With low protein concentrations in the feed (2 g/l) the accelerated fouling did not occur.

In an internal study Hunt et al. [44] showed that the productivity of the P-CAC system for the separation of Lysozyme and BSA by size exclusion chromatography is five times higher compared to conventional batch chromatography. In that study two feed inlets spaced 180° apart from each other could be used in the P-CAC, while still achieving baseline separation of the two proteins. Figure 5 represents the unwrapped annular cylinder showing the two feed inlets (Feed and Feed II) placed 180° apart. Using two feed inlets is especially useful when the chosen resin has a very low capacity for the substances to be separated, which is normally the case in size exclusion chromatography where the feed volume is typically 1 – 5% of the total column volume. Figure 6 shows the chromatogram of the separation of BSA and lysozyme when two feed inlet ports were used. Through both inlet ports 5 ml/min of the of BSA/lysozyme-mixture were pumped into the P-CAC column. PBS-buffer was used as the main eluent. Four clearly separated peaks (two peaks of BSA and Lysozyme respectively) each of which was baseline separated from the others could be recovered at the column outlet. Using only one feed inlet port and doubling the feed flow rate to 10 ml/min resulted in a dramatically decreased resolution.

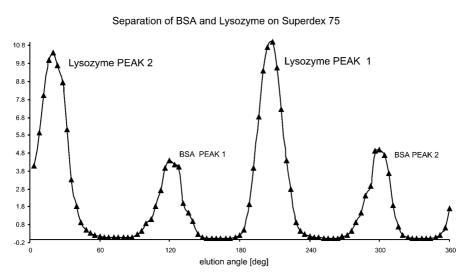


Fig. 6. Separation of BSA and lysozyme on a laboratory P-CAC type 2 (see Table 1 for details)

6.2 Continuous Annular Reversed Phase Chromatography

Blanche et al. [45] showed that the P-CAC technology is very promising for the purification of Plasmid DNA at preparative scale especially when resins with low binding capacities for the product of interest are used. The aim of the study was to purify the Plasmid DNA out of a clear lysate of *E. coli*. The lysate containing RNA, nicked DNA, as well as the Plasmid DNA was loaded onto the annular column filled with Poros 20 R2 beads as the stationary phase. The chromatographic process for the purification is shown in Fig. 7.

The feed is introduced at the top of the annular column at the 0° position. The feed solution is followed by a wash buffer, which is introduced to the annular column through the main inlet port. A 1 vol.% mixture of 2-propanol in a 100 mmol/l ammonium acetate buffer was used as wash buffer. In the washing zone the nicked DNA followed by the RNA are eluted from the column according to their affinity to the resin. At 180° offset from the feed nozzle the elution buffer (5 vol.%) 2-propanol in 100 mmol/l ammonium acetate) was pumped to the annulus of the column. The elution buffer was used to strip off the bounded Plasmid DNA. Regeneration of the column was achieved by a 20 vol.% mixture of 2-propanol in 100 mmol/l ammonium acetate buffer. All of the above-mentioned steps, i. e., feed, wash, elution, and regeneration, were done simultaneously and continuously on the P-CAC system.

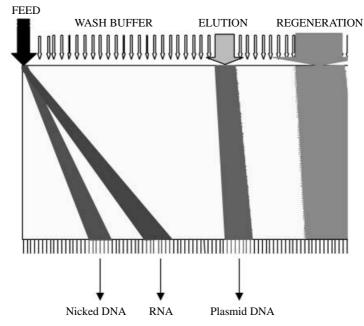


Fig. 7. Unwrapped P-CAC cylinder showing the configuration for the Plasmid DNA purification

Figure 8 compiles some pertinent analytical data of the obtained fractions. Most of the nicked DNA (chromatogram C in Fig. 8: pooled nicked DNA fraction from the wash zone of the P-CAC) as well as the RNA (chromatogram D in Fig. 8 pooled RNA fraction from the wash zone of the P-CAC) were removed in the wash zone. Chromatogram A in Fig. 8 represents the composition of the P-CAC feedstock. Chromatogram B in Fig. 8 demonstrates the purity of the pooled plasmid DNA fraction obtained with the P-CAC.

The application of the P-CAC technology to the purification of plasmid DNA by reversed phase chromatography using Poros 20 R2 as the stationary phase proofed to be very simple. The conversion of the batch chromatography parameters into continuous chromatography parameters was straightforward. In addition, no deterioration (in terms of plasmid recovery and purity) of the separation performances occurred when switching from batch to continuous modes. In terms of throughput it turned out that the P-CAC column had a 20-fold higher productivity then a batch column with the same resin volume.

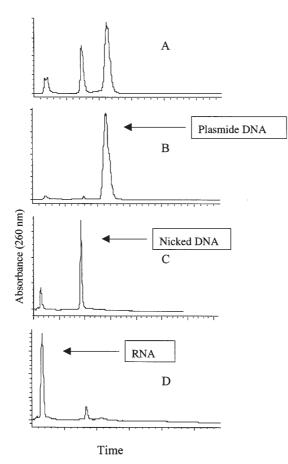


Fig. 8. Analysis of P-CAC eluates by anion exchange HPLC

6.3 Mixed Mode Continuous Annular Chromatography

Recently, the simultaneous chromatographic inter-separation of the PGMs (platinum group metals) and base metals by continuous annular chromatography has been demonstrated [46]. The basic configuration of the P-CAC is used to separate the PGMs from base metals and to inter-separate them in the same apparatus is shown in Fig. 9.

The P-CAC used in the study was a laboratory scale P-CAC type 1 (see Table 1) filled with a Size-Exclusion Chromatography (SEC) gel such as the Toyopearl HW 40 resin to 60% of its height. The SEC gel was overlaid with an inert layer of glass beads (250 μ m in diameter). This glass bead layer prevented a mixing of the SEC gel and the cation-exchange resin (Dowex 50), which was filled on top. The ion exchange layer itself was also covered with an inert layer of inert glass beads (250 μ m in diameter) to prevent as much as possible the dispersion of the feed and the step eluent, which were introduced into the annulus through the feed nozzles.

The feed, containing $RhCl_6^{3-}$, $PdCl_4^{2-}$, $PtCl_6^{3-}$, and $IrCl_6^{2-}$ as well as Fe(II + III), Ni(II), and Co(II) in a hydrochloric acid solution (3–4 mol/l) was introduced through a feed nozzle directly into the annulus. The main eluent, 0.4 mol/l HCl, assured that the PGMs passed the cation-exchange resin without any interaction, since PGMs in hydrochloric acid solution are present as anionic complexes. The base metals, Fe, Ni, and Co, however, are adsorbed by the cation-exchange resin under such conditions. The PGMs running through the cation-exchange layer were then separated in the SEC gel layer according to the size of their complexes in the elution order rhodium – palladium – platinum – iridium. At an angular po-

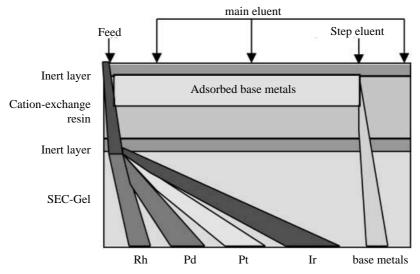


Fig.9. Basic principle of a mixed mode P-CAC; the unwrapped cylinder shows the two chromatographic layers separated by an inert layer



Fig. 10. Photograph of the separation of the PGM and base metals in a two-phase (mixed mode) P-CAC system

sition after iridium as the last precious metal to leave the annular column, the base metals were stripped of the cation-exchange resin by using a step-eluent (2-3 mol/l HCl). In this eluent the base metals were not retained by either of the two stationary phases. Therefore a fraction consisting of the sum of the base metals could be collected finally at the end of the annular column (Fig. 10).

It has been found from batch experiments that the base metals Fe, Ni and Co are fully adsorbed by the cation-exchange resin when the hydrochloric acid concentration of the eluent does not exceed 0.4 M. If the concentration exceeds 0.4 M the base metals start to break through. The same thing happens when the hy-

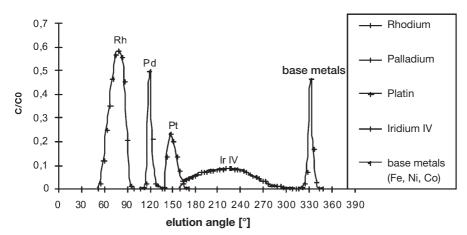


Fig. 11. Experimental chromatogram of a separation of a solution containing PGMs and base metals using a mixed mode P-CAC system1

drochloric acid concentration of the feed solution exceeded 4 M. The minimum height of the cation-exchange resin in the P-CAC depends on the concentration of the base metals present in the feed solution. The height is directly proportional to the maximum capacity of the resin. The maximum capacity of the resin for the mixture of all three cations was calculated from the adsorption isotherm. The adsorption isotherm represents the equilibrium of a compound between the liquid and the solid phase in chromatography; isotherms can be estimated by batch shaking experiments.

It was also shown that the feed inlet band of the PGMs broadens when it passes through the cation-exchange resin layer. This means that the concentration of the platinum group metals in the sample decreases accordingly, which depending on the exact conditions results in dilution factors between 2 and 10. Figure 11 shows the experimental chromatogram of the separation of a mixture used in the studies.

7 Conclusion

Several applications throughout the last two decades have shown that starting from batch chromatography experiments a scale-up to a continuous annular chromatograph is easy and straightforward. It has also been shown that many operating modes, including isocratic, step and displacement elution are possible on a CAC. The apparatus retains its relative mechanical simplicity in comparison with fixed-bed processes. No precise timing of a valve system for the introduction of feed and the product removal are needed. The key advantages of annular chromatography over fixed-bed operations are likely the simplicity of the apparatus, its productivity and resolution improvement, and its truly continuous operational capabilities.

A very promising application of the P-CAC technology, which at the time this article was written was undergoing intensive studies, is to couple the continuous chromatograph to a continuous fermenter system. Continuous bioreactors are receiving attention as an efficient method of producing biochemicals. For this application it was necessary to develop a P-CAC unit where the column can be autoclaved by steam. The coupling of a continuous fermentation to a continuous capturing step promises a significant improvement in terms of throughput and product yield.

Compared to the SMB system the annular chromatography allows the continuous separation of a multicomponent mixture as it is most often the case in biopharmaceutical separations.

8 Pofor

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