Molded Rigid Monolithic Porous Polymers: An Inexpensive, Efficient, and Versatile Alternative to Beads for the Design of Materials for Numerous Applications

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A simple molding process carried out within the confines of a closed mold has been used for the preparation of porous polymer monoliths. The polymerization is carried out using a mixture of monomers, porogenic solvent, and free-radical initiator under conditions that afford macroporous materials with through-pores or channels large enough to provide the high flow characteristics required for their applications. The versatility of the preparation technique is demonstrated by its use with both hydrophobic and hydrophilic monomers such as styrene, (chloromethyl)styrene, glycidyl methacrylate, alkyl methacrylates, 2-(acrylamido)-2-methyl-1-propanesulfonic acid, acrylic acid, and acrylamide. Techniques have been developed that allow fine control of the porous properties of the polymers. These, in turn, determine the hydrodynamic properties of the devices that contain the molded media. Since all of the mobile phase must flow through the monolith, the mass transport within the molded material is accelerated considerably by convection, and the monolithic devices perform well even at much higher flow rates than those used with packed columns. This is particularly important for the chromatographic separations of large molecules such as proteins, for which diffusion is a serious problem that significantly slows down the separation processes. Similarly, this convection provides faster transport of substrates to active sites with immobilized enzymes, and it increases significantly the apparent activity of the conjugate. The molded separation media have been used for the chromatographic separation of biological compounds using modes such as reversed-phase, hydrophobic interaction, and ion-exchange chromatography, synthetic polymers and oligomers in reversed-phase and precipitation−redissolution mode, for electrochromatography, enzyme immobilization, and molecular recognition, and in advanced detection systems. Grafting of pores with selected polymer chains has led to "smart" materials that quickly respond to external stimuli and change the permeability and/or polarity of the monolith.

Introduction

Different types of macroporous (macroreticular) polymers emerged simultaneously in the late 1950s as a result of the search for polymeric matrixes suitable for the manufacture of ion-exchange resins with better osmotic shock resistance and faster kinetics (Abrams, 1964; Corte and Meier, 1971; Meitzner and Olin, 1980). 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 cross-linking. These polymers are typically produced as spherical beads by a suspension polymerization process that was invented in 1912 (Hofman and Delbruck, 1912; Brooks, 1990; Yuan et al., 1991), which uses a polymerization mixture containing both a cross-linking monomer and an inert diluent, the porogen (Seidl et al., 1967; Kun and Kunin, 1968; Sederel and DeJong, 1973; Guyot and Bartholin, 1982; Hodge and Sherrington, 1989). Porogens can be solvating or nonsolvating solvents for the polymer that is formed or soluble non-cross-linked polymers or mixtures of polymers and solvents.

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 and catalysts, and chromatographic packings fit well into the latter (Helfferich, 1962; Arshady, 1991). 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 (Arshady, 1991).

While suspension polymerization has already been analyzed in the literature (Seidl et al., 1967; Guyot and Bartholin, 1982), little was known until recently (Svec and Fréchet, 1992, 1996) on how to prepare macroporous polymers by bulk polymerization within a mold.

Mechanism of Pore Formation

The "classical" mechanism of pore formation that occurs during the polymerization process depends on the type of porogen used for the creation of the porous structure. For example, during a typical polymerization in the presence of a precipitant (nonsolvating solvent), the mechanism is the following (Seidl et al., 1967; Guyot and Bartholin, 1982): The organic phase contains both monovinyl and divinyl monomers, an initiator, and a porogenic solvent. The free-radical initiator decomposes at a particular temperature, and the initiating radicals start the polymerization process in solution. The polymers thus formed precipitate after they become in-

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soluble in the reaction medium as a result of both their cross-linking and the choice of porogen (poor solvent for the polymer). In this process, the monomers are thermodynamically better solvating agents for the polymer than the porogen. Therefore, the precipitated insoluble gellike species (nuclei) are swollen with the monomers that are still present in the surrounding liquid. The polymerization then continues both in solution and within the swollen nuclei. Polymerization within the latter is kinetically preferred because the local concentration of monomers is higher in the individual swollen nuclei than in the surrounding solution. Branched or even cross-linked polymer molecules formed in the solution are captured by the growing nuclei and further increase their size. The cross-linked character of the nuclei prevents their mutual penetration and loss of their individuality through coalescence. The nuclei, enlarged by the continuing polymerization, associate in clusters being held together by polymer chains that cross-link the neighboring nuclei. The clusters remain dispersed within the liquid phase rich in the inert solvent (porogen) and continue to grow. In the later stages of the polymerization, the size of the clusters is large enough to allow contact with some of their neighbors, thereby forming a scaffolding-like interconnected matrix within the polymerizing system. The interconnected matrix becomes reinforced by both interglobular cross-linking and the capture of chains still polymerizing in solution, which leads to the final porous polymer body. The fraction of voids within the final porous polymer (macropores) is, at the end of the polymerization, close to the volume fraction of the porogenic solvent in the initial polymerization mixture because the porogen remains trapped in the voids of the cross-linked polymer.

This mechanism of pore formation during the heterogeneous polymerization of monomers in the presence of porogens does not yet allow a prediction of the sizes of the pores that should result. The current knowledge of factors that control pore size in macroporous polymers is mostly empirical.

Mass Transport and Space Utilization in Packed Beds

Porous beads with well-defined size, shape, and chemistry are generally packed into columns that are subsequently used in diverse applications. The technology for the preparation of macroporous beads has been developed to such a degree that excellent control over their properties, including bead size, chemistry, and porosity, is routinely achieved. Despite the many advantages that led to their widespread use, columns and reactors packed with typical particulate materials also have some limitations. The slow diffusional mass transfer of high molecular weight solutes into the stagnant phase present in the pores of the beads and the large void volume between the packed particles are the most important and best known limitations of present day technology (Martin and Synge, 1941; Unger, 1990).

The passage of molecules within the pores of a standard macroporous material is controlled by diffusion. Typically, small entities such as gases, small organic molecules, and ions move relatively quickly, whereas the motion of large molecules such as proteins, polysaccharides, or synthetic polymers is considerably slower because their diffusion coefficients are several orders of magnitude smaller than those of low molecular weight compounds. This effect is detrimental to processes where the speed of the mass transfer limits the overall rate, as is the case in chromatography, catalysis, or adsorption.

The resistance to mass transfer is further accented by the discontinuity typical of packed beds. The lowest theoretical interparticle volume of perfectly packed uniformly sized spherical beads is calculated to be about 27% of the total volume. A liquid forced through a bed packed of macroporous particles flows readily through the relatively large interstitial voids between the particles, where resistance against its flow is the smallest. In contrast, there is no flow of the liquid present in the very small pores, which therefore remains stagnant. When a compound is introduced into the stream of the mobile phase, for example, injected in a way typical of chromatographic separations, this compound is also carried through the voids. However, because of the concentration gradient between the solution in the voids and the stagnant liquid within the pores, diffusion occurs, causing transport of these compounds into the pores until an equilibrium concentration is reached in both stream and pores. Once the concentration "pulse" has passed by the bead, the amount of compound in the main stream decreases steeply and the concentration gradient is reversed. The compound then diffuses back from the pores into the surrounding liquid, and eventually only the original stagnant phase remains within the pores (Snyder and Kirkland, 1979).

Because the diffusion rate for small molecules is quite high, the equilibrium concentration within the pores is reached quickly. However, in the case of macromolecules, the situation is quite different. The slow diffusional mass transfer of macromolecules within a macroporous matrix may be illustrated with the example of biocatalysis in a reactor packed with an immobilized proteolytic enzyme. Once added, the macromolecular substrate (protein) diffuses slowly from the bulk solution into the pores, where it interacts with the active sites of the bound enzyme and reacts to form product. This product then desorbs and diffuses back to the main stream. If diffusion is slower than the enzymatic reaction, it becomes the rate-determining step and only those active sites that are located in close proximity to the support surface are supplied with the substrate. Because of hindered diffusion, no substrate reaches the sites located deeper in the bead and, therefore, the theoretical enzymatic activity cannot be utilized completely. As a result, the overall activity of the immobilized biocatalyst does not reach its full potential, and a larger volume of the supported catalyst has to be used to achieve the expected throughput (Cheetham, 1985).

Similarly, the diffusion problem encountered with large molecules affects the performance of classical HPLC separations. The detrimental effect of a slow rate of diffusional mass transfer observed for large molecules within the chromatographic separation medium was already recognized by Van Deemter in the mid-1950s (Van Deemter et al., 1956). The efficiency of the whole system deteriorates rapidly as the flow rate increases. As a result, longer columns or slower flow rates must be used to achieve the desired separation (Unger, 1990). Several approaches directed toward improving the mass transfer for high-performance liquid chromatography (HPLC) are discussed in the recent literature (Chen and Horváth, 1995).

Enhancement of Mass Transfer by Convection

The positive effect of convection on the efficiency of heterogeneous catalysts was recognized in the late 1970s. Nir and Pismen (1977) demonstrated a considerable improvement in catalytic activity when a large-pore inorganic support was used. In contrast to diffusion, for which the concentration gradient is the driving force, convection uses flow to dramatically accelerate the mass transfer of compounds. This suggests that macroporous supports might be well suited for applications requiring rapid mass transfer. However, a major problem is that most pores with a size up to about 100 nm found in typical macroporous polymers are too small to allow convection.

In the 1980s, Polymer Laboratories developed macroporous poly(styrene-*co*-divinylbenzene) beads, with some pores larger than 400 nm, that were later duplicated by Regnier (1991) for applications in chromatography of biopolymers. Though these pores allow some convection, flow around the beads is still preferred, as the convective flow through the pores accounts for less than 2% of the total (Afeyan et al., 1990). However, even this small proportion of the flow through the beads is claimed to be sufficient to significantly enhance the speed of separations of biopolymers as compared to conventional packing materials. Theoretical treatment of processes including flow through large pores indicates that the maximum effect of convection can be achieved only if all of the mobile phase is forced to flow through the porous medium (Rodrigues et al., 1993; Liapis, 1993). Unfortunately, beds packed with particulate materials always contain a large void volume between the packed particles. Therefore, it is not realistic to envision beds for which flow would occur through pores within the beads when a neighboring large interparticle void volume is available for essentially unimpaired flow. Obviously, new material designs that either incorporate very little or, preferably, none of the discontinuity typical of packed beds are required for total convection.

Design of Porous Media with Limited Discontinuity

The ideal implementation of the idea of complete elimination of flow through the voids leads to a system consisting of only one single piece of porous material. Such systems have already been treated theoretically (Liapis, 1993), but experimental work remained scarce as a result of the lack of better alternatives to the beads.

The first attempt to make a "single-piece" separation medium dates back to the late 1960s and early 1970s. For example, swollen polymer was prepared by a free-radical polymerization of an aqueous solution of 2-hydroxyethyl methacrylate with 0.2% ethylene dimethacrylate (cross-linking monomer) (Kubin et al., 1967). The gel was inserted into a 220 × 23.5 mm i.d. glass tube, and this column was used for size-exclusion chromatography. The authors claimed that the effectiveness of fractionation was rather low as a result of a longitudinal diffusion resulting from very slow flow rate (only 4 mL/h). In contrast, the permeability of monolithic open-pore polyurethane foams was excellent; however, excessive swelling in some solvents and softness were deleterious characteristics that prevented their use in both high-performance liquid and gas chromatography (Ross and Jefferson, 1970; Hileman et al., 1973; Hansen and Sievers, 1974; Lynn et al., 1974).

It is well-known that the problem of interparticular volume does not exist in processes in which a membrane is used as the separation medium. Both theoretical calculations (Su et al., 1992) and experimental results (Klein, 1991; Su et al., 1992) clearly document that membrane systems can be operated in a "dead-end", or filtration mode, at much higher flow rates than those of packed beds. This is because all of the substrate solution flows through the support and mass transfer is much faster as a result of this convective flow (Su et al., 1992). This is particularly true for separations in which macromolecular compounds are involved. Although membranes may have a binding capacity per unit volume similar to that of particles, a rather large membrane area is required to achieve a capacity equivalent to that of a packed column (Klein, 1991). Therefore, thin membranes based upon modified cellulose, nylon, or other polymers have to be stacked into a pile to minimize the size of the separation units (Roper and Lightfoot, 1995). Similarly, porous sheets in which macroporous beads are embedded into a web of polymer (Hagen et al., 1990; Manganaro and Goldberg, 1993) and porous cellulose monoliths (Noel et al., 1993) have been stacked in a cartridge to simulate a column with almost no voids.

Macroporous disks (Tennikova et al., 1990; Josic et al., 1992; Tennikova and Svec, 1993), rolled woven matrixes (Yang et al., 1992, 1993), and compressed soft poly(acrylamide) gels (Hjertén et al., 1989, 1992) placed in a cartridge or column also represent examples of media that exhibit almost no interstitial porosity. These elegant approaches are described in detail in the other review articles of this series. In the early 1990s, we developed novel *rigid* macroporous monoliths formed by a very simple "molding" process in which a mixture of monomers and solvent is polymerized within a closed tube or other container under carefully controlled conditions (Svec and Fréchet, 1992).

Porous inorganic materials are very popular supports widely used in catalysis and chromatography (Unger, 1990). This is one of the reasons why silica-based monoliths were developed almost simultaneously with the organic polymers. Although the single-piece open-pore silica foams had already been prepared by the end of the 1970s (Pretorius et al., 1979), useful monolithic inorganic materials for chromatographic applications have only emerged recently (Fields, 1996; Minakuchi et al., 1996, 1997).

Preparation of Rigid Polymer Monoliths

The preparation of the totally new class of rigid macroporous organic polymers produced by a simple "molding" process is simple and straightforward (Figure 1). The mold, typically a tube, is sealed at one end, filled with a polymerization mixture, and then sealed at the other end. The polymerization is then triggered frequently by heating in a bath at a temperature of 55−80 °C. The seals are removed, the tube is provided with fittings and attached to a chromatographic pump, and a solvent is pumped through the column to remove the porogens and any other soluble compounds that remained in the polymer rod after the polymerization was completed. A broad variety of tube sizes and materials, such as stainless steel, poly(ether ether ketone) (PEEK), and glass tubes, have been used as molds for the preparation of monoliths (Wang et al., 1993; Svec and Fréchet, 1995b,c; Fréchet and Svec, 1994, 1995; Peters et al., 1997b).

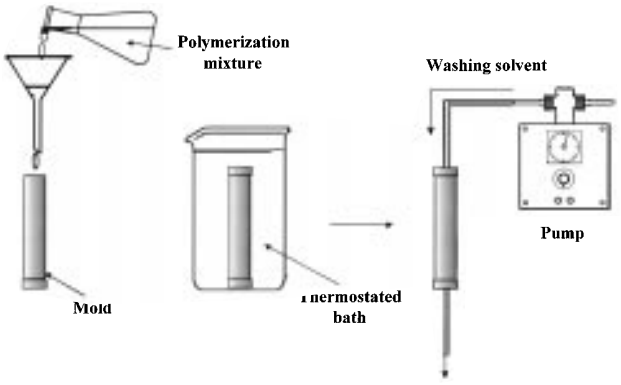


Figure 1. Preparation of macroporous monolith by a "molding" process (Svec and Fréchet, 1996).

While the preparation of cylindrical monoliths with a homogeneous porous structure and diameter up to about 10−25 mm is readily achieved in a single-step polymerization, larger size monoliths are somewhat more difficult to prepare. Dissipation of the heat of polymerization is frequently slow, and the exotherm 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 reaction proceeds continuously minimizes the exotherm and allows the preparation of very large diameter monoliths with homogeneous porous structures (Peters et al., 1997b).

Control of Porous Properties

Many applications of porous materials such as catalysis, adsorption, ion exchange, chromatography, solid-phase synthesis, etc., rely on intimate contact with a surface that supports the active sites. 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 come from the 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 a 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 Figure 2. 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 bulklike 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

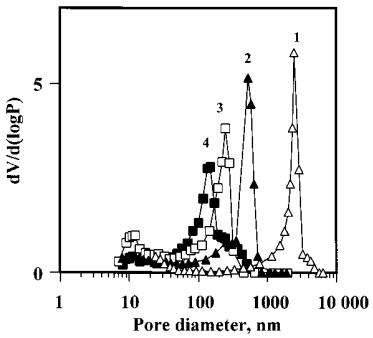


Figure 2. Effect of dodecanol in the porogenic solvent on differential pore size distribution of molded poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) monoliths. Conditions: polymerization time, 24 h; temperature, 70 °C. Polymerization mixture: 24% glycidyl methacrylate, 16% ethylene dimethacrylate , and cyclohexanol and dodecanol contents in mixtures 60/0 (1), 57/3 (2), 54/6 (3), 45/15 vol % (4) (Viklund et al., 1996).

organic phases and the absence of dynamic forces that are typical of stirred dispersions (Svec and Fréchet, 1995d).

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 the cross-linking divinyl monomer allow the tuning of the average pore size within a broad range spanning at least 2 orders of magnitude from tens to thousands of nanometers.

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 composition. The effect of 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 (Svec and Fréchet, 1995a; Viklund et al., 1996).

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 because of 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 (Viklund et al., 1996).

In contrast, increasing the proportion of the cross-linking agent present in the monomer mixture affects the chemical composition of the final monoliths. At the same time, it also decreases their average pore size as a result of early formation of highly cross-linked globules with a reduced tendency to coalesce. The experimental results imply that, in this case, the pore size distribution is controlled by limitations in *swelling of cross-linked nuclei* (Viklund et al., 1996).

Morphology of Monoliths

The morphology of the monoliths is closely related to their porous properties, and is also a direct consequence

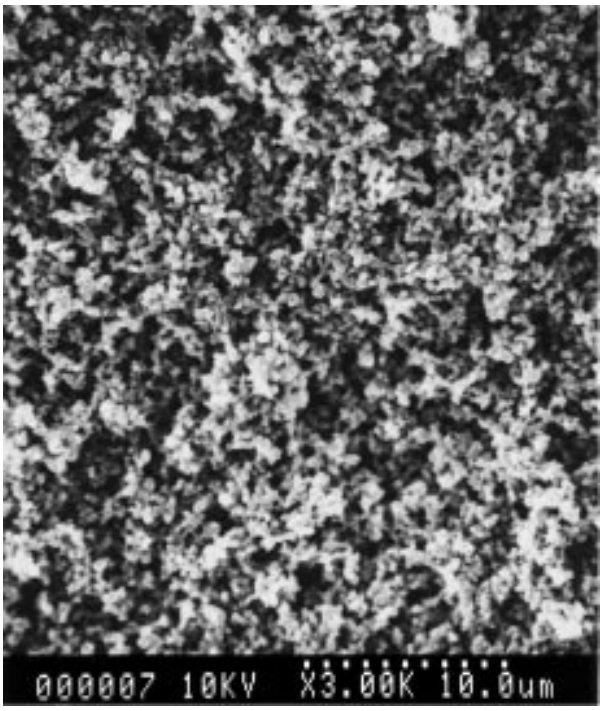


Figure 3. Scanning electron micrograph of the inner part of the poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) monolith.

of the quality of the porogenic solvent as well as the percentage of cross-linking monomer and the ratio between the monomer and porogen phases. The presence of interactive effects between these reaction conditions was verified using multivariate analysis (Viklund et al., 1997a).

In general, the morphology of macroporous materials is rather complex. The scanning electron micrograph shown in Figure 3 reveals the details of the globular internal structure of a molded monolith of porous poly(glycidyl methacrylate-*co*-ethylene dimethacrylate). Although this morphology featuring individual microglobules and their irregular clusters is similar to that found for beads (Pelzbauer et al., 1979), the sizes of both the clusters and the irregular voids between clusters are much larger.

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 crushed by the extremely high pressures required for flow. 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 large 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 reactive capacity.

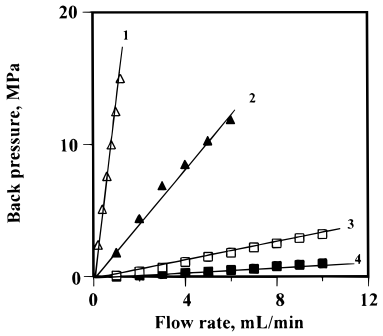


Figure 4. Effect of flow velocity on backpressure in the molded poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) 100 × 8 mm monolithic column. Conditions: Mobile-phase tetrahydrofuran. Polymerization mixture: 24% glycidyl methacrylate, 16% ethylene dimethacrylate, and cyclohexanol and dodecanol contents in mixtures 54/6 with temperature 80 °C (1), 54/6 with 70 °C (2), 54/6 with 55 °C (3), and 57/3 with 55 °C (4) (Viklund et al., 1996).

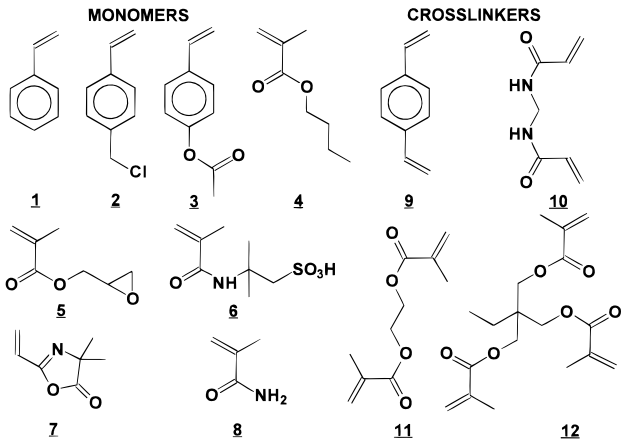
Figure 4 shows the backpressure per unit of flow rate (flow resistivity) as a function of the flow rate. Typically, the pressure needed to sustain even a very modest flow rate is quite high for materials that have a mean pore diameter of less than about 500 nm, while high flow rates can be achieved at low pressures with materials that have pores larger than 1000 nm. Although the shape of the pores within the monoliths is very different from that of a tube, the Hagen−Poiseuille equation essentially holds also for the flow through the molded porous poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) and poly(styrene-*co*-divinylbenzene) monoliths because, as expected, flow does not depend on the chemistry of the material (Viklund et al., 1996).

Surface Chemistries

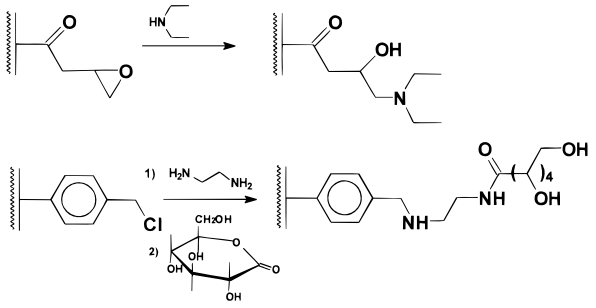
The number of monomers that may be used in the preparation of polymer monolith is much larger than that used for classical suspension polymerization because there is only one phase in the mold. Therefore, almost any monomer, including water-soluble hydrophilic monomers which are not suitable for standard polymerization in aqueous suspensions, may be used to form a monolith. This greatly increases the variety of surface chemistries that can be obtained. However, the polymerization conditions optimized for one system cannot be transferred directly 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 (Wang et al., 1994). A few examples of monomers (**1**−**7**) and cross-linking agents (**8**−**11**) that have been used for the preparation of porous rigid monoliths are shown in the Chart 1. The list of monomers includes a broad variety of chemistries varying from very hydrophilic [acrylamide (**8**) and 2-(acrylamido)-2-methyl-1-propanesulfonic acid (**6**)] through reactive [glycidyl methacrylate (**5**), (chloromethyl)styrene (**2**), 2-vinyl-4,4-dimethylazlactone (**7**)] and protected functionalities [4-acetoxystyrene (**3**)] to rather hydrophobic monomers [styrene (**1**) and butyl methacrylate (**4**)] (Wang et al., 1994; Viklund et al., 1996; Xie et al., 1997a).

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

Chart 1



Scheme 1



reactions are easily performed using monoliths prepared from monomers containing reactive groups such as **2** and **5**. For example, Scheme 1 shows the reaction of glycidyl methacrylate monoliths with diethylamine, which leads to an ion exchanger (Svec and Fréchet, 1992), and the reaction of poly[(chloromethyl)styrene-*co*-divinylbenzene] with ethylenediamine and then with γ-gluconolactone, which completely changes the surface polarity from hydrophobic to highly hydrophilic (Wang et al., 1995).

Using these 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 (1990) 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. A similar reaction was used to graft poly[(acrylamido)-methylpropanesulfonic acid) (**6**) onto the internal surface of hydrolyzed poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) monoliths (Viklund et al., 1997b).

Grafting can also provide the monolithic polymers with rather unexpected properties. For example, the two-step grafting procedure summarized in Scheme 2, which involves the vinylization of the pore surface by reaction of the epoxide moiety with allylamine, and a subsequent in situ radical polymerization of *N*-isopropylacrylamide (NIPAAm) initiated by azobis(isobutyronitrile) (AIBN) within these pores leads to a composite

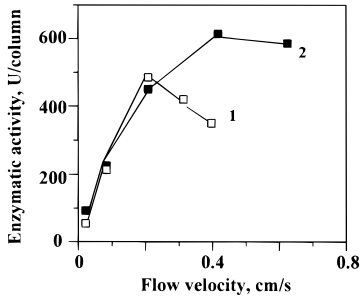
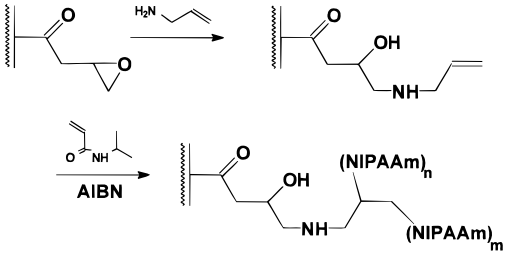


Figure 5. Effect of linear flow velocity of a L-benzoyl arginine ethyl ester solution (0.2 mol/L) on enzymatic activity of trypsin immobilized on the poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) beads (1) and monolith (2). Conditions: reactor < 50 × 8 mm i.d.; temperature, 25 °C (Petro et al., 1996).

Scheme 2



that changes its properties in response to external temperature (Peters et al., 1997a).

Use of Rigid Polymer Monoliths

High-Throughput Bioreactors. Because the monoliths allow total convection of the mobile phase through their pores, mass transfer is also dramatically increased. On the basis of 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 performance as supports for immobilization of biocatalysts, thus helping to extend 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 biocatalysts and also facilitates workup 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, faster mass transfer should translate into higher activity. Comparative studies with trypsin immobilized onto both macroporous beads and fully permeated poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) (Petro et al., 1996c) and poly[2-vinyl-4,4-dimethylazlactone-*co*-methylenebis(acrylamide)] (Xie at al., 1997b) 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 (Figure 5). 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 dimethacrylate) monolith re-

Table 1. Porous Properties and Enzymatic Activities of Monolithic Poly(2-vinyl-4,4-dimethylazlactone-*co*-acrylamide-*co*-ethylene dimethacrylate) Reactors[a] (Xie et al., 1997b)

	monolith			
	A	B	C	D
VAL/AA[b]	16/4	12/8	8/12	4/16
porogenic solvent	tetradecanol	tetradecanol	tetradecanol	decanol + oleyl alcohol (1:1)
$D_{p,med}$,[c] μm	2.92	2.72	2.57	2.65
V_p,[d] mL/g	1.52	1.51	1.44	1.48
A,[e] μmol/min/mL	103	203	221	136

[a] Polymerization mixture: 20 wt % ethylene dimethacrylate, 20% vinylazlactone + acrylamide, 60% porogenic solvent, and azobis(isobutyronitrile) (1% with respect to monomers). Conditions: temperature, 65 °C; polymerization time, 24 h. [b] Percentage of vinylazlactone (VAL) and acrylamide (AA) in the 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.

actor 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 capability of the pump used (Petro et al., 1996c). In contrast, the range of available flow rates for the packed column is limited by the exponential growth in the backpressure that must not exceed the upper limits of the equipment used. Not only does the enzyme bound to the monolith have 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 matrixes, the 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 μmol/min per 1 mL of support is obtained with the support containing 20% azlactone and 30% acrylamide. Although this activity for 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 attachment of the enzyme to the azlactone moieties of the monolith is achieved in a single step (Xie et al., 1997b).

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 and affinity chromatography. For example, Figure 6 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 rods (Petro et al., 1996c).

Solid-Phase Detection. Peroxyoxalate chemiluminiscence is one of the most efficient methods for the direct detection of hydrogen peroxide (Kwakman and Brinkman, 1992). This approach can be further extended to the indirect detection of some other compounds. The original experimental setup consists typically of a reactor packed with a solid particulate support with a bound fluorophore such as 3-aminofluoranthene attached to. In contrast, Pontén et al. (1996) used a bulk polymerization in a glass mold initiated by UV light for the preparation of solid-phase macroporous poly(glycidyl methacrylate-*co*-trimethylolpropane trimethacrylate) monolithic reactors. 3-Aminofluoranthene immobilized onto the monolithic supports exhibited a light generation efficiency twice 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.

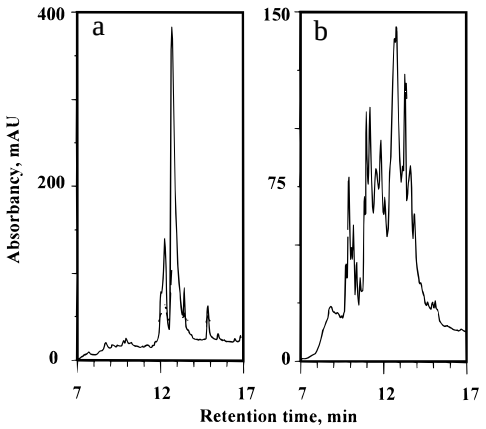


Figure 6. Reversed-phase separations of cytochrome *c* digests obtained with trypsin-modified beads (a) and trypsin-modified monolithic reactor (b) in a tandem with a chromatographic column. Conditions for digestion: (a) trypsin-modified beads; reactor, 50 × 8 mm i.d.; 0.2 mg of cytochrome *c*; digestion buffer; flow rate, 0.2 mL/min; 25 °C; residence time, 15 min; (b) trypsin immobilized onto molded monolith; other conditions the same as those with trypsin-modified beads. Reversed-phase chromatography column, Nova-Pak C18, 150 × 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 (Petro et al., 1996).

Macroporous Monoliths as Fluid Gates and Valves. Some polar polymers dissolve in water only when the temperature is below the lower critical solution temperature (LCST) while they precipitate once the temperature exceeds this mark. The chains undergo a rapid and reversible phase transition from extended hydrated helices below the LCST to collapsed hydrophobic coils above the LCST. Poly(*N*-isopropylacrylamide) (PNIPAAm) is an example of a temperature-sensitive polymer, which has a LCST of 32 °C. These polymers are recently finding numerous applications in drug delivery systems, polymer purification, encapsulated enzyme bioreactors, and membranes with controlled permeability (Dusek, 1993).

The volume transition exhibited by poly(*N*-isopropylacrylamide) in response to changes in temperature can be used to control liquid flow through polymer monoliths. For example, PNIPAAm chains grafted to the pore surface of a monolith using the process shown in Scheme 2 exist in their collapsed form at a temperature of 40

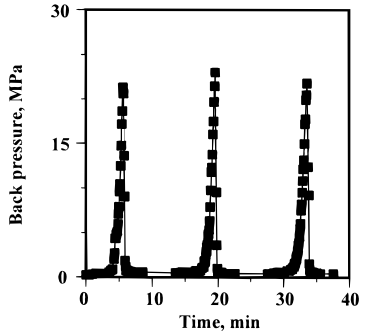


Figure 7. Thermal gate behavior of porous poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) monolith grafted with poly(*N*-isopropylacrylamide). Conditions: monolithic cylinder, 10 × 10 mm in a stainless steel cartridge; flow rate of water, 1 mL/min. The cartridge is removed from a 40 °C bath at a backpressure close to 0 MPa and reimmersed into the bath after the backpressure exceeds 20 MPa (Peters et al., 1997).

°C and do not obstruct flow through the pores. Once the material is cooled to room temperature, which is well below the LCST, the chains expand and fill the pores completely, thus preventing the flow of a liquid. This on/off behavior is readily monitored in a pumped system by the rapid increase in backpressure which opposes flow through the monolith. When the column is reimmersed in the bath at 40 °C, the backpressure returns almost immediately to its original value. Figure 7 clearly documents that this gate effect is rapid, reversible< and reproducible (Peters et al., 1997a).

The extent of "swelling" of the grafted chains within the pores can be controlled by an addition of a cross-linking monomer such as methylenebis(acrylamide) to the polymerization mixture used for the grafting. Even only 1% cross-linking does not allow the chains to swell at a temperature below their LCST to an extent sufficient to completely fill the pores. Therefore, the flow through such a monolith is only slowed but not shut off fully, a process in which the monolith acts as a gate to decrease flow rate in response to a change in temperature (Peters et al., 1997a).

Molecular Recognition. Materials with an enhanced selectivity toward specific substrate molecules can be produced using the technique of molecular imprinting in which interacting monomer(s) and a cross-linker are polymerized in the presence of a template molecule. 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 (Wulff, 1995). However, until recently, all of them relied on the use of particles that very often have an irregular shape and poor flow characteristics.

In contrast to the particulate materials, the molding technique has some advantages when used for the preparation of molecularly imprinted monoliths. Matsui et al. (1993) was the first to prepare an imprinted monolith. 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 (1994a,b) 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. 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 (Nilsson et al., 1994).

Separation Processes. (1) High-Performance Liquid Chromatography. (a) 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 (Wang et al., 1994). The average retention increase, which reflects the contribution of one methylene group to the overall retention of a particular solute and is defined as the separation factor (or selectivity, α), has a value of 1.42. This value is close to that published in the literature for typical polystyrene-based beads (Tanaka et al., 1989). 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 (Wang et al., 1994).

The efficiency of the monolithic polymer 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 (Minakuchi et al., 1996, 1997). The penalty paid for their more regular internal structure and higher efficiency is the more complicated method used for the preparation of the silica-based monolithic columns.

To simplify the preparation, Fields (1996) prepared a monolithic silica column directly within a capillary. Although his process works well 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. (1997) for silica and that shown by Viklund et al. (1997a) for organic polymers. Efficiencies achieved with this in situ prepared monolithic silica capillary were again only 5000−13 000 plates/m, i.e., in the range observed for molded polymer monoliths. This suggests that the efficiency is directly related to the morphology 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 (Unger, 1990). Similarly, the efficiency of capillary electrophoresis separations reaches its maximum for a specific capillary diameter and then decreases steeply for both larger and smaller sizes (Stedry et al., 1995). 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.

(b) 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

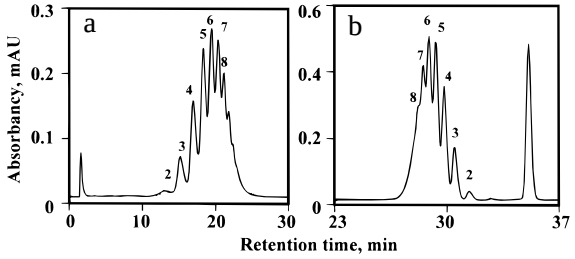


Figure 8. Separation of styrene oligomers by reversed-phase (a) and size-exclusion (b) chromatography. Conditions: (a) column, molded poly(styrene-*co*-divinylbenzene) monolithic column, 50 × 8 mm i.d.; mobile phase, linear gradient from 60 to 30% water in tetrahydrofuran within 20 min; flow rate, 1 mL/min; injection volume, 20 μL; UV detection, 254 nm; (b) series of four 300 × 7.5 mm i.d. PL gel columns (100, 500, and 105 Å and Mixed C); mobile phase, tetrahydrofuran; flow rate, 1 mL/min; injection volume, 100 μL; toluene added as a flow marker; UV detection, 254 nm; temperature, 25 °C; peak numbers correspond to the number of styrene units in the oligomer (Petro et al., 1996a).

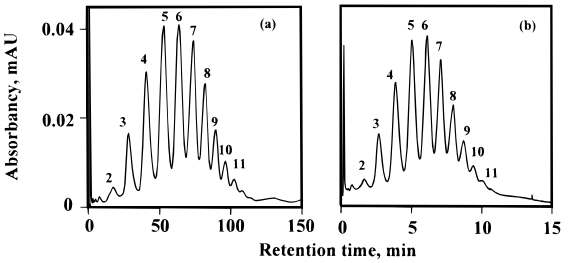


Figure 9. Effect of flow rate and gradient time on the separation of styrene oligomers in a molded poly(styrene-*co*-divinylbenzene) monolithic column. Conditions: column, 50 × 8 mm i.d.; (a) mobile phase, linear gradient from 60 to 30% water in tetrahydrofuran within 200 min; flow rate, 1 mL/min; (b) 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 μL; UV detection, 254 nm; peak numbers correspond to the number of styrene units in the oligomer (Petro et al., 1996a).

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 is considerably faster, because of 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), is achieved within 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 (Wang et al., 1994).

Excellent performance for the elution of another peptide, insulin (molecular weight 5800), was also observed using silica-based monoliths (Minakuchi et al., 1996). 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.

(c) Separation of Styrene Oligomers. Early examples of the separation of styrene oligomers by HPLC on reversed-phase octadecylsilica columns in a gradient of the mobile phase follow the normal course well-known for reversed-phase chromatography of small molecules (Larmann et al., 1983). Their retention depends both on the composition of the mobile phase and on the number of repeat units in the oligomer. Larger polystyrene oligomers, being much more hydrophobic, exhibit longer retention times. This means that shorter oligomers elute prior to longer ones, quite unlike size-exclusion chromatography for which larger molecules elute first.

Figure 8 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 chromatographic mode. The chromatograms are mirror images and exhibit a number of peaks that can be assigned to the individual styrene oligomers (Petro et al., 1996a). The resolution achieved with the molded rod column is very good, with the chromatogram in Figure 8 even indicating the presence of an undecamer.

In general, an increase in the resolution of a SEC system can only be achieved with a better column packing or a longer column. In contrast, the gradient elution method provides more options that can contribute to improvements in the separation. If variables such as the range of mobile-phase composition remain constant for a specific column and specific 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. Figure 9 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 of 200 mL is the same for both separations and, indeed, no significant differences can be seen 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 (Petro et al., 1996a). 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 typical C18 silica-based reversed-phase packed columns.

(d) 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 composition accelerates the elution, allowing separation of the sample components to be achieved 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 (Snyder, 1990). 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, and thus high-throughput separations can be

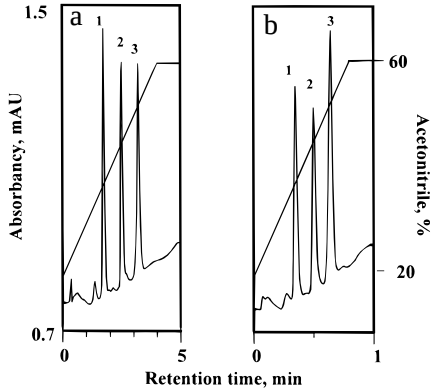


Figure 10. Separation of cytochrome *c* (1), myoglobin (2), and chicken egg albumin (3) by reversed-phase chromatography on the continuous poly(styrene-*co*-divinylbenzene) monolithic column at flow rates of 5 mL/min (a) and 25 mL/min (b). Conditions: column, 50 × 8 mm i.d.; mobile phase, linear gradient from 20 to 60% acetonitrile in water (Wang et al., 1994).

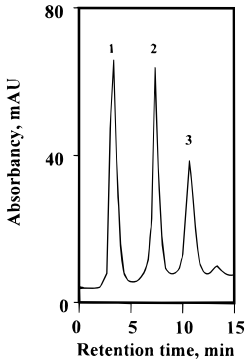


Figure 11. Separation of myoglobin (1), conalbumin (2), and soybean trypsin inhibitor (3) by ion-exchange chromatography on a diethylamine-modified molded poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) monolithic column. Conditions: column, 60 × 16 mm i.d.; mobile-phase gradient, from 0.01 mol/L TRIS−HCl buffer pH 7.6 to 1 mol/L NaCl in the buffer in 30 min; flow rate, 2 mL/min; total protein loading, 20 mg; UV detection, at 280 nm (Svec and Fréchet, 1995a−d).

achieved easily. Figure 10 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. All individual proteins are baseline separated in sharp and narrow peaks. No significant differences are 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. This, together with the low backpressure observed even at very high flow rate, enables rapid separations to be achieved simply with an increase in the flow rate. A further decrease in the time needed for the separation can be achieved by using an even steeper gradient, as shown by four proteins being separated in less than 25 s (Wang et al., 1993; Svec et al., 1995).

In contrast to reversed-phase chromatography, the separation in *ion-exchange mode* occurs under mild conditions using an entirely aqueous mobile phase. The elution from the monolith, which must contain charged ion-exchange functionalities, is achieved using an increasing concentration gradient of salt in the mobile phase. The epoxide groups of a molded poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) monolith readily react with many compounds to form ion exchangers (Azanova et al., 1990, 1991; Hradil and Svec, 1990). For example, the reaction with diethylamine leads to an analogue of the (diethylamino)ethyl (DEAE) chemistry (Scheme 1) which is well-suited even for large-scale separations. Figure 11 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. All of the proteins are baseline separated within a reasonable period of time, and the symmetry of the peaks is very good.

The breakthrough curves measured for the monolithic columns with different proteins are very sharp and confirm the fast mass transport kinetics of the monoliths (Svec and Fréchet, 1995b,c). 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 monolith at 1% breakthrough is 324 mg of ovalbumin and represents the specific capacity of 40.0 mg/g of the separation medium or 21.6 mg/mL of the column volume which is in a range similar to that found for megaporous perfusive beads (Afeyan et al., 1990).

The ultimate goal in the development of any separation medium, i.e., its use for the separation of actual field samples, has also been demonstrated by the separation of baker's yeast (*Saccharomyces cerevisiae*) extract leading to several sharp peaks (Svec and Fréchet, 1995b). This separation compares favorably to those obtained with commercial packed ion-exchange columns and even to recently introduced acrylamide-based compressed monolithic media (BioRad, 1997).

To further accelerate the ion-exchange separations discussed above, 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. 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 unit bears the required functionality. This increases the local concentration of ion-exchange groups and improves the separation properties of the matrix. Figure 12a shows the separation of myoglobin, chymotrypsinogen, and lysozyme in a monolithic column with strong acid grafts using an increasing gradient of sodium chloride. Because of the low resistance to flow, elution can be carried out at a flow rate of 7 mL/min, and the separation of the 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 gradients may accelerate it even more. Figure 12b shows the same separation using a stepwise gradient. 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.

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

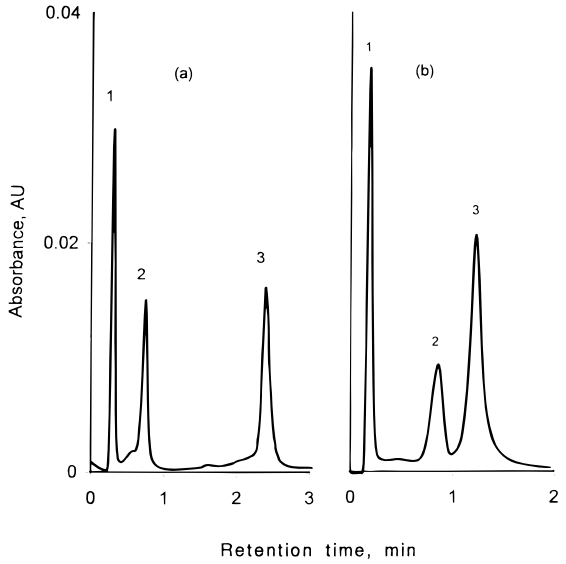


Figure 12. Separation of myoglobin (1), chymotrypsinogen (2), and lysozyme (3) by ion-exchange chromatography on a poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) monolithic column grafted with poly[2-(acrylamido)-2-methyl-1-propanesulfonic acid] chains. Conditions: column, 50 × 8 mm i.d.; mobile phase A, 0.02 mol/L of phosphate buffer, pH 6; mobile phase B, 1 mol/L of sodium chloride in A; flow rate, 7 mL/min; UV detection, 280 nm; (a) gradient linear from 5% to 80% B in A in 3 min; (b) one-step gradient from 5% to 80% B at time 0.

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.

Because the hydrophobicity of styrene or alkyl methacrylate based monolithic matrixes is too high to make them useful for hydrophobic interaction chromatography, Xie et al. (1997a,c) developed porous monoliths based on highly hydrophilic copolymers of acrylamide and methylenebis(acrylamide). 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 Figure 13, 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, to operate satisfactorily, a typical HIC column must be reequilibrated 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 chromatographic separation of proteins in a simple *isocratic* mode (Peters et al., 1997a).

The use of monoliths with PNIPAAm chains grafted to the internal pore surface as gates and valves to regulate liquid flow was discussed previously. The

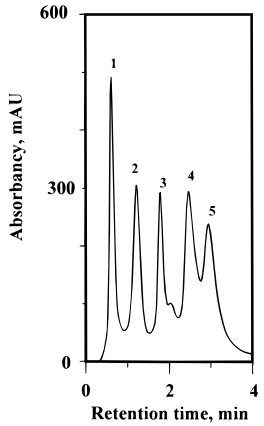


Figure 13. Separation of cytochrome (1), ribonuclease, (2), carbonic anhydrase (3), lysozyme (4), and chymotrypsinogen (5) by hydrophobic interaction chromatography on a molded poly[acrylamide-*co*-butyl methacrylate-*co*-*N*,*N*′-methylenebis(acrylamide)] monolithic column. Conditions: column, 50 × 8 mm i.d.; 10% butyl methacrylate; mobile-phase gradient, from 1.5 to 0.1 mol/L of ammonium sulfate in 0.01 mol/L of sodium phosphate buffer (pH 7) in 3 min; gradient time, 3.3 min, flow rate, 3 mL/min.

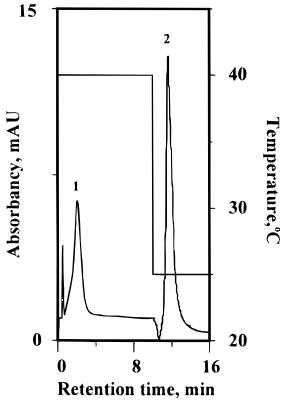


Figure 14. Temperature-controlled hydrophobic interaction chromatography of carbonic anhydrase (1) and soybean trypsin inhibitor (2) using a porous poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) monolith grafted with poly[*N*-isopropylacrylamide-*co*-methylenebis(acrylamide)]. Conditions: monolithic cylinder, 10 × 10 mm in stainless steel cartridge; mobile phase, 1.4 mol/L of ammonium sulfate in 0.01 mol/L of phosphate buffer (pH 7); flow rate, 1 mL/min (Peters et al., 1997).

extended solvated chains that are present below the LCST are more hydrophilic, while the collapsed chains that prevail above the LCST are more hydrophobic. In contrast to isothermal separations in which the surface polarity remains constant throughout the run (Hosoya et al., 1995; Kanazawa et al., 1996), HIC separation of proteins can be achieved at *constant salt concentrations* while utilizing the hydrophobic−hydrophilic transition of the grafted chains of PNIPAAm which occurs in response to *changes in temperature*. Figure 14 shows the isocratic separation of carbonic anhydrase and soybean trypsin inhibitor. 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.

(e) Precipitation−Redissolution Separation of Synthetic Polymers. In this technique originally

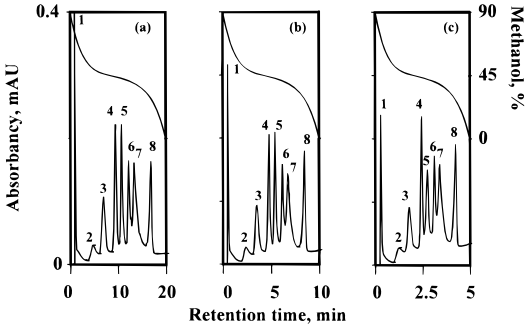


Figure 15. Separation of polystyrene standards in a molded poly(styrene-*co*-divinylbenzene) monolithic column. Conditions: column, 50 × 8 mm i.d.; mobile phase gradient, methanol in tetrahydrofuran; shape of gradient shown in each chromatogram; flow rate, 2 (a), 4 (b), and 8 mL/min (c); polystyrene standards with a molecular weight of 519 (1), 1250 (2), 9200 (3), 34 000 (4), 68 000 (5), 170 000 (6), 465 000 (7), and 2 950 000 (8); 3 mg/mL of each in tetrahydrofuran; injection volume, 30 mL (5); UV detection, 254 nm (Petro et al., 1996a).

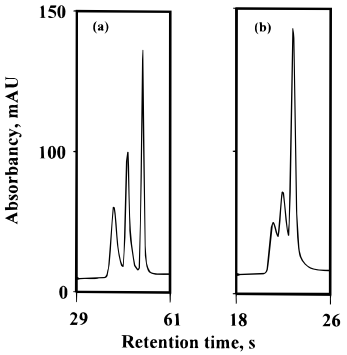


Figure 16. Effect of gradient steepness on the very fast separation of polystyrene standards in a molded monolithic poly(styrene-*co*-divinylbenzene) column. Conditions: column, 50 × 8 mm i.d.; mobile phase, linear gradient from 100% methanol to 100% tetrahydrofuran within 1 min (a) and 12 s (b); flow rate, 20 mL/min; analytes, molecular weight 9200, 34 000, 980 000 (order of elution); 3 mg/mL of each standard in tetrahydrofuran; injection volume, 20 μL (5); UV detection, 254 nm (Petro et al., 1996b).

developed for packed columns (Glockner, 1991), 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. Because 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 again. 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. Figure 15 shows the separations of a mixture of narrow polystyrene standards with molecular weights ranging from 519 to 2 950 000 at different flow rates in a gradient of methanol in tetrahydrofuran. All eight standards are well-separated even at the flow rate of 8 mL/min despite the very short length (5 cm) of the column used. Once again, the separation can be achieved 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 compromising 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 are obtained using mobile phases in which acetonitrile and water were used as precipitants (Petro et al., 1996a). In a "field" test, the molecular weight distribution profiles of a polystyrene sample with a broader molecular weight distribution ($M_w = 239\,000$; $M_w/M_n = 1.90$) obtained both by precipitation chromatography on a single 5-cm-long monolithic column and by a "classical" SEC determination in a series of four 30-cm-long columns matched quite well, although the two methods rely on different properties of the macromolecules (solubility and hydrodynamic volume, respectively) (Petro et al., 1996a).

Generally, gradient separations can be performed faster by using higher flow rates and steeper gradients. This also applies to the precipitation−redissolution chromatography of synthetic polymers. Figure 16 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. In contrast to the column packed with porous beads, a good separation is achieved even within a mere 4 s in a 12 s gradient of the mobile phase. An attempt to further accelerate the separation with a mobile-phase gradient that changes from pure methanol to tetrahydrofuran in 6 s partly failed as the chromatogram exhibited only two peaks and a shoulder for the smallest polystyrene standard (Petro et al., 1996b). Because the backpressure in the molded monolith for tetrahydrofuran at a flow rate of 20 mL/min (flow velocity 3300 cm/h) is only 2.6 MPa (380 psi), the separation could be significantly improved if a chromatographic system providing gradients at higher flow rates was available.

(2) Capillary Electrochromatography. Capillary electrochromatography (CEC) is a "hybrid" separation method in which uncharged molecules are separated in a fused-silica capillary, typically packed with C18-modified silica beads as in reversed-phase HPLC, but where the mobile phase is driven through the capillary by the electroosmotic flow characteristic of all electrophoretic methods. In theory, extremely high efficiencies can be obtained for CEC separations because of the plug flow profile of the mobile phase, which leads to smaller zone broadening. Although CEC was invented in the early 1970s (Pretorius et al., 1974) and its potential for packed capillary columns demonstrated in the 1980s

(Jorgenson and Lukacs, 1981; Tsuda et al., 1982; Stevens and Cortes, 1983), serious technical problems have slowed the development of this promising separation technique. These problems include the difficult fabrication of frits within a capillary, the packing of beads into a tube with a very small diameter, and the limited stability of packed capillary columns.

Many of these problems can be solved by the direct molding of rigid, monolithic columns for reversed-phase electrochromatography within the confines of untreated fused-silica capillaries. The molding proceeds in a single step by a simple copolymerization of ethylene dimethacrylate, butyl methacrylate, and 2-(acrylamido)-2-methyl-1-propanesulfonic acid in the presence of a porogenic solvent (Peters et al., 1997c). The composition of the specifically designed ternary porogenic solvent allows the fine control of the porous properties of the monolithic material over a broad range. Thus, optimal properties of the monolithic capillary are easily adjusted. While the electroosmotic flow through these capillary columns increases with both increasing pore size of the monolith and content of charged functionalities, better chromatographic properties have been observed for monoliths with larger surface area and hydrophobicity. For example, an optimized monolith with 0.3% AMPS, 420 nm large pores, and 9.7 m^2/g of surface area affords an efficiency of up to 160 000 plates/m and provides baseline separation of all nine benzene derivatives within only 6 min.

Chiral monolithic columns for the capillary electrochromatographic separation of enantiomers in reversed-phase mode can also be prepared by a simple "molding" method using polymerization mixtures containing a chiral monomer. Since molecular recognition is a delicate process relying on very specific interactions, nonspecific effects that might deteriorate the enantioseparation must be minimized by optimizing both the chiral separation medium and the mobile phase. The extent of hydrophobic interactions can be controlled through the choice of the monomers used for the preparation of the separation medium. Because finding the conditions for the direct incorporation of highly hydrophilic monomers may be difficult, the polymerization of a latent hydrophilic monomer such as glycidyl methacrylate followed by the hydrolysis of its epoxide ring to a diol functionality appears to be a very productive method for the preparation of very efficient hydrophilic chiral stationary phases for capillary electrochromatography (Peters et al., 1998).

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 confirms the great potential of these new molded continuous supports. Besides reversed-phase, hydrophobic interaction, ion-exchange, and precipitation chromatography, these materials have also been used for capillary electrochromatography characterized by a very high efficiency. The fast mass transport substantially contributes to an increase in the enzymatic activity of immobilized enzymes, and high flow rates can be used to achieve high throughputs. The approach has also been exploited for the preparation of molecularly imprinted molded materials, and their capability for molecular recognition has been demonstrated clearly. Among numerous advantages of the continuous polymer monoliths are their ease of preparation and ruggedness, the versatility of their chemistries, and their excellent efficiency in the separation of biological and synthetic molecules. The high speed of these separations indicates that these monoliths can be used for real-time process control, for the design of smaller units with the very high throughput required for industrial separations, for fast diagnostics, for sensors, and for many other applications.

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