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Stellan Hjertén's contribution to the development of monolithic stationary phases

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Abstract

This overview is presented to celebrate birthday of one of the luminaries of the separation science and my friend – Stellan Hjertén. He made significant contributions to a variety of areas in the separation science such as electrophoresis, liquid chromatography, and capillary electrochromatography to name just a few. Since the scope of his work was enormous, this review will focus only on a single aspect of his scientific activities, design and applications of monolithic materials. During the years starting from 1989, Stellan Hjertén published many excellent papers concerning the preparation of acrylamide chemistry-based monoliths and their use in both micro-HPLC and capillary electrochromatography. The following text details his works in field.

Keywords

Monolith; Continuous bed; HPLC; Electrochromatography; Microfluidic devices

1. Audaces Fortuna Iuvat

The development of monoliths in Hjertén's lab did not arrive as a lightning from the blue skies. Stellan was prepared and, as said in the heading, fortune helped him. Already at the end of the 1950s, he sought gels for molecular sieve electrophoresis and use of a mixture of acrylamide with a higher percentage of methylene bisacrylamide has led to a white granular materials [1]. Although these irregular particles could be used for the chromatographic separation, their performance as a stationary phase was poor and experiments with them were discontinued.

In the late of 1980s, he carried out studies aiming at an improvement in performance of both electrophoresis and chromatography. They started with agarose beads that were dehydrated to shrink to half of their size, crosslinked, and these non-porous beads were modified at their surface with alkyl chains to enable separation of proteins in reversed phase mode. In contrast to the established theory of chromatography, they observed that efficiency of a column packed with these particles increased with increasing flow rate. This unexpected effect has been assigned to the compression of the bed and decrease in the volume of interparticular voids between the particles leading to a more uniform internal structure of the packed bed [2-4].

At the same time, Stellan also asked the following question [5]: "Is it possible to design a chromatographic bed by bulk polymerization directly in the chromatographic tube?" The general notion in the late 1980s was that a chromatographic bed must be formed from discrete particles. Since even monodisperse beads could not be perfectly packed in a column, it was

impossible to reach the maximum resolution predicted by theory. Stellan also realized additional disadvantages of packed beds such as the need for the preparation of the beads, their classification to obtain the desired size with a narrow distribution, and their packing in the column. He also suggested solution to this challenge [5]: “A continuous gel plug with channels sufficiently large to permit an hydrodynamic flow might be the ideal chromatographic column”.

At this point, Stellan remembered his 20 years old experiments with highly crosslinked polyacrylamide mentioned above and the answer came soon after. The first communication presenting results obtained with a new type of the highly compressed poly(N,N'-methylenebisacrylamide-co-acrylic acid) stationary phase and shown in Fig. 1 demonstrated that his assumptions were correct and the resolution of proteins separated in ion exchange mode was independent of flow rate. This short first paper did not revealed any experimental details that would enable reproduction of his experiments but coined a term “*continuous polymer bed*” for this type of separation medium.

2 Compressed monolithic columns for HPLC

2.1 Standard size columns

More detailed description of the preparation procedures was published two years later affording beds for the separations of proteins in both ion exchange and hydrophobic interaction modes [6]. The polymerization reaction leading to the ion exchange column started with about 4% solution of monomers, methylenebisacrylamide and allyldimethylamine or acrylic acid, in a phosphate buffer containing a significant percentage of ammonium sulfate (ca. 5%). After addition of ammonium persulfate and N,N,N',N'-tetramethylethylenediamine, the mixture was transferred to a 350 × 6 mm tube and the polymerization reaction allowed to proceed for 5 h. At this point, a loose gel was formed. The tube was then attached to a pump and phosphate buffer pumped through at a flow rate of 0.5 mL/min. The hydrodynamic pressure compressed the gel to 6 cm long plug. Further compression to the final length of 3.5 cm was achieved with a manually powered piston that also held the bed from expansion during its applications. Using similar procedure but different monomer mixture containing methylenebisacrylamide and butyl acrylate in proportion 6:1, compressed gel structure for the separations in hydrophobic interaction mode was also prepared. The trick that helped to create the desired gel structure is addition of ammonium sulfate to the monomer solution. This salt is thought to increase the hydrophobic interaction between the polymer chains formed during the polymerization process and to enable creation of “channels” between them [6]. These channels must be rather large in the gel prior to the compression. Unfortunately, no pictures of these have been published although it might be possible to get them after freeze drying the gel. In contrast, electron micrographs of the compressed column clearly showed aggregated 0.5 µm particles and 3-4 µm large flow-through channels between the clusters. The particles, we sometime also call microglobules, are not porous as demonstrated by the independence of elution times on molecular weight found for a series of monodisperse dextran standards. While the separations of five proteins using these monolithic stationary phases in both ion exchange modes were competed in about 10 min, significantly more time of up to 30 min was required to achieve the separations in hydrophobic interaction mode. Clearly, the gradient time of 35 min for the latter was much longer than that for 10 min of the former [6].

Further improvement came another two years later [7] and consisted in the use of a new crosslinker, piperazine diacrylamide together with methacrylamide, and in the fine tuning of the porous structure. The percentage of methacrylamide in its mixture with the crosslinker was found to significantly affect the permeability to flow. Fig. 2 shows that the minimum at the back pressure plot was achieved at a piperazine diacrylamide/methacrylamide ratio of 1.3 representing 55.9% crosslinking. Another variable affecting the flow through properties was

the amount of added ammonium sulfate and the lowest back pressure was monitored for monoliths prepared at a concentration of 60 mg/mL. This material resisted any degradation for at least 3 weeks at pH ranging from 1 to 11 and for 6 h at pH 12. This resistance is much better than that typical of the original methylenebisacrylamide monolithic structures that were quite susceptible to hydrolysis even at pH 5.5 and 9.

Using the optimized composition of the polymerization mixture to which was added a very small amount of acrylic acid, a cation exchanger was prepared. Interestingly, the gel obtained by polymerization in a test tube was diluted with water prior to packing in the column tube [7]. Since continuous crosslinked gel cannot be dispersed in a liquid, this fact indicates that the product of this polymerization was not a monolithic structure but rather could have a particulate character. The compressed bed formed from these particles was then used for the separation of proteins at different flow rates keeping the gradient volume constant. Fig. 3 shows that the separation was significantly accelerated compared to the previous studies and an excellent resolution of five proteins was achieved in about 2 min. Similarly, the height of the continuous packed beds was varied in range 15-155 mm to demonstrate, that even a bed as short as 2.7 cm is sufficient to baseline separate all analytes. The last variable changed in this set of experiments was the total monomer concentration in the polymerization mixture. Using otherwise identical preparation conditions, the column exhibited different permeability to flow with a minimum at 15% of monomers. After modification with dextrane and octadecylation (*vide infra*), this column could be used even at a flow rate of 10 mL/min representing a flow velocity of 60 cm/min enabling the separation of five proteins to be completed in about 2 min.

To further improve the permeability of the columns, another series of optimization experiments included variations in polymerization temperature, content of ammonium sulfate, pH of the buffer solution used for the preparation of the polymerization mixture, and concentration of tetramethylethylenediamine and ammonium persulfate in the initiating system [8]. The results helped to design the optimum conditions. One of the best systems included polymerization of a mixture consisting of 0.84 g piperazine diacrylamide, 0.66 g methacrylamide, 1 mg ammonium persulfate, 1.9 mg tetramethylethylenediamine, and 600 mg ammonium sulfate dissolved in 10 ml of 50 mmol/L potassium phosphate buffer pH 7.0 at a temperature of 34 ° C. Proteins eluted from a 2.1 × 0.6 cm column packed with this material and run at a flow rate of 10 mL/min in less than 10 s.

Although hydrophobic interaction chromatography has been demonstrated very early on with a bed consisting of butyl acrylate and N,N'-methylenebisacrylamide [6], a new chemistry enabling separation in this chromatographic mode has been introduced in 1996 [9]. The mixture of monomers used for the preparation of these columns consisted of piperazine diacrylamide, methacrylamide, and N-isopropylacrylamide. Fig. 4 illustrates the performance with the separation of 7 proteins.

Most of the columns discussed above were prepared from water soluble monomers and used for the separations in completely aqueous mobile phases. However, reversed phase chromatography that is very popular mode for the separation of proteins requires stationary phase containing highly hydrophobic ligands to achieve the desired interactions and a mobile phase containing an organic modifier. Since polymerization of hydrophobic monomers in aqueous system is difficult, Hjerten's group prepared the columns in two steps [10]. The first step was the preparation of a gel from a mixture consisting of piperazine diacrylamide, methacrylamide, and 10% allylglycidylether using the typical polymerization conditions. The gel particles were then dehydrated by washing with acetone and diethyl ether. After dispersion in another portion of ether and addition of an ether solution of octadecanol and BF₃, the reaction of the epoxy functionality with alcohol produced the desired hydrophobic groups attached to the polymer gel. The optimum reaction time was 4 h.

Alternatively, the gel was first modified with hydrophilic functionalities using reaction with glucose, trimethylolpropane, dextran (mol. weight 10,000), and poly(vinyl alcohol) (mol. weight 49,000). This reaction was carried out in an aqueous solution which pH was adjusted to 10.5 using sodium hydroxide. As a result, the gel now contained a large number of hydroxyl groups that could be used for the post-modification with hydrophobic ligand. The argument that this coating also helped to prevent the nonspecific hydrophobic interactions does not hold since the material was eventually hydrophobized to enable separations in reversed phase mode. The next step was then dehydration described above followed by reaction with 1,2-epoxyoctane or 1,2-epoxyoctadecane. The resulting hydrophobic particles were then packed in a column and compressed to the final length [10]. The performance demonstrated only with separations of various digests appeared to be decent but without mass spectrometric assignment of the peaks not very useful.

2.2 Capillary columns

Being a visionary, Stellan realized already in the early 1990s, long before the onset of all of the current omics, that the several millimeters I.D. columns may not be the best choice for the separations of very small samples. Therefore, his group was developing monolithic capillary columns simultaneously with their larger counterparts [6]. The first attempts represented just a miniaturized approach used previously and comprised *in situ* polymerization followed by compression. Once again, the decrease in length was significant. A 60 cm long plug was compressed to mere 7 cm using hydrodynamic pressure of up to 10 MPa provided by a HPLC pump. Since the capillary chromatography was in its infancy at that time, Stellan developed a simple yet elegant method to create the ammonium sulfate gradient required for the separation. His “prototype” 70 × 0.3 mm poly(N,N'-methylenebisacrylamide) column then separated five proteins in hydrophobic interaction mode [6].

Despite some success of the initial experiments with capillaries, they also found some challenges. Stellan wrote [11]: “Although preliminary experiments with continuous beds prepared directly in narrow capillary were promising, visual inspection of the beds indicated that they were no homogeneous. Typically, two or three 0.5 to 1 mm long segments of buffer (without stationary phase) were observed. These dead spaces, which occurred only in the microcolumns, were created during compression of the continuous bed.” Solution to this problem was found in attachment of the monolith to the capillary wall after its functionalization with 3-(trimethoxysilyl)propyl methacrylate in and changed composition of the polymerization mixture affording monoliths enabling separations similar to those in compressed beds. Obviously, covalent link of the polymer to the capillary did not allow compression and this step could be eliminated [11]. Instead, they used a higher concentration of the monomers in the polymerization mixture to decrease the size of the channels. The recognition that monolithic structures prepared from piperazine diacrylamide and methacrylamide allowed for higher flow velocities than those made of N,N'-methylenebisacrylamide has led to a complete abandoning of the latter.

To obtain chemistry suitable for the separations in the hydrophobic interaction mode, the capillary columns were prepared from mixtures of piperazine diacrylamide, methacrylamide, and N-isopropylacrylamide similar to those used in the standard size columns [9]. Fig. 5 shows effect of concentration of methacrylamide in the mixture on permeability of the capillary column measured by the back pressure at two different flow rates. The most permeable column was obtained from a mixture consisting of 0.125 g piperazine diacrylamide, 0.075 g methacrylamide, and 0.1 g N-isopropylacrylamide dissolved in 50 mmol/L sodium phosphate buffer pH 7.0. This 120 × 0.32 mm column then separated proteins. As seen from Fig. 6, the separation performance measured at constant gradient volume was largely independent of the flow rate.

Using similar approach, they also prepared monolithic capillary columns for the separation of proteins in cation exchange mode [11]. The polymerization mixtures used in this study included piperazine diacrylamide, methacrylamide, and acrylic acid. An increase in the total monomer concentration from 19.3 to 31.5% afforded columns exhibiting higher back pressure but simultaneously providing for a better performance. Fig. 7 illustrates separation of four proteins in ion exchange mode using a 100 mm $\times$ 25 μ m I.D. capillary column prepared from the mixture producing the most dense monolith.

In addition to the typical both *on-capillary* and *off-capillary* UV detections, they also introduced detection of proteins by transferring them on a blotting membrane followed by staining [12]. Use of fast atom bombardment mass spectrometry in combination with the separation of neuropeptides using cation exchange chromatography with monolithic capillary column is another detection technique introduced by Hjertén's group [13].

Monolithic capillary columns with hydrophobic chemistry for the reversed phase separation were prepared using technique similar to that described above [10]. First, a monolith was prepared by copolymerization of piperazine diacrylamide and methacrylamide. After dehydration, the polymer was activated by filling the pores with boron trifluoride etherate followed by 1,2-epoxybutane or 1,2-epoxyoctane. The mechanism of this reaction path was not disclosed. Finally, egg L- α -phosphatidylcholine liposomes were immobilized to the pore surface via hydrophobic interaction [14]. The effect of these liposomes was demonstrated by comparison of separation of four drugs using the original hydrophobic column and the liposome modified counterpart. While no separation was observed with the original monolith with controlled hydrophobicity, Fig. 8 demonstrates the separation of all components in isocratic mode using 10 mmol/L sodium phosphate buffer pH 7.4 as the mobile phase. These experiments could be used as model to predict the partitioning or absorption properties of drugs [14].

Normal phase separations using monolithic capillaries were another chromatographic mode examined by Hertén's group [15]. They prepared a series of monoliths using combinations piperazine diacrylamide with either methacrylamide and N-isopropylacrylamide or 2-hydroxyethyl methacrylate and vinylsulfonic acid in different proportions while also varying the content of monomers in the polymerization mixture. Since the conventional HPLC pump was not able to produce constant flow rate at the microliter level they split the flow and kept the system at constant pressure. Permeability of all prepared columns was very good. The least permeable 175 mm $\times$ 100 μ m I.D. column prepared from a mixture of 125 mg piperazine diacrylamide, 50 mg methacrylamide and 300 mg N-isopropylacrylamide dissolved in 1 mL of 50 mmol/L sodium phosphate buffer containing 10 mg ammonium sulfate afforded a flow rate of over 250 nL/min at a constant pressure of 8 MPa. At the same time, this column separated very well several small molecules with efficiencies reaching up to 150,000 plates/m as shown in Fig. 9.

In yet another approach, the monolithic column has been prepared by polymerization of piperazine diacrylamide with methacrylamide in the presence of hemoglobin [16]. After a careful removal of the hemoglobin by washing of the 90 $\times$ 6 mm I.D. column with sodium phosphate buffer, and aqueous acetic acid containing sodium dodecylsulfate, this imprinted monolithic column enabled the separation of hemoglobin from ribonuclease and cytochrome c. In contrast, similar column prepared without addition of hemoglobin did not separate this protein from the mixture.

3 Capillary electrochromatography

3.1 Typical monolithic columns

Early work on highly crosslinked acrylamide-based continuous beds for CEC was published by Hjertén et al. in an obscure journal in 1995 [17]. The approach that was disclosed in detail in 1997 was complex, requiring a multiplicity of steps including the modification of the capillary surface with 3-(trimethoxysilyl)-propyl methacrylate, two individual polymerizations, and a chemical functionalization [18]. The initial matrix was formed by copolymerizing a dilute aqueous solution containing 2-hydroxyethyl methacrylate and piperazine diacrylate initiated using a peroxodisulfate- N,N,N',N'-tetramethyl ethylenediamine system in the presence of a high concentration of ammonium sulfate. The inorganic salt was again added to obtain a porous structure with the desired permeability. The pores of this matrix were then filled with another polymerization mixture consisting of allyl glycidyl ether (20%), 2-hydroxyethyl methacrylate (50%), and piperazine diacrylate (30%) to which dextran sulfate had been added. Initiated again by the same redox system, the second polymerization proceeded within the pores of the matrix and led to the “immobilization” of the charged polysaccharide within the newly formed polymer, and activation of the surface for additional functionalization. Reaction of both epoxide and hydroxyl functionalities located within the monolith with 1,2-epoxyoctadecane catalyzed by BF_3 led to the covalent functionalization of the matrix with an unquantified number of octadecyl chains. Several measurements of the effect of flow rate on efficiency as well as of flow velocity at different field strengths were carried out using 12,5 cm long capillaries (9,5 cm active length). In contrast, the best separations, such as that shown in Fig. 10, were obtained using a 55 cm long capillary column. Retention times in excess of 20 min were required for the elution of aromatic hydrocarbons from this column.

In order to simplify the preparation described previously, a new procedure was developed [19]. The polymerization mixture consisted of an aqueous solution of acrylamide, piperazine diacrylamide, and vinylsulfonic acid. The desired hydrophobicity of the gel was achieved by admixing a hydrophobic monomer such as stearyl methacrylate or butyl methacrylate. Since neither of these non-polar monomers is soluble in water, a surfactant was added to the mixture, followed by sonication to form an emulsion of the hydrophobic monomer in the aqueous solution. Once initiated, the mixture was immediately drawn into an acryloylsilanized capillary where the polymerization was completed. The presence of the strongly acidic sulfonic acid functionalities affords an electroosmotic flow (EOF) of a magnitude that remains constant over a broad pH range.

Although preliminary separations using these continuous gel beds were good, an elegant trick involving the strength of the mobile phase enabled a much better resolution of five polycyclic aromatic hydrocarbons to be achieved. A solution containing the analytes dissolved in 50:50 acetonitrile/aqueous buffer was injected electrokinetically into the capillary column, and the separation was started. After a short period of time, the solvent in the inlet vial was replaced with a 70:30 acetonitrile/buffer mixture, and the elution was completed under these conditions. A comparison of the standard isocratic run with the stepwise gradient elution (Fig. 11) shows that the latter procedure led to a significant sharpening of the peaks. The authors assumed that this improvement resulted from the gradient of the mobile phase generated by diffusion across the strong solvent/weak solvent interface [19].

Addition of a surfactant such as sodium dodecyl sulfate at levels below the critical micelle concentration was also reported to improve isocratic CEC separations [19]. In explaining this phenomenon Hjertén assumed that the dodecyl alkyl groups of the surfactant interact with the octadecyl chains of the stationary phase, thus increasing both the density of the interacting hydrophobic moieties at the surface as well as the number of sulfonic acid groups that support

the EOF. While the higher hydrophobicity led to stronger retention, the increase in EOF counteracted this effect by accelerating flow. Thus, the net migration time for retained PAHs did not change, but the resolution improved significantly. However, these assumptions have not been confirmed unambiguously, although the accelerating effect of the increased number of charged functionalities could easily be demonstrated using a non-retained marker.

Ericson and Hjertén later described another method of preparation of a monolithic capillary column that was used for gradient separation of proteins in CEC mode [20]. The first step involved a polymerization initiated by ammonium persulfate/tetramethylethylenediamine system in a system consisting of two phases: aqueous, comprising of a solution of acrylamide and piperazine diacrylamide in a mixture of a buffer solution and dimethylformamide, and immiscible highly hydrophobic octadecyl methacrylate. Continuous sonication was applied for 40 min in order to emulsify the highly hydrophobic octadecyl methacrylate and form a dispersion of fine polymer particles. In the remaining steps of this preparation process, another portion of initiator was added to the system to restart the polymerization of two newly added monomers, dimethyldiallylammonium chloride and piperazine diacrylamide. The resulting partly polymerized dispersion was then forced into a methacryloylsilylated capillary using pressure and, finally, the polymerization process was re-started and carried out to completion.

Understanding and controlling the forces that drive the movement of charged molecules during CEC separations is extremely important. Proteins may be particularly difficult to separate, since, depending on their net charge as determined by both the protein pI and the pH value of the mobile phase, the same molecule can move electrophoretically towards either the anode or the cathode. In contrast, EOF proceeds in only one direction as determined by the charge of the mobile counterions in the Stern layer. Thus, in Hjertén's approach, using immobilized quaternary ammonium cations, EOF always proceeds from the anode to the cathode. Electroosmotic flow was also used by the same group to introduce the mobile phase gradient generated using an HPLC instrument to mix the gradient, into the capillary. Two overall flow scenarios shown schematically in Fig. 12 were discussed [20]:

- i. In the monolith containing a high level of charged moieties, electroosmotic flow outweighs the electrophoretic migration. In this "normal flow gradient" situation, both the EOF and the net migration of the protein molecules occur in the same direction, provided that all of the proteins have net charges of equal sign. This was achieved by using a mobile phase consisting of 80% acetonitrile and 20% buffer with a pH value of 2. This pH is well below the pI of the proteins, ensuring that the biopolymers were positively charged. Samples were injected at the cathode, and as with other typical gradient systems, the percentage of acetonitrile was higher at the capillary inlet (cathode) than at the outlet, with detection occurring at the anodic end of the column.
- ii. In contrast, in the monolith containing a moderate number of charged moieties, electromigration of the charged proteins was faster than the electroosmotic flow. In this "counter flow gradient" system, the EOF proceeded in the same direction. However, proteins were injected at the anode end, and migrated in a direction opposite to the gradient due to their electrophoretic mobilities. Accordingly, the detector was positioned at the cathodic end of the capillary column.

Fig. 13 shows excellent separations of four proteins in both moderate and high EOF columns using electrically driven flow, and compares the separation with that achieved using standard HPLC methodology. Since the separations were primarily governed by the nature of the mobile phase gradient, all three chromatograms are very similar. This comparison also demonstrates that the separation is achieved via reversed-phase partitioning rather than electrophoresis.

It should be noted that this successful approach to monolithic CEC column for the separation of proteins employs a polymeric matrix containing positively charged moieties. Since the

overwhelming majority of proteins also possess net positive charges at pH 2, the danger of undesired electrostatic interactions is greatly diminished.

In continuation of the development of monolithic columns for CEC, a series of columns was prepared using different combinations of monomers aiming at a stationary phase suitable for CEC separation of basic tricyclic antidepressants [21]. A polymerization mixture comprising 0.30 g piperazine diacrylamide, 0.14 g acrylamide, 0.15 g N-isopropylacrylamide, 5 μ L vinylsulfonic acid, and 0.08 g ammonium sulfate dissolved in 1.7 mL 50 mmol/L sodium phosphate buffer pH 7 afforded columns enabling baseline separation of all three compounds.

Another group of compounds that has been embraced by Stellan's group were chiral amino acids and hydroxy acids [22,23]. The chiral stationary phase (CSP) for the CEC enantioseparation in the ligand exchange mode was prepared by copolymerization of piperazine diacrylamide, vinylsulfonic acid, and N-(2-hydroxy-3-allyloxypropyl)-L-4-hydroxyproline which served as a selector. While experiments leading to the eventually used CSP were not revealed, the separation conditions including applied voltage and pressure, and the column length were found to affect the resolution. A variety of racemic amino acids were resolved with selectivity factors α ranging from 1.21 (asparagine) to 2.65 (α -methyl-2,4-dihydroxyphenylalanine) using a mobile phase consisting of 0.1 mmol/L copper (II) sulfate in 50 mmol/L sodium phosphate buffer pH 4.6 [21]. In contrast, selectivity factors for hydroxy acids were lower than those found for amino acids with most of them being smaller than 1.1. As always, exceptions prove the rule and α value of 2.0 was observed for 3-(4-hydroxyphenyl)-lactic acid [23].

3.2 Homogeneous continuous gels

Based on previous experience with albumin immobilized in a gel matrix and used for enantioseparations [24], Hjertén's group extended the concept of homogeneous gel separation media for chiral CEC by incorporating a functionalized cyclodextrin and charged monomer in poly(acrylamide-co-methylenebisacrylamide) gel [25]. More specifically, the polymerization mixture was prepared by dissolving 40 mg acrylamide, 1.4 mg N,N'-methylenebisacrylamide, in 0.5 mL of 100 mmol/L Tris / 150 mmol/L boric acid buffer pH 8.2 to which 0.5 mL of 2-hydroxy-3-allyloxypropyl- β -cyclodextrin selector solution and 2-acrylamido-2-methyl-1-propanesulfonic acid or dimethyldiallylammonium chloride are added. The last two compounds support EOF to drive the flow. Stellan describes this type of stationary phase in the following way [25]: "A homogeneous separation medium is visually transparent or only slightly opalescent, i.e. no elements are large enough to be visible in a light microscope and/or cause sufficient light scattering to make the medium strongly opalescent." A vast variety of racemic drugs was resolved with these CSP. For example, the anionic gels afforded α values varying between 1.11 for ibuprofen and 3.17 for hydrobenzoin and column efficiencies of 2,600 (mephobarbital)-560,000 (hexobarbital) found for the first eluting peak. Cationic gels generally afforded better selectivity but only low column efficiencies.

4 Microfluidic devices

Despite a significant number of studies concerning capillary electrochromatography using monoliths in capillaries, the first monolithic material prepared in channel of a very simple chip was demonstrated by Hjertén's group in 2000 [26]. This first polymer-based monolithic stationary phase was prepared in 30.6 cm long and approximately 40 μ m wide and 20 μ m deep semicircular serpentine microchannels etched into quartz substrates and sealed with a "roof" formed by 4 μ m thick layer of silicon dioxide (Fig 14). Monolithic stationary phase for reversed-phase electrochromatography was obtained by polymerization of methacrylamide, isopropyl acrylamide, piperazine diacrylamide, and vinylsulfonic acid mixtures in an aqueous buffer solution using the common redox initiating system ammonium peroxosulfate/

tetramethylethylenediamine. In addition, similar polymerization mixtures were used to create polymer monoliths in straight channel for pressure-driven anion-exchange liquid chromatography with the exception that isopropyl acrylamide and vinylsulfonic acid were used instead of diallyldimethylammonium chloride. Since polymerization begins once the initiator is added, channels have to be filled immediately after the mixture is prepared. Before formation of the polymer monolith, the channels were treated just as the fused silica capillaries with [3-(methacryloyloxy)propyl] trimethoxysilane to provide them with pendant methacryloyl groups that are incorporated during polymerization into the growing polymer monolith thus covalently attaching it to the channel walls.

The location of the polymer bed could be controlled to a certain extent by using fluid plugs containing poly(ethylene glycol), tetramethylethylenediamine, and ammonium peroxydisulfate but no monomers. These fluid plugs displaced the polymerization mixture to keep certain sections of the microchannel clear of monolith. The plugs also helped to create sharp ends to the monolith. The completed chips were attached to a special clamp apparatus for fluid interfacing and operated using a highly modified CE instrument capable of UV detection.

The performance of the acrylamide-based monolithic stationary phase in the chip was compared to that in a fused silica capillary containing monolith prepared from the same mixture. Not surprisingly, van Deemter plots were virtually identical for both devices since the channel etched in quartz, which did not have on-chip injection arms, closely resembled the capillary. Neutral alkyl phenones and positively charged antidepressants were separated in the serpentine channel in 18 and 9 min, respectively. A faster separation was demonstrated with a rapid separation of uracil, phenol, and benzyl alcohol in a Y-shaped channel containing only a 1.8 cm long monolith. The separation was completed in less than 20 s, with all three peaks eluting within a 7 s window. A column efficiency calculated for unretained peak of uracil was 6300 plates, which represents 350,000 plates/m.

Overall, this implementation using the expensive and difficult to fabricate quartz microchips does not represent a significant advantage compared to typical CEC in much cheaper fused silica capillaries placed in standard instruments. Also, carrying out these experiments required laborious manual changing of contents of the inlet reservoir to achieve the sample injection. Therefore, Hjertén's group developed what they call a hybrid microdevice as an alternative to quartz microchips [27]. This device consisted of a short, fused silica capillary column containing a 2.8 cm long polymer gel prepared from acrylamide, 2-acrylamido-2-methyl-1-propanesulfonic acid and allyl- β -cyclodextrin as crosslinker. This capillary was set in a groove on a polyvinyl chloride support plate that included sample reservoirs, electrodes, and a slit for "on-tube" UV-detection. In addition, a ball lens was mounted beneath the detection slit to focus the UV beam. Gel electrophoresis [27,28] and electrochromatography [27] were then performed in this system. For example, several alkyl phenones were separated in the electrochromatographic mode in less than 200 s. The suggested separation mechanism was related to interactions between the hydrophobic cavity of the β -cyclodextrin and the analytes. However, no direct evidence for this mechanism was presented.

Claimed advantages of this hybrid device compared to traditional microchips include lower cost in terms of materials and preparation, UV-detection with no need for fluorescent labels, the ability to change the separation column by simply switching the capillary, and the ability to directly utilize the existing body of experience available for capillary separations. Although elegant, this approach is limited to very simple geometries and is less suitable for the more complex layouts typical of μ -TAS.

5 Conclusions

The above lines validate what has been said at beginning. Stellan Hjertén's contribution to the development of monolithic columns is significant. His domain is chemistry related to (meth) acrylamide types of monomers and polymerizations in aqueous solutions. A vast variety of different polymerization approaches has been developed in his group to meet the requirements of the aqueous chemistry and the desired separation modes. Some of his columns were commercialized by BioRad Laboratories under trade name UNO-columns. Although they did not enjoyed the fame of other monolithic technologies, in their time UNO columns were the first monolithic *column* technology (second to the monolithic discs manufactured by BIA Separation) on the market and represented an interesting alternative to packed columns enabling rapid separations of proteins.

Stellan's enormous extent of work is often referred to in scientific papers and placed him on a high pedestal in numerous review articles and books [29-31]. Since he is still very active and continues to present his newest results on numerous conferences, it is likely, that we may soon see some new exciting developments perhaps even related to monolithic structures.

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Abbreviations

CEC, capillary electrochromatography; EOF, electroosmotic flow; CSP, chiral stationary phase.

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Figure 1.

High-performance cation-exchange chromatography of alcohol dehydrogenase, horse myoglobin, whale myoglobin, ribonuclease, and cytochrome c using a compressed continuous gel at the flow-rates indicated (Reprinted with permission from ref. [5]. Copyright 1989 Elsevier B.V.). Mobile phase: Linear gradient from 0.01 mol/L sodium phosphate buffer pH 6.4 to 0.25 mol/L sodium chloride in the same buffer. Gradient volume 5 mL.

Figure 2.

Relationship between pressure per cm bed height and the PDA/MA ratio (also expressed as cross-linker concentration C , %) for the compressed continuous beds (Reprinted with permission from ref. [7]. Copyright 1993 Elsevier B.V.). Total monomer concentration $T = 5\%$ (w/v); bed dimensions, $3.5 \text{ cm} \times 0.6 \text{ cm}$ I.D. (5 ml monomer solution). Ammonium sulphate, 0.25 g per 5 ml of monomer solution.

Figure 3.

Cation-exchange chromatography at various flow rates at constant gradient volume (Reprinted with permission from ref. [7]. Copyright 1993 Elsevier B.V.). Bed dimensions, 15.5 cm $\times$ 0.6 cm I.D. Sample: 1 = ovalbumin; 2 = horse skeletal muscle myoglobin; 3 = whale myoglobin; 4 = cytochrome c; 5 = lysozyme. Comparison of the chromatograms shows that the resolution is independent of the flow-rate.

Figure 4.

Chromatograms showing the separation of model proteins on columns filled with C_{30} -containing continuous hydrophobic interaction chromatographic bed (Reprinted with permission from ref. [9]. Copyright 1996 Elsevier B.V.). The model proteins included cytochrome c (C), myoglobin (M), ribonuclease (R), lysozyme (L), trypsinogen (T), chymotrypsinogen A (A) and ferritin (F). Bed dimensions: (a) 10.5 cm $\times$ 6 mm I.D., (b) 12 cm $\times$ 0.32 mm I.D. The absorbance measurements were made at 220 nm.

Figure 5.

Relationship between flow resistance (back pressure) and the content of methacrylamide (MA) in the monomer solution (w/v) used for the preparation of the hydrophobic interaction chromatographic gel (Reprinted with permission from ref. [9]. Copyright 1996 Elsevier B.V.). The concentrations of PDA and IPA were kept constant at 125 and 100 mg/ml, respectively. Bed dimensions: 12 cm $\times$ 0.32 mm I.D.. Flow-rate: 6 μ L/min. Mobile phase: 10 mM phosphate buffer, pH 7.0.

Figure 6. Hydrophobic-interaction chromatography at various flow-rates at constant gradient volume 120 μL (Reprinted with permission from ref. [9]. Copyright 1996 Elsevier B.V.). Bed dimensions: 12 cm $\times$ 0.32 mm I.D.. Flow-rate: (a) 6 $\mu\text{L}/\text{min}$, blank gradient, (b) 3, (c) 6, (d) 15, (e) 30 $\mu\text{L}/\text{min}$. Comparison of the chromatograms shows that the resolution is independent of the flow-rate.

Figure 7.

Separation of horse skeletal muscle myoglobin, whale myoglobin, cytochrome c, and lysozyme using 10 cm $\times$ 0.025 mm I.D. column prepared from piperazine diacrylamide, methacrylamide, and acrylic acid (Reprinted with permission from ref. [11]. Copyright 1994 Elsevier B.V.). Mobile phase 0.06 mol/L sodium chloride in 10 mmol/L sodium phosphate pH 6.2; flow rate 0.11 μ L/min; back pressure 2.8 MPa.

Figure 8.

Elution profile of aspirin, salicylic acid, warfarin and pindolol (from left to right) on liposomes immobilized via C 4 ligands in a 120 mm long capillary continuous bed (1.0 μmol phospholipid) (Reprinted with permission from ref. [14]. Copyright 1996 Elsevier B.V.). The peaks were identified by runs with different drug concentration ratios. In the absence of liposomes the drugs were eluted in a single sharp peak at the retention volume indicated by an arrow.

Figure 9.

Normal-phase capillary chromatography of polar aromatic compounds (Reprinted with permission from ref. [15]. Copyright 1999 Elsevier B.V.). Peaks: pyridine (1), 4-pyridylmethanol (2), 4-methoxyphenol (3), 2-naphthol (4), catechol (5), hydroquinone (6), resorcinol (7), 2,7-dihydroxynaphthalene (8). UV detection at 220 nm; mobile phase: pure methanol, pressure: 5.6 MPa.

Figure 10.

Chromatograms obtained by reversed-phase liquid chromatography on a continuous bed with C18 ligands and immobilized dextran sulfate (Reprinted with permission from ref. [17]. Copyright 1997 Elsevier B.V.). Capillary column: 550 mm (effective length 525 mm) $\times$ 25 μ m I.D. $\times$ 375 μ m O.D. Elution order: naphthalene; 2-methylnaphthalene; fluorene; phenanthrene; anthracene. Mobile phase: 5 mmol/L borate buffer (pH 8.7) containing 62% acetonitrile. Pressure: 5.2 MPa.

Figure 11.

Effect of gradient elution and zone sharpening of the starting zone upon electrochromatography of polycyclic aromatic hydrocarbons on a C18-derivatized continuous bed containing sulfonic acid groups (Reprinted with permission from ref. [18]. Copyright 1996 American Chemical Society). Dimensions of the capillary: 20.0 cm (16.0 cm effective length) $\times$ 75 μ m i.d. Applied voltage: 3.0 kV (4.2 μ A). Mobile phase at the start: 50% (v/v) acetonitrile in 4 mmol/L phosphate (pH 7.4). Sample: naphthalene (1), 2-methylnaphthalene (2), fluorene (3), phenanthrene (4), and anthracene (5). After 6 min, the mobile phase was replaced by 70% (v/v) acetonitrile in 4 mmol/L phosphate buffer (pH 7.4). The electrochromatogram is shown in panel b. A control experiment performed without zone sharpening under isocratic conditions in 60% (v/v) acetonitrile in 4 mmol/L sodium phosphate (pH 7.4) is presented in panel a. A comparison between the electrochromatograms shows that zone sharpening, along with gradient elution, decreases the widths of the peaks and thereby affords a baseline separation.

Figure 12.

Diagram illustrating the principle for counterflow (a) and normal-flow (b) gradients (Reprinted with permission from ref. [19]. Copyright 1999 American Chemical Society). The direction of electroosmotic flow is opposite to that of the electrophoretic movement in both methods and is opposite to the net migration velocity, $v_{migr} (=v_{eo} - v_{elph})$, in (a) and coincides with the net migration direction in (b). v_{eo} is not constant along the capillary, and v_{elph} is higher in the direction of the electrophoretic migration.

Figure 13.

Separation of proteins using gradient electrochromatography (a, b) and reversed-phase μ HPLC (c) (Reprinted with permission from ref. [19]. Copyright 1999 American Chemical Society). Peaks: ribonuclease A (R); cytochrome c (C); lysozyme (L); chymotrypsinogen (Ch). Protein concentration: 0.6 mg/mL of each protein except for ribonuclease A (1.8 mg/mL); mobile phase: a linear gradient from 5 to 80% acetonitrile in 5 mmol/L sodium phosphate pH 2.0; Detection at 280 nm. Columns: 8 cm (6 cm effective length) $\times$ 50 μ m i.d.; voltage, 5.5 kV (700 V/cm); (a) Moderate-EOF column, (b) high-EOF column, (c) conventional capillary RP-HPLC; pressure 5 MPa.

Figure 14.

Chip layout, cross section of the monolith, and fast anion-exchange chromatography of uracil (1), phenol (2), and benzyl alcohol (3) using the Y-shaped chip format. (Reprinted with permission from ref. [25]. Copyright 2000 Elsevier B.V.). Conditions: straight monolithic column 18 mm long, mobile phase 5 mmol/L sodium phosphate buffer (pH 7.4) containing 15% acetonitrile, applied voltage, 2.4 kV (500 V/cm), EOF 2.1 mm/s, UV detection at 254 nm, electrokinetic injection 100 V for 2 s.