2006-12-04

Development of Polymer Monoliths for the Analysis of Peptides and Proteins

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DEVELOPMENT OF POLYMER MONOLITHS FOR THE ANALYSIS OF PEPTIDES AND PROTEINS

by

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A dissertation submitted to the faculty of

Brigham Young University

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Department of Chemistry and Biochemistry

Brigham Young University

December 2006

BRIGHAM YOUNG UNIVERSITY

GRADUATE COMMITTEE APPROVAL

of a dissertation submitted by

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This dissertation has been read by each member of the following graduate committee and by majority vote has been found to be satisfactory.

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As chair of the candidate's graduate committee, I have read the dissertation of Binghe Gu in its final form and have found that (1) its format, citation, and bibliographical style are consistent and acceptable and fulfill university and department style requirements; (2) its illustrated materials including figures, tables, and charts are in place; and (3) the final manuscript is satisfactory to the graduate committee and is ready for submission to the university library.

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ABSTRACT

DEVELOPMENT OF POLYMER MONOLITHS FOR THE ANALYSIS OF PEPTIDES AND PROTEINS

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Doctor of Philosophy

Several novel polymer monoliths for the analysis of peptides and proteins were synthesized using polyethylene glycol diacrylate (PEGDA) as crosslinker. Photo-initiated copolymerization of polyethylene glycol methyl ether acrylate and PEGDA yielded an inert monolith that could be used for size exclusion liquid chromatography of peptides and proteins. This macroscopically uniform monolith did not shrink or swell in either water or tetrahydrofuran. More importantly, it was found to resist adsorption of both acidic and basic proteins in aqueous buffer without any organic solvent additives.

A strong cation-exchange polymer monolith was synthesized by copolymerization of 2-acrylamido-2-methyl-1-propanesulfonic acid (AMPS) and PEGDA. A ternary porogen (water, methanol and ethyl ether) was found suitable to prepare a flow-through monolith with moderate pressure drop in aqueous buffer. The resulting monolith showed

excellent ion exchange capillary liquid chromatography of peptides using a simple salt gradient. Extremely narrow peaks were obtained for the analysis of synthetic peptides, natural peptides and a protein digest. A peak capacity of 179 was achieved.

Although the poly(AMPS) monolith demonstrated extraordinary performance, one main drawback of this monolith was its relatively strong hydrophobicity. A decrease in hydrophobicity was achieved by using more hydrophilic monomers (e.g., sulfoethyl methacrylate or vinyl sulfonic acid). The most hydrophilic poly(vinyl sulfonic acid) monolith provided high resolution cation-exchange liquid chromatography of protein standards and lipoproteins. Use of the new PEGDA biocompatible crosslinker over the conventional ethylene glycol dimethacrylate crosslinker for the preparation of polymer monoliths was found to be advantageous for the analysis of biological compounds in several chromatography modes.

ACKNOWLEDGMENTS

I am very grateful for the support throughout this project from my advisor Dr. Milton L. Lee. He has been a great resource for suggestions and encouragement on the project as well as advice and help for my career path. I am honored that I could have an opportunity to study in his group. What I have learned from him is invaluable to me and my future career development.

I would also like to thank professors in the chemistry department for teaching me fundamentals and experimental skills in analytical chemistry. My graduate committee members, Drs. Harrison, Linford, Peterson and Woolley, have provided critical evaluation and useful suggestions in my progress reports during my five-year PhD study.

I especially thank Susan Tachka for her excellent administrative support for all of us in Dr. Lee's group. Her hard work made things go smoothly and rapidly. Members in Dr. Lee's group are also greatly acknowledged. We constantly helped each other and had very friendly discussions that led to solutions to problems and new ideas.

Most of all, I would like to express my appreciation to my wife, Koudi, for her love and support. She was also a graduate student in organic chemistry in the chemistry department. It was very challenging for both of us to pursue graduate studies while taking care of our two-year-old boy Kenneth. She tried her best to do a good job both at school and at home. Without her support and personal sacrifice, it would have been impossible

for me to successfully finish this project. It is to her and my two lovely sons, Kenneth and newborn George, that this dissertation is dedicated.

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LIST OF ABBREVIATIONS

2-D	two-dimensional
AETC	2-acryloyloxyethyltrimethylammonium chloride
AIBN	2,2’-azobisisobutyronitrile
AMPS	2-acrylamido-2-methyl-1-propanesulfonic acid
AMT	accurate mass tag
APS	ammonium peroxosulfate
BA	butyl acrylate
BMA	butyl methacrylate
CLC	capillary liquid chromatography
DEAEM	diethylaminoethyl methacrylate
DMPA	2,2-dimethoxy-2-phenylacetophenone
EDMA	ethylene glycol dimethacrylate
FITC-BSA	bovine serum albumin fluorescein isothiocyanate conjugate
GMA	glycidyl methacrylate
HDL	high density lipoprotein
HEMA	2-hydroxyethyl methacrylate
HPLC	high performance liquid chromatography
IAC	immunoaffinity chromatography
IE	ion-exchange

i.d.	inner diameter
IMAC	immobilized metal affinity chromatography
ISEC	inverse size exclusion chromatography
LAC	lectin affinity chromatography
LC	liquid chromatography
LIF	laser induced fluorescence
METC	2-methacryloyloxyethyltrimethylammonium chloride
MS	mass spectrometry
Mud-PIT	multi-dimensional protein identification technology
PEG	polyethylene glycol
PEGDA	polyethylene glycol diacrylate
PEGMEA	polyethylene glycol methyl ether acrylate
ROMP	ring-opening metathesis polymerization
RP	reversed-phase
RSD	relative standard deviation
SCX	strong cation-exchange
SEC	size exclusion chromatography
SEM	scanning electron microscopy
SEMA	sulfoethyl methacrylate
SFR	stable free radical
SP	swelling propensity
SPE	N,N-dimethyl-N-methacryloxyethyl-N-(3-sulfopropyl) ammonium betaine
TEMED	N,N,N’,N’-tetramethylethylenediamine

TEMPO	2,2,6,6-tetramethylpiperidyl-1-oxy
THF	tetrahydrofuran
TPM	3-(trimethoxysilyl)propyl methacrylate
TRIM	trimethylolpropane trimethacrylate
VAL	2-vinyl-4,4-dimethylazlactone
VS	vinyl sulfonic acid

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CHAPTER 1 BACKGROUND AND SIGNIFICANCE

1.1 Proteomics

After approximately 13 years of extensive research, a working draft of the human genome was decoded.[1-3] This was a great achievement towards the understanding of the complexity of human biology. The human genome is believed to be a rich source of information for a variety of purposes, such as disease diagnosis, early detection of geneic pre-dispositions to diseases, gene therapy, and rational drug design, including pharmacogenomic custom drugs. However, the true complexity of human biology is at the level of proteins, not genes. This is because protein diversity cannot be fully characterized by gene expression analysis alone, primarily due to alternative splicing and posttranslational modification of proteins. For example, it is estimated that approximately 1,500,000 different proteins exist in the human body while the total gene count in the human body is around 22,000.[4,5] Furthermore, the raw genetic sequence cannot predict a protein's function and expression level in different cells. Thus, the analysis of proteins is the next key step to understand the complexity of human biology. The term, "proteome", was coined in analogy to genome by Wilkins et al. in 1996.[6] Proteomics is a term that refers to the characterization of all proteins expressed by a group of active genes in a given cell or tissue.[7]

The analysis of the human proteome is an extremely challenging task due to the large number of proteins present in the human body. Moreover, marker proteins, whose

expressions change during the progression of a disease or under drug treatment, are the targeted proteins to be analyzed. Most of these proteins exist at trace levels, while other more abundant proteins interfere with detection. Therefore, techniques that can handle a very broad concentration dynamic range are required for proteomics studies.

Currently, the most popular method for resolving a large number of proteins is two-dimensional (2-D) gel electrophoresis.[8] This technique separates proteins based on their isoelectric points in the first dimension, and molecular weights using denaturing gel electrophoresis in the second dimension. The combination of these two orthogonal techniques can resolve up to 10,000 proteins, making 2-D gel electrophoresis a very powerful technique for profiling proteins in human body fluids.[9-12] With the recent development of MALDI and ESI mass spectrometry, the identification of proteins in a 2-D gel has become a less demanding task, further increasing the popularity of 2-D gel electrophoresis in proteomics research.[13-16] Although very successful for proteomics, 2-D gel electrophoresis suffers some limitations. The most serious problem is its inability to detect low abundance proteins,[17,18] although some controversy exists.[19] This aspect is particularly problematic because most proteins of interest are in the trace level range. In addition, 2-D gel electrophoresis can be problematic in the detection of very hydrophobic and/or basic proteins.[17] It is also labor-intensive and time-consuming. These limitations make it necessary to develop alternative or complementary techniques for proteomics. Liquid chromatography (LC) is one of the most promising methods, and it will be outlined in the following section.

1.2 Liquid Chromatography in Proteomics

Due to its high resolving power, excellent reproducibility, online preconcentration capability, ability of detecting both hydrophilic and hydrophobic proteins/peptides, and ease of interfacing with mass spectrometry (MS), high performance liquid chromatography (HPLC) has gained increasing interest in proteomics studies.[20] Two basic methodologies have been proposed for the use of LC in proteomics research. The first is a "top-down" approach in which proteins are separated first, followed by enzymatic digestion (e.g., tryptic digestion) of the separated proteins and subsequent identification by peptide profile mapping.[21] The other is the "bottom-up" approach, also called "shotgun" proteomics. In this method, all proteins in a sample are digested first, followed by separation of peptides and subsequent identification of the original proteins by software searching.[22] In both cases, separation by chromatography plays a key role in the analysis of proteins.

1.2.1 Reversed-phase LC of Proteins

At present, most LC of proteins is performed using the reversed-phase (RP) mode of chromatography coupled with MS for detection and identification. This is mainly due to the compatibility of RP LC with MS. In RP LC, samples can be easily desalted, and water and water-miscible volatile organic solvents are typically used as mobile phases. Although conventional RP LC has been applied in proteomics studies, an improvement in resolution and peak capacity is required due to the complexities of proteomic samples. Several approaches have been proposed for using RP LC for proteomics research. These include ultrahigh pressure liquid chromatography,[23-26] accurate mass tag measurement by high resolution MS,[27-28] and elution-modified displacement chromatography.[29,30]

With ultrahigh pressure equipment in LC, small particles can be used, resulting in high column efficiency and high resolution. For example, efficiencies up to 570,000 plates/m were obtained using 1.5 μm nonporous silica particles under a column back-pressure of 20,000 psi.[23] The high efficiency achieved by ultrahigh pressure LC has been extended to proteomics research. Shen et al. used 3 μm particles packed in a fused silica capillary for the analysis of soluble yeast proteins, and obtained a peak capacity of 1,000 at a column pressure of 10,000 psi.[25] Further improvement in sensitivity was achieved by the same group by incorporating micro solid-phase extraction, enabling analysis of ng protein samples.[26]

Another elegant approach to facilitate the identification of proteins is to use the so-called "accurate mass tag" (AMT) technique. This is based on the concept that a protein can be theoretically identified by detection of several unique peptides by tandem MS, provided that unambiguous detection of the peptides of interest can be made. Using high resolution Fourier-transform ion cyclotron resonance MS, it is feasible to resolve a mass difference of 1 ppm, which makes possible the accurate mass measurement of such unique peptides.[27] The AMT approach has been successfully applied to the analysis of *Saccharomyces cerevisiae, Caenorhabditis elegans,* and *Deinococus radiodurans* proteomes.[27,28] A high coverage (61%) of proteins was reported using the AMT method.[28]

Another unique RP LC approach for proteomics is to use elution-modified displacement chromatography.[29,30] The main advantage of displacement chromatography over conventional linear elution chromatography is the characteristic enrichment of low abundance analytes. For example, trace marker peptides were detected in the fmol range in a model study of the rhGH proteome.[29,30] Another favorable feature is relatively fast

analysis since enrichment and separation are performed at the same time. One main disadvantage of this novel technique is the requirement of large amount of sample, which is often difficult to obtain in proteomics.

1.2.2 Two-Dimensional LC

Although RP LC has achieved some success in certain proteomic studies (*vide supra*), the peak capacity is insufficient to resolve most extremely complex proteome samples. One method to dramatically improve peak capacity is to use 2-D or multi-dimensional LC modes, where overall peak capacity is the product of the peak capacities in each dimension.[31-34] For proteomics studies, the second dimension is typically a RP mode directly coupled with MS. The first dimension, which should be an orthogonal mode to RPLC, could be ion-exchange (IE), affinity, or size exclusion chromatography (SEC). The most widely used combination is IE-RP LC coupled to MS. For example, using 2-D IE-RP LC with column switching, a total peak capacity of 600 was achieved for the separation of complex protein mixtures.[35] This approach was applied to human brain glioma cells and the A431 epidermal cancer cell proteome, leading to the identification of 213 and 280 proteins, respectively.[36,37] Recently, a remarkable 1504 proteins were identified in the yeast proteome using the IE-RP LC approach.[38] Similarly, 490 proteins were detected in human blood serum by combining IE and RP LC.[39]

In the IE-RP LC combination, each fraction of the first dimension would be either collected and injected offline to the second RP column, or online to the second dimension by column switching. Sometimes, a trapping column before the second dimension is required for desalting, preconcentration, and injection to the second dimension. A main disadvantage of this approach is that it is time-consuming. It may take several hours or

days to complete the IE-RP run. An elegant approach, termed "multi-dimensional protein identification technology" (Mud-PIT), was introduced to run the 2-D separation in relatively short time.[22,40] In this method, a single capillary column was packed with IE silica particles in one section and RP silica particles in another section in tandem. Although not strictly comprehensive 2-D, Yates' group used this approach and identified a remarkable 1484 proteins in the yeast proteome.[40] The Mud-PIT approach was also proven useful for the identification of membrane proteins from crude rat brain homogenate.[41]

In addition to IE as the first dimension in 2-D chromatography of a proteome, affinity or SEC is also used for specific applications. Due to the strong binding between antibody and antigen, affinity chromatography is particularly useful for preconcentration of low abundance analytes. It is well known that posttranslational modifications of proteins, such as phosphoration and glycosylation, are very common and play important roles in the behavior and interactions of cellular proteins. Such modified proteins often exist at very low concentrations and, thus, are difficult to identify in complex mixtures containing highly abundant proteins. Immobilized metal affinity chromatography (IMAC)[42,43] and immunoaffinity chromatography (IAC)[44,45] are useful for trapping phosphopeptides. Lectin affinity chromatography (LAC) is applicable to the analysis of glycoproteins. In IMAC, a transition metal, such as Fe(III) or Ga(III) ions, is immobilized in the stationary phase and used to selectively isolate and concentrate phosphopeptides due to high affinity binding between the transition metal and serine, threonine or tyrosine in the phosphopeptides. Using the IMAC approach, analysis of bovine casein at the level of 30 pmol was achieved.[42] IAC is similar to IMAC except that an antibody (e.g.,

antiserine, antithreonine or antityrosine) rather than a transition metal is used to capture the phosphopeptides. Because antibody-antigen interaction is typically stronger than metal chelating interaction, the selectivity and preconcentration factors of IAC are better than IMAC.[44,45] For the analysis of glycoproteins, LAC has proven to be one of the most powerful techniques to isolate and enrich specific classes of glycoproteins based on the lectin ligands used.[46,47] Xiong et al. used LAC coupled with RPLC to study lymphosarcoma in dogs, and identified two proteins that are related to cell adhesion and cancer cell migration.[46]

SEC has also been used as the first dimension in 2-D LC for proteomics research, although little attention has been paid to this mode. This is probably due to the low resolving power and low loading capacity of SEC. However, since separation in SEC is determined by protein size, a feature similar to the second dimension in 2-D gel electrophoresis, it should be very useful for fractionating proteins. Several reports have been found using SEC-RP LC for proteomics studies.[48-50]

The previously described studies confirmed the power of the 2-D approach, and addressed some of the limitations of conventional protein analysis techniques, such as reproducibility, automation, and convenience in interfacing with MS. However, much remains to be done to further improve the capabilities of current separation media and techniques to meet the needs of proteomics. Most techniques described above utilized packed columns, i.e., columns packed with μm-size spherical particles. To prepare such columns requires multiple steps, starting with the synthesis of small spherical particles, followed by sieving and introduction of functionality by chemical derivatization. Finally, the particles are packed into a chromatographic column and retaining frits are fabricated.

Especially for proteomics studies in which capillary columns are used to improve mass sensitivity, packing of the column is a tedious process, and frits are hard to make reproducibly. Another more fundamental limitation of packed columns is that they have large void volume. Even the best packed columns have ~40% external porosity. The existence of such large external porosity limits the speed of chromatography due to the resistance to mass transfer. As commented on by Regnier, the most significant advances in HPLC have always followed the introduction of enhanced support matrices.[51] Nearly 20 years ago, a novel chromatographic support, called a monolith, was introduced to overcome some of the limitations of packed columns and to achieve fast separations.[52,53] In the following sections, monolith technologies will be reviewed in detail.

1.3 Introduction to Monoliths

HPLC became available only after the introduction of microparticulate porous silica particles in the 1960s.[54,55] However, it has been proven difficult to apply HPLC to the chromatography of macromolecules with conventional microporous particles. The limiting factor in chromatography of macromolecules is the mass transfer resistance due to the small diffusion coefficients of macromolecules. This results in both low column efficiency and limited speed of analysis. Biomolecules were first separated in HPLC in the mid 1970s only when macroporous silica particles became available (see reviews 56 and 57). Further improvements in the HPLC of macromolecules were achieved with the availability of hydrophilic rigid organic resins.[58-60] To alleviate mass transfer resistance for fast separation of macromolecules, more advanced separation media have been introduced, which include nonporous particles,[61] hybrid separation media[62] and perfusive beads.[63] The highly original perfusive beads introduced by Regnier's group allow a small

portion of the mobile phase to flow through the pores of the beads.[63] This convective flow greatly enhances the mass transfer of macromolecules, resulting in a significant improvement in the speed and chromatographic efficiency for macromolecules. It is natural to anticipate that further improvements will be achieved if all of the mobile phase is forced to flow through the separation media. Realizing that this approach was not realistic for packed columns, led to the development of membrane chromatography.[64-66] Another reason for the rapid development of membrane chromatography was the finding that proteins could be separated using very short columns. In fact, a membrane can be viewed as a column with extremely short length and large diameter. The introduction of membrane chromatography enabled the rapid and extremely fast separation of biopolymers.[65] Several other alternatives to a membrane have also been introduced and used as chromatographic supports. These include cellulose sheets,[67] woven fabrics[68] and macroporous discs.[69] However, they have not gained widespread application mainly due to fabrication difficulties and lack of suitable accessories for sample introduction.

Although fast separation can be achieved using membrane chromatography, one limitation of a membrane is the low sample loading capacity per unit volume compared to porous particles. Around the year 1990, monolith technologies were introduced as novel chromatographic supports by two groups independently.[52,53] A monolith, originally called a continuous polymer bed,[52] is a continuous rod with canal-like μm-sized through-pores and nm-sized pores in its skeletal structure. At first glance, a monolith appears to be analogous to a membrane, except that a monolith has a longer length and smaller diameter. In fact, these two media have great differences. A membrane does not have distinct through-pores and nanopores. Furthermore, the specific loadabilty of a monolith

is much greater than a membrane. Monolithic columns have received considerable interest due to their favorable features, such as ease of preparation, abundance of functional group chemistries available and, most importantly, enhanced mass transfer. After approximately 17 years of study, monoliths have become key alternatives to packed beds. Guichon once commented that "the invention and development of monolithic columns is a major technological change in column technology, indeed the first original breakthrough to have occurred in this area since Tswett invented chromatography, a century ago."[70] Iberber also called the monolith the 4th generation of chromatographic sorbent for the analysis of biomolecules.[71]

1.3.1 History of Monoliths in Chromatography

The first article that describes the preparation of a monolith for use in chromatography can be dated back to 1967.[72] The synthesis of this monolith was done using a molding process. An aqueous solution of 2-hydroxyethyl methacrylate (HEMA) with 0.2% ethylene glycol dimethacrylate (EDMA, as a crosslinker) was introduced into a glass tube (22 cm $\times$ 23.5 mm i.d.). After free radical polymerization, a highly swollen polymer hydrogel with a continuous structure was obtained and used in size exclusion chromatography of water soluble polymers. Due to significant swelling and tendency to be compressed upon applying pressure, chromatography was performed using gravity. As a result, a very low mobile phase flow rate (only 4 mL/h) was used, which in turn resulted in low column efficiency due to excessive longitudinal diffusion. The low permeability of the column was primarily due to the use of a very low amount of crosslinker (only 0.2%). Nevertheless, this was the first attempt to prepare a polymer monolith for use in chromatography.

Several years later, monoliths with improved permeability were obtained using polyurethane chemistry.[73-77] In contrast to the swollen poly(HEMA) gel reported above, the continuous polyurethane monolithic columns maintained their permanent macroporous structure even in the dry state. These columns have been demonstrated in both GC[73-76] and LC[73,77] modes. Unfortunately, inferior chromatographic performance of these columns prevented their wide acceptance, presumably due to swelling and softness in some solvents.

Two decades passed before modern monoliths were successfully introduced, which competed favorably with packed columns.[52,53] The first was based on polyacrylamide.[52] In this approach, acrylic acid and bisacrylamide were copolymerized in a stainless steel column with the use of redox initiation (N,N,N',N'-tetramethylethylenediamine, TEMED and ammonium peroxosulfate, APS). After polymerization, the monolith was compressed by applying a high pressure using an HPLC pump. This step was important to produce a uniform/homogeneous bed to improve column efficiency and resolution. Fast and efficient separation of several model proteins was demonstrated using cation-exchange chromatography. An interesting finding about this monolithic column was that resolution increased with an increase in mobile phase flow rate.

In contrast to the soft polyacrylamide based monolith, a rigid monolith based on methacrylate polymers was introduced in 1992.[53] In this approach, glycidyl methacrylate (GMA) was used as monomer and EDMA was used as crosslinker. The monomer solution also contained a large amount (60%) cyclohexanol and dodecanol as porogens (pore-forming agents), and 1% 2,2'-azobisisobutyronitrile (AIBN) as initiator. Using

thermally initiated polymerization, a rigid polymer monolith with a high degree of crosslinking was formed inside a stainless steel column. Tetrahydrofuran (THF) was pumped through the resulting monolith to flush out porogens and any unreacted monomers. The epoxide group in the monolith was subsequently reacted with diethylamine to produce an anion-exchange monolith for protein analysis. Comparable chromatographic performance to packed columns was obtained using this type of monolith.

Following the introduction of the polymer monolith, silica monoliths were also synthesized using sol-gel chemistry several years later.[78,79] In contrast to the typical one-step in-situ preparation of polymer monoliths, the synthesis of a silica monolith often requires multiple steps. Another feature of silica monoliths is that they are mostly applied to the analysis of small molecules.

1.3.2 Preparation of Polymer Monoliths

The preparation of polymer monoliths is often a one-step in-situ process, typically via free radical polymerization. Several initiation techniques have been developed to prepare monoliths in different molds for specific applications. In addition, surface chemistry (functionality) can be easily controlled in polymer monoliths. Since a one-phase transparent monomer solution is the starting point to prepare a monolith, both hydrophilic and hydrophobic monomers can be used by choosing appropriate porogens. Chemical derivatization of some reactive monoliths (e.g., GMA) increases the range of available functionalities dramatically. More importantly, the recently introduced grafting technique allows nearly unlimited introduction of a variety of surface functionalities. Furthermore, the porosity of the polymer monolith can be controlled by varying the

components in the monomer solution as well as by using different initiation techniques. This characteristic is particularly important for fast separation in that a monolith with very high permeability can be easily prepared while maintaining good chromatographic performance. Finally, the crosslinker is equally important in the design and preparation of polymer monoliths. Because crosslinker to total monomer ratio is typically in the range of 30-70 wt%, the crosslinker will affect both the mechanical strength and the backbone polarity of the resulting monolith.

Initiation of polymerization. The early development of polymer monoliths followed analytical packed column technology in which stainless steel tubing with i.d. of 4.6 mm is typically used. As a result, thermal initiation dominated the preparation of polymer monoliths. For example, the first successful poly(GMA) monolith was prepared in a stainless steel tube via thermal initiation.[53] The thermal initiator AIBN is widely used in the preparation of polymer monoliths. Upon heating at ~60 ºC, AIBN decomposes to form free radicals (Figure 1.1), which can initiate the polymerization of most vinyl-containing monomers. Because the decomposition temperature is low, any solvent with boiling point above 60 ºC can potentially be used as a porogen to prepare the monoliths. The most widely used and effective porogens for the preparation of polymer monoliths are either long-chain alcohols[53,80-82] (e.g., cyclohexanol and dodecanol) or short chain alcohols[83-85] (e.g., 1-propanol and 1,4-butanediol). In addition to AIBN, benzoyl peroxide has been used as a thermal initiator for monolith preparation, although it has not been very popular.[81]

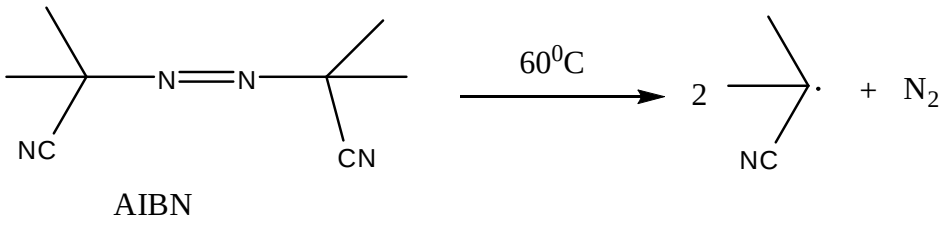


Figure 1.1. Radical formation through thermal decomposition of AIBN.

Besides the conventional AIBN thermal initiator, stable free radical initiators were also introduced to synthesize novel monoliths with "living" surface properties. For example, 2,2,6,6-tetramethylpiperidyl-1-oxy (TEMPO) has been used as initiator to initiate the copolymerization of styrene and divinylbenzene.[86] In contrast to AIBN or benzoyl peroxide where 55~80 ºC is sufficient to form initiator radicals, a higher temperature of 130 ºC is required to decompose TEMPO. As a result of the slow initiation of TEMPO, quite different monolith morphology with very high surface area was obtained, which could be potentially used in size exclusion chromatography of synthetic polymers. Although TEMPO-initiated polymerization resulted in a monolith with high surface area (300 m^2/g), the permeability of the resulting monolith was low. Further improvement of flow-through properties was achieved by using other stable free radicals or a mixture of them. 3-Carboxy-2,2,5,5-tetramethylpyrrolidinyl-1-oxy and 4-carboxy-2,2,6,6-tetramethylpiperidinyl-1-oxy have been used to prepare polystyrene monoliths with a through-pore medium diameter of 1 μm, thus enabling fast flow under moderate column backpressure.[87] The main advantage of using a stable free radical initiator is the ease of post-column modification. After polymerization, there are still "living" free radicals on the surface of the resulting monolith, which can be used to graft other vinyl-containing monomers. In this approach, it is straightforward to introduce new surface chemistries from the same bulk monolith that has optimized mechanical properties.

Another unique branch of polymer monolith can be prepared via ring-opening metathesis polymerization (ROMP).[88,89] Here, the initiator, more accurately called a catalyst, is a transition metal complex (e.g., a Grubbs-type catalyst with a general formula

of $Cl_2(PR_3)_2Ru(=CHPh)$, R = phenyl or cyclohexyl). Polymerization is performed at low temperature (<0 ºC) and under an inert environment (nitrogen protection). Monomers are cycloolefins, and crosslinkers are olefins with two or more vinyl groups in two or more rings. The unique property of ROMP is that polymer chain growth is via ring opening while vinyl groups remain intact. Another feature of ROMP is its "living" characteristic. After polymerization, the initiator is still active on the surface of the polymer, which allows flexible grafting of various chromatographic ligands. Buchmeiser's group pioneered the preparation of polynorbornene-based monoliths using the ROMP approach. Several chromatographic modes including RP, IE and chiral recognition have been demonstrated. [88,89]

With the recent burgeoning interest in proteomics research, the capillary column has received more attention due to increased mass sensitivity. Another trend is the development of monoliths for use in planar microchip formats.[90] Both of these factors have led to the development of photoinitiation for the preparation of polymer monoliths. For photoinitiation, the most widely used initiator is 2,2-dimethoxy-2-phenylacetophenone (DMPA). Upon UV irradiation at 365 nm, DMPA decomposes into three radicals that subsequently initiate the polymerization of vinyl-containing monomers (Figure 1.2).

Several requirements must be followed for the use of photoinitiation. First, a mold that is UV transparent is required. Fluoropolymer-coated fused silica, quartz, and glass microchips fulfill this requirement. In addition, porogens must not absorb UV radiation in any significant degree. Fortunately, the UV transparency requirement is satisfied by most

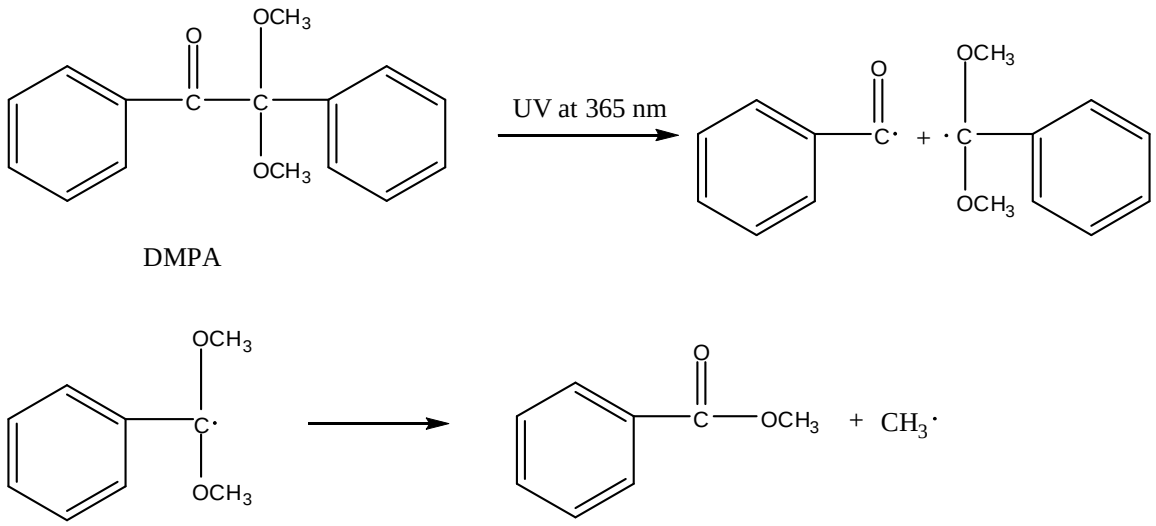


Figure 1.2. Radical formation from photo-decomposition of DMPA.

organic solvents that would be selected for use. Finally, the diameter must be sufficiently small that UV radiation can penetrate the whole diameter of the mold. Capillaries with i.d. smaller than 320 μm and most microchips can be safely used. Despite the limitations described above, photoinitiation has several distinct advantages, which have spurred its rapid development.[91-101] Probably, the most important characteristic is the improvement in monolith uniformity over heat initiation. It is well known that free radical polymerization is an exothermic process. Using thermal polymerization, the heat generated cannot dissipate well, resulting in a temperature gradient along the radial direction of the tube (higher in the center than periphery). Because the morphology of the monolith, such as surface area and through-pore diameter, is very sensitive to the temperature,[82] the temperature gradient under thermal initiation leads to nonuniformity, which in turn affects column efficiency.

In addition, another attractive feature of photoinitiation is the selective patterning of monoliths in desired regions. Polymerization occurs only in the region that is exposed to UV irradiation. Using a suitable UV mask, a monolith with advanced pattern can be obtained. For example, a capillary with dual functionalities has been prepared for peptide mapping using suitable masks.[95,98] Photoinitiation is especially suited to microchip applications.[90,93] In a microchip, the functional monolith is ideally placed only in the separation channel, while reservoirs and injection channels are void of monolith. This geometry is hard, if not impossible, to achieve using other types of initiation. Third, photoinitiation operates much faster than thermal initiation. Complete conversion of monomers to polymer can occur in minutes using photoinitiation, in sharp contrast to >20 h using thermal initiation.[94,95, 99-101] The fast reaction rate using photopolymerization is

very useful in the optimization of the monolith. It is not uncommon that an acceptable recipe results only after hundreds of screening experiments. Finally, since photoinitiated polymerization is performed at room temperature, low boiling point organic solvents, such as methanol and ethyl ether,[99-101] can be safely used as porogens. This adds more control over the adjustment of the pore size distribution of a monolith, which is a key property of a monolith for use in flow-through applications.

Due to the polarity of acrylamide-based monomers, redox initiation is often used for the preparation of acrylamide-based monoliths.[52,102] For this initiation technique, APS and TEMED have been used as initiators (Figure 1.3). Polymerization using this redox system occurs at room temperature. The APS-TEMED system has also been used to prepare polymethacrylate monoliths, although this is not well recognized as an alternative to the widely used thermal initiation.[103]

A less common initiation technique using a high energy irradiation source (e.g., γ-radiation) was reported recently for the preparation of polymethacrylate monoliths.[104] High energy irradiation breaks down chemical bonds (e.g., C-H bonds), yielding a radical that can be used to initiate the polymerization of vinyl-containing monomers. This process is similar to UV initiated photografting (*vide infra*) for which deep UV (e.g., 210 nm) is typically used, except that high energy (γ-radiation) is used. Thus, the radiation can penetrate deeply into the monomer, making it possible to prepare up to 4 mm diameter monoliths. Other advantages of high energy radiation initiation include fast reaction rate and no chemical initiator required. The main disadvantage, however, is the extremely dangerous γ-radiation, making it difficult to perform in conventional laboratories.

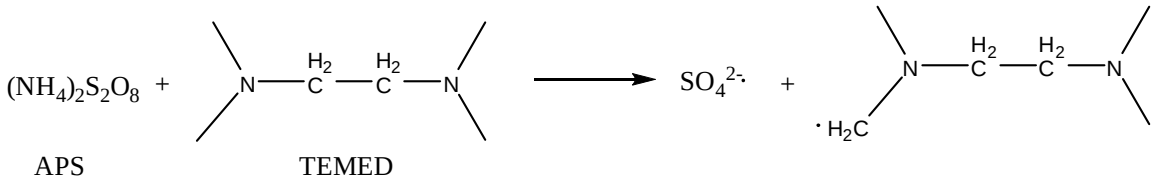


Figure 1.3. Mechanism of redox initiation.

Functionalization of the monolith surface. Three methods have been developed for the introduction of functionalities during the synthesis of polymer monoliths. The widely used, originally adopted, and still most popular one is copolymerization. During the 17 years of development of the monoliths, numerous examples can be found using copolymerization to introduce desirable chemistry. For some monomers that are not readily available or difficult to synthesize, chemical derivatization of some bulk reactive monolith (e.g., GMA) introduces new surface chemistry. This approach is particularly useful in affinity chromatography and enzyme immobilization. Actually, derivatization is a well established method to prepare silica-based particles used in conventional HPLC.[54,55] For example, monodisperse bare silica particles are prepared and silanized with alkylsilanes to generate reversed-phase particles. The third approach, grafting, is another powerful method to introduce new chemistries. In particular, grafting by deep UV has recently gained in popularity.[94-96,98]

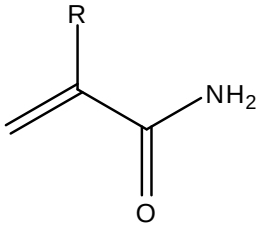
Numerous functional monomers have been used to prepare polymer monoliths via copolymerization, which include hydrophilic, hydrophobic, reactive and ionizable monomers. Typical hydrophilic monomers include acrylamide, N-isopropylacrylamide, N-ethylacrylamide or their methacrylamide analogs, HEMA and polyethylene glycol methyl ether acrylate (PEGMEA). Hydrophobic monomers mainly comprise butyl methacrylate (BMA) or butyl acrylate (BA), and styrene as well as norbornene. The widely used reactive monomer is GMA or chloromethylstyrene, or 2-vinyl-4,4-dimethylazlactone (VAL). Ionizable monomers include 2-acrylamido-2-methyl-1-propane sulfonic acid (AMPS), 2-acryloyloxyethyltrimethylammonium chloride (AETC)

or 2-methacryloyloxyethyltrimethylammonium chloride (METC). The chemical structures of the monomers described above are shown in Figure 1.4.

In addition to copolymerization, chemical derivatization is another powerful method to introduce new surface functionality. Among the three reactive monomers, GMA has gained the most widespread application. The epoxide group in the poly(GMA) monolith can be potentially attacked by any nucleophiles, such as amines or amino groups in proteins or sodium sulfite. For example, the first polymethacrylate monolith was based on GMA.[53] After the epoxide was reacted with diethylamine, diethylaminoethyl chemistry was introduced, which was successfully used for anion exchange chromatography of acidic proteins. The reactive epoxide can also be modified with sodium sulfite to generate a cation-exchange monolith. For example, Ueki et al. has used this approach for strong cation-exchange chromatography of inorganic cations.[105] The more useful function of the poly(GMA) monolith is for immobilization of enzymes (e.g., trypsin) for preparation of microbioreactors. Immobilization of enzymes can be achieved by direct reaction of the enzyme with poly(GMA), or by inserting an arm between poly(GMA) and the enzyme. A detailed review on using poly(GMA) to immobilize enzymes has been recently published.[106]

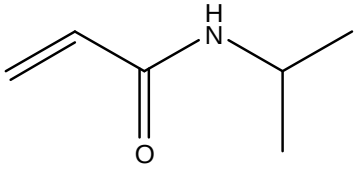
Grafting of a functional monomer has several advantages as compared to either copolymerization or derivatization. First, only one optimization process is sufficient. In the grafting approach, a generic monolith [e.g., poly(BMA)] with good mechanical strength and flow properties is prepared and optimized. Then a new monomer (e.g., those shown in Figure 1.4) with suitable initiator is loaded into the pores of the general

Hydrophilic monomers:

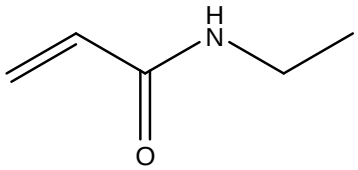


R = H or CH_3

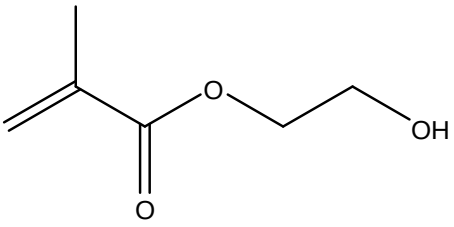
acrylamide or methacrylamide



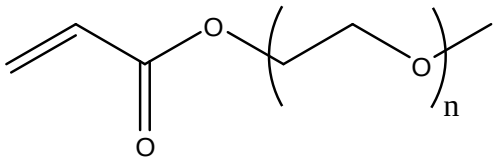
N-isopropylacrylamide



N-ethylacrylamide

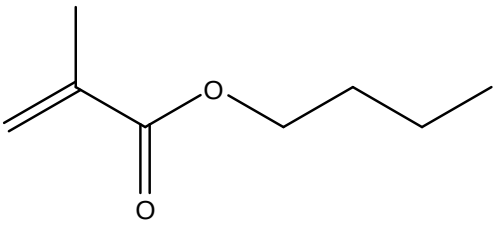


HEMA

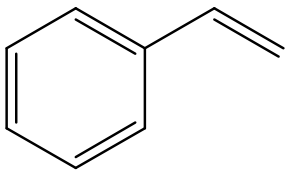


PEGMEA

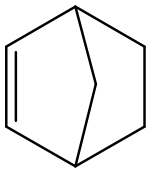
Hydrophobic monomers:



BMA

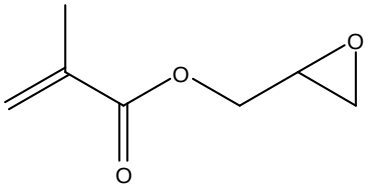


styrene

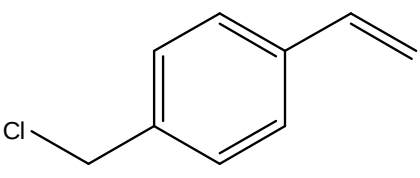


norbornene

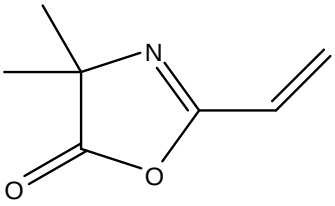
Reactive monomers:



GMA

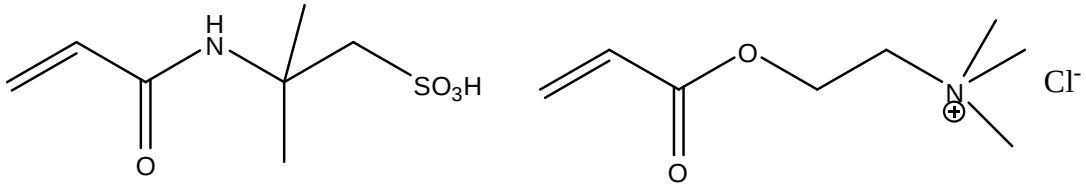


chloromethylstyrene



VAL

Ionizable monomers:



AMPS AETC

Figure 1.4. Chemical structures of common monomers used for the preparation of polymer monoliths in copolymerization.

monolith and grafted on the surface of the monolith. Thus, different chemistries can be introduced through one general monolith. This feature is very attractive. The optimization of a new monolith is a time-consuming process. Although some theoretical aspects of the preparation of macroporous particles have been proposed,[107-109] the main approach to optimize a new monolith system is via trial-and-error at the present. By grafting, tedious optimization of each of the new monomers is avoided. Second, a better surface coverage is expected as compared with copolymerization. Because grafting occurs from the monolith surface and extends into the pores of the monolith, all of the functionalities are accessible for interaction. Furthermore, the grafted chains can also serve as new loci from which new chains can grow, ultimately leading to a highly branched structure. This further increases the binding capacity of the resulting monolith. Finally, grafting is typically fast, and the degree of grafting can be easily controlled.

Grafting can be realized in several ways. First, the nature of the ROMP method determines the ease of surface grafting.[88,110] Because the initiator is attached to the surface of the resulting monolith after polymerization, grafting can be initiated after the introduction of a new cycloolefin. A variety of functionalities such as carboxylic acid, tertiary amine and cyclodextrin, have been grafted into a base polynorbornene monolith. Second, grafting can be achieved via the use of stable free radicals (SFR).[86,87] This approach is very similar to the ROMP method. SFR on the surface of the monolith can be activated upon heating, and they initiate the graft polymerization of new monomers. For example, chloromethylstyrene and vinylpyridine have been successfully grafted to polystyrene monoliths.[111] Third, grafting is performed through the immobilization of initiators. This approach is similar to the ROMP and SFR methods; however, it is not a

universal method. To immobilize an initiator on the surface of a monolith, the bulk monolith should be reactive. For example, Tripp et al. immobilized a free radical azo initiator through the reaction of 4,4'-azobis(4-cyanovaleric acid) with the chloromethyl functionality in the polychloromethylstyrene monolith.[112,113] This free radical initiator was used to graft VAL onto the monolith to separate amines. Fourth, grafting is achieved by the introduction of vinyl-containing chemicals. This is not a universal approach, either. For the poly(GMA) monolith, allylamine can be reacted with the epoxide group to form a pendant vinyl group. If one adds a new monomer solution with initiator, grafting from the vinyl group in the poly(GMA) monolith will occur. Peters et al. used this approach to graft N-isopropylacrylamide, and obtained a unique monolith that was thermally responsive.[114] The hydrophobicity of this grafted monolith changes from hydrophilic to hydrophobic upon an increase in temperature. Finally, grafting is realized via UV irradiation. This is a universal approach and very popular today. If a polymer is irradiated with deep UV (e.g., < 200 nm), hydrogen abstraction occurs, leaving an active radical on the polymer surface.[115,116] Using this approach, AMPS, VAL and BMA have been grafted onto the poly(BMA) monolith.[94,96] Furthermore, N,N-dimethyl-N-methacryloyloxy-ethyl-N-(3-sulfopropyl)ammonium betaine (SPE) has been grafted onto the same poly(BMA) monolith for rapid and efficient separation of proteins in capillary electrochromatography.[117]

Control of monolith porosity. In flow-through applications, such as chromatography, online enzyme microreactor, and online solid phase extraction, both large surface area and good permeability are desirable. A large surface area provides more active sites for effective interactions, and good permeability allows faster

processing and low back-pressure. For polymeric monolithic columns, the surface area is mainly determined by the nanopores (<2 nm) and mesopores (between 2 and 50 nm), while the macropores (>50 nm) provide little contribution. The permeability, on the other hand, is mainly determined by the average diameter of the macropores (through-pores). Unfortunately, in most cases, a monolith with good permeability typically has low surface area, and vice versa. Thus, a balance must be met between surface area and flow-through properties. This requires the optimization of the pore size distribution to fit each application.

The pore size distribution of a polymer monolith can be adjusted by several variables. These include initiator concentration, total monomer to total porogen ratio, monomer to crosslinker ratio, porogen nature and ratio of porogens if more than one porogen is used. Although a decrease in initiator can decrease the pressure drop of the monolith, a longer time is required to complete the polymerization. A decrease in total monomer to total porogen ratio is a straightforward method to decrease the pressure drop of the monolith; however, it decreases the homogeneity and rigidity of the monolith as well. A change in monomer to crosslinker ratio can have an effect on the pressure drop of the resulting monolith, although it also changes the rigidity and homogeneity of the monolith. The most powerful factor to control the pressure drop of the monolith is the selection of porogens since they do not affect the composition and rigidity of the monolith.

The selection of porogen(s) is unlimited. Good solvent, poor solvent and linear polymers have proven useful for the preparation of macroreticular polymer resins in suspension polymerization.[107-109] The combination of good solvent, poor solvent and/or

linear polymer is also effective in preparing polymer monoliths. There are several requirements for the design and selection of porogens. First, they must dissolve all reagent components (i.e., initiator, monomer and crosslinker). A transparent monomer solution is a prerequisite for developing a good monolith. Second, the polarity of the porogen must be easily adjustable. Porogens generally include both a good solvent and a poor one. In this way, porogen mixtures with different solvent strengths can be obtained by varying the ratio between the good and poor solvents. This is particularly important because it controls the onset of phase separation (i.e., polymer chain precipitation from the porogens as it grows longer), which determines the pore size distribution of the resulting monolith. In general, good solvents will generate a monolith with small through-pores due to later onset of phase separation. Poor solvents, on the other hand, yield monoliths with large through-pores, resulting in good permeability. Finally, the porogen must be compatible with the initiation technique. Depending on the initiation technique used, different organic solvents have been used for monolith synthesis (see Section 1.3.2). For example, in thermal initiation, low boiling point solvents will not work as porogens. Similarly, only UV transparent solvents can be utilized if photoinitiation is used.

In addition to common organic solvents as porogens, linear polymers can also work as porogens. For example, polyethylene glycol (PEG) of different molecular weights has been used as porogen to prepare polyacrylamide monoliths.[117] A systematic study using PEG as coporogen has also appeared recently for the preparation of polymethacrylate monoliths for hydrophobic interaction chromatography of proteins.[118] Another atypical porogen is supercritical carbon dioxide. This porogen is attractive in

that it is nontoxic, nonflammable and inexpensive. Furthermore, the solvating power can be adjusted by applying different pressures. Thus, supercritical carbon dioxide itself rather than a mixture of solvents can effectively work as porogen, making optimization somewhat straightforward. Using EDMA and trimethylolpropane trimethacrylate (TRIM) as model monomers, monoliths with a broad range of through-pore diameters (20 nm - 8 μm) have been prepared.[119,120]

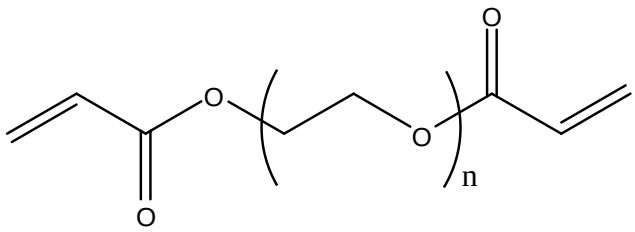
Among all types of polymer monoliths developed, the polyacrylamide monolith is unique. In addition to the common porogen approach to control the pore size distribution,[117,121] ionic strength is another powerful method to adjust the through-pore diameter.[52,102] In this approach, no organic solvents are required. The monomer mixture is a transparent aqueous solution. The formation of through-pores is achieved by promoted hydrophobic interaction of the polymer backbone in the presence of a high concentration of salt (e.g., ammonium sulfate). For polyacrylamide monoliths containing hydrophobic ligands, a surfactant is often used to emulsify the monomer mixture, and the same principle can be applied to control the formation of through-pores.[102]

Crosslinking during monolith polymerization. In contrast to various monomers used for preparation of polymer monoliths,[52,53,80,88,121-124] the number of crosslinkers is much more limited. Very little effort has been directed toward study of crosslinker effects on chromatographic performance. This is quite surprising since the crosslinker is an integral part of the resulting monolith, typically accounting for 30-70% by weight. As a result, the crosslinker should be expected to significantly affect both the rigidity of the resulting monolith and its overall polarity.

In Chapter 2, I report a new crosslinker, polyethylene glycol diacrylate (PEGDA), for the preparation of acrylate-based polymer monoliths for aqueous SEC of peptides and proteins.[99] The PEGDA crosslinker demonstrated superior biocompatibility compared to conventional ethylene glycol dimethacrylate. At the same time, several other crosslinkers, including polyethylene glycol dimethacrylate, were copolymerized with BMA for RPLC of proteins.[125] Although the advantage of biocompatibility of the crosslinker was not demonstrated in these studies due to the use of the RP mode of chromatography; nevertheless, the feasibility of using crosslinkers other than conventional EDMA to prepare methacrylate-based polymer monoliths was clearly shown.

For analysis of biological samples, such as peptides and proteins, the use of PEGDA is helpful to suppress nonspecific interactions. As shown in Figure 1.5, PEGDA has an acrylate group at each end of the molecule, with a PEG chain between. According to a systematic study conducted by Ostuni et al.,[126] a molecule that contains ≥ 3 ethylene glycol units effectively resists the adsorption of proteins. PEG or PEG-containing materials have been widely used as slab gel matrix, capillary electrophoresis coating, capillary gel electrophoresis matrix, and artificial organ coating.[127-130] A unique feature of PEG is that it does not denature proteins, even during precipitation at high concentration, which is in sharp contrast to other organic solvents (e.g., acetonitrile) which tend to denature proteins.[131]

With the use of PEGDA as a crosslinker, a strong cation-exchange (SCX) polymer monolith was recently introduced for capillary liquid chromatography of peptides, as described in Chapter 3.[101] Using simple one-step copolymerization of AMPS and PEGDA, the resulting monolith provided extremely narrow peaks and high peak



PEGDA

Figure 1.5. Structure of PEGDA.

capacity. Although not completely understood, the extraordinary chromatographic performance is believed to be related to the use of the biocompatible crosslinker PEGDA.

1.3.3 Application of Polymer Monoliths

Due to ease of preparation and enhanced mass transfer, polymer monoliths have found numerous applications in a variety of fields related to the analysis of biological samples. These include preconcentration and solid-phase extraction,[132] enzyme bioreactor for protein digestion,[106] capillary electrochromatography,[133-136] chip electrochromatography,[90] and liquid chromatography (both in analytical and capillary formats).[137-139] A detailed description of all of these applications is beyond the scope of this dissertation. Instead, applications of polymer monoliths to the analysis of biological analytes in capillary liquid chromatography will be briefly reviewed.

Acrylamide-based monoliths. The first monolithic capillary column was based on polyacrylamide.[140] A small amount of butyl acrylate was copolymerized with a large amount of methylenebisacrylamide in a 300 μm i.d. capillary via typical APS-TEMED redox initiation. The resulting monolith was successfully used for hydrophobic interaction chromatography of model proteins. One main problem of this early approach was the compressibility of the monolith. For example, an initial length of 60 cm was compressed to 7 cm upon applying pressure. To improve the mechanical strength of the polyacrylamide-based monolith, two modifications were made. One was to treat the inner wall of the capillary with 3-(trimethoxysilyl)propyl methacrylate (TPM) to fix a pendant double bond on the inner wall. The other was to use a new crosslinker, piperazine diacrylamide, to replace the conventional methylenebisacrylamide crosslinker. Using these improved methods, stable polyacrylamide monoliths (no compression during usage)

were prepared in both 320 μm and 10 and 25 μm i.d. capillaries. The monoliths were demonstrated for fast weak cation-exchange chromatography of model basic proteins.[141,142]

Acrylate or methacrylate-based monoliths. It is somewhat surprising that development of capillary acrylate or methacrylate-based monoliths for protein analysis lagged far behind that of acrylamide-based monoliths. In 2004, a polymer monolith synthesized from the copolymerization of BMA and EDMA using photoinitiation was reported.[97] The monolith was prepared in a 200 μm i.d. UV transparent fused silica capillary and used for fast RP LC of proteins. As a result of the through-pore diameter of 2.24 μm, the monolith had extremely low flow resistance. Baseline separation of ribonuclease A, cytochrome c, myoglobin and ovalbumin was achieved in 40 s using a flow rate of 100 μL/min and an optimized steep gradient.

Lee's group was the first to prepare acrylate-based monolithic capillary columns for LC of peptides and proteins.[99,101] The main contribution of this work was the development of a novel PEGDA crosslinker, which effectively resists adsorption of proteins and peptides. As a result, the contribution of the crosslinker to monolith hydrophobicity was minimized. The PEGDA crosslinker is particularly useful for separation techniques that can analyze biomolecules in their native states (e.g., SEC, IE, and affinity chromatography). Detailed descriptions of these studies will be provided in Chapters 2-4.

Affinity capillary LC of glycoproteins and glycans using polymethacrylate monoliths was recently reported by El Rassi's group.[143-145] Both neutral poly(GMA) and cationic poly(GMA-co-METC) monoliths were synthesized in 100 μm i.d. fused silica

capillaries using thermal initiation. Two lectins including concanavalin and wheat germ agglutinin were used to selectively trap very low concentration (~10^{-8} M) glycoproteins and glycans that contained sugar sequences recognized by the lectins. By synthesizing and coupling another polyacrylate monolith (based on pentaerythritol diacrylate monostearate) that could be used for RP LC, a 2-D separation scheme was demonstrated.[144]

Norbornene-based monoliths. Buchmeiser's group developed norbornene-based monoliths via the ROMP approach.[88,89,110,146] A decrease in i.d. from analytical (3 mm)[89] to capillary (200 μm)[146] increased the resolution of oligodeoxynucleotides by two times, presumably due to better temperature control in the capillary during the preparation of the monolith. A variety of biomolecules, such as oligodeoxynucleotides, double-stranded DNA fragments and proteins, were separated using monolithic capillary columns in the RP LC mode.[146]

Styrene-based monoliths. The first demonstration of preparing a styrene-based monolith in a capillary was reported in 1998.[147] The capillary had an i.d. of 150 μm with a pulled 5-10 μm needle tip at one end, which served both as a separation unit and an electrospray device. The polystyrene monolith was formed inside the pulled capillary by thermal initiation at 65 ºC and used for separation and electrospray ionization of peptides and proteins. Equal or better chromatographic performance (i.e., resolution and signal strength) was obtained using the monolithic pulled capillary compared to a similar capillary packed with either C18 silica particles or polystyrene beads. Due to their hydrophobic nature, polystyrene monoliths are exclusively used in the RP chromatography mode.

Using a similar approach, several other groups have prepared polystyrene monoliths in the capillary format for RPLC of peptides, proteins and nucleic acids.[123,124,147-157] The preparation of polystyrene monoliths typically involved toluene and decanol as porogens. Huber's group improved polystyrene monoliths significantly by using THF and decanol as porogens.[124] They claimed that a large number of mesopores were formed because THF is a worse solvent than toluene. As a result, mass transfer resistance became much smaller. In fact, they demonstrated extremely efficient separation of proteins and peptides using their polystyrene monolithic columns. The peak width at half height for most proteins was <10 s.[148] Another noteworthy development of the polystyrene monolith was reported by Karger's group.[151] By preparing monoliths in 20 μm i.d. capillaries, the detection sensitivity was improved by 20 fold over 75 μm i.d. columns, enabling the detection of 10-40 fmol peptides. Excellent efficiency (100,000 plates/m) was obtained for RPLC of peptides. Attempts were also made to increase the hydrophobicity of the bare polystyrene monolith.[150] After the polystyrene monolith was prepared, an additional derivatization step, which involved Friedel-Crafts alkylation with chlorooctadecane in the presence of aluminum chloride as a catalyst, was performed to introduce C18 chemistry onto the monolith. However, as compared to the original polystyrene monolith, only marginal improvement in retention and peak shapes was obtained.

1.4 Significance and Content of this Dissertation

1.4.1 Significance

Liquid chromatography of proteins can be performed in two different ways: under denaturing conditions and under native conditions. The RP mode is in the first category while other modes of chromatography, such as ion exchange, affinity, size exclusion, and

hydrophobic interaction, belong to the second category. As reviewed in Section 1.2, RPLC is the most popular mode for proteomics studies. This is reasonable because RPLC can be directly coupled with MS, the most powerful detector for proteomics research at the present. In addition, RPLC is a well developed technique that has superior selectivity, efficiency, and resolution compared to other modes of chromatography. The rapid development of polystyrene monoliths for RP capillary liquid chromatography (CLC) of biomolecules (Section 1.3.3) confirmed the usefulness of this mode. However, other modes of chromatography are equally important for proteomics studies (see 2-D LC for proteomics in Section 1.2.2) because they are complementary to the RP mode. At present, polymer monoliths for these chromatographic modes are less developed.

For analysis of proteins in their native states, hydrophobic interaction is detrimental to most separations except for hydrophobic interaction chromatography. The PEGDA crosslinker has been proven to effectively resist nonspecific hydrophobic interactions of proteins (see Section 1.3.2). As a result, it contributes negligible hydrophobicity if used as a crosslinker to synthesize a monolith. It was another aim of this dissertation to investigate the use of PEGDA as a biocompatible crosslinker to prepare polymer monoliths for the analysis of peptides and proteins in their native states. Because PEGDA contributes insignificant hydrophobicity to the polymer monolith backbone, I hoped that highly efficient monoliths could be prepared using this novel crosslinker. With the development of highly efficient, high resolution capillary LC methods based on polymer monoliths, enhanced resolving power of 2-D LC should be realized, which would encourage more applications of LC in proteomics research.

1.4.2 Overview of this Dissertation

Chapter 2 reports the design, preparation and evaluation of an inert polymer monolith for use in SEC. PEGMEA was used as monomer and PEGDA as crosslinker. A macroscopically uniform monolith with low flow resistance was obtained using methanol and ethyl ether as porogens via photoinitiation. The optimized monolith was successfully applied to SEC of peptides. By replacing PEGMEA with ionizable monomers, monoliths with IE functionalities could be obtained. Chapters 3 and 4 report the synthesis of SCX monoliths for resolution of peptides and proteins. In Chapter 3, AMPS was used as the functional monomer. A ternary porogen, water/methanol/ethyl ether, was found suitable to prepare the poly(AMPS) monolith. Due to the use of the biocompatible PEGDA crosslinker, extremely sharp peaks were obtained for LC of both synthetic and natural peptides including a tryptic digest. A peak capacity of 179 was obtained using a shallow salt elution gradient. Although very successful, the main drawback of the poly(AMPS) monolith was its relatively strong hydrophobicity, i.e., 40% acetonitrile was required to suppress hydrophobic interactions for hydrophobic peptides. Chapter 4 deals with improvements in hydrophilicity by using two other commercially available sulfonic acid-containing monomers to prepare SCX monoliths: sulfoethyl methacrylate (SEMA) and vinyl sulfonic acid (VS). The hydrophobicities of the resulting monoliths were systematically evaluated. Results show that the poly(VS) monolith was the least hydrophobic among the three SCX monoliths studied. The poly(VS) monolith was applied to the separation of various proteins including lipoproteins using capillary LC. Five subclasses of high density lipoproteins were separated under a linear salt gradient. Chapter 5 outlines future proposed research using the PEGDA crosslinker.

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CHAPTER 2 PREPARATION AND EVALUATION OF POLY(POLYETHYLENE GLYCOL METHYL ETHER ACRYLATE-CO-POLYETHYLENE GLYCOL DIACRYLATE) MONOLITH FOR PROTEIN ANALYSIS[*]

2.1 Introduction

Minimal interaction of support matrix and analytes is desirable for separations such as gel electrophoresis and size exclusion chromatography of proteins. Proteins are well known to exhibit hydrophobic and/or ionic interactions with a variety of surfaces. Therefore, an inert material, which can significantly reduce or eliminate adsorption of proteins, would be very useful.

Known materials that resist protein adsorption include polysaccharide and polyacrylamide polymers; these enjoy wide application in gel electrophoresis and size exclusion separation of proteins.[1] An efficient method to address adsorption problems in capillary electrophoresis is to coat the capillary surface with such polymers.[2,3] In addition to polysaccharide and polyacrylamide, other neutral hydrophilic polymers have been investigated and found useful in capillary electrophoresis, such as polyvinyl alcohol,[4] polyethylene oxide,[5,6] polyvinylpyrrolidinone[7] and a copolymer of polyethylene glycol and polypropylene glycol.[8] All of these polymers are neutral and hydrophilic. A systematic study of protein adsorption on a variety of surface structures resulted in the conclusion that materials are protein compatible if they are neutral, hydrophilic, proton acceptors and not proton donors.[9-11]

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Other materials used in gel electrophoresis reported in 1992 by Zewert and Harrington are polyhydroxy methacrylate, polyhydroxy acrylate, polyethylene glycol methacrylate and polyethylene glycol acrylate.[12,13] To avoid the toxicities of acrylamide and bisacrylamide, and the difficulties associated with polyacrylamide gel electrophoresis of very hydrophobic proteins, such as bovine serum albumin or zein, polyethylene glycol methacrylate 200 in hydroorganic solvents was evaluated. Although there was no direct evidence to show the inertness of this material, successful electrophoresis of proteins demonstrated the protein compatibility of such polymers.

The inert polymers mentioned above are polymer gels that are soft in nature. These polymers can only be used in their swollen states because such polymers lose their permeabilities upon drying. Attempts have been made to prepare rigid beads with permanent porous structures from such polymers. Among these hydrophilic polymers, polyacrylamide is the only one that could form rigid beads by inverse suspension techniques using a high content of bisacrylamide as a crosslinker.[14] The use of a higher level of crosslinker accounted for the formation of rigid beads instead of soft particles.

As introduced in Chapter 1, monolithic materials offer an alternative to columns packed with small particles or beads. Attempts have been directed towards the synthesis of polyacrylamide monoliths. The first demonstration of preparing a poly(acrylic acid-co-methylene bisacrylamide) monolith was performed in 1989 by Hjertén's group.[15] However, the monolith was soft. Several years later, a rigid poly(acrylamide-co-bisacrylamide) monolith was reported in 1997 by Svec's group.[16] Several variables were studied to prepare a flow-through monolith with a mean pore diameter of ~ 1 μm. The porogens used for preparing the acrylamide-co-bisacrylamide monolith were dimethyl

sulfoxide and a long chain alcohol, such as heptanol or dodecanol. The concentration of initiator was also investigated to adjust the medium pore diameter of the monolith; a lower concentration of initiator increased the permeability of the resulting monolith as expected. Unfortunately, thermally initiated polymerization was used to prepare the monolith. As a result, 24 h was required to complete the polymerization at 1% initiator concentration.

In this Chapter, a protein compatible poly(polyethylene glycol methyl ether acrylate co-polyethylene glycol diacrylate) monolith (PEGMEA-co-PEGDA) was prepared by photo-initiated polymerization. Physical properties, such as pressure drop and swelling or shrinking in organic solvents, were characterized first, and then inertness in LC was evaluated by using a series of both acidic and basic model proteins under a variety of buffer conditions.

2.2 Experimental

2.2.1 Chemicals

Anhydrous methanol, anhydrous ethyl ether and ACS reagent hexanes were purchased from Mallinckrodt Chemicals (Phillipsburg, NJ), Fisher Scientific (Fair Lawn, NJ) and EMD Chemicals (Gibbstown, NJ), respectively. HPLC grade toluene and THF were from Mallinckrodt Chemicals, and Curtin Matheson Scientific (Houston, TX), respectively. All other solvents (cyclohexanol, dodecanol and dimethyl sulfoxide) were of analytical grade or better. Phosphate buffer solutions were prepared with deionized water from a Millipore water purifier (Molsheim, France) and filtered through a 0.22 µm filter. Thiourea (99.9%), 2,2-dimethoxy-2-phenylacetophenone (99%), 3-(trimethoxysilyl)propyl methacrylate (98%), ethylene glycol dimethacrylate (EDMA,

98%), polyethylene glycol methyl ether acrylate (PEGMEA, average molecular weight, Mn, ~454), and polyethylene glycol diacrylate (PEGDA, Mn ~575 and ~258) were supplied by Sigma-Aldrich (Milwaukee, WI) and used without further purification. Proteins {pepsin from porcine stomach mucosa, bovine serum albumin (>99%), myoglobin from horse skeleton muscle, α-chymotrypsinogen A from bovine pancreas, lysozyme from turkey egg white, and bovine serum albumin fluorescein isothiocyanate conjugate (FITC-BSA)} and peptides {neurotensin, angiotensin II fragment 3-8 and leucine enkephalin} were also obtained from Sigma-Aldrich.

2.2.2 Capillary Liquid Chromatography

UV transparent fused silica capillary tubing with 75 µm i.d. and 365 µm o.d. was supplied by Polymicro Technologies (Phoenix, AZ). Capillary LC experiments were performed with an ISCO Model 100 DM syringe pump (Lincoln, NE), 60 nL Valco internal sample loop (Houston, TX), a Linear Scientific UVis 203 detector (Reno, NV) and a Thermo Separations PC 1000 V3.0 software work station (Fremont, CA) for data collection and treatment. The PC 1000 provided retention times, peak heights, peak areas, asymmetry factors and column plate counts. On-column UV detection was performed at 214 nm. Chromatograms were transferred to an ASCII file and redrawn using Microsoft Excel (Redmond, WA).

2.2.3 Preparation of Polymer Monoliths

Before filling the UV transparent capillary with monomer mixture, the capillary inner surface was treated with 3-(trimethoxysilyl)propyl methacrylate (commercial identification number Z-6030) to ensure covalent bonding of the monolith to the capillary wall.[3,17] Briefly, the capillary was rinsed sequentially with acetone, water, 0.2 M NaOH,

water, 0.2 M HCl, water and acetone using a syringe pump for 30 min each at a flow rate of 5 µL/min. The washed capillary was then dried in an oven at 120 ºC for 1 h, filled with a 30% Z-6030 acetone solution, sealed with a rubber septum and placed in the dark for 24 h. The vinylized capillary was then washed with acetone at a flow rate of 5 µL/min for 10 min, dried using a stream of nitrogen for 3 h, and sealed with a rubber septum until used.

Four monolith recipes shown in Table 2.1 were designed to test protein compatibility, and the monoliths were prepared as follows. The monomer mixture was prepared in a 1 dram (4 mL) glass vial by admixing in sequence the initiator, monomer, crosslinker and porogens, and ultrasonicating for 5 min before use. Because of the low viscosity of the monomer solution, the introduction of monomer solution into the UV transparent capillary was facilitated by capillary surface tension. The capillary was then placed under a Dymax 5000AS UV curing lamp (Torrington, CT) for 10 min. For measurement of polymerization conversion (*vide infra*), a series of irradiation times were used. The UV curing lamp can produce an irradiation intensity of 200 mW/cm^2 in the wavelength range of 320 ~ 390 nm.

2.2.4 Laser Induced Fluorescence Imaging of FITC-BSA

Laser induced fluorescence (LIF) imaging of FITC-BSA in a series of capillary columns was performed in a device described elsewhere.[18] Briefly, the 488 nm line from an Ar ion laser was used to excite the sample, and the fluorescence was imaged using a Nikon Coolpix 995 digital camera (Tokyo, Japan).

2.2.5 Pressure Drop Measurements

Pressure drop measurements were performed using a Fisons Phoenix 20 CU HPLC pump (Milano, Italy) in the constant flow mode. Methanol and THF were pumped

Table 2.1. Composition of reagent solution for various monoliths used in this study. Recipes for monoliths 1 and 4 reagents were optimized. Units are in g.

No.	DMPA	PEGMEA	EDMA	PEGDA	Ethyl ether	Other
#1	0.008	0.32	0.48	-	-	0.38 cyclohexanol + 0.58 dodecanol + 0.24 hexanes
#2	0.008	-	0.8	-	1.20	-
#3	0.006	-	-	0.6	1.40	-
#4	0.006	0.15	-	0.45	1.10	0.30 methanol

through the monolithic column at flow rates of 4, 6, 8 and 10 µL/min, respectively, and the pressure drop for water was measured at 4 µL/min. After stabilizing, the pump pressure was recorded.

2.2.6 Polymerization Conversion Evaluation and Scanning Electron Microscopy

A bulk solution of 10 g optimized monomer mixture (monolith #4, Table 2.1) was prepared based on the procedure outlined in Section 2.2.3. An aliquot of 0.3 g of the monomer mixture was dispensed into a series of 1 dram (4 mL) glass vials and irradiated under the UV lamp for 10 s, 20 s, 30 s, 1 min, 2 min, 5 min, 10 min, and 30 min, respectively. The bulk monolith was carefully removed by breaking the glass vial, sliced into sections, Soxhlet extracted with methanol overnight and placed in a vacuum oven at 60 ºC overnight. The dried monolith material was weighed and compared with the combined weight of the monomer and crosslinker to obtain the conversion of monomer to polymer.

One of the dry monoliths (i.e., with 10 min irradiation time) was also used to obtain the SEM images. The monolith was sputtered with ~20 nm gold, and SEM images were taken using an FEI Philips XL30 ESEM FEG (Hillsboro, OR).

2.2.7 Inverse Size Exclusion Chromatography (ISEC)

The same liquid chromatographic system as described in Section 2.2.2 was used to run the ISEC. The mobile phase was THF and detection was at 254 nm. Narrow distribution polystyrene standards with molecular masses of 201, 2 460, 6 400, 13 200, 19 300, 44 100, 75 700, 151 500, 223 200, 560 900, 1 045 000, 1 571 000 and 1 877 000 were purchased from Scientific Polymer Products (Ontario, NY). A solution of 1 mg/mL polystyrene and toluene each in THF were chromatographed.

2.2.8 Protein Recovery Determination

A monolithic column with a total length of 80 cm and effective length of 60 cm was prepared with one detection window at 19 cm and the other at 60 cm from the column inlet. The detection window at 19 cm was created by carefully introducing an air bubble during introduction of the monomer solution. A mixture of protein and thiourea (an internal standard to calibrate any detection window response variation due to different background absorbances of the two detection windows) was injected into the monolithic column. Protein recovery was calculated by comparison of the calibrated protein peak area from the second detection window with that from the first one. The calibrated peak area of a protein was obtained by dividing the protein peak area by that of thiourea from the same detection window.

2.3 Results and Discussion

2.3.1 Crosslinker Influence on Inertness of the Monolith

Initially, EDMA was chosen as a crosslinker to prepare the PEGMEA monolith because EDMA has been widely used in the preparation of rigid porous polymer monoliths, such as butyl methacrylate, glycidyl methacrylate and 2-hydroxyethyl methacrylate.[19] However, the resultant monolith (monolith #1, Table 2.1) exhibited strong adsorption of FITC-BSA as shown in the LIF images (see Figure 2.1, A panels). To investigate the cause of adsorption of BSA in the poly(PEGMEA-co-EDMA) monolith, monolith #2 composed of pure EDMA was prepared with ethyl ether as porogen. Not surprisingly, the EDMA monolith had a strong fluorescence residue after introducing FTIC-BSA and flushing with 0.1 M phosphate buffer (pH 7.0) containing 0.5 M NaCl buffer (Figure 2.1, B panels). Because polyethylene glycol is known not to

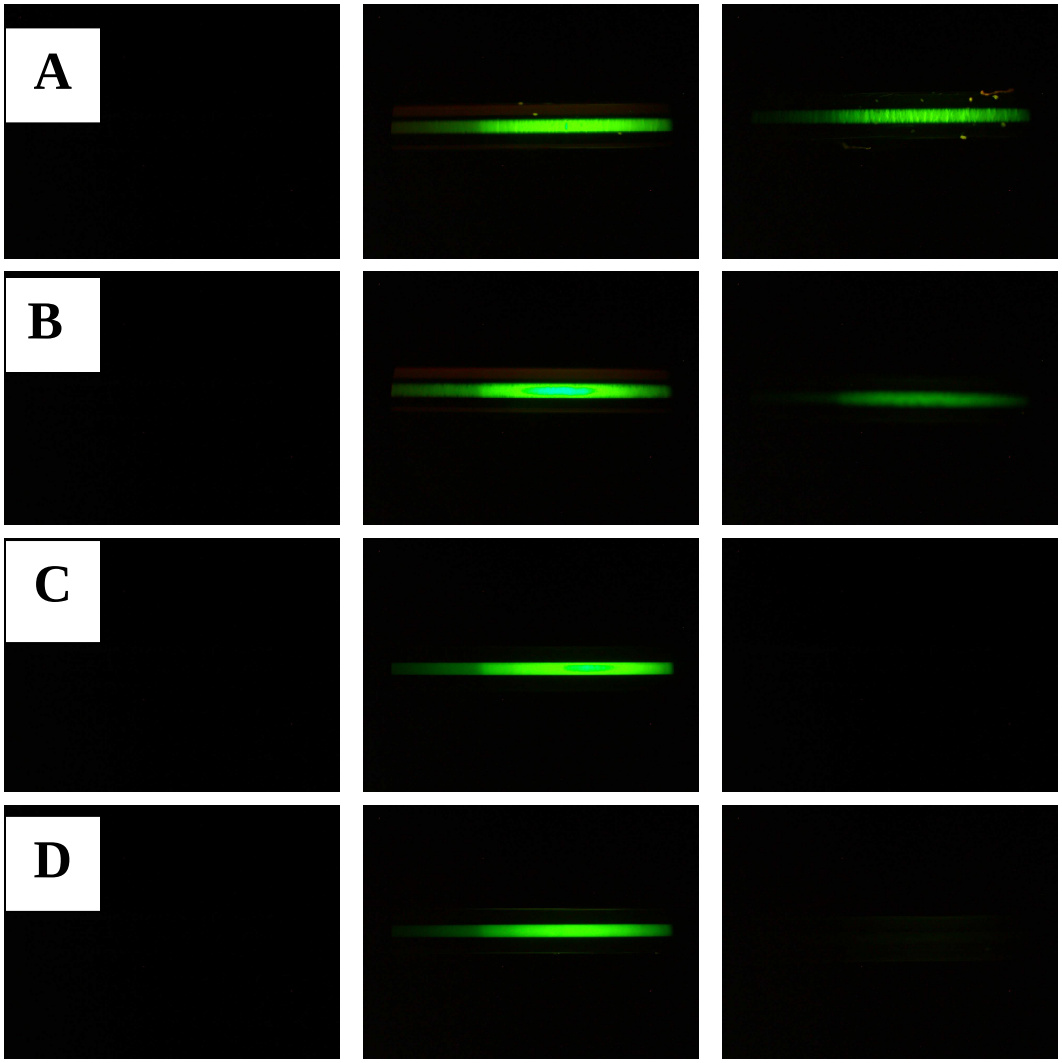


Figure 2.1. LIF images of the monolith before, during and after loading of FITC-BSA. Procedures: the LIF image was first recorded before loading of FITC-BSA for which a dark background was obtained for all monoliths; the monolithic column was loaded with 0.01 mg/mL FITC-BSA and the fluorescence image was taken; the monolithic column was then flushed with 100 mM (pH 7.0) phosphate buffer containing 0.5 M NaCl for 5 min under a linear flow velocity of ~4 mm/s, and the LIF image was obtained again. (A) PEGMEA/EDMA monolith; (B) EDMA monolith; (C) PEGDA Mn ~258 monolith; (D) PEGMEA/PEGDA monolith; the monomer recipes for all of the monoliths are listed in Table 2.1.

adsorb proteins, PEGDA was chosen as a crosslinker for the preparation of the PEGMEA monolith. Results of the use of PEGDA with Mn ~575 as crosslinker showed that the PEGMEA/PEGDA monolith did resist the adsorption of proteins (data not shown). Unfortunately, the resultant monolith was compressible upon application of >1000 psi buffer even though 75% crosslinker was used in the monomer recipe. This indicates that the PEGMEA monolith with long-chain PEGDA crosslinker yielded a soft monolith. However, replacement of PEGDA Mn ~575 with PEGDA Mn ~258 dramatically improved the rigidity of the monolith. From the fluorescence images (Figure 2.1, C panels) of this new polymer monolith #3, no obvious adsorption of FITC-BSA was observed. Therefore, PEGDA Mn ~258 was finally selected as the crosslinker to prepare the PEGMEA/PEGDA monolith (monolith #4, Table 2.1). A fluorescence test of the optimized PEGMEA/PEGDA monolith also showed no adsorption of FITC-BSA (see Figure 2.1, D panels).

2.3.2 Optimization of Porogen Composition

To be useful in flow-through applications, the monolith must have low flow resistance. Furthermore, for chromatographic use, a homogeneous monolith is critical for achieving high efficiency. Here, homogeneity refers to the uniformity of monolithic bed along both radial and axial directions. Because polymer monoliths are made of tiny globules which are connected together to form the continuous rod, they are microscopically heterogeneous. Thus, homogeneity in this dissertation refers to the uniformity of monolithic bed macroscopically. If the monolith is free of voids or cracks and its color is uniform (some monoliths had dark and light spots along the axial direction of the column, indicating that they were inhomogeneous) under examination of a microscope, this monolith is referred to as homogeneous, and *vice versa*. Therefore,

optimization was performed to prepare a homogeneous monolith with flow resistance as low as possible.

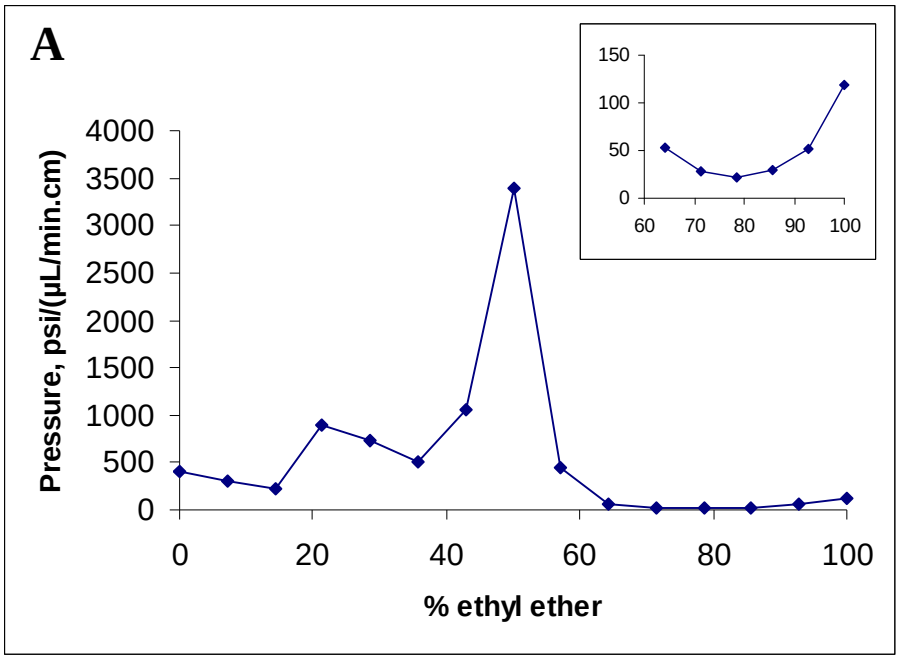
Five factors can be adjusted to change the pressure drop of the polymer monolith (see Section 1.3.2.3 for a detailed discussion about control of the porosity of the polymer monoliths). For the preparation of the PEGMEA/PEGDA monolith, when ethyl ether was used as porogen, the crosslinker had to be greater than 70% to make a rigid monolith. As a result, 75% PEGDA (crosslinker) and 25% PEGMEA (monomer) were used throughout the optimization of the monolith. The total monomer to porogen ratio was kept constant at 3:7 and the initiator concentration was 1% of the monomers. A variety of solvents were evaluated to prepare the PEGMEA/PEGDA monolith. First, 30% PEGMEA or PEGDA solutions (containing 1% DMPA as photoinitiator) in ethyl ether, hexanes, cyclohexanol, dodecanol, dimethyl sufoxide, methanol, toluene or THF were prepared and placed under the UV lamp to find the best porogens for the PEGMEA/PEGDA monolith. PEGMEA and PEGDA both dissolved well in all solvents except hexanes. For PEGMEA, dodecanol formed a white solid material, and dimethyl sulfoxide resulted in a transparent soft gel. All other solvents formed a dense liquid after 10 min UV irradiation. For PEGDA, dimethyl sulfoxide and THF resulted in transparent solid materials, which indicated the formation of an extremely small pore structure. All other solvents yielded a white solid, except toluene which formed a yellow rigid solid.

A 2 cm long monolith prepared in a UV transparent capillary was used to test the pressure drop of the monolith composed of only PEGDA. Ethyl ether and methanol porogens yielded a porous monolith, whereas all others would not allow flow at 4500 psi methanol. This is also in contrast to other reported monoliths for which a long-chain

alcohol, such as cyclohexanol or dodecanol, was used to prepare a porous monolith.[16,17,20] Therefore, methanol and ethyl ether were selected as porogens to optimize the preparation of the PEGMEA/PEGDA monolith. Since both PEGMEA and PEGDA do not dissolve in hexanes, and both dissolve in mixtures of hexanes and methanol or ethyl ether, hexanes was selected as a macroporogen for the monolith. Thus, the final porogens selected were methanol, ethyl ether and hexanes.

Three porogen mixtures, i.e., methanol/hexanes, ethyl ether/hexanes and methanol/ethyl ether, were optimized for the desired homogeneity and flow resistance of the monolith. The pressure drop of the monolith was found to be insensitive to the ratio of methanol and hexanes or ethyl ether and hexanes. Fortunately, the flow resistance of the monolith was found to be strongly dependent on the ratio of methanol and ethyl ether (see Figure 2.2, panel A). For the optimized recipe (monolith #4), i.e, 7.5% PEGMEA, 22.5% PEGDA, 15% methanol and 55% ethyl ether, the pressure drop was 21 psi/(μL/min·cm) when methanol was used as pumping liquid in a 75 μm i.d. monolithic capillary. For a 20 cm × 75 μm i.d. capillary, this corresponds to a linear flow velocity of 3.78 mm/s of methanol at a pressure of 420 psi.

The SEM images of the optimized PEGMEA/PEGDA monolith are shown in Figure 2.3. From the images, a rough estimation of 0.2~0.3 μm diameter globule size could be made. If these globules were tightly packed as in a packed column, pressure drop would be tremendously high. Thus, the low flow resistance (21 psi/(μL/min·cm)) would be contributed from large through-pores or large porosity of the monolith. It may also be a result of a high degree of connectivity of the through-pores, which has been shown to be an important factor affecting the permeability of a monolith in theoretical



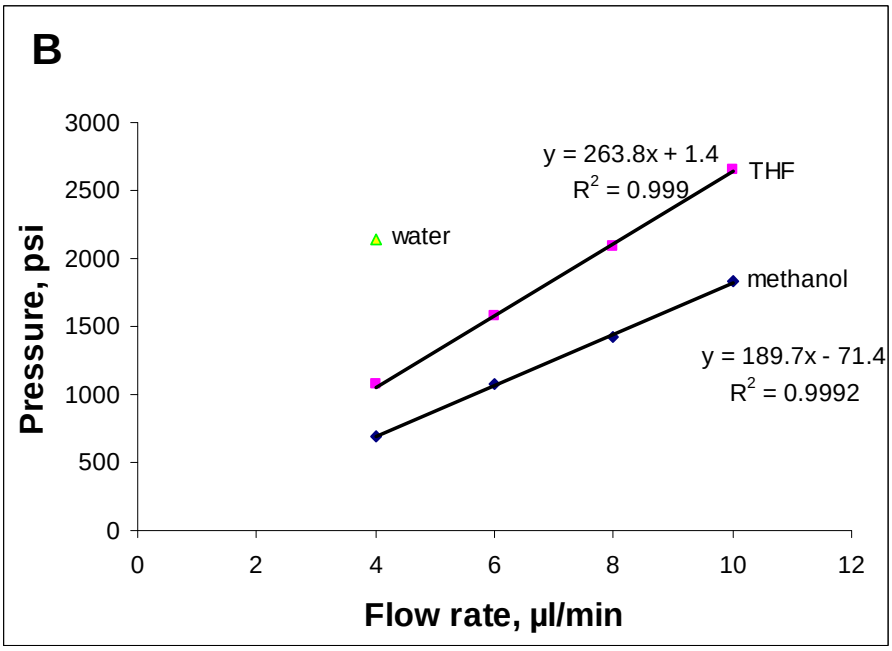


Figure 2.2. Flow resistance of the PEGMEA/PEGDA monolith. (A) Pressure drop dependence of the monolith on the percent of ethyl ether; inset is the magnification of the section for ethyl ether of 60 ~ 100%; (B) linear pressure dependence of the optimized PEGMEA/PEGDA monolith on the flow rates of water, THF and methanol.

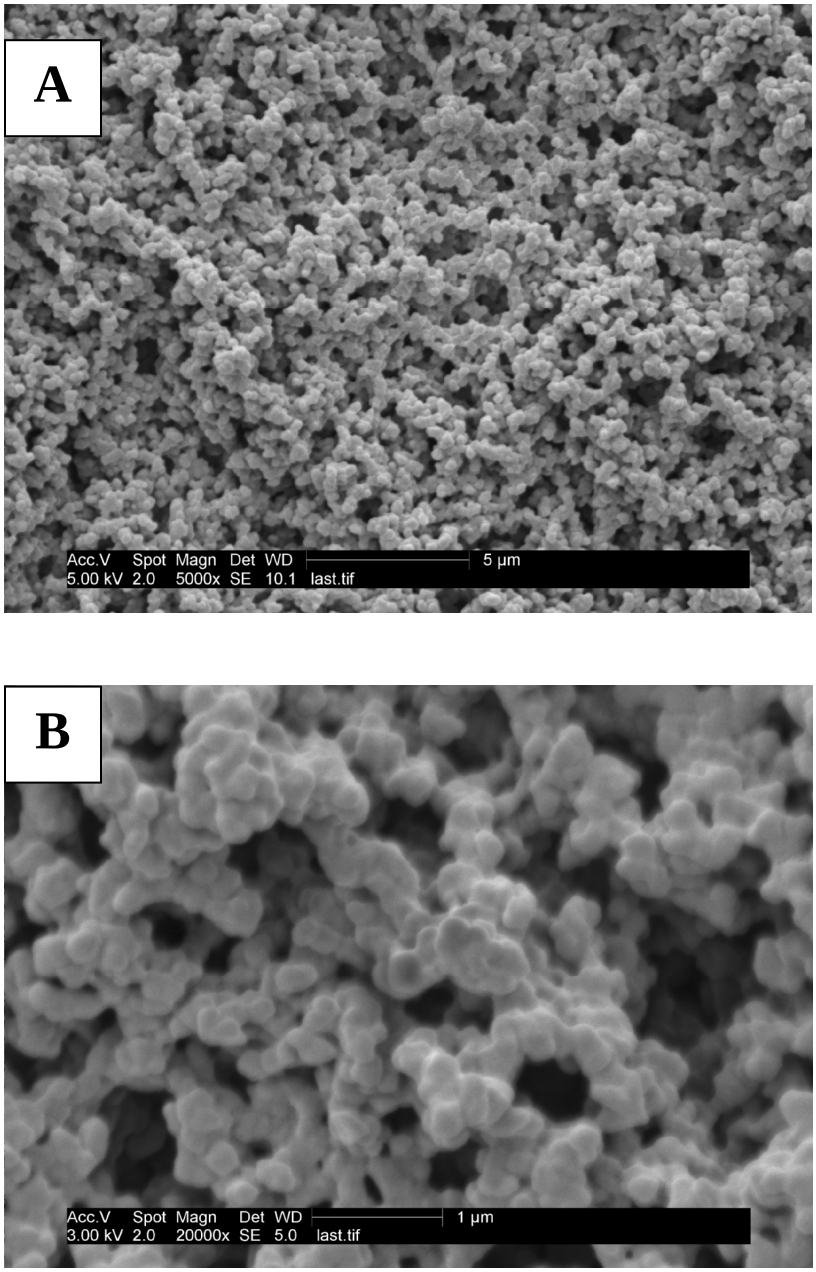


Figure 2.3. SEM images of the optimized PEGMEA/PEGDA monolith. (A) 5000 magnification; (B) 20000 magnification.

studies.[21,22] The shrinking of the monolith in methanol (*vide infra*), could also lead to low flow resistance.

2.3.3 Kinetics of Polymerization of PEGMEA/PEGDA

Both thermal and UV-initiated polymerization can be used to prepare polymer monoliths. Typically, thermally initiated polymerization uses AIBN as initiator, and polymerization proceeds slowly, normally taking 24 h.[16,17] In contrast, photo-initiated polymerization can be finished in minutes.[20] The kinetics of polymerization of PEGMEA/PEGDA are shown in Figure 2.4. Over 90% of the monomer was converted into polymer in 2 min, and complete conversion of the monomer was finished in ~10 min. The high irradiation intensity (200 mW/cm^2) used in my experiments, which is ~10 fold greater than a previously reported UV curing system,[20] contributed to the fast polymerization of the monomer solution.

2.3.4 Physical Properties of the PEGMEA/PEGDA Monolith

A quantitative index, the swelling propensity (*SP*), was defined by Nevejans and Verzele[23] to characterize the swelling and shrinking properties of a packed bed:

$$SP = \frac{p(solvent) - p(H_2O)}{p(H_2O)}$$

where *p* takes into account the viscosities of the solvent, and is defined as the ratio of pressure over solvent viscosity. By definition, $SP = 0$ if no swelling or shrinking occurs, $SP > 0$ if there is swelling, and $SP < 0$ if the packed bed shrinks. From Figure 2.2B, the *SP* values for methanol and THF were calculated to be -0.44 and -0.08, respectively, assuming viscosities for water, methanol and THF of 1.025, 0.59 and 0.55 cP, respectively, at room temperature (data from the online CRC Handbook at 25 ^{o}C). This indicates that no significant shrinking or swelling of the PEGDA/PEGMEA monolith in

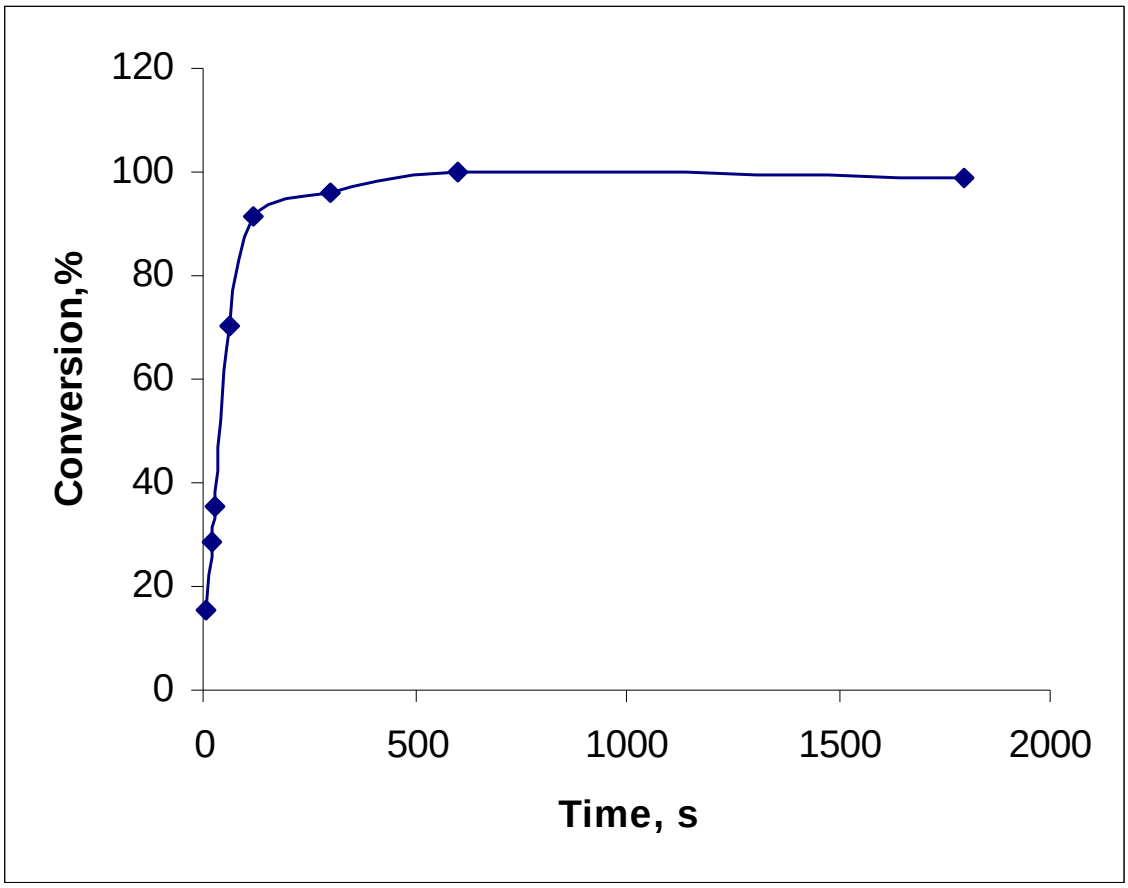


Figure 2.4. Rate of conversion of monomers to polymer. For experimental conditions, see Section 2.2.6.

THF was observed. Since THF can dissolve most hydrophobic polymers, the stability of the monolith in THF indicates that the monolith is relatively non-hydrophobic. However, shrinking of the monolith did occur in methanol, which unexpectedly had a positive effect because it improved the column permeability while maintaining a rigid structure. As shown in Figure 2.2B when 2600 psi THF was applied to the monolithic column (4 cm × 75 μm i.d.), no change in pressure drop was observed. This indicates high stability of the monolith, which is a result of the high concentration of crosslinker used in the monomer recipe.

2.3.5 Chromatographic Evaluation of the Monolith

Proteins were carefully selected to investigate the possibility of hydrophobic or ionic interaction with the monolithic material. Acidic (pepsin), basic (lysozyme) and hydrophobic (BSA) proteins were included. Several peptides with different molecular masses were also used to explore the elution mechanism of the monolithic column. Table 2.2 lists the molecular masses and pIs of the proteins and peptides used in this study.

Phosphate buffers, (a) pH 7.0 with concentrations of 10, 20, 50, 100, 200, and 500 mM; (b) 10 mM concentration with pH values of 2.0, 4.0, 6.0, 8.0, 10.0, and 12.0; and (c) 100 mM concentration (pH 7.0) with additives of 0.5 M Na_2SO_4, 0.5 M NaCl, 10% ethylene glycol or 10% acetonitrile, were used to elute the proteins. Buffers (a) and (c) were used to explore the possible hydrophobic interaction of the proteins with the monolith, and buffer (b) was used to investigate the possibility of any ionic interactions. In all cases, the proteins eluted earlier than thiourea. This indicates an SEC elution mode.

When buffer (a) was used, splitting of all of the protein peaks was observed when the buffer concentration was increased to 500 mM. However, the elution times were kept

Table 2.2. Proteins and peptides used in this study.

Analyte	Molecular mass	pI
bovine serum albumin[a]	68 000	4.7
pepsin[a]	34 000	< 1
α-chymotrypsinogen A[a]	24 000	8.8
myoglobin[a]	17 500	7.1
lysozyme[a]	14 000	11.0
neurotensin[b]	1 672.9	9.5
angiotensin II fragment 3-8[b]	774.9	7.8
Leucine enkephalin[b]	555.6	5.9

[a] The molecular mass and isoelectric point (pI) of proteins were obtained from "Schmidt, Jr., D. E.; Giese, R. W.; Conron, D.; Karger, B. L. *Anal. Chem.* **1980**, *52*, 177-182."

[b] The molecular mass of peptides were read from the label of the chemicals provided by Sigma-Aldrich, and the pI values were obtained from EMBL Heidelberg European Molecular Biology Laboratory program http://www.embl-heidelberg.de/cgi/pi-wrapper.pl).

nearly constant for the proteins investigated within experimental error (except for the 500 mM buffer because two retention times were obtained due to peak splitting). Buffer (c), 0.5 M Na_2SO_4 in 100 mM (pH 7.0), also caused splitting of the protein peaks. This indicates possible hydrophobic interaction of the proteins with the monolith. However, 10% ethylene glycol or even 10% acetonitrile in buffer (c) (in which α-chymotrypsinogen A forms a precipitate in the buffer with acetonitrile as an additive) provided elution of proteins in a similar manner as 0.5 M NaCl additive. Not only were protein profiles similar to each other when buffer (c) was used, but the elution times were also close to each other within experimental error. This strongly suggests that hydrophobic interactions, if any, would not be very significant.

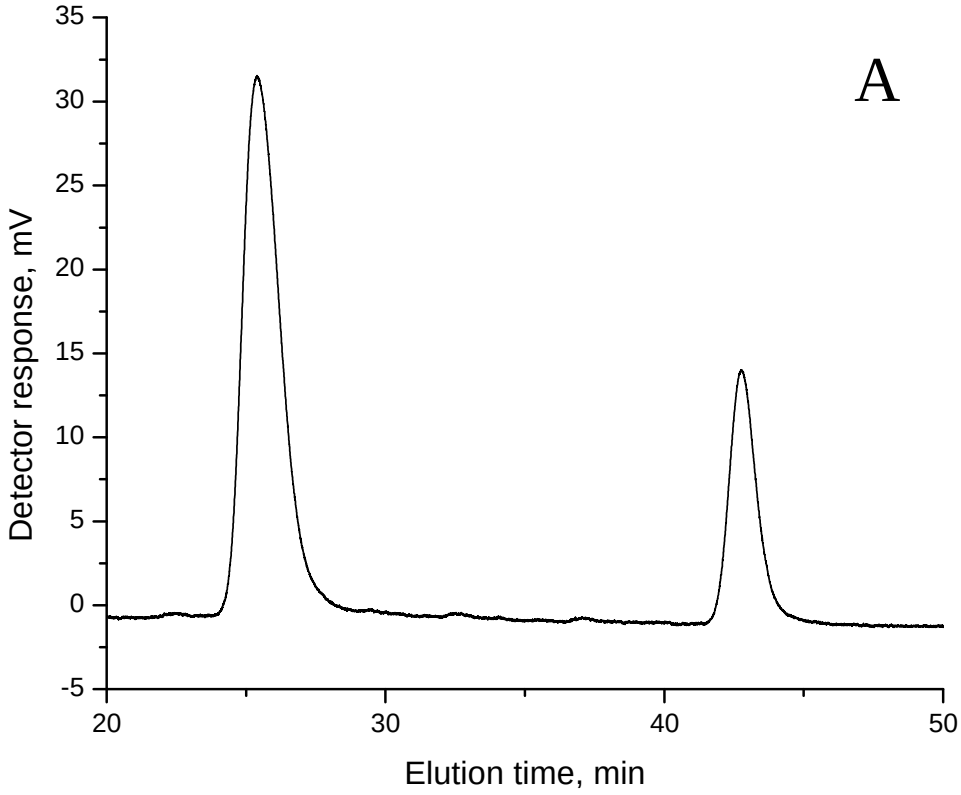
The pH of buffer (b) was found to strongly affect the protein peak profiles. At pH 2.0, all proteins showed some degree of tailing, and α-chymotrypsinogen A and lysozyme exhibited peak splitting. Above pH 4.0, the symmetry of the protein peaks improved, except that lysozyme split into two peaks at all pH values. This indicates a possible ionic interaction between lysozyme and the monolith. However, as shown above, this weak ionic interaction disappeared when buffer (c) with 0.5 M NaCl additive (weak buffer ionic strength) was used.

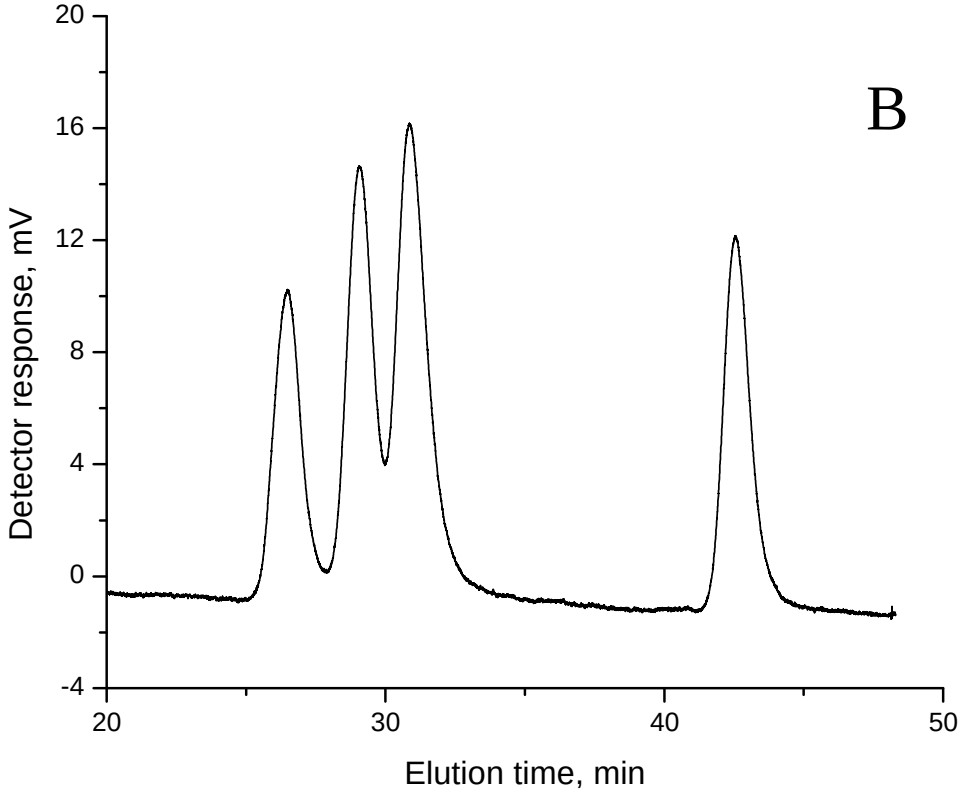
In summary, good peak symmetries for all of the proteins were obtained with the use of buffer (c) with 0.5 M NaCl additive, i.e, 100 mM phosphate (pH 7.0) buffer containing 0.5 M NaCl, a condition often employed in high performance SEC of proteins. This indicates that the PEGMEA/PEGDA monolith had insignificant hydrophobic or ionic interactions with the proteins. It should be mentioned that all of the experiments described above employed high mobile phase flow rate (~1.10 mm/s) so that proteins

eluted within ~3 min from a ~20 cm monolithic column. Such a flow rate facilitates the screening of buffers at the expense of skewing protein peaks. If a lower flow rate was used, improvement in peak symmetry could be achieved.

Figure 2.5A shows a chromatogram of a mixture of proteins and thiourea using low mobile phase flow rate. No separation between these proteins was observed. Injections of each protein in the same column under the same chromatographic conditions revealed that all of the five proteins with different molecular masses and pIs had almost the same elution time (Figures not shown). In contrast, for the chromatography of three peptides, a moderate separation was achieved, although they were not baseline resolved (see Figure 2.5, panel B). A mixture of α-chymotrypsinogen A, the three peptides and thiourea was thus injected into the column, and the chromatogram is shown in Figure 2.5 panel C. Although the elution time for proteins was a little earlier than neurotensin (compare Figures 2.5 panels A and B), coelution of α-chymotrypsinogen A and neurotensin was observed. Since I aim at developing an inert monolith with pressure drop as low as possible while keeping it homogeneous macroscopically, no further optimization of pore size distribution was attempted for SEC of proteins.

It should be mentioned that the protein peak shown in Figure 2.5A was a coelution profile of five proteins, and thus it was relatively broad. Chromatography of each of the five proteins revealed column efficiencies of 6 000 ~ 8 000 plates/m and asymmetric factors of 1.3 ~ 1.5. For peptides and thiourea, elution of each of them separately under otherwise the same chromatographic conditions resulted in column efficiencies of 9 000 ~ 20 000 plates/m and asymmetric factors of <1.1. This roughly follows the trend of SEC. In the SEC of proteins, significantly lower plate counts for


A
Detector response, mV
35
30
25
20
15
10
5
0
-5
20
30
40
50
Elution time, min


B
Detector response, mV
20
16
12
8
4
0
-4
20
30
40
50
Elution time, min

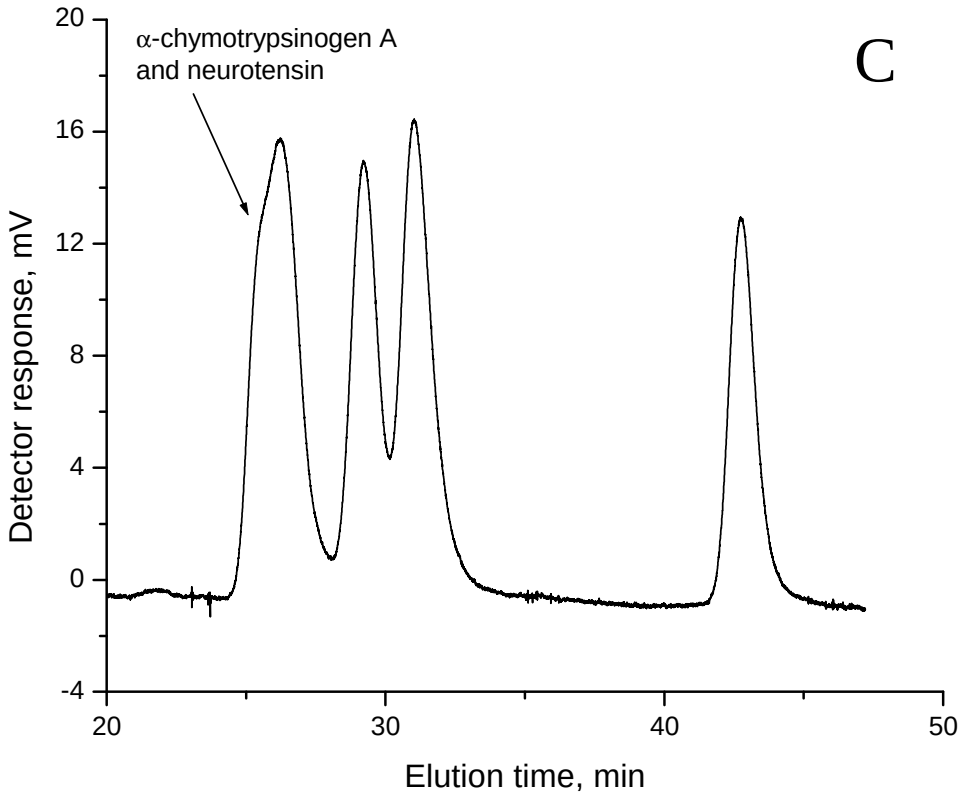


Figure 2.5. Chromatograms of mixtures of several peptides, proteins and thiourea under isocratic elution. Conditions: 60 cm × 75 μm i.d. PEGMEA/PEGDA monolithic column; 100 mM phosphate buffer (pH 7.0) containing 0.5 M NaCl operated under 600 psi constant pressure; online UV detection at 214 nm; 0.15 mg/mL of thiourea, 0.8 mg/mL of each protein, and 0.5 mg/mL of each peptide; (A) mixture of bovine serum albumin, pepsin, α-chymotrypsinogen A, myoglobin, lysozyme and thiourea; (B) mixture of neurotensin, angiotensin II fragment 3-8, leucine enkephalin and thiourea (in elution order); (C) mixture of α-chymotrypsinogen A, neurotensin, angiotensin II fragment 3-8, leucine enkephalin and thiourea; for physical properties of the proteins and peptides, see Table 2.2.

proteins than for small molecules have been observed due to the lower diffusion coefficients of the macromolecules. The typical plate counts in modern conventional SEC (column dimensions of 250 mm × 4.6 mm i.d.) ranged from 8,000 plates/m for proteins (i.e., amylase) to 34,000 plates/m for small molecules (i.e., glycyl tyrosine).[24] For example, a plate count in SEC for α-chymotrypsinogen A was estimated to be ~5,600 plates/m based on a previously published chromatogram.[25] Thus, the plate counts achieved for proteins in this study with the use of the polymer monolith are acceptable. Furthermore, a plate count of 2240 ~ 6400 plates/m was reported in monolithic SEC of polystyrenes in THF.[26]

2.3.6 ISEC Characterization of the PEGMEA/PEGDA Monolith

To clarify the separations of proteins and peptides as shown in Figure 2.5, the porosity and pore size distribution of the PEGMEA/PEGDA monolith were investigated by ISEC. ISEC was initially used to characterize the structure of a packed bed with known probe compounds, e.g., polystyrene standards with narrow molecular mass distribution.[27] Guiochon and coworkers were among the first to use ISEC to characterize the porous structure of silica monoliths.[28] They defined several terms to describe the structure of a monolithic bed, such as total porosity ε_t, external porosity ε_e and internal porosity ε_i. Based on the ISEC plot, a pore size distribution of a monolith could also be derived assuming a simple correlation of $M_w = 2.25(10d)^{1.7}$, where M_w is the molecular mass of the polystyrene standard, and *d* is the diameter of the polystyrene standard in nm. Following the method of Gouichon et al.,[28] I obtained the ISEC plot of the PEGMEA/PEGDA monolith which is shown in Figure 2.6A.

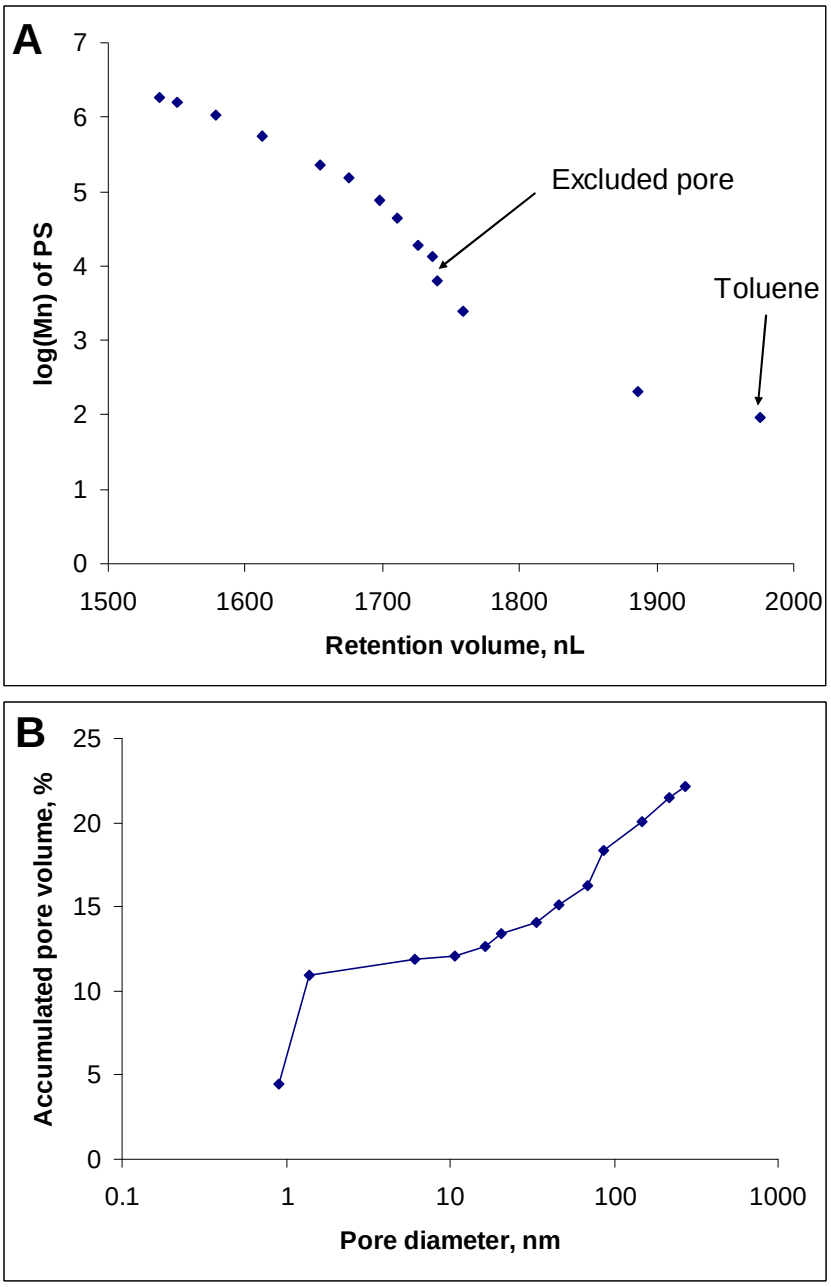


Figure 2.6. ISEC plot (A) and accumulated pore size distribution (B) for the PEGMEA/PEGDA monolithic column. Conditions: 59.3 cm × 75 µm i.d. monolithic column; THF mobile phase operated under a constant pressure of 1500 psi, resulting in a flow rate of 0.45 µL/min; online detection at 254 nm; in (A), toluene was used as a small molecule to determine the total porosity of the column; the excluded pore was approximately the intersection point of the interpolated straight lines corresponding to the internal and external pore zones.

The retention volumes shown in Figure 2.6 were the corrected retention volumes taking into account the extracolumn volume of the chromatographic system, which was measured to be 248 nL including the 60 nL internal sample loop. From Figure 2.6A, the total porosity was calculated to be 75.4%, which is in agreement with the percent of porogen content in the monomer recipe (monolith #4 in Table 2.1, 70% porogen). The excluded molecular mass was estimated to be 10^4, which corresponds to 14 nm. The external porosity was thus calculated to be 66.3% and the internal porosity was 9.1%. The relatively large total porosity (75.4%) accounts for the low flow resistance of the monolithic column.

The accumulated pore size distribution curve was derived from the ISEC calibration curve, and is shown in Figure 2.6B. The pore volume fraction corresponding to pores larger than 304 nm was 77.8% (not drawn in the figure), and 7.0% for pores between 50 and 304 nm. The pore volume fraction for micropores (< 2 nm) was 10.9%, and only 4.2% for mesopores (2 nm ~ 50 nm). It can be seen that most of the pore volume fraction came from pores larger than 304 nm. The mesopore volume fraction was very small (4.2%), and the pore volume fraction in the range of 1.4 ~ 10.8 nm was only 1.1%. Because the Stokes' radius for proteins in the molecular mass range of 10 K ~ 70 K is between 1.5 ~ 3.6 nm (data are from http://itsa.ucsf.edu/~hdeacon/Stokesradius.html), the monolith would predict no separation of the proteins used in this study. This explains the coelution of the proteins shown in Figure 2.5A. In contrast, the pore volume fraction of micropores was relatively large (10.9%), and the curve (Figure 2.6B) in this pore size range was sharp. These two characteristics explains the separation of peptides (Figure 2.5B). Although the molecular mass difference between proteins and peptides is huge, the difference between the pore volumes corresponding to excluded proteins and peptides is

nevertheless small, as can be seen in Figure 2.6B. This unique pore size distribution of the monolith clarifies that α-chymotrypsinogen A coeluted with neurotensin (Figure 2.5C).

In summary, the PEGMEA/PEGDA column shows SEC elution of peptides and proteins. The larger the molecule, the earlier the elution. However, due to the small pore volume fraction in the mesopore range of the monolith, separation of proteins could not be achieved for this monolithic column.

2.3.7 Protein Recovery Evaluation

To further evaluate the protein adsorption properties of the PEGMEA/PEGDA monolith, a protein recovery experiment was performed. In conventional HPLC, the peak areas of a compound eluted from a packed column and stainless steel tubing are compared.[25,29] Because a strong dependence of peak area on mobile phase flow rate was observed in my capillary liquid chromatographic experiments, a direct comparison of the protein peak areas from monolithic and open tubular fused silica capillaries would not provide reliable data for calculating protein recovery. In contrast, the two detector method[30] or modified two detection window method[31,32] in capillary electrophoresis would be applicable for measuring protein recovery in the capillary format because peak areas are measured in one run and variations in detector or detection window responses are taken into account.

In my work, the two detection window method was used to perform recovery experiments. Thiourea was used as an internal standard to calibrate the detection window response variation. The recoveries for pepsin, BSA, myoglobin, α-chymotrypsinogen A, and lysozyme were 98.0, 99.6, 103.5, 99.2, and 98.7%, respectively. This provided direct

evidence that the PEGMEA/PEGDA monolith does not adsorb any significant amount of proteins under the conditions of 100 mM phosphate buffer (pH 7.0) containing 0.5 M NaCl.

2.4 Conclusions

A non-adsorptive monolith for proteins, PEGMEA/PEGDA, was prepared using methanol and ethyl ether as porogens. Complete conversion of the monomer to the polymer monolith could be finished in 10 min. The polymer monolith had very low flow resistance, and was macroscopically homogeneous. Protein recovery approached 100% if 100 mM phosphate pH 7.0 buffer containing 0.5 M NaCl was used as mobile phase. No significant ionic or hydrophobic interactions with proteins were found.

Another feature of this monolith is that it did not discriminate the elution of several proteins (molecular weights from 14 K to 67 K) studied. Together with the homogeneity and low flow resistance characteristics, the monolith would be very useful in situations requiring an inert material for protein analysis, such as in flow counteracting capillary electrophoresis[33,34] or electric field gradient focusing,[18] in which the required hydrodynamic flow produces band broadening. By incorporating an inert material in the separation channel, sharpening of the protein bands is expected while maintaining the original separation/focusing mechanism. Currently, the incorporation of such a monolith into the separation/focusing channels of electric field gradient focusing devices[18] is under investigation. For SEC of proteins using this monolith, a reduction in through-pore diameter and optimization of the pore volume in the mesopore range must be accomplished. Unfortunately, this would be accomplished with a concomitant increase in flow resistance of the monolith.

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CHAPTER 3 EFFICIENT POLYMER MONOLITH FOR STRONG CATION-EXCHANGE CAPILLARY LIQUID CHROMATOGRAPHY OF PEPTIDES[*]

3.1 Introduction

Strong cation exchange (SCX) is an extremely important mode of ion exchange chromatography for analyzing peptides and proteins.[1] The utility of an SCX column, which often contains sulfonic acid groups, lies in its ability to maintain negative charge even under acidic buffer pH conditions (e.g., pH ~3). Under such conditions, most peptides bear positive charge due to the presence of positively charged basic residues (e.g., Arg, His and Lys), terminal amino groups, uncharged acidic residues (e.g., Glu and Asp), and terminal carboxyl groups. SCX chromatography is, therefore, generally applicable for peptide analysis when operated in acidic buffer pH. On the other hand, when the buffer pH is in the neutral pH range, SCX can only be applied to the analysis of basic peptides or proteins.

Particle based SCX columns received considerable interest for peptide analysis in the 1980s because of the complementary selectivity to reversed-phase chromatography.[2-9] For example, the retention of peptides on the PolySulfoethyl A SCX column was found to be monotonically related to the charge of the peptides.[5,7] Hodges et al.[2] designed several types of peptide standards to evaluate three commercially available SCX columns. They found that retention of peptides was not only related to charge, but also

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peptide chain length. The retention of peptides was empirically linearized under conditions in which hydrophobic interactions were suppressed. Peptide mapping of protein digests was also investigated by the use of two or three-dimensional chromatography, in which ion exchange was often followed by reversed-phase chromatography.[3,6,8,9]

Monolithic materials have received considerable interest due to ease of preparation and enhanced mass transfer.[10-15] Excellent reviews[11-13] have appeared describing applications of polymer monoliths in liquid chromatography of both small molecules and macromolecules. To date, a variety of polymer monoliths have been developed,[16-23] with efforts directed mainly towards reversed-phase chromatography.

Polymer monoliths have been extended to include SCX chromatography. To introduce sulfonic acid groups into the monolith backbone, several approaches have been reported, including adsorption of surfactants,[24-26] grafting of a sulfonic acid-containing monomer,[27-29] functionalization of a reactive monolith with sodium sulfite,[30] and copolymerization.[17,18,31-35] For example, surfactant (e.g., sodium dodecyl sulfate) has been dynamically adsorbed into an in-situ synthesized polymer monolith by hydrophobic interaction, where the other end of the surfactant serves as the SCX functional group.[26] Although this approach was simple, it was demonstrated for ion exchange of small ions only, likely due to the inherent strong hydrophobicity of the long surfactant chain.

Another method to introduce the sulfonic acid group is to graft a sulfonic acid-containing monomer on a bulk polymer monolith.[27-29] Svec et al.[27] demonstrated the feasibility of grafting 2-acrylamido-2-methyl-1-propanesulfonic acid (AMPS) onto poly(butyl methacrylate-co-ethylene glycol dimethacrylate) monolith by photoinitiated

hydrogen abstraction. Using catalyst initiated free-radical grafting polymerization,[28] AMPS was also grafted into a hydrolyzed poly(glycidyl methacrylate-co-ethylene glycol dimethacrylate) monolith, and the grafted monolith was successfully used for SCX of proteins. Furthermore, thermally initiated grafting of a zwitterionic sulfobetaine into a poly(trimethylolpropane trimethacrylate) monolith was also performed and investigated for protein separation.[29]

Functionalization of a reactive monolith is another strategy to introduce a sulfonic acid group. Ueki et al.[30] synthesized a poly(glycidyl methacrylate-co-ethylene glycol dimethacrylate) monolith. The reaction of the epoxy group in the glycidyl methacrylate with sulfite introduced the desirable sulfonic acid functionality onto the polymer monolith surface. The functionalized monolith was used for SCX of inorganic cations.

A more straightforward method to introduce the sulfonic acid group into a polymer monolith is by copolymerization. A sulfonic acid-containing monomer was copolymerized with a crosslinker in the presence[17,18,31-34] or absence[35] of a bulk monomer. AMPS was copolymerized with a bulk monomer butyl methacrylate and a crosslinker ethylene glycol dimethacrylate to generate electroosmotic flow in electrochromatography.[31,32] However, no attempts were made to use these monoliths for SCX liquid chromatography. This is probably due to the low amount of AMPS used (typically < 5%), which is insufficient for SCX of cations. The high percentage of butyl methacrylate would also lead to unwanted strong hydrophobic interaction. Interestingly, after ionic adsorption of aminated latex nanoparticles, such monoliths were successfully demonstrated for ion exchange of small anions[33] and saccharides.[34]

Although direct copolymerization without the use of a bulk monomer is the simplest method for monolith synthesis, the only reported example of this is the copolymerization of 2-(sulfooxy)ethyl methacrylate and ethylene glycol dimethacrylate for electrochromatography of peptides.[35] However, due to the relatively low amount of 2-(sulfooxy)ethyl methacrylate used in the monolith recipe (~17% total monomers), the resulting monolith showed strong hydrophobicity. The separation of model peptides (2 or 3 residues) exhibited reversed-phase rather than ion exchange behavior.

The lack of reports on direct copolymerization of a sulfonic acid-containing functional monomer with a crosslinker for SCX mainly results from two reasons. First, a new optimization must be performed in order to obtain the new polymer monolith although the composition of the monolith is simpler. Second, a sulfonic acid-containing monolith is believed to swell excessively in aqueous buffer.[30-32,34] Thus, the stability of a monolith composed of a high percentage of sulfonic acid-containing monomer is questionable. In this study, the preparation of a stable polymer monolith by direct copolymerization of polyethylene glycol diacrylate and a high amount (40%) of AMPS was demonstrated for SCX liquid chromatography of peptides for the first time. It was hoped that this new polymer monolith could dramatically improve peak capacity of ion exchange chromatography in which ion exchange of peptides is often considered relatively slow and less efficient than reversed-phase liquid chromatography for proteomics studies.[36]

3.2 Experimental Section

3.2.1 Chemicals and Reagents

2,2-dimethoxy-2-phenylacetophenone (DMPA, 99%), 3-(trimethoxysilyl)propyl methacrylate (98%), AMPS, polyethylene glycol diacrylate (PEGDA, Mn ~258), and

ethylene glycol dimethacrylate (EDMA) were purchased from Sigma-Aldrich (Milwaukee, WI) and used without further purification. Synthetic peptide standard CES-P0050 was obtained from Alberta Peptides Institute (Edmonton, Alberta, Canada). Bradykinin fragment 1-7, peptide standard P2693 and its nine components were from Sigma-Aldrich. Protein standards (myoglobin from equine skeletal muscle, cytochrome c from bovine heart, and lysozyme from chicken egg white) were also obtained from Sigma-Aldrich. Porogenic solvents for monolith synthesis and chemicals for mobile phase buffer preparation were HPLC or analytical reagent grade.

For digestion of β-casein (Sigma-Aldrich), 1 mL of β-casein digestion solution, which contained 50 μL of 1 M Tris pH 8.0 (99.9% purity, Fisher Scientific, Fair Lawn, NJ), 10 μL of 0.1 M $CaCl_2$ (EM Science, Cherry Hill, NJ), 20 μL of sequencing grade modified trypsin (Promega, Madison, WI), 100 μL of 2 mg/mL β-casein, and 820 μL of Mili Q water, was incubated at 37 ºC in a Shake' N' Bake hybridization oven (Boekel Scientific, Feasterville, PA) overnight. The digest was quenched by acidifying with formic acid. The β-casein digest was then desalted using a Strata-X 33 μm polymeric sorbent column (Phenomenex, Torrance, CA), following the manufacturer's protocol. The eluent from the desalting column was lyophilized in a Centrivap cold trap (LabConco, Kansas City, MO), re-suspended in 20 μL of gradient elution starting buffer, and centrifuged using an Eppendorf centrifuge (Brinkmann, Westbury, NY) at 10,000 rpm for 3 min before injection.

3.2.2 Polymer Monolith Preparation

Before filling the UV transparent capillary (75 μm i.d., 360 μm o.d., Polymicro Technologies, Phoenix, AZ) with monomer solution, the capillary inner surface was

treated with 3-(trimethoxysilyl)propyl methacrylate to ensure covalent bonding of the monolith to the capillary wall.[37] The bulk monomer solution was prepared in a 1 dram (4 mL) glass vial by mixing 0.008 g DMPA, 0.32 g AMPS, 0.48 g PEGDA, 0.20 g water, 0.55 g methanol and 1.70 g ethyl ether. The monomer mixture was vortexed and ultrasonicated for 5 min to help dissolve AMPS and eliminate oxygen. Because of its low viscosity, the monomer solution was introduced into the UV transparent capillary by capillary surface action. The capillary (22 cm total length and 16.5 cm monomer length, unless otherwise specified) was then placed perpendicular to a UV dichroic mirror from Navitar (Newport Beach, CA), which was operated 45^{o} directly under a Dymax 5000AS UV curing lamp (Torrington, CT) for 3 min. The resulting polymer monolith inside the capillary was connected to an HPLC pump, and flushed with methanol and water sequentially to remove porogens and any unreacted monomers. The prepared polymer monolith was then equilibrated with buffer solution before use. Care was taken to avoid drying the monolith by storing it filled with water or mobile phase. After the completion of all chromatographic experiments, a small section (2 cm) of the monolith inside the capillary was dried under vacuum for scanning electron micrography (SEM) analysis (FEI Philips XL30 ESEM FEG, Hillsboro, OR).[38] The same procedure was also applied to synthesize poly(AMPS-co-EDMA) monoliths.

3.2.3 Capillary Liquid Chromatography (CLC)

CLC of peptides was performed using a system previously described, with some modifications.[38] Briefly, two ISCO Model 100 DM syringe pumps with a flow controller (Lincoln, NE) were used to generate a two-component mobile phase gradient. Due to the nL/min flow required for the monolithic capillary, the gradient flow from the pump was

split with the use of a Valco splitting tee (Houston, TX), which was installed between the static mixer of the syringe pumps and the 60 nL Valco internal loop sample injector. A 33 cm long capillary (30 μm i.d.) was used as the splitting capillary, and a 5 cm long capillary (30 μm i.d.) was connected between the splitting tee and the injector to minimize extracolumn dead volume. The mobile phase flow rate was set at 69 μL/min. The actual flow rate in the monolithic capillary column was measured by monitoring movement of a liquid meniscus through 100 cm long open tubular capillary (75 μm i.d.), which was connected to the monolithic capillary using a Teflon sleeve (Hamilton, Reno, NV). Depending on the mobile phase used, the flow rate in the monolithic capillary was 70-100 nL/min, resulting in split ratios from 700:1 to 1000:1.

For CLC of peptides with gradient elution, mobile phase A was a 5 mM phosphate buffer (pH 2.7 or 7.0) with various amounts of acetonitrile. Mobile phase B was the same composition as mobile phase A plus 0.5 M NaCl, and a gradient rate of 1-5% B/min was typically used. All mobile phases were filtered through a 0.2 μm Nylon membrane filter (Supelco, Bellefonte, PA) and ultrasonicated before use. The apparent pH of the mobile phase was measured using a pH meter (Omega, Stamford, CT). On-column UV detection was performed at 214 nm. Chromatograms were transferred to an ASCII file and redrawn using Microcal Origin (Northampton, MA). The monolithic column was also used for CLC of proteins using aqueous buffers.

For measurement of the dynamic binding capacity of the monolithic column, 1 mg/mL bradykinin fragment 1-7 in 5 mM phosphate containing 40% acetonitrile (pH 2.7) was pumped under constant pressure of 2000 psi through the monolithic column (18.6 cm long, 75 μm i.d.) using one syringe pump. No splitter was used for these measurements.

Because of the low amount (<1 mL) of the bradykinin fragment 1-7 solution available, it was preloaded into a sample loop capillary (2 m long, 320 μm i.d.), with one end connected to the Valco injector and the other end to the monolithic column using Upchurch unions (Oak Harbor, WA). The flow rate was measured to be 91 nL/min. Following the same procedures, the dynamic binding capacity based on uptake of protein (cytochrome c) was also performed on a new monolithic column (7 cm long, 75 μm i.d.). A solution of 4 mg/mL cytochrome c in 5 mM phosphate (pH 6.2) was pumped through the column under constant pressure of 850 psi, resulting in a column flow rate of 91 nL/min.

For studying the swelling/shrinking properties of the polymer monolith, different organic solvents were pumped through a 10 cm long monolith segment inside a capillary at different pressures. A splitter and detector were not used for these measurements. The flow rate was measured as described above.

Safety Considerations. AMPS monomer is listed as a suspected carcinogen, and PEGDA is a sensitizing agent. Appropriate MSDS information should be consulted for physical handling of these materials. Sunglasses that block UV light and gloves should be worn to avoid sunburns caused by the high power UV curing system during the preparation of the monolith.

3.3 Results and Discussion

3.3.1 Polymer Monolith Preparation

AMPS, a commercially available acrylamido derivative, was chosen as monomer to synthesize the SCX monolithic column because it contains the desirable sulfonic acid group. PEGDA, which is an acrylate based crosslinker with three ethylene glycol units,

has been shown to resist adsorption of peptides and proteins.[38] Therefore, it was selected as crosslinker for the synthesis of the monolith. PEGDA was used instead of EDMA as crosslinker to prepare a monolith with more hydrophilicity.

The most widely used porogen strategy was adopted to control the through-pores in the monoliths in this study. To date, choice of porogens has been mainly achieved by trial-and-error, although some theoretical aspects for porogen selection have been derived for macroporous particle synthesis using suspension polymerization.[39-41] Because the solubility of AMPS in common organic solvents is low, water was selected as one of the porogens to help dissolve AMPS. Methanol was selected as another porogen because it was proven efficient for the formation of macroporous through-pores in a poly(PEGDA) monolith.[38] Unfortunately, any combination of water and methanol (with 0.32 g AMPS and 0.48 g PEGDA) yielded a nonporous or microporous translucent gel structure which allowed no flow of mobile phase. The same results were also observed for combination of water, methanol and 1-propanol. Since ethyl ether is another powerful porogen for PEG-based monoliths,[38] it was finally chosen as the third porogen. After simple optimization, a recipe (25% monomers, composed of 40:60 wt% AMPS and PEGDA, and 75% porogens, composed of 8:23:69 wt% water, methanol and ethyl ether) was finalized, and the resulting monolith supported considerable flow under moderate pressure in aqueous buffer. Noteworthy was the incorporation of 40% AMPS, which represents the highest reported percentage of AMPS copolymerized into a polymer monolith backbone. Due to the one-step in-situ synthesis protocol, the rate of success in preparing such monolithic capillary columns approached 100%.

A scanning electron micrograph of the optimized monolith is shown in Figures 3.1A and 3.1B. It can be immediately observed that the morphology of the

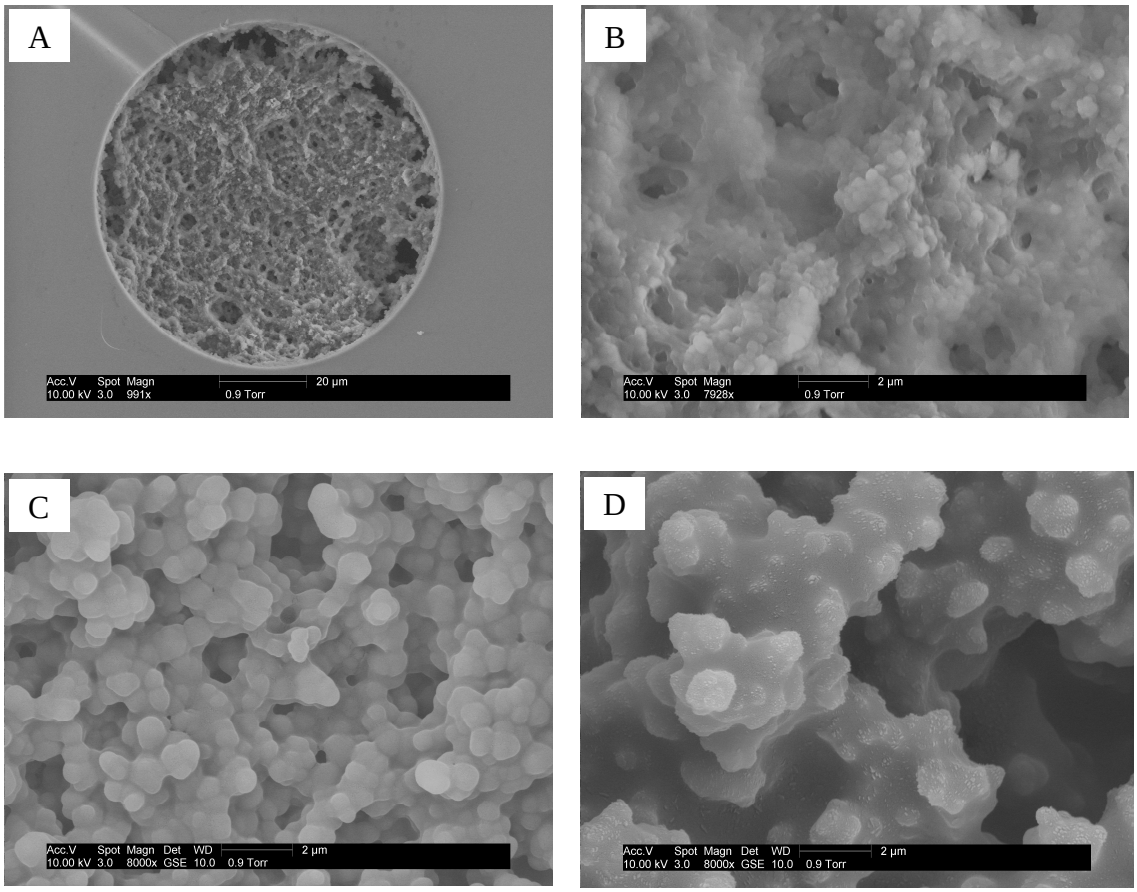


Figure 3.1. SEM images of several monoliths synthesized. (A) Optimized poly(AMPS-co-PEGDA) monolith used in this study (scale bar = 20 µm); (B) higher magnification of the monolith in (A) (scale bar = 2 µm); (C) poly(AMPS-co-PEGDA) monolith that has the same composition as (A) except that methanol and ethyl ether were 0.85 and 1.40 g, respectively (scale bar = 2 µm); (D) poly(AMPS-co-EDMA) monolith (recipe: 0.008 g DMPA, 0.35 g AMPS, 0.40 g EDMA, 0.35 g water, 1.10 g methanol, scale bar = 2 µm).

poly(AMPS-co-PEGDA) monolith is unique. It was composed of fused microglobules, with no distinct microspheres. It appeared intermediate between a conventional polymer monolith with a distinct particulate structure[11-13] and a silica monolith with a skeletal structure.[14-15] The through-pores of the monolith were obvious. Cracks along the circumference of the monolith (Figure 3.1A) were presumably due to shrinking of the monolith upon drying when SEM images were taken.

To explore variables that could result in the formation of this unique morphology, two other monoliths were prepared and their SEM images are shown in Figures 3.1C and 3.1D. With an increase in methanol in the porogen composition, conventional polymer monolithic morphology with discrete and more "regular" microglobules was formed (Figure 3.1C). If EDMA was used as crosslinker, the resulting poly(AMPS-co-EDMA) monolith exhibited similar fused but more porous structure (compare Figures 3.1B and 3.1D). Based on these micrographs, it seems that porogens rich in methanol or the use of EDMA as crosslinker favored the formation of conventional polymer monolithic morphology, while a monolith formed from porogens rich in ethyl ether, or that used PEGDA as crosslinker tended to form a fused structure. Both porogen and crosslinker are important factors that control the morphology of poly(AMPS) monoliths.

3.3.2 Effect of Acetonitrile on the Elution of Synthetic Peptides

An ideal SCX column for LC of peptides should be moderately hydrophilic, able to retain weakly charged analytes (e.g., +1 charged peptides), and exhibit retention of analytes independent of buffer pH from acidic to neutral.[2] In addition, high binding capacity is another favorable feature which improves peptide resolution.

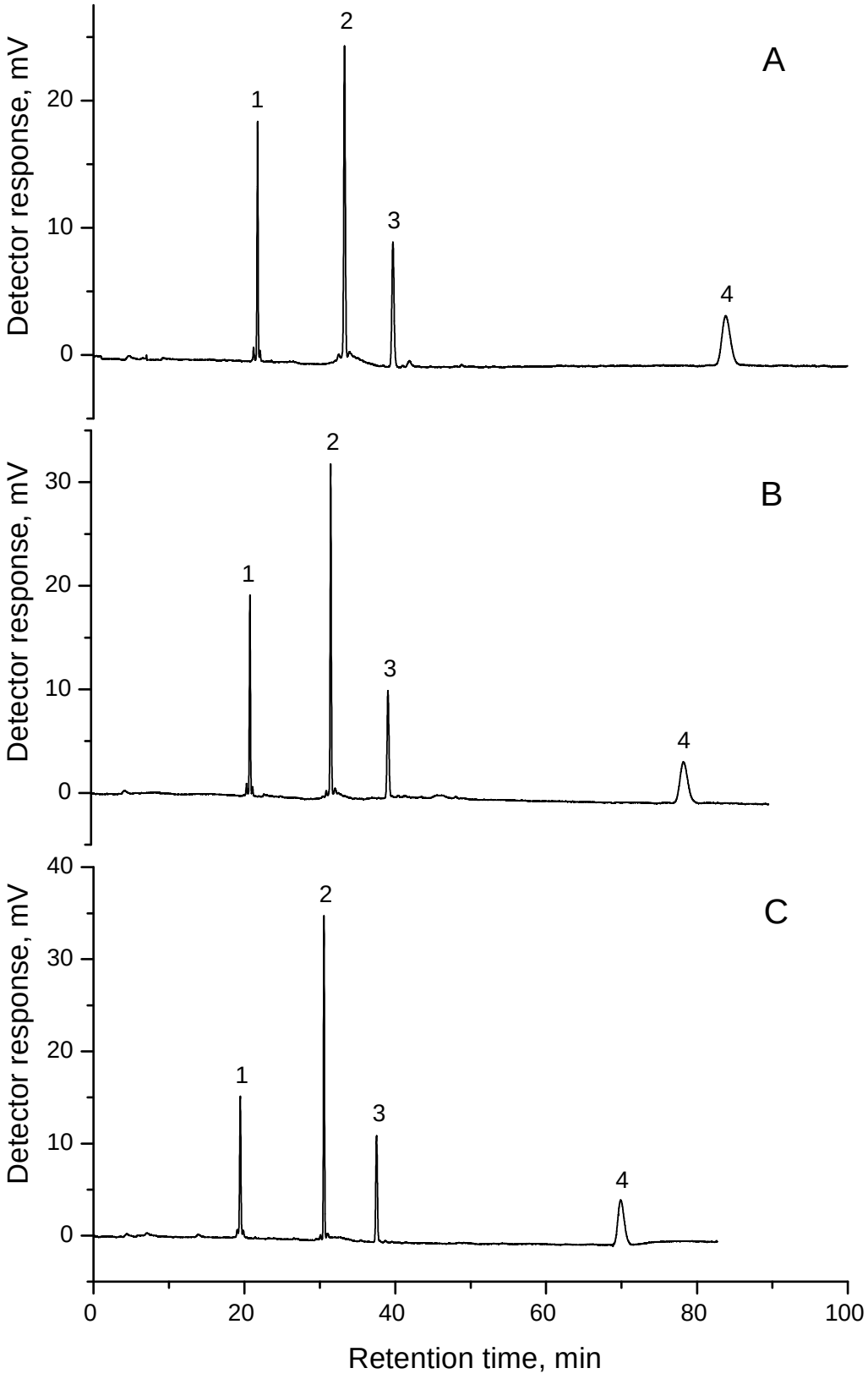
Table 3.1. Properties of synthetic peptides.

Analyte	Amino acid sequence[a]	Charge at pH 2.7	Charge at pH 7.0	Hydrophobicity index at pH 2.0[b]	Hydrophobicity index at pH 7.0[c]
1	Ac-Gly-Gly-Gly-Leu-Gly-Gly-Ala-Gly-Gly-Leu-**Lys**-amide	+1	+1	14.7	18.6
2	Ac-**Lys**-Tyr-Gly-Leu-Gly-Gly-Ala-Gly-Gly-Leu-**Lys**-amide	+2	+2	17.5	23.4
3	Ac-Gly-Gly-Ala-Leu-**Lys**-Ala-Leu-**Lys**-Gly-Leu-**Lys**-amide	+3	+3	21.4	30.2
4	Ac-**Lys**-Tyr-Ala-Leu-**Lys**-Ala-Leu-**Lys**-Gly-Leu-**Lys**-amide	+4	+4	24.2	35.0

[a] Amino acid sequence was from ref [42]. Ac = N_{α}-acetyl; Amide = C_{α}-amide. Positively charged residues are indicated in bold font. [b] Hydrophobicity index was calculated based on ref [43]. [c] Data were from ref [42].

Hodges et al.[2,42] designed several synthetic peptides to evaluate particle based SCX columns. The synthetic peptide standard, CES-P0050, was composed of four peptides (see Table 3.1) which possess certain characteristics for SCX column evaluation. These peptides are all undecapeptides having similar chain length to those most commonly encountered in protein tryptic digests, and they do not have any acidic residues (the C-terminal groups are amides), so they possess the same charge in acidic to neutral buffers. The hydrophobicity index of these peptide standards has been compiled for pH 7.0.[42] However, they were re-tabulated in Table 3.1 for easy reference, along with other properties (e.g., amino acid sequence).

Figure 3.2 shows a gradient elution chromatogram of the synthetic peptides under different buffer conditions using the poly(AMPS-co-PEGDA) monolithic SCX column. With an increase in acetonitrile in the mobile phase from 0% to 40% (see Figures 3.2A to 3.2E), the elution times for peptides 1-4 were monotonically decreased. For peptide 4, addition of 40% acetonitrile in the elution buffer was required to suppress hydrophobic interactions (compare Figures 3.2D and 3.2E). For the less hydrophobic peptides 2 and 3, 20-30% acetonitrile could effectively eliminate hydrophobic interactions, as evidenced by the very sharp peaks obtained. For the least hydrophobic peptide 1, no acetonitrile was required because no significant hydrophobic interactions were observed. The minor differences in retention times for peptide 1 were likely due to differences in mobile phase column flow rate. The dramatic decrease in retention time and improvement in peak shape for peptide 4 indicates relatively strong hydrophobicity of the poly(AMPS-co-PEGDA) monolith. This feature is not desirable for two-dimensional LC (e.g., ion exchange followed by reversed-phase) for proteomics, in which an aqueous buffer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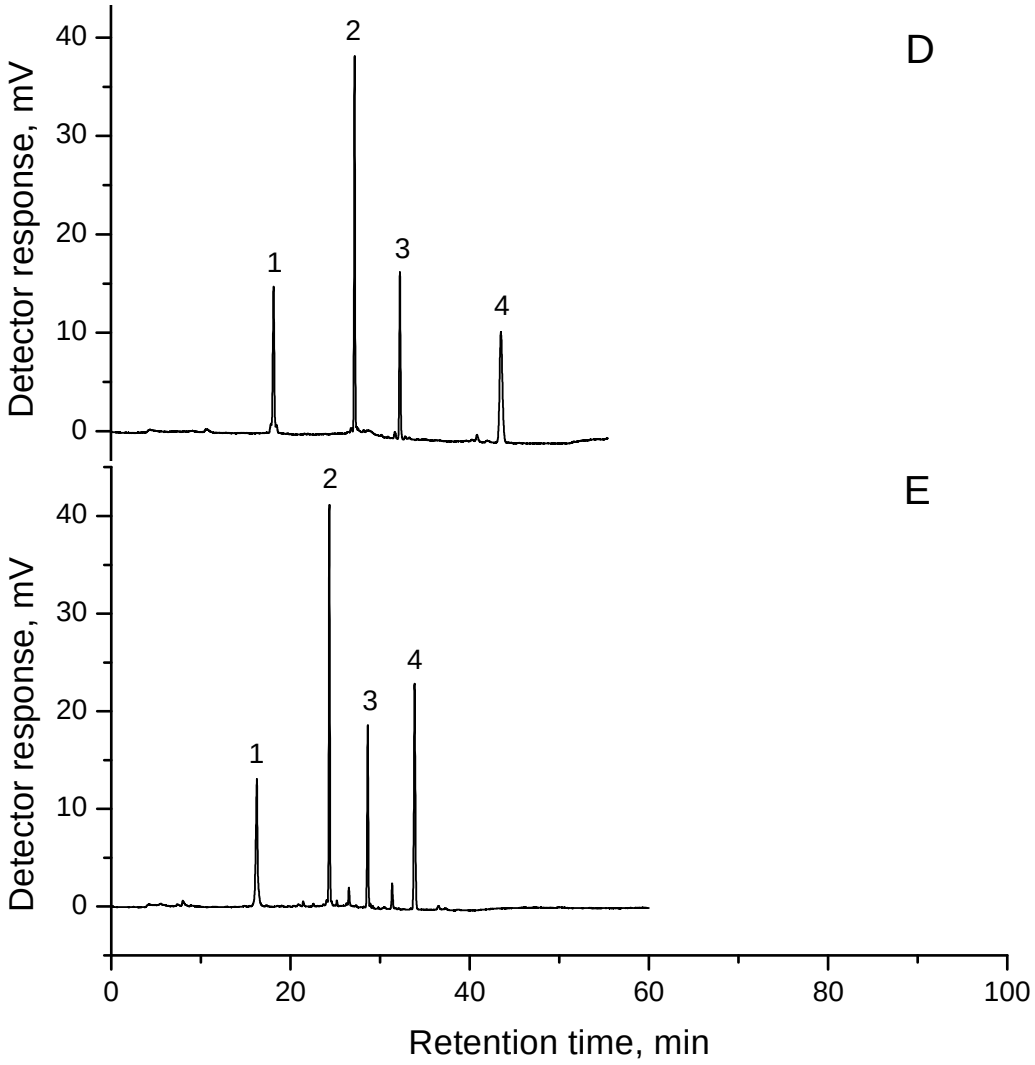


Figure 3.2. SCX chromatography of synthetic peptides. Conditions: 16.5 cm × 75 μm i.d. monolithic column; buffer A was 5 mM NaH_2PO_4 (pH 2.7) and buffer B was buffer A plus 0.5 M NaCl, both buffers containing 0, 10, 20, 30, or 40% (v/v) acetonitrile (panels A, B, C, D, and E, respectively); 2 min isocratic elution of 1% B, followed by a linear AB gradient (5% B/min, equating to 25 mM salt/min) to 100% B and various times of isocratic elution of 100% B until peptide 4 was eluted; ~10 min gradient delay time; mixture of peptides 1-4 (see Table 3.1 for sequence) in CES-P0050, which was dissolved in 400 μL buffer A with 0% acetonitrile, resulting in a concentration of 0.44 mM for peptide 3; 69 μL/min pump master flow rate; 76, 83, 85, 89 or 100 nL/min column flow rates (panels A, B, C, D, and E, respectively); online UV detection at 214 nm.

without acetonitrile is required in the first dimension to effect retention of peptides in the second dimension before separation.

The relatively strong hydrophobicity of the poly(AMPS-co-PEGDA) monolith was surprising. The biocompatible crosslinker PEGDA was specially designed and used to decrease unwanted polymer backbone hydrophobicity. To further confirm the biocompatibility of PEGDA, a poly(PEGDA) monolith was prepared following a previously published protocol,[38] and peptides 1-4 were eluted from the monolith using buffers containing various amounts (0-40%) of acetonitrile. Results (data not shown) indicated negligible differences in peptide elution with the use of different buffers. Therefore, the relatively strong hydrophobicity of the poly(AMPS-co-PEGDA) monolith must be due to the monomer AMPS itself. In fact, the AMPS molecule contains an isobutyl arm, which connects to the sulfonic acid group on one end and the acrylamido group on the other end. Alpert et al.[5] found that PolySulfoethyl A columns were superior to the more hydrophobic sulfopropyl columns.[8,9] In analogy, it is expected that the monolithic sulfobutyl phase possesses stronger hydrophobicity than desired due to the butyl segment in the side groups.

Despite the strong hydrophobicity of the poly(AMPS-co-PEGDA) monolith, it was shown to retain strongly the +1 charged peptide (see Figure 3.2E). This positive feature is uncommon for commercially available particulate SCX columns where only the PolySulfoethyl A column could retain the peptide.[2,42] For 40% acetonitrile, where any hydrophobic interaction was greatly eliminated, retention of the peptide on the monolith would be expected from ionic interaction only. This strong ionic interaction can be attributed to the use of a high amount of AMPS (40%) in the copolymerization.

With hydrophobic interactions suppressed (i.e., with the use of 40% acetonitrile), the four synthetic peptides were eluted as extremely sharp peaks (see Figure 3.2E), with an average peak width at baseline of 0.28 min. According to the simple definition of peak capacity in gradient elution (peak capacity = time of gradient/peak width),[44] the peak capacity was calculated to be 71, a value surpassing most particulate based SCX columns[1-9] (peak capacities of 24~66 were estimated based on several chromatograms provided in these references) and other polymer monolithic SCX columns[10,28,30,33,34] [Peak capacities of 5~32 were again estimated; in cases of isocratic elution, the peak capacity was calculated as $n = (\sqrt{N}/4)\ln(t_2/t_1)$, where N is the column efficiency, and t_2 and t_1 are the retention times of the last and the first eluting peaks, respectively]. The asymmetry factors calculated at 10% peak height for peptides 1-4 were 1.01, 0.94, 0.90, and 0.99, respectively. The sharp peaks together with minimal fronting or tailing indicated a highly efficient SCX monolithic column.

The run-to-run reproducibility of the poly(AMPS-co-PEGDA) column was good. For three consecutive runs using conditions the same as in Figure 3.2E, the relative standard deviation (RSD) of the retention times for peptides 1-4 were 1.9, 0.7, 0.3, and 0.4%, respectively. For peak height, the RSD values for peptides 1-4 were 4.6, 2.3, 2.0 and 1.7%, respectively. These data clearly demonstrate that good reproducibility could be readily achieved if the column was equilibrated with starting buffer for a sufficient period (typically ~10 column volumes) between runs, although the polymer monolith exhibited swelling in aqueous buffers (*vide infra*).

Column-to-column reproducibility measurements gave retention time RSD values (n=3) for peptides 1-4 of 1.3, 1.6, 2.2, and 2.4%, respectively. However, significant

deviation was observed for peak height measurements; the RSD values for peptides 1-4 were 18.5, 18.6, 34.6, and 21.9%, respectively.

3.3.3 Effect of Buffer pH on the Resolution of Synthetic Peptides

With an increase in buffer pH from 2.7 to 7.0, greater retention with similar sharp peaks was observed for synthetic peptides 1-4 under otherwise identical conditions as in Figure 2.2E (data not shown). Because the peptides bear the same charges in both buffer pHs (see Table 3.1), this indicates an increased negative charge density of the monolith upon an increase in buffer pH. Although AMPS is a strong organic acid with pKa of 1.2,[45] the pKa of poly(AMPS) shifts to a higher value due to the absence of electron-withdrawing vinyl groups upon polymerization.[46] An increase in metal-poly(AMPS) retention was observed with an increase in buffer pH from 1 to 7.[47] Thus, the lower acidity of poly(AMPS) over AMPS accounts primarily for the increased retention of peptides at pH 7.0 compared to pH 2.7. Another contributing factor is the presence of acrylic acid, an impurity found in both AMPS and PEGDA monomers, which can be copolymerized into the monolith backbone. However, no confirmation of this was sought. The stronger retention of peptides upon increase of buffer pH was also observed for most particulate based SCX columns.[2]

3.3.4 Dynamic Binding Capacity

One of the most important properties of an ion exchange column is the binding capacity,[48] which determines the resolution, column loadability, and gradient elution strength. For the measurement of dynamic binding capacity of an SCX column, proteins (e.g., lysozyme or hemoglobin) are often used. Although the monolithic column could elute and separate proteins using buffers with high ionic strength (*vide infra*), it did not

elute lysozyme, cytochrome c or hemoglobin within 2 h under conditions typical for SCX chromatography of peptides [e.g., 5 mM phosphate (pH 2.7) containing 40% acetonitrile and 0.5 M NaCl]. Therefore, bradykinin fragment 1-7, which bears +2 charge at pH 2.7, was used to determine the monolithic column dynamic binding capacity. During frontal analysis, a sharp increase in baseline was observed, indicating fast kinetic interaction of the peptide with the column. With the use of 1 mg/mL peptide, it took an amazingly long time (1074 min) to saturate the column. Based on the measured flow rate of 91 nL/min, the dynamic binding capacity was 119 mg/mL, corresponding to 157 μequiv/mL. From the monolith recipe (see Section 3.2.2), this 40% AMPS / 60% PEGDA monolith had a theoretical binding capacity of 475 μequiv/mL. This indicates that ~33% of AMPS in the monolith backbone was accessible for ionic interaction. The major portion (67% in this case) of AMPS is most likely buried in the polymer monolith, due to the direct copolymerization method used. Nevertheless, the dynamic binding capacity of the poly(AMPS-co-PEGDA) monolith was high. This was supported by the elution of the +4 charged peptide 4 as shown in Figure 3.2E after a 20 min gradient step. For simple comparison with other SCX columns, the dynamic binding capacity was also measured based on cytochrome c uptake although such measurement might be inappropriate and inaccurate due to hydrophobic binding. It took 282 min to saturate the 7 cm long monolith, resulting in a binding capacity of 332 mg/mL.

The dynamic binding capacity of this monolith was compared with other columns. Alpert et al.[5] reported that the PolySulfoethyl A column had a dynamic binding capacity of 100 mg hemoglobin/mL packing material, corresponding to ~3 μequiv/mL. Because 157 μequiv peptide/mL or 332 mg protein/mL was achieved for the current monolithic

column, the binding capacity was greater than that of the PolySulfoethyl A column. For the poly(glycidyl methacrylate-co-ethylene glycol dimethacrylate) monoliths[28,29] grafted with AMPS for SCX chromatography of proteins, the dynamic binding capacity was found to be typically lower than 100 mg protein/g monolith. For the functionalized poly(glycidyl methacrylate-co-ethylene glycol dimethacrylate) monolith,[30] the dynamic binding capacity was 90-300 μequiv/mL, albeit based on copper ion uptake. The binding capacity was very low (~1 μequiv/mL) for the anion exchange polymer monolith,[33] which was prepared by agglomeration of aminated latex particles to a monolith prepared through the copolymerization of a small amount of AMPS, a large amount of BMA and EDMA. This was presumably due to the lower amount of AMPS used in the copolymerization. In summary, the dynamic binding capacity of the current monolith, which was prepared from direct copolymerization of 40% AMPS and 60% PEGDA, was greater than the particulate-based SCX PolySulfoethyl A column and most of the other polymer monolithic SCX columns.

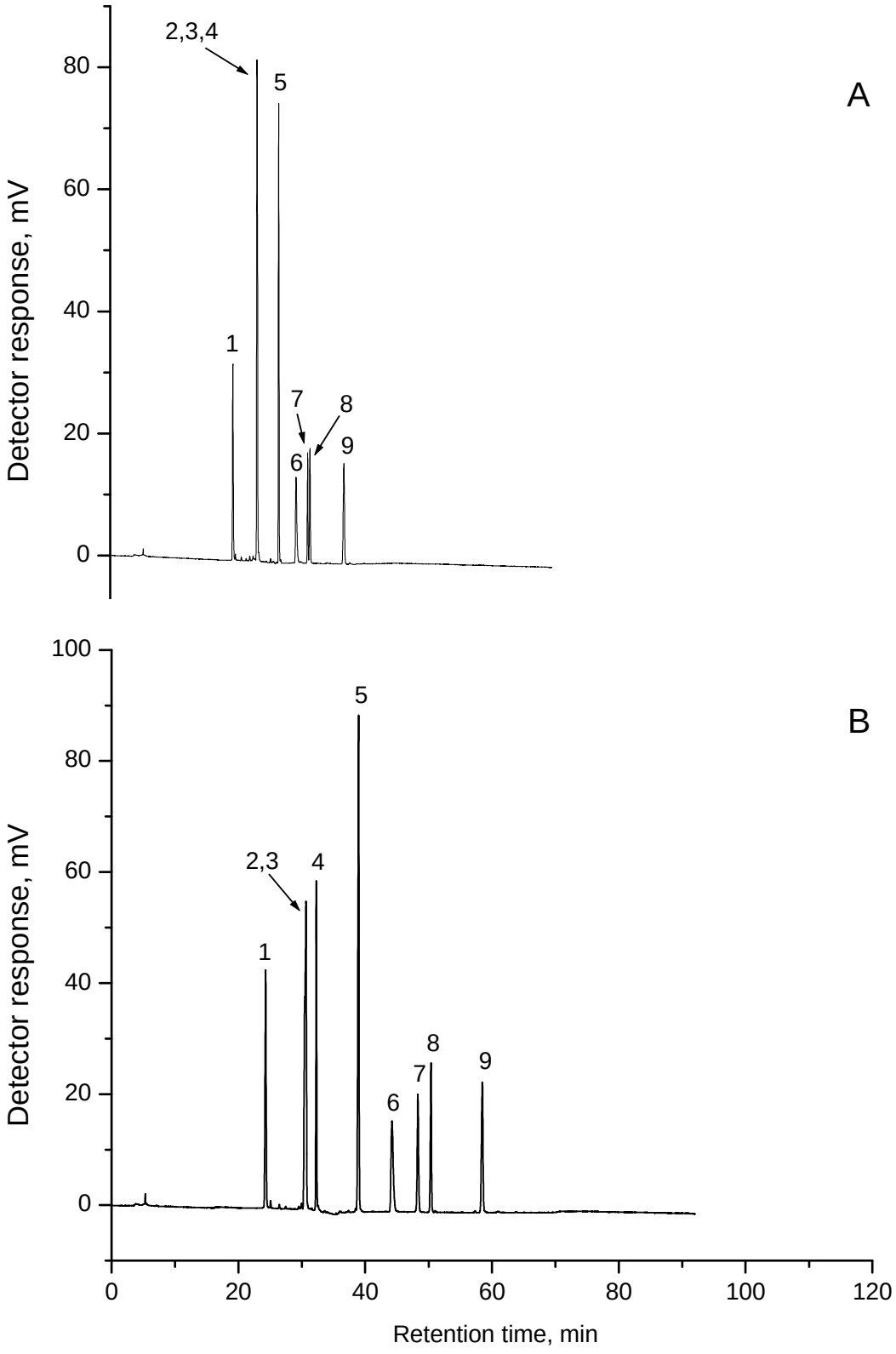
3.3.5 SCX Chromatography of a Complex Peptide Mixture

To demonstrate the general utility of the poly(AMPS-co-PEGDA) monolith for peptide analysis, a more complex peptide mixture P2693 composed of 9 natural peptides (see Table 3.2) was chromatographed using buffer containing 40% acetonitrile under different gradient rates (Figure 3.3). As seen in Figure 3.3A, 7 out of the 9 peptides were resolved when 5% B/min gradient rate was used. By decreasing the gradient rate to 2% B/min, 8 peaks were baseline separated (Figure 3.3B). A further decrease in the gradient rate to 1% B/min resolved all 9 peptides, although peptides 2 and 3 were not baseline separated (Figure 3.3C and inset). Thus, it is convenient to use a shallow gradient to

Table 3.2. Properties of the nine peptides in the P2693 standard.

No	Analyte	Amino acid sequence[a]	Molecular weight	No. of residues	Charge at pH 2.7	Hydrophobicity index at pH 2.0[b]
1	Oxytocin	Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2	1007.19	9	+1	19.5
2	Methionine enkephalin	Tyr-Gly-Gly-Phe-Met	573.70	5	+1	10.0
3	Leucine enkephalin	Tyr-Gly-Gly-Phe-Leu	555.62	5	+1	12.6
4	Bombesin	pGlu-Gln-**Arg**-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-**His**-Leu-Met-NH_2	1619.85	14	+2	34.9
5	Luteinizing hormone releasing hormone	pGlu-**His**-Trp-Ser-Tyr-Gly-Leu-**Arg**-Pro-Gly	1183.27	10	+2	20.4
6	[Arg8]-Vasopressin	Cys-Tyr-Phe-Gln-Asn-Cys-Pro-**Arg**-Gly-NH_2	1084.23	9	+2	11.5
7	Bradykinin fragment 1-5	**Arg**-Pro-Pro-Gly-Phe	572.66	5	+2	7.5
8	Substance P	**Arg**-Pro-**Lys**-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2	1347.70	11	+3	27.9
9	Bradykinin	**Arg**-Pro-Pro-Gly-Phe-Ser-Pro-Phe-**Arg**	1060.20	9	+3	16.8

[a] Amino acid sequence was from Sigma website. Positively charged residues were indicated in bold font. Free N-terminal bears +1 charge while pyroed N-terminal with glu (pGlu) is neutral. [b] Hydrophobicity index was calculated based on ref [43].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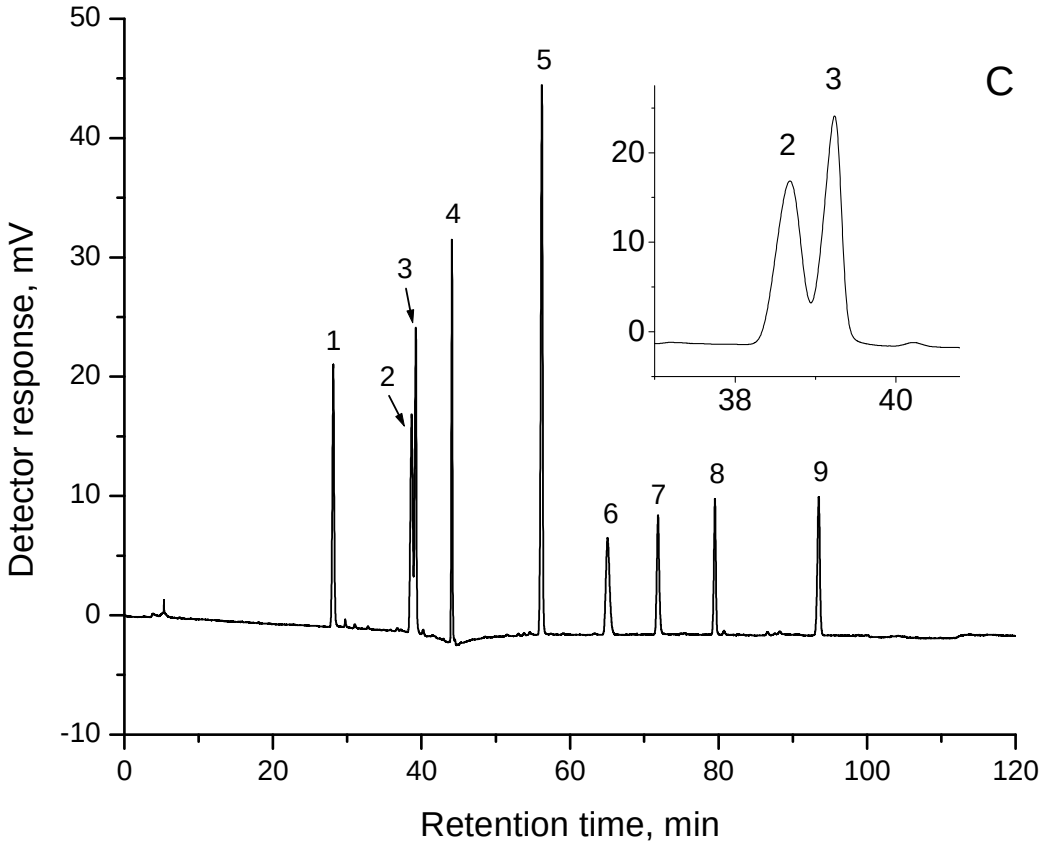

Figure 3.3. SCX chromatography of natural peptides. Conditions were the same as those in Figure 3.2E with the following exceptions: mixture of nine natural peptides (see Table 3.2) dissolved in 25 μL buffer A to make each peptide ~1 mg/mL; gradient rate of (A) 5% B/min; (B) 2% B/min; (C) 1% B/min.

improve resolution for analyzing complex samples. The separation shown in Figure 3.3C was governed by an ion exchange mechanism. Following the empirical relationship between retention time and charge-to-chain length ratio developed by Hodges et al.,[2] a straight line [$t_R = 66.03 \times N/\ln(n) - 2.05$] was obtained with a regression coefficient of 0.96, where t_R is the peptide retention time, N is the charge, and n is the number of amino acid residues. This confirmed a pure ionic interaction of the polymer monolith for SCX of natural peptides with 5 to 14 residues and a hydrophobicity range from 7.5 to 34.9 (see Table 3.2).

It is interesting that the elution order in Figure 3.3C is the reverse of that in capillary zone electrophoresis (CE) (cf technical bulletin for P2693 from Sigma, http://www.sigmaaldrich.com/sigma/datasheet/p2693dat.pdf) except for peptides 7 and 8. This is not unexpected because retention in SCX is based on the charge-to-ln(chain length) ratio while in CE, migration is determined by analyte charge-to-size ratio. Thus, an analyte with more charge and smaller size will migrate earlier in CE, and elute later in SCX. As compared with separation in CE, better resolution (with the exception of peptides 2 and 3) was generally obtained for SCX chromatography, although longer time was required. Peak widths were somewhat narrower in SCX chromatography than in CE. This demonstrates that comparable or better resolution and efficiency were achieved for peptide analysis with the use of the poly(AMPS-co-PEGDA) monolithic column than for CE.

The average peak width at baseline in Figures 3.3A (excluding the second peak due to coelution of three peptides), 3B (excluding the second peak due to coelution of two peptides) and 3C (excluding the second and third peaks due to incomplete resolution)

were 0.27, 0.38 and 0.56 min, resulting in peak capacities of 74, 130 and 179 for the gradient rates of 5%, 2% and 1% B/min, respectively. As discussed above, the peak capacity calculated from Figure 3.2E was 71 where a gradient rate of 5% B/min was used for SCX of four synthetic peptides. It seems that the peak capacity depends on the salt gradient rate and not on the analytes used. A shallower gradient resulted in a greater peak capacity. This was due to the use of the unique monolith, for which the peak width increased less proportionally upon an increase in the gradient elution time. This feature is attractive for resolving complex peptide samples (e.g., protein digests).

Noteworthy was the resolution between methionine enkephalin and leucine enkephalin (inset in Figure 3.3C). These two peptides bear the same charge and have the same chain length (see Table 3.2). They also have very similar molecular weight and hydrophobicity. Due to the use of 40% acetonitrile in the mobile phase, it is not likely that the resolution was based on differences in hydrophobicity. Instead, the separation was primarily due to differences in ionic interaction resulting from a minor difference in molecular weight. Because methionine enkephalin has a greater molecular weight than leucine enkephalin, the ionic interaction between methionine enkephalin and the monolith would be expected to be somewhat smaller, leading to earlier elution. The successful separation of methionine enkephalin and leucine enkephalin emphasizes the exceptional resolution provided by the poly(AMPS-co-PEGDA) monolith.

Further evaluation of the monolith was conducted for SCX chromatography of a β-casein digest (Figure 3.4). Once again, very nice separation was obtained. Based on several completely resolved peaks (indicated on Figure 3.4), the peak capacity was

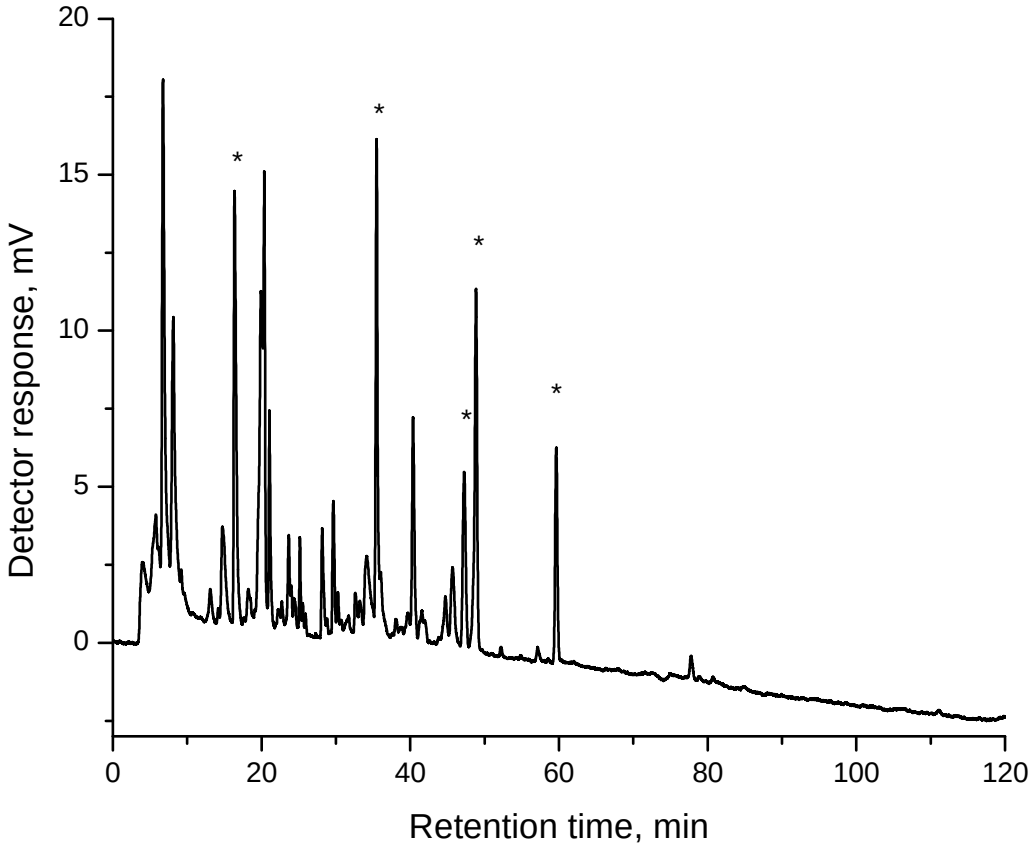

Figure 3.4. SCX chromatography of β-casein digest. Conditions were the same as in Figure 3.3C.

estimated to be 167, close to 179 measured using peptide standard P2693. This confirmed that peak capacity was not dependent on the sample analyzed, but on the gradient rate. It should be mentioned that the protein digest had to be desalted. If the β-casein digest was not desalted (see Section 3.2.1), the peptides coeluted in 15 min (data not shown). This is expected because peptides will not be strongly retained if they are dissolved in a high concentration of salt buffer. During the experiment, it was also important to use freshly prepared peptides and to store them in a refrigerator. For example, peptide standard CES-P0050 degraded if dissolved in the starting buffer and stored at 2-8 ^{o}C for more than 2 months. Figure 3.5 shows a separation of a degraded sample. In addition to the main four peptides, eight other peptides could be clearly seen. This, once again, demonstrates the high resolution of the poly(AMPS-co-PEGDA) monolith for SCX liquid chromatography of peptides. It opens the possibility of using SCX chromatography for quality analysis (e.g., purity) of peptides, although such analyses are almost exclusively performed using reversed-phase liquid chromatography.

3.3.6 SCX Chromatography of Protein Standards

Attempt was also made to perform SCX chromatography of basic proteins, and the result is shown in Figure 3.6. As mentioned before, proteins did not elute from the monolithic column when 5 mM phosphate (pH 2.7) containing 40% acetonitrile and 0.5 M NaCl was used as eluent. This is likely due to stronger binding of proteins than peptides, as confirmed by the elution of proteins when NaCl concentration was increased to 2.0 M. However, due to the poor solubility of NaCl in 40% acetonitrile, a buffer that contains no acetonitrile must be used. Thus, the separation in Figure 3.6 was based on a mixed-mode mechanism. An increase in buffer salt concentration resulted in a decrease

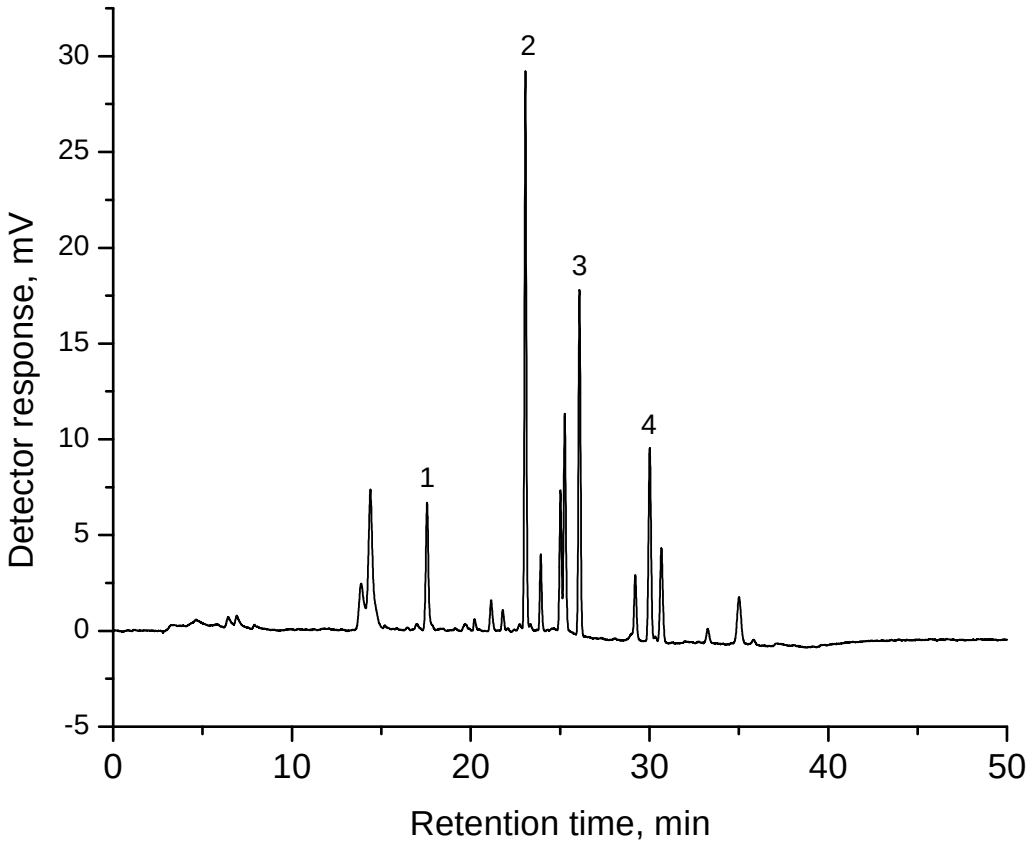

Figure 3.5. SCX chromatography of old synthetic peptide sample. Conditions were the same as in Figure 3.2E.

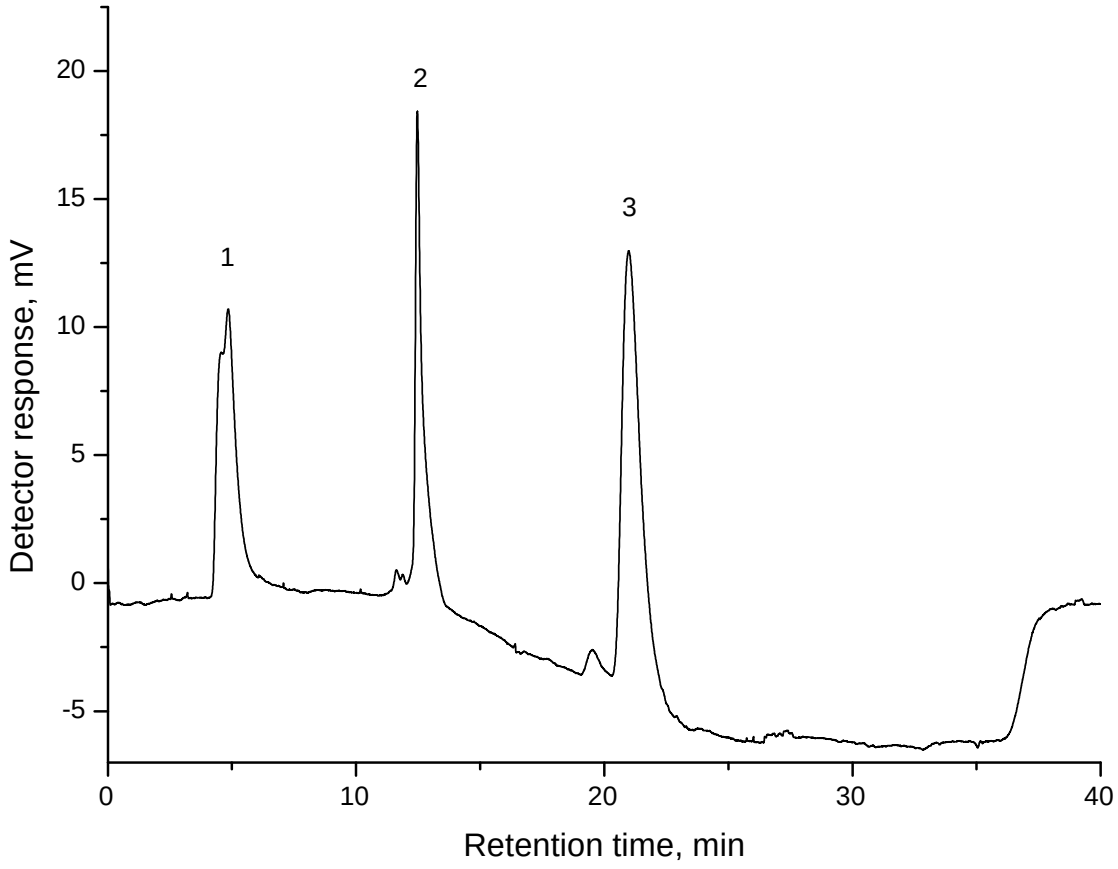

Figure 3.6. SCX chromatography of proteins. Conditions were the same as in Figure 3.2E except that different buffers were used; buffer A was 5 mM phosphate (pH 6.2) and buffer B was buffer A plus 2.0 M NaCl; analytes: (1) myoglobin, (2) cytochrome c, and (3) lysozyme. The baseline drift during gradient elution and the rise of the baseline at the end of gradient were due to the difference in UV absorbances of buffers A and B.

in ionic interaction and an increase in hydrophobic interaction. As a result, protein peaks were broadened by the increased nonspecific hydrophobic interaction during salt gradient elution. Although the SCX column exhibited worse chromatographic performance for proteins than for peptides, it was comparable to other monolithic SCX columns for protein analysis.[28]

3.3.7 Stability of the Poly(AMPS-co-PEGDA) Monolith

Permeability is a good index to reflect swelling or shrinking of the monolith. If a monolith swells, its through-pores will decrease in size, resulting in lower permeability, and *vice versa*. From Table 3.3, the permeability was approximately an order of magnitude lower in aqueous buffer than in some organic solvents. With the use of organic solvents, the permeability decreased roughly with an increase in solvent relative polarity, except that ethyl ether and acetone had the highest permeability. This indicates that the monolith swells in more polar solvents and shrinks in less polar solvents.

Although the poly(AMPS-co-PEGDA) monolith swelled in aqueous buffer and shrank in organic solvents, no detachment of the monolith from the capillary wall was observed under any condition, likely due to covalent attachment to the capillary wall. Furthermore, the column flow rate reached a constant value after equilibration with a new solvent. This indicated reversible shrinking or swelling of the monolith under a variety of solvent conditions. For the SCX liquid chromatography of peptides reported in this study, the column flow rate measured was 70-100 nL/min when the backpressure read from the pump panel was between 2000 and 2300 psi during the gradient run. This indicates that a considerable flow was generated at moderate pressure even though the monolith swelled.

Table 3.3. Permeability of the poly(AMPS-co-PEGDA) monolith.

Flushing fluid	Relative polarity[a]	Viscosity, η (cP)[b]	Column backpressure, Δp (psi)	Linear velocity, u (mm/s)	Permeability, k ($\times 10^{-15}$ m^2)[c]
Hexane	0.009	0.300	800	5.52	30.0
Ethyl ether	0.117	0.224	800	12.09	49.1
THF	0.207	0.456	800	2.51	20.8
Acetone	0.355	0.306	800	9.09	50.4
Acetonitrile	0.460	0.369	800	3.30	22.1
Methanol	0.762	0.544	800	1.17	11.5
Water	1.000	0.890	1200	0.27	2.9
Buffer A	/	0.846	1200	0.33	3.4
Buffer B	/	0.890	1200	0.47	5.1

[a] Relative polarity data were from http://virtual.yosemite.cc.ca.us/smurov/orgsoltab.htm. [b] Viscosity data were from online CRC Handbook of Chemistry and Physics, 85th edition, 2004-2005. For buffer A which contains 40% aceonitrile, the viscosity is ~95% water (Sadek, P. C., in HPLC Solvent Guide, 2nd ed., John Wiley and Sons: New York, 2002). For buffer B which contains both 40% acetonitrile and 0.5 M NaCl, the viscosity is assumed to be 0.89×0.95×1.052 = 0.890 because 0.5 M NaCl is 1.052 times the viscosity of pure water. [c] Permeability $k = \eta L u/\Delta p$, where η is the viscosity, L is the column length (10 cm in this case), u is the solvent linear velocity, and Δp is the column backpressure.

The polymer monolith could be used continuously over 1 month under a pressure of >2000 psi. Excessive swelling of the sulfonic acid-containing polymer monolith in aqueous buffer, which would result in no flow, was not observed for the poly(AMPS-co-PEGDA) monolith reported in this study.

3.3.8 Tentative Explanation of the Sharp Peaks Obtained

It is interesting that the permeabilities of the monolith in aqueous buffers A and B were different (see Table 3.3). An increase in permeability was observed with the use of the same buffer with 0.5 M NaCl additive. This reflects a responsive property of the poly(AMPS-co-PEGDA) monolith upon contact with salt. Viklund et al.[29] reported that poly(TRIM) monolith with a surface grafted with SPE showed a salt dependant permeability. However, the permeability decreased with an increase in NaCl concentration in the range of 0-0.2 M. Interestingly, no such trend was observed for the monolith prepared by copolymerization of TRIM and SPE.

The salt dependant permeability of the poly(AMPS-co-PEGDA) monolith is expected to have an influence on the chromatography of peptides. The mobile phase flow rate in the monolithic column increased in my system during the salt gradient run because the nano flow gradient in the column was generated by a passive splitter (see Section 3.2.3). Thus, two gradients effected the elution of peptides from the monolithic column. One was a simple salt gradient, which narrowed the peptide bands during elution. The other was a naturally formed flow gradient. The flow gradient would provide an effectively sharper salt gradient than set in the program. As seen in Figure 3.3, the sharper the salt gradient, the narrower the peak widths. Double gradient elution was previously demonstrated in ion exchange liquid chromatography of small ions, where a

flow gradient was intentionally employed to achieve fast separation.[49] It should be emphasized that although a natural flow rate gradient existed in these studies, it did not contribute significantly to the sharpening of peptide bands, especially under shallow (e.g., 1% B/min) salt gradient conditions, where a flow rate increase of ~1.4 times (based on Table 3.3) was estimated for a 100 min interval.

It is hypothesized that the extremely sharp peaks achieved in this study are primarily due to the nature of the poly(AMPS-co-PEGDA) monolith. While the poly(AMPS-co-PEGDA) monolith was shown to exhibit strong hydrophobicity, the hydrophobicity was mainly derived from the side chains of the monolith that attached the functional AMPS monomer. The backbone of the polymer monolith contributed negligible hydrophobicity due to the use of both a biocompatible crosslinker PEGDA and a biocompatible acrylamido group in the AMPS. Thus, no nonspecific hydrophobic interaction between the polymer backbone and peptide would occur. Because the side chains are located on the surface of the polymer monolith upon contact with aqueous buffer, mass transfer resistance would be small, resulting in high column efficiency. To test this hypothesis, SCX chromatography of synthetic peptides 1-4 on a poly(AMPS-co-EDMA) monolith was performed under the same conditions as in Figure 3.2E. Although well separated, the peaks for all four peptides were broad and tailing (data not shown). This observation confirms that the extremely narrow peaks obtained in this study were primarily due to the use of the biocompatible crosslinker PEGDA.

3.4 Conclusions

A poly(AMPS-co-PEGDA) monolith containing as high as 40% AMPS was prepared by one-step copolymerization. The monolith had several favorable features,

such as high binding capacity, extraordinarily high resolution and high peak capacity, making it ideal for resolving complex peptide samples, such as protein digests. Due to its excellent chromatographic performance and ease of preparation, the poly(AMPS-co-PEGDA) monolith is expected to find many applications.

A unique structural feature of the new monolith is the use of PEGDA instead of the conventional EDMA crosslinker, which is believed to result in the high resolution and sharp peaks obtained for peptide analysis. Due to the hydrophobicity of the AMPS monomer, a better monolith could be obtained if a more hydrophilic functional monomer was used. For example, if acrylamido methanesulfonic acid or 2-acrylamido-1-ethanesulfonic acid was used in place of AMPS, the hydrophobicity of the resulting monolith would be dramatically decreased. This should, in turn, provide even better separation of peptides and make efficient SCX of proteins possible with aqueous buffers containing no acetonitrile. Unfortunately, neither of the two monomers is commercially available. I am currently investigating their synthesis.

3.5 References

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CHAPTER 4 POLYMER MONOLITHS WITH LOW HYDROPHOBICITY FOR STRONG CATION-EXCHANGE CAPILLARY LIQUID CHROMATOGRAPHY OF PEPTIDES AND PROTEINS

4.1 Introduction

High performance liquid chromatography has grown in importance for proteomics research due to its high resolving power, excellent reproducibility and ease of interfacing with mass spectrometry.[1] Because of the extreme complexities of peptide mixtures in "shotgun" proteomics,[2] orthogonal two-dimensional (2-D) liquid chromatography is required for which overall peak capacity is the product of the peak capacities of each dimension. The most widely used 2-D liquid chromatography combination is ion-exchange chromatography [especially strong cation-exchange chromatography (SCX)] followed by reversed-phase (RP) chromatography.[3,4] For this combination, it is important to use a hydrophilic SCX column that possesses negligible mixed-mode (i.e., ion-exchange and hydrophobic interaction) retention of peptides. Otherwise, the resultant 2-D liquid chromatography is not strictly orthogonal and the final overall peak capacity is compromised. In the worst case, some very hydrophobic peptides will not elute from the first dimension SCX column. Currently, the Polysulfoethyl A stationary phase, which was developed in the late 1980s,[5-7] is used most widely for SCX chromatography of peptides. However, although relatively hydrophilic, the Polysulfoethyl A column has been found to exhibit some hydrophobicity, and 15-25% acetonitrile is required to suppress hydrophobic interactions to improve both peptide peak shapes and resolution.[5-7]

Polymer monoliths that have comparable chromatographic performance to particle packed columns were introduced in approximately 1990.[8,9] To date, a variety of polymer monoliths with a broad range of surface chemistries have been introduced for use in liquid chromatography (please refer to Chapter 1 for detailed review).[8-15] In contrast to monomers used for preparation of polymer monoliths, the number of crosslinkers is much more limited. Very little effort has been directed toward study of crosslinker effects on chromatographic performance. This is quite surprising since the crosslinker is an integral part of the resulting monolith, typically accounting for 30-70% by weight. As a result, the crosslinker should be expected to significantly affect both the rigidity of the resulting monolith and its overall polarity.

Lee's group was the first to use polyethylene glycol diacrylate (PEGDA) as a biocompatible crosslinker to synthesize an inert polymer monolith for the analysis of peptides and proteins (see Chapter 2).[16] A polyethylene glycol methacrylate-based crosslinker has also been used to prepare RP monolithic columns.[17] The biocompatibility of PEGDA is mainly a result of the PEG segment in the molecule. PEG is a well known material that can effectively resist the adsorption of biomolecules,[18,19] and has found application in various fields related to protein resistance.[20-23]

Using PEGDA as crosslinker, I recently prepared an SCX polymer monolith for capillary liquid chromatography of peptides (see Chapter 3).[24] Using simple one-step copolymerization of 2-acrylamido-2-methyl-1-propane sulfonic acid (AMPS) and PEGDA, the resulting monolith provided extremely narrow peaks and high peak capacity. Although not completely understood, the extraordinary chromatographic performance is believed to be related to the use of the biocompatible crosslinker PEGDA.

In addition, it was demonstrated that excessive swelling could be avoided by using a high percentage (60 wt%) of crosslinker.

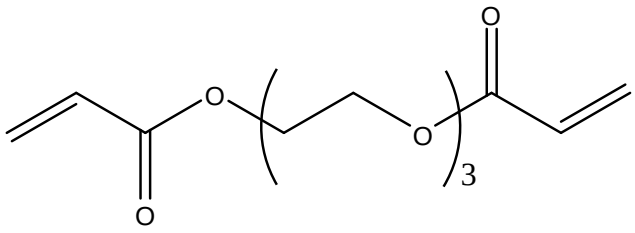
Although quite successful, an obvious drawback of the poly(AMPS-co-PEGDA) monolith is its relatively strong hydrophobicity, i.e., 40% acetonitrile is required to suppress hydrophobic interactions with hydrophobic peptides. I believe that the hydrophobicity mainly comes from the AMPS monomer because it has a 4-carbon moiety (C4) in the molecule (see the structure of AMPS, Figure 4.1).

In an attempt to decrease the hydrophobicity of the poly(AMPS-co-PEGDA) monolith, two other commercially available sulfonic acid-containing monomers, sulfoethyl methacrylate (SEMA) and vinyl sulfonic acid (VS), were investigated to prepare SCX monoliths. It was hoped that by decreasing the hydrocarbon character of the group that linked the sulfonic acid functionality and the acrylate or vinyl group, a monolith with decreased hydrophobicity would result. The final goal of this study was to apply the more hydrophilic monoliths to the resolution of various proteins, including lipoproteins.

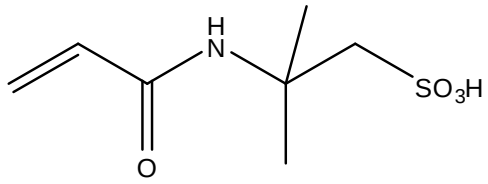
4.2 Experimental Section

4.2.1 Chemicals and Reagents

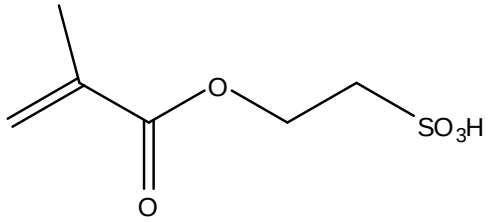
2,2-dimethoxy-2-phenylacetophenone (99%), 3-(trimethoxysilyl)propyl methacrylate (98%), and PEGDA (Mn ~258) were purchased from Sigma-Aldrich (Milwaukee, WI) and used as received. SEMA was obtained from Polysciences (Warrington, PA), and VS (sodium salt, 30% aqueous solution) was purchased from Sigma-Aldrich. Both of the monomers were used without further purification. Porogenic solvents for monolith



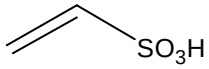
PEGDA



AMPS



SEMA



VS

Figure 4.1. Chemical structures of PEGDA crosslinker and several sulfonic acid-containing monomers.

synthesis and chemicals for mobile phase buffer preparation were HPLC or analytical reagent grade.

Bradykinin fragment 1-7 and proteins (myoglobin from equine skeletal muscle, cytochrome c from bovine heart, α-chymotrypsinogen A from bovine pancreas and lysozyme from chicken egg white) were obtained from Sigma-Aldrich. Synthetic peptide standard CES-P0050 was purchased from Alberta Peptides Institute (Edmonton, Alberta, Canada). High density lipoprotein (HDL) was from Calbiochem (La Jolla, CA). Ethylenediaminetetraacetic acid (EDTA, disodium salt, dihydrate, ultrapure grade) was provided by Invitrogen (Carlsbad, CA).

4.2.2 Polymer Monolith Preparation

UV transparent fused silica capillary tubing (75 μm i.d., 375 μm o.d., Polymicro Technologies, Phoenix, AZ) was silanized with TPM to provide a pendant vinyl group for anchoring of polymer monoliths following a procedure developed by Vidič et al.[25] with slight modifications. Briefly, a 5 m long capillary was rinsed sequentially with ethanol and water. The capillary was then filled with 2 M HCl, and heated at 110 ^{o}C for 3 h in a GC oven with both ends sealed with a union (Upchurch, Oak Harbor, WA). After surface activation, the capillary was rinsed again with water and ethanol, and dried at 120 ^{o}C for 1 h under a nitrogen gas purge. Silanization of the surface-activated capillary was performed with 15% (v/v) TPM in dry toluene at 35 ^{o}C overnight. After silanization, the capillary was washed with toluene and acetone sequentially, and then dried under a nitrogen gas purge at room temperature overnight. Both ends of the silanized capillary were sealed with rubber septa until further use.

Monomer solutions (see Table 4.1 for reagent composition) were prepared in

Table 4.1. Reagents and dynamic binding capacities of poly(AMPS), poly(SEMA) and poly(VS) monoliths.

	Reagent							Dynamic binding capacity[a]	
	DMPA (g)	Monomer (g)	PEGDA (g)	Water (g)	Methanol (g)	Ethyl ether (g)	UV time (min)	Peptide (μequiv/mL)	Protein (mg/mL)
AMPS[b]	0.008	0.32	0.48	0.20	0.55	1.70	3	157	332
SEMA	0.008	0.32	0.48	/	/	0.80	30	62	8
VS	0.0008	1.07[c]	0.48	/	0.75	/	3	11	32

[a] Dynamic binding capacity was measured based on the uptake of bradykinin fragment 1-7 (peptide) or cytochrome c (protein). For experimental conditions, please refer to Section 3.2.3. [b] The reagents and dynamic binding capacity for poly(AMPS) monolith are from Sections 3.2.2 and 3.3.4, respectively. [c] The VS monomer is a 30 wt% water solution.

1-dram (4 mL) glass vials by admixing initiator, monomer, crosslinker and porogens. The monomer solutions were ultrasonicated for 10 s, introduced into the surface silanized capillary by capillary action, and irradiated for a certain amount of time using a UV curing system reported in Section 3.2.2. After the monoliths were prepared, they were connected to an HPLC pump and flushed with methanol and water to remove porogens and any unreacted monomers. Scanning electron micrographs (SEM) of the monoliths were obtained as previously described in Section 3.2.2.

4.2.3 Capillary Liquid Chromatography (CLC)

The CLC system used in this study was described in detail in Section 3.2.3. To decrease the system delay time and set the split ratio to ~1:1000, the splitter capillary was changed to 40 cm long × 30 μm i.d., and the original stainless steel tubing (100 cm long × 1/32 inch o.d. × 200 μm i.d.) from the mobile phase mixer was replaced with an open tubular capillary (70 cm long × 360 μm o.d. × 75 μm i.d.). The chromatographic conditions are given in the figure captions. The dynamic binding capacities of the test peptides and proteins were measured, following exactly the procedure previously described in Section 3.2.3.

Safety Considerations. The SEMA and VS monomers, and the PEGDA crosslinker are sensitizing agents. Appropriate MSDS information should be consulted for handling of these materials. Sunglasses that block UV light and gloves should be worn to avoid burns caused by the high-power UV-curing system during the preparation of the monoliths.

4.3 Results and Discussion

4.3.1 Preparation of Polymer Monoliths

The proper selection of porogen is of paramount importance in the preparation of a monolith for use in chromatography. Because PEGDA was used as crosslinker and sulfonic acid-containing monomers similar to AMPS were used, the initial choice of porogen was a mixture of water, methanol and ethyl ether as used in Section 3.2.2. Although polymer monoliths were formed using this recipe, cracks along the axis of the capillary were observed under an optical microscope. This resulted in monoliths with extremely low flow resistance and poor column efficiency, because of channeling of the mobile phase through the cracks in the monolith. New porogens had to be found to prepare the poly(SEMA) monolith. After extensive screening, a binary porogen composed of ethyl ether and hexanes yielded monoliths that were macroscopically uniform and possessed very low flow resistance. Further optimization, however, revealed that ethyl ether was unnecessary to be included as a coporogen. The optimized reagent composition for the poly(SEMA) monolith is given in Table 4.1. Figure 4.2 shows an SEM image of the poly(SEMA) monolith. The monolith was attached to the capillary wall, and no cracks were observed. A morphology typical to conventional polymethacrylate monoliths was obtained.

The preparation of the poly(VS) monolith was originally thought to be somewhat challenging because the VS monomer could only be obtained as a 30 wt% water solution and not in the neat form. This introduced the requirement that water must be included in the monolith recipe and the weight ratio between VS and water had to be equal to (no addition of water) or less than 3/7 (with addition of water). Fortunately, the preparation of

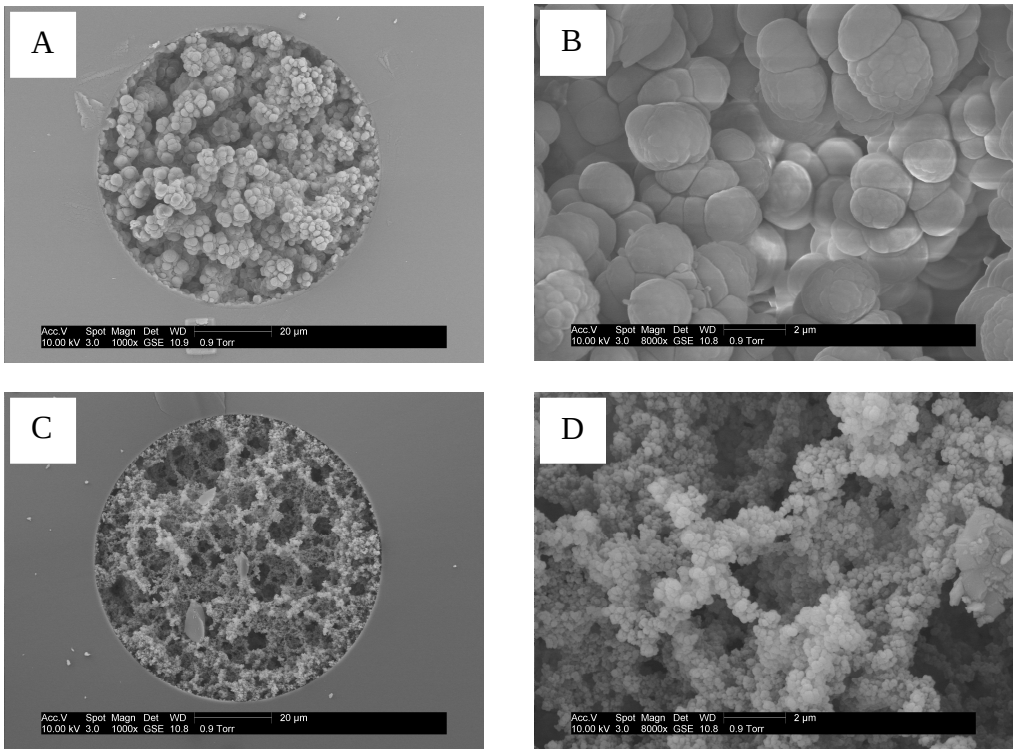


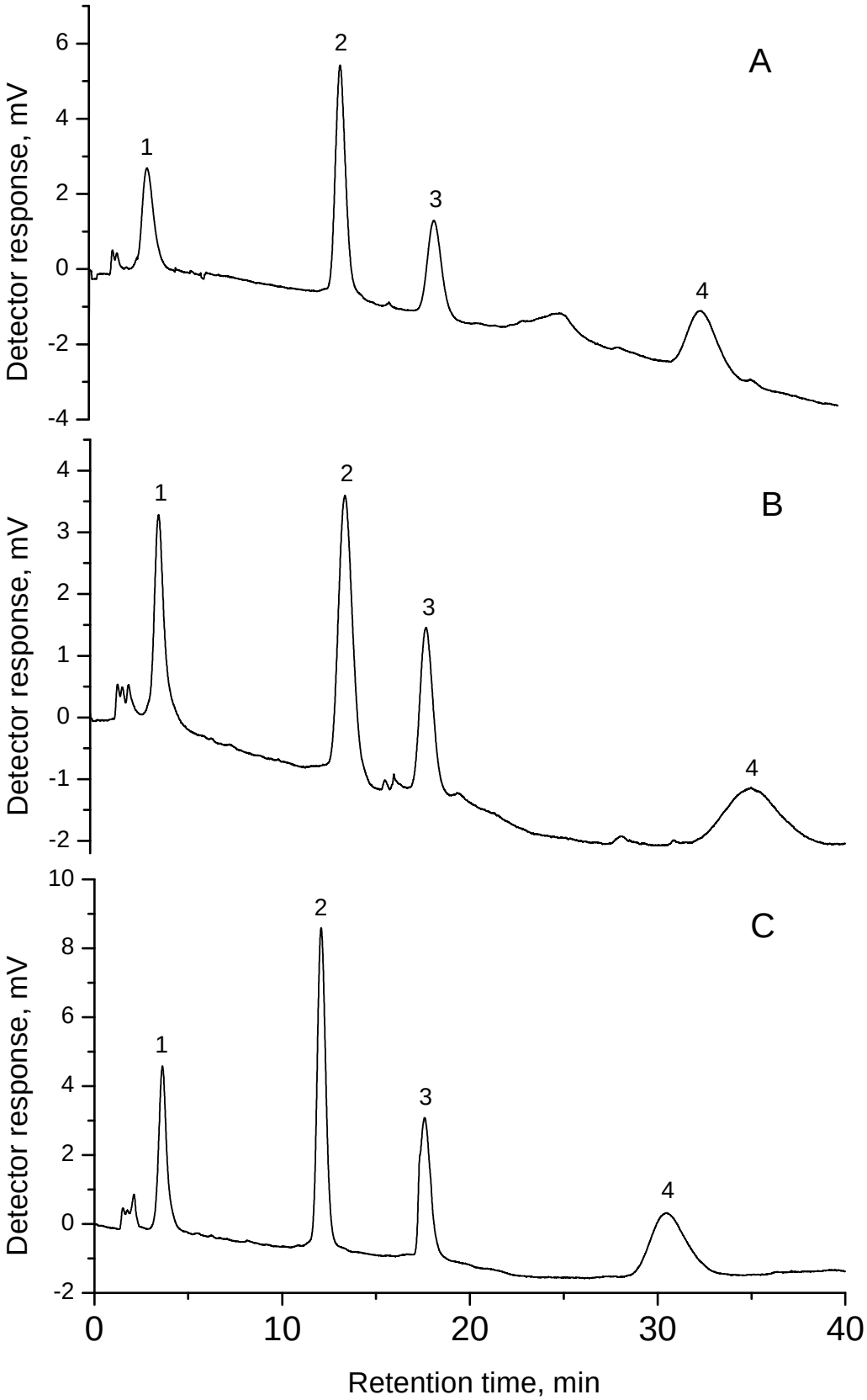
Figure 4.2. SEM images of poly(SEMA) and poly(VS) monoliths. (A) poly(SEMA) monolith (scale bar = 20 μm); (B) higher magnification of the monolith in (A) (scale bar = 2 μm); (C) poly(VS) monolith (scale bar = 20 μm); (D) higher magnification of the monolith in (C) (scale bar = 2 μm).

the poly(VS) monolith was far less difficult than anticipated. A combination of water and methanol was found effective in generating a stable flow-through monolith. The optimized reagent composition is listed in Table 4.1. SEM of the optimized poly(VS) monolith (Figure 4.2C-D) revealed a different morphology compared to the poly(SEMA) monolith, but a similar morphology to the poly(AMPS) monolith.

4.3.2 Hydrophobicity of the Poly(SEMA) Monolith

Four synthetic undecapeptides (see Table 3.1) were used to determine the hydrophobicity of the poly(SEMA) and poly(VS) monoliths. Figure 4.3 shows a gradient elution separation of the four synthetic peptides using buffers that contain different amounts of acetonitrile. For the most hydrophobic peptide 4 with hydrophobicity index 24.2 at pH 2.0,[24] 40% acetonitrile was required to suppress the hydrophobic interaction between the monolith and the peptide. For the other three peptides, there was negligible difference between the elution patterns between 0% to 20% acetonitrile additives. However, when higher concentrations of acetonitrile (e.g., 30 or 40%) were used in the mobile phase, narrower peaks were observed. In general, the elution pattern of the four synthetic peptides using poly(SEMA) were similar to that of the poly(AMPS) monolith. However, much lower column efficiency was observed for the newly prepared poly(SEMA) monolith, although resolution of the four peptides was acceptable. A peak capacity of 21 was achieved for the poly(SEMA) column using buffers containing 40% acetonitrile, in contrast to 71 for the poly(AMPS) monolith.

It is surprising that the hydrophobicity of poly(SEMA) is similar to that of poly(AMPS) although there is less hydrocarbon character in the SEMA molecule.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Detector response, mV
Retention time, min
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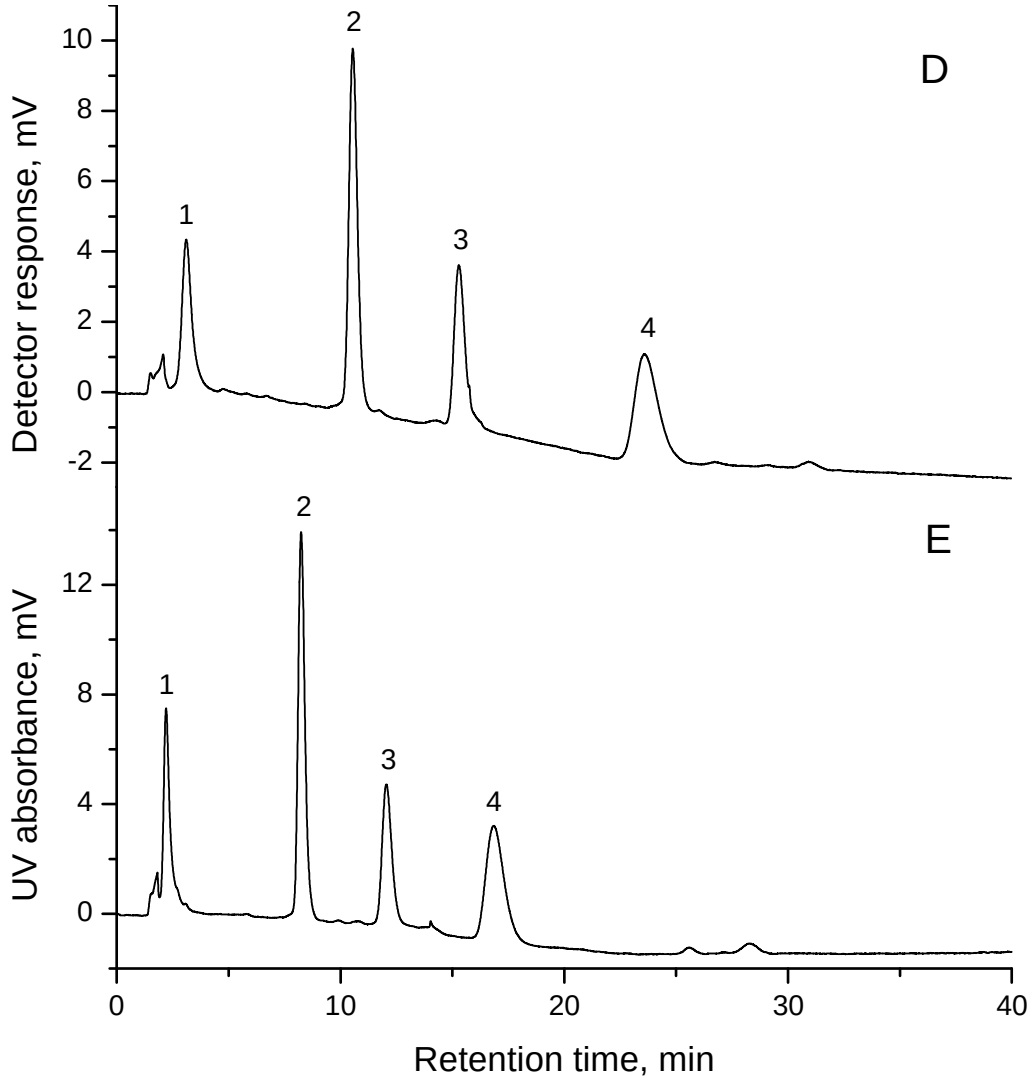


Figure 4.3. SCX chromatography of four synthetic undecapeptides. Conditions: 15 cm × 75 μm i.d. poly(SEMA) monolithic column; buffer A was 5 mM NaH_2PO_4 (pH 2.7) and buffer B was buffer A plus 1.0 (panel A) or 0.5 M NaCl (panels B, C, D and E), both buffers containing 0, 10, 20, 30, or 40% (v/v) acetonitrile (panels A, B, C, D, and E, respectively); 2 min isocratic elution of 1% B, followed by a linear AB gradient (5% B/min for panels B, C, D and E, and 2.5% B/min for panel A) to 100% B and various times of isocratic elution with 100% B until peptide 4 was eluted; 1.8 min gradient delay time; mixture of peptides 1-4 (Table 3.1); 12 μL/min pump master flow rate; 510, 460, 440, 440 or 440 nL/min column flow rates (panels A, B, C, D, and E, respectively); online UV detection at 214 nm.

Therefore, for overall hydrophobic interaction, other factors must be considered. Due to the single bond connection to the monolith backbone, the sulfonic acid functional group would rotate freely into and out of the backbone. In some circumstances, analytes could directly interact with the backbone of the monolith. Although the contribution to hydrophobicity by the biocompatible PEGDA crosslinker was found to be insignificant, the carbon-carbon linkage resulting from polymerization of vinyl groups in the monomer could lead to some hydrophobic interactions. Thus, the overall hydrophobicity must result from the sum of the hydrocarbon components of the side chains of the functional groups and the backbone of the polymer.

The backbone hydrophobicity is mainly determined by the type of vinyl group and the surface coverage by the functional groups. At present, there is no good methodology available to directly measure the surface coverage by the functional groups. One indirect method is to use dynamic binding capacity to estimate the surface coverage. The dynamic binding capacity of the poly(SEMA) monolith was measured to be 62 μequiv/mL, based on the uptake of bradykinin fragment 1-7 (see Table 3.1). This value is smaller than that of the poly(AMPS) monolith (157 μequiv/mL), indicating a lower surface coverage by the sulfonic acid groups; this results in less hydrophobicity. However, another more important factor that affects the backbone hydrophobicity is the type of vinyl groups in the monomer. The backbone hydrophobicity of poly(AMPS) is low because of the use of the biocompatible acrylamido group. As a result, the overall hydrophobicity of poly(SEMA) (from the C2 sulfonic acid linkage and the backbone) is comparable to that of poly(AMPS) (mainly from the C4 linkage).

Although disappointing for decreasing column hydrophobicity, the poly(SEMA) monolith had very low flow resistance, which made it useful in performing fast separations. Figure 4.4 shows a separation of the four undecapeptide standards in 5 min using a fast flow rate (linear velocity of 43 cm/min) and a sharp gradient. The total analysis time could be further decreased to 2 min by simply using a sharper gradient rate (50% B/min). However, the peak for peptide 4 became somewhat skewed under these conditions.

4.3.3 Hydrophobicity of the Poly(VS) Monolith

Figure 4.5 shows the elution of the four synthetic peptides under various acetonitrile concentrations. It is obvious that the overall hydrophobicity of the poly(VS) column is much less than either poly(AMPS) or poly(SEMA) monoliths. As is seen in Figure 4.5A for which no acetonitrile was used, peptide 4 could be eluted in 40 min, although a tailing peak was observed due to nonspecific hydrophobic adsorption. With an acetonitrile concentration of 30%, hydrophobic interactions could be suppressed, and 40% acetonitrile narrowed the peptide 4 peak somewhat further. The resolution of peptides 2 and 3 was improved with the addition of 20% acetonitrile. Although improvement was made with the addition of acetonitrile, the effect of acetonitrile on the peak profiles for peptides 2 and 3 was not as dramatic as for either poly(AMPS) or poly(SEMA) monoliths, indicating decreased hydrophobicity of the poly(VS) monolith. Peak capacity was increased from 20 (Figure 4.5A) to 27 (Figure 4.5D) with the addition of 30% acetonitrile compared to no acetonitrile, and decreased to 24 (Figure 4.5E) when 40% acetonitrile was used. Therefore, 20-30% acetonitrile is sufficient to suppress the hydrophobic interaction of the poly(VS) monolith. The hydrophobicity of the poly(VS)

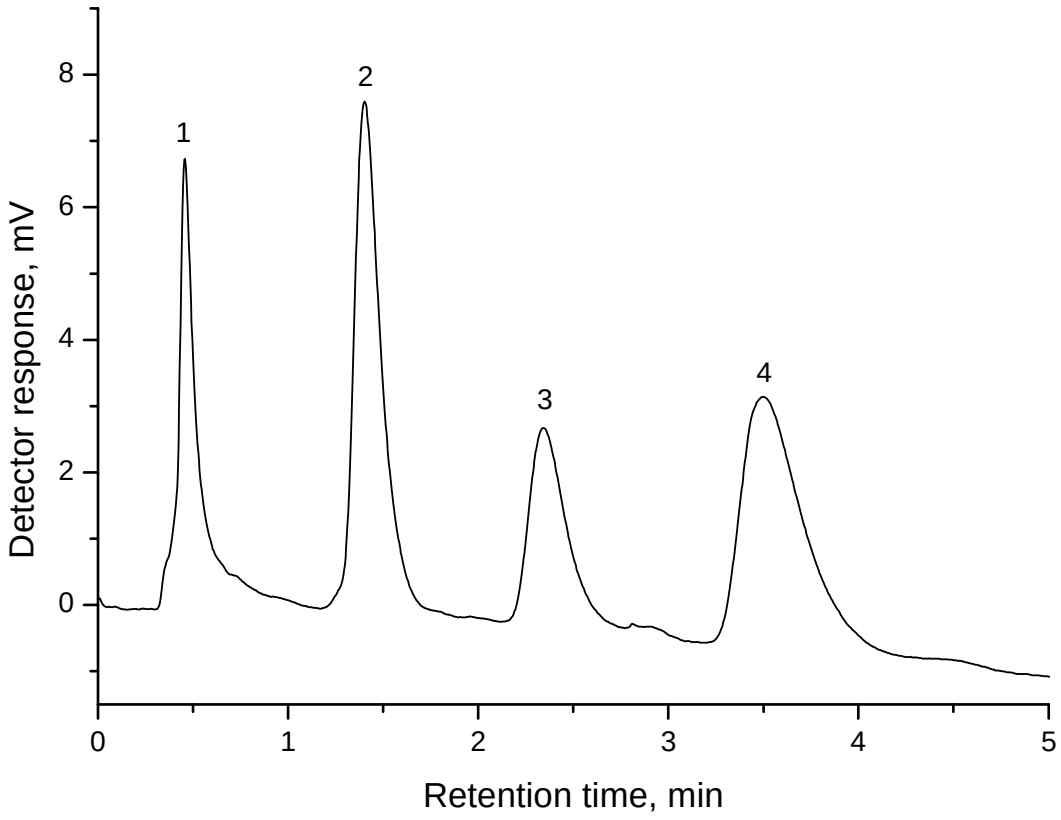
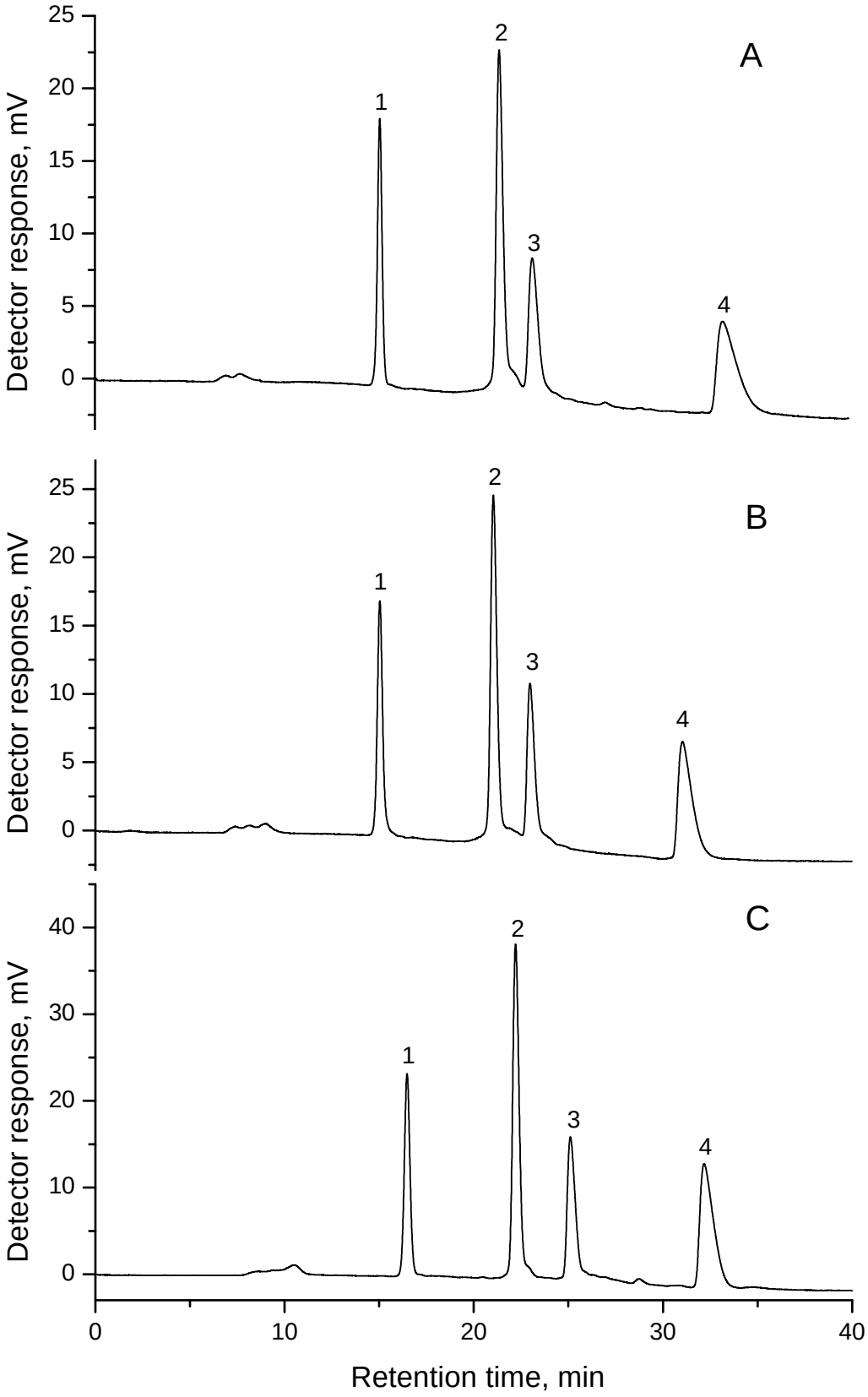


Figure 4.4. Fast SCX chromatography of synthetic peptides using a poly(SEMA) monolithic column. Conditions were the same as in Figure 4.3E except that a faster pump master flow rate of 48 μL/min, column flow rate of 1.9 μL/min and 20% B/min gradient rate were used.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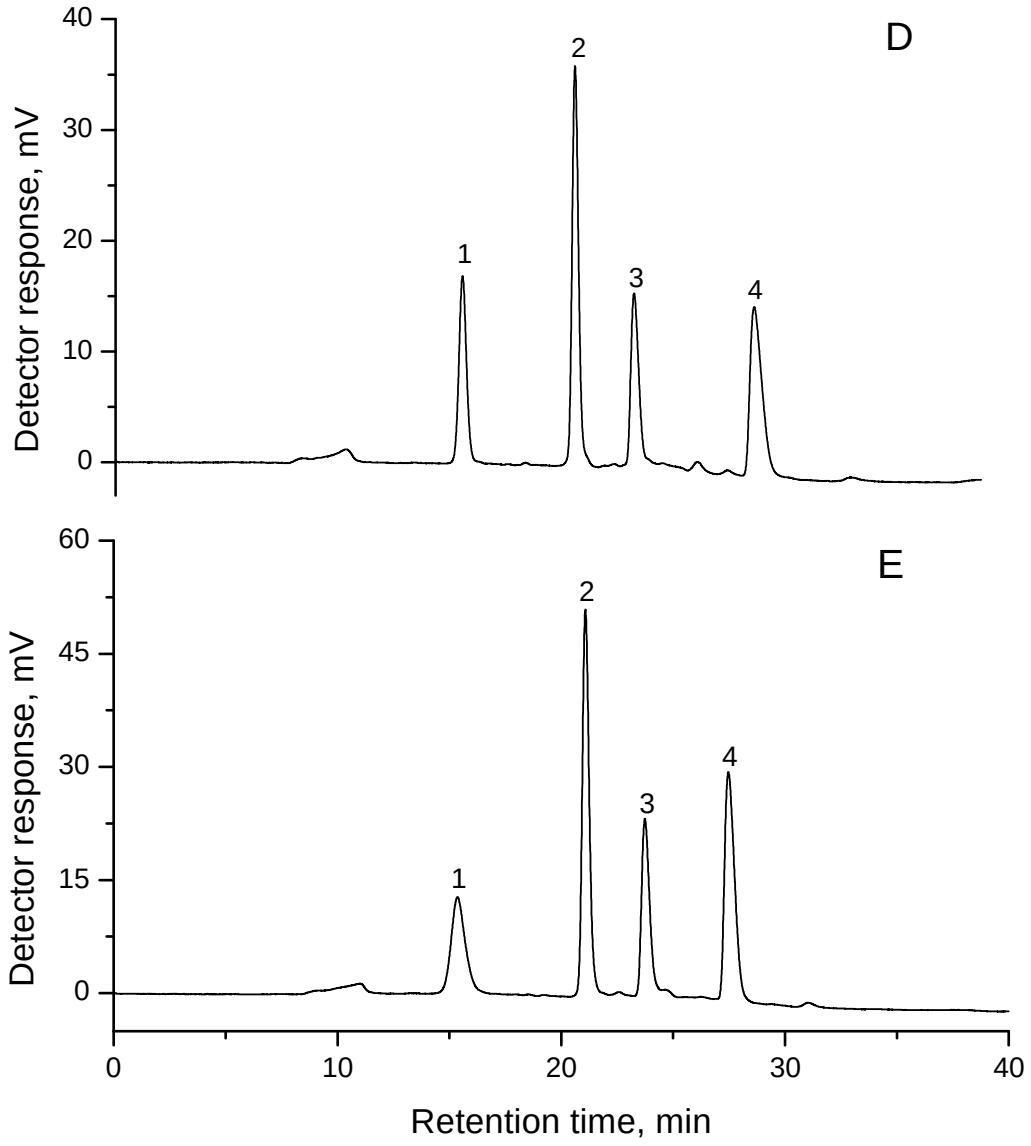

Figure 4.5. SCX chromatography of synthetic peptides using a poly(VS) monolithic column. Conditions were the same as in Figure 4.3 with the following exceptions: 16 cm × 75 μm i.d. poly(VS) monolithic column; buffer B in panel A contained 0.5 M NaCl; pump master flow rate was 24 μL/min; gradient delay time was 8 min; 102, 98, 83, 83 or 78 nL/min column flow rates (panels A, B, C, D, and E, respectively).

monolith must come from the backbone of the monolith because the VS monomer does not have any extra carbon atoms in the linking group. While the dynamic binding capacity of the poly(VS) monolith was smaller than that of the poly(AMPS) monolith, indicating less hydrophobicity, a significant contribution to column hydrophobicity could still come from the backbone of the monolith. Although somewhat hydrophobic, it should be noted that the poly(VS) monolith could elute the most hydrophobic peptide 4 in relatively short time without the addition of acetonitrile, making it useful as a first dimension in proteomics studies.

The column stability and reproducibility of the poly(VS) monolith are excellent. The poly(VS) monolith was continuously used at ~1000 psi head pressure for two months without deterioration of column performance (i.e., resolution, efficiency and peak shape). This confirms that it is feasible to prepare a stable SCX monolith by copolymerization of a sulfonic acid-containing monomer and a crosslinker if high percentage of crosslinker is used. An evaluation of run-to-run reproducibility with buffers containing 30% acetonitrile, gave relative standard deviation values (RSD, n = 5) of retention times and peak heights for the four synthetic peptides of 1.5, 1.0, 0.8, and 0.5, and 2.5, 1.2, 2.0, and 1.6, respectively. Column-to-column reproducibility was also good; the RSDs (n = 3) for retention times and peak heights were 2.5, 1.4, 1.6, and 3.0, and 2.3, 2.8, 1.6, and 4.0, respectively.

4.3.4 Strong Cation-Exchange Liquid Chromatography of Proteins

Figure 4.6 shows SCX chromatography of protein standards using the hydrophilic poly(VS) monolith. Sharp peaks were obtained for all four proteins. Although the poly(VS) monolith generated lower peak capacity for the four undecapeptides than did

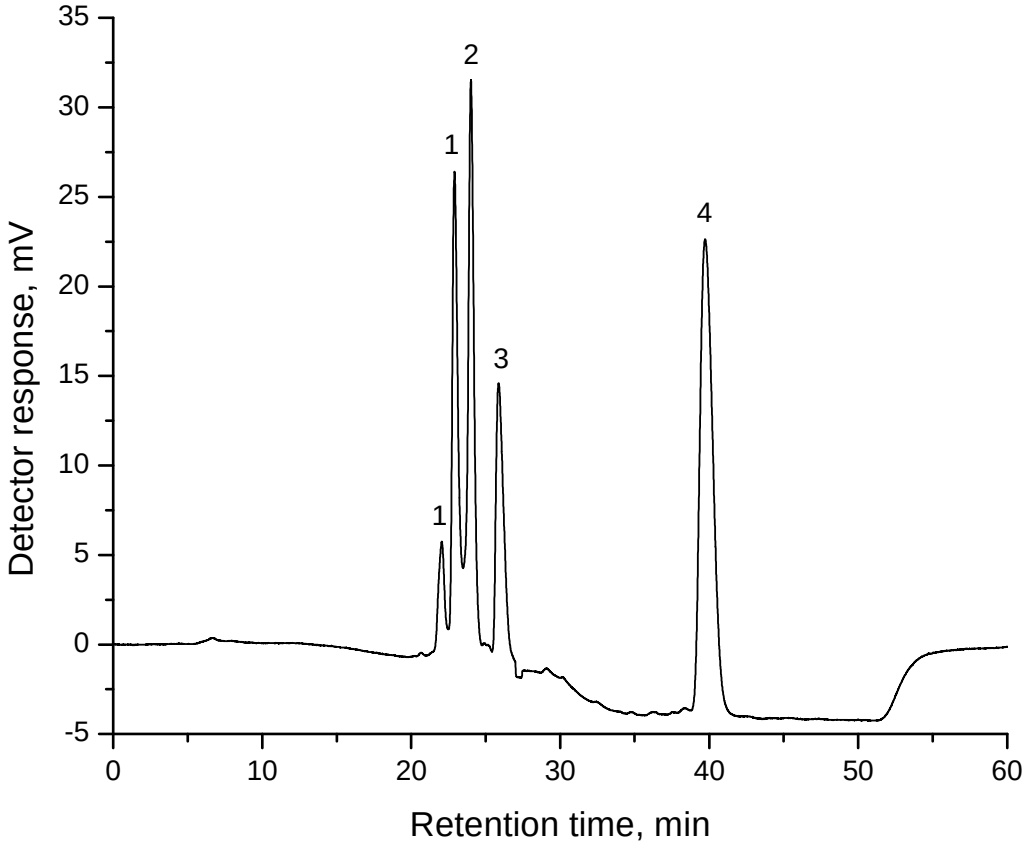

Figure 4.6. SCX chromatography of proteins. Conditions: 16 cm × 75 μm i.d. poly(VS) monolithic column; buffer A was 5 mM phosphate (pH 6.2) and buffer B was buffer A plus 1.0 M NaCl; pump master flow rate, 24 μL/min pump master flow rate; column flow rate was 104 nL/min; gradient delay time was 8 min; linear gradient from 1% B to 50% B in 20 min, ramped to 100% B in 2 min and followed by 20 min isocratic run of B; analytes: (1) 1.14 mg/mL of cytochrome c, (2) 1.60 mg/mL of α-chymotrypsinogen A, (3) 1.10 mg/mL of ribonuclease A and (4) 1.50 mg/mL of lysozyme; the baseline drift during gradient elution and the rise of the baseline at the end of the gradient were due to the difference in UV absorbances of buffers A and B.

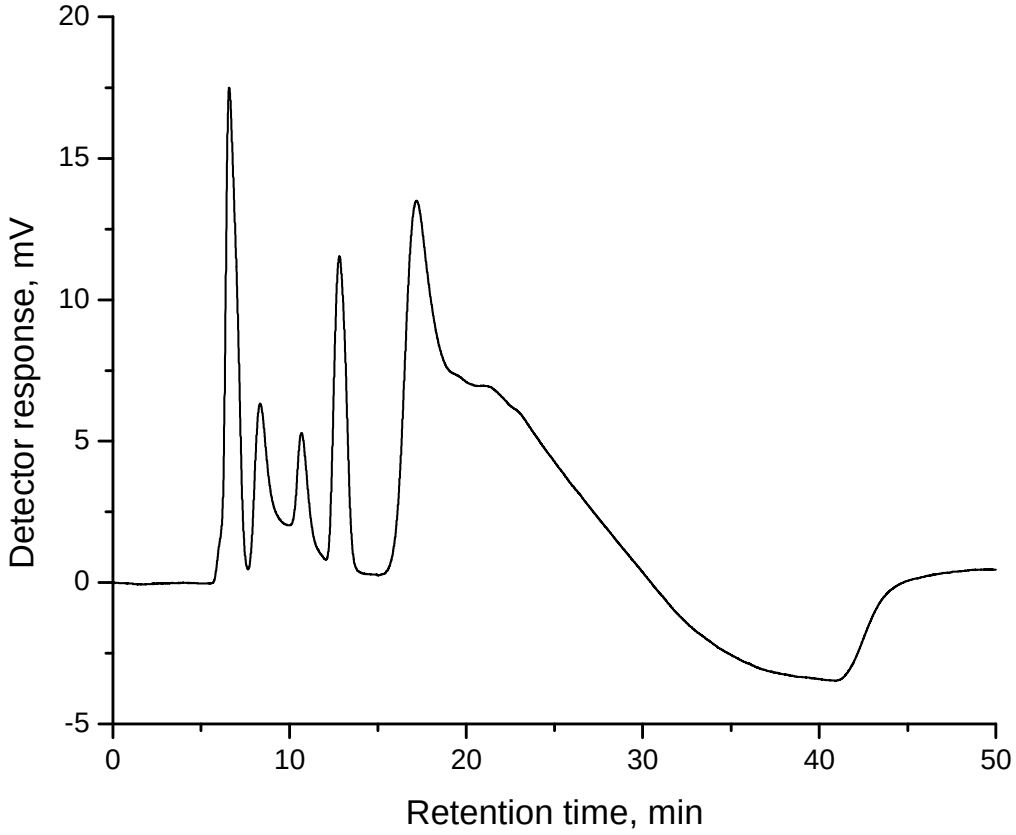

Figure 4.7. SCX chromatography of high density lipoprotein. Conditions were the same as in Figure 4.6 with the following exceptions: buffer A was 10 mM citrate (pH 5.0) containing 0.01% EDTA, and buffer B was buffer A plus 1.0 M NaCl; 2 min 1% B, 20 min gradient from 1% to 100% B, and 12 min 100% B; analyte: 11 mg/mL HDL; online UV detection at 214 nm.

the poly(AMPS) monolith, it yielded better peak profiles for proteins. This indicates that a polymer monolith with less hydrophobicity was prepared. It also demonstrates that a monolith with carefully designed hydrophilicity is beneficial for SCX chromatography of proteins.

The usefulness of the poly(VS) monolith was further demonstrated by SCX chromatography of hydrophobic proteins. Lipoproteins are important biological macromolecular complexes of lipids and apolipoproteins which function to transport lipids in blood.[26] Disorders in lipoprotein metabolism are one of the most important risk factors for the development of coronary heart disease. Because they contain lipids and are bulky, lipoproteins are very hydrophobic and, thus, difficult to analyze using conventional SCX columns.[27] HDL is a very complex mixture that has been resolved into 12 subclasses using 2-D gel eletrophoresis.[28] Using the hydrophilic poly(VS) monolith, five subclasses of HDL were resolved (Figure 4.7). Further optimization of chromatographic parameters for this application is underway.

4.4 Conclusions

In this study, I prepared stable SCX monoliths by copolymerizing sulfonic acid-containing monomers and PEGDA crosslinker. In the design of SCX polymer monoliths for peptides and proteins, it is important to control the overall hydrophobicity to decrease nonspecific interactions. The overall hydrophobicity of the monolith can be tuned by the use of appropriate crosslinkers and monomers. The contribution of hydrophobicity from the monomer mainly results from the linking group that connects the sulfonic acid functionality with the polymerization functionality. The type of polymerization functionality (e.g, vinyl or methacrylate or acrylamido) also results in different backbone

hydrophobicity. Among the three monomers (AMPS, SEMA and VS) studied, VS resulted in a monolith with the least hydrophobicity.

Further improvement should be achieved with the use of more suitable monomers. For example, if acrylamido methanesulfonic acid is used as a functional monomer and PEGDA as a crosslinker, an SCX monolith with negligible hydrophobicity would be expected. Although I have already synthesized this monomer, I have not been able to purify it sufficiently. Another potentially useful monomer would have acrylate or methacrylate at one end, PEG in the middle, and sulfonic acid at the other end. By using PEGDA as a crosslinker, an ideal monolith with backbone completely comprised of PEG and surface comprised of sulfonic acid would be obtained. Detailed description of the improvement in hydrophilicity of SCX monoliths is provided in Chapter 5.

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CHAPTER 5 FUTURE DIRECTIONS

5.1 Optimization of Pore Volume Distribution of the Poly(PEGMEA-co-PEGDA) Monolith for SEC of Proteins

To date, only two reports have been found for preparing polymer monolithic SEC columns.[1,2] The first report was based on poly(styrene-co-divinylbenzene) prepared via stable free radical initiation.[1] Although the monolith possessed relatively broad pore volume distribution, the separation of model linear polystyrene standards (Molecular masses of 3,200,000, 210,500 and 580) indicated that column efficiency was too low to achieve moderate resolution. Only marginal separation was achieved for SEC of the three polystyrenes. Another potential problem is the use of very hydrophobic polystyrene chemistry. Although surface modification of the polystyrene could render it somewhat hydrophilic, it is very difficult to completely modify the surface via on-column modification. As a result, it is very challenging to perform SEC of proteins using this type of chemistry.

Lubbad et al. used the ROMP method to prepare a polynorbornene monolith for SEC of synthetic polymers.[2] By using a mixture of suitable crosslinkers, a monolith, which possesses both good mechanical strength and, more importantly, continuous pore size distribution, was obtained. Due to the hydrophobic nature of polynorbornene, the monolith was evaluated for SEC of polystyrene standards. Although the monolith was used for fast separation, resolution of standard polystyrenes was still insufficient, as

observed from overlay of chromatograms of several polystyrene standards. The norbornene-based monolith has the same limitation as the polystyrene-based monolith for aqueous SEC of proteins, as described in the previous paragraph.

There is clearly a need to develop novel polymer monoliths for aqueous SEC of proteins. In Chapter 2, an inert poly(PEGMEA-co-PEGDA) monolith that exhibited negligible hydrophobic and ionic interactions with proteins was successfully developed. The monolith was applied to SEC of peptides. However, no separation of globular proteins in the molecular weight range of 10-100 kDa was observed. Inverse size exclusion chromatography with the monolith revealed that the mesopore volume, which determines the resolution of proteins, accounted for only 4.2% of the total pore volume, while most of the pore volume was contributed from the macropores (77.8%) and micropores (10.9%). Because of the 10.9% micropore volume, separation of peptides was achieved. Thus, for SEC of proteins using this type of inert monolith, a mesopore volume of at least 10% must be obtained. It is also well known that the efficiency in SEC is much lower for macromolecules (e.g., proteins) than small molecules (e.g., peptides) due to the smaller diffusion coefficients of macromolecules.[3] This implies that a mesopore volume much greater than 10% would be required to effectively separate proteins. It would be desirable if a mesopore volume >20% was obtained.

I propose to synthesize polymer monoliths for aqueous SEC of proteins using the inert PEGMEA chemistry. Two approaches will be explored to optimize the mesopore volume. The first is to use a template in the porogen design. I plan to investigate PEG as a porogen for the preparation of a poly(PEGMEA-co-PEGDA) monolith. PEG has been used as porogen or coporogen for the preparation of other polymer monoliths.[4,5] However,

in these two examples, the reasons for using PEG as a porogen were mainly to adjust the through-pore diameter and to control the surface hydrophilicity. The PEG I plan to use is intended to control the mesopore volume. During the formation of the polymer monolith, portions of the porogen will be trapped inside globules and between globules. After polymerization and flushing, the porogen will be washed out, leaving the desirable pores. By choosing a suitable chain length of PEG porogen, which has a Stokes's diameter that is comparable to the diameters of globular proteins, monoliths with templated porous structure will be obtained. My preliminary results show that PEG (Mw of 3300) is a very promising porogen to provide a monolith with large number of mesopores. SEC of two model proteins, bovine serum albumin and thyroglobulin, was recently obtained (data not shown). Although not baseline separated, this demonstrates the potential of using PEG to engineer the pore volume distribution. Further optimization (e.g., adjusting the ratio between PEG and coporogen, tuning the ratio between total monomer to total porogen, and increasing the column diameter and length) will improve the SEC separation.

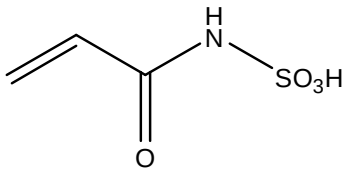
The other approach to adjust the mesopore volume is to increase the crosslinker to monomer ratio in the monolith recipe. Based on a related study involving solid phase extraction using a poly(styrene-co-divinylbenzene) monolith, a significant increase in surface area was obtained if the divinylbenzene crosslinker to styrene monomer ratio was increased.[6] The surface area of typical polymer monoliths is in the range of 1-20 m^2/g. By using 80 wt% of divinylbenzene in the recipe, a monolith with surface area as high as 400 m^2/g was obtained.[6] This increase in surface area indicates an increase in micro and/or mesopore volume. I plan to greatly increase the PEGDA ratio in the poly(PEGMEA-co-PEGDA) monolith recipe for increasing the mesopore volume. It is likely that a

combination of both approaches proposed in this dissertation will ultimately result in a highly original polymer monolith that can be used for fast, efficient and high resolution of proteins in the SEC mode.

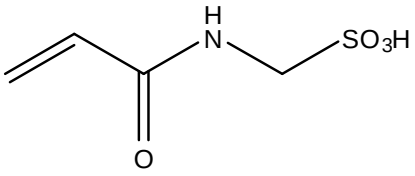
5.2 Further Improvement in SCX Monolith Hydrophilicity

In Chapters 3 and 4, three polymer monoliths were prepared and used for SCX chromatography of peptides and proteins. The poly(AMPS) monolith generated the highest resolution and peak capacity for the analysis of peptides. However, it was not well suited for protein analysis due to its strong hydrophobicity. The poly(SEMA) monolith surprisingly possessed the same strong hydrophobicity as poly(AMPS). An attractive feature, however, was the low flow resistance, making it possible to perform fast SCX chromatography of peptides. The least hydrophobic monolith was obtained when VS was used as the functional monomer. The poly(VS) monolith was useful for SCX chromatography of both peptides and proteins including lipoproteins. Although relatively hydrophilic, the poly(VS) monolith still possessed some hydrophobicity, i.e., 20-30% acetonitrile was required to suppress hydrophobic interactions for highly hydrophobic peptides.

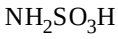
Clearly, a further decrease in hydrophobicity will result in a better SCX monolith for the analysis of biological analytes. This can be achieved by designing and using more suitable monomers. I propose to use two types of monomers to achieve this goal. The first monomer will be acrylamidosulfonic acid or acrylamidomethane sulfonic acid (Figure 5.1). These two monomers, analogs to AMPS, have shorter linking groups between the acrylamido and sulfonic acid functionalities. According to the studies described in Chapters 3 and 4, the hydrophobicities of monoliths prepared from such



Acrylamidosulfonic acid



Acrylamidomethane sulfonic acid



Sulfamic acid

Figure 5.1. Chemical structure of the proposed monomers.

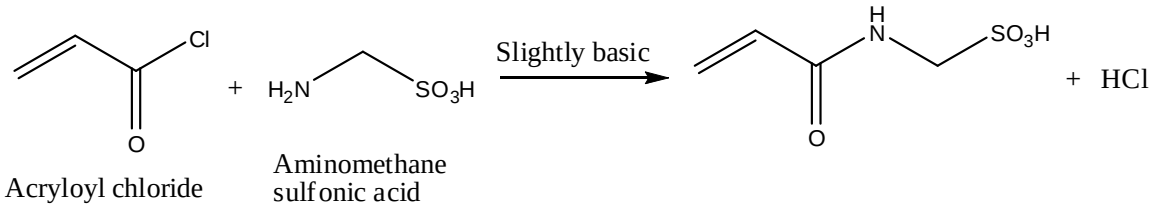


Figure 5.2. Scheme for the synthesis of acrylamidomethane sulfonic acid.

monomers will be greatly decreased. A similar porogen system (e.g., water, methanol, ethyl ether and hexanes) will be used to synthesize the monolith. A challenging task, however, will be the synthesis of such monomers. I plan to use acryloyl chloride and sulfamic acid or aminomethylsulfonic acid as reactants (Figure 5.2). Preliminary results indicate that acrylamidomethane sulfonic acid can be synthesized. I plan to purify the product (monomer) from reactants by using preparative ion exchange chromatography.

Another type of monomer that could be potentially useful for preparing SCX monolith with negligible hydrophobicility is shown in Figure 5.3. Because the poly(PEGMEA-co-PEGDA) monolith has proven to be an inert monolith for proteins (Chapter 2), the monomer I propose here will provide a monolith that has negligible hydrophobicity. This will in turn result in one of the best SCX polymer monoliths for biological compound analysis.

5.3 Preparation of Anion-exchange Polymer Monoliths Using PEGDA as Crosslinker

With the recent successful development of SCX monoliths, future efforts will be naturally directed towards the synthesis of anion-exchange monoliths. Once again, I will use the biocompatible PEGDA crosslinker to decrease backbone nonspecific hydrophobic interactions during the anion-exchange process. Both direct copolymerization and surface derivatization approaches will be explored to introduce the amine functionality (Figure 5.4).

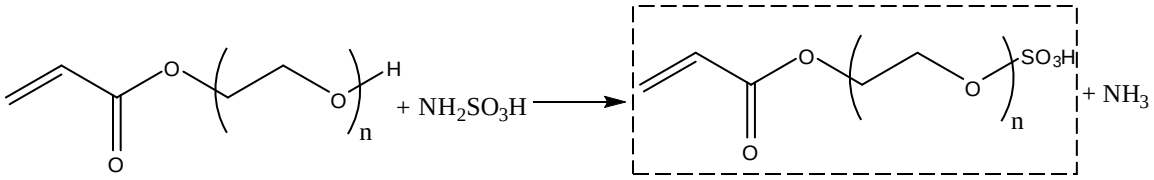


Figure 5.3. Scheme for the synthesis of the proposed novel monomer.

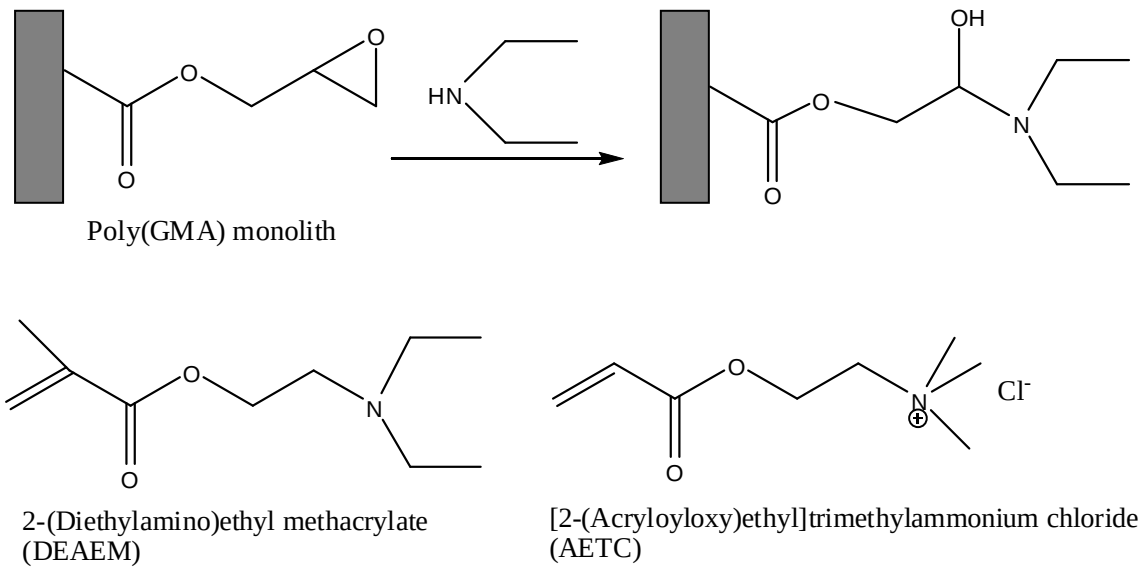


Figure 5.4. Approaches for the preparation of anion-exchange monoliths.

In the derivatization approach, a poly(GMA) monolith using PEGDA as crosslinker will be prepared first, followed by chemical modification with diethylamine to provide the desirable weak anion-exchange monolith. Alternatively, direct copolymerization of amine-containing functional monomers such as DEAEM or AETC with PEGDA will yield monoliths with weak and strong anion-exchange functionalities, respectively. A systematic study will be performed to evaluate the different approaches for the preparation of anion-exchange monoliths using model acidic protein standards such as myoglobin, conalbumin, ovalbumin and soybean trypsin inhibitor. My preliminary results indicate that the poly(AETC) monolith had the least hydrophobicity and exhibited the highest column efficiency. Application of the poly(AETC) monolith to lipoproteins gave very encouraging results; 12 subclasses of high density lipoproteins were resolved using simple gradient elution anion-exchange chromatography. I will optimize the synthesis variables and chromatographic parameters for improvements in both mechanical stability and chromatographic performance.

5.4 Preparation of Other Types of Polymer Monoliths Using PEGDA as Crosslinker

It will be straightforward to prepare a monolith using PEGDA as crosslinker for use in hydrophobic interaction chromatography. This can be easily achieved by using a large amount of PEGDA and a small amount of hydrophobic monomer (e.g., butyl acrylate) in the monolith recipe. The resulting monolith will have a hydrophobicity that is small enough to retain proteins only under high concentration of sodium sulfate. As a result, separation will be guided by the hydrophobic interaction chromatography mechanism. On the other hand, a reversed-phase monolith could be prepared if I use a

large percentage of hydrophobic monomers and a small portion of PEGDA in the monolith recipe. Considering the lack of good polymethacrylate or polyacrylate monoliths for use in reversed-phase capillary LC of proteins or peptides,[7] my aim is to prepare a RP monolith that has very good chromatographic performance, comparable to the well developed polystyrene-based monolith. This ambitious goal could be achieved through optimization of the synthesis recipe. A porogen that can provide abundant mesopores will be useful to achieve such a goal because mass transfer resistance is much smaller in mesopores than in micrpores in the monolith. PEG holds promise as a porogen for such purposes.

In chiral separation, nonspecific hydrophobic and/or ionic interactions are detrimental. Chiral stationary phases mainly include three types: three-point interaction, protein, and cavity phases.[8,9] The most popular stationary phase is the three-point interaction type. Any chemicals that possess the required chiral recognition capability and have polymerizable vinyl groups could be potentially copolymerized with PEGDA to yield chiral monoliths. With the use of the biocompatible PEGDA crosslinker, I hope that significant advances in chiral polymer monolithic stationary phases will be obtained.

Finally, the PEGDA crosslinker is also suitable for preparation of affinity monoliths. For example, poly(GMA), a widely used monolith, is often used as a base material for immobilizing affinity ligands. By using PEGDA to replace conventional EDMA or TRIM crosslinkers, nonspecific binding from the backbone will be decreased.

5.5 Application of PEGacrylate-based Monoliths to Proteomics Research

The application of polymer monoliths in proteomic research is scarce, compared with packed columns. With the recent commercialization of capillary polystyrene and

polymethacrylate monoliths, more applications should appear in the future. My approach of using a biocompatible PEGDA crosslinker has led to the development of efficient SEC and SCX capillary monolithic columns. The proposed work described in Sections 5.1-5.4 will enable the development of other novel and efficient monoliths that can be used in a variety of chromatographic modes. This opens the possibility to use PEG acrylate-based monolithic capillary columns for 2-D LC of proteins, currently the most promising method for proteomics research.

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