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Development and Optimization of Organic Based Monoliths for Use in Affinity Chromatography

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DEVELOPMENT AND OPTIMIZATION OF ORGANIC BASED MONOLITHS FOR USE IN AFFINITY CHROMATOGRAPHY

by

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DEVELOPMENT AND OPTIMIZATION OF ORGANIC BASED MONOLITHS FOR USE IN AFFINITY CHROMATOGRAPHY

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Affinity chromatography is an important and useful tool for studying biological interactions, such as the binding of an antibody with an antigen. Monolithic supports offer many advantages over traditional packed bed supports in affinity chromatography, including their ease of preparation, low back pressures and good mass transfer properties. Monoliths can be broken down into two basic categories: organic (polymer) and inorganic (silica) monoliths. There are many varieties of polymer based monoliths; however, a large focus has been on co-polymers of glycidyl methacrylate (a functional monomer) and ethylene dimethacrylate (a cross-linking agent). The solvents of choice for making this type of monolith are typically 1-dodecanol and cyclohexanol. The combination of monolith supports with biological ligands of interest in affinity chromatography has given rise to a technique known as affinity monolith chromatography (AMC).

In order to study the conditions needed for preparing affinity monolithic supports, a combinatorial library was prepared in which the polymerization temperature and relative ratio of cyclohexanol to1-dodecanol was varied to determine the effects on the

total protein content that could be achieved with such materials. In the first of this work, glycidyl methacrylate was used along with a cross linking agent that was either ethylene dimethacrylate or trimethylolpropane trimethacrylate. It was found that changing the ratio of these agents could be used to obtain a high protein content for monoliths containing immobilized human serum albumin (HSA). It was also found that these materials could be used for the separation of chiral substances such as (*R/S*)-warfarin and (D/L)-tryptophan. The second study utilized a monolith comprised of a co-polymer of glycidyl methacrylate and ethylene dimethacrylate to examine the effectiveness of this material to entrap carbon-based nanomaterials for eventual use in characterizing such materials or using them in separations based on biologically-relevant proteins or ligands.

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Chapter 1

Introduction

Affinity chromatography is an important approach for studying and utilizing solute–ligand interactions in biological systems. This method makes use of the selective and reversible interactions of many biological compounds, such as the binding of an antibody with an antigen or the binding of an enzyme with a substrate [1, 2]. Affinity chromatography makes use of these interactions by placing one of the binding partners within a column and applying the complementary partner, or analyte. Experiments can then be conducted with this system to obtain information on both the kinetics and thermodynamics of the interaction. The combined use of supports designed for high performance liquid chromatography with an affinity ligand results in a technique known as high performance affinity chromatography (HPAC), which has been shown to be an effective tool for the separation and analysis of biological compounds that can bind to various immobilized ligands [3-8].

One type of support that has recently been used in HPAC is a polymethacrylate-based monolith. Monolithic columns have been of great recent interest for use in HPAC because of their low back pressures, their ease of preparation and their good mass transfer properties [3-5, 9-11]. The combination of affinity ligands with monolith columns is a technique known as affinity monolith chromatography (AMC) [6, 12-14]. Various types of monoliths have been used in AMC, with many such reports using co-polymers of glycidyl methacrylate (GMA) and ethylene glycol dimethacrylate (EDMA) [3-5, 9-11].

The co-solvents utilized to prepare these monoliths are usually cyclohexanol (CyOH) and 1-dodecanol (DoOH), which are used to generate the pores within the monoliths during their preparation. Monolith columns are typically prepared through the use of an initiator and heat. Monoliths are attractive alternatives to packed bed supports in chromatography because of the high external porosity of monoliths. Like packed bed supports, monoliths have also been shown to be effective in immobilizing proteins such as human serum albumin (HSA) [5, 10, 15].

This thesis will focus on the development and optimization of organic-based monoliths for use in HPAC. The first part of this thesis will discuss experiments that were completed in an attempt to improve the total amount of protein that could be immobilized within monolithic supports that were based on a GMA/EDMA co-polymer or a co-polymer of GMA and trimethylolpropane trimethacrylate (TRIM). The protein HSA was used as a model ligand for this work. The second part of this thesis will address new applications that have been explored for monoliths in HPAC and in reversed-phase liquid chromatography (RPLC), with the latter being based on the inclusion of carbon-based nanomaterials.

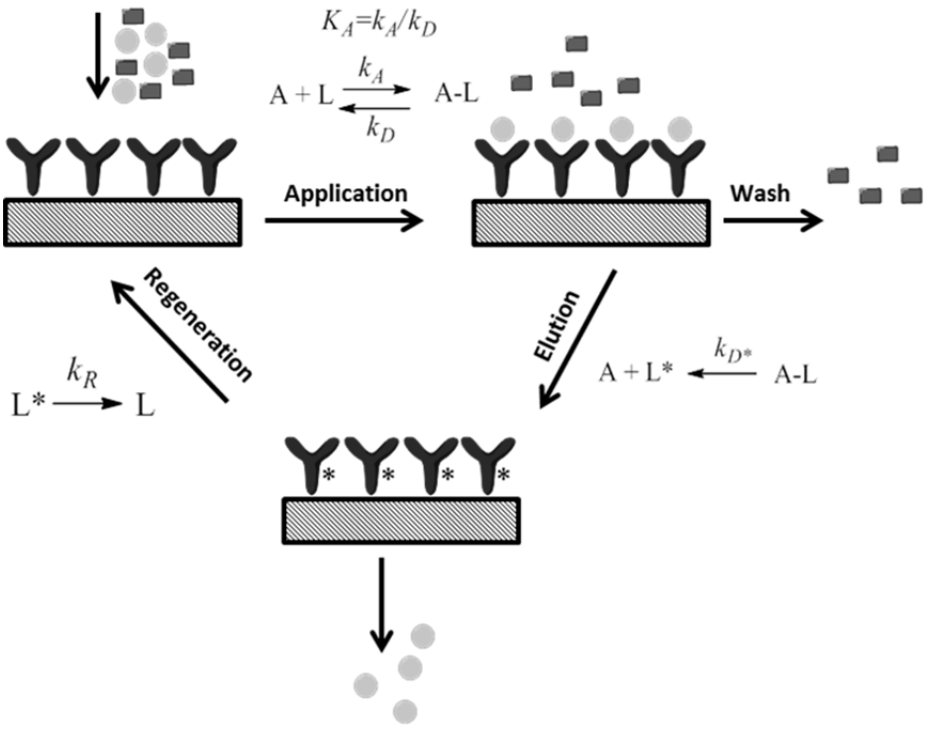
AFFINITY CHROMATOGRAPHY

There are several approaches that can be utilized to perform chemical separations. One useful tool is a technique referred to as affinity chromatography. Affinity chromatography makes use of specific and reversible interactions for the separation and purification of many biologically relevant compounds [16]. These interactions are commonly used in affinity chromatography by immobilizing one of a pair of interacting

compounds, such as a ligand or antibody, to a solid support; this immobilized agent is what comprises the stationary phase and is commonly referred to as the affinity ligand [16]. There are numerous ways to attach an affinity ligand to a solid support, including covalent immobilization, adsorption, or entrapment [16]. After a column containing the desired ligand has been prepared, the complementary agent can then be applied to this column in an on/off elution approach for a selective separation or to examine the binding properties of the ligand with this applied agent.

Figure 1-1 illustrates the traditional approach of sample application and elution that is commonly used in affinity chromatography and HPAC. In this figure, a small plug of analyte is applied to the affinity column in the presence of an application buffer which has the appropriate pH and ionic strength to promote binding between the immobilized ligand and applied target [16]. During this step, the sample components which are complementary to the affinity ligand will bind to the column while the other sample components will be washed from the column and give a peak that contains these non-retained components. This separation will occur because the interaction between the complementary target and ligand is quite selective, with a typical association equilibrium constant of greater than 10^6 M^{-1} when a binding agent such as an antibody is utilized as the affinity ligand. After the non-retained sample components have been eluted, a second mobile phase, known as the elution buffer, is applied that will elute the bound target. As it elutes, this target is typically characterized through the use of an appropriate detection format such as UV-Vis, fluorescence, or mass spectrometry. The column is then regenerated by re-applying the original application buffer [16].

Figure 1-1. A traditional scheme utilized in performing affinity chromatography. In this approach an analyte is applied to the affinity column in an appropriate application buffer. As the compounds that are complementary to the immobilized affinity ligand are allowed to bind to the column, the non-retained sample components elute from the column. Next, an elution buffer is applied that will elute the bound target for collection, measurement or characterization. Following elution of the bound target, the application buffer is re-applied to regenerate the column. Adapted with permission from ref. [16].


$K_A=k_A/k_D$
A + L
k_A
k_D
A-L
Application
Wash
Regeneration
Elution
k_R
L* → L
k_{D^*}
A + L* ← A-L

The on/off elution scheme shown in Figure 1-1 is the most common approach used in affinity chromatography and HPAC, but it is also possible to perform these methods by utilizing isocratic conditions. This second approach involves employing the same mobile phase for elution as is used during application of the target. Isocratic conditions can be utilized if the target only binds to the affinity ligand with moderate to weak affinity, as occurs for a ligand-target interaction that has an association equilibrium constant less than or equal to approximately 10^6 M^{-1} [15].

Like traditional affinity chromatography, in HPAC the immobilized ligand has the ability to bind specifically to the analyte of interest. One example of such an interaction, and one that will be used later in this thesis, is binding of the anti-coagulant drug warfarin with the protein HSA [9, 10] HPAC can be used for studying biological interactions, for protein purification and for chiral separations [5]. It has many advantages over traditional affinity chromatography, such as ease of automation, high specificity, good speed, and high reproducibility [4, 5, 8, 17-20]. In each of these methods it is generally desirable to have a reasonable amount of immobilized ligand to provide for strong retention of the target analyte.

The goal will be to produce supports that contain high amounts of immobilized ligands such as proteins to afford affinity columns with high retention for use in HPAC. One possible use of these supports is in the development of miniaturized HPLC systems [5].

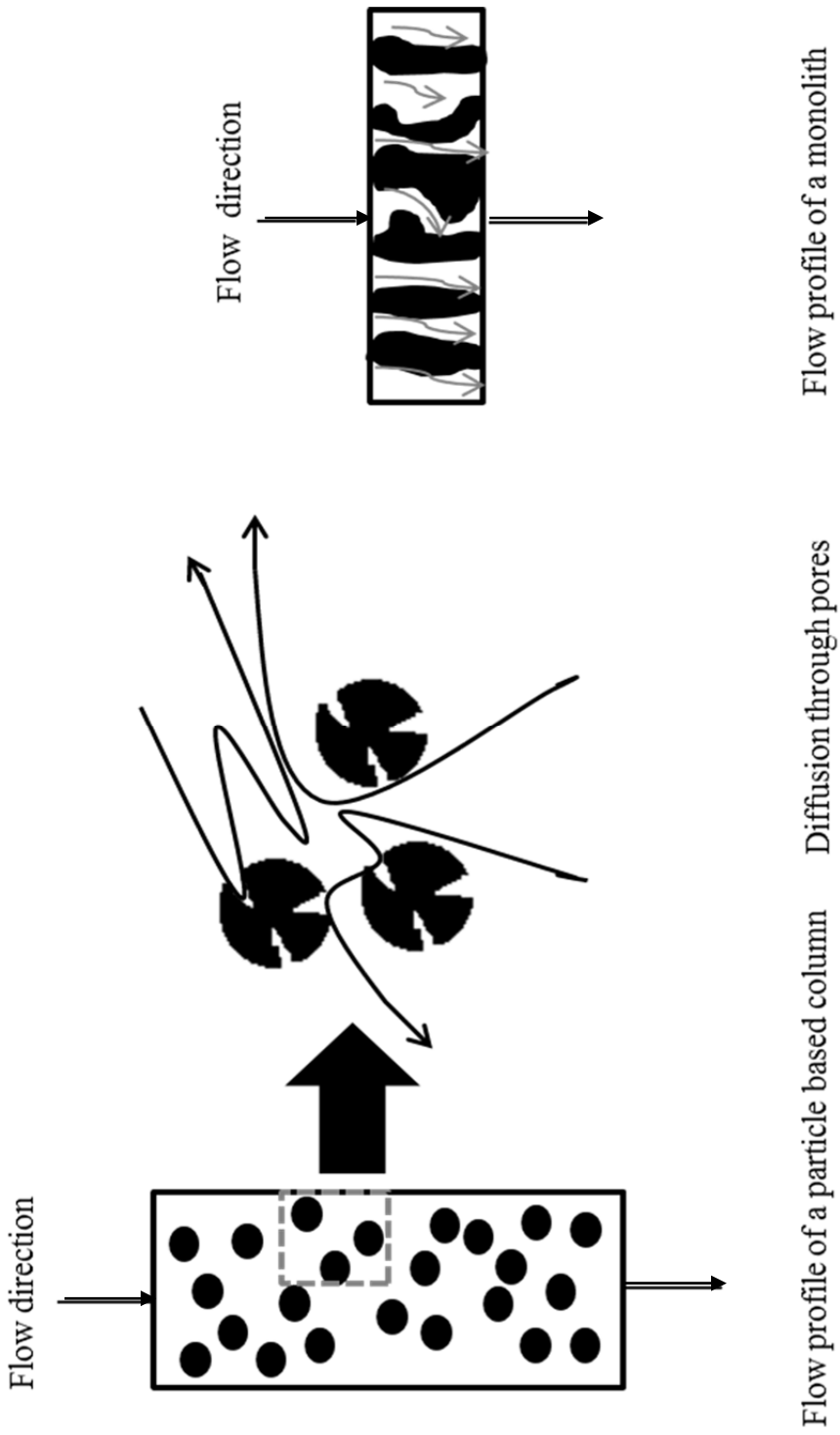
MONOLITH SUPPORTS

Monolithic supports are continuous bed supports that have a higher external porosity than particle-based supports, which also gives monolithic supports higher permeability and lower back pressures [10, 15]. Monoliths are advantageous because they have been shown to decrease band broadening when compared to particle-based supports, thus allowing for more efficient separations [21-23]. An additional advantage in the use of monoliths is that these materials can be prepared in a variety of formats, depending on the desired application. When monoliths are utilized in a chromatographic system flow occurs predominately through the large macropores, with most target interactions with the stationary phase occurring at or near the surface of the macropores. However, when traditional porous particles are utilized in chromatography, the fluid within the pores is considered to be stagnant and the majority of the interactive surface is located within the pores; as a result, the movement of the target to the stationary phase in traditional porous particles tends to occur via diffusion [24]. This difference between monoliths and traditional porous particles is illustrated in Figure 1-2.

There are two main classes of monoliths that are most commonly used in chromatography: polymer-based monoliths (organic) and silica monoliths (inorganic). Organic-based monoliths were first introduced in the 1980s; this development was followed by the development and implementation of silica monoliths in the 1990s [10, 15]. Monoliths generally contain two types of pores. The first group of pores are called "flow-through pores", and the second group are typically referred to as smaller "diffusion" pores. These two groups are also known as macropores and mesopores, respectively [15]. There are several types of materials that have been utilized to prepare

monoliths for affinity chromatography. These materials include cryogels, polymethacrylate, silica, and agarose [25]. Polymethacrylate based monoliths will be the specific type of monolith that will be addressed in this thesis.

Figure 1-2**.** Graphical representation of flow through a particle-based column and a monolith column.


Flow direction
Flow direction
Flow profile of a particle based column
Diffusion through pores
Flow profile of a monolith

IMMOBILIZATION TECHNIQUES

There are several ways to covalently attach a ligand to a chromatographic support. One popular technique for accomplishing this is through the use of the Schiff base method [3-5]. In this approach the support is typically modified to have diol functional groups. Next, the diol groups are oxidized to aldehydes using an oxidizing agent such as periodic acid. Once the aldehyde groups are present it is fairly simple to then attach them to amine containing ligands, such as a protein in the presence of sodium cyanoborohydride via a reductive amination mechanism [16]. This particular approach is easily accomplished on organic monoliths comprised of glycidyl methacrylate (GMA) and ethylene glycol dimethacrylate (EDMA).

The epoxide group present in GMA is commonly used either directly for ligand attachment, as is done with the epoxy immobilization technique, or it can be converted to a diol group through the use of sulfuric acid and heat and then used in other immobilization methods [26]. There are additional types of immobilization techniques that have been utilized for ligand attachment, such as the carbonyl diimidazole (CDI) and disuccinimidyl carbonate (DSC) methods [5].

AFFINITY MONOLITH CHROMATOGRAPHY

It was stated earlier that the combination of affinity ligands with monolith columns is known as affinity monolith chromatography (AMC) [6, 12-14]. Various types of monoliths have been used in AMC, with many of these reports using GMA/EDMA co-polymers as the support material [3-5, 9-11]. The co-solvents utilized to prepare these monoliths are usually cyclohexanol and 1-dodecanol, which are used to generate the

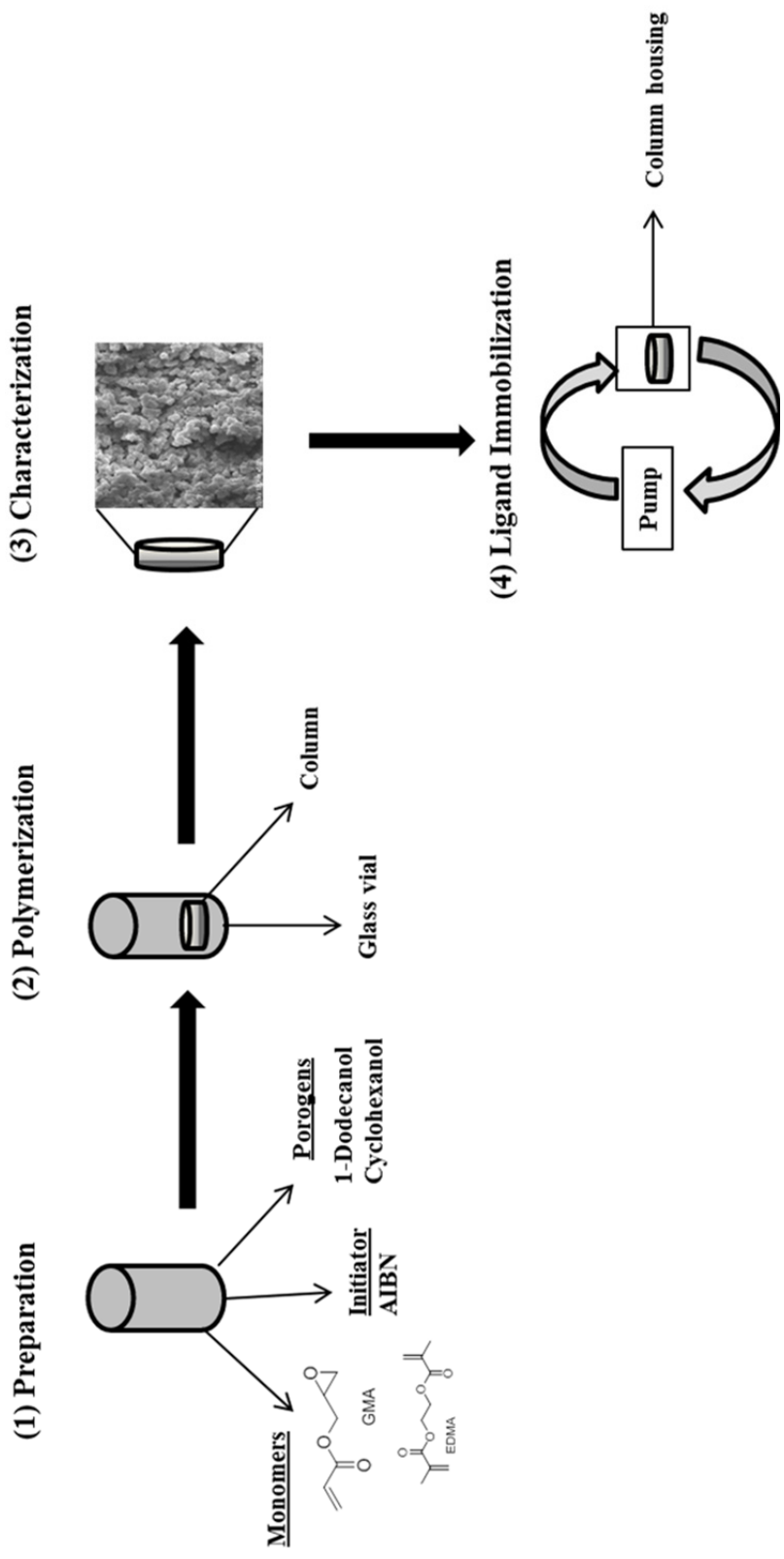
pores within the monolithic column. These solvents are commonly referred to as porogenic solvents or "porogens". Monolith columns are often prepared through the use of an initiator and heat. However, there have also been recent examples in which photo-initiation has proven successful for the polymerization of monoliths in capillaries and within the channels of a microchip [27].

The general procedure for preparing a GMA/EDMA monolith for use in AMC is illustrated in Figure 1-3. The polymerization mixture is first mixed prior to being introduced into the desired casing (i.e., a capillary, disk, chip, or column). This mixture is then allowed to react at the preferred temperature for a given amount of time (e.g., 24 h). After the monolith has been synthesized, it is removed from the casing, if needed, and placed into the appropriate column housing. Typical column housings that are used with such monoliths are comprised of stainless steel and can be made in a variety of lengths. The column is then washed to remove unreacted reagents and excess porogenic solvents. This step is accomplished by applying organic solvents such as methanol or acetonitrile in a continuous, flow-based approach to the column.

One advantage to using GMA/EDMA monoliths in AMC is that the epoxy groups in the polymer can be directly used for immobilization of various ligands (e.g., proteins or other amine-containing ligands) [3, 21, 22]. Not only can polymethacrylate monoliths be used directly for the immobilization of ligands such as proteins, but it is also possible to convert these epoxy groups into diol groups and then to implement various other immobilization methods, such as the Schiff base method, carbonyldiimidazole method, cyanogen bromide method, or *N*-hydroxysuccinimide technique [3, 5]. The ligand of interest is most commonly immobilized within an activated GMA/EDMA monolith by

cycling a solution of the ligand in the appropriate buffer through the monolith support through the use of a reciprocating pump [3, 5]. One way to alter the quantity of immobilized ligand is to adjust the relative amounts of porogenic solvents that are used to generate the monolith. This approach has been proven to have a significant influence on the pore size of the monolith. This change, in turn, will affect the surface area and the amount of ligand that can be attached to the support. This effect was demonstrated in a previous study in which it was found that altering the ratio of dodecanol- to-cyclohexanol during monolith formation had a large effect on the amount of immobilized immunoglobulin G that could be attached to these materials [3].

Figure 1-3. General scheme for the preparation of polymethacrylate-based monoliths. The monomers utilized here are glycidyl methacrylate (GMA) and ethylene dimethacrylate (EDMA). The porogenic solvents are 1-dodecanol (DoOH) and cylcohexanol (CyOH). The initiator is azobisisobutyronitrile (AIBN). Modified with permission from ref [5].


(1) Preparation
Monomers
GMA
EDMA
Initiator
AIBN
Porogens
1-Dodecanol
Cyclohexanol
(2) Polymerization
Column
Glass vial
(3) Characterization
(4) Ligand Immobilization
Pump
Column housing

Overall Goal and Summary of Work

The overall goal of this thesis is to optimize polymethacrylate monoliths based on GMA/EDMA or GMA/TRIM for use in affinity chromatography and reversed phase liquid chromatography. **Chapter 2** will compare and optimize these two types of monoliths for use in protein immobilization. This study will use a combinatorial approach to optimize and improve upon these organic monoliths for use in the immobilization of HSA or similar proteins for AMC. Two different immobilization techniques (i.e., the epoxy method and Schiff base method) will be utilized with these materials and compared to deterime the total protein content that can be obtained in each type of monoliths. Imaging techniques will be utilized to examine the structures of the monoliths and HPLC will be used to study the binding and elution of model analytes with an immobilized protein within the monoliths.

The work in **Chapter 3** will explore various applications of the optimized monoliths for use in HPAC or RPLC. In **Chapter 3,** the monoliths will be prepared to investigate the effectiveness of entrapping carbon based nanomaterials within a monolith. These stationary phases will then be used in RPLC experiments to study the separation of nitrotoluene isomers.

REFERENCES

1. Hage, D.S., *High-performance affinity chromatography and immobilized serum albumin as probes for drug- and hormone-protein binding.* J. Chromatogr. B, 2000. **739**: 39-54.
2. Hage, D.S., *High-performance affinity chromatography: a powerful tool for studying serum protein binding.* J. Chromatogr. B, 2002. **768**: 3-30.
3. Jiang, T., Mallik, R., Hage, D. S., *Affinity Monoliths for Ultrafast Immunoextraction.* Anal. Chem., 2005. **77**: 2362-2372.
4. Mallik, R., Hage, D. S., *Affinity monolith chromatography.* J. Sep. Sci., 2006. **29**: 1686-1704.
5. Mallik, R., Jiang, T., Hage, D.S., *High-performance affinity monolith chromatography: Development and evaluation of human serum albumin columns.* Anal. Chem., 2004. **76**: 7013-7022.
6. Pan, Z., H. Zou, W. Mo, X. Huang, R. Wu, *Protein A immobilized monolithic capillary column for affinity chromatography.* Anal. Chim. Acta, 2002. **466**: 141-150.
7. Tetala, K.K.R., van Beek, T. A., *Bioaffinity chromatography on monolithic supports.* J. Sep. Sci., 2009. **33**: 422-438.
8. Xuan, H., D.S. Hage, *Immobilization of a1-acid glycoprotein for chromatographic studies of drug-protein binding.* Anal. Biochem., 2005. **346**: 300-310.
9. Guiochon, G., *Monolithic columns in high-performance liquid chromatography.* J. Chromatogr. A, 2007. **1168**:101-168.

10. Ikegami, T., Tanaka, N., *Monolithic columns for high-efficiency HPLC separations.* Curr. Opin. Chem. Biol., 2004. **8**:527-533.

11. Nordborg, A., Hilder, E.F., *Recent advances in polymer monoliths for ion-exchange chromatography.* Anal. Bioanal. Chem., 2009. **394**:71-84.

12. Okanda, F.M., Z. El Rassi, *Affinity monolithic capillary columns for glycomics/proteomics: 1. Polymethacrylate monoliths with immobilized lectins for glycoprotein separation by affinity capillary electrochromatography and affinity nano-liquid chromatography in either a single column or columns coupled in series.* Electrophoresis, 2006. **27**: 1020-1030.

13. Urban, J., P. Jandera, *Polymethacrylate monolithic columns for capillary liquid chromatography.* J. Sep. Sci., 2008. **31**: 2521-2540.

14. Uzun, L., Yavuz, H., Say, R., Ersoez, A., Denizli, A., *Poly(ethylene dimethacrylate-glycidyl methacrylate) monolith as a stationary phase in dye-affinity chromatography.* Industrial & Engineering Chemistry Research, 2004. **43**: 6507-6513.

15. Yoo, M.J., Hage, D.S. , *Affinity monolith chromatography:principles and recent developments*, in *Monolithic Chromatography and Its Modern Applications*, P. Wang, Editor 2010, ILM Publications: St Albans. p. 648.

16. Hage, D.S., Ruhn, P., *Handbook of Affinity Chromatography, 2nd Ed.*, D.S. Hage, Editor 2006, Taylor & Francis/CRC Press: Boca Raton.

17. Mallik, R., Xuan, H., Hage, D. S., *Development of an affinity silica monolith containing α1-acid glycoprotein for chiral separations* J. Chromatogr. A, 2007. **1149**: 294-304.

18. Mallik, R., Hage, D. S. , *Development of an affinity silica monolith containing human serum albumin for chiral separations* J. Pharm. Biomed. Anal., 2008. **46**: 820-830.

19. Wu, R., Hu, L., Wang, F., Ye, M., Zou, H., *Recent development of monolithic stationary phases with emphasis on microscale chromatographic separation.* J. Chromatogr. A, 2008. **1184**: 369-392.

20. Yoo, M.J., Hage, D. S., *Evaluation of silica monoliths in affinity microcolumns for high-throughput analysis of drug-protein interactions.* J. Sep. Sci., 2009. **32**: 2776-2785.

21. Kalashnikova, I.I., Natalia; Tennikova, Tatiana, *Macroporous monolithic layers as efficient 3-D microarrays for quantitative detection of virus-like particles.* Anal. Chem., 2007. **79**: 5173-5180.

22. Platonova, G.A., Pankova, G. A., Il'ina, I.Y., Vlasov, G.P., Tennikova, T.B., *Quantitative fast fractionation of a pool of polyclonal antibodies by immunoaffinity membrane chromatography.* J. Chromatogr. A, 1999. **852**:129-140.

23. Podgornik, A., Barut, M., Jancar, J., Strancar, A., *Isocratic separations on thin glycidyl methacrylate-ethylenedimethacrylate monoliths.* J. Chromatogr. A, 1999. **848**: 1-60.

24. Tennikova, T.B., Freitag, R., *An introduction to monolithic disks as stationary phases for high performance biochromatography.* J. High Resolut. Chromatogr., 2000. **23**: 27-38.

25. Champagne, J., Delattre, C., Shanthi, C., Satheesh, B., Duverneuil, L., Vijayalakshmi, M. A., *Pseudoaffinity chromatography using a convective interaction media -disk monolithic column.* Chromatographia, 2007. **65**: 639-648.

26. Josic, D., Buchacher, A., Jungbauer, A., *Monoliths as stationary phases for separation of proteins and polynucleotides and enzymatic conversion.* J. Chromatogr. B., 2001. **752**: 191-205.

27. Mao, X., Luo, Y., Dai, Z., Wang, K., Du, Y., Lin, B., *Integrated lectin affinity microfluidic chip for glycoform separation.* Anal. Chem., 2004. **76**: 6941-6947.

CHAPTER 2

OPTIMIZATION OF HUMAN SERUM ALBUMIN MONOLITHS FOR HIGH PERFORMANCE AFFINITY CHROMATOGRAPHY

I. INTRODUCTION

Monolithic columns have been of great recent interest for use in high performance affinity chromatography (HPAC) because of their low back pressures, ease of preparation and good mass transfer properties [1-6]. Monoliths generally contain two types of pores. The macropores are the flow-through pores and are responsible for the majority of analyte interactions are usually in the size range of micrometers for typical chromatography applications. Mesopores are smaller in size and are considered the diffusion pores [2].

The combination of affinity ligands with monolith columns is known as affinity monolith chromatography (AMC) [4-10]. Various types of monoliths have been used in AMC, with many such reports using co-polymers of glycidyl methacrylate (GMA) and ethylene glycol dimethacrylate (EDMA) [1-6]. The co-solvents utilized to prepare these monoliths are usually cyclohexanol and 1-dodecanol, which are used to generate the pores within the monolith column. These columns are typically prepared through the use of an initiator and heat. GMA/EDMA monoliths are advantageous because they have been shown to be effective in immobilizing proteins such as human serum albumin (HSA), antibodies such as IgG, and other miscellaneous ligands of importance, (e.g.

protein A) [3, 4, 8]. An alternative crosslinking agent besides EDMA that will also be discussed in this thesis is trimethylolpropane trimethacrylate (TRIM). TRIM has been shown to be a successful alternative to EDMA in the polymerization of methacrylate based monoliths and has been further used in the separation of large biological agents such as bacteria [11].

HPAC is an effective tool for the separation and analysis of many compounds that can bind to various biologically-related ligands [3-5, 8, 12, 13]]. HPAC is a type of high performance liquid chromatography in which the biological binding agent of interest (i.e., the affinity ligand) is immobilized within a column. This ligand usually has the ability to bind with moderate-to-strong affinity and with good specificity to the analyte of interest, such as occurs between the drug warfarin and the protein HSA [14, 15]. HPAC can be useful for studying many biological interactions, for protein purification, and for chiral separations [4]. This method has many advantages, such as its ease of automation, high specificity, speed, and good reproducibility. In many of the applications of HPAC (e.g., binding studies), the total amount of ligand that is immobilized in the column is vital to the success of this method by providing good retention and high resolution between retained and non-retained sample components.

The work in this chapter will focus on improving the total amount of protein that can be immobilized in monolithic supports based on co-polymers of GMA/EDMA or GMA/TRIM. The optimization of protein content in monolithic columns for use with HPAC has been shown to be important in allowing for these columns to be used for studying drug-protein interactions involving proteins such as HSA [4]. In this research, the amount of monomers (GMA and EDMA or GMA and TRIM) will be held constant at

a specific value that is dependent upon the two polymerization temperatures (60 ºC and 80º C). This study will use a combinatorial approach to optimize and improve upon the resulting organic monoliths for the immobilization of HSA for use in AMC. The relative amounts of cyclohexanol to 1-dodecanol will also be varied to generate a library of monoliths. Two different immobilization techniques (i.e., the epoxy method and Schiff base method) will be utilized to study the relative change in the total protein content of the different monoliths under the various tested conditions. Imaging techniques will be utilized to depict the pore structure of the column materials that are generated. HPLC will be used to study the binding and elution of model analytes to the immobilized HSA within each monolith. The results should make it possible to determine the optimum ratio of 1-dodecanol to cyclohexanol for monolith synthesis and the optimum temperature for monolith preparation with respect to achieving the highest total amount of immobilized protein. These results, in turn, should make it possible to generate more effective monolithic columns for use in future HPAC experiments that involve HSA or similar proteins.

II. EXPERIMENTAL SECTION

Reagents

The GMA (97% pure), EDMA (98%), cyclohexanol (> 99%), 1-dodecanol (98%), 2,2'-azobisisobutryonitrile (AIBN, 98% pure), TRIM (98%), HSA (Cohn fraction V, essentially fatty acid free, >96%), sodium cyanoborohydride (94%, a mild reducing agent), sodium borohydride (98%, a strong reducing agent), periodic acid (> 99%, an oxidizing agent), and racemic warfarin (3-(α-acetonylbenzyl)-4-hydroxycoumarin, >98%) were from Sigma-Aldrich (St. Louis, MO, USA). Reagents for the bicinchoninic acid (BCA) protein assay were from Pierce (Rockford, IL). All aqueous reagents, solutions and buffers were prepared using water from a Nanopure system (Barnstead, Dubuque, IA) and were filtered using 0.2 μm GNWP nylon filters from Millipore (Billerica, MA, USA).

Apparatus

The monoliths were prepared in 4.6 mm i.d. x 5 cm stainless steel columns with PEEK inner liners from Alltech (Deerfield, IL). These columns included a special frit that could be used to compress the monoliths and to avoid the formation of gaps within the columns during and after their preparation. The monoliths were placed into 4.6 mm i.d. x 1 mm PEEK disks, which were prepared as described previously [3]. The immobilization of protein within the monoliths was accomplished through the use of a reciprocating 501 Waters HPLC pump from Millipore (Milford, MA). The HPLC system used in the chromatographic studies consisted of a 200 gradient pump and 200 UV detector from Perkin Elmer (Waltham, MA). Samples were injected using a Rheodyne

Lab Pro valve (Cotati, CA) and a 20-μL loop constructed from PEEK tubing. Chromatographic data were collected using LabView 5.1 (National Instruments, Austin, TX) and processed using PeakFit 4.12 (SeaSolve Software, San Jose, CA, USA). Scanning electron microscopy (SEM) was performed using a Hitachi S4700 Field-Emission Scanning Electron Microscope with a W95/NT based computerized operating system (Pleasanton, CA).

Preparation of Monolithic Columns

Figure 2-1 shows the general procedure that was used for the preparation of the monolith columns and for the immobilization of HSA in these columns. This procedure began with the polymerization of the monomers, cross-linkers and initiator. Following completion of monolith formation, this support material was washed for 2 h at 0.5 mL/min with acetonitrile, followed by a 1 h wash with water. There were two types of immobilization techniques utilized in this study (Note: These will be addressed in the next section). In the first method (i.e., the Schiff base immobilization method), activation of the support was required and was accomplished by first converting the monolith into a diol form through treatment with 0.5 M sulfuric acid and heat. This step was not needed in the second immobilization technique (i.e, the epoxy method). Thus, in these two immobilization approaches the monolith was used either directly or after activation prior to immobilization. In each case, a solution of 5 mg/mL of HSA, dissolved in the desired buffer, was cycled through the column during the immobilization step.

The general reaction scheme for the preparation of the monolith columns is illustrated in Figure 2-2. There were two types of monoliths prepared in this work, with each type being synthesized at two distinct temperatures. The first type of monolith was

prepared by combining GMA (the functional monomer) and EDMA (the cross linking agent) in either a 50:50 (v/v) mixture for polymerization at 60ºC or in a 60:40 (v/v) mixture for preparation at 80º C. These reagents were combined with various ratios of cyclohexanol and 1-dodecanol, which were used as the porogenic solvents.

Figure 2-1. General scheme for the preparation of affinity monoliths. The monomers utilized here were glycidyl methacrylate (GMA) and ethylene glycol dimethacrylate (EDMA) or trimethyloypropane trimethacrylate (TRIM). The porogenic solvents were 1-dodecanol (DoOH) and cylcohexanol (CyOH). The initiator was azobisisobutyronitrile (AIBN).

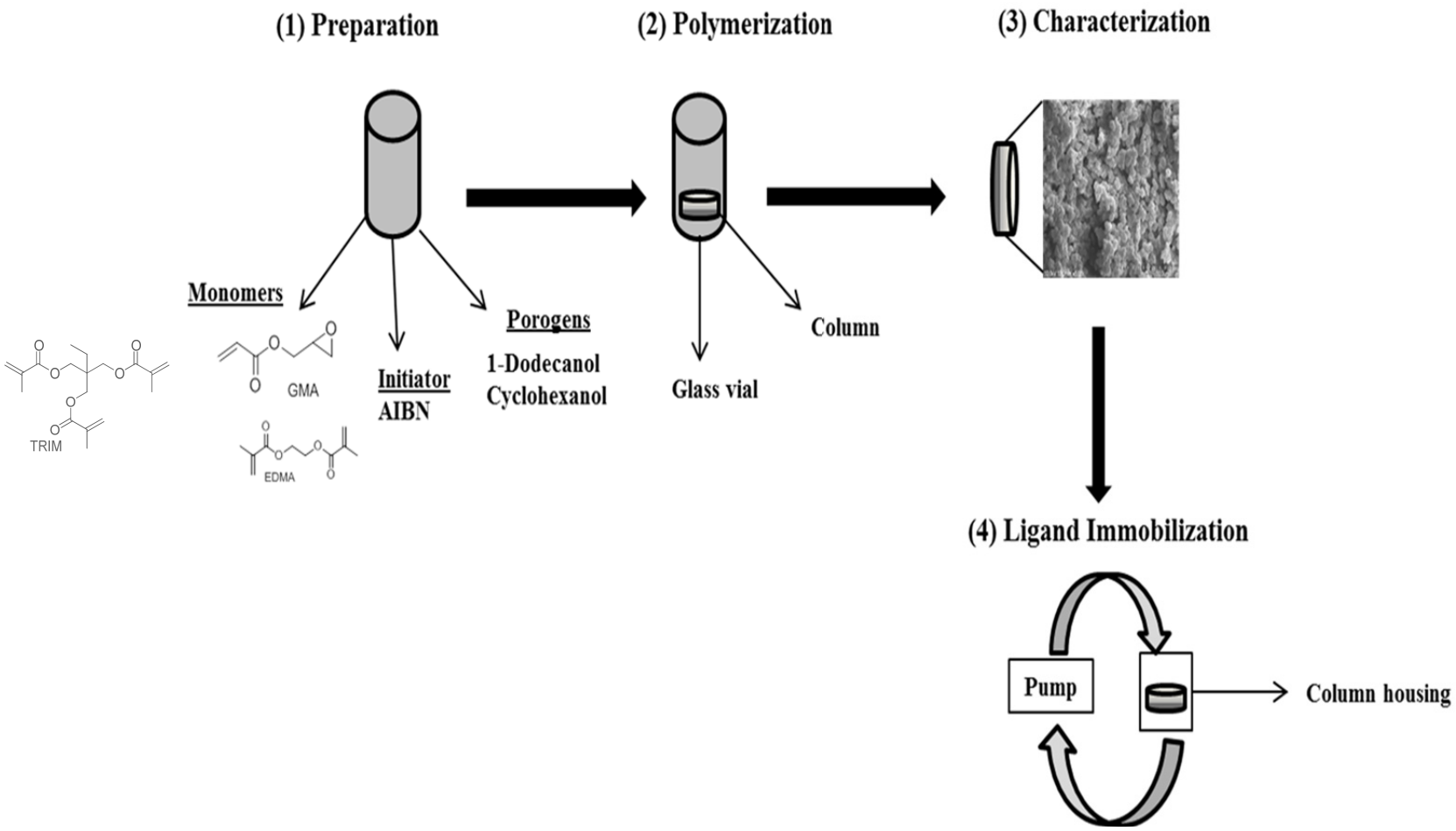
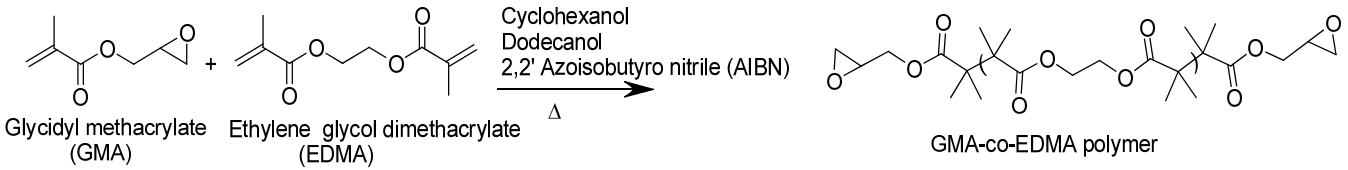
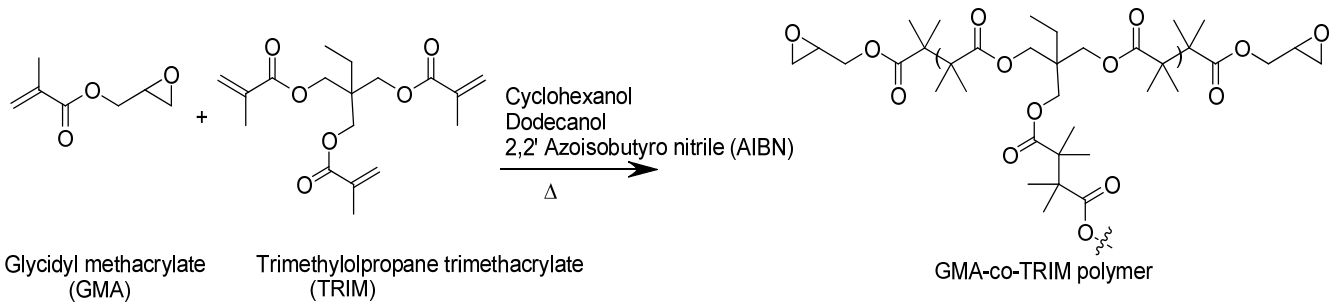

(1) Preparation
(2) Polymerization
(3) Characterization
Monomers
Porogens
1-Dodecanol
Cyclohexanol
Initiator
AIBN
GMA
TRIM
EDMA
Glass vial
Column
(4) Ligand Immobilization
Pump
Column housing

Figure 2-2. General reaction schemes for the preparation of a (a) GMA/EDMA monolith or (b) a GMA/TRIM monolith.

(a)


Cyclohexanol
Dodecanol
2,2' Azoisobutyro nitrile (AIBN)
Δ
Glycidyl methacrylate
(GMA)
Ethylene glycol dimethacrylate
(EDMA)
GMA-co-EDMA polymer

(b)


Cyclohexanol
Dodecanol
2,2' Azoisobutyro nitrile (AIBN)
Δ
Glycidyl methacrylate
(GMA)
Trimethylolpropane trimethacrylate
(TRIM)
GMA-co-TRIM polymer

The relative amount of GMA/EDMA compared to the porogenic solvents was held constant at 40:60 (v/v). AIBN was used as an initiator and the relative amount of this agent was approximately 1% of the total monomer weight. The reagents were combined in a 5 mL glass vial and sonicated for 10 min. A stream of nitrogen gas was then placed through the vial for 15 min to remove any trapped air bubbles. A 1 mL syringe was used to place the reagents into an empty 4.6 mm i.d. x 5 cm PEEK lined column housing or 4.6 mm i.d. x 1 mm PEEK disks. One end of the column was sealed with a column plug prior to the addition of the reagents; once the reagents were added, the other end was sealed with the same type of column plug. The sealed column was then held upright in a sonicator for 5 min to remove any trapped bubbles. The sealed column was then placed in a water bath at either 60ºC or 80º C for 24 h. After polymerization, the column was placed into a standard column housing. For the 4.6 mm i.d. x 5 cm columns, a special frit-insert was used to compress the monolith against the wall and reduce the effects of polymer shrinkage. If a 4.6 mm i.d. x 1 mm disk column was used, a Delrin housing and PEEK lined frits were used to place the column into the housing.

The second type of monolith that was prepared in this study consisted of GMA but used TRIM as the cross linker, with these monomers being added in a ratio of 70:30 (v/v). Cyclohexanol and 1-dodecanol were again used as the porogenic solvents and the ratio of GMA and TRIM to porogens was the same as described earlier. The ratio of the porogenic solvents was varied to achieve an optimum pore size and total protein content for the final monolithic supports. The amount of added AIBN was 1% of the total weight of the GMA. As described previously, each of the GMA/TRIM supports were made at either 60 ºC or 80ºC. The reagents were mixed together in a flask and sonicated for 10

min. A stream of nitrogen was then passed through the flask for 15 min to remove any trapped air bubbles. The mixture was loaded into a stainless steel column (4.6 mm i.d. x 5 cm) via a 1 mL syringe or into PEEK disks with dimensions of 4.6 mm i.d. x 1 mm. Once the polymerization mixture was loaded into the appropriate type of column, this mixture was again sonicated for 5 min by holding the column in a vertical position to remove any trapped air bubbles. The column was next placed in either a 60 ºC or 80 º C water baths for 24 h. Once polymerization was finished, the column was placed into an appropriate housing and a frit insert was placed into the column to prevent shrinkage or gap formation in the 4.6 mm i.d. x 5 cm columns. A Delrin housing was utilized for the 4.6 mm i.d. x 1 mm PEEK disk columns, with two PEEK lined frits being placed on either side of these columns. After the columns were assembled, they were washed with acetonitrile for 2 h at 0.5 mL/min, followed by a 1 h wash with water at the same flow rate.

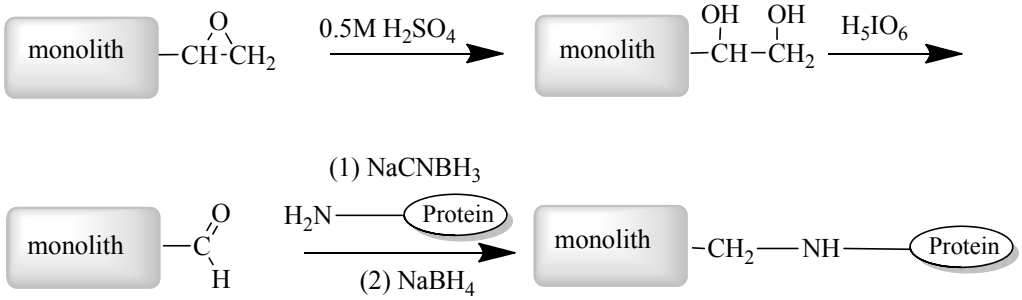
Protein Immobilization

The reaction for the immobilization of HSA by the Schiff base method is depicted in Figure 2-3. A previous example of this method involved the hydrolysis of the GMA/EDMA monolith epoxy groups to form diols through the use of sulfuric acid; this reaction was followed by oxidation of the diol groups with periodic acid to produce aldehyde groups on the monolith's surface [4]. These aldehyde groups were then reacted with primary amine groups on HSA (or other proteins with similar functionalities) to form a Schiff base. The Schiff base was then converted to a more stable secondary amine through the use of a mild reducing agent (i.e. sodium cyanoborohydride). Any unreacted

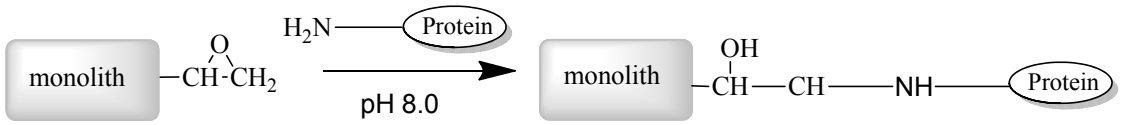
aldehydes were later converted into alcohol groups through the addition of sodium borohydride.

Figure 2-3. Reaction schemes for the Schiff base and epoxy immobilization methods.

Schiff base Immobilization



Epoxy Immobilization



The Schiff base method utilized in this study was carried out as previously reported [4]. After a monolith column was assembled into a Delrin housing and washed thoroughly with acetonitrile and water, a 5 mL solution of 0.5 M sulfuric acid was passed through the column at 0.5 mL/min. The column was sealed and placed in a water bath at 60ºC for 4 h. The column was then washed thoroughly with 100 mL of water at 0.5 mL/min. A 40 mL solution containing 2 g of periodic acid in a 90:10 (v/v) mixture of acetic acid and water was circulated through the column for approximately 4 h at 0.5 mL/min and room temperature. The column was then washed with 100 mL water at 0.5 mL/min. Next, a 10 mL solution of 5 mg/mL of HSA in pH 6.0, 1.5 M potassium phosphate buffer, which contained 25 mg sodium cyanoborohydride, was circulated through the column at 0.5 mL/min for 3 days, after which a fresh solution of the same reagents was applied at 0.5 mL/min for an additional 3 days at room temperature. A 20 mL solution containing 2.5 mg/mL of sodium borohydride in pH 8.0, 0.1 M potassium phosphate buffer was applied to the column in a circulating manner for 2 h at 0.5 mL/min. The column was then washed with pH 7.4, 0.067 M potassium phosphate buffer for 4 h at 0.5 mL/min and room temperature. The final affinity column was stored in the pH 7.4 buffer at 4ºC until use.

The epoxy immobilization method is shown in Figure 2-3. This procedure was adapted from a previously reported method [4]. The reaction mechanism involved a nucelophilic attack by a primary amine group present on a protein such as HSA, leading to the base opening of the epoxide groups on the monolith, generating a stable secondary amine linkage. In this method, a 10 mL solution containing 6 mg/mL HSA in pH 8.0, 1.5 M potassium phosphate buffer was cycled through a monolith column for 3 days at room

temperature. After 3 days, this solution was replaced with a fresh solution containing the same reagents and cycled through the column for an additional 3 days. After the immobilization step, any unreacted epoxy groups were blocked by passing through the column a 60 mL portion of pH 8.0, 0.2 M Tris buffer at 0.5 mL/min for 2 h at room temperature [4]. The column was then washed for 4 h with pH 7.4, 0.067 M potassium phosphate buffer and stored in this buffer at 4ºC until use.

Assessment of Monoliths

The relative ratio of dodecanol to cyclohexanol was varied to create a library of monoliths at 60ºC and 80ºC. The optimized monoliths were then characterized through the use of scanning electron microscopy (SEM). The total amount of protein was determined for each monolith via a BCA assay [16]. For this assay, each monolith was prepared in triplicate during the optimization studies and washed with 100 mL of water at 0.5 mL/min for 3 h at room temperature. The monolith column was then removed from the housing and ground to a fine powder through the use of a mortar and pestle. The powder was placed on a watch glass and dried in a vacuum oven overnight at room temperature. This same procedure was repeated for a control monolith that did not contain any immobilized protein. All samples were analyzed and prepared in triplicate, with HSA being utilized as the standard in the BCA assay.

SEM images were obtained on several columns after the polymerization process. These samples were prepared in either vials using 4.6 mm i.d. x 1 mm PEEK disks or in stainless steel column housings with dimensions of 4.6 mm i.d. x 5 cm that were washed with 100 mL acetonitrile at 0.5 mL/min for 3 h. The monoliths were removed from the

housing and sliced into thin disks through the use of a razor blade. The samples were placed on a watch glass and dried under vacuum at 100ºC for 8 days before imaging. Prior to imaging, chromium was used to sputter coat the sample for a period of 5 min.

The chromatographic studies were performed at room temperature using pH 7.4, 0.067 M potassium phosphate buffer as the mobile phase. The mobile phase buffer was degassed and sonicated for approximately 30 min prior to use. A sample containing a 20 μM solution of racemic warfarin was prepared in pH 7.4, 0.067 M potassium phosphate buffer. A 20 μM solution of D/L-tryptophan was also prepared in pH 7.4, 0.067 M potassium phosphate buffer. All samples were used within one day of preparation, and a 20 μL injection of each analyte or a void marker (i.e., sodium nitrate) was made in triplicate at flow rates ranging from 0.1-1.0 mL/min. The elution of *R*- and *S*-warfarin was monitored at 308 nm. The D- and L-tryptophan were monitored at 280 nm. A 20 μL injection of 0.2 mM sodium nitrate was monitored at 205 nm. The extra-column void time was determined by injecting sodium nitrate onto a zero dead volume connector and monitoring the elution at 205 nm.

III. RESULTS AND DISCUSSION

Optimization of Monoliths

Although the general procedure for the polymerization of monoliths was modified from a previous reported method [4], during these current studies several parameters were held constant while the relative amounts of the porogenic solvents were varied. For the GMA/EDMA monoliths prepared at 60ºC, the amount of monomers to porogens was held constant at a ratio of 40:60. The relative amounts of the GMA and EDMA were 20:20 (v/v) at 60ºC and the relative amounts at 80ºC were 24:16 (v/v). The relative amounts of GMA and TRIM were 28:12 (v/v) at both 60ºC and 80ºC.

Figures 2-4 and 2-5 show the overall trends noted in the total amount of protein that could be immobilized as the amount of 1-dodecanol to cyclohexanol was varied in the monoliths. It was found that higher levels of cyclohexanol compared to 1-dodecanol for the GMA/TRIM monolith gave a slightly higher total protein content. It was also found that the optimum amount of 1-dodecanol compared to cyclohexanol for the GMA/TRIM monolith at 60ºC was 13 (v/v)%, and the maximum protein content did not change substantially when going from 60ºC to 80ºC, as is shown in Figure 2-5**.** The optimum amount of 1-dodecanol for the GMA/EDMA monolith at 60ºC was roughly 10 (v/v)%, as is shown in Figure 2-4, and at 80ºC it was 20 (v/v)%, as is also shown in Figure 2-4. The precision associated with the measured amount of total immobilized protein in these studies was typically between ±10 and ±15%.

Figure 2-4. Effects of varying the porogen composition on the amount of HSA that could be attached to GMA/EDMA monoliths prepared at 60ºC and utilizing (a) the epoxy method or (b) Schiff base method for immobilization or to GMA/EDMA monoliths prepared at 80ºC and using (c) the epoxy method or (d) Schiff base method for immobilization. The error bars represent the standard deviation of the mean.

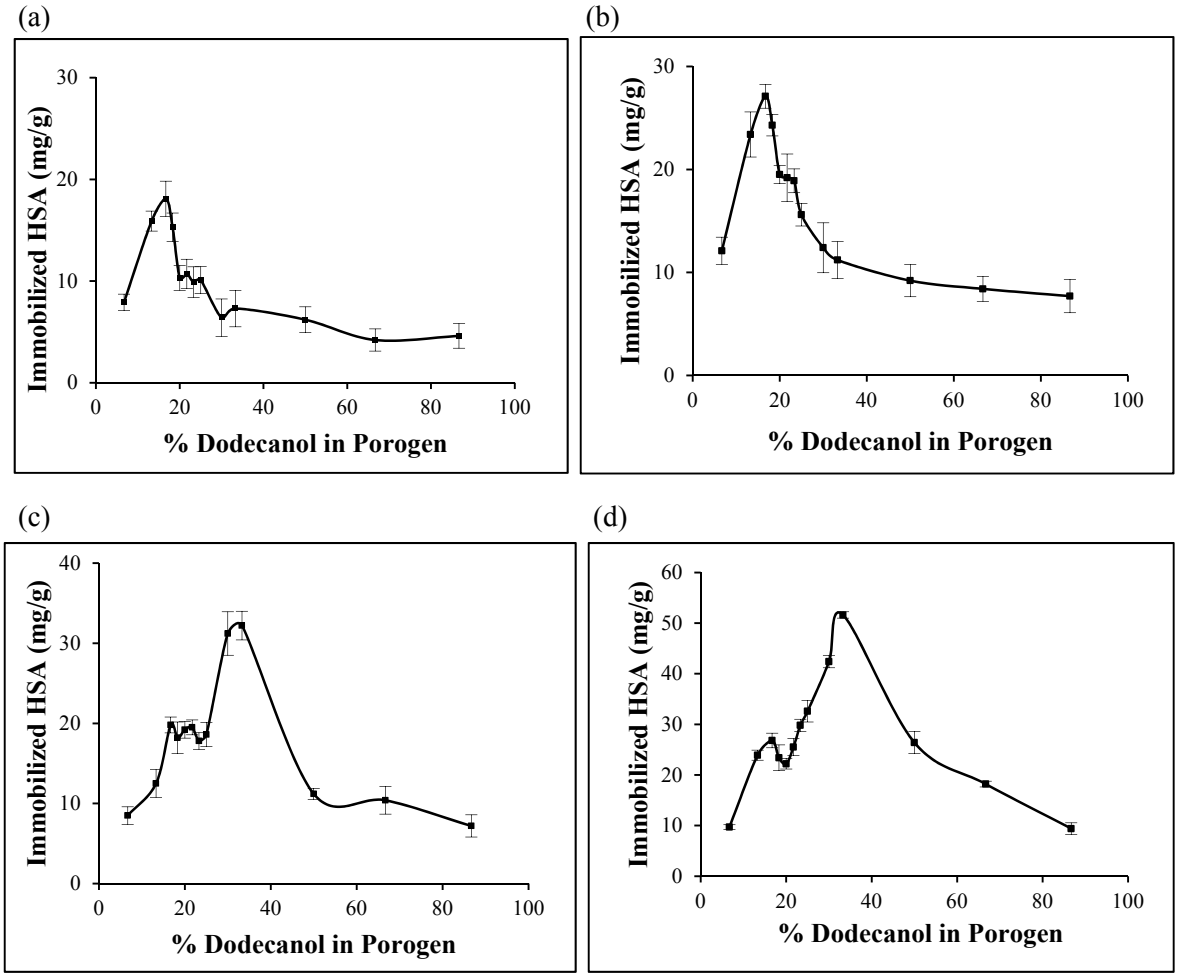
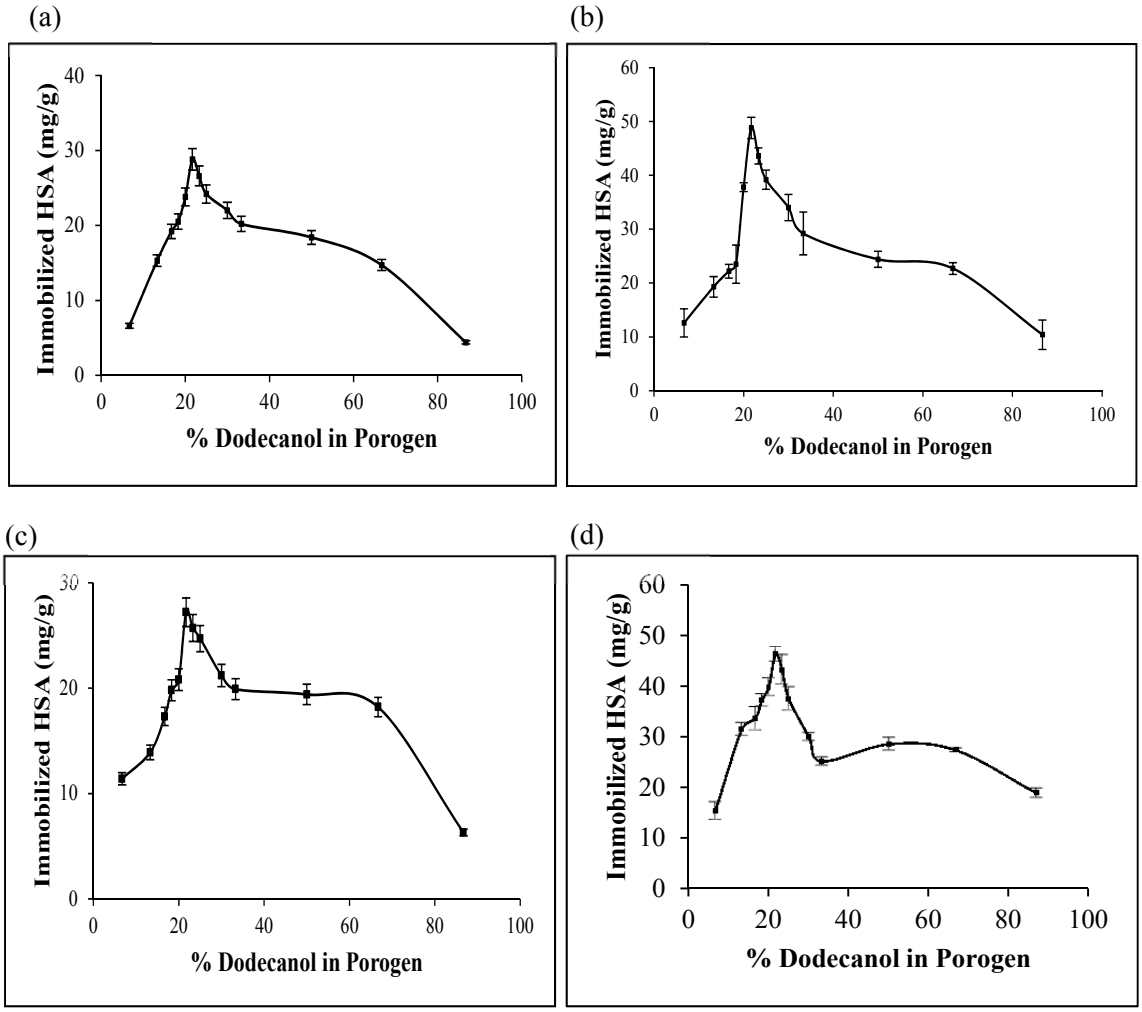

(a)
Immobilized HSA (mg/g)
% Dodecanol in Porogen
(b)
Immobilized HSA (mg/g)
% Dodecanol in Porogen
(c)
Immobilized HSA (mg/g)
% Dodecanol in Porogen
(d)
Immobilized HSA (mg/g)
% Dodecanol in Porogen

Figure 2-5. Effects of varying the porogen composition on the amount of HSA that could be attached to GMA/TRIM monoliths prepared at 60ºC and utilizing (a) the epoxy method or (b) Schiff base method for immobilization, and to GMA/TRIM monoliths prepared at 80ºC and utilizing (c) the epoxy method or (d) Schiff base method for immobilization. The error bars represent the standard deviation of the mean.


(a)
Immobilized HSA (mg/g)
% Dodecanol in Porogen
(b)
Immobilized HSA (mg/g)
% Dodecanol in Porogen
(c)
Immobilized HSA (mg/g)
% Dodecanol in Porogen
(d)
Immobilized HSA (mg/g)
% Dodecanol in Porogen

The relative amount of protein that was immobilized to the columns varied depending upon the immobilization technique that was utilized. On average, the epoxy immobilization technique gave less total protein than the Schiff base method. The Schiff base typically gave 30-50% more immobilized protein. For the GMA/EDMA monoliths, it was noted that monoliths prepared at 60ºC provided a lower total protein content compared to the monoliths prepared at 80ºC. For the GMA/EDMA column showing the highest total protein content, the column prepared at 60ºC gave 50% less immobilized protein compared to the column prepared at 80ºC. The final, optimized polymerization conditions allowed for reproducible columns to be prepared with relatively low back pressures. This back pressure was typically less than 80 psi when the GMA/EDMA monolith was prepared at 60ºC and was typically less than 120 psi for a monolith prepared at 80ºC when measured using columns that were 5 cm in length and using a flow rate of 0.5 mL/min when the mobile phase was pH 7.4, 0.067 M potassium phosphate buffer. In the Appendix, Tables 2.1a-2.8a indicates the total protein content with the standard deviation for each experiment. These tables were used to generate the trends in Figures 2-4 and 2-5.

The relative stability of the GMA/EDMA columns prepared both in the 1 mm disk form and the 5 cm PEEK stainless column was at least up to at least 6 months. For disk columns that were 1 mm in length, the typical back pressures for the GMA/EDMA monoliths prepared at 60ºC were less than 30 psi and the back pressure for the GMA/EDMA monoliths prepared at 80ºC was less than 50 psi at a flow rate of 0.5 mL/min and in the presence of pH 7.4, 0.067 M potassium phosphate buffer.

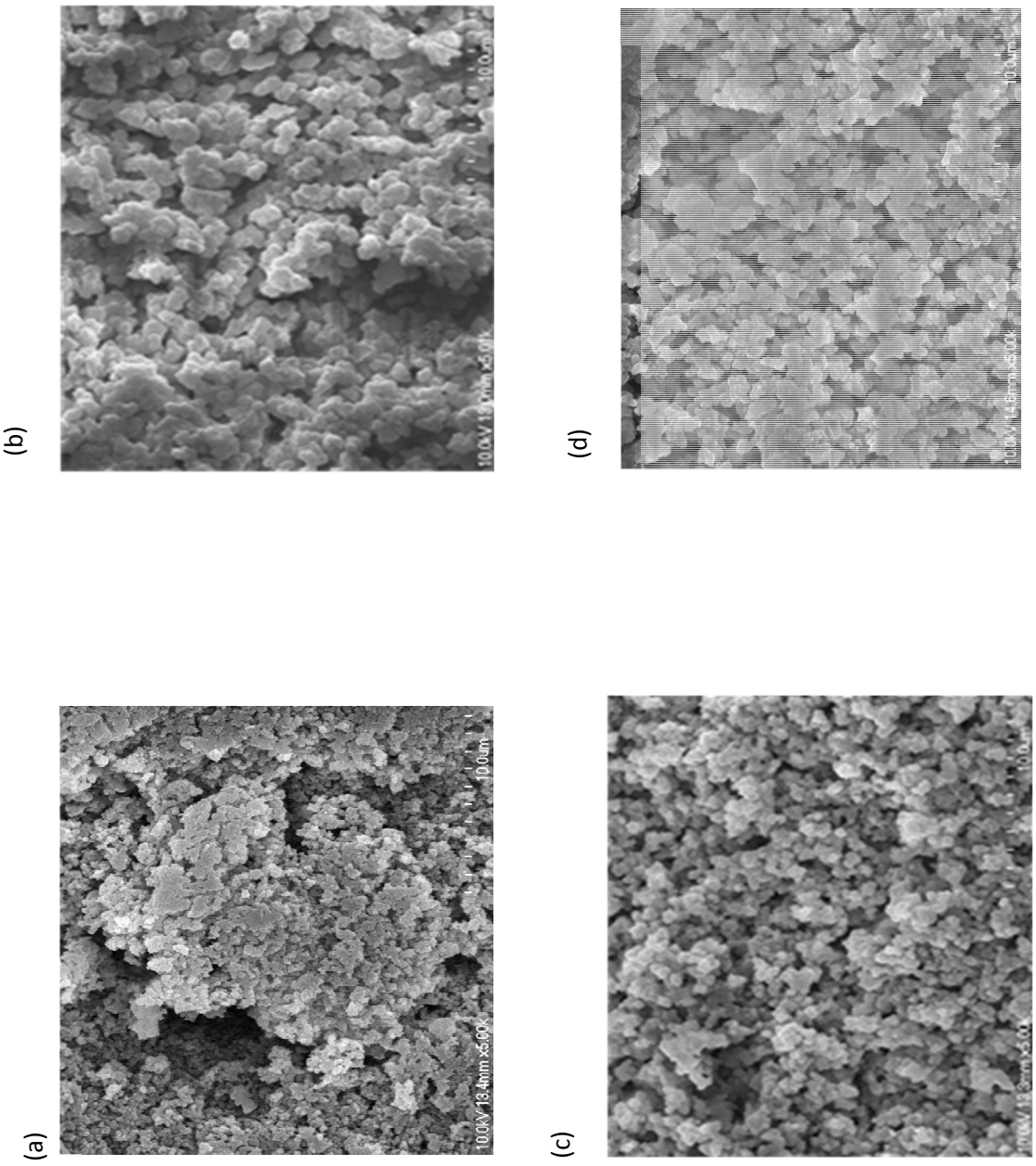
The GMA/TRIM columns gave similar results, producing reproducible columns that showed no change in retention for up to at least 3 months. The GMA/TRIM columns prepared at 60ºC showed an average back pressure that was less than 90 psi and the columns prepared at 80ºC had a back pressure that was typically less than 180 psi for columns that were 5 cm in length when measured at a flow rate of 0.5 mL/min and using a mobile phase that was pH 7.4, 0.067 M potassium phosphate buffer. For disk columns that were only 1 mm in length, the back pressure was significantly lower. For GMA/TRIM disks prepared at 60ºC, the average back pressure was less than 40 psi and for the GMA/TRIM disks prepared at 80ºC the average back pressure was less than 60 psi, as measured at a flow rate of 0.5 mL/min and in the presence of pH 7.4, 0.067 M potassium phosphate buffer.

Assessment of Monoliths

In an attempt to analyze the structures of the GMA/TRIM monoliths and GMA/EDMA monoliths, SEM was performed. To do this, columns were prepared according to the procedures mentioned earlier, with these columns then being washed and dried under vacuum prior to acquiring SEM images. Figure 2-6 shows the structural

changes in the monoliths that were seen upon increasing the polymerization temperature from 60ºC to 80ºC for the GMA/EDMA and GMA/TRIM monoliths.

Figure 2-6. Scanning electron micrographs for GMA/TRIM monoliths prepared at (a) 80ºC or (b) 60ºC and GMA/EDMA monoliths prepared at (c) 80ºC or (d) 60ºC. Other conditions are given in the text.


(a)
(b)
(c)
(d)

It was found in these results that the pore size of the throughpores became smaller as the polymerization temperature was increased from 60ºC to 80ºC. The pore structure for the GMA/TRIM monoliths was also found to be different when compared to the GMA/EDMA monoliths, as is evident in Figure 2-6.

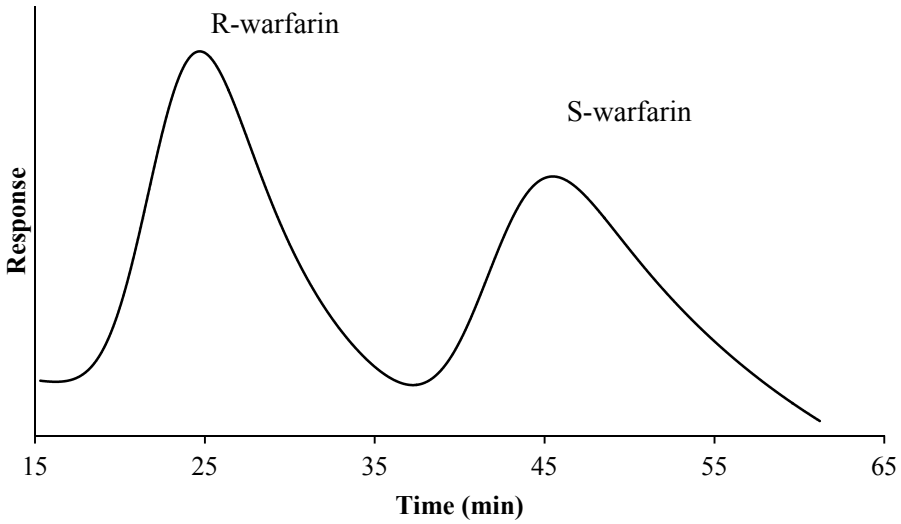
Another study that was conducted to test the final conditions utilized in the preparation of the monoliths was to analyze the binding properties of these supports when using immobilized HSA for the retention and chiral separation of two model analytes: (*R/S*)-warfarin and (D/L)-tryptophan. (*R/S*)-Warfarin is an important anti-coagulant that is often used as a model analyte to examine the binding of solutes to Sudlow site I of HSA [15, 17]. L-Tryptophan is similarly used as a model analyte to examine the binding of solutes to Sudlow site II of HSA. One application of HSA in HPAC columns has been in the chiral separation of various pharmaceutical agents. For instance, HSA columns based on particulate supports have been shown to effectively separate (*R/S*)-warfarin or (D/L)-tryptophan [4].

These separations were first carried out using GMA/EDMA monolith which corresponded to the use of 20% 1-dodecanol in the total solvent mixture and which was prepared at 80ºC. The separations were also carried out using a GMA/TRIM monolith which corresponded to the use of 13% 1-dodecanol in the total solvent mixture and which was prepared at 80°C. In both cases, HSA was immobilized to the monolith via the Schiff base method. The monolith was prepared in both a 4.6 mm i.d. x 5 cm stainless steel PEEK lined housing and in a 4.6 mm i.d. x 1 mm disk form. A 20 µL sample of 20 µM (*R/S*)–warfarin in pH 7.4, 0.067 M potassium phosphate buffer was injected at various

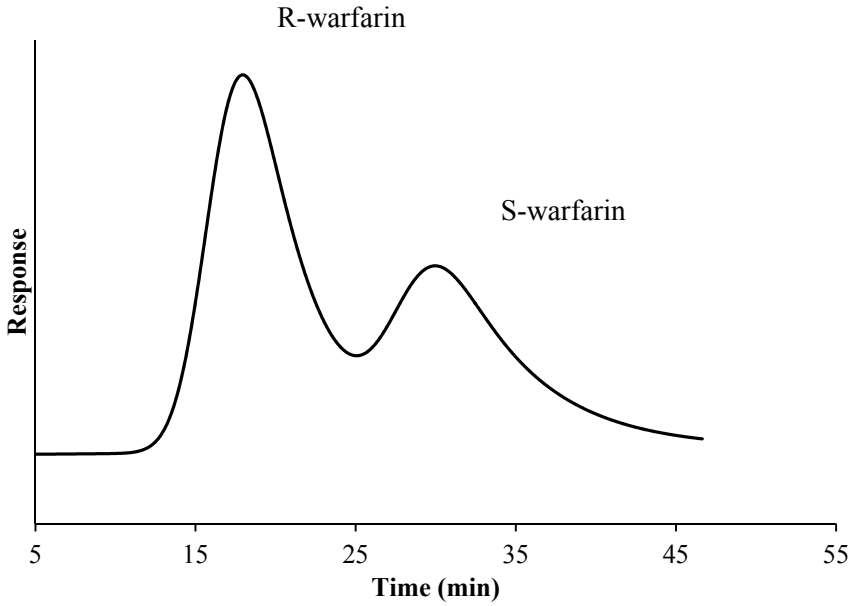
flow rates onto these monoliths, as shown in Figure 2-7. The retention factors for the chiral separation of (*R/S*)-warfarin for these monoliths are provided in Table 2-1

Figure 2-7**.** Chiral separation of *R*- and *S*-warfarin on a 4.6 mm i.d. x 5 cm GMA/EDMA monolith (a) or GMA/TRIM monolith (b) column prepared at 80ºC and containing HSA immobilized by the Schiff base method. For exact conditions for column preparation please refer to the Appendix, tables 2.4a and 2.8a. The mobile phase was pH 7.4, 0.067 M potassium phosphate buffer and the flow rate was 0.5 mL/min.

(a)



(b)



The next part of this study consisted of performing a similar separation with (D/L)-tryptophan. A 20 µL injection of 20 µM (D/L)-tryptophan in pH 7.4, 0.067 M potassium

phosphate buffer was injected on the same GMA/EDMA columns (both the 4.6 mm i.d. x 5 cm and the 4.6 mm i.d. x 1 mm disk) that were utilized in the study for the chiral separation of (*R/S*)-warfarin. The retention factors were determined for (D/L) tryptophan and are reported in Table 2-1.

Table 2-1.= The retention factors for D/L-tryptophan and (*R/S*)-warfarin on a 4.6 mm i.d. x 5 cm GMA/EDMA monolith and on a 4.6 mm i.d. x 1 mm GMA/EDMA disk.

Column	**R-Warfarin** **Retention factor, *k***	**S-Warfarin** **Retention factor, *k***	**D-Tryptophan** **Retention factor, *k***	**L-Tryptophan** **Retention factor, *k***
GMA/EDMA 80 5 cm	37 (± 2)	55 (± 1).	0.68 (± 0.03)	2.72 (± 0.05)
GMA/EDMA 80 5 cm (Control)	0.29 (±0.03)	0.29 (± 0.03)	0.06 (± 0.02)	0.06 (± 0.02)
***GMA/EDMA 80 1 mm**	29.5 (± 0.4)	29.5 (± 0.4)	1.18 (± 0.09)	1.18 (± 0.09)
GMA/EDMA 80 1mm (Control)	0.67 (± 0.07)	0.67 (± 0.07)	0.02 (±0.01)	0.02 (± 0.01)

*Note: Chiral separations were not achieved on the 1 mm GMA/EDMA columns for either analyte
*Note: Values in the parentheses indicate the standard deviation associated with the experiment.

One important conclusion made from the analysis of the retention factors that were calculated for both the GMA/EDMA monolith prepared with dimensions of 4.6 mm i.d. x 5 cm and 4.6 mm i.d. x 1 mmis that they were not the same and that the 1 mm disk columns gave a lower retention factor in both cases for the analysis of the D/L-tryptophan and the *R/S*-warfarin. The experiment for the 1 mm disk monoliths were completed at various flow rates ranging from 0.5 mL/min to 0.1 mL/min. However, with a decrease in flow rate there was a decrease in efficiency. As, the flow rate decreases, as was expected, the peaks became very broad and more difficult to accurately examine.

The values obtained for the retention factors on the GMA/EDMA monoliths did give good correlation to previously reported results [4]. When comparing the GMA/TRIM results for the separation of *R/S*-warfarin and the GMA/EDMA column, the peaks were better resolved for the GMA/EDMA column. This could be attributed to the change in structure of the crosslinking agent and the mechanism by which the protein was immobilized. Even though there were similar results for the total protein content achieved for the GMA/EDMA compared to the GMA/TRIM prepared at 80°C, better retention was achieved on the GMA/EDMA column.

It is important to note that when utilizing the 1 mm disk columns for the GMA/EDMA column the analysis of racemic warfarin could be completed in just a few minutes with retention factors that were similar to those for the 5 cm columns. However, chiral separation could not be achieved on the 1 mm disk columns because of the fewer number of theoretical plates and lower efficiencies than the 5 cm columns.

IV. CONCLUSIONS

In this study the goal was to optimize polymerization conditions to maximize the immobilization content that could then be obtained for HSA or similar proteins on methacrylate based monoliths. The monoliths were prepared in triplicate and evaluated in triplicate by a BCA assay for their total protein content. The precision associated with the total amount of measured protein was typically between 10-15%. It was found that higher levels of cyclohexanol compared to 1-dodecanol for the GMA/TRIM monolith gave a slightly higher total protein content. It was also found that the optimized amount of 1-dodecanol compared to cyclohexanol for the GMA/TRIM monolith at 60ºC was 13 (v/v)% and that the maximum protein content for this support did not change substantially when going from 60ºC to 80ºC. The GMA/EDMA gave different behavior, in which the optimized amount of 1-dodecanol for the GMA/EDMA monolith at 60ºC was roughly 10 (v/v)% and at 80ºC it was 20 (v/v) %. The optimized monoliths for each condition were successfully imaged via SEM. The Schiff base immobilization method afforded a higher total protein content when compared to the epoxy immobilization method.

The results reported in this study are easily applied to other protein systems for studying drug-protein interactions, as well as to the development of protein supports for chiral separations of relevant pharmaceutical agents. It was found that fast separations (i.e., under 3 min) could can be achieved when disk columns were utilized for this work. The microcolumns that were developed in this work should be useful in chemical separations because they offer advantages such as good speed and ease of automation when included

in an HPLC system. These supports are also relatively inexpensive to make, so they should be cost effective for use in the development of new chiral separation methods.

REFERENCES

1. Guiochon, G., *Monolithic columns in high-performance liquid chromatography.* J. Chromatogr. A, 2007. **1168**: 101-168.

2. Ikegami, T., N. Tanaka, *Monolithic columns for high-efficiency HPLC separations.* Curr. Opin. Chem. Biol., 2004. **8**: 527-533.

3. Jiang, T., R. Mallik., D.S. Hage, *Affinity Monoliths for Ultrafast Immunoextraction.* Anal. Chem., 2005. **77**: 2362-2372.

4. Mallik, R., T. Jiang, D.S. Hage, *High-performance affinity monolith chromatography: Development and evaluation of human serum albumin columns.* Anal. Chem., 2004. **76**: 7013-7022.

5. Mallik, R., D.S. Hage, *Affinity monolith chromatography.* J. Sep. Sci., 2006. **29**: 1686-1704.

6. Nordborg, A., E.F. Hilder, *Recent advances in polymer monoliths for ion-exchange chromatography.* Anal. Bioanal. Chem., 2009. **394**: 71-84.

7. Okanda, F.M., Z. El Rassi, *Affinity monolithic capillary columns for glycomics/proteomics: 1. Polymethacrylate monoliths with immobilized lectins for glycoprotein separation by affinity capillary electrochromatography and affinity nano-liquid chromatography in either a single column or columns coupled in series.* Electrophoresis, 2006. **27**: 1020-1030.

8. Pan, Z., H. Zou, W. Mo, X. Huang, R. Wu, *Protein A immobilized monolithic capillary column for affinity chromatography.* Anal. Chim. Acta, 2002. **466**: 141-150.

9. Urban, J., P. Jandera, *Polymethacrylate monolithic columns for capillary liquid chromatography.* J. Sep. Sci., 2008. **31**: 2521-2540.

10. Uzun, L., Yavuz, H., Say, R., Ersoez, A., Denizli, A., *Poly(ethylene dimethacrylate-glycidyl methacrylate) monolith as a stationary phase in dye-affinity chromatography.* Industrial & Engineering Chemistry Research, 2004. **43**: 6507-6513.

11. Buszewski, B., Szumski, M., Klodzinksa, E., Jarmalaviciene, R., Maruska, A., *Migration of bacteria through a monolith.* J.Chromatogr.A, 2009. **1216**: 6146-6150.

12. Tetala, K.K.R., T.A. van Beek, *Bioaffinity chromatography on monolithic supports.* J. Sep. Sci., 2009. **33**: 422-438.

13. Xuan, H., D.S. Hage, *Immobilization of a1-acid glycoprotein for chromatographic studies of drug-protein binding.* Anal. Biochem., 2005. **346**: 300-310.

14. Hage, D.S., *High-performance affinity chromatography and immobilized serum albumin as probes for drug- and hormone-protein binding.* J. Chromatogr. B, 2000. **739**: 39-54.

15. Hage, D.S., *High-performance affinity chromatography: a powerful tool for studying serum protein binding.* J. Chromatogr. B, 2002. **768**: 3-30.

16. Smith, P.K., R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujmoto, N.M. Goeke, B.J. Olson, D.C. Klenk, *Measurement of protein using bicinchoninic acid.* Anal. Biochem. , 1985. **150**:76-85.

17. Fehske, K.J., W.E. Muler, *High-affinity binding of ethacrynic acid is mediated by the two most important drug binding sites of human serum albumin* Pharmacol., 1986. **32**: 208-213.

V. APPENDIX

Two figures provided in this chapter were established based upon a set of data. Figure 2-4 and Figure 2-5. The data utilized to generate these plots. The data shown below provide the relative amounts (v/v)% of the cyclohexanol and 1-dodecanol utilized in each experiment. Protein content (mg/g support) represents the average of each column that was made in triplicate. The (±) indicate the standard deviation of the experiment.

Table 2.1a: GMA-20 (v/v) % and EDMA-20 (v/v) %, AIBN 1% weight of monomers. Protein content represents the average of each column that was made in triplicate. The (±) indicates the standard deviation of the experiment.

Monolith[a,b]	% CyOH	% DoOH	HSA (mg/g support)
1	42	18	6.4 (± 3.2)
2	52	8	15.9 (± 1.7)
3	8	52	4.6 (± 2.1)
4	40	20	7.3 (± 3.1)
5	30	30	6.2 (± 2.2)
6	20	40	4.2 (± 1.9)
7	48	12	10.3 (± 2.1)
8	50	10	18.1 (± 3.0)
9	49	11	15.3 (± 2.4)
10	47	13	10.7 (± 2.5)
11	46	14	9.9 (± 2.6)
12	45	15	10.1 (± 2.3)
13	56	4	7.9 (± 1.4)

[a]. Polymerization temperature: 60° C
[b]. Epoxy immobilization method

Table 2.2a: GMA-20 (v/v) % and EDMA-20 (v/v) %, AIBN 1% weight of monomers. Protein content represents average of each column that was made in triplicate. The (±) indicates the standard deviation of the experiment.

Monolith[a,b]	% CyOH	% DoOH	HSA (mg/g support)
1	42	18	12.4 (± 4.2)
12	52	8	23.4 (± 3.8)
3	8	52	7.7 (± 2.8)
4	40	20	11.2 (± 3.1)
5	30	30	9.2 (± 2.7)
6	20	40	8.4 (± 2.1)
7	48	12	19.5 (± 1.5)
8	50	10	27.1 (± 2.0)
9	49	11	24.3 (± 1.8)
10	47	13	19.2 (± 4.0)
11	46	14	18.9 (± 2.0)
12	45	15	15.6 (± 1.9)
13	56	4	12.1 (± 2.3)

[a]. Polymerization temperature: 60^{o} C
[b.] Schiff base immobilization method

Table 2.3a: GMA-24 (v/v) % and EDMA-16 (v/v) %, AIBN 1% weight of monomers. Protein content represents average of each column that was made in triplicate. The (±) indicates the standard deviation of the experiment.

Monolith[a,b]	% CyOH	% DoOH	HSA (mg/g support)
1	42	18	31.2 (± 4.7)
2	52	8	12.5 (± 3.0)
3	8	52	7.2 (± 2.4)
4	40	20	32.2 (± 3.1)
5	30	30	11.2 (± 1.2)
6	20	40	10.4 (± 3.0)
7	48	12	19.2 (± 1.8)
8	50	10	19.8 (± 1.7)
9	49	11	18.2 (± 3.4)
10	47	13	19.5 (± 1.6)
11	46	14	17.8 (± 1.8)
12	45	15	18.6 (± 2.6)
13	56	4	8.5 (± 1.9)

[a]. Polymerization temperature: 80^{o} C
[b]. Epoxy immobilization method

Table 2.4a**:** GMA-24 (v/v) % and EDMA-16 (v/v) %, AIBN 1% weight of monomers. Protein content represents average of each column that was made in triplicate. (*) Indicates preparation utilized for chromatographic conditions. The (±) indicates the standard deviation of the experiment.

Monolith[a,b]	**% CyOH**	**% DoOH**	**HSA (mg/g support)**
1	42	18	42.4 (± 2.1)
2	52	8	23.9 (± 1.7)
3	8	52	9.4 (± 2.0)
***4**	40	20	51.6 (± 1.1)
5	30	30	26.4 (± 3.8)
6	20	40	18.2 (± 1.0)
7	48	12	22.2 (± 1.8)
8	50	10	26.8 (± 2.5)
9	49	11	23.4 (± 4.4)
10	47	13	25.5 (± 2.9)
11	46	14	29.8 (± 2.1)
12	45	15	32.6 (± 3.7)
13	56	4	9.7 (± 0.8)

[a]. Polymerization temperature: 80^{o} C
[b.] Schiff base immobilization method

Table 2.5a: GMA-28 (v/v) % and TRIM-12 (v/v) %, AIBN 1% weight of monomers. Protein content represents average of each column that was made in triplicate. The (±) indicates the standard deviation of the experiment.

Monolith	% CyOH	% DoOH	HSA (mg/g support)
1	42	18	22.0 (± 5.5)
2	52	8	15.3 (± 7.7)
3	8	52	4.4 (± 2.3)
4	40	20	20.2 (± 6.0)
5	30	30	18.4 (± 0.4)
6	20	40	14.7 (± 2.3)
7	48	12	23.8 (± 4.4)
8	50	10	19.2 (± 3.3)
9	49	11	20.5 (± 2.1)
10	47	13	28.8 (±1.4)
11	46	14	26.6 (± 6.2)
12	45	15	24.2 (± 1.9)
13	56	4	6.6 (± 2.9)

[a]. Polymerization temperature: 60^{o} C
[b.] Epoxy immobilization method

Table 2.6a**:** GMA-28 (v/v) % and TRIM-12 (v/v) %, AIBN 1% weight of monomers. Protein content represents average of each column that was made in triplicate. The (±) indicates the standard deviation of the experiment.

Monolith[a,b]	% CyOH	% DoOH	HSA (mg/g support)
1	42	18	34.0(± 4.2)
2	52	8	19.3 (± 3.3)
3	8	52	10.4 (± 4.7)
4	40	20	29.2 (± 6.9)
5	30	30	24.4 (± 2.6)
6	20	40	22.7 (± 1.9)
7	48	12	37.8 (± 1.4)
8	50	10	22.2 (± 2.2)
9	49	11	23.5 (± 6.1)
10	47	13	48.8 (± 3.4)
11	46	14	43.6 (± 2.1)
12	45	15	39.2 (± 3.1)
13	56	4	12.6 (± 4.5)

[a]. Polymerization temperature: 60^{o} C
[b.] Schiff base immobilization method

Table2.7a**:** GMA-28 (v/v) % and TRIM-12 (v/v) %, AIBN 1% weight of monomers. Protein content represents average of each column that was made in triplicate. The (±) indicates the standard deviation of the experiment.

Monolith[a,b]	% CyOH	% DoOH	HSA (mg/g support)
1	42	18	21.2 (± 2.4)
2	52	8	13.9 (± 2.1)
3	8	52	6.3 (± 1.5)
4	40	20	19.9 (± 3.0)
5	30	30	19.4 (± 2.9)
6	20	40	18.2 (± 1.8)
7	48	12	20.8 (± 2.7)
8	50	10	17.3 (± 3.0)
9	49	11	19.8 (± 3.2)
10	47	13	27.2 (± 2.2)
11	46	14	25.7 (± 3.9)
12	45	15	24.7 (± 3.1)
13	56	4	11.4 (± 2.6)

[a]. Polymerization temperature: 80^{o} C
[b.] Epoxy immobilization method

Table 2.8a: GMA-28 (v/v) % and TRIM-12 (v/v) %, AIBN 1% weight of monomers. Protein content represents average of each column that was made in triplicate. (*) Preparation used for chromatographic experiments. The (±) indicates the standard deviation of the experiment.

Monolith	% CyOH	% DoOH	HSA (mg/g support)
1	42	18	30.0 (± 1.3)
2	52	8	31.5 (± 2.2)
3	8	52	19.0 (± 1.6)
4	40	20	25.2 (± 1.4)
5	30	30	28.6 (± 2.2)
6	20	40	27.4 (± 0.7)
7	48	12	39.8 (± 3.0)
8	50	10	33.6 (± 4.0)
9	49	11	37.2 (± 2.1)
***10**	47	13	46.3 (± 2.6)
11	46	14	43.2 (± 5.0)
12	45	15	37.5 (± 4.0)
13	56	4	15.5 (± 3.0)

[a]. Polymerization temperature: 80^{o} C
[b.] Schiff base immobilization method

CHAPTER 3

ENTRAPMENT OF CARBON BASED NANOMATERIALS WITHIN MONOLITHIC CHROMATOGRAPHIC SUPPORTS

I. INTRODUCTION

The emerging field of carbon based nanomaterials is of recent interest in the areas of chromatography and separation science. The widely accepted definition of a nanomaterial states that such a material has a particle size of 100 nm or less in at least one dimension [1]. Nanomaterials have unique size-dependent characteristics, such as their large surface-to- volume ratio (S/V). For example, if the size of the nanoparticle is less than 2 nm, the S/V ratio can exceed 50% [2].

The inclusion of carbon-based nanomaterials such as graphene, multi-walled carbon nanotubes (MWCNTs), and single walled carbon nanotubes (SWCNTs) have been shown to improve the mechanical, thermal and electrical stability of polymer-based nanocomposites [3, 4]. SWCNTs are prepared by having a single layer of graphite folded onto itself; the resulting edge that is formed is then joined together [5]. These nanostructures have been prepared in lengths that range from several nanometers to a few micrometers, with a typical diameter of 0.4-2 nm [5]. MWCNTs consist of several layers of graphite and can be prepared in a variety of lengths (from nanometers to micrometers) and diameters (typically in the nanometer size range) [1].

One of the first applications of nanomaterials, and in particular within separations, occurred in the 1960s. This took place when Kirkland et al. developed porous silica microspheres by using a nonporous glass bead core with nanometer-sized silica spheres affixed to

the glass bead core; this material was used to enhance gas chromatography (GC) separations by allowing for a higher optimum linear gas velocity when compared to traditional GC columns [6, 7]. Following this development, the inclusion of other nanomaterials based on carbon nanotubes, gold and silver nanoparticles were demonstrated [4, 8, 9].

One type of chromatographic support that is attractive for the incorporation of nanomaterials is an organic based monolith. Organic based monoliths are polymer based materials that are advantageous for use in high performance liquid chromatography (HPLC) due to their good mass transport properties and high permeability [8, 10-16]. A recent example utilized gold nanoparticles that were coated on the surface of such a monolith for the pre-concentration of thiol-containing peptides and the separation of proteins [17]. Another recent example utilized MWCNTs entrapped within glycidyl methacrylate/ethylene glycol dimethacrylate (GMA/EDMA) monoliths, or attached to the surface of these monoliths for the separation of uracil and other alkylbenzene derivatives using reversed-phase liquid chromatography (RPLC) [8].

This chapter will focus on the development of monoliths comprised of GMA/EDMA that will be used for the entrapment of carbon based nanomaterials such as carbon onions and reduced graphene oxide multilayer flakes. The supports obtained after physical entrapment will be characterized through a variety of techniques, such as infrared spectroscopy (IR), scanning electron microscopy (SEM), and thermogravimetric analysis (TGA). A mixture of nitrotoluene isomers will be used as model analytes to test the use of these materials for RPLC. The possible use of such materials to study the binding of nanomaterials or functionalized nanomaterials to other agents will also be discussed.

II. EXPERIMENTAL SECTION

Reagents

The GMA (97% pure), EDMA (98%), cyclohexanol (> 99%), 1-dodecanol (98%), 2,2'-azobisisobutryonitrile (AIBN, 98% pure), tetrahydrofuran (>99.0%), methanol, 2-nitrotoluene (99%), 3-nitrotoluene (99%), and 4-nitrotoluene (99%) were from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile was from Honeywell Burdick & Jackson (Muskegon, MI, USA). Graphene nanopowder multilayer flakes MO-1 were acquired from Graphene-Supermarket (Ronkonkoma,NY). The graphene nanopowder multilayer flakes are actually comprised of a mixture of reduced graphene oxide and graphene with a specific surface area of 60m/g^2, an average purity of 99.9% and an average flake thickness of 28 nm.

Carbon onions were graciously donated from the laboratory of Dr. Yongfeng Lu in the Electrical Engineering Department at UNL. All aqueous reagents, solutions and buffers were prepared using water from a Nanopure system (Barnstead, Dubuque, IA) and were filtered using 0.2 μm GNWP nylon filters from Millipore (Billerica, MA, USA).

Apparatus

The monoliths were polymerized in 4.6 mm i.d. x 1 mm PEEK disks within a 5 mL glass vial, as described previously [3]. The washing of the monoliths was accomplished through the use of a reciprocating 501 Waters HPLC pump from Millipore (Milford, MA). The HPLC system used in the chromatographic studies consisted of a Model 200 gradient pump and Model 200 UV detector from Perkin Elmer (Waltham, MA). Samples were injected using a Rheodyne Lab Pro valve (Cotati, CA) and a 20-μL loop constructed from PEEK tubing. Chromatographic data were collected using LabView 5.1 (National Instruments, Austin, TX) and processed using PeakFit 4.12 (SeaSolve Software, San Jose, CA, USA). Fourier transform infrared (FT-IR)

analysis was completed using a 360 FT-IR ESP, Thermoelectron Corporation (currently Thermo Fisher Scientific, Madison, WI). Thermogravimetric analysis (TGA) was performed using a Perkin Elmer STA 6000 Simultaneous Thermal Analyzer (Elmer, Waltham, MA). Scanning electron microscopy (SEM) was performed using a Hitachi S4700 Field-Emission Scanning Electron Microscope with a W95/NT based computerized operating system (Pleasanton, CA).

Preparation of Monolithic Columns

In Chapter 2, the procedure for the preparation of the monolithic columns was described in detail. In this current chapter, the preparation of monoliths for use in the entrapment of carbon-based nanomaterials will be addressed. This procedure began with the polymerization of the monomers, cross-linkers and initiator. Several of the conditions that were most commonly utilized in this study are shown in Table 3-1.

The monoliths were prepared by combining GMA (the functional monomer) and EDMA (the cross linking agent) in a 60:40 (v/v) mixture at either 55ºC or 80ºC. These reagents were combined with the porogenic solvents: cyclohexanol (CyOH) and 1-dodecanol (DoOH), as listed in Table 3-1. Each reagent was combined in a vial and allowed to mix for 5 min prior to the addition of the nanomaterial. Each nanomaterial was carefully weighed into a 5 mL glass vial that contained the monomers, porogens and initiator. The amount of nanomaterial that was utilized in each preparation was approximately 1.0 wt % . This particular amount was based upon a prior literature method for the use of GMA/EDMA monoliths in the entrapment of carbon nanotubes [8].

Assessment of Monoliths

SEM images were obtained on several monolith materials after the polymerization process. These samples were prepared in 5 mL vials using 4.6 mm i.d. x 1 mm PEEK disks. Following polymerization, the monoliths were washed with 100 mL acetonitrile at 0.5 mL/min

for 3 h. The monoliths were then removed from their PEEK disk housings and ground to a fine powder. The samples were placed onto a watch glass and dried under vacuum at 100ºC for 8 days before imaging. Prior to imaging, chromium was used to sputter coat each sample for a period of 5 min to generate a conductive surface.

Table 3-1. Polymerization conditions used for physical entrapment of the carbon based nanomaterials at both 55ºC and 80ºC.

Reagent	**Weight %**
GMA	24
EDMA	16
CyOH	54
DoOH	6
*AIBN (1.0 wt % monomers)	
* Carbon nanomaterials (1.0wt % total)	

FT-IR spectroscopy and TGA were used to examine each monolith sample. These monoliths were prepared in 5 mL vials using the 4.6 mm i.d. x 1 mm PEEK disks and washed as described earlier. The monoliths for the FT-IR analysis were ground to a fine powder and were dried at room temperature under vacuum for 3 days prior to analysis. Typically, 16 FT-IR scans were performed. The monoliths for TGA were ground to a fine powder and allowed to dry under vacuum for 1 week prior to analysis. The temperature program utilized in the TGA experiments went from 30ºC to 900ºC at a rate of 20ºC/min.

The chromatographic experiments utilized a mobile phase that was comprised of 45% acetonitrile (ACN), 50% water, and 5% tetrahydrofuran (THF). The flow rate utilized was 0.1 mL/min and the nitrotoluene isomers were detected at 254 nm. A stock solution was prepared with a concentration of 20 mg/mL for each nitrotoluene isomer in methanol. The solution was then diluted to 0.10 mg/mL using water. All chromatographic experiments were conducted at room temperature. Each nitrotoluene isomer was injected individually to determine its elution time on the modified monolith. A mixture of the isomers was then injected at several different flow rates that initially ranged from 0.01 mL/min-5.0 mL/min. The flow rate selected for use in later studies was 0.1 mL/min, which provided a back pressure of approximately 54 psi.

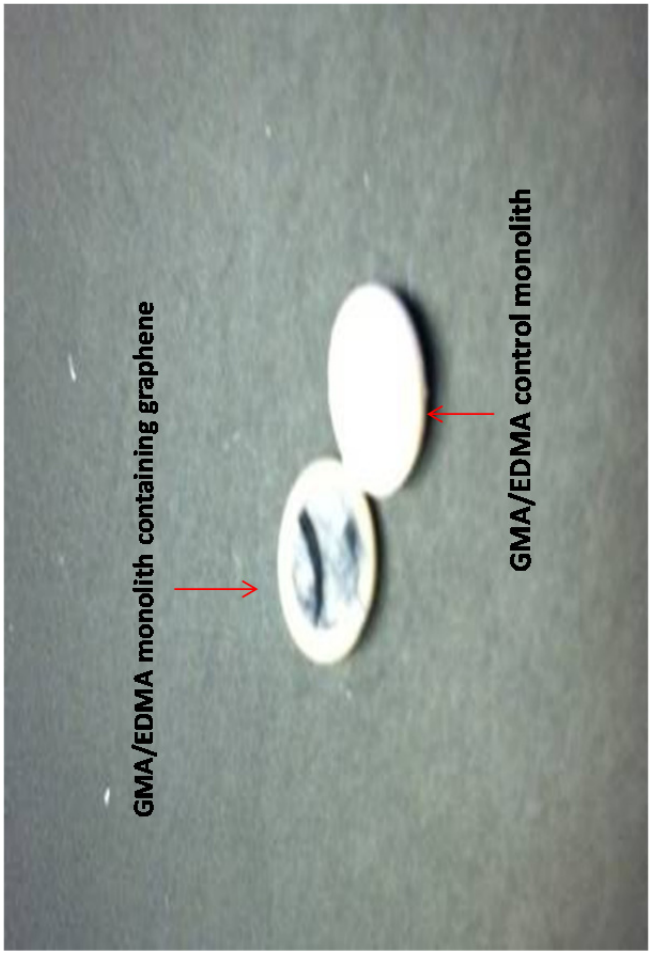
III. RESULTS AND DISCUSSION

The purpose of this study was to examine the entrapment of carbon-based nanomaterials such as reduced graphene oxide or carbon onions within GMA/EDMA monoliths for later use in chromatographic methods or flow-based interaction experiments. Several different combinations of monomers and cross linkers were initially utilized in combination with carbon onions. However, due to the limited supply of these nanomaterials, a cheaper alternative nanomaterial, was employed for the development and optimization of such supports. Reduced Graphene oxide nanopower multi-layer flakes were instead chosen for use during the optimization of a method for the direct inclusion of carbon-based nanomaterials within a GMA/EDMA monolith.

There were several factors to consider during the creation of these hybrid materials. One of the biggest issues surrounded the placement and homeogenity of the carbon nanomaterial in the polymers. When the carbon nanomaterials were placed into a mixture of GMA and EDMA there was an almost immediate separation and sedimentation of the carbon nanomaterials away from the monomers. This separation was probably related to the hydrophobic nature of the carbon nanomaterials in the presence of the other, relatively polar reagents. However, upon addition of the solvents (i.e., 1-dodecanol and cyclohexanol) and after a period of 5 min of shaking, a fairly homogenous dispersion of the carbon nanomaterials, monomers, and porogenic solvents was obtained.

During the preparation of each nanomaterial/monolith hybrid material, a control support was prepared from the same mixture except with no nanomaterials being added to the polymerization mixture. There was a clear distinction between these supports both before and after polymerization. The control monolith was white in color. The solution containing the carbon materials prior to polymerization appeared dark gray in color. Polymerization of this material at either 55 ºC or 80ºC produced monoliths that were light gray, as shown in Figure 3-1.

Figure 3-1. GMA/EDMA monoliths prepared at 80ºC both with and without the addition of reduced graphene oxide multi-layer flakes to the polymerization mixture.


GMA/EDMA monolith containing graphene
GMA/EDMA control monolith

It was clear from this visual inspection that carbon nanomaterials could be entrapped within the GMA/EDMA monoliths.

One important observation made after polymerization dealt with the appearance of the monoliths prior to the isolation of a column from the overall block of the final polymerized mixture. The polymerized block did vary in the distribution of the carbon based nanomaterials, with a significant proportion of these nanomaterials settling at the bottom of the vial. However, the PEEK rings that surrounded the monolith column also settled to the bottom, which allowed a large percentage of the nanomaterials to remain contained and polymerized within the matrix in the region of these disks. The biggest difference that was noted in the appearance of the monolith support was when the temperature was varied between 55ºC and 80ºC. When prepared at 55 ºC, the monoliths were less robust compared to the monoliths prepared at 80ºC. The monoliths made at 80ºC that contained the carbon nanomaterials also were more rigid than those prepared from the same polymerization mixture at 55ºC.

Once visual confirmation of the entrapped carbon nanomaterials had been made, further techniques were used to image this material and measure the amount of entrapped nanomaterials. For example, TGA was performed on the monolith samples and control supports prepared at 55ºC and 80ºC, as well as on control samples of the nanomaterial (e.g., reduced graphene oxide). Figure 3-2 shows some typical plots that were obtained for these materials, in which the change in weight percentage is plotted versus temperature during a temperature ramp from 30 to 900ºC at a rate of 20ºC/min. Table 3-2 shows the final data that were obtained for the TGA of the monoliths that were prepared at 55ºC and 80ºC.

Figure 3-2. Thermogravimetric analysis plots for reduced graphene oxide multilayer flakes, a GMA/EDMA monolith prepared at 55ºC containing the reduced graphene oxide multilayer, and a control GMA/EDMA monolith prepared at 55ºC.

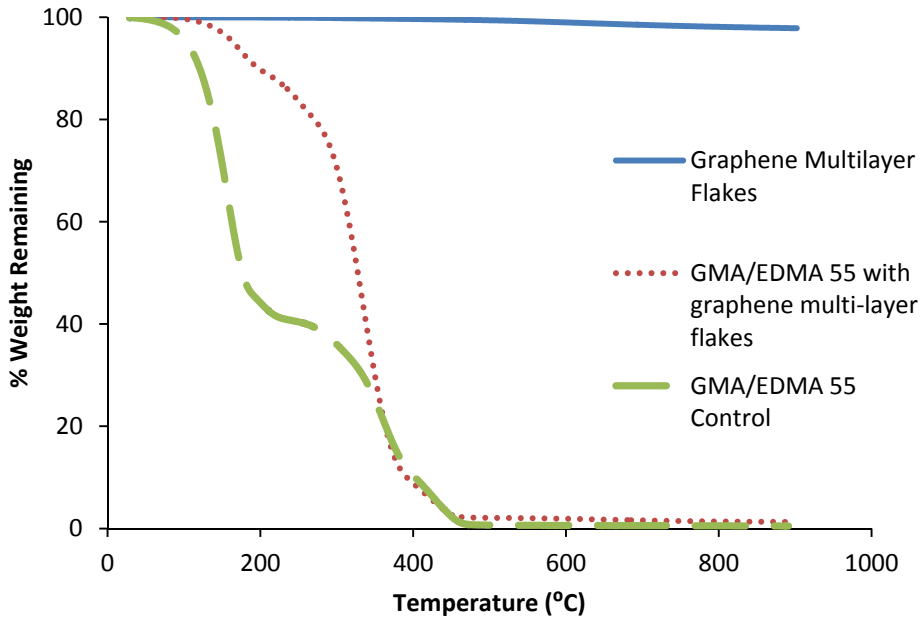

100
80
60
40
20
0
% Weight Remaining
0
200
400
600
800
1000
Temperature (°C)
Graphene Multilayer Flakes
GMA/EDMA 55 with graphene multi-layer flakes
GMA/EDMA 55 Control

Table 3-2. TGA data obtained for the monoliths prepared at 55ºC or 80ºC

Monolith	Run#	Initial Mass (mg)	Final Mass (mg)	Char yield (%)	Average (%)	Standard Deviation (%)
Control 55	1	46.82	0.22	0.48	**0.46**	0.04
	2	24.94	0.12	0.49		
	3	24.24	0.10	0.41		
Graphene 55	1	76.49	0.55	0.72	**1.03**	0.30
	2	20.82	0.24	1.17	(**1.23**)[a]	(0.08)[a]
	3	21.76	0.28	1.29		
Control 80	1	26.75	0.21	0.77	**0.76**	0.06
	2	26.35	0.25	0.81		
	3	31.24	0.22	0.70		
Graphene 80	1	23.32	0.27	1.15	**1.12**	0.05
	2	30.09	0.33	1.08		
	3	29.92	0.35	1.12		
Carbon Onion 80	1	19.91	0.18	0.90	**0.99**	0.13
	2	20.01	0.23	1.14		
	3	22.32	0.21	0.94		

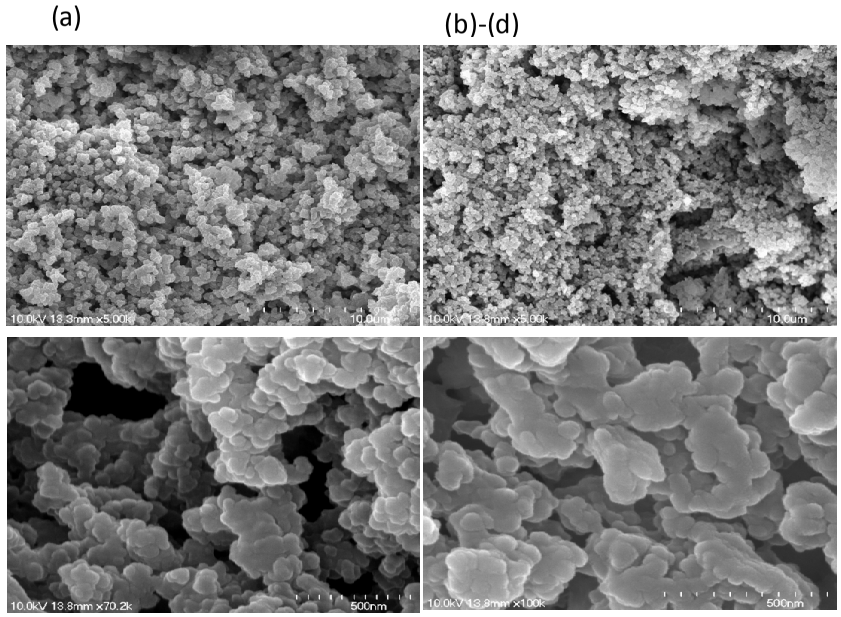
[a]Values based on only runs 2 and 3 for the Graphene 55 samples.

As can be seen in Table 3-2, there was a clear difference in char yield% for the monoliths containing carbon nanomaterials that were prepared at both 80ºC and 55 ºC. When comparing the percentage of mass remaining for the control monolith prepared at 55 ºC and the monolith prepared at 55 ºC containing the reduced graphene oxide multilayer flakes, there was approximately 0.57 (± 0.30)% weight remaining when all samples were considered; the result was 0.77 (± 0.08)% when only runs 2 and 3 were considered. When comparing the percentage of mass remaining for the control monolith prepared at 80ºC and the monolith containing the reduced graphene oxide multilayer flakes, there was 0.36 (± 0.08)% weight remaining which could be attributed to the entrapped reduced graphene oxide flakes. For the control monolith prepared at 80ºC and the monolith containing carbon onions, there was a difference of 0.23 (± 0.14)% weight due to the carbon onions.

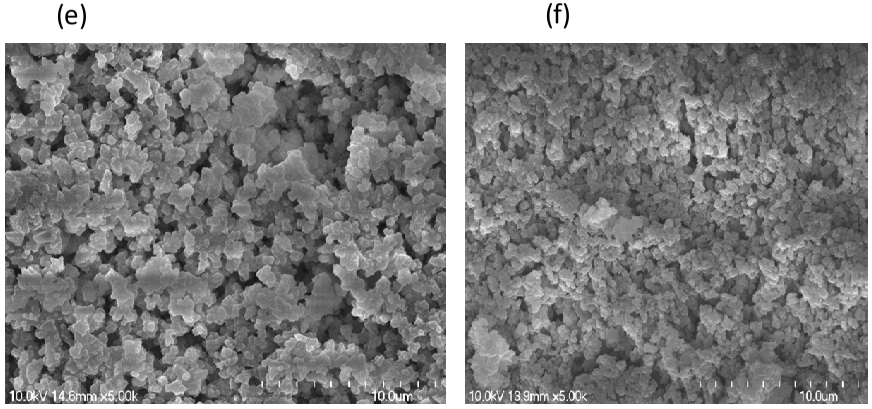
The TGA results indicated that this approach could be used to quantify the amount of entrapped carbon nanomaterials within the monolith supports with reasonable precision. The results also indicated that the polymerization temperature did affect the amount of carbon nanomaterials that could be entrapped in these supports. In particular, the TGA data suggested that lower polymerization temperatures may have given a greater percentage of carbon nanomaterials in the final support. This difference could be due to the mechanism by which the carbon nanomaterials are entrapped within the macropores or transverse between pores during polymerization. This agrees with previous results in which the surface area and pore structure of GMA/EDMA monoliths have been shown to vary significantly with the polymerization temperature. For instance, at a polymerization temperature of 80ºC the pore size of the GMA/EDMA monoliths is known to decrease when compared to similar monoliths that are prepared at 60ºC [12].

SEM imaging techniques were next used to examine the monoliths containing the carbon nanomaterials. Figure 3-3 shows some images that were obtained for monoliths that were prepared at a polymerization temperature of 80ºC or 55ºC.

Figure 3-3. SEM images of monoliths prepared at a polymerization temperature of 55 or 80º C and with or without the addition of carbon onions to the polymerization mixture.



(a) Control and (b-d) GMA/EDMA monoliths prepared at 80ºC containing carbon onions



(e) Control and (f) GMA/EDMA monolith prepared at 55ºC containing graphene

The SEM images obtained allowed for the visualization of the overall pore structure of the monoliths but did not afford images in which the carbon onions or reduced graphene oxide multilayer flakes could be directly identified. However, the images obtained for the supports prepared at 55ºC were at a low resolution. Higher magnification was not achieved during this analysis or any of the subsequent analyses. One way to address this issue would be to allow the support to dry for a longer period of time under vacuum and at a higher temperature. The monoliths prepared at 80ºC allowed for high magnification to be achieved; however, at a high magnification it was still difficult to distinguish the pores of the monoliths from entrapped carbon onions. Future work will involve utilizing other imaging techniques such as transmission electron microscopy (TEM) to examine these materials.

FT-IR was also utilized to obtain the IR spectrum of the monoliths containing entrapped carbon onions, control supports, and carbon onions. A spectrum was obtained for each monolith that was prepared at 55ºC or 80ºC. Some of the monoliths made at 55ºC contained reduced graphene oxide multilayer flakes. A spectrum of the reduced graphene oxide multilayer flakes was also taken. However, the results obtained were inconclusive and not definitive.

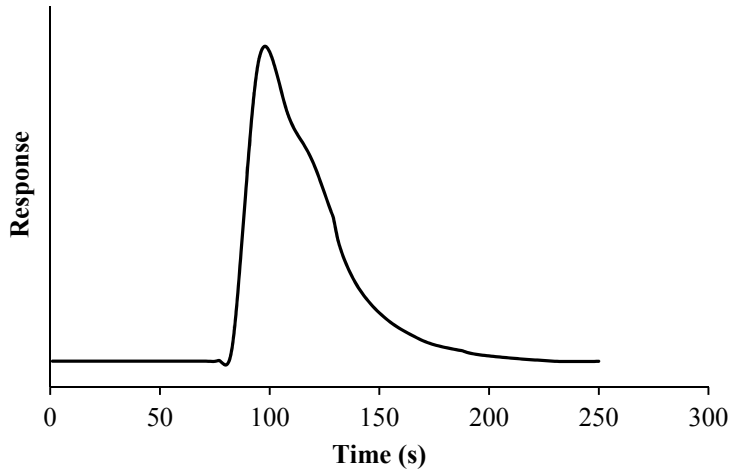
Previous work with monoliths and nanomaterials has suggested that carbon nanomaterials may enhance chromatographic separations for RPLC when using supports that are based on organic based monoliths. This has been recently illustrated using benzene derivatives as model analytes [8]. This type of application was explored by using a monolith support that was prepared at 80ºC and that contained carbon onions, as described previously. Figure 3-4 shows a representative chromatogram that was obtained in preliminary experiments that used this type of monolith for a RPLC separation of nitrotoluene isomers.

This set of preliminary experiments used a mobile phase that contained 45% acetonitrile, 50% water and 5% THF, as applied to the column at a flow rate of 0.1 mL/min. As is shown in

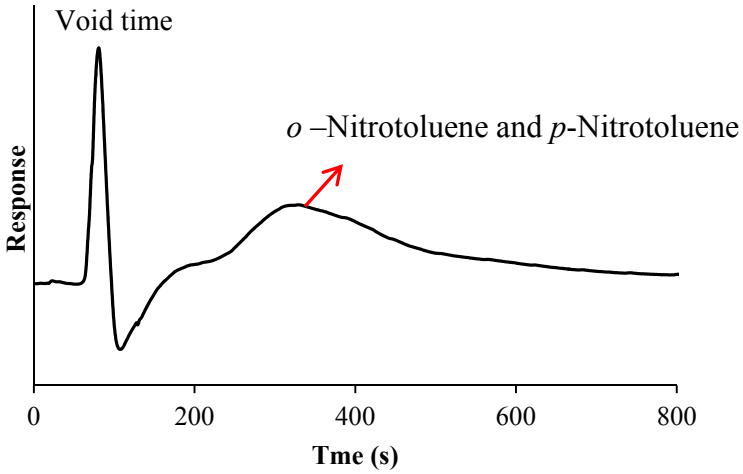
Figure 3-4, there was no significant separation of the nitrotoluene isomers on the control monolith column. However, the monolith that contained the carbon onions and that was present in only a 1 mm long disk column did give a partial separation of the meta-isomer from the ortho- and para-isomers. Although the mobile phase composition and flow rate have not been fully optimized for such work, these results again confirmed that carbon onions were entrapped within the support. These results also indicted that the monolithic supports and a flow-based format could be used to examine the interactions of these nanomaterials with other substances that were injected onto the system. It is important to note that even with the small column dimensions (4.6 mm i.d. x 1 mm) a retention time of over 300 seconds was achieved using an isocratic solvent system. Finally, these results indicate that these nanomaterial-based interactions could be used as the basis for a chemical separation in a small flow-based device.

Figure 3-4**.** Chromatograms for nitrotoluene isomers that were obtained on a control monolith prepared at 80ºC and a monolith prepared at 80ºC that contained entrapped carbon onions. Other conditions are given in the Experimental section.

Control monolith prepared at 80^{o}C



Monolith containing carbon onions prepared at 80^{o}C



IV. CONCLUSIONS

The purpose of this study was to study and optimize the conditions needed for the preparation of GMA/EDMA monoliths that contained entrapped nanomaterials, such as reduced graphene oxide multilayer flakes or carbon onions. There were several factors considered during this study, including the methods that could be used for characterizing such materials. TGA provided a fairly simple means for determining the amount of nanomaterials that were entrapped in the GMA/EDMA monoliths. IR provided qualitative evidence for such entrapment, and SEM allowed the overall pore structure of the hybrid materials to be examined. It was also found that a flow-based format using chromatography could be used with such supports to examine interactions of other substances with the entrapped nanomaterials.

This latter observation means that these materials could be useful in future work aimed at using chromatography with entrapped hybrid nanomaterials for measuring properties such as binding constants or rate constants for biological ligands that may be attached to these materials. The results of this study also indicate that small columns based on GMA/EDMA mixtures with carbon nanomaterials may be a useful platform for incorporating these materials into small flow-based devices for chemical separations or sensors. One goal of future work will be to characterize these materials through the use of Raman spectroscopy, which is commonly used to study materials with non-polar bonds. The further optimization of this entrapment approach and its use with other nanomaterials is also of interest, such as work with silver nanoparticles and carbon based nanomaterials that have been modified for ligand or protein attachment.

References

1. Zhang, Z., Wang, Z., Liao, Y., Liu, H., *Applications of nanomaterials in liquid chromatography: Opportunities for separation with high efficiency and selectivity.* J. Sep. Sci., 2006. **29**:1872-1878.
2. Sapsford, K.E., Tyner, K.M., Dair, B.J., Deschamps, J.R., Medintz, I.L., *Analyzing nanomaterial bioconjugates: A Review of current and emerging purification and characterization techniques.* Anal. Chem., 2011. **83**:4453-4488.
3. Hu. H., W., X., Wana, L., Liu, F., Zheng, H., et al., *Preparation and properties of graphene nanosheets-polystyrene nanocomposites via in situ emulsion polymerization.* Chem. Phys. Lett., 2010. **484**:247-53.
4. Kuila, T., Bhadra, S., Yao, D., Kim, N.H., Bose, S., Lee, J.H., *Recent advances in graphene based polymer composites.* Prog. Polym. Sci, 2010. **35**:1350-75.
5. Katz, E., Willner, I., *Biomolecule-functionalized carbon nanotubes: applications in nanobioelectronics* Chem. Phys. Chem., 2004. **5**:1084–1104.
6. Kirkland, J.J., *Porous thin-layer modified glass bead supports for gas liquid chromatography.* Anal. Chem., 1965. **37**:1458-1461.
7. Kirkland, J.J., *Controlled surface porosity supports for high-speed gas and liquid chromatography.* Anal.Chem., 1969. **41**:218-220.
8. Chambers, S.D., Svec, F., Frechet, J.M.J., *Incorporation of carbon nanotubes in porous polymer monolithic capillary columns to enhance the chromatographic separation of small molecules.* J. Chromatogr. A, 2011. **1218**:2546-2552.
9. Liu, J., White, I., DeVoe, D.L., *Nanoparticle-functionalized porous polymer monolith detection elements for surface-enhanced raman scattering.* Anal. Chem., 2011. **83**:2119-2124.
10. Guiochon, G., *Monolithic columns in high-performance liquid chromatography.* J. Chromatogr. A, 2007. **1168**:101-168.

11. Ikegami, T., Tanaka, N., *Monolithic columns for high-efficiency HPLC separations.* Curr. Opin. Chem. Biol., 2004. **8**:527-533.

12. Jiang, T., Mallik, R., Hage, D. S., *Affinity monoliths for ultrafast immunoextraction.* Anal. Chem., 2005. **77**:2362-2372.

13. Nordborg, A., Hilder, E.F., *Recent advances in polymer monoliths for ion-exchange chromatography.* Anal. Bioanal. Chem., 2009. **394**:71-84.

14. Svec, F., Frechet, J.M.J., *Continuous rods of macroporous polymer as high-performance liquid chromatography separation media.* Anal. Chem., 1992. **64**:820-822.

15. Wang, Q., Svec, F., Frechet, J.M.J., *Macroporous polymeric stationary-phase rod as continuous separation medium for reversed phase chromatography.* Anal. Chem., 1993. **65**:2243-2248.

16. Wang, Q., Svec, F., Frechet, J., *Reversed-phase chromatography of small molecules and peptides on a continuous rod of macroporous poly(styrene-co-divinylbenzene).* J. Chromatogr. A, 1994. **669**:230-5.

Porous polymer monolithic column with surface-bound gold nanoparticles for the capture and separation of cysteine-containing peptides. Anal. Chem., 2010. **82**:3352-3358.