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SURFACE MODIFICATIONS OF CAPILLARY-CHANNELED POLYMER (C-CP) FIBERS FOR APPLICATIONS IN HIGHLY SELECTIVE SEPARATIONS

Jennifer Pittman
Clemson University, jjpittm@clemson.edu

SURFACE MODIFICATIONS OF CAPILLARY-CHANNELED POLYMER (C-CP) FIBERS FOR APPLICATIONS IN HIGHLY SELECTIVE SEPARATIONS

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Jennifer J Pittman
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Accepted by:
R. Kenneth Marcus, Committee Chair
Kenneth A Christensen
George Chumanov
William T Pennington

ABSTRACT

High Performance Liquid Chromatography (HPLC) is a key component in the purification and separation of biological samples. Stationary phases in HPLC are generally silica based porous particles or monoliths designed with high surface area and high capacities in mind. However, in the field of macromolecule separations, specifically proteins, the porous based stationary phases have inherent issues that include slow mass transfer, high operating back pressures, and analyte carryover. Recent research has looked to non-porous polymeric materials as stationary phases in HPLC to overcome these challenges. Specifically, fibrous based polymer stationary phases exhibit significant benefits for macromolecules that include improved mass transfer, decreased operating pressures, and improved chemical robustness.

Capillary-Channeled Polymer (C-CP) fibers have been under investigation in the Marcus laboratory for their application as stationary phases in HPLC. C-CP fibers are extruded from standard textile polymers through a spinneret. The spinneret shape provides the unique structure of the fibers, which consists of eight channels that run the length of the fiber. These C-CP fibers have been successfully employed for macromolecule separations due to the increased surface area over cylindrical fibers, an improved mass transfer due to their non-porous nature, and reduced back pressures allowing for operation at higher linear velocities.

C-CP fibers come in a variety of base polymers; polyester, polypropylene, and nylon, providing a wide array of chemical interactions (ionic, π-π, hydrophobic) and therefore separation mechanisms to occur. However, the ability to generate HPLC stationary phase surfaces with a high degree of analyte specificity is desired. The focus of this research is on modification of C-CP fibers, specifically to generate a high density functional group surface for analyte selective interactions. All three available base polymers of C-CP fibers were evaluated for their ability to undergo chemical modification while maintaining the structural integrity and characteristics of the fibers. Several modification approaches, including plasma grafting, covalent modification, and lipid adsorption, were utilized and their performance evaluated in order to obtain metal or protein selective HPLC stationary phases.

DEDICATION

This dissertation is dedicated to all those who believed in me. It is with your love, support, and encouragement that I was able to reach this point in my academic career.

To my loving husband, Daniel Pittman, without you this would have been an impossible journey, thank-you for your endless supply of patience, encouragement, and LOVE.

To my family (Mom and Duane, Dad, Erica and Rob, Adam, Grace, Grandma and Grandpa, Jay and Patti, Karen and Keith, Stacey, Mark and Katie, April and Michael) thank-you for all your unconditional love, support, and the constant encouragement you provided. It gave me the courage to keep going when things got tough.

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TABLE OF CONTENTS

Table of Contents (Continued)

Page

Table of Contents (Continued)

Page

Table of Contents (Continued)

Page

LIST OF TABLES

LIST OF FIGURES

List of Figures (Continued)

Figure Page

List of Figures (Continued)

Figure Page

List of Figures (Continued)

Figure Page

List of Figures (Continued)

Figure Page

List of Figures (Continued)

Figure Page

List of Figures (Continued)

Figure Page

CHAPTER 1

INTRODUCTION

Introduction to Separation and Chromatography

The need to analyze and understand complex, real world, mixtures is universal across many disciplines. In order to better understand these systems and quantitatively measure their components a variety of separations tools and methodologies must be developed. The purpose of developing these separations is to enhance the ability to isolate, analyze and quantify the components of interest. Separations in their simplest forms have existed for hundreds of years and likely began with selective precipitation of metal species[1]. Analytical separations techniques have evolved substantially over the past century and separation methodologies now include techniques such as electrophoresis, distillation, field flow fractionation, solid and liquid extractions, and chromatography. For the purpose of the work presented here, the primary focus is chromatographic separation techniques.

Liquid chromatography (LC) is a separation technique that requires flow of a liquid (i.e. the mobile phase) through a solid material (i.e. the stationary phase). To be effective, LC techniques must be capable of resolving the components of a mixture to the point that individual species can be differentiated. During LC analysis, the complex analyte mixture is applied as a liquid sample to the beginning of a column containing a solid stationary phase. A mobile phase is passed through the column via either gravity flow or a pump, moving the sample

through the column[2]. The components of the mixture partition between the stationary phase and mobile phase differently based on their structure (e.g. steric limitations on surface sorption) and reactivity (e.g. chemical interactions with the stationary phase functional groups), resulting in the separation of the components.

LC was first developed and published for the separation of environmental samples, specifically plant extracts, in the early 1900s by a Russian researcher Mikhail Tswett[3]. These initial studies were based on a gravity flow fed system and since this time chromatographic instrumentation has evolved to allow for separations based on pumping of a mobile phase at a controlled velocity (flow rate). These velocity controlled systems generate a back pressure resulting from the forced fluid movement through the stationary phase of the chromatography column. The back pressure generated by the system is a function of the column diameter, stationary phase particle size and porosity, viscosity of the mobile phase, and the flow rate[4]. Pump fed chromatographic separations fall into two categories, low pressure LC and high pressure LC. Low pressure LC systems are traditionally used in preparative scale separations where large amounts (gram quantities) of analyte need to be processed[5]. The generation of a "substantial" backpressure, >500-1000psi, has led to a variation of LC called high pressure liquid chromatography (HPLC)[2]. HPLC uses the same basic mechanism of separation as LC but under higher backpressures[4]. The need to continually perform improved separations has resulted in the development of stationary

phases that could perform faster separations on higher capacity columns with better selectivity, but these separations result in higher backpressures[2, 4]. HPLC eventually evolved from high pressure liquid chromatography to *high performance liquid chromatography* based on the realization that performance, not pressure, was the central issue in the separations[4]. Today HPLC allows for the separation of milligram (mg) or less quantities of complex mixtures at high linear velocities with decreased analyte diffusion, improved mass transfer kinetics, and separations that occur in minutes instead of hours compared to a low pressure LC separation (i.e. better resolution and higher throughput).

To improve upon separation performance three major components of a chromatographic separation system can be manipulated: the mobile phase, the stationary phase, and the instrumentation. Changes in mobile phase composition, gradient rates, flow rates, and additives all play a role in the transport and partitioning of the analyte between the mobile phase and the stationary phase. These properties are generally evaluated on a per separation/column basis. Research into improvements of the stationary phase includes evaluating components such as stationary phase material, packing density, porosity, particle size, and surface functional groups. The utilization of smaller particles with higher porosity while still employing higher linear velocities has led to a need for improved instrumentation. Advancements in the instrumentation include more control of flow and gradient rates, as well as the ability of the hardware to handle these increased backpressures.

The acquisition of quality separations primarily relies on the development of a highly selective stationary phase. Selectivity is the ability of a stationary phase to distinguish between the components of an analyte mixture (i.e. increased column selectivity equals an improved separation). The selectivity (α) of a column is defined by the ratio of the capacity factors of the most retained analyte to the less retained analyte in the mixture, Equation 1.1[2, 6]. Therefore, in order to determine the selectivity of a stationary phase one must have an understanding of capacity factor. Capacity factor (k') is a measure of analytes relative retentions, which is the ability of the column stationary phase to interact and bind the analyte components of the mixture, as defined by Equation 1.2[5, 6]. Where t_r is the retention time of the analyte and t_0 is the time it takes an unretained molecule to pass through the column. Thus, selectivity of a separation is dependent upon the interactions of an analyte with the stationary phase. In order to increase the selectivity of a separation, changes in mobile phase (i.e. chemical composition) or stationary phase (i.e. surface functional group type and density) are required[2]. Development of a highly selective stationary phase for proteins and metal ions is the focus of this research.

$$\alpha = \frac{k'_B}{k'_A} \qquad \text{(Eqn. 1.1)}$$

$$k' = \frac{t_R - t_0}{t_0} \qquad \text{(Eqn. 1.2)}$$

Separation Theory

The goal of a chromatographic separation is to resolve the components of the complex mixture in the shortest amount of time possible. In order to achieve this a variety of parameters or chromatographic figures of merit, are used to determine the quality of the separation. The ultimate goal, and most evident evaluation parameter on the success of a separation, is the resolution of the analyte peaks in the chromatogram. The resolution (R_s) of two peaks is determined by evaluating their retention time (t_r) and their peak width (*w*), Eqn. 1.3[1, 4].

$$R_s = \frac{2\,\Delta t_r}{(w_A + w_B)} \qquad \text{(Eqn. 1.3)}$$

As discussed previously, to increase resolution of the separation an increase in selectivity, or an increased Δt_r, is one parameter that can evaluated. Another approach to increasing the resolution of a separation would be to increase the efficiency (decrease *w*)[4]. However, increasing the retention of the analyte is associated with an increase in peak width, which is discussed below. In order to decrease the peak width (*w*), or increase column efficiency, an increase in the number of theoretical plates within the column is desired. Equation 1.4 shows that the number of plates (*N*) available in the separation is a function of peak width (*w*) and retention time (*tr*)[1, 4]. The higher the number of theoretical plates, the narrower the peak width and the greater resolving capacity of the column. Over the course of a column length (*L*) one would want to increase the number of plates and decrease the plate height (*H*) (Eqn. 1.5)[4].

$$N = 15\left(\frac{t_r}{w}\right)^2 \quad \text{(Eqn. 1.4)}$$

$$H = \frac{L}{N} \quad \text{(Eqn. 1.5)}$$

As shown in the equations discussed above, peak width plays a predominant role in the quality of separations obtained; smaller peak widths equal an increased number of plates and, therefore, better resolution. The height equivalent to a theoretical plate (*H, HETP)* is inversely proportional to the total plates (*N*) of the stationary phase, for that reason in order to increase the number of plates (*N*) and resolution (R_s) one must evaluate ways to decrease the plate height (*H*). HETP takes into account a variety of parameters related to mobile phase velocity and stationary phase characteristics to evaluate peak broadening, described in equation 1.6, the van Deemter equation[2, 4].

$$H = A + \frac{B}{u} + C * u \quad \text{(Eqn. 1.6)}$$

Each variable in this equation evaluates a parameter of the separation (particle size, diffusion, mass transfer effects of mobile phase and stationary phase, and flow dynamics) to better understand the total column bandspreading[2]. The “A” term in Eqn. 1.6 relates to particle size and packing of the stationary phase, while the “B” and “C” terms are a function of linear velocity (*u*) and relate to diffusion and mass transfer respectively. Figure 1.1 is a van Deemter plot demonstrating how each term independently affects plate height as a function of linear velocity[4].

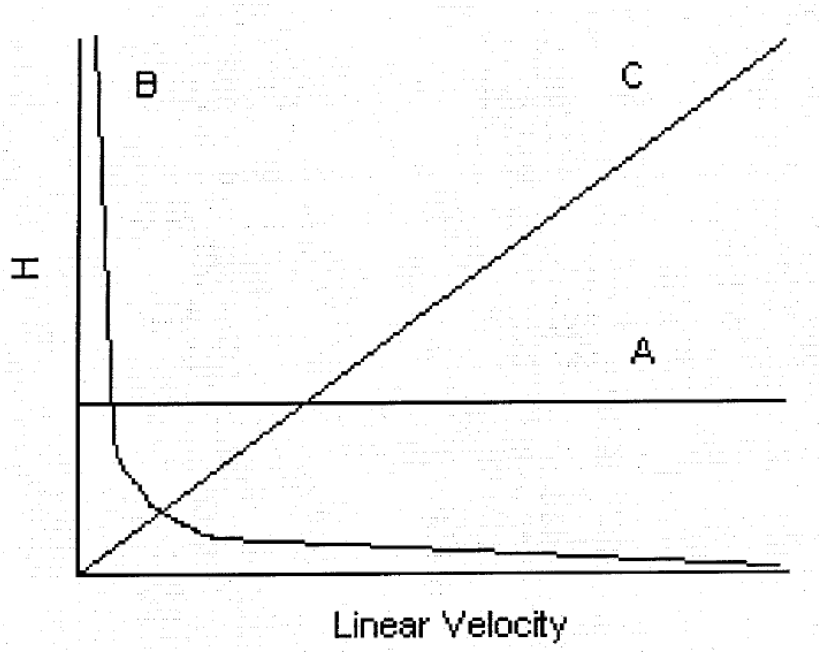

Figure 1.1 van Deemter plot showing contributions of each individual term to band broadening as a function of linear velocity[6]

The “A” term is a result of the multiple flow paths and variations in flow velocity along the column. In order to minimize the contributions of “A” to the overall plate height a homogenous packing of the stationary phase is required. Poorly packed stationary phases causes flow inequality due to increases in eddy diffusion and changes in laminar flow[2, 5]. The “B” term of this equation is inversely related to linear velocity (*u*) and influences band broadening associated with longitudinal diffusion. Longitudinal diffusion of the analyte band occurs as a function of time and is related to the diffusion coefficient of the molecule. Therefore, separations performed at increased linear velocities decrease the amount of time the molecules have to diffuse axially in the column[2, 5]. The “C” term is the mass transfer term and includes mass transfer of the molecule in the stationary phase and the mobile phase. This term takes into account three

processes: transfer of the molecule from the mobile phase to the stationary phase; interaction of the molecule with the stationary phase (on/off mechanism); and in the case of porous particle stationary phases this term also includes the transfer of the molecule through the stagnant mobile phase within the pores to the surface of the stationary phase. These transfer properties are a significant contribution to the band broadening and are a driver for developing improved stationary phases[2, 5].

Metal Separations

Ion chromatography (IC), the separation of anions and cations by passing the analyte through an oppositely charged stationary phase, was first researched in the 1930s but gained momentum during the Manhattan project in the 1940s where extensive research into the application was performed using polystyrene-divinylbenzene resins (PS-DVB)[7]. One of the most significant advances in the history of IC was the development of the conductivity detector in 1975 by Smalls, Stevens, and Baumann allowing for the universal detection of anions and cations marking what is known as the real birth of IC[7-9]. Modern day IC plays a significant role in the environmental (e.g. drinking and waste water), semiconductor and electronics industries, and the food and dietary supplements industry (e.g. heavy metal contaminants)[9]. IC is a form of LC and for the same reasons discussed earlier, extensive research into the advancement of stationary phases has occurred since its inception. IC and LC stationary phases have evolved to include porous particles, smaller diameter polymer and silica based beads, capillary

columns, monolithic columns, and a variety of surface modifications all designed with the same goal of generating more selective stationary phases. The field of IC has evolved since its conception to not only include binary systems, ion-exchange, ion-pairing, and ion-exclusion[10]; but to also include tertiary separation systems like immobilized metal affinity chromatography. These tertiary separations utilize a charged stationary phase surface with which the metal interacts allowing the remaining metal coordination sites to interact with the analytes (e.g. proteins) of interest; in these separations metal ions act as a bridge between the stationary phase and the analytes[5, 11].

Protein Separations

The human genome codes for over 20,000 proteins[11]. Add to this number the post translational modifications that proteins undergo and an overwhelming number of proteins exist in the human body. The field of proteomics is the study of these proteins, their structure and function. Researchers study proteins to gain insight into cells, organisms, biological pathways and diseases. The presence of certain proteins or changes in the levels of proteins can signify diseases; therefore the ability to understand these proteins and their functions is of great importance in understanding and diagnosing such diseases[11]. Proteins are only a portion of a very complex mixture of biomolecules consisting of lipids, nucleic acids, enzymes, and other cellular material[5]. The ability to purify classes of proteins and isolate specific ones is crucial to advancing the field of proteomics. Protein separations can be achieved through a variety of interactions (e.g.

hydrophobic, ion exchange, affinity, etc.) and techniques (e.g. extractions, immunoassays, electrophoresis, chromatography, etc.)[2]. The focus of this dissertation is on the chromatographic approach.

Several chromatographic separation mechanisms are employed in protein purifications including reversed-phase (RP), ion-exchange (IEC), bio-recognition (affinity), hydrophobic interaction (HIC), and size exclusion (SEC)[2, 5]. The mechanisms underlying each of these techniques play an important role in protein purification and extraction process. Chromatography is generally implemented as part of a multistep process that includes purification of a group of proteins, followed by extraction or isolation of a smaller subset or even single protein[5]. Several of these approaches that are pertinent to this dissertation are outlined briefly below.

Reversed-phase (RP) chromatography allows for separation of proteins based on hydrophobicity. RP stationary phases consist of aliphatic, aromatic (or both) surface moieties generating a hydrophobic surface for analyte interactions, protein elution from the surface occurs with organic solvents. Selectivity in a RP separation comes from the available surface moieties (C4, C8, C18, phenyl), strength of organic solvent mobile phase, ion pairing agents, and surfactants. RP separations are a powerful technique, however, the primary drawback to using RP chromatography is the denaturation and loss of protein function as a result of organic solvent exposure[2].

IEC separation of proteins is considered a mild separation mechanism, wherein the protein functionality and conformation are maintained, and is a popular technique for all stages of the purification process. IEC separates proteins based on their overall charge and utilize stationary phases with oppositely charged surface groups; anionic, cationic, or even zwitterionic in some cases. Selectivity in IEC separations can be controlled by manipulating the type of charged surface groups (strong versus weak), ionic strength, and pH[2].

Bio-recognition or affinity chromatography has a wide range of applicability and is based on a specific affinity or interaction of an analyte of interest with a ligand on the stationary surface[2, 12]. Affinity chromatography stationary phases exist for the separation of a wide range of biological molecules using recognition elements immobilized to the surface; such as lectins for the extraction or polysaccharides, antibodies for extraction of the corresponding antigens, and biotin for the extraction of streptavidin[2, 5]. Affinity chromatography provides a high specificity and selectivity relative to the other available chromatographic techniques. This technique is a very powerful isolation and purification technique for proteins and yields highly purified proteins in a single step[2].

Immobilized metal affinity chromatography (IMAC) was first demonstrated for its ability to separate proteins by Porath in the mid 1970's[12]. IMAC is a form of affinity chromatography that utilizes a stationary phase surface capable of chelating a metal ion, while the target analyte interacts with the remaining coordination sites of the metal ion. A general schematic is presented in Fig 1.2.[13,

[14] Traditionally, iminodiacetic acid (IDA) is attached to the surface of the stationary phase as the metal chelation ligand (A in Fig. 1.2) and the selectivity of the separation is influenced by the metal ion affixed (B in Fig. 1.2) to surface of the stationary phase.[2]

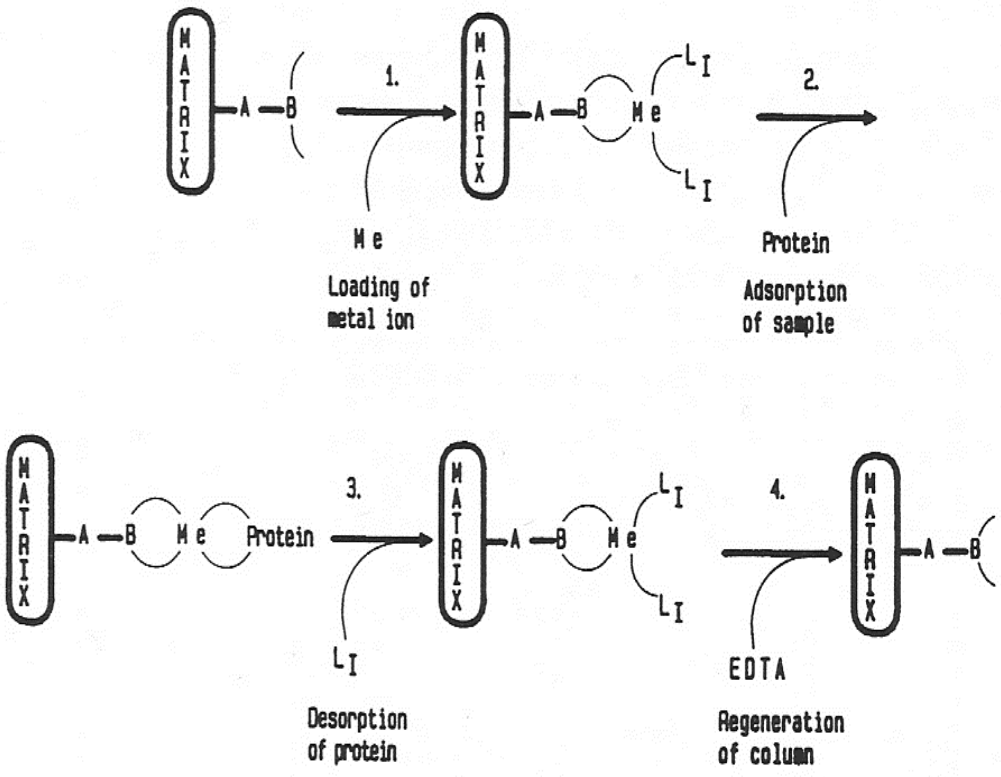

Figure 1.2. Immobilized Metal Affinity Chromatography (IMAC) Mechanism[2]

Emphasis in this research will be placed on the development of an immobilized metal affinity chromatography (IMAC) stationary phase as it is versatile and group specific technique (i.e. application for the extraction of a group or variety of proteins including serum proteins, interferon, and fusion proteins with histidine tails)[2].

Stationary Phases

Traditional stationary phases are designed for their ability to efficiently separate analyte molecules of interest. In order to do this, optimization of the stationary phase parameters such as plate height, functional group densities, packing density, surface area, and porosity (or lack thereof) are required to increase mass transfer kinetics, resolution, and selectivity[1, 6]. Traditionally, stationary phases consisted of silica based supports that were chemically derivatized generating a surface coating that interacted with the analytes in the mobile phase. A wide variety of silica modified stationary phase chemistries exist depending on the desired type of interaction (e.g. hydrophobic, ion-exchange, ect) and have been employed for HPLC separations of macromolecules. However, the silica based sorbents are not considered chemically robust, have a narrow working pH range, and carry over effects associated with undesirable analyte -silanol interactions [4]. The drive to improve upon these silica stationary phases has led researchers to investigate polymeric stationary phases[15].

Implementation of a polymer based stationary phase provides the advantages of low cost, chemical and physical robustness, and the extensive availability of polymeric materials. A wide variety of polymer based stationary phases have been explored for protein separations including polymer beads[16-18], polymer based monoliths[19, 20], and textiles[21, 22]. The focus of this work is based on polymer fiber stationary phases, specifically textile materials due to cost, flexibility in material type (i.e. woven versus aligned fibers), and the variety of

chemical functionality readily available allowing for both direct implementation and a solid foundation for further functionalization techniques[15, 23].

The first reports on the implementation of polymer based fiber stationary phases for liquid chromatography came from Kirby and Cates who utilized fiber segments packed into a gravity column for the separation of dye molecules[24]. Their studies were designed in order to gain a deeper understanding of the role the structural and physical properties of polyethylene terephthalate (PET) contributed to the separation process. Reports indicated that crystallinity of the PET fibers was an important parameter in the adsorption and separation of the dye molecules.

Ladisch and coworkers were the first to report implementation of continuous textile fiber materials as a stationary phase for liquid chromatography[22, 25-28]. Specifically, demonstrating the use of woven fabrics, rolled cylindrically and inserted into column hardware as a continuous fibrous matrix or rolled stationary phase (RSP). These stationary phases were extensively studied for their application in protein separations[22]. Ladisch et al. used a variety of polymer materials to generate these RSPs demonstrating the ability of continuous textile polymers to separate analytes under a variety of conditions, ion-exchange, hydrophobic, size exclusion, all based on the chemical characteristics of the fibers chosen[25-28]. Investigation into the derivatization of these RSPs for protein separations was evaluated through use of diethylaminoethyl (DEAE) modified cotton/polyester blended fabrics generating

an anion-exchange stationary phase[25]. This research set the stage for the implementation of continuous textile polymer materials in HPLC of biomolecules.

Capillary-channeled polymer (C-CP) fibers, the stationary phase employed in this research, have been studied since 2003 by the Marcus laboratory for their implementation as stationary phases primarily for the separation of macromolecules[29]. C-CP fibers are melt extruded polymers and come in a variety of base polymers depending on the chemical properties desired. Nylon-6, poly(ethylene terephthalate*) (*polyester or PET), and polypropylene (PP) are examples of polymers that have been utilized in this laboratory. These fibers have a unique structure to which their name is derived that includes eight channels that run the periphery of the entire fiber length. C-CP fibers have a nominal diameter of 35-50 µm and channel diameters of 5-20 µm, as shown in Figure 1.3a[29].This fiber structure provides several benefits over a traditionally round fiber of the same diameter including an increased surface area and packing efficiency due to interdigitation of the fibers, Figure 1.3b[30].

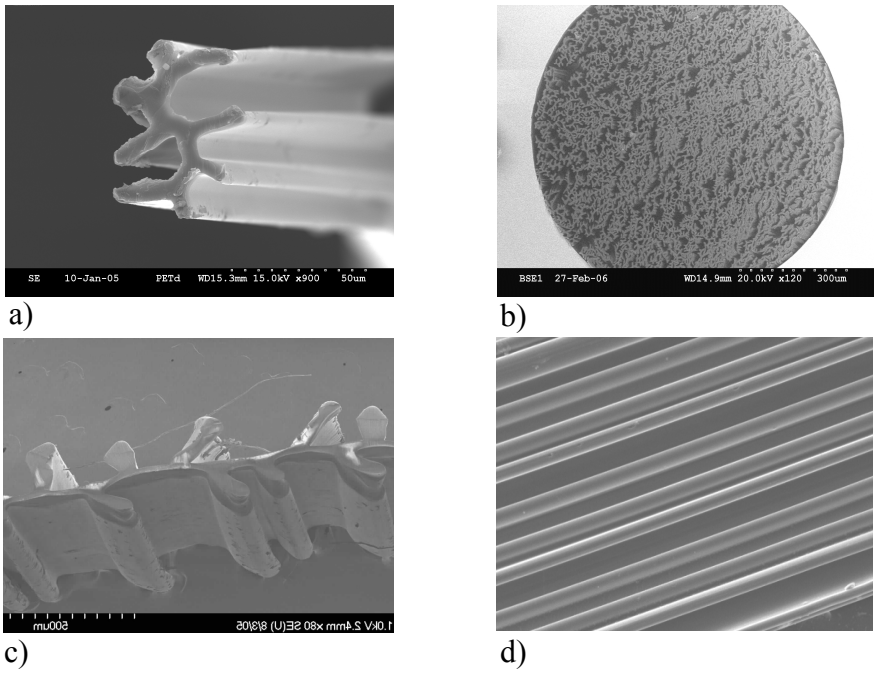

Figure1.3. a) SEM Image of a C-CP Fiber[31] b) End view image of a C-CP Fiber Packed Column c) SEM cross sectional image of a C-CP film[32] d) SEM image demonstrating channel structure of C-CP film[32]

The physical structure of the C-CP fibers is such that a spontaneous wicking of liquids along single fibers or packed columns occurs resulting in an efficient fluid transportation with decreased backpressures allowing for operation of C-CP fiber columns at increased linear velocities (40 mm s^{-1})[23, 33]. C-CP fibers have been employed for the separation of a variety of molecules including polyaromatic hydrocarbons[34], inorganic compounds[30], amino acids[34], polymers[35], and proteins[29, 31, 36-41]. The availability of a wide chemical composition of these polymers has led to the exploitation of a variety of interactions including, hydrophobic, anionic, cationic, and π-π allowing for application of C-CP fiber separations in reversed phase, hydrophobic interaction, ion exchange[40, 42-45]. The

ability to change the base polymer allows for changes in selectivity and increase in application. Additional increases in application come from the versatility of the C-CP format itself. The fiber format affords flexibility in the packing of standard HPLC and microbore columns[38, 43, 46, 47], as well as employment as a fiber tip based SPE phase[42]. Additionally, the channel format of the C-CP polymers has been utilized in a film format (Fig. 1.3c,d) acting as an efficient TLC support for the separation and desalting of proteins[32]. However the ability to take it a step further and increase the type of selectivity even specifically targeting analyte species is the purpose of this research.

Modification of C-CP Fibers

The ability to modify C-CP fibers is the technique utilized in this work to increase capacity and selectivity desired for implementation into high affinity, specifically IMAC, separations. C-CP fibers are readily available to the Marcus lab in PP, PET, and nylon-6 as discussed previously and their varying functional composition affords opportunities to affix capture ligands to the surface, providing a wide range of potential modifications. All three available C-CP fibers were evaluated in this dissertation for their modification and implementation as a stationary phase for highly selective extractions.

PET C-CP fibers are considered versatile in their employment due to the variety of chemical interactions they provide, including hydrophobic, π-π, and ion exchange interactions with the potential end groups. The presence of the ester bond in the backbone structure of PET and the amide bond in nylon-6 allows for

a variety of covalent modifications to be synthesized. PP C-CP fibers have no distinct moieties for which chemical interactions can occur, but their hydrophobic backbone allows for the investigation of adsorption based modifications.

Summary

The importance of protein separations and the complexity associated with the separation and extraction of proteins from biological solutions was discussed in the previous sections. Several methodologies were discussed for the separation of complex mixtures of proteins along with the challenges that researchers face when using these methods. Additionally, the parameters that can be tuned to increase selectivity of extractions was discussed. C-CP fibers were presented for their application as a stationary phase in HPLC for the separations of macromolecules.

The focus of the work presented in this dissertation is based on the chemical modification of C-CP fibers for the selective extraction of analyte molecules (proteins and metals). The primary goal of the research presented here was to generate a high surface density of charged functional groups on the C-CP fiber surface in a manner that the physical characteristics of the fibers were maintained. It was then anticipated that these charged groups would be capable of chelating transition metals allowing for the extraction of hexa-histidine tagged (his-tag) proteins through immobilized metal affinity chromatography (IMAC). Several approaches to modification were evaluated, some successful and some

not for their ability to generate this desired high density metal chelation surface. The following chapters will present each modification method explored.

Chapter 2 looks at employment of polyethylene terephthalate (PET, polyester) capillary-channeled polymer (C-CP) fibers as the base structure for surface modification to generate a sorbent for solid phase extraction (SPE) of aqueous cations. Surface functionalization with polyacrylic acid (PAA) was achieved using a "grafting to" approach, yielding a stable sorbent that has the potential of being employed over a large range of pH values and a variety of sample matrices. PAA was chosen for surface modification because the repeating acrylic acid monomeric unit, which contains a carboxylic acid, has the ability to bind metal ions. In a micropipette-tip SPE format, these PAA-functionalized C-CP fibers showed the ability to efficiently bind several metals (Cu^{2+}, Cu^{+}, Ni^{2+}, Fe^{3+}, UO_2^{2+}). The results of this chapter were published in Analytical Methods (J. J. Pittman, V. Klep, I. Luzinov and R. K. Marcus, *Analytical Methods*, 2010, **2**, 461-469).

Chapter 3 demonstrates the development and evaluation of a covalent solution based chemistry to modify C-CP fiber packed columns generating a high density functional group stationary phase for high performance liquid chromatography (HPLC). Specifically, polyethylene terephthalate (polyester, PET) C-CP fibers were modified with ethylenediamine (EDA) as an HPLC stationary phase having primary amine, carboxylic acid, amide, and alcohol functional groups. These fiber surfaces, coined "super nylon" based on the

similarity with the functional groups present on nylon fiber surfaces, allow for the separation of proteins to occur on a single stationary phase under both anionic and cationic ion exchange conditions. Quantification of the functional group densities on the fiber surfaces was accomplished using textile dyes. These "super" modified stationary phases, were evaluated for their ability to separate ribonuclease A, cytochrome c, and lysozyme under ion exchange conditions.

Chapter 4 describes the employment of Nylon-6 C-CP fibers as a base polymer for modification in order to generate a high capacity and analyte specific stationary phase. The functional composition of the base polymer fiber affords opportunities to affix capture ligands to the surface, providing a wide range of potential separation methodologies. A novel synthetic approach to the covalent coupling of iminodiacetic acid (IDA) ligands to the surface of nylon-6 C-CP fibers is described. The common textile coupling agent, 2,4,6-trichloro-1,3,5-triazine (TCT), is employed as the linker molecule to the fiber surface. A triazine-based reactive dye was first employed to test the availability of the reactive groups on polyester and nylon-6 C-CP materials, with the native nylon-6 providing high amounts of loading. The development of a chelating functionality, specifically a di-substituted iminodiacetic acid based on a 1,3,5-triazine linker molecule is presented. Once generated, the triazine-based chelating ligand couples through the amide functionality on the nylon-6 fiber surface. Results of this work will be submitted to Journal of Chromatography A.

Chapter 5 presents the modification of polypropylene (PP) capillary-channeled polymer (C-CP) fibers with functionalized lipids (PEG-lipids) to generate analyte-specific surfaces for application as a stationary phase in high performance liquid chromatography (HPLC) or solid phase extraction (SPE). PP surfaces were modified using an aqueous phase adsorption of a head group-modified lipid. The aliphatic moiety of the lipid adsorbs strongly to the hydrophobic PP surface, with the active head groups orienting themselves toward the more polar mobile phase, thus allowing for interactions with the desired solutes. Specifically presented here is the proof of concept achieved utilizing the affinity of biotin and streptadivin. Additionally, a PE-DTPA lipid was utilized for modification of PP C-CP fibers, demonstrating their application as a immobilized metal affinity chromatography (IMAC) stationary phase with successful detection through chelation of his-tag labeled green fluorescent protein (his-tag GFP).

Chapter 6 describes the use of polypropylene (PP) capillary-channeled polymer (C-CP) films as a thin layer chromatographic (TLC) support for the separations of proteins. C-CP films have parallel, micrometer-sized channels that induce solution wicking via capillary action. The basic premise by which C-CP films can be used as media to manipulate analyte solutions (e.g. proteins in buffer), for the purpose of desalting or chromatographic separation prior to MALDI-MS analysis is presented here. Cytochrome c and myoglobin prepared in a Tris-HCl buffer, and ribonuclease A, lysozyme, and transferrin prepared in

phosphate buffered saline (PBS), were used as the test solutions to demonstrate the de-salting and separation abilities of PP C-CP films. Chapter 5 results are published in the Journal of American Society of Mass Spectrometry (J. J. Pittman, B. T. Manard, P. J. Kowalski and R. K. Marcus, *J Am Soc Mass Spectrom*, 2012, **23**, 102-107).

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CHAPTER 2

EXTRACTION OF METALS FROM AQUEOUS SYSTEMS EMPLOYING CAPILLARY-CHANNELED POLYMER (C-CP) FIBERS MODIFIED WITH POLY(ACRYLIC ACID) (PAA)

Introduction

Solid phase extraction (SPE) is a proven method for sample pretreatment, the removal of matrix interferences and contaminants, and the pre-concentration of analytes prior to trace analysis.[1, 2] SPE originated in the 1950s with sorbent systems designed from charcoal packed into cartridges for extraction of organic contaminants from aqueous samples.[3] The applications of SPE showed significant growth in the 1970s, in parallel with high performance liquid chromatography (HPLC), as the use of silica-based sorbents evolved.[3, 4] A variety of fields employ SPE, including biological, geological, and food analysis, and is of importance in the analysis of heavy metals in the fields of environmental remediation and trace contaminant detection. The ability to detect metals in aqueous environmental samples is of great concern as it has been shown that, while some metals at appropriate concentrations are beneficial, trace-level exposure to toxic metals (or high concentrations of otherwise beneficial metals) can cause health problems.[4-6] The application of SPE to environmental samples requires the ability to immobilize trace amounts of elements across a variety of sample matrices. The complexity of sample matrices has changed dramatically

over time with increases in industrialization and population, and therefore increases in pollution.[7, 8] The types of aqueous samples that require testing and monitoring are numerous, including natural, potable, and industrial waste waters. Even among these, there are large disparities in the matrix (e.g., salt and fresh waters) that require different processing approaches prior to instrumental analysis.

As the diversity of SPE applications grows, the demand for improved extraction media drives research and development of new approaches.[9] Silica-based resins are the most commonly used and commercially available SPE phases.[9, 10] However, silica-based sorbents exhibit several disadvantages resulting in limitations when employed as a sorbent phase, such as a narrow pH window of 4-7 and residual surface silanol groups leading to undesired interactions. The porous nature of the silica sorbents is a two-edged sword; providing very high specific surface areas (a reflection of equilibrium loading capacities) but poor adsorption/desorption kinetics and recoveries which can lead to carry over and sample contamination[11]. Researchers have extended the boundaries of what types of sorbents are considered typical (i.e. silica) to non-conventional materials as unique as human hair and bone powder.[9] Recent research efforts have focused towards inorganic sol-gels,[12-14] polyurethane foams,[15] polymer sorbents[4, 6, 16] and functionalized silica resins[17, 18] to provide high binding capacity and/or metal-specific sorbents. Relevant to the work described here, both polymer and silica-based fibers have also been used as

SPE media for a variety of analyte species.[19-23] Key driving forces here are the inherent low cost of fiber production, the ease of device packing, and the diversity of potential surface chemistries that can be exploited for effective adsorption.

The development of sorbents that possess fast kinetics, large binding capacities, high recovery, are reusable, can be adapted to any type format (micropipette tip, syringe, column), and uses dilute and/or small amounts of elution solvents is a continuing theme. [6, 9] We describe here the initial evaluation of polyacrylic acid (PAA) derivatized capillary-channeled polymer (C-CP) fiber solid phase extraction (SPE) tips for metal ion binding. The C-CP fiber format is under investigation in this laboratory for use as stationary phases in HPLC and SPE, primarily for proteomics applications.[24-28] The fibers are melt-extruded from common textile polymers including polypropylene, polyester, and nylon-6 in such a manner that they have eight channels running along the entire fiber length.[29] As suggested in the SEM micrograph in Fig. 2.1, the structure of the fibers results in interdigitation of the “fingers” within the column format, resulting in efficient use of space and a high packing density, while maintaining very high solvent transport efficiencies. In fact, single fibers (as well as fiber bundles) have the inherent ability to wick solutions due to the capillary action within the channels. Important for SPE applications, they exhibit ~3 times the surface area of a circular fiber of the same nominal diameter,[26] though the specific surface (2 – 5 m^2 g^{-1}) is low by typical SPE standards, but on par with monolithic materials

used in chromatography and SPE.[1] On the other hand, the non-porous C-CP fibers have improved mass transfer kinetics when compared to porous materials, thus there is more accessible surface area in a dynamic situation. It is also worthy to note that C-CP fibers have demonstrated increased recovery/decreased carryover for proteins in both HPLC and SPE applications.[24, 29] The chemical nature of the base polymers allows operation across wide pH ranges (1-14),[26, 27] making them applicable as an extraction media for a wide range of sample matrices. Finally, as evidenced by the extremely wide portfolio of textile dye chemistries, polymer fibers present a plethora of potential means of surface modifications to affect high selectivity in SPE applications.

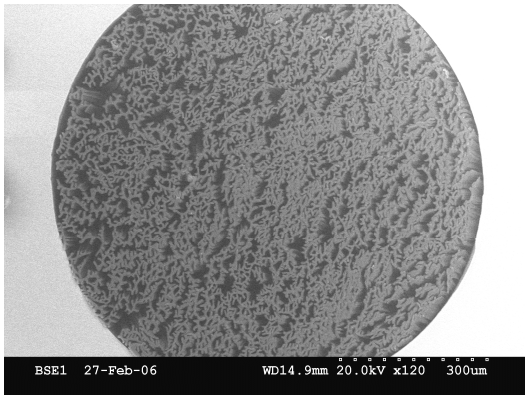

Figure 2.1 SEM micrograph of the cross section of a C-CP fiber packed SPE tip

The ability to control surface interactions is of great importance and can be exploited in C-CP fibers by choosing the base polymer (polypropylene, polyethylene terepthalate (PET, polyester), nylon-6) to affect π-π, hydrophobic, and electrostatic interactions to varying degrees, allowing for their use in different modes of HPLC and SPE separations. For example, the application of nylon-6 as a base polymer allows for charge-based separations of proteins as the end

groups provide a zwitterionic surface.[30] As one means to enhance the charge density of the C-CP fiber surface, functionalization using a “grafting-to” approach that deposits a polyacrylic acid (PAA) layer on the surface of PET has been employed.[31] In this approach, polymer chains with reactive functionalities react with complementary functional groups located on the surface of the substrate to form a layer of covalently anchored macromolecules. PAA was chosen for surface modification due to the presence of acrylic acid monomeric units in the macromolecule which results in a high density of accessible carboxylate groups (pK_a = 4.3-4.5[32-34]) on the surface after the grafting. Previous reports in the literature have demonstrated the use of PAA for metal chelation.[35-40] The PAA modification of C-CP fibers provides a high density of cationic binding sites that can be tailored based on the molecular weight of the grafted polymer and the time over which grafting is allowed to occur. In addition to their high density of charged groups, PAA-coated C-CP fibers are well suited for trace metal extraction as a result of their stability/robustness over a wide range of pH values, nonporous structure, and low leachable impurities.

Presented here is the initial application of PAA-modified C-CP fibers as a sorbent material for metal ion extraction from aqueous media. Specifically, the PAA-modified fibers are employed in solid phase extraction micropipette tips. Detailed experiments involve determination of metal binding capacity and the ability of the PAA-Functionalized C-CP fiber tips to bind a variety of metals both in single and multi-element solutions. In addition, experiments were designed to

study the result of varying parameters such as the rate of loading (sample flow rate), exposure time, and the strength of the eluent on the adsorption/desorption and recovery of copper (Cu^{2+}) from aqueous solutions. We suggest that PAA-functionalized C-CP fibers are a versatile, inexpensive, reusable sorbent for SPE that allows for recoveries greater than 88% for metal ions in aqueous media.

Experimental

Chemicals and Reagents

Stock metal ion solutions, 3 µg mL^{-1} unless otherwise specified, were prepared from water soluble salts (chloride or nitrate) obtained from Acros (Fair Lawn, NJ) using milliQ water (18.2 MΩ/cm) derived from a NANOpure Diamond Barnstead/Thermolyne Water System (Dubuque, IA). Uranium solutions, 3 µg mL^{-1}, were prepared from uranium oxide dissolved in trace metal grade nitric acid (VWR (West Chester, PA)) and diluted to the appropriate volume with milliQ water. Poly(glycidyl methacrylate), PGMA (synthesized as described elsewhere[41]) and polyacrylic acid from Sigma Aldrich (St. Louis, MO, USA) were used for functionalization of the sorbent phase. Standards used for calibration and hydrochloric acid used for desorption of metals from the fiber tips were obtained from VWR (West Chester, PA).

Instrumentation

Metal ion concentration determinations were performed using a Jobin-Yvon Horiba Ultima 2 (Longjumeau, France) inductively coupled plasma-optical emission spectrometer (ICP-OES) (Edison, NJ) and Analyst 5.2 software. The

manufacturer default settings were used for ICP control and are presented in Table 2.1. The ICP is equipped with a single view radial plasma, a Meinhard concentric glass nebulizer, a cyclonic spray chamber, and a 1.0 m Czerny-Turner monochromator containing 2400 grooves mm^{-1} holographic grating. Emission acquisition occurred with an integration time of 0.5 sec, five wavelength points and five replicates per sample. Typical analytical response characteristics for the analytes determined in this study are presented in Table 2.2.

Table 2.1 ICP-OES operation conditions

Parameters	Condition
Power (W)	1000
Ar gas flow rate (L/min)	12.0
Nebulizer (L/min)	0.02 at 1.0 bar
Sheath gas flow rate (L/min)	0.20
Peristaltic pump speed (rpm)	20.0
Replicates	5

Table 2.2 Analytical response characteristics for target elements by ICP-OES

Element	Wavelength (nm)	Response Function	R^2	Detection Limits (ng/mL)
Cu	223.008	y= 28161x + 2475	0.9998	11
Ni	221.647	y= 27689x + 724.3	0.9999	3.6
Fe	259.940	y= 265924x + 5354	0.9999	0.30
U	385.958	y= 23996x – 2939	0.9968	18

Functionalization Process

C-CP fibers are obtained from Clemson University School of Materials Science and Engineering in spool lengths greater than a 1000 m[42]. Fibers are manually wound onto a rotary counter with a circular frame to produce a fiber

bundle[28] which is taken through the functionalization process involving PGMA anchoring layer.[31] Surface functionalization can be performed on C-CP fibers, regardless of the base polymer, using a "grafting-to" approach.[31] In the present experiments, polyethylene terephthalate (PET, polyester) is used as the base polymer as it is readily extruded into the C-CP format, and present multiple opportunities for surface functionalization. Atmospheric pressure plasma treatment, using a Harrick Scientific Corporation (Model PDC-32G) apparatus, of the PET C-CP fibers, at a power of 7 watts for 10 min creates reactive carboxyl and hydroxyl functional groups on the surface. An anchoring layer of PGMA was then placed on the activated surface of the fibers using a dip coating method.[31] The fibers were dried in air followed by annealing at 60℃ for 90 minutes to promote chemical tethering of the PGMA to the activated fiber surface.[31] The excess PGMA was then removed by rinsing with acetone. The PAA was deposited by wetting the fibers with an ethanolic solution of PAA of the desired molecular weight. In these experiments, two different molecular weights of PAA were investigated; 5,000 Da and 100,000 Da. PAA was reacted with PGMA-coated C-CP fibers at various conditions; specifically three combinations were explored in detail. A grafting time and temperature combination of 80ºC for 120 min provides a high density of functional groups while a combination such as 60ºC for 10 min provides a lower density of functional groups. After the desired grafting time was reached, the excess PAA was removed by rinsing with water. The combination of grafting density and molecular weights of PAA employed,

yielded three combinations of functionalized C-CP fibers; high molecular weight-high grafting density (HMWHD), high molecular weight-low grafting density (HMWLD) and low molecular weight-high grafting density (LMWHD). The amount of the grafted to PET surface polymer was determined using ellipsometry for model PET films (deposited on silicon wafer) as described elsewhere.[31] The ultimate metal binding capacity of the fibers is determined by the number of available binding sites, which is controlled by the functionalization conditions. As the molecular weight of the PAA increases, so to does the thickness of the layer and, therefore, more functional carboxylic groups are placed on the surface. In addition, as the grafting time is increased the grafting density of the polymer increases as well, increasing the amount of polymer placed on the fibers. Figure 2.2 pictorially depicts the HMWHD, HMWLD, and LMWHD functionalized fibers and how metal binding may be affected by the structure of the PAA layer.

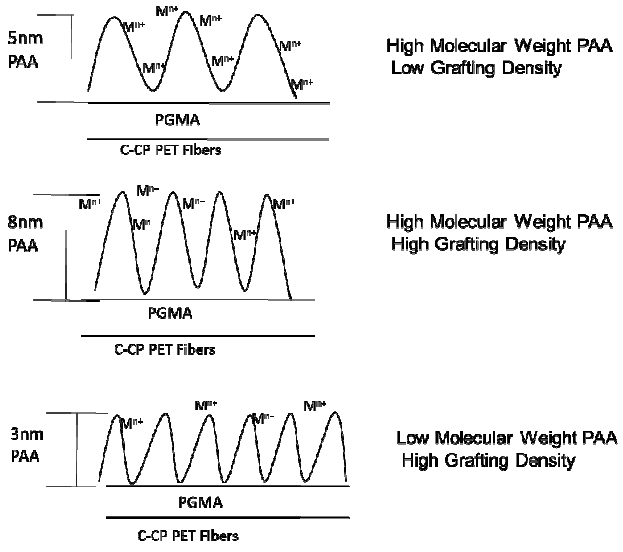

Figure 2.2 Diagrammatic representation of the assumed binding site distributions for the three PAA coating conditions

Tip Preparation for Solid Phase Extraction

Once the functionalization is complete, a monofilament is looped through the fiber bundle and then pulled through an approximately 300 mm segment of a 0.8 mm i.d. fluorinated ethylene polypropylene (FEP) capillary tubing (Cole Palmer (Vernon Hills, IL)) as described previously.[28] This packing technique and the shape of the fibers result in collinear alignment of the fibers inside of the FEP tubing, as shown in Fig. 2.1. Once packed inside the tubing, a 6 mm gap is formed in the end of the tubing by pulling the fibers farther through the tube. The capillary is then cut to a 1 cm length of packed fibers (~3.5 mg of fibers) and the 6mm gap left in the FEP tubing is press-fit to the end of the micropipette tip (Fisher Scientific, Pittsburgh, PA), Fig. 2.3. The compression of the FEP tubing around the end of the micropipette tip is sufficient to hold the tip in place during the sample adsorption and desorption steps.

Figure 2.3. Photograph of C-CP fiber packed SPE tip attached to micropipette tip

Solid Phase Extraction Procedure

To avoid the errors resulting from irreproducible exposure of analyte to the stationary phase during a manual aspiration procedure,[28] a centrifugation process was employed to pass the sample and eluate solutions through the tips in a controllable fashion. Very simply, the prepared tips were set in 15 ml centrifuge tubes (VWR, West Chester, PA) for processing. This approach also allows for multiple sample extractions simultaneously, limited only by the capacity of the centrifuge. However, for centrifugation to be successfully employed, the relative centrifugal force (RCF) needs to be evaluated. RCF is a measurement of the acceleration, in *g* (acceleration due to gravity), applied to a sample during centrifugation. The RCF controls the fluid flow rate (i.e., resident time) of the various solutions through the tip. Slow centrifugation rates permit greater transit times for solute interaction with the fiber surface, but there is some minimum level required for solution to pass through the tip based on the fiber packing density. If the applied RCF is too large, the sample will pass quickly through the tip and the system would not be able to reach equilibrium, limiting the binding of the analyte and the efficiency of the elution.

For the sake of consistency, all experimental data presented here was acquired for centrifugation performed using the same protocol. All of the test, rinse, and elution solutions were loaded in 1ml aliquots because the fiber packed tip is attached to a 1 ml commercial pipette tip, yielding collected volumes sufficient to be analyzed on the ICP-OES. More specifically, 1 ml of sample

solution was loaded into the tips and centrifuged for 5 min; this process was repeated 3 times for a total volume (V_t) of 3 ml. The tips were then rinsed and centrifuged with three, 1 ml aliquots of milliQ water, to remove non-specifically bound analyte. Finally, three 1 mL aliquots of the hydrochloric acid elution solutions were passed through the SPE tips for desorption of the immobilized metals from the surface, V_t = 3 ml. The entire analytical procedure described here, from loading of the sample to elution for ICP-OES analysis, takes 45 min to complete, though performed in parallel batches. RCFs ranging from 575-1900 were examined using a Clinical 50 Centrifuge (VWR, West Chester, PA), it was determined that the adsorption/desorption efficiencies remained relatively constant over this range as the percentage recoveries did not vary significantly. A nominal centrifugation rate of 1200 RCF, was quite sufficient to obtain quantitative recoveries, with the solution transient times under these conditions of ~1 min, and so the total process could be completed in less than 10 min on sample batches.

A key variable when evaluating the PAA-functionalized C-CP fibers as an SPE sorbent is to determine the lifetime of the tips. Due to the efficiency of the acid elution process, there is no requirement for regeneration of the PAA-fiber surfaces prior to reuse of the tips, therefore the SPE tips are ready for loading of the next sample immediately following the acid elution step. Even so, the robustness of the polymer coating itself must be evaluated. A single control SPE tip was generated from a HMWHD functionalization of PAA, which was run under

the exact same conditions, 3 ml of 3 μg ml^{-1} Cu^{2+} loaded and elution with 5% HCl, in parallel with each experimental variation performed on the remaining experimental tips. The control tip's recovery was evaluated after each experiment to ensure that the data collected during each experimental set was valid, while also determining the stability of the PAA-functionalized C-CP fiber tips.

Results and Discussion

Qualitative Aspects

The PAA-functionalized surface provides anionic binding sites that should be capable of binding cationic metal species. Literature reports confirm that copper binds strongly to both linear and cross-linked polyacrylic acid polymer chains.[36, 38, 43] Qualitative experiments were performed by soaking functionalized fibers in concentrated aqueous solutions of copper. When exposed to an aqueous Cu^{2+} solution, the fiber surfaces visibly showed that copper was bound to the surface. Figure 2.4 is an optical photograph of PAA- functionalized C-CP fiber packed SPE tips that have bound Cu^{2+}, which is visible by the blue-green surface coloration. To investigate whether the copper was bound electrostatically to the fiber surface, the fibers were subsequently rinsed with DI-H_2O with no visual evidence in the wash solution that copper was released, nor was there a decrease in the intensity of the fiber color; suggesting that the Cu^{2+} was indeed bound to the surface functional groups. The fibers were subsequently treated with hydrochloric acid, resulting in removal of copper from

the PAA- functionalized C-CP fiber surface apparent by the resulting blue green solution and white fiber surface.

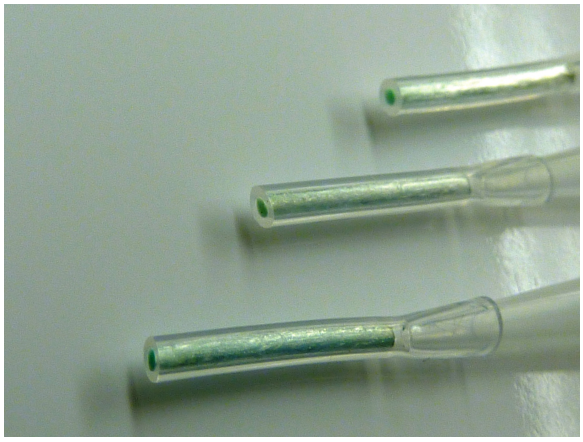

Figure 2.4. Photograph of PAA-functionalized C-CP fiber tips with Cu^{2+} bound to the surface

Each of the three variations of PAA functionalization (i.e. HMWHD, HMWLD, LMWHD) was evaluated to determine extent of the polymer surface coverage. Ellipsometry is a common approach used to characterize polymer layer thicknesses of chemically similar, dielectric materials.[44] Because of the roughness of the C-CP fibers due to the presence of the channel structure, thickness measurements were performed instead on silica wafers covered with PET film and functionalized using the same “grafting-to” approach used on the C-CP fibers.[31] Based on the determined layer thickness and the theoretical number of chains per unit length, the number of expected carboxylate groups per unit surface area can be calculated. From that number and the computed total fiber surface area of fiber in the tips, the binding capacity of Cu^{2+} can be projected based on an assumed 1:2 Cu^{2+}:carboxylate binding stoichiometry. A comparison of the anticipated binding capacity (mass) of Cu^{2+} based on calculations from the

ellipsometry measurements and those determined experimentally through Cu^{2+} binding to three corresponding PAA-C-CP fiber tips (as determined by ICP-OES detection) is presented in Table 2.3.

Table 2.3. Evaluation of binding sites on PAA C-CP SPE tips reported as amount of bound Cu^{2+}

	HMWHD		**HMWLD**		**LMWHD**	
Method	Thickness (nm)	Mass (µg)	Thickness (nm)	Mass (µg)	Thickness (nm)	Mass (µg)
Ellipsometery	8	7.7[1]	5	4.7[1]	3	3.0[1]
SPE	---	6.9[2]	---	5.5[2]	---	3.8[2]

[1] Values computed based on thickness of functional layer
[2] Values obtained by SPE using ICP-OES detection

As can be seen, the values are in quite good agreement. Discrepancies between theory and SPE binding measurements can be explained by taking into consideration that SPE looks at carboxyl groups actually accessible to Cu^{2+} for binding and the actual coordination of Cu^{2+} to PAA on the fiber surface. The ellipsometry-based calculations assume that *all* carboxylate groups are available for binding, that the calculated number is the same as the actual number of groups present on the surface, and that a 1:2 Cu^{2+}:carboxylate coordination occurs. However, significant fraction of acrylic acid groups (responsible for PAA anchoring to the surface via reaction with epoxy groups in the PGMA chain) is not available for the attachment of Cu^{2+}. It is also known that Cu^{2+} coordinates with carboxylate groups; as bi-dentate or higher-order complexes.[37] However, the extent to which coordination occurs at a surface depends on conditions, such as the distance between surface ligands and the pH of the solution.[37, 38] The coordination of Cu^{2+} to these specific PAA- functionalized C-CP fiber surfaces

has not been studied, but PAA complexation of Cu^{2+} in solution has been studied and was determined that at a minimum of two carboxylates are involved per metal ion (i.e. bi-dentate).[37] Since the coordination assumptions made to perform the ellipsometry calculations (i.e., 1:2 Cu^{2+}:carboxylate) and the number of available carboxylates may not be completely valid, it makes sense that the computed loading masses are biased-high in comparison to the actual binding seen in the SPE numbers.

Quantification

Experiments were designed to focus on optimizing the binding/elution conditions, determination of binding capacity, metal-specific binding vs. competitive binding, and process recovery. The metal ion solutions were loaded, tips rinsed with DI-H_2O, and analyte eluted with hydrochloric acid in three 1 mL fractions as previously described. The recovered loading solution (i.e., the test solution passed through the tip) was analyzed to determine the total amount of metal ions bound to the surface by calculating the difference between the stock solution and the flow through from the loading solution. The rinse solution was analyzed for non-specifically bound metal ions and the acid eluate solution analyzed for specifically bound metal ions.

Control Tip

One of the most outstanding attributes of the PAA-functionalized C-CP fiber SPE tips is that the cost associated with the fiber in a single tip is only ~$0.03 US. In addition, the capillary packing and micropipette tip fitting is quite

simple in comparison to that of bead-form sorbents. Even so, it would be beneficial to be able to use single tips through multiple metal ion extractions and to know the extent to which there is carryover or polymer degradation following acid elution. In order to test these potentialities, and to ensure that the results obtained throughout the course of the experiments were valid and not related to exhaustion of the tip lifetime, a single control tip was analyzed repeatedly under identical conditions throughout the course of this study. The control tip was made from HMWHD PAA-functionalized C-CP fibers and was analyzed each time an experimental variable was altered and studied (i.e., a new experimental set, as denoted by the name). A 3.0 µg mL^{-1} Cu^{2+} solution, applied in three 1 mL aliquots (a total of 9 µg of Cu^{2+}) was used as the analyte in the control tip. (The sole exceptions to this method were in the case of the competitive binding experiments where 0.9 µg of Cu^{2+} was applied.) Based on preliminary studies, this amount of Cu was enough to overload the PAA SPE micropipette tips. Elution was accomplished using three 1 ml aliquots of a 5% HCl elution solvent, instead of the lower HCl concentrations used for elution in all other experiments. The use of 5% HCl represents an extreme case in terms of testing the chemical robustness of the adsorbed PAA layer as well as the physical structure of the fiber-packed tip. Observation of decreased recoveries here would have allowed the tips to be replaced before decreased efficiency actually affected those other experiments. Table 2.4 summarizes the experimental sets for which the control tip was run in conjunction, the amount of metal bound to the fibers, the efficiency

of that process (% Cu^{2+} adsorbed), and the efficiency of the desorption of the captured metal (% Cu^{2+}desorbed). As can be seen the % Cu^{2+}adsorbed in each experiment is very consistent, averaging 87.3 % with a variation of 6.4 %RSD across the 19 exposures. This number is less than 100 % because the tips were intentionally, slightly overloaded. The control SPE tip averaged a 97 % overall recovery of the adsorbed Cu^{2+} over 19 applications and showed no signs of either chemical or physical degradation over a 12 month period. These results clearly illustrate the stability of the PAA-functionalized C-CP fiber SPE phases, even under the fairly challenging 5 % HCl elution conditions and indeed drying in between experiments. As such, there is a high degree of confidence of the relative validity of the results obtained across the range of the various test conditions.

Table 2.4. Control HMWHD on PAA-Functionalized C-CP SPE tips as determined by ICP-OES detection

Experimental Set[1]	Mass Adsorbed (µg)	% Cu^{2+} Adsorbed	% Cu^{2+} Desorbed
5.0% HCl	6.9	83	100
0.5% HCl	8.3	85	94
0.1% HCl	7.1	85	100
0.05% HCl	8.1	80	93
0.1% HCl	7.9	80	97
0.005% HCl	8.0	85	95
0.001% HCl	7.7	85	96
Ni^{2+} Run 1	8.7	89	100
Ni^{2+} Run 2	8.5	90	97
Cu^{+} Run 1	7.4	90	98
Cu^{+} Run 2	7.3	90	100
UO_2^{2+} Run 1	7.1	81	94
UO_2^{2+} Run 2	8.9	89	100
Fe^{3+} Run 1	7.8	100	100
% Recovery Run 1	7.8	88	97
% Recovery Run 2	8.1	80	88
Competitive Binding Run 1	0.84	93	93
Competitive Binding Run 2	0.90	97	97
% Recovery Run 3	7.8	88	98

[1]Experimental parameter that was varied to correspond to control tip data

Cu^{2+} Elution Characteristics

Elution of metal from the PAA surface takes place by employing hydrochloric acid, so that the pH decreases below the pKa of the PAA resulting in protonation of the carboxylate functional groups on the fiber surface, displacing the metal ions into solution. In order to make an SPE application more environmentally friendly and consumer affordable, low concentrations/low volumes of elution solvent are preferable. Three 1 ml aliquots of the 3 µg ml^{-1} solution of $CuCl_2$ were run through the tips, exposing a total of 9 µg Cu^{2+} to the fiber surface, which had been previously determined to be in excess of the binding capacity of the amount of fiber in the present tips. The tips were exposed to the excess to ensure that the full binding capacity of the tips was achieved. The respective solutions were passed through the tips using centrifugal force of 1215 RCFs. The eluting acid concentrations were varied between 0.001% and 5% (in 3, 1 ml aliquots) in order to evaluate the minimum solvent strength necessary for elution of Cu^{2+} ions from the three densities of PAA-functionalized C-CP SPE tips. The experimental results presented in Table 2.5 show that there is no difference within experimental error for elutions performed in the range 0.005%-to-5% HCl for the HMWHD and HMWLD polymers. In the case of LMWHD coating, the amount of copper desorbed from the surface varies with the elution strength, but follows no discernible trends. Statistical analysis (student t-test), at a 95% confidence interval, of the LMWHD recoveries showed no significant difference among the solvent elution strengths (i.e. HCl

concentrations). It is also clear that the HMWHD PAA fibers, covered with the thickest PAA layer, showed the greatest amount of variability within experimental sets. We suppose that the observed phenomenon can be connected to limited solubility (swellability) of the grafted PAA chains in the water with acidic pH.

Table 2.5. Desorption of Cu2+ from the surface of PAA-Functionalized C-CP SPE tips as determined by ICP-OES detection

% HCl	HMWHD (µg)	HMWLD (µg)	LMWHD (µg)
5	6.9 ± 0.9	5.5 ± 0.2	3.8 ± 0.2
0.5	8.1 ± 2.5	5.2 ± 0.3	5.2 ± 0.3
0.1	6.9 ± 1.7	5.3 ± 0.2	5.3 ± 0.2
0.05	6.2 ± 1.3	3.9 ± 1.2	3.9 ± 0.2
0.005	5.8 ± 2.3	5.4 ± 0.3	5.4 ± 0.3
0.001	3.9 ± 1.1	3.8 ± 0.4	3.8 ± 0.4

Therefore, for the relatively thick grafted layer diffusion limited, non-equilibrium extraction may occur. This phenomenon will require further investigation. When elution is attempted with 0.001% HCl, approximately 60% recovery occurs in HMWHD and HMWLD, with a second elution of 5% HCl allowing complete recovery. Statistically there is no dissimilarity in the recovery with varying HCl strength until the 0.001% HCl was used with the exception of a single outlier in both the HMWHD and HMWLD cases (at 0.5% and 0.05%, respectively). The 5% upper limit of exposure was chosen because the stability of PAA on the C-CP fibers had not been evaluated above this point. In addition, the amount of copper exposed to the surface can be accounted for with recoveries > 90% when 5% HCl was used for elution. Therefore, it was unnecessary to explore higher concentrations of HCl as an increase in cost and potential degradation of the

functionalized surface layer are all negatives that will not be offset by a substantial increase in recovery. However, this study only looked at the strength of elution solvent necessary to elute Cu^{2+} from the surface; other metals may need higher concentrations, stronger acids, or a chelator to affect efficient removal.

Metal Species' Binding Characteristics

In order to begin to evaluate the performance of the PAA-functionalized C-CP fiber tips for metal ion extractions from complex matrices, several factors need to be explored including the ability to bind a variety of metal ions, the extent of competitive binding, and the total recovery of metals. Several transition metals were evaluated, in individual solutions, for their binding capacities to the PAA-functionalized fiber tips; Cu^{2+}, Cu^{+}, Ni^{2+}, Fe^{3+}, and the lanthanide oxide complex, UO_2^{2+}. Table 2.6 lists the metal loading/recovery for the three PAA surface layers for the metals initially applied as three 1 ml aliquots of 3 µg mL^{-1} concentration solutions, resulting in a total of 9 micrograms exposed. There is an initial trend that is obvious regardless of the metal bound to the surface; the HMW PAA tips have the highest binding capacities. In fact the higher molecular weight grafted layers are thicker, therefore more functional groups are available for binding. The relative binding capacities for the individual metal ions varied, though the relative amounts for each metals were consistent between the PAA C-CP fiber surface types. The concept of hard soft acid base theory (HSAB) can help explain some of the binding trends among the metal ions. Carboxylate ions (the

ligand species on the PAA surface) are considered hard bases. On the other hand, PAA is a weak polyelectrolyte, that as the molecular weight of the polymer increases becomes a weaker acid, i.e. borderline base.[37, 40]

Table 2.6. Metal Concentration on PAA-Functionalized C-CP SPE tips as determined by ICP-OES detection

	HMWHD (µg)	**HMWLD (µg)**	**LMWHD (µg)**
Cu^{2+}	6.9 ± 0.9	5.5 ± 0.2	3.8 ± 0.2
Cu^{1+}	5.5 ± 1.1	4.8 ± 0.8	4.2 ± 0.2
Ni^{2+}	2.1 ± 0.6	1.7 ± 0.1	0.76 ± 0.02
Fe^{3+}	2.9 ± 1.1	2.4 ± 0.3	2.5 ± 0.2
UO_2^{2+}	2.8 ± 0.8	1.9 ± 0.4	1.2 ± 0.4

The metals investigated are a combination of soft (Cu^{1+}), borderline (Ni^{2+}, Cu^{2+}), and hard (Fe^{3+},UO_2^{2+}) character. The best binding capacity comes with Cu^{2+}, a borderline metal, in all three degrees of functionalization. The higher level of complexation of Cu^{2+} with the $–COO^-$ surface ligands of PAA is also in line with the Irving Williams series relationship.[45] Overall, the differences observed in the binding capacity of the remaining metals are believed to be related to some combination of the extent of metal complexation (i.e. mono-, bi-dentate, etc. with PAA ligands), coordination geometry, and hydration; all factors that need to be explored in further detail. The differences observed here are most surely in the adsorption step as opposed differences in the elution characteristics as the elution recoveries and mass balance values are quite uniform.

Table 2.7 shows a comparison of the PAA functionalized C-CP fiber tips to other silica and polymer based metal sorbents. The capacities reported in Table 2.7 for all sorbent phases are based on Cu^{2+} binding. The binding capacity of the

HMWHD PAA fibers is comparable to the other fiber sorbent phase, polyacrylonitrile based, presented. Most of the sorbent materials presented in Table 2.7 are small and porous particles whereas the PAA sorbent exhibits lower binding capacity due to the nonporous nature of the fibers (resulting in much lower (single $m^2\ g^{-1}$) specific surface area). Comparisons of total binding sites on each surface (which are probably the greatest determining factor) are difficult to make based on the available literature. On the other hand, the non-porous nature of the fibers results in increased mass transfer efficiencies during the adsorption desorption steps as well as puts much lower backpressure constraints in terms of fluidics than in the case of pack-bed media. These latter two characteristics are of high relevance in high throughput analyses.

	Capacity ($\mu mol\ g^{-1}$)	Sorbent Base Material	Particle Size (µm)	Sorbent Format	Analyte Volume (mL)	Adsorption Time (min)	Total Extraction Time (min)
3-methyl-1-phenyl-4-stearoyl-5-pyrazolone	43	Silica Gel	149-420 (70Å)	Column	1000	200	208
Salicylaldoxime	80	Silica Gel	60 (120Å)	Column	25	3	28
Resacetophenone	186	Silica Gel	125-250	Column	50	15-50	65-225
LMWHD PAA	*17*	*PET Polymer Fibers*	--	*Micropipette Tip*	*3*	*3-15*	*9-45*
HMWLD PAA	*25*	*PET Polymer Fibers*	--	*Micropipette Tip*	*3*	*3-15*	*9-45*
HMWHD PAA	*31*	*PET Polymer Fibers*	--	*Micropipette Tip*	*3*	*3-15*	*9-45*
Poly(acrlyamino phosphonic-dithiocarbamate)	32	Polyacrylonitrile Fiber	---	Column	1000	200	210
o-Aminophenol	53	Amberlite XAD-2 (polystyrene copolymer)	250-841 (90Å)	Column	1000	50	510
Chromotropic Acid	134	Amberlite XAD-2 (polystyrene copolymer)	250-841 (90Å)	Column	100	33-50	70-105
Iminodiacetic Acid 3M Empore	1900	Poly (styrenedivinylbenzene)	8	Disk	100	1	24

Table 2.7. Comparison of PAA-Functionalized C-CP fibers with other sorbent phases in terms of Cu^{2+} binding

Metal Species Selectivity

The single solution binding experiments showed the tips had an appreciably higher capacity for Cu^{2+} ions. In order to explore the potential selectivity/bias of the PAA-functionalized C-CP fiber tips, experiments were performed using Cu^{2+} and Ni^{2+} solutions. The loading solution was prepared to contain equal amounts of copper and nickel, 0.9 µg each. This total mass is appreciably below the maximum capacity of the tips to prevent overloading. Table 2.8 shows both the flow through (unbound metal) and the recovered amounts for Cu^{2+} and Ni^{2+} for the mixed-metal solutions. It is apparent that when the SPE tips are presented with a dual element solution the tips exhibit a preference for Cu^{2+}, as all 0.9µg applied was bound/eluted from each of the fiber tip surfaces. On the other hand, there is appreciable flow-through of the Ni^{2+} species, for each of the tips. That said, those amounts of Ni^{2+} adsorbed are recovered in the elution step. Clearly, one must consider both kinetic and thermodynamic effects here, and so static adsorption studies are in order to better understand adsorption biases.

Table 2.8. Metal Concentration on PAA-Functionalized C-CP SPE tips as determined by ICP-OES detection

	Flow Through		**Desorption**	
	Cu^{2+}	**Ni^{2+}**	**Cu^{2+}**	**Ni^{2+}**
HMWHD (µg)	0.00 ± 0.00	0.37 ± 0.14	0.88 ± 0.04	0.43 ± 0.14
HMWLD (µg)	0.02 ± 0.04	0.57 ± 0.06	0.89 ± 0.09	0.24 ± 0.06
LMWHD (µg)	0.01 ± 0.01	0.71 ± 0.03	0.96 ± 0.11	0.13 ± 0.02

Recovery

In order for the PAA-functionalized C-CP fibers to be useful in applications such as trace contaminant detection, it is important that they can process large volumes of low concentration metals, concentrating them through elution into smaller volumes. In order to evaluate this ability, 10 ml of a dilute solution of Cu^{2+} was run through the tips, totaling 2.2 µg and 4.4 µg for Trial 1 and 2 respectively. The tips were then washed with water, followed by elution with a total of 3 ml of 1% HCl, with analysis performed on the eluate by ICP-OES. This experiment was repeated twice (trial 1 and 2) and results are presented in Table 2.9. This data shows that with the currently utilized conditions all tips are capable of producing recoveries of at least 88%.

Table 2.9. % Recovery of Cu^{2+} on PAA-Functionalized C-CP SPE tips as determined by ICP-OES detection

	Flow Through (µg)	**Amount Desorbed (µg)**	**% Recovery**
	Trial 1 (2.2 µg loaded)		
HMWHD	0.00 ± 0.00	2.8 ± 0.0	104
HMWLD	0.00 ± 0.00	1.8 ± 0.2	92
LWMHD	0.00 ± 0.00	2.0 ± 0.2	100
	Trial 2 (4.5 µg loaded)		
HMWHD	0.05 ± 0.05	4.6 ± 0.4	102
HMWLD	0.21 ± 0.11	3.8 ± 0.2	88
LMWHD	0.26 ± 0.06	3.8 ± 0.3	90

Conclusions

The functionalization of polyester C-CP fibers with polyacrylic acid (PAA) results in a high density, anionic surface capable of metal binding. It is evident from the results presented here that binding and elution of several metals from the SPE tips surface can occur with low concentrations of HCl, resulting in an environmentally safe and cheap method for metal detection. The non-porous structure of the C-CP fibers shows its advantages in the fast binding kinetics and high recoveries of analyte from the tips. The C-CP fibers were functionalized with a high molecular weight (HMW) and a low molecular weight (LMW) PAA, after evaluation of the tips for copper binding it is apparent that the HMW coated tips have a higher density of functional groups. When exposed to dual-element solutions the PAA functionalized C-CP fiber tips exhibit a binding preference for Cu^{2+}. PAA functionalized C-CP fiber tips are low cost (<$0.03/tip) and reusable as was demonstrated in the ability to use the fiber tips for 19 experiments over a 1 year period while still maintaining 96% recovery on the control tip. Future experiments will look at the role of adsorption conditions, including static studies, to better understand the binding of individual metals and any preferential binding that occurs. Expansion of the desorption studies to include other eluents (i.e. complexation agents, EDTA) will also be undertaken. On a more fundamental level, experiments to investigation the surface interactions of the metals with the functionalized surface, i.e. PAA-M^{n+} complexation stoichiometry, will be performed. Finally, given the high fluidic transport efficiencies of the

functionalized C-CP fibers, use of flow-through loops and cartridges seems to be an area of great potential.

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CHAPTER 3

SURFACE MODIFICATION OF POLYESTER (PET) C-CP FIBERS UTILIZING ETHYLENEDIAMINE TO GENERATE AN ION EXCHANGE STATIONARY PHASES FOR HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) SEPARATIONS OF PROTEINS

Introduction

Ion exchange chromatography (IEC) is a prominent method of separation and purification of biomolecules (specifically proteins), primarily due to the non-denaturing conditions employed during separation[1]. The ability to separate proteins of interest while maintaining the native conformation of the protein, and therefore experience no loss of biological activity, allows for downstream processing and protein use based on function (e.g., protein therapeutics)[2]. The basic retention mechanism of proteins to the stationary phase in IEC is based on electrostatic interactions, both anionic and cationic[3-6]. IEC columns involve a variety of support phases, but focus primarily on porous polymeric beads/resins having strong and weak exchange characteristics. Anion exchange resins utilize nitrogen based functional groups, the specific ligand depends on if a strong (quaternary amine) or a weak (primary amine) ion exchanger is desired[1]. For cation exchange resins, a strong ion exchange group is usually based on a sulfonic acid while weak exchange resins utilize carboxylic acid groups[7]. Strong exchange resins differ from weak resins not only in what the surface group is, but also in how the surface group responds to certain buffers and pH changes. The

functional groups on a strong exchange resin remain ionized across a wide range of solution conditions, whereas the functional groups on the surface of a weak exchange resin are operable over a limited range of pH values. Commercially available stationary phases utilize both strong and weak functional groups, which are generally formed through derivatization of traditional silica or polymer (polystyrene, polyacrylate) supports.

In recent years research has focused on ways to improve upon the traditional stability-riddled silica and polymer bead supports by looking at new materials that provide an increased mechanical and chemical stability. Researchers have recently evaluated alternative inorganic supports such as zirconia[8], as well as other porous acrylamide polymers[9-12], monoliths[13, 14], and non-porous polymeric materials[15-21] for the separation of biomolecules. These approaches address limitations in the mechanical stability resulting from compression due to the high back pressures associated with the traditional porous stationary phases. The implementation of polymeric stationary phases alleviates the undesirable protein interactions with the exposed silanol groups that are common place in silica-based supports that result in peak broadening and analyte carry over. Monolithic and non-porous polymer materials are being developed for the added advantage of increased mass transfer efficiencies over porous particles.

This laboratory has focused on the application of capillary-channeled polymer (C-CP) fibers as stationary phases for the separation of proteins and

polymers in HPLC[22-28]. C-CP fibers are melt extruded from readily available textile polymers, specifically polyethylene terephthalate (polyester, PET), polypropylene (PP) and nylon-6. The extrusion process generates a very unique shape of fiber (Fig. 3.1), from which the name comes, that consists of eight channels running along the entire fiber length[29]. The shape of these C-CP fibers allows for the interdigitation of the channels within a column structure (Fig. 3.1c), resulting in a high packing density and efficient use of space. This interdigitation and the inherent shape of the fibers, i.e. the channels, results in a high solvent transport efficiency, thus allowing C-CP fiber columns to be operated at high linear velocities with low back pressures. C-CP fibers are nonporous at the size scale of polypeptides and proteins and so no intraphase diffusion occurs, resulting in improved mass transfer kinetics in comparison to porous materials. The nonporous nature of the fibers also decreases ghosting and carry-over effects while demonstrating an increased recovery for proteins in HPLC applications[30].

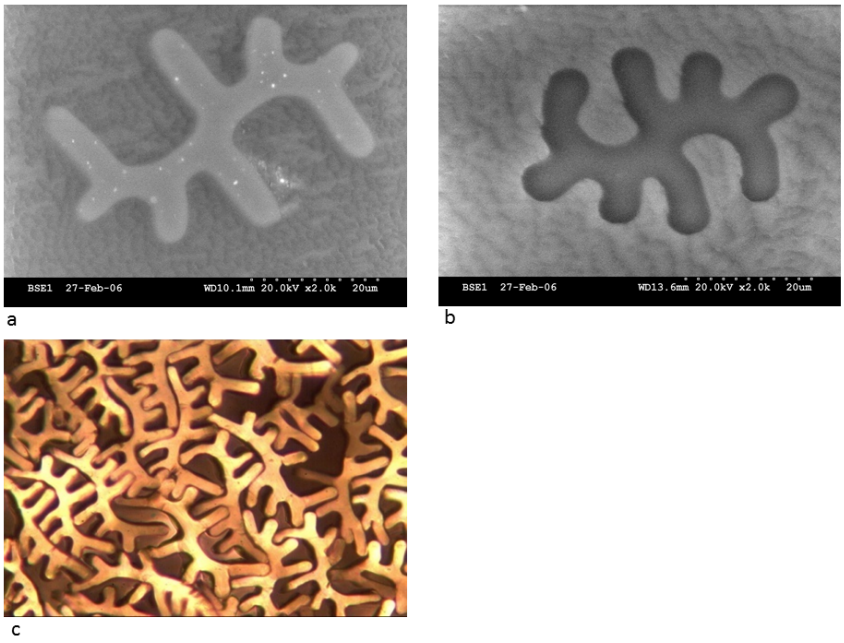


Figure 3.1. SEM Cross sectional view of a) Nylon and b) PET C-CP fibers c) SEM Image of a packed C-CP fiber column showing interdigitation of the fibers[23]

The ability to control surface chemistries and therefore exploit analyte stationary phase interactions, while maintaining the physical attributes of the fibers and their behavior in a column, is of great importance. In C-CP fiber applications, selectivity can be obtained through the choice of the base polymer, PP, PET, or nylon-6, affecting hydrophobic, π-π, and electrostatic interactions. This wide range of potential interactions allows for their application in different modes of HPLC separations. Specifically, nylon C-CP fibers have been utilized in biological compatible separations including ion-exchange (IEC)[31, 32] and hydrophobic interaction chromatography (HIC)[33]. The charged surface groups available on nylon (amines and carboxylic acids) are a result of the discontinuity of the polymer chains (end groups) as depicted in Fig. 3.2. The number of these surface groups is sufficient to perform separation of proteins[28] but will vary based

on the nature of extrusion process and the molecular weight of the starting polymer from which a fiber spool is extruded[34].

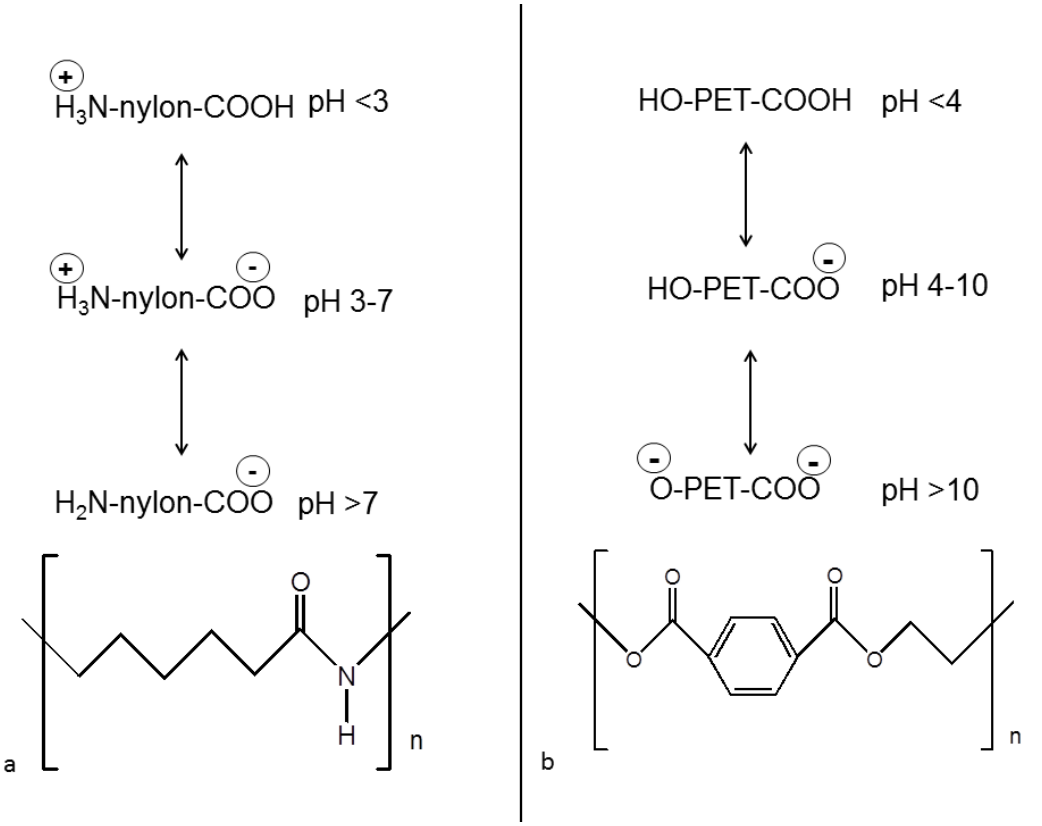


Figure 3.2. a) Structure of nylon-6 and a depiction of naturally occurring end groups and their charges as a function of pH b) Structure of PET and a depiction of the naturally occurring end groups and their charges as a function of pH

A particular benefit of the nylon C-CP fiber functional groups is that both cationic and anionic exchange mechanisms are possible due to the zwitterionic nature of the surface,[28, 33] illustrated in Fig. 3.2. As can be seen, the ionic state of the nylon-6 surface end groups can be "tuned" as a function of the pH that the solution in which the fibers are immersed[35]. Generally IEC retention is explained through the "net charge" model that implies that at a protein's pI the net charge of the protein is zero, while above the pI the protein is negatively charged and

below this pI a positively charged protein exists[7]. However, what the “net charge” model does not take into account are that pockets of charge or surface groups that carry a charge opposite the “net charge” exist. These are important factors and will affect the IEC chromatography, therefore generation of a high density zwitterionic stationary phase for the separation of proteins through an IEC mechanism is an attractive feature. The generation and implementation of zwitterionic stationary phases has also been evaluated by other researchers where such stationary phases have been utilized to separate metals on porous particles[36], phenolic acids have been separated on monolithic zwitterionic stationary phases[37], and separation of proteins on a nonporous polymer bead based zwitterionic surface[38]. Zwitterionic stationary phases have become prevalent in hydrophilic interaction chromatography (HILIC)[39-44]. However, to date, the benefits of a zwitterionic polymeric fiber stationary phases have not been explored.

As depicted in Fig. 3.2, the end groups present in polyester are also pH active, providing the possibility of cation exchange separations above pH ≈ 4. Evaluation of the available textile polymer modification chemistries leads to a plethora of potential means of surface modifications that can affect charged surfaces. In this instance, exploration of the surface modification of PET-based C-CP fibers is particularly intriguing because of the relative ease of PET fiber extrusion and chemical versatility. This is due primarily to the ease of modification associated with the ester present in the backbone structure of PET.

The surface modification presented here focuses on an approach presented to develop bioactive textile materials,[45, 46] specifically, the exposure of PET fibers to ethylenediamine (EDA) to affect a surface composed of primary and secondary amines, as well as primary alcohols. EDA breaks down the PET C-CP fiber backbone through nucleophilic acyl substitution, an addition – elimination mechanism that can occur in neat solutions at room temperature. The rate of modification depends on the crystallinity of the fiber and can be controlled through the EDA solution concentration, temperature, and fiber exposure time[47, 48].

As depicted in Fig. 3.3, the primary process by which EDA modifies the PET surface is aminolysis, a simple, fast, and straightforward reaction (Fig. 3.3a). Generation of the amine functional groups, in addition to the already existing carboxylic acid end groups on the surface, results in the desired dual charge pH sensitive zwitterionic surface comparable to nylon-6. However, due to the hygroscopic nature of EDA, in combination with the functionalization occurring under atmospheric conditions, hydrolysis of the PET C-CP fiber backbone can occur as well (Fig. 3.3b).

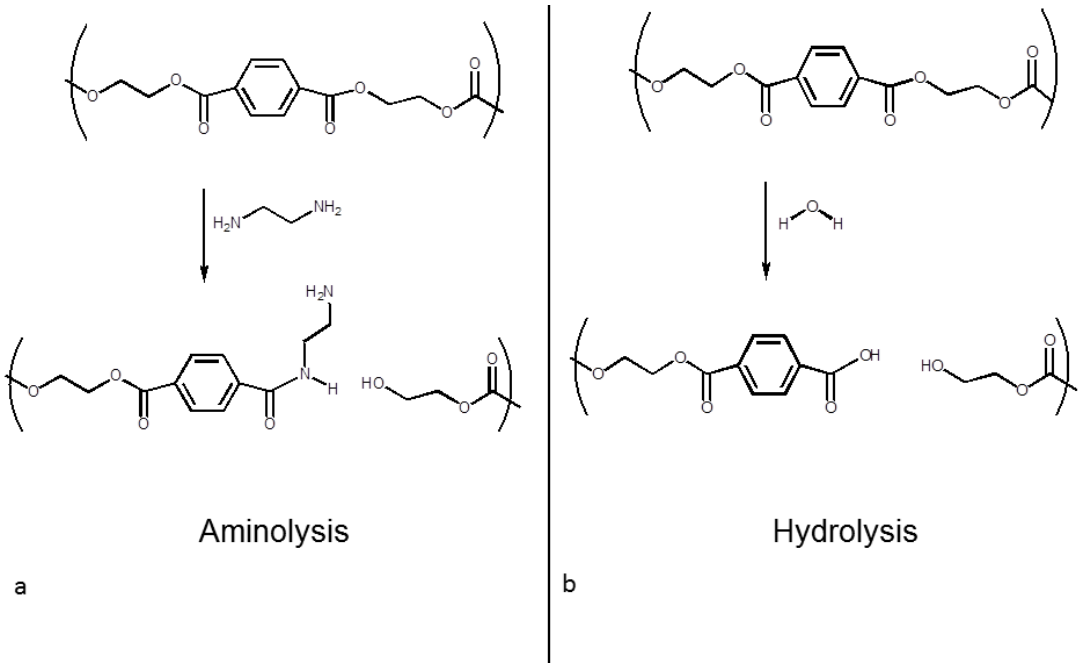


Figure 3.3 a) Aminolysis Reaction with PET b) Hydrolysis Reaction with PET

In an effort to minimize the amount of hydrolysis, the functionalization here was carried out in neat EDA solutions, though not completely eliminating the generation of the carboxylic acid functional group. Aminolysis, Fig. 3.3a, breaks down the fiber backbone through nucleophilic acyl substitution, an addition – elimination mechanism that occurs at room temperature. Generally, the nucleophilic reaction of EDA with PET occurs rapidly, but when allowed to react for extended periods of time will react with every ester bond present, thus destroying the physical integrity of the C-CP fibers. The most desirable set of experimental conditions would allow for uniform coverage of the functional groups generated on the surface, while maintaining the bulk integrity of the fiber. Once generated these EDA modified columns are coined "*super nylon*" due to

the expected high density of functional groups generated relative to PET or native nylon-6 itself.

Research presented here focuses on the use of the EDA modification chemistry to affect high density C-CP fiber surfaces that have application (as in the case of nylon-6) in IEC separations of proteins. (It cannot be understated that such a surface would provide outstanding opportunities for subsequent modifications based on the coupling of species-specific ligands to affect affinity separations[49, 50].) The *super nylon* surfaces were evaluated for their functional group density using textile dye chemistries. The use of these dyes not only allows for quantification of functional groups but also allow for a visual confirmation of the uniformity of the functional groups. Anionic and cationic functional group densities were compared to those of native PET and nylon-6 C-CP fibers. Columns packed with *super nylon* C-CP fiber stationary phases are evaluated and compared to native (unreacted) PET and nylon-6 C-CP fiber columns with regards to the separation of a three protein mixture using a standard Tris-HCl buffer mobile phase with NaCl as the displacing agent. The functional groups of each of the C-CP fiber surfaces examined here are considered “weak”; therefore separations occurring over a range of pH values (i.e., surface charge characteristics) are evaluated as well. It is believed that the synthetic strategy described here can have applicability not only for the generation of zwitterionc IEC phases, but also as a starting point to further surface modifications for affinity-based separations.

Experimental

Chemicals and Reagents

Protein stock solutions were prepared with protein concentrations of 0.25 mg mL^{-1} each of ribonuclease A (Ribo) and cytochrome c (Cyto), and 0.5 mg mL^{-1} lysozyme (Lyso) purchased from Sigma-Aldrich (Milwaukee, WI). MilliQ water (18.2 MΩ cm^{-1}) derived from a NANOpure Diamond Barnstead/Thermolyne Water System (Dubuque, IA) was used for all dilutions and sample preparations. Buffers used in the preparation and separation of proteins were prepared from Tris-HCl (Teknova, (Hollister, CA)) and sodium chloride (NaCl) obtained from Sigma-Adrich. Column modification took place using ethylenediamine (EDA) while quantification of functional groups on the surface occurred using acid red 1 (AR1) and methylene basic blue (MB), each from Sigma-Aldrich. Dye solutions were prepared in acetic acid and ammonium hydroxide (Fisher Scientific (Pittsburgh, PA)) respectively. Isopropanol (IPA) (Fisher Scientific) was used as an additive in the chromatographic buffers. The adjustment of buffer pH values employed hydrochloric acid (HCl) purchased from VWR (West Chester, PA).

Chromatographic System and Instrumentation

Separation of the three protein suite was performed on a high performance liquid chromatograph (HPLC) system, initiated with analyte injection using a 6-port Rheodyne (Cotaiti, CA) injector fitted with a 10 µL loop. A Waters (Milford, MA) chromatographic system composed of a 600S controller connected to a 626 pump with a 996 photodiodarray (PDA) detector was employed. C-CP

fiber columns, made in house, were placed in line, where a traditional commercial column would be located. Data from the PDA was collected using Empower 2 Software (Waters (Milford, MA)) and processed using Microsoft Excel (Seattle, WA).

Column Packing

Capillary-channeled polymer (C-CP) fibers (nylon-6 and polyester) were obtained from Fiber Innovations Technology (FIT) (Johnson City, TN) on spools measuring more than 1000m in length. The method of C-CP fiber column construction, initially described by Marcus et al. was employed here[51]. A monofilament pulled the fiber loop through a PEEK-Lined™ 2.1 mm I.D. x 150 mm long stainless steel column (GRACE (Deerfield, IL)) resulting in a collinear alignment of the fibers in the column[51]. The fibers extend the entire 150 mm column length and were trimmed flush with the ends of the column using a surgical scalpel. A 2 µm frit was added to each end, followed by capping of the column ends. The number of fiber rotations is directly related to the interstitial fraction (IF) of the column, in these experiments the column IF's were 55-62 %. These interstitial fractions were chosen based on the predetermined optimal working range for macromolecules. In order to obtain these IF's, 3150 nylon fibers and 4800 PET fibers were packed per column; with the variation in the number of fibers between nylon and PET resulting from differences in fiber perimeter. The total surface area in the nylon C-CP column is 980 cm^2 while the total surface area in the PET packed C-CP fiber columns is 1667 cm^2,

differences between the fibers are visible in the micrographs of Figs. 1 a and b, for nylon-6 and PET, respectively.

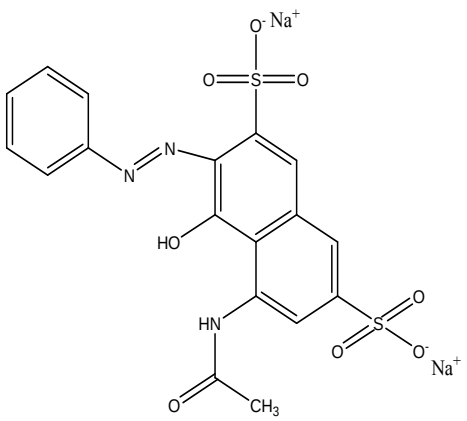
On-Column Functionalization Process

Polyester (PET) C-CP fibers were first packed into a 2.1 x 150 mm i.d. column using the process described above. However, the fibers are initially cut flush with only the front end of the column, leaving the fibers hanging out of the tail end of the column. This allows the C-CP fibers to potentially shrink in length during the functionalization process without leaving a gap inside of the column. The column was then attached to a Shimadzu LC-10AT HPLC pump (Kyoto, Japan), in which the EDA solution was pumped through the HPLC column at a flow rate of 0.1 mL min^{-1}, no substantial backpressure was realized, allowing the column to remain packed with C-CP fibers during functionalization. Once the EDA eluent reached the uncapped end of the column, it was assumed that all fibers had been thoroughly wetted. EDA was pumped through the column for 3 or 10 minutes after initial wetting, generating fiber types designated as Super-3 and Super-10, respectively. Upon completion of functionalization, the fibers were cut flush with the end of the column, the frit put in place and the column capped. Flushing of the column with MilliQ H_2O for 30 minutes at 1 mL min^{-1} removed any residual EDA from the column.

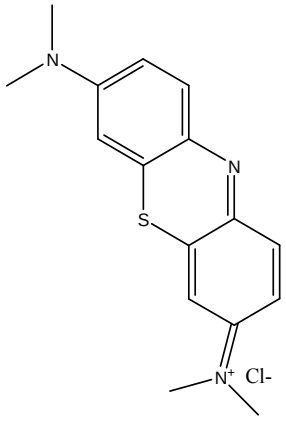
Quantification of Functional Groups

Nylon and PET C-CP fibers, native and functionalized, were bundled into ~3 cm long segments. The number of rotations, number of fibers per rotation,

weight of each bundle, and length of each bundle were recorded to allow for calculations of surface area and functional group density, etc. Segments of PET C-CP fibers were functionalized using EDA by soaking in a 10:1 liquor ratio (LR) at room temperature for the desired amount of time (3 or 10 minutes). The cationic and anionic functional groups were visualized and quantified utilizing acid red 1 (AR1) and methylene blue (MB), respectively (Figure 3.4). Individual dye solutions were made at a concentration of 0.1 g L^{-1} in a 1 g L^{-1} solution of either acetic acid or ammonium hydroxide for AR1 or MB respectively[45]. The dye solutions were combined with the fiber bundles in a 50:1 LR and 0.5 % on mass of fiber (OMF) to generating a dye bath and were allowed to soak for 1 hour at 60 ℃. The fibers were subsequently washed using 0.5 % OMF of either the 1 g L^{-1} acetic acid solution or the 1 g L^{-1} ammonium solution as appropriate, followed by a water wash. UV-VIS measurements of the dye bath solutions were obtained for MB at 660 nm, while AR1 was analyzed at 557 nm. Quantification of functional groups was accomplished using calibration curves generated for both AR1 and MB at 557 nm and 660 nm respectively. These calibration curves and the absorbance values obtained from analysis of the dye bath are utilized to calculate the dye remaining in solution which allows for back calculations to obtain the amount of dye adsorbed to the fiber surface.



Acid Red 1 (AR1)



Methylene Blue (MB)

Figure 3.4. Chemical structure of acid red 1 and methylene blue dyes

Protein Separation Methods

Ion exchange separations of a protein suite containing ribonuclease A (Ribo), cytochrome c (Cyto) and lysozyme (Lyso) were evaluated on the four different HPLC C-CP fiber columns (native nylon-6 and PET, Super-3, and Super-10). Separation conditions were optimized for the standard Nylon C-CP fiber column, 2.1 x 150 mm with a 58 % IF based on previous work in this laboratory[28]. Evaluation of gradient rates and flow rates resulted in an optimized separation of the protein suite occurring at 4 mL min^{-1} with a gradient rate of 16.7 % increase in elution solvent per minute. Slight variations in retention times between the columns are expected as the linear velocities range from 14-18 mm s^{-1}. A 20 mM Tris-HCl solution at pH 8.2 was held for 2 minutes prior to initiation of the gradient, 0-100 % 20 mM Tris-HCl, 1 M NaCl at pH 8.2. Evaluation of the addition of an organic modifier to minimize hydrophobic interaction used the same conditions, with the addition of 0-3 % isopropyl alcohol (IPA) to both

buffers. To evaluate the effect of pH, 6 M HCl and 0.1M NaOH were used to adjust the loading and elution buffers to equivalent pH values across the range of pH = 7.2 - 9.2.

Results and Discussion

PET C-CP fibers were modified using ethylenediamine (EDA), a modification chemistry employed for the alteration of PET in the textile industry[45, 46], to generate alcohols, primary amines, and amides (Fig. 3.3). In order to gain a general understanding of the degree of functionalization and optimal reaction time, fibers were reacted with EDA at controlled intervals of up to 10 minutes. The physical integrity of the fibers and uniformity of the subsequent coverage was visually evaluated, as illustrated in the photographs presented in Fig. 3.5.

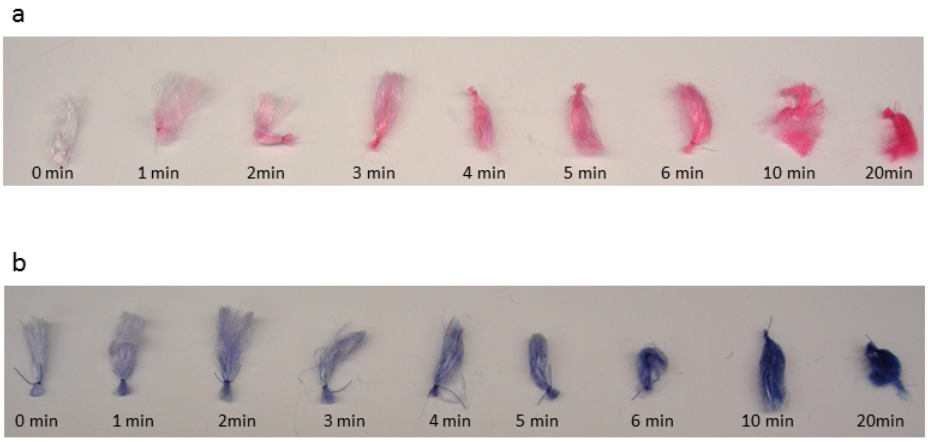


Figure 3.5. PET C-CP fibers modified with EDA at controlled exposure times of 0, 1, 2, 3, 4, 5, 6, 10, 20 minutes a) anionic surface functional groups dyed with C.I. acid red 1 b) cationic surface functional groups dyed with methylene blue (basic blue 9).

The density and availability of the functional groups are revealed through the indicator dye adsorption, with the acid red 1 reflective of the presence of the

anionic (-COO^-) groups and the methylene blue for the cationic (NH_3^+) groups [45, 46]. The increasing density and uniformity of the dye adsorption is visible in Fig. 3.5, as the functionalization time increases from 0 to time 20 minutes. As discussed previously, excessive exposure of the PET fibers to EDA results in significant degradation of the fiber backbone; this damage is visible after 20 minutes of modification. Based on the visual uniformity and integrity of the fibers in Fig. 3.5, it was determined that 3 and 10 minute PET C-CP fiber exposures would be used to conduct further experiments. Based on the fact that the EDA functionalization process should result in the presence of functional groups similar to those on nylon-6, but in a higher density, these PET C-CP fibers are designated as “Super-3” and “Super-10”. As an additional experimental variable, modification experiments were performed using diethylenetriamine (DETA), a weaker base than EDA. Just as the case of EDA, DETA modifies the PET surface through aminolysis. The results are not presented here as the modification was not as successful as the EDA case, with the fibers resultant from a 20 min DETA exposure having the general appearance of those of Fig. 3.5 with a 3 min EDA exposure.

The cationic and anionic functional groups were quantified for the four test fiber types (native PET, nylon-6, and Super 3 and 10) using absorbance measurements as previously discussed[45], with the results presented in Tables 3.1 and 3.2, respectively.

Table 3.1. Cationic Functional Group Density

	0% Organic Modifier (IPA)				10% Organic Modifier (IPA)			
	mol / cm^2	% RSD	meqv / g	% RSD	mol / cm^2	% RSD	meqv / g	% RSD
PET	1.11E-09	5.54	1.01E-03	6.96	5.60E-10	11.3	9.66E-04	11.2
Nylon	2.69E-09	5.83	1.59E-03	0.98	3.68E-09	1.33	4.52E-03	2.38
Super-3	1.62E-09	2.03	1.39E-03	13.4	7.63E-10	30.1	1.02E-03	27.6
Super-10	1.73E-09	7.90	1.57E-03	18.1	2.84E-09	4.28	4.08E-03	2.62

Table 3.2. Anionic Functional Group Density

	0% Organic Modifier (IPA)				10% Organic Modifier (IPA)			
	mol / cm^2	% RSD	meqv / g	% RSD	mol / cm^2	% RSD	meqv / g	% RSD
PET	6.78E-09	1.84	1.22E-02	4.72	4.54E-10	10.8	1.57E-03	14.1
Nylon	5.87E-09	5.23	6.67E-03	8.70	4.68E-09	9.20	1.14E-02	11.0
Super-3	5.68E-09	11.6	9.48E-03	11.6	5.13E-10	16.9	1.30E-03	14.2
Super-10	3.82E-09	2.36	5.83E-03	5.16	2.11E-09	4.54	5.06E-03	4.34

Table 3.1 shows the available primary amine (-NH_3) surface groups for both the native C-CP fibers (nylon and PET) and the EDA modified PET C-CP fibers. The same EDA exposure conditions were used here as were used in the dying of the fibers shown in Fig. 3.5. Once complete, the fibers were removed from the adsorption bath, carefully squeezed to remove excess dye solution, washed with ~8 mL of water. The wash solutions were each diluted to 10 mL prior to the absorbance measurements performed at 557 nm. The functional group concentrations were calculated based on calibration curve responses, the exposed fiber mass, and their respective surface areas. The total number of

available surface groups is lower in both of the surface modified versions, Super-3 and Super-10, in comparison to nylon. As presented in Table 3.1, the overall trend in cationic (-NH_3) functional groups from highest to lowest is nylon, Super-10, Super-3, and PET. Of course, based on the structure shown in Fig. 3.2, PET does not possess these functional groups. The higher density functional groups present on Super-10 relative to Super-3 are logical and expected purely due to the longer reaction time and exposure of the fibers to EDA in the case of Super-10. Since the purpose of this modification approach was to generate a high density, super capacity *super nylon* it was anticipated that the number of functional groups in Super-10 would have been higher than those present in native nylon-6.

Since the fiber surface generated is zwitterionic, the total capacity of the column is dependent upon both the cationic and anionic functional groups. The density of anionic functional groups was determined using the adsorption of MB, absorbance measurements taken at 660 nm, following the same guidelines as those utilized for the cationic groups presented earlier. The generation of the anionic groups, -COOH, is an unavoidable side reaction of the desired modification, therefore understanding the degree at which this reaction occurs is essential. Table 3.2 shows that the fiber stationary phase with the highest number of anionic groups is PET, followed by nylon, Super-3, Super-10. It could be reasonably assumed that the extrusion process of PET would generate more carboxylic acid end groups than those of nylon. However, the reaction of PET

with EDA will result in more carboxylic acid groups than PET has initially, consequently the number of –COOH groups present on the surface of both Super-3 and Super-10 should be greater than native PET. By the same token, the quantification of functional groups under these conditions might be effected by the adsorption of the aromatic dye to the PET surface through π - π interactions. In order to decrease these potential interactions the experiments were run with the addition of 10 % isopropyl alcohol (IPA). The addition of IPA in low concentrations is enough to reduce hydrophobic interactions while still allowing the ionic interactions to occur. As presented in Table 3.2, quantification of the anionic groups on the fiber surfaces in the presence of 10% IPA yields functional group densities that fall into a reasonable pattern, nylon, Super-10, Super-3, and PET. Nylon again has more functional groups as a result solely of the extrusion process than either of the PET EDA modified (Super) versions. Since there was a substantial rearrangement in the anionic functional group density trend among the fibers with the addition of IPA, cationic groups were re-evaluated in the presence of IPA (Table 3.1). There is some variation in the concentration of functional group densities, but no change in the trend among the C-CP fibers evaluated. These trends are supported visually in Figure 3.6 as the fibers evaluated are dyed directly with AR1 and MB.

Figure 3.6. a) C-CP fibers (PET, Super-1, Super-3, Super-10, Nylon) dyed with C.I. acid red 1 to visually reflect the anionic functional groups. b) C-CP fibers (PET, Super-1,Super-3, Super-10, Nylon) dyed with methylene blue (basic blue 9) to visually reflect the cationic functional groups

The initial purpose of the modification procedure was to create a stationary phase with a higher functional group density than either native fiber (PET or nylon) possessed. EDA-functionalization of PET under these time restrains was not successful in achieving this goal. It is feasible that, with increased time exposure of the PET fibers to EDA, the functional group density would increase such that a “super density” stationary phase was developed but at the cost of fiber strength and stationary phase stability (20 minute EDA exposure, Figure 3.5). However, evaluation of these C-CP fibers proceeded in order to evaluate if any chromatographic benefits existed when using the anionic/cationic functional groups on the PET backbone, since the potential for π-π interactions exists. Functionalization of PET C-CP fiber columns was

performed *in situ*, ensuring that any surface with potential exposure to analyte was exposed to and modified with EDA. Separations occurred using a Tris-HCl buffer with NaCl as the displacing salt. A standard three protein mixture of ribonuclease A (Ribo), cytochrome c (Cyto), and lysozyme (Lyso) was separated.

An important factor in ion exchange separations is the mobile phase pH, as this controls the charge of both the stationary phase and the proteins. The C-CP fiber stationary phases being explored here, with the exception of the native PET, can be zwitterionic as the result of the anionic and cationic functionality, Fig. 3.2. The performance of the fibers was evaluated over the range of 7.2 to 9.2 in 0.5 pH intervals, allowing for evaluation of the stationary phases under both a dual charge (zwitterionic) and an cationic separation mode. The proteins being evaluated are all basic proteins, having pIs in the range of 8.7-11.3, meaning the separations are expected to occur primarily with a cation exchange mechanism.

The “net-charge” model says that proteins have a positive net charge at pHs below their pI and no net charge at their pI. Keeping in mind the net-charge on a protein has no direct relation to the surface charges or any pockets of charge, both of these things are important in chromatography and will determine the actual separation mechanism. However, as the pH of the buffer is increased, the charged sites within the proteins begin to deprotonate, as the charges on the amino acids change the electrostatic interactions change and result in changes in the tertiary structure, the folding, of the protein. As the folding changes the exposed charged sites vary and the proteins show changes in separation and

interaction with varying surface chemistries. Figures 3.7-3.9 show the separations of the protein suite on all four columns as a function of pH.

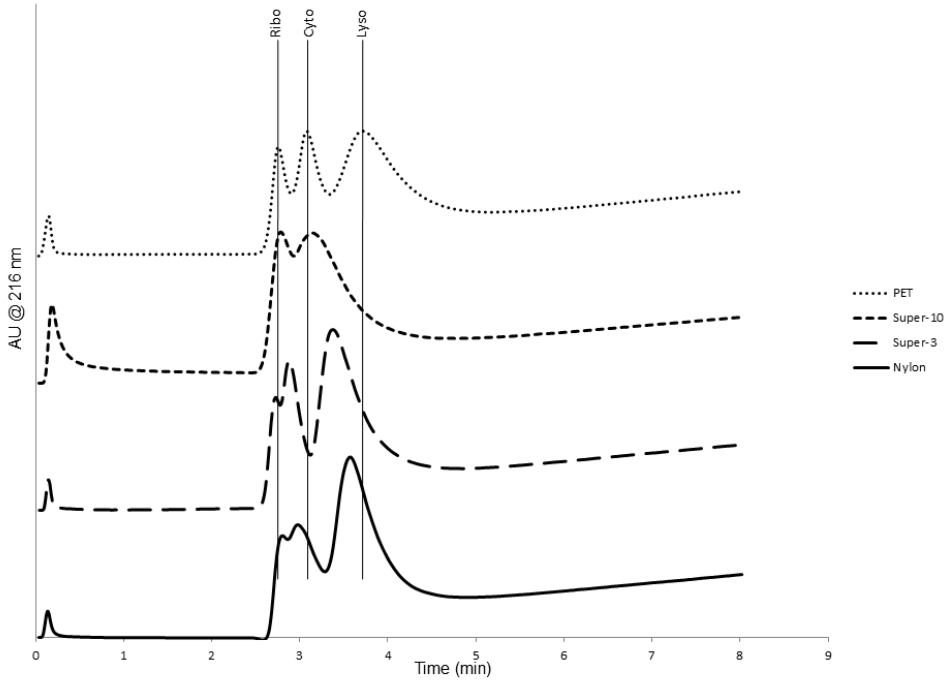


Figure 3.7. Separation of the three protein suite (Ribo, Cyto, Lyso) under IEC conditions at pH 7.2 on Nylon, Super-3, Super-10, and PET C-CP hplc columns. Separations occurring using a mobile phase of 20 mM Tris-HCl with a 2 minute hold after injection followed by a gradient separation of 0-100% 1 M NaCl in 6 minutes with a flow rate of 4 mL min-1.Protein Concentration= 0.25 mg mL^{-1} Ribo, 0.25 mg mL^{-1} Cyto, 0.5 mg mL^{-1} Lyso. Injection Volume = 10 µL

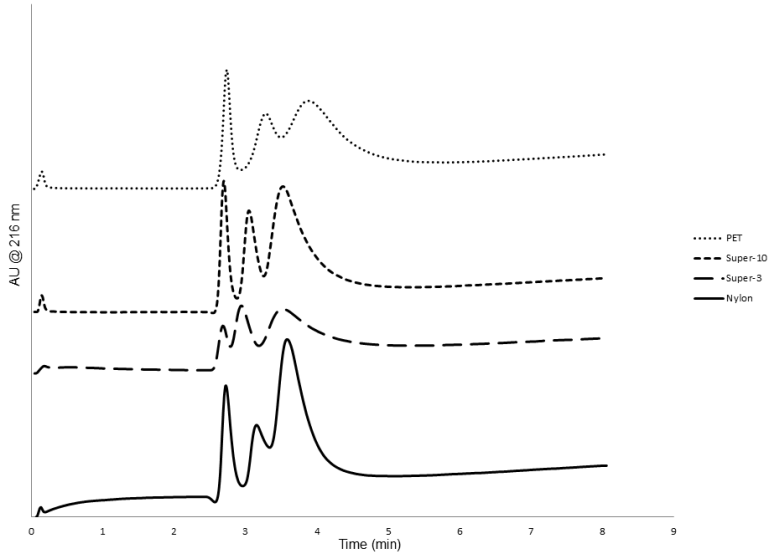


Figure 3.8. Separation of the three protein suite (Ribo, Cyto, Lyso) under IEC conditions at pH 8.2 on Nylon, Super-3, Super-10, and PET C-CP hplc columns. Separations occurring using a mobile phase of 20 mM Tris- with a 2 minute hold after injection followed by a gradient separation of 0-100% 1 M NaCl in 6 minutes with a flow rate of 4 mL min^{-}1.Protein Concentration= 0.25 mg mL^{-1} Ribo, 0.25 mg mL^{-1} Cyto, 0.5 mg mL^{-1} Lyso. Injection Volume = 10 µL

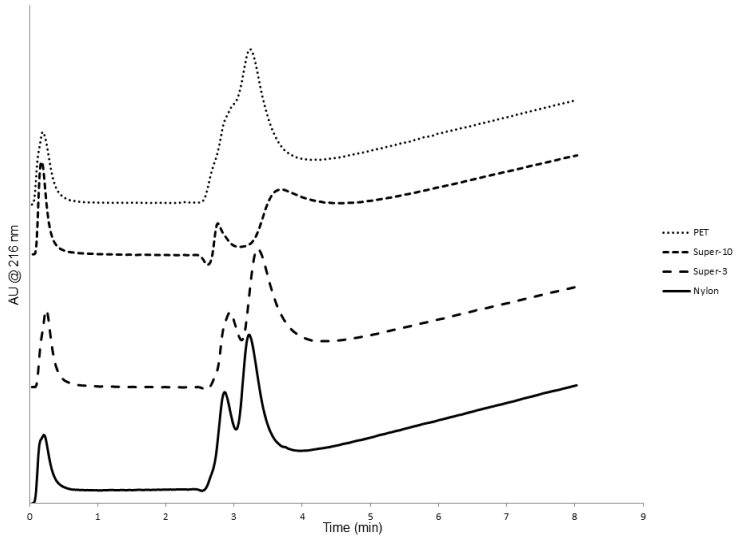


Figure 3.9. Separation of the three protein suite (Ribo, Cyto, Lyso) under IEC conditions at pH 9.2 on Nylon, Super-3, Super-10, and PET C-CP hplc columns. Separations occurring using a mobile phase of 20 mM Tris-HCl with a 2 minute hold after injection followed by a gradient separation of 0-100% 1 M NaCl in 6 minutes with a flow rate of 4 mL min^{-}1.Protein Concentration= 0.25 mg mL^{-1} Ribo, 0.25 mg mL^{-1} Cyto, 0.5 mg mL^{-1} Lyso. Injection Volume = 10 µL

Demonstrated in these figures, the optimal protein separation on all columns occurs at pH = 8.2, Figure 3.8. Evaluation of all four C-CP fiber columns at pH 8.2 shows that the optimal separation occurred using a Super-10 C-CP fiber column as the highest overall resolution was achieved. At pH = 8.2 the surface of the nylon C-CP fibers is no longer zwitterionic, i.e. the separation is primarily cation exchange. Comparison of the native PET to the Super-10 fibers show that the addition of anionic groups to the stationary phase is beneficial in the separation of proteins, as would be expected since you have an increase the total anionic capacity. As the pH shifts above and below 8.2 a visible reduction in the ability to separate these three proteins occurs. A decrease in the resolution is visible beginning at pH = 8.7 for all columns and further degradation is visible at pH = 9.2, Fig 3.9, such that Ribo is now unretained. At these pH values the charge on the stationary phase isn't changing so the separation changes are based solely on charge and conformational changes of the protein. It is not remarkable that at pH = 9.2 Ribo is completely unretained, based on a pI of 8.7 Ribo has an overall negative charge matching that of the stationary phase.

pH values below the optimal separation at pH = 8.2 were also evaluated, with rather poor separations of the protein suite occurring. Of all the columns evaluated, the best resolution of proteins in this pH range was the PET C-CP fiber column, whereas the other C-CP stationary phases have no resolution at pH = 7.2, (Fig. 3.7). At this pH the proteins are all still below their pI, therefore maintaining an overall positive charge. At pH = 7.2 nylon, Super-3, and Super-10

stationary phases are all zwitterionic due to the presents of primary amines and carboxylic acids present on the fiber surface. Therefore, the potential for protein stationary phase repulsion exists, something that is not an issue for the PET C-CP fiber column due to the only surface charge present being anionic ($-COO^-$).

PET C-CP fibers have been utilized in previous work for the separation of proteins using reversed-phase chromatography[26]; therefor it is feasible that hydrophobic interactions are occurring in these ion-exchange separations. This was evaluated by using the addition of organic solvents in the ion exchange buffers. It has been previously proven that in order to minimize undesired hydrophobic interactions occurring in ion exchange chromatography the addition of an organic solvent additive up to concentrations of 10 % are acceptable[1]. In ion exchange separations that utilize commercial columns hydrophobic interactions are generally undesirable as this means the analyte is interacting with the column support or the cross-linking agent and not the stationary phase as desired. When C-CP fibers are utilized in HPLC separations the fibers are not only the stationary phase but also the support so interactions with the support do not have to be considered undesirable, as long as these interactions are understood. In fact, previous research has shown that C-CP fibers provide a successfully separation of proteins when utilized in a mixed-mode[28].

An evaluation of the C-CP fiber ion exchange separations in the presence of IPA under the optimum conditions described earlier, pH = 8.2 (Fig. 3.8), with the addition of 0.1, 1.0, and 3 % IPA occurred. The addition of 0.1% IPA had no

substantial effect on the resolution of the proteins. The addition of the higher concentrations of IPA does indeed affect the retention of proteins and reduce resolution. Visible in Fig. 3.10 is a complete loss of resolution between cytochrome c and lysozyme in the case of the amine (nylon and *super nylon*) columns after increasing the concentration of the IPA additive to 3% IPA.

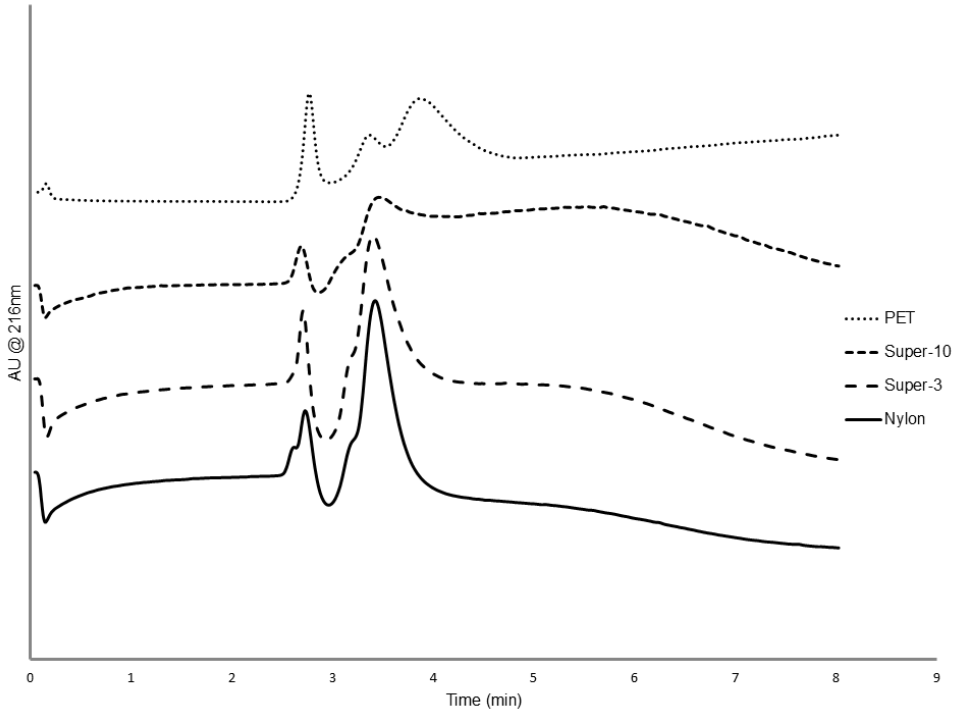


Figure 3.10. The effect of 3% IPA on the separation of the three protein suite (Ribo, Cyto, Lyso) under IEC conditions at pH 8.2 on Nylon, Super-3, Super-10, and PET. Separations occurring using a mobile phase of 20 mM Tris-HCl, 3% IPA with a 2 minute hold after injection followed by a gradient separation of 0-100% 1 M NaCl, 3% IPA, 20 mM Tris-HCl in 6 minutes with a flow rate of 4 mL min-1.Protein Concentration= 0.25 mg mL^{-1} Ribo, 0.25 mg mL^{-1} Cyto, 0.5 mg mL^{-1} Lyso. Injection Volume = 10 µL

Ribonuclease and lysozyme appear to be the least affected by the addition of IPA to the separation buffers (i.e. no shift in retention time), indicating that their primary mode of separation is through ionic interactions. Also depicted in Figure

3.10 is the separation of the protein suite on PET is unaffected by the organic additive, indication again that this separation is primarily through ionic interactions. The most affected of the proteins in the suite is cyto, the second protein in the elution. The addition of 3 % IPA to the separation buffers causes and increased retention of cyto resulting in co-elution of cyto and lyso. It is expected that if cyto was interacting through a low level of hydrophobic interactions there would be a decrease in the retention time upon addition of IPA, Fig 3.10 shows the opposite occurs in our separation. The exact cause of the increased retention is unknown but changes in the solvation of cytochrome c in the IPA/buffer eluent could cause a change in the desired interactions with the stationary phase (i.e. cyto interaction become more preferable with the stationary phase). Additionally, the increased retention could be a result of the low levels of IPA causing slight changes in the tertiary structure of the protein resulting in exposure of additional ionic interaction sights. These separations indicate that all of the C-CP fiber columns evaluated in these protein separation studies have the potential to exhibit some degree of mixed-mode interactions (reversed-phase, anion and cation exchange). The generation of the Super-10 and Super-3 stationary phases appear to have a greater extent of potential hydrophobic interactions than the PET C-CP fibers. This potential increase in hydrophobic character of the stationary phase could be partially attributed to the increased alkyl character as a result of the addition of two carbon chain associated with the EDA molecule.

Conclusions and Future Work

The modification of PET with EDA through nucleophilic acyl attack was successful at generating a stationary phase containing primary amine functional groups. Unfortunately, the attempt to generate a “super-nylon” stationary phase was not successful as the quantification of functional groups using anionic/cationic textile dyes showed that the native nylon C-CP fiber surface possesses the highest density of functional groups. The implementation of all four C-CP fibers as stationary phases showed that in preliminary studies optimal separation on all HPLC columns occurred at pH = 8.2. At the optimal pH, the Super-10 column demonstrated the best separation and peak shape due to the presence of increased carboxylic acid groups relative to PET, it is also true the Super-10 has a higher density of amine groups but at this pH they play no role in the separation. The separations presented here occur below the pI of all proteins, i.e. proteins possess a net positive charge. This net positive charge does not preclude there from being pockets of negative charge on the proteins or negatively charged surface groups available for interaction. Therefore, the separations presented here may not fully utilize the zwitterionic nature of the stationary phase. It is safe to assume, though, that the nylon and super-nylon columns operate on mixed-mode mechanism using RP-AEX-CEX interactions.

Future work will look at exploiting the mixed-mode abilities of the modified C-CP fiber columns especially Super-10 by evaluating a variety of displacing salts. The variation of displacing salts both the anion and cations, since it is a

zwitterionic stationary phase, will change the separation ability of the columns by affecting the interaction of the proteins with the bulk buffer solutions. Additionally experiments will be performed to analyze the tertiary protein structure under differing salt and pH conditions to understand in greater detail the degree of hydrophobic vs ionic interactions occurring on the C-CP fiber surface. Evaluation of acidic proteins under the above described conditions will be evaluated as well.

Future work will also look at implementation of the modified PET, *super nylon*, C-CP fibers as a stationary phase in hydrophobic interaction chromatography (HIC) as a result of the increased alkyl character associated with the polymer backbone of the EDA. Additionally, there is potential for employment of these modified C-CP fibers as stationary phases in HILIC chromatography since the increased functional groups, primary amines, carboxylic acids, amides and alcohols all increase the polarity and hydrophilic nature of the stationary phase. The presence of these functional groups on the fiber surface also allows for exploration of further modification chemistries to be employed, generating a higher density analyte specific stationary phase using “click chemistries”. Utilizing C-CP fibers as a base for functional group generation and modification open the doors to a range of opportunities in the HPLC separation of proteins and macromolecules.

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CHAPTER 4

COVALENT IMINODIACETIC ACID LIGAND ATTACHMENT TO A NYLON-6 CAPILLARY-CHANNELED POLYMER (C-CP) FIBER SUPPORT PHASE THROUGH A 2,4,6-TRICHLORO-1,3,5-TRIAZINE (TCT) TETHER

Introduction

Driven by the growing use of protein therapeutics, preparative chromatography (aka, downstream processing) is becoming an increasingly important challenge in the field of separation science [1]. The primary challenges come about by the sheer chemical and physical complexity presented by the matrices from which specific proteins must be extracted, e.g. from fermentation broths. Simply, the target proteins/monoclonal antibodies are present at relatively low concentrations in matrices/solutions that exhibit far from ideal fluidic characteristics. In order to attain high levels of efficiency of target protein recovery, there must be a high level of specificity in the adsorption/capture of the target compound, while operating under conditions of high overall throughput. Affinity chromatography is based on (ideally) highly-specific interaction between the target protein and the chromatographic stationary phase, such that non-specific interactions are minimized and concomitant species are passed through the column or are easily washed off. High recovery percentages of the target proteins from the stationary phase, without compromising biological activity, is

obviously the primary objective. Additional pragmatic aspects include the cost and robustness of the stationary phase, susceptibility to phase fouling, and column clogging. Finally, one must be concerned with the high operational costs which some abut due to the high backpressures required of high volume columns. A significant portion of affinity stationary phase supports consist of various forms of agarose and silica materials (e.g. particles, monoliths, sol gels) modified using covalent immobilization to attach affinity ligands to the surface [2, 3].

The limiting aspects in preparative protein separations generally have more to do with the physical/hydrodynamic aspects of available stationary phases, rather than the isolation chemistries per se. It would be desirable to have supports that provide better mass transfer characteristics at higher flow rates, without the penalties of prohibitively high backpressures. Certainly, the ability to tailor surface chemistries to affect the desired separation in a facile and robust fashion is also required. To this end, fibrous support/stationary phases hold some promise as the materials are readily available at low costs, there is a wide range of well-established surface modification chemistries, and mass transfer is driven by convective diffusion in fairly porous column structures [4-10]. Marcus has recently reviewed the physical and chemical rationale for the consideration of natural and synthetic fibers for chromatographic separations [11, 12].

Capillary-channeled polymer fibers (C-CP) are a family of uniquely-shaped textile polymers generated through a melt extrusion process. The novelty of

these fibers is a result of the wide variety of shapes (>40) in which they can be extruded through a simple change of the extrusion spinneret [12]. Current research in this laboratory focuses on the use of the C-CP fiber shape which consists of eight channels that run the entire length of the fiber [12, 13]. Specifically, these C-CP fibers are being developed for separations by high performance liquid chromatography (HPLC). When packed into an HPLC column structure, interdigitation of the fibers forms a "monolithic" type structure of parallel, open capillaries that run the entire length of the column. C-CP fiber columns exhibit substantially lower backpressures than packed-bed phases [14, 15], resulting in the ability to operate columns at high linear velocities in comparison to commercial columns; thus interest in their application at preparative scales. Additionally, the shape and nonporous nature of the fiber (versus the size of protein molecules) leads to improved mass transfer, decreased carry-over, and increased analyte recovery [14-17]. These combined characteristics lead to the most appropriate application of C-CP fibers as stationary phases for biomacromolecule (i.e. protein) separations [12-15, 18-21]. The C-CP fiber stationary phase application has been primarily in a traditional analytical HPLC column format, with the mechanism of separation based on the choice of base polymers including nylon-6, polyethylene terephthalate (PET, a polyester), and polypropylene (PP). Protein separations have been accomplished via reversed phase (RP), hydrophobic interaction (HIC), ion-exchange (IEC), and hydrophilic interaction (HILIC) methods. As the benefits of employing C-CP fiber stationary phases for protein

separations become clearer, the ability to apply them in analyte-specific separations becomes of greater interest.

A particularly versatile form of affinity chromatography is immobilized metal affinity chromatography (IMAC), first employed as a protein purification and separation technique in the late 1970s [22]. IMAC is considered to be one of the primary modes of purification for proteins from extracts due to the high degree of extraction efficiency and mild elution conditions [3, 22]. IMAC purification of proteins is based on the affinity of histidine residues on the surface of natural proteins, and for recombinant proteins having engineered histidine tags (his-tags), for transition metal ions coordinated to the stationary phase surface. IMAC stationary phases commonly consist of iminodiacetic acid (IDA), a tridentate chelating surface group, however some applications utilize other bidentate and pentadentate ligands [3, 23]. The experimental design for IMAC starts with loading the surface with the chelated metal, generally Ni^{2+}, though Co^{2+}, Cu^{2+}, and Zn^{2+} have been used based on the selectivity of the target analyte [3, 22]. After loading of the stationary phase, the column is equilibrated and exposed to the sample, the protein(s) of interest are held to the surface of the stationary phase through interaction with his-tag, while the remaining sample flows through. As with any surface interaction, some non-specific binding can occur, so the column is washed usually using a low concentration solution of imidazole or salt. Once the non-specifically bound portions of the sample have been removed, the protein of interest can be eluted from the column using an imidazole gradient. When

necessary the column can be regenerated by metal ion removal upon exposure to aqueous ethylenediaminetetraacetic acid (EDTA).

A synthetic methodology to modify the surface of C-CP fibers towards applications in the preparative separation of proteins by IMAC was desired as a first approach to downstream protein processing. To this end, surface modifications that do not destroy the chemical structure of the fiber, while presenting a robust surface chemistry, are necessary. The physical structure of the C-CP fibers must also be maintained to realize the aforementioned physical/hydrodynamic benefits versus packed-bed phases. In order to achieve these targets, the use of a covalent-coupling modification technique was desired. Nylon-6 and PET fibers are amenable to modification due to the amine, hydroxyl, carboxylic acid, and amide groups naturally present on the fiber surface. Because PET and nylon-6 are common textile fibers, initial research into their modification focused on textile chemistries, specifically reactive dyes. Reactive dyes have been used commercially since the late 1950s as a way to dye (primarily) cellulose/cotton, though applications on wool and nylon textile materials exist [24, 25]. Examples of commercially available reactive dyes include Cibacron and Procion (Sigma Aldrich, St. Louis, MO), which provide robust finished surfaces as a result of the direct covalent fixation of the dye molecule with the amine or hydroxyl positions on the base polymers. The covalent attachment of these dyes occurs via the use of a highly reactive molecule, 1,3,5-trichloro-2,4,6-triazine (trichlorotriazine, TCT), also known as cyanuric chloride,

as a tether to the fiber surface. These dyes have been extensively studied and employed for a variety of additional applications, providing a thorough understanding of their efficiency, fixation, and exhaustion/saturation capacity [26-30].

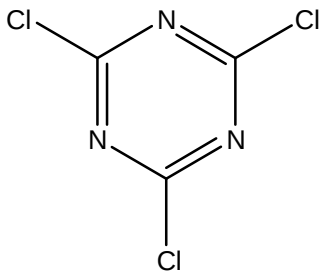

Figure 4.1. Structure of 1,3,5-trichloro-2,4,6-triazine (trichlorotriazine, TCT)

Trichlorotriazine has been described in the chemical literature as a reagent for a variety of uses, including the formation of amides and β-lactams, dehydration reactions, oxidation reactions, and as a coupling agent, affecting applications ranging from agriculture to pharmaceutical and biological applications [31, 32]. In addition to these solution-based synthetic uses, successful anchoring of TCT to activated carbon, epoxy resins, and textile material (cellulose, PET) surface(s) has also been demonstrated [33]. For example, Ohe et al. utilized TCT to tether sugar moieties to the surface of PET fabric [34]. Additionally, Hou and co-workers demonstrated that cellulosic materials modified with trichlorotriazine functionalized with multi-cationic benzyl groups can yield antibacterial fabrics [35]. The wide use of TCT-based dyes points to their

availability at reasonable prices, a key consideration in the development of large-scale chromatographic stationary phases.

We describe here the synthesis of a *bis*-IDA substituted TCT linker molecule and proof of concept for using this construct to affect a surface that readily coordinates transition metal ions, suggesting a means of performing IMAC separations on nylon-6 C-CP fibers. Demonstrated first is the use of a triazine-based reactive dye to illustrate the potential suitability of various polymer fiber surfaces towards the proposed triazine-based modifications. The synthetic methodology, including the IDA functionalization of TCT and coupling to the amide functionality of a nylon-6 surface, was first developed using the more easily characterizable small molecule model *N*-methylacetamide to simulate the nylon-6 surface. This allowed for the application of NMR and mass spectrometry methods to characterize the reaction products. Ultimately demonstrated is a two-step synthesis of a disubstituted version of triazine having IDA ligands that can be tethered to the surface of nylon-6 C-CP fibers. The ligand was successfully tethered to nylon-6 C-CP fibers, generating a chelating surface on the C-CP fiber (hereafter referred to as nylon-IDA C-CP fibers). The successful coupling was verified visually after exposure of the fibers to an aqueous Cu^{2+} solution. Subsequent regeneration of the surface was affected by use of an EDTA wash, with the metal ion removal confirmed by the presence of the anticipated spectral features in the UV-VIS absorbance spectrum of that wash solution. In no portion of the synthesis are the fibers subjected to chemical conditions that are

detrimental to the physical aspects of the nylon-6 C-CP fiber structure. Based on the overall simplicity of the surface modification and the low cost of the reactant chemicals, this is viewed as a viable methodology to pursue in the development of C-CP fibers in the general area of affinity chromatography, and IMAC in particular.

Experimental

Fiber Materials

Nylon-6 C-CP fibers were obtained from the Clemson University School of Material Science and Engineering (CU-MSE) [36]. C-CP fibers are melt-extruded in quantities greater than 1000 meters and stored on spools for ease of use. The experiments performed here also utilized PET C-CP films; melt extruded at CU-MSE. The PET C-CP films were utilized instead of PET C-CP fibers solely for demonstration and visualization purposes; the chemical attributes are identical to PET C-CP fibers. Finally, a cotton/polyester blend fabric was excised from a bulk lot, again obtained from CU-MSE.

Reagents

All reagents were purchased from Sigma Aldrich (St. Louis, MO) and included *N*-methylacetamide, 2-chloro-4,6-dimethoxy-1,3,5-triazine, diethyl iminodiacetate, 2,4,6-trichloro-1,3,5-triazine, Procion Red MX-5B (reactive red 2), sodium sulfate, sodium acetate, ethylene diamine and diisopropylamine. Methanol, acetonitrile, sodium hydroxide, trifluoroacetic acid and hydrochloric acid were purchased from Fisher Scientific (Pittsburgh, PA). MilliQ water (18.2

MΩ/cm) derived from a NANOpure Diamond Barnstead/Thermolyne Water System (Dubuque, IA) was utilized in solution makeup and fiber washing. Tetrahydrofuran (Fisher Scientific) that was used in reactions occurring in the dry box was purified by passing through alumina columns under a dry N_2 atmosphere with a MBraun (Stratham, NH) solvent purification system. NMR samples were prepared with either chloroform-d or deuterium oxide both purchased through Sigma Aldrich.

General Equipment and Methods

A MBraun dry box was employed for all reactions involving substitution of the triazine molecule due to its moisture sensitivity. Upon completion of substitution reactions, samples were collected and analyzed. NMR analysis was performed to validate the synthesis reactions presented using a Jeol 300 Spectrometer (Peabody, MA) operating at 75 MHz for ^{13}C and 300 MHz for ^{1}H, all NMR spectra were collected at 25 ℃ and referenced to TMS.

Mass spectrometric analysis was performed on a Microflex MALDI-TOF (Bruker Daltonics (Billerica, MA)) in the positive ion, reflectron mode. The TOF-MS is equipped with a nitrogen laser (337 nm) operated at a pulse rate of 60 Hz, with spectral acquisitions occurring at 60% power and 800 shots. The Microflex control and data analysis occurred using Compass^{TM} software (Bruker Daltonics). The samples of interest were dissolved in a solution of 0.1% trifluoroacetic acid in 50:50 acetonitrile:water (ACN:H_20) and were spotted in 1 µL aliquots onto a standard stainless steel target and allowed to dry completely. A sinapinic acid

(15 mg mL^{-1}) solution made up in 0.1% trifluoroacetic acid in 50:50 acetonitrile:water was added in a 1 µL aliquot directly on top of the analyte spot and allowed to dry. Additionally, samples were submitted to the Chemistry and Biochemistry Mass Spectrometry Center at the University of South Carolina for high-resolution mass analysis.

Synthetic Methods

Anchoring of Reactive Red 2 Dye to Fiber Surfaces

Covalent fixation of reactive red 2 took place under conditions typical of the textile dye industry, i.e. neutral pH [26]. The fibers (~ 500 mg) were placed in an aqueous solution consisting of 80 g L^{-1} sodium sulfate and 40 g L^{-1} sodium acetate at pH 7-8. A liquor ratio (LR) of 50:1 and a 2 % OMF (on mass of fiber) were employed, the fibers were placed in solution at 25 ºC and the dye bath temperature ramped to 60 ℃ at a rate of approximat ely 2 ℃ min^{-1} and held for 30 minutes. The materials being dyed were then removed from the dye bath, washed with MilliQ water, and soaked in MilliQ water for 30 minutes at 60 ℃ to remove any non-covalently bound dye. The dyed C-CP fibers were further soaked in the salt solution consisting of 80 g L^{-1} sodium sulfate and 40 g L^{-1} sodium acetate at pH 7-8 and an additional aliquot of water at 60 ℃ for 30 minutes to remove any non-covalently bound dye, neither of these aliquots showed a significant amount of dye.

Preparation of n-(4,6-dimethoxy-1,3,4-trazin-2-yl)-n-methylacetamide

In the dry box, 2-chloro-4,6-dimethoxy-1,3,5-triazine (1.41 mmol) was weighed and dissolved in 10 mL of tetrahydrofuran (THF), and to this solution an equal molar amount of N-methylacetamide (1.41 mmol) was added. Upon combination of the two reactants, diisoproplyamine (DIPA, 7.14 mmol) was added to the mixture along with an additional 10 mL of THF. The reaction was stirred at 25 ℃ for 16 hours in the dry box, after which time the hazy white solution was vacuum filtered. The filtrate was removed from the dry box and all volatiles were removed under reduced pressure to yield a white solid (0.382 g, 82.2 %).

Preparation of Intermediate 1 (I1)

Trichlorotriazine (TCT, 1.31 g, 7.1 mmol) was dissolved in THF (10 mL) inside of the dry box, with two molar equivalents of diethyl iminodiacetate (3.40 mL, 18.9 mmol) added to the TCT solution. To this solution, 42.7 mmol of DIPA (6.0mL) was added and the reaction stirred under nitrogen for 16 hours at 25 ℃. At completion, the hazy white solution is vacuum filtered, followed by THF removal under vacuum yielding a yellow/orange oil. This oil is dried at 50 ℃ and 27 mm Hg for 12 hours, yielding a semicrystalline solid (3.04 g, 87.4 %). A portion of the sample was dissolved in chloroform-d and analyzed by ^{1}H NMR.

Preparation of Product 1 (P1)

Cleavage of the ethyl groups from **I1** generates a molecule capable of chelating metals through the generated carboxylic acid moieties. **I1** (0.1 mmol) was refluxed at 80 ºC with 3 mmol sodium hydroxide (NaOH) in 50 mL of methanol. After 24 hours the reaction was first gravity then vacuum filtered, followed by solvent removal with a rotary evaporator apparatus. The sample is then prepared for NMR spectrometric analysis using a solution of 1% (v/v) HCl in D_2O.

Preparation of Intermediate 2 (I2)

N-methylacetamide (0.14 g, 1.9 mmol) and **I1** (0.45 g, 0.92 mmol) were combined in the glove box and dissolved in 10mL of THF. Upon dissolution, DIPA (0.65 mL, 4.6 mmol) was added and the reaction stirred under nitrogen for 16 hours at 25 ℃. The hazy white solution was vacuum f iltered under nitrogen. The solvent was removed from the amber colored solution using a rotary evaporator to, yield an amber colored crystalline solid (0.395 g, 81.2 %). A portion of the sample was dissolved in chloroform-d and analyzed by ^{1}H NMR spectrometry.

Preparation of Product 2 (P2)

Base catalyzed ester hydrolysis of **I2** was undertaken in methanolic sodium hydroxide under reflux. **I2** (0.1 g, 0.2 mmol) was refluxed at 80 ℃) with 6.8 mmol NaOH (0.27 g) in 40 mL of methanol. After 24 hours, the reaction was filtered twice, first using gravity then vacuum filtration, followed by solvent

removal under reduced pressure. The dried product was then sampled and prepared for MALDI analysis as described earlier.

Generation of Nylon-IDA C-CP Fiber Surface

Covalent fixation of **I1** to the nylon-6 C-CP fiber surface occurred using typical textile dye conditions as described in the *Anchoring of Reactive Red 2 Dye to Fiber Surfaces* section [26]. Once **I1** was tethered to the nylon-6 C-CP fibers the ethyl groups were cleaved to allow for metal chelation. Cleavage of the ethyl occurred using a methanol- sodium hydroxide reflux. **I1** tethered to nylon-6 fibers (assuming a tethering efficiency of 100%, 0.17 mmol of **I1** would be present) were refluxed in 30 mL of methanol containing 10.5 mmol NaOH for 16 hours, after which time the fibers were washed with copious amounts of water and allowed to air dry.

Metal Interaction with Nylon-IDA C-CP Fibers

Nylon-IDA C-CP fibers were allowed to soak for 10 minutes in a 0.1 M $CuCl_2$ solution, after which time the excess solution was squeezed back into the beaker. Fibers were rinsed under running milliQ water to remove unbound Cu^{2+}. After rinsing, the fibers were soaked for 10 minutes in a 0.1 M EDTA solution to remove chelated metal. To determine the extent of metal chelation to the fiber surface, the water rinse and the EDTA desorption solution were analyzed on a Genesys UV-VIS absorbance spectrophotometer (Thermo Scientific, Waltham,

MA) with the concentration calculations performed using a molar absorptivity of 15.5 x 10^3 L mol^{-1} cm^{-1} at 730 nm [37].

Results and Discussion

Assessment of native polymer surface activity

Initial investigations into the use of trichlorotriazine as a covalently-tethered linker molecule to C-CP fiber support phases involved an evaluation of the availability/density of the necessary functional groups (i.e. amide, carboxylic acid, hydroxyl, esters, amines) on the fiber surface. Based on their native functionality, PET and nylon-6 based C-CP film and fiber materials were initially tested. A cotton/polyester blend textile material was utilized as a control fabric as the tethering chemistry of reactive dyes to cotton is well understood. Based on its use of a triazine linker group, Procion Red MX-5B (reactive red 2) dye was selected for probing of the film/fiber surfaces. Successful fixation of reactive red 2 to the surface of these materials demonstrates the availability of the active surface groups. Reactions took place under standard conditions employed for such reactive dyes [26]. The current system exhibits reactivity similar to commercial textile materials, taking place at neutral pH to avoid the undesired C-CP material degradation that results in highly caustic solutions. In addition to probes of the native PET and nylon-6 surfaces, well characterized treatments of the PET surfaces were employed to affect greater activity toward the triazine-based dye binding (i.e., generation of greater numbers of active groups on the fiber surfaces). It is well known that exposure of PET to sodium hydroxide

results in the generation of hydroxyl and carboxylic acid end groups [34, 38, 39]. This strategy was expected to produce similar reactive groups at the surface of the PET C-CP fibers, but such benefit could be accompanied by potential fiber degradation. In order to assess the balance between surface reactivity and fiber degradation, PET C-CP films were exposed to 1 M sodium hydroxide for 1, 2, and 3-hour increments. Based on reports by Bide [38], PET films were also exposed to ethylene diamine (EDA) as an additional means to activate the surface. In this case generation of an amide, a primary amine, and the hydroxyl group was expected to occur.

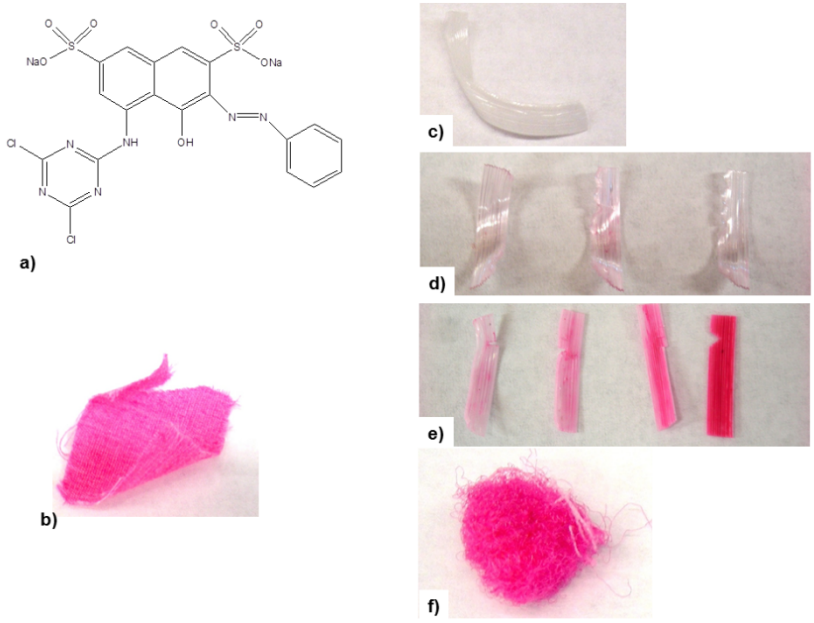

Figure 4.2. Photographs depicting the coupling of Procion Red MX-5B (reactive red 2) dye and C-CP film and fiber materials. a) chemical structure of the triazine-based dye molecule, b) commercial cotton polyester blend fabric following exposure to the dye, c) native PET C-CP film following exposure to the dye, d) PET C-CP films digested with 1 M sodium hydroxide (NaOH) for 1, 2, and 3 hours (left to right) and treated with the dye, e) PET C-CP films digested with a 100% ethylene diamine (EDA) solution for 10, 20, 30, and 60 seconds (left to right) and treated with the dye, and f) native nylon- 6 C-CP fibers after exposure to the dye.

As seen in the photographs presented in Fig. 4.2, there is a range of responses for the different film/fiber surfaces to the triazine-based commercial dye whose structure is presented in Fig. 4.2a. In the case of the positive control (Fig. 4.2b), the cotton/polyester fabric shows clear, yet heterogeneous, dye fixation. Closer examination of the fabric reveals the expected dye modification of the cotton threads making up the swatch, while the polyester threads remain unaffected. Simply, the cotton fibers contain large numbers of active hydroxyl groups, while the polyester (as processed in this fabric) do not. Consistent with the control sample, there is no appreciable indication that the dye reacts with the untreated PET C-CP film (Fig. 4.2c). Exposure of the PET films to 1M NaOH for progressively longer periods of time does yield a surface that is mildly accommodating for dye fixation (Fig. 4.2d). While such a treatment would be easy to employ in practice, treatment of micron-diameter PET C-CP fibers for such extended periods of time results in embrittlement of the fibers to the end effect of lower loading levels than seen for the cotton fibers in the fabric. It is only upon performing the amine-based activation, utilizing EDA, that appreciable amounts of dye fixation are achieved (Fig. 4.2e). The treatment of the PET surface using EDA results in a breakdown of the fiber backbone to produce the necessary functional groups for reactivity. It is important to note that the reaction of EDA with the PET surface is rapid and efficient (1 minute), however, as in the case of NaOH activation, excessive exposure results in formation of a brittle film. Different from the case of the native PET C-CP materials, uniform tethering of the

reactive red 2 dye to the surface of a *native* nylon-6 C-CP fiber is demonstrated in Fig. 4.2f. The bundle of fibers exposed to the dye is readily wet in aqueous solution, with the fixation occurring under the desired ambient conditions. Since these experiments demonstrate that nylon-6 C-CP fibers can be utilized in their native form, the remainder of this work focuses solely on their implementation as the polymer support for modification approaches using triazine-based chemistry.

Use of N-methylacetamide as a model compound for TCT-nylon-6 coupling

As is well known throughout the history of textile materials, organic moieties can interact with the surfaces of natural and synthetic polymers through a wide variety of interactions. In order to achieve the desired result of having a tethered, solution accessible chelate, the adhesion to the surface must be through the TCT linker molecule, and not random adsorption, to facilitate solute/chelator interactions. By the same token, fixation of the TCT to the surface followed by attachment of the chelates would face kinetic limitations as sequential substitutions of the remaining chlorine atoms become disproportionally more difficult. There might also be the expectation of steric effects if such reactions were run on a saturated surface. Therefore, a strategy was employed to generate the TCT-IDA species prior to fixation to the fiber surface. In order to ensure that the fundamental ligation chemistry of the IDA to the triazine linker molecule could be appropriately affected, a solution-based approach to the proposed coupling chemistry was explored. Specifically, the basic coupling chemistry was tested using a small-molecule model compound.

This involved reacting commercially available dimethoxytriazine with *N*-methylacetamide, a small molecular reactivity mimic of the primary amide units in the target nylon-6. Figure 4.3 shows the reaction scheme using 2-chloro-4,6-dimethoxy-1,3,5-triazine and *N*-methylacetamide. The degree of substitution of the trichlorotriazine molecule influences its overall reactivity, so 2-chloro-4,6-dimethoxy-1,3,5-triazine provides an accurate and convenient model for the target reactions.

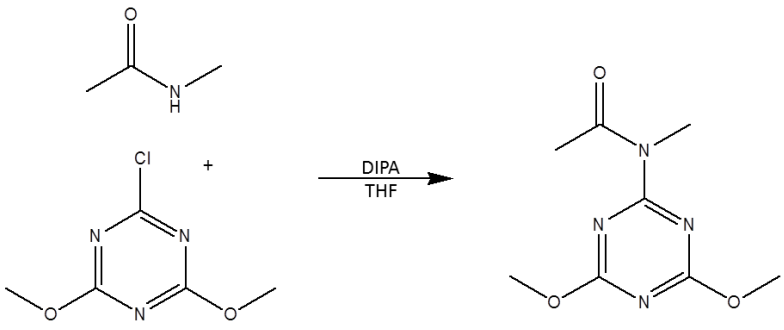

Figure 4.3. Proof of concept reaction scheme, coupling 2-chloro-4,6-dimethoxy-1,3,5-triazine and the n-methylacetamide “nylon” surrogate.

The ^{1}H NMR spectrum (Supplementary Data section (A-1)) of the product, *N*-(4,6-dimethoxy-1,3,4-trazin-2-yl)-*N*-methylacetamide indicates successful product formation with minor residual diisopropylamine (DIPA). As this was a proof of concept reaction without desire to isolate analytically pure material, no additional purification steps were undertaken. The results indicated that the proposed coupling between the di-substituted TCT and the amide moiety could be readily affected.

Synthesis of di-substituted TCT having iminodiacetic acid chelating groups

The dye fixation experiment (Fig. 4.2f) demonstrated that the nylon-6 surface was capable of triazine modification while the solution based chemistry utilizing n-methylacetamide demonstrated the coupling chemistry should occur through the amide bond on the nylon-6 surface. With this knowledge a synthetic path forward for the generation of the TCT-based chelating ligands was pursued as depicted in Fig. 4.4.

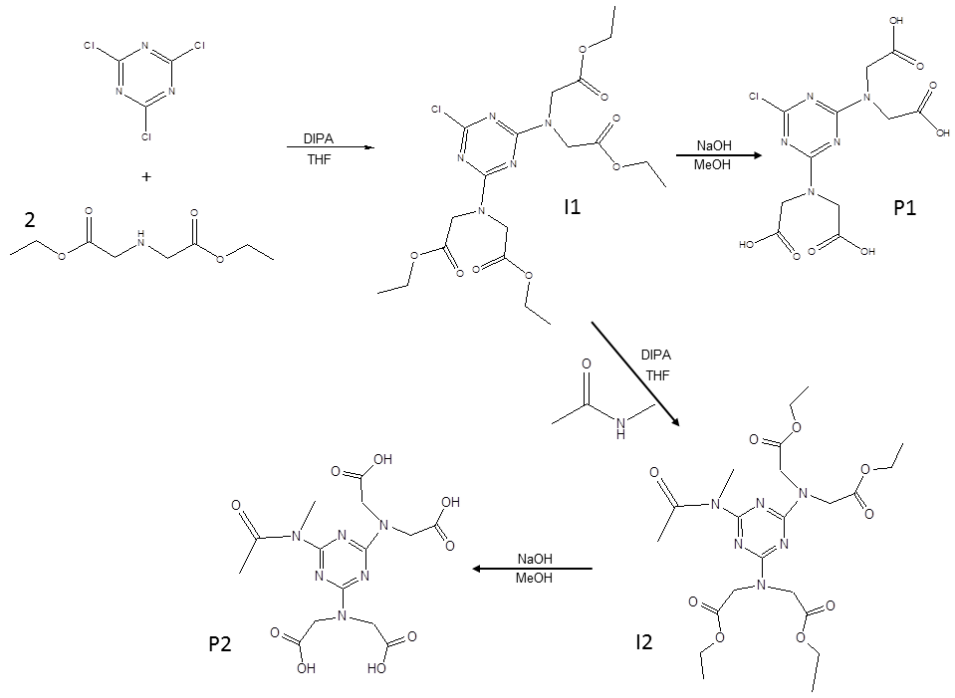

Figure 4.4. Reaction schemes for generation of the metal chelating, triazine-based linker molecule.

In order to ensure that the linkage of the chelator (IDA) occurred through the secondary amine and not one of the carboxylic acid groups, the substitution reactions were employed using the protected form of IDA, diethyl iminodiacetic acid. The formation of the desired ligand is a two-step process that starts by

linking the ethyl-protected chelator moiety, diethyl iminodiacetate, to the trichlorotriazine molecule. The second step involves the cleavage of the ethyl groups on the diethyl iminodiacetate to generate the carboxylic acids necessary for chelation. Due to the moisture-sensitive nature of TCT, reactions were carried out in an inert atmosphere to minimize hydrolysis.

As discussed previously, the trichlorotriazine molecule is highly reactive due to the decreased electron density of the carbon atoms. Therefore, the replacement of a chlorine atom with a more electron donating group, in this case the nitrogen of the diethyliminodiacetic acid, decreases the reactivity of the triazine molecule. This results in the sole generation of the mono-substituted form before appreciable di-substitution occurs. Therefore, addition of the proper number of equivalents of diethyliminodiacetic acid to trichlorotriazine (2:1) over a sufficiently long period of time, suggests that the di-substituted intermediate would be produced (designated as I1 in Fig. 4.4). As demonstrated by the ^{13}C NMR spectrum presented in A-2, the anticipated product is indeed recovered with no appreciable indications of side-products. High resolution mass spectrometry (HRMS) analysis of the product yielded the expected molecular mass of 490.1704 Da, with an error (E) equal to 1.2 ppm.

In order to affect the primary chelation of the his-tag capturing metal ion, there species must be a primary coordination with the desired tridentate IDA species. Therefore the ester I1 was subjected to a sodium methoxide reflux, resulting in cleavage of the ethyl groups and generation of the desired carboxylic

acids to yield the product designated at P1. The supporting ^{13}C NMR spectrum is presented in A-3, reflecting the successful transformation of the ester to the acid form of IDA. As might be anticipated, the reaction conditions employed in the ester cleavage could also result in the hydrolysis of the remaining chloro-group. There were no indications of the unwanted conversion from a C-Cl to a C-O bond in the NMR spectra, but indeed that product was confirmed by HRMS analysis (E=0.3 ppm). Unfortunately, this particular strategy rendered the triazine effectively passivated towards the desired goal of further coupling of the IDA-TCT ligand to the nylon-6 fiber surface.

To avoid this synthetic dead end, tethering of the diethyl iminodiacetate-modified triazine to the nylon-6 surface prior to cleavage of the ethyl groups is required. As described previously, the n-methylacetamide coupling reaction (Fig. 4.3) is proof of concept in terms of the solution chemistry. As depicted in the lower portion of Fig. 4.4, successive reaction of n-methylacetamide with intermediate I1 would be anticipated to yield intermediate I2, with a subsequent sodium methoxide reflux hydrolyzing the ester to the desired di-substituted iminodiacetic acid-triazine molecule bound to the N-methylacetamide product, P2. The desired intermediate I2 was indeed confirmed via ^{13}C-NMR as presented in A-4. The subsequent ethyl group cleavage was successful (without hydrolysis of the amide bond) resulting in the di-substituted iminodiacetic acid based triazine ligand tethered to the “nylon monomer” (P2), as confirmed by MALDI-MS as presented in A-5.

Upon successful completion of the solution-based synthesis, tethering of the TCT portion of the ligand to the nylon fiber surface was attempted. Intermediate 1 was tethered to the nylon-6 C-CP fiber surface under the same conditions discussed previously for the reactive red 2 dye. Upon completion of the tethering experiments the C-CP fibers were refluxed in a sodium methoxide solution to cleave the ethyl groups, generating the carboxylic acid functional groups, yielding the nylon-IDA C-CP fibers necessary for metal chelation. While there are a number of metal ions capable of affecting an IMAC separation, the nylon-IDA C-CP fibers were exposed to a 0.1 M $CuCl_2$ solution to incorporate Cu^{2+} onto the surface. The selection of Cu^{2+} was made because the green color of that ion in aqueous solution means that chelation can be readily quantified by UV-VIS spectroscopic analyses. Figure 4.5a is a digital photograph of the nylon-IDA C-CP fibers after exposure to the copper solution, while Fig. 4.5b shows the results following exposure of un-modified nylon fibers to the copper solution as a control case. The nylon-IDA C-CP fibers have a blue-green tint to them, while the unmodified nylon fibers remain bright white, visually demonstrating successful tethering of the IDA-triazine chelating ligand to the nylon C-CP fiber surface. Aqueous washing of the modified fibers resulted in no visible change in color.

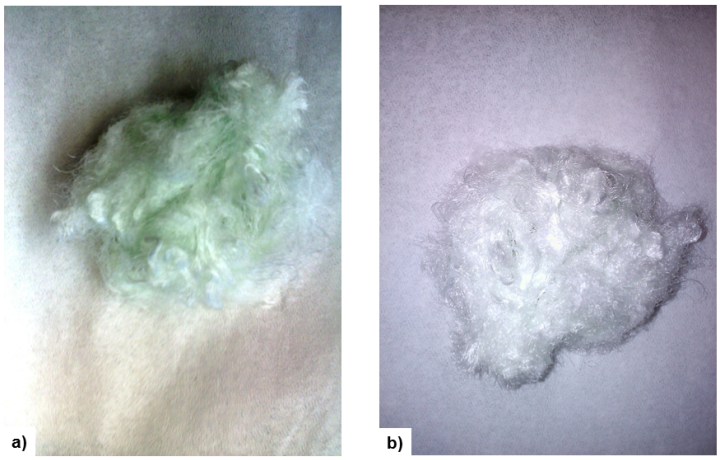

Figure 4.5. Digital photograph of A) nylon-IDA C-CP fibers and B) native nylon-6 C-CP fibers after exposure to Cu^{2+} through soaking in a 0.1 M $CuCl_2$ solution.

The final assessment in the concept of preparing a nylon-IDA surface that could be suitable for use in IMAC separations involved the demonstration that the bound Cu^{2+} species could be recovered by conventional means (an EDTA wash), as well as suggesting the surface density of ions. UV-VIS adsorption spectra were acquired from 400-800 nm, with the characteristic adsorption band for the EDTA-Cu^{2+} complex at 730 nm being the means of performing the quantitative measure. Three different controls, EDTA solution alone, native nylon-6 following Cu^{2+} exposure and a water rinse, the IDA-modified surface with Cu^{2+} exposure and a water rinse, and the ultimate EDTA rinse of the bound Cu^{2+}, were all evaluated. In order to minimize kinetic effects in the metal loading process, both types of nylon-6 C-CP fibers (native and IDA-modified) were soaked in excess $CuCl_2$ (0.1 M) for 10 minutes. Once chelation had occurred, the fibers were soaked in water to remove any non-chelated copper ions followed by elution using a 0.1 M EDTA solution. Figure 4.6 presents the adsorption responses for

the water and EDTA desorption washes for both the nylon-IDA and the bare nylon surfaces along with a scan of the EDTA solution that was not exposed to the fibers. Only in the case of the EDTA elution of copper from IDA-modified nylon-6 C-CP fibers is there any spectroscopic indication of Cu^{2+} in the wash solutions.

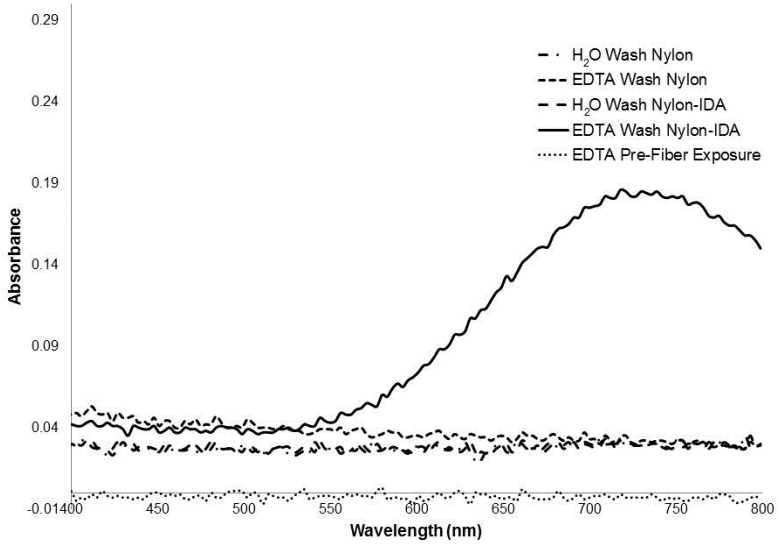

Figure 4.6. UV-VIS adsorption spectra of wash solutions in the region of the EDTA-Cu^{2+} absorbance signature; native nylon-6 C-CP fibers exposed to Cu^{2+} and treated with DI-H_2O and EDTA washes, nylon-IDA C-CP fibers exposed to Cu^{2+} and treated with DI-H_2O and EDTA washes

The amount of copper chelated and eluted from the fiber surface is relatively low, 16.5 mg Cu^{2+} (~0.26 µmol) per g of nylon C-CP fiber, in comparison to previous poly(acrylic acid)-modified PET C-CP fiber tips [40]. Additional EDTA washes revealed insignificant further release. This capacity translates into a ligand modification density of 0.23 µmol/m^2. Each triazine-modified chelating ligand tethered to the surface of the nylon-6 fibers possesses

two IDA moieties each capable of chelating one Cu^{2+} ion. Based on the aqua color of the Cu^{2+} exposed fibers (Fig. 4.5a), it is suggested that the ions are coordinated in an octahedral geometry (tridentate with regards to a single IDA), with the remaining three coordination sites occupied by water molecules. By this observation, coordination between two IDA moieties, either on the same or different TCT linkers, is considered highly unlikely. There are certainly many potential reasons for the low binding density at this point, including non-optimized TCT binding conditions (LR and OMF), incomplete cleavage of the protecting ethyl groups, or the fact that the IDA surface groups were not fully loaded with Cu^{2+} (based on kinetic or steric factors). All of these aspects will be investigated in future works directed specifically at IMAC applications.

Conclusions and Future Work

Presented here is a synthetic approach to the successful generation of a di-substituted iminodiacetic acid triazine based chelating ligand. The synthesis approach taken here generates the protected chelating molecule prior to attachment to the nylon monomer or nylon-6 C-CP fiber surface. There are several benefits to this general approach, primarily the ability to perform solution-based characterization of the chelating ligand to ensure a pure di-substituted triazine molecule is achieved prior to attachment to the fiber surface. Chelation of the IDA disubstituted triazine molecule to nylon-6 fiber was completed successfully under conditions which pose no threat to the physical nature of the fibers themselves. However, the low chelation capacity must be increased before

these fibers are capable of being employed as an IMAC stationary phase. The separate synthesis of the IDA-TCT complex prior to tethering allows for greater levels of overall control of the final product. In order to increase the overall ligand loading capacity experimental parameters will be evaluated starting with the OMF and LR combinations. This will include fixation of the chelating ligand under a variety of conditions to include both neutral dye fixation and organic solvent conditions. In theory, for each chelating ligand attached to the fiber surface the extraction of two protein molecules is possible using IMAC conditions, and so even greater capacity may be realized than mono-substituted ligands that are commonly employed. This assumes that there is no steric hindrance or protein interaction between the adjacent molecules. To evaluate this and ensure the highest capacity IMAC HPLC stationary phase is generated the mono- and di-substituted forms of the chelating ligand will be tethered to the surface and evaluated as well.

As noted in the Introduction, the use of TCT-based immobilization chemistries has found a number of uses in chemical separations. This general strategy, when employed to C-CP fibers, may present a very attractive path towards the general goals of affecting affinity chromatography stationary phases having low production costs, high permeability (avoiding fouling and clogging), and highly efficient mass transfer characteristics. By the same token, this methodology can be implemented on other stationary phases, so long as they contain electronegative functionalities, such as amide and hydroxyl groups.

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CHAPTER 5

FUNCTIONALIZED LIPID MODIFICATION OF CAPILLARY-CHANNELED POLYMER (C-CP) FIBERS FOR HIGHLY SELECTIVE ANALYTE EXTRACTIONS

Introduction

Polymeric stationary phases, specifically fiber based polymers, for high performance liquid chromatography (HPLC) have been under investigation for over fifty years.[1] Polymer phases have been evaluated and implemented for the separation of ions, small molecules, and macromolecules[2-7]. In the most common implementations, polymeric stationary phases take the form of hollow[8, 9] or cylindrical solid fibers[10, 11], rolled fabric[4, 12, 13], fiber staples[14], and continuous fiber phases[5, 15, 16]. These polymeric phases generally offer the same benefits as the more traditionally accepted HPLC stationary phases of porous silica based materials; including chemical robustness and ease of chemical derivatization. In a fiber format, additional features include improved mass transfer due to their nonporous nature and convective diffusion through the column structure[17, 18].

The specific type of polymer fiber under investigation here for applications in HPLC[7, 19-24] and solid phase extraction (SPE)[25-27] is the capillary-channeled polymer (C-CP) fiber. C-CP fibers are effectively non-porous (relative to the size of proteins), having average diameters of 35 - 50µm, and a very unique shape that includes 8 channels (Fig. 5.1a).

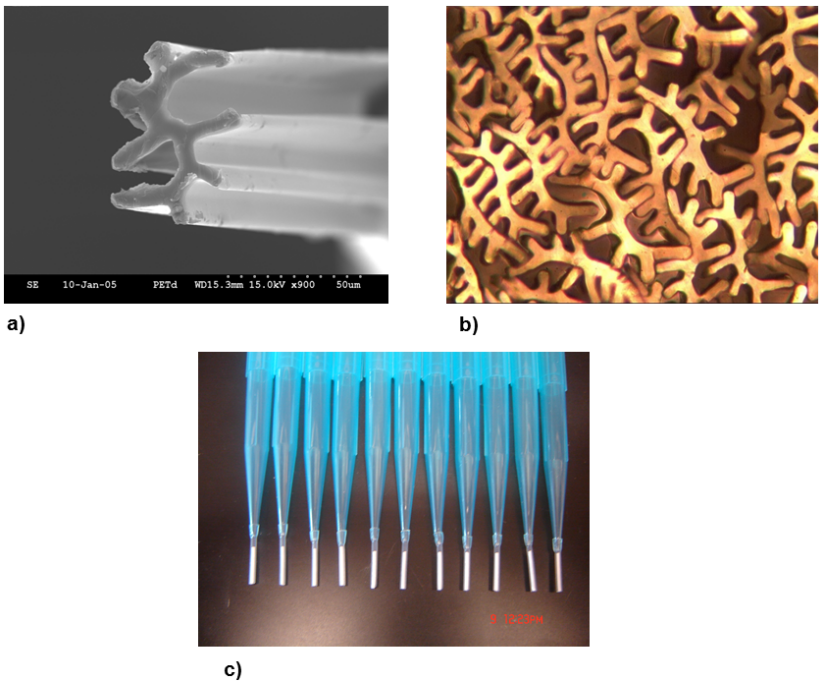

Figure 5.1. a) Scanning electron micrograph (SEM) image of the cross section of a capillary-channeled polymer (C-CP) fiber. b) Image of a packed column of C-CP fibers demonstrating the interdigitation C-CP fibers exhibit c) Image demonstrating SPE tip C-CP fiber format

These channels on the perimeter of the fibers allow for self-alignment of the fibers when packed into an HPLC column, with interdigitation of the fibers (Fig. 5.1b) resulting in a distribution of micron-sized open channels that run the entire length of the column. C-CP fibers have 2-3x more surface area in comparison to circular cross section polymer fibers of the same nominal diameter[18, 21, 28]. The capillary channels of the C-CP column are very efficient at fluid transport, allowing for traditional HPLC column sizes to be operated at linear velocities of > 25 mm s^{-1}, while maintaining back pressures of < 2000psi [6, 29]. As a result, these C-CP columns provide a high throughput and high efficiency separation media for biomacromolecules.

C-CP fibers are melt-extruded from a variety of base polymers, with nylon-6[24, 30, 31], polyester (PET)[7, 21, 28], and polypropylene (PP)[28] used in applications of macromolecule separations. The variety of base polymer choices allows application of C-CP fibers as an HPLC stationary phase in reversed phase (RP), ion-exchange (IEC) and hydrophobic interaction (HIC) chromatography modalities. Beyond the variety of interactions obtained solely through the polymers' native form, there is a desire to perform highly selective separations and extractions. Recent research has focused on the ability to modify the surface of the C-CP fibers, allowing for more specific analyte-surface interactions[27]. Surface modifications to C-CP fibers capitalize on the high mass transfer rates, ease of column assembly, and low cost to generate a stationary phase that can be tailored to specific analyte interactions without sacrificing the fluidic properties of the fibers/columns. While there exists a large portfolio of surface modification methodologies found in both the textile and bioanalytical chemistry literature, most of these tend to be quite specific[32-40]. It would be desirable that a more generalized approach to surface modifications, while still imparting specificity, could be realized.

C-CP fiber modification has been approached through active end group generation including aminolysis, hydrolysis, and exposure to strong bases (i.e. NaOH or permanganate)[32, 34, 41, 42]. These straightforward approaches produce a high, in some cases too high, density of –COOH, -NH, -OH, -CONH on the fiber surface for either analyte interaction or further modification processing under mild

ambient conditions. However, these approaches to fiber modification are detrimental to the physical structure of the C-CP fibers by breaking down the basic polymer/fiber backbone[35]. The well-established approach of plasma grafting to polymer surfaces has been evaluated as a means to generate tailored surfaces[43, 44]. The ability to graft a functional moiety to a polymer surface provides for a large variety of possible functional groups and generally speaking does not destroy the polymer backbone. For example, a grafting-to approach has been evaluated utilizing polyacrylic acid (PAA) as a means of generating an anionic surface to immobilize transition metal ions from aqueous solution[27]. The C-CP fiber and column structure provided a challenge in the grafting process, resulting in a non-uniform oxidation and therefore heterogeneous distributions of active sites throughout the column.

The solution-based functionalized lipid adsorption approach reported here is advantageous since modification of the PP C-CP fibers was performed after assembly in a column format and subsequently segmented for attachment to micropipette tips following functionalization of the C-CP fiber surface. Functionalized lipids are prominent in biologically-based research[45-55]. These amphipathic molecules have been used in diverse applications including diagnostic assays[54], coatings on hydrophobic nanoparticles[50], and lipid bilayer modification of capillary electrophoresis (CE)[47, 56, 57]. The commercial availability of these functionalized lipids and the wide range of available head groups provide easy access to many potential surface modifications using one basic

approach. The use of functionalized lipids as means of modifying polymer surfaces for selective capture has not been described previously.

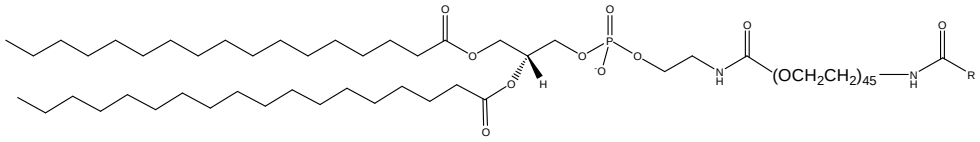

Figure 5.2. Structure of a head group functionalized polyethylene glycol lipid

We report the results for the surface modification of polypropylene (PP) C-CP fibers using functionalized lipids to impart significantly greater selectivity than the predominantly hydrophobic interactions between biomolecules and the PP surface. PP C-CP fibers were chosen for their relative chemical inertness and highly hydrophobic surfaces, which should provide an ideal support for the adsorption of the aliphatic tails of the functionalized lipids. Specifically, PP C-CP fibers were modified with two variants of lipids, demonstrating a high level of selectivity for protein capture via two types of affinity interactions. A biotin PEG-lipid was used to capture streptavidin, labeled with Texas Red (SaV-TR), based on its high affinity for the biotinylated surface. Additionally, a lipid modified with a metal-chelating head group was utilized to modify the PP C-CP fibers as a metal affinity chromatography (IMAC) stationary phase. The two lipid head groups utilized here, affecting different means of chemical selectivity, demonstrate a simple, generalized approach to the functionalization of C-CP fibers to affect high-specificity stationary phases. Surely, the diversity of potential functionalized lipid head groups, and further derivatization of same, suggests broad chemical applicability. It is envisioned that this approach can be implemented in many

different systems, ranging from clinical diagnostics on fiber tips to C-CP fiber columns used in preparative chromatography.

Experimental

Chemicals and Reagents

Polyethylene glycol lipids (PEG) modified to contain either a biotin (1,2-Dimyristoyl-sy-Glycerol-3-phosphoethanolamine-N-[biotinyl (polyethylene glycol)-2000]) or a methoxy (1,2-Dimyristoyl-sy-Glycerol-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000]) head group, (referred to as PEG-Biotin and PEG-OMe) were purchased from Avanti Polar Lipids (Alabaster, AL). Additionally, PE-DTPA (1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-N-diethylenetriaminepentaacetic acid) pre-coordinated with Cu^{2+} was purchased from Avanti Polar Lipids. Working solutions, 5 µg mL^{-1}, of each lipid were prepared in a 50:50 ethanol:water solution. Ethanol was obtained from Fisher Scientific (Pittsburgh, PA) and MilliQ water (18.2 MΩ cm^{-1}) was derived from a NANOpure Diamond Barnstead/Thermolyne Water System (Dubuque, IA). Bovine serum albumin (BSA) was obtained from Sigma Adrich (Milwaukee, WI) and prepared to a working concentration of 5 µg mL^{-1} in phosphate buffered saline (PBS) consisting of 140 mM NaCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, and 2.7 mM KCl at pH 7.3 (Sigma Aldrich, Milwaukee, WI). Two fluorescent proteins (5 µg mL^{-1}) were utilized for detection, streptavidin labeled with Texas Red (SAv-TR) and enhanced green fluorescent protein with a hexahistidine-tag (his-tag EGFP) (obtained from an in-house *E. coli* cell culture), were employed as the

capture species on the PP C-CP fiber surfaces modified with PEG-Biotin and PE-DTPA respectively. Modification studies utilizing the affinity of SAv protein solutions for biotin were prepared in a PBS solution. Experiments performed to reduce nonspecific binding to the C-CP fiber surface utilized a SAv protein solution in 0.1% Tween20 (Rockland-inc.com, Gilbertsville, PA) PBS (PBST) solution. Modification studies utilizing the PE-DTPA employed a his-tag GFP prepared in a 20 mM Tris-HCl buffer (Teknova, Hollister, CA).

C-CP Fiber Tip Preparation

The procedure for first preparing microbore C-CP fiber columns, and subsequent implementation as SPE tips has been described previously. [25, 27, 58] Previous research has shown that C-CP columns having interstitial volumes of $\varepsilon_i \approx 60\%$ are optimal for macromolecule separations. In this case, a total of 658 PP fibers were packed to extend entirely through a 300 mm long, 0.8 mm i.d. fluorinated ethylenepropylene (FEP) capillary tube (Cole Palmer, Vernon Hills, IL), referred to here as the *fiber column*. The initial fiber column format can be employed readily in-line with an HPLC pump to clean and rinse the fibers from any latent spin finish from the extraction process. By the same token, this is an ideal format for modifying large segments of fiber for either HPLC or SPE applications. In order to minimize chemical use in these demonstrations, the fiber column was segmented and the fibers used in a *tip* format. Following cleaning, the column was cut into 1 cm lengths with an additional 6mm gap at one end. This gap allows the FEP tubing to be press-fit on to the end of the

commercial low-retention micropipette tip (Redi-tip, Fisher Scientific, Pittsburgh, PA) as described previously[25, 27], Fig 5.1c. It should be noted that PP C-CP fiber tips of these geometries have been shown to have binding capacities on the order of 1.5 μg, for a range of proteins[26].

Fiber Functionalization

PP packed C-CP fiber tips were functionalized through a basic adsorption process, utilizing the affinity of the aliphatic lipid side chains for the hydrophobic polypropylene surface. Three lipids were employed in these experiments, PEG-Biotin, PEG-OMe, and PE-DTPA based on the desired analyte interactions. The lipid solutions were prepared to working concentrations in a 50% water:ethanol solvent, balancing their solubility with the tendency for surface adsorption. The lipid solutions were exposed to the fiber tips in 1 mL aliquots and centrifuged (Clinical 50, VWR, West Chester, PA) at 1200 relative centrifugal force (RCF) for 3 minutes. This approach allows for the solvents to pass uniformly through the fiber interstices so that surface modification can occur uniformly. As noted above, chemical modification of complete fiber columns would increase the experimental throughput, with the individual tip modification process limited by the capacity of the centrifuge.

Experimental Outline

The demonstration experiments presented here utilized three different fiber columns per experiment; an analyte specific column (PEG-Biotin or PE-DTPA), a control column (PEG-OMe), and a bare PP column. The functionalized

columns were washed with PBS and then a BSA block was applied. In each case, 1 mL solutions were passed through the tips as described above. The BSA block ensures that the analyte protein exposed to the surface during the experiments does not adsorb itself to the bare PP due to hydrophobic interactions. Cursory studies indicated that future work will be able to disregard the BSA block once the adsorption capacity is determined and optimal PEG-lipid surface coverage is achieved. After a buffer wash the fibers are exposed to the fluorescently labeled protein test solutions. Finally, all tips were washed with buffer prior to imaging to remove residual, nonspecifically bound, fluorescent protein from the fiber tips.

Images and Statistical Data

C-CP fiber packed tips were removed from the commercial micropipette tip and taped onto a glass microscope slide. Fluorescent images of the fiber tips were generated on an Olympus IX71, 4x/0.13 UPlanFI (infinity corrected) objective (Olympus, Center Valley, PA). Fluorescent excitation was achieved using a Xe arc lamp with spectrophotometer filters (Chroma, Bellows Falls, VT) set at excitation 575/25 and emission 624/40 for SAv-TR or excitation 494/20 and 531/22 for EGFP. An Ocra-ER (Hamamatsu) CCD camera was used for detection, images were processed and statistical data extracted using Slidebook 5.0 (Denver, CO).

Results and Discussion

C-CP fibers were evaluated as a support for functionalization through hydrophobic interactions of the PP surface and the aliphatic portion of the functionalized lipid. It is assumed that the lipid tail will lay along the fiber surface while the functionalized head group would remain in free solution, allowing for analyte interaction. Initial proof of concept was demonstrated utilizing the well understood interaction and high affinity of biotin and SAv. PP C-CP fibers were coated with a PEG-biotin lipid, as previously described. Additionally, two controls (PEG-OMe and PP) were utilized during these experiments to assure the fluorescence measurements obtained were a result of the biotin-SAv specific interactions and not due to nonspecific binding. All tips were exposed to 1 mL of the SAv-TR solution and a 1 mL buffer wash. Figure 5.3 depicts representative fluorescence images of the tips for the three test cases (PEG-biotin, PEG-OMe, and PP, 5.3a – c respectively). As can be seen, there are pronounced differences in the intensities of the fluorescence images, which are quantified in the graph of Fig. 5.3d for triplicate experiments. A statistically-significant difference between the columns modified with PEG-Biotin and the two control columns analyzed is presented. The two control columns are not significantly different, but the trend that the BSA-PP surface affects more non-specific binding suggests that hydrophobic and ionic interactions play a role. The amount of BSA exposed to the surface was ~200 times the expected binding capacity of a 1 cm packed PP C-CP tip[26], sufficient to coat the fiber surface. It is likely at this

concentration that protein-protein stacking occurred between the BSA molecules coating the fiber surface. In addition, protein-protein interactions between the SAv capture protein and the BSA already on the surface of the C-CP fibers is likely.

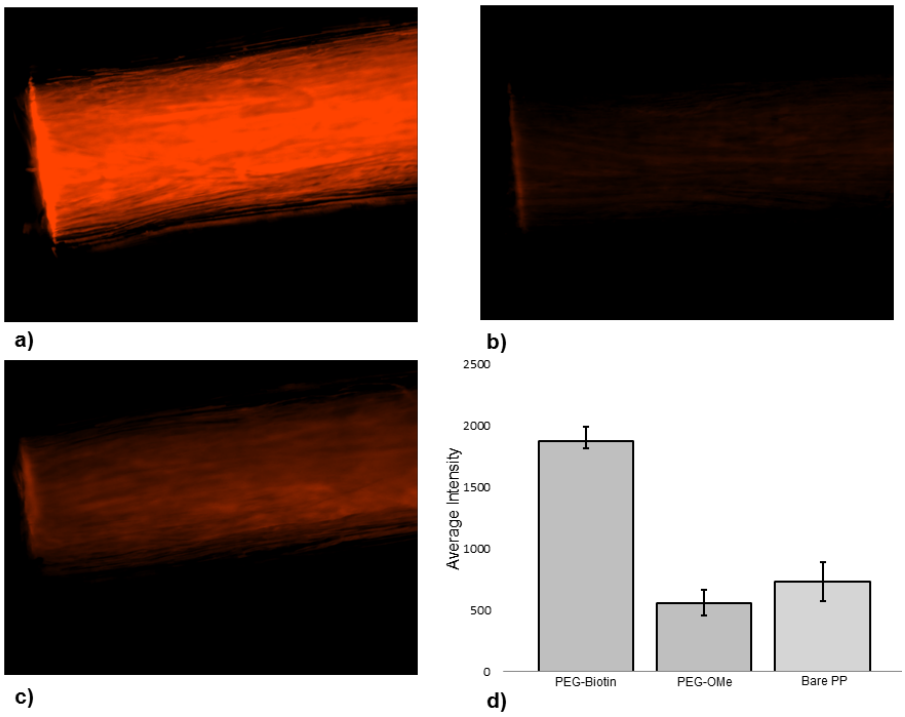

Figure 5.3. Fluorescent Images of PP C-CP fibers after modification using head group modified PEG lipids, experiment employed a BSA block to decrease nonspecific binding, all fibers were exposed to SAv-TR. a) Biotin modified PP C-CP fibers. b) PEG-OMe modified PP C-CP fibers. c) Native, unmodified PP C-CP fibers. d) Average intensity response for each column, collected from an identical size mask down the center of each image

Given there is a directional aspect to the initial PEG deposition, as well as to the loading of the target analytes, there is a natural question as to the homogeneity of the overall processes as a function of distance down the fiber tips. In order to assess the uniformity of the surface modification/analyte immobilization, three representative images were taken along the 1 cm fiber tips.

The statistical data depicted in Fig. 5.4 indicate, that for all three surface-types, the variability in the amount of immobilized SAv-TR among the three regions (entrance, middle, and exit) is statistically insignificant. While the uniformity observed is an important characteristic, future studies as to the actual density of the reactive groups, and ultimately that of the target proteins, are certainly in order.

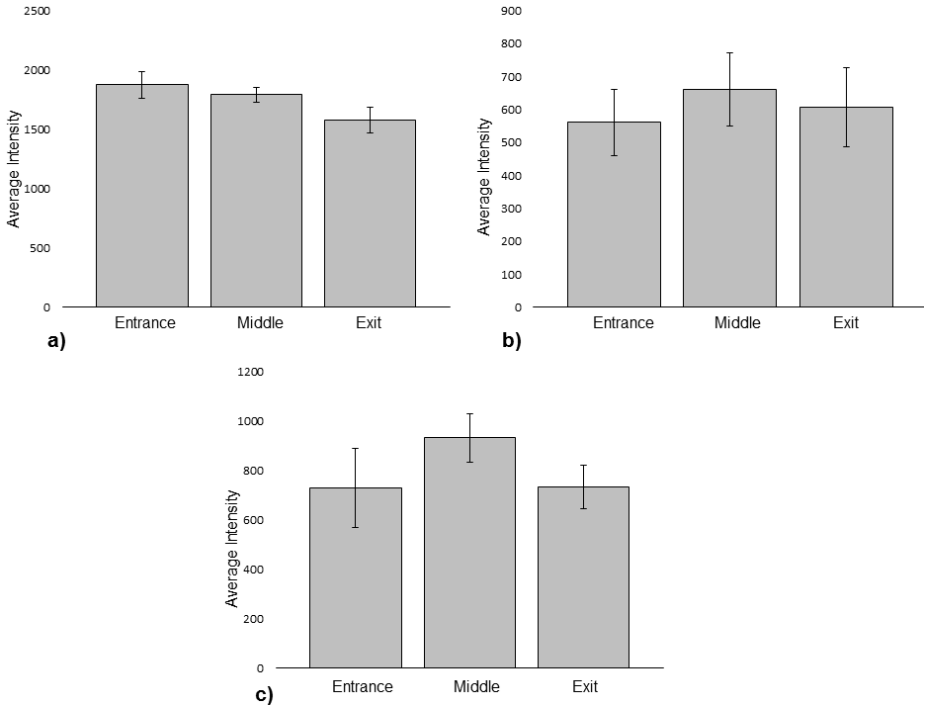

Figure 5.4. Demonstration of the uniformity of modification collected from three positions along the length of the C-CP fibers from Figure 5.3. Figure 5.4a) PEG-Biotin modified PP C-CP fibers. b) PEG-OMe modified PP C-CP fibers. c) Native unmodified PP C-CP fibers.

In order to evaluate if a significant portion of the non-specific binding (Fig 5.3b,c) was a result of hydrophobic interaction or the protein-protein interactions between the BSA coated fibers and the SAv-TR the experiment was repeated substituting the BSA block with a surfactant wash. In this experiment a 0.1%

Tween20 in PBS (PBST) buffer was used to prepare the fiber surface after lipid loading, additionally the SAv-TR capture protein was loaded on the lipid modified fibers in the PBST buffer. Application of Tween20 as a blocking agent is common practice in immunoassays and as depicted in Figure 5.5 was successful at minimizing non-specific binding of the SAv-TR with the PP and PEG-OMe surfaces of the C-CP fibers. Tween20 completely prevented non-specific binding from occurring on the fiber surface while allowing complete and uniform coverage of the SAv-TR with the PEG-Biotin coated fibers, Fig 5.5a.

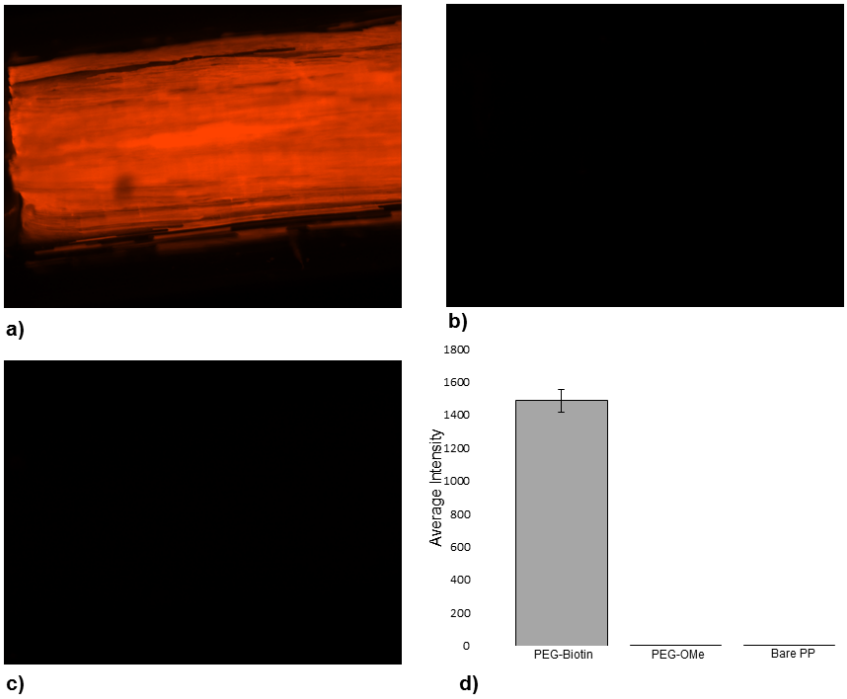

Figure 5.5. Fluorescent Images of PP C-CP fibers after modification using head group modified PEG lipids, experiment utilized a 0.1% Tween20 to decrease nonspecific binding, all fibers were exposed to SAv-TR. a) Biotin modified PP C-CP fibers. b) PEG-OMe modified PP C-CP fibers. c) Native, unmodified PP C-CP fibers. d) Average intensity response for each column, collected from an identical size mask down the center of each image.

Demonstrated in Figure 5.6 is the statistical representation of the entire fiber tip surface (entrance, middle, exit) of the 1cm PP C-CP fiber tip demonstrating the PEG-Biotin coating was fairly homogenous.

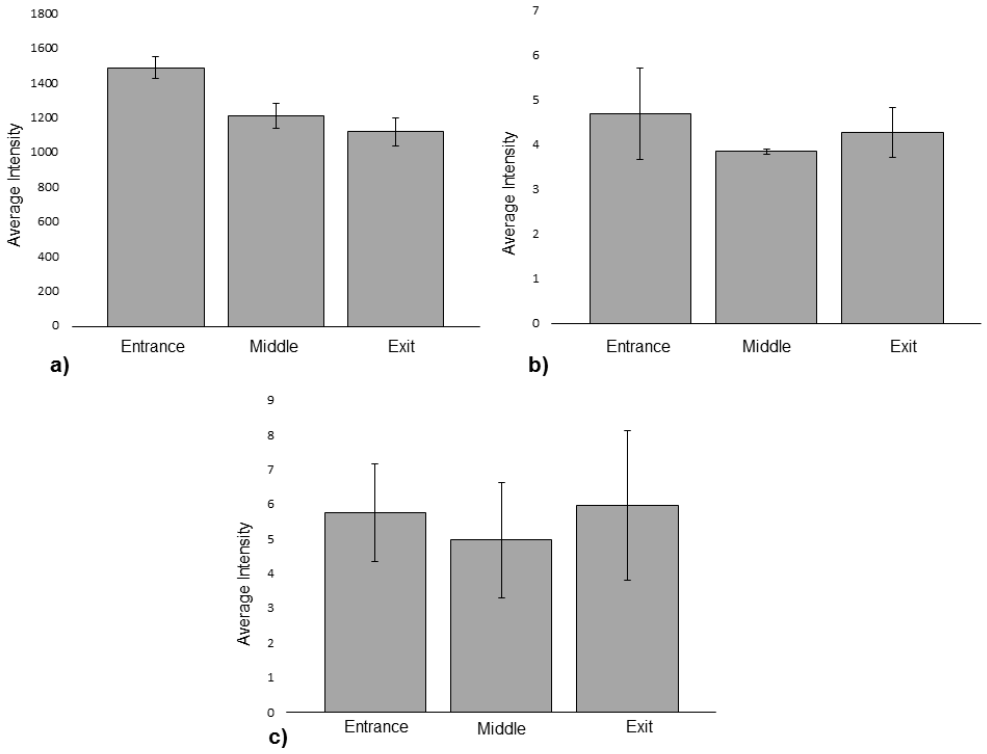

Figure 5.6. Demonstration of the uniformity of modification collected from three positions along the length of the C-CP fibers from Figure 5.5. Figure 5.6a) PEG-Biotin modified PP C-CP fibers. b) PEG-OMe modified PP C-CP fibers. c) Native unmodified PP C-CP fibers

It is expected that as the loading conditions are evaluated and particularly as the loading capacity of the PP C-CP fiber surface is determined the slight gradient visible in Fig 5.6a will be removed and a very uniform binding will occur. Statistical data for the PEG-OME (Fig 5.6b) and the bare PP (Fig 5.6c) surface show that there was unequivocally no non-specific binding occurring on either control tip fiber surface. It is significant to note, the addition of 0.1% Tween to the protein loading buffer is sufficient to overcome all undesired binding affects,

therefor the need for specific pre-treatment of the fiber surface or employment of analyte specific post exposure washing steps is eliminated.

The second demonstration of the employment of lipids for fiber surface modification utilizes PE-DPTA to affect an IMAC-type interaction. IMAC is one of the most powerful approaches in the process-scale purification of proteins from *E. coli* mixtures. When utilized in this fashion the proteins targeted for extraction have been expressed with a poly-histidine tag (his-tag). His-tags are optimal for IMAC chromatography as the high density of histidines, usually hexa his-tags, are optimal for transition metal coordination. The PE-DTPA is pre-chelated with Cu^{2+} therefore the stationary phase is functionalized and ready for application in IMAC is a single, straightforward step. In this case, Cu^{2+} coordinated with the deprotonated acetic acid groups on the DPTA is available for protein capture via the his-tags.

Initial efforts utilizing PE-DTPA-modified C-CP surfaces employed a fluorescein isothiocyanate (FITC)-labeled anthrax n-terminal lethal factor (LFn) as the detection or target capture protein. LFn was expressed in *E. coli* to contain a fusion tag of a hexa-histidine amino acid sequence. Initial purification of the LFn occurred utilizing a commercial IMAC column prior to labeling with fluorescent FITC tag, demonstrating the fidelity of the his-tag capture. After modification of the fiber surface with PE-DTPA, as previously described, fiber columns were washed with buffer, BSA blocked, and then exposed to 5 µg mL^{-1} LFn in 20 mM Tris-HCl. Analysis of the collected images revealed high amounts

of non-specific binding on the control fiber tips (PEG-OMe and PP). The addition of the FITC post-purification leads to some ambiguity as to whether the poor performance was the result of the FITC tag non-specifically adsorbing or the LFN interaction with the fiber surfaces non-selectively.

To alleviate the questions posed above, the experiments were carried out utilizing a his-tagged green fluorescent protein (GFP). Since GFP is a naturally fluorescent protein there is no label to potentially interfere with the his-tag chelation or adsorption as a result of the fluorescent labeling. The same experimental steps applied previously were employed: a buffer wash of the C-CP fiber tips, a BSA block, and exposure to 5 μg mL^{-1} GFP in 20 mM Tris-HCl. The resulting images showed the same high levels of non-specific binding, suggesting that the FITC tag on LFn was not responsible for the poor selectivity but a result of the experimental conditions. Potential contributions from the FEP tubing at this combination of excitation /emission wavelengths was ruled out by removing the fibers and illuminating the tubing alone, wherein there was no indication that any spectroscopically-active components were present. The inability to distinguish between the controls and the modified C-CP fibers means the experimental conditions under which his-tag proteins are exposed to the fibers surfaces allows for a large amount of non-specific binding.

Protein-protein interactions, hydrophobic interactions with the support surface or the BSA coatings, electrostatic interactions, and interactions between other amino acid residues in the protein (not the his-tag sequence) and the

chelating head group are all potential sources of non-specific binding across the spectrum of immobilization-based separations and diagnostics. A final possibility is the fact that there are the intended chelation interactions occurring with the PE-DPTA surface, but additionally occurring non-specific retention on the control surfaces. Thus, it is possible that the simple rinse step employed (1 mL of 20 mM Tris-HCl) was not effective at removing non-specifically bound protein. A wide range of experimental variables were evaluated, including lipid loading conditions, BSA blocking, exposure conditions for GFP, concentrations of lipids and proteins, none of which yielded the desired immobilization performance.

Two sets of experimental conditions did result in successful modification with a significant response relative to the controls utilized. Figures 5.7 a-c depict the fluorescent miscroscope images obtained for the PE-DTPA Cu^{2+}chelated, PEG-OMe, and the PP fibers that were exposed to the his-tag EGFP, with the variation of using a 1 M NaCl wash. The PP C-CP fibers were exposed to the lipid, the BSA block applied, and EGFP exposure under the same set of experimental conditions as previously described. The NaCl wash presents a relatively high ionic strength environment in the mobile phase, thereby decreasing the propensity for electrostatic interactions on the control surfaces. Clearly the 1 M salt wash decreases the relative extent of nonspecific binding, but as evident in Figs. 5.7b-c, a higher than desired level is still occurs.

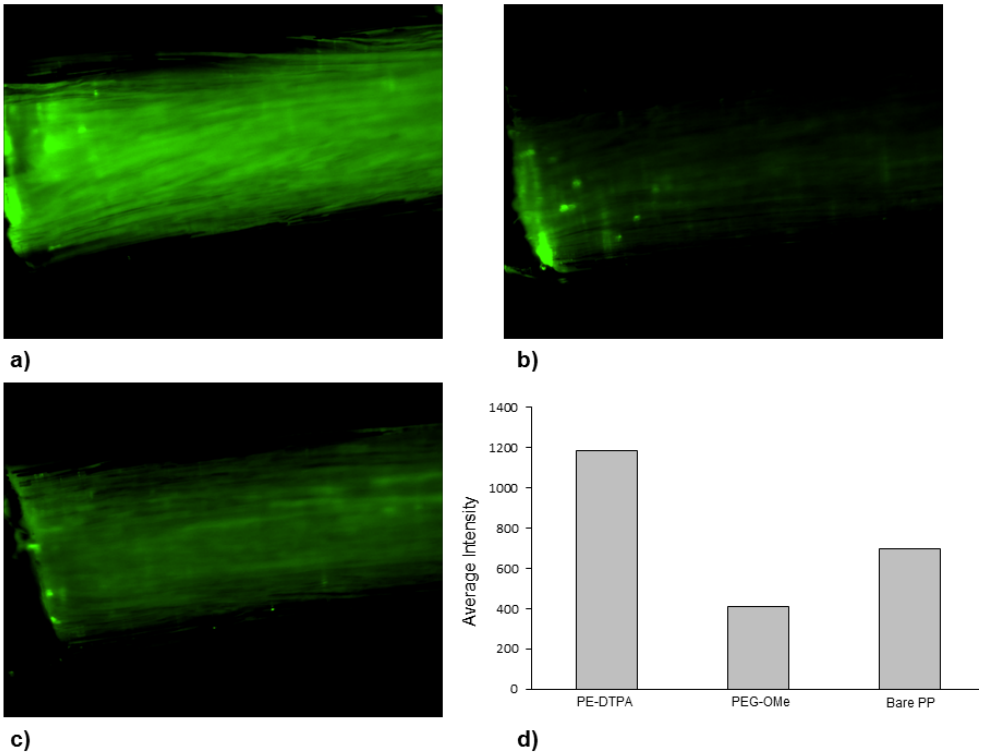

Figure 5.7. Fluorescent Images of PP C-CP fibers after modification using head group modified lipids, experiment utilized green fluorescent protein (EGFP) that contained a his-tag. Prior to image collection all columns were washed with 1 M NaCl to eliminate non-specific binding.
a) 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-N-diethylenetriaminepentaacetic acid (PE-DTPA) modified PP C-CP fibers. b) PEG-OMe modified PP C-CP fibers. c) Native, unmodified PP C-CP fibers. d) Average intensity response for each column, collected from an identical size mask down the center of each image.

The second successful approach to reducing the amount of nonspecific binding involved the loading of the EGFP protein in the presence of 20 mM Tris buffer that containing 20 mM of imidazole. This particular situation would be expected to decrease nonspecific interactions, and is a common practice on commercial IMAC columns[59, 60]. Taken a step further, an alternative washing step using the same buffer combination of 20 mM Tris and 75 mM imidazole was evaluated as a means to decrease the amount of non-specific binding in each of

the fiber/ligand systems. Clearly depicted in the fluorescent images in Fig. 5.8, residual non-specific binding to the control surfaces has been all but totally eliminated, as visible in Figs. 5.8b and c.

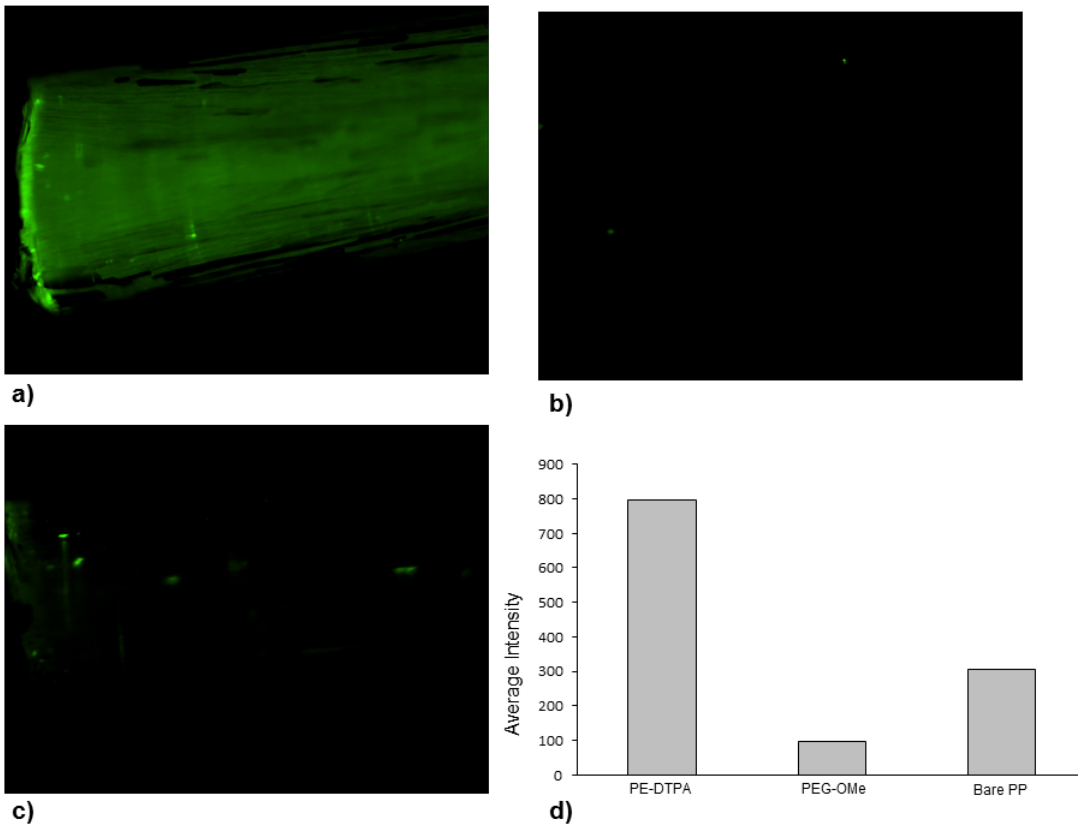

Figure 5.8. Fluorescent Images of PP C-CP fibers after modification using head group modified lipids, experiment utilized green fluorescent protein (EGFP) that contained a his-tag. Prior to image collection all columns were washed with 75mM Imidazole to eliminate non-specific binding. a) PE-DTPA modified PP C-CP fibers. b) PEG-OMe modified PP C-CP fibers. c) Native, unmodified PP C-CP fibers. d) Average intensity response for each column, collected from an identical size mask down the center of each image.

As seen in the bar graphs of Fig. 5.8d, the use of the imidazole rinse reduces the amount of binding in the case of the IMAC chelate by ~30% relative to what was seen in Fig. 5.7a, which likely reflects nonspecific binding, in general, or perhaps reflects an overloading of the stationary phase. On the other hand, the

integrated intensities for the PEG-OMe and PP controls have been reduced by ~80% and 60%, respectively. Taken a step farther, it is easy to see that the background fluorescence levels are seen in Figs 5.8b and c originate from anomalous “hotspots”, and are not distributed homogeneously as seen in images in Fig 5.3. In fact, these artifacts are also seen in the fluorescent images of Fig.5.7. These intense regions, existing at the entrance end of the tips, are likely the result of underivatized PP base fibers or particulate matter from the test solutions captured at the fiber heads.

Conclusions and Future Work

Presented here is a straightforward approach to modification of a polymer fiber surface, specifically PP C-CP fibers to affect highly selective protein separations. The C-CP fibers hold a number of very positive attributes in terms of proteins separations, including high solution transport and solute mass transfer efficiencies as demonstrated previously[6, 21, 22, 61]. Several advantages exist using this modification approach in comparison to others previously employed in this laboratory. First, the fibers are packed into the column or tip prior to modification therefore ensuring uniform exposure of the lipid to the fiber surface during modification, generating a uniform functional group modification of the stationary phase surface. Second, this approach employs chemistries that occur under ambient conditions. Finally, the wide array of available lipid head groups presents a rich portfolio of separation mechanisms. The results presented here demonstrate the flexibility of this modification approach; any lipid should be

absorbable to the PP C-CP fibers generating a modified fiber surface in a single step application. The ability to change the analyte specific interaction is as easy as a lipid substitution. The only experimental procedure changes required would be specific to the analyte being extracted and any minimization of nonspecific binding.

Paths forward will look at the more practical aspects of this generalized methodology. For example, a potential limitation to this approach is the fact that the lipid is only a semi-permanent modification and can be removed from the fiber surface using strongly hydrophobic solvents. However, this limitation is of minor concern as the primary application of the modified fibers takes place under biologically-relevant conditions in which organic solvents would be detrimental. The total binding capacity, first of the lipids and then the captured proteins, will be a particular issue of interest in terms of preparative chromatography. The need for the BSA blocking step is also in question. More fundamental studies will look towards gaining greater insights into factors controlling protein accessibility to the active lipid head groups, such as primary lipid orientation and use of linker molecules of varying length and chemical makeup between the lipid tails and the functional head group. Ultimately, it is believed that the combination of the physical attributes of the C-CP fiber column/tips coupled with the general utility of the lipid surface modification methodology holds a great deal of promise in fields ranging from clinical diagnostics to downstream protein processing.

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CHAPTER 6

CAPILLARY-CHANNELED POLYMER (C-CP) FILMS AS PROCESSING PLATFORMS FOR PROTEIN ANALYSIS BY MATRIX-ASSISTED LASER/DESORPTION IONIZATION MASS SPECTROMETRY (MALDI-MS)

Introduction

Thin layer chromatography (TLC) provides benefits over column chromatography methods due to the simplicity and versatility of the technique [1, 2]. One of the primary downsides of TLC is the ambiguous nature of spot identification. Analytical determinations often require excision and/or dissolution prior to assay; resulting in non-quantitative recoveries, increased risk of contamination and analysis time, and analysis (solely) of removed spots. Online coupling of TLC with mass spectrometry detection began in the 1990s, involving a number of ion/atom/photon bombardment methods [3-5]. Key in each of these methods is the need to ablate analyte species that exist *within* the sorbent matrix. Therefore, the probe beam must penetrate the medium or some form of extraction/transfer of the solutes (i.e., blotting) from the medium must be employed. The advent of matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) provided further impetus for the TLC profiling of biomacromolecules [6-9]. MALDI-MS analysis generally involves extraction of solutes to the secondary sorbent followed by application of the MALDI matrix to

affect co-crystallization. Direct MALDI from TLC plates has also been described, addressing the transfer issues [9].

Buffers typically employed in the processing of biological samples can be detrimental in MALDI (and electrospray ionization (ESI)) mass spectrometry, resulting in solute ionization suppression and formation of adducts that complicate spectral interpretation. The removal of buffer species (aka, de-salting) is achieved by use of a sorbent/chromatographic solid phase extraction (SPE) medium, followed by elution of proteins into solvents compatible with the ionization process [10, 11]. This occurs *de facto* in TLC due to different migration rates of macromolecules and salts [12, 13].

Capillary-channeled polymer (C-CP) fibers have been explored in this laboratory for their use in protein chromatography and SPE [14-19]. C-CP fibers have several positive attributes towards macromolecular separations, most importantly enhanced mass transfer efficiencies that allow very rapid separations with high recoveries [15, 17]. C-CP fibers can be extruded from a number of common polymers, affecting a range of surface chemistries with low materials cost. Relevant here, the C-CP fibers have also been implemented in a micropipette tip format as a sorbent for desalting proteins prior to ESI-MS [18]. C-CPs can also be extruded in an ~5 mm wide film format, with parallel channels as presented in the electron micrographs of Fig 6.1. The channels (~5 – 40 μm in width) create capillary forces that result in the ability to spontaneously move solutions along the film.

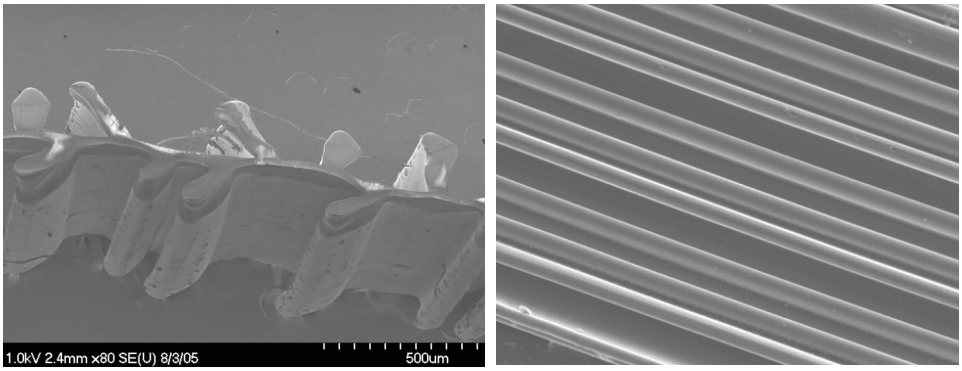

Figure 6.1. SEM micrographs of a polypropylene (PP) C-CP film. a) cross section and b) channel structure

Radial diffusion in the channels promotes efficient solute-surface interactions; effectively yielding parallel TLC channels. Thus, different from Lebrilla and coworkers who used a single open CE channel [14], the films present parallel channels of smaller dimension affording solute desalting and separations via wicking action rather than electrophoretic forces. In addition, there is no buffer/electrolyte residue in the ablation zone as in the case of the CE channel.

Demonstrated here is the concept of using C-CP films as processing platforms for protein analysis by MALDI-MS. Different from the aforementioned applications of TLC-MALDI-MS, the proteins reside *on* the surface of the polymer channels; therefore no extraction step is necessary. Samples can be de-salted or separated on C-CP films via wicking action in seconds. The long-term goal is to generate a phase that is more practical than conventional TLC plates, can be tailored to specific analytes, allows for low sample and solvent consumption (µL), and can be imaged directly.

Experimental

Chemicals and Reagents

A working solution containing 2.5 µM each of cytochrome c and myoglobin, (Sigma Aldrich (St. Louis, MO, USA)) was prepared in 100 mM Tris-HCl (Teknova, Hollister, CA, USA) and milliQ water (18.2 MΩ/cm) derived from a NANOpure Diamond Barnstead/Thermolyne Water System (Dubuque, IA). Individual protein solutions of 2.5 µM ribonuclease A and transferrin were prepared in 150 mM phosphate buffered saline (PBS) at pH 7.3. Protein separations were achieved using a 50:50 acetonitrile:water (ACN:H_2O) eluent. MALDI matrix solutions consisting of 15 mg/mL of sinapinic acid (Fisher Scientific (Pittsburgh, PA)) were prepared in 0.1% trifluoroacetic acid (TFA) (Sigma Adrich), 50:50 ACN:H_2O (VWR (West Chester, PA)).

Film Preparation and Sample Spotting

Polypropylene (PP) C-CP films were melt-extruded at the Clemson University School of Materials Science and Engineering (Clemson, SC). Films are also available from a commercial source, Specialty & Custom Fibers, LLC (Clemson, SC). Film segments were conditioned sequentially with water, methanol, and acetonitrile to remove any production residues. The films were attached via double-sided tape to a conventional steel MALDI target prior to application of the solutions. For all on-film analysis, 2 µL of protein solution was spotted and allowed to dry (<1 min.) before any additional treatments occurred. Protein solution residues were washed by applying 9 µL of DI-H_2O to the loaded

protein. After 5 seconds, the water was pipetted back off of the film. In the case of the protein separation and film imaging (Figure 6.3), a 2 µL aliquot of the working protein solution was applied to one end of a 2 cm long film and washed as previously described, with the separation of the proteins initiated using 9 µL of eluent, resulting in transport down the plurality of the channels. The respective solutions were dried under ambient conditions and the matrix solution applied using a chromatography reagent sprayer (Sigma Aldrich). Use of a sprayer (as opposed to use of wicking) ensures the integrity of the chromatographic separations.

MALDI Analysis

Analyte spectra were obtained on a Bruker Daltonics (Billerica, MA) microflex LRF, MALDI-TOF mass spectrometer in the positive ion, linear mode. Instrument control and data processing occurred in the Compass™ (Bruker Daltonics) software environment. The microflex is equipped with a nitrogen laser (337 nm) operating at a pulse rate of 60 Hz. Mass spectral acquisitions occurred using 200 laser shots at 79% laser power. C-CP film imaging utilized a beam diameter of ~100 µm, and a 200 µm raster, recording spectra at every other coordinate position (e.g., 1, 3, 5, etc.). Spectral background (B) and noise (N) used in calculating S/B and S/N values in Table 6.1 are based on the average and standard deviation of the signals in a 100 Da mass window centered 2000 Da below that of the respective protein molecular ions.

Target/substrate	Buffer	Expected MW[1] (Da)	Determined MW (Da)	Peak Width ($w_{0.5}$) (Da)	Signal (S)	Signal-to-Noise (S/N)	Signal-to-Background (S/B)	Determined MW (Da)	$w_{0.5}$ (Da)	S	S/N	S/B
			In Buffer matrix					**De-salted**				
Steel												
Cytochrome c	Tris	12,384	12,192.82	33.26	7617	134	12					
Myoglobin	Tris	17,500	16,969.46	463.14	1220	68	5					
On Flat PP Film												
Cytochrome c	Tris	12,384	12323.31	116.69	281	49	35					
Myoglobin	Tris	17,500	17091.07	136.20	68	32	58					
On C-CP Film												
Cytochrome c	Tris	12,384	12289.92	24.52	33	34	38	12312.04	51.54	623	291	123
Myoglobin	Tris	17,500	ND[2]	ND[2]	ND[2]	-	-	17081.55	231.93	62	47	17
Ribonuclease A	PBS	13,700	13893.22	53.52	50	47	34	13845.75	109.94	140	69	157
Lysozyme	PBS	14,300	ND[2]	ND[2]	ND[2]	-	-	14447.93	289.65	80	65	24
Transferrin	PBS	79,550	81749.6	2860.29	24	35	28	81794.62	2093.65	207	114	26

[1]MW values obtained from supplier; Sigma Aldrich (St. Louis, MO)
[2]ND – not detected, S/N < 3

Table 6.1. MALDI-MS responses for test proteins on different substrates before and after de-salting. (2.0 µL aliquots of 2.5 µM protein in buffer.)

Results and Discussion

In order to assess the potential utility of the C-CP films as MALDI substrates, an initial comparison between the responses using a standard steel target and a cast polypropylene film was performed. In each case, 2 µL of the protein test solution (2.5 µM each of cytochrome c and myoglobin in 100 mM Tris-HCl) was allowed to dry, followed by the spray application of matrix. The results of these experiments are presented in Table 6.1 using normalized, arbitrary units. Clearly seen is the superior ion yield of the well-developed metal target. Ablation from the flat PP film results in a significant reduction in analyte ion intensities, though the film-ablated protein responses do show better signal-to-background (S/B) values. The lower protein ion yields from the polymer film are likely due to a number of reasons, including photon penetration/absorption into the polymer, differences in the extraction height (film is 0.5 mm above the metal target), and distortions of the electrostatic fields in the near surface region affecting extraction. Introduction of the channel structure of the C-CP films results in further degradation of the observed spectral quality as depicted in the entries for cytochrome c and myoglobin in the left-hand portions of Table 6.1 and shown in Fig 6.2a. In this case, there is no discernible signal for the myoglobin constituent, and so MW and S values are not reported. In addition to the reduced ablation/ionization efficiencies described for the cast PP film substrate, there are likely geometric aspects limiting the incident photons reaching the

channel bottoms as well as potential disturbances of the resultant plume formation. It is important to note that in each of these cases where desalting is not performed, large amounts of continuum and discrete, low-mass signals are present, reflecting the presence of buffer constituents.

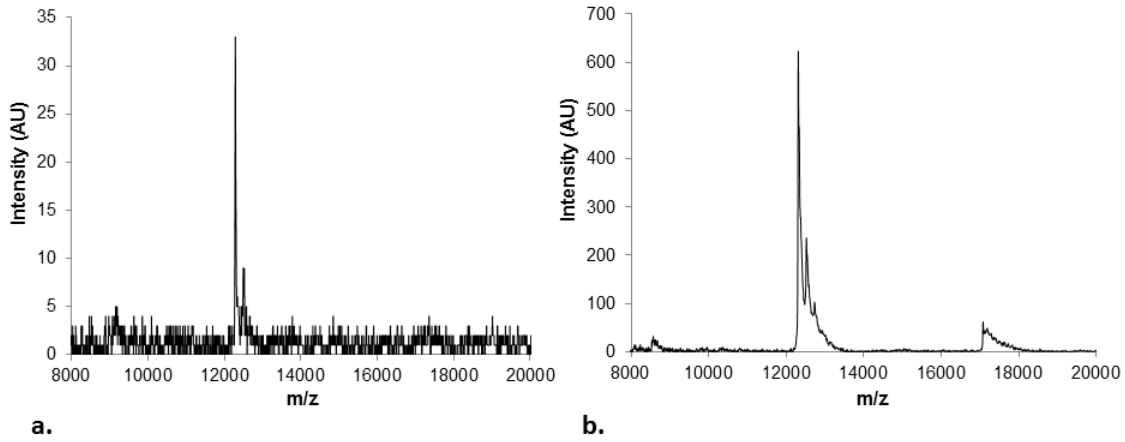

Figure 6.2. MALDI-MS spectra of 2 µL sample aliquots containing 2.5 µM (each) of cytochrome c and myoglobin in 100 mM Tris buffer deposited on a PP C-CP film. a) as deposited and b) following the H_2O wash step.

The ability to selectively isolate protein species within the C-CP film channels is demonstrated in Fig 6.2b, with the quantitative figures of merit included in the right-hand portion of Table 6.1. In this case, 9 µL of DI-H_2O was added to the film segment after the initial solution had dried, with the solution pipetted back off the film after 5 seconds of interaction. The proteins are selectively retained via their hydrophobic interaction with the polymer while the water-soluble buffers are removed. While the overall intensities (S) here do not match those of the metal target (Table 6.1), there is an appreciable improvement in S, S/N and S/B upon completing the simple desalting step. The myoglobin signal, previously indistinguishable from spectral background values of ~1 AU

shows a particularly impressive improvement of >50X. While not seen in the mass spectrum (Fig 6.2b) there is also a dramatic decrease in the mass-dependent continua and buffer related signals upon on-film desalting of the sample.

To further demonstrate utility of the C-CP films, three additional proteins (ribonuclease A, lysozyme, and transferrin) present in a different buffer solution, 150 mM PBS, were evaluated before and after desalting. As presented in Table 6.1, the improvement in signal intensity is ~3X for ribonuclease A, while that for transferrin is far more substantial. As was seen previously for myoglobin, the lysozyme signal increased from a non-detectible value of ~1 to 80, the result of removing the suppressing salt species. Across the group of proteins, the spectral background levels do increase upon desalting, while the standard deviation of the backgrounds do not change as much. Thus the improvements in S/N are generally better upon desalting than S/B. As would be hoped, the films' desalting abilities appear to be equally effective for these five proteins in two different buffer/salt systems.

Included in Table 6.1 are the determined molecular weights (MW) and respective peak widths for each of the test proteins and target materials. The peak widths for this MALDI-MS should result in resolving powers ($m/\Delta m$) of ~1,000. In the case of the two-protein mixture in Tris buffer on the steel target, it is clear that the presence of buffer has led to an erroneous MW assignment due to adduct formation as well as pronounced peak broadening beyond what would

be expected for neat protein solutions. As described above, it would not be unexpected that the insulating nature of the polymer films would perturb the extraction efficiencies of the target ions. This is seen first in the MW and peak widths for the flat PP film, and also for the C-CP films. Errors in mass accuracy could also be attributed from the fact that the mass scale was calibrated for the metal target. Mass calibration from a film target and recessing the film into the metal substrate (to have equivalent laser focus and ion extraction distances) might be expected to alleviate some of the problems. It is clear, though, that the use of the C-CP film does not detract from the mass analyzer performance figures of merit beyond the flat film itself, though offering the capacity for greatly improved signal characteristics. One final point of relevance for transferrin is the fact that the broad peak is not unexpected for MALDI-MS of glycoproteins. In fact, the presence of intact, glycolsylated protein may indicate a particular attractive feature of this method.

The second potential advantage of using C-CP films as MALDI-MS substrates lies in the ability to achieve chromatographic separations. The realization of this aspect is demonstrated in Fig. 6.3. Seen in Fig. 6.3a are the MALDI-MS images of C-CP films wherein the first 3 mm of the film is used as an initial deposition zone. The film images appear as colored dots (red and green representing cytochrome c and myoglobin, respectively), generated by the Compass^{TM} software, using a 25% threshold. In film A, 2 µL of the protein:buffer solution was applied, allowed to dry, and a 9 µL aliquot of DI H_2O applied to the

end of the film using a syringe as described above for the short film segment. Mass spectra derived from this region are ostensibly the same as Fig 6.2b, reflecting the desalting of the test solution.

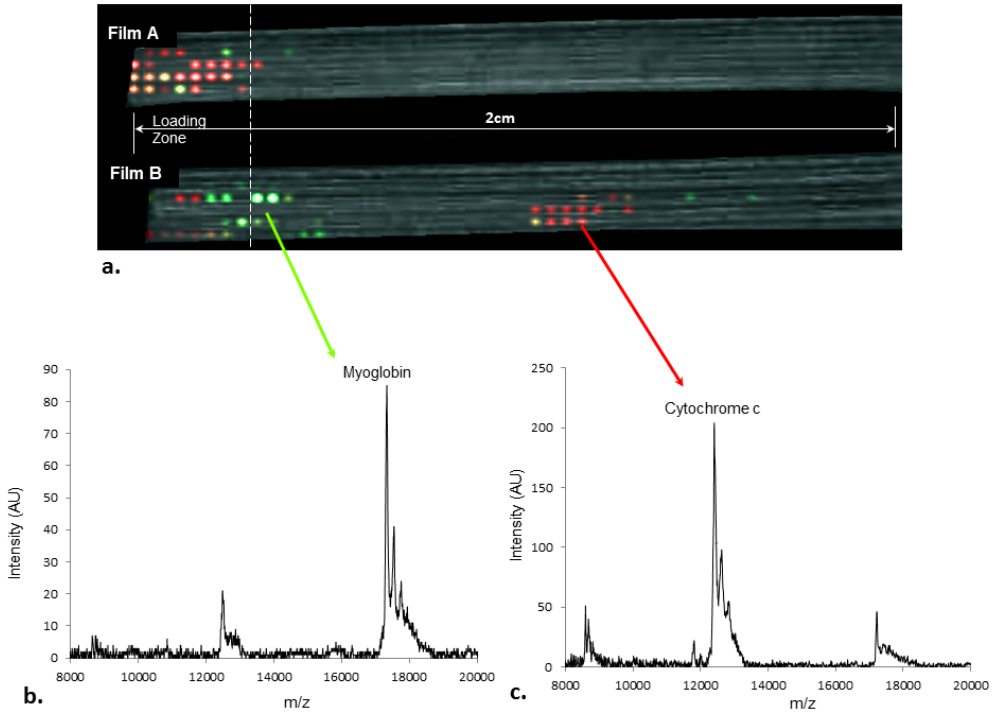

Figure 6.3. MALDI-MS imaging of proteins on PP C-CP film used as a chromatographic stationary phase. a) cytochrome c and myoglobin distributions on films depicted as red and green dots respectively from Compass^{TM} software; film A following the H_2O wash step and film B following the chromatographic elution, b) extracted mass spectrum representing myoglobin following elution with 50:50 ACN: H_2O with 0.1% TFA, and c) extracted mass spectrum representing cytochrome c following elution with 50:50 ACN: H_2O with 0.1% TFA

As in the case of conventional TLC, spatial separation/segregation of proteins is achieved on the C-CP films by elution with organic solvents. In reversed-phase HPLC on PP C-CP fiber columns, separations occur via gradient elution using an ACN:H_2O combination containing 0.1% TFA as an ion paring agent [17]. To demonstrate the potential to affect differential mobility of the

myoglobin/cytochrome c pair, a simple 50:50 ACN:H_2O solvent composition was chosen. As shown in the MALDI-MS image of film B in Fig 6.3a, as well as the extracted mass spectra of Figs. 6.3b and c, this solvent system results in separation of the two proteins. Specifically, cytochrome c is seen to elute approximately 1.1 cm down the film, while myoglobin, which has a greater affinity for the film surface moves only a short distance from the deposition zone. These results are in agreement with previous HPLC separations wherein cytochrome c elutes at lower solvent strength than myoglobin [17].

Conclusions and Future Work

The principle of using C-CP films as processing platforms to affect de-salting and separation of proteins has been demonstrated. While there are reductions in overall sensitivity in comparison to metal target deposition, as well as some degradation of MS performance, there are many paths forward toward improving the overall response including use of films of fewer channel number to spatially restrict the solution, detailed study of the role of channel:laser beam orientation, and modification of ion extraction parameters. By the same token, there is a wealth of opportunities in the realm of protein solution processing, both from the simple desalting perspective as well as achieving higher resolution separations. Many challenges remain, but the overall simplicity, flexibility, and practicality of the approach is believed to warrant further development and applications.

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CHAPTER 7

SUMMARY

The basis of the research in this dissertation was to generate a functionalization process for the chemical modification of C-CP fibers for the selective extraction of analyte molecules (proteins and metals). The goal was to generate a high surface density of functional groups through a modification process that would maintain the physical characteristics of the C-CP fibers and therefore retain their highly desired attributes. Several modification approaches were taken that evaluated grafting, adsorption, and covalent modification of the C-CP fibers. These approaches generated a variety of surface functional groups, all with the capability of retaining metals and proteins with high levels of selectivity.

Chapter 2 described the employment of polyethylene terephthalate (PET, polyester) capillary-channeled polymer (C-CP) fibers as the base structure for surface modification using the “grafting to” approach. This grafting approach successfully deposited a polyacrylic acid (PAA) surface layer, which resulted in a high density of carboxylic acids available for analyte interactions. These interactions were explored through implementation of the PAA C-CP fibers as a sorbent in solid phase extraction (SPE) for selective removal of metal ions from aqueous solutions. Several cations, including Cu^{2+}, Cu^{+}, Ni^{2+}, Fe^{3+}, UO_2^{2+}, were successfully bound, concentrated, and efficiently eluted using low level

concentrations of hydrochloric acid (0.005%) with recoveries greater than 95% on average. A control tip utilized for 19 extractions over the course of one year had recoveries greater than 96% and demonstrates the excellent reusability and stability of the chemically modified surface of the PET C-CP fibers.

Chapter 3 presented the generation of an ion-exchange surface through covalent modification of the polyethylene terephthalate (PET, polyester) fibers. Specifically, evaluation of a covalent solution based chemistry employing ethylenediamine (EDA) for in-column modification of the C-CP fibers. This modification approach results in generation of a high density of surface functional groups on the PET C-CP fibers. These generated functional groups consist of primary amines, carboxylic acids, amides, and alcohols. The EDA modified PET surface is zwitterionic as the result of the amine and carboxylic acid functional groups resulting in a pH responsive stationary phase. This type of zwitterionic surface exists naturally on nylon-6, therefore the EDA modified PET fibers are coined “super nylon.” The functional group density of the super nylon stationary phases were evaluated using standard ionic textile dyes with concentrations of anionic groups (e.g., $COOH^-$) ranging from 4.54×10^{-10} to 4.68×10^{-9} mol cm^{-2}, and cationic groups (e.g. NH_3^+) ranging from 5.60×10^{-10} to 3.68×10^{-9} mol cm^{-2}. Comparison of the super nylon stationary phases to native base polymer PET and nylon-6 occurred via ion exchange chromatography to separate a suite of proteins, including ribonuclease A, cytochrome c, and lysozyme. Separations were run at 4 mL min^{-1} with the optimal protein separation occurring at pH 8.2

and the best resolution occurring on the super nylon column after 10 minutes of functionalization. Use of isopropyl alcohol as an additive at 3 % resulted in significant changes in retention for cytochrome c, implying that interactions other than ion exchange are occurring, and may potentially be hydrophobic or π-π interactions. Modification of the PET C-CP fibers using EDA was successful at generating a zwitterionic stationary phase, however, the reaction of EDA with the PET backbone resulted in a decrease in fiber integrity, and consequently additional solution based covalent modifications were explored.

Chapter 4 demonstrated the employment of nylon-6 C-CP fibers as a base polymer for modification generating a metal chelating stationary phase. A synthetic approach that utilizes the coupling agent trichlorotriazine (TCT) to link the chelating ligand iminodiacetic acid (IDA) ligands to the surface of nylon-6 C-CP fibers was presented. Textile reactive dyes possessing a triazine linker moiety were employed for visualization of the modification process, demonstrating the capability of triazine to react with the amide bond of the nylon fibers. The desired chelating ligand, a di-substituted iminodiacetic acid based 1,3,5-triazine molecule, was synthesized in solution prior to tethering to the nylon-6 surface. This solution based synthetic approach allowed for evaluation of each step through NMR and HRMS to ensure generation of the intermediates occurred with low levels of impurities. The successful coupling of this IDA based chelating ligand was evaluated through the ability of the surface to chelate Cu^{2+} ions and be regenerated with an EDTA wash, evaluated through UV-VIS analysis

of the solutions. The successful generation of a metal chelating surface has set the stage for implementation of this strategy for immobilized metal affinity chromatography (IMAC) separations of his-tagged proteins on C-CP fiber columns.

Chapter 5 presents the modification of polypropylene (PP) capillary-channeled polymer (C-CP) fibers with head group modified lipids generating highly selective analyte-specific surfaces for application as a stationary phase in high performance liquid chromatography (HPLC) or solid phase extraction (SPE). The aliphatic portion of the lipid adsorbs strongly to the hydrophobic PP surface, allowing for the active head groups to orient themselves into the polar mobile phase. This orientation allows for interaction of the functional moiety, the head group, to interact with the desired analytes. Specifically, this chapter demonstrated two proof of concept applications using different head group modified lipids. The first utilized the well know affinity of biotin and streptadivin (SAv). A PEG-Biotin lipid was adsorbed to the surface of the PP C-CP fibers, with confirmation of surface functionalization and uniformity evaluated through Texas Red (SAv-TR) binding. Additionally, the generation of a highly analyte specific surface utilized another lipid moiety, PE-DTPA, a chelating head group modified lipid. This modification approach allowed for the PP C-CP fibers to be employed as a chelating stationary phase in immobilized metal affinity chromatography (IMAC), which was successfully detected through chelation of the stationary phase and a his-tag labeled green fluorescent protein (his-tag

EGFP). These modification approaches provide a quick, inexpensive, efficient, and universally applicable functionalization with little change in the modification procedure of the fiber surface due to the large array of readily available head group modified lipids.

Chapter 6 evaluated the application of a new type of C-CP format, films, for their ability to separate proteins based on the natural wicking of the C-CP film, much like thin layer chromatography (TLC). The format of the films is novel and their micrometer-sized channels induce solution wicking via capillary action resulting in efficient mass transport from the solution phase to the channel surface allowing for adsorption of hydrophobic protein. These C-CP films were presented for their ability to desalt protein solutions prior to analysis with MALDI-MS. C-CP films were successful in improving the S/N ratio of several proteins (e.g. cytochrome c, myoglobin, ribonuclease A, and lysozyme) with two biologically relevant buffers (Tris-HCl and PBS) through a basic de-salting concept. An additional application of these C-CP films for the separation of two proteins was shown to be successful as well. The separation of cytochrome c and myoglobin occurred using the natural wicking abilities of these films to separate the proteins using a standard reversed-phase solvent composition (acetonitrile with 0.1% trifluoroacetic acid), and was confirmed through imaging of the C-CP film using MALDI-MS. These films demonstrate another avenue of application that will be further explored for the functionalization and development of highly selective protein processing platforms.

The future of surface modifications of C-CP fibers for selective separations will revolve around the trichlorotriazine and lipid based modifications presented in Chapters 4-5. Specifically, the ability to improve the ligand density on the modified nylon-6 C-CP fiber will be investigated. Once optimal surface modification is achieved evaluation of the loading capacity of the ligand along with metal selectivity will be completed. Upon achieving optimal tethering (a high capacity surface) the iminodiacetic acid functional group fibers will be employed as a stationary phase in immobilized metal affinity chromatography (IMAC) for the extraction and purification of his-tag proteins. Future avenues of research will evaluate the ability to tether a variety of ligands to the trichlorotriazine molecule increasing the variety of analyte specific extractions available.

This laboratory will continue researching surface modifications through basic adsorption mechanisms. The lipid based modification presented here shows great strides in developing a single step homogenous surface modification. The work presented here only evaluated one set of loading conditions (50% ethanol) and while successful modification was achieved additional conditions may provide a higher capacity and a more uniform coverage along the length of the fiber surface. Swelling of the polymer through exposure to organic solvent prior to surface modification will also be evaluated. This allows for investigation into the potential of the aliphatic lipid chains to slightly embed in the polymer structure resulting in a more rugged surface modification. Additionally, the capacity of the modified surfaces will be evaluated for a variety

of analyte specific extractions. Once the capacity and stability of the modified fibers characterized their implementation as a stationary phase in high performance liquid chromatography (HPLC) for analyte extraction will be evaluated.

APPENDICES

APPENDIX A

Supplemental Data for Chapter 4

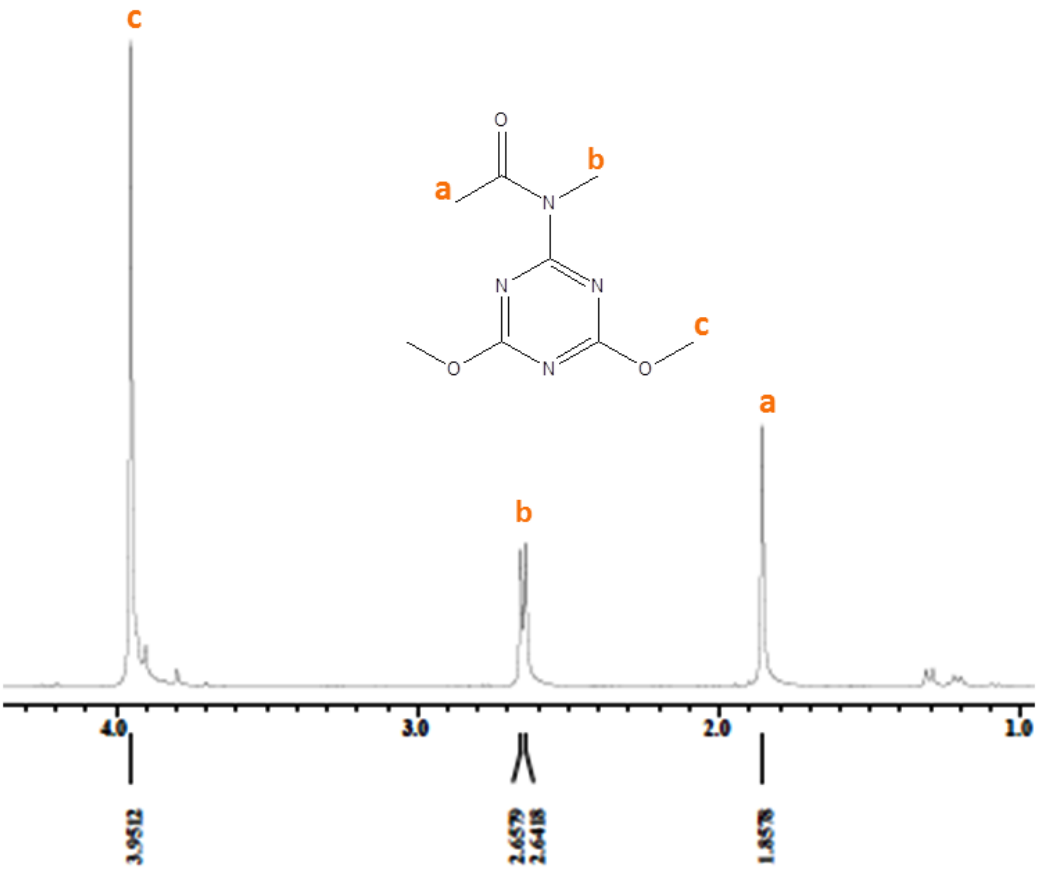

Figure A-1: ^{1}H NMR spectrum of *n-(4,6-dimethoxy-1,3,4-trazin-2-yl)-n-methylacetamide*.

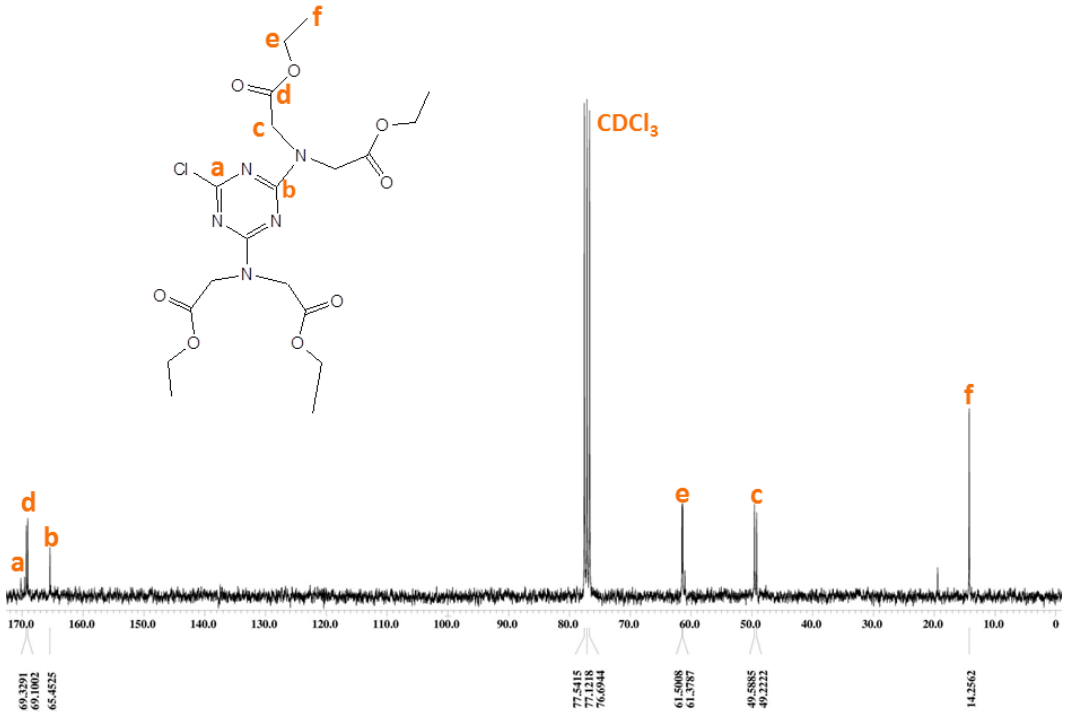

Figure A-2: ^{13}C NMR spectrum of Intermediate 1 (I1).

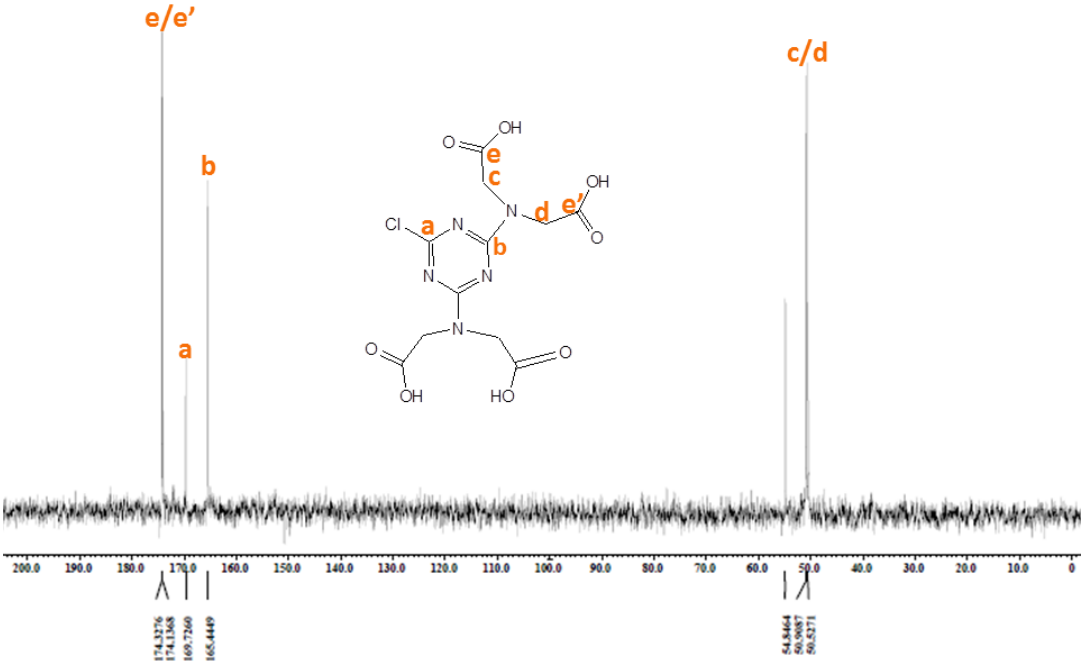

Figure A-3: ^{13}C NMR spectrum of Product 1 (P1).

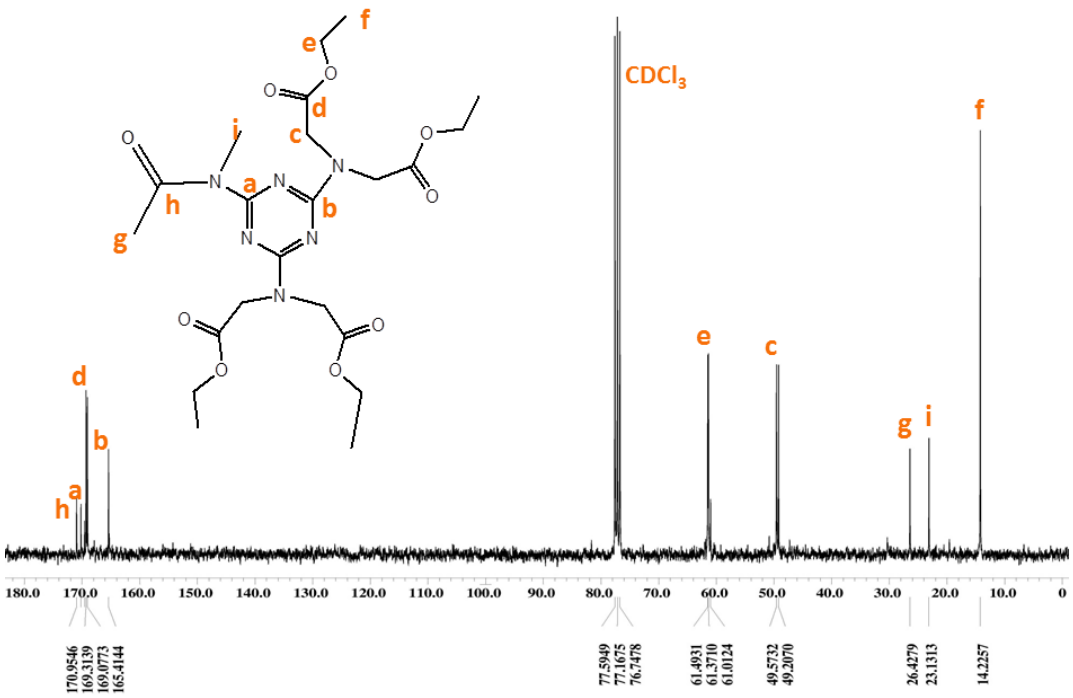

Figure A-4: ^{13}C NMR spectrum of Intermediate 2 (I2).

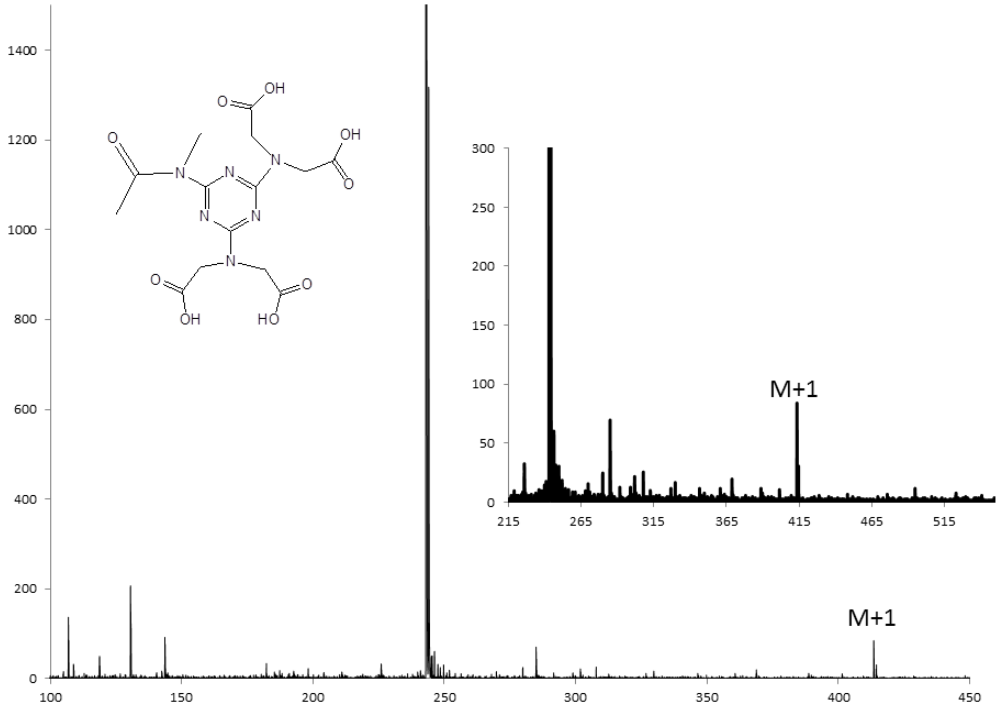

Figure A-5: MALDI-MS spectrum of Product 2 (P2).

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