Development of Functionalized Capillary-Channeled Polymer (C-CP) Fibers as Stationary Phase for Affinity Chromatography

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DEVELOPMENT OF FUNCTIONALIZED CAPILLARY-CHANNELED POLYMER (C-CP) FIBERS AS STATIONARY PHASE FOR AFFINITY CHROMATOGRAPHY

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Chemistry

by
Hung Khiem Trang
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Accepted by:
R. Kenneth Marcus, Committee Chair
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ABSTRACT

At the heart of the chromatography technique, the stationary phase is the essential component that dictates multiple aspects of the separation process. Diverse chemistry of the stationary phases, in combination with the choice of appropriate base support materials, allows chromatography to be used in a wide range of applications ranging from the high-performance separations of trace analytes in complex biological samples for chemical detection and quantification to the large-scale purification of a recombinant protein from a complex media. Traditional chromatographic stationary phases mostly utilize porous materials for the high surface area and versatility for chemical modification. However, in many cases, these materials suffer from high operating back-pressure and inefficient mass transfer. Joining the efforts to investigate alternative phases, Marcus research group (Clemson University) has investigated the potentials of capillary-channeled polymer (C-CP) fibers for the past 15 years. These fibers are characterized by eight capillary channels running down the length of the fiber, allowing the fibers to interdigitate to form well-aligned micrometer-sized channels when packed into a column format. It is the unique shape that allows separations to be conducted at high linear velocities (>75 mm s\(^{-1}\)) with high column permeability (<0.14 MPa cm\(^{-1}\)). The highly efficient fluid movement through the narrow channels composed of non-porous C-CP fibers gives rise to favorable mass transfer rates, facilitating fast protein separations and processing. Moreover, another advantage of C-CP fiber supports over other commercial sorbents is that these fibers are quite stable over a wide pH range and are fairly inexpensive (<$
100 lb\(^{-1}\)). Additionally, packing a C-CP fiber column is a simple process that can be conveniently performed without any special equipment.

The concepts of using natural and synthetic polymer fibers as chromatographic stationary phases are not new. Potential advantages cited include low costs, ease in column fabrication, low operation backpressure, facile solute mass transfer, and a general ease of tailoring fiber surfaces to affect high levels of chemical specificity. There have been several surface modification techniques reported for polymer fibers in the literature. However, many of these modification methods can be detrimental to the physical properties of the polymer by destroying the polymer backbone. In order to further exploit the advantageous fluidic properties of C-CP fiber columns without compromising the fiber integrity, milder modification approaches have been a focus of this group recently, including covalent coupling and direct ligand adsorption methods. The research studies reported in this work focus on the development of functionalized C-CP fiber for a variety of affinity chromatography applications. The ultimate goal is to investigate non-invasive modification schemes that do not compromise the mechanical strength and fluidic properties of C-CP fibers. Affinity ligands are immobilized on the fiber surface through one of the following schemes:

1. **Scheme 1:** Physical adsorption of recombinant protein A (rSPA) ligands on polypropylene (PP) C-CP fibers for immunoglobulin (IgG) capture
2. **Scheme 2:** Microwave-assisted grafting polymerization of Glycidyl Methacrylate (GMA) onto nylon C-CP fibers as a ligand binding platform
with applications in Immobilized Metal-ion Affinity Chromatography (IMAC) protein separations and uranium capture

3. **Scheme 3**: Polydopamine-coated C-CP fiber for phosphopeptides capture and analyses
DEDICATION

I dedicate this dissertation to my parents, my siblings and especially my husband for their endless love and encouragement throughout this long journey.

To my dear husband, Bobby Cook, I love you with all my heart. You brought light into the darkest corner of my life. Thank you for protecting and supporting me every step of the way. Thank you for always staying by my side and loving me unconditionally. Thank you for taking me out of my shell and teaching me how to love and appreciate life. Thank you for just being you, a fun, loving and sweet man that always put a smile on me after a long hard day. Thank you for preparing myself to step outside into the real world on my own. I am a better and stronger person because of you. I would not trade our times for anything in the world and will carry you with me for the rest of my life.

This PhD degree is our joint effort and accomplishment. Without you, I would not have been able to go through this long and tough journey. I made it for you, my dear.
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I would like to acknowledge my research advisor, Dr. R. Kenneth Marcus, for his support throughout my graduate career. I am thankful for being a part of such a well-funded lab which allows me the opportunities to pursue my research interests. I also would like to send my deep appreciation for being so understanding during the toughest time of my life.

I would like to acknowledge my committee members, Dr. George Chumanov, Dr. Carlos D. García and Dr. Sarah W. Harcum as well as the Clemson University Chemistry Department. I also greatly appreciate the help and support from all Marcus group’s past and present members.

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CHAPTER ONE

INTRODUCTION

1.1 Introduction to affinity chromatography

Emerging as one of the most powerful techniques in modern research, chromatography is widely employed in the separation of analytes ranging from small molecules such as drug or hormone in blood sample to the purification of large biomolecules such as recombinant proteins or antibody from complex cell cultures. Among different modes of chromatography, affinity chromatography is one of the most versatile techniques. It can be defined as a chromatographic approach involving the selective and reversible interaction between the targeted analytes and the specific binding ligand immobilized to a chromatographic matrix, the process of which is schematically shown in Fig 1.1.

The unique feature of separating biomolecules based on their biological function or individual chemical structure allows affinity chromatography to be employed in purification process that would be time-consuming or challenging otherwise. Not only does it offer high selectivity but also high capacity for the analytes of interest with purity levels achievable in the order of several thousand-fold. The binding between the analytes and affinity ligands in affinity chromatography is the result of combined weak intermolecular forces including electrostatic, hydrophobic interactions, van der Waals’ forces and/or hydrogen bonding [1-2]. These reversible interactions can be reversed either specifically
through using a competitive binding reagent or non-specifically through changing the pH, ionic strength or polarity of the mobile phase to elute the bound analytes from the affinity stationary phases. Table 1.1, which is compiled based on [1-2], lists some typical biological interactions between the ligands and the targeted solutes that are frequently employed in affinity chromatography.

**Figure 1.1** Typical separation procedures in affinity chromatography
1.2 Support materials for affinity chromatography

An ideal support material for affinity chromatographic stationary phase is characterized by the following factors: chemical inertness, chemical stability, mechanical stability, pore size and particle size.

1.2.1 Chemical inertness

The most important feature of an ideal support material for affinity chromatography is chemical inertness, which means the support needs to be inert to any solutes including the analytes to avoid nonspecific binding. This feature requires that the support materials have a chemical property as close as possible to that of the aqueous medium in which it is used for. In other words, they should be as hydrophilic as possible [1]. Moreover, most affinity separations are performed at a low ionic strength, therefore, in order to prevent nonspecific ionic interactions, there should be as few charges on the support as possible. As these criteria are best satisfied by hydroxyl or amide containing support, the most commonly used media in affinity chromatography are the neutral polysaccharides and polyacrylamide [2].

Polysaccharide-based materials, including cellulose, crosslinked dextran and agarose, are the most commonly used support for affinity chromatography [2]. These polysaccharide-based supports are characterized by a porous and hydrophilic network composed of multiple hydroxyl groups facing the aqueous medium without attracting the components in the samples [1]. Cellulose was first introduced by Peterson and Sober in 1956 [3] and commercially provided under
<table>
<thead>
<tr>
<th>Targets</th>
<th>Ligands</th>
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<tr>
<td>Antigens (drugs, hormones, peptides, proteins, viruses or cell components)</td>
<td>Antibodies</td>
</tr>
<tr>
<td>Antibodies against the antigens</td>
<td>Antigens</td>
</tr>
<tr>
<td>Enzymes</td>
<td>Substrates, substrate analogues, inhibitors, cofactors, coenzymes</td>
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<tr>
<td>Sugar, sugar binding proteins, polysaccharide, glycoprotein, glycolipid, cell surface receptor</td>
<td>Lectins</td>
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<td>Receptor, carrier proteins</td>
<td>Hormones, vitamins</td>
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<td>DNA/RNA binding proteins, complementary nucleotides</td>
<td>DNA or RNA</td>
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<tr>
<td>Glutathione-S-transferase or GST fusion proteins</td>
<td>Glutathione</td>
</tr>
<tr>
<td>Synthetic dyes</td>
<td>Proteins and enzymes</td>
</tr>
<tr>
<td>Boronates</td>
<td>Carbohydrates, nucleosides, nucleotides, nucleic acids, glycoproteins, and catechols</td>
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<tr>
<td>Protein A, Protein G, Protein L and related ligands</td>
<td>Antibodies and antibody fragments</td>
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<tr>
<td>Poly (His) fusion proteins, native proteins with histidine, cysteine and/or tryptophan residues on their surfaces.</td>
<td>Metal ions</td>
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different trade names including Whatman by GE Healthcare, Cellufine by JNC Corporation, Sartobind by Sartorius and Kaneka KanCap by Kaneka [1-2]. Cross-linked dextran, which was introduced in 1959 by Porath and Flodin [4], is commonly used as a gel filtration media and better known under the trade name Sephadex produced by GE Healthcare [1-2]. Together with cellulose, cross-linked dextran is also widely used as a matrix for ion exchangers [2]. The most popular support, not only among polysaccharide-based media but also in general, is cross-linked agarose. Since its introduction as the support for affinity chromatography in 1960s, agarose, a polysaccharide extracted from seaweed agar, still retains its popularity [1]. Commercial cross-linked agarose support is provided by under various trade names such as Sepharose Fast Flow from Amersham Biopolymeric sciences or Affi-Gel from Bio-Rad Laboratories [1-2].

Known for its matrix rigidity, silica-based materials are ideal for applications that require high operating backpressure. Their use in the methods of high-performance liquid affinity chromatography (HPLAC) or high-performance affinity chromatography (HPAC) was first reported in 1978 [5]. Even though silica is highly hydrophilic, its native surface is predominantly covered with weakly acidic silanol groups, which give silica a strongly negative charged surface at neutral pH [1]. Along with other binding forces, these negative charges irreversibly bind large biomolecules such as proteins to the native silica surface. In order to “inactivate” these silanol groups and provide the matrix with functional groups for ligand attachment, the silica surface can either by coated with polymer or reacted with alcohols or trialkoxysilane [1]. Silanization with trialkoxysilane such as γ-
aminopropylsilane or γ-glycidoxypropylsilane is a commonly used procedure for this purpose [2].

Newer generations of support materials utilized in affinity chromatography include polymeric support made of polystyrene. In its native form, polystyrene/divinylbenzene exhibits a strong hydrophobic property; hence is unsuitable for affinity chromatography support [1]. These polymeric supports must be coated with hydrophilic surfaces that minimize non-specific binding and provide functional groups for ligand attachment [6-7]. Other polymers employed as support materials for affinity chromatography includes polyacrylamide and polymethacrylate [1-2].

1.2.2 Chemical stability

Another important feature of an ideal support material for affinity chromatography is that they must remain chemically stable under normal operation conditions. First of all, the support must not be susceptible to biodegradation or bio-fouling by enzymes and microbes in the process stream. In addition, it must also withstand the harsh components in the elution buffers, regenerating solvents and cleaning agents that are more than often used in every operating cycle. Polysaccharide-based supports such as cross-linked agarose or cellulose are popular because these materials satisfy most of the above criteria in terms of chemical stability. These materials are not attacked by enzymes, usable within the pH range between 3 and 12, and experience no physical or chemical damage in all commonly used aqueous eluants [8]. Moreover, cross-linked agarose is also
resistant to 0.5 M sodium hydroxide, which is the gold standard cleaning in place (CIP) step commonly used in the downstream processing.

Silica-based materials are ideal for HPLC protein separations due to the rigidity of the particles. However, despite efforts to enhance stability by using, for example, aluminum oxide [9] or zirconium oxide [10], these materials still experience hydrolytic degradation at alkaline pH [8]. As a result, silica-based materials are not suitable for operating pH range higher than 8, unless they are shielded with an inert coating which provides a hydrophilic surface and protect the silica from hydrolysis [8]. Ongoing research and development of alternative rigid materials has turned to porous synthetic organic polymers such as polystyrene/divinyl benzene or inorganic materials such as hydroxyapatite [2].

1.2.3 Mechanical stability

High-throughput affinity chromatography columns expected to operate at high flow rates must be able to withstand the pressure drop during normal operations without collapsing. Especially in downstream processing where the feed streams more than often are filled with large particulate contaminants that may plug up the separation bed, resulting in high backpressures. Packed bed made of soft porous gels such as agarose beads cannot sustain such high pressures and will collapse [1]. Silica-based supports or cross-linked polymers are more resistant to high back pressures often extending up to several hundred bars [1-2].
1.2.4 Pore size

One of the main targets in many affinity chromatography applications is large biomolecules, especially proteins. Therefore, the pore size is an important feature to be considered when choosing a material support. Preferably, the pore size of the support must be large enough for the biomolecules of interest to pass through and fully interact with the affinity ligand. According to Renkin equation [11], which allows the estimation of the effective diffusion coefficient ($D_{\text{eff}}$) of a solute in a porous material, the pore diameter of the support should be at least 5 times larger than the diameter of the solute. Therefore, for a protein with the average size of 60 Å diameter, the support need to have a pore size of at least 300 Å [1-2]. Another consideration for the pore size is the surface area of the materials which can be maximized for ligand attachment.

1.2.5 Particle size

As the binding capacity of affinity media is directly related to the ligand density, it is desirable that the support materials have a large surface area available for the immobilization of a large number of ligands. Matrices with small particle sizes not only offer just that but also minimize the mass transfer effects and band broadening. However, trade-offs in terms of high operating backpressure, potential contaminant particulate blockage and column fouling must be considered. The preferable particle size commonly used in preparative scale range from 30-100 µm [1-2].
1.3 **Immobilization methods for affinity chromatography**

As affinity chromatography purifies biomolecules on the basis of selective binding to the affinity ligand, it is important that the bio-specificity of the ligands be maintained upon being immobilized onto the chromatography matrix. Multi-site attachment, mis-orientation of the affinity ligand and steric hindrance are common issues that must be taken into consideration in the immobilization procedure.

The issue of multi-site attachment results from the affinity ligand being attached through more than one site on the ligand. In some cases, these extra sites offer stronger ligand linkage than single-site attachment. However, when denaturation or distortion is caused to the ligand through multi-site attachment, the ligand is susceptible to reduced binding affinity [12]. In order to overcome this issue, ligand attachment through more site-specific functional groups is recommended. Improper orientation, which occurs when the attachment site is located near or at the ligands active binding regions, can also result in reduced or lost ligand’s activity. This problem is the most obvious when a protein is immobilized onto a support through its pervasive primary amine groups. Immobilization methods that aim at sites away from a ligand’s active region should be considered to minimize the improper orientation of the ligand attachment. One familiar example showcasing how the issues of multisite attachment and improper orientation are overcome is the use of the carbohydrate chains in the Fc region on antibodies for the covalent immobilization onto hydrazide-containing supports, which not only minimize the multisite attachment but also expose the binding regions (Fab) for optimal binding [13-14]. The last immobilization effect that might
impact the ligand’s binding affinity is steric hindrance, which involves the blockage of the active site of a ligand by the support or neighboring ligands. Spacing of ligands adequately on the support and the use of spacer arms or tether between the ligand and support should be considered. The latter is especially critical for effective binding when a small ligand is used for the capture of larger biomolecules.

Different approaches for immobilizing affinity ligands onto stationary phases include non-covalent methods like non-specific and bio-specific adsorption, covalent coupling techniques, entrapment, and molecular imprinting. Non-specific adsorption of ligands onto support is among the earliest ligand immobilization methods reported. This technique relies purely on the physical adsorption of the ligand to a support through intermolecular forces including Coulombic interactions, hydrogen bonding, and hydrophobic interactions [15]. Due to its simplicity and convenience, nonspecific immobilization has been still commonly used and applied to various supports including alumina, silica, carbon or charcoal, collagen and metals [16]. The basis of this method involves the exposure of the support to a solution of the ligand. Adsorption of ligands can occur either through passive diffusion or with agitation or shaking. Dynamic in-column loading which feeds the ligand solution through the media with mechanical pumps, is another approach often used in the industry. As the forces involved in adsorption methods are all non-covalent and reversible in principle, desorption of ligands may occur, leading to ligand activity loss. External factors such as the pH, ionic strength and temperature that affect physical and chemical properties of both supports and ligands are to be considered for strong adsorption [1-2].
Another immobilization technique that falls into the same non-covalent category is bio-specific adsorption, which takes advantage of the non-covalent but strong and specific binding between the primary ligand and a secondary ligand immobilized onto the support [17]. The most commonly used method involves the utilization of avidin or streptavidin for the attachment of biotinylated ligands. Avidin is a glycosylated protein in egg whites while streptavidin is a non-glycosylated bacterial counterpart of avidin isolated from *Streptomyces avidinii* [17-18]. Both proteins are characterized by 4 identical biotin-binding subunits with strong affinity, \(10^{15} \text{ M}^{-1}\) for avidin and \(10^{13} \text{ M}^{-1}\) for streptavidin [17-18]. Both species can be covalently immobilized onto supports through amine reactive methods. The carbohydrate moiety on the glycosylated avidin offers another venue for a more site-specific attachment [17-18].

Non-covalent immobilization based on nonspecific adsorption of ligands onto support surface suffers most, if not all, of the potential issues regarding the attachment and subsequent activity of the ligands. While this relatively simple approach is suitable for the immobilization of inexpensive and abundant ligands, more robust methods utilizing covalent immobilization are preferred in most cases, especially in the downstream processing. Diverse coupling chemistries have long been well established targeting different functional groups including amine, sulfhydryl, hydroxyl, or carbonyl groups [1]. Typical procedures involve the activation of the ligand and/or the support. Ligand activation allows a more specific attachment through targeted regions on the ligand. This is the case in the example given above about the covalent attachment of the \(F_c\) region on antibodies onto...
hydrazide-containing supports. Though less specific, support activation is much more commonly used for ligand covalent immobilization due to the availability of commercial activated supports with various reactive moieties such as N-hydroxysuccinimide, carbonyldiimidazole or oxirane.

1.4 Capillary-Channeled Polymer (C-CP) fibers and current development

At the heart of the chromatography technique, the stationary phase is the essential component that dictates multiple aspects of the separation process. Diverse chemistry of the stationary phases, in combination with the choice of appropriate base support materials, allows chromatography to be used in a wide range of applications ranging from the high-performance separations of trace analytes in complex biological samples for chemical detection and quantification to the large-scale purification of a recombinant protein from a complex media. Traditional chromatographic stationary phases mostly utilize porous materials for their high surface area and versatility for chemical modification. However, in many cases, these materials suffer from high operating back-pressure and inefficient mass transfer. Joining the efforts to investigate alternative phases, Marcus research group (Clemson University) has investigated the potentials of capillary-channeled polymer (C-CP) fibers for the past 15 years. As shown in Fig. 1.2, these fibers are characterized by eight capillary channels running down their length, allowing them to interdigitate to form well-aligned micrometer-sized channels when packed into a column format [19-20]. It is their unique shape that allows separations to be done at high linear velocities (>75 mm s$^{-1}$) with high column
permeability (<0.14 MPa cm$^{-1}$) [21-22]. The highly efficient fluid movement through the narrow channels composed of non-porous C-CP fibers gives rise to favorable mass transfer rates, facilitating fast protein separations and processing [23-26]. Moreover, another advantage of C-CP fiber supports over other commercial sorbents is that they are quite stable over a wide pH range and are fairly inexpensive (< $100 lb^{-1}$). The packing a C-CP fiber column shown in Fig. 1.2 is a simple process that can be conveniently performed without any special equipment.

The key feature of “non-porosity” allows C-CP fibers to be used for macromolecule separations at high mobile phase velocities. When studying the relationship between the plate height and mobile phase linear velocities, the van Deemter C term is virtually non-existent. Though this is not the case for small molecules, this trend is observed for large macro-biomolecules that C-CP fibers are applied to. In other words, C-CP fibers are “non-porous” to those large biomolecules. As a result, separations of these large molecules at very high mobile phase flow rates (up to 100 mm s$^{-1}$ in microbore columns) does not lead to band broadening penalties. Recent study by the group determined the pore sizes of C-CP fibers utilizing inverse size-exclusion chromatography (iSEC) with various probe molecules ranging in size from small metal ions to 150-kDa proteins under non-retaining mobile phase conditions [26]. The nominal pore size was determined to be on the order of $\sim$2-4 nm, which is relatively small and insignificant compared to the >30 nm typical of porous phases [26].

Among the three most commonly used base fiber supports (polypropylene PP, polyterephthalate PET and nylon), PP has the most inert and hydrophobic
chemistry. While there are limited options when it comes to chemical modification, its extreme hydrophobicity allows physical adsorption to be conveniently applied for a simple surface modification to improve its application modalities. Demonstration of this simple modification is provided in recent publications where commercially available head group-modified poly(ethylene glycol) lipids (PEG-lipids) [27], which is characterized by long aliphatic tails providing a means for its attachment to the hydrophobic fiber surface. The hydrophilic head group containing affinity ligands giving PEG-lipid, hence the PP fiber surface, selectivity. The proof-of-concept for this adsorption modification of PP fiber using lipid tethered ligands (LTL lipids) was demonstrated through the capture of Texas red-labelled streptavidin spiked into TEM-mCit (endothelial marker) lysate using a biotinylated PEG-lipid. The preparation of the modified fiber can be conveniently done through spin-down procedures using micro-centrifuge and fibers packed in tip format. Another approach for greater throughput and better control of the adsorption process is through on-column loading, which is also demonstrated.

Due to its highly hydrophobic surface chemistry, the adsorption of the hydrophobic moiety of PEG-lipid exhibit excellent robustness under various common HPLC solvents and are only disrupted with 50% acetonitrile or hexane [27-28]. The fairly strong interaction between the lipid moiety of PEG-Lipid and fiber chain is investigated using the environmentally sensitive chromophore 7-nitro-2-1,3-benzoxadiazol-4-yl (NBD), either attached to the head group or the lipid tails [29]. It was determined that the lipid part of PEG-lipids intercalate into the fiber
matrix, hence, explaining its strong and robust immobilization onto C-CP PP fiber with respect to common HPLC solvents.

The physical adsorption approach is also utilized to modify C-CP PP fibers with recombinant *Staphylococcus aureus* protein A (rSPA) [30]. Demonstration of rSPA-modified PP C-CP fiber in a SPE tip format for the capture and recovery of IgG was also made. Simple spin-down procedure in a microcentrifuge was
conveniently performed to coat the fiber with rSPA ligands. The optimal loading condition was determined to generate the rSPA-modified SPE fiber tip with 5.7 mg mL\(^{-1}\) (bed volume) ligand capacity [30]. The resulting modified fiber tip exhibited stability across numerous solvent environments. The hydrophobicity of PP C-CP fiber was taken advantage for the protein A ligand adsorption. However, this chemical property of PP also presents a challenge as any uncovered surface on PP may serve as potential sites for non-specific binding of non-IgG entities in complex matrices. In fact, those works demonstrated that in the presence of a model host cell protein, myoglobin, non-specific binding was not occurring. The same conclusion could be made with the target IgG. The protein A ligand in that case adhered to the hydrophobic PP fiber surface without compromising the desired \(~3:1\) IgG: protein A capture stoichiometry [30]. The bound IgG was found to be successfully eluted by 0.1 M acetic acid with an 89% recovery [30]. Regeneration with 10 mM NaOH did not disrupt the rSPA ligand adsorption [30]. Although the study was only conducted in the micropipette tip format as an initial proof-of-concept, the potential was clearly demonstrated and deserves further investigation.

On the chemical modification front to immobilize functional groups to C-CP fiber surface through covalent binding, following are the group’s most recent efforts. PET C-CP fibers, in their native form, have been applied for protein separations in both reversed phase (RP) and hydrophobic interaction chromatography (HIC) mode [31]. The most recent attempt to apply this kind of fiber as a HIC stationary phase has been made to capture exosomes [32]. The
ester bond in PET can be conveniently modified through reaction with homo or hetero-functional amine compounds. Ethylenediamine (EDA) was used to form high densities of primary amine groups (13.9-60 μmol m⁻²) on PET surface [33]. In-situ solid phase peptide synthesis (SPPS) approach was then utilized to link 8-amino-3,6-dioxaocatanoic acid to the EDA-treated PET surface as a hydrophilic spacer, which subsequently serve as anchoring sites for D-biotin ligands [33]. High selectivity of the biotin functionalized PET C-CP fiber was demonstrated through the capture of streptavidin SAv-TR from an E. coli cell lysate spiked with SAv-TR and EGFP.

In a separate attempt, PET was functionalized with polyethyleneimine (PEI) on-column under ambient conditions to induce aminated surface [34]. 1,4-butandiol diglycidylether (BUDGE) was used as a cross-linking reagent between subsequent PEI modification steps to generate layers of high-density primary and secondary amine functional groups (74.8-154.9 μmol g⁻¹) [34]. When used as weak anion exchange phase for the capture of bovine serum albumin as the model protein, the functionalized PEI/BUDGE PET fibers show high dynamic binding capacity of 1.99–8.54 mg mL⁻¹ bed volume, at linear velocities of 88–438 cm min⁻¹ [34]. The chromatographic performance of PEI and PEI/BUDGE PET fibers was compared to that of native nylon. All the configurations showed the ability to separate a common protein mixture of bovine serum albumin, hemoglobin and lysozyme at high mobile phase linear velocity (~70 mm s⁻¹). Different elution profile and characteristics were observed, reflecting different modes of protein-surface interactions.
Recently a new microwave-assisted polymerization grafting method using potassium persulfate (KPS) as the radical initiator has been developed to functionalize nylon 6 C-CP fibers with acrylic acid (AA) and acrylamido-2-methylpropanesulfonic acid (AMPS), providing ligands for weak cation exchange (WCX) [35] and strong cation exchange (SCX) [36] protein separation respectively. The resultant grafted fibers show a significant increase of 12-13 folds in the dynamic binding capacity of lysozyme ($\sim$12-13 mg mL$^{-1}$) compared to that of the native nylon fiber ($\sim$1 mg mL$^{-1}$) [35-36]. Moreover, the highly efficient mass transfer of C-CP fiber is not affected by the modification process. High protein binding capacity is achievable even at extremely high mobile phase linear velocity ($\sim$7-70 mm s$^{-1}$) [35-36] unheard of in commercial stationary phases.

1.5 Summary

The concepts of using natural and synthetic polymer fibers as chromatographic stationary phases are not new [37-38]. Potential advantages cited include low costs, ease in column fabrication, low operation backpressure, facile solute mass transfer, and a general ease of tailoring fiber surfaces to affect high levels of chemical specificity. There have been several surface modification techniques reported for polymer fibers in the literature [39-43]. However, many of these modification methods can be detrimental to the physical properties of the polymer by destroying the polymer backbone. In order to further exploit the advantageous fluidic properties of C-CP fiber columns without compromising their mechanical strength, milder modification approaches have been a focus of this
group recently, including covalent coupling [33-34, 44] and direct ligand adsorption methods [27-28, 30, 45]. This study will work towards this end with a narrowed focus on affinity chromatography. This powerful and versatile chromatographic technique was estimated to be involved in over 60% of all purification applications [46]. Utilizing the specific but reversible interaction between the targeted analytes and the immobilized binding sites called affinity ligands on the stationary phases, this technique offers high selectivity and usually high capacity for the compounds of interest [1]. Moreover, the wide variety of available ligands makes affinity chromatography a powerful method for both small- and large-scale purification of compounds from complex samples.

The research studies reported in this work focus on the development of functionalized C-CP fiber for a variety of affinity chromatography applications. The ultimate goal is to investigate non-invasive modification schemes that do not compromise the mechanical strength and fluidic properties of C-CP fibers. Affinity ligands are immobilized on the fiber surface through one of the following schemes:

1. **Scheme 1**: Physical adsorption of recombinant protein A (rSPA) ligands on polypropylene (PP) C-CP fibers for immunoglobulin (IgG) capture

2. **Scheme 2**: Microwave-assisted grafting polymerization of Glycidyl Methacrylate (GMA) onto nylon C-CP fibers as a ligand binding platform with applications in Immobilized Metal-ion Affinity Chromatography (IMAC) protein separations and uranium capture

3. **Scheme 3**: Polydopamine-coated C-CP fiber for phosphopeptides capture and analyses
Modification scheme 1 was investigated in chapter two [47] and three [48], the graphic abstract of which is provided in Fig. 1.3. Specifically, in chapter two, the loading characteristics of recombinant *Staphylococcus aureus* protein A (rSPA) on polypropylene (PP) capillary-channeled polymer (C-CP) fibers were investigated through breakthrough curves and frontal analysis [47] The dynamic adsorption data was fit to various isotherm models to assess the possible mode of rSPA-PP fiber adsorption. Among them, the Langmuir-linear model fit the experimental data best, suggesting a two-stage mechanism of adsorption. The first stage involves the formation of a monolayer coverage, which follows the Langmuir isotherm. When the adsorbate concentration increases, solute starts to adsorb onto the already adsorbed layer, following a linear adsorption response. The relationship between the rSPA loading and flow rate and column length was also investigated. These two parameters are related through the residence time of rSPA in the column. It was determined that loading at the flow rate of 0.5 mL min\(^{-1}\) (∼28 mm s\(^{-1}\)) with a 1 × 10\(^{-5}\) M (0.5 mg mL\(^{-1}\)) rSPA feed concentration on a 30-cm (0.762 mm i.d.) column could conveniently produce a reasonable binding capacity of rSPA on PP surface within only 6 min. Under those conditions, the rSPA binding at 50% breakthrough was found to be ∼2.1 mg g\(^{-1}\) fiber. Operation of the rSPA-modified columns across ten complete processing cycles using clean-in-place conditions (including urea, guanidine HCl, and NaOH) commonly used in the bioprocessing industry allows assessment of the robustness of the rSPA capture layers. In all cases, the robustness was quite good, with the relative responses providing insights to the rSPA/PP surface structure. In chapter three,
Figure 1.3 Modification scheme 1: Protein A-modified C-CP PP fiber for IgG capture and analysis.
rSPA-modified PP C-CP fibers were used as an affinity chromatography stationary phase for the quantitation of immunoglobulin G (IgG) in complex biological matrices [48]. Optimization of the chromatographic method regarding mobile phase components and load/elution conditions was performed. The six-minute analysis, including a load step with 12 mM phosphate at pH 7.4, an elution step with 0.025% phosphoric acid and a re-equilibration step, was employed for quantitation of IgG₁ from 0.075 to 3.00 mg mL⁻¹ in an IgG-free CHO cell supernatant matrix. Quantification of IgG₁ content in a different CHO cell line was accomplished using the external calibration curve as well as using a standard addition approach. The high level of agreement between the two approaches suggests that the protein A-modified C-CP fiber phase is immune from matrix effects due to concomitant species such as host cell proteins (HCPs), host cell DNA, media components and other leachables and extractables. The inter-day and intra-day precision of the method were 3.1 and 3.5% RSD respectively for a single column. Column-to-column variability was 1.3 and 6.6% RSD for elution time and peak area, respectively, across columns prepared in different batches. The method reported here is well-suited for IgG analysis in complex harvest cell culture media in both the development and production environments.

Modification scheme 2 was investigated in chapter four [49], the graphic abstract of which is shown in Fig. 1.4. In this project, nylon 6 capillary-channeled polymer (C-CP) fibers were grafted with glycidyl methacrylate (GMA) as a monomer with ceric ammonium nitrate (in 0.1 M nitric acid) used as the initiator. The polymerization reaction occurs rapidly (15 min) in a residential microwave.
Figure 1.4 Modification of nylon-iminodiacetic acid (nylon-IDA) C-CP fibers for IMAC stationary phases
Iminodiacetic acid (IDA) is then attached to the grafted GMA polymers by reacting with the reactive terminal epoxide groups. Different parameters regarding the grafting time, initiator concentration and conversion time were investigated to find the optimal conditions for the entire modification process. The resulting nylon-IDA fibers were characterized by attenuated total reflection-Fourier transform infrared spectroscopy (ATR-FTIR) and scanning electron microscopy (SEM). The resulting carboxyl density and copper binding capacity were determined to be $0.61 \pm 0.02 \, \mu\text{mol g}^{-1}$ and $0.38 \pm 0.01 \, \mu\text{mol g}^{-1}$, respectively. When charged with Cu$^{2+}$ ions and packed in a column format, the nylon-IDA fibers can be applied as an IMAC stationary phase for the separation of histidine rich proteins. The performance of this novel phase was evaluated through the separation of a mixture of model proteins (cytochrome C, α-chymotrypsinogen A and lysozyme) and a recombinant histidine-tagged protein (his-tagged ubiquitin). Despite multi-step modifications, columns of the modified fibers still maintain the anticipated high levels of throughput and efficiency, with binding capacities of $6.9 \pm 0.6 \, \text{mg lysozyme g}^{-1} \, \text{fiber}$ and $6.3 \pm 0.1 \, \text{mg His-tagged ubiquitin g}^{-1} \, \text{fiber}$.

Chapter five explores further the application of modification scheme 2, the graphic abstract of which is provided in Fig. 1.5. Nylon 6 C-CP fibers were once again used as a base support on which the grafting of GMA polymer was performed in a domestic microwave with ceric ammonium nitrate (in dilute nitric acid) being used as the initiator. The reactive epoxide groups of the GMA polymer serve as anchoring points for the attachment of iminodirpropionitrile (IDPN), providing the dinitrile groups on the fiber surface. Subsequent reaction with
Figure 1.5 Modification of nylon-amidoxime (nylon-AO) C-CP fibers for uranyl ions capture and analysis
Figure 1.6 Modification of nylon-polydopamine (nylon-PDA) C-CP fiber for phosphopeptide capture and analysis
hydroxylamine NH$_2$OH converts these dinitriles into amidoxime functional groups that are capable of binding uranium ions with high binding affinity. The resulting nylon-amidoxime (nylon-AO) fibers were characterized by attenuated total reflection-Fourier transform infrared spectroscopy (ATR-FTIR) and Scanning electron microscopy/Energy Dispersive X-Ray Spectroscopy (SEM/EDX). The uranium binding capacity of the nylon-AO was determined both through dynamic binding experiments. Applications of nylon-AO to sample clean-up and preconcentration of uranium in water samples will be investigated.

Finally, the modification scheme 3 reported in chapter six takes advantage of the oxidative self-polymerization of dopamine under alkaline conditions [50]. The graphic abstract of this modification scheme is given in Fig. 1.6. A simple system involving a dilute aqueous solution of 0.2% w/v dopamine hydrochloride in 0.15% w/v TRIS buffer, pH 8.5 was utilized to coat polydopamine onto C-CP nylon surface. The reaction, upon exposure to air, occurs instantaneously with a clear color change of the dopamine solution from clear to pinkish shade. After 72 hours, the reaction mixture turns dark brown. Upon thorough washing, the modified C-CP nylon fiber shows a uniform dark brown coating of polydopamine on the surface. Confirmation of the coating, though, will also be made through ATR-FTIR analysis. Imaging using scanning electron microscopy (SEM) will also be performed to examine the fibers’ morphology and topography. The polydopamine-coated C-CP nylon fiber will then be packed into a microbore column format. Loading of Fe$^{3+}$ ions will be performed through frontal analysis with iron (III) nitrate solution. The saturation of the polydopamine-coated fiber will be monitored at 300 nm and used
for the calculation of the Fe$^{3+}$ binding capacity. The presence of Fe$^{3+}$ can also be confirmed by Scanning Electron Microscopy/Energy Dispersive X-Ray Spectroscopy (SEM/EDX). The Fe$^{3+}$-bound polydopamine-coated C-CP fibers will then be tested for their ability to capture phosphopeptides from a protein digest mixture. This experiment will probably be done in the SPE tip format. The phosphopeptide eluate will then be confirmed using MALDI-TOF.
1.6 List of publications

The following chapters in this dissertation are based on these papers.


**Chapter Four:** Trang, H. K.; Jiang, L.; Marcus, R. K., Grafting polymerization of Glycidyl methacrylate onto capillary-channeled polymer (C-CP) fibers as a ligand binding platform: Applications in immobilized metal-ion affinity chromatography (IMAC) protein separations. *J. Chromatogr. B* 2019, 1110, 144-154.

**Chapter Six:** Trang, H. K.; Marcus, R. K., Application of Polydopamine-Coated Nylon Capillary-Channeled Polymer (C-CP) Fibers as a Stationary Phase for Mass Spectrometric Phosphopeptide Analysis. *Electrophoresis* 2019, *Submitted for publication*. 
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50. Hung K Trang; Marcus, R. Kenneth, Application of Polydopamine-Coated Nylon Capillary-Channeled Polymer (C-CP) Fibers as a Stationary Phase for Mass
CHAPTER TWO

EVALUATION OF LOADING CHARACTERISTICS AND IgG BINDING PERFORMANCE OF STAPHYLOCOCCAL PROTEIN A ON POLYPROPYLENE CAPILLARY-CHANNELED POLYMER FIBERS

2.1 Introduction

The past decade has witnessed dramatic growth in the therapeutic monoclonal antibody (mAbs) industry. According to Biopharmaceutical Benchmarks 2014, mAb sales reached a total of $63 billion in that year, representing almost 50% of the total biopharmaceutical global market, and included six of the ten top-selling products [1]. New insights into bioprocessing methods have led to dramatic increases in productivity. The titer of industry-scale recombinant cell cultures, from which antibodies are produced, has increased significantly from less than 1 g L$^{-1}$ to the common 5 g L$^{-1}$ (or even 10 g L$^{-1}$ in some reports) in the past few years, and in much larger-scale bioreactors of 20-25 kL capacity [2-4].

During the early development phase of recombinant mAb production, screening of large numbers of harvest cell culture samples for immunoglobulin G (IgG) titer is necessary. Towards this purpose, affinity chromatography utilizing the protein A ligand is most often the method of choice to determine the mAb concentration due to the high selectivity of the Fc region of IgG toward protein A [5, 6]. This method is also widely employed as one of the steps in mAb downstream
processing. However, as the initial capture of IgG using the immobilized protein A must be followed by several polishing steps to assure safety and efficacy, downstream processing has been a bottleneck in the antibody production, adding up to 80% of the total manufacturing cost [1].

Commercially available protein A sorbents come on a wide variety of supports including agarose, polystyrene, porous glass, and macroporous polymers [7–9]. They also differ in how protein A ligands are immobilized onto the sorbents, mostly through covalent binding such as CNBr, epoxy, amine, and thioester chemistry. However, these commercialized sorbents are expensive and limited in the operating flow rate (throughput) due to high backpressures and low mass transfer rates. Considering the ever-growing mAb market, there is a high need for the analysis and processing of high volumes of clones and samples; hence the high demand for fast, reproducible and cost-efficient platform methods for mAb titer analysis and characterization. Ideally, these methods could be scaled-up for industrial manufacturing; these needs inspire this work.

The purpose of this study is to investigate further the potential of polypropylene (PP) capillary-channeled polymer (C-CP) fibers as support phases for protein A affinity chromatography. C-CP fibers are characterized by eight capillary channels running down their length, allowing them to interdigitate to form well-aligned micrometer-sized channels when packed into a column format [2-3]. It is their unique shape that allows separations to be done at high linear velocities (>75 mm s⁻¹) with high column permeability (<0.14 MPa cm⁻¹) [4-5]. The highly efficient fluid movement through the narrow channels composed of non-porous C-
CP fibers gives rise to favorable mass transfer rates, facilitating fast protein separations and processing [6-9]. Moreover, another advantage of C-CP fiber supports over other commercial sorbents is that they are quite stable over a wide pH range and are fairly inexpensive (< $100 lb⁻¹).

The concepts of using natural and synthetic polymer fibers as chromatographic stationary phases are not new [10-11]. Potential advantages cited include low costs, ease in column fabrication, low operation backpressure, facile solute mass transfer, and a general ease of tailoring fiber surfaces to affect high levels of chemical specificity. There have been several surface modification techniques reported for polymer fibers in the literature [12-16]. However, many of these derivatization methods can be detrimental to the physical properties of the polymer by destroying the polymer backbone. In order to further exploit the advantageous fluidic properties of C-CP fiber columns without compromising their mechanical strength, milder modification approaches have been a focus of this group recently, including covalent coupling [17-19] and direct ligand adsorption methods [20-22]. Among recent work was the demonstration of a simple modification of PP C-CP fibers with protein A by direct adsorption for the capture of IgG [23]. The ligand in this case adheres to the hydrophobic PP fiber surface without compromising the desired ~3:1 IgG: protein A capture stoichiometry. Although the study was only done in the micropipette tip format as an initial proof-of-concept, the potential was clearly demonstrated and deserves further investigation.
As a follow-up study, in this work, the loading characteristics of recombinant *Staphylococcus aureus* protein A (rSPA) ligands on PP C-CP fiber surfaces and their resulting IgG binding capacity are investigated across complete processing cycles. The study was performed using a microbore polyether ether ketone (PEEK) column format on an analytical HPLC system. The dynamic loading characteristics of rSPA on PP C-CP fibers were determined at seven concentration levels and five volumetric flow rates on columns of different lengths through frontal analysis. Evaluation of these parameters provides a better understanding of how rSPA dynamically interacts with PP C-CP fibers and helps determine the loading conditions for the optimal rSPA density on PP surface in the most efficient way. The second part of the study evaluated the IgG processing performance of the adsorbed rSPA ligands on the PP fibers. IgG binding capacity was determined indirectly through the elution peak areas following the frontal loading. Changes in IgG dynamic binding capacity and recovery on the rSPA modified columns were determined over 10 operating cycles with and without clean-in-place (CIP) steps, allowing a realistic evaluation of the potential of using PP C-CP fibers as a new IgG-capture stationary phase.

2.2 Materials and methods

2.2.1 Chemicals and instrumentation

HPLC-grade acetonitrile (ACN) and methanol (MeOH) were purchased from EMD Millipore (Darmstadt, Germany). Phosphoric acid (85%) was purchased from Fisher Scientific (Pittsburg, PA). Sodium hydroxide and potassium chloride
were purchased from Fisher Scientific (Fair Lawn, NJ). Biotechnology-grade sodium chloride was purchased from Amresco (Solon, Ohio). Guanidine hydrochloride (guanidine HCl), potassium phosphate monobasic and sodium phosphate dibasic were purchased from Sigma Aldrich (St. Louis, MO). Urea was purchased from J.T. Baker (Center Valley, CA). DI water (18.2 MΩ.cm) obtained from a Millipore water system (Billerica, MA) was used to prepare all aqueous solutions. Phosphate buffered saline (PBS) was prepared by dissolving NaCl (8.18 g), Na$_2$HPO$_4$ (1.42 g), KCl (0.20 g) and KH$_2$PO$_4$ (0.24 g) in 1.0 L of DI water. Protein A derived from recombinant Staphylococcus aureus (rSPA) was obtained from Syd Labs (Malden, MA) in the bulk stock solution of 49.4 mg mL$^{-1}$. Further dilutions were prepared from this stock in PBS. Purified IgG from human serum from Sigma-Aldrich was prepared in stock solutions of 5 mg mL$^{-1}$ in PBS.

2.2.2 C-CP fiber microbore column construction and instrumentation

Polypropylene C-CP fibers were manufactured through a melt extrusion process from PP pellet feedstock in the Clemson University, School of Materials and Engineering (Clemson, SC) [24]. The size of PP fiber used in the study is 3 deniers per filament (dpf); i.e., 3 grams per 9000 meters of single fiber. The perimeter determined through electron micrographic imaging of cross-sectioned fibers is 199 µm. Based on these measurements, and assuming that the fiber is completely non-porous (as is the case relative to the protein A ligand and IgG here [7]), the specific surface area of the fibers is calculated to be approximately 0.6 m$^2$ g$^{-1}$. 
The procedure for packing C-CP fibers into microbore columns has been described previously [4-5]. In order to construct the microbore columns used in this study, 2 rotations of PP fiber bundles (480 single fibers) were wound from the spool onto a rotary counter, stretched on a dye fork, and rinsed sequentially with near-boiling H₂O, ACN, MeOH and DI H₂O. Fibers were pulled through 0.762 mm i.d. PEEK tubing (IDEX Health & Science LLC (Oak Harbor, WA)). The columns were then flushed with ACN and DI-H₂O at 1 mL min⁻¹ on the HPLC system until a stable UV absorbance detector baseline at 216 nm was reached. This ensures the complete stripping of residual antistatic surfactant coatings applied during the manufacturing process. Once cleaned, the columns were stored at 4°C and cut into desired lengths. To confirm uniformity, the interstitial fraction (ε₁) determined with uracil retention of all the columns constructed was 0.65 with less than 5% variability. Cleaning and chromatographic experiments were performed on a Dionex Ultimate 3000 HPLC system operated by Chromeleon 6.80 software and consisted of an LPG-3400SD pump, a WPS-3000TSL autosampler and a VWD-3400RS UV-Vis absorbance detector.

2.2.3 rSPA loading characteristics

2.2.3.1 rSPA dynamic binding capacity

The dynamic binding characteristics for rSPA on the PP C-CP fibers was obtained through frontal analysis [9]. A stream of mobile phase containing a known concentration of rSPA was continuously fed through the microbore column until breakthrough was observed at the column outlet as depicted in Fig. 2.1. Frontal
Figure 2.1 a) Demonstration of the basic principles of frontal analysis; b) Breakthrough curves obtained with and without column determined at UV 230 nm, flow 0.5 mL min⁻¹ (~28 mm s⁻¹), 0.5 mg mL⁻¹ rSPA on 30-cm PEEK column. The area between the two curves is equal to the gray rectangle and represents the amount of rSPA adsorbed on the fiber.

analysis was first performed in the column by-pass position to obtain the elution profile of the protein solution under non-retained conditions. The area under this elution curve represents the mass of the protein flowing through the system, non-retained. The same experiment was then performed on the microbore column packed with PP fiber to obtain similar breakthrough curve. In this case, the area under the curve represents the mass of the protein left after some amount was bound onto the PP fiber. The difference in the area between the two breakthrough curves is the amount of adsorbed protein on the fiber, which can be calculated in two ways. The first approach involves the integration of the area under the two curves and taking their difference. The second approach is to determine the values of $V_0$ and $V$ through the integration of the breakthrough profiles (equal area
method). The area of interest is equal to the area of the rectangle defined by \( [A]_0 \) and \( (V-V_0) \) as demonstrated in Fig. 2.1a. Fig. 2.1b depicts actual breakthrough curves reflecting the mobile phase protein optical absorbance at 230 nm, performed on a 30-cm microbore column, representative of all the results obtained in the study.

### 2.2.3.2 rSPA adsorption isotherms

Frontal analysis was performed on 10-cm columns at a volume flow rate of 0.1 mL min\(^{-1}\) (equivalent to 5.6 mm s\(^{-1}\)) with rSPA concentrations from 0.125 to 2 mg mL\(^{-1}\). The UV detector was set at 230 nm for the breakthrough curve recording. Determination of the dynamic binding capacity was done as described above. The obtained data were then fit to various isotherm models using MATLAB (Natick, MA). In order to evaluate the fit, both the correlation coefficient (\( R^2 \)) and the residual sum of squares (RSS) between the experimental and fit isotherm models were used [25].

\[
RSS = \sum_{i=1}^{n}(q_{exp,i} - q_{calc,i})^2 \tag{Eq. 1}
\]

In this equation, \( q_{exp,i} \) is experimental binding capacity, \( q_{calc,i} \) is the predicted corresponding to the fit isotherm model and \( n \) is the number of data points. To be clear, these models should by no means reflect \textit{a priori} the rSPA-PP fiber adsorption process(es). However, evaluating their fits to the experimental data should provide insights into possible adsorption mechanisms/processes.

### 2.2.3.3 Effects of flow rate and column length on rSPA loading
The effect of the volume flow rates on rSPA loading was investigated by feeding $1 \times 10^{-5}$ M (0.5 mg mL$^{-1}$) rSPA through 10-cm columns at 0.10, 0.25, 0.5, 0.75, and 1.0 mL min$^{-1}$ (equivalent to linear velocities of 5.6, 14.0, 28.1, 42.2, and 56.3 mm s$^{-1}$). In another similar set of experiments, frontal analysis was also performed at a constant flow rate at 0.5 mL min$^{-1}$, on column lengths ranging from 5 to 33 cm.

### 2.2.4 IgG binding characteristics of the adsorbed rSPA ligands

Following parametric optimization of the ligand application, rSPA-modified columns were prepared by loading rSPA at a concentration of $1 \times 10^{-5}$ M at a flow rate of 0.5 mL min$^{-1}$ onto PP fibers packed in 30-cm PEEK columns. The area between the two IgG breakthrough profiles obtained when the system was run with and without rSPA-modified column was too small (Fig. 2.1) for accurate determination using the approaches described in section 2.3.1. As a result, the IgG binding capacity of the columns was evaluated based on the recovered amount of IgG, represented by the elution peak area. The elution peak areas of 10 complete cycles were converted into a percentage relative to the first cycle being 100%. Data were collected for three different columns that were run under the same cycling conditions and averaged to better reflect trends and evaluate the precision of the methodology. Complete processing cycles, including load, elution, equilibration, and CIP steps were then tested on 10-cm columns cut from those longer ones. Loading was done with $\sim 7 \times 10^{-8}$ M (0.01 mg mL$^{-1}$) IgG solutions in PBS (pH 7.4) until saturation was reached (5 min), followed by elution with 0.025%
phosphoric acid (2.5 min). The loading and elution steps were recorded at the wavelength of 216 nm. Fig. 2.2 depicts typical absorbance traces for these two steps. The column was then re-equilibrated with PBS (2.5 min) and cleaned-in-place as would be done in industrial settings (2.5 min). Finally, the column was re-equilibrated until the baseline was reached before another cycle started, with the total cycling time being less than 20 min in all cases. The cleaning reagents used in this study included 2 M guanidine HCl, 2 M urea, 0.1 M NaOH and 1 M NaOH. The whole cycle was performed at a flow rate of 1.0 mL min$^{-1}$.

2.3 Results and discussion

2.3.1 rSPA adsorption isotherms and postulated adsorption process

![Absorbance traces for load and elution steps of IgG on rSPA modified column](image)

**Figure 2.2** Typical temporal responses for the load and elution steps of IgG on 10-cm rSPA modified column. Loading solution: 10 g mL$^{-1}$ IgG. Flow rate: 1 ml min$^{-1}$. UV detection at 216 nm.
Typical adsorption isotherms reflect the amount of adsorbate bound to the surface of the adsorbent ($q$, mg g$^{-1}$) as a function of the concentration ($C$, mg mL$^{-1}$) under equilibrium conditions at a constant temperature [26]. Therefore, adsorption isotherms are most commonly constructed from static (equilibrium) binding capacity measurements [26]. Alternatively, the concept of an adsorption isotherm can be also be generated from dynamic binding capacity obtained from performing frontal analysis [9, 21]. This latter approach was used in this study to obtain the adsorption isotherms. The experimental data employed in the adsorption isotherms shown in Fig. 2.3 is the result of consecutive frontal analysis experiments performed at various input (not equilibrium) concentration levels ranging from 0.125 to 2 mg mL$^{-1}$. Breakthrough curves were obtained through frontal analysis at the slowest possible flow rate of 0.1 mL min$^{-1}$ (5.6 mm s$^{-1}$ in linear velocity) that can be accurately executed by the present HPLC pump. It is worth mentioning that this flow rate, equivalent to about 3 column void volumes per minute, is comparatively higher than those normally used for frontal analysis in common porous stationary phases (1-2 mm s$^{-1}$). Herein, lies the advantage of C-CP fibers which affect virtually no mass transfer resistance in comparison to traditional stationary phases [6].

Based on the measurements of the fiber perimeter of the cross-section and the mass per single fiber, the specific surface area is estimated to be about 0.6 m$^2$ g$^{-1}$ fiber. Calculation using the Stokes radius of protein A (5 nm) [27] produces a theoretical saturation binding capacity for a PP fiber surface in the range of $\sim$ 0.48 mg g$^{-1}$ fiber. According to Bjork, the Staphylococcal protein A is not a typical
globular protein, instead it has a markedly elongated shape [27]. This calculation assumes that there is only one layer adsorbed onto the fiber surface; rSPA attaches to the fiber on its shorter edge and there are no conformational changes or interactions between the adjacent rSPA molecules. It is also assumed that the PP fiber surface is planar, which is not unreasonable based on the known porosity [7]. All of the above are simplest-case assumptions. The fact that this calculated value is lower than the dynamic binding capacities obtained in this study means that there is more to the adsorption of rSPA to PP fiber surface than those assumptions suggest. The data display a close-to-linear relationship across the tested concentration range as shown in Fig. 2.3a. In fact, fitting the obtained data points to a linear isotherm produced a very satisfactory correlation coefficient ($R^2$) value of 0.9962 as presented in Table 2.1. Similar close-to-linear isotherms were previously reported for the adsorption of FITC-PEG lipids (0.005-3.0 mg mL$^{-1}$) on PP C-CP fibers [21] and lysozyme (0.005-5.0 mg mL$^{-1}$) and BSA (0.025-1.5 mg mL$^{-1}$) on PET C-CP fibers [28]. The similarity of the adsorption behavior in these studies can possibly be attributed to the hydrophobic nature of the interaction between the adsorbate and the fiber surfaces. On the contrary, the adsorption of BSA on nylon C-CP fibers, which are more hydrophilic, does not display the close-to-linear isotherm presented here [9]. Instead, its isotherm was more typical of the classic Langmurian model and reached saturation within a smaller concentration range (below 1 mg mL$^{-1}$) [9]. This initial comparison to previous work demonstrates the need to evaluate various isotherm models to obtain mechanistic insights.
regarding the adsorptive behavior of different solutes on to C-CP fibers of different base materials.

The classic Langmuir isotherm is the most common model used by chromatographers to explain surface adsorption [29-30]. Its equation is expressed below

\[ q = q_s \frac{bc}{1+bc} \]  

(Eq. 2)

Where \( C \) is the protein feed concentration (mg mL\(^{-1}\)), \( q \) is the binding capacity (mg g\(^{-1}\)) of the column at the protein concentration \( C \), \( q_s \) (mg g\(^{-1}\)) is the maximum DBC at infinite protein loading concentration and \( b \) (mL mg\(^{-1}\)) is the adsorption-desorption equilibrium constant on the surface [30-32]. Fitting the data from Fig. 2.3a to Eq. 2 yields \( q_s = 7.455 \) mg g\(^{-1}\) and \( b = 0.885 \) mL mg\(^{-1}\), with the least-squares goodness-of-fit \( R^2 = 0.9319 \) as presented in Table 2.1. As shown in Fig. 2.3a, the Langmuir isotherm underestimated binding of rSPA ligands on PP fiber surface at the lower end of the concentration range (below 0.5 mg mL\(^{-1}\)), while overestimating in the higher concentration levels.

Originally developed to explain gas-solid adsorption behavior, the Langmuir model requires the following assumptions to be met [33]: (i) the adsorbent surface is perfectly flat with no corrugations, (ii) all adsorbing sites on the surface are equivalent; i.e. the surface is completely homogeneous, (iii) only monolayer coverage is allowed, and (iv) there are no adsorbate-adsorbate interactions. In order to test the validity of the Langmuir model, the Scatchard plot of \( q/C \) versus \( q \) was generated. In theory, if the isotherm follows the Langmuir model perfectly, the
Scatchard plot is expected to display linearity with a negative slope [30], as demonstrated in the following equation:

\[
\frac{q}{C} = -\frac{q}{K_d} + \frac{Q_{max}}{K_d}
\]  

(Eq. 3)

Figure 2.3 a) Experimentally determined dynamic binding capacities and adsorption isotherm fits for rSPA loading of PP C-CP fibers with fits to linear, Langmuir, Freundlich and Moreau isotherm models. b) Scatchard plot of the adsorption isotherm data. c) Experimentally determined dynamic binding capacities and adsorption isotherm fits for rSPA loading of PP C-CP fibers with fits to bi-Langmuir, BET and Langmuir-linear isotherm models.
However, the Scatchard plot Fig. 2.3b is clearly not linear over the operable concentration range, therefore the Langmuir isotherm is not likely an appropriate model for the rSPA on PP C-CP fibers.

Table 2.1 Derived parameters for different rSPA adsorption isotherm models

<table>
<thead>
<tr>
<th>Isotherm model</th>
<th>Parameters</th>
<th>Correlation coefficient $R^2$</th>
<th>RSS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linear</td>
<td>$a = 1.975 \text{ mg g}^{-1}$, $b = 1.181$</td>
<td>0.9962</td>
<td>0.4112</td>
</tr>
<tr>
<td>Langmuir</td>
<td>$q_e = 7.455 \text{ mg g}^{-1}$, $b = 0.885 \text{ ml mg}^{-1}$</td>
<td>0.9319</td>
<td>0.7454</td>
</tr>
<tr>
<td>Freundlich</td>
<td>$a = 3.357 \text{ ml g}^{-1}$, $n = 0.548$</td>
<td>0.9839</td>
<td>0.1757</td>
</tr>
<tr>
<td>Bi-Langmuir</td>
<td>$q_{e,1} = 31.82 \text{ mg g}^{-1}$, $b_1 = 0.07164 \text{ ml mg}^{-1}$, $q_{e,2} = 1.085 \text{ mg g}^{-1}$, $b_2 = -18420 \text{ ml mg}^{-1}$</td>
<td>0.9972</td>
<td>0.0703</td>
</tr>
<tr>
<td>Moreau</td>
<td>$q_e = 9.354 \text{ mg g}^{-1}$, $b = 0.7972 \text{ ml mg}^{-1}$, $l = 0.4247$</td>
<td>0.9420</td>
<td>0.6344</td>
</tr>
<tr>
<td>BET</td>
<td>$q_e = 2.641 \text{ mg g}^{-1}$, $b_a = 6.029 \text{ ml mg}^{-1}$, $b_l = 0.2543 \text{ ml mg}^{-1}$</td>
<td>0.9968</td>
<td>0.0347</td>
</tr>
<tr>
<td>Langmuir-Linear</td>
<td>$q_e = 1.486 \text{ mg g}^{-1}$, $b_1 = 24.9 \text{ ml mg}^{-1}$, $b_2 = 1.243 \text{ ml mg}^{-1}$</td>
<td>0.9991</td>
<td>0.0010</td>
</tr>
</tbody>
</table>

The Scatchard plot provides other insights into the adsorption between rSPA and PP fiber surface. First, the characteristic concave upward form of the Scatchard plot is indicative of negative cooperativity [34], which can be explained using the random sequential adsorption (RSA) theory, also commonly referred to as “random car parking” [35-37]. According to this theory, a protein molecule only adsorbs to a surface area that does not overlap with any other already adsorbed protein. In the case of encountering an adsorbed protein on its way to the surface, it reflects back into the bulk solution. As the surface is increasingly covered, the gaps between the adsorbed proteins are too small to accept incoming proteins. Consequently, it takes longer periods of time for a molecule to access the
remaining spaces that are large enough to attach (i.e., slow kinetics). A second reason for the Scatchard plot curvature is the presence of multiple classes of surface binding sites; simply binding that is other than those between the rSPA ligands and a homogeneous PP fiber surface. PP fibers are characterized by extremely hydrophobic nature, which is the only driving force behind the interaction with rSPA. Unless there were significant amounts of impurities on/in the PP fiber surface/matrix, the chances for binding to these “other” sorts of support sites are extremely low.

No matter the causes, the characteristic curvature of the Scatchard plot suggests that heterogeneous isotherm models have better chances to fit, and adequately explain, the experimental data [38-39]. The first adsorption model for heterogeneous surface adsorption examined was the Freundlich isotherm [26, 30, 40], the equation of which is presented as:

\[ q = aC^{1/n} \]  

(Eq. 4)

where \( a \) (mL g\(^{-1}\)) is the Freundlich coefficient which is an indicator of equilibrium adsorption capacity and \( n \) is the Freundlich exponent which is smaller than unity and reflects how the isotherm deviates from the linear model. Treating the raw data in Fig. 2.3a with Eq. 4 yields coefficient values of \( a = 3.357 \) (mL g\(^{-1}\)) and \( n = 0.548 \) (Table 2.1). The correlation coefficient \( R^2 \) of the fitting is 0.9839, which is higher than that of the Langmuir model. This Freundlich isotherm model was originally proposed by Boedeker [41] for the adsorption of polar compounds on polar adsorbents and was later popularized by Freundlich [40, 42]. The Freundlich isotherm is quite accurate in modeling the adsorption on heterogeneous surfaces.
However, this model cannot predict the plateauing trend of the adsorption response and it also fails to explain the linearity at the low concentrations [26]. In fact, the Freundlich isotherm has an infinitely large initial slope [32]. As the concentration approaches zero, the limit of the ratio \( q/C \) is infinite, which is clearly demonstrated by the isotherm’s tangential approach to the vertical axis [30]. This shows the trend of strong adsorption at low concentration and means that the retention under analytical conditions is infinite. In the previous study where the protein A modification on PP fibers was initially evaluated with the tip format [23], it was found that the loading of rSPA at the modest concentration of 0.1 mg mL\(^{-1}\) leads to a strongly adsorbed ligands that were not washed off by various solvent systems including PBS pH 3-12, 1 M NaCl, 0.01M NaOH, 0.1 M citrate buffer pH 3-5, 0.1 M phosphate buffer pH 5-7 and 0.5 M acetate buffer pH 3-5.

According to Gritti and Guiochon, concave upward Scatchard plots similar to the one displayed on Fig. 2.3b suggest that the bi-Langmuir isotherm should be evaluated [38-39]. First suggested by Graham in 1953 [43], this isotherm is considered the simplest model for adsorption onto a non-homogeneous surface [30]. It assumes that the surface is composed of patches of two different homogeneous surfaces, which may have similar interactions with a given adsorbate while exhibiting different interaction energies [30]. The equation for the bi-Langmuir model is presented as:

\[
q = q_{s,1} \frac{b_1 C}{1 + b_1 C} + q_{s,2} \frac{b_2 C}{1 + b_2 C}
\]  
(Eq. 5)

in which the subscripts refer to the two adsorption sites [30, 32]. Fitting of the raw data to the Eq. 5 generated \( q_{s,1} = 31.82 \) mg g\(^{-1}\), \( b_1 = 0.07164 \) mL mg\(^{-1}\), \( q_{s,2} = 1.085 \)
mg g⁻¹ and \( b_2 = -18420 \text{ mL mg}^{-1} \) with the correlation coefficient \( R^2 \) of 0.9972 (Table 2.1). As shown in Fig. 2.3c, the bi-Langmuir isotherm fits the experimental adsorption data well (as would be expected in any case by adding more variables). As the adsorption on each site is independent of the other, breaking Eq. 5 into two separate equations can provide some useful information regarding the fit of the model. Supposedly, both sites should follow the Langmuir isotherm [30]. However, as observed from Fig. 2.3c, only site 1 shows a typical Langmuirian response, while site 2 yields a fairly linear isotherm. Contributions from site 1 at concentration levels up to 0.5 mg mL⁻¹ are dominant. However, as the concentration of rSPA increases beyond that point, binding at site 1 reaches a plateau. When the rSPA concentration is \( \sim 1.0 \text{ mg mL}^{-1} \), the contributions from the two sites are about equal, and at higher concentrations, binding at site 2 quickly dominates.

PP fibers are made through melt extrusion from pure polypropylene pellet. Considering the homogeneity of the material, it is hard to comprehend how it could generate such different binding surfaces that facilitate both Langmuirian and linear adsorption. The same situation was also reported by Sainio when the bi-Langmuir isotherm was used for the adsorption of benzyl(dimethyl)dodecyl ammonium chloride and benzyl(dimethyl)tetradeccyl ammonium chloride on polystyrene-divinylbenzene adsorbent [25]. Considering the possibility of adsorbate aggregation in that system, the authors suggested that the bi-Langmuir model could describe two different adsorption stages rather than the adsorption of two independent sites [25]. The first stage involves the formation of monolayer coverage, which follows the Langmuir isotherm. When the adsorbate
concentration increases, solute adsorbs onto the already adsorbed layer (adsorbate-adsorbate), which is believed to follow a linear adsorption response [25]. It is important to point out that the proposed two-stage mechanism breaks the bi-Langmuir assumption on monolayer coverage and independent adsorption from two binding sites [30]. This two-stage mechanism provides the premise for another model, namely the Langmuir-linear model that will be discussed in subsequent paragraphs.

Despite having reasonable agreement with the experimental data, both the Freundlich and the bi-Langmuir models share one common assumption regarding the formation of monolayer coverage on the surface that cannot be validated. The interaction between protein molecules leading to multilayering is documented in many studies in the literature [37, 44-48]. Therefore, the possibility of multilayering should be considered. In fact, previous work on the adsorption of BSA and lysozyme onto PET C-CP fibers provided evidence for this multilayering behavior through atomic force microscopy (AFM) images of the fiber surfaces before and after adsorption [28]. The work suggested that proteins were not uniformly adsorbed on the fiber surface. Instead, the adsorption seemed to crowd certain areas leading to an “island growth process” of protein multilayering [28]. Therefore, isotherm models dealing with multilayering adsorption are considered as follows.

The Moreau isotherm is the most common, and also the simplest, model for adsorption on a homogeneous surface coupled with adsorbate-adsorbate interactions [30, 49]. The equation is presented as:

\[
q = q_s \frac{bC + b^2C^2}{1 + 2bC + b^2C^2}
\]  
(Eq. 6)
where $q_s$ (mg g\(^{-1}\)) is the monolayer saturation capacity, $b$ (mL mg\(^{-1}\)) is equilibrium constant at infinite dilution and $I$ is the adsorbate-adsorbate interaction parameter. The Moreau isotherm shows a convex upward curvature when the adsorbate-adsorbate interactions are low ($I < 1$) [30, 32]. In the case of very weak adsorbate-adsorbate interactions ($I \to 0$), the Moreau equation basically breaks down to the Langmuir isotherm model [30]. If such interactions are significant ($I > 1$), the isotherm is initially convex downward and may display an inflection point at the higher concentration range [30, 32]. The parameter $I$ is dependent on the conditions of binding capacity determination. It was reported in a study of BSA adsorption onto nylon C-CP fiber that the difference between this parameter for two sets of binding capacity (static and dynamic) was of three orders of magnitude [9]. Fitting the data in Fig. 2.3a into this Moreau isotherm equation yielded $q_s = 9.354$ (mg g\(^{-1}\)), $b = 0.7972$ (mL mg\(^{-1}\)) and $I = 0.4247$, with a modest correlation coefficient of $R^2 = 0.942$ (Table 2.1).

The second multilayering adsorption model assessed was the Brunauer, Emmett, and Teller (BET) isotherm. Based on the theory used to develop the original BET model for gas-solid adsorption isotherms, the extended liquid-solid BET isotherm assumes that molecules in the first adsorbed layer form the adsorption sites for outer layers [31, 50]. As a result, the solute molecules can adsorb onto either the bare surface of the adsorbent or a layer of solute already adsorbed, inducing multilayer adsorption [30]. The model assumes that molecules in the subsequent layers other than the first one behave essentially as those in the bulk liquid. It is important to note that each layer does not need to be completely
covered before the adsorbate-adsorbate interactions are allowed to occur [51]. The expression for this isotherm is derived from the first order kinetic adsorption-desorption relationships [30-31, 50].

\[ q = q_s \frac{b_s C}{(1-b_l C)(1-b_l C+b_s C)} \]  

(Eq. 7)

In this equation, \( q_s \) (mg g\(^{-1}\)) is the monolayer saturation capacity and \( b_s \) and \( b_l \) (mL mg\(^{-1}\)) are the equilibrium constants of adsorption of the adsorbate on the bare surface and on the previously adsorbed layers, respectively [31]. Fitting the experimental data to the BET model produced \( q_s = 2.641 \) mg g\(^{-1}\), \( b_s = 6.029 \) mL mg\(^{-1}\), \( b_l = 0.2543 \) mL mg\(^{-1}\) and an \( R^2 \) of 0.9968 (Table 2.1).

Considering the best fit of the dynamic binding data was found for the bi-Langmuir model, another modified version of the Langmuir model that describes the heterogeneous surface adsorption and takes into account the adsorbate-adsorbate interaction was evaluated. The Langmuir-linear model describes the adsorption process in two different stages. In this system, the first stage occurs with rSPA adsorbed directly on the PP fiber surface to form the first layer, which follows the Langmuir adsorption isotherm:

\[ q = q_s \frac{b_s C}{1+b_l C} \]  

(Eq. 8)

where \( S^* \) denotes the adsorption on the bare PP fiber surface. Each adsorbate molecule on the first layer then can become an active center for the subsequent molecules that lead to the formation of the subsequent layers. According to Sainio who suggested the model [25], the assumption for the model is that the interaction energy between the adsorbate in the bulk liquid phase and the first layer is constant. The second assumption is that there is no restriction on the number of
molecules attached to a surface aggregate and this distribution follows a linear isotherm. As a result, the amount of adsorbate attached to the surface aggregate $q_{s***}$ is related to $q_{s*}$ and $C$ in the following equation:

$$q_{s***} = b_2 q_{s*} C$$  \hspace{1cm} (Eq. 9)

The total binding capacity of the adsorbate is the sum of the adsorbate attached to the bare surface and the subsequent aggregate layers is as follows:

$$q = q_{s*} + q_{s***} = \frac{q_s b_1 C}{1 + b_1 C} + \frac{q_s b_1 b_2 C^2}{1 + b_1 C} = \frac{q_s b_1 C}{1 + b_1 C} (1 + b_2 C)$$  \hspace{1cm} (Eq. 10)

The Langmuir-linear isotherm model predicts an almost linear adsorption at finite values of $C$, which fits the trend shown in the experimental data. Fitting the experimental data to this model produced $q_s = 1.486$ mg g$^{-1}$, $b_1 = 24.9$ mL mg$^{-1}$, $b_2 = 1.243$ mL mg$^{-1}$ and $R^2$ of 0.9991 (Table 2.1).

Close examination of the experimental adsorption data reveals that the experimental isotherm actually has a slightly upward curvature and downward curvature that lie before and after the rSPA concentration level of 0.5 mg mL$^{-1}$, generating a mildly S-shaped isotherm. This small deviation from linearity was observed previously in adsorption isotherms of FITC-PEG lipid on PP fibers and lysozyme on PET fibers [21, 52]. As a result, it is clearly seen in Fig. 2.3a and 2.3c that the Moreau and the bi-Langmuir models, with their convex upward curvature, miss compensating for the S-shaped character of the experimental data. On the contrary, the BET and the Langmuir-linear models show much better fits, both graphically and mathematically, yielding close-to-unity $R^2$ values and very low RSS.
In terms of prediction ability, the evaluation of the three multilayering adsorption models can be made by expanding their isotherms to infinite concentration of rSPA. The BET fit displays a transition from convex to concave curvature. Beyond the concentration range investigated in the study, the binding capacity predicted by BET quickly increases towards infinity as the concentration of rSPA approaches \( \sim 3.93 \text{ mg mL}^{-1} \), which is the positive asymptote of the isotherm. Beyond this value, the BET equation produces negative q values and approaches to 0 when C goes to infinity. The Langmuir-linear does not produce the typical Langmuirian isotherms with a plateau. Instead, it shifts from convex curvature to linear and as the concentration goes to infinity, as does the binding capacity. The Moreau model, on the other hand, follows a close-to-Langmuir isotherm with the predicted plateau obtained at \( q_s = 9.354 \text{ mg g}^{-1} \) as the rSPA concentration goes to infinity. As a result, only the Moreau and the Langmuir-linear models allow the prediction of the rSPA binding capacity beyond the tested range.

In summary, the Langmuir-linear model not only gives the best mathematical description of the experimental data with close-to-unity \( R^2 \) and very low RSS but also allows the prediction of the rSPA binding capacity beyond the tested range. Therefore, this model is considered to be well suited to model the adsorption of rSPA on PP C-CP fibers. Thus, a mechanistic picture evolves in which an initial rSPA layer is formed on the fiber surfaces, followed by continuous addition of protein as the load concentrations are increased.
2.3.2 rSPA loading characteristics with respect to linear velocity, column length and residence time

While the isotherm responses provide insights into the adsorption processes, loading of the fiber much beyond the monolayer would not likely provide greater IgG binding capacity. As noted above, the transition point between the Langmuirian formation of a monolayer and the onset of multilayering was at a solute concentration of $\sim 1 \times 10^{-5} \text{ M (0.5 mg mL}^{-1})$ rSPA, yielding a density of $\sim 2.1 \text{ mg rSPA g}^{-1} \text{ fiber}$. Note that this value is $\sim 4x$ that calculated based on a space filling model, which is likely within reason. This concentration was used in experiments to evaluate the effect of linear velocity and column length on rSPA binding capacity.

Dynamic binding capacity can be affected by adjusting two parameters, either the flow rate or the column length, both of which effect the residence time of the solute inside the column [53-54]. As shown in Fig. 2.4a, as the linear velocity increases, the dynamic binding capacity of rSPA onto PP fiber decreases for this 10-cm long column. Depicted in this system is a $\sim 50\%$ drop in capacity whilst the linear velocity was increased by a factor of 6X. This response corroborates previous protein loading experiments on C-CP fibers, wherein the column hydrodynamic and solute mass transport efficiencies provide great benefits in terms of processing throughput; i.e., the amount processed per unit time [8-9]. This counterintuitive response is due to the lack of intra-phase diffusion of proteins to access the entirety of the phase surface, coupled with the improved mass transport
that results from operation at high shear rates as predicted by Leveque and verified in other protein adsorption studies [55-57].

Figure 2.4 a) Effects of linear velocity on the loading of rSPA. Feed concentration of 0.5 mg mL\(^{-1}\) rSPA at various flow rates on 10-cm microbore columns. b) Effects of column length on loading of rSPA. Feed concentration of 0.5 mg mL\(^{-1}\) rSPA at flow rate of 0.5 mL mL\(^{-1}\); c) Effects of residence time on the loading of rSPA. Feed concentration of 0.5 mg mL\(^{-1}\) rSPA.

A related, but independent, experiment was done to evaluate the relationship between the column length and the dynamic binding capacity of rSPA at the concentration of 0.5 mg mL\(^{-1}\) and the flow rate of 0.5 mL min\(^{-1}\) (∼28 mm s\(^{-1}\)). So long as there are not kinetic limitations, the provision of greater support surface area should not affect the resultant binding capacities (on a per unit mass
basis). As can be seen in Fig. 2.4b, the binding capacity for columns of all lengths
tested, except for the shortest (5-cm) column, yield essentially the same DBC of
1.9 mg rSPA per gram of fiber. This is clear evidence that there is some minimum
residence time that is required to effectively load the columns. In this case, that
value is ∼3.6 s (100 mm/28 mm s⁻¹) for the 10-cm long column. Clearly, solute
mass transport in the channel structure of the C-CP fiber columns is far more
efficient than any sort of static (diffusion limited) deposition process.

The data sets depicted in Figs. 2.4a and 2.4b can be re-graphed in units of
time and overlayed to better demonstrate the effects of residence time on rSPA
binding capacity. Calculated from column lengths, column void volumes and
volumetric flow rates, residence time reflects how long the rSPA resides on-
column. Intuitively, one would expect that longer residence times would mean
higher probabilities for rSPA to adsorb onto the fiber surface. However, as depicted
in Fig. 2.4c, this is only true up to ∼3.6 s of rSPA residence time, beyond which all
of the columns reach full capacity. Another important point gleaned from the
overlayed graphs is the excellent agreement between the two independent data
sets. Changes in the column length or the flow rate that resulted in the same rSPA
residence times generate very similar rSPA binding capacities. Thus, loading at a
flow rate of 0.5 mL min⁻¹ with a 0.5 mg mL⁻¹ rSPA feedstock concentration and a
30-cm column could conveniently produce dense, uniform rSPA phases on PP C-
CP fiber surfaces within 1 minute. These columns were then cut into 10-cm
segments used for evaluation of IgG capture, release, and column robustness.
2.3.3 Binding performance of the adsorbed rSPA ligands

Adsorption relies on the physical attachment of a ligand (rSPA in this case) to a support that is not treated for covalent immobilization [26]. This immobilization approach has been used in many applications [58-62]. The non-specific adsorption is dependent on the chemical properties of the ligand and the support material, with forces involved in this process including Columbic interactions, hydrogen bonding and hydrophobic interactions. Considering the highly hydrophobic nature of PP fibers, the latter is surely the predominant force behind the physical adsorption of rSPA ligands onto the fiber surface. It is important to point out that this adsorption results from the collective interaction at multiple contact sites between the rSPA molecules and the fiber surface. Because the chance of breaking all of these weak forces at the same time is small, the protein adsorption is only partially reversible [48].

On-column loading, which involves the constant feeding of a ligand reservoir to a support by a mechanical pump is one of the most commonly used methods for adsorptive ligand loading due to its ease-of-use, time-efficiency and precision [26]. Considered the most straight-forward and easiest method of immobilization, non-specific adsorption does have one major potential drawback versus directed, covalent attachment; the randomness of the adsorption may cause improper orientation as ligands are attached at or close to their active sites, leading to a reduced or complete loss of ligand activity. Steric hindrance is another immobilization effect of concern. It happens when the access to the ligand’s active site is obstructed by either the support or the neighboring ligands. As a result, the
activity of affinity phases prepared through non-specific adsorption should be carefully evaluated, particularly following exposure to solvents that may affect the ligand binding and geometry. Therefore, the second part of this study is devoted to the investigation of the IgG binding capacity of the adsorbed rSPA ligands under complete operating cycles commonly employed in the bioprocessing industry. The whole cycle was performed at the flow rate of 1 mL min\(^{-1}\) (~56 mm s\(^{-1}\)). At such a high flow rate, IgG travels very fast along the already crowded rSPA-modified surface, which should minimize further the propensity to bind non-specifically to any exposed regions of the PP surface. As a result, it is believed that IgG is essentially bound only to active rSPA sites on the PP surface. Even if non-specific immobilization is occurring, the mild IgG elution solvents employed here would not release the protein from the PP surface. As such, the eluted IgG can be used as a "read-out" for the apparent active ligand density on the rSPA-modified columns.

The cost of commercial protein A stationary phases can be comparatively high. However, as the number of cycles increases, the initial purchase cost is reduced in relation to other operational costs such as labor, process buffers and plant overhead [63]. As the key factor in the cost impact of protein A step is the lifetime of the media, cleaning is an important step to maintain the integrity of the column and extend its useful lifetime. On a large industrial scale, media reuse requires routine cleaning and that cleaning validation be well documented for the resin lifetime studies [64]. The choice of CIP approaches in terms of reagent, time and frequency is very much dependent on the properties of the media comprised of two components, the stationary support and the affinity ligands (rSPA). It is
important to differentiate between the stability of these two components in designing an appropriate cleaning scheme. Some commercial support phases have a limited range of pH recommended for operation. For example, the silica backbone of ProSep-vA Ultra is not stable under alkaline conditions [65] while those made of cross-linked agarose (Sepharose) are recommended for use over the pH range of 3.0-11.0 [26]. The PP C-CP fiber support can withstand a wide range of pH and is resistant to virtually all of the common reagents used in the downstream processing. It is the stability of protein A ligands and their adhesion to the fiber support that are of concern here. Staphylococcal protein A is a robust protein known for its stability against heat and denaturing agents. The specific binding of IgG is still maintained even after treatment with 6 M guanidine hydrochloride, 4 M urea, 4 M thiocyanate, low pH (as low as 1.50) or low concentrations of non-ionic detergents [63, 66]. However, it is susceptible to degradation at high pH conditions. In spite of enhanced stability upon immobilization, the use of high concentrations of NaOH is still not practical for the sanitization of some protein A media. For the rSPA-modified PP fiber investigated in this study, it is important to evaluate how harsh cleaning conditions affect the rSPA activity, the overall surface ligand arrangement and IgG binding capacity.

The results of the cycling experiments are presented in Fig. 2.5, wherein the recoveries of IgG are plotted for each cycle, as a function (percentage) of the amount recovered in the first cycle of the suite. Also presented for each test are the breakthrough curves that were obtained for the IgG load step of each experiment. In doing so, differences between the load characteristics and the
eventual recoveries are easily seen. As a benchmark, 10 load-elute cycles without a CIP step in between were performed to assess any changes occurring through this simple cycling. As observed in Fig. 2.5a, the recovered amount of IgG as reflected by the elution peak areas initially decreases and seems to be stabilized at around 63% of the first cycle value, with the vast majority of the decrease occurring between the first and second cycles. Protein A ligands are adsorbed onto the PP fiber surface through hydrophobic interaction. However, as discussed above, there is a high probability that multi-layering occurs. As protein A is known to have a high percentage of glutamic and aspartic amino acid residues, it is expected that Coulombic attraction would be one of the main forces that allows multi-layering to happen. Considering the change in pH from the loading step (pH 7.4) to the elution step (pH ~2.5), it is possible that the interaction needed for the formation of multi-layers in the first place weakens, hence the outer rSPA ligand layers are lost. The change in pH may also induce the reorientation of the adsorbed rSPA ligands, making the binding sites not available for IgG. However, after the second complete cycle, the arrangement of the rSPA ligand phase seems to be stabilized as seen in the reproducibility of the breakthrough curves and recovery statistics.

Urea is a well-known protein denaturant that can induce the release of weakly adsorbed host cell proteins. When 2 M urea was used as the cleaning step in between the consecutive cycles, the change in the recovered IgG, as demonstrated in Fig. 2.5b, is qualitatively similar to the response seen for the case of no CIP step. Here again, the recovery is stabilized after the second complete
cycle, with an average yield of \( \sim 52\% \) that of the initial cycle. No appreciable changes are seen in the uptake as depicted in the breakthrough curves. As a chaotropic reagent, urea is capable of denaturing rSPA ligands, which may enhance their binding to the fiber surface. At the same time the new orientation of the denatured ligands is not conducive to refolding and, the binding capacity decreases, but this process is not supported in terms of the breakthrough curves. However, as seen Fig. 2.5c, a different trend is observed when another denaturant, guanidine HCl of 2 M concentration was used and the same reasoning apparently is not applicable. Instead of the IgG recovery decreasing between the first and second cycle, and then remaining stable, here the initial drop is followed by a

**Figure 2.5** Relative amount of eluted IgG over 10 complete operating cycles and their representative breakthrough profiles, plotted as a function of the first cycle recovery. All processes performed at a solution flow rate of 1 mL min\(^{-1}\), with an IgG load concentration of 10 µg mL\(^{-1}\). a) No CIP, b) 2 M Urea CIP, c) 2 M guanidine HCl CIP, d) 0.1 M NaOH CIP, and e) 1 M NaOH CIP.
steady increase. After the first cycle, IgG recovery decreases by 45% on average, increasing to just below 70% at the last cycle. What complicates this response more is the fact that, as seen in the breakthrough curves, the amount of captured IgG actually decreases. This is all the more interesting considering guanidine HCl is a stronger denaturant than urea.

The difference in the trends observed when urea and guanidine HCl were used may stem from the difference in their denaturing activity. Protein A lacks cysteine [27] so its conformation is mostly dependent on non-covalent forces. Considering its high composition of glutamic acid, aspartic acid and lysine residues, columbic forces are expected to play a significant role in protein A’s native structure. The pH of 2 M urea and 2 M guanidine HCl are 6.5-7.0, at which point these amino acids remain charged. Occurring in the solution as the guanidinium and chloride ions, guanidinie HCl is expected to have a different pathway in its interaction with the adsorbed rSPA ligands than urea does. In fact, it has been reported that guanidine HCl at low concentration up to 2 M has the ability to stabilize the protein [67]. Moreover, immobilized rSPA ligands show higher stability than free rSPA in solution [63]. However, without further analytical data, it is hard to determine what actually happen to the adsorbed rSPA ligands when urea and guanidine were used as the cleaning step.

Revisiting Fig. 2.5a, 2.5b and 2.5c shows that there is some disagreement between the eluted IgG percentage graphs and their corresponding breakthrough profiles. In all three cases (where there was no cycling involved and where urea and guanidine HCl were used as CIP reagents), the decrease of 30-40% of the
eluted IgG compared to the first cycles was not well reflected by their corresponding representative breakthrough profiles. While the breakthrough profiles show that there are more or less similar amounts of IgG bound to the columns for each cycle, the eluted amount changed more drastically. This contradictory trend may possibly be due to the questionable recovery rate of the IgG. However, as there is so little difference between the two IgG breakthrough profiles obtained when the system was run with and without an rSPA-modified column, it is impossible to determine the amount of bound IgG accurately. Therefore, it is undecided if the decrease in eluted IgG was due to the low recovery rate or not.

If the first or even second cycles are ignored and the remaining cycles are normalized to the second or third cycle respectively, then the trends in the eluted IgG and breakthrough profiles are in better agreement. It is worth mentioning that before the first cycles, these modified columns were only exposed to the loading buffer. Therefore, during the first couple of cycles when the modified columns are exposed to buffers of different pH and even CIP reagents, the ligand phase may experience some rearrangement and stabilization. Loosely bound rSPA ligands on the outermost layers, upon binding the IgG, may be washed out from the column. It is likely that the eluted peak observed in the first couple of cycles may have contained those washed off, loosely-bound rSPA ligands. Therefore, the first elution peak may actually be an overestimation of the amount of bound and eluted IgG. The implication of this stabilization of the ligand bed after a few cycles means that for future use, whether it be for IgG quantitation or IgG purification, the rSPA-
modified C-CP fiber columns should be “conditioned” with at least a few cycles before their use.

The greater potential stress to the rSPA phase surely occurs under caustic CIP conditions, which are virtually a necessity in downstream processing applications where cellular debris and other host cell proteins are present. When NaOH at a low concentration of 0.1 M was tested as a cleaning reagent, an interesting trend was recorded. Protein A is widely known for not being stable in high pH environments so it is intuitive to expect the recovered IgG amount would drastically decrease with increasing numbers of cycles, which is contradicted by the increase shown in Fig. 2.5d. Initially, the ~20% decrease between the first and second cycles, seems to reflect the same processes seen in the other CIP methods, but after the first cycle followed by consecutive increase exceeding the recovery of the first cycle. The trend shown here is confirmed by a shift of the breakthrough curves to the right, demonstrating there is a higher binding capacity of IgG. After 10 cycles with NaOH being used as a cleaning reagent, there is a significant change in the IgG breakthrough profiles that allows the quantification of the bound IgG. The amount of eluted IgG was also quantified for the 10th cycle based on the elution peak area. The IgG recovery rate was then calculated as the ratio of the eluted amount to the bound amount. After 10 cycles operated and cleaned with 0.1 M NaOH, the recovery rate of the rSPA-modified column was found to be 105 ± 4%. This high recovery rate suggests that there is no significant non-specific binding involved and the rSPA ligands do not show any evidence of deterioration.
When the concentration of NaOH was increased to 1 M, the trend initially shows an increase in the amount of recovered IgG followed by a gradual decrease towards a seemingly plateau as shown in Fig 2.5e. Investigating the 50% breakthrough profiles of the 10 cycles shows that after the third cycle, the IgG binding capacity of the modified column has increased appreciably, but this is not reflected in the amount of recovery. This suggests that the eluted fraction is smaller than the amount of captured IgG. In fact, this explanation is supported by the calculated IgG recovery rate at the 10th cycle, which was found to be 85 ± 2%. There are a few factors that could cause such a decrease in the recovery rate. Firstly, the alkaline tolerance of rSPA quickly plummeted at elevated NaOH concentration. Secondly, the possible removal of the deteriorated rSPA ligands could expose the bare PP surface, which would induce non-specific binding to the IgG. Third, it has been reported in the literature that the exposure to the strong alkaline condition enhanced the affinity of the immobilized Protein G ligands for IgG, which makes it difficult to elute all the bound IgG [64, 68-69]. Even though this phenomenon has not been reported for the immobilized protein A ligands, this could have happened to the remaining active rSPA ligands on the C-CP PP fibers, leading to a low recovery rate of IgG.

Taken as a whole, it seems reasonable to propose a unified interpretation to the behavior across multiple capture/elute/CIP cycles. As clear from the rSPA adsorption experiments, that phase exists as a fairly uniform ligand monolayer, with some amount of multi-layering taking place. Following initial exposure to IgG and its subsequent elution, it appears that the loosely bound rSPA (presumably
the overlayer) is removed with the IgG. In the case of the urea and guanidine HCl CIP steps, the system effectively remains in a steady state, though in the latter situation it appears that the elution step becomes more facile. As suggested in the literature, CIP in 0.1 M NaOH seems to have an activating effect in terms of affecting greater IgG capture and recovery. Increasing the NaOH concentration has the initial effect of increasing capture efficiency, but this is followed by some loss of ligand.

2.4 Conclusions

The physical adsorption of rSPA ligands on polypropylene C-CP fibers is a straight-forward method to produce a protein A stationary phase that can be used in the analytical determination of IgG in high titer or in IgG downstream processing on the industrial scale. The loading characteristics of rSPA ligands on C-CP fibers were evaluated through frontal analysis. Among different models fit to the experimental adsorption data, the Langmuir-linear isotherm yielded the best fit. This model suggests that the adsorption of the rSPA ligands to the bare fiber surface follows the Langmuir isotherm. Subsequent adsorption onto this first layer is proportional to the amount of rSPA in the first layer and follows a linear isotherm. Residence time, which can be adjusted through either flow rate or column length, can affect the dynamic binding capacity of rSPA onto C-CP fiber. In essence, though, column residence times of greater than 5 s are sufficient to allow the mass transfer/adsorption processes to be effectively completed. In this study, loading of
rSPA at a solution concentration of 0.5 mg mL\(^{-1}\) at a flow rate of 0.5 mL min\(^{-1}\) onto 30-cm column yields an rSPA density of \(\sim 2.1\) mg g\(^{-1}\) fiber.

The processing characteristics of the rSPA-modified columns were evaluated through the amount of IgG recovered in complete operating cycles with loading, eluting, cleaning and re-equilibrating steps. The results reflect a situation where overlayers of rSPA are lost in the initial capture/elute cycles, with the efficiencies being invariant over 10 complete cycles when urea or guanidine HCl are used for CIP. Enhanced capacity is realized under mild caustic washes but use of strong NaOH conditions does lead to some diminishing of the adsorbed rSPA ligand layer. While a more extensive cycling life time study is needed, the results obtained from this study suggest that the rSPA modified C-CP fibers do have a great deal of practical potential.

Future efforts will focus on the assembly of rSPA C-CP columns directed towards application at-reactor in the rapid assessment of titer levels and purity and in the assembly of gram-quantity columns for practical downstream processing. The demonstrated ability to perform rapid, small-scale separations brings confidence into the former application. The high permeability of the C-CP fiber columns and demonstrated high throughput capabilities support the latter efforts.

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CHAPTER THREE

APPLICATION OF PROTEIN A-MODIFIED CAPILLARY-CHANNELED
POLYMER POLYPROPYLENE FIBERS TO THE QUANTITATION OF IgG IN
COMPLEX MATRICES

3.1. Introduction

The advent of the FDA-approved murine IgG2a CD3 specific transplant rejection drug OKT3 (or muromonab) in 1986 opened an ever-growing market for the therapeutic monoclonal antibodies (mAbs) [1]. Reaching the total sales of $63 billion in 2013, mAbs represented almost 50% of the total biopharmaceutical global market and including six of the ten top-selling products [2]. Increasing product demands have driven significant progress in upstream processing over the last two decades [3]. Many facilities are equipped with production bioreactors with volumes ranging from 5 kL to 25 kL that are capable of producing mAb titers of 1-5 g L\(^{-1}\) in mammalian cell cultures [2-3], with some reports as high as 10-13 g L\(^{-1}\) for an extended culture growth [4]. In practice, harvest cell cultures must be continuously screened and monitored for Immunoglobulin (IgG) expression across the complete growth cycle in both the development and production phases.

Cell cultures are teeming with a wide range of contaminants including host cell proteins (HCPs), host cell DNA, cell metabolites, media components, chemical additives and leachables, which will be referred to as non-IgG contaminants throughout this paper [5]. Methods for mAb quantification are expected to have
sufficient specificity and sensitivity to handle complex matrices that may contain low target concentrations in the presence of large amounts of these concomitant species [6]. Other attributes include efficient sample utilization, short analysis time and high throughput [6]. Current IgG quantification methods are fundamentally categorized into two different approaches: immunological-based and separation-based techniques [7]. Immunoassay involve the labeling of one of the two components in the interaction (either the antigen or antibody) with either an enzyme, a fluorophore or a chemiluminescent probe [7]. More modern approaches rely on the direct monitoring of the immunological interaction through a wide range of transduction techniques including surface plasmon resonance, acoustic waveguide, atomic force microscopy and quartz crystal microbalance [7]. Among those, enzyme-linked immunosorbent assay (ELISA), radial immunodiffusion (RID) and immune-nephelometry are the most common methods. However, these methods are rather time-consuming and labor-intensive.

In terms of separations, representative techniques commonly used for IgG quantification include various high-performance liquid chromatography techniques. Rapid methods have been developed on ion exchange (IEC), reversed phase (RP) and size-exclusion chromatography (SEC) supports [7]. These modes of chromatography are commonly used to characterize antibodies’ aggregate and fragment content, glycosylation patterns and charged isoforms for process monitoring and quality control [3, 8]. Among them, RP chromatography, with the ability to separate species with minor structural heterogeneities and the compatibility to in-line mass spectrometry, has become a powerful tool [9-10].
However, the complex and hydrophobic nature of antibodies can lead to poor recovery and limited resolution in RP chromatography [10]. One common drawback across all of these methods is that there is no common procedure suitable for the analysis of all monoclonal antibodies, a characteristic of which is their inherent variability [6, 11]. Even minor changes in the variable regions can result in a substantial deviation in the mAbs’ isoelectric point (pI) or hydrophobicity [11]. Therefore, it is impossible to devise a generic procedure suitable for all monoclonal antibodies of interest [6].

Combining the attributes of separation and immunoassay techniques, affinity chromatography (AC) employing bacteria-derived protein A or protein G [6] is a practical alternative for IgG quantification [7]. Widely known for its high affinity for the Fc region of IgG, protein A derived from Staphylococcus aureus has long been utilized to capture IgG from complex matrices such as cell culture supernatant. As a result, affinity chromatography employing the protein A ligand has been a method of choice to quantify the mAb or to fraction it for further downstream aggregate and charge variant determinations [12].

This laboratory has developed polypropylene (PP) capillary-channeled polymer (C-CP) fibers as supports for protein A affinity separations [13-14]. Characterized by eight axial channels running along their length, C-CP fibers inter-digitate in column format to form micrometer-sized channels that facilitate highly efficient protein separations at linear velocities of up to 100 mm s⁻¹ with high column permeability (<0.14 MPa cm⁻¹). Previous work demonstrated that the rSPA ligands retained not only their high binding specificity but also the desired ~3:1
IgG: protein A capture stoichiometry [13]. Loading rSPA at the flow rate of 0.5 mL min\(^{-1}\) (\(~28\) mm s\(^{-1}\)) with a \(1 \times 10^{-5}\) M (0.5 mg mL\(^{-1}\)) feed concentration on a 30-cm (0.762 mm i.d.) column results in a complete coverage of rSPA on the PP surface within only 6 minutes [14]. The present study investigates the potential application of rSPA-modified C-CP PP columns to the quantification of IgG in complex biological matrices.

3.2 Materials and methods

3.2.1 Materials and chemicals

HPLC-grade acetonitrile (ACN) and methanol (MeOH) were purchased from EMD Millipore (Billerica, MA). Phosphoric acid (85%) was purchased from Fisher Scientific (Pittsburg, PA). Potassium phosphate monobasic (\(\text{KH}_2\text{PO}_4\)) and sodium phosphate dibasic (\(\text{Na}_2\text{HPO}_4\)) were purchased from Sigma Aldrich (St. Louis, MO). Potassium chloride was purchased from Fisher Scientific (Fair Lawn, NJ) and biotechnology-grade sodium chloride was purchased from Amresco (Solon, OH).

All aqueous solutions were prepared with ultra-pure Milli-Q water (18.2 MΩ.cm) obtained from a Millipore water system (Billerica, MA). Protein A derived from recombinant \(\text{Staphylococcus aureus}\) protein A (rSPA) in the bulk stock solution obtained from Syd Labs (Malden, MA) at a concentration of 49.4 mg mL\(^{-1}\) was diluted to 0.5 mg mL\(^{-1}\) in phosphate buffered saline (PBS).

Human IgG\(_1\) at a stock concentration of 3.0 mg mL\(^{-1}\) was purchased from Calbiochem (Darmstadt, Germany). Spiking experiments were performed using
the supernatants of two Chinese Hamster Ovary (CHO) cell cultures. The cell lines used in this study included CHO DP (12445 ATCC® CRL-TM) and CHO-PF K1 (Sigma, Cat. No. 00102307). The former expresses IgG\textsubscript{1} while the latter does not. The CHO cell culture was grown in the Harcum laboratory (Dept. of Bioengineering, Clemson University) as suspension cultures in 125 mL shake flasks containing 30 mL of EX-CELL® CD CHO serum-free medium (Cat. No.14361C). The cultures were grown in a humidified incubator maintained at 5% \text{CO}_2 and 37°C with shaking at 100 RPM, to a final cell density of \sim 1.5 \times 10^6 \text{cells mL}^{-1}. Harvested cells were centrifuged at 1000 g for 5 min, after which the supernatant was aspirated and stored at -20°C until needed. The frozen supernatant was thawed, re-centrifuged and filtered through 0.45 µm PTFE membranes before IgG\textsubscript{1} analysis.

3.2.2 Microbore C-CP fiber column construction

PP C-CP fibers were obtained from the Clemson University, School of Materials Science and Engineering, manufactured via melt extrusion from polypropylene pellets. PP C-CP fibers used in this study were characterized by following values: 3 deniers per filament (dpf) or 3 grams per 9000 meters of single fiber; a cross section perimeter of \sim 200 µm and a specific area of 0.6 m\textsuperscript{2} g\textsuperscript{-1}.

The process of packing C-CP fibers into microbore columns has been described [13-14]. Two rotations of PP fibers (equaling 480 single fibers) are stretched on to a dye fork. After being rinsed with hot water, ACN, MeOH then Milli-Q water, the fibers were pulled through 30-cm polyether-ether-ketone (PEEK)
tubing, 0.03" (0.762 mm) inner diameter (IDEX Health & Science LLC, Oak Harbor, WA). Further cleaning was done by flushing the columns with ACN and Milli-Q water at 1 mL min\(^{-1}\) on the HPLC system until a stable absorbance baseline was reached. Columns produced in this study have the interstitial fraction (\(\epsilon_I\)) (determined with uracil retention) of 0.65 with less than 5% RSD variability between different column lots.

### 3.2.3 rSPA loading

Breakthrough experiments and frontal analysis are the most common means of assessing the solute loading of chromatographic media [14]. Absorbance measurements monitor solutes introduced on-column in a continuous fashion, with column saturation identified where the absorbance of the eluent reaches a plateau value corresponding that of the load solution (\(A_{\text{sat}}\)). The amount of loaded solute is computed in terms of the volume and concentration of solution having passed through the column at breakthrough (\(A/A_{\text{max}} = 1\)). rSPA-modified C-CP fiber columns were eventually prepared by loading rSPA at a concentration of 500 µg mL\(^{-1}\) (\(\sim 1 \times 10^{-5}\) M) at a flow rate of 0.5 mL min\(^{-1}\) for \(\sim 6\) minutes.

### 3.2.4 Chromatographic procedure development

All chromatographic experiments were performed at room temperature on a Dionex Ultimate 3000 HPLC system operated by Chromeleon 6.80 software and consisted of an LPG-3400SD pump, a WPS-3000TSL auto-sampler and a VWD-3400RS UV-Vis absorbance detector. UV detection at both 216 nm and 280 nm
were used, though as discussed in subsequent sections, 216 nm provides the best compromise among considerations dealing with selectivity, sensitivity, and background species interference.

### 3.2.4.1 Elution buffer composition

Elution of IgG from protein A-modified substrates is typically affected in acidic media. A number of the common elution buffers were evaluated, including 0.1 M citrate (pH = 2.5); 0.1 M acetate (pH = 2.8); 0.5 M acetate (pH = 2.5); 0.1 M glycine HCl (pH = 2.8) and 0.025% H₃PO₄ (∼4.5 mM H₃PO₄, pH = 2.5). The equilibrating buffer was 12 mM phosphate buffer at a pH = 7.4 and a 1.0 µL injection of 1.0 mg mL⁻¹ IgG₁ in PBS was used as the test sample.

### 3.2.4.2 Sample load/column equilibration buffer composition

Two buffers, (A) 12 mM phosphate at pH 7.4 and (B) 12 mM phosphate and 1 M NaCl at pH 7.4 were employed to study the effects of salt concentration on the loading efficiency. The concentration of NaCl in the equilibrating buffer was adjusted by modifying the percentage of these two buffers. In all cases, the eluting buffer was 0.025% H₃PO₄ [14] and a 1.0 µL injection of CHO-PF K1 supernatant spiked with 1.0 mg mL⁻¹ IgG₁ was used as the test sample.

### 3.2.4.3 Flow rate optimization

The effects of the mobile phase flow rate on the chromatographic behavior of IgG binding and elution events were investigated as follows. The flow rate of the
equilibrating buffer (and *de facto* the injection) was investigated at 0.1, 0.5 and 1.0 mL min\(^{-1}\) while the eluting buffer flow rate was kept at 1.0 mL min\(^{-1}\). In a similar manner, the flow rate of the eluting buffer was investigated at 0.5, 1.0, 1.5 and 2.0 mL min\(^{-1}\). The complete range of solvent flow rates correspond to linear velocities of 5.6 – 112 mm s\(^{-1}\).

### 3.2.5 Chromatographic method validation

The optimized chromatographic conditions were used for the construction of analytical response curves. The equilibrating and eluting buffers were 12 mM phosphate at pH 7.4 and 0.025\% \(\text{H}_3\text{PO}_4\), respectively. Due to the inavailability of certified reference materials for IgG in cell culture media, quantification was performed using calibration functions derived for injections of IgG in buffer media as well as standard addition analysis of culture samples. The stock solution of monoclonal IgG\(_1\) was diluted to different concentration levels in the equilibrating buffer to prepare “neat” calibration curves. CHO supernatant samples spiked with IgG\(_1\) were used to evaluate the standard addition methodology.

### 3.3 Results and discussion

#### 3.3.1 rSPA loading

The adsorption of rSPA on the PP C-CP fiber is through physical interaction (hydrophobic adsorption) with no covalent binding involved [13-14]. Previous work yielded a linear-Langmuir model to explain the adsorption process of rSPA onto the fiber surface [14]. The random manner of rSPA ligand adsorption could lead to
issues of ligand mis-orientation and steric hindrance. In fact, on a weight basis, the previous 2.9:1 IgG:rSPA ratios observed imply that the ligand utilization is extremely high [13]. Therefore, loading rSPA ligands beyond the monolayer coverage would only waste the expensive ligands and risk ligand leakage from the column during elution. It was noted that the transition point between the Langmuirian monolayer coverage to the onset of multilayering occurred at a rSPA ligand concentration of $\sim 1 \times 10^{-5}$ M (0.5 mg mL$^{-1}$) [14]. This rSPA feedstock concentration yields a density of $\sim 2.1$ mg rSPA g$^{-1}$ fiber, which is $\sim 4X$ the amount calculated based on a space filling model on the fiber surface.

Changes in either column length or flow rate that result in the same rSPA residence time were found to produce very similar rSPA binding capacities. Loading 0.5 mg mL$^{-1}$ rSPA feedstock concentration onto a 30-cm column at a flow rate of 0.5 mL min$^{-1}$ produced a density of $\sim 2.1$ mg rSPA g$^{-1}$ fiber. Under these conditions, 95% breakthrough was achieved after only 1.5 mL of load volume, equal to $\sim 3$ minutes of loading. In order to ensure complete loading, the loading was performed for 6 minutes (or 3.0 mL in volume) in total. Frontal analysis across 6 columns revealed a loading precision of 1.6 %RSD, suggesting that the ligand coverage is uniform and reproducible.

3.3.2 Elution buffer composition

The release of IgG from the surface-adsorbed protein A ligands is typically performed under acidic conditions [15]. Linear elution schemes introducing gradual changes in pH or chaotropic agent presence have been used for isotype
separations [16]. However, these linear elution gradients lead to excessive IgG band broadening, compromising the method’s sensitivity [16]. Step gradients from 100% equilibration buffer to 100% eluting buffer are better suited for high-sensitivity IgG analysis. Some common eluting buffer systems include glycine HCl [11], citrate [16-17], acetate [17], phosphate [16], and hydrochloric acid [18] at different concentrations [19-20] to yield acidic conditions with pH <3.0. The choice of elution buffer represents a compromise between detection capabilities, elution efficiency, and general operational considerations. Previous efforts had shown that UV-vis absorbance monitoring of IgG elution at 216 nm provided sensitive, reproducible results [14]. As demonstrated in Fig. 3.1, baseline contributions in the elution absorbance is a serious issue for 0.1 M citrate at pH 2.5; 0.1 M acetate at pH 2.8 and 0.5 M acetate at pH 2.5. A similar situation was also observed when 0.1 M glycine HCl at pH 2.5 was used, though there was a small peak for the eluting IgG. These large background absorbance responses present an additional issue as longer equilibration times would be required for the absorbance to return to baseline levels prior to the next run.

The problems associated with baseline perturbations can be addressed by using a more UV-transparent mobile phase additives, at lower concentration. As seen in Fig. 3.1, phosphoric acid and hydrochloric acid at modest concentrations of ~4.5 mM (0.025% v/v) and 10 mM respectively yield very sharp elution profiles with rapid returns to background absorbance values. Based on the retention times for the IgG elution, glycine HCl 0.1 M at pH 2.5 has the strongest elution strength, followed by 10 mM HCl at pH 2.0 and the 0.025% v/v H₃PO₄ at pH 2.5. In terms of
peak shape, HCl produced the sharpest and most symmetric elution peak, and $\text{H}_3\text{PO}_4$ was the second best. These two elution buffers would be expected to yield high quantification performance. Additionally, these two mineral acids are conveniently washed off the column within one minute of column equilibration time prior to subsequent runs.

The choice between these two elution buffers comes down to their compatibility with the HPLC hardware, the majority of which here is made of 316
grade stainless steel [21]. This type of stainless steel has a generally high resistance to solvent corrosion induced by various chemicals including phosphoric acid [21]. However, hydrochloric acid is corrosive to this material [22]. Unless all HPLC parts are made of corrosion-resistant materials such as titanium or PEEK as employed in “biocompatible” systems, the use of halide ions at low pH is not recommended by manufacturers [21]. With the intention of devising a method that can be done on a routine basis, phosphoric acid is chosen over hydrochloric acid as the elution solvent in this study.

3.3.3 Specificity of rSPA-modified column

IgG is just one of many proteins present in cell culture supernatant. There is an absolute need for high levels of specificity in quantifying the IgG titer in production systems, alleviating concomitants which would lead to erroneous IgG values. The specificity of the rSPA-modified C-CP fiber column was evaluated based on its ability to isolate IgG from complex cell culture supernatant. The supernatant of CHO-PF K1, which does not express mAb, was used as the complex matrix for the spiking of monoclonal IgG\textsubscript{1}. Fig. 3.2 depicts the load/elution chromatograms of neat IgG\textsubscript{1} (1 mg mL\textsuperscript{-1} in PBS), CHO cell supernatant, and the supernatant spiked with 1 mg mL\textsuperscript{-1} IgG\textsubscript{1}. In the case of the neat IgG\textsubscript{1} in PBS, the injection peak (∼1 min) represents un-retained antibody under these specific conditions, with a sharp release of the captured IgG following the change to the elution buffer at t = 2 min. In the case of the CHO supernatant, the magnitude of the injection peak is far greater as all of the un-retained concomitants pass through
the column, as expected and seen on commercial protein A columns [11, 18, 20, 23]. Key here is the fact that no solute species are seen following the buffer change. This suggests that either the amount of non-specific retention of non-IgG contaminants is quite minimal, or they are simply not removed in the change to acidic conditions. Non-retention is not surprising based on previous efforts [13-14], as the adsorbed protein A presents a fairly hydrophilic surface, which is not conducive to protein retention. Finally, in the case of the spiked supernatant, the injection peak includes the concomitants and any un-retained IgG₁, with the release of captured IgG upon the buffer change. Even for this singular set of chromatograms, the quantitative figures are quite impressive. Specifically, when converted into mAu*mL to account for the difference in the volumetric flow rates in the loading and elution steps, the sum of the injection peaks (neat IgG and supernatant) yields a value of 14.4 mAu*mL and the measured value is 13.9 mAu*mL, and the IgG recovery from the supernatant is 7.8 mAu*mL versus a value of 8.5 mAu*mL seen for the neat IgG injection. The better than 90% agreement in quantitative values suggests that the method does indeed yield the high selectivity.

3.3.4 Loading/equilibration buffer composition

The loading buffers used in many protein A and G affinity chromatography methods include salt (NaCl) at a moderate concentration (most often 150 mM) [6, 11, 18, 20, 23], while some others do not call for its use at all [17, 19]. In one study, high concentrations of NaCl were shown to be critical in reducing the non-specific binding to the affinity ligands [18]. In general, the role of NaCl is not properly
explained, other than it is an additive used out of habitual practice to match the ionic strength of the supernatant and possibly stabilize the proteins. The contradictions are seen as well in computational modeling of the IgG-rSPA system, in some cases showing improved binding that is salt-specific, and in others no effect whatsoever [18, 24].

![Figure 3.2 Load/elute chromatograms for 1 µL injections of 1.0 mg mL\(^{-1}\) IgG\(_1\) in PBS buffer, CHO-K1 (IgG-free) supernatant, and CHO-K1 spiked with IgG to a concentration of 1 mg mL\(^{-1}\). Elution solvent = 0.025 % H\(_3\)PO\(_4\), injection flow = 0.1 mL min\(^{-1}\), elution flow = 1.0 mL min\(^{-1}\)](image)

The conflicting experimental and computational results regarding IgG loading buffer require it to be evaluated on the C-CP fiber system. Fig. 3.3a
illustrates the effects of loading buffer composition on the resultant IgG elution profiles for the CHO DP cell supernatant spiked with 1 mg mL$^{-1}$ IgG$_1$. The concentrations reflect the NaCl content added to the 12 mM phosphate (pH = 7.4) buffer present throughout. In these experiments, the change from the loading buffer to elution buffer occurs at $t = 2.0$ min. The effects for the rSPA-modified polypropylene C-CP fiber column system are quite pronounced. The binding of IgG$_1$ seems to be stronger, as visually illustrated in longer elution times, as the salt content is increased. (The absorbance baseline is changed as well, but this is a minor effect.) There is also an apparent loss in the product recovery based on the absorbance peak areas. Of course, lower amounts of recovered protein could also be due to lower loadings from that same buffer. As seen in Fig. 3.3b, the integrated areas of the un-retained fractions do not change across the buffer compositions, reflecting no change in the IgG loading. Therefore, it is the elution/recovery of IgG from the spiked samples that is suppressed with increasing salt content, suggesting stronger affinity of the IgG for the protein A under these conditions.

The use of NaCl has also been mentioned as a means to reduce non-specific binding to the affinity chromatography stationary phase. Exposure to extremely high amounts of the previously listed non-IgG concomitants in the complex matrix poses a higher risk of non-specific binding, resulting in lower recovery rates of IgG and fouling of preparative columns. In the analytical applications, fouling is less of an issue. More importantly, there is possible interference due to non-specific binding with the accuracy of the IgG quantification if non-IgG concomitants co-elute from the column [16]. For example, due to the
substantial non-specific binding of host cell protein in the cell supernatant interfering with the IgG elution signal, 1 M NaCl has been included in the binding buffer to suppress the non-specific binding [18]. As was shown in Fig. 3.2 here, there is no suggestion of concomitant elution when the non-spiked IgG CHO supernatant was injected. As a result, no salt is employed here in the binding/equilibrating buffer.

### 3.3.5 Flow rate optimization

Very different from the case of porous chromatographic support phases, the virtually non-porous C-CP fibers allow for extremely high linear velocities when performing both analytical and preparative protein separations [25]. This translates to the ability to use high volumetric flow rates, yielding greater analytical throughput. The volumetric flow rates investigated in this study ranged from 0.1 mL min$^{-1}$ to 2.0 mL min$^{-1}$, equating to linear velocities of $\sim 6 – 112$ mm s$^{-1}$ and a maximum backing pressure of $\sim 1200$ psi on this HPLC system. Considering the minor difference in the viscosity of the loading and eluting buffer, the pressure is mostly dependent on the flow rate itself. A linear relationship between flow rate and pressure ($y = 576x + 33$, $R^2 = 0.993$) reflects a lack of bed perturbation as flow rate is increased. If the flow rate is increased up to 4.0 mL min$^{-1}$, the column will incur the pressure of about $\sim 2300$ psi (as calculated from the fitted linear equation), which is still lower than the pressure limit allowed by the HPLC system used in this study (up to 6000 psi). The operating pressure of the columns used in the study is actually limited by the column packing material itself as PEEK can only
Figure 3.3. Role of NaCl concentration in 12 mM phosphate buffer load/equilibration solvent on IgG recovery. a) absorbance transients reflecting recoveries of 1 μL injections of 1.0 mg mL⁻¹ IgG spikes in CHO-DP supernatant into different solvents followed by elution with 0.025 % H₃PO₄, b) Relative recoveries of injection (un-retained IgG) peaks and elution peak areas.
withstand the pressure of up to 3000 psi. This upper flow rate limit is comparable to those reported for TSKgel Protein A-5PW column by Tosoh Science [26] and POROS® A20 column by AB Applied Biosystems [20].

**Table 3.1** Roles of load and elution buffer flow rates on IgG1 retention and recovery for triplicate 1 μL injections of 1 mg mL⁻¹ IgG1 in CHO-PF K1 supernatant matrix.

<table>
<thead>
<tr>
<th>Load flow rate (mL min⁻¹)</th>
<th>Elution flow rate (mL min⁻¹)</th>
<th>Unretained peak area (mAU*μL)</th>
<th>Eluted peak area (mAU*μL)</th>
<th>Total peak area (mAU*μL)</th>
<th>Percent recovery (%)</th>
<th>Recovery precision (% RSD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>1.0</td>
<td>2.2</td>
<td>8.2</td>
<td>10.4</td>
<td>79</td>
<td>0.7</td>
</tr>
<tr>
<td>0.5</td>
<td>1.0</td>
<td>3.4</td>
<td>6.2</td>
<td>10.4</td>
<td>64</td>
<td>7.2</td>
</tr>
<tr>
<td>1.0</td>
<td>1.0</td>
<td>3.8</td>
<td>5.4</td>
<td>9.6</td>
<td>59</td>
<td>23</td>
</tr>
<tr>
<td>0.1</td>
<td>0.5</td>
<td>2.1</td>
<td>7.8</td>
<td>10.0</td>
<td>79</td>
<td>1.3</td>
</tr>
<tr>
<td>0.1</td>
<td>1.5</td>
<td>2.2</td>
<td>6.9</td>
<td>9.1</td>
<td>76</td>
<td>1.8</td>
</tr>
<tr>
<td>0.1</td>
<td>2.0</td>
<td>2.2</td>
<td>6.5</td>
<td>8.7</td>
<td>75</td>
<td>1.3</td>
</tr>
</tbody>
</table>

The binding of IgG to the rSPA ligands on the C-CP surface is a complex issue as it depends on a variety of factors, including the accessible ligand density on the surface, steric hindrance and potential mis-orientation of the ligand. Of course, there is a kinetic aspect as well as protein solute must diffuse from the solution bulk flow to the fiber surface and be captured on the time scale of the column residence time. As a result, their interaction with the ligands is time dependent. As shown above in section 3.3 and Fig. 3.2, there is an un-retained peak at the beginning of the chromatogram, either due to insufficient residence time or column overload. In the case of bound protein elution, the linear velocity and desorption kinetics will limit the recoveries and column recycling times. Therefore, the binding and elution flow rates must be evaluated.
Table 3.1 summarizes the quantitative results obtained by varying the load and elution flow rates in terms of the integrated areas of the injection (un-retained IgG) and the elution peaks, the percentage of mAb recovery, and the precision of \( n=5 \) measurements. Due to the difference in the investigated load and elution flow rates, the peak area is converted into mAu*\( \text{mL} \) for the same scale comparison. The \% recovery is the percentage of the IgG elution peak out of the total area of the un-retained and elution peaks, basically representing the fraction of IgG retained and eluted, versus the amount injected. To be fair, this measurement is biased negatively as the columns are operating in conditions where 100\% retention is assured. In the first set of experiments, the load flow rate was changed, while maintaining a constant elution rate of 1 mL min\(^{-1}\). Clearly, the lowest load rate yields the highest retention (based on the lowest injection peak area), while also yielding the highest overall recovery (79\%) and best precision (0.7\% RSD). Using the 0.1 mL min\(^{-1}\) load rate (the lowest reliably delivered by this HPLC), variations in the elution recoveries are somewhat better for the low flow rates. The highest flow rate accomplishes full elution <15 s after buffer change versus >2 min for the lowest flow rate. While not the highest throughput, the loading and eluting flow rates of 0.1 mL min\(^{-1}\) and 1.0 mL min\(^{-1}\) produce the highest and most reproducible IgG\(_\text{1}\) binding and recovery. These flow rates are chosen for the remainder of the studies.

3.3.6 Method validation

3.3.6.1 Accuracy
The use of the protein A-modified C-CP fibers in a process development/characterization context relies on the fact that a straightforward quantification methodology can be applied to monitor the IgG production through the course of the CHO cell culture process. Such a method should allow for potential changes in reaction conditions and culture media changes. Ideally, the use of an affinity phase that is selective for the analyte and not prone to non-specific binding should allow for the use of external standardization employing calibration functions constructed in representative host matrix material. As demonstrated in Fig. 3.2, this would appear to be a valid approach for the present system. Calibration functions were generated using IgG\textsubscript{1} in PBS (i.e., matrix free), covering a concentration range of 0.25-3.0 mg mL\textsuperscript{-1} with the integrated absorbance of the elution peaks determined for triplicate injections at each of the five spike 

<table>
<thead>
<tr>
<th>Sample</th>
<th>External Calibration function</th>
<th>IgG\textsubscript{1} (mg mL\textsuperscript{-1})</th>
<th>Precision (%RSD)</th>
<th>Standard addition function</th>
<th>IgG\textsubscript{1} (mg mL\textsuperscript{-1})</th>
<th>Precision (%RSD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG\textsubscript{1}-spiked CHO-PF K1</td>
<td>$y = 9.71 x - 1.35; R^2 = 0.9989$</td>
<td>1.01</td>
<td>1.7</td>
<td>$y = 9.81x + 0.46; R^2 = 0.9994$</td>
<td>0.15</td>
<td>5.5</td>
</tr>
<tr>
<td>CHO DP “A”</td>
<td>$y = 9.09 x - 0.64; R^2 = 0.9982$</td>
<td>0.16</td>
<td>2.6</td>
<td></td>
<td>0.15</td>
<td>5.5</td>
</tr>
<tr>
<td>CHO DP “B”</td>
<td>$y = 9.09 x - 0.64; R^2 = 0.9982$</td>
<td>0.15</td>
<td>3.9</td>
<td>$y = 9.78x + 0.52; R^2 = 0.9994$</td>
<td>0.15</td>
<td>3.6</td>
</tr>
</tbody>
</table>

Table 3.2 Quantitative performance of external calibration function and standard addition methods for IgG\textsubscript{1} determinations of spiked CHO-PF K1 supernatant and CHO DP culture sample supernatants. 1 μL injections, n = 3 per calibration point and analytical determination.
concentrations. The response curve had a high degree of linearity as listed in Table 3.2. Across the concentration range, the triplicate determinations varied by <11.2% RSD. An initial evaluation of the accuracy and precision using a 1.00 mg mL\(^{-1}\) spike to the IgG-free supernatant yielded a concentration of 1.013 mg mL\(^{-1}\) (a relative error of 1.3 %) with a precision of 1.7% RSD for triplicate injections. There is no statistically significant difference between the calculated and known spike concentration at the significance level \(\alpha=0.05\).

In the absence of available standard CHO cell supernatant reference materials, the IgG1 concentrations in two separate, duplicate cultures were determined using the external calibration function as well as through a standard addition method. The CHO supernatant samples (coded A and B) harvested from CHO DP cultures expressing human IgG1 were analyzed. The two cultures were processed via the same protocols and so near-equivalent IgG1 concentrations were expected. Standard addition response curves were prepared by spiking the samples with four concentrations of IgG1 equating to 0.06 – 0.6 mg mL\(^{-1}\). In that concentration region, the calibration function for IgG1 spiked into CHO-PF K1 cell supernatant yielded a well-behaved response function (Table 3.2). The general agreement between the slopes of the standard calibration curve and those of the standard additions reflects a lack of matrix effects. The quantitative results of the external calibration and the standard addition analyses are presented in Table 3.2. Use of external calibration yielded values that differ by less than 1 % relative, with quantitative precision of \(\sim 2.5-4\%\), for triplicate measurements. There is no statistically significant difference between the calculated concentrations produced
by both methods at the significance level \( \alpha=0.05 \). The agreement between the 
external calibration and the standard addition quantification clearly demonstrates 
a self-consistency.

### 3.3.6.2 Linearity

The IgG response curves used in quantification were linear (as presented 
in Table 3.2) with \( R^2 > 0.99 \) over the concentration range of 0.075 – 3 mg mL\(^{-1}\) for 
1 \( \mu \)L sample injection volumes, corresponding to the loading of 0.075 to 3 \( \mu \)g of 
IgG. It is also relevant to determine the maximum column mass loading for which 
a Beer’s Law relationship is valid. In order to investigate the maximum amount of 
IgG that can be injected, different volumes were investigated for a fixed IgG 
concentration of 1.0 mg mL\(^{-1}\). A linearity in response (\( R^2 > 0.99 \)) was maintained 
to an injection volume \( \sim 6 \mu \)L of the stock solution, beyond which the recovered 
amount of IgG begins to reach a plateau. At this point, the column loading is 6 \( \mu \)g 
of IgG. The apparent saturation could be due to multiple causes, departure from 
linearity due to high (>1 AU) peak absorbance values, column overloading, or 
decreased recoveries. The first situation may come into play as the maximum peak 
absorbance was \( \sim 1.2 \) AU for the highest injection volume. Of the other two causes, 
poor recoveries would be expected to lead to greater levels of inter-injection 
imprecision versus lower injection volumes and some sort of increasing bias. This 
is not the case. Indeed, based on previous studies [13, 27] a maximum loading of 
6 - 10 \( \mu \)g of protein is quite reasonable.
3.3.6.3 Limits of detection

The limits of detection (LOD) and quantification (LOQ) were determined based on the standard deviation of the response curve and its slope [18], as follows

\[
LOD = \frac{10\sigma_{RSS}}{3k} \quad (1)
\]

\[
LOQ = \frac{10\sigma_{RSS}}{k} \quad (2)
\]

where \( \sigma_{RSS} \) is the residual standard deviation of the response function and \( k \) is the slope of the calibration curve in the lower concentration range (0.075 – 0.6 mg mL\(^{-1}\)). Based on the linear regression statistics presented in Table 3.1 for 1 \( \mu \)L injections of IgG\(_1\)-spiked CHO-PF K1, a limit of detection of 0.017 mg mL\(^{-1}\) (17 ng absolute) was calculated, with a limit of quantification of 0.056 mg mL\(^{-1}\) realized.

So long as the injection volume and the concentration of the standards are combined so that the column is not overloaded, the LOD/LOQs of the method can be further improved by injecting higher volumes of the samples. As an example, a calibration function was generated for 40 \( \mu \)L injections, which was linear \( (R^2 > 0.99) \) over a concentration range of 0.016 – 0.125 mg mL\(^{-1}\). Above this concentration level, the onset of response curve saturation was observed. Using equations 1 and 2, the calculated LOD was reduced to 0.004 mg mL\(^{-1}\), with an LOQ of 0.013 mg mL\(^{-1}\). It must be admitted, though, that the calculated LOD concentration could not be visually discerned on a single-injection basis. Ultimately, the present method provides limits of detection that address the complete range of antibody concentrations of relevance in the IgG expression cycle.

3.3.6.4 Column reproducibility
Figure 3.4 Multiple load/elute chromatograms for 1 µL injections of 1.0 mg mL\(^{-1}\) IgG\(_1\) in PBS buffer illustrating a) Intra-day and Inter-day precision for a single column and b) batch-to-batch reproducibility. Load solvent = 12 mM phosphate, flow rate = 0.1 mL min\(^{-1}\), elution solvent = 0.025 % H\(_3\)PO\(_4\), flow rate = 1.0 mL min\(^{-1}\).
The protein A-modified C-CP fiber columns must be operated over multiple cycles and multiple days, hopefully without the need for extensive re-calibration. As an example of the reproducibility of the columns, a single column was put through six (6) load/elute/equilibration cycles within a given day, for multiple days. Fig. 3.4a presents the chromatographic traces for the replicate analytical cycles over a period of three consecutive days for 1 µL injections of 1 mg mL\(^{-1}\) IgG\(_1\). The quantitative aspects of these chromatograms, and one additional day after one week of dormancy are presented in Table 3.3. The intra-day precision of the retention times agrees to better than 0.6% RSD for each individual day, with the variability of the analytical recoveries on most days being less than 3.5% RSD. There is not a significant difference in performance following the column storage with a buffer fill for one week. Taken a step further, the cumulative inter-day statistics show a variability that is completely in line with the intra-day

<table>
<thead>
<tr>
<th>Day #</th>
<th>Elution time (min)</th>
<th>Elution peak area (mAU*min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intra-day average</td>
<td>Intra-day % RSD</td>
</tr>
<tr>
<td>1</td>
<td>3.32</td>
<td>0.31</td>
</tr>
<tr>
<td>2</td>
<td>3.30</td>
<td>0.17</td>
</tr>
<tr>
<td>3</td>
<td>3.32</td>
<td>0.33</td>
</tr>
<tr>
<td>4 (1 week later)</td>
<td>3.29</td>
<td>0.59</td>
</tr>
<tr>
<td>Inter-day average</td>
<td>3.31</td>
<td></td>
</tr>
<tr>
<td>Inter-day %RSD</td>
<td>0.54</td>
<td></td>
</tr>
</tbody>
</table>
reproducibility. It must be emphasized that the data presented here represent quantitative figures derived from a single calibration function generated at the beginning of the study. Surely even greater precision could be realized via daily re-calibration.

### 3.3.6.5 Colum-to-column reproducibility

**Table 3.4** Batch-to-batch reproducibility characteristics for the recovery of 1 mg mL\(^{-1}\) IgG\(_1\) in PBS matrix. 1 μL injections. \(n = 6\) for each column

<table>
<thead>
<tr>
<th>Column #</th>
<th>Elution time (min)</th>
<th>Intra-column %RSD</th>
<th>Elution peak area (mAU*min)</th>
<th>Intra-column %RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.27</td>
<td>0.07</td>
<td>7.29</td>
<td>2.15</td>
</tr>
<tr>
<td>2</td>
<td>3.29</td>
<td>0.06</td>
<td>7.64</td>
<td>1.60</td>
</tr>
<tr>
<td>3</td>
<td>3.32</td>
<td>0.31</td>
<td>8.22</td>
<td>3.32</td>
</tr>
<tr>
<td>4</td>
<td>3.37</td>
<td>0.02</td>
<td>8.43</td>
<td>0.78</td>
</tr>
<tr>
<td>Inter-column average</td>
<td>3.31</td>
<td></td>
<td>7.89</td>
<td></td>
</tr>
<tr>
<td>Inter-column %RSD</td>
<td></td>
<td></td>
<td>6.62</td>
<td></td>
</tr>
</tbody>
</table>

It is expected that the IgG quantitation results obtained in one location can be translated to other laboratories; i.e., there must be a high level of column-to-column reproducibility. To this end, the reproducibility in the performance of rSPA-modified C-CP fiber columns prepared in different batches was evaluated for the recovery of 1 μL injections of 1 mg mL\(^{-1}\) IgG\(_1\). Fig. 3.4b presents equivalent load/elute/equilibrare chromatograms for columns prepared in four (4) batches. There are multiple challenges in achieving equivalent performance; most crucial are the quality of the chemistry and the reproducibility of column packing. The
robustness of the chemical modification procedure is reflected in the yields of the capture/release process and potential non-specific binding issues. Variability in the packing process may cause differences in the column residence times and the retention time of IgG analyte. As demonstrated in the quantitative data of Table 3.4, the intra-column precision in the IgG₁ elution times and recoveries are outstanding. As in the case of the single-column metrics of Table 3.3, the retention times for single columns vary by less than 0.3% RSD, and the recoveries vary by less than 3.3% RSD. Taken across multiple columns, the variation in the elution times is 1.3% RSD, while the variability in the recoveries is on the order of 6.5% RSD. On the surface, this suggests that the control over the column packing process is greater than the chemical modification processes. It is also worth mentioning that these columns were actually prepared and stored over a course of eight months before being tested, which proves their stability for long time use.

3.4 Conclusions

In this work, the potential application of the rSPA-modified C-CP PP fiber [14] to the IgG quantitation was investigated. Relevant chromatographic parameters were investigated to develop a method for the quantitation of IgG in complex biological matrices, CHO cell supernatants. Ultimately, a loading buffer containing 12 mM phosphate at pH 7.4 and an elution buffer of 0.025% phosphoric acid were used as the mobile phases. Further investigations into the loading and elution conditions indicated that the optimal flow rates for IgG recovery included loading at 0.1 mL min⁻¹ and elution at 1.0 mL min⁻¹. Method precision was validated
through intra-day and inter-day reproducibility of the elution peak area, which were 3.1% and 3.5% RSD respectively. The inter-column reproducibility of ~6.6% demonstrates uniform surface chemistries and reproducible column packing and rSPA ligand loading.

The quantitative figures of merit obtained here are quite comparable to those reported for the CIM protein A HLD disk produced by BIA Separations (Ljubljana, Slovenia) [18]. One problem with that protein A phase is its susceptibility to non-specific binding of the HCPs to the disk backbone made of poly(glycidyl methacrylate-co-ethylene dimethacrylate) [18]. As a result, a high salt concentration load/equilibration buffer (up to 1 M NaCl) was required. The Agilent bio-monolith protein A column was reported to have a linearity range covering 0.02 to 2 mg mL\(^{-1}\) at 5 µL injection volumes while the range reported for the MAbPac Protein A column by Thermo Scientific is 0.01 to 5 mg mL\(^{-1}\) at a 20 µL injection volume [17, 23]. These ranges are wider than the one investigated in this study mostly due to their higher loading capacity, allowing higher injection volumes. The LOD reported for Agilent bio-monolith protein A column was 0.5 µg, for a 50 µL injection of mAb concentration of 10 µg mL\(^{-1}\) [17], which is close to the value reported in this study.

With such merits, the rSPA-modified C-CP fiber columns fit in the tool box for Process Analytical Technologies (PAT), which is defined by FDA as “Systems for analysis and control of manufacturing processes based on timely measurements of critical quality parameters and performance attributes of raw and in-process materials” [28]. These columns can provide quantitative information
reflecting the cell culture process that is important for the optimization of basal media formulation and seed culture selection and quality control during the early development phase of recombinant mAb production. Real-time monitoring of the cell culture growth allows harvest decisions to be timely made. Moreover, when followed by other modes of chromatography such as SEC or IEC two-dimensional liquid chromatography (2D-LC) can be implemented for further characterization (e.g., glycosylation, charge variants) of the purified mAbs [29-30]. Despite its potential, the rSPA-modified C-CP PP columns have certain limitations in some practical application aspects. The short linear dynamic of the method reported here can be restrictive. To be clear, columns of greater length, and therefore higher binding capacities, can be readily implemented. Another issue with this method is the carry-over of the bound IgG that cannot be fully eluted from the column in previous runs. The average % carry-over for IgG\textsubscript{1} at the concentration of 1.0 mg mL\textsuperscript{-1} was determined to be around 2.5%. Therefore, it may be advantageous to use a low-volume CIP (clean-in-place) step with 0.1 M NaOH between analyses under high titer conditions. Such a CIP has been demonstrated to have very little effect in terms of ligand bleed from the C-CP column in previous studies. It is recommended that the calibration standards should be run from low to high concentrations and the column should be washed for 2 minutes with the elution buffer and re-equilibrated with the loading buffer before the sample is injected. In practice, the method described here can be accelerated greatly in terms of the time periods between the various load/elute/equilibration functions. Another issue of the rSPA-modified C-CP PP columns that needs to be addressed is their overall
recovery. The highly efficient fluid movement feature of C-CP fiber is a critical factor here. Maximizing residence time of the sample injection through flow rate optimization can only help so much. Increasing column length or scaling up to larger column format will be investigated.

Acknowledgements

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CHAPTER FOUR

GRAFTING POLYMERIZATION OF GLYCIDYL METHACRYLATE ONTO CAPILLARY-CHANNELED POLYMER (C-CP) FIBERS AS A LIGAND BINDING PLATFORM: APPLICATIONS IN IMMOBILIZED METAL-ION AFFINITY CHROMATOGRAPHY (IMAC) PROTEIN SEPARATIONS

4.1 Introduction

Over the past couple of decades, the pharmaceutical industry has dramatically expanded its market of therapeutic drugs produced from biological sources. These biotherapeutics can be of recombinant peptides, proteins or monoclonal antibodies. Among them, therapeutic drugs of protein origin are the most common entities in the biopharmaceutical industry. Upstream and downstream processing are the two pivotal processes in the manufacture of protein-based therapeutics. While the yield of upstream processing has dramatically increased over the past two decades [1], downstream processing, on the other hand, is still the rate limiting step. Recovery, separation, and purification of the protein therapeutics may account for 50 to 90% of the total product manufacturing cost [2]. As these clean-up/polishing steps in the downstream processing rely heavily on chromatographic techniques, there is continuous interest in the development of stationary phases that allow high-throughput and cost-effective protein purification.
After being introduced by Porath and co-workers in 1975 [3], immobilized metal-ion affinity chromatography (IMAC) has been extensively studied and developed into one of the more popular approaches for protein purification [4]. IMAC utilizes the affinity of electron-rich amino acids (most commonly histidine) towards certain transition metal-chelate complexes that are immobilized onto a support [5]. Among the most popular metal ions employed in IMAC are the borderline transition metals including Cu$^{2+}$, Zn$^{2+}$, Ni$^{2+}$ and Co$^{2+}$ [5-6]. It is a powerful chromatographic technique that allows easy, single-step purification of native histidine-rich proteins as well as recombinant histidine (His)-tagged proteins [7]. For the most part, commercially available IMAC stationary phases are divided into two groups based on their operating pressure [4]. Low-pressure IMAC support materials are generally made of cross-linked agarose matrices which are permeable to macromolecules due to their highly porous structure. On the other hand, high-performance (HP-IMAC) supports employed at elevated pressures in HPLC systems are based on hydrophilized silica [4]. Drawbacks of these traditional phases include slow diffusion of protein molecules into and out of the pores and the chance of pore clogging due to the complexity of the feed samples. The latter is particularly true for HP-IMAC phases, where high pressures and flow rates are employed to affect high-throughput purification on both analytical and preparative scale. In addition, there is also a limitation in the operating pH range of silica-based phases versus polymeric ones.

Many monolith IMAC phases employing silica [8], polymethacrylate [9-11] or cryogels [12-13] have been reported. These alternative support phases have
large though-pores for enhanced permeability, which allows for fast protein separation with high mass transfer efficiency. The applications reported for these monolith-based IMAC phases are largely for smaller biomolecules such as phosphopeptides [8, 11] or plasmid DNA purification [14]. In the applications using the cryogel IMAC for protein separation, low dynamic binding capacity of (His)$_x$ lactate dehydrogenase (0.44 mg mL$^{-1}$) were reported due to its low surface area [13]. In another application using cryogel for cytochrome C purification, higher dynamic binding capacity was reported at 20 mg mL$^{-1}$ [15]. A commercial epoxide-activated methacrylate Convective Interaction Media (CIM) disk (i.e., monolithic membrane) manufactured by BIA Separations (Ljubljana, Slovenia) was reported to have a dynamic binding capacity of 18-30 mg mL$^{-1}$ of stationary phase, with a linear velocity range of 53 –530 cm h$^{-1}$ (~0.15-1.5 mm s$^{-1}$) [9]. These values are close to those reported for the other commercially available IMAC resins as shown subsequently in Table 4.1.

Another support format that has received attention and studied quite extensively by several research groups is hollow fiber membranes [16-20], either non-porous or porous and of different base chemistries including polyethylene, polypropylene, polysulphone and nylon. While the modified membranes are reported to have rapid and high adsorption rates, they tend to be difficult to use in conventional batch-wise or columnar adsorption modes [2, 21]. By the same token, membrane formats are not generally employed on the analytical scale.

Depending of the functional groups available on the support surface and the chelating agents, different immobilization methods can be used to produce IMAC
stationary phases. The most common is the epoxide activated support which is commercially available for convenient and simple immobilization of not only IMAC ligands but also various affinity ligands [4]. This epoxidation approach employs the long-established polymerization grafting of glycidyl methacrylate [2, 16-20]. In this scheme, there are different radical initiator systems and means of polymerization grafting such as plasma [22], ozone [23] or UV light [24]. As effective as they are, these methods are difficult to impart on to non-uniform surfaces and shapes (such as the fibers described here). Moreover, concerns about mechanical degradation of the polymers need to be considered. Among possible explored techniques, microwave irradiation is a promising approach which has been widely studied [25-28].

Capillary-channeled polymer (C-CP) fibers have been studied in this laboratory as an alternative stationary phase for analytical separations and downstream processing. Each C-CP fiber is characterized by the eight axial channels running along its length. When packed into a column format, the wings from neighboring fibers inter-digitate along their length to form aligned micro-channels that facilitate highly efficient fluid movement [29-30]. Their non-porous structure, combined with high column permeability, allow analytical-scale separations to be performed at high linear velocities (up to 100 mm s\(^{-1}\)) with high column permeability (<0.14 MPa cm\(^{-1}\)) [31-32]. Moreover, these materials are more stable over a wide pH range and less expensive (< $100 lb\(^{-1}\)) than the conventional stationary phases. This laboratory has utilized C-CP fibers in a variety of chromatographic modes including reversed-phased [32], ion-exchange [33],
hydrophobic interaction [34] and affinity [35-36]. One of the positive aspects for using polymer phases is ready surface modification. Recently a new microwave-assisted polymerization grafting method using potassium persulfate (KPS) as the radical initiator has been developed to functionalize nylon 6 C-CP fibers with acrylic acid (AA) and acrylamido-2-methylpropanesulfonic acid (AMPS), providing ligands for weak and strong cation exchange protein separation respectively [37-38].

In this study, the surface of nylon C-CP fibers are covalently modified with the iminodiacetic acid (IDA) ligand. The nylon-IDA C-CP fibers are then charged with Cu$^{2+}$ ions and investigated for their potential in the purification of native proteins and recombinant His-tagged proteins. The present effort is a continuation of the microwave-assisted modification approach in which ceric ammonium nitrate (NH$_4$)$_2$Ce(NO$_3$)$_6$ (CAN) in dilute nitric acid was used as an alternative radical initiator system to graft nylon 6 C-CP fiber with glycidyl methacrylate (GMA). The reactive epoxide groups on the GMA residues were converted into metal-chelating iminodiacetate (IDA) groups. Various reaction parameters were investigated to achieve the highest degree of epoxide grafting and IDA conversion rate possible. The resulting nylon-IDA fibers were characterized by attenuated total reflection-Fourier transform infrared spectroscopy (ATR-FTIR) to confirm the modification process. Scanning electron microscopy (SEM) was also performed to examine the fiber’s morphology and topography. The IDA ligand density and copper binding capacity of the modified fibers were also determined. Once packed into the microbore column format and charged with Cu$^{2+}$ ions, the nylon-IDA fibers were evaluated for their protein binding capacity using native lysozyme and a
recombinant his-tagged ubiquitin as test proteins. The simplicity of microwave-assisted grafting polymerization coupled with the chemical versatility of the epoxide group on the GMA monomer provides a convenient platform for the development of C-CP fiber based stationary phases with diverse chemistry suitable for both analytical and preparative scale separations.

4.2 Materials and methods

4.2.1 Chemicals and instrumentation

HPLC-grade acetonitrile (ACN), methanol (MeOH) and 70% ethyl alcohol were purchased from EMD Millipore (Billerica, MA). Potassium phosphate monobasic (KH$_2$PO$_4$), sodium phosphate dibasic (Na$_2$HPO$_4$) and concentrated nitric acid were purchased from Sigma Aldrich (St. Louis, MO). Phenolphthalein (2% in ethanol) was purchased from Fluka Analyticals (St. Louis, MO). Activated alumina powder was purchased from Polysciences, Inc. (Warrington, PA). Sodium hydroxide pellets were purchased from Fisher Scientific (Fair Lawn, NJ). Concentrated hydrochloric acid (HCl) was purchased from JT Baker (Phillipsburg, NJ). Sodium carbonate was purchased from Acros Organics (Bridgewater, NJ). Molecular biology grade HEPES, free acid was purchased from BDH (West Chester, PA). Ceric ammonium nitrate (NH$_4$)$_2$Ce(NO$_3$)$_6$ (CAN) was purchased from Macron Fine Chemicals (Center Valley, PA). Iminodiacetic acid (IDA), dimethyl sulfoxide (DMSO), imidazole, sodium chloride and copper (II) sulfate pentahydrate were purchased from Alfa Aesar (Ward Hill, MA). Glycidyl methacrylate (GMA) and 4-(4-Nitrobenzyl) pyridine (NBP) were purchased from
TCI (Tokyo, Japan). Cytochrome C from equine heart, α-Chymotrypsinogen A from bovine pancreas and Lysozyme from chicken egg white were all purchased from Sigma-Aldrich (St. Louis, MO). Recombinant human histidine-tagged ubiquitin (HisU) was purchased from Boston Biochem (Cambridge, MA). Standardized sodium hydroxide solution (0.1142 M) and hydrochloric acid (0.1 M) were purchased from Ricca Chemical Company (Arlington, TX) and Fisher Scientific (Fair Lawn, NJ), respectively. All aqueous solutions were prepared with ultra-pure Milli-Q water (18.2 MΩ.cm) obtained from a Millipore water system (Billerica, MA). Nylon 6 C-CP fibers were provided by Material Science and Engineering department, Clemson University. The denier per filament is 2.67 grams per 9000 meters of a single fiber. The cross-section perimeter of each fiber is ∼207 µm.

All chromatographic experiments, including the protein separation and loading/elution characterization, were performed on a Dionex Ultimate 3000 HPLC system (Thermo Fisher Scientific Inc., Sunnyvale, CA) consisted of an LPG-3400SD pump, a WPS-3000TSL auto-sampler and a VWD-3400RS UV-Vis detector and was operated under Chromeleon 6.80 software. The microwave polymerization grafting of GMA onto the nylon C-CP fiber was performed in an Emerson MW7300W microwave oven, without any modifications of the commercial unit.

The cell lysate of *E. coli* (NEB 5-alpha *E. coli*) used for the HisU spiking experiment was prepared as follows. *E. coli* culture was grown in 1 L Luria-Bertani broth at 37 °C to an O.D. of 0.8 before being pelletized at 13,000 g for 10 min. The cell pellet was then re-suspended in phosphate buffered saline and re-centrifuged
4.2.2 Preparation of IDA-modified nylon fiber

We have described the methods for preparing native fibers and the column packing procedure in detail previously [29, 31]. The nylon 6 C-CP fibers employed here were produced in the Clemson University Department of Materials Science and Engineering, having nominal diameters of ~40 µm, with the unique fiber shape yielding a perimeter of ~210 µm. For convenience, the three kinds of fibers described in this paper will be referred to as native nylon, the intermediate stage nylon-GMA and the final product nylon-IDA. The entire chemical modification process for the nylon-IDA fiber is detailed in Fig. 4.1. Glycidyl methacrylate (GMA) was filtered through an activated alumina bed to remove any residual stabilizing agent (4-methoxyphenol). A solution of 0.1 M HNO₃ was purged with nitrogen for at least 30 minutes to deplete any dissolved oxygen before use. The polymerization grafting solution was composed of 0.6 g (3% w/v) GMA and various amounts of ceric ammonium nitrate, ranging from 0.125% to 2.0% w/v in 20 mL 0.1 M HNO₃. In order to prepare the native fibers for further modification, they must be washed to remove residual surface species and stretched to inhibit in-column changes in the packing [29, 31]. Nine rotations of nylon-6 C-CP fibers (540 fibers) were removed from the spool and stretched on a dying fork. The fiber bundles were rinsed with hot water for better alignment. Further rinsing with excess amounts of DI-water, MeOH and ACN was done to remove any residual chemicals left on the
fibers during the manufacturing process. The fibers were then placed into 50-mL beakers containing the polymerization solution. To ensure even microwave and heat distribution, two beakers containing the fibers and the reaction mixture were placed in the middle of the glass turntable plate. The polymerization grafting reaction was performed in the Emerson microwave oven at its lowest power level (70 W) for the pre-planned time periods. The GMA-grafted fiber bundles were thoroughly washed to remove excess visible homopolymer and then placed in a glass jar containing 10 mL of 0.5 M IDA in 1:1 DMSO:1 M Na₂CO₃.
It would be natural to expect that the coupling of the IDA to the GMA-nylon fibers would be accelerated under microwave irradiation, and it was. However, the solvent and high pH of the reaction mixture damage the fibers which make it impossible to be packed into a column format. The coupling of IDA through the epoxides groups on GMA was executed in a standard laboratory oven at 100°C for different pre-planned periods to determine the optimal reaction time. The fibers were then thoroughly cleaned with DI-water to remove residual DMSO. The, now nylon-IDA, fibers were placed in a beaker containing 30 mL 0.1 M HCl and left stay overnight at room temperature to hydrolyze remaining unreacted epoxide groups. After that, the nylon-IDA fiber bundles are ready to be packed into 0.03” (0.762 mm) i.d. polyether ether ketone (PEEK) tubing (IDEX Health & Science LLC (Oak Harbor, WA)) for chromatographic implementation in column format. As presented previously [29, 31], the fiber loop was fed through the tubing using 50-lb test fishing line to arrive at a matrix of nominally-aligned C-CP fibers.

To determine the grafting degree and conversion rate, the modified fibers at each stage were vacuum dried and weighed. The grafting degree G (%) was calculated as follows [17]:

$$G(\%) = \left[ \frac{W_1 - W_0}{W_0} \right] \times 100\% \quad \text{(Eq. 1)}$$

where $W_0$ and $W_1$ are the weights of the native nylon and nylon-GMA respectively. The conversion rate ($X$) of the epoxide groups into IDA is related to the weight changes of the fibers as follows [17]:

$$X = \frac{142 \times (W_2 - W_1)}{133 \times (W_1 - W_0)} \quad \text{(Eq. 2)}$$
where $W_2$ is the weight of the nylon-IDA fiber and the factors 142 and 133 correspond to the molecular weights of GMA and IDA, respectively.

The optimal scheme for the graft polymerization of GMA onto the native nylon fiber and the conversion of the GMA’s epoxides into IDA was as follows: grafting in 0.125% w/v of CAN for 15 minutes at a 70 W microwave power and conversion for 10 hours at 100°C. The final nylon-IDA C-CP fibers were then thoroughly washed and packed into a ∼30 cm segment of the 0.762 mm i.d. PEEK tubing. After packing, the columns were mounted on the HPLC system and washed with DI-water and 0.2 M NaCl for at least 1 hour to remove all residual chemicals left from the modification steps.

### 4.2.3 Preussmann test for the detection of epoxide groups

To test the uniformity of the grafting layer under different reaction conditions, nylon-GMA fibers were vacuum dried and weighed before being placed into a test tube containing 2 mL 0.02 M 4-(4-nitrobenzyl)pyridine (NBP) in 70% ethanol and 1 mL 1 M Na$_2$CO$_3$. The reaction was run at room temperature overnight. The same experimental procedure was also applied to nylon-IDA fibers to determine the IDA conversion rate. The concentrations of NBP before and after the reaction were determined through an HPLC method. 1 µL of the samples and standards were injected and separated on a 150 x 4.6 mm YMC-Pack Pro C18 column in an isocratic mobile phase containing 60% 20 mM phosphate buffer (pH 6.5) and 40% ACN at the flow rate of 1.0 mL min$^{-1}$. Detection of the residual NBP was made at 265 nm.
4.2.4 Bulk characterization of the modified nylon 6 C-CP fibers

The scanning electron microscope (SEM) images of the fibers were taken using a Hitachi SU6600 operating in the variable pressure mode, with a 10-kV accelerating voltage at the Clemson University Electron Microscopy Laboratory. The attenuated total reflection-Fourier transform infrared spectroscopy (ATR-FTIR) characterization of the fibers was performed on a Thermo-Nicolet Magna 550 FITR in the Analytical Testing Lab of Material Science & Engineering Department, Clemson University.

4.2.5 Acid-base titration

The carboxyl functional group densities of the nylon-IDA C-CP fibers were determined through an acid-base titration, based on a similar procedure reported in the literature for polyacrylamide gels [39]. Likewise, titrations have been employed in this laboratory for carboxylic acid group density determinations on C-CP fibers, with single-percent precision [37]. The fibers were placed in a beaker with ~20 mL of 0.1 M HCl and shaken for 20 minutes. After thorough washing with DI-H$_2$O to remove residual HCl, the excess water from was removed using a syringe and the beaker placed in a vacuum oven to allow the fibers to reach complete dryness. The dried fibers were weighed, placed in DI-water and titrated against a standardized NaOH 0.1142 M solution with a few drops of 2% phenolphthalein being used as the indicator.
4.2.6 Copper binding capacity

The copper density was determined by stripping the bound copper ions in acidic conditions and coupling to IDA for color intensification. This procedure was modified based on similar methods using ethylenediamine tetraacetic acid (EDTA) for both stripping and coupling purposes [13, 40]. The IDA-fibers were placed in \( \sim 10 \text{ mL of } 0.1142 \text{ M NaOH} \) and shaken for 6 minutes to convert the carboxyl groups into their anionic form. The fibers were then thoroughly rinsed with DI-H\(_2\)O. After excess water was removed using a syringe, the charged fibers were placed in \( \sim 10 \text{ mL of } 0.5 \text{ M CuSO}_4 \) and shaken for 30 minutes. The fibers were then washed thoroughly to remove the non-bound CuSO\(_4\). The fibers were dried under vacuum overnight to complete dryness. Upon drying, the fibers were weighed and loaded into a 1-mL syringe. The bound copper content was stripped off the fiber three times using three 1 mL portions of 1 M HCl. To intensify the color/response of the stripped copper ions, each eluted solution was added into an equal volume of 0.2 M IDA in 1.2 M NaOH. A serial concentration of CuSO\(_4\) solutions was treated the same way for color intensification. The samples and standards were directly injected into the HPLC system without a column at flow rate of 1 mL min\(^{-1}\) of water and analyzed at 730 nm. Absorbance peak areas (mAu*min) were used to construct a response curve from which the concentration of copper extracted from the copper bound nylon-IDA fibers in each portion was determined.

4.2.7 Protein dynamic binding capacity
The dynamic binding capacity of the nylon-IDA fibers was determined through frontal analysis, as is common and described previously [37-38], with lysozyme being used as the test protein. The loading buffer (A) was 20 mM HEPES, 0.2 M NaCl, pH 7.0 and the eluting buffer (B) is A with 0.1 M imidazole (Im), also at pH 7.0. Lysozyme solutions of different concentrations in buffer A were fed through the column until the UV absorbance at 280 nm reached a plateau. The column was then washed with buffer A to remove the non-bound proteins. After the baseline was re-established, buffer B was used to induce the elution of the bound lysozyme from the column. For the adsorption isotherm data collection, various concentrations of lysozyme (0.05-1.0 mg mL\(^{-1}\)) were fed to the column at the constant flow rate of 1.0 mL min\(^{-1}\)\((U_0\sim 70\ mm\ s^{-1})\). Another set of experiments, wherein the lysozyme concentration was kept at 1.0 mg mL\(^{-1}\) while the flow rate was varied (0.1-1.0-mL min\(^{-1}\), \sim 7-70 mm s\(^{-1}\)), was performed to investigate the effect of the flow rate on the column protein binding capacity.

To investigate the application of the nylon-IDA fibers to the purification of his-tagged protein, 20 µg mL\(^{-1}\) of recombinant human His\(^{6}\)-Ubiquitin (HisU) in loading buffer A was fed to the column at 0.5 mL min\(^{-1}\) flow rate. Due to the strong binding, an elution buffer made of A containing 0.5 M imidazole at pH 7.0 was used instead of the 0.1 M imidazole at pH 7.0 used for the native protein efforts.

### 4.2.8 Protein separation and purification

Demonstration of the nylon-IDA column’s ability to separate a mixture of four model proteins was made using a step gradient procedure. Mobile phase A is
composed of 20 mM HEPES, 0.2 M NaCl, pH 7.0 and the eluting buffers (B) and (C) contain A with 0.1 M and 0.5 M imidazole (Im), respectively, both also at pH 7.0. Separation was performed at 1.0 mL min\(^{-1}\) flow rate with a step gradient program: 100%A: 0%B (0-2 min), 95%A: 5%B (2-5 min), 75%A: 25%B (5-8 min), 100%C (8-11 min) and back to 100%A (11-15 min). A 50 µL injection containing chymotrypsinogen A (0.1 mg mL\(^{-1}\)), cytochrome C (0.3 mg mL\(^{-1}\)) and lysozyme (0.3 mg mL\(^{-1}\)) and His-U (0.3 mg mL\(^{-1}\)), prepared in mobile phase A, was used as the protein test mixture.

The ability of nylon-IDA column to purify a histidine-tagged protein was demonstrated through the separation of recombinant human His\(_6\)-Ubiquitin (HisU) (1.5 mg mL\(^{-1}\)) spiked into \(E.\) coli cell lysate. The separation was performed at 2.0 mL min\(^{-1}\) flow rate with a step gradient program: 98%A: 2%C (0-1 min), 100%C (1-3 min) and back to 98%A: 2%C (3-5 min).

All of the chromatographic separations were recorded at 280 nm. The gradient baseline absorbance was obtained by running the same chromatographic programs with no sample injection. This background absorbance was then subtracted from the sample chromatogram.

4.2.9 Column permeability

The column hydrodynamic properties were evaluated by running 1 M NaCl through a 30-cm long column at different flow rates. Column back pressure is defined as the difference between the pressure of the HPLC system with and without the installed column. Linear velocity was calculated based on column
length and volumetric flow rate, with the column interstitial fraction determined using uracil as the marker compound, as is typical with packed-bed formats. (The combination of the ionic mobile phase and ligated surface results in no uracil retention.) The permeability of the column at different flow rates was given by the following equation:

$$\frac{\Delta P}{L} = \frac{u \mu}{k_w} \quad \text{(Eq. 3)}$$

where $\Delta P$ is the column pressure drop (Pa); $L$ is the column length (m), $u$ is the linear velocity of the mobile phase (m s$^{-1}$), $\mu$ is the mobile phase viscosity (Pa s) and $k_w$ is the column permeability (m$^2$) [41]. The viscosity of 1 M NaCl was obtained from Ref [42].

### 4.3 Results and discussion

#### 4.3.1 Preparation of the nylon-IDA fibers

C-CP nylon-IDA fibers were prepared by microwave-assisted grafting polymerization and subsequent chemical modifications. The preparation scheme as illustrated in Fig. 4.1 consists of the following steps: (1) grafting of GMA onto the C-CP nylon fibers, (2) conversion of the epoxide groups on the nylon-GMA into metal-chelating groups, (3) hydrolysis of the remaining epoxide groups, and (4) packing fibers into a microbore column and charging it with copper ions. We have described the column packing procedure in detail previously [29, 31], with 540 fibers employed in each column here to yield an interstitial fraction of $\sim 0.6$. Column-to-column variability in terms of back pressures and protein retention times were less than 5% RSD in this case. The following sections describe the
optimization of the processes and characterization of the respective surface properties.

4.3.1.1 Grafting of GMA onto the nylon C-CP fiber surface

In previous studies, poly-AA and poly-AMPS were grafted from the nylon 6 C-CP fiber surface through the microwave-assisted radical polymerization method using potassium persulfate (KPS) as the initiator [37-38]. Initially, the same procedure was performed to graft poly-GMA. However, the greater reactivity of GMA in combination with the powerful microwave free radical generation from KPS resulted in excessive formation of insoluble homo-polymer blocks that so strongly adhered within the fiber channels that their removal was impossible without damaging the fiber. As a result, ceric ammonium nitrate (CAN) in dilute nitric acid was used as an alternative redox initiator [43]. The reaction scheme of the graft polymerization (as well as homo-polymerization) is summarized as follows [44].

Initiation:

\[
\text{Ce}^{4+} + \text{nylon-H} \rightleftharpoons \text{Complex} \rightleftharpoons \text{nylon} \cdot + \text{Ce}^{3+} + \text{H}^+ \\
\text{nylon} \cdot + M \rightarrow \text{nylon-M} \cdot \quad (M = \text{GMA monomer}) \\
\text{Ce}^{4+} + M \rightarrow M \cdot + \text{Ce}^{3+} + \text{H}^+
\]

Propagation:

\[
\text{nylon-M}_n \cdot + M \rightarrow \text{nylon-M}_{n+1} \cdot \\
M_m \cdot + M \rightarrow M_{m+1} \cdot
\]
Termination:

\[
\text{nylon-M}_n + \text{Ce}^{4+} \rightarrow \text{nylon-M}_n + \text{Ce}^{3+} + \text{H}^+ \\
\text{M}_m + \text{Ce}^{4+} \rightarrow \text{M}_m + \text{Ce}^{3+} + \text{H}^+ \\
\text{nylon} \cdot + \text{Ce}^{4+} \rightarrow \text{Oxidative products} + \text{Ce}^{3+} + \text{H}^+
\]

The use of lower microwave power (70 W vs. 100 W previously), together with slower initiation rate, provides better control of the GMA grafting for this redox initiator system. Homo-polymers in this case occur in a gel-like form and are easily washed off the fibers. The acidification of redox initiator solutions has been shown to improve the grafting degree of vinyl monomers on cellulosic substrates [45-46] with the effectiveness decreasing in the order HNO₃>H₂SO₄>HCl>CH₃COOH [47]. However, an elevated H⁺ concentration was reported to suppress the generation of radicals, which consequently reduced the grafting polymerization [47-49]. Nitric acid, at a concentration of 0.1 N, was used here to acidify the CAN redox initiator. Due to the low solubility of GMA, the highest concentration that could guarantee complete dissolution of GMA in the polymerization grafting mixture was 3% w/v. Higher concentrations result in incomplete dissolution, resulting in the formation of homo-polymer blocks that are strongly attached to the fibers and extremely hard to remove without damaging the fiber.

The original color of CAN in the grafting mixture is bright orange. As the redox reaction occurs, the required radicals in the fiber are formed, allowing the grafting to proceed, with the color of the fading as Ce⁴⁺ is gradually reduced to Ce³⁺. Different concentrations of the initiator were tested at the fixed GMA concentration of 3% w/v. The reaction time was set for 30 minutes to guarantee
reaction completion, which is indicated by the disappearance of the orange color. As shown in Fig. 4.2a, higher amounts of initiator (CAN) do not necessarily yield higher degrees of grafting, in fact the uniformity of the grafting layer is also of concern. To investigate the surface modifications, a reaction between 4-(4-nitrobenzyl) pyridine (NBP) and the accessible epoxide groups on the grafted layer, which results in the development of an intense blue color, was utilized as an indicator. As shown in Fig. 4.3a, the nylon-GMA fibers on the left (low initiator %) have a uniform coloration while their counterparts on the right (high initiator %) show visible patches of inhomogeneity. Higher initiator concentrations lead to the predominance of the competitive homo-polymerization process, which in turn

![Graphs showing optimization of modification process](image)

**Figure 4.2** Optimization of the modification process: (a) Initiator concentration study; (b) Grafting time study and (c) Epoxide conversion time study
results in non-uniform grafting on the fiber surface. The highest degree of grafting and uniformity was achieved with the lowest concentration, 0.125% w/v, of initiator.

In consideration that the 30-minute reaction time may be excessive with this low initiator concentration, further study of reaction time was conducted. It was found that at least 15 minutes was required for the initiator to be completely utilized based on the change in the solution color. Fig. 4.2b shows fairly low grafting degrees at the 5 and 10-minute reaction times, suggesting incomplete reaction. The grafting degree appears to reach a plateau after 15 minutes of reaction. Beyond the 15-minute irradiation time, a great deal of variability is seen. This may be reflective of the thickening of the homopolymer gel, restricting the access of the monomer to the surface. As the highest level of reproducibility and efficiency was achieved at the 15-minute grafting time, it was selected for further grafting procedures. Consequently, the optimized grafting conditions were found to be 3% w/v of GMA, 0.125% w/v of the CAN initiator in 20 mL 0.1 N nitric acid and 15 min of grafting at the lowest energy setting (70 W) of the microwave.

4.3.1.2 Optimization of the conversion of epoxides into IDA

Upon surface grafting, the versatile GMA units provide the native nylon with reactive epoxide end groups that can be converted into a variety of functional ligands [2]. The conversion of epoxide groups to IDA ligands has been described in the literature. Hemdan et al. reported that the reaction efficiency increased with the reaction pH, reaching a maximum at around pH 10.5 before abruptly dropping [50]. Yamagishi et al. reported that the addition of DMSO to the IDA solution greatly
The procedure employed in this study involved the use of 0.5 M IDA dissolved in an equimolar mixture of 1 M Na$_2$CO$_3$ and DMSO, with the final pH adjusted to 10.5. It is believed that the presence of DMSO helps enhance the conversion [16]. The procedure employed in this study involved the use of 0.5 M IDA dissolved in an equimolar mixture of 1 M Na$_2$CO$_3$ and DMSO, with the final pH adjusted to 10.5. It is believed that the presence of DMSO helps
relax the grafted GMA polymer as well as swell the fiber trunk so that the reagents have better access to the reactive epoxide groups [16]. Moreover, this polar aprotic solvent has been reported to enhance the $S_N2$ reaction between IDA and the epoxide group [16].

Elevated temperature is required for the IDA coupling reaction to proceed well. However, in order to retain the mechanical strength of the nylon fiber, the most appropriate temperature needs to be determined. In such alkaline conditions, and in the presence of the organic solvent at high temperature, damage to the bulk fiber may be expected. Literature reports include the reaction being conducted at 80°C on polyethylene hollow fiber membranes grafted with GMA [16]. However, nylon has a lower tolerance to degradation in the presence of DMSO at such temperatures. In the scope of this project, the fiber (upon modification) will be mechanically pulled into a column so they are required to maintain certain strength to survive the frictional force through the microbore tubing. It was found that as long as the fiber does not reach a temperature of greater than 100°C, the fiber integrity is well preserved.

The IDA conversion efficiency was initially determined based on the change in the weight between the nylon-GMA and the modified nylon-IDA fiber. As presented in Fig. 4.2c (orange points), the conversion percentage after 2 hours is ~46%, with the efficiency increasing very little as the reaction time was lengthened up to 10 hours. The efficiency at that point is close to 60%, which is approximately the same as the optimized conversion rate reported in the literature [16]. The conversion rate was also determined chemically, which would better reflect the
chemically-accessible functional groups. The density of the epoxide groups was determined utilizing their reactivity with NBP under alkaline conditions. Fig. 4.3b provides a visual comparison of the progression of the conversion reaction over time. The intensity of the deep blue color reflects the epoxide group density on the fiber surface. The color is the most intense in the nylon-GMA at the beginning of the IDA conversion reaction. As the reaction time increases, the epoxide groups are converted to IDA groups and the color fades away to near invisibility at the end of the 6-hour interval. The remaining NBP indicator after the color development was quantified via an absorbance method to determine the epoxide conversion as reflected in in the blue data points in Fig. 4.2c. The trend observed here is essentially the same as the conversion rate based on the fiber weight gain, though with greater overall efficiency and precision of measurement. It is worth noting that not all grafted GMA units are accessible for the IDA conversion reaction due to steric hindrance. Therefore, the efficiency determined by the NBP reaction reflects a more realistic picture of the IDA conversion process. As shown in Fig. 4.2c, close-to-unity conversion of the accessible epoxide groups to IDA is achieved after the 10-hour reaction time.

4.3.2 Characterization of the modified fibers

4.3.2.1 ATR-FTIR

Infrared spectrophotometry is a valuable tool in the characterization of fiber surfaces. The characteristic spectroscopic features of nylon 6 fibers were reported earlier [38, 51], all of which are visible in the bottom spectrum of Supplementary
Material, Fig. A.1 (native nylon), including 3300 cm\(^{-1}\) (N-H stretch), 2931 cm\(^{-1}\) (CH\(_2\) asymmetric stretch), 2861 cm\(^{-1}\) (CH\(_2\) symmetric stretch) and 1639 cm\(^{-1}\) (amide stretch). After the grafting polymerization to immobilize the epoxide functionality, the ATR-FTIR of the nylon-GMA fiber shows a distinctive addition of the C=O stretch at 1729 cm\(^{-1}\), and less prominent peaks are observed in the region of \(\sim 900–1300\) cm\(^{-1}\), which are indicative of the presence of the epoxide groups from GMA and the C-O and C-O-C functionality of the GMA. Each of these reflects the functional units presented in Fig. 1. The top spectrum of Supplementary Material Fig. A.1 shows the spectral features of nylon-IDA fibers. The distinctive peak characteristic of C=O stretch is still present; however, the region characteristic of the epoxide group is minimized. Additionally, there is a broad band around 3500 cm\(^{-1}\), which is representative of the hydroxyl groups, either from the IDA carboxylate groups or from hydrolyzed epoxide groups.

### 4.3.2.2 SEM imaging

The microscopic pre- and post-modification fiber morphology was investigated through SEM imaging as presented in Supplementary Material, Fig. A.2. The cross-sectional column micrographs of native nylon and its IDA-modified counterparts show the preservation of the characteristic shape of C-CP fibers. A closer look reveals that the modified fibers seem to be slightly more tightly-packed than the native nylon 6, even though the same number of fibers are employed for all cases. The fiber surface micrographs show that there is no visible damage or stretching. One unique feature is observed in the highest magnification of the Cu\(^{2+}\)-
bound fibers, where some level of “hot spotting” is seen, presumably reflective of formation of metal nanoparticles.

4.3.2.3 Column permeability

As the fibers are subject to multiple steps of chemical modification, there is a natural concern regarding any potential compromises in the hydrodynamic efficiency of the packed columns. The SEM images of the modified fibers suggested very little perturbation to the channel structure dimensions or geometry. In order to confirm that there is negligible stationary phase compression and bed perturbation to the fibers, the permeability of a native nylon and nylon-IDA column were compared. As presented in Supplementary Material, Fig. A.3, well-behaved linear correlation between the packed-column pressure drops (kPa cm⁻¹) and the linear velocity of the mobile phase (1 M NaCl) are indeed observed. The slopes of the respective trend lines represent the term \( \mu/k_w \) in equation (3), which are calculated to be 8.5 x 10⁻¹² m² and 6.1 x 10⁻¹² m², respectively, for the native and modified-fiber columns. Considering that the fibers were subjected to multiple steps of chemical modification, this change in permeability is fairly modest, and is inconsequential in terms of chromatographic performance.

4.3.2.4 Ligand and copper binding density

The density of COOH groups on the nylon-IDA fiber prepared under the optimized conditions was determined through an acid-base titration to be 0.61 ± 0.02 mmol g⁻¹ fiber as described in Section 2.5. Assuming that all of the acidic
moieties are due to the presence of IDA, which itself consists of two carboxyl groups each, the density of IDA units on the modified nylon would be $0.31 \pm 0.01$ mmol g$^{-1}$ fiber. Since each Cu$^{2+}$ ion is, in turn, surface-coordinated by two carboxyl groups, one would expect the copper binding capacity of the IDA fiber to be in the range of the density of the IDA units. The density of the bound copper was determined (as described in Section 2.6) in triplicate to be $0.38 \pm 0.01$ mmol g$^{-1}$ fiber, which is slightly higher than the predicted value based on the carboxyl group density. This may imply that some metal ions may be coordinated with single carboxylic acid functionalities. As shown in Table 4.1, the copper binding capacity of nylon-IDA fiber is much higher than the reported values of some commercially available IMAC stationary phases, in fact a factor of 10 or more on a mole-per-mL stationary phase basis.

4.3.3 Protein separation

When Porath and co-workers first introduced IMAC as a protein purification technique in 1975, it was postulated that the electron-donating amino acid residues histidine, tryptophan and cysteine were capable of forming stable coordination bonds with chelated metal ions at or near neutral pH [3]. Sometimes referred to as “Porath’s triad”, these amino acid residues are believed to play an important role in the driving force behind the binding in IMAC [5]. Of these residues, histidine has been shown to be the predominant amino acid that is responsible for the binding of proteins via IMAC processes [4]. The overall binding strength (i.e., retention) is determined not only by the number of histidine residues on a protein but also their
surface accessibility and the nature of their microenvironment [6]. The elution order of the three model proteins shown in Fig. 4.4a reflects just that.

Fig. 4.4a is a sequential, step-gradient chromatogram of a synthetic mixture containing 1) α-chymotrypsinogen A, 2) cytochrome C, 3) lysozyme, and (4) HisU on the Cu\textsuperscript{2+}-charged nylon-IDA C-CP fiber column. Chymotrypsinogen A, has two histidine residues on the protein surface, having different degrees of accessibility. His-57 is involved in the catalytic site while His-40 is positioned near the edge of the active site. Despite the presence of these histidine residues, chymotrypsinogen A does not bind appreciably to the IMAC support as demonstrated in Fig. 4.4a. To be clear, in the absence of odd interactions with the fiber support, the elution characteristics (i.e., order) for this phase should be like any other IMAC phase employing the same IDA surface ligand. Sulkowski reported the difference in the interaction behavior between chymotrypsinogen and its active form chymotrypsin on IMAC support [52]. At pH 7, the former did not bind to the support while the latter was retained and subsequently eluted at pH 6 [52]. Further molecular modelling study showed that even though both histidines on chymotrypsinogen occur on the protein surface, they have different degrees of accessibility [53]. With higher accessible surface area, His-57 was predicted to participate in the metal recognition process [53]. However, this catalytic residue is involved in strong hydrogen bonding, rendering it too rigid to engage in the coordination bond with the metal complex [53]. The side chain of His-40 is buried in the crystal structure of chymotrypsinogen and is involved in the hydrogen bonding [53]. As a result, the two histidine residues in this protein cannot coordinate with the metal chelate
complex. During the transformation to the active chymotrypsin, the microenvironment near His-40 changes to free up this residue for the co-ordination bond with the IMAC support [53].

The importance of the histidine surface accessibility and their microenvironment is demonstrated through the elution order of the other two proteins: horse heart cytochrome C and lysozyme from hen egg white, both of which have only a single histidine residue. Horse cytochrome c was retained on the IDA column and eluted at 5% of elution buffer B (containing 5 mM imidazole). The retention of this protein is due to the presence of the histidine residue His-33 on its surface that is readily available for coordination with the metal-chelate complex [5]. Lysozyme, on the other hand, was retained more strongly than cytochrome C and was eluted at a composition of ∼25% elution buffer B (∼25 mM imidazole). There is a correlation between the elution order of these two proteins and the pKa values of their histidyl residues: cytochrome C (His-33, pKa 6.5) and lysozyme (His-15, pKa 5.2) [54]. Because histidine coordinates to IDA-Cu$^{2+}$ in its unprotonated form [3, 55], lysozyme forms stronger bond to the IMAC support than cytochrome C at pH 7. The stronger binding of lysozyme to the IDA-Cu ligands is also believed to be accentuated by the tryptophan residues on its surface [5]. The last peak in Fig. 4.4a is the recombinant human His$_6$-Ubiquitin (HisU). Due to its six consecutive histidine residues on the surface (the his-tag), HisU binds more strongly to IDA-Cu$^{2+}$ than the naturally occurring proteins in the mixture. Therefore, a stronger elution buffer (0.5 M imidazole, pH 7.0) was required for its release.
The selectivity of the nylon-IDA column is further evaluated using an *E. coli* cell lysate sample spiked with HisU (1.5 mg mL\(^{-1}\)). Fig. 4.4b shows the chromatograms of the lysate with and without HisU spike. Polyhistidine tag is one of the most convenient and widely used purification tags for genetically engineered proteins expressed in Escherichia coli or other prokaryotic expression systems [4]. In many applications, this purification can be achieved through a single chromatography step, as demonstrated in the isolation of HisU from the *E. coli* cell lysate matrix in Fig. 4.4b. Seen clearly here, the diverse lysate products, including other proteins and peptides, are not retained in the hydrophilic nylon-IDA surface. It is worth noting that this demonstration was performed at the flow rate of 2.0 mL min\(^{-1}\) (linear velocity of \(\sim\)120 mm s\(^{-1}\)), suggesting the potential for high throughput purification in downstream processing of his-tagged recombinant proteins.

### 4.3.4 Protein binding capacity

The dynamic binding capacity (DBC) of the Cu\(^{2+}\)-charged, IDA-modified nylon C-CP fibers was determined through frontal analysis experiments with lysozyme being used as a model protein. Previous works from this laboratory have also employed lysozyme as a model protein in the case ion exchange on microwave-modified nylon-6 C-CP fiber columns [37-38]. Breakthrough curves obtained through loading increasing concentrations of lysozyme (0.05-1.00 mg mL\(^{-1}\)) at a flow rate of 1.0 mL min\(^{-1}\) (\(\sim\)70 mm s\(^{-1}\)) are shown in Fig. 4.5a. The steepness and smooth/monotonic nature of the curves suggests high efficiency of mass transfer to the C-CP nylon-IDA fiber surfaces. Fig. 4.5b shows the resulting
Figure 4.4 (a) The baseline-subtracted chromatogram of 50 µL 1) α-chymotrypsinogen A (0.1 mg mL\(^{-1}\)), 2) cytochrome C (0.3 mg mL\(^{-1}\)), 3) lysozyme (0.3 mg mL\(^{-1}\)) and 4) HisU (0.3 mg mL\(^{-1}\)) on the Cu\(^{2+}\)-charged nylon-IDA C-CP fiber column. Chromatographic conditions: Mobile phase A (20 mM HEPES, 0.2 M NaCl, pH 7.0), B (A + 0.1 M imidazole, pH 7.0) and C (A + 0.5 M imidazole, pH 7.0). Orange dashed line depicts the gradient run at 1.0 mL min\(^{-1}\) flow rate. UV detection at 280 nm; (b) The baseline-subtracted chromatogram of *E. coli* cell lysate with and without the HisU (1.5 mg mL\(^{-1}\)) spike. Mobile phases are the same as above. Orange dashed line depicts the gradient run at 2.0 mL min\(^{-1}\) flow rate. UV detection at 280 nm.
adsorption isotherm for lysozyme binding. The DBC of nylon-IDA fibers varies from 5.2 to 6.9 mg g$^{-1}$ fiber (equivalent to 2.60 – 3.29 mg mL$^{-1}$ fiber bed volume), across the lysozyme feed concentrations of 0.05 - 1.0 mg mL$^{-1}$. Considering that lysozyme has only one accessible histidine on the surface, there is only one binding event expected to occur between each lysozyme molecule and its corresponding binding site on the charged nylon-IDA fibers. Assuming that there are no protein-protein interactions, a Langmuir isotherm (Eq. 4) can be fit to the experimental DBC data using MAT-LAB (Natick, MA) to model the binding behavior.

$$q = q_s \frac{bC}{1+bC} \quad (\text{Eq. 4})$$
In this equation, \( q \) is the binding capacity (mg g\(^{-1}\)) of the column at the protein feeding concentration \( C \) (mg mL\(^{-1}\)), \( q_s \) (mg g\(^{-1}\)) is the maximum DBC at the infinite protein loading concentration and \( b \) (mL mg\(^{-1}\)) is the adsorption-desorption equilibrium constant on the surface. Fitting the data from Fig. 4.5b to the Langmuir isotherm equation yields \( q_s = 6.33 \text{ mg g}^{-1} \) and \( b = 65.97 \text{ mL mg}^{-1} \). The adsorption isotherm has a steep profile at the low concentration range and there is no appreciable difference between the binding capacity achieved between the lowest and highest feed concentrations. These isotherm-determined binding capacities are \(~1/3\) those determined for lysozyme on WCX- and SCX-modified nylon C-CP fiber column [23].

Another set of frontal analysis experiments was conducted wherein a constant concentration (1.0 mg mL\(^{-1}\)) of lysozyme was fed through the Cu\(^{2+}\)-charged nylon-IDA column at different volumetric flow rates 0.1-1.0 mL min\(^{-1}\). The breakthrough curves in Fig. 4.5c are still characterized by a steep, continuous profile. Moreover, there is only a slight deviation in the positions of the curves on the volume scale, indicating that the binding capacity achieved at these different flow rates does not vary significantly. Considering the wide range of flow rates, which corresponds to the linear velocity of \(~7-70 \text{ mm s}^{-1}\), the variation in the DBC, as reflected in Fig. 4.5d, is not very significant; \(~30\%\) decrease in DBC for a 10-fold increase flow rates; i.e. a 7x increase in throughput. This underscores an advantage of C-CP fibers; the binding capacity, hence the yield, is not compromised even at very high velocity.
While these performance parameters show potential for the C-CP nylon-IDA fibers in the preparative scale, a concern about their performance consistency needs to be addressed. Shown in Fig. 4.6 are the stacked chromatograms of 10 complete load/elute cycles (1.0 mg mL\(^{-1}\) of lysozyme) being performed without a clean-in-place (CIP) step in between. The highly reproducible performance of the

![Chromatograms of 10 consecutive load/elute cycles of lysozyme.](image)

Figure 4.6 Chromatograms of 10 consecutive load/elute cycles of lysozyme. The loading buffer (A) was 20 mM HEPES, 0.2 M NaCl, pH 7.0 and the eluting buffer (B) was A with 0.1 M imidazole, also at pH 7.0. Lysozyme (1.0 mg mL\(^{-1}\)) in loading buffer A was fed to the column at flow rate of 1.0 mL min\(^{-1}\) for 10 minutes until saturation reached. The column was then washed with loading buffer A for 5 minutes. Finally, the bound lysozyme was eluted with eluting buffer B for 5 minutes before the column was equilibrated in A for another 5 minutes for the next cycle. UV detection at 280 nm.
column is demonstrated by the nearly overlapped breakthrough profiles and elution peaks. The lysozyme binding capacities differ by 2.2% RSD (avg = 5.32 mg g⁻¹, n=10), while the recoveries vary by just 0.5% RSD. This illustrates high efficiency in both the bind and elute steps towards protein separations as well as the stability of the C-CP fiber IMAC phase in the case of sequential saturation events.

As the modified C-CP nylon-IDA fibers in this project will be ultimately used as an IMAC stationary phase, HisU was used to demonstrate their ability in to separate histidine-tagged proteins. Based on the previously-determined loading capacities, 20 µg mL⁻¹ of HisU was fed to the nylon-IDA column at the flow rate of 0.5 mL min⁻¹. With six consecutive histidine residues on its surface, HisU is more strongly bound to IDA than other naturally occurring histidine-rich proteins. As a result, an eluting buffer containing 0.5 M imidazole at pH 7.0 was used to guarantee complete elution of the bound HisU proteins. Using the data obtained from the breakthrough curve (not shown here), the binding capacity of HisU was calculated to be 6.3 ± 0.1 mg g⁻¹ fiber.

In this study, nylon 6 C-CP fibers were grafted with GMA through a microwave-assisted polymerization approach to affix reactive epoxide groups to their surface, which were in turn converted into IDA units. The resulting nylon-IDA fibers are characterized as having a high density of carboxyl end groups and subsequent copper binding capacity. When applied as an IMAC stationary phase, they also show high protein binding capacity. Moreover, in spite of going through multiple modification steps, the nylon-IDA fiber columns still maintain high
permeability. The overall method of grafting was proven to be an easy and efficient way to uniformly modify the surface of the C-CP fibers.

4.4 Conclusions

Table 4.1 compares the properties of the C-CP nylon-IDA fibers developed in this work with commercially available IMAC stationary phases. In terms of protein dynamic binding capacity, C-CP nylon-IDA fibers only outperform the ProPac IMAC-10 (Dionex) and rank in the lower range when compared to the other phases in the table. However, these values just tell one side of the story. As discussed previously, the binding capacity of C-CP nylon-IDA fibers only decreases by 30% when the linear velocity is changed 10-fold. In other words, its performance is hardly compromised at the expense of higher flow rates. In fact, the linear velocity at which the C-CP nylon-IDA developed here can be operated is about 20-60 times higher than those reported by any other commercially-available IMAC phases shown in the table. It is worth mentioning that the highest flow rate of 2.0 mL min\(^{-1}\) (or 120 mm s\(^{-1}\)) recommended here is actually due to the operational pressure restrictions of the hardware, specifically the PEEK tubings and fittings which only sustain pressures of up to 2500 psi. Another particular advantage of the nylon 6 fiber as a chromatographic support are their extreme pH stability [56], as demonstrated by stable operation following steps involving exposure to 0.1 M HNO\(_3\) and 0.5 M imidazole (pH = 10.5). High throughput, hence high turnover is the primary operational advantage of the IMAC C-CP fibers developed here in comparison to commercially-available IMAC phases.
Table 4.1 Comparison of physical attributes of Nylon-IDA C-CP fiber columns with commercial IMAC phases

<table>
<thead>
<tr>
<th></th>
<th>IMAC stationary phases</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Nylon-IDA</td>
</tr>
<tr>
<td>Base support</td>
<td>C-CP Nylon fiber</td>
</tr>
<tr>
<td>Bead size</td>
<td>N/A</td>
</tr>
<tr>
<td>Porosity</td>
<td>Non-porous</td>
</tr>
<tr>
<td>Ligands</td>
<td>Bidentate iminodiacetic acid (IDA)</td>
</tr>
<tr>
<td>Metal Binding capacity</td>
<td>Cu²⁺ 375 µmol g⁻¹ fiber (~240 µmol mL⁻¹ fiber)</td>
</tr>
<tr>
<td>Protein binding capacity</td>
<td>6.0-8.0 mg g(^{-1}) fiber (~3.0-4.0 mg mL(^{-1}) fiber)</td>
</tr>
<tr>
<td>--------------------------</td>
<td>--------------------------------------------------------</td>
</tr>
<tr>
<td>Linear velocity</td>
<td>0.1-2.0 mL min(^{-1}) (~6.0-120 mm sec(^{-1}))</td>
</tr>
<tr>
<td>Operating pressure</td>
<td>&gt;2500 psi</td>
</tr>
<tr>
<td>pH stability</td>
<td>2-14</td>
</tr>
</tbody>
</table>
The microwave-assisted polymerization grafting proves to be a simple method to modify the surface of C-CP nylon fibers. The monomer glycidyl methacrylate (GMA), once grafted on C-CP fibers, provides a convenient platform for further modification using epoxide coupling chemistry. The terminal epoxide can be easily hydrolyzed in acidic condition to form diols, making the fibers more hydrophilic and at the same time providing available hydroxyls for various well-established ligand immobilization methods. Furthermore, these epoxides are also reactive to ligands containing sulfhydryl, amine, or hydroxyl. This versatility will allow future attempts to attach various affinity ligands to the C-CP fiber for a wide range of both analytical and preparative scale separations.

Acknowledgments

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CHAPTER FIVE

INITIAL EVALUATION OF NYLON-AMIDOXIME (NYLON-AO) CAPILLARY-CHANNELED POLYMER (C-CP) FIBERS FOR THE PRECONCENTRATION AND ANALYSIS OF URANIUM IN LOW CONCENTRATION SAMPLES

5.1 Introduction

Uranium is a naturally occurring radioactive heavy metal that can be found in soil, rock, groundwater, freshwater, seawater and other biota samples [1]. Its concentration in these samples is not only dependent on mineral composition in the environment and natural water sources but also on the incidental releases from anthropogenic activities (mainly involving the uranium nuclear fuel cycle) [1]. All uranium isotopes emit alpha particles with weak penetrating power [1-2]. Moreover, due to their long half-lives and slow rates of decay, uranium radiotoxicity is relatively low [2-3]. However, like many other heavy metals, uranium and its compounds are highly toxic upon ingestion or inhalation [1-2]. As the amount of uranium in air is usually small, inhalation is not a significant exposure channel. Ingestion, on the other hand, of uranium from dietary sources such as drinking water or food (especially root vegetables) is the main route through which the general population is exposed to uranium. No matter what the sources are, the exposure risks substantially increase for those working or living near factories that process phosphate ores for phosphate fertilizers, government facilities making or testing nuclear weapons, modern battlefield using depleted uranium weapons,
coal-fired power plants or facilities processing uranium ore or enriching uranium for reactor fuel [4]. Regarding the radioactivity of uranium, human exposure at normal or background levels does not pose any adverse health risks [5]. However, like any other heavy metals such as lead or cadmium, exposure to high levels of uranium may cause progressive or irreversible renal injury, potentially leading to kidney failure and death. In addition, uranium exposure also severely affects normal functioning of other human organs such as brain, liver, heart, and reproductive systems [6]. The World Health Organization (WHO) established the tolerable daily intake of uranium at 0.6 µg kg$^{-1}$ body weight per day [3]. Health Canada drinking water guidelines fixed the maximum contaminant level for uranium at 20 µg L$^{-1}$ [2] while regulations by the Environmental Protection Agency (EPA) in the US set the threshold at 30 µg L$^{-1}$ for the public water systems [1].

Improvements in modern analytical techniques has brought down the limit of detection of uranium well below the level that it occurs in almost all realistic samples [1]. However, in some cases, sample clean-up procedures that remove impurities, eliminate matrix effects and pre-concentrate uranium from the samples are necessary to improve analytical detection limits, offer higher degree of selectivity and sensitivity and enhance the measurement accuracy [7]. Liquid-liquid extraction (LLE), extraction chromatography and solid phase extraction (SPE) are the most common techniques used to separate and pre-concentrate uranium from different matrices [7]. LLE of uranium is based on the formation of uncharged uranium complexes in aqueous phase either through chelation or ion-association with a selective extractant of choice, which is then extracted into a suitable
immiscible organic solvent [7]. The distribution factor of uranium-extract complex between the aqueous and organic phases is determined by the proper choice of extractant, pH or acidity of the aqueous phase, organic solvents, masking agents, salting out agents and modifiers [7]. While LLE offers fast kinetics and relatively high capacities, the need for large volumes of organic solvents/extractants with limited regeneration compromise its applicability [8].

Extraction chromatography takes advantage of both the favorable selectivity of solvent extraction and the ease of operation in multistage column chromatography [9]. Typical extraction chromatographic resins are developed by adsorbing liquid extractants, either single compounds or mixtures, onto inert supports made of porous silica or organic polymers [7, 9]. Separation achieved through selective distribution of metal ions between the organic liquid extract stationary phase layer and the aqueous phase is especially useful to preconcentrate the analytes in relative small amounts of sample for radiochemical analyses [9]. This technique has gained more popularity in sample clean-up for the analysis of actinides thanks to commercially available extraction chromatographic resins with different selections of liquid extractants. The most widely-known commercial provider, Eichrom Technologies LLC, manufactures RE, TEVA®, UTEAVA® and TRU resins which have been used in standard methods developed by accredited laboratories and regulatory agencies worldwide (e.g., the US Environmental Protection Agency and the International Atomic Energy Agency) for the analysis of actinides. However, no matter how widely used it is, the extraction chromatography technique still suffers from following drawbacks. First,
chromatographic extraction resins are susceptible to the bleeding of liquid extractants from the column during sample clean-up and preconcentration. Secondly, as the stationary phase used in extraction chromatography is based on porous materials, different factors regarding the resins including the particle size, resin porosity, particle and pore size distribution, uniformity in packing and the extractant and diluent loading may contribute to variations in mobile phase flow pattern, analyte diffusion in the stationary phase and extraction kinetics. As a result, no matter how highly selective the extracts are, poor column efficiency leads to excessive band broadening and essentially no practical separation of the target analytes.

As an alternative to extraction chromatography, solid phase extraction (SPE) works on selective binding uranium ions via complexing or ion exchanging to the active sites immobilized on a stationary solid support [7]. In comparison to LLE, SPE offers more flexibility, higher enrichment factors, lower consumption of reagents and reuse of the stationary phase [7]. As the ligands are immobilized through covalent binding onto the support, there is no leaching issue as in extraction chromatographic resins. In this work, a novel SPE for uranium preconcentration was constructed using capillary-channeled polymer (C-CP) fibers instead of conventional porous silica or organic polymers as the support materials. C-CP fibers have been investigated as stationary/support phases for HPLC and solid phase extractions (SPE) in Marcus laboratory (Clemson University) for the past 15 years in a variety of chromatographic modes [10-14]. When packed into a column format, the characteristic eight axial channels of the C-CP fibers inter-
digitate lengthwise to form aligned micro-channels which allow highly efficient fluid movement and favorable mass transfer rates [15-16]. Analytical-scale separations on the C-CP fiber packed column occur at high linear velocities (up to 100 mm s\(^{-1}\)) with high column permeability (<0.14 MPa cm\(^{-1}\)) [10, 17]. Moreover, these fibers show stability over a wide pH range and are less expensive (< $100 lb\(^{-1}\)) than the conventional supports.

In this study, nylon C-CP fibers were grafted with glycidyl methacrylate (GMA) in a microwave-assisted grafting polymerization [18]. The epoxide groups on GMA polymers were then converted into amidoxime groups through two additional chemical modification steps involving iminodipropionitrile and hydroxylamine. The resulting nylon amidoxime C-CP fibers were characterized for the proof-of-concept online preconcentration and analysis of low concentration uranium samples.

### 5.2 Materials and methods

#### 5.2.1 Chemicals and instrumentation

HPLC-grade acetonitrile (ACN) and methanol (MeOH) were purchased from EMD Millipore (Billerica, MA). Concentrated nitric acid HNO\(_3\), trace metal analysis grade were purchased from Sigma Aldrich (St. Louis, MO). Activated alumina powder was purchased from Polysciences, Inc. (Warrington, PA). Sodium hydroxide pellets were purchased from Fisher Scientific (Fair Lawn, NJ). Concentrated hydrochloric acid (HCl) was purchased from JT Baker (Phillipsburg, NJ). Sodium carbonate was purchased from Acros Organics (Bridgewater, NJ).
Ceric ammonium nitrate (NH₄)₂Ce(NO₃)₆ (CAN) was purchased from Macron Fine Chemicals (Center Valley, PA). Dimethyl sulfoxide (DMSO) and sodium chloride were purchased from Alfa Aesar (Ward Hill, MA). Glycidyl methacrylate (GMA) was purchased from TCI (Tokyo, Japan). 3, 3’-Iminodipropionitrile and Arsenazo III (2,2′-(1,8-Dihydroxy-3,6-disulfonaphthylene-2,7-bisazo)bisbenzenearsonic acid, 2,7-Bis(2-aronophenylazo)chromotropic acid) were purchased from TCI America (Portland, OR). Hydroxylamine hydrochloride NH₂OH.HCl was purchased from MP Biomedicals, LLC (Solon OH). Uranium standard at 1000 µg mL⁻¹ (ppm) in 2% HNO₃ was purchased from High-Purity Standards (Charleston, SC). The pH of lower uranium concentration solutions was adjusted to pH ~5 with 4% w/v NaOH and 6 M HCl. All aqueous solutions were prepared with ultra-pure Milli-Q water (18.2 MΩ.cm) obtained from a DI water system (Billerica, MA). Nylon 6 C-CP fibers were provided by Material Science and Engineering department, Clemson University. The denier per filament is 2.67 grams per 9000 meters of a single fiber. The cross-section perimeter of each fiber is ~207 µm. The microwave polymerization grafting of GMA onto the nylon C-CP fiber was performed in an Emerson MW7300W microwave oven, without any modifications of the commercial unit.

Mock seawater was prepared by dissolving 25.6 g of NaCl and 0.2 g of NaHCO₃ in 1 L of DI water and adjusted to pH 7.8. Mock urine was prepared by dissolving 19 g urea, 7.7 g NaCl, 0.9 g MgSO₄.7H₂O and 0.6 g CaCl₂.2H₂O in 1 L of DI water and adjusted to pH 7.8. These two matrices together with tap water
were used in a spiking experiment to evaluate the recovery of the nylon-amidoxime (nylon-AO) C-CP fibers.

5.2.2 Preparation of C-CP nylon-amidoxime (nylon-AO) fiber

The four versions of the fibers used in the study will be referred to as 1) native nylon, the intermediate stage 2) nylon-GMA, 3) nylon-IDPN and the final product 4) nylon-AO. The entire chemical modification process for the IDA-nylon fiber is detailed in Fig. 5.1. Glycidyl methacrylate (GMA) was filtered through an activated alumina bed to remove any residual stabilizing agent (4-methoxyphenol). A solution of 0.1 M HNO$_3$ was purged with nitrogen for at least 30 minutes to deplete any dissolved oxygen before use. The polymerization grafting solution was composed of 0.6 g (3% w/v) GMA and 0.025 g (0.125% w/v) ceric ammonium nitrate in 20 mL degassed 0.1 M HNO$_3$. Nine rotations of nylon-6 C-CP fibers (540 fibers) were removed from the spool and stretched on a dying fork. The fiber bundles were rinsed with hot water for better alignment. Further rinsing with excess amounts of DI-water, MeOH and ACN was done to remove any residual (anti-static) chemicals left on the fibers during the manufacturing process. The fibers were then placed into 50-mL beakers containing the polymerization solution. To ensure even microwave and heat distribution, two beakers containing the fibers and the reaction mixture were placed in the middle of the glass turntable plate. The polymerization grafting reaction was performed in the Emerson microwave oven at its lowest power level (70W) for the pre-planned time periods. The GMA-grafted fiber bundles were thoroughly washed to remove excess visible homopolymer and
then placed in a glass jar containing 15 mL of 0.25 M IDPN in 1:1 DMSO: 1 M Na₂CO₃. The coupling of IDA through the epoxides groups on GMA was executed
in a standard laboratory oven at 100°C for 16 hours. The fibers were then thoroughly cleaned with DI-water to remove residual DMSO. The, now nylon-IDPN, fibers were placed in another glass jar containing 15 mL 3% w/v NH₂OH.HCl in 50% MeOH in the oven at 100°C for the final step of converting the nitriles on IDPN into amidoxime groups. After that, the nylon-AO fiber bundles are ready to be packed into 0.03” (0.762 mm) i.d. polyether ether ketone (PEEK) tubing (IDEX Health & Science LLC, Oak Harbor, WA) for chromatographic implementation in column format.

5.2.3 Bulk characterization of the modified nylon 6 C-CP fibers

The scanning electron microscope (SEM) imaging and energy-dispersive X-ray spectroscopy (EDX) analysis of the fibers were performed on using a Hitachi S-3400N operating in the variable pressure mode, with a 10kV accelerating voltage at the Clemson University Electron Microscopy Laboratory. The attenuated total reflection-Fourier transform infrared spectroscopy (ATR-FTIR) characterization of the fibers was performed on a Thermo-Nicolet Magna 550 FITR in the Analytical Testing Lab of Material Science & Engineering Department, Clemson University.

5.2.4 Offline preconcentration of uranium in spiked matrices

Due to the large volume of samples used in this spiking experiment, the nylon-AO C-CP fibers were packed in a bigger tubing than usually used. Thirty rotations of nylon-AO C-CP fibers were prepared using the same procedure presented in section 2 and packed into 10-cm, 1/8” OD, 1/16” ID fluorinated
ethylene propylene (FEP) tubing (Cole-Parmer, Vernon Hills, IL). The packed column was then connected between two 60-mL luer-lock syringe mounted onto two Fusion 100 syringe pumps (Chemyx Inc, Stafford, Texas). The whole sample clean-up scheme is presented in Fig. 5.2. In order to facilitate the strongest binding between uranium and the amidoxime functional group, the pH of all spiked samples was adjusted to 5.5. The syringe containing 50 mL of spiked samples (tap water, mock seawater and mock urine) was programed by the syringe pump to load into the nylon-AO C-CP fibers packed column at the flow rate of 10.0 mL min\(^{-1}\). The eluate transferred into the receiving syringe was then pumped at the same flow rate to the original syringe to ensure maximal binding of uranium from the samples. The fiber column was then treated with three 1-mL portions of 1 M HNO\(_3\) to elute the bound uranium.

Figure 5.2 Setup for the offline preconcentration of uranium from spiked samples

The recovery of uranium from the spiked samples was determined through iCAP 7200 Inductively coupled plasma-Optical emission spectroscopy (ICP-OES) system (Thermo Scientific, Cambridge, UK) in radial view mode. The auxiliary gas (Ar) flow rate, nebulizer gas (Ar) flow rate and plasma gas (Ar) flow rate were 1.0, 0.4 and 12 L min\(^{-1}\), respectively. Uranium was detected at 385.958 nm.

5.2.5 Column permeability
The column hydrodynamic properties were evaluated by running DI water through a 30-cm long column at different volumetric flow rates ranging from 1.0 to 10 ml min\(^{-1}\). Column back pressure is defined as the difference between the pressure of the HPLC system with and without the installed column. Linear velocity was calculated based on column length and volumetric flow rate, with the column interstitial fraction determined using 0.1% acetone. The permeability of the column at different flow rates was given by the following equation:

\[
\frac{\Delta P}{L} = \frac{u\mu}{k_w}
\]  
(Eq. 1)

where \(\Delta P\) is the column pressure drop (Pa); \(L\) is the column length (m), \(u\) is the linear velocity of the mobile phase (m s\(^{-1}\)), \(\mu\) is the mobile phase viscosity (Pa\(\cdot\)s) and \(k_w\) is the column permeability (m\(^2\)) [19]. The viscosity of water at 25\(^\circ\)C, 8.90 \(\times\) 10\(^{-4}\) Pa\(\cdot\)s, was obtained from Ref [20].

5.2.6 Dynamic binding capacity

The dynamic binding capacity of the nylon-AO fibers was determined through frontal analysis, the detailed procedure of which was given in [12]. Briefly, 100 ppm uranium solution was continuously fed through the system at the flow rate of 1.0 mL min\(^{-1}\) with and without the column being mounted. The breakthrough curves were obtained at 414 nm. The amount of bound uranium ions was determined through the difference in the area between the two breakthrough curves.

5.2.7 Online preconcentration and post-column derivatization systems
The online preconcentration system was put together as described in Fig. 5.3. Besides the Dionex Ultimate 3000 HPLC (Thermo Fisher Scientific Inc., Sunnyvale, CA) which consists of an LPG-3400SD pump, a WPS-3000TSL autosampler and a VWD-3400RS UV-Vis absorbance detector and is operated under Chromeleon 6.80 software, there are two more pumps used in the system. For convenience, the three pumps will be referred to as pump A (Dionex LPG-3400SD), pump B (Shimadzu LC-20AD) and pump C (Shimadzu LC-10AT). Mobile phase (DI water) from pump A is connected to the autosampler and the nylon-AO C-CP fiber column through a 6-port switching valve (VICI Valco Instruments Co. Inc., Houston TX). The eluent from the column is then mixed with freshly prepared 0.005% Arsenazo III in 1 M HNO₃ solution delivered by pump B at the confluence union tee, which is consequently directed towards the UV-Vis detector. On the other side of the switching valve, pump C was used to load the sample into a 1.0 mL PEEK sample loop (IDEX Health & Science LLC, Oak Harbor, WA). When the switching valve is in load position, the two sides of the system was not connected.

5.3 Results and discussion

5.3.1 Preparation of nylon-AO C-CP fibers

There are various chemical modification methods reported in the literature for nylon materials. Those that rely on the cleavage of the amide bonds [21-22] potentially cause severe physical damage to the nylon bulk structure. In order to maintain the mechanical strength and fluidic properties of C-CP fibers, the non-invasive surface grafting approach, which is sub-classified into “grafting onto” and
“grafting from” methods, was considered to introduce functional ligands onto polymer base support surfaces [23-25]. The former is a straightforward technique involving the attachment of functional polymer chains onto the base polymer. The attachment process is affected not only by the chemical and physical properties of the attached polymer chains and the base support but also by those of the solution phase in which the attached polymer chains are dissolved. Despite its simplicity and convenience, this approach does not generate high grafting ligand density due to self-inflicting steric hindrance of subsequent polymer chain grafting. On the other hand, the “grafting from” method which relies on the polymerization of a monomer containing the desired functional groups directly from the base polymer support surfaces results in higher grafting density [26-27]. There are different systems that generate free radicals which initiate and propagate the grafting polymerization from the base support surface such as plasma [28], ozone [29] or UV light [25]. As effective as they are, these methods are difficult to impart on to non-uniform surfaces and shapes (such as the fibers described here). Moreover, concerns about mechanical degradation of the polymers need to be considered. Among possible explored techniques, microwave irradiation is a promising approach which has been widely studied [30-33]. Marcus research group has reported the application of the microwave-assisted polymerization grafting method using potassium persulfate (KPS) as the radical initiator to functionalize nylon 6 C-CP fibers with acrylic acid (AA) [34] and acrylamido-2-methylpropanesulfonic acid (AMPS) [35], providing ligands for weak and strong cation exchange protein separation respectively. Further optimization for the grafting polymerization of the
monomer glycidyl methacrylate (GMA) onto nylon C-CP fiber was made using ceric ammonium nitrate \((\text{NH}_4)_2\text{Ce(NO}_3)_6\) (CAN) in dilute nitric acid as an alternative radical initiator system [36]. The resulting nylon-GMA C-CP fibers equipped with the reactive epoxide groups serve as a platform for the covalent immobilization of various ligands. The attachment of iminodiacetic acid (IDA) was made recently to generate an immobilized metal-ion affinity chromatography (IMAC) stationary phase for histidine-tagged recombinant protein separations [36].

The modification scheme utilized in this study is another demonstration of the versatility of nylon-GMA C-CP fibers [36] as a convenient platform for the generation of stationary phase with various functional ligands. The process of making nylon-AO C-CP fibers shown in Fig. 5.1 consists of three main steps: (1) grafting of GMA onto the C-CP nylon fibers, (2) attachment of the iminodipropionitrile (IDPN) to the epoxide groups on the nylon-GMA, (3) conversion the nitriles into amidoxime groups. As shown in Fig. 5.1b, upon exposure to uranium solution, the resulting nylon-AO fibers exhibit a noticeable yellow tint, indicating the binding of uranium and confirming the successful functionalization of the amidoxime groups onto the C-CP nylon fibers.
Figure 5.3 System setup for online uranium preconcentration and post-column arsenazo iii complexation and detection
5.3.2 Characterization of the modified fibers

5.3.2.1 ATR-FTIR

Fig. 5.4 shows the ATR-FTIR spectra of C-CP nylon fibers at four different stages in the modification process. The native nylon shows all the characteristic spectroscopic features that were reported earlier [35, 37], including 3300 cm\(^{-1}\) (N-H stretch), 2931 cm\(^{-1}\) (CH\(_2\) asymmetric stretch), 2861 cm\(^{-1}\) (CH\(_2\) symmetric stretch) and 1639 cm\(^{-1}\) (amide stretch). After the grafting polymerization to immobilize the epoxide functionality, the ATR-FTIR of the nylon-GMA fiber shows a distinctive addition of the C=O stretch at 1729 cm\(^{-1}\), and less prominent peaks are observed in the region of \(\sim 900 – 1300\) cm\(^{-1}\), which are indicative of the presence of the epoxide groups from GMA and the C-O and C-O-C functionality of the GMA [18].
The IR spectrum of nylon-IDPN fibers still maintain the distinctive peak characteristic of C=O stretch. However, the region characteristic of the epoxide group almost disappears. Additionally, there is a broad band around 3500 cm\(^{-1}\), which is representative of the hydroxyl groups resulting from hydrolyzed epoxide groups [18]. There is also a small peak at \(\sim 2200\) cm\(^{-1}\), which indicates the presence of the C≡N groups from the attachment of iminodipropionitrile [38-39]. All of these added spectral features are still visible in the IR spectrum of the finalized product, nylon-AO. There is a slight increase in the intensity of the broad hydroxyl band around 3500 cm\(^{-1}\) and N-H stretch band at 3300 cm\(^{-1}\).

5.3.2.2 SEM imaging-EDS

The morphology and topography of C-CP fiber surface before and after modification process was shown in Fig. 5.5. The surface of nylon-AO fiber seems to be rougher than that of the native nylon, possibly due to the modification process. However, there is no visible damage or stretching observed here. One unique feature is observed in the highest magnification of the UO\(_2^{2+}\)-bound fibers, where some level of “hot spotting” is seen, presumably reflective of formation of metal nanoparticles.

The change in the chemical composition throughout the modification process was semi-quantitatively determined using energy dispersive X-ray spectroscopy (SEM-EDX). The relative composition of carbon, nitrogen and oxygen in native nylon fiber and nylon-AO is changed significantly. The successful coupling step of cyano groups (from IDPN) to the nylon-GMA, which was then
converted to amidoxime functional groups lead to an increase in the nitrogen content observed here. The oxygen content not only results directly through these.

Figure 5.5  SEM imaging and EDX analysis of native nylon, nylon-AO and uranium-bound nylon-AO C-CP fibers
modification steps but also through the concomitant hydrolysis of the epoxides on nylon-GMA to form abundant hydroxyls, which were clearly shown in the broadening band around 3500 cm$^{-1}$ in the FTIR spectra. The binding of uranium ions nylon-AO was confirmed through the presence of uranium peaks. Moreover, as uranium is a dioxycation of uranium (VI), there is a significant increase in the relative oxygen content also.

5.3.3 Offline preconcentration of uranium in spiked matrices

The first pKa of the amidoxime functional group was about 5 [40], which explains why the binding of uranium to amidoxime is negligible at pH below 3 and highest in the pH range of 5 to 8 [41-42]. Real samples such as urine or seawater are characteristically basic with pH around 8 [1], at which uranium occurs in one of its most stable complexes with carbonate [43]. In order to facilitate the strongest binding of uranium to the nylon-AO C-CP fibers, the pH of all spiked samples was adjusted to pH 5.5. Table 5.1 shows the concentration of uranium in the spiked samples versus the values determined after the preconcentration using nylon-AO C-CP fibers. Although the recovery is reasonable with values in the range of high 80% to low 90%, there is still room for improvement. The sample loading rate was 10.0 mL min$^{-1}$ or 123.4 mm s$^{-1}$ in linear velocity, which is in the lower range of the values investigated in section 5.3.6. Moreover, the loaded uranium (ranging from 15 to 25 µg in 50-mL samples) is well within the lower binding capacity range of the packed fibers. Therefore, the less than optimal recovery rates may have resulted from the elution step instead of the loading/capture step. Three 1-mL
volumes of 1 M HNO$_3$ was used to elute the bound uranium in this procedure. Increasing the elution volume may help enhance the recovery rate; however, at the expense of lower preconcentration factors. Higher concentration of nitric acid [44] or the use of alternative acids [45] such as hydrochloric or sulfuric acid will be investigated in future study. As uranium forms a strong complex with carbonate [43, 46], high concentration of carbonate salt in combination with H$_2$O$_2$ [47-48] or is also a good candidate reagent.

### Table 5.1 Results of uranium preconcentration and recovery from spiked samples

<table>
<thead>
<tr>
<th>Spiked samples</th>
<th>Uranium spiked (µg L$^{-1}$)</th>
<th>Uranium found (µg L$^{-1}$)</th>
<th>Recovery (%)</th>
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<tr>
<td><strong>DI water</strong></td>
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<td>350</td>
<td>311.4 ± 8.6</td>
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<td>400</td>
<td>363.2 ± 11.6</td>
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<td><strong>Tap water</strong></td>
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<td>300</td>
<td>269.2 ± 7.0</td>
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<td>448.4 ± 5.4</td>
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<td><strong>Mock seawater</strong></td>
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<td>328.4 ± 13.0</td>
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<td>440.1 ± 6.6</td>
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<td><strong>Mock urine</strong></td>
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<td></td>
<td>450</td>
<td>417.0 ± 15.3</td>
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<td>500</td>
<td>424.1 ± 8.1</td>
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#### 5.3.4 Arsenazo III method
The proof of concept for an online uranium preconcentration and analysis was investigated using a commercial HPLC system equipped with a UV/Vis detector. Uranium (VI) has the maximum absorbance at 414 nm, which was used for the determination of nylon-AO’s dynamic binding capacity. Beyond this purpose, this wavelength has limited use due to its extremely low sensitivity. The elution of uranium does not register for any concentration below 1.0 µg level. As such, other means of detection to enhance the detection limit were explored. Simple and selective method using azo-dyes for the spectrophotometric determination of uranium and other actinides have been widely reported in the literature [49-51]. Among these organic dyes, arsenazo III, a bis-diazo reagent based on chromotropic acid and o-aminophenylarsonic acid [52], is the most commonly used due to its higher sensitivity than other chromogenic reagents of this type, such as arsenazo I and arsenazo II [52-55].

Initially, effort to couple both the elution of bound uranium ions and photometric complexation with arsenazo III into one single step was made. Arsenazo III was dissolved freshly into 1 M HNO₃ at the concentration of 0.005% w/v. In acidic condition, arsenazo III solution exhibits a pinkish or red-crimson color, depending on the concentration, with the maximum absorbance occurring at around 530 nm. Concentrations of 0.01-0.1% w/v aqueous solution of arsenazo III are generally used [50]. As the complexation with uranium ions proceed, the solution turns blue with the absorbance at 530 nm significantly decreasing in proportion to an increase in the absorbance at around 650 nm [52]. In order to reduce background absorbance, a much lower concentration of arsenazo III,
0.005% w/v in 1 M HNO₃, was used. However, the dye appears to adsorb onto the fiber and takes more than a few wash steps to remove. As the absorbance of the dye itself changed with pH, the background absorbance varied drastically until the dye is completely washed off the column. Consequently, the attempt to have elution and complexation in one single step failed. Post-column reaction was then setup according to Fig 5.3 to allow the simultaneous complexation of the eluted uranium ions with arsenazo III for subsequent detection by the UV/Vis detector.

5.3.5 Uranium binding kinetics at different sample loading flow rates

1.0 mL of 100 ppb U was pumped into a 1mL sample loop and then loaded onto the nylon-AO C-CP fiber column at different volumetric flow rates ranging from 1.0 mL min⁻¹ up to 10 mL min⁻¹. The elution of the bound uranium was induced by an injection of 100 µL of 1 M HNO₃ at 1.0 mL min⁻¹ after the switching valve was moved back to the Load position. The effluent from the column was then mixed with the 0.005% w/v Arsenazo III solution at the confluence union tee before being detected at 655 nm by the UV-Vis detector. The peak area of the uranium elution peak was compared at different flow rates. As shown in Fig. 5.6a, the peak areas do not change significantly across the flow rate range. In fact, the peak area even grows bigger at the higher end of flow rates, which is intuitively in contrast to what is expected considering the inverse relationship to the residence time of the analyte staying inside the column. At the highest flow rate investigated, 10 mL min⁻¹, the analyte only stays inside the column for ~0.6 second but the peak area is hardly changed at all. This points to the fast binding kinetics of uranium to
Figure 5.6 (a) Peak area and peak height of the eluted uranium that was preconcentrated from 100 ppb samples loaded at different flow rates; (b) Breakthrough curves of 100 ppm uranium loading at 1mL min\(^{-1}\)
amidoxime ligands on the nylon-AO fibers, which is contributed by following factors. Firstly, the dynamic binding capacity of a 30-cm, 0.03” PEEK column packed with 9 rotations of nylon-AO C-CP fibers, was determined to be 75.4 ± 3.9 µg uranium or 1.19 ± 0.06 mg g⁻¹ fiber, which is more than 4 order of magnitudes higher than the amount loaded onto the column from the loading of 1.0 mL 100 ppb uranium (~10 ng uranium in every column volume, ~0.1 mL). The high binding capacity allows the nylon-AO C-CP fibers to preconcentrate uranium from large volume samples containing low concentration such as ground water, seawater or urine. Secondly, the breakthrough curve in Fig. 5.6b which was obtained by loading 100 ppm uranium at 1 mL min⁻¹ show a steep profile, suggesting the high efficiency of the mass transfer of uranium to the accessible amidoxime ligands on the C-CP nylon-AO fiber surfaces. Moreover, the binding constant of uranium ion and amidoxime is relatively high, being cited in the range of 10⁻¹⁵ M. As a result, the binding is roughly independent of the loading flow rates, hence residence time of the analytes. Thirdly, under conditions of high shear (γ=linear velocity/channel gap), the diffusion layer thicknesses decrease with γ¹/³ [56-57], which consequently increases the adsorption rates and surface ordering of the adsorbates [57-58]. Shortened diffusion distances to nonporous fiber surfaces where convective diffusion likely controls mass transfer [59] significantly enhance protein binding efficiency onto C-CP fibers, which has been clearly demonstrated in previous work [10, 60]. In this particular application of nylon-AO C-CP fibers to the capture of uranium, the effects of high shear on the binding is even more
striking considering the high density of amidoxime binding ligand and a much higher diffusion coefficient of uranium ions than those of proteins.

5.3.6 Column permeability

Even though the nylon C-CP fibers are subject to multiple steps of chemical modification, SEM imaging of nylon-AO fibers shows very little perturbation to the channel structure dimensions or fiber geometry. Permeability testing was also performed to study the hydrodynamic efficiency of the nylon-AO packed column. Every data point on the curves in Fig. 5.7 represents the column pressure drops at the increment of 1.0 mL min\(^{-1}\) in volumetric flow rates. Both native nylon and nylon-AO C-CP fiber columns exhibit well-behaved linear correlation between the packed-column pressure drops (kPa cm\(^{-1}\)) and the linear velocity of the mobile phase (DI water) within the range from 1.0 to 6.0 mL min\(^{-1}\). Beyond this range, a somewhat nonlinear behavior was observed in both kinds of fibers. For native nylon, the pressure curves up while for nylon-AO, the pressure drop slightly curves down at the extreme flow rates. The term \(\mu/k_w\) in equation (1) represents the slope of the trend lines between the column backpressure drop and the linear velocity. Plugging in the viscosity \(\mu\) of the mobile phase water (8.9 x 10\(^{-4}\) Pa·s), the permeability \(k_w\) for native nylon is calculated to be 8.5 x 10\(^{-12}\) m\(^2\), which agrees with values reported earlier 8.8 x 10\(^{-12}\) m\(^2\) [35] and 8.5 x 10\(^{-12}\) m\(^2\) [36] for flow rates below 1.5 mL min\(^{-1}\). Despite multiple steps of chemical modification, there was only a minor decrease in the permeability of nylon-AO which is determined to be 6.1 x 10\(^{-12}\) m\(^2\) for the whole flow rate range from 1.0 to 10.0 mL min\(^{-1}\). C-CP fibers have
been well known for facilitating highly efficient fluid movement with low backpressure. However, this is the first time that C-CP fiber packed column is investigated over such a wide range of flow rates (from 1.0 to 10 mL min$^{-1}$). Here is confirmed that C-CP fibers are even more advantageous to run at high flow rates. The pressure drops observed at the highest flow rate tested was still well within the lower end of the normal HPLC operating pressures.

![Graph showing pressure drop vs linear velocity for native nylon 6 and nylon-AO fibers packed in microbore column format (300 mm long, 0.762 mm i.d.) as function of the flow linear velocity. Mobile phase: DI Water.](image)

**Figure 5.7** Column back pressure of native nylon 6 and nylon-AO fibers packed in microbore column format (300 mm long, 0.762 mm i.d.) as function of the flow linear velocity. Mobile phase: DI Water.

### 5.3.7 Effects of sample injection volume on LOD/LOQ

The limits of detection (LOD) and quantification (LOQ) were calculated from the standard deviation of the response curve and its slope, as reported earlier [13]

$$LOD = \frac{10\sigma_{RSS}}{3K} \quad (\text{Eq. 2})$$
where $\sigma_{RSS}$ is the residual standard deviation of the response function and $k$ is the slope of the calibration curve. Fig. 5.8 shows the calibration curves of different ranges of uranium concentrations injected in various volumes (10 µL and 1.0 mL) and eluted with 100 µL 1 M HNO$_3$. The LOD and LOQ for 10 µL injection calibration curve are 0.83 ppm and 2.5 ppm, respectively. These limits are comparable to the values reported in literature for colorimetric determination of uranium in a complex with arsenazo III [61-62]. When the sample volume was increased from 10 µL to 1.0 mL, the LOD and LOQ of the method were improved about 20-fold, to 0.03 ppm.

Figure 5.8 Calibration curves achieved from the injection of 10 µL and 1.0 mL of uranium standard at different concentration levels
ppm and 0.09 ppm, respectively. Moreover, the corresponding sensitivity, demonstrated by the slopes, also increased significantly from 0.26 to 7.02. As a result, higher injection volumes allow the analysis of low uranium concentration samples. Consistent injection volumes can be achieved using a fixed large volume sample loop shown in Fig. 5.3 or through a programmable pump. The uranium loading and binding efficiency is independent of the mobile phase flow rates at least up to 10 mL min\(^{-1}\), as demonstrated in section 5.3.4. Therefore, loading of high-volume samples can be performed at high flow rates to improve throughput without the penalty of low binding efficiency.

It is widely known that the UV-Vis detector is concentration sensitive, instead of mass sensitive, detector which responds to the analyte mass per unit volume of the mobile phase [63]. The calibration curve must be constructed using the analyte concentration, not its absolute mass. In this application, a small amount of uranium is retained on the fiber packed inside a microbore column and then consistently eluted using the same volume of elution solution (100 µL). Therefore, it is reasonable to expect that, essentially the absolute mass, instead of concentration, can also be used for the generation of a standard curve. However, the data collected showed that was not the case. When the x-axis in Fig. 5.8 is converted into the absolute mass of uranium by multiplying the corresponding concentration by the injection volume, mass-based response, instead of concentration-based response, curve (not shown here) indicates there is a discrepancy in the peak areas of supposedly the same amount of uranium in the injection. Since 10 µL of 10 ppm and 20 ppm contain the same amount of uranium
Figure 5.9 (a) Peak area of the same amount of uranium pre-concentrated from different injection volumes at different concentrations; (b) Blank-corrected chromatograms of the eluted peaks
as in 1 mL of 0.1 ppm and 0.2 ppm, respectively, there should be no difference in
the peak area for the same amount of uranium delivered by 10 µL and 1.0 mL
sample injection. Yet is what was not observed. For better comparison, Fig. 5.9a
show the peak area of the uranium elution peak preconcentrated from the same
theoretical mass delivered by different injection volumes of different uranium
concentration levels.

This phenomenon is contributed by various factors. Firstly, the large
injection volume leads to more significant band broadening effects. Once the
sample is injected and starts dispersing into the mobile phase stream, two
processes occur contributing to sample band spreading demonstrated in Fig. 5.10.
The first process, convection due to the laminar flow of the mobile phase causes
a parabolic flow profile which is characterized by stagnant linear velocity at the
tube’s wall and twice the velocity of the carrier stream at the center of the tube [64].
The second process is diffusion due to the concentration gradient between the

![Figure 5.10](image)

**Figure 5.10** Bandspreading of the injected samples due to convection and
diffusion.
linear velocity at the center of the tubing and at the edge of the tubing experiences, respectively, which in turn maintains the integrity of the sample’s flow profile [65]. Convection is the main mechanism by which dispersion occurs in the first 100 ms after the sample injection while both processes significantly contribute to dispersion in between 3-20s after the sample injection [65]. Beyond 25 s, diffusion is the only predominant dispersion contributor [65]. At the flow rate of 1.0 mL min\(^{-1}\), it takes \(~6\) s for the sample to travel the whole column (30-cm 0.03” ID column packed with nylon-AO with void volume \(~90\) µL). For injected volumes less than \(100\) µL, most of the sample is expected to sweep through the column within \(~6\)s. During this time, both convection and diffusion contribute to the sample band broadening. For 1.0-mL injection, it takes at least a whole minute for the sample to sweep through the column; therefore, diffusion is expected to be the main cause of the sample bandspreading. The effect of diffusion is increasingly dominant with increasing injected sample volumes. This explains why at higher flow rates, which reduce the effects of diffusion, the peak area of the eluted uranium increases as shown in Fig. 5.6a. The width \(W_0\) of the sample injection plug is broadened to \(W\) at the column exit. The larger the injection volume, the larger \(W_0\) and \(W\) are. Different injection volumes containing essentially the same amount of uranium generate different preconcentration footprint inside the column, which in turn produce different elution profiles.

Secondly, the molar absorptivity of arsenazo III dye and its complex with uranium is highly sensitive to the changes in pH [52, 66]. The dispersion of \(100\) µL 1 M HNO\(_3\) injection for the elution of bound uranium itself is manifested as a pH
gradient, due to the two processes discussed above as it sweeps through the column and mixes with the arsenazo III dye solution. This phenomenon is clearly shown in the blank run profile in Fig. 5.11. When the injection of 100 μL 1 M HNO₃ after a blank run was mixed with the 0.005% arsenazo III dye in 1M HNO₃, the slight change in the pH results in a significant background peak. Not only does the molar absorptivity of the free arsenazo III dye itself varies with pH [51, 67] but also does that of the UO₂²⁺-arsenazo III complex [66]. In Fig. 5.11, the elution of the preconcentrated uranium from 10 μL of 10 ppm uranium solution generates a uranium concentration gradient along the elution profile reflective of its
preconcentration footprint inside the column. In combination with the pH gradient and the resultant changes in the molar absorptivity of both arsenazo III dye and its complex with uranium, the elution peak has a shoulder.

As a result of the two factors discussed above, the responses from the same amount of uranium preconcentrated from different injection volumes are not the same, which is clearly displayed in the chromatograms in Fig. 5.9b. Consequently, the preconcentration of uranium in this case does not result in mass sensitive responses. However, it is worth noticing that the peak widths of the eluted uranium in Fig. 5.9b do not vary much. In order to confirm that these eluted peaks contain the same mass of bound uranium preconcentrated from different injection volumes of uranium concentrations, future study involving a mass sensitive detection such as mass spectrometry will be conducted. If this is the case, a mass-based response calibration curve can be constructed. This means a small volume of high uranium concentration standards can be used to cover the needed calibration range, which will not only save the standards but also reduce waste generation. Depending on the LOD/LOQ of the mass spectrometry detector, the loading volume of samples containing low concentration of uranium such as seawater, groundwater or even digested urine can be adjusted so that the preconcentrated amount would fall into the pre-calibrated range.

5.4 Conclusions

This study further explores the application of glycidyl methacrylate (GMA) polymerization grafting onto C-CP nylon fibers as a ligand binding platform [18].
The reactive exopoxide groups of the GMA polymer serve as anchoring points for the attachment of iminodipropionitrile (IDPN), providing the dinitrile groups on the fiber surface. Subsequent reaction with hydroxylamine NH$_2$OH converts these dinitriles into amidoxime functional groups that are capable of binding uranium ions with high binding affinity. The resulting nylon-amidoxime (nylon-AO) fibers packed in column format still maintains high permeability despite multiple modification steps. Advantageous features of C-CP fibers including highly efficient fluid movement and favorable mass transfer allow the nylon-AO fibers to preconcentrate uranium at extremely high linear flow velocities. The potential of this online SPE was investigated through a post-column Arsenazo III colorimetric method. In this initial proof-of-concept demonstration, 1.0 mL of uranium standard solutions (more than 10 times the column void volume) at different concentrations were preconcentrated using the online SPE system. With the preconcentration step, the LOD/LOQ was improved more than 30-fold compared to those reported in the literature for the conventional colorimetric determination of uranium in a complex with arsenazo-III method [61-62]. Higher injection of samples can be achieved using a fixed large volume sample loop shown in Fig. 5.3 or through a programmable pump, which helps improve the method sensitivity and allows the analysis of low uranium concentration samples. As the binding efficiency is not only compromised but even improved at higher flow rates, loading of high-volume samples can be performed at high flow rates to improve sample throughput.
Acknowledgements

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6.1 Introduction

Protein phosphorylation and dephosphorylation (by kinases and phosphatases in eukaryotic cells, respectively) plays an important role in protein function and interactions [1-2]. As an important regulator of cellular signaling networks, phosphorylation is estimated to affect $\sim30\%$ of the proteome [3]. Detailed analyses of phosphorylated proteins can provide a better understanding of biological processes at the molecular level. To this end, cellular proteins are often digested to yield the phosphopeptides which are then identified using mass spectrometry. Even though improvements in modern mass spectrometric techniques allow for high-throughput phosphorylated protein analyses, these studies are still challenging due to the complexity of phosphorylated proteins and the low stoichiometry of protein phosphorylation [4]. In addition, phosphopeptides are known for their poor ionization efficiencies due to their associated negative charge. Moreover, the low abundance of phosphopeptides in complex peptide mixtures is also a challenge. In order to overcome these two technical difficulties, use of some form of sample clean-up is critically required to isolate and pre-concentrate phosphopeptides from complex peptide matrices [5].
Among the different approaches of selective enrichment of phosphopeptides are immobilized metal ion affinity chromatography (IMAC) and metal oxide affinity chromatography (MOAC) [6]. IMAC utilizes the affinity of the phosphate groups in phosphopeptides to metal ions (Fe$^{3+}$ or Ga$^{3+}$) immobilized on the stationary phase via the acidic chelating ligands of iminodiacetic acid (IDA) or nitriloacetic acid (NTA) [7-8]. The conventional IMAC technique has two main disadvantages. First, it experiences possible leakage of metals during sample loading and washing steps. Secondly, the selectivity of the traditional IMAC adsorbents (which is typically low) is interfered by the non-phosphopeptides containing acidic amino acid residues (such as glutamate and aspartate) [8-9]. The latter can be partially addressed by methyl esterification of carboxylic acid groups of peptides; however, this extra process may invite more side reactions and involves laborious steps, which limit applications on the preparative scale [10-11]. Researchers have been looking into other metal ions and chelating agents relative to possible leakage and perhaps greater selectivity towards phosphopeptides species. Among them, Ti$^{4+}$ and Zr$^{4+}$ have gained popularity in the past decade [8]. Zhou and co-workers reported the development of new IMAC phases that contain either a phosphate or phosphonate group [9, 12], which show strong interactions for the binding of Ti$^{4+}$ and Zr$^{4+}$, while also taking care of the metal ion leakage issue. On top of that, the developed IMAC phases also display higher selectivity towards phosphopeptides when applied to complex peptide mixtures. The majority of supports used for these IMAC phases includes silica, polymers, carbon-based materials, magnetic nanoparticles, mesoporous beads, monolithic materials
(polymer or hybrid organic silica) and metal-organic frameworks [13-15]. These materials are complicated to fabricate and so far, have found limited applications in the online phosphopeptide enrichment for mass spectrometric analysis.

Joining the efforts to develop alternative chromatographic phases for use in proteomic applications, this laboratory has investigated the potential of capillary-channeled polymer (C-CP) fibers through diverse separation modalities. The C-CP fibers are characterized by eight capillary channels that allow them to interdigitate to form well-aligned micrometer-sized channels when packed into a column format [16-17]. C-CP fibers are extruded from commodity polymers (nylon 6, polyester, polypropylene) which tend to be quite stable over wide pH range and solvent strengths and are very inexpensive (<$100 lb\textsuperscript{−1}). It is this unique structure that allows separations at high linear velocities (>75 mm s\textsuperscript{−1}) with very low back pressure (<0.14 MPa cm\textsuperscript{−1}) [18-19]. The highly efficient fluid movement through the narrow channels composed of non-porous C-CP fibers gives rise to favorable mass transfer rates, facilitating fast protein separations and processing [20-23]. This laboratory has utilized C-CP fibers for protein separations across the spectrum of reversed-phased [19], ion-exchange [24-25], hydrophobic interaction [26-27] and affinity [28-30] chromatographies.

The neurotransmitter dopamine has been reported to be involved in the cross-linking and resultant high affinity of marine adhesives through oxidative self-polymerization [31-32]. This phenomenon has been utilized in the formation of adherent polydopamine coatings on a variety of substrate surfaces [31, 33]. The polymerization system simply involves a dilute aqueous solution of dopamine
buffered to pH 8.5 by TRIS [31, 33]. The versatility of polydomapine also comes from various functional groups incorporated into its chemical structure including catechols, quinones, amines and imines which can serve as reactive sites for covalent modification with desired moieties [34]. Polydopamine-coated materials, immobilized with metal ions through the strong chelating ability of catechol hydroxyl groups, have found applications as the new IMAC stationary phases for phosphopeptide enrichment from complex biomatrices [35-38]. However, such studies so far have focused on off-line sample clean-up and enrichment where the target phosphopeptides are collected and spotted onto target plates for subsequent MALDI-MS analysis. Taking advantage of the highly efficient fluid movement of C-CP fibers presented above, and the ready modification of the platform, this work aims towards the initial demonstration of polydopamine-coated C-CP fibers that can be used as a platform for online enrichment and mass spectrometric analysis of phosphopeptides. Specifically, Fe$^{3+}$-bound nylon-PDA fibers packed in a microbore column format were evaluated. It is believed that the phase holds promise in providing high fidelity phosphopeptide isolation as well as enhancing the throughput of phosphopeptide MS analysis.

6.2 Materials and methods

6.2.1 Chemicals and instrumentation

HPLC-grade acetonitrile (ACN) and methanol (MeOH) were purchased from EMD Millipore (Billerica, MA). Glacial acetic acid (CH$_3$COOH), o-phosphoric acid (H$_3$PO$_4$) 85%, concentrated ammonium hydroxide (NH$_4$OH), sodium
hydroxide (NaOH) pellets and ferric nitrate (Fe(NO₃)₃) were purchased from Fisher Scientific (Fair Lawn, NJ). Concentrated hydrochloric acid (HCl) was purchased from J.T. Baker (Center Valley, PA). High purity formic acid (HCOOH) was purchased from VWR Amresco Life Science (Solon, OH). Sodium carbonate (Na₂CO₃) was purchased from Acros Organics (Bridgewater, NJ). Ammonium bicarbonate (NH₄HCO₃) was purchased from MP Biomedicals, LLC (Solon, OH). Sodium bicarbonate (NaHCO₃), HPLC grade was purchased from EMD (Gibbstown, NJ). Dopamine hydrochloride, trifluoroacetic acid (CF₃COO, TFA) and 2,5-dihydroxybenzoic acid was purchased from Sigma Aldrich (St. Louis, MO). Tris-(hydroxymethyl) aminomethane (TRIS base), biotechnology grade was purchased from G Biosciences (St. Louis, MO). Bovine serum albumin (BSA) and bovine β-casein were purchased from Sigma-Aldrich (St. Louis, MO). Sequencing grade modified trypsin was purchased from Promega (Madison, WI). The MALDI matrix solution containing 2% w/v dihydroxy benzoic acid was prepared in 90:9:1 mixture of ACN:DI-H₂O: 85% H₃PO₄.

All aqueous solutions were prepared with ultra-pure Milli-Q deionized (DI) water (18.2 MΩ.cm) obtained from a Millipore water system (Billerica, MA). Nylon 6 C-CP fibers were provided by the Material Science and Engineering Department, Clemson University. The denier per filament was 2.67 grams per 9000 meters of a single fiber. The cross-section perimeter of each fiber is ~207 µm.

All chromatographic experiments were conducted using a Dionex Ultimate 3000 HPLC system (Thermo Fisher Scientific Inc., Sunnyvale, CA) consisting of
an LPG-3400SD pump, a WPS-3000TSL auto-sampler and a VWD-3400RS UV-Vis absorbance detector and operated under Chromeleon 6.80 software.

6.2.2 Preparation of polydopamine-modified C-CP nylon fibers:

For convenience, the polydopamine-modified C-CP nylon fibers will be referred to here as nylon-PDA. The entire preparation process of the nylon-PDA fibers is detailed in Fig. 1. Native nylon-6 C-CP fiber bundles (consisted of 9 rotations or 540 fiber strands) were stretched on a dying fork and rinsed with hot water for better fiber alignment. The fibers were then thoroughly washed consecutively with excess amounts of DI-water, MeOH and ACN remove any residual (anti-static) chemicals left on the fibers during the manufacturing process. The fiber bundles were then placed into a 20 mL solution of 0.2% w/v dopamine hydrochloride in 0.15% w/v TRIS buffer, pH 8.5 contained in a 2-ounce glass jar for 1 hour. Then, the fiber bundles were placed into a 20 mL solution of 0.2% w/v dopamine hydrochloride in 0.15% w/v TRIS buffer, pH 8.5 contained in a 2-ounce glass jar for 72 hours. The iron-binding process is shown in Fig. 6.1.

Figure 6.1 Process of making polydopamine-coated nylon C-CP fibers

For convenience, the polydopamine-modified C-CP nylon fibers will be referred to here as nylon-PDA. The entire preparation process of the nylon-PDA fibers is detailed in Fig. 1. Native nylon-6 C-CP fiber bundles (consisted of 9 rotations or 540 fiber strands) were stretched on a dying fork and rinsed with hot water for better fiber alignment. The fibers were then thoroughly washed consecutively with excess amounts of DI-water, MeOH and ACN remove any residual (anti-static) chemicals left on the fibers during the manufacturing process. The fiber bundles were then placed into a 20 mL solution of 0.2% w/v dopamine hydrochloride in 0.15% w/v TRIS buffer, pH 8.5 contained in a 2-ounce glass jar for 1 hour. Then, the fiber bundles were placed into a 20 mL solution of 0.2% w/v dopamine hydrochloride in 0.15% w/v TRIS buffer, pH 8.5 contained in a 2-ounce glass jar for 72 hours. The iron-binding process is shown in Fig. 6.1.
open to the air. The coating process was allowed to occur at room temperature and atmospheric pressure undisturbed for 72 hours. The nylon-PDA fibers were then removed from the jar and thoroughly rinsed with DI water and packed into a 30-cm long, 0.03” (0.762 mm) i.d. polyether ether ketone (PEEK) tubing (IDEX Health & Science LLC (Oak Harbor, WA)) as described previously [19].

6.2.3 Preparation of Fe$^{3+}$-bound polydopamine-modified C-CP nylon fibers:

Fe$^{3+}$ is by far the most common metal ion used in IMAC for phosphopeptide enrichment [6, 8]. It is easy to prepare in solution and available in most labs. On the other hand, Ti$^{4+}$ and Zr$^{4+}$ are more difficult to work with due to their strong tendency towards hydrolysis [39-40]. These ions reportedly have higher selectivity but at the same time also have stronger binding to multi-phosphorylated peptides, leading to poor recoveries of those types of peptides [14]. The peptides generated in β-casein digest used in this demonstration contain up to four phosphate groups, therefore, Fe$^{3+}$ has been used in this example. To be clear, in real applications, the choice of metal ions or combinations thereof, are typically optimized for the capture and recovery of the targeted phosphopeptides, depending on the nature of the specific protein samples.

To generate the ultimate IMAC column, the nylon-PDA packed column was mounted onto the HPLC system and charged with Fe$^{3+}$ ions through multiple 100 μL injections of a 1 M Fe(NO$_3$)$_3$ solution at a 1.0 mL min$^{-1}$ flow rate. The loading was monitored through the absorbance at 304 nm until saturation was reached, a reflection of the fact that the surface ligands had been completely charged. The
column was then ready for analytical separations following equilibration with the chromatographic buffer A (250 mM acetic acid).

6.2.4 Preparation of protein digests

In order to assess the ability to selectively extract and preconcentrate phosphopeptides to the exclusion of other peptides, two primary proteins were evaluated. Specifically, β-casein, which has 3 phosphopeptides in a typical digest, and BSA, which has no phosphopeptides, were digested separately using the same procedure. Each protein was weighed and dissolved into a 15-mL plastic centrifuge tube with 0.1 M NaHCO₃, pH 8.2 and urea was added to denature the protein. The final concentrations of urea and the denatured stock protein was 8 M and 10.0 mg mL⁻¹, respectively. Following denaturation, 1.0 mL of the denatured protein solution was transferred to another 15-mL plastic tube and diluted 10X with 0.1 M NaHCO₃, pH = 8.2 to reach the final concentration of 0.8 M urea and 1.0 mg mL⁻¹ protein. The stock sequencing grade modified trypsin (0.5 ± 0.05 mg mL⁻¹, according to the manufacturer) was reconstituted in 100 µL of 50 mM acetic acid, 65 µL of which was added to 10 mL of denatured 1.00 mg mL⁻¹ protein digest. The mixture was incubated at 37°C for 24 hours. The digest was transferred to 1.5-mL centrifuge tubes and stored at -20°C. For BSA, β-mercaptoethanol was added to the stock solution at the beginning to the final concentration of 10 mM to dissociate the intramolecular disulfide bonds.

6.2.5 Characterization of modified nylon 6 C-CP fibers
The scanning electron microscope (SEM) imaging and energy-dispersive X-ray spectroscopy (EDX) analysis of the fibers were performed on using a Hitachi S-3400N operating in the variable pressure mode, with a 10kV accelerating voltage at the Clemson University Electron Microscopy Laboratory. The attenuated total reflection-Fourier transform infrared spectroscopy (ATR-FTIR) characterization of the fibers was performed on a Thermo-Nicolet Magna 550 FITR in the Analytical Testing Lab of Material Science & Engineering Department, Clemson University.

6.2.6 MALDI and ESI

6.2.6.1 Offline sample clean-up for MALDI-MS analysis

The chromatographic isolation of phosphopeptides from the digested β-casein was performed as follows. The mobile phase includes 250 mM acetic acid (pH = 2.8) as the loading buffer (A), NH₄OH solution (pH = 10.5) as the elution buffer (B) and DI water as the wash buffer (C). The nylon-PDA column was prepared by packing 9-rotation nylon bundles (540 fibers) coated with polydopamine into the 30 cm long, 0.03” i.d. PEEK tubing, as described in Section 2.2. The chromatographic program includes the loading step 0-2 min (100% A), washing step 2-6 min (100% C), elution step 6-8 min (100% B) and re-equilibration step 8-10 min (100% A), all at the volumetric flow rate of 1.0 mL min⁻¹ (∼60 mm s⁻¹). Two samples, 5 µL of the β-casein digest and 20 µL of the 1:100 mixture of the β-casein and BSA digests, were injected with absorbance detection employed at 216 nm. The elution peak (∼400 µL) was collected, acidified with 10 µL glacial acetic acid and mixed 1:1 with the MALDI matrix solution. For comparison, the
unenriched β-casein digest sample and the 1:100 β-casein and BSA digest mixture were also prepared the same way. 1 µL aliquots of all samples were deposited on a MSP 96 MALDI target plate (Bruker Daltonik GmbH, Germany) and loaded into a Bruker Daltonics (Billerica, MA, USA) microflex LRF MALDI-TOF mass spectrometer, controlled and processed by Compass, a Bruker Daltonics software. The MALDI spectra of the samples were obtained in the positive ion, reflectron mode using a nitrogen laser (337 nm) at a pulse rate of 60 Hz. Mass spectral acquisitions occurred using 100 laser shots at 35% laser power.

6.2.6.2 Online sample-cleanup for ESI-MS analysis

Similar chromatographic conditions for isolating phosphopeptides from the digest samples (described in Section 2.5.1) were also employed here with some modification. The column effluent was directed to the ESI source attached to Thermo Q-Exactive Focus Hybrid Quadrupole-Orbitrap Mass Spectrometer. Instead of the 250 mM acetic acid (pH = 2.8), a formic acid solution (pH = 2.0) was used as the loading buffer (A). The switch to formic acid was done to achieve the lower pH with a smaller volume of the protic acid. Formic acid would also be expected to provide better ESI-MS yields. The other mobile phases included NH₄OH solution (pH = 10.5) as the elution buffer (B), acetonitrile (C) and DI water (D) as the wash buffer. The flow rate was adjusted to 0.5 mL min⁻¹ for optimal coupling to the ESI source. The total run time was extended to 15 minutes to allow for a more extensive washing step. The chromatographic program includes the loading step 0-1 min (100% A), washing step 1-7 min (100% D), eluting step 7-11
min (100% B) and re-equilibration step 11-15 min (100% A). Two samples, 5 µL of β-casein digest and 20 µL of a mixture of 1:100 β-casein and BSA digest, were injected onto the iron-bound nylon-PDA column. The detected ion range was set from 1000-3000 m/z in both (+) and (-) modes. The capillary temperature was 320°C and the spray voltage was 4.0 kV. The auxiliary gas and sheath gas flow rates were set at 0 and 40 (arbitrary units), respectively.

In order to present a greater challenge to the isolation of the phosphopeptides, a 100 µL of 1:1000 mixture of the β casein and BSA digests was investigated. In order to remove the abundant non-phosphopeptide fragments containing acidic amino acids, which are weakly retained on the polydopamine phase, an intermediate wash step of 50% aqueous ACN was applied from 1-7 min (50% C: 50% D), instead of 100% water, prior to passage of the elution buffer. To be clear, the methodology here is designed to isolate the phosphopeptides and does not include a subsequent LC separation of those species; at this point.

6.3 Results and discussion

6.3.1 Preparation of polydopamine C-CP nylon fiber

Dopamine, upon self-polymerization under alkaline conditions or in the presence of oxidants, forms a polydopamine coating rich in catechol groups that are capable of forming hydrogen bonds, metal–ligand complexes, and quinhydrone charge-transfer complexes. As a result, this strong coating can adhere to almost any solid surfaces including metals, oxides, polymers and semiconductors without surface pretreatment [41-45]. While the reaction itself
seems simple, the exact molecular mechanism behind the polymerization, which involves complex redox steps with a series of intermediates along the process, is still a topic of debate. Figure 2 shows one of the early proposed mechanisms which is based on the synthetic pathway of the melanin (eumelanin) in living organisms [34]. In alkaline conditions, dopamine first undergoes oxidation to dopaminequinone, which then cyclizes to form leucodopaminechrome. Further oxidation and rearrangement results in 5, 6-dihydroxyindole and its oxidized counterpart 5,6-indolequinone. Polymerization and branching from these 2 reactive monomers eventually form a cross-linked network of polydopamine. In a different model proposed by Bielawski and co-workers [46], instead of being formed from covalent bonds between the aryl rings of the monomers, polydopamine is a supramolecular aggregate of monomers held together through noncovalent forces such as hydrogen bonding, charge transfer, and π-stacking. Other researchers suggest that the formation polydopamine is comprised of both covalent cross-linking and non-covalent self-assembly [47].

The entire process of making polydopamine-coated nylon C-CP fibers from the fiber spool to the packed column format is detailed in Fig. 1. In this work, a simple system involving a dilute aqueous solution of 0.2% w/v dopamine hydrochloride in 0.15% w/v TRIS buffer, pH 8.5 was utilized to coat polydopamine
onto C-CP nylon surface. The reaction, upon exposure to air, occurs instantaneously with a clear color change of the dopamine solution from clear to a pinkish shade. As shown in Fig. 1, after 72 hours, the reaction mixture turns dark brown and fibers likewise. Upon thorough washing, the modified C-CP nylon fibers exhibit a visually-uniform coating of polydopamine on the surface. At this point, the fibers were loaded with the Fe$^{3+}$ employed for the IMAC capture process.

6.3.2 Characterization of the modified C-CP fibers

6.3.2.1 ATR-FTIR

Infrared spectroscopy is an essential tool in characterizing the chemical functionality of materials. The ATR-FTIR spectra shown in Fig. 3, display the characteristic spectroscopic features of native nylon 6 fibers reported earlier.

**Figure 6.3** ATR-FTIR spectra of the native and polydopamine-coated nylon (nylon-PDA) C-CP fibers.
[25, 48], including 3300 cm\(^{-1}\) (N-H stretch), 2931 cm\(^{-1}\) (CH\(_2\) asymmetric stretch), 2861 cm\(^{-1}\) (CH\(_2\) symmetric stretch) and 1639 cm\(^{-1}\) (amide stretch). Upon the polydopamine coating, nylon-PDA shows a broad band around 3500 cm\(^{-1}\), which is representative of the hydroxyl groups from dopamine. The indole or indoline-based features proposed as possible structures occurring in polydopamine [49] are characterized by N–H (\(~3400\) cm\(^{-1}\)), C–H (2800-3050 cm\(^{-1}\)) and C═O (\(~1750\) cm\(^{-1}\)) stretching frequencies [50]. However, all of these characteristic features cannot be discerned from the strong overlapping peaks from the native nylon 6 fibers. When zooming in the IR spectra within the range of 1700-1750 cm\(^{-1}\) (inscribed by the dashed lines), there is a small structural peak occurring in nylon-PDA and iron-bound nylon IDA, which may be due to the indoline structures. The lack of absorbance intensity in the IR region that indicates the grafting of polydopamine on native nylon surface is in stark contrast with the strong absorbance of the visible light that gives nylon-PDA fibers a distinctively dark brown coating. This may result from the major difference in the thickness of the PDA coating (in the range of tens of nanometers [31, 51]) compared to that of the bulk nylon materials. Fig. 3 also shows the IR spectrum of iron-bound nylon-PDA, which is identical to the unbound counterpart. As a result, the confirmation of the iron binding to nylon-PDA was made through the energy-dispersive X-ray spectroscopy (EDX) analysis discussed below.

6.3.2.2. SEM imaging and EDX analysis
The electron microscopy images in Fig. 4 show that there are virtually no differences in morphology and topography of C-CP nylon fibers before and after the coating of polydopamine. The modified fibers do not have any damage or structural stretching. The surface of the iron-bound PDA nylon is smooth with no visible spots reflective of iron nanoparticles, indicating a uniform and consistent coating of the whole fiber. The chemical composition change throughout the

**Figure 6.4** SEM imaging and EDX analysis of native nylon, nylon-PDA and iron-bound nylon PDA C-CP fibers
modification process was semi-quantitatively determined using energy dispersive X-ray spectroscopy (SEM-EDX). After the coating of polydopamine, there is an increase in the relative composition of nitrogen and oxygen. The presence of iron on the PDA-coated nylon was also confirmed, as shown in Fig. 4.

6.3.3 Phosphopeptide sample clean-up for off-line MALDI analysis

As the primary challenges to the newly-developed phases, a well-known phosphoprotein, β-casein, and one without phosphorylation, BSA, were used as test species. Digests of the former provide well-known phosphopeptide MS signatures while the latter presents a peptide-rich spectrum from which to isolate minor species. Figure 5a depicts the mass spectrum of the raw β-casein digest. The peptide profile of unenriched β-casein tryptic digest is fairly simple with 3 dominant ions of > m/z = 2000, two of which are the phosphopeptides β₁ and β₂, as listed in Table 1. The other smaller fragments are all below the m/z of 1500. The base peak m/z 2186 in the spectrum obtained from the unenriched β-casein digest is the non-phosphopeptide fragment β₄, which contains an acidic aspartic residue at its N-terminus. The corresponding mass spectrum for the C-CP column-enriched β-casein digest is shown in Fig. 5b. The spectrum shows three prominent peaks, marked with the asterisk (*), each of which is assignable to expected phosphopeptides. After enrichment, the intensity of the β₁, β₂ and β₃ phosphopeptides all increase, with β₂ being the predominant species. There is also another peak at m/z 1561, which is another ion species formed by the phosphopeptide β₂.
In order to showcase the selectivity of Fe\textsuperscript{3+}-nylon-PDA towards phosphopeptide enrichment, the 1:100 mixture of the tryptic digests of β-casein and BSA was also evaluated. Without enrichment (Fig. 5c), the sample contains mostly peptide fragments below m/z 1500, as would be expected from a BSA digest [52-53]. The presence of the higher-mass peptides characteristic of a β-casein digest, which are present in the tryptic digest mixture at the picomole level (∼0.4 pmol), are barely noticeable. In contrast, the MALDI spectrum obtained after the Fe\textsuperscript{3+}-nylon-PDA fiber sample clean-up (Fig. 5d) clearly shows a much more simplified profile, where the non-phosphopeptide peaks are virtually non-existent.

**Figure 6.5** MALDI mass spectra of β-casein digest sample and 1:100 mixture of β-casein and BSA digest sample before (a and c) and after (b and d) sample clean-up. Peaks marked with the asterisk (*) indicate the phosphopeptide peaks.
The spectrum in this case is composed, almost exclusively, of the phosphopeptide species \( \beta_1 (m/z \ 2061) \), \( \beta_2 (m/z \ 1561 \text{ and } 3122) \) and \( \beta_3 (m/z \ 3042) \), with much higher intensity and signal to noise ratio. There is also a prominent peak at \( m/z=2186 \) Da, attributed (Table 6.1) to the \( \beta_4 \) peptide which is an acidic peptide unit. Acidic peptides are commonly found in phosphopeptide separations, often requiring sample-specific tuning of the isolation conditions including choice of metal ion, pH, etc. Such efforts were beyond the scope of this demonstration.

The entire process of capturing, washing, and eluting of phosphopeptides from the Fe\(^{3+}\)-nylon-PDA C-CP fiber column was conveniently performed within 10 minutes using a standard HPLC system. In comparison to other protocols that involve the incubation of materials or use in spin-down format [9, 54-55], the sample clean-up using the nylon-PDA C-CP fiber columns offers enhanced convenience and higher throughput. As the column volume is fairly small (less than 100 µL), the eluted phosphopeptide peak can be easily captured in a very small volume without appreciable dilution. As a result, the extraction is so efficient that the eluted phosphopeptide fraction does not require further freeze-drying to concentrate the eluate, as is common in other protocols [9, 54-55]. Indeed, the elution was directly mixed with MALDI matrix solution and immediately ready for MALDI analysis. At the end of the isolation run, the column was washed, re-equilibrated and ready for additional separation cycles within minutes.

6.3.4 Direct on-line sample-cleanup for ESI-MS analysis

The ultimate goal of this project is to create a convenient on-line sample
**Table 6.1** Peptide fragments from β-casein digest observed in the mass spectra

<table>
<thead>
<tr>
<th>ID</th>
<th>Residues</th>
<th>Sequence</th>
<th>[M+H]^+</th>
<th>[M+2H]^{2+}</th>
<th>[M+3H]^3+</th>
</tr>
</thead>
<tbody>
<tr>
<td>β₁</td>
<td>16-40</td>
<td>FQSpEEQQTEDELQDK</td>
<td>2061.83</td>
<td>3122.27</td>
<td>1041.43</td>
</tr>
<tr>
<td>β₂</td>
<td>48-63</td>
<td>RELEELNVPGEVESSLSpSpEEESI</td>
<td>1031.42</td>
<td>1581.64</td>
<td>1094.09</td>
</tr>
<tr>
<td>ITR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β₃</td>
<td>48-63</td>
<td>RELEELNVPGEVESSLSpSpEEESI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β₄</td>
<td>184-202</td>
<td>DMPQAFLYQEPVILGPVR</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

No. of phosphorylation events: 0 0 1 0 0 0
cleanup that serves as an integrated platform for mass spectrometric analysis of phosphopeptides. In order to demonstrate the potential of nylon-PDA C-CP fiberstowards this end goal, a demonstration of on-line sample cleanup and capture of phosphopeptides from β-casein digest was made directly for ESI MS analysis. The chromatographic protocol described above for off-line MALDI analysis was used here with some modification. Instead of 250 mM acetic acid (pH = 2.8), a formic acid solution (pH = 2.0) was used as the loading buffer (A). In order to avoid ionization suppression, it is recommended that the concentration of additives used in ESI-MS should be low, likely less than 10 mM. As formic acid is a stronger acid (pKa = 3.76) than acetic acid (pKa = 4.75), much less formic acid is needed (∼2% v/w or 550 mM) to induce pH 2.0 mobile phase solution [56]. The flow rate was adjusted to 0.5 mL min$^{-1}$ for optimal coupling to ESI source based on the ion source solvent loading limitations. The total chromatographic run time was extended to 15 minutes to allow a more extensive washing step.

As seen in Fig. 6a, the mass spectrum of the eluted fraction from the clean-up of β-casein digest sample shows three phosphopeptide ion fragments at m/z 1031 (peptide β1 with z=1), m/z 1041 (peptide β2 with z=2) and m/z 1561 (peptide β2 with z=3). The predominant non-phosphopeptide fragment m/z 2186 seen in Fig. 5b and 5d MALDI spectra has an aspartic residue at the N-terminus. With a pKa of ∼3.65 at its carboxyl side chain, this amino acid residue is not completely protonated in the 250 mM acetic loading buffer, which in turn accounts for the stronger binding of the non-phosphopeptide fragment β4 to the Fe$^{3+}$-nylon-PDA. In order to reduce its binding, the pH of the loading buffer used in the on-line ESI
Figure 6.6 The total ion chromatogram and the elution mass spectra of (a) β-casein digest and (b) 1:100 mixture of β-casein and BSA digest sample. In Fig. 6c, an intermediate wash step was added to further remove non-phosphopeptides from the 1:1000 mixture of β-casein and BSA digest sample. Peaks marked with the asterisk (*) indicate the phosphopeptide peaks.
MS experiment was lowered to 2.0, which further suppresses the dissociation of the aspartic end. As a result, less β4 fragment, which shows up at m/z 1094 (z=2) on the mass spectrum, bound and came out in the eluted fraction. The binding of acidic non-phosphopeptide is a common issue in the IMAC clean-up and enrichment of phosphopeptides. As demonstrated here, this can be resolved through the use of lower pH loading and wash buffer. However, as most IMAC stationary phase relies on carboxyl- containing chelators such as imminodiacetic acid (IDA) or nitriloacetic acid (NTA), potential leaching of metal ions at low pH must be taken into consideration. Therefore, most procedures utilizing IDA- or NTA-based IMAC phases still call for a mildly acidic loading buffer at pH 2.8-3.5. On the other hand, as the complexation between Fe$^{3+}$ and the catechols on PDA has one of the highest known binding constants of metal-ligand chelates (K$_d$ $\sim$ 1 x 10$^{-37}$ to 1 x 10$^{-40}$ M) [57-60], there is no concern about the leaching of Fe$^{3+}$ ions from the nylon-PDA, even at a much lower operating pH.

In order to present a greater challenge to the chromatographic isolation of phosphopeptides, the 1:1000 mixture of the β casein and BSA digests was also evaluated. The lower pH loading buffer and the washing step with water was not sufficient to remove the abundant nonphosphopeptide fragments in the sample, which results in a huge elution peak shown in Fig. 6b. The mass spectrum has a complex profile with no discernable phosphopeptide peaks. Fig. 6c shows the chromatogram of the same sample, however, with an extra step of washing with 50% ACN. The unretained non-phosphopeptide species were washed out before
the final step phosphopeptide elution. The mass spectrum obtained shows much cleaner profile with high intensity of phosphopeptide fragments.

It is also worth mentioning that the sample injection volume here is 100 uL, (approximately the column volume) and loaded with salts and denaturants, which potentially cause severe interference with ESI ionization, consequently suppressing the analyte signals. This is indeed the case observed in the signal of the flow through peaks, the total intensity of which is expected to be at least 2 orders of magnitude higher than the elution peaks. However, due to high salt and denaturant concentration, most of the peptides in the sample were not detected. Standard practice in peptide digest sample preparation for mass spectrometric analysis usually calls for the tedious and time-consuming steps of desalting. Herein lies another advantage of nylon-PDA C-CP fibers with respect to their application towards phosphopeptide enrichment. Highly efficient fluid movement through microchannels in packed C-CP fiber column, along with the mildly hydrophobic Fe$^{3+}$-PDA-nylon 6 surfaces, allows small molecules abundant in protein digest sample to flow through unretained in the loading step. As a result, the signal of the eluted target analytes is not compromised.

6.4 Conclusions

This study demonstrates a simple method of modifying nylon C-CP fibers for the capturing of phosphopeptides in complex protein digest samples prior to MALDI and ESI-MS analysis. The modification process takes advantage of the spontaneous oxidative polymerization of dopamine to form a uniform
polydopamine coating on the fiber surface. The binding of Fe$^{3+}$ ions onto this polydopamine coating is strong and can be used for the phosphopeptide enrichment. Nylon-PDA packed in a column format can be mounted onto an HPLC system for the isolation of phosphopeptides which can either be captured for off-line analysis using MALDI or coupled to simultaneous on-line ESI-MS analysis. Indeed, the use of the Fe$^{3+}$-PDA-nylon 6 fibers to isolate phosphopeptides followed by phosphopeptide LC-MS analysis is expected to hold great potential.

Beyond the basic demonstrations presented here, there are certainly challenges as one moves to actual biological matrices. As seen in the mixed protein-digest samples above, there is a tendency for acidic peptides to be carried through the process. Changes in metal ion identity and processing pH are common parts of method optimization. As demonstrated in the case of the on-line separation, an intermediate wash using mild organic solvents can also pay benefits in the realized selectivity and sensitivity. These methods and approaches are common in the field and can be readily exercised on the C-CP fiber phase.

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CHAPTER SEVEN

SUMMARY AND FUTURE WORK

Summary

Capillary-channeled polymer (C-CP) fibers have been extensively studied in this laboratory as an alternative stationary phase for analytical separations and downstream processing. When packed into a column format, their characteristic eight axial wings inter-digitate along their length to form aligned micro-channels that facilitate highly efficient fluid movement [1-2]. Advantages of C-CP fibers include low costs, ease in column construction, high column permeability, efficient mass transfer and low operating backpressure. In their native forms, C-CP fibers made of polypropylene (PP), polyethylene terephthalate (PET) and nylon have been utilized as stationary phase in a variety of chromatographic modes including reversed-phased [3], ion-exchange [4] and hydrophobic interaction [5-6]. In order to further expand the scope of their applications, this laboratory has recently investigated different approaches to modify the surface of C-CP fibers so that they can be used in various other chromatographic modalities. This research works towards this end with a narrowed focus on a special mode of separation called affinity chromatography. The ultimate goal is to produce a high density of ligands or functional groups through mild modification processes that do not compromise the physical characteristics as well as their highly desired attributes. Chapter II and III investigated a simple physical adsorption of recombinant Staphylococcus
*aureus* protein A (rSPS) onto the surface of PP C-CP fibers to generate an affinity stationary phase for the capture of immunoglobulins [7-8]. In chapter IV and V, nylon C-CP fibers were functionalized through the free-radical polymerization of glycidyl methacrylate (GMA) in a residential microwave using ceric ammonium nitrate (in dilute nitric acid) as the initiator. The reactive epoxide groups on GMA polymer serve as the anchoring point for the attachment of different ligands. In chapter IV, imminodiacetic acid ligands, upon being coupled to nylon-GMA, were charged with copper (II) ions and used as stationary phase for immobilized metal ion affinity chromatography (IMAC) protein separation. In chapter V, imminodipropionitrile was attached to nylon-GMA, which was then converted into amidoxime through a reaction with hydroxylamine. Nylon-amidoxime was then applied to the sample clean-up and preconcentration of uranium ions from ground water or urine samples. Chapter VI reported a simple modification system utilizing a dilute aqueous solution of dopamine in TRIS buffer, pH 8.5 to coat polydopamine onto C-CP nylon surface. The resulting polydopamine-coated nylon C-CP was then charged with \( \text{Fe}^{3+} \) ions and used for the capture and enrichment of phosphopeptides for mass spectrometric analysis.

**Future directions**

The microwave-assisted polymerization grafting proves to be a simple method to modify the surface of C-CP nylon fibers. The monomer glycidyl methacrylate (GMA), once grafted on C-CP fibers, provides a convenient platform for further modification using epoxide coupling chemistry. As the modification
scheme involving many complicated and lengthy reactions, the fibers were chemically modified as a loose bundle during the whole process for the convenience of washing and handling. However, after going through multiple steps, the modified fibers may become twisted or folded, which result in inhomogeneous column packing and bad column performance. An alternative solution to this issue can be addressed by performing modification steps on fibers already packed in columns. As the fibers are well-aligned throughout the modification process, the integrity of the packing is expected to go through minimal perturbations. This in-column modification technique can be applied to the first step of glycidyl methacrylate polymerization grafting onto C-CP nylon fibers to produce to epoxy-activated fibers already packed in column format ready for subsequent ligand attachment. The ligands of choice can then be coupled to the expoxy-activated fibers by continuously pumping the ligand reaction media through the column. This modification step, which can be conveniently done using an HPLC pump at optimal flow rate depending on the reaction rate, will ensure the formation of uniform and maximal ligand attachment on the expoxy-activated fiber surface.

As the epoxide groups on GMA polymer are rather stable at pH below 8, nylon-GMA can be pre-made and stored for an extended period of time before use [9]. These epoxides are reactive towards ligands containing sulfhydryl, amine, or hydroxyl groups, allowing future attempts to attach various affinity ligands to the epoxide-activated nylon-GMA C-CP fiber for a wide range of both analytical and preparative scale separations [9-10]. The reactivity of the terminal epoxide to these functional groups follows the order \(-\text{SH} > \text{–NH} > \text{–OH}\) [10]. Strong alkaline
conditions (pH 11-12) are required for the coupling of hydroxyl containing ligands while milder conditions (pH 7-9) are sufficient to target the amine and sulhydryl groups on the ligands [9-10].

Most well-established ligand immobilization methods target hydroxyl groups, which are abundant on the popular polysaccharide-based stationary support used in affinity chromatography [9]. The lack of hydroxyl groups limits the options of coupling methods that can be applied for the attachment of affinity ligands on the surface of native C-CP fiber. This limitation can be overcome through the hydrolysis of the terminal epoxides on the GMA polymer grafted on nylon C-CP fibers into diols, which not only make the fibers more hydrophilic but also provide available hydroxyls for various well-established ligand immobilization methods such as cyanogen bromide [11-12], carbonyldiimidazole [13], carbodiimide [14] or Schiff base (reductive amination) method [15-16].
References


6. Lei Wang; Marcus, R Kenneth, Evaluation of protein separations based on hydrophobic interaction chromatography using polyethylene terephthalate


APPENDICES
Figure A.1  ATR-FTIR spectra of the native and sequentially-modified nylon 6 C-CP fibers.
**Figure A.2** SEM images of native nylon and sequentially-modified nylon 6 C-CP fibers and the column cross-sections.
Figure A.3  Column back pressure of native nylon 6 and nylon-IDA fibers packed in microbore column format (300 mm long, 0.762mm i.d.) as function of the flow linear velocity. Mobile phase = 1 M NaCl.