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CAPILLARY-CHANNELED POLYMER FIBERS AS STATIONARY PHASES FOR PROTEIN CHROMATOGRAPHY

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ABSTRACT

Capillary-channeled polymer (C-CP) fibers have been studied in this laboratory as stationary phases for protein separations in high-performance liquid chromatography (HPLC). C-CP fibers are uniquely shaped so as to include eight continuous capillary-channels which interdigitate once packed into a column. The packed column resembles a monolithic structure of unobstructed flow through capillary channels which reduces backpressure and increases linear velocity, reducing separation time. Fibers are effectively nonporous with respect to macromolecules, resulting in fast mass transfer and high sample recovery. C-CP fibers made from polypropylene (PP) yield a fairly homogenous hydrophobic surface suitable for reversed phase (RP) chromatography. In a microbore C-CP column, separations can be done very quickly, in less than ten minutes, even at low flow rates, saving time as well as money in column cost, solvent cost and waste generation.

Electrospray ionization mass spectrometry (ESI-MS) analysis provides more analytical information than multi-wavelength detectors. However, the low flow rates needed (<0.5 mL min⁻¹) for optimal spectral clarity generally imply low linear velocities and slow separations if liquid chromatography (LC) in tandem with mass spectrometry (MS) is desired. To this end, decreased C-CP column diameters (0.5 mm i.d.) were employed to increase linear velocity, and therefore speed separation time, without the need to increase flow rates. Ribonuclease A, cytochrome c, myoglobin and lysozyme were loaded in phosphate buffered...
saline and urine. Matrix was removed using a water loading phase before applying a gradient of ACN to elute proteins according to hydrophobicity into ESI-MS.

The truly interesting and research relevant proteins are often found in small concentrations in serum. Analysis of biofluids for potential biomarkers (i.e. proteins) has been of great interest in medicinal research; however, most biomarkers are typically found in minute concentrations and masked by more abundant proteins, prominently, serum albumin. A large portion of albumin hinders the collection of the proteins of interest. Modifying nylon C-CP fibers with cibacron blue dye, an albumin-specific ligand has the potential to selectively remove albumin from serum samples.
DEDICATION

I would like to dedicate this thesis to my family, my husband and my two best friends. To my family, thank you for being my audience from age 3 to 25. Thank you, my dear siblings, for fueling my sibling rivalry and for listening to my scientific ramblings even though I was probably boring you to tears. Thank you to my parents for the sacrifices you have made so that I might live to learn for a lifetime without limits. Thank you to my G-Ma, for being my example that women are strong and smart and capable, especially under pressure.

To Jane, for supporting my ambitions and always picking up where we left off like no time has passed at all. Kirstin, this place would have crushed me if you were not here with me. If Clemson gave me one thing, it was you.

To Chuck, there is not space enough to thank you here. You have given me everything. And while this thesis is not the gift you deserve, it is what I give to you, for now.
ACKNOWLEDGMENTS

I would like to acknowledge the many mentors in chemistry that have encouraged me to pursue higher levels of scientific understanding. Notably, I extend many thanks to my very first mentor in chemistry Dr. Dominic Frollini of Upper St. Clair High School. His willingness to answer my many questions, even when I was not the most capable student, opened my eyes to the avenues of chemistry beyond general curriculum and inspired my undergraduate major decision. I would also like to extend my gratitude to the dedicated chemistry professors at Indiana University of Pennsylvania. I learned invaluable techniques and concepts. I truly appreciate the time and genuine support given to not just me, but all chemistry students.

I would also like to acknowledge my fellow graduate students at Clemson University. Through all of the stress and frustration, I am eternally thankful for my friends who always lent an ear or a shoulder or a glass of wine.

I would also like to acknowledge my advisor, Dr. Marcus, without whom I would not be earning this Master’s degree.
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Understanding the proteome has become an area of ever increasing interest in the scientific and medical communities. Since the term proteomics was first coined in the early 1990’s scientists have been working to uncover the many truths that proteins hold [1]. It is generally known that proteins are foundational in living beings; they make up tissues, exist in, on and around cells, and perform functions based on their shape. Proteins are integral in every function in the body, from contracting a muscle to fighting a disease. It is for this reason that illuminating the many intricacies of protein mechanisms has become so important. Knowing the roles proteins play allows for the manipulation and exploitation of the proteome to a medical advantage. One of the fastest growing areas of the pharmaceutical industry is that of protein therapeutics, especially and most commonly disease treatment with antibodies [2, 3]. On the other hand, with a more fully understood proteome, protein diagnostics are possible, using either the presence of a protein, the absence of a protein or the abnormal level of a protein [4, 5]. What is more, monitoring the specific protein levels can also help guide treatments. All of these benefits of understanding the proteome rely on a depth of understanding. In order to understand and use proteins to our advantage we need ways to study protein interactions as well as detection methods. To look at individual proteins demands the ability to separate.

The Marcus Lab has, over the past 12 years, explored the use of capillary channeled polymer (C-CP) fibers as stationary phases for protein separations [6-
Utilizing fibers generated in the Clemson University materials science department, fibers are packed into microbore columns for use in high performance liquid chromatography (HPLC) and as solid phase extraction (SPE) spin-down tip formats [11, 12]. The fibers are made from several available polymers; polypropylene (PP), nylon and polyester (PET), which can be used in various modes of chromatography; reversed phase, ion exchange, hydrophobic interaction chromatography and chemical modifications for other modes, respectively [11, 13, 14]. Each fiber is extruded with a unique cross-sectional shape with protrusions forming eight collinear channels which run down the length of the fiber. As these fibers are mechanically drawn into microbore or standard inner-diameter columns, the protrusions interlock with those of adjacent fibers forming a series of much smaller channels.

The channels promote unobstructed fluid flow, which in HPLC reduces back pressure and increases linear velocity; this is especially useful with viscous samples. Another exploited property of the C-CP fibers is the nearly non-porous surface. C-CP fibers have been specifically employed to separate proteins, as the pore size is small enough that no protein can enter, thus virtually removing the C-term broadening with respect to the van Deemter equation [14, 15]. The downside to this feature is a severe reduction in surface area, and thus binding capacity, when compared to porous stationary phases. Another potential advantage of polymer stationary phases is the chemical stability over a broad range of pH.

One clear benefit of the C-CP fibers has been for solid phase extraction (SPE) format separations [12, 16-19]. SPE is a standard sample clean-up step
that serves to remove matrix salts prior to instrumental data collection; this is especially the case in mass spectrometry (MS) [20, 21]. SPE works by adsorbing analyte to a stationary phase while salts and matrix are washed off of the sample and stationary phase, then a suitable eluent removes the analyte and can proceed to further analysis. In order to analyze a sample in MS, analyte must first be ionized. If the sample is in an ionic solution, salts are almost indefinitely more ionizable and will be preferentially ionized; this can result in noisy spectra or the loss of analyte signal all together. In previous publications, a small length of C-CP fiber column was attached to a micropipette tip so as to form an SPE-tip which could be used in a centrifuge to provide a driving force for fluid movement [12, 22]. Protein samples in buffer and matrices are loaded into the tip, spun, washed with water, spun, and finally eluted with 50/50 ACN/Water. Eluted proteins can be spotted and analyzed using MALDI-MS. Similarly, full length columns have been used as injection loops in electrospray ionization (ESI) mass spectrometry to desalt inline [23]. The resulting spectra generally have an overall increase in signal to noise ratio. SPE using C-CP fibers is fast, simple and significantly less expensive than commercially available SPE cartridges, with a single SPE-tip costing less than $0.25.

Capitalizing on the hydrophobic nature of the polypropylene fiber surface allows not only SPE type separations, but reversed phase HPLC as well. It has been demonstrated that three proteins can be separated using a gradient from 90:10 H₂O:ACN, to 50:50 H₂O:ACN over 2.5 minutes [24]. Optimum fiber packing, in terms of interstitial fraction, was determined to be ~0.63 for reversed phase chromatography on polypropylene. This fiber packing is translated into
rotation count for ease of understanding in the lab, and these rotation counts will
be specific to column inner diameter necessarily. A range of column inner
diameters and flow rates were also analyzed for resolution and plate height.

The old adage that “time is money” is a driving force in industry and
developmental research. Higher throughput, i.e. analyses per minute, increases
money made per minute. The low flow rates used to obtain low plate heights on
large i.d. columns do not promote high throughput. Meanwhile the high flow rates
are typically only suitable for absorbance spectral monitoring. Ideally, these fiber
columns could be coupled with electrospray ionization (ESI) mass spectrometry
(MS). ESI tends to ionize better at reduced flow rates, with preferred flow rates
between 200–600 μl min⁻¹ [25]. This reduced flow rate also decreases solvent
consumption and waste generation. The benefit of using mass spectrometry for
detection is the added benefit of chemical and structural identification. Using
retention time as the only indicator of an analyte of interest leaves significant
room for error in identification. The challenge is to efficiently separate a sample in
a short period of time using a flow rate compatible with ESI.

The focus of this thesis will highlight a method of protein analysis using
microbore C-CP fiber columns coupled with ESI-MS. Multiple points of interest
will be combined to result in an ideal separation scheme. These points include;
reduced flow rates for use with ESI, reduced column i.d. for increased linear
velocity at low flow rates, and optimized gradient conditions for efficient protein
separation. Currently many lines of research in protein LC-MS use monolithic
stationary phases [26-29]. In general the comparisons of the C-CP fibers unique
to this research group to monolithic stationary phases include reduced back
pressure, as a result of through-pores, and chemical stability of the polymers. The glaring difference tends to be the cost; monolithic columns tend to be difficult to make with appreciable column to column variability, thus making commercial columns very expensive, even for a very small column. C-CP columns, conversely, are very easy to make with significantly inexpensive materials. Fibers are generated in kilometers long spools such that the fibers from a single batch are the same, decreasing the column variability.

Employing polypropylene C-CP fibers as reversed phase columns in line with LC-MS and their potential as the first dimension in LC-LC-MS could impact the fields of proteomics and urinomics as a method which reduces cost through materials and increased throughput. Proteomics aims to discover and study biomarkers as diagnostics and therapeutics for disease. Urinomics hopes to apply proteomics and other “omics” to the study of urine as a non-invasive method of biomarker collection. But the added challenges of protein separations in high salt matrices demands a cost effective method of separation that includes an inline analysis. Coupling C-CP fibers to LC-MS provides a possible answer to these challenges through the removal of salts, reduced back pressure, higher linear velocity, reversed phase separations all while in-line with an established method of identification.
CHAPTER TWO
POLYPROPYLINE CAPILLARY-CHANNELED POLYMER (C-CP) FIBERS FOR
REVERSED PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY
MASS SPECTROMETRY SEPARATION AND ANALYSIS OF
PROTEINS FROM BIOLOGICAL MATRICES

Introduction

One of the largest fields of biomedical research is the field of proteomics. With the realization that proteins can serve as markers in disease diagnostics and treatment, it is logical that research has aimed to decode the information contained in protein compositions. With more complexity than the human genome, the Human Proteome Project has attempted to map the vast protein landscape [30]. The search for biomarkers and the thresholds of the marker status; presence, absence, abundance, interaction as well as the structure of proteins have been some of the goals of current research. In order to find and use proteins as biomarkers, a sample must be drawn and prepared for analysis, examples include urine, serum, saliva, tissue and sexual fluids [31]. While all of these sample sources are of interest, urine has become increasingly popular as a protein analyte source.

Urine’s popularity is due to the fact that collection is non-invasive, sample size in most cases is large and the normal protein levels remain relatively stable in a subject, so changes observed reveal information [32], [33]. Urine protein levels have been used to profile pediatric cystinuria, diabetic nephropathy progression among other diagnostics and health monitoring [34], [35], 7. One of
the biggest challenges in the analysis of urine and other complex protein samples is matrix effects. Proteins of interest are generally in low concentrations and can be overshadowed by interfering compounds like salts and high abundance serum proteins which provide little diagnostic value [36].

Because of the challenges in protein monitoring and analysis due to the complexity of samples [37, 38], a separation step or multiple steps are standard practice in proteomics [20, 39]. Current methods include solid phase extraction (SPE), 2D gels, ligand binding assays, precipitation steps and filtration steps [40-43]. Each of these methods adds time to analysis as the sample components are prepared prior to a detection method such as mass spectrometry. SPE is often used as a method of desalting to remove matrix and is usually a bottleneck point in the throughput for sample analysis. After SPE preparation, sample is separated using reversed phase chromatography or capillary electrophoresis prior to coupling with ESI-MS for detection [20, 39]. This added step and the inherent challenges of LC-MS have left room in the area of protein LC-MS for novel research.

As a proposed method of filling this gap, novel capillary-channeled polymer (C-CP) fiber stationary phases have been studied. The fibers unique 8 channeled shape allows for dense packing by interdigitation. But while packing is dense and uniform, there is not an extreme increase in backpressure as there are straight channels running the length of the column. The channels promote fluid transport and are therefore especially well-suited for analysis of viscous biological samples. Another advantage of the C-CP fiber columns is their virtually non-porous surface [15]. The lack of pores reduces mass transport limitations for
large molecules, thus reducing the van Deemter c-term broadening. This allows for increased linear velocities without significant peak broadening.

The current parallel to these unique fibers and their interlocking structure is the field of monolithic stationary phases. Monoliths boast low backpressures as the stationary phase is one piece which suffers little to no compression under pressure, unlike packed bed columns. Monoliths can also be synthesized to allow different modalities including ion exchange, reversed phase and affinity chromatography. The monoliths are often generated in column; however the formation of the structure can be non-uniform, irreproducible, incomplete and expensive. Monolithic stationary phases have paired well with ESI-MS yielding very fast baseline separation of a variety of compounds, including proteins [44].

Often the challenges in LC-MS come from physical limitations. Flow rates that are ideal in chromatography are too voluminous to efficiently ionize and desolvate in an ESI source. Ion pairing agents in chromatography, like TFA, are ion suppressants in mass spectrometry [45]. Developing methods that are reliable and maintain the sensitivity and speed can pose its own challenges [46]. Coupling liquid chromatography to mass spectrometry usually involves some sample preparation method like filtration or SPE which quickly becomes the analysis bottleneck because the time to prepare a sample is longer than the instrument run time, and is usually not automated in line with detection [32]. In some cases, differences in matrices can shift the retention time of an analyte [47].

While the challenges in mass spectrometry can pose problems, the advantages far outweigh the option of UV/Vis absorbance detection. UV/Vis does
not provide specific information about the structure or identity of an analyte, merely its presence. A major goal of proteomics is to identify an analyte as well as gain some understanding of the structure and function of the protein of interest [48, 49].

Current trends in fast protein separations use monolithic columns and reversed phase gradients. Nischang et. al. used a 15 cm poly(BuMA-co-EDMA) monolithic capillary columns with a 0-50% ACN+0.1% formic acid gradient to separate three proteins [50]. They were able to achieve baseline resolution in 6 minutes at a flow rate of 1.6 μL min⁻¹. Similarly, Terborg et. al. added gold nanoparticles to poly(glycidyl methacrylate-co-ethylene dimethacrylate) capillary columns for separations of 3 proteins. The gradient conditions 5-70% ACN + 0.1% TFA in water over 7.5 min was unable to baseline resolve all proteins in 8 min at a flow rate of 2 μL min⁻¹ [51]. Finally, Vaast et. al. using a poly(styrene-co-divinylbenzene) monolithic capillary column separates a 5 protein suite in under 4 minutes with nearly baseline separation at a flow rate of 4 μL min⁻¹ [52].

We describe here a method of protein separation using polypropylene C-CP fibers for analysis using UV/Vis absorbance and ESI-MS detections.

**Experimental**

Polypropylene capillary channeled polymer fibers were melt-extruded by the Clemson University College of Material Science and Engineering (Clemson, SC, USA). Polypropylene, by in-house designation, PP4, was chosen based on previous publications in this lab [11, 53]. With a uniform shape and maximum cross-sectional area of 40 μm, it provides the highest separation efficiency. PP4
fibers were provided to the Marcus lab on a spool containing a continuous length of fibers over 5 km. Fibers were bundled using a rotary counter to translate optimum packing to a rotation count. After bundling, fibers are heat shrunk using boiling water and then cleaned sequentially in water, acetonitrile and methanol. Clean fibers are pulled by hand using a monofilament through a length of PEEK tubing (Cole-Parmer, USA) of two different inner diameters; 0.8mm and 0.4mm. Columns were packed to an interstitial fraction of ~0.63, determined by uracil injection.

Stock protein solutions of 500 μg mL⁻¹ each ribonuclease A, lysozyme, myoglobin, transferrin and cytochrome C from Sigma Aldrich (St. Louis, MO, USA) were made up in MilliQ water (conductivity 18 mΩ/cm) + 0.1% TFA and kept frozen between uses. Mobile phase components of the same MilliQ water + 0.1% TFA and HPLC grade acetonitrile (ACN) +0.1% TFA were prepared in house. For matrix studies, proteins were dissolved in 100 mM phosphate buffered saline (PBS) with reagents from (Sigma), and synthetic urine matrix at 100 μg mL⁻¹. Synthetic urine mimics the salt composition and pH of a human sample.

Gradient conditions studied varied but all began at 100% MiliQ water + 0.1% TFA and ended at 50:50 H₂O:ACN. Linear velocity and interstitial packing studies were performed on a Dionex Ultimate 3000 HPLC system with a multi-wavelength UV/Vis detector controlled by Chromeleon 6.7 software (Thermo). The remaining experiments were performed on a Thermo Scientific Surveyor+ LC-ESI-MS system.
Results & Discussion

Previous work in this group has demonstrated the ability of C-CP fibers to separate bio-molecules. In general, these separations have been done on 4.6 mm, 2.1 mm and 1.0 mm inner diameter (ID) C-CP fiber columns. One of the driving factors of the fiber column success is the low back pressure exhibited which allows for very high flow rates. As flow rates increase, so must linear velocity, which in the case of virtually non-porous stationary media, increases the rate of mass transfer, and ultimately the speed of separation [54]. It is with this logic the following study was presupposed. By decreasing the inner diameter of the column, linear velocity of a particular volume flow rate must increase. Figure 2.1 demonstrates this phenomenon. Uracil injections on columns of equal length and interstitial fraction of approximately 0.63, were used to calculate linear velocity at various flow rates. At a flow rate of 0.5 mL min\(^{-1}\) the microbore column, 1.0mm ID, shows a linear velocity of about 33 mm sec\(^{-1}\), while the column with half the inner diameter exhibiting a linear velocity of over 77 mm sec\(^{-1}\). At flow rates over the 0.5 mL min\(^{-1}\), the 0.5 mm ID column exhibits significant back pressure. This is not considered a serious draw back because the intended application of this system is to couple to electrospray ionization mass spectrometry, which ionizes best in a range of flow rates between 0.25-0.5 mL min\(^{-1}\).
C-CP fiber columns have been studied for the use of reversed phase separations of biomolecules for several years in the Marcus lab [7, 8, 11, 55]. In order to couple the fiber columns to an HPLC-ESI-MS system, several parameters needed to be optimized. A comparison of the separation of three proteins was used to understand the effect of flow rate. Figure 2.2 shows the separation of ribonuclease A, cytochrome c and human transferrin at 100 μg mL⁻¹ each in PBS buffer, with a single gradient program of 100% MilliQ water + 0.1% TFA to 100% ACN + 0.1% TFA in 10 minutes. The flow rate of the separation was varied from 0.1-0.5 mL min⁻¹. Overall, peak width decreases and resolution increase with increasing flow rate. The resolution of the first and second peaks,
ribonuclease A and cytochrome c respectively, reach 2.04 +/- .03 at the maximum flow rate, as seen in Table 2.1.

Table 2  Resolution calculated from peak 1; ribonuclease A, and peak 2; cytochrome c, in Figure 2.2.

<table>
<thead>
<tr>
<th>Flow Rate (mL min⁻¹)</th>
<th>Linear Velocity (mm sec⁻¹)</th>
<th>Resolution +/-</th>
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<tr>
<td>0.5</td>
<td>62.0438</td>
<td>2.04 0.03</td>
</tr>
<tr>
<td>0.4</td>
<td>50.8982</td>
<td>1.76 0.06</td>
</tr>
<tr>
<td>0.3</td>
<td>37.7778</td>
<td>1.63 0.02</td>
</tr>
<tr>
<td>0.2</td>
<td>24.8538</td>
<td>1.38 0.02</td>
</tr>
<tr>
<td>0.1</td>
<td>12.14286</td>
<td>1.15 0.03</td>
</tr>
</tbody>
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The overall time of separation is also reduced by more than half for the 0.5mL min⁻¹ compared to the 0.1 mL min⁻¹ example. While it is clear that optimally a higher flow rate is preferred, this figure also serves to support the use of a flow rate near 0.25 mL min⁻¹ as peak shape and resolution remain. As this experiment used the larger 0.8 mm ID column, the pressures extolled on a 0.5 mm ID column could become problematic. To use 0.25 mL min⁻¹ provides a compromise between separation characteristics and pressure constraints as well as ESI operational flow rates.
Figure 2.2 Gradient elution (100:0→0:100, H₂O+0.1% TFA:ACN+0.1% TFA) of 100 µg mL⁻¹ each: ribonuclease A, cytochrome c and transferrin from a 30cm x 0.4mm ID column packed with polypropylene C-CP fibers. Flow rate was varied. After choosing the flow rate, gradients were varied at the 0.25 mL min⁻¹ flow rate. Each gradient program had a one minute loading segment before the initiation of the gradient. Gradients in this experiment were kept linear, that is, at the initiation of the gradient, the rate was constant over the time period. Gradients each ran from 100% MilliQ water + 0.01% TFA to 50% ACN + 0.01% TFA. Figure 2.3 shows the effects each gradient had on the pairs of peaks. Resolution of each peak-pair was calculated using the resolution equation:

$$R = \frac{2(t_2-t_1)}{w_2 + w_1}.$$  

Four proteins separated in each experiment; ribonuclease a, cytochrome c, lysozyme and myoglobin. While there is a trend toward increased resolution with decreasing gradient rate, there are some points where the trend is not followed.
**Figure 2.3** Resolutions calculated between neighboring peak pairs. Gradients were varied linearly by change in ACN+0.01%TFA and are represented by %B/min, which is a gradient rate of 25 %B/min would take 2 minutes to reach 50% B.

These instances, such as gradient rates 6.25 and 5 %B min⁻¹, in which the resolution remains the same or decreases even at decreasing gradient rate can be explained by longitudinal broadening. As it takes longer for proteins to elute in the mobile phase, the proteins diffuse and broaden the peak shape, reducing the resolution.
Figure 2.4 Chromatographic traces of the a) UV at 280nm and b) Total ion chromatogram (TIC) of the four protein suite solution made in synthetic urine matrix. Flow rate of 0.25 mL min$^{-1}$ gradient conditions: 1 min hold in 100% MilliQ water + 0.01% TFA, 0-10% ACN + 0.01% TFA in 30 seconds, 10%-50% ACN in 5 min.

Figure 2.5. Mass spectra shown are representative of each of the four peaks in Figure 2.4, peak 1 represented by a), peak 2 by b) and so on. nH$^+$ charge states are labeled. X-axis denotes m/z.
Figures 2.4 and 2.5 show the ability to separate a four protein mixture; ribonuclease A, cytochrome c, lysozyme, and myoglobin, at 100 µg mL\(^{-1}\) each, from undiluted urine matrix for analysis by ESI-MS. Figure 2.4 shows the chromatographic traces obtained. There is a shift in retention time and a broadening of peak shape from the UV trace to the total ion chromatogram (TIC), this is a result of a change in system tubing ID. Figure 2.5 are the peaks analyzed by MS with the nH\(^+\) charge states labeled. Each peak’s spectrum is determined by averaging the spectra of the center of the elution peak, while subtracting the spectra of the neighboring baselines. The ability to deconvolve individual proteins from a chromatograph increases the chances of identifying the analyte. Typically a UV chromatogram gives little to no identification or structural information. With the data collected the peaks can be correctly identified as a) ribonuclease a, 13,700 Da, b) cytochrome c, 12,327 Da c) lysozyme, 14,300 Da and d) myoglobin, 17,000 Da. It is also noteable that the charge states of the eluted proteins are, in general, higher than those of proteins that are directly injected into the system. Proteins tend to denature on the surface of the fibers and remain this way upon elution. It is well understood that a protein will exhibit higher charge states in ESI when denatured as more protonation sites are available [56].

The ability to couple HPLC to ESI-MS has important advantages, and its own set of challenges. Flow rate restrictions being one large factor, and conditional constraints being another. In this case, flow rates were kept at 0.25
mL min\(^{-1}\) to maintain optimal ionization. Trifluoroacetic acid (TFA) is a very common ion pairing agent used in reversed phase chromatography to control mobile phase pH and peak shape. However, TFA is a known ion suppressant in ESI-MS\[^{45}\]. In order to reduce the ion suppressant effects of TFA, the concentration has been reduced from a typical 0.1 v/v% to 0.01%, rather than replacing with a different ion pairing agent like formic acid \[^{44, 45}\]. In practice, the loss in resolution from 0.1% TFA to 0.01% TFA is approximately 0.046. Because of the minimal loss in resolution, 0.01% TFA was used in all ESI-MS experiments.

**Conclusion**

This work has served to compile many past findings surrounding the use of C-CP fibers as stationary phases for protein separations. Previously, this four protein suite was separated over 25 min at 1.5 mL min\(^{-1}\) flow rate \[^{55}\]. It is notable that in the case of C-CP fiber columns, increased flow rates result in increased resolution. This is attributed to the more favorable mass transfer rates at higher flow rates allowing a protein that finds a favorable mobile phase composition to be “swept” off the fiber quickly, resulting in better peak shape and resolution. Decreasing column diameter has served to reduce the solvent consumption in protein separations. While the times for resolved separations may not be as short as those previously reported, solvent consumption is decreased as flow rates are significantly reduced \[^{57}\].

Further, the use of C-CP fibers as SPE desalting matrices has shown promise in many practices. Tip format desalting prior to MALDI and ESI MS analysis has
shown significant reduction in noise and increased spectral clarity [12, 22, 58, 59]. Following these publications, the application of the C-CP fiber column as an in-line desalting injection loop was demonstrated as a simple in-line clean-up step to reduce noise in protein MS due to matrix effects [23]. Compiling these methods of reversed phase chromatography using gradients, decreasing column inner diameter and inline SPE desalting of matrices prior to ESI-MS has shown the ability of the ever-cost efficient C-CP fiber column to provide a single step method of protein sample preparation (SPE), separation (RP-HPLC) and analysis (ESI-MS) in under 10 minutes. This is a comparable timescale to current reversed phase protein separations on monolithic columns [27].

In the future, work would include employing these methods to quantify proteins in matrices to further the application of C-CP fibers for proteomic analysis. It would also be interesting to see the effect that temperature would have on C-CP fiber column separations. A further reduction in back pressure would be expected, but it would be interesting to see the effect on resolution, peak shape and separation time, as the solvation strength should increase with temperature. Other future studies should look into the transfer kinetics and whether, in the case of C-CP fibers, the flow rate or the linear velocity is the driving force of the separation efficiency. While the two parameters are clearly dependent upon one another, it seems that even when the linear velocity is comparable, if the flow rates are different, the separation time is also different.

Literature searches surrounding this project have also spawned questions into glycoproteomics and whether the C-CP fibers have the capacity to separate
glyoproteins to further diagnostics [60, 61]. Current trends in proteomics show a
desire to create single step bioreactor/analyzers by immobilizing trypsin on the
surface of a column to digest a protein and simultaneously separate the digest
products. Research in this group has shown promise in the field of surface
modifications via lipid-tethered ligands (LTL) [62, 63]. It would be conceivable to
immobilize a trypsin enzyme to the LTL to digest proteins on column.
CHAPTER THREE

CIBACRON BLUE TRIAZINE DYE LIGAND MODIFIED CAPILLARY-CHANNELED POLYMER (C-CP) FIBERS FOR THE REMOVAL OF SERUM ALBUMIN

Abstract

The affinity ligand quality of the triazine dye, cibacron blue, toward serum albumin has been well studied [64-68]. By immobilizing the ligand to a stationary phase, affinity separations can be performed in order to remove bulk albumin from serum samples. This decrease in albumin concentration may serve as a direct advantage for proteomics as large amounts of serum albumin hinder the study of low abundance proteins of interest [69]. Capillary-channeled polymer (C-CP) fiber columns have shown promise in protein separations as they demonstrate low backpressures ideal for viscous protein samples, virtually non-porous surfaces with respect to proteins which reduce van Deemter C-term effects, and high mass transfer rates to increase solute interactions at the stationary phase surface. Combining these attributes with the specificity of cibacron blue would generate a useful depletion column that could be used in spin-down or column formats. Nylon’s terminal amine can react directly with the chloride moiety of the triazine dye covalently. Complementarily, head-group-modified PEG-lipids can be used on polypropylene (PP) fibers for the same end goal [62, 63]. While this may be a useful study, results were far exceeded by commercial and previous publications [64, 67-72].
Introduction

The ever growing field of proteomics is in need of evolving technologies in order to handle the complexities and problems inherent in biological samples. The study of proteins aims to solve medical problems and explain biological processes, aiding in the progress of many fields including, pharmaceuticals, cell biology and biotechnology. In general, the most abundant protein in any whole serum sample will be albumin [73]. However, the truly interesting and research worthy proteins are found in minute concentrations in serum. With the large proportion of albumin in samples, collecting the proteins of interest can pose serious problems.

The Marcus group has been developing novel stationary phases for the separation of proteins via HPLC [7, 8, 10, 74]. With the help of the Materials Science Department of Clemson University, several different versions of capillary-channeled polymer (C-CP) fibers have been created [6]. The polymers which make up the fiber backbone include nylon, polypropylene (PP), and polyester (PET). These plastics are melt-extruded so as to provide channels which run the length of the fibers, increasing the available surface area. When pulled through an HPLC column, the fibers clasp together to form even smaller channels that run straight through the column. The fibers are effectively non-porous, when used with macromolecules [15]. The closest current comparable technology is monolithic solid phases [75]; the advantages C-CP fibers have over the monoliths are low cost, low back-pressure, reproducibility and both pH and
physical stability. The polymeric structure of the fibers allows for reversed phase (RP), hydrophobic interactions (HIC) as well as ion exchange (IEC).

With a focus on affinity chromatography, we have been able to functionalize the fibers in order to achieve more specific separations [76]. Preliminary data and future publications include Cibacron Blue dyeing of nylon fibers and PEG-lipid functionalization of PP fibers using hydrophobic interactions. PEG-lipids have been shown to adsorb to the fibers through hydrophobic interactions, leaving a functionalized head group to “hang” in the mobile phase to interact with the analyte of interest. Cibacron Blue is known to have an affinity to albumin [68, 77, 78]. Functionalizing the fibers with the dye should add the affinity of the CB-albumin complex to the fibers’ already efficient fluid transport and high surface area with negligible pore size for optimum mass transport. This research is significant because the already defined separation capabilities of C-CP fibers will be further specified via affinity interactions to provide simple yet effective albumin depletion from samples. With fast and inexpensive sample cleanup, those interested in proteins other than albumin can easily deplete the abundant albumin from the bulk.

**Experimental**

Dying Fibers

Extruded nylon-6 fibers were obtained from the Materials Science Department of Clemson University. Fibers were placed in a dye bath containing ~1mg mL⁻¹ Cibacron Blue dye (Sigma Aldrich, St. Louis, MO, USA) kept at pH 8 with calcium carbonate. Bath was heated to 80° C with stirring, fibers soaked for
8hrs. Dyed fibers were washed successively with water, acetonitrile, acetone, ethanol and methanol before being packed into columns.

Column Construction

550 cibacron blue dye-modified nylon-6 fibers were pulled collinearly through fluorinated fluorinated ethylene propylene (FEP) microbore (0.8 mm inner diameter) tubing from Cole Parmer, resulting in an interstitial fraction of about 0.63. After a full wash using water, acetonitrile and ethanol, columns were cut into 1 inch “tips” and press fit onto micropipette tips in order to facilitate use in a centrifuge.

Experimental Setup

Column tips are loaded with protein solutions at 100 µg mL\(^{-1}\) concentrations and solution is driven through the column using centrifugal force at 1900 rcf. Effluent is collected and analyzed using a Tecan microplate reader at 280 nm.

Dye Determination

In order to determine the mass of dye on the fibers a simple colorimetric test was performed. 1 inch dyed nylon tips were dissolved in 1 mL concentrated HCl. At extremely acidic pH, cibacron blue becomes pink, so dye quantification was determined using a Thermo Genysis 10S UV/Vis spectrophotometer at 500 nm.

**Results & Discussion**

Determination of dye on nylon
Nylon-6 fibers were spooled and washed with water, acetonitrile and ethanol before dying. Fibers were left in a dye bath containing 1 mg mL\(^{-1}\) cibacron blue dye, MilliQ water and the pH was adjusted to ~9 with sodium bicarbonate. The bath was maintained at 60°C and fibers were submerged for 6-8 hours. Following dye treatment, fibers were washed heavily again with water, acetonitrile and ethanol before packing into FEP tubing. In order to determine the best method of removing adsorbed dye, several solvents were passed through tips three times in 800 µL aliquots. Figure 3.1 shows 3 different tips, the left tip shows a bare nylon fiber tip, center is a tip exposed to dye via solution load and centrifugation, the right tip is dye-bath-dyed nylon. The disparity in color between the center and right tips may indicate that the dye has merely adsorbed to
the fiber during simple exposure and that the dye bath allows bonding and 
maximum exposure of the fiber to the dye. Absorbance of eluent was read using

![Graph](image)

**Figure 3.2**: 5 wash solvents; DMSO, PBST, NH$_4$OH, EtOH and H$_2$O, were loaded in 800uL aliquots. Each was reloaded in order to pass a total of 3 times. Eluent absorbance was read at 280nm on the Tecan plate reader.

a Tecan plate reader at 280 nm. Each solvent was tested on 3 tips and 3 
absorbance readings from each elution taken. **Figure 3.2** confirms the use of 
ethanol as a wash medium. Ammonia was added as a wash step as well.

Ammonia is suggested by textile dye manufactures as a way to remove cross 
dye from laundered fabrics, so logic follows that it would be useful to remove 
non-bonded dye here. A challenge that arises here is that, even though it is 
apparent that ethanol is the best for removing non-bonded dye, it is not obvious 
when all dye has been removed. It is worth mentioning here that the UV 
absorbance intensity is much greater for the cibacron blue dye than for BSA. And 
so any stray dye in subsequent steps may have an effect on BSA quantification 
results.
Subsequent to removing non-bonded dye, total dye was determined. 2.5 cm tip portions, 5 mg nylon on average, were extracted from the FEP tubing and the dyed nylon was dissolved in 1mL concentrated hydrochloric acid. At extremely low pH, cibacron blue appears pink/red in color as seen in Figure 3.3. Absorbance readings were taken using a Genysis 10S UV/Vis spectrometer at 500 nm. 5mg mL⁻¹ Nylon in concentrated HCl was used as stock to dissolve/dilute CB. Fibers extracted from dyed nylon tip, dissolved in 1 mL HCl and read, shows 0.134 (+/- 0.0002) mg cibacron blue per dyed tip.

BSA Removal with CB-Nylon in tip format

Dyed and cleaned tips were compared to native nylon tips for the removal of BSA from a PBS solution. Figure 3.4 shows the calculated mass of the load, which is mass loaded minus mass eluted using UV/Vis calibration, the mass
washed, using a PBS + 0.1% tween 20 solution, and the calculated difference. The difference between the load and the wash represents the mass of BSA bound to the surface of the fibers. Native tips will bind BSA through hydrophobic interactions while CB-dyed tips should bind BSA based on the ligand interaction plus the hydrophobic interaction. In this case it seems that the dyed fibers actually reduce the binding capacity. Previous papers published report the use of tween20 as a method for the reduction in non-specific binding of proteins [79]. However, this data suggests that even tween20 will not completely remove adsorbed BSA. It is also worth noting the overlap of the error bars which may also suggest a statistically insignificant difference between the two sets of fibers.

**Figure 3.4:** Mass of BSA loaded on cibacron blue dyed and native nylon 1 inch tips. 100 ppm BSA loaded in PBS, washed with PBS+.1% tween 20. Difference suggests mass remaining on fiber.
In an attempt to further confirm or negate the presence of BSA on the fiber surface, fluorescent imaging using fluorescently labeled proteins was used as a qualitative control. Rhodamine-red labeled BSA (R-BSA) and yellow fluorescent protein (YFP), were graciously provided by Dr. Kenneth Christensen. **Figure 3.5** is a comparison of BSA loading between a native nylon tip and a dyed nylon tip. Image (a) is a native nylon tip with no exposure to R-BSA while (b) is the same tip after 100 µg mL\(^{-1}\) R-BSA in PBS has been passed through the tip 3 times. Image (c) is a dyed nylon tip with no R-BSA exposure with image (d) is the same tip exposed to the same R-BSA solution. There is an obvious increase in
fluorescence intensity between the native nylon tips before and after R-BSA loading, i.e. a positive control for the R-BSA loading and imaging. There was a background of fluorescence contributed from the dye itself as evidenced in (c). There is no change in fluorescence count between (c) and (d), suggesting that no BSA is binding to the dyed nylon, even via hydrophobic interaction. There is a slight possibility that the rhodamine dye attaches near or in the di-nucleotide fold of BSA which is the identified area via which cibacron blue interacts.

Ligand Interaction

Some steps were taken to ensure that the interactions expected were, in fact, foundational. First, that the dye-protein complex exists without a support phase was confirmed. A simple incubation and separation was performed using buffer exchange and ultra-centrifugation. A PBS solution of BSA and CB was incubated for 30 min with stirring to allow the protein and dye to interact. An aliquot of the incubated solution was loaded onto a PD10 buffer exchange column with an exclusion limit of 5000 Da. The blue void fraction, 3.5 mL, was collected as it should contain BSA (~65kDa) and any BSA+CB complex (~65kDa + 800 Da). Figure 3.6 (a)

Figure 3.6: PBS solution of BSA and CB was incubated for 30 min and then separated using a PD10 SEC column (5000 Da exclusion limit). Image (a) shows the second fraction containing CB. Image (b) shows an aliquot of the first 3.5 mL void fraction following ultra-centrifugation (1000 Da exclusion limit). Upper blue solution would be excluded BSA-CB complex.
shows the remaining fraction containing only the CB (~800 Da). To confirm the strength of the interaction, ultra-centrifugation was employed as verification. A fraction of the first fraction was loaded into an ultra-centrifugation filter and spun down. **Figure 3.6 (b)** shows the final separation with the upper portion maintaining its blue color from the dye. CB falls below the exclusion limit of the filter (1000 Da) and would therefore pass through if not complexed with the much larger BSA. Because the eluent in the ultra-filtration step remains uncolored, while the filtrate is blue, the BSA and cibacron blue are concluded to interact because without an interaction between the two, the cibacron blue would have passed through the filter, coloring the eluent.

Cibacron Blue Head Group Modified PEG-Lipid

The final efforts in this exploration were following in the footsteps of other methods studied in this lab to use poly ethylene glycol (PEG) lipids with a modified head group. In this case, commercial PEG-lipids with a hydroxyl modified head group were purchased from Nanocs. Triazine dyes have the ability to react with both amine and hydroxyl reactive centers, though CB does so only at elevated temperatures and pH. In this case the time and conditions needed to generate this lipid-tethered ligand (LTL) would compromise the PEG-lipid. So a complementary ligand, Reactive Blue 4 (RB4), was chosen as it has the same ligand interaction with BSA but can react at a significantly lower temperature (30°C vs. 60°C). After generation of LTLs, polypropylene (PP4) tips were exposed to the LTL solution and then a PBS solution of 100 ppm R-BSA. **Figure 3.7** shows controls of native PP4, and PP4 exposed to just the LTL (no
fluorophore). Each of those tips was exposed to the R-BSA solution imaged and then washed with PBS + 0.1% tween20 and imaged again. Side by side comparison shows that both the native and LTL treated tips capture R-BSA, while only LTL treated PP4 retains any BSA after a PBS + 0.1% tween20 wash. The retention is very low and may be considered insignificant in comparison to more robust stationary phases with the same ligand. This may be due to non-optimal lipid loading. There was also no separation of PEG-lipid+CB from the reaction solution which would also contain unreacted dye and PEG-lipid. The unreacted PEG-lipid would also adsorb to the fiber surface, reducing protein capture.

**Figure 3.7** Fluorescent microscope images of native PP-4 tips and those treated with cibacron blue LTL. Second row images show those tips with rhodamine red modified BSA loaded on the surface. Bottom row images show the tips after the r-BSA has experienced a wash in PBS+0.01% Tween-20.

**Conclusions**

The results of the experiments performed in this study led to the conclusion that at this point, albumin removal using the dye modified fiber system fails to function in the expected manner. With the surety that the dye indeed acts as a ligand for BSA, the addition of the dye to the fiber does not maintain this interaction to the point of BSA depletion from a bulk solution as desired. The postulated reasoning behind this is a surface ligand deficiency.
As a demonstration, 1 inch of nylon packed FEP tubing contains, on average, 5 mg of fiber. While the exact monomer length is not known, for these purposes assume 10,000 monomers per polymer length. Converting the mass of the fiber divided by the molecular mass of nylon monomers, 226.32 g mol$^{-1}$, the moles of nylon in a tip amount to about 2.2 nanomole. The challenge arises because each polymer has only 1 tertiary amine group which can bond to the triazine dye, thus only 2.2 nanomoles of cibacron blue may bond. This is nearly consistent with the 0.134 mg amount reported in Figure 3.3. There are two orders of magnitude difference, which may be a result of the 10,000 monomer assumption, or incomplete washing of the fiber. Then, based on the assertion that biomolecules only interact at the surface, the number of “visible” ligands is further reduced. Accounting for all of these and the fact that not all reactive centers will react with the dye and not all dye molecules will interact with a BSA means that the system may, in fact, capture BSA, but not at a detectable level and not efficiently enough to be implemented.

Two future systems could possibly overcome these limitations. The first, being a new nylon fiber using a shorter polymer length, which would increase the number of end-groups per mass. The second would be to implement the lipid tethered ligand format currently being studied in this lab. While attempts have been made to use this format, a fundamental problem with the organic chemistry may have interfered with results. The temperature and pH at which the reactive dye needs to bond will degrade the PEG-lipid. It may be possible to create a synthetic LTL with the dye which could subsequently increase ligand density and perform as a functional system.
Should either of those systems perform efficiently, this opens a full line of possible dye-protein ligand modes for selective capture of an array of proteins.
CHAPTER FOUR

CONCLUSIONS AND FUTURE WORK

The preceding thesis has contained two studies for the further application of C-CP fibers to separate proteins. Through the fibers’ unique eight channeled design, fluid flow is unobstructed and allows for the use of high linear velocities without significant increases in backpressure. Because of this property, high throughput analysis is the most notable advantage. Another key property for high throughput protein analysis is the low porosity of the fibers. The innability of macromolecules to enter the pores allows for increased mass transfer rates and high sample recovery. The high throughput sample analysis and the low materials cost to produce C-CP fibers make the fibers an extremely affordable and efficient option for protein separations.

Increasing the speed of analysis is a common goal in any method. C-CP fiber columns allow for increased flow rates because of their unobstructed fluid flow, additionally, increased flow rates result in increased resolution. This is attributed to the more favorable mass transfer rates at higher flow rates. While the times for resolved separations may not be as short as those reported elsewhere, solvent consumption decreases as flow rates are significantly reduced [57]. Further, the use of C-CP fibers as SPE desalting matrices has shown promise in many practices. Tip format desalting prior to MALDI and ESI MS analysis has shown significant reduction in noise and increased spectral clarity [12, 22, 58, 59].
Following these publications, the application of the C-CP fiber column as an in-line desalting injection loop was demonstrated as a simple in-line clean-up step to reduce noise in protein MS due to matrix effects [23]. Compiling these methods of reversed phase chromatography using gradients, decreasing column inner diameter and inline SPE desalting of matrices prior to ESI-MS has shown the ability of the ever-cost efficient C-CP fiber column to provide a single step method of protein sample preparation (SPE), separation (RP-HPLC) and analysis (ESI-MS) in under 10 minutes.

In the future, work would include employing these methods to quantify proteins in matrices to further the application of C-CP fibers for proteomic analysis. It would also be interesting to see the effect that temperature would have on C-CP fiber column separations. A further reduction in back pressure would be expected, but it would be interesting to see the effect on resolution, peak shape and separation time, as the solvation strength should increase with temperature. Other future studies should look into the transfer kinetics and whether, in the case of C-CP fibers, the flow rate or the linear velocity is the driving force of the separation efficiency. While the two parameters are clearly dependent upon one another, it seems that even when the linear velocity is comparable, if the flow rates are different, the separation time is also different.

Literature searches surrounding this project have also spawned questions into glycoproteomics and whether the C-CP fibers have the capacity to separate glyoproteins to further diagnostics [60, 61]. Current trends in proteomics show a desire to create single step bioreactor/analyzers by immobilizing trypsin on the
surface of a column to digest a protein and simultaneously separate the digest products. Research in this group has shown promise in the field of surface modifications via lipid-tethered ligands (LTL) [62, 63]. It would be conceivable to immobilize a trypsin enzyme to the LTL to digest proteins on column.

Another application of the C-CP fibers is deployment as affinity ligand substrates for affinity chromatography. The results of the experiments performed in the cibacron blue study led to the conclusion that at this point, albumin removal using the dye modified fiber system fails to function in the expected manner. With the surety that the dye indeed acts as a ligand for BSA, the addition of the dye to nylon fibers does not maintain this interaction to the point of BSA depletion from a bulk solution as desired. The postulated reasoning behind this is a surface ligand deficiency, due to the fact that the dye may only attach at the terminal amine.

Two future systems could possibly overcome these limitations. The first, being a new nylon fiber using a shorter polymer length, which would increase the number of end-groups per mass. The second would be to implement the lipid tethered ligand format currently being studied in this lab. While attempts have been made to use this format, a fundamental problem with the organic chemistry may have interfered with results. The temperature and pH at which the reactive dye needs to bond will degrade the PEG-lipid. It may be possible to create a synthetic LTL with the dye which could subsequently increase ligand density and perform as a functional system.

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