AN INVESTIGATION OF A NOVEL MONOLITHIC CHROMATOGRAPHY COLUMN, SILICA COLLOIDAL CRYSTAL PACKED COLUMNS

by

Douglas Scott Malkin

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SIGNED: Douglas Scott Malkin
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TABLE OF CONTENTS

LIST OF TABLES............................................................................................................................................14

LIST OF FIGURES..............................................................................................................................................15

ABSTRACT..........................................................................................................................................................25

CHAPTER 1: ROLE OF SEPARATION SCIENCE IN PROTEOMICS

1.1 CURRENT SEPARATION PRACTICES AND PROBLEMS.................................................................27

1.1.1 STATEMENT OF THE PROTEOMIC PROBLEM..............................................................................27

1.1.2 CURRENT CHROMATOGRAPHIC SEPARATION PRACTICES.........................................................29

1.2 METHODS TO IMPROVE SEPARATIONS............................................................................................31

1.2.1 INCREASING THE RESOLUTION.......................................................................................................31

1.2.2 INCREASING THE SENSITIVITY......................................................................................................33

1.3 SEPARATION EFFICIENCY AND KINETIC PLOTS A REVIEW.........................................................34

1.3.1 PLATE NUMBER (N)............................................................................................................................34

1.3.2 PLATE HEIGHT (H)............................................................................................................................35

1.3.3 VAN DEEMTER EQUATION AND PLOTS.........................................................................................37

1.3.4 KINETIC PLOTS................................................................................................................................39

1.4 SILICA COLLOIDAL CRYSTALS AS A SEPARATION MEDIUM.....................................................41

1.5 SUMMARY...............................................................................................................................................42
# TABLE OF CONTENTS – CONTINUED

1.6 REFERENCES ............................................................................................................. 44

1.7 TABLES ..................................................................................................................... 46

1.8 FIGURES ................................................................................................................... 48

CHAPTER 2: CHEMICAL STABILIZATION AND FUNCTIONALIZATION OF SILICA COLLOIDAL CRYSTALS ON SLIDES AND IN CAPILLARIES

2.1 ABSTRACT ............................................................................................................... 58

2.2 INTRODUCTION ..................................................................................................... 58

2.3 EXPERIMENTAL .................................................................................................... 61

   2.3.1 CHEMICALS AND MATERIALS ...................................................................... 61

   2.3.2 SILICA COLLOIDAL CRYSTALS (SCCs) .................................................... 62

   2.3.3 CHEMICAL MODIFICATION AND STABILIZATION OF SCC ............. 63

   2.3.4 SCANNING ELECTRON MICROSCOPY .................................................. 64

   2.3.5 FT-INFRARED SPECTROSCOPY .............................................................. 64

   2.3.6 MICROARRAY SPOTTING ............................................................................ 65

   2.3.7 SONICATION TEST ....................................................................................... 65
### TABLE OF CONTENTS - CONTINUED

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.3.8 NANOINDENTATION</td>
<td>66</td>
</tr>
<tr>
<td>2.3.9 PRESSURE VS. FLOW RATE</td>
<td>66</td>
</tr>
<tr>
<td><strong>2.4 RESULTS AND DISCUSSION</strong></td>
<td>67</td>
</tr>
<tr>
<td>2.5 CONCLUSIONS</td>
<td>72</td>
</tr>
<tr>
<td>2.6 REFERENCES</td>
<td>74</td>
</tr>
<tr>
<td>2.7 FIGURES</td>
<td>78</td>
</tr>
</tbody>
</table>

**CHAPTER 3: SUB-MICRON PLATE HEIGHTS FOR CAPILLARIES PACKED WITH SILICA COLLOIDAL CRYSTALS**

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1 ABSTRACT</td>
<td>96</td>
</tr>
<tr>
<td>3.2 INTRODUCTION</td>
<td>96</td>
</tr>
<tr>
<td><strong>3.3 EXPERIMENTAL</strong></td>
<td>98</td>
</tr>
<tr>
<td>3.3.1 CHEMICALS AND MATERIALS</td>
<td>98</td>
</tr>
<tr>
<td>3.3.2 SILICA COLLOIDAL CRYSTALS (SCCs)</td>
<td>98</td>
</tr>
<tr>
<td>3.3.3 CHEMICAL MODIFICATION OF SCCs</td>
<td>99</td>
</tr>
<tr>
<td>3.3.4 CAPILLARY HOLDER FABRICATION</td>
<td>99</td>
</tr>
<tr>
<td>3.3.5 SCANNING ELECTRON MICROSCOPY</td>
<td>100</td>
</tr>
</tbody>
</table>
TABLE OF CONTENTS- CONTINUED

3.3.6 MEASURING POROSITY ................................................................. 100

3.3.7 ELECTROCHROMATOGRAPHY ..................................................... 100

3.3.8 PRESSURE VS. FLOW RATE ....................................................... 101

3.4 RESULTS AND DISCUSSION .......................................................... 101

3.5 CONCLUSIONS ............................................................................. 105

3.6 REFERENCES ................................................................................ 106

3.7 TABLES ....................................................................................... 108

3.8 FIGURES ..................................................................................... 109

CHAPTER 4: SUB-MICRON PLATE HEIGHTS FOR THE SEPARATION OF INTACT PROTEINS IN CAPILLARIES PACKED WITH SILICA COLLOIDAL CRYSTALS

4.1 ABSTRACT .................................................................................. 121

4.2 INTRODUCTION .......................................................................... 121

4.3 EXPERIMENTAL .......................................................................... 124

4.3.1 CHEMICALS AND MATERIALS .................................................. 124

4.3.2 PROTEINS ............................................................................. 124

4.3.3 SILICA COLLOIDAL CRYSTALS (SCCs) ....................................... 124
### TABLE OF CONTENTS - CONTINUED

4.3.4 CHEMICAL MODIFICATION OF SCC ......................................................... 125

4.3.5 CAPILLARY HOLDER FABRICATION ....................................................... 126

4.3.6 ELECTROCHROMATOGRAPHY .............................................................. 126

4.4 RESULTS AND DISCUSSION .................................................................... 127

4.5 CONCLUSIONS ......................................................................................... 131

4.6 REFERENCES .......................................................................................... 132

4.7 TABLES .................................................................................................. 135

4.8 FIGURES ................................................................................................. 136

### CHAPTER 5: RPLC OF BIOMOLECULES IN SILICA COLLOIDAL CRYSTALS

5.1 ABSTRACT .............................................................................................. 146

5.2 INTRODUCTION ......................................................................................... 146

5.3 EXPERIMENTAL ...................................................................................... 148

   5.3.1 CHEMICALS AND MATERIALS ......................................................... 148

   5.3.2 SILICA COLLOIDAL CRYSTALS (SCCs) .............................................. 149

   5.3.3 CHEMICAL MODIFICATION OF SCC ............................................... 150
TABLE OF CONTENTS- CONTINUED

5.3.4 INSTRUMENTATION ..........................................................150

5.3.5 PRESSURE VS. FLOW RATE .............................................150

5.3.5 FORMATION OF DIFFERENT COLUMN TIPS .....................150

5.3.6 FORMATION OF DIFFERENT COLUMN TIPS .....................151

5.3.7 MIGRATIONS/SEPARATIONS ..........................................151

5.4 RESULTS AND DISCUSSION .............................................152

5.5 CONCLUSIONS ...............................................................156

5.6 REFERENCES .................................................................157

5.7 TABLES ..........................................................160

5.8 FIGURES ..............................................................163

APPENDIX A: OTHER CONTRIBUTIONS

A.1 ISOELECTRIC FOCUSING (IEF) IN A PACKED CAPILLARY ........177

A.2 THIN LAYER CHROMATOGRAPHY (TLC) IN A SCC ...............177

A.3 PACKING LARGER BORE COLUMNS .....................................178

A.4 SEPARATION OF GLYCOFORMS ..........................................178
TABLE OF CONTENTS - CONTINUED

A.5 REFERENCES........................................................................................................180

A.6 FIGURES.............................................................................................................181

REFERENCES

CHAPTER 1 REFERENCES...........................................................................................186

CHAPTER 2 REFERENCES...........................................................................................188

CHAPTER 3 REFERENCES...........................................................................................190

CHAPTER 4 REFERENCES...........................................................................................191

CHAPTER 5 REFERENCES...........................................................................................193

APPENDIX A REFERENCES.........................................................................................196
# LIST OF TABLES

| Table 1.1 | Comparison of HPLC, CE, and Slab-Gel Electrophoresis (SGE). | 46 |
| Table 1.2 | Modes of separation by SGE, HPLC, and CEC. | 47 |
| Table 3.1 | Electrochromatographic plate heights for nonporous colloidal silica spheres. | 108 |
| Table 4.1 | Table summarizing experimental conditions. | 135 |
| Table 5.1 | Gradient conditions used for the SCC column. | 160 |
| Table 5.2 | Peak capacity ($n_c$) of TAMRA labeled angiotensin II calculated using $n_c = 1 + \frac{t_g}{4\sigma}$. | 161 |
| Table 5.3 | Peak capacity ($n_c$) of TAMRA labeled angiotensin II calculated using $n_c = \frac{t_{r,n}-t_o}{4\sigma}$. | 162 |
LIST OF FIGURES

Figure 1.1  A general schematic of the “bottom-up” proteomic method. Where proteins are digested before the separation, and the digested peptides are then separated and detected using MS. 48

Figure 1.2  A general schematic of the “top-down” proteomic method. Where intact proteins are separated from one another and detected using MS. 49

Figure 1.3  The effect of decreasing the plate height on the resolution of a mixture. Illustrated is the effect of reducing the plate height while maintaining the same length of separation. Decreasing the plate height leads to sharper peaks and this leads to higher resolution. 50

Figure 1.4  The effect of decreasing the plate height on the sensitivity. Illustrated here is the effect of reducing the plate height while maintaining the same amount of analyte on the column. Decreased plate heights lead to sharper peaks and this leads to proportionally higher sensitivity. 51

Figure 1.5  Schematic diagrams of the three major band broadening effects at the molecular level. 52

Figure 1.6  A van Deemter plot illustrating the effect of the eddy diffusion term (A), longitudinal diffusion term (B), and the resistance to mass transfer term (C) individually and cumulatively. 53

Figure 1.7  Kinetic plots of plate number versus analysis time for varying particle sizes. 54
LIST OF FIGURES-CONTINUED

**Figure 1.8** Opal in mother nature. Photograph and SEM micrograph of a gem quality opal.  

**Figure 1.9** Color photographs of gem quality opals in capillaries with varying particle sizes. A scanning electron micrograph of an opal in a capillary (330nm particles). All of these images are from our lab.  

**Figure 1.10** Van Deemter plots for acetophenone on various commercial columns. The minimum plate height was found to be ~2d₀.  

**Figure 2.1** A schematic diagram illustrating chemical sintering.  

**Figure 2.2** Mixed horizontal polymerization reaction of ((chloromethyl)phenylethyl)-trichlorosilane and methyltrichlorosilane followed by atom transfer radical attachment (ATRA) of 1,2-epoxy-5-hexene (Sigma).  

**Figure 2.3** Silylation reaction with (3-Glyciodxypropyl) Trimethoxysilane.  

**Figure 2.4** Mixed horizontal polymerization reaction of octadecyltrichlorosilane (C18) and methyltrichlorosilane (C1).  

**Figure 2.5** Photograph of SCCs, made of 0.33 µm particles, on (a) a planar glass coverslip and (b) in a 100 µm I.D. fused silica capillary.
LIST OF FIGURES-CONTINUED

**Figure 2.6**  SEM images of a SCC made from 0.35 μm particles and modified using the trichlorosilanes on (a) a glass coverslip and (b) in a fused silica capillary. The polymer visible in the top micrograph of both sets is an image of what happens if too much water (80 % humidity) is present in the mixture. The bottom micrograph in both sets shows the effect of having 50% humidity.

**Figure 2.7**  FTIR spectra of the mixed horizontally polymerized surface using ((chloromethyl)phenylethyl)-trichlorosilane and methyltrichlorosilane and the ATRA product.

**Figure 2.8**  FTIR spectra of the (3-Glycidoxypropyl)Trimethoxysilane surface.

**Figure 2.9**  Microarray spotting of fluorescein conjugated bovine serum albumin (BSA-FITC) onto SCC. The total average fluorescence of a single spot on the SCC is reported in both tabular and graph form. The data indicates that the epoxy rings from the ATRA and Glycidoxy reactions are both active on the SCC and are able to bind protein.

**Figure 2.10**  Fluorescence micrographs and white light micrographs of BSA-FITC spotted on a (3-Glycidoxypropyl)Trimethoxysilane modified and an ATRA modified surface.

**Figure 2.11**  Photograph showing the effect of sonication on modified silica colloidal crystals.

**Figure 2.12**  Photograph showing the effect of sonication on a trichloro modified silica colloidal crystal after the organic portion was removed using an UV-ozone plasma cleaner.
**LIST OF FIGURES-CONTINUED**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.13</td>
<td>Load (P) versus displacement (h) plot of nanoindentation data on an unmodified SCC. The dotted lines represent the qualitative point that the crystal starts to break down.</td>
</tr>
<tr>
<td>2.14</td>
<td>Load (P) versus displacement (h) plot of nanoindentation data on a Trimethoxy (glycidoxy) modified SCC. The dotted lines represent the qualitative point that the crystal starts to break down.</td>
</tr>
<tr>
<td>2.15</td>
<td>Load (P) versus displacement (h) plot of nanoindentation data on a Trichloro modified SCC. The dotted lines represent the qualitative point that the crystal starts to break down.</td>
</tr>
<tr>
<td>2.16</td>
<td>Load (P) versus displacement (h) plot of nanoindentation of a bare glass substrate, used as a control.</td>
</tr>
<tr>
<td>2.17</td>
<td>Pressure vs. flow rate data for SCCs packed in fused silica capillaries.</td>
</tr>
<tr>
<td>2.18</td>
<td>Spray from SCC packed fused silica capillary.</td>
</tr>
<tr>
<td>3.1</td>
<td>Schematic and photograph of electrochromatography experiment.</td>
</tr>
<tr>
<td>3.2</td>
<td>Images of a silica colloidal crystal packed in a capillary. Photographs through an optical microscope for a) end-on view and b) side-on view. c) SEM image of the end-on view of a region of the capillary.</td>
</tr>
<tr>
<td>3.3</td>
<td>SEM image of the full capillary cross-section.</td>
</tr>
</tbody>
</table>
LIST OF FIGURES-CONTINUED

Figure 3.4  Graph of volume flow rate vs. pressure for two 100 µm i.d. capillaries packed with silica colloidal crystals. The error bars are standard deviations from multiple measurements. 112

Figure 3.5  Histogram of porosity values. Ten measurements were made for the volume of water that wicked into colloidal crystals of known length in 100 µm i.d. capillaries. 113

Figure 3.6  Raw electrochromatograms for DiI-C_{12} at four different electric fields, superimposed on the same time axis, without removing the contribution from injected width. The mobile phase was 90:10 MeOH:water and 0.1 % formic acid, and the stationary phase was horizontally polymerized C_{18}/C_{1}. 114

Figure 3.7  Raw data (o) for chromatograms of DiI-C_{12} on expanded time scales at four electric fields over the range that was used, showing high quality of the Gaussian fit (-). The injected width is included in the overall peak profile. The fitting parameters, separation distances and plate heights are listed for each chromatogram. 115

Figure 3.8  Plots of typical injected peaks (o) and fit to a Gaussian (-) for each. The plate heights for the entire set of 16 injections ranged from 48 to 110 nm. 116

Figure 3.9  Plot of plate height vs. analyte migration rate. The circles are experimental data and the solid curve is $H=2γD/v$. Regression showed that $A=0±20$ nm. 117
LIST OF FIGURES-CONTINUED

Figure 3.10  Plot of the peak variance vs. time, as a plug of DiI-C$_{12}$ underwent diffusion in the capillary in the absence of an electric field. A least-squares fit to determine the slope, which is equal to $2\gamma D$, revealed the diffusion coefficient to be $\gamma D = 9.2(\pm 0.7) \times 10^{-8} \text{ cm}^2/\text{s}$.

Figure 3.11  Plot of migration rate of DiI-C$_{12}$ vs. applied electric field reveals nonlinearity at high fields, which is likely due to heating of the capillary. The error bars are standard deviations from multiple measurements.

Figure 3.12  Knox plot of the same data that are provided in Figure 3.9. The solid line is a plot of the equation shown in the graph, $h = 2/\nu$.

Figure 4.1  Schematic of double reservoirs made of PDMS used in electrochromatography experiments. The capillary (blue) spanned the two inner reservoirs, while the Pt electrodes were placed in the outer reservoirs.

Figure 4.2  Electrochromatogram of a mixture of three proteins, which elute in the order of lysozyme, ribonuclease A and cytochrome C. The separation was performed isocratically using a 1000 V/cm electric field and a mobile phase of 40:60 ACN:water with 0.1% formic acid. The separation distance was 1.5 cm.

Figure 4.3  Fluorescence image of a 75 um i.d. capillary 2 min after the three labeled proteins were injected as plug, showing that they separated. The mobile phase composition was 40:60 ACN:water with 0.1% formic acid., and E=1,000 V/cm. The corresponding electrochromatogram with respect to length is also shown.
LIST OF FIGURES-CONTINUED

Figure 4.4  Migration rate of lysozyme as a function of electric field. The mobile phase composition was the same as in Figure 4.2.  139

Figure 4.5  Top: Raw images of the lysozyme zones in the 75 um i.d. capillary at varying electric fields each with a migration distance of 1.5 cm. Bottom: The same raw data on the same spatial scale, but converted into chromatograms (O), and showing the best fit to a Gaussian (-).  140

Figure 4.6  Top: Raw images of the lysozyme zones in the 75 um i.d. capillary at a migration distance of 1.5 cm for each replicate measurement at 1000 V/cm. Bottom: The same raw data on the same spatial scale, but converted into chromatograms (O), and showing the best fit to a Gaussian (-).  141

Figure 4.7  Plate height vs. migration rate for lysozyme. The error bars represents standard deviations from three replicate measurements.  142

Figure 4.8  Comparison of plate height vs. migration rate for lysozyme and DiIC_{12}.  143

Figure 4.9  A sequence of fluorescent images of the electrophoretic migration (500V/cm) of BSA through the colloidal crystal column packed with 385 nm particles. A mobile phase of 50:50 ACN:Water, 0.1 % formic acid was used.  144

Figure 4.10  A sequence of electrochromatograms of the electrophoretic migration (500V/cm) of BSA through the colloidal crystal column packed with 385 nm particles. A mobile phase of 50:50 ACN:Water, 0.1 % formic acid was used.  145
LIST OF FIGURES-CONTINUED

**Figure 5.1**  Schematic of the UHPLC-fluorescence detector system. 163

**Figure 5.2**  Schematic of the UHPLC-nanoESI-MS system. The dashed box region is shown in a photograph in Figure 5.3. 164

**Figure 5.3**  Photograph of the UHPLC-nanoESI-MS system. The SCC column and MS-inlet are circled in red. 165

**Figure 5.4**  Photographs of the different tip styles and the spray achieved from each tip after being packed with a SCC. All fabricated in our lab. 166

**Figure 5.5**  Photograph of the New Objective Picotip and the spray achieved from the tip after being packed with a SCC. 167

**Figure 5.6**  Graph of volume flow rate vs. pressure for two 100 µm i.d. capillaries packed with silica colloidal crystals. The error bars are standard deviations from multiple measurements. 168

**Figure 5.7**  Chromatogram of ribonuclease A. The separation was performed isocratically using a flow rate of ~150 nL/min and a mobile phase of 40:60 ACN:water with 0.1% formic acid. The separation distance was 1.5 cm. 169

**Figure 5.8**  Mass Spectrum of ribonuclease A from Figure 5.7. 170
LIST OF FIGURES-CONTINUED

Figure 5.9  Chromatogram of a mixture of three neuropeptides, which elute in the order of met-enkephalin, leu-enkephalin, and angiotensin II. The separation was performed using gradient elution using a flow rate of ~150 nL/min. The separation distance was 1.5 cm. Gradient conditions are listed in Table 5.1. 171

Figure 5.10  Mass Spectrum of met-enkephalin from Figure 5.9. 172

Figure 5.11  Mass Spectrum of leu-enkephalin from Figure 5.9. 173

Figure 5.12  Mass Spectrum of angiotensin II from Figure 5.9. 174

Figure 5.13  Comparison of chromatograms of a mixture of three neuropeptides, which elute in the order of met-enkephalin, leu-enkephalin, and angiotensin II. 175

Figure 5.14  Gradient elution of TAMRA labeled Angiotensin II with on column fluorescence detection. Separation distance ~1.5 cm. Gradient conditions were the same as in Figure 5.9 and are listed in Table 5.1. 176

Figure A.1  Fluorescence vs. time for the pressure-driven remobilization, measured at a point near the end of the capillary, after focusing at 500 V/cm. The red dotted line is a Gaussian fit to the trypsin peak, revealing a standard deviation of 0.90 s., which corresponds to 0.10 pH units. (Data courtesy of Yimin Hua) 181

Figure A.2  Fluorescence micrograph of a TLC separation of three-proteins over a length of 1 cm. (Data courtesy of Zharou Zhang) 182
LIST OF FIGURES-CONTINUED

**Figure A.3**  Colored photographs of a chromatography column packed with a silica colloidal crystal formed from 330 nm particles. The blue color arises from the Bragg diffraction, as the particles are on the order of the wavelength of light.  

**Figure A.4**  Reaction schematic of surface-initiated atom transfer radical polymerization (ATRP) of polyacrylamide with addition of the acrylamido phenylboronic acid (APBA) monomer to form the new hydrophilic interaction stationary phase. (Schematic courtesy of Saliya Ratnayaka)

**Figure A.5**  Pressure driven flow separation of ribonuclease b glycoforms. The column was 1.2 cm long and packed with 500 nm particles. The column was modified to have 200:1 acrylamide to APBA and a mobile phase of 70:30 ACN:water with 0.5 % formic acid was used. (Data courtesy of Saliya Ratnayaka)
ABSTRACT

Many researchers have investigated ways to improve the separation power of conventional chromatography, most notable is the development of ultra-high performance liquid chromatography (UHPLC). However, only slight improvements in separation efficiency have been achieved up to this point, and unfortunately, modern reversed phase liquid chromatography (RPLC) methods do not have high enough resolving power to analyze complex proteomic mixtures.

Uniformly sized silica particles from 10 nm to 1 micron are known to self-assemble into a highly ordered face centered cubic crystal. Silica colloidal crystals have shown recent promise in biological applications such as permselective nanoporous membranes, DNA sieving, reversed phase separation of small molecules on planar substrates, protein sieving, microarrays, total internal reflection fluorescence microscopy of live cells, and 3-D scaffolds for supported lipid films. In this work, silica colloidal crystals packed in capillaries are explored for their potential improvement in the efficiency of reversed phase chromatography.

The silica colloidal crystal columns were chemically stabilized with trichlorosilanes. The trichlorosilanes form chemical bonds between the particles and the substrate creating an increase in mechanical stability, and at the same time, providing an excellent chromatographic monolayer. After stabilization the fritless columns were able to withstand the pressure limit of the commercial UHPLC. Next, the columns were characterized using a small dye molecule, 1,1’-Didodecyl-3,3,3’,3’-tetramethylindocarbocyanine (DiIC$_{12}$). The dye was run under capillary
electrochromatography (CEC), and sub-micron plate heights were achieved. Further, a
van Deemter plot of the dye molecule indicates that the plate height is largely due to the
molecule’s diffusion. This result suggests that the plate heights for proteins would be
even smaller, since proteins have diffusion coefficients an order of magnitude smaller.
The analysis of proteins by CEC yielded nanometer plate heights. Finally, pressure driven
flow separations coupled with nano-electrospray ionization (n-ESI) MS have also been
explored. The Poiseuille flow profile has been shown not to perturb the low plate heights.
Gradient elution of peptides was also achieved, and the results demonstrate the highest
chromatographic peak capacities for short analysis times to date.
CHAPTER 1. ROLE OF SEPARATION SCIENCE IN PROTEOMICS

1.1 CURRENT SEPARATION PRACTICES AND PROBLEMS

1.1.1 STATEMENT OF THE PROTEOMIC PROBLEM

With the completion of the Human Genome Project in the early 2000s, it is often stated that the next challenging analytical problem for separation scientists to solve is the analysis of the proteome.1-10 Proteomics research has developed into one of the fastest growing areas in biological research, with the main goal of proteomics being to assign the function to proteins. The wealth of information that is to be gained through proteomics will provide a deeper understanding of the roles that proteins play in a biological system. Important applications include: the comparison of a cancerous cell’s proteins to a normal cell’s proteins, understanding how diseases start and progress, and investigating a cell’s proteins response to pharmaceuticals.3

Comparing a cancerous cell’s proteins to a normal cell’s proteins makes it possible to identify a protein or proteins that are distinctive to cancer.3-5 These particular proteins are often called biomarkers. The identification and detection of these biomarkers will have a profound effect on early disease diagnosis as well as drug discovery. Early disease diagnosis is especially important for diseases such as cancer where oftentimes early detection provides a much better chance for survival.4, 5

Much of the research in the field of proteomics has centered on complete cell lysates.6-8, 11 Unlike the static genome, the proteome differs between cell types, and the
proteins present differ from one cell to the next. Not only are protein structures dictated by the genome, the environment and cell history also play a key role. The environment and cell’s history are known to lead to post translational modifications and sequence variants.\textsuperscript{1-3} The dynamic nature of the proteome poses a challenging analytical problem for separation scientists because of the extreme complexity of the samples. Furthermore, the concentration range of proteins in a cell is known to span six orders of magnitude.\textsuperscript{1-3,9,10} The complexity and concentration range are even more pronounced when human blood plasma is used.\textsuperscript{10} To address this separation problem we require high sensitivity, high resolution, and high throughput.\textsuperscript{2,9,10}

There are two common methods used in proteomics. The most common method is “bottom-up” (Figure 1.1), which is performed by direct analysis of a mixture of peptides from a proteolytic (trypsin, pepsin) digest.\textsuperscript{6,7} The protein is first digested and the resulting peptides are separated and detected using mass spectrometry. The analysis of peptides by MS and tandem MS-MS is preferred, as much research has been put into algorithms for identifying proteins from their peptide fragments.\textsuperscript{3} However, since “bottom-up” methods involve digestion of proteins into peptides, the sample is more complex since there will always be more peptides than the parent proteins. This requires even higher resolution separations. Additionally, the “bottom-up” method does not provide a large amount of molecular information about the intact protein,\textsuperscript{3} and does not give insight into post translational modifications.

The second less common method is “top-down” (Figure 1.2), which is done by direct separation and detection of the intact proteins.\textsuperscript{8} Although “top-down” protein MS
has recently gained interest, it is still more difficult than peptide analysis.\textsuperscript{1-4, 8, 9} From the perspective of separations under reversed phase chromatography, the analysis of peptides is more established than the separation of intact proteins. “Top-down” should be advantageous however for the detection of differential post translational modifications.\textsuperscript{8} Furthermore, the mixture is far less complex in the “top-down” method. In both methods, separation science plays a critical role in protein identification and analysis.

\textbf{1.1.2 \textit{CURRENT CHROMATOGRAPHIC SEPARATION PRACTICES}}

There are many reviews and articles summarizing the methods of separation for proteome analyses.\textsuperscript{1-10} The chemical diversity of proteins and peptides makes their separation achievable with a wide variety of techniques. Historically, the most prominent type of proteomic analysis is slab gel electrophoresis (SGE), where multiple samples can be run in parallel in 1D format.\textsuperscript{1, 2} Further, in the 2D format, where the proteins are separated by their isoelectric point in the first dimension followed by sieving electrophoresis in the second dimension, thousands of proteins can be resolved. Despite the high resolving power, SGE has poor sensitivity, poor quantitation, is not a high throughput technique, and sample automation and interfacing with MS is very cumbersome.\textsuperscript{1, 2} Research has been moving toward high resolution capillary chromatographic analyses for in-line integration with MS.\textsuperscript{1-10} Capillary chromatographic analysis is especially appealing as it attacks many of the problems exhibited by conventional SGE. Capillary chromatographic analysis has higher sensitivity, better quantitation, can be automated, is easily coupled to MS with electrospray ionization
(ESI), and has shorter analysis times lending itself to high throughput. Table 1.1 summarizes the advantages and disadvantages of the two techniques.

While recent work in the field of proteomics has focused on the improvement of the identification of proteins with various MS methods, chromatography has also played a critical role in both intact protein and peptide analyses.\textsuperscript{1-10} Many modes of chromatography exist and are summarized in Table 1.2. Of these modes, reversed-phase liquid chromatography (RPLC) is the most widely used.\textsuperscript{1-10} This can be attributed to reversed-phase chromatography’s high compatibility with MS detection with ESI or nanoESI. RPLC columns have a hydrophobic stationary phase (often C18 or C4) and as the peptides or proteins elute, hydrophobicity controls the separation. The fast mass transfer of the analyte between the stationary phase and mobile phase yields the highest separation efficiencies when compared with other chromatographic modes.\textsuperscript{2} Separation efficiency of RPLC is highly dependent on the length of the column and the particle size. While RPLC can be used as a standalone separation technique, it is often coupled to another orthogonal dimension because the general postulation is that no single chromatographic separation is capable of resolving the complex mixtures.\textsuperscript{1,10} The most prevalent example of a 2D chromatographic separation is multidimensional protein identification technology (MUDPIT)\textsuperscript{11} which is a combination of strong cation exchange (SCX) with RPLC. RPLC offers tremendous potential in the separation of proteins and peptides and is likely to remain a very important component of proteomics and biomarker discovery in the future.
This work will discuss the use of sub-micron nonporous inorganic silica colloidal spheres as a mean of increasing the efficiency of reversed-phase chromatography column. Of particular interest is the potential improvement in separation efficiency over that of conventional RPLC columns and their applications in proteomics and biomarker discovery.

1.2 METHODS TO IMPROVE SEPARATIONS

1.2.1 INCREASING THE RESOLUTION

Resolution ($R_s$) is the most important indication of the success or failure of an analytical separation.\textsuperscript{12,13} Resolution describes the amount of overlap of two adjacent peaks in a separation. When examining ways to increase the resolution it is useful to relate the resolution to the plate height ($H$), the selectivity factor ($\alpha$) and the retention factors ($k$) of the two adjacent species where $L$ is the separation length.

$$R_s = \frac{\sqrt{L/H}}{4} (\alpha - 1) \left(\frac{k}{1+k}\right)$$  \hspace{1cm} (1)

To increase the resolution, the $H$, $\alpha$ and $k$ have to be optimized. Controlling the retention factor is achieved in liquid chromatography by changing the composition of the mobile phase. Manipulation of the selectivity factor can also improve separations. The selectivity factor can be increased by changing the mobile phase composition, the column temperature, or the stationary phase composition.\textsuperscript{12-13} For existing stationary phases, mobile phases have been thoroughly studied to optimize $\alpha$ and $k$. By increasing the
length, higher resolution can be achieved, but at the expense of speed. Over the past few decades researchers have moved toward reducing the plate height instead of increasing the length of the column.\textsuperscript{13} The plate heights for protein HPLC today is on the order of 10 to 20 \( \mu \text{m} \). Figure 1.3 illustrates conceptually the effect that reducing the plate height by a factor of 50 would have on resolution. In practice, plate heights have only decreased by about a factor of two per decade.

The present approach to decrease the plate height of an analyte is to use smaller diameter particles at elevated pressure.\textsuperscript{14-17} The decrease in plate height does not come without a penalty. The decrease in particle size results in an increase in the back pressure of the column that is needed to drive the separation. The Kozeny-Carman equation,\textsuperscript{12} describes the pressure drop (\( \Delta P \)) across a packed bed

\[
\Delta P = \frac{180 \eta L_0 (1 - \varepsilon)^2}{d_p^2 \varepsilon^2} \quad (2)
\]

where \( \eta \) is the solvent viscosity, and \( \varepsilon \) is the interparticle porosity. This equation explains very nicely how the pressure limit of commercial HPLC equipment can quickly be achieved, and hence higher pressure pumps (UHPLC) are needed to benefit from using smaller particles. Particles as small as 1.7 \( \mu \text{m} \) in diameter are presently the state-of-the-art.
1.2.2 INCREASING THE SENSITIVITY

Another analytical requirement in the field of proteomics is high sensitivity for detecting low abundance proteins. Decreasing the plate height increases the overall signal to noise ratio. This can be explained by examining the Gaussian function

\[ f(x) = \frac{A}{2\sigma\sqrt{\pi}/2} e^{\frac{(-x-x_c)^2}{2\sigma^2}} \]  

(3)

Where \( A \) is the Area under the curve, \( \sigma \) is the standard deviation of the peak, and \( x_c \) is the position of the center of the peak. For example, by keeping the area under the curve constant the result of decreasing the standard deviation of the peak 10-fold will result in a proportional increase in the intensity of 10-fold. This is illustrated in Figure 1.4 where the position of the center of the peak has been set to 0. This increase in the signal to noise ratio is especially important for example in the field of biomarker discovery. The standard deviation of the peak is related to the plate height through equation 4.

\[ H = \frac{\sigma^2}{L} \]  

(4)

This indicates that the plate height decreases as the square of the standard deviation. A 100 fold decrease in the plate height will result in a 10-fold decrease in the standard
deviation and a 10 fold increase in the signal intensity. This means that the plate height would need to be drastically improved for the sensitivity to be improved significantly.

1.3 SEPARATION EFFICIENCY AND KINETIC PLOTS A REVIEW

1.3.1 PLATE NUMBER (N)

The plate number is often used as a measure of a column’s efficiency. N can be expressed by

\[ N = \frac{L}{H} \]  \hspace{1cm} (5)

Since both the length of the separation and the plate height are in units of length, N is dimensionless. By substituting equation 4 into equation 5, N can further be expressed as

\[ N = \frac{L^2}{\sigma^2} \]  \hspace{1cm} (6)

When N is expressed as in equation 6 it shows that the efficiency increases with the ratio of the separation length to the standard deviation of the peak. N is used to describe the separation power of a column.\(^{10, 12}\) More commonly in chromatography the bands elute off the column and emerge in time instead of distance and hence it is more useful to describe N in time units.
Where $t_r$ is the retention time of the emerging peak. Plate numbers for typical chromatographic separations of small molecule are in the tens of thousands, and for proteins are in the thousands.

1.3.2 PLATE HEIGHT ($H$)

Plate height is also often used as a measure of the columns efficiency. $H$ can be used as an index to indicate the effect of zone broadening in a chromatography column.\textsuperscript{12} There are many mechanisms of zone broadening in chromatography that will increase the plate height.

Flow alone through a packed column leads to zone broadening. This is because the individual analyte molecules can take paths of varying length through the bed. This is often referred to as eddy diffusion and is illustrated in Figure 1.5. The contribution to $H$ from Eddy diffusion can be estimated as $H_A$.

$$H_A = 2\lambda d_p$$

Where $\lambda$ is a constant related to packing structure and $d_p$ is particle diameter.
An analyte is more concentrated at the center of the band than at the edges and as such, the analyte diffuses out to the edges from the center, which leads to zone broadening. This is referred to as longitudinal diffusion and is illustrated in Figure 1.5. The contribution to $H$ from longitudinal diffusion is $H_B$.

$$H_B = \frac{2\gamma D_m}{u}$$  \hspace{1cm} (9)

Where $\gamma$ is the obstruction factor, $D_m$ is the diffusion coefficient in the mobile phase, and $u$ is the mobile phase linear flow rate. For low mobile phase velocities the analyte will spend a lot of time in the column which yields a large plate height value. At high velocities the analyte spends less time in the column which decreases the broadening effect of longitudinal diffusion.

The diffusion of the analyte in and out of a pore takes a certain amount of time. If the analyte take a long time to diffuse out of a pore and the mobile phase velocity is high than the analyte in the pore will trail the analyte in the mobile phase. This will broaden the zone. This is referred to as the resistance to mass transfer and is also illustrated in Figure 1.5. $H$ for the resistance to mass transfer can be estimated as $H_{C_m}$.

$$H_{C_m} = \omega \frac{d^2}{D_m} u$$  \hspace{1cm} (10)
Where $\omega$ is a constant related to the particle structure. The mobile phase velocity is proportional to the plate height, and the higher the velocity, the more broadening observed.

In chromatography, an amount of analyte is injected onto the column and this injection has a defined plug size. An injected plug has a particular width which will also affect the observed plate height. The contribution to the observed plate height from the injection can be described by $H_{inj}$.12

$$H_{inj} = \frac{l^2}{12L}$$

(11)

Where $l$ is defined as the full width of the square injection plug. An analogous would be used for describing the contribution from the detection window.

1.3.3 VAN DEEMTER EQUATION AND PLOTS

The observed plate height is a combination of the eddy diffusion term ($A$), longitudinal diffusion term ($B$), the resistance to mass transfer term ($C_m$) and the injection and detection widths. This can be expressed as the van Deemter equation.
By substituting equation 8, 9, 10 & 11 into equation 12 the extended van Deemter equation can be written as follows lumping injection and detection together.

\[ H = A + \frac{B}{u} + C_m u + H_{inj} + H_{det} \]  \hspace{1cm} (12)

The extended van Deemter equation is the common mathematical expression used to express the observed plate height for a chromatographic peak. More often people express the plate height independently of injection.\(^\text{12}\)

\[ H = 2\lambda d_p + \frac{2\gamma D_m}{u} + \omega \frac{d_p^2}{D_m} u + \frac{l^2}{12L} \]  \hspace{1cm} (13)

A van Deemter plot is a plot of the observed plate height vs. the average linear velocity of mobile phase.\(^\text{12}\) Van Deemter plots are often used to determine the optimum mobile phase flow rate. Figure 1.6 shows a general van Deemter plot illustrating the effect of the A, B and C terms individually as well as cumulatively.

\[ H = A + \frac{B}{u} + C_m u \]  \hspace{1cm} (14)

\[ H = 2\lambda d_p + \frac{2\gamma D_m}{u} + \omega \frac{d_p^2}{D_m} u \]  \hspace{1cm} (15)
For a fair comparison of the efficiency of columns with varying particle sizes the reduced parameters can be used,\textsuperscript{14,16} which shows the plate height independent of the particle size. The reduced parameter plate height ($h$) is defined as.

$$h = \frac{H}{d_p}$$  \hspace{1cm} (16)

The reduced parameter mobile phase velocity ($v$) can be defined as.

$$v = \frac{d_p u}{D_m}$$  \hspace{1cm} (17)

Substitution of equation 16 and 17 into equation 15 leads to the van Deemter equation in reduced parameters.

$$h = 2\lambda + \frac{2\nu}{v} + \omega v$$  \hspace{1cm} (18)

The reduced plate height is independent of particle size and analyte diffusion coefficient.\textsuperscript{12}

\textbf{1.3.4 KINETIC PLOTS}

A kinetic plot is an alternative to the van Deemter plot to include an assessment of analysis time. In its simplest form, it is a plot of the observed plate number vs. the
When dealing with kinetic plots, the length of the separation is taken as freely adjustable, with a fixed-pressure limit determined by the LC instrument. Hence, kinetic plots relate the compromise between analysis time and the separation efficiency which can be achieved at a defined pressure and particle size. This leaves the particle size to define. Figure 1.7 compares the kinetic plots of varying particle sizes. Upon examination of the kinetic plots, it can be concluded, that at short analysis times, smaller particles yield better column performance. However, if the analysis time is long, the larger particles yield a better overall performance. Undoubtedly, the main goal of separations is to obtain high resolution with as short of an analysis time as possible. Recall that in the field of proteomics high resolution and high throughput are required, and, kinetic plots validate the result of decreasing particle diameter.

While kinetic plots of this fashion allow for insight into isocratic separations, they do not define gradient elution separations. In 2006 Wang et al. presented a novel variation to the kinetic plot which allowed for insight into the kinetics of a separation under gradient conditions. They proposed plotting the conditional peak capacity, which they describe as the difference in the retention time of the last eluting and first eluting peaks divided by the average width of the peaks, versus the gradient time. Hence, kinetic plots in this form relate the compromise between analysis time and the peak capacity which can be achieved at a defined pressure and particle size.
1.4 SILICA COLLOIDAL CRYSTALS AS A SEPARATION MEDIUM

Uniformly sized silica particles have been made in sizes from 10 nm to 1 μm. It is well known that these uniformly sized silica particles self-assemble into a highly ordered face centered cubic crystal when placed on a solid substrate with varying thicknesses. Xia et al. review the use of silica colloidal crystals as photonic band gap crystals, removable templates to fabricate macroporous materials, masks in lithographic patterning, and optical sensors. Silica colloidal crystals have also showed promise to have many uses in biological analysis. These include their use in permselective nanoporous membranes, separations of DNA, small molecules, peptides and proteins, high surface area substrates for microarrays of DNA and protein analysis, porous substrates for total internal reflection fluorescence microscopy of live cells, and their use as 3-D scaffolds for supported lipid films. Silica colloidal crystals are also geologically prevalent as opals, as illustrated in Figure 1.8. As high crystallinity is achieved, the substrate will act as a diffraction grating and an opalescence character can be observed. This diffraction is called Bragg diffraction and the color observed is indicative of the size of the particles that are involved. Figure 1.9 illustrates the particle size dependence on color. As the particle size increases from 150 to 500 nm the observed color goes from violet to teal respectively. The SEM image is shown below, detailing the high crystallinity.

Silica has been used as a packing material for decades. Conventional HPLC columns are typically packed with porous 3.5 or 5 μm silica particles. Typical UHPLC columns are packed with porous 1.7 μm silica particles. This small decrease in particle
size has a predictable increase in the efficiency of the columns, giving plate heights that scale with particle size.\textsuperscript{15, 17} Figure 1.10 illustrates the commercially available columns and their resulting plate heights for a small molecule where on average the minimum plate height achieved is $2d_p$. This work will discuss the use of sub-micron nonporous particles to gain further reductions in plate height.

1.5 SUMMARY

Proteomic analysis requires fast, sensitive and high resolution separations. While slab gel electrophoresis may show high resolution, it is a slow and cumbersome process. Chromatography allows for the analysis of samples with mass spectrometry because of the convenient interface, but lacks resolution and high throughput. The reduction of the particle diameter is the most straightforward and most time proven way to decrease the plate height in liquid chromatography. Hence it is the most logical approach to increasing the overall resolution and sensitivity while also decreasing the total analysis time. The background literature shows that improvements in reducing plate heights have been glacial, with the introduction of UHPLC giving only a factor of two improvement in plate height. Further, there are presently no UHPLC materials designed for protein separations. In the face of this slow pace of progress, we have set out to make a quantum-leap improvement in plate height.

Our lab has experience applying silica colloidal crystals to separations of DNA, small molecules, and peptides on planar substrates, we had yet to explore the ability to pack these silica colloidal crystals into capillary columns. The work presented in this
dissertation will explore the fabrication, stabilization and characterization of capillaries packed with silica colloidal crystals.
1.6 REFERENCES


### Table 1.1 Comparison of HPLC, CE, and Slab-Gel Electrophoresis (SGE). (Adapted from reference 1)

<table>
<thead>
<tr>
<th>Function</th>
<th>Slab Gel</th>
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<tr>
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<td>mM</td>
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<tr>
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<td>nL-uL</td>
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<td>Off-Column fluorescence, UV or MS</td>
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<td>No</td>
</tr>
<tr>
<td>Multidimensional</td>
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<td>Yes, low n</td>
</tr>
<tr>
<td>Interfacing to MS</td>
<td>Possible, but not simple</td>
<td>Simple</td>
</tr>
<tr>
<td>Slab Gel modes</td>
<td>HPLC modes</td>
<td>CEC modes</td>
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<tr>
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**Table 1.2** Modes of separation by SGE, HPLC, and CEC. (Adapted from reference 1)
1.8 FIGURES

“Bottom-up”

**Figure 1.1** A general schematic of the “bottom-up” proteomic method. Where proteins are digested before the separation, and the digested peptides are then separated and detected using MS.
“Top-down”

Figure 1.2 A general schematic of the “top-down” proteomic method. Where intact proteins are separated from one another and detected using MS.
**Figure 1.3** The effect of decreasing the plate height on the resolution of a mixture. Illustrated is the effect of reducing the plate height while maintaining the same length of separation. Decreasing the plate height leads to sharper peaks and this leads to higher resolution.
Figure 1.4 The effect of decreasing the plate height on the sensitivity. Illustrated here is the effect of reducing the plate height while maintaining the same amount of analyte on the column. Decreased plate heights lead to sharper peaks and this leads to proportionally higher sensitivity.
Figure 1.5 Schematic diagrams of the three major band broadening effects at the molecular level. (copied from http://www.currentprotocols.com/protocol/et0601)
Figure 1.6 A van Deemter plot illustrating the effect of the eddy diffusion term (A), longitudinal diffusion term (B), and the resistance to mass transfer term (C) individually and cumulatively.
Figure 1.7 Kinetic plots of plate number versus analysis time for varying particle sizes. (Copied from reference 17)
Figure 1.8 Opal in mother nature. Photograph and SEM micrograph of a gem quality opal. (Copied from http://minerals.caltech.edu/Silica_Polymorphs/index.html)
Figure 1.9 Color photographs of gem quality opals in capillaries with varying particle sizes. A scanning electron micrograph of an opal in a capillary (330nm particles). All of these images are from our lab.
Figure 1.10 Van Deemter plots for acetophenone on various commercial columns. The minimum plate height was found to be $\sim 2d_p$. (copied from reference 31)
CHAPTER 2. CHEMICAL STABILIZATION AND FUNCTIONALIZATION OF SILICA COLLOIDAL CRYSTALS ON SLIDES AND IN CAPILLARIES

2.1 ABSTRACT

Silica colloidal crystals have been demonstrated to be useful in applications such as microarrays, permselective nanoporous membranes, total internal reflection microscopy, photonics, sensors, and chemical separations. Typically, silica colloidal crystals are sintered to increase their ruggedness. An alternative approach using horizontal polymerization is presented. Silica colloidal crystals, made of 330 nm particles and modified by horizontal polymerization with trichlorosilanes, are formed on coverslips as well as packed into capillaries and characterized via scanning electron microscopy (SEM), FTIR, fluorescence and optical microscopy, sonication, nanoindentation and UPLC to study stability, chemical composition, and packing density. The final material is shown to be durable, crack free, and chemically modified. This development allows for an in situ stabilization method for more rigorous handling of a chromatography column, microarray slide or other device.

2.2 INTRODUCTION

Silica colloids are on the order of 10 nm to about 1 μm.\textsuperscript{1,37} It is well known that silica colloids self-assemble into a highly ordered face centered cubic crystal\textsuperscript{39} when placed on a solid substrate, with varying thicknesses.\textsuperscript{40} Xia et al. review the use of silica
colloidal crystals as photonic band gap crystals, removable templates to fabricate macroporous materials, masks in lithographic patterning, and optical sensors. Silica colloidal crystals are also used in biological analyses, including their use in permselective nanoporous membranes, separations of DNA, small molecules, peptides and proteins, high surface area substrates for microarrays of DNA and protein analysis, porous substrates for total internal reflection fluorescence microscopy of live cells, and 3-D scaffolds for supported lipid films. However, for many of these applications the silica colloidal crystals need to be chemically modified, and they are also far too fragile for any practical use as deposited.

Silica is readily chemically modified via silylation with chlorosilanes. Sagiv was one of the first scientists to form self-assembled C<br>monolayers from trichlorosilanes on glass substrates. The formation and density of these organic monolayers was characterized by Wasserman et al. Commercial chromatographic bonded phases are not usually made from trichlorosilanes because these are not as reproducible as those from monofunctional silanes. Our group has previously discussed the preparation of mixed self assembled organic monolayers using a controlled amount of surface water to combat these problems. Too much water has been shown to easily lead to the formation of oligomers in solution and not enough water has been shown to yield an insufficient monolayer. Mixed layers containing octadecyltrichlorosilane and methyltrichlorosilane lead to a very dense self-assembled monolayer and decreases the amount of surface silanol groups. Self-assembly occurs via Si-Cl bonds reacting with the silanol groups and adsorbed water present on the surface of the silica,
leading to the formation of a network of Si-O-Si bonds.\textsuperscript{13} The result is a stable monolayer that has multiple covalent bonds to the other reagent silanes, and occasionally a covalent bond to the substrate.\textsuperscript{20} The density of bound silanes is coincidentally the same as the density of silanol groups on the surface, which is 8 \( \mu \text{m}/\text{m}^2 \).\textsuperscript{24} It was also previously demonstrated that upon the formation of these self-assembled monolayers there is an observed improvement in the hydrolytic stability under acidic conditions.\textsuperscript{12, 13, 14, 15, 16, 17, 18, 19} This result is explained by the fact that multiple Si-O-Si bonds are formed for each organotrichlorosilane, which forms a polymer network.\textsuperscript{20}

It is known that silica colloidal crystals are held together by van-der Waals forces, and the crystals easily break upon outside agitation or contact.\textsuperscript{21} The fragile nature of the crystals prevents aggressive handling in their use in microarrays and separations. Our lab previously described a method to stabilize silica colloidal crystals by sintering with high temperature.\textsuperscript{21, 23} One disadvantage of this method of stabilization is the requirement of high temperature, which could result in a disturbing of the order of the crystal lattice. Miguez et al. designed a possible stabilization alternative by using layer by layer growth of silica via chemical vapor deposition of silicon tetrachloride, which reacts with the hydration layer.\textsuperscript{22} It was observed that an increase in mechanical stability could be achieved. SEM images showed that the deposited layer gave thicker connections between particles. In chromatography, these deposits decrease the pore sizes. Since it is difficult to achieve reproducibility with silicon tetrachloride, and reproducible pore size is critical in chromatography, silicon tetrachloride is not an attractive candidate for stabilizing silica colloidal crystals in our applications.
The use of mixed trichlorosilanes to increase the mechanical stability of colloidal crystals via chemically sintering the particles was investigated. The idea is that chemical bonds form between the particles and the particles and the substrate comprising the crystal to increase stability (Figure 2.1), and at the same time, they provide an excellent chromatographic monolayer. SEM, FTIR, fluorescence and optical microscopy, sonication, nanoindentation and UPLC were used to characterize the modification and test the mechanical stability of the crystal. An increase in mechanical stability of the silica colloidal crystal should aid with their use in microarrays or multiwall plate assays, substrates for immunoassays, electrophoresis media, HPLC and UPLC or molecular sieving.

2.3 EXPERIMENTAL SECTION

2.3.1 CHEMICALS AND MATERIALS. Trichlorosilanes were purchased from Gelest Inc., Morrisville, PA, and toluene, methanol, and nitric acid were purchased from Sigma Aldrich, St. Louis, MO. Silica particles (0.350 μm) were purchased from Fiber Optic Center Inc., New Bedford, MA. Albumin from bovine serum (BSA), fluorescein conjugate was purchased from Molecular Probes, Carlsbad, CA.

Fused silica capillary was obtained from Polymicro, Phoenix, AZ (TSP100375, 100 μm I.D.). Glass coverslips purchased from VWR (48300-025, 25 x 75 mm) were cut into 25 x 25 mm slides. Double polished silicon wafers (100 mm) were purchased from SUMCO USA, Phoenix, AZ.
2.3.2 SILICA COLLOIDAL CRYSTALS (SCCs). The silica particles (0.35 μm) were calcined at 600 °C three times, for 6 hours each, in a box furnace (Lindberg/Blue, BF1766A). To ensure minimal aggregation between each calcination step, the particles were suspended in ethanol by sonication (Advanced Sonic Processing Systems, Sonochemical Reaction Vessel w/ VWR 6000 series chiller). The particles are then acid rehydroxylated after calcination using 50:50 nitric acid/water. The resulting particles were found to be 0.33 μm in diameter by SEM.

Colloidal crystals were formed on glass coverslips and silicon wafers by spin coating (Brewer Science, CEE model 200). Coverslips and silicon wafers were cleaned with 7x detergent and lens tissue, followed by 90 minutes in 50:50 nitric acid/water. The clean coverslips were then placed on the spin coater and a 200 μL suspension of 10 % (wt %) calcined colloids in a mixture of acetone and water is used to spin. After deposition, the crystals were rehydroxylated using 50:50 nitric acid/water.

Colloidal crystals can be packed in fused silica capillaries in a variety of ways. I chose to pack by a variation of gravity packing previously discussed by Colón et al. The capillary was cleaned by slowly pumping (Harvard Apparatus PhD 2000) sodium hydroxide (Sigma) through the capillary, followed by ultrapure water (Millipore, Milli-Q), and a final rinse with 100 % ethanol.

The capillary is then cut into smaller capillaries each 10 cm long and is put in a vacuum oven (VWR) for 20 minutes at 70 °C. The clean capillaries are then placed into a mixture of 25 % (wt %) rehydroxylated particles in water and the slurry fills the column
via capillary forces. Upon completion of the slurry filling the capillary, the capillary is placed vertical in a vial and allowed to dry at room temperature (~23 °C). The drying was done at room temperature because it has been demonstrated that the quality of the colloidal crystal can be improved by the controlling of drying.\(^{28}\) This process typically yields colloidal crystals about 2 cm in length.

### 2.3.3 CHEMICAL MODIFICATION AND STABILIZATION OF SCC

Prior to the silanization reactions, the silica colloidal crystals (SCCs) on planar substrates were placed in the UV ozone system, at a constant humidity of 50 %, for 15 minutes to allow for the proper amount of surface adsorbed water. The SCC is then placed directly into the reaction flask containing the silanes in anhydrous toluene. ((chloromethyl)phenylethyl)-trichlorosilane and methyltrichlorosilane were mixed in the ratio of 20:1 (v/v). The reaction (Figure 2.2) was performed at room temperature (~23 °C) over three hours. The bonded phase was then washed with dry toluene and allowed to dry at 120 °C for one hour. Atom transfer radical attachment of 1,2-Epoxy-5-hexene was then performed. A flask containing 2.25 mL 1,2-Epoxy-5-hexene, 100 mL DMF and 125 \(\mu\)L Methyl TREN was bubbled with Ar for 30 minutes to remove oxygen. The SCC was placed in a reaction flask and 100 \(\mu\)mols of CuCl was added to a schlenk flask. Using a schlenk line, an inert atmosphere was established in both the reaction flask and the schlenk flask. The epoxy-hexene solution was transferred into the schlenk flask with the copper salt. Once the copper salt was completely dissolved, this solution was transferred into the reaction flask and left for 1 h. The slides were removed from the solution and rinsed with DMF.
The reaction with (3-glycidoxypropyl)trimethoxysilane (Figure 2.3) was performed under refluxing conditions for three hours. As with the trichlorosilanes the bonded phase was then washed with dry toluene and allowed to dry at 120 °C for one hour.

Prior to silanization reactions, the SCCs in fused silica capillaries were placed in a humidity chamber, at a constant humidity of 50 %, for one hour. The SCC is then placed directly into the reaction flask containing the silanes in anhydrous toluene.

Octadecyltrichlorosilane and Methyltrichlorosilane were mixed in the ratio of 4:1 (v/v). The reaction (Figure 2.4) was performed at room temperature (~23 °C) over a period of three hours. The bonded phase was then rinsed with dry toluene using an Agilent 1100 series HPLC and allowed to dry at 120 °C for one hour.

It is important that the trichlorosilane reaction is not done under too high of humidity as self-polymerization is possible in the presence of too much water.19

2.3.4 SCANNING ELECTRON MICROSCOPY. Samples were coated with platinum prior to electron microscopy. Field emission SEM micrographs were collected using a Hitachi S-4800 Type II using ThermoNORAN NSS EDS.

2.3.5 FT-INFRARED SPECTROSCOPY. Infrared spectra were collected for colloidal crystals on silicon using a Nicolet 4700 FTIR from Thermo Electron Corporation. The FTIR spectra were collected at 2-cm⁻¹ resolution and 512 scans, with a bare silicon wafer
as the blank. The baselines in the spectra were corrected. The spectra were acquired in transmission mode at 55° incidence with vertical polarization to avoid interference fringes. The plates were cleaned with boiling HPLC grade methanol and dried with nitrogen before the spectra were obtained.

2.3.6 MICROARRAY SPOTTING. All microarrays were done using BSA-FITC. The total average fluorescence data for spots was generated by spotting the SCC using a Whatman microcaster 8-pin system hand printer. One pin was used to deliver protein multiple times and the surface was then rinsed and blocked before the data was collected using a Nikon Eclipse TE2000-U with a Nikon model C-SHG1 mercury lamp power supply, and a Cascade 512B CCD camera from photometrics.

The glycidoxy SCC and the ATRA SCC for imaging were generated by the Genomics Core at the Arizona Cancer Center. The fluorescence and white light images were collected with a Nikon Eclipse TE2000-U with a Nikon model C-SHG1 mercury lamp power supply, and a Cascade 512B CCD camera from photometrics.

2.3.7 SONICATION TEST. Modified SCCs on glass coverslips were placed into glass vials containing water and placed into a VWR model 75T sonicator. The time the individual coverslips spent in the sonicator was varied from 0 – 60 seconds. Both the mixed horizontally polymerized surface and the (3-Glycidoxypropyl)Trimethoxysilane
modified surface were studied. Unmodified SCCs were also studied and used as a reference.

2.3.8 NANOINDENTATION. Nanoindentation data was obtained for SCC on glass coverslips using a NANO UTM from Agilent Technologies by Eric Ross of Gonzaga University. Three trials were run on each of the trichloro, glycidoxy, and unmodified SCC. The data were collected at a maximum force of 50 mN.

2.3.9 PRESSURE VS. FLOW RATE. Three ~2-cm octadecyltrichlorosilane/methyltrichlorosilane modified SCC were tested on a MicroTech Scientific XtremeSimple Dual Binary System. The SCC were tested using 100 % methanol with 0.1 % formic acid. Valco 1/16” stainless steel ferrules (Pt# ZF1S6) with PEEK 1/16” OD X 0.020” ID tubing (UpChurch Pt# 1532) and Valco stainless steel nuts (Valco Pt# ZN1) were used in conjunction with a narrow bore stainless steel union (Valco Pt# ZU1XC) to provide a connection to the LC, 25µM ID fused silica exit tubing (Polymicro Pt#TSP025375) able to withstand approximately 15,000 psi. In addition to the flow rate setting on the LC system, a SGE 25-µL LC syringe with a 26 gauge needle was attached to the column exit using a stainless steel union (Valco Pt# ZU1XC or similar), an Upchurch One-piece PEEK finger-tight fitting (Pt# F130), and a piece of 1/16” OD Teflon tubing used to secure the syringe needle.
2.4 RESULTS AND DISCUSSION

Figure 2.5a shows an image of a colloidal crystal on a glass substrate that was made from spin coating pre-calcined 330 nm silica particles and modified via horizontal polymerization with trichlorosilanes. Figure 2.5b shows an image of a colloidal crystal made from the same particles but packed in a capillary. Both colloidal crystals are a bluish green color, characteristic of Bragg diffraction for 330 nm particles. The Bragg peak is based on the particle diameter, refractive index, and crystal thickness.23

SEM was used to study the horizontal polymerization modification reactions. Micrographs in Figure 2.6 demonstrate that water plays a major role in the reactions. As previously reported by Fairbank et al., at too low of humidity the trichlorosilanes are believed to react directly with surface silanols.19 At too high of humidity it is believed that oligomers can form.19 As shown in the Figure 2.6a top and Figure 2.6b top microscopic silane polymers will result at 80% humidity. With these micrographs and supporting literature it is very important to note that, while a full surface water layer is very important when trichlorosilanes are going to be used for horizontal polymerization, too much water will lead to complications. Since this is the case, I incubated the crystals in a humidity chamber at 50 % humidity before modification which results in a complete surface layer of water and results in a good SCC19 (Figure 2.6a & b bottom).

Modification with the mixed trichlorosilanes should correlate with an increase in the C-H stretching, and this was confirmed by FTIR measurements. The FTIR spectrum
of the mixed ((chloromethyl)phenylethyl)-trichlorosilane (benzyl chloride, or BC initiator) and methyltrichlorosilane (C1) surface is shown in Figure 2.7. After the initiator is attached to the surface, atom transfer radical attachment (ATRA) of the epoxy is performed. A visual comparison of these spectra with each other and an unmodified SCC (Figure 2.7) shows that the C-H stretching increases upon the addition of the silanes and further increases upon the addition of epoxy. The major peaks are methylene symmetric and asymmetric stretches at 2580 and 2917 cm\(^{-1}\), respectively. The smaller peak at 2959 cm\(^{-1}\) is assigned to the asymmetric methyl stretch.\(^{14}\) The SCCs were also found to bead water after the silylation reactions with trichlorosilanes. Upon completion of the ATRA the slides become more hydrophilic.

The FTIR spectrum of (3-glyciodxypropyl)trimethoxysilane modified SCC is shown in Figure 2.8. A comparison of the spectrum with an unmodified SCC shows that the C-H stretching increases upon the addition of silane. Unlike the trichlorosilane modified surfaces, the glyciodxy surface does not have the methyl stretch present, which is expected based on the structure of the molecule.

To check for activity of the epoxide rings, an unmodified SCC and modified SCC with BC -C1 and ATRA of epoxy and glycidoxy were all printed with protein in-house using a Whatman microcaster hand printer. A total of five spots (BSA-FITC) were imaged using fluorescence microscopy, and an average total fluorescence of the spots was determined. Figure 2.9 shows the average total fluorescence of the spots, which is background corrected for the scatter from the silica colloidal crystal itself. The ATRA
and Glycidoxy modified crystals show significant fluorescence, since the amines from the protein covalently bond to the surface by reaction with the epoxide ring. The epoxy and glycidoxy crystals give total average fluorescence values of $1.6 \times 10^8$ and $3.8 \times 10^8$, respectively. The unmodified and BC-C1 modified SCC showed nonspecific adsorption, with average fluorescence values of $6.7 \times 10^6$ and $3.4 \times 10^7$, respectively. One possible reason for the ATRA modification having less average fluorescence than the glycidoxy-modified crystal could be related to the methyl spacers. Methyl spacers are known to be very reactive and could make up a large portion of the total surface coverage. The methyl spacers have been added since it has been shown to increase the total surface coverage which means a more complete self-assembled monolayer is achieved. Another possibility, but less likely, is that the ATRA reaction had not come to completion.

Glycidoxy and ATRA modified slides were sent to the Genomics Core at the Arizona Cancer Center where they were printed with BSA-FITC. Both fluorescence and optical micrographs were taken of the spotted surfaces (Figure 2.10). The fluorescence and optical images are from the same region on the crystal. The fluorescence images again show the activity of the epoxide ring to bind the amine group of proteins, giving easily observable spots. The spot size is much smaller for the BC-C1 surface because the methyl spacers allow for a more hydrophobic surface that the conventional glycidoxy surface, and hence impede wicking of the drop. The optical images further show that the ATRA surface has less pronounced divots, which indicate an increase in mechanical stability. The glycidoxy surface had been made from trimethoxysilanes, which do not
self-assemble as well. This result establishes that the horizontal polymerization significantly improves mechanical stability.

The increase in mechanical stability was further tested by using sonication on crystals that had been chemically modified with BC-C1, and Glycidoxy. The results of the sonication of the modified crystals and an unmodified crystal are shown in Figure 2.11. It can be seen that the stability increases after both modifications, however, it is much more pronounced for the mixed horizontal polymerization of trichlorosilanes. This increase in mechanical stability is likely due to the better connectivity of the particles to one another and to the silica substrate itself. Silicon tetrachloride has been shown to increase the mechanical stability via connection of the silica particles to one another. It is therefore reasonable to expect both dichlorosilanes and trichlorosilanes to aid in the connection of the colloids to one another and to the silica substrate.

To test whether the connectivity arises from the siloxane network created when horizontal polymerization occurs a slide modified with a mixed phase of trichlorosilanes was placed into a UV-Ozone plasma cleaner to remove the organic portion of the silane after modification. The same sonication test was performed (Figure 2.12). A similar result was observed for the trichlorosilane modification as was observed in Figure 2.11. An important note is that upon removal of the organic layer, we are able to modify the surface with any silane of our choice, e.g., glycidoxy. This is advantageous because few silanes are available in the trichloro form.
An independent quantitative assessment of mechanical stability was carried out via nanoindentation.\textsuperscript{29, 30} Nanoindentation allows for plots of the load (P) versus displacement (h) for unmodified (Figure 2.13) and modified (Figure 2.14 & 2.15) crystals and a plain glass substrate (Figure 2.16). The dotted lines represent the point at which either a sudden jump in displacement is observed or when a sudden drop in load is observed at a fixed displacement. It has been shown in the literature that during nanoindentation, a break-down of the initial crystal structure can result in a discrete jump in the P-h curve.\textsuperscript{29} This would suggest that the higher the load at which the crystal breaks down the higher the mechanical stability. The order of mechanical stability of our crystals is trichloro > trimethoxy > unmodified with an average load at the break down point of 20 mN, 11 mN, and 8 mN, respectively. The data may also reflect the way that the surface breaks down. In other words, it seems like both the unmodified and the trimethoxy modified crystals break down in larger chunks this is suggested by the multiple break points. It also suggests that the trichloro modified crystals collapse all at once.

Mechanical stability of the SCCs in fused silica capillaries were tested using UHPLC. Flow rate versus pressure plots were obtained for three 2 cm long SCCs packed into 100 μm ID fused silica capillaries (Figure 2.17). It is important to note that no frit is used, and the crystals were pressurized between 12,000 and 13,000 psi to test if they could withstand high pressures. At least five flow-rate measurements were taken at each pressure and the average reported as a single data point. Multiple separate flow-rate experiments were also performed on each crystal. Crystals #1 and #2 had nearly identical
profiles while crystal #3 had a steeper slope with greater flow rates at the same pressures as crystals #1 and #2. Crystals #1 and #2 had stationary phase beds of 2.40 and 2.31 cm respectively, while crystal #3 had a shorter bed length of 2.18 cm. The differences in the bed length can be used to explain the differences in slope from crystal to crystal. It is known that the back pressure exhibited on a chromatographic bed is directly related to the length. All of the plots exhibit linear character, which is indicated by the very small standard deviation associated with the slope from the flow rate against pressure plots. The flow rates obtained through the crystals are also on the scale that is needed to perform nanospray ionization. Figure 2.18 is an image of the spray from one of the crystals.

Work is being performed in our lab in conjunction with Phoenix S&T to use these crystals as nanocolumns for separations in the field of proteomics. It has been shown that a decrease in particle diameter will lead to a decrease in plate height since the contributions from the eddy diffusion term as well as the mass transport term of the van Deemter equation are dependent on particle diameter. With the introduction of ultra-high pressure pumps, smaller particles will now be able to be used in separations. This is already being demonstrated by the decrease in plate height from UHPLC (1.7 µm particles) to conventional HPLC (5 µm particles) for commercial columns.

2.5 CONCLUSIONS

Silica colloidal crystals modified by horizontal polymerization with trichlorosilanes are shown to be more durable than unmodified, or those modified with trimethoxysilanes. Stabilization occurs because of the siloxane network that is formed via horizontal
polymerization. The organic portion of the trichlorosilane may be removed using an UV-ozone plasma cleaner, and the SCC will still exhibit increased stability. Fused silica capillaries packed with SCCs were able to withstand pressures above 12,000 psi without a frit. The ability to both stabilize and modify silica colloidal crystals at the same time extends their ruggedness while also extending their range of applications.

ACKNOWLEDGEMENTS

I thank Dr. George Watts at the Genomics Core at the Arizona Cancer Center for spotting the glycidoxy and ATRA slides and Eric Ross of Gonzaga University for performing the nanoindentation measurements. This work was supported by the National Institutes of Health.
2.6 REFERENCES


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2.7 FIGURES

Figure 2.1 A schematic diagram illustrating chemical sintering.
Figure 2.2 Mixed horizontal polymerization reaction of ((chloromethyl)phenylethyl)-trichlorosilane and methyltrichlorosilane followed by atom transfer radical attachment (ATRA) of 1,2-epoxy-5-hexene.
Figure 2.3 Silylation reaction with (3-Glyciodxypropyl)Trimethoxysilane.
Figure 2.4 Mixed horizontal polymerization reaction of octadecyltrichlorosilane (C18) and methyltrichlorosilane (C1).
Figure 2.5 Photograph of SCCs, made of 0.33 μm particles, on (a) a planar glass coverslip and (b) in a 100 μm I.D. fused silica capillary.
Figure 2.6 SEM images of a SCC made from 0.35 µm particles and modified using the trichlorosilanes on (a) a glass coverslip and (b) in a fused silica capillary. The polymer visible in the top micrograph of both sets is an image of what happens if too much water (80 % humidity) is present in the mixture. The bottom micrograph in both sets shows the effect of having 50% humidity.
**Figure 2.7** FTIR spectra of the mixed horizontally polymerized surface using \((\text{chloromethyl})\text{phenylethyl})\text{-trichlorosilane and methyltrichlorosilane and the ATRA product.}
Figure 2.8 FTIR spectra of (3-Glycidoxypropyl)Trimethoxysilane surface.
Figure 2.9 Microarray spotting of fluorescein conjugated bovine serum albumin (BSA-FITC) onto SCC. The total average fluorescence of a single spot on the SCC is reported in both tabular and graph form. The data indicates that the epoxy rings from the ATRA and Glycidoxy reactions are both active on the SCC and are able to bind protein.

<table>
<thead>
<tr>
<th>Coating</th>
<th>Average Total Fluorescence</th>
<th>Sigma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unmodified</td>
<td>6.7E+06</td>
<td>4.6E+06</td>
</tr>
<tr>
<td>Initiator/C1</td>
<td>3.4E+07</td>
<td>1.6E+07</td>
</tr>
<tr>
<td>ATRA</td>
<td>1.6E+08</td>
<td>2.9E+07</td>
</tr>
<tr>
<td>Glycidoxy</td>
<td>3.8E+08</td>
<td>1.3E+08</td>
</tr>
</tbody>
</table>
Figure 2.10 Fluorescence micrographs and white light micrographs of BSA-FITC spotted on a (3-Glycidoxypropyl)Trimethoxysilane modified and an ATRA modified surface.
Figure 2.11 Photograph showing the effect of sonication on modified silica colloidal crystals.
Figure 2.12 Photograph showing the effect of sonication on a trichloro modified silica colloidal crystal after the organic portion was removed using an UV-ozone plasma cleaner.
Figure 2.13 Load (P) versus displacement (h) plot of nanoindentation of an unmodified SCC. The dotted lines represent the qualitative point where the crystal starts to break down.
Figure 2.14 Load (P) versus displacement (h) plot of nanoindentation of a Trimethoxy (glycidoxy) modified SCC. The dotted lines represent the qualitative point where the crystal starts to break down.
Figure 2.15 Load (P) versus displacement (h) plot of nanoindentation of a Trichloro modified SCC. The dotted lines represent the qualitative point where the crystal starts to break down.
Figure 2.16 Load (P) versus displacement (h) plot of nanoindentation of a bare glass substrate, used as a control.
Figure 2.17 Pressure vs. flow rate for SCCs packed in fused silica capillaries.
Figure 2.18 Spray from SCC packed fused silica capillary.
CHAPTER 3. SUB-MICRON PLATE HEIGHTS FOR CAPILLARIES PACKED WITH SILICA COLLOIDAL CRYSTALS

3.1 ABSTRACT

Extremely uniform packing of colloidal silica in capillaries is shown. Reversed-phase electrochromatograms of DiI-C12 exhibit plate heights as low as 0.13 μm and a reduced plate height as low as 0.4, using 75 μm i.d. capillaries packed with 330 nm silica particles. The contribution from the A term is 0±20 nm in electrochromatography. The particles are shown to form colloidal crystals inside the capillaries. Optical images show Bragg diffraction, indicative of crystallinity; SEM images show face-centered cubic crystallinity; and the porosity is 0.25±0.01, which is in agreement with that of face-centered cubic crystals. The capillaries are fritless, and 100 μm i.d. capillaries packed with silica colloidal crystals withstand pressures of at least 12,400 psi.

3.2 INTRODUCTION

The introduction of sub-2 μm particles to reversed phase liquid chromatography (RPLC) has delivered improved speed and resolution to separations.1, 2 These improvements are explained by the contribution of the particle diameter, $d_p$, to the plate height, $H$,3

$$H = A + \frac{B}{v} + Cv$$

(1)
where the $A$ and $C$ parameters are dependent upon the diameter, $d_p$, of the porous particles.

$$A = 2\lambda d_p; \quad B = 2\gamma D; \quad C = \frac{\omega d_p^2}{D}$$  \hspace{1cm} (2)

This model neglects contributions from injection, detection, and slow desorption from the surface. The parameters $\lambda, \gamma$ and $\omega$ are due to details of the particles and the packing. Using smaller particle diameters increases the optimal velocity and gives lower plate heights; as a result, sub-2 $\mu$m particles have had a significant impact on the practice of HPLC.\textsuperscript{4}

Detailed studies have shown that $\lambda$ increases as the particle size becomes smaller,\textsuperscript{3} indicating that efficiency is limited by nonuniform packing. This arises from a radial distribution of packing densities in the cylindrical tube, giving a radial distribution of velocities, which broadens peaks.\textsuperscript{5-8} Recent experiments using nonporous 1.0 $\mu$m particles in capillaries of varying diameter show that the $A$ term is primarily due to the radial heterogeneity of the packing.\textsuperscript{9}

The purpose of this paper is to investigate whether better packing is possible with silica colloidal crystals because they form face centered cubic lattices.\textsuperscript{10} Slabs of silica colloidal crystals have been used for separations of small molecules,\textsuperscript{11} and for DNA and protein sieving.\textsuperscript{12} The plate heights are well above 1 $\mu$m,\textsuperscript{11,12} yet the colloidal crystals should achieve sub-micron plate heights by virtue of the crystalline packing. In this
work, silica colloidal crystals packed inside capillaries\textsuperscript{13} are used to determine whether sub-micron plate heights are attainable.

3.3 EXPERIMENTAL

3.3.1 CHEMICALS AND MATERIALS. Silanization reagents were purchased from Gelest Inc., Morrisville, PA, and the toluene, methanol, and nitric acid were purchased from Sigma Aldrich, St. Louis, MO. The silica colloids (0.350 \( \mu \text{m} \)) were purchased from Fiber Optic Center Inc., New Bedford, MA. The DiI molecules were purchased from Molecular Probes, Carlsbad, CA. Fused silica capillary was obtained from Polymicro, Phoenix, AZ.

3.3.2 SILICA COLLOIDAL CRYSTALS (SCCs). 0.35 \( \mu \text{m} \) colloids were calcined at 600 °C three times for 6 hours each in a box furnace (Lindberg/Blue, BF1766A). To eliminate aggregation between each calcination step, the colloids were suspended in ethanol by sonication (Advanced Sonic Processing Systems, Sonochemical Reaction Vessel w/ VWR 6000 series chiller). The colloids were then acid rehydroxylated using 50:50 nitric acid/water. The resulting colloids are 0.33 \( \mu \text{m} \) in diameter.

The capillary was cleaned by slowly pumping (Harvard Apparatus PhD 2000) sodium hydroxide (Sigma) through the capillary, followed by ultrapure water (Millipore, Milli-Q), and a final rinse with 100 \% ethanol. The capillary was then cut into smaller
capillaries each 10 cm long and is put in a vacuum oven (VWR) for 20 minutes at 70 °C. The clean capillaries were then placed into a mixture of 25 % (wt %) rehydroxylated colloids in water and the slurry fills the column via capillary forces. The capillary was then placed vertical in a vial and allowed to dry at room temperature (~23 °C). This typically yields colloidal crystals of about 2 cm in length.

3.3.3 CHEMICAL MODIFICATION OF SCCs. Prior to the silanization reactions, the SCCs in fused silica capillaries were placed in a humidity chamber at a constant relative humidity of 50 %, for thirty minutes. The colloidal crystals were then chemically modified by horizontal polymerization of trichlorosilanes bearing the functional groups C\textsubscript{18} and C\textsubscript{1}, to give a C\textsubscript{18} density of 3 μm/m\textsuperscript{2}.\textsuperscript{14} The reaction was performed overnight at room temperature (~23 °C) to allow for the mixed self-assembled monolayer to form. The bonded phase was then rinsed with dry toluene using an Agilent 1100 series HPLC and allowed to dry at 120 °C for two hours.

3.3.4 CAPILLARY HOLDER FABRICATION. Polydimethylsiloxane (PDMS) molds were made by pouring a 10:1 mixture of PDMS base (sylgard 184, Dow Corning, Midland, MI) to curing agent into a petri dish, followed by drying overnight at 40 °C. The PDMS was then removed from the petri dish and reservoir holes were fabricated using a cork bore set. All of the holders were assembled by sealing PDMS to clean glass slides with oxygen plasma oxidization. All of the holders used in this study have the design
illustrated in Figure 3.1. The capillary columns that were used were ~2 cm long and either 100 μm or 75 μm in inner diameter.

3.3.5 SCANNING ELECTRON MICROSCOPY. SEM images were obtained with a JEOL Neoscope.

3.3.6 MEASURING POROSITY. Porosity was measured from the volume of water that wicks into a known length of colloidal crystal. A water plug was wicked into the open region of a capillary, and its length was measured. It was then pushed by syringe to touch the colloidal crystal, where it then wicked into the crystal. After filling the full length of the colloidal crystal, which is evident from the increase in transparency, the length of the remaining water plug was measured. For a change in the length of the plug of water, \( \Delta l_w \), and a length of colloidal crystal \( l_c \), the porosity, \( \varepsilon \), is the ratio of the two values.

\[
\varepsilon = \frac{\Delta l_w}{l_c}
\]

3.3.7 ELECTROCHROMATOGRAPHY. Columns are initially cleaned in boiling methanol for 15 minutes; this allows for the boiling methanol to wick into the capillary
via capillary forces and rid the stationary phase of any adsorbed organics. The mobile phase is sonicated to remove pre-existing air bubbles. The mobile phase is then pumped through the capillary columns using an Agilent 1100 series HPLC. The capillary columns were then placed into the holder and conditioned at 500 V/cm, using a Matsusada high voltage power supply, for 15 minutes. Injection was done by quickly dipping the capillary, into a 13 µM solution of DiI (1,1-didodecyl-3, 3, 3,3-tetramethyl-
indocarbocyanine perchlorate) in methanol, which allowed the analyte to adsorb at the entry of the capillary. The electromigration was driven using Pt electrodes in the reservoirs, with electric fields ranging from 500 to 2000 V/cm. The peaks were imaged using an inverted fluorescence microscope equipped with a fluorescence cube for Cy3 and a 2x objective. Movies of the migrating dye were acquired using a sensitive CCD camera (Princeton Instruments Photonmax) to view the migration from injection to final separation. The same equipment was used for measuring diffusion coefficients.

3.3.8 PRESSURE VS. FLOW RATE. Two ~2-cm octadecyltrichlorosilane/methyltrichlorosilane modified SCC were tested on a MicroTech Scientific XtremeSimple Dual Binary System.

3.4 RESULTS AND DISCUSSION

Figures 3.2a and 3.2b show photographs of a typical packed capillary through a zoom optical microscope with color camera. The photographs illustrate the blue color,
which is indicative of crystalline packing of the particles. The color is due to Bragg
diffraction from the even spacing of particles on the order of the wavelength of blue
light. Figure 3.2c shows the SEM image of a small region of the capillary. The
packing is polycrystalline, with two different crystal faces evident: the (111) face, which
has hexagonal symmetry, and the (100) face, which has square symmetry. The packing
exhibits mostly domains of hexagonal symmetry, with occasional domains of square
symmetry. The full SEM image (Figure 3.3) shows that this polycrystalline packing
extends all the way across the capillary. The region near the wall is dominated by square
symmetry, while hexagonal symmetry is more prevalent for the bulk, but both are face-
centered cubic colloidal crystal. In short, what is different about this capillary is that
crystalline structure extends all the way across the capillary up to the walls, whereas
conventional columns exhibit order only near the wall, while the bulk is amorphous. If
this polycrystalline packing that is visible at the end of the capillary persists throughout
the length of the capillary, it would avoid the radial heterogeneity of packing density,
yielding smaller plate heights.

One cannot image the interior of the colloidal crystal, but resistance to flow and
porosity can reveal whether the particles are close-packed. Figure 3.4 provides a plot of
volume flow rate vs. pressure for a pair of capillaries having inner diameters of 100 µm
i.d. and packing lengths of 2.31 and 2.40 cm. The highest volume flow rates are 200
nL/min at 12,400 psi, which are more than sufficient to achieve stable nanospray
ionization. The dimensionless resistance to flow, $\phi$, is calculated from the linear velocity,
\(v\), through the area \(\pi r_c^2\), for capillary radius \(r_c\), pressure, \(P\), length, \(L\), viscosity, \(\eta\), and particle diameter, \(d_p\).\(^{16}\)

\[
\phi = \frac{P d_p^5}{v \eta L} \quad (4)
\]

For the two capillaries, the values of \(\phi\) are 747 and 733. To compare with pressure-packed capillaries, for the nonporous 1.0 \(\mu m\) particles,\(^{17}\) \(\phi=363(\pm10)\), which is two-fold lower. The flow resistance thus indicates that the colloidal crystal is packed more densely. A quantitative indicator of close-packed structure is the porosity, which is 0.39 for pressured-packed particles\(^{18}\) and 0.26 for face-centered cubic crystals.\(^{19}\) A histogram of porosity values (Figure 3.5) shows that \(\varepsilon=0.25\pm0.01\) (95% CI). This is in agreement with the porosity of face-centered cubic crystals, revealing that the entire volume of the packing is a colloidal crystal.

To determine plate height, DiI-C\(_{12}\) was electromigrated over distances on the order of 1.5 mm at varying electric fields. The plate height was determined by fitting each peak to a Gaussian after traveling to the end of the field of view of the fluorescence microscope. Raw data for representative electrochromatograms are shown in Figure 3.6, covering the range of electric fields. This raw data shows plate heights that are well under 1 \(\mu m\). Figure 3.7 shows the same electrochromatograms, plotted on an expanded scale, with their corresponding Gaussian fits which were used to calculate the plate height.
values. The injected widths were then corrected for at the varying electric fields (Figure 3.8), which were typically found to contribute ~100 nm to the plate height. The contribution from the detection was also calculated and found to contribute negligibly (~3 nm).

The plate-height, with the contribution from injected width subtracted out, is plotted vs. migration rate in Figure 3.9. The velocity axis is the analyte migration rate, rather than flow rate, to facilitate an independent determination of the $B$ term. The $B$ term was measured by migrating the peak well into the packed capillary, and monitoring peak variance vs. time with no field. This plot (Figure 3.10) shows $B=2\gamma D=18.4(\pm0.02) \mu m^2/s$. Knowing the $B$ term allows $A$ as the only fitting parameter. The best-fit curve from the nonlinear regression, shows that $A=0\pm20$ nm. There is evidence of heating (Figure 3.11), but this does not affect plate height, in agreement with our previous report.\textsuperscript{11} This is perhaps a consequence of the high thermal conductivity of a densely packed material. At the highest electric field, a plate height of 0.13 $\mu$m is observed after correction for the contribution from injection.

One might wonder if the sub-micron plate heights are simply from using sub-micron particles. Table 3.1 compares the plate height, as well as the reduced plate height ($h=H/d_p$), with earlier reports for electrochromatography with colloidal silica. The plate height for this material is significantly lower in every case. A Knox plot is given in Figure 3.12. The lower values of the plate height and reduced plate height reveal that the
ultrahigh efficiency is not simply due to using smaller particles, instead, more uniform packing is achieved.

3.5 CONCLUSIONS

These results show that silica colloidal crystals made of 330 nm diameter silica spheres give extremely low plate heights. Lengths of 1.5 mm were used in this study, and the plate height values are as low as 130 nm which yield about 9000 theoretical plates. Upon independently determining \( \gamma D = 9.2(\pm 0.7) \times 10^{-8} \text{ cm}^2/\text{s} \), this value was used to fit the van Deemter plot and yielded an Eddy diffusion term of 0±20 nm. Now that the plate height is known to be diffusion limited, molecules with lower diffusion coefficients (such as proteins) can be used which would allow for even smaller plate heights. Research will be done to investigate the use of these materials for their possible improvements in the field of proteomic analysis.

Acknowledgments

This work was supported by NIH under grants R01GM065980 and R44 RR21799.
3.6 REFERENCES


### 3.7 TABLES

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**Table 3.1.** Electrochromatographic plate heights for nonporous colloidal silica spheres.
3.8 FIGURES

Figure 3.1. Schematic and photograph of electrochromatography experiment.
Figure 3.2. Images of a silica colloidal crystal packed in a capillary. Photographs through an optical microscope for a) end-on view and b) side-on view. c) SEM image of the end-on view of a region of the capillary.
Figure 3.3. SEM image of the full capillary cross-section.
Figure 3.4. Graph of volume flow rate vs. pressure for two 100 µm i.d. capillaries packed with silica colloidal crystals. The error bars are standard deviations from multiple measurements.
Figure 3.5. Histogram of porosity values. Ten measurements were made for the volume of water that wicked into colloidal crystals of known length in 100 μm i.d. capillaries.

95\% \text{ CI}: \varepsilon=0.25 \pm 0.01
Figure 3.6. Raw electrochromatograms for DiI-C$_{12}$ at four different electric fields, superimposed on the same time axis, without removing the contribution from injected width. The mobile phase was 90:10 MeOH:water and 0.1 % formic acid, and the stationary phase was horizontally polymerized C$_{18}$/C$_{1}$. 
Figure 3.7. Raw data (o) for chromatograms of DiI-C$_{12}$ on expanded time scales at four electric fields over the range that was used, showing high quality of the Gaussian fit (—). The injected width is included in the overall peak profile. The fitting parameters, separation distances and plate heights are listed for each chromatogram.
Figure 3.8. Plots of typical injected peaks (o) and fit to a Gaussian (-) for each. The plate heights for the entire set of 16 injections ranged from 48 to 110 nm.
Figure 3.9. Plot of plate height vs. analyte migration rate. The circles are experimental data and the solid curve is $H=2\gamma D/v$. Regression showed that $A=0\pm20$ nm.
Figure 3.10. Plot of the peak variance vs. time, as a plug of DiI-C\textsubscript{12} underwent diffusion in the capillary in the absence of an electric field. A least-squares fit to determine the slope, which is equal to 2\(\gamma D\), revealed the diffusion coefficient to be \(\gamma D = 9.2(\pm0.7) \times 10^{-8}\) cm\textsuperscript{2}/s.
Figure 3.11. Plot of migration rate of DiI-C$_{12}$ vs. applied electric field reveals nonlinearity at high fields, which is likely due to heating of the capillary. The error bars are standard deviations from multiple measurements.
Figure 3.12. Knox plot of the same data that are provided in Figure 3.9. The solid line is a plot of the equation shown in the graph, \( h = \frac{2}{\nu} \).
CHAPTER 4. SUB-MICRON PLATE HEIGHTS FOR THE SEPARATION OF INTACT PROTEINS IN CAPILLARIES PACKED WITH SILICA COLLOIDAL CRYSTALS

4.1 ABSTRACT

Fused silica capillaries packed with silica colloidal crystals formed from 385 nm nonporous silica particles were evaluated for their potential increase in efficiency for protein separations. Reversed-phase electrochromatograms of a three-protein mixture, lysozyme, ribonuclease A, and cytochrome C, exhibit submicron plate heights and excellent resolution ($R_s \approx 5$) in a separation length of 1.5 cm. A van Deemter plot for lysozyme shows that the contribution from the eddy diffusion term is again on the nanometer scale. The overall plate height was below 50 nm for all of the different migration rates. BSA was also migrated through the column, and plate heights of 50 nm or lower were achieved. The results indicate that silica colloidal crystals are well suited for electrophoretic separations of proteins, and they could potentially be valuable for the field of proteomics.

4.2 INTRODUCTION

Protein analysis is a vital area of interest in the field of medical research as well as clinical diagnostics. Many diseases are believed to be described by their unique set of proteins and relative concentrations $^{35}$. The human genome has code for over 20,000 proteins, many of which undergo post translational modifications while in the body. This
complexity actually makes protein separations a challenging analytical task for a separation scientist.\textsuperscript{1,15-17} Many researchers have investigated ways to improve the separation power of conventional chromatography.\textsuperscript{2,15-16,18} It has been demonstrated that packing smaller inner diameter columns is one way to increase efficiency,\textsuperscript{3} however, many runs on these columns can take several hours.\textsuperscript{2} Monolithic materials have also been utilized to increase separation efficiency due to their smaller diffusion distances.\textsuperscript{2,4,18-20} However, monoliths only offer slight improvements in separation efficiency, less than two-fold, up to this point in time.

Researchers have shown that one method to increase the separation efficiency is to reduce the particle diameter of the silica packing material.\textsuperscript{2,5-6,21-24} Not only is there a demand for higher efficiency in the field of proteomics but there is also a need for much higher speed separations, and fast separations have been demonstrated using capillary zone electrophoresis however the separation lacks high selectivity.\textsuperscript{9-12} Reversed phase separations are much more selective; however the separation cannot exceed the rate of analyte diffusion between the stationary phase and mobile phase.\textsuperscript{12} The Jorgenson research group has demonstrated faster HPLC by using smaller nonporous particles, but this requires ultrahigh pressures.\textsuperscript{2,5-6,22-23} An alternative to using ultrahigh pressures is capillary electrochromatography (CEC). CEC has become a widely used separation technique for electrophoretic-driven separations. This electrophoretic mode is commonly used since it exploits two effects, namely differences in the partition coefficients of analytes between a stationary phase and mobile phase; and differences in the migration velocities of free analytes and analytes interacting with the stationary phase under the
influence of an electric field. As a result, the nature of the stationary phase has a critical influence on the separation. There is a wide range of possibilities and analytical conditions available with CEC which should allow for the resolution of a wide variety of compounds including neutral species.

Our group has previously shown that the migration velocity, \( u \), does in fact have contributions from both the reversed-phase retention factor, \( k' \), and the electrophoretic mobility, \( \mu_e \), where \( E \) is the field.

\[
 u = \frac{\mu_e E}{1 + k'}
\]

A priori, molecules that have only small differences in either electrophoretic mobility or reversed-phase retention factor are still able to be separated from one another.

Silica colloidal crystals also have applications in permselective nanoporous membranes, DNA sieving, reversed phase separation of small molecules on planar substrates, protein sieving, microarrays, total internal reflection fluorescence microscopy of live cells, and 3-D scaffolds for supported lipid films. In this work, silica colloidal crystals packed in capillaries are explored for their potential improvement in the efficiency of protein separations. This platform has previously been shown to yield plate heights as low as 150 nm in the electrochromatography of a fluorescent dye, when corrected for injection width. It was found that the plate height was largely due to the diffusion of the analyte. This suggests that the plate heights for proteins could be even smaller, since proteins have diffusion coefficients an order of magnitude smaller. If this
material was to yield low plate heights for proteins it could potentially be very useful for the field of “top-down” proteomics.

### 4.3 EXPERIMENTAL SECTION

#### 4.3.1 Chemicals and Materials

Silylation reagents were purchased from Gelest Inc., Morrisville, PA. Toluene, acetonitrile, formic acid, and nitric acid were purchased from Sigma Aldrich, St. Louis, MO. Water was supplied from an ultrapure water system from Millipore, Milford, MA. Silica particles (nominally 0.50 μm) were purchased from Fiber Optic Center Inc., New Bedford, MA. Fused silica capillary was obtained from Polymicro, Phoenix, AZ.

#### 4.3.2 Proteins

Ribonuclease A, cytochrome C, and lysozyme were purchased from Sigma Aldrich St. Louis, MO, and were labeled with Alexa Fluor 546 using a commercial labeling kit (Invitrogen, Carlsbad, CA). The concentration of proteins after labeling was $10^{-4}$ M. The protein samples were diluted by 1:100 (v/v) with 0.1 % formic acid to give a final solution concentration of $10^{-6}$ M. Alexa Fluor 647 labeled BSA was purchased from (Invitrogen, Carlsbad, CA), and diluted to be $10^{-6}$ M.

#### 4.3.3 Silica Colloidal Crystals (SCCs)

0.5 μm colloids were calcined three times at 600 °C for 6 hours each time in a box furnace (Lindberg/Blue, BF1766A). To eliminate
aggregation between each calcination step, the colloids were suspended in ethanol by sonication (Advanced Sonic Processing Systems, Sonochemical Reaction Vessel w/ VWR 6000 series chiller). The colloids were then acid rehydroxylated using 50:50 nitric acid/water. The resulting colloids were \(0.385 \mu m\) in diameter.

The capillary was cleaned by slowly pumping (Harvard Apparatus PhD 2000) 0.1 M sodium hydroxide (Sigma) through the capillary, followed by ultrapure water (Millipore, Milli-Q), and the final rinse was with 100 % ethanol. The capillary was then cut into smaller capillaries each 10 cm long and is put in a vacuum oven (VWR) for 20 minutes at 70 °C. The clean capillaries were then placed into a colloid of 25 % (wt %) rehydroxylated silica particles in water, and the colloid filled the column via capillary forces. The capillary was then placed vertical in a vial and allowed to dry at room temperature (~23 °C). This typically yields colloidal crystals of about 2 cm in length.

4.3.4 Chemical Modification of SCCs. Prior to the silanization reactions, the SCCs in fused silica capillaries were placed in a humidity chamber at a constant humidity of 50 %, for thirty minutes. The colloidal crystals were then chemically modified by horizontal polymerization of trichlorosilanes bearing the functional groups C\(_4\) and C\(_1\), to give a C\(_4\) density of \(3 \mu m/m^2\).\(^{14}\) The reaction was performed overnight at room temperature (~23 °C) to allow for the mixed self-assembled monolayer to form. The bonded phase was then rinsed with dry toluene using an Agilent 1100 series HPLC and allowed to dry at 120 °C for two hours.
4.3.5 Capillary Holder Fabrication. Polydimethylsiloxane (PDMS) molds were made by pouring a 10:1 mixture of PDMS base (sylgard 184, Dow Corning, Midland, MI) to curing agent into a petri dish, followed by drying overnight at 40 °C. The PDMS was then removed from the petri dish and reservoir holes were fabricated using a cork bore set. All of the holders were assembled by sealing PDMS to clean glass slides with oxygen plasma oxidization. All of the holders used in this study have the design illustrated in Figure 4.1. The capillary columns that were used were ~2 cm long and either 100 µm or 75 µm in inner diameter.

4.3.6 Electrochromatography. Packed columns were initially cleaned in boiling methanol for 15 minutes; this allows for the boiling methanol to wick into the capillary via capillary forces and rid the stationary phase of any adsorbed organics. The mobile phase was sonicated to remove pre-existing air bubbles. The mobile phase was then pumped through the capillary columns using an Agilent 1100 series HPLC. The capillary columns were then placed into the holder and conditioned at 300 V/cm, using a Matsusada high voltage power supply, for 15 minutes. The protein samples were injected from the positive electrode side from PBS solution by applying a 50 V/cm electric field for 5 min. After washing the reservoir with the mobile phase and refilling it with fresh mobile phase, an electric field ranging from 500 to 1500 V/cm was applied. The separation processes were monitored by using an inverted fluorescence microscope equipped with a
mercury lamp and a 2x objective (Nikon Eclipse TE2000U). A Cy3 filter (Omega Optical, Brattleboro, VT) was used for detection of the Alexa Fluor 546 conjugated protein samples. The emission was collected by a high sensitivity CCD camera (ProEm, Princeton Instruments) using an acquisition time of 0.2 s, controlled by the Winview software provided by the camera manufacturer. Data were analyzed using Origin (Microcal, Northampton, MA). A summary of the experimental conditions for each separation/migration is given in Table 4.1.

4.4 RESULTS AND DISCUSSION

Figure 4.2 shows the electrochromatogram of a mixture of three proteins: lysozyme, ribonuclease a and cytochrome c. The elution order was determined by injecting each protein separately. The proteins are well resolved, with $R_s \approx 5$, within a separation length of only 1.5 cm using an isocratic mobile phase composition of 40:60 ACN/water with 0.1% formic acid. The lysozyme elutes, first which is different from what one would observe in a RPLC separation, and this is because the electrophoretic component contributes to the separation, in addition to the reversed phase retention. Figure 4.3 shows a raw image and an electrochromatogram with respect to distance instead of time. As would be expected, the same resolution is observed in both the case for distance and time. The resolution is achieved over such a short separation distance because of the low plate heights (100-400 nm) for the three proteins. The low plate heights will allow for miniaturized separations, and shorter analysis times by using shorter separation distances. They could find use in lab-on-a-chip devices. The equation...
for resolution shows the relationship between the plate height, $H$, resolution, $R_S$, and separation length, $L$.

$$R_S = \frac{\sqrt{L/H}}{4} \frac{\Delta t}{<t>} \quad (2)$$

A van Deemter plot is used to determine the minimum plate height, and in electrochromatography, the migration rate and over-all flow rate are directly proportional to the electric field that is used. The increased current associated with higher electric fields could cause heating, which could introduce an apparent C term. Heating would cause a decrease in the mobile phase viscosity thus causing a disproportionate increase in the observed migration rate when increasing the electric field. Figure 4.4 shows the migration of lysozyme measured as a function of the electric field for the same mobile phase composition as that of Figure 4.2. The migration rate follows the electric field linearly, which indicates that no significant heating is observed over the range of 500-1500 V/cm. The linearity would further suggest that the silica colloidal crystals exhibit good power dissipation, which could be due to the higher thermal conductivity of the packed silica particle bed. The packed capillaries were shown to have interstitial volume fractions of ~25 %, hence the majority of the medium is solid silica, and the thickness that power must dissipate in the liquid portion is far less than the $d_p$ of 385 nm. The lack of heating allowed for the plate height to be studied for electric fields up to 1500 V/cm.
The biggest problem in evaluating the plate height quantitatively for protein separations is that proteins are typically heterogeneous. Upon further examination of Figures 4.2 and 4.3, both ribonuclease a and cytochrome c have shoulders due to other underlying species. Lysozyme exhibited a narrow peak and no shoulder, therefore, lysozyme was used for studying the plate height quantitatively. Because the detector was only sampling at 5 Hz and the lysozyme peak was so narrow at the high electric fields, there were limited points in the time domain to fit the peak to a Gaussian. As an alternative, as shown previously, the image of the zone at a given point in time can be used to plot the electrochromatogram in space, which was used to accurately characterize the peak width and plate height. These spatial electrochromatograms were used to calculate the spatial standard deviation, \( \sigma \), which was then used to calculate the plate height from equation 3 the known position of the center of the zone, \( L \), which was always set to 1.5 cm.

\[
H = \frac{\sigma^2}{L} \quad (3)
\]

Figure 4.5 shows the raw images of lysozyme for three runs at varying electric fields, 500, 1000, and 1500 V/cm, all at the 1.5 cm position for each peak. The height of each of the peaks is 75 \( \mu \)m, which is the capillary inner diameter. In the images, the lysozyme zone has moved from the left to the right. Axially, their spatial standard deviations are 19.7 \( \mu \)m, 16.6 \( \mu \)m, and 18.4 \( \mu \)m for 500, 1000, and 1500 V/cm.
respectively. Beneath each image is a plot of the numerical data from the image, on the same spatial scale fit to a Gaussian. Figure 4.6 shows the raw images of lysozyme for three replicate runs at 1000 V/cm, all at the 1.5 cm position for each peak. Like in the case with Figure 4.5, the height of each of the peaks is 75 \( \mu \text{m} \), which is the capillary inner diameter. Axially, their spatial standard deviations are 19.0 \( \mu \text{m} \), 16.6 \( \mu \text{m} \), and 16.6 \( \mu \text{m} \).

Again, beneath each of the raw images is a plot of the numerical data with the Gaussian fit. The average plate height of 20 nm achieved at the 1000 V/cm gives a total of \( \sim 750,000 \) theoretical plates in a length of 1.5 cm.

A plot of plate height vs. analyte migration rate is shown in Figure 4.7. This plot is analogous to a van Deemter plot. The plate height at all the migration rates is well below 100 nm and is as low as 20 nm for the optimal migration rate, which was at 1000 V/cm. When plotted on the same scale as DiIC\(_{12}\) (Figure 4.8) one can see that the smaller diffusion coefficient yields lower plate heights. The smaller diffusion coefficient also allows for smaller plate heights to be achieved at the lower electric fields, which was not possible in the case of the small dye molecule. The plate height reaches values as low as 20 nm at the optimal electric field. This lowest plate height is 300-fold lower than that for capillary electrochromatography of proteins\(^{33}\) and is 50-fold less than that for capillary zone electrophoresis.\(^{34}\)

To further test the efficiency of the colloidal crystal columns, BSA was run at 500 V/cm with a 50:50 ACN:water mobile phase with 0.1 % formic acid. Figure 4.9 shows raw image freeze frames of BSA labeled with alexa fluor 647 migrating through the silica colloidal crystal column. For each of these points in time, an individual
electrochromatogram can be observed and is plotted with the Gaussian fit in Figure 4.10. After fitting to a Gaussian peak width can be described quantitatively. The results indicate that BSA, which is a large relatively acidic protein, yields low plate heights as low as ~30 nm. This plate height means that we have about 500,000 theoretical plates in a distance of 1.57 cm.

4.5 CONCLUSIONS

These results show that silica colloidal crystals made of 385 nm diameter silica spheres give extremely low plate heights. Lengths of ~1.5 cm were used in this study, and plate height values as low as 20 nm (about 750,000 theoretical plates) at a migration rate of ~1 mm/s were achieved. Silica colloidal crystal columns could have a significant advantage over conventional capillary electrophoresis and capillary electrochromatography by providing shorter analysis times, higher resolution, or a combination of the two. Further research will be done to investigate the use of these materials under pressure driven flow to investigate their potential use as HPLC columns.

ACKNOWLEDGMENTS

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4.6 REFERENCES


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### 4.7 TABLES

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**Table 4.1.** Table summarizing experimental conditions.
4.8 FIGURES

Figure 4.1. Schematic of double reservoirs made of PDMS used in electrochromatography experiments. The capillary (blue) spanned the two inner reservoirs, while the Pt electrodes were placed in the outer reservoirs.
Figure 4.2. Electrochromatogram of a mixture of three proteins, which elute in the order of lysozyme, ribonuclease A and cytochrome C. The separation was performed isocratically using a 1000 V/cm electric field and a mobile phase of 40:60 ACN:water with 0.1% formic acid. The separation distance was 1.5 cm.
Figure 4.3. Fluorescence image of a 75 um i.d. capillary 2 min after the three labeled proteins were injected as plug, showing that they separated. The mobile phase composition was 40:60 ACN:water with 0.1% formic acid, and E=1,000 V/cm. The corresponding electrochromatogram with respect to length is also shown.
**Figure 4.4.** Migration rate of lysozyme as a function of electric field. The mobile phase composition was the same as in Figure 4.2.
Figure 4.5. Top: Raw images of the lysozyme zones in the 75 um i.d. capillary at varying electric fields each with a migration distance of 1.5cm. Bottom: The same raw data on the same spatial scale, but converted into chromatograms (O), and showing the best fit to a Gaussian (-).
Figure 4.6. Top: Raw images of the lysozyme zones in the 75 µm i.d. capillary at a migration distance of 1.5 cm for each replicate measurement at 1000 V/cm. Bottom: The same raw data on the same spatial scale, but converted into chromatograms (O), and showing the best fit to a Gaussian (−).
**Figure 4.7.** Plate height vs. migration rate for lysozyme. The error bars represent standard deviations from three replicate measurements.
Figure 4.8. Comparison of plate height vs. migration rate for lysozyme and DilC$_{12}$.
Figure 4.9. A sequence of fluorescent images of the electrophoretic migration (500V/cm) of BSA through a colloidal crystal column packed with 385 nm particles. A mobile phase of 50:50 ACN:Water, 0.1 % formic acid was used.
Figure 4.10. A sequence of electrochromatograms of the electrophoretic migration (500V/cm) of BSA through the colloidal crystal column packed with 385 nm particles. A mobile phase of 50:50 ACN:Water, 0.1 % formic acid was used.
CHAPTER 5: RPLC OF BIOMOLECULES IN SILICA COLLOIDAL CRYSTALS

5.1 ABSTRACT

Fused silica capillaries packed with silica colloidal crystals (SCCs) formed from 330 nm nonporous silica particles were evaluated for their potential use as reversed phase liquid chromatography (RPLC) columns. The SCC columns achieved adequate flow to perform nano-electrospray ionization mass spectrometry (n-ESI-MS). A reversed phase chromatogram of ribonuclease a exhibited a plate height of 100 nm, indicating that the plate height is not affected by the pressure driven Poiselle flow profile. Reversed phase gradient elution of a three peptide mixture, met-enkephalin, leu-enkephalin, and angiotensin II, exhibits comparable resolution to the best commercial nanocolumn with a 10X decrease in the total analysis time. The results indicate that SCCs can be used under pressure driven flow and could potentially be valuable for high-throughput applications such as proteomics.

5.2 INTRODUCTION

Biomarker discovery is a vital area of interest in proteomics, medical research and clinical diagnostics. Many diseases are believed to be described by their set of proteins and the relative concentrations of these proteins. The human genome codes for over
20,000 proteins, many of which undergo post translational modifications while in the body. The complexity of the proteome makes the separation and identification of possible biomarkers a challenging analytical task for a separation scientist. Many researchers have investigated ways to improve the separation power of conventional chromatography. However, many runs on these columns can take several hours, and to date only slight improvements have been achieved in separation efficiency.

Researchers have shown that one method to increase separation efficiency and decrease the analysis time is to reduce the particle diameter of the silica packing material, hence the development of ultra-high performance liquid chromatography (UHPLC). UHPLC uses a smaller particle size of 1.7 μm to increase the separation efficiency. A common way used to evaluate the performance of a column is to determine the height equivalence of a theoretical plate, which is commonly referred to as the plate height, H. A lower H results in a more efficient column/separation. The van Deemter equation (equation 1) can be used to describe the efficiency of a column as a function of the linear flow rate of the mobile phase (u).

\[ H = 2\lambda dp + \frac{2yD}{u} + \left(\frac{\omega dp^2}{D}\right)u \]  

A decrease in plate height can be predicted using the van Deemter equation expressed using \(d_p\) as the particle diameter and \(D\) as the diffusion coefficient in the mobile phase.
Where $\lambda$ is a constant having to do with the packing structure, $\gamma$ is the obstruction factor, and $\omega$ is a constant related to the particle structure.

Uniformly sized silica particles from 10 nm to 1 $\mu$m are known to self assemble into a highly ordered face centered cubic crystal, commonly referred to as a silica colloidal crystal. Silica colloidal crystals have been used in biological applications such as permselective nanoporous membranes, DNA sieving, reversed phase separation of small molecules on planar substrates, protein sieving, microarrays, total internal reflection fluorescence microscopy of live cells, and 3-D scaffolds for supported lipid films. In this work, silica colloidal crystals packed in capillaries are explored for their potential improvement in the efficiency of reversed phase chromatography. This platform has previously been shown to yield plate heights as low as 130 nm in the electrochromatography of a fluorescent dye, and 20 nm in the electrochromatography of proteins. It was found that the major contribution to plate height was the diffusion of the analyte. If this platform was to yield low plate heights under pressure driven flow then it could potentially be very useful for in field of proteomics and biomarker discovery.

5.3 EXPERIMENTAL

5.3.1 CHEMICALS AND MATERIALS. Silanization reagents were purchased from Gelest Inc., Morrisville, PA. Toluene, acetonitrile, formic acid, and nitric acid were purchased from Sigma Aldrich, St. Louis, MO. Water was supplied from an ultrapure water system from Millipore, Milford, MA. LCMS grade solvents were purchased from
Fisher Scientific, Pittsburgh, PA. Silica colloids (0.350 µm) were purchased from Fiber Optic Center Inc., New Bedford, MA. Fused silica capillary was obtained from Polymicro, Phoenix, AZ. Met-enkephalin, leu-enkephalin, angiotensin II, and ribonuclease A were purchased from Sigma Aldrich, St. Louis, MO. TAMRA labeled angiotensin II was purchased from ANASPEC, Fremont, CA. High pressure unions, tees, and fittings were purchased from Valco Instruments, Houston, TX.

5.3.2 SILICA COLLOIDAL CRYSTALS (SCCs). 0.35 µm colloids were calcined at 600 °C three times for 6 hours each time in a box furnace (Lindberg/Blue, BF1766A). To eliminate aggregation between each calcination step, the colloids were suspended in ethanol by sonication (Advanced Sonic Processing Systems, Sonochemical Reaction Vessel w/ VWR 6000 series chiller). The colloids were then acid rehydroxylated using 50:50 nitric acid/water. The resulting colloids were 0.33 µm in diameter.

The capillary was cleaned by slowly pumping (Harvard Apparatus PhD 2000) sodium hydroxide (Sigma) through the capillary, followed by ultrapure water (Millipore, Milli-Q), and a final rinse with 100 % ethanol. The capillary was then cut into smaller capillaries each 10 cm long and put in a vacuum oven (VWR) for 20 minutes at 70 °C. The clean capillaries were then placed into a mixture of 25 % (wt %) rehydroxylated colloids in water. The slurry fills the column via capillary forces. The capillary was then placed vertical in a vial and allowed to dry at room temperature (~23 °C). This typically yields colloidal crystals of about 2 cm in length.
5.3.3 CHEMICAL MODIFICATION OF SCCs. Prior to the silanization reactions, the SCCs in fused silica capillaries were placed in a humidity chamber at a constant relative humidity of 50 %, for thirty minutes. The colloidal crystals were then chemically modified by horizontal polymerization of trichlorosilanes bearing the functional groups C\textsubscript{18} and C\textsubscript{1} or C\textsubscript{4} and C\textsubscript{1} to give a C\textsubscript{18} or C\textsubscript{4} density of 3 \textmu m/m\textsuperscript{2}.\textsuperscript{28} The reaction was performed overnight at room temperature (\textasciitilde 23 °C) to allow for the mixed self-assembled monolayer to form. The bonded phase was then rinsed with dry toluene using an Agilent 1100 series HPLC and allowed to dry at 120 °C for two hours.

5.3.4 INSTRUMENTATION. Separations were run using an Accela UHPLC from Thermo Fisher Scientific. The flow was split using a high pressure compatible stainless steel tee and the column was hooked up via a high pressure compatible union. The column was then either coupled to an upright Nikon fluorescence microscope equipped with a fluorescence cube for Cy3 and a 2X objective (Figure 5.1) or MS via nano-electrospray ionization (Figures 5.2 & 5.3). The capillary columns that were used were at least 1.5 cm long and either 100 \textmu m or 75 \textmu m in inner diameter.

5.3.5 PRESSURE VS. FLOW RATE. Two \textasciitilde 2-cm SCCs modified with octadecyltrichlorosilane/methyltrichlorosilane were tested on a MicroTech Scientific
XtremeSimple Dual Binary System. The SCC columns were tested using 100 % methanol with 0.1 % formic acid. Valco 1/16” stainless steel ferrules (Pt# ZF1S6) with PEEK 1/16” OD X 0.020” ID tubing (UpChurch Pt# 1532) and Valco stainless steel nuts (Valco Pt# ZN1) were used in conjunction with a narrow bore stainless steel union (Valco Pt# ZU1XC) to provide a connection to the LC, 25µM ID fused silica exit tubing (Polymicro Pt#TSP025375) able to withstand approximately 15,000 psi. In addition to the flow rate setting on the LC system, a SGE 25-µL LC syringe with a 26 gauge needle was attached to the column exit using a stainless steel union (Valco Pt# ZU1XC or similar), an Upchurch One-piece PEEK finger-tight fitting (Pt# F130), and a piece of 1/16” OD Teflon tubing used to secure the syringe needle.

5.3.6 FORMATION OF DIFFERENT COLUMN TIPS. Blunt ended columns, tapered columns and pulled tip columns were used in this study (Figure 5.4). Tapered columns have a tapered tip, which was made by dremeling the end of the column with 120 grit sand paper. Pulled tip columns were fabricating in-house by using a propane torch. Picotips (commercially available pulled tips) have also been used (Figure 5.5) and were purchased from New Objective, Woburn, MA.

5.3.7 MIGRATIONS/SEPARATIONS. Columns were initially cleaned in boiling methanol for 15 minutes; this allows for the boiling methanol to wick into the capillary via capillary forces and rid the stationary phase of any adsorbed organics. The protein and
peptide samples were prepared in 100% H2O with 0.1 % FA and injected using the Accela auto sampler. The separation processes were then monitored by the upright fluorescence microscope or the LTQ-MS. Data were analyzed using Origin (Microcal, Northampton, MA).

5.4 RESULTS AND DISCUSSION

Volume flow rate through the SCCs in fused silica capillaries were tested using UHPLC. Flow rate versus pressure plots were obtained for two ~2 cm long SCCs packed in 100 μm I.D. fused silica capillaries (Figure 5.6). The crystals were pressurized between 12,000 and 13,000 psi to test if they could withstand high pressures, all of the separations were then performed at ~10000 psi. A minimum of five flow-rate measurements were taken at each pressure, and the average reported as a single data point. Multiple separate flow-rate experiments were also performed on each crystal. Both of the crystals had nearly identical profiles with stationary phase beds of 2.40 and 2.31 cm. It is known that the back pressure exhibited on a chromatographic bed is directly related to the length. Both of the crystal’s flow rates versus pressure plots exhibit linear character, which is indicated by the very small standard deviation associated with the slope from their flow rate versus pressure plot. The flow rates obtained through the crystals are also on the scale that is needed to perform nano-spray ionization. Figure 5.4 shows images of the spray from silica colloidal crystal columns with varying tip geometries fabricated in-house. The blunt tip column, while the easiest to fabricate,
unfortunately leads to an overwhelming amount of broadening due to droplet formation on the tip. This occurs due to the low contact angle of the aqueous mobile phase on the glass tip. One approach to fix this problem was to use a tapered tip column. However, the rough surface that is achieved by the fabrication process often caused the tip to fracture under the application of the electric field necessary for nESI. The fracturing often caused sporadic spray in multiple directions. The most successful geometry was found to be that of pulled tips. Pulled tips fixed not only the problem of droplet formation, but they also did not suffer from the fracturing of the tip under an electric field. Because the crude method used to pull tips in-house did not always yield the same uniform tips, commercial pulled tips were purchased from New Objective. Figure 5.5 shows an image of the spray from a New Objective Picotip silica colloidal crystal column.

To determine the plate height, ribonuclease a was migrated through the 1.5 cm long column using an isocratic mobile phase composition of 40:60 ACN/water with 0.1% formic acid and detected by the LTQ-mass spectrometer. The plate height was then determined by fitting the peak to a Gaussian. Raw data for a representative chromatogram is shown in Figure 5.7. This raw data yields a plate height that is ~100 nm. Figure 5.8 shows the mass spectrum of the ribonuclease a from Figure 5.7. The characteristic protein envelope can be used to calculate charge states and molecular weight. The adjacent members \((x_1\) and \(x_2\)) of the mass to charge envelope can be expressed as

\[
x_1 = (M + n)/n
\]  

(2)
and

\[ x_2 = \frac{(M + n + 1)}{(n + 1)} \]  

(3)

where M is the molecular weight and n is the charge. Combining the two equations gives

\[ n = \frac{(x_2 - 1)}{(x_2 - x_1)} \]  

(4)

and allows for the estimation of M. \(^{30}\)

Figure 5.9 shows the first ever gradient elution chromatogram on a SCC column, a mixture of three peptides: met-enkephalin, leu-enkephalin, and angiotensin II. The peptides are well resolved with a separation length of only 1.5 cm and a 6 minute gradient. Figures 5.10, 5.11, and 5.12 show the mass spectra of the three individual peptides from Figure 5.9. Both met and leu enkephalin show a large peak for their corresponding [M+H] peak. Anigotensin II, which has multiple basic sites, yields peaks corresponding to its doubly and triply charged states. The gradient conditions used for the SCC column are shown in Table 5.1 and were chosen based on the optimized gradient conditions used for an agilent nano-column. \(^{31}\) A steeper gradient was chosen upon examination of kinetic plots. One can conclude that shorter analysis/gradient times are required for smaller particles to yield comparable column performance to conventional
LC columns. Hence, a 6 minute gradient in a SCC column yields comparable resolution to that of a 60 min gradient in an Agilent nano-column (Figure 5.13). When comparing the total analysis time, the SCC column has the ability to run 10 samples in the time that it takes to do one sample on the Agilent nano-column.

A common way used to evaluate the performance of a separation under gradient conditions is by peak capacity, \( n_c \). To determine the peak capacity, TAMRA labeled angiotensin II was migrated through the 1.5 cm long column using the same gradient conditions as in Figure 5.9. Figure 5.14 shows a plot of three runs and their temporal peak widths (4\(\sigma\)) 2.12 s, 2.20 s, and 2.08 s. Peak capacity has been calculated in a variety of ways. The formula for peak capacity is most often expressed mathematically as

\[
n_c = 1 + \frac{t_G}{4\sigma}
\]

(5)

where \( t_G \) is the gradient time, or

\[
n_c = \frac{t_{r,n} - t_o}{4\sigma}
\]

(6)

where \( t_{r,n} \) is the retention time of the last eluting peak and \( t_o \) is the elution time of an unretained peak. Calculated peak capacity values using equation 4 are listed in Table 5.2.
and the results from equation 5 are listed in Table 5.3. At an operating pressure of 
~10000 psi the SCC columns achieve a peak capacity of ~165 in only 6 minutes. When 
compared to a 1.8 \( \mu \)m particle column,\textsuperscript{35} which at best is able to achieve \( n_c=100 \) at 15000 psi in the same amount of time, the peak capacity for the SCC column is unprecedented. On-going work in conjunction with bioVidra Inc. is being performed toward possible commercialization of this platform as an UHPLC column.

5.5 CONCLUSION

The results show that silica colloidal crystals made of 330 nm diameter silica spheres maintain the extremely low plate heights that were observed with these materials under electrophoretic separations previously. Lengths of 1.5 cm were used in this study, and the plate height value for a protein was found to be 100 nm, which yields about 150,000 theoretical plates. Upon running the gradient elution of angiotensin II peak capacities of 165 were achieved in a 6 minute gradient time, which is at least 10X faster than the commercially available column from Agilent Technologies. Silica colloidal crystal columns could have a significant advantage over conventional chromatography columns by providing shorter analysis times, higher resolution, or a combination of the two. Further research will be done to optimize the proper gradient times, column length, particle size, and injection.
5.6 REFERENCES


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## 5.7 TABLES

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<th>Time (min)</th>
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<th>% ACN</th>
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**Table 5.1.** Gradient conditions used for the SCC column.
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<th>Run 3</th>
<th>Average</th>
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**Table 5.2.** Peak capacity \(n_c\) of TAMRA labeled angiotensin II calculated using \(n_c = 1 + \frac{t_G}{4\sigma}\).
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<th>Run 2</th>
<th>Run 3</th>
<th>Average</th>
<th>Stdev</th>
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<td>4s</td>
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<td>2.20 s</td>
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<td>2.13 s</td>
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<td>( t_{r,n} - t_o )</td>
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<td>343.1 s</td>
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<td>( n_c )</td>
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</table>

**Table 5.3.** Peak capacity \((n_c)\) of TAMRA labeled angiotensin II calculated using

\[
n_c = \frac{t_{r,n} - t_o}{4\sigma}.
\]
Figure 5.1. Schematic of the UHPLC-fluorescence detector system.
**Figure 5.2.** Schematic of the UHPLC-nanoESI-MS system. The dashed box region is shown in a photograph in Figure 5.3.
Figure 5.3. Photograph of the UHPLC-nanoESI-MS system. The SCC column and MS-inlet are circled in red.
Figure 5.4. Photographs of the different tip styles and the spray achieved from each tip after being packed with a SCC. All fabricated in our lab.
Figure 5.5. Photograph of the New Objective Picotip and the spray achieved from the tip after being packed with a SCC.
Figure 5.6. Graph of volume flow rate vs. pressure for two 100 μm i.d. capillaries packed with silica colloidal crystals. The error bars are standard deviations from multiple measurements.
Figure 5.7. Chromatogram of ribonuclease A. The separation was performed isocratically using a flow rate of $\sim 150 \text{ nL/min}$ and a mobile phase of 40:60 ACN:water with 0.1% formic acid. The separation distance was 1.5 cm.

\[
N = 150,000, \quad L = 1.5 \text{ cm}, \quad H = 100 \text{ nm}
\]

\[
2.7 \times 10^7 \exp\left(-\frac{(x-1.92)^2}{(2 \times 0.005^2)}\right)
\]
Figure 5.8. Mass Spectrum of ribonuclease A from Figure 5.7.
Figure 5.9. Chromatogram of a mixture of three neuropeptides, which elute in the order of met-enkephalin, leu-enkephalin, and angiotensin II. The separation was performed using gradient elution using a flow rate of ~150 nL/min. The separation distance was 1.5 cm. Gradient conditions are listed in Table 5.1.
Figure 5.10. Mass Spectrum of met-enkephalin from Figure 5.9.
Leu-enkephalin is Tyr-Gly-Gly-Phe-Leu MW= 556 Da

Figure 5.11. Mass Spectrum of leu-enkephalin from Figure 5.9.
**Figure 5.12.** Mass Spectrum of angiotensin II from Figure 5.9.
Figure 5.13. Comparison of chromatograms of a mixture of three neuropeptides, which elute in the order of met-enkephalin, leu-enkephalin, and angiotensin II.
**Figure 5.14.** Gradient elution of TAMRA labeled Angiotensin II with on column fluorescence detection. Separation distance ~1.5 cm. Gradient conditions were the same as in Figure 5.9 and are listed in Table 5.1.
APPENDIX A: OTHER CONTRIBUTIONS

A.1 ISOELECTRIC FOCUSING (IEF) IN A PACKED CAPILLARY

Capillary IEF allows for the high speed separation of proteins, giving peak capacities of 50 and pI resolution of 0.1 pH units. In the field of proteomics the focused proteins must be removed from the capillary to be analyzed by mass spectrometry. Unfortunately, remobilization to remove the proteins is often done by pressurized flow, which exhibits a Poiselle flow profile. The Poiselle flow profile leads to band broadening of the focused protein zones. Research in our group has been to use capillaries packed with 1 μm silica particles, which would allow for an even flow profile across the capillary. To date it has been shown that pressure-driven remobilization in a packed bed can be completed in two minutes, with a resolution of 0.10 pH units (Figure A.1).

A.2 THIN LAYER CHROMATOGRAPHY (TLC) IN A SCC

Capillary and planar TLC allows for quick experiments to screen possible isocratic separation conditions while also offering the ability to run multiple analytes at the same time in the planar platform. Unfortunately, the technology in the field is quite archaic. We proposed the use of silica colloidal crystals (SCCs) formed with 250 nm particles up to 1 μm particles to allow for high resolution separations of biological
molecules. To date the results indicate the first ever high resolution separation of proteins by TLC (Figure A.2).

A.3 PACKING LARGER BORE COLUMNS

Conventional liquid chromatography columns have a larger bore, commonly 4.6 mm or 2.1 mm in diameter. All of the work discussed in this dissertation was taken on nanocolumns with an inner diameter of 75 or 100 μm. In the field of proteomics, many people are familiar and comfortable with the use of nanocolumns; however, larger bore columns allow for easier use with commercial equipment. To date we have developed a process to pack these columns in a highly crystalline manner (Figure A.3), which is in the process of being patented.

A.3 SEPARATION OF GLYCOFORMS

Unlike the static genome, the proteome differs between cell types, and the proteins present differ from one cell to the next. Not only are protein structures dictated by the genome, the environment and cell history also play a key role. The environment and cell’s history are known to lead to post translational modifications and sequence variants.\textsuperscript{6-8} One common type of post translational modification is glycosylation, where a glycan (sugar chain) is attached to the protein. Glycoproteins are of interest because they are thought to be common biomarkers to diseases such as cancer. We are trying to
increase the resolution of the separation of intact glycoforms so the separation could be useful in early disease diagnostics. To date we have fabricated a hydrophilic interaction liquid chromatography (HILIC) column, a mixture of polyacrylamide with acrylamido phenylboronic acid (APBA) (Figure A.4). With this new column 3 of the 5 glycoforms of ribonuclease B have been resolved under pressure driven flow (Figure A.5).
A.5 REFERENCES


Figure A.1. Fluorescence vs. time for the pressure-driven remobilization, measured at a point near the end of the capillary, after focusing at 500 V/cm. The red dotted line is a Gaussian fit to the trypsin peak, revealing a standard deviation of 0.90 s., which corresponds to 0.10 pH units. (Data courtesy of Yimin Hua)
**Figure A.2.** Fluorescence micrograph of a TLC separation of three proteins over a length of 1 cm. (Data courtesy of Zharoui Zhang)
**Figure A.3.** Colored photographs of a chromatography column packed with a silica colloidal crystal formed from 330 nm particles. The blue color arises from Bragg diffraction, as the particles are on the order of the wavelength of light.
Figure A.4. Reaction schematic of surface-initiated atom transfer radical polymerization (ATRP) of polyacrylamide with addition of the acrylamido phenylboronic acid (APBA) monomer to form the new hydrophilic interaction stationary phase. (Schematic courtesy of Saliya Ratnayaka)
Figure A.5. Pressure driven flow separation of ribonuclease b glycoforms. The column was 1.2 cm long and packed with 500 nm particles. The column was modified to have 200:1 acrylamide to APBA and a mobile phase of 70:30 ACN:water with 0.5 % formic acid. (Data courtesy of Saliya Ratnayaka)
REFERENCES

CHAPTER 1 REFERENCES


CHAPTER 2 REFERENCES


CHAPTER 3 REFERENCES


**CHAPTER 4 REFERENCES**


**CHAPTER 5 REFERENCES**


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**APPENDIX A REFERENCES**


