SYNTHESIS AND CHROMATOGRAPHIC EVALUATION OF SUPERFICIALLY POROUS PARTICLES FOR ULTRAHIGH PRESSURE LIQUID CHROMATOGRAPHY

James William Treadway

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Approved by:
James W. Jorgenson
Mark H. Schoenfisch
J. Michael Ramsey
Gary J. Pielak
Dorothy A. Erie
ABSTRACT

James William Treadway: Synthesis and Chromatographic Evaluation of Superficially Porous Particles for Ultrahigh Pressure Liquid Chromatography (Under the direction of James W. Jorgenson)

Chromatographic separations play a vital role in the separation and analysis of biological samples. Superficially porous particle have emerged as a useful particle architecture for liquid chromatography. These particles have received significant attention recently for the chromatographic efficiency gains that they display. Ultrahigh pressure liquid chromatography employs small particles and high pressures to also yield highly efficient separations. Increasingly, the field of chromatography had employed a combination of these two methods for creating highly efficient columns. One portion of this work details the work with micron-sized superficially porous particles for the separation of intact proteins and other large molecules. Superficially porous particles specifically tailored for the separation of large molecules are not currently commercially available in the single micron size range. One aspect of this work describes the synthetic route that was utilized to create these particles in such a small overall particle size. Another aspect of this work details an evaluation of the separation capabilities of these particles when packed into capillary columns. These columns were utilized for the separation of small molecules, peptides and proteins.

Another portion of this work concerns the utilization of commercially manufactured superficially porous particles. One chapter focuses on the creation of highly efficient capillary columns packed with commercial prototype superficially porous particles. The capillary
columns created in that chapter exhibit isocratic efficiencies for the separation of small molecules rivaling those seen for capillary columns packed with fully porous particles. This is the first time that such efficient separations have been demonstrated using superficially porous particles packed into capillary columns. A final section of the dissertation focuses on the comparison between commercially available fully and superficially porous particles packed into capillary columns. These particles were bonded with either conventional or charged-surface reversed-phase bondings. The separation characteristics for these columns were evaluated in terms of separating small molecules, peptides, and proteins. It was seen that both the bonded phase and the particle morphology contributed to separation differences between the columns. This work provides insight into which particle and bonding type should be pursued for future investigations.
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Figure 3.21: Same particle size distribution as seen in Figure 3.20 but with the damage state of the particles noted (n=144).

Figure 3.22: Pore size distribution for the final batch of superficially porous particles. Note the peak in incremental pore volume around an average pore size of 480 Å.

Figure 3.23: Example isocratic chromatogram from the characterization of a 16.6 cm X 75 µm ID column packed with 1.06 µm C8 bonded superficially porous particles. This separation was performed at ambient temperature at 16,700 psi in 80/20 v/v water/acetonitrile with 0.1% TFA added. The peaks are, in order of elution, ascorbic acid, hydroquinone, resorcinol, catechol, and 4-methyl catechol. Plate counts for this separation are 93,000 for hydroquinone, 84,000 for resorcinol, 80,000 for catechol, and 73,000 for 4-methyl catechol.

Figure 3.24: $h\nu$ plot for the characterization of four 75 µm ID capillary columns packed with C8 bonded superficially porous particles. All were characterized at ambient temperature in an 80/20 v/v water/acetonitrile mobile phase with 0.1% TFA added. The columns were, in name order, 19.6, 16.6, 17.7, and 18.0 cm long respectively. Efficiencies are shown for hydroquinone, $k' \sim 0.1$. The parameters shown are the fits of the experimental data to the reduced van Deemter equation.

Figure 3.25: Example chromatogram from the characterization of a 19.2 cm X 75 µm capillary column packed with 1.06 µm C4 bonded superficially porous particles. This separation was performed at ambient temperature at 17,800 psi in 90/10 v/v water/acetonitrile with 0.1% TFA added. The peaks are, in order of elution, ascorbic acid, hydroquinone, resorcinol, catechol, and 4-methyl catechol. Plate counts for this separation are 72,000 for hydroquinone, 63,000 for resorcinol, 65,000 for catechol, and 59,000 for 4-methyl catechol.

Figure 3.26: $h\nu$ plot for the characterization of 3 capillary columns of 75 µm ID packed with 1.06 µm diameter C4 bonded superficially porous particles. The columns were characterized at ambient temperature in 90/10 v/v water/acetonitrile with 0.1% TFA added. The columns were, in name order, 18.7, 19.2, and 17.1 cm long, respectively. Efficiencies
are shown for hydroquinone, k’ = 0.15. The parameters shown for each of the columns are the result of fitting the experimental data to the reduced van Deemter equation.

Figure 3.27: Loading comparison using the 1.06 µm C8 bonded superficially porous particles. The separation was accomplished at 40 °C with a 16.5 µL 0.5-40% acetonitrile gradient. The sample was a tryptic digest of alcohol dehydrogenase loaded at either 60 fmol (A) or 20 fmol (B) on column. The notated ions are the peaks used to mark the beginning and the end of the elution window for peak capacity calculation.

Figure 3.28: Retention time comparison for the separation of a tryptic digest of alcohol dehydrogenase on a column packed with C18 bonded Cortecs particles (60 fmol, 33.0 µL gradient) and a column packed with in-house synthesized C8 bonded particles (20 fmol, 32.5 µL gradient). The equation of the linear regression is shown above. Error bars are ± 1 standard deviation.

Figure 3.29: Peak capacity as a function of gradient length on in-house synthesized superficially porous particles. All separations were carried out at 40 °C with a 20 fmol sample of alcohol dehydrogenase tryptic digest standard. Error bars are ± 1 standard deviation.

Figure 3.30: Separation of E. coli whole cell lysate on a 19.1 cm X 75 µm column packed with 1.06 µm C8 bonded superficially porous particles. All separations performed at 40 °C with a 25 µL 0.5-60% acetonitrile gradient. (A) has 0.2% formic acid in the mobile phase with 250 ng injected onto the column, (B) has 0.3% formic acid and 0.025% trifluoroacetic acid in the mobile phase with 250 ng injected onto the column, and (C) has 0.3% formic acid and 0.025% trifluoroacetic acid in the mobile phase with 100 ng injected onto the column.

Figure 3.31: Select deconvoluted masses from the separations of E. coli on the 19.1 cm X 75 µm column packed with 1.06 µm C8 bonded superficially porous particles. Separation was performed using a 25 µL gradient from 0.5-60% acetonitrile with 0.3% formic acid and 0.025% trifluoroacetic acid in the mobile phase. These masses cover a range of molecular weights and retention times. The top trace (A) is the 250 ng injection on column, while the bottom trace (B) is the 100 ng injection on column. The leading edge of the peaks is very steep, so that the protein trace often doesn’t start until appreciably far up the peak. The deconvoluted masses of the peaks are written above the respective peaks, retention order is the same on both columns.

Figure 3.32: Peak profile from 20 ng ribonuclease A injected onto a 19.1 cm X 75 µm ID column packed with 1.06 µm C8 bonded superficially porous particles.
Figure 3.33: Summed mass spectra from the ribonuclease A peak. (A) is summed over the entire peak, and (B) is summed over just the leading edge of the peak. The minority component peak is clearly present in the spectrum summed over just the leading edge of the peak.

Figure 3.34: MaxEnt deconvoluted masses of the spectra seen in the previous figure. (A) is deconvoluted from the entire peak, while (B) only represents the leading edge of the peak.

Figure 3.35: Reconstructed chromatogram for the different masses from Figure 3.34 from the ribonuclease A peaks. The plotted data was taken from the autoME output. The black trace is 13682 Da, red trace is 13698 Da, green trace is 13714 Da, and blue trace is 13730 Da.

Figure 3.36: Comparison between the two peaks. (A) is the total ion current from the ribonuclease A peak, while (B) is the m/z 1918-1919 extracted ions, showing the narrow early eluting peak.

Figure 4.1: Example packing progress plot for a 50 µm ID column packed with a 50 mg/mL acetone slurry at 30,000 psi. Fit parameters are shown ± 1 standard deviation.

Figure 4.2: Example packing progress plot with square root of time axis for a 50 µm ID column packed with a 50 mg/mL acetone slurry at 30,000 psi. Fit parameters are shown ± 1 standard deviation.

Figure 4.3: SEM image of the prototype superficially porous particles employed in this investigation.

Figure 4.4: Packing progress plot for the first set of 30 µm ID capillaries packed at 30 mg/mL. Black circles, red squares, green triangles, and blue inverted triangles are the first, second, third, and fourth packed columns respectively. All experimental data were fit to the packing modeling equation.

Figure 4.5: Packing progress plot for the second set of 30 µm ID capillaries packed at 30 mg/mL. Black circles, red squares, green triangles, and blue inverted triangles are the first, second, third, and fourth packed columns respectively. All experimental data were fit to the packing modeling equation.

Figure 4.6: Packing progress plot for the set of 50 µm ID capillaries packed at 30 mg/mL. Black circles, red squares, green triangles, and blue inverted triangles are the first, second, third, and fourth packed columns respectively. All experimental data were fit to the packing modeling equation.
Figure 4.7: Packing progress plot for the set of 50 µm ID capillaries packed at 50 mg/mL. Black circles, red squares, green triangles, and blue inverted triangles are the first, second, third, and fourth packed columns respectively. All experimental data were fit to the packing modeling equation.

Figure 4.8: Packing progress plot for the set of 75 µm ID capillaries packed at 30 mg/mL. Black circles, red squares, green triangles, and blue inverted triangles are the first, second, third, and fourth packed columns respectively. All experimental data were fit to the packing modeling equation.

Figure 4.9: h-ν plot for the characterization of the first batch of 30 µm ID columns packed at 30 mg/mL. Efficiency data is shown for hydroquinone (k’~0.2). As with the packing progress plots, black circles, red squares, green triangles, and blue inverted triangles are the first, second, third, and fourth packed columns respectively. Packing order is also indicated by the numbers on the right of the figure.

Figure 4.10: h-ν plot for the characterization of the second batch of 30 µm ID columns packed at 30 mg/mL. Efficiency data is shown for hydroquinone (k’~0.2). As with the packing progress plots, black circles, red squares, green triangles, and blue inverted triangles are the first, second, third, and fourth packed columns respectively. Packing order is also indicated by the numbers on the right of the figure.

Figure 4.11: Example chromatogram from a highly efficient separation performed on a 31.7 cm X 30 µm ID column. The separation was performed at 16,000 psi at ambient temperature in 60/40 water/acetonitrile with 0.1% trifluoroacetic acid added. The peaks are, in order of retention, ascorbic acid (dead time marker), hydroquinone, resorcinol, catechol, and 4-methyl catechol.

Figure 4.12: h-ν plot for the characterization of the 50 µm ID columns packed at 30 mg/mL. Efficiency data is shown for hydroquinone (k’~0.2). As with the packing progress plots, black circles, red squares, green triangles, and blue inverted triangles are the first, second, third, and fourth packed columns respectively. Packing order is also indicated by the numbers on the right of the figure.

Figure 4.13: h-ν plot for the characterization of the 50 µm ID columns packed at 50 mg/mL. Efficiency data is shown for hydroquinone (k’~0.2). As with the packing progress plots, black circles, red squares, green triangles, and blue inverted triangles are the first, second, third, and fourth packed columns respectively. Packing order is also indicated by the numbers on the right of the figure.
Figure 4.14: h-ν plot for the characterization of the 75 µm ID columns packed at 30 mg/mL. Efficiency data is shown for hydroquinone (k’~0.2). As with the packing progress plots, black circles, red squares, green triangles, and blue inverted triangles are the first, second, third, and fourth packed columns respectively. Packing order is also indicated by the numbers on the right and top of the figure.

Figure 5.1: Gradient profile employed for the first column comparison. Note that the gradient is loaded from right to left, for sample elution from the column the gradient is played back from left to right.

Figure 5.2: Example chromatograms from equivalent alcohol dehydrogenase tryptic digest separations on (A) BEH C18 and (B) Cortecs C18+ showing the peaks used to calculate separation window. Data shown are for the low energy scans.

Figure 5.3: Sample gradient profile employed for the second column comparison. This gradient is the intermediate length gradient employed, 33.0 µL. Note that the gradient is loaded from right to left, for sample elution from the column the gradient is played back from left to right.

Figure 5.4: Sample gradient profile employed for the separation of intact proteins, in this case the 25 µL gradient. Note that the gradient is loaded from right to left, for sample elution from the column the gradient is played back from left to right.

Figure 5.5: Example chromatogram showing the separation of intact proteins from E. coli whole cell lysate on a 50.2 cm X 75 µm ID column packed with 1.9 µm BEH C18 particles. For this separation, 250 ng of sample was placed on the column and was then separated using a 25 µL 0.5 to 60% acetonitrile gradient, with 0.2% formic acid in the mobile phase. Column was held at 40 °C during the separation. Deconvoluted masses used for separation window determination (11185 and 32904 Da) and peak width determination (6856, 8325, 11185, 11673, and 21135 Da) are indicated.

Figure 5.6: Particle size distributions as imaged by SEM for the Cortecs C18 (A, n=142) and for the Cortecs C18+ (B, n=117) particles.

Figure 5.7: Example chromatograms obtained from the four particle types: (A) BEH C18, (B) BEH C18+, (C) Cortecs C18, and (D) Cortecs C18+. For all the separations, the peaks are, in ascending elution order: ascorbic acid (dead time marker), hydroquinone, resorcinol, catechol, and 4-methyl catechol. Note that the x and y axes change to account for differences in the specific separations. Separations on the fully porous BEH particles were performed in 50/50 water/acetonitrile while separations performed on the superficially porous Cortecs particles were
performed in 60/40 water/acetonitrile, with both mobile phases having 0.1% trifluoroacetic acid added. Separations were performed at ambient temperature. .................................................................238

Figure 5.8: Isocratic performance comparison between capillary columns packed with the superficially porous Cortecs particles showing fits to the reduced van Deemter equation. The columns packed with C18 particles are shown as the solid markers and lines while the particles functionalized with the C18+ bonding are shown with the open markers and dotted lines. Performance is shown for the first retained compound, hydroquinone. All of the columns were evaluated at ambient temperature using a 60/40 v/v water/acetonitrile mobile phase with 0.1% trifluoroacetic acid added.................................................................239

Figure 5.9: Isocratic performance comparison between capillary columns packed with fully porous BEH particles showing fits to the reduced van Deemter equation. The columns packed with the C18 functionalized particles are shown with the solid markers and lines while the columns packed with particles functionalized with the C18+ bonding are represented by the open markers and dotted lines. Performance is shown for the first retained compound, hydroquinone. All of these columns were evaluated at ambient temperature using a 50/50 v/v water/acetonitrile mobile phase with 0.1% trifluoroacetic acid added..........................240

Figure 5.10: Separation comparison between the three columns employed. Separations are shown on (A) a BEH C18 column, (B) a Cortecs C18 column, and (C) a Cortecs C18+ column. All separations were performed with a 19 µL 4-40% acetonitrile gradient at 40 °C with a 200 fmol sample of alcohol dehydrogenase tryptic digest standard. The m/z 817 peak is noted in each chromatogram to illustrate retention differences between the columns. Data is shown from the low energy scans.................................................................241

Figure 5.11: Separation comparison between the (A) low (20 fmol) and (B) high (200 fmol) loadings of alcohol dehydrogenase tryptic digest on the Cortecs C18 column. All separations were performed with a 19 µL 4-40% acetonitrile gradient at 40 °C. Data shown is from the low energy scans.................................................................242

Figure 5.12: Impact of sample diluent on retention of early eluting peaks with a column packed with Cortecs C18+ particles. All separations performed with a 4-40% acetonitrile with 0.1% formic acid added. The 200 fmol alcohol dehydrogenase tryptic digest standard was prepared in either (A) 3% acetonitrile, (B) 0.5% acetonitrile, or (C) 0% acetonitrile with 0.1% formic acid added. Data is shown for the low energy scans.................................................................243
Figure 5.13: Impact of gradient initial conditions on peak retention with a column packed with Cortecs C18+ particles. All separations were performed with 200 fmol alcohol dehydrogenase tryptic digest standard prepared in 100% water with 0.1% formic acid added. Initial gradient conditions were varied between (A) 3%, (B) 0.5%, and (C) 0% acetonitrile with the balance made up with water, with 0.1% formic acid added. All gradients ended at 40% acetonitrile. Data is shown for the low energy scans.

Figure 5.14: Schematic cutaway drawing of the pigtail-electrospray tip connection for the Waters QTOF Premier instrument nanospray source. (A) shows the way the connection is made, with a 20 μm ID capillary ‘pigtail’ coming from the column (at left) and the 20 μm ID capillary electrospray tip (at right) both coming through PEEK fingertight nuts and forming a connection within the metal union, shown as a dark square. Panel B) depicts a properly made connection, with both capillaries connected end-to-end within the metal union with zero dead volume.

Figure 5.15: Example chromatograms from consecutive, identical runs. These are the same set of peaks for each separation, With the x-axis slightly shifted for (A) to better line up the peaks. (A) shows a separation with ceramic-cut capillaries used for the pigtail-spraytip connection. (B) shows the identical separation with commercially cut components used for the pigtail-spraytip connection.

Figure 5.16: Impact of data acquisition rate on calculated peak capacity. Data from the first day are shown with open circles, data from the second day shown with closed circles. All points were taken in duplicate, error bars are ± 1 standard deviation.

Figure 5.17: Impact of calculated points per 4σ peak width on calculated peak capacity. This assumes a 4.7 4σ peak width, consistent with the observed data. Data from the first day are shown with open circles, data from the second day shown with closed circles. All points were taken in duplicate, error bars are ± 1 standard deviation.

Figure 5.18: Comparison between the separations on the four columns employed running 33.0 μL 0.5-40% acetonitrile gradients, 60 fmol alcohol dehydrogenase tryptic digest standard on column. The separation is shown on a (A) BEH C18 column, (B) BEH C18+ column, (C) Cortecs C18 column, and (D) Cortecs C18+ column. First 30 minutes removed to allow emphasis on actual separation space.

Figure 5.19: Comparison between the retention times of the monitored analytes on the BEH C18+ column and the BEH C18 column. All points were taken in duplicate, error bars are ± 1 standard deviation. No alteration
has been made to the data to compensate for slight variations run-to-run in flow rate, etc. The sample was 60 fmol alcohol dehydrogenase tryptic digest standard, and it was separated using a 33 µL 0.5-40% acetonitrile gradient at 40 °C.

Figure 5.20: Comparison between retention times of the monitored analytes on the BEH C18+ column (red squares), Cortecs C18 column (green triangles), and Cortecs C18+ (blue inverted triangles) compared to their retention times on the BEH C18 column. All points were taken in duplicate, error bars are ± 1 standard deviation. No alteration has been made to the data to compensate for slight variations run-to-run in flow rate, etc. The sample was 60 fmol alcohol dehydrogenase tryptic digest standard, and it was separated using a 33 µL 0.5-40% acetonitrile gradient at 40 °C.

Figure 5.21: Relation between measured peak capacity and measured elution window for the four column types: BEH C18 (black circles), BEH C18+ (red squares), Cortecs C18 (green triangles), and Cortecs C18+ (blue inverted triangles). The three elution windows are created by 16.5, 33.0, and 66.5 µL gradients of 0.5-40% acetonitrile, all with 60 fmol injections of alcohol dehydrogenase tryptic digest standard. All points were taken in duplicate, error bars are ± 1 standard deviation.

Figure 5.22: Relation between measured peak capacity and sample loading for the four column types: BEH C18 (black circles), BEH C18+ (red squares), Cortecs C18 (green triangles), and Cortecs C18+ (blue inverted triangles). Either 20, 60, or 180 fmol of alcohol dehydrogenase tryptic digest standard was injected on column using a 2 µL injection. All separations were carried out using a 33.0 µL 0.5-40% acetonitrile gradient. All points were taken in duplicate, peak capacity error bars are ± 1 standard deviation.

Figure 5.23: Ratio of peak widths of selected analytes between the Cortecs and BEH material as a function of molecular weight (MH+) of the analyte on the C18 stationary phase. This work used separations of 60 fmol of alcohol dehydrogenase tryptic digest standard separated using a 33 µL 0.5-40% acetonitrile gradient at 40 °C.

Figure 5.24: Ratio of peak widths of selected analytes between the Cortecs and BEH material as a function of molecular weight (MH+) of the analyte on the C18+ stationary phase. This work used separations of 60 fmol of alcohol dehydrogenase tryptic digest standard separated using a 33 µL 0.5-40% acetonitrile gradient at 40 °C.

Figure 5.25: Example chromatograms from the separation of intact proteins from E. coli whole cell lysate (250 ng) on each of the column types using a 25 µL 0.5-60% acetonitrile gradient with 0.2% formic acid in the
mobile phase at 40 °C. The columns were, in order from top to bottom, (A) BEH C18, (B) BEH C18+, (C) Cortecs C18, and (D) Cortecs C18+. All of the chromatograms except the Cortecs C18+ column have been vertically offset to provide ease of viewing.

Figure 5.26: Deconvoluted protein mass base peak intensity chromatograms for separations using capillary columns packed with the four particle types employed. These were created using the same separations shown in Figure 5.26. The first and last peaks used for the set analysis window are indicated by arrows. All show the separation of intact proteins (250 ng) from *E. coli* whole cell lysate using a 25 µL 0.5-60% acetonitrile gradient with 0.2% formic acid in the mobile phase at 40 °C. The columns were, in order from top to bottom, (A) BEH C18, (B) BEH C18+, (C) Cortecs C18, and (D) Cortecs C18+. All chromatograms except for the Cortecs C18+ have been vertically offset for ease of viewing.

Figure 5.27: Deconvoluted protein mass base peak intensity chromatograms for separations using capillary columns packed with the four particle types employed. These are the same chromatograms shown in Figure 5.27 but translated to approximately match the retention of the first peak used (12518 Da) for the second peak capacity comparison. This allows easier visual comparisons of the separations. The chromatograms have been horizontally translated but not stretched or shrunk in either direction.
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>°C</td>
<td>Degrees Celsius</td>
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<tr>
<td>µL</td>
<td>Microliter</td>
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<tr>
<td>µm</td>
<td>Micrometer</td>
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<tr>
<td>1D</td>
<td>1-dimensional (referring to a separation)</td>
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<tr>
<td>2D</td>
<td>2-dimensional (referring to a separation)</td>
</tr>
<tr>
<td>2D-SDS-PAGE</td>
<td>Two-dimensional polyacrylamide gel electrophoresis with sodium dodecyl sulfate</td>
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<tr>
<td>Ag/AgCl</td>
<td>Silver/silver chloride</td>
</tr>
<tr>
<td>Ala</td>
<td>Alanine</td>
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<tr>
<td>BEH</td>
<td>Bridged-ethyl hybrid</td>
</tr>
<tr>
<td>BET</td>
<td>Brunauer-Emmet-Teller method</td>
</tr>
<tr>
<td>BJH</td>
<td>Barrett-Joynner-Halenda method</td>
</tr>
<tr>
<td>C18</td>
<td>Bonding with a saturated hydrocarbon containing 18 carbon atoms</td>
</tr>
<tr>
<td>C18+</td>
<td>C18 bonded phase with positive surface charge</td>
</tr>
<tr>
<td>C18TAB</td>
<td>Octadecyltrimethylammoniumbromide</td>
</tr>
<tr>
<td>C4</td>
<td>Bonding with a saturated hydrocarbon containing 4 carbon atoms</td>
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<tr>
<td>C8</td>
<td>Bonding with a saturated hydrocarbon containing 8 carbon atoms</td>
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<tr>
<td>cm</td>
<td>Centimeter</td>
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<tr>
<td>CSH</td>
<td>Charged surface hybrid</td>
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<tr>
<td>CTAB</td>
<td>Cetyltrimethylammonium bromide</td>
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<tr>
<td>Da</td>
<td>Dalton</td>
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<tr>
<td>dB</td>
<td>Decibel</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td><em>Escherichia coli</em></td>
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<tr>
<td>EMG</td>
<td>Exponentially modified Gaussian</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>-----------------</td>
<td>---------------------------------------------------------------------------</td>
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<tr>
<td>eV</td>
<td>Electron-volt</td>
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<tr>
<td>FA</td>
<td>Formic acid</td>
</tr>
<tr>
<td>fmol</td>
<td>Femtomole</td>
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<tr>
<td>FWHM</td>
<td>Full width at half maximum</td>
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<tr>
<td>Glu</td>
<td>Glutamic acid</td>
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<tr>
<td>$h_{\text{min}}$</td>
<td>Minimum reduced plate height</td>
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<tr>
<td>HMW PDDA</td>
<td>High molecular weight poly(diallyldimethylammonium) chloride</td>
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<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
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<tr>
<td>Hz</td>
<td>Hertz</td>
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<tr>
<td>ID</td>
<td>Internal diameter</td>
</tr>
<tr>
<td>LC-MS</td>
<td>Liquid chromatography coupled to mass spectrometry</td>
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<tr>
<td>LED</td>
<td>Light emitting diode</td>
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<tr>
<td>Lys</td>
<td>Lysine</td>
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<tr>
<td>m</td>
<td>Meter</td>
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<tr>
<td>M</td>
<td>Molar (moles per liter)</td>
</tr>
<tr>
<td>m/z</td>
<td>Mass to charge ratio</td>
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<tr>
<td>$m^2/g$</td>
<td>Square meters per gram</td>
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<tr>
<td>mg</td>
<td>Milligram</td>
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<tr>
<td>MH+</td>
<td>Molecule with a single positive charge (from a proton)</td>
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<tr>
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<td>Millimeter</td>
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<td>Molecular weight</td>
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<td>Nanoliter</td>
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xxxii
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<th>Abbreviation</th>
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<tr>
<td>nm</td>
<td>Nanometer</td>
</tr>
<tr>
<td>OD</td>
<td>Outer diameter</td>
</tr>
<tr>
<td>P123</td>
<td>Triblock copolymer $\text{EO}<em>{70}\text{PO}</em>{70}\text{EO}_{70}$ (ethylene oxide, propylene oxide, ethylene oxide)</td>
</tr>
<tr>
<td>PAH</td>
<td>Poly(allylamine) hydrochloride</td>
</tr>
<tr>
<td>PEEK</td>
<td>Polyetheretherkeytone</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PEI</td>
<td>Polyethyleneimine</td>
</tr>
<tr>
<td>PSD</td>
<td>Particle size distribution</td>
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<tr>
<td>psi</td>
<td>Pound per square inch</td>
</tr>
<tr>
<td>PTFE</td>
<td>Polytetrafluoroethylene</td>
</tr>
<tr>
<td>Q-TOF</td>
<td>Quadrupole time-of-flight mass spectrometer</td>
</tr>
<tr>
<td>RSD</td>
<td>Relative standard deviation</td>
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<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>Ser</td>
<td>Serine</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>V</td>
<td>Volt</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume/volume mixture</td>
</tr>
<tr>
<td>Val</td>
<td>Valine</td>
</tr>
<tr>
<td>W</td>
<td>Watts</td>
</tr>
<tr>
<td>w:v</td>
<td>Weight to volume ratio (kilogram:liter)</td>
</tr>
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LIST OF SYMBOLS

\( \Delta P \) Pressure drop
\( \Delta P_{\text{opt}} \) Pressure required to operate a column at optimum linear velocity
\( \Delta \alpha_{\text{prop},j_1,j_2,\text{obs}} \) Experimentally observed proportional change in \( \alpha \) from earlier packed column \( j_1 \) to later packed column \( j_2 \)
\( \text{Å} \) Angstrom
\( A \) Eddy diffusion van Deemter coefficient
\( a \) Reduced eddy diffusion van Deemter coefficient
\( B \) Longitudinal diffusion van Deemter coefficient
\( b \) Reduced longitudinal diffusion van Deemter coefficient
\( c \) Reduced resistance to mass transfer van Deemter coefficient
\( C \) Resistance to mass transfer van Deemter coefficient
\( D_M \) Diffusion coefficient for the analyte in the mobile phase
\( d_p \) Particle diameter
\( F \) Volumetric flowrate
\( F_p \) ‘Particle volumetric flowrate’ or volumetric flowrate of particles
\( F_s \) Volumetric flowrate of slurry toward the packed bed
\( g \) Acceleration due to gravity
\( H \) Plate height
\( h \) Reduced plate height
\( j \) Indicates packing order of a particular column during sequential column packing
\( K \) Equilibrium coefficient
\( k' \) Retention factor
\( L \) Column length
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$M_2$</td>
<td>Second central moment</td>
</tr>
<tr>
<td>$m_{p,c,r}$</td>
<td>Mass of particles required to pack a column of a certain length and radius</td>
</tr>
<tr>
<td>$m_{p,s}$</td>
<td>Mass of particles in the packing vessel</td>
</tr>
<tr>
<td>$m_{p,s,0}$</td>
<td>Initial mass of particles in the slurry</td>
</tr>
<tr>
<td>$m_{p,s,j,calc}$</td>
<td>Mass of particles in the packing vessel at the beginning of packing the $j$th column</td>
</tr>
<tr>
<td>$N$</td>
<td>Plate count</td>
</tr>
<tr>
<td>$n_c$</td>
<td>Peak capacity</td>
</tr>
<tr>
<td>$P$</td>
<td>Power</td>
</tr>
<tr>
<td>$r_o$</td>
<td>Internal diameter of the column</td>
</tr>
<tr>
<td>$t$</td>
<td>Time</td>
</tr>
<tr>
<td>$t_0$</td>
<td>Retention time of an unretained analyte (dead time)</td>
</tr>
<tr>
<td>$t_g$</td>
<td>Gradient length</td>
</tr>
<tr>
<td>$t_{max}$</td>
<td>Retention time of the last retained peak</td>
</tr>
<tr>
<td>$t_{min}$</td>
<td>Retention time of the first retained peak</td>
</tr>
<tr>
<td>$t_r$</td>
<td>Retention time</td>
</tr>
<tr>
<td>$u$</td>
<td>Mobile phase linear velocity</td>
</tr>
<tr>
<td>$u_t$</td>
<td>Particle sedimentation velocity</td>
</tr>
<tr>
<td>$V_M$</td>
<td>Volume of the mobile phase</td>
</tr>
<tr>
<td>$V_{por,rel}$</td>
<td>Relative porous volume</td>
</tr>
<tr>
<td>$V_S$</td>
<td>Volume of the stationary phase</td>
</tr>
<tr>
<td>$V_{slurry}$</td>
<td>Volume of the packing vessel</td>
</tr>
<tr>
<td>$w_{med}$</td>
<td>Median peak width</td>
</tr>
<tr>
<td>$w_p$</td>
<td>Average peak width</td>
</tr>
<tr>
<td>$\alpha$</td>
<td>Packing rate descriptor</td>
</tr>
</tbody>
</table>
\( \beta \) Column length packed before reaching maximum pressure

\( \gamma_e \) Obstruction factor caused by the presence of particles to diffusion in the bulk mobile phase

\( \varepsilon_e \) External column porosity

\( \varepsilon_f \) Fraction of the total hydrodynamic particle volume filled with fluid

\( \varepsilon_{p,i} \) Particle porosity

\( \eta \) Viscosity of the fluid

\( \kappa_2 \) Particle-solvent constant

\( \nu \) Reduced linear velocity

\( \nu_i \) Interstitial velocity

\( \nu_p \) Instantaneous rate of bed formation

\( \rho \) Ratio of nonporous core diameter to overall particle diameter for superficially porous particles

\( \rho_f \) Density of the fluid

\( \rho_p \) Particle density

\( \rho_{skel} \) Density of the particle skeleton

\( \sigma \) Standard deviation

\( \sigma_t^2 \) Temporal variance

\( \tau \) Exponential component of the exponentially modified Gaussian fit

\( \varphi \) Flow resistance parameter

\( \varphi_{part} \) Volume fraction of the particles

\( \Omega \) The ratio of the effective diffusivity of an analyte in the porous shell relative to its bulk diffusion coefficient
CHAPTER 1: INTRODUCTION

1.1 Proteomic Separations

The separation of biomolecules, including proteins and peptides, is fundamental to the field of proteomics. Proteomics, which is the study of proteins levels within a biological system, offers the promise of a better understanding of biological processes and disease states[1]. This field deals with great sample complexity, and therefore requires analytical separation prior to further analysis and identification. For example, the human genome has around 20,500 different protein-coding genes[2]. A variety of separation approaches are employed in proteomic research including gradient liquid chromatography, capillary electrophoresis, isoelectric focusing, gel electrophoresis, and tube gel electrophoresis [3, 4]. The detection and identification of the analyte molecules is often performed using mass spectrometry[5]. Overall, the proteomics field is divided into top-down and bottom-up proteomics, depending on the state of the protein during analysis[6].

1.1.1 Top-Down Proteomics

One area of proteomic research is known as top-down proteomics. Top-down proteomics interrogates intact proteins. This methodology has the advantage that the protein is analyzed in its whole form, often preserving post-translational modifications that can be critical for full protein characterization[6]. It is also a less complex sample than that seen for bottom-up proteomics, since the proteins have not been digested into peptides. The methodology is not without its downsides, however. Accurate mass detection is critical to accurately identify intact
proteins. This requires the highest resolution mass spectrometric instrumentation available, limiting its broad utility[7].

Top-down proteomics offers different separation challenges than those seen for bottom-up proteomics. While the sample is much less complex, the separation of intact proteins is much more difficult than the separation of peptides. Two-dimensional gel electrophoresis with sodium dodecyl sulfate (2D-SDS-PAGE) can be employed, often following isoelectric focusing, but suffers from low resolution, reproducibility, and recovery[4]. Tube gel electrophoresis and isoelectric focusing have been seen to give better results than traditional gel electrophoresis[6]. Capillary electrophoresis is a useful technique for intact protein separation, and it can be directly coupled to a mass spectrometer[8]. Liquid chromatographic techniques have seen utility for intact protein separations, but they have issues with recovery and protein carryover or ghosting run-to-run[9]. Liquid chromatography does have the ability to be directly interfaced to mass spectrometry via electrospray. Specialized columns and stationary phases must be employed for liquid chromatographic separation of intact proteins. The first portion of experimental work in this dissertation (Chapters 2 and 3) focuses on the creation of such specialized material intended for the reversed-phase separation of intact proteins.

1.1.2 Bottom-Up Proteomics

Bottom-up proteomics consists of analyzing peptides from digested proteins in a sample [10]. One advantage of this approach is that the separation of peptides is often more efficient than that of intact proteins. Additionally, peptide samples are more readily solubilized and handled than intact protein samples[11]. However, the analytical complexity of the experiment is increased with this approach, since each protein is cleaved into multiple peptides. This increased complexity imposes more stringent requirements on the separation and detection of
these samples. After data collection, the data is analyzed to take the peptide identifications and
determine the original intact proteins from which they were obtained, often by searching against
a database of known proteins [12]. While there are clear shortcomings to this methodology,
much of the current work in the field is done using bottom-up methodology.

Bottom-up proteomic separations often require conventional separation modalities but
require very high efficiency separations to maximize the number of peaks resolved. For gradient
separations, separation performance is measured peak capacity. Peak capacity is defined as the
maximum number of peaks which can be resolved at a given resolution for a given separation
window[13]. For bottom-up proteomics, it is impossible with a 1D separation (shotgun
proteomics) to achieve the peak capacity necessary to fully separate all of the components of a
tryptic digest of more than a few proteins. The use of mass spectrometric detection can help
mitigate this issue, since the mass spectrometer can detect multiple components at one time. For
example, a shotgun methodology has been employed for the analysis of an entire digested yeast
proteome in around an hour using a 1-dimensional separation and a high resolution mass
spectrometer[14]. Another approach is the use of 2-dimensional systems to separate the sample
via two orthogonal separations, which lead to the final peak capacity for the separations being
the product of the peak capacities from the two separations, so long as the resolution is
maintained when transferring between dimensions[13]. This can either be done with two liquid
chromatography separations of different modes, or bringing in other separation techniques such
as capillary electrophoresis or isoelectric focusing. 2D approaches have been used extensively in
the last 30 years with many different chromatographic modes[15]. Even with 2D approaches,
however, improving the peak capacity of both dimensions of separation is important to resolve
the most sample components possible. The second portion of this dissertation (Chapters 4 and 5)
describes work towards the creation of and evaluation of columns with high peak capacity which could be employed in bottom-up proteomic analyses.

1.2 A Brief Introduction to Chromatographic Efficiency

When designing columns for high efficiency separations, a theoretical understanding of the band broadening processes at work during the separation is important. While the preceding section on proteomic separations dealt with chromatographic efficiency in terms of observed peak capacity, the most insightful methodology of evaluating separations employing packed beds of particles is through the use of isocratic separations. Such separations allow for the straightforward characterization of peak profile. A chromatographic peak can be characterized by its plate count, N. N is defined by Equation 1.1[16]

\[ N = \frac{t_r^2}{\sigma_t^2} \]

where \( t_r \) is the peak’s retention time and \( \sigma_t^2 \) is the temporal variance of the peak. A higher value of N corresponds to a thinner peak, which allows for increased resolution between peaks for a separation. While N is a useful metric for characterization of a specific peak, it also does not explicitly take any of the characteristics of the column into consideration. For the characterization of packed beds, a more useful metric is plate height, H, defined by Equation 1.2

\[ H = \frac{L}{N} \]

where L is the length of the column. This allows for the performance of the column to be understood independent of its length. Another variable that must be accounted for is the mobile phase linear velocity, u. The same column operated at varying linear velocities can give peaks of differing variance. This relation of plate height to mobile phase linear velocity is most easily understood through the van Deemter equation, Equation 1.3[17]:

\[ \text{Equation 1.3[17]} \]
\[ H = A + \frac{B}{u} + C \cdot u \]  
\text{Equation 1.3}

where the A term refers to band broadening processes (eddy dispersion) independent of mobile phase linear velocity (u), the B term refers to band broadening processes (axial diffusion) inversely proportional to u, and the C term refers to band broadening processes (mass transport) directly proportional to u. One immediate observation from this relation is that the various contributions to plate height or band broadening are additive. An illustration of the different contributions to plate height as a function of mobile phase linear velocity is shown in Figure 1.1. Exhaustive treatments exist for the full expressions of these terms which are beyond the scope of this dissertation[18-22]; relevant band broadening contributions will be discussed below as they pertain to specific attributes of superficially porous particles.

The van Deemter equation is a useful way to understand plate height as a function of mobile phase linear velocity, but it is difficult to use plate height to compare columns packed with differing particle sizes or operated with differing analyte diffusivities (different mobile phase or analyte). It is therefore useful to use reduced parameters for such comparisons, as defined in Equation 1.4 and Equation 1.5 below, where \( d_p \) is the particle diameter and \( D_M \) is the diffusion coefficient for the analyte in the mobile phase[17].

\[ h = \frac{H}{d_p} \]  
\text{Equation 1.4}

\[ \nu = \frac{u \cdot d_p}{D_M} \]  
\text{Equation 1.5}

Reduced parameters can also be used to write a reduced form of the van Deemter equation, Equation 1.6, with reduced a, b, and c terms analogous to the A, B, and C terms from the van Deemter equation.
The use of reduced parameters allows for fair comparisons of different length columns packed with different sized particles operated under different conditions. This utility underlies the frequent use of reduced variables for the experimental evaluation of columns in this dissertation.

1.3 Superficially Porous Particles

A stationary phase support morphology which has attracted much attention recently is the superficially porous particle. Such a particle is, in its most general sense, a nonporous core surrounded by a porous layer or shell. This is the reason for an alternate name for these particles, core-shell particles.

Since 2007, there has been an interest in the performance offered by modern embodiments of superficially porous particles[23, 24]. The difference in particle morphology between these particles and the widely used fully porous particles impact their utility in separations. The following is an overview of separation efficiency considerations that pertain to columns packed with superficially porous particles, grouped by term from the traditional van Deemter equation. For excellent overview of the efficiency considerations for superficially porous particles, [25] is a good reference.

1.3.1 Eddy Dispersion: The A Term

The A term describes eddy dispersion, or band broadening based upon the inherently nonuniform structure of a packed bed. When assessing the eddy dispersion (A) term for liquid chromatography, it is instructive to have a more nuanced understanding than that provided by the van Deemter equation. Giddings actually described five different regimes for eddy dispersion, all of which have at least some dependence on velocity of the mobile phase[18]. Much more
recent work has shown that one of these factors, trans-column eddy dispersion, controls the efficiency for many reversed-phase chromatographic separations, including those is capillary columns[20]. This kind of eddy dispersion arises from velocity differences across the column. Packing density is radially nonuniform in packed beds of all but the smallest diameter columns. This has been seen in packed capillary columns using confocal laser scanning microscopy[19, 26], indicating a wall region near the outside of the bed and a bulk region of differing packing structure. Such systematic radial heterogeneity leads to a radial velocity differential within the column due to the difference in permittivity of the differing packing regions. A similar phenomenon has been observed in analytical millimeter-bore columns, with detection microelectrodes placed at the column’s outlet both at the center of the column and near the outer edge[27-29]. For these columns it was observed that analytes eluted later near the wall, indicating that for these columns mobile phase velocity was lower near the column wall than through the middle of the column. It was also observed that the separations efficiencies measured near the wall were lower than those observed near the center of the column. Thus for both capillary columns and millimeter bore columns radial packing density biases often exist.

Packing more homogenous columns would help to alleviate this issue of radial velocity bias. Some studies with superficially porous particles packed into millimeter bore columns suggest that columns packed with superficially porous particles have significantly lower eddy dispersion than columns packed with traditional fully porous particles[30, 31]. One study using confocal laser scanning microscopy of capillary columns found that there was less of an abrupt transition from wall region to bulk region for superficially porous particles as opposed to fully porous particles, perhaps explaining the reduced eddy dispersion seen for these particles[26].

One argument that has been put forth is that the smaller particle size distribution (PSD) of
superficially porous particles than that of most superficially porous particles is responsible for the reduction in eddy dispersion seen with superficially porous particles[32]. However, experimental studies with columns packed with both low and high PSD fully porous particles suggest that in fact the PSD of a material does not impact the eddy dispersion observed in columns[33, 34]. Computer simulation results support these observations, showing that overall column porosity has a much stronger influence on column performance than PSD does[35]. If the narrow PSD of superficially porous particles is not the reason for their decreased eddy dispersion, perhaps another aspect of their morphology is. One major difference between some superficially porous particles and fully porous particles is the greater external roughness seen for superficially porous particles as opposed to most fully porous particles. Greater external roughness could limit the ability of particles to slip past each other and rearrange during packing[36]. In this way particle external roughness may contribute to a more radially homogenous bed structure. Independent of the exact reason, which is not yet fully understood, columns packed with superficially porous particles show less eddy diffusion than columns packed with fully porous particles do.

### 1.3.2 Longitudinal Diffusion: The B Term

The next area that needs to be considered is longitudinal diffusion within the packed bed. This term, the B term, is often overlooked when assessing the efficiency of a column. This term is related to the relaxation of axial concentration gradients within the eluting band. With their nonporous cores, superficially porous particles should exhibit lower B-term broadening since the overall porosity of the packed bed should be lower. This behavior can be simply described by Equation 1.7 for the reduced B term:
\[ b = 2 \left( \gamma_e \frac{1 - \epsilon_i}{\epsilon_i} \frac{1 - \rho^3 \Omega}{1 + \frac{\rho^3}{2}} \right) \]

Equation 1.7

where \( \epsilon_i \) is the external column porosity, \( \rho \) is the ratio of nonporous core diameter to overall particle diameter, \( \Omega \) is the ratio of the effective diffusivity of an analyte in the porous shell relative to its bulk diffusion coefficient, and \( \gamma_e \) is the obstruction factor caused by the presence of particles to diffusion in the bulk mobile phase[37]. For a general understanding of the impact of relative core diameter on \( b \)-term, \( \gamma_e \) is set to 0.6, \( \epsilon_i \) is set to 0.4, and \( \Omega \) is set to 1 for a moderately retained solute. Reduced \( B \) term (\( b \)) is then plotted as a function of \( \rho \) in Figure 1.2. It is apparent that with increasing relative core diameter, the \( b \)-term should significantly decrease, especially for \( \rho \) values larger than 0.4 or 0.5, which are what are employed for superficially porous particles. The experimental data supports these theoretical expectations. Separations of small molecules on sub-2 \( \mu \)m fully porous and superficially porous particles showed lower \( B \) terms for the superficially porous material than the fully porous material[38].

1.3.3 Resistance to Mass Transfer: The C Term

When superficially porous particles were first developed, the initial expectation was that they would improve the mass transfer term for the particles. With the more recent superficially porous particles, there has been a renewed interest in understanding the impact of the superficially porous morphology on mass transfer[39]. When considering the morphology of a superficially porous particle relative to that of a fully porous particle, the expectation is that the nonporous core should limit the diffusion length of an analyte into and out of the particle. A theoretical treatment of the separation process was employed by Horvath et al. [40] to study this process. Using the general rate model, expressions were determined for the film mass transfer and for mass transfer within the stagnant mobile phase as a function of \( \rho \) parameter. These
expressions were then used, while taking into account the effect of shell thickness on retention via the phase ratio, to create theoretical plots of expected resolution between two components of varying molecular weight. As the thickness of the porous layer decreases, the phase ratio between the stationary and mobile phases also decreases, decreasing retention and relative retention between analytes. It was found that superficially porous particles show greater resolution vs. fully porous particles as the molecular weight of the analyte goes up. The superficially porous particles were also found to have increasingly superior resolution when compared to fully porous particles as the linear velocity increased above the optimum for the separation. It was of note, however, that comparing the operation of fully porous and superficially porous particles at the optimum flowrate for the separation on the respective columns, only negligible improvement was seen for the resolution with the use of superficially porous particles. Another finding of this theoretical study was that compensating for the loss in retention as the shell thickness decreased allowed for much greater resolution improvement to be realized than would otherwise have been realized. In practice, this could be done be decreasing the strength of the mobile phase. Without compensating for retention loss, \( \rho \) values between 0.8 and 0.9 were seen to give best improvement in resolution for large peptides and proteins. From this study, it is shown that the mass transfer benefits expected from superficially porous particles are most beneficial for large molecular weight analytes. Such improvement has been seen for the elution of an intact protein from columns packed with a series of superficially porous particles with different shell thicknesses[41].

1.4 Desirable Particle Characteristics for Proteomic Separations

The preceding understanding of superficially porous particles supports the decision to pursue superficially porous particles as a particle morphology for biological separations. When
designing superficially porous particles tailored toward the separation of large molecules, additional requirements must be taken into consideration beyond those required for small molecule separations. These considerations reach beyond the typical efficiency considerations highlighted in the previous section. To this end, four major attributes of particles for proteomic separations are discussed in greater detail: pore diameter, surface area, overall particle size, and bonded stationary phase.

1.4.1 Pore Diameter

For efficient mass transfer into and out of the particles in a chromatographic bed, the pores in the particle have to be sufficiently large to allow unhindered analyte diffusion. To allow for unrestricted diffusion of the analyte through a pore, the pore width of the particles should be at least ten times the diameter of the solute[17]. A study of the diffusion of proteins in aqueous solution across nanoporous membranes indicates that this rule of thumb may not even be adequate for large molecules. In this study, proteins showed much slower diffusion rates than for bulk solution in pores which were at least 10 times the diameter of the solute[42].

Insufficient pore size is usually judged by experimental observation in reversed-phase chromatography of macromolecules. For one manufacturer of superficially porous particles, both a small pore (90 Å) and wider pore (160 Å) are offered in the otherwise same particle makeup. When comparing the performance of the material to the gradient separation of peptides and small proteins, it was seen that the smaller pore material showed less efficient peaks than the wider pore material[43]. For proteins, pore size should be even larger due to the larger size of intact proteins. In a comparison between the gradient separation of a range of molecular weight proteins (25, 200, 250, and 465 kDa) on superficially porous particles with either 200 or 400 Å pores (2 different vendors), it was seen that pore size was influential to peak shape[41]. For the
200 Å pore column compared to the 400 Å pore column, the 25 kDa peak is comparable, the 200 and 250 kDa peaks are a bit wider, and the the 465 kDa peak is substantially wider. In another comparison from that same paper, 400 Å pore thin shell particles were substantially better at separating both light and heavy chain antibody components than 160 Å thicker shell particles. Overall, commercial particles intended for the separation of proteins have pores at least 300 Å in diameter[17].

1.4.2 Surface Area

From a mass transfer perspective, it is advantageous to have wide pores to allow for mass transfer. The resultant surface area of the particle must be taken into consideration though. As a general rule, the specific surface area of a particle decreases in direct proportion to the increase in pore diameter[17]. A decrease in particle specific surface area has a direct impact upon the retention of analytes on the column. The retention factor (k’) can be expressed in a thermodynamic sense by Equation 1.8, where K is the equilibrium coefficient for the analyte between the stationary and mobile phases, V_S is the volume of stationary phase, and V_M is the volume of the mobile phase[17].

\[ k' = \frac{K \cdot V_S}{V_M} \]  
Equation 1.8

Retention factor is also related to the retention time of an analyte on a column in isocratic mode (t_r) by Equation 1.9, where t_0 is the retention time of an unretained analyte.

\[ t_r = t_0 \cdot (1 + k') \]  
Equation 1.9

Assuming a constant coating thickness for the bonded stationary phase, decreasing the surface area of the particles in the column leads to a proportionate decrease in stationary phase volume and also analyte retention.
This issue of lower surface area is also salient in the use of superficially porous particles, since their solid cores do not contribute to the overall porosity of the particle. The proportion of the particle volume taken up by the nonporous core can be defined by the simple geometric relation shown in Equation 1.10, where the relative porous volume, \( V_{\text{por,rel}} \), is expressed as a function of \( \rho \).[39]

\[
V_{\text{por,rel}} = 1 - \rho^3
\]

Equation 1.10

This result is plotted in Figure 1.3, where it can be seen that relative porous volume decreases drastically as the shell becomes very thin, in the same regime where large improvements are expected for mass transfer. From the previous relation for \( k' \), it can be seen that as the fractional volume of the particle comprised of porous material decreases, the retention of analytes will decrease as well. This is the constraint from the earlier analysis of mass transfer in superficially porous particles that resulted in a maximum protein resolution for a superficially porous particle with a core comprising 80-90% of the radius, instead of a completely nonporous particle[40].

Another consideration for low surface area materials is decreased loading capacity. Reversed-phase separations are usually thought of as consisting of analytes interacting with the chromatographic system independent of other analytes. As the concentration of analyte increases, however, at some point the analyte molecules start interacting with each other, leading to decrease in peak symmetry and efficiency. The mass of analyte required to detrimentally overload the column is directly related to the amount of the stationary phase in the column[44, 45]. This imposes practical experimental limitations on the mass of analyte that can be injected on widepore materials, especially if they are superficially porous. In a recent study, protein overloading effects were assessed for both fully porous and thin shell superficially porous particles, both with wide pores[46]. It was seen that the column packed with superficially porous
particles was more sensitive to overload effects in terms of peak width and peak tailing. In another study, several superficially porous particles with the same overall particle diameter but different porous layer thicknesses were assessed for overloading characteristics with the intact protein myoglobin [41]. In that study, it was seen that as the porous layer became thinner, the particles became more susceptible to overloading.

1.4.3 Bonded Phase

When considering the separation of biological molecules, especially proteins, the bonded stationary phase can have impact on the separation as well. In this treatment, only stationary phases for reversed-phase separation are considered. Some evidence exists that the use of shorter chain straight chain hydrocarbons (C4 or C8) can give sharper peaks than longer chain hydrocarbons (C18) [19, 41]. This effect is not always especially strong, and this fact is reflected by the marketing of both C4 and C18 bonded widepore superficially porous and fully porous particles for protein separations. In a comparison between fully and superficially porous particles for proteins bonded with either C4 or C18, it was seen that the effect of bonded phase itself was influenced by the content of unreacted silanols on the particle surface [46]. Silica based particles are usually bonded via a condensation reaction with a chlorosilane or other reactive silane [17]. Not all of the surface silanols are reacted, however, leaving these acidic groups available for ion-exchange type mechanisms during separation. Another issue in stationary phase chemistry is hydrolytic cleavage. The previously stated bonding reaction results in a siloxane bond, which is vulnerable to hydrolytic cleavage. An approach to counteract this is the use of sterically protected bonding agents, monofunctional silicon bonding agents with two bulky side groups (i-propyl or i-butyl) and one longer reversed-phase ligand attached [41]. This
approach may not only decrease hydrolytic cleavage of the stationary phase, it may also reduce the impact of secondary analyte interactions with surface silanols on the separations.

### 1.4.4 Overall Particle Size

One last attribute of the particle that should be considered when designing a particle for biological separations is the overall particle diameter. One impact of the particle size is its influence on chromatographic efficiency. From the traditional van Deemter equation, both the A and C term depend upon the particle diameter, with the C term depending upon the particle diameter squared[17]. Thus decreasing the particle diameter should decrease the H term and improve the separation efficiency. A theoretical van Deemter plot of this phenomenon is included in Figure 1.4, where it can be seen that both the plate height decreases and that the velocity at which the minimum plate height occurs shifts out to higher velocity. The advantage of the velocity shift is that the separation is not only more efficient, but also taking place more quickly. These advantages come at the cost of higher pressure, though, since the pressure required for a set linear velocity is shown by Equation 1.11, where \( \Delta P \) is the pressure drop across the column, \( \phi \) is a flow resistance parameter, \( \eta \) is the viscosity of the liquid, \( d_p \) is the particle diameter, \( L \) is the column length, and \( u \) is the linear velocity of the mobile phase[13].

\[
\Delta P = \frac{\phi \cdot \eta \cdot u \cdot L}{d_p^2} \tag{Equation 1.11}
\]

Furthermore, the linear velocity at which the minimum plate height is obtained is inversely proportional to the particle diameter, leading to the expression shown in Equation 1.12 for the pressure required to operate a column at the optimum linear velocity[47].

\[
\Delta P_{opt} \propto \frac{1}{d_p^3} \tag{Equation 1.12}
\]
This shows that significantly elevated pressures are required for the utilization of smaller diameter particles. This was initially realized in the Jorgenson lab by the operation of capillary columns packed with 1.5 µm nonporous particles at pressures up to 60,000 psi for the isocratic separation of small molecules[47]. With further work in the Jorgenson lab, pressures over 100,000 psi have been employed for the isocratic separation of small molecules on 1.0 µm nonporous particles[48].

Elevated pressures have the potential to lead to column heating due to the power generated by the pressure drop across the column P, shown in Equation 1.13 where F is the volumetric flowrate through the column and ∆P is the pressure drop across the column[47].

\[ P = F \cdot \Delta P \]  
Equation 1.13

As higher pressures have been employed in commercial chromatography, the issue of viscous heating has become apparent and limits the power regime accessible to conventional millimeter bore columns[49]. Work in the Jorgenson lab has typically been with small diameter capillary columns, which have been shown to effectively dissipate heat and counteract the negative effects of thermal gradients[50]. Thus these viscous heating issues are not an issue with the use of capillary columns. It is also worth mentioning that superficially porous particles, with their solid cores, have been found to have a higher thermal conductivity than fully porous particles, making it more easy for them to conduct heat out of the bed to the column wall where it can dissipate[51]. It has been observed that columns packed with superficially porous particles show lower susceptibility to the negative effects arising from frictional heating than do columns packed with fully porous particles[51, 52].

The above discussion is general for most analyte classes. Specific effects have been observed at elevated pressures specifically for proteins. The use of elevated pressure for protein
separations can influence their retention under isocratic conditions, due to the changes in molecular volume between being in solution and being associated with the stationary phase[53]. It has also been shown that protein recovery for reversed phase separations can be increased by going to higher pressure[54]. This is an advantage in terms of sensitivity, as well as eliminating carryover run-to-run, which is often seen for the reversed-phase separation of intact proteins. Such so-called ghosting can affect subsequent separations and would be beneficial to avoid.

As seen in this section, other aspects of chromatography affect the separation of large molecules, especially proteins, beyond what is often considered for small molecule separation. Due to these competing constraints such as surface area, pore size, and shell thickness, practical limits are put upon the materials which can be successfully employed. In view of this, the first area of investigation in this dissertation is the creation of superficially porous particles specifically tailored for the separation of large molecules such as intact proteins.

1.5 Dissertation Overview

The rest of the dissertation is split into four chapters (2-5). Chapters 2 and 3 involve the synthesis and characterization of micron sized superficially porous particles for intact protein separations. Chapters 4 and 5 involve the preparation and evaluation of capillary columns with superficially porous particles.

Chapter 2 covers initial studies in particle synthesis for these particles though early characterization with small molecules. The emphasis in this chapter is upon improving the morphology of the porous layer for these particles. Chapter 3 covers continued studies in the synthesis of the micron size superficially porous particles for protein separation. The synthetic emphasis in this chapter is reducing particle adhesion and particle damage throughout the synthetic route. This chapter concludes with the utilization of the particles for small molecule,
peptide, and intact protein separations. Chapter 4 focuses on the preparation of highly efficient capillary columns packed with commercial prototype superficially porous particles. As part of this chapter, a methodology for modeling packing progress in capillary columns is described and it is applied to understanding sequential capillary column packing. Chapter 5 covers the comparison of capillary columns packed with both fully and superficially porous commercial particles with either traditional or charged surface reversed-phase bondings. These columns are evaluated in isocratic mode with small molecules, and in gradient mode with both peptides and intact proteins. Insight is gained into the differing effects of particle architecture and surface functionalization upon separations.
Figure 1.1: Example van Deemter plot, showing the additive contributions from the A (red trace), B (green trace), and C (blue trace) terms to the overall plate height \( H \) (black trace).
Figure 1.2: Theoretical influence of $\rho$ parameter on reduced B term. The b-term is plotted relative to the b-term at $\rho=0$ (fully porous particle).
Figure 1.3: Impact of solid core to full particle radius (ρ) on relative porous volume of superficially porous particles. Note the similarity to Figure 1.2.
Figure 1.4: Theoretical change in column efficiency with decreasing particle diameter. The black, red, and green traces represent 3, 2, and 1 µm diameter particles, respectively. Note the decrease in overall plate height and the movement of the mobile phase velocity at minimum plate to higher linear velocity.
1.7 References


CHAPTER 2: SUPERFICLALLY POROUS PARTICLES FOR MACROMOLECULE SEPARATIONS – INITIAL FINDINGS

2.1 Introduction

2.1.1 Synthetic Approaches to Creating Superficially Porous Particles

There are several synthetic routes which have been employed to create superficially porous particles. The original pellicular particles created by Horvath and coworkers were prepared by crosslinking polystyrene-divinylbenzene on the outside of 50 µm glass beads[1]. Most of the subsequent work with superficially porous particles, however, involves the use of particles made entirely of silica. All of the following methods make use, in one way or another, of the Stöber process which consists of the hydrolysis and condensation of alkyl silicates under basic conditions to yield spherical silica particles[2]. This process allows for the creation of spherical silica particles of uniform size which can be used as the nonporous cores of superficially porous particles.

One approach to creating superficially porous particles is to attach colloidal silica particles to the outside of a substantially larger core silica particle, forming a porous layer where the pores are created by the interstices between the colloids. In 1969, Iler described a method of depositing small silica colloids, around 100 nm in diameter, onto a silica surface using alternating layers of silica and a positively charged organic species[3]. In 1970 Kirkland was awarded a patent for a method of creating superficially porous particles specifically for chromatography by depositing alternating layers of inorganic colloids and positively charged organic polymers, then thermally treating the particles at elevated temperature to remove the
polymer and improve the mechanical strength of the overall particle[4]. This approach was used to create Du Pont’s Zipax 30 µm diameter superficially porous silica particles[5]. In 1984, Bergna and Kirkland described another method of creating superficially porous particles, by initially attaching a layer of colloidal silica to the core particle surface using a charged polymer and then spray drying a mixture of these cores and more colloidal silica to more easily deposit a reasonably thick layer of colloidal silica on the core particle surface[6]. This general synthetic route was employed by Kirkland to create the 5 µm Poroshell particles in 1992[7]. The use of spray drying to create superficially porous particles often resulted in the creation of unwanted fully porous microspheres, however, making it disadvantageous[5]. Both the more recent 5 µm Poroshell particles marketed by Agilent Technologies and the Halo 2.7 µm superficially porous particles manufactured by Advanced Materials Technologies are created using a simple layer-by-layer approach using alternating layers of positively charged organic polymer and negatively charged colloidal silica particles[5, 8]. The particles are subsequently heated to remove the polymer layers and enhance mechanical strength.

Another approach to creating superficially porous particles is the creation of a porous layer via hydrolysis and condensation of a silica precursor such as tetraethylorthosilicate (TEOS) in the presence of a templating agent. For these the templating agent is usually a triblock copolymer such as EO70PO70EO70 (P123) with a co-surfactant such as cetyltrimethylammonium bromide (CTAB) and co-solvent of ethanol[9]. This synthetic route was originally developed to create fully porous silica particles. It can be easily adapted to make superficially porous particles by starting off with nonporous silica cores and then condensing the silicates species in the presence of the templating agent to form a porous shell on top of the nonporous core[10]. Octadecyltrimethylammoniumbromide (C18TAB) was used as the co-sufactant for the previous
synthesis to help increase pore size. This step can be repeated to grow the porous shell, and this stepwise growth pattern is evident in cross-section electron microscopy images of some commercially created superficially porous particles[11]. The templating agent is then removed at elevated temperature and the pores can be further expanded with hydrothermal treatment for use as a chromatographic support[10]. In a similar methodology, a superficially porous particle can be created by taking a nonporous silica particle, partially dissolving it under basic conditions, then redepositing the silica on the particles using a templating agent[12]. This eliminates the need to add a silica precursor to the reaction mixture.

A different approach to the creation of superficially porous particles is based upon one-step base catalyzed condensation of mercapto-containing silica precursors in the presence of a polymer and cationic surfactant which yields microparticles covered in nanoparticles[13-15]. Further improvement to the synthetic route has allowed the deposition of multiple layers of nanoparticles on the microparticle surface, allowing the porous layer to grow in size[15, 16]. Particles of this type are extensively nanoporous, but it is believed that most of the separation takes place in the interstices between the nanoparticles on the surface. These particles have shown much better performance for large molecule analytes than for small ones, which may be due to their thin shell, wide pore morphology. This also may be a consequence of small analytes experiencing restricted diffusion within the nanopores while the large molecules are completely excluded from the nanopores.

Yet another approach to the creation of superficially porous particles is the use of a sol-gel synthetic route, first developed to create fully porous chromatographic stationary phase supports. This involves the hydrolysis and co-condensation of tetraethoxysilane and an n-alkyltrimethoxysilane using a Stöber-like process to give embedded n-alkyl groups in the
resulting spherical silica particles[17]. These alkyl groups are then removed via thermal treatment, leaving a porous structure behind. Some work has been previously undertaken within the Jorgenson group to extend the application of this sol-gel synthetic route to the creation of superficially porous particles[18, 19]. In this case, a mixture of tetraethyl orthosilicate and octadecyltrimethoxysilane was employed to create the porous layer. The reactants were hydrolyzed and polymerized on the surface of nonporous silica cores, which after thermal treatment yielded superficially porous particles. Applying these particles to ultrahigh pressure separations met with limited success however, and was curtailed with the advent of readily available fully porous particles for use at ultrahigh pressures.

2.1.2 Current Trends in Superficially Porous Particles

Over the last decade, superficially porous particles have seen continued, sustained growth. As many commercial manufacturers have come out with their own lines of superficially porous particles, some trends are emerging, many mirroring those seen in fully porous particles.

One trend is the creation of superficially porous particles for large biomolecule separations. Such commercial particles have been around since the early 1990’s with the Poroshell superficially porous particle with 300 Å pores marketed by Agilent Technologies[7]. Subsequently in 2010, Advanced Materials Technology Company introduced a particle featuring 160 Å pores intended for peptide and small protein separations with the same shell thickness used for their small pore particles [20]. Phenomenex began offering a wide pore, thin shell superficially porous particle in 2012 with 200 Å pores[21, 22]. This was followed up the next year by Advanced Materials Technology with the development of a superficially porous particle with 400 Å pores and a thin shell intended for macromolecule separations[23, 24]. As can be seen, this is a major area of growth within the superficially porous particle market. It should also
be noted that the above particles are fairly large, with the Advanced Materials peptide particles having a diameter of 2.7 μm and all the other particles having diameters greater than 3 μm.

Another recent trend within superficially porous particles, mirroring the trends in liquid chromatography stationary phase supports overall, is the movement toward the utilization of smaller particles. The Halo particles, which started the current sustained interest in superficially porous particles, were introduced in 2006 with a particle diameter of 2.7 μm[8, 25, 26]. Soon after, Kinetex 2.6 and 1.7 μm diameter superficially porous particles were introduced by Phenomenex in 2010[27-29]. Also in 2010, Omamogho and coworkers described a synthesis of 1.7 μm superficially porous particles as well[10, 30, 31]. In 2011 Blue and Jorgenson described the creation of 1.1 μm superficially porous particles, the smallest reported superficially porous particles to date[32]. In 2013 the currently smallest commercial superficially porous particle, the Kinetex 1.3 μm particle, was introduced by Phenomenex[33]. Waters Corporation released a 1.6 μm superficially porous particle in 2013, adding to the number of column manufacturers offering a sub-2 μm superficially porous particle[34, 35].

It is interesting to note in closing that to date there is little confluence between these two recent trends. Commercial superficially porous particles smaller than 2 μm all have pores no greater than 100 Å in diameter, while commercial superficially porous particles with thin shells and pore sizes greater than 200 Å are all greater than 3 μm in diameter. The one exception to this trend is the superficially porous particles created by Blue, who created 1.1 μm particles with 187 Å pores and 1.6 μm particles with 248 Å pores[36].

2.1.3 Synthetic Objective

Bearing the proceeding sections in mind, a reasonable synthetic objective can be defined for a superficially porous particle intended for use in separating large molecules. In terms of
particle morphology, the particle should have a thin porous shell ($\rho>0.8$) to allow for facile mass transfer. In keeping with the convention in the field, the pores should be at least 200 Å in diameter to allow for easy access of the analyte into the porous layer. It should be noted, however, the commercial particles already exist that satisfy these conditions, as noted in Section 2.1.2. In addition to this, however, the particles should be around 1 µm in diameter to take advantage of the expected efficiency and protein recovery gains[37]. A schematic diagram of this synthetic objective particle is show in Figure 2.1. In term of synthetic route, the layer-by-layer method will be utilized, as significant know-how already exists within the Jorgenson lab on the implementation of this synthetic strategy for superficially porous particles[32, 36].

### 2.2 Materials and Methods

#### 2.2.1 Initial Synthesis Method

To create micron sized superficially porous particles, the layer-by-layer method from Kirkland described earlier in this chapter was employed[8]. When initially setting out to create these particles, the conditions used were similar to those found by Blue to be useful for the synthesis of micron-sized superficially porous particles[32, 36]. To begin, 1.0 µm silica spheres obtained from Fiber Optic Center Inc (New Bedford, MA) were heated to 1000 °C in a Lindburg Blue M Box Furnace (Thermo Scientific, Waltham, MA) for 24 hours to densify the particles into ~0.95 µm cores. These cores were then rehydroxylated by refluxing them with a 10% v/v aqueous solution of nitric acid (Fisher Scientific, Fair Lawn, NJ) while stirring for 20 hours. All water used in these studies was purified using a Barnstead NANOpure system (Thermo Scientific, Waltham, MA). The particles were then washed by repeated suspension with deionized water followed by centrifugation and removal of the supernatant until a neutral pH was obtained. The particles were then dried and ready for use in synthesis.
These core particles were then suspended in deionized water in a 1:10 w:v (kilogram:liter) ratio using 10 min sonication employing a Cole-Parmer 8891 bath sonicator (Vernon Hills, IL) in addition to shaking. A 0.5% w/w aqueous solution of 65 kDa poly(allylamine) hydrochloride (PAH) (Sigma Aldrich, St. Louis, MO) was added to the core solution in a 4.5:1 v/v polyelectrolyte solution:core solution ratio and the mixture was shaken by hand in a polypropylene centrifuge tube for 10 minutes to coat the particles with polyelectrolyte. The particles were then centrifuged down using either a Fisher Scientific Safety Centrifuge (Fair Lawn, NJ) or an International Clinical Centrifuge, model CL (International Equipment Co., Needham, MA) and the supernatant subsequently removed. Further excess polyelectrolyte was removed by resuspending the particles in deionized water using shaking and sonication and then centrifuging the particles down and removing the supernatant. This process was iterated 4 times. After the excess polyelectrolyte was removed, the particles were resuspended in deionized water (same volume used for initial core suspension) using sonication and shaking.

To coat the particles with colloidal silica, an aqueous solution of 5% w/w Nexsil-85 (manufacturer reported dynamic light scattering diameter 75-95nm) colloidal silica (Nyacol Nano Technologies Inc., Ashland, MA) was prepared. The solution pH was adjusted to between 2.5 and 3 with 10% v/v nitric acid as measured by a Corning pH Meter 430 (Sigma Aldrich, St. Louis, MO). An equal volume of pH adjusted colloidal silica solution to that of the water originally used to suspend the cores was added to the suspended polyelectrolyte covered cores and shaken by hand for 15 minutes. Excess colloidal silica was removed via centrifugation and removal of the supernatant. The particles were then resuspended in deionized water and washed 5 more times via centrifugation. To follow the coating process, the particles were imaged at various points during the synthesis process using scanning electron microscopy (SEM). A small
aliquot of the suspended particles would be periodically spotted onto SEM stubs and allowed to dry. The particles were then coated with a layer of gold/palladium using a Cressington 108 Auto Sputter Coater (Cressington Scientific Instruments Ltd., Watford, UK) to allow them to be imaged using a Hitachi S-4700 cold cathode field emission scanning electron microscope (Tokyo, Japan).

The polyelectrolyte coating step and the colloidal silica coating step can by alternated multiple times to yield multiple layers of attached colloidal silica and grow the porous shell using the methodology described above. When the desired number of colloidal silica layers were attached to the particles, the particles were then suspended at 10 or 20 mg/mL, split into 1.5 mL centrifuge tubes, flash frozen and then lyophilized to dryness while under centrifugation using a Savant Speed Vac Concentrator (Thermo Scientific, Waltham, MA) attached to an Edwards 18 2-stage rotary vacuum pump (Edwards Vacuum, Crawley, UK) equipped with an in-line cold trap to collect the water.

After the particles have dried, they are removed and heated in the box furnace for 16 hours at 540 °C to remove the polyelectrolye layer. The particles are then sintered at around 940 °C for 18 hours to impart mechanical strength to the particles. After sintering, the particles are rehydroxylated by refluxing them in a 1M aqueous solution of hydrochloric acid (Fisher Scientific, Fair Lawn, NJ) for 20 hours while stirring. The particles were then ready for bonding and endcapping for use in reversed-phase separations.

Bonding and endcapping was carried out generally following a protocol laid out in US patent application 2002/0070168[38]. Particles were reacted with $1 \times 10^{-4}$ moles of silane per square meter of silica present in the reaction, which represents a 10X excess of bonding reagent in terms of expected silanol groups. In addition to the silane, 1.2 equivalents of a weak base
were used to allow the reaction to proceed by neutralizing the acid formed by the silane bonding reaction. When the particle specific surface area was unknown, an estimate erring on the side of high surface area was used to ensure adequate bonding. This particular reaction utilized the octadecyldimethylchlorosilane (Gelest, Morrisville, PA) in refluxing toluene (Fisher Scientific, Fair Lawn, NJ) with imidazole (Sigma Aldrich, St. Louis, MO) as base activator for 4 hours. After reaction, the particles are successively washed with toluene, acetone (Fisher Scientific, Fair Lawn, NJ), 1:1 v/v mixture of deionized water and acetone, then finally resuspended in acetone. After removing the liquid acetone via evaporation, the particles were further dried under vacuum at 80 °C for 16 hours using an Edwards 18 2-cycle pump (Edwards Vacuum, Crawley, UK) connected to a National Appliance Co. Model 5831 vacuum oven (Portland, OR). The bonded particles were then reacted in an analogous manner using trimethylchlorosilane (Gelest, Inc., Morrisville, PA) to help endcap previously unreacted surface silanol groups. The particles were then washed and dried as previously, yielding material for use as a reversed phase chromatographic material.

2.2.2 Variation of Polyelectrolyte Type

To determine the best polyelectrolyte type to use in the synthesis of single layer superficially porous particles, high molecular weight (400-500 kDa) poly(diallyldimethylammonium) chloride (HMW PDDA) and 2 kDa polyethyleneimine (PEI), both obtained from Sigma Aldrich (St. Louis, MO), were employed in addition to the previously mentioned PAH. All were used to create 0.5% w/w aqueous solutions which were subsequently used in the above synthesis through the colloidal silica washing step to determine the influence on polyelectrolyte type on colloidal silica coating.
2.2.3 Impact of pH on Colloidal Silica Coating

To determine the impact of pH on the coating of colloidal silica onto the core particles, 5% w/w aqueous solutions of Nexsil-85 colloidal silica were prepared with pH values of 3, 2.5, and 2 using a 10% v/v nitric acid solution. The synthesis was analogous to the previous method except with the use of HMW PDDA which had been found to be advantageous in the previous section.

2.2.4 Particle Centrifugation Studies

To study the influence of particle collection technique during washing upon particle morphology, a more modern centrifuge was employed than previously, an Eppendorf Centrifuge 5424 R (Hamburg, Germany). This centrifuge allowed the controlled variation of centrifugal force applied to the particles. To test the use of sedimentation via gravity, the particles were placed into a centrifuge tube and allowed to sediment under gravity with the centrifuge tube standing straight up.

2.2.5 Influence of Particle Lyophilization Technique

For particle lyophilization, the particles were flash frozen in 1.5 mL polypropylene snap-top centrifuge tubes (Fisher Scientific, Fair Lawn, NJ) using either liquid nitrogen or a dry ice/acetone slurry. Before freezing the particles were usually slurried at 20 mg/mL in deionized water. Particle lyophilization studies were undertaken with the Speed Vac concentrator and rotary pump mentioned earlier. This setup has the vacuum pump attached to a sealed chamber containing a centrifuge, allowing the water to be removed via sublimation while the tubes are spinning to ensure no loss of non-volatile sample (particles in this case). After lyophilization, samples of the particles were either re-suspended in water and placed on an imaging stub to dry
or tapped down dry onto an imaging stub covered in conductive tape. These particles were then imaged using SEM.

2.2.6 Capillary Column Packing and Evaluation

Superficially porous particles created by the above methodology, including the aforementioned polymer removal at 540 °C and sintering at 940 °C, were created for use in column packing. The particles were rehydroxylated, then were bonded and endcapped as stated previously. These particles were then analyzed by obtaining SEM images then taking two measurements across the diameter of each particle using ImageJ software (National Institutes of Health, Bethesda, MD). The two measurements for each particle were number averaged to give the average particle diameter for the particle. These diameter measurements were then used to determine the average particle diameter for the sample and the relative standard deviation for the sample, often plotted in a histogram using Igor Pro 6.0 to help visualize the distribution.

Column packing conditions have been set forth elsewhere[39-42], but will be enumerated here to reflect those conditions used during this study. For this study all columns were packed in polyimide coated fused silica capillary (Polymicro Technologies, Phoenix, AZ) with 360 µm outer diameter (OD) and 30 µm inner diameter (ID). An outlet frit was created by using a tungsten wire to push a 50-100 µm plug of 2.5 µm diameter nonporous silica particles (Bangs Laboratory, Fishers, IN) 100-200 µm into the capillary, using methodology similar to a previous report[43]. The particles were then sintered into place using an electric arcing device[44] to allow a 100-200 µm long carbon fiber detection microelectrode to be inserted into the column outlet, allowing for negligible extra column band broadening[45].

For high pressure packing, a high pressure packing vessel was employed consisting of a slurry reservoir and an attachment for a high pressure fitting to hold the capillary inlet in the
packing reservoir during column packing. Pressure was applied to the reservoir using a Haskel DSHF-300 pneumatic amplifier pump (Burbank, CA) connected to the packing vessel. The slurry was prepared at 2-3 mg/mL in acetone and sonicated before use. The push solvent was acetone as well. Packing was initiated at low pressure, then pressure was slowly ramped up over the course of the packing process, often up to 30 - 35 kpsi. Pressure was determined by using the inlet air pressure multiplied by the pump’s amplification ratio. Packing progress was followed by the use of a microscope equipped with a 100X oil immersion objective (Carolina Biological Supply Company, Burlington, NC). After the column had reached a sufficient length, pressure was removed and the column was allowed to slowly vent to atmospheric pressure.

After packing, the column was attached to an in-house constructed high pressure injector[42] and pressure was applied to the inlet of the column for solvent exchange and bed consolidation. The injector was attached to a DSXHF-903 pneumatic amplifier pump (Haskel, Burbank, CA) to supply the high pressure necessary for bed consolidation. The column was flushed in the mobile phase it was intended to run in, in this case 90/10 v/v water/acetonitrile (Fisher Scientific, Fair Lawn, NJ) with 0.1% trifluoroacetic acid (TFA) added (Fisher Scientific, Fair Lawn, NJ). The column was gradually pressurized to 42 kpsi, held there for 1 hour, then allowed to depressurize. After the column depressurized, it was repressurized to 10 kpsi and a temporary frit was created using a heated wire stripper (Teledyne Interconnect Devices, San Diego, CA). The column was then depressurized and removed from the high pressure system. The temporary frit was removed and a frit was created by applying a 1:1 v:v mixture of potassium silicate (PQ Corporation, Valley Forge, PA) and formamide (Sigma Aldrich, St. Louis, MO) to a glass fiber filter (Whatman, Pittsburgh, PA) and then repeatedly pushing the
inlet of the column onto it[46]. The frit could be quickly set by the use of the electric arcing
device or allowed to dry overnight at 50 °C.

Isocratic column evaluation has also been described elsewhere[39-42], but a brief
overview will be included to reflect the conditions for this specific experiment. The capillary
column is attached to the high pressure injector using a high pressure fitting, which is connected
to the outlet of the DSXHF-903 pneumatic amplifier pump. A DSTV-100 pneumatic amplifier
pump (Haskel, Burbank, CA) is connected in series before the DSXHF-903 pump to allow for
low pressure injections. The sample consists of the electroactive compounds hydroquinone,
resorcinol, catechol, and 4-methyl catechol (All from Sigma Aldrich, St. Louis, MO) with L-
ascorbic acid (Fisher Scientific, Fair Lawn, NJ) used as the dead time marker. The sample is
injected onto the head of the column by filling the ultrahigh pressure injector with sample
solution, then sealing the injector and applying 1000 psi for 3 seconds using the DSTV-100
pump. After this, the injector is flushed out with mobile phase and then pressurized to the
analysis pressure using the DSXHF-903 pump. The compounds are detected at the end of the
column using by an 8 µm diameter carbon fiber microelectrode inserted up to the specially
formed outlet frit described previously in this chapter[43, 45]. The electrode was held at +1.1 V
relative to an Ag/AgCl reference electrode to allow for amperometric detection of the analytes.

The signal was amplified using a SR570 Current Amplifier (Stanford Research Systems,
Sunnyvale, CA) employing a 12dB lowpass filter set to 3 Hz. The signal was recorded by in-
lab written program, Stripchart Recorder, within Labview 6.1 (National instruments, Austin,
TX). After acquisition, the signal was digitally filtered to remove high frequency noise and
baseline subtracted to account for low-frequency baseline drift using Igor Pro 6.0 (WaveMetrics,
Inc., Lake Oswego, OR). The peaks were assessed using the method of iterative statistical
moments with ± 3σ integration limits within Igor Pro 6.0 to generate retention times and plate counts[47]. These data points were used to create h-ν plots and the data were fit using a reduced Van Deemter equation in Igor Pro 6.0. Diffusion coefficients for hydroquinone in the mobile phase to determine ν were calculated for the pressures employed in the separations based upon previous literature[48]. This allows calculation of the reduced minimum plate height and a, b, and c terms, which are used for column evaluation.

2.2.7 Multiplicity Evaluation and Refinement

To better understand whether the superficially porous particles are adhered to each other, in-solution optical microscopy was employed. Recently employed by Blue[36, 49], this method involves slurrying the particles in a suitable solvent, often water for unbonded particles and acetone for reversed-phase bonded particles. A small aliquot of the slurry is then placed onto a glass microscope slide (Fisher Scientific, Fair Lawn, NJ) and then quickly covered with a coverslip. The particles were then observed using the oil immersion microscope described previously and images were captured using a camera mounted in the microscope and GrabBee software (VideoHome Technology Corp., New Taipei City, Taiwan). This allows the visualization of the micron sized particles within a slurry environment where they can be observed moving around and vibrating due to Brownian motion.

From these images, it should be noted that observed particle multiplets may either be physically adhered or merely aggregated in solution. In-solution optical imaging represents an alternative method for determining particle adhesion over imaging of dry samples via SEM. To give a more careful understanding of the nature of the particles, several images are taken and then the particles are counted and binned based upon how many particles are observed to be stuck together. These observations are weighted based upon the number of particles adhered (ie
one aggregate particles counts as 8 in the total) together to give an idea of the mass proportion found in each particle adhesion multiplicity, which is relevant in terms of yield. The n values reported for these multiplicity distributions reflects all particles seen, such that seeing two single particles and an aggregate of 8 particles would give n=10.

To improve the multiplicity distribution of the particles, sedimentation was employed. This involved slurrying the particles in an appropriate solvent and then allowing them to sediment via gravity. Larger particles will sediment more quickly than lower diameter particles, most simply described by the sedimentation equation for porous particles[50, 51], Equation 2.1, where \( u_t \) is the particle’s sedimentation velocity, \( d_p \) is the particle’s hydrodynamic radius, \( \varphi_{\text{part}} \) is the volume fraction of the particles, \( \kappa_2 \) is a particle-solvent constant, \( \rho_{\text{skele}} \) is the density of the particle skeleton, \( \varepsilon_{p,i} \) is the particle porosity, \( \rho_f \) is the density of the fluid, \( \varepsilon_f \) is the fraction of the total hydrodynamic particle volume filled with fluid, \( g \) is the acceleration due to gravity, and \( \eta \) is the viscosity of the fluid.

\[
\begin{align*}
    u_t &= \frac{d_p^2 (1-\varphi_{\text{part}})^{-\kappa_2} \left[ \rho_{\text{skele}} (1-\varepsilon_{p,i}) + \rho_f (\varepsilon_f - 1) \right] g}{18\eta} \\
\end{align*}
\]

Equation 2.1

By this relation, adhered particles will sediment faster than single particles since they have a larger effective hydrodynamic radius, and also larger particle multiplets will sediment more quickly than smaller multiplets will. This particle separation technique results in a frontal separation. Due to the low degree of polydispersity in the single particles, distinct particle fronts can be seen as the particles sediment. A schematic depiction of this process is given in Figure 2.2. After allowing the particles to sediment, the top of the suspended particles is removed and saved, and then the particles are resuspended and the process is repeated. After several iterations, a sample consisting of single particles and small multiplets can be obtained.
2.2.8 Packing and Evaluation of Multiplicity Refined Particles

When packing and characterizing the column created using the multiplicity refined particles, the same general column preparation and evaluation processes were employed. The columns were packed in fused silica capillaries with a 30 µm internal diameter (ID) and 360 µm outer diameter (OD).

2.3 Results and Discussion

2.3.1 Initial Synthesis Method

The coating of core particles with colloidal silica using the initial synthesis method was followed by SEM to determine particle morphology. Images obtained after the addition of 1, 2, and 3 layers of colloidal silica during the coating process can be seen in Figure 2.3. It can be seen that the particles appear to stick together to an increasing degree over the course of the synthesis. This confirms the findings of Blue that the coating micron sized cores with relatively large diameter colloidal silica can lead to an undesirable degree of particle adhesion[36]. Fortunately, taking the thin particle shell requirement into consideration from Chapter 1, it was determined that 1 coat of colloidal silica could give the kind of large $\rho$ parameter desired in the synthetic objective. Thus the rest of the efforts on this project center upon the creation of superficially porous particles with only one layer of attached silica.

2.3.2 Influence of Polyelectrolyte on Colloidal Silica Coating

It has been found previously that the type of polyelectrolyte used for the synthesis method can have an impact upon the resultant particle morphology[36]. Thus it was useful to determine if the choice of polyelectrolyte impacted the colloidal silica coating for this particular synthetic system. The results of coating the cores with either PAH, PEI, or HMW PDDA followed by coating on one layer of colloidal silica and then washing away the excess can be
seen in the SEM images in Figure 2.4. The PAH particles appear to have the least coating, with some of the particles visible showing bare patches. The PEI particles appear to be more evenly coated, though they are still not as heavily coated as the particles made with the use of HMW PDDA. On one level this may be an effect of the PDDA having a much larger molecular weight than the other polyelectrolytes employed and thus being able to interact more strongly with the large colloidal silica. Another difference between the polyelectrolytes is that the PDDA derives its charge from a quaternary amine, so that it will always bear the same positive charge while the other polyelectrolytes rely upon primary or secondary amines to achieve their charge, meaning that under some reaction conditions their charge may vary. It is believed that the first reason (higher molecular weight) is the most plausible, since at the reaction conditions employed amine functionalities should retain a positive charge. Moving forward HMW PDDA was used as the polyelectrolyte to facilitate colloidal silica coating.

2.3.3 Impact of pH on Colloidal Silica Coating

When coating colloidal silica onto the core particles, some irreproducibility in colloidal silica coverage was seen batch-to-batch and the pH of the colloidal silica solution was suspected of being the cause. Thus the pH of the colloidal silica solution was varied between pH 2 and 3 and the colloidal silica coating was investigated via SEM imaging. The results can be seen in Figure 2.5. As can be seen, decreasing the pH to either 2.0 or 2.5 allows the colloidal silica to coat the core particle more heavily. It is expected that as the pH of the solution drops, the colloidal silica lose some of their negative surface charge due to protonation of some of their acidic surface silanol groups. This allows more colloidal silica to coat the core particle without running into as much detrimental colloid-colloid electrostatic repulsion. Further studies involving decreasing the colloidal silica solution pH below 2 were carried out but no real
improvement in surface coverage was seen. Thus moving forward the pH of the colloidal silica solution was adjusted to pH 2 before addition to the core solution.

2.3.4 Influence of Centrifugation on Particle Morphology

When looking at the particles being created, it was observed that many of the particles were clumping together or had undesirable surface morphology (clumps of colloidal silica extending out beyond the expected monolayer). This was suspected of occurring during the wash steps after both polyelectrolyte and colloidal silica coating. Efforts were made to resuspend the particles after each instance of centrifugation during the wash procedure, but resuspension was often difficult. This observation led to the thought that the particles may form small aggregates which are carried forward through the synthesis. Also since the colloidal silica at this point in the synthesis is only held onto the surface of the core particle by the polyelectrolyte, moving to larger colloidal silica increases the chances that the colloids could get sheared off or rearranged due to interparticle interactions during the centrifugation process.

Analogous particle syntheses were undertaken using the aforementioned synthetic route except that for the excess polyelectrolyte and colloidal silica wash steps either 450Xg or 50Xg or simple sedimentation (1Xg) was used to remove the particles from the supernatant.

The particles were imaged using SEM after coating with colloidal silica and washing away the excess colloidal silica, and can be seen Figure 2.6. It is noticeable that with the highest force used for sedimentation there are more colloidal silica protrusions on the particles than are seen with the lower forces used for sedimentation. Cutting down on the number of protrusions means a more uniform particle surface and a more uniform porous layer thickness. One advantage of smooth particles with an even coating of colloidal silica is that it should provide fairly constant mass transfer characteristics, hopefully leading to better separation. Particle shell
roughness may also play a part in the packing process, though the exact impact is difficult to determine. Another observation is that the particle pellet obtained using gravitational sedimentation is much easier to resuspend after sedimentation compared with high centrifugation forces. This is in line with the observation that with high force centrifugation the particles are being smashed together into each other. For the sedimentation route, this may speak to a decrease in particle adhesion and aggregation, which would be a definite benefit. The one downside of using gravitational sedimentation is that it takes much longer than the use of centrifugation. It was found that using centrifugation for the polyelectrolyte wash steps and sedimentation using gravity for the colloidal silica washes provided reasonable time savings while still allowing gentle treatment of the particles after the colloidal silica was attached. This general experimental paradigm was carried forward for the future particle syntheses.

2.3.5 Influence of Particle Lyophilization Technique

After working to improve the particle morphology during the in-solution portion of the synthesis, it was also important to ensure that the desirable particle morphology was retained during the drying step. Initial lyophilization efforts did allow the particles to dry, but upon imaging showed particle surface damage as can be seen in Figure 2.7 panel A. Caked particles that appeared to have been forced up against the side of the tube were occasionally seen, as seen in Figure 2.7 panel B. The particle damage process is believed to be similar to that seen with centrifugation during the colloidal silica wash steps. In hopes of alleviating this issue, the particles were lyophilized without the use of centrifugation using the same general lyophilization setup employed previously. Without centrifugation, the particles appear to have less surface damage, as seen in Figure 2.7 panel C. The resultant pellet in the centrifuge tubes after drying is noticeably fluffier to the eye than that seen when centrifugation was employed. Thus moving
forward for the lyophilization step the particles were not subject to centrifugation while being lyophilized.

2.3.6 Initial Column Packing and Evaluation

After the improvements to the particle synthesis process, superficially porous particles were created for chromatographic testing. In short summary, SEM images of the particles through some of the synthesis steps are shown in Figure 2.8 showing the particles before lyophilization, after sintering, after bonding, and after endcapping. The particles were found to have a number average diameter of 1.07 µm with a relative standard deviation of 2.4% (n=70). A histogram of the particle size distribution is included in Figure 2.9. To determine the ρ parameter, a sample of the cores before the synthesis but after densification was also sized and found to have a number average diameter of 0.94 µm with a relative standard deviation of 5.1% (n=100). A histogram of the particle size distribution of these core particles is shown in Figure 2.10. Some of the particles are of unusual shape or multiple cores that fused together during creation. Removing these large outliers from the distribution yields a number average diameter of 0.94 µm and a relative standard deviation of 1.9% (n=98), similar to that seen in the final superficially porous material. This allows the calculation of the ρ parameter for the superficially porous particles, which is 0.88. This falls within the expected useful range for large molecule separations.

The next step was to create packed capillary columns with the in-house synthesized superficially porous particles to determine their suitability for chromatographic separations. When the slurry was prepared, some very large chunks were seen to settle out quickly, so the slurry was allowed to stand a few minutes before use. Column packing proceeded slowly, taking around 22 hours to pack with multiple resuspensions, additions of new slurry, and pressure
cycling. After flushing, the 14.3 cm long column was characterized in 90/10 v/v water/acetonitrile + 0.1% TFA. An example chromatogram for this separation is shown in Figure 2.11 for a separation at 3500 psi. Though the peaks are broader than would be hoped for, they do exhibit reasonably good peak symmetry. To assess the performance of the column in a more systematic way, an h-v plot for this column can be seen in Figure 2.12 for hydroquinone. It can be seen that the column does indeed exhibit poor performance, with a reduced minimum plate height of around 10 and a very steep c-term region. Thus more work was needed to improve the performance of columns packed with these particles.

2.3.7 Multiplicity Evaluation and Refinement

When determining methodology to improve the performance of columns packed with the particles, the presence of particles which had adhered together sometime during the synthesis process (multiplets) appeared to be an area for improvement. Some understanding of these multiplets can be gained from SEM imaging, as seen in Figure 2.13 where both large particle chunks and free particles can be seen in two different images of the same sample. In-solution imaging of dilute slurry had been found to give a more straight forward understanding of the multiplicity distribution of samples. Using in-solution imaging of the particles in acetone, it could be seen that many multiplets appear to exist in the material, as seen in Figure 2.14 panel A. When a multiplicity distribution was generated for those particles, shown in Figure 2.15 panel A, it was seen that only a small portion of the particles were single. It should also be noted that for samples with large proportions of very large particle clumps his method is believed to underrepresent the number of particles in large chunks since they fall out of solution very easily.
To help improve the proportion of single particles within a sample, sedimentation was employed to yield a frontal separation of the particles, with the single particles on top. Two separate, nonsequential attempts were made to refine samples of the original superficially porous particles suspended in acetone (Sedimentation 1 and 2) with sedimentation 2 being more selective than sedimentation 1. These sedimentations yielded much improved results, as seen in the images in Figure 2.14 panels B and C where many of the particles can be seen to be single. To get a better understanding of the multiplicity distribution of the samples, multiplicity distribution charts were created for the two sedimentation attempts and can be seen in Figure 2.15 panels B and C. It can be seen that both sedimentation attempts showed an increase in the proportion of single particles and small multiplets, and a decrease in the proportion of large multiplets. Sedimentation 1 showed just less than 40% of the particles to be single, while the more careful sedimentation and sampling of sedimentation 2 resulted in around 50% of the particles imaged being single.

2.3.8 Packing and Evaluation of Multiplicity Refined Particles

Once the particles were refined, they were slurried in acetone and packed into 30 µm ID fused silica columns. Both particle samples took between 1 and 2 days to pack. The sedimentation 1 particle column was 11.9 cm long when evaluated in 90/10 water/acetonitrile +0.1% TFA. It was found to give much higher efficiency than the column created with the unrefined particles, with the sedimentation 1 column showing a reduced minimum plate height for hydroquinone of around 2.7. An example chromatogram from the column packed with sedimentation 1 particles is shown in Figure 2.16. The sedimentation 2 column was 14.0 cm long when characterized in the same mobile phase as the previous column. While there were some difficulties during the characterization, a few data points were able to be obtained which showed
the sedimentation 2 column to be more efficient than the other columns characterized, with the data points near a reduced plate height of 1.8. A comparison between the two columns packed with refined particles and the column packed with unrefined particles is shown in Figure 2.17. It can be seen that the refined particle columns showed much higher efficiency than the unrefined particle column.

2.4 Conclusions

Through this investigation, superficially porous particles were successfully synthesized bearing at least some of the desirable characteristics for large molecule separations. It was found that improvements to the colloidal silica coating, particle washing, and particle lyophilization steps improved the overall morphology of the particles created. This is necessitated by the much larger size of colloidal silica employed in this investigation relative to the colloidal silica size previously employed in the lab for the successful creation of micron sized superficially porous particles. With this large colloidal silica size, it was seen that the colloids showed a tendency to rearrange if subjected to excessive force. This issue is also probably due to the core size employed. Blue fabricated 1.6 µm superficially porous particles employing 1.4 µm diameter cores using a synthetic route similar to the one described here and reported not having as much trouble as when 0.9 µm diameter cores were employed[36]. With the difficulty seen in the present embodiment, the present synthesis may be approaching the maximum feasible colloid to core diameter ratio for this synthetic route.

While these particles were successfully synthesized with good surface characteristics, it was found that a large amount of interparticle adhesion was occurring. It was found that these strongly adhered particles were detrimental to column performance, with the best performing column being packed with the particles having the largest proportion of single particles and not
showing large multiplets. Thus it appears that to create particles capable of packing efficient columns, it is important for the particles to be unattached to each other. The promising performance of the column packed with the most highly refined particles illustrated that the particles themselves have the potential to create very efficient capillary columns. It also appeared to be worth the significant effort required to either reduce the number of adhered particles during the synthesis or to refine the synthesized particles to improve the final multiplicity distribution. Efforts in both these areas of synthetic improvement as well as chromatographic applications of these particles are covered in Chapter 3.
2.5 Figures

Figure 2.1: Schematic diagram of the superficially porous particle synthetic objective for this study.
Figure 2.2: Schematic of particle refinement via sedimentation using gravity in centrifuge tubes with the left tube being the original slurry and the right tube being the slurry after undergoing sedimentation for a suitable length of time.
Figure 2.3: Particle images after 1 (A), 2 (B), and 3 (C) layers of colloidal silica have been added. Note the increasing degree of aggregation of the superficially porous particles with subsequent colloidal silica attachment steps.
Figure 2.4: Coverage comparison of colloidal silica on core particles with the use of PAH (A), PEI (B), or HMW PDDA (C) as the polyelectrolyte. All particles imaged after one colloidal silica coating step and then washing away the excess colloidal silica.
Figure 2.5: Comparison of particle coverage by colloidal silica at colloidal silica solution pH values of 3.0 (A), 2.5 (B), and 2.0 (C).
Figure 2.6: Particle morphology of superficially porous particles after washing away excess colloidal silica with the polyelectrolyte and colloidal silica washes being performed at either 450Xg (A), 50Xg (B), or 1Xg (C). Note the difference in colloidal silica morphology, specifically decreasing colloidal silica protrusions being seen with decreasing force used for sedimentation.
Figure 2.7: Particle morphology of particles after lyophilization. (A) was lyophilized with centrifugation, reconstituted in water, then spotted and dried for SEM. (B) is the same sample as (A) but was sampled dry after lyophilization, and the select chunk pictured illustrates the potential issue of particle merging during lyophilization. (C) shows particles which have been lyophilized without the use of centrifugation.
Figure 2.8: Superficially porous particles before lyophilization (A), after sintering (B), after bonding with octadecyldimethylchlorosilane (C), and after endcapping with trimethylchlorosilane (D).
Figure 2.9: Size distribution of first large batch of superficially porous particles created. Each particle diameter represents the number average of two measurements across the particle. The particles (n=70) were found to have a number average diameter of 1.07 µm with a relative standard deviation of 2.4%.
Figure 2.10: Size distribution of core particles used to synthesize the first large batch of superficially porous particles. Each particle diameter represents the number average of two measurements across the particle. The particles were found to have a number average diameter (n=100) of 0.94 µm with a relative standard deviation of 5.1%. Removing the large outliers gives a number average diameter (n=98) of 0.94 µm with a relative standard deviation of 1.9%.
Figure 2.11: Example chromatogram for the first packed capillary column using the in-house superficially porous particles. Column was 14.3 cm long, 30 µm ID, run at ambient temperature in 90/10 water/acetonitrile + 0.1% TFA. This specific injection was run at 3500 psi, just above the column’s optimum linear velocity. The five peaks are, in order of retention: ascorbic acid, hydroquinone, resorcinol, catechol, and 4-methyl catechol.
Figure 2.12: $h$-$\nu$ plot for hydroquinone for the first packed column using the in-house synthesized superficially porous particles. The column was 14.3 cm long, 30 µm ID, run at ambient temperature in 90/10 water/acetonitrile + 0.1% TFA. Efficiency data shown for hydroquinone, $k'$ ~0.1
Figure 2.13: SEM images of bonded and endcapped particles showing what appeared to be physically adhered particles (A) and much less adhered particles (B).
Figure 2.14: Visualization of bonded and endcapped superficially porous particles as shown by in-solution imaging. Shown are unsized particles (A), sedimentation 1 (B), and sedimentation 2 (C). The white dots are particles, notice the greater number of singlets seen for the multiplicity refined samples (B) and (C).
Figure 2.15: Multiplicity distributions obtained from in-solution imaging of the superficially porous particles without refinement (n=366) (A), sedimentation 1 (n=486) (B), and sedimentation 2 (n=503) (C). ‘M’ stands for particles associated with aggregates containing more than 10 particles.
Figure 2.16: Example chromatogram from the column packed with the ‘sedimentation 1’ 1.07 µm diameter particles. Column was 11.9 cm long, 30 µm ID, run at ambient temperature in 90/10 water/acetonitrile + 0.1% TFA. This specific injection was run at 13,200 psi, near the column’s optimum linear velocity. The five peaks are, in order of retention: ascorbic acid, hydroquinone, resorcinol, catechol, and 4-methyl catechol.
Figure 2.17: h-ν plot showing the efficiency comparison between the unrefined column and the columns created using the multiplicity refined particles. Unrefined column (14.3 cm) shown as black circles, sedimentation 1 column (11.9 cm) shown as red boxes, and sedimentation 2 column (14.0 cm) shown as green triangles. All columns had a 30 µm ID and were characterized at ambient temperature in 90/10 v/v water/acetonitrile mobile phase with 0.1% TFA added.
2.6 References


3.1 Introduction

3.1.1 Methods for Particle Size Refinement

As demonstrated at the end of Chapter 2, particle size refinement is often necessary to improve the particle size distribution of a material. Refining the particle size distribution (creating a sample enriched in unadhered particles) has been shown to greatly improve chromatographic performance. It is therefore useful to take a survey of particle size refinement techniques.

3.1.1.1 Sedimentation and Centrifugation

One of the most straightforward particle size refinement techniques is sedimentation, which was employed in Chapter 2. In this process, particles are allowed to fall through a fluid at the terminal velocity of the particle, where the gravitational force on the particle balances the particle’s resistance to motion due to motion through a viscous fluid. This process can be most simply understood by Stokes law (Equation 3.1)[1]

\[ u_t = \frac{d_p^2 g (\rho_p - \rho_f)}{18 \eta} \]  

Equation 3.1

where \( u_t \) is the terminal velocity of the particle, \( d_p \) is the particle diameter, \( g \) is the acceleration due to gravity, \( \rho_p \) and \( \rho_f \) are the densities of the particles and the fluid respectively, and \( \eta \) is the viscosity of the fluid. It can therefore be easily seen that particles with larger diameter will
sediment more quickly than particles with smaller diameter. This condition only holds for particles that exhibit a low Reynolds number, that are well dispersed, and that are in a dilute solution[1]. This relation can be further modified to account for the sedimentation of porous particles in organic solvents with only partial solvent filling of the intraparticle volume to give Equation 3.2[2, 3], previously introduced in Chapter 2.

\[ u_s = \frac{d_p^2 (1-\phi_{part})^{-\kappa_2} [\rho_{skel}(1-\varepsilon_{p,i}) + \rho_f (\varepsilon_f - 1)]g}{18\eta} \]  

Equation 3.2

where \( \phi_{part} \) is the volume fraction of the particles, \( \kappa_2 \) is a particle-solvent constant, \( \rho_{skel} \) is the density of the particle skeleton, \( \varepsilon_{p,i} \) is the particle porosity, and \( \varepsilon_f \) is the fraction of the total hydrodynamic particle volume filled with fluid. Using either of the relations for sedimentation velocity, it can be seen that it is a viable method for the separation of particles of constant density based upon particle diameter. This method can be applied to the refinement of a material, or even fractionating a sample, but often multiple iterations are required for satisfactory results in terms of separation efficiency[1].

3.1.1.2 Elutriation

A particle size refinement similar to sedimentation is elutriation. In this technique, particles are allowed to sediment through a fluid, but the fluid is moving upwards in direct opposition to the sedimentation[1]. In one embodiment of this system, particles are suspended in steep-walled inverted cone with fluid flowing up from the bottom, so that very large particles sediment out of the chamber, intermediate particles are retained in the vessel, and small particles flow out of the top of the vessel. This type of system was employed by Scott to separate an ion-exchange resin in the 5-40 µm size range[4]. More recently, Kirkland has reported the use of elutriation to remove unwanted particles from superficially porous particles in the 2.6 to 6 µm size range[5, 6]. Another way to set the elutriation up is to have a series of connected chambers
of increasing diameter (same volumetric flowrate) to allow for sample fractionation due to the progressively lower fluid velocity in the chambers[1].

3.1.1.3 Filtration

Another methodology to refine particle size is to the use of particle sieving or filtration. In this method, a screen or mesh is used to only allow particles below a certain diameter through the filter, yielding to two populations of particles. This methodology is useful and convenient for larger particles, but can be difficult for small particles[1]. A multi-screen filtration system was developed in the Jorgenson lab for the size refinement of particles between 1 and 2 µm in diameter, but suffered from low throughput and contamination of the sample by filter material[7]. Other methods of particle size refinement may be more useful for particles in this small size range.

3.1.1.4 Hydrodynamic Chromatography

Hydrodynamic chromatography is another method of particle refinement. As a review on the subject has recently been published[8], only a simple overview will be given here. The process was first described theoretically in 1969-1971[9-12] and was first proven experimentally in 1974[13]. This method of particle size separation is facilitated by the parabolic flow profile of pressure driven flow within narrow channels, sometimes within a packed bed of nonporous particles. As the particles to separate get larger, they cannot access the lower-velocity flow regions near the walls of the channels. Thus larger particles experience a higher overall velocity and elute from the column sooner than smaller particles. This method does not have a minimum particle size like some of the previous methods have, with recent reports of hydrodynamic chromatography being employed for the separation of nanoparticles[14, 15]. Aside from particle analysis, this technique has been used for the preparative refinement of particle size distribution
in the Jorgenson lab[16, 17]. This preparative methodology uses a long, large-bore column to fractionate particle samples. While this is a powerful particle separation technique, it is somewhat experimentally involved and suffers from low throughput.

3.1.2 Recent Applications of Superficially Porous Particles to Biological Separations

When attempting to employ superficially porous particles in biological separations, it is helpful to understand previous and current examples of similar work. As discussed previously in Chapter 1, superficially porous particles offer unique advantages for high molecular weight analytes, such as biological samples, due to their shorter diffusion length than fully porous particles[18]. Investigators have successfully employed small pore (90-100 Å) superficially porous particles of varying size for the separation of peptide mixtures[19-22]. One investigator reported a very high peak capacity for peptides using these particles, with a 90 cm total column length and 2.1 mm ID packed with 2.6 µm superficially porous particles achieving a peak capacity of 1360 in 480 minutes[21]. Though many manufacturers of superficially porous particle columns originally only offered small pore particles, in 2010 Advanced Materials Technology Company put out the first modern superficially porous particle tailored for peptide separations[23]. These particles have 160 Å pores, and were shown to provide superior separation efficiency to analogous particles with 90 Å pores and fully porous widepore particles. It should be noted that these larger pore particles have the same core to particle diameter ratio as the corresponding 90 Å pore material, both manufactured by Advanced Materials Technology. Similar particles are now available from other vendors as well. Even wider pore superficially porous particles, 5 µm particles with 300 Å pores, can also be used for the separation of peptides[24].
With the proliferation and differentiation of superficially porous particles in recent years, more particles designed for the separation of even larger analytes than peptides have emerged. These particles have very wide pores (at least 200 Å) and also differ from other superficially porous particles in that they have a larger nonporous core to particle diameter ratio ($\rho$) than particles designed for small molecule or peptide separations. The widepore Poroshell particles offered by Agilent Technologies have been used for several decades for the separation of proteins\cite{5, 25}. Recently, Patrie and coworkers have utilized these Poroshell particles in both 75 and 500 µm bore columns for the separation of intact proteins for proteomic separations\cite{26-28}. Phenomenex has recently introduced Aeris widepore (200 Å pores, 3.6 µm) particles for the separation of large molecules\cite{29}. These particles have been employed for the separation of both intact proteins and monoclonal antibodies \cite{29-31}. Another superficially porous particle designed for intact protein separation has been recently introduced by Advanced Materials Technology (3.4 µm diameter, 400 Å pores) and has been shown to be useful for the separation of intact proteins and monoclonal antibodies\cite{32, 33}. Many of the applications listed above are still in the realm of academic labs or particles manufacturers. As biological investigators become accustomed to these relatively new superficially porous particles designed for biological separations, their utilization in the application field appears likely to increase.

3.2 Materials and Methods

The initial synthetic route for this set of work was the final synthesis protocol from Chapter 2 used to create the batch of particles used for column packing and chromatographic characterization. The following methodologies are derived from this initial method.
3.2.1 Impact of Colloidal Silica to Core Ratio

It is important to make sure that the original core particles have as little adhesion as possible, since adhered core particles will lead to adhered particles later on. Thus it was decided to use undensified cores for the synthesis, since densifying the cores has been seen to sinter the cores together. The cores should densify during the sintering process. The same nominally 1 µm silica spheres from Fiber Optic Center Inc (New Bedford, MA) used in the previous study were employed but were not densified prior to coating with colloidal silica. They were hydroxylated in a similar manner to that described in Chapter 2, where the cores were refluxed in 10% v/v aqueous nitric acid (Fisher Scientific, Fair Lawn, NJ) for 20 hours, then washed with deionized water (Barnstead NANOpure system, Thermo Scientific, Waltham, MA) until they showed a neutral pH. They were then dried and used for particle synthesis.

For the following syntheses, it had been found that the number of wash steps could be reduced from 5 to 3 each for both the polyelectrolyte and the colloidal silica coating steps without detriment to the synthetic process. This allowed for reduction in synthesis time, largely because the colloidal silica was washed via sedimentation.

To investigate the impact of the colloidal silica to core particle ratio, hydroxylated cores were coated with HMW PDDA (Sigma Aldrich, St. Louis, MO), washed, and resuspended in aqueous slurry as described in Chapter 2. A 5% w/w aqueous solution of Nexsil-85 colloidal silica (Nyacol Nano Technologies Inc., Ashland, MA) was created and adjusted to pH 2 with 10% v/v nitric acid. The cores were then allowed to react with the colloidal silica in 2:1, 1:1, 2:3, 1:2, 1:3, and 1:5 colloidal silica solution to core solution volume ratios. For reference the initial method uses a 1:1 volume ratio for this reaction.
3.2.2 Improvements in Core Dispersion

It was found useful to invest in a more powerful sonicator than that which was currently in lab, a Cole-Parmer 8891 bath sonicator (Vernon Hills, IL) which is said to have 100 W power and 42 kHz frequency. A more powerful sonication bath could help ensure that the cores are fully dispersed in solution. To this end, an Elma P30H sonication bath (Elma Schmidbauer, GmbH, Singen, Germany) was purchased, which can operate at either 37 or 80 kHz and has a peak power of 400 W. It is also useful that the power of the sonicator can be adjusted to tune the power output to the application. For most of the following work, unless otherwise noted, the P30H sonicator was used at 100% power, 80 kHz, pulse mode (highest power). The ability of the sonicator to disperse particles was assessed using the in-solution imaging method outlined in Chapter 2.

3.2.3 Sonication During Polyelectrolyte Coating

It was found that cores aggregate during the polyelectrolyte coating step, believed to be through the interaction of partially coated core particles with each other. An illustration of this is shown in Figure 3.1 from an earlier synthesis using hydroxylated, densified cores and HMW PDDA with a 10 minute coating time. As a note the images in this figure were collected before image capture software had been purchased and thus were captured by outputting the microscope camera output to a television screen and the images was captured using a cellular phone camera. The cores look relatively well dispersed before addition of PDDA, but after addition of PDDA they appear much more aggregated, especially after the washing steps. 15 minutes of shaking and sonication in the Cole-Parmer 8891 sonication bath after particle washing proved ineffective at dispersing the aggregates, as seen in Figure 3.1, panel D.
A model for the proposed interaction in shown in Figure 3.2 where during the polyelectrolyte coating step partially coated core particles could interact and become electrostatically bound. As the polyelectrolyte coating progressed they would be fully coated in polyelectrolyte as a doublet. Particles bound in this way would be carried forward as a doublet (or larger multiplet) through the colloidal silica coating step, where colloidal silica would coat the outside but not coat the interface where the particles were adhered. If the particles broke apart after this, however, there would be circular bare patches on both of the particles. This theory was supported by the common observation of such circular bare patches on particles after colloidal silica coating.

To counteract this issue, it would be advantageous to make sure that during the polyelectrolyte coating step the particles did not bind together. Thus it was proposed to sonicate the cores using the P30H sonicator during the polyelectrolyte coating reaction to see if the particles would aggregate. The idea was that if any particles bound together they could be broken apart by the sonication long enough to become more fully coated in polyelectrolyte and would not re-aggregate. For this study hydroxylated cores were coated with HMW PDDA while the solution was being sonicated in the P30H sonicator at 80 kHz, 100% power, pulse mode. The particles were in-solution imaged before, during, and after the PDDA coating. The particles were washed by centrifugation with 10 minutes of sonication to suspend them after each wash and then 15 minutes after the last wash. The particles were also in-solution imaged after washing and resuspension. Multiplicity distributions were prepared for all times imaged.

3.2.4 Use of Polyethylene Glycol During Lyophilization

Much of the work up to this point with these particles has concerned the improvement of particle coating and dispersal in the solution phase. However, the particles need to be dried in
such a way as to retain their ease of dispersion later on in the synthesis. When comparing in-solution images of particles just before lyophilization and resuspended after lyophilization, as seen in Figure 3.3, it was seen that many more of the particles appear to be adhered together after lyophilization. It is thought that the freezing process, though done as quickly as possible in liquid nitrogen, may still be forcing particles together in solution, thus cramming them together into the observed clumps.

To prevent the particles from being pushed together, and to generate some room between particles, an aqueous solution of polyethylene glycol (PEG) was added to the lyophilization solution prior to lyophilization. This hydrophilic polymer should both dissolve easily in the aqueous lyophilization slurry and coat the particles, hopefully preserving a more dispersed slurry during freezing.

The first study in the use of PEG during lyophilization involved the creation of colloidal silica covered particles using the 1:3 v:v colloidal silica to cores ratio outlined earlier in this chapter. These particles were chosen since they had already shown potential as less adhered particles. The particles were slurried at 20 mg/mL (in reference to theoretical core masses) in deionized water. Two differing molecular weights of PEG were used: 3400 (Aldrich, Milwaukee, WI) and 8000 (Sigma, St. Louis, MO). Solutions of each were prepared in deionized water, either 0.5 or 5% w/w. 0.5 mL of the above particle solution was combined with 0.5 mL of each of the 4 PEG solutions and allowed to sit with intermittent shaking for 15 minutes. The particles were then flash frozen in liquid nitrogen, after which time they were lyophilized while the centrifuge was spinning. Spinning was done since the particles should be held apart by the PEG and because some particle losses had been seen without spinning the centrifuge in previous experiments. When the particles were dry, they were removed from the
lyophilization setup. The four batches of particles were heated to 550 °C for 16 hours to remove the polymer then 830 °C for 18 hours to sinter them together. Lowering the sintering temperature was done to hopefully decrease the degree of particle-particle adhesion being created and reinforced during the sintering process. This specific temperature was chosen since it was close to the temperature cited by Kirkland in the 2007 patent for superficially porous particles[6].

A subsequent PEG-assisted lyophilization study was undertaken to better understand desirable lyophilization conditions for the creation of non-adhered superficially porous particles. The PEG used for this study was the 8000 MW PEG since it appeared to be slightly better in the last study. The particles used for this study were coated with the standard amount (1:1 v/v) of colloidal silica reported earlier. PEG solutions of 2, 5, and 10% w/w were combined with particle slurries of 20, 60, and 200 mg/mL in a 1:1 v/v ratio and allowed to mix for 20 min with intermittent gentle agitation. A table of the differing reaction pairs and the mass ratios between particles and PEG can be seen in Table 3.1. The samples were then flash frozen in liquid nitrogen, after which time the samples were dried using lyophilization with the centrifuge spinning. The samples were then heated to 550 °C for 16 hours to remove the polymers and then heated to 820-840 °C for 18 hours to sinter the particles to improve their mechanical strength.

3.2.5 Impact of Rehydroxylation Technique

After deciding to use PEG for lyophilization, an attempt was made to create a large batch of superficially porous particles. When thinking about the most useful time to multiplicity refine the particles, it is useful to do so before bonding. This should utilize the surface charge on the particles (allow them to repel each other) and also use water as the sedimentation liquid, which has some advantages over using organic solvents. To improve the multiplicity separation
technique, it was also found useful to alter the sedimentation strategy somewhat from that described in Chapter 2. The new sedimentation scheme involves doing initial suspensions of the crude sample, with most of the suspended particles being taken off after a short sedimentation time of perhaps an hour and placed in new tubes. The crude fractions are then resuspended and allowed to sediment in a more careful fashion, with only the top of the supernatant being removed. This allows for the removal of the largest chunks quickly and helps ensure that the slurry is sufficiently dilute not to aggregate and crash prematurely out of solution during the careful sedimentations. With several iterations of this technique, a useful amount of low multiplicity material could be generated.

To investigate the cause of particle damage during the rehydroxylation step, various rehydroxylation techniques were used. All particles used in this study came from the same batch of sintered superficially porous particles. The rehydroxylation solution was used at either 1 or 0.1 M concentrations of hydrochloric acid (Fisher Scientific, Fair Lawn, NJ) for 20 hours at reflux with stirring. Two rehydroxylation times using 1 M hydrochloric acid were used: either 4 or 20 hours. Additionally, a rehydroxylation was attempted using a 20 hour reflux in 1 M hydrochloric acid but without the use of stirring. All rehydroxylation trials were subsequently washed to neutral pH and then imaged using SEM as described previously.

3.2.6 Final Particle Analysis

Using the synthetic route laid out over the preceding sections, superficially porous particles were synthesized for eventual chromatographic evaluation. The main difference was the thermal treatment was carried out at 540 °C for polymer removal and at 840 °C for particle strengthening, slightly different from previous methods. This was due to slight alterations that
were made to the furnace. The particles were initially analyzed by in-solution optical imaging and SEM imaging.

The particles were further analyzed using nitrogen analysis with a TriStar II V1.03 instrument (Micrometrics Instrument Corporation, Norcross, GA). Specific surface area was calculated using Brunauer-Emmet-Teller (BET) method[34], and pore volume parameters were calculated using the nitrogen adsorption data and the Barrett-Joyner-Halenda (BJH) method[35].

3.2.7 Column Packing and Isocratic Column Characterization

Once the final batch of silica particles were synthesized, they were bonded using a method similar to that outlined in Chapter 2. However, in this embodiment the entire process was kept under dry nitrogen to help keep water out of the reaction. The particles were bonded before specific surface area data was available, so the particles were estimated to have a specific surface area of 16 m²/g. This ended up being a reasonable assumption in light of the subsequently measured specific surface area. Another change from the previous method was that the bonding reaction was not stirred during bonding, due to the particle damage seen earlier from stirring. Finally, the bonding and endcapping steps are done sequentially, with all the imidazole (Sigma-Aldrich, St. Louis, MO) for both the bonding and the endcapping reactions being added at the beginning along with the bonding agent. After the reaction proceeded for 4 hours, the endcapping reagent was added directly to the reaction vessel. This allows the bonding and endcapping process to be streamlined along with hopefully cutting down on water contamination during the bonding process and particle loss during washing. Two batches of the particles were bonded, one with n-butyldimethylchlorosilane (C4) and the other with octyldimethylchlorosilane (C8), both purchased from Gelest (Morrisville, PA). Both batches of particles were endcapped with trimethylchlorosilane purchased from Gelest (Morrisville, PA). The use of the shorter
ligand (C4 and C8) than the previous octadecyl (C18) silane bonding was in the hopes of improving performance with large molecules.

Column packing was previously detailed in Chapter 2. In the current work, the particles were packed into 75 μm ID 360 μm OD fused silica capillaries (Polymicro Technologies, Phoenix, AZ) rather than 30 μm ID capillaries as previously. This is because these columns were expected to be used for LC-MS studies and the larger capillary ID allows for better sample loading and better signal. The 75 μm ID capillaries employ different outlet frits than the 30 μm ID capillaries used in Chapter 2. The frits are prepared using a 1:1 v/v mixture of formamide (Sigma-Aldrich, St. Louis, MO) and potassium silicate (PQ Corporation, Valley Forge, PA) used to wet a glass fiber filter (Whatman, Pittsburgh, PA) and then the column is then pressed down repeatedly into the wetted glass fiber filter to form a frit in the outlet of the column[36]. The fritted column is then heated overnight at 50 °C to set the frit.

Column packing is performed using a slightly modified packing reservoir, similar to a previous design but with a smaller internal volume of 0.5-0.6 mL. This smaller amount of slurry allowed for the conservation of particles. In this instance, columns were packed using slurries of particles between 10 and 20 mg/mL in acetone. The slurries were sonicated for 5 minutes prior to use with P30H bath sonicator at 100% power, 80 kHz frequency, in pulse mode with occasional gentle agitation by hand. Columns were packed up to 30 kpsi with maximum pressure usually being reached within 5 minutes of packing initiation. Packing was often followed by back illuminating the column with a white LED light. Another difference between this method and the previous method is that for packing the columns stirring was kept to a minimum to avoid damage to the particles. The slurry reservoir was stirred with a magnetic
stirbar for only 3-5 second every 10-30 minutes. Due to the small size of the particles, this was sufficient to maintain packing.

After packing, the particles were flushed in a manner similar to that described in Chapter 2. The C8 bonded particles were generally flushed and run in 80/20 v/v water/acetonitrile (Fisher scientific, Fair Lawn, NJ) with 0.1% TFA added (Fisher Scientific, Fair Lawn, NJ) while the C4 bonded particles were used with 90/10 v/v water/acetonitrile with 0.1% TFA added. The columns were generally flushed at 52 kpsi with the pressure being immediately applied to the column as opposed to the gradual addition of pressure previously. Otherwise flushing proceeded in a manner similar to that set forth in Chapter 2. One major difference, however, was the high pressure injector used for column flushing and characterization. A schematic figure is shown in Figure 3.4. All of the metal tubing, valves, and tees were stainless steel and purchased from High Pressure Equipment Company (Erie, PA). The tubing was al 1/8 inch OD, 0.020 inch ID, and the valves and tees were purchased to work with this tubing. Flexible PTFE tubing is used for the injection and waste ports. The injector was assembled in a planar fashion and secured to a piece of wood, which was in turn secured to the top of a lab jack to allow for incremental injector height adjustment. The capillary column was securely attached to the outlet of the injector with the inlet of the column usually positioned within the first few centimeters into the tubing between the two tees. This capillary positioning allows for sample to both be injected onto the column and easily washed away from the column inlet before high pressure elution.

Column characterization was undertaken using the mobile phases described above, using the same protocol described in Chapter 2 but with the new high pressure injector. For the C4 columns, retention loss was initially seen when using the columns for multiple days. For the retention loss experiments, retention factor was measured for the last eluting component, 4-
methyl catechol, at similar pressures, usually between 15 and 25 kpsi. C4 bonded particles show very little retention change as a function of pressure, so this allows comparison between columns. One way to counteract the loss of retention on these columns was to fill the column with ethanol before removing the column from the high pressure system. For the ethanol column storage experiments, 95% ethanol (AAPER Alcohol and Chemical Company, Shelbyville, KY) was used.

3.2.8 Gradient Characterization – Peptides

The next objective was to assess the performance of these particles with a peptide sample. The analysis was carried out using a home-built system for running gradients at pressures higher than those achievable with commercial instrumentation. The details of this instrument have been reported elsewhere[37-39], so it will not be discussed in detail here. In brief, a Waters nanoAcquity instrument (Waters Corporation, Milford, MA) is utilized to load a gradient in reverse followed by a sample injection all into a high aspect ratio gradient storage loop through a tee with the column. The nanoAcquity is then isolated from the system and a pneumatic amplifier pump (Haskel, Burbank, CA) is used to apply high pressure through the loop. This pushed the sample and gradient onto the column in the correct direction and order. It should be noted that this system is a constant pressure system instead of a constant flow system, so gradients are reported as a volume rather than as a time.

For this study, the mobile phase consisted of Optima LC-MS grade solvents of water and acetonitrile (both purchased from Fisher Scientific, Fair Lawn, NJ) both with 0.1% mass spectrometry grade formic acid added (Fluka, Sigma-Aldrich, St. Louis, MO). The sample was a MassPrep standard consisting of a tryptic digest of yeast alcohol dehydrogenase (Waters Corporation, Milford, MA) prepared in Optima LC-MS grade water with 0.1% formic acid.
added. The sample was made up at either 10 or 30 fmol/µL. All injections were 2µL in volume with a full loop injection. The sample was eluted from the column using a 0.5 to 40% acetonitrile gradient of either 8, 16.5, or 32.5 µL volume. All gradients had a 1.5 µL pre-gradient hold and a 2 µL wash after the gradient at 85% acetonitrile. The column was then re-equilibrated at 0.5% acetonitrile before the next sample was injected. A sample gradient profile for the 16.5 µL gradient is shown in Figure 3.5. During analysis the column was maintained at 40 °C using a Waters Temperature Control Module II (Waters Corporation, Milford, MA). Pressure was adjusted to give a measured volumetric flowrate of around 300 nL/min, as measured using an isocratic 80/20 water/acetonitrile mixture. The column was connected to a 25 cm length of 20 µm ID fused silica capillary (the pigtail) using an PicoClear zero dead volume connector (both from New Objective, Woburn, MA). This pigtail was then connected to a 20 µm ID capillary pulled to a 10 µm ID spray tip (New Objective, Woburn, MA).

The mass spectrometer was a Waters Q-TOF Premier (Waters Corporation, Milford, MA) operated in positive ion mode. Electrospray ionization was carried out at +3 kV. The mass spectrometer was operated in continuum mode with a 5 eV collision energy. Data was acquired with a 0.02 second interscan delay using a 0.28, 0.36, or 0.53 second scan time (3.3, 2.6, and 1.8 Hz) for the 8, 16.5, and 32.5 µL gradients, respectively. This is to make sure each peak is well sampled and that a set acquisition rate does not unduly bias the results. Data was acquired over an m/z range of 50-1990. A lockspray solution of 70/30 v/v water/acetonitrile with 0.1% formic acid added, containing [Glu\textsuperscript{1}]-fibrinopeptide B and leucine encephalin acetate hydrate (Sigma Aldrich, St Louis, MO) was employed. The lockspray solution was introduced and ionized through the reference spray needle and used for mass correction.
Data acquisition was performed by Waters (Milford, MA) MassLynx Version 4.1 software. Data acquisition began a few minutes before the high pressure pump was engaged to begin the separation. Chromatographic full width half max peak height (FWHM) data were obtained from the Apex peak fitting algorithm output generated using ProteinLynx version 2.5 data processing software (Waters Corporation, Milford, MA). All acquisitions were done in duplicate and the arithmetic mean of the 4σ peak width measurements for the two runs was used for peak characterization. 4σ peak widths were calculated by multiplying the FWHM values by 1.7 (approximation for $4/2.35 \approx 1.7021$, the ratio of standard deviations present at FWHM and 4σ peak widths for a Gaussian peak[40]). Peak identities were assigned based on low energy m/z values and peak intensities. Table 3.2 shows the 16 ions used for peak width calculations to determine peak capacity. Peak capacity for a resolution of 1 was calculated according to Equation 3.3[41]

$$n_c = \frac{t_g}{w_p} + 1$$  \hspace{1cm} \text{Equation 3.3}$$

where $n_c$ is the peak capacity, $t_g$ is the gradient length, and $w_p$ is the average peak width. The elution window was used in place of gradient length $t_g$ since it could be accurately measured though the true gradient length was always longer than the elution window. For these separations, the m/z 844.45 ion (for the 8 and 16.5 µL gradients) or the m/z 549.29 ion (for the 32.5 µL gradient) was used for the beginning of the elution window and the m/z 656.85 ion was used for the end of the elution window. The peak width for a given separation condition, $w_p$, was the arithmetic mean of the calculated 4σ peak widths described earlier. In some instances the results of this investigation are compared to results obtained using a 48.6 cm X 75 µm capillary column packed in-house with 1.77 µm Cortecs C18 bonded superficially porous
particles (Waters Corporation, Milford, MA). This column was operated in a similar manner to the current study. For a detailed description, see Chapter 5.

For this work, a 19.1 cm X 75 µm column packed with 1.06 µm C8 bonded superficially porous particles was employed. 32,000 psi pressure was used for this separation, provided by a DSXHF-903 pneumatic amplifier (Haskel, Burbank, CA). The loading study involved injecting either 20 or 60 fmol of MassPREP yeast alcohol dehydrogenase tryptic digest standard (Waters Corporation, Milford, MA) onto the column and eluting the sample using a 16.5 µL 0.5-40% acetonitrile gradient. For the retention time study, the in-house column was used to separate a 20 fmol sample using a 32.5 µL gradient while the Cortecs column was used to separate a 20 fmol sample using a 33.0 µL gradient. The gradient length study involved injecting 20 fmol of alcohol dehydrogenase tryptic digest on the column packed with in-house C8 bonded particles and eluting it using either an 8.0, 16.5, or 32.5 µL gradient from 0.5-40% acetonitrile.

3.2.9 Gradient Characterization – Proteins

To assess the performance of columns packed with the in-house particles, proteins from a whole cell lysate from *Escherichia coli* were separated on a 19.1 cm X 75 µm ID column packed with 1.06 µm C8 bonded particles. This study employed the same high pressure gradient system discussed in the previous section using acetonitrile and water, both with 0.2% formic acid added. Separations were performed at 32 kpsi with a 25 µL 0.5-60% acetonitrile gradient, incorporating a 1.5 µL pre-gradient hold at 0.5% acetonitrile, a 2 µL increase from 60 to 85% acetonitrile, a 2 µL hold at 85% acetonitrile, and a 2 µL decrease from 85 to 0.5% acetonitrile, after which point the column was allowed to recondition before performing another separation. A schematic of the gradient profile is shown in Figure 3.6. As previously, injections were 2.0 µL full loop injections. The mass spectrometer settings were also the same except that data was acquired at
1.9 Hz in continuum mode over the mass range of m/z 400 to 1990. Lock mass was not used to correct the masses obtained for the protein study.

The *E. coli* sample was diluted using water with 0.2% formic acid to give two samples measured to be around 50 and 125 ng/µL by Bradford assay, resulting in 100 and 250 ng injected on column per 2 µL injection. The samples were analyzed using the above gradient condition. When that did not produce satisfactory results, the mobile phase was changed to be acetonitrile and water, but both with 0.3% formic acid and 0.025% trifluoroacetic acid (Optima LC-MS grade, Fisher Scientific, Fair Lawn, NJ) added. This was done following the work of Roth et al.[26] who have seen good results using that mobile phase additive for intact protein separations on silica based superficially porous particles. The measured ion signal was lowered by the use of trifluoroacetic acid. For the separations employing the new mobile phase additives, the sample diluent was changed as well.

The raw protein data was processed using the autoME V 4.1.2 program developed by Waters Corporation (Milford, MA). Processing protein data using this approach has been documented previously[42, 43], so only a brief overview will be given here. The autoME program uses functions available within MassLynx 4.1 to automatically sum spectra together and then deconvolute the summed spectra to give zero charge spectra showing molecular mass. This deconvolution is achieved through the MaxEnt 1 algorithm in MassLynx[44]. The processing parameters employed in this experiment are based upon those used previously in the lab[42, 43], and are the same as those used by Richardson[43] except that for the current work the scan interval was 3, the input mass range was the same as the acquisition, the output mass range was 3,000-80,000, the maximum number of MaxEnt iterations was set to 9, and the de-harmonization tolerance was 5. After processing, a table is generated by autoME showing mass spectral
information for each of the deconvoluted spectra. This allows single masses to be extracted and plotted to give peak profiles for individual proteins.

The peak profile of ribonuclease A was also investigated. A 10 ng/µL sample of ribonuclease A from bovine pancreas (Type XII-A, Sigma-Aldrich, St. Louis, MO) was prepared in water with 0.3% formic acid and 0.025% trfluoroacetic acid. It was eluted from the column using a 12.5 µL 0.5-60% acetonitrile gradient otherwise following the previous gradient profile. The trifluoroacetic acid containing mobile phase employed in the E. coli lysate separation was employed for this separation as well. The peak was analyzed using MaxEnt 1 in Masslynx 4.1 on its own to investigate summed spectra obtained from the ribonuclease A peak. The maxEnt parameters were the same as those employed for the autoME processing, but only used the input mass range of m/z 1100 to 1990, and the calculation was done using 0.1 Da/channel.

3.3 Results and Discussion

3.3.1 Impact of Colloidal Silica to Core Ratio

In trying to create well coated particles, the colloidal silica to core reaction ratio was investigated to determine its impact upon the final particles. The results of the study are seen in Figure 3.7. It is seen that at high colloidal silica to core ratios, the cores are completely covered in colloidal silica and there is little difference, though the 1:1 and 2:1 volume ratio reactions appear to give the thickest coating. At the lowest ratio coating, there is not enough colloidal silica and the particles were poorly coated. One interesting result was the coating results from the 1:3 colloidal silica to core volume ratio, shown in Figure 3.7 panel E. There the particles were lightly but substantially uniformly covered in colloidal silica and there appears to be very little particle-particle adhesion. While these particles may not have enough of a porous layer for some applications, it did appear that the porous structure was very open, perhaps yielding an
advantageous pore size. For most of the following experiments, it was decided to use the original colloidal silica coating ratio (1:1) since it gave a good thick coating of colloidal silica on the core particle. Additionally, the 1:3 reaction ratio was revisited a couple times in later studies to evaluate its impact upon particle adhesion. This reaction type may be suitable to revisit at a later date to assess any impact it might have on very large molecule separations.

3.3.2 Improvements in Core Dispersion

The new sonicator, the P30H, was tested to determine if it was able to disperse core particles. To illustrate the efficacy of this particle dispersal method, Figure 3.8 shows a solution of hydroxylated silica cores being dispersed in deionized water by the P30H sonicator. After 90 minutes of sonication at 80 kHz, 100% power, pulse mode, the cores appear to be largely single. One advantage of operating at 80 kHz is that it generated much less noise than even a conventional ultrasonic bath, which is convenient for the long sonication times required to disperse core particles.

This improvement in core dispersal technique also allows for a more rigorous hydroxylation of the silica cores. The 1 µm cores as received from Fiber Optic Center, Inc. should be hydroxylated to ensure that the surface is fully covered in silanol groups. This was done loosely following the procedure outlined in Chapter 2, where the particles were placed into 10% v/v aqueous nitric acid (Fisher Scientific, Fair Lawn, NJ) but in this case were sonicated for 150 minutes prior to refluxing for 20 hours. After this initial reflux step, the particles are left in nitric acid solution and sonicated for 150 minutes in the P30H sonicator to ensure that any particle aggregates have dispersed. It was found to be easier to disperse particles after hydroxylation. The particles were then subjected to another 20 hour reflux in 10% v/v nitric acid with stirring, after which time they were sonicated again for 60 minutes then washed by
centrifugation with water until the water reached a neutral pH. They were then dried and ready for use in synthesis. With this core preparation method, all of the cores stayed suspended in solution. Using the original hydroxylation method, some of the particles would quickly sediment, indicating that large particle masses were still present. The two-step hydroxylation method for the core particles was used for all subsequent syntheses.

3.3.3 Sonication During Polyelectrolyte Coating

Hydroxylated cores were coated with HMW PDDA while in the P30H sonicating bath and in-solution imaged at various times. The results of this work can be seen in Figure 3.9 where the multiplicity distribution of the particles before coating with HMW PDDA, after 7 minutes of coating with sonication, after 15 min coating with sonication, and after washing and resuspension after HMW PDDA coating reaction. It can be seen that the cores do form aggregates early on, as can be seen by the large number of small multiplets in that distribution. After 15 minutes of coating with sonication, it can be seen that most of the particles are single, with a few doublets seen as well. Over the course of washing and resuspension, however, it can be seen that the cores become almost as well dispersed as the initial cores. A similar study had been conducted earlier with regard to sonicating the particles during the HMW PDDA coating reaction, but it was unsuccessful, probably due to insufficient sonication strength provided by the previous sonication bath. The use of strong sonication during the polyelectrolyte coating step appears to be useful in keeping the particles dispersed. This methodology was carried forward in future particle syntheses in hopes of improving particle coating and dispersal.

3.3.4 Use of Polyethylene Glycol During Lyophilization

The next study involves the addition of PEG before the freezing step to help reduce particle-particle adhesion. For the first PEG study, aqueous slurries of a batch of superficially
porous particles were mixed with either 5% or 0.5% w/w solutions of PEG with average molecular weights (MW) of either 3400 or 8000. After flash freezing, the samples were lyophilized. The samples that used the 5% PEG solutions of both types looked like fluffy white masses and were much larger than the leftovers from just lyophilizing particles. The samples which used the 0.5% PEG solutions were much smaller but still looked larger than that seen when just lyophilizing particles. After sintering, the particles were analyzed via SEM, as can be seen in Figure 3.10. The particles lyophilized with the 0.5% PEG solutions show many large particle chunks, while the particles lyophilized with the 5% PEG solutions appear to be more single via SEM. The particles were also in-solution imaged, which can be seen in Figure 3.11. As with the SEM images, the particles lyophilized with the 0.5% PEG solutions appear to have a greater proportion large particle chunks than the particle samples lyophilized with the 5% PEG solutions. The high concentration of 8000 MW PEG appeared to give the best results in this preliminary study.

These encouraging results prompted a second lyophilization study with the use of PEG. This study varied both the overall concentration of particles and PEG in the solution as well as the ratio of particles to PEG. After the particles were combined in various ratios with PEG, flash frozen, and then lyophilized, they all appeared to be fluffy white masses. After thermal polymer removal and sintering, the particles were imaged using SEM. Some representative images can be seen in Figure 3.12. The higher ratios samples of particles to PEG showed many particle clumps, both to the eye and with SEM imaging, sometimes seeing large rods of adhered particles. Additionally, the lower ratios of particles to PEG showed melting of the colloidal silica on the particle surface, which would sometimes make the samples very difficult to suspend in water. At right around a 1:1 w/w ratio, the particles appeared to be relatively single and the
particle surface retained the desired morphology. A summary of these observations is shown in Table 3.3. A further assessment of the particles was carried out using in-solution imaging. It should be noted that for all samples there were at least some large particle agglomerates seen by eye which are not accounted for in the in-solution images. The particles were imaged after sintering for 3 samples and for one of the highly attached samples it was imaged after sintering and rehydroxylation in the hope that it would free more particles up. The multiplicity distributions generated from these observations are shown in Figure 3.13. These observations corroborate the SEM observations that with particle to PEG ratios of around 1:1, a large proportion of the imaged particles are single (panels A and B), a much larger proportion than either of the clumped samples (Panels C and D). Thus it was found that using PEG during the lyophilization process can be beneficial, but that the proper ratio must be maintained to see the benefits of this approach. Moving forward, it was decided to use a 5% aqueous MW 8000 PEG solution mixed 1:1 with a 60 mg/mL aqueous slurry of particles since it showed good particle singularity and morphology and allowed reasonable throughput for the drying process.

To test the use of PEG during lyophilization against the original lyophilization (no PEG, 10 mg/mL), one batch of particles was created where some of the particles were frozen using the preferred PEG some were frozen in water at 10 mg/mL. The particles were then dried using lyophilization and then had polymer removed and were sintered using the previously outlined methodology. The particles were then suspended in deionized water, sonicated 15 minutes in the Cole-Parmer 8891 sonicator, and then in-solution imaged. Multiplicity distributions for the two samples are shown in Figure 3.14. It can be seen that for the portion able to be suspended that the use of PEG during the freezing process increases the proportion of free particles after sintering.
3.3.5 Impact of Rehydroxylation Technique

One batch of superficially porous particles was created and multiplicity refined using a simple sedimentation scheme. These particles were then bonded and endcapped as previously stated in Chapter 2, and then a size distribution for individual particles was generated using the SEM images of the particles, seen in Figure 3.15. The particles had a number average diameter of 1.09 µm and relative standard deviation of 4.8% (n=155). It can be seen from this distribution that there is a significant minority of particles at lower diameter than the rest. To investigate this more closely, the counted particles were observed and listed as undamaged, slightly damaged, moderately damaged, or bare in reference to the degree of colloidal silica coating seen. These observations were then used to generate a second size distribution that took into account particle damage as a function of particle size, as seen in Figure 3.16 which shows that 50% of the particles were undamaged, 26% slightly damaged, 20% moderately damaged, and 4% were bare. It can also be seen in Figure 3.16 that the particles of lower diameter than the main distribution were all either moderately damaged or bare. Thus it became apparent that more precautions should be taken to prevent particle damage during the synthesis.

The rehydroxylation step appeared to be where many of the bare particles were originating. One hypothesis was that the damaged particles were adhered particles being ripped apart during the rehydroxylation process either by the treatment or by mechanical action of the stirbar. To test this, superficially porous particles were synthesized and then multiplicity refined soon after sintering, so that a population of mostly single particles was rehydroxylated. After the rehydroxylation process, however, widespread particle damage was seen in this batch of particles. SEM images of the particles before and after the rehydroxylation step are shown in Figure 3.17, where it is clear that the particles show much more damage after the
To further probe the particle damage conditions, four rehydroxylation conditions were tried: stirring, 20 hour reflux, 1 M hydrochloric acid (condition 1); stirring, 4 hour reflux, 1 M hydrochloric acid (condition 2); stirring, 20 hour reflux, 0.1 M hydrochloric acid (condition 3); no stirring, 20 hour reflux, 1 M hydrochloric acid (condition 4). After rehydroxylation and washing, the particles were SEM imaged, as seen in Figure 3.18. Looking at the particles, it is clear that the unstirred sample showed the least amount of particle damage, suggesting that long term stirring of the particles causes mechanical stress on the particles resulting in the loss of attached colloidal silica. Moving forward the particles were rehydroxylated without the use of stirring.

3.3.6 Final Particle Analysis

A final synthesis of several replicate batches of superficially porous particles was undertaken. New cores had to be purchased to allow for the continued synthesis and they were found to be slightly smaller than the previous cores, thus slightly reducing the overall size of the final particles. Several synthetic batches were combined after multiplicity refinement but before bonding. This represented the final superficially porous particle batch. This final particle batch was characterized in several ways. The particles were in-solution imaged to determine their multiplicity distribution, which can be seen in Figure 3.19 panel A. It was found that the particles imaged were roughly 95% single (n=716) with only a few small multiplets seen. SEM images were also taken of the particles which revealed that the particles were well coated in colloidal silica, as can be seen in Figure 3.19 panel B. SEM images were then analyzed to give a particle size distribution, seen in
Figure 3.20, yielding a number average particle diameter of 1.06 µm with a relative standard deviation (RSD) of 2.9% (n=144). Similar to a previous investigation, the damage state of the particles was assessed and particle damage as a function of particle size was plotted in Figure 3.21 (n=144). It was found that 75% of the particles were undamaged, 22% were slightly damaged, and only 3% were moderately damaged. This represents an improvement over the previous particle damage distribution shown in Figure 3.16. The cores of the particles measured 0.88 µm in diameter (2.3% RSD, n=59), leading to an overall particle core to total particle diameter ratio (ρ value) of 0.83, consistent with the desired particle morphology.

The particles were then further analyzed by subjecting them to nitrogen analysis to yield specific surface area and pore data. The BET analysis indicated that the particles have a specific surface area of 11.8 m²/g. BJH analysis of the adsorption data indicated a specific pore volume of 0.079 mL/g. The nitrogen adsorption data was used to yield a pore size distribution, shown in Figure 3.22, revealing a peak in incremental pore volume at a 480 Å average pore width. Very small pores below 40-50 Å are seen in the pore distribution as well, but they are small enough to hopefully not interfere with the separation of proteins. A peak in the pore distribution is also observed around a 1050 Å average pore width, which is expected to correspond to interparticle spaces in the bulk material being analyzed. For comparison to the data obtained for the in-house particles, some published wide pore superficially porous particles designed for protein separations are have a specific surface area of 15 m²/g, a specific pore volume of 0.113 mL/g, a median pore width of around 400 Å, and a particle diameter of 3.4 µm[33]. Thus the particles synthesized in-house appeared to have similar pore size and surface area to the known material, but with a much smaller overall particle diameter.
3.3.7 Column Packing and Isocratic Column Characterization

Column packing with the final batch of superficially porous particles proceeded much more smoothly and quickly than previously. The column often would pack non-stop, without the need to re-start packing due to an obstruction in the capillary. It would generally take half an hour to over two hours to pack columns between 15 and 20 cm long, a major improvement over the previous packing of in-house superficially porous particles. This improvement in packing speed may in part have been due to the higher slurry concentration employed, along with fewer large, irregularly shaped particles which may have promoted column clogging.

The C8 bonded particles showed good performance with isocratic characterization. An example chromatogram is shown in Figure 3.23 for the characterization of a 16.6 cm X 75 µm ID column characterized in 80/20 v/v water/acetonitrile + 0.1% TFA. The separation was performed at ambient temperature. This chromatogram shows good peak symmetry and separation for all of the components. h-v plots for four 75 µm capillary columns packed with 1.06 µm C8 bonded superficially porous particles are shown in Figure 3.24. The columns are labeled C8-1, 2, 3, and 4 having lengths of 19.6, 16.6, 17.7, and 18.0 cm respectively. It can be seen that all the columns performed well, with reduced minimum plate heights ranging from just below 1.7 to just below 2.2. This confirms the findings of Chapter 2 which indicated that these particles had the potential to create efficient columns if properly synthesized. Looking at the best fit parameters to the reduced van Deemter equation for the four columns shown in Figure 3.24, it can also be seen that the columns exhibit somewhat similar characteristics column-to-column. The average terms for the columns are an a-term of 0.1, b-term of 1.4, and c-term of 0.5. Comparing the four columns, it was seen that three of the columns (1, 2, 4) have very similar performance, with one of the columns (3) showing slightly worse performance. This
decrease in performance appeared to be primarily influenced by the c-term, which was larger for this column than for the others. While slight differences did exist between the columns, these columns did exhibit good column-to-column reproducibility and efficiency, meaning that a more careful column packing study was not warranted.

The C4 particles were also isocratically characterized, but used a 90/10 v/v water/acetonitrile mobile phase with 0.1% TFA added do to their expected lower retention than the C8 particles. An example chromatogram is shown in Figure 3.25 for an isocratic separation in 90/10 v/v water/acetonitrile with 0.1% TFA added on a 19.2 cm X 75 µm column packed with 1.06 µm C4 particles. The separation was performed at ambient temperature. This chromatogram shows good peak symmetry, similar to that seen for the C8 particles in Figure 3.23, though some slight differences in selectivity are seen. Three columns packed with the C4 bonded material were packed and characterized, with the h-v plot seen in Figure 3.26 comparing their performance. The columns named C4-1, 2, and 3 were 18.7, 19.2, and 17.1 cm long respectively. Similar to the C8 columns run previously, there is reasonable column-to-column repeatability in terms of efficiency. The three columns had reduced minimum plate heights between about 2.4 and 2.6. This represents poorer performance than that seen for the C8 bonded particles. As with the C8 particles, the columns packed with C4 bonded particles exhibit reasonable agreement column-to-column in terms of the best fit parameters to the reduced van Deemter equation, seen in Figure 3.26. For the three C4 bonded columns, the average a-term was 0.1, the average b-term was 1.5, and the average c-term was 1.0. The a- and b-terms are in agreement with those found for the C8 bonded particles, but the c-term is roughly double that seen for the C8 bonded particles. This may be due to a difference in packing structure or a function of the C4 bonding.
During the characterization of the C4 particles, it was found that the columns would lose a significant amount of retention if the same column was characterized on different days. Columns were not seen to lose appreciable retention over the course of characterization, which is often several hours long. Evidence of this can be seen in Table 3.4, which shows the measured retention factor for 4-methyl catechol as a function of number of uses for the column and the number of days each use of the column came after the first one. It can be seen for the first use, the retention is similar for all three of the columns, right around 1.0. It should be noted that column C4-0, the first C4 column packed, was originally flushed and run in 80/20 v/v water/acetonitrile with 0.1% TFA added. Its first use with 90/10 water acetonitrile came the next day. For all three of the columns investigated, however, successive uses of the column reduce the column’s retention significantly. This trend indicated that the stationary phase is being removed, probably through hydrolytic cleavage.

It was desirable to find a way to reduce this issue so as to retain the retention of the column for later use. One hypothesis was that the stationary phase was being cleaved off by storing the column with 0.1% TFA in it. TFA is a reasonably strong acid, and as the column dries out it may become much more concentrated than it originally was in the mobile phase. Whatever the mechanism, it was decided to minimize the interaction of the stationary phase with the mobile phase used for characterization. This was achieved by ensuring that after the column was characterized, flushed, or tested using a TFA containing mobile phase, it was flushed out before removal from the system with 95% ethanol injected though the high pressure injector. Thus the column was always stored in a solvent that did not contain TFA.

The changes in retention for these columns were measured and are shown in Table 3.5. First, it can be seen that the initial retention for these columns is greater than that seen for the
previous C4 bonded particle columns in Table 3.4. This may reflect the removal of TFA from the column after flushing, since not doing so may remove stationary phase before the column’s retention has been assessed. The other observation that was clear was that these more carefully used columns showed much less retention loss use-to-use as compared to the previous investigation. This suggests that the retention losses seen previously were due to the presence of the 90/10 v/v water/acetonitrile mobile phase with 0.1% TFA added during column storage. It has not been determined the exact cause of the stationary phase loss, whether it occurs in solution or as a result of the column bed drying out after being filled with mobile phase. The present methodology, however, has been found to be useful for improving the longevity of the C4 bonded phase. This phenomenon of retention loss was seen but to a much lesser extent with the C8 bonded particles, probably due to their greater chain length and resultant resistance to hydrolytic cleavage. Another difference, which was not investigated, was the observation that the C8 particles were used with an 80/20 v/v water/acetonitrile mobile phase while the C4 bonded particles were used with a 90/10 v/v water /acetonitrile mobile phase. Moving forward, columns created with either C4 or C8 bonded particles were used in such a way as to avoid storage in strongly acidic mobile phases.

3.3.8 Gradient Separation Characterization – Peptides

After assessing the utility of the columns to small molecule, isocratic separations, it was next needed to determine the utility of these columns to peptide separations. First, a loading study was carried out to determine impact of loading upon these particles. Either 20 or 60 fmol of alcohol dehydrogenase tryptic digest standard was injected on column. Example chromatograms from the loading study can be seen in Figure 3.27. It can be seen that the lower column loading produces noticeably narrower peaks. At 60 fmol loading, an average peak
capacity of 108 (±4) was observed, while at 20 fmol loading an average peak capacity of 149 (±2) was observed. Thus a 28% decrease in peak capacity is seen going to the higher loading level. For comparison, the Cortecs C18 column showed a 12% decrease in peak capacity (310 ± 0 to 272 ± 0) going from 20 to 60 fmol of tryptic digest of alcohol dehydrogenase on column. It is unsurprising that sample loading has a large impact upon the performance of the in-house C8 column, since this packing material had a relatively low specific surface area compared to the Cortecs material (12 m²/g vs 100 m²/g for the Cortecs material). Further reduction in sample size would probably further improve peak width for the column packed with in-house synthesized superficially porous particles. To ensure good signal-to-noise for peak characterization, a 20 fmol loading was used for the gradient length study.

When comparing the results for the in-house particles to the Cortecs particles, it was observed that there is a difference in selectivity, manifested as a difference in peak elution order. To understand this further, a plot was created comparing the elution time for the sixteen ions on the different columns. Since the gradients are very similar, phases showing the same retention mechanism should show a linear regression with a slope of 1. The results are shown in Figure 3.28. The times have not been adjusted in any way. The regression line with a slope near one appears to indicate similar retention characteristics, but it can be seen that there is a wide spread of points on either side of the line, indicating a difference in selectivity.

The next investigation was assessing the impact of gradient length on peak capacity. The results of the gradient length study are seen in Figure 3.29. It can be seen that the peak capacity increases modestly with an increase in separation time. A 42% increase in peak capacity was seen for a 210% increase in the separation window. Thus increasing the gradient length (decreasing the gradient steepness) is a way to improve peak capacity at the expense of
analysis time. This is a similar result to that seen with the Cortecs C18 particles using a 60 fmol loading of tryptic digest of alcohol dehydrogenase. For that separation, a 43% increase in peak capacity (220 ± 1 to 314 ± 4) was seen for a 260% increase in the separation window (27.4 ± 0.0 min to 98.1 ± 0.7 min). Overall the in-house C8 bonded superficially porous particles showed applicability to peptide separations, though improvements in detector sensitivity would be advantageous to allow for decreased particle loading.

3.3.9 Gradient Separation Characterization – Proteins

The next step in the characterization of these particles was their use in the separation of intact proteins. Initial separations of *E. coli* whole cell lysate were done using mobile phases with 0.2% formic acid added, but the separation did not look promising. This data is shown in Figure 3.30 panel A. Most of the protein came out in a wide lump for the 250 ng injection and very little was seen with a 100 ng injection. Therefore the mobile phase additive was changed to 0.3% formic acid and 0.025% trifluoroacetic acid to hopefully improve the chromatography. The results of this investigation are shown in Figure 3.30 panels B and C. One improvement over the previous mobile phase was that many peaks were seen with both the high and low loading conditions. The new mobile phase additive allowed much better separation to occur, as can be seen by the partially resolved peaks at both loading levels. To further investigate the separation, extracted masses from the autoME processing were plotted for the lower and higher loading levels for the separations. The selected masses were, in order of elution: 8324, 6855, 11202, 21132, 11672, and 12204 Da, covering a range of molecular weights and retention times. The traces can be seen in Figure 3.31. A variety of peak shapes are seen, most are appreciably tailed. The largest molecular weight analyte has the widest peak. Peak widths appear to improve slightly with the lower sample loading, but the effect was not substantial. From this initial
investigation, it was seen that these particles have some utility in the separation of intact proteins.

To assess this column’s performance with intact proteins further, a 10 ng/µL sample of ribonuclease A was injected onto the same column as used previously. For this separation, a 12.5 µL 0.5-60% acetonitrile gradient with 0.3% formic acid and 0.025% trifluoroacetic acid was employed. An example of the peak obtained is seen in Figure 3.32. The peak shows significant tailing. When assessing the summed mass spectrum from the peak, seen in Figure 3.33, it was noticed that at the very front of the ribonuclease A peak there is a minority component that appears to have a charge envelope very similar to that of ribonuclease A. To gain further insight into this peak, MaxEnt deconvolution was performed on both the summed spectra shown in Figure 3.33. The results of the MaxEnt processing are shown in Figure 3.34. Heterogeneity is apparent in the main peak, for which a series of peaks 16 Da in mass apart from one another exist near the expected intact protein mass. This suggests that the protein is partially degraded due to oxidation. From the figure it is seen that there is more oxidation early in the eluting ribonuclease A peak, suggesting that perhaps sample heterogeneity accounts for the broad peak seen. To test this, the different constituent mass peaks from the main peak cluster were plotted as a function of time, shown in Figure 3.35. It can be seen that the oxidized peaks elute at the beginning of the main peak, and tail off over the rest of the peak. The unoxidized peak profile can also be seen, appearing to be similar to the overall recorded peak profile from Figure 3.32. This indicates that the observed wide peak shape is not caused by sample heterogeneity.

Figure 3.34 also shows that early in the peak a minority component exists, with a deconvoluted mass of 13423.3 Da as compared with a deconvoluted mass of 13680.6 for the main mass peak found in the ribonuclease A chromatographic peak. This minority peak is
257.3 Da lower in mass than the unoxidized ribonuclease A peak. In this case, it is informative to consider the amino acid sequence of the protein[45]. Either the loss of the first two n-terminal residues (H$_2$N-Lys-Glu) or the last three c-terminal residues (-Ala-Ser-Val-COOH) from ribonuclease A would result in proteins with masses 257.2863 Da lower (using average masses for the residues[46]) than the intact protein, corresponding very closely to the mass difference seen in the deconvoluted masses. This indicates that the minority peak may be a degraded ribonuclease A. Further work would be required to determine if this was in fact the identity of the minority peak.

Most interestingly, however, when an extracted ion range (m/z 1918-1919, representing the minority analyte) is plotted, as shown in Figure 3.36 panel B, it is seen that this minority component exhibits a very sharp chromatographic peak. This peak is around 3 seconds wide at half height. The peak’s thin profile may be due to its low loading, since it is present at much lower levels than the main peak. This reinforces the assertion that loading probably plays a major role in the performance of these particles, and sensitive detection is critical to realize their full separation potential.

3.4 Conclusions

In conclusion, superficially porous particles with the desired morphology laid out at the beginning of Chapter 2 were synthesized. Many approaches were found to improve the number of single particles created by the synthesis. Through these improvements and careful size refinement of the final material, enough particles were obtained to allow for characterization and chromatographic analysis. The small molecule data obtained from columns packed with these particles suggests that the particles can be used to created well-packed capillary columns. The analysis with peptides and proteins shows substantial promise for the particles as well. Peak
shape was not always optimal, however, indicating that other improvements could be made. One way in which this could be done would be the use of the C4 bonded particles for biomolecule separations. The C4 bonding would perhaps have to be improved from a stability standpoint since the biological separations are carried out at elevated temperature in acidic mobile phases. This may be able to be accomplished through the use of a multifunctional bonding reagent, though such bonding raises its own issues. Improvements in particle bonding may lead to improvements for both the C4 and C8 functionalized material when used for biological separations in capillary columns. Another desirable improvement would be to use a more sensitive mass spectrometer for detection, which would cut down on the loading requirements for the column.
3.5 Tables

<table>
<thead>
<tr>
<th></th>
<th>20 mg/mL Particles</th>
<th>60 mg/mL Particles</th>
<th>200 mg/mL Particles</th>
</tr>
</thead>
<tbody>
<tr>
<td>2% PEG</td>
<td>1:1</td>
<td>3:1</td>
<td>10:1</td>
</tr>
<tr>
<td>5% PEG</td>
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<td>10% PEG</td>
<td>1:5</td>
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</tr>
</tbody>
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Table 3.1: Reaction matrix for the second PEG-assisted lyophilization. The concentrations listed are the initial solutions, before lyophilization they are mixed in a 1:1 v/v ratio. Particle concentration based upon starting core mass. The ratios reflect the ratio of particle mass to PEG mass.
<table>
<thead>
<tr>
<th>Observed m/z</th>
<th>Molecular Weight (MH+) (Da)</th>
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<tr>
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<td>549.30</td>
</tr>
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<td>675.36</td>
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<td>598.33</td>
<td>598.33</td>
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<tr>
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</tr>
<tr>
<td>724.41</td>
<td>1447.80</td>
</tr>
<tr>
<td>950.48</td>
<td>950.48</td>
</tr>
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Table 3.2: The 16 ions used for peak capacity calculations in this study.
<table>
<thead>
<tr>
<th></th>
<th>20 mg/mL Particles</th>
<th>60 mg/mL Particles</th>
<th>200 mg/mL Particles</th>
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<tbody>
<tr>
<td><strong>2% PEG</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1:1</td>
<td>3:1</td>
<td>10:1</td>
</tr>
<tr>
<td></td>
<td>Good</td>
<td>Clumps</td>
<td>Clumps</td>
</tr>
<tr>
<td><strong>5% PEG</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2:5</td>
<td>6:5</td>
<td>4:1</td>
</tr>
<tr>
<td></td>
<td>Slight Melt</td>
<td>Good</td>
<td>Clumps</td>
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<tr>
<td><strong>10% PEG</strong></td>
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<td></td>
<td></td>
</tr>
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<td></td>
<td>1:5</td>
<td>3:5</td>
<td>2:1</td>
</tr>
<tr>
<td></td>
<td>Melt</td>
<td>Melt</td>
<td>Clumps</td>
</tr>
</tbody>
</table>

Table 3.3: Observations overlaid upon the reaction matrix from the previous figure. As in the previous table, the listed ratios reflect the ratio of particle mass to PEG mass. Note that around a 1:1 ratio of PEG to particles appears to be the best for PEG assisted lyophilization.
<table>
<thead>
<tr>
<th></th>
<th>Use Number</th>
<th>( k' ) for 4-methyl Catechol</th>
<th>Days Since 1(^{st}) Use</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Column C4-0</strong></td>
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<td>0.96</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.65</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.54</td>
<td>88</td>
</tr>
<tr>
<td><strong>Column C4-1</strong></td>
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<td>0.97</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.56</td>
<td>10</td>
</tr>
<tr>
<td><strong>Column C4-2</strong></td>
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<td>0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.39</td>
<td>18</td>
</tr>
</tbody>
</table>

Table 3.4: Retention losses seen for C4 bonded columns when used successive times with 90/10 v/v water/acetonitrile with 0.1% TFA added. Retention factors were recorded for 4-methyl catechol at ambient temperature.
<table>
<thead>
<tr>
<th></th>
<th>Use Number</th>
<th>k’ for 4-methyl Catechol</th>
<th>Days Since 1&lt;sup&gt;st&lt;/sup&gt; Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column C4-3</td>
<td>1</td>
<td>1.06</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.03</td>
<td>4</td>
</tr>
<tr>
<td>Column C4-4</td>
<td>1</td>
<td>1.05</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.03</td>
<td>8</td>
</tr>
</tbody>
</table>

Table 3.5: Retention changes for columns packed with C4 bonded 1.06 µm superficially porous particles. These columns had been created and used so as to minimize interaction with acidic mobile phases. Retention factors are given for 4-methyl catechol at ambient temperature.
3.6 Figures

Figure 3.1: In-solution images of hydroxylated, densified core particles in solution (A), after 15 minute reaction with HMW PDDA (B), after washing 4 times (C), after postwashing resuspension for 15 minutes with sonication in the Cole-Parmer 8891 sonicator (D). The glare in the pictures is due to the fact that the images are taken of a television screen using a cellphone camera.
Figure 3.2: Illustration of proposed core adhesion route during the polyelectrolyte coating step. The large black circles with negative signs indicate negatively charged core particles, the red plus signs stand for polyelectrolyte, and the small black circles with negative signs represent the negatively charged colloidal silica. Two partially coated silica cores in solution (A) while polyelectrolyte coating is occurring electrostatically interact and stick together (B). The resultant doublet is then more fully coated with polyelectrolyte as the coating process progresses (C). This polyelectrolyte bound and covered doublet then is coated with colloidal silica (D) during the colloidal silica coating step, yielding a doublet superficially porous particle.
Figure 3.3: In-solution images of particles just before lyophilization (A) and the same batch of particles resuspended in water after lyophilization (B). It can be seen that there are many more adhered particles after lyophilization.
Figure 3.4: Schematic diagram of new high pressure injector. The injector is not drawn to scale.
Figure 3.5: Example gradient profile for the peptide separations, in this case a 16.5 µL volume 0.5 to 40% acetonitrile gradient. Note that the gradient is loaded from right to left, for sample elution from the column the gradient is played back from left to right.
Figure 3.6: Example gradient profile for the protein separation a 25.0 µL volume 0.5 to 60% acetonitrile gradient. Note that the gradient is loaded from right to left, for sample elution from the column the gradient is played back from left to right.
Figure 3.7: Impact of colloidal silica to core solution volume ratio. Shown are the results for the 2:1 (A), 1:1 (B), 2:3 (C), 1:2 (D), 1:3 (E), and 1:5 (F) colloidal silica to core volume ratios.
Figure 3.8: Dispersal of hydroxylated silica cores in water using the Elma P30H bath sonicator. (A) shows the particles after 30 second of sonication, (B) shows the particles after 30 minutes of sonication, and (C) shows the particles after 90 minutes of sonication. The particles appear to be largely single after 90 min of sonication.
Figure 3.9: Multiplicity distributions illustrating the use of sonication during the polyelectrolyte coating step. ‘M’ stands for more than ten adhered particles. (A) shows the multiplicity distribution of the hydroxylated cores before coating with HMW PDDA (n=144), (B) is 7 minutes after reaction with PDDA with sonication (n=231), (C) shows the cores after 15 minutes of coating with PDDA with sonication (n=353), and (D) shows the core multiplicity distribution after washing and resuspension (n=413). The resuspended cores appear to be almost as well dispersed as the original cores.
Figure 3.10: Images taken via SEM of the particles lyophilized with the use of PEG, after the particles were sintered. (A) used 0.5% 3400 MW PEG solution, (B) used 5% 3400 MW PEG solution, (C) used 0.5% 8000 MW PEG solution, and (D) used 5% 8000 MW PEG solution. Many more large chunks can be seen for the 0.5% samples than for the 5% sample.
Figure 3.11: In-solution images of the PEG-lyophilized particles after sintering. (A) used 0.5% 3400 MW PEG solution, (B) used 5% 3400 MW PEG solution, (C) used 0.5% 8000 MW PEG solution, and (D) used 5% 8000 MW PEG solution. More large chunks can be seen with the 0.5% PEG solutions than with the 5% PEG solutions.
Figure 3.12: SEM images of particles after sintering showing various morphologies. The particles shown in (A) were lyophilized in a combination of 10% PEG and 60 mg/mL particles, showing extensive melting. The particles shown in (B) were a lyophilized in combination of 2% PEG and 60 mg/mL particles, showing significant clumping. The particles in (C) were lyophilized in a combination of 5% PEG and 60 mg/mL particles, showing good surface morphology and not as many particle clumps as some other samples.
Figure 3.13: Particle multiplicity distributions after sintering for selected lyophilization schemes obtained from in-solution imaging. ‘M’ stands for more than ten adhered particles. (A) was a mix of originally 20 mg/mL particle and 2% PEG solutions (n=982), (B) was a mix of originally 60 mg/mL particles and 5% PEG (n=1282), (C) was a mix of 60 mg/mL particles and 2% PEG (n=841), and (D) was a mix of 200 mg/mL particles and 2% PEG after rehydroxylation to try to free more particles (n=248). It can be seen that (A) and (B) show a much larger proportion of single particles than (C) and (D), indicating that (A) and (B) appear to be better lyophilization conditions.
Figure 3.14: Particle multiplicity distributions to compare particle lyophilization techniques after particle sintering. ‘M’ stands for more than ten adhered particles. (A) shows particles lyophilized without the use of PEG, simply flash frozen at 10 mg/mL (n=261), and (B) shows particles lyophilized using the preferred PEG method (n=701).
Figure 3.15: Particle size distribution of bonded and endcapped superficially porous particles. They were found to have a number average diameter of 1.09 µm and a relative standard deviation of 4.8% (n=155).
Figure 3.16: Same size distribution as shown in Figure 3.15 but with the observed particle damage notated (n=155). The percent of the total population represented by each damage class is notated in parentheses. Note that the undamaged and slightly damaged particles show a relatively tight distribution while the badly damaged and bare particles contribute exclusively to the observed low diameter population.
Figure 3.17: SEM images showing the comparison of multiplicity refined particles before (A) and after (B) rehydroxylation while stirring. Notice the increase in particle surface damage after rehydroxylation.
Figure 3.18: SEM images of the rehydroxylation study. (A) shows the particles before rehydroxylation, (B) shows particles after 20 hour stirred reflux in 1 M hydrochloric acid, (C) shows particles after 4 hour stirred reflux in 1 M hydrochloric acid, (D) shows particles after 20 hour stirred reflux in 0.1 M hydrochloric acid, and (E) shows particles after 20 hour unstirred reflux in 1 M hydrochloric acid.
Figure 3.19: Initial characterization of the final batch of superficially porous particles created. ‘M’ stands for more than ten adhered particles. (A) shows the particle multiplicity distribution from in-solution imaging (n=716). (B) shows an SEM image of the particles, indicating that the good coverage of the particles by the colloidal silica.
Figure 3.20: Size distribution of the final batch of superficially porous particles (n=144).
Figure 3.21: Same particle size distribution as seen in Figure 3.20 but with the damage state of the particles noted (n=144).
Figure 3.22: Pore size distribution for the final batch of superficially porous particles. Note the peak in incremental pore volume around an average pore size of 480 Å.
Figure 3.23: Example isocratic chromatogram from the characterization of a 16.6 cm X 75 µm ID column packed with 1.06 µm C8 bonded superficially porous particles. This separation was performed at ambient temperature at 16,700 psi in 80/20 v/v water/acetonitrile with 0.1% TFA added. The peaks are, in order of elution, ascorbic acid, hydroquinone, resorcinol, catechol, and 4-methyl catechol. Plate counts for this separation are 93,000 for hydroquinone, 84,000 for resorcinol, 80,000 for catechol, and 73,000 for 4-methyl catechol.
Figure 3.24: h-v plot for the characterization of four 75 µm ID capillary columns packed with C8 bonded superficially porous particles. All were characterized at ambient temperature in an 80/20 v/v water/acetonitrile mobile phase with 0.1% TFA added. The columns were, in name order, 19.6, 16.6, 17.7, and 18.0 cm long respectively. Efficiencies are shown for hydroquinone, k’ ~0.1. The parameters shown are the fits of the experimental data to the reduced van Deemter equation.
Figure 3.25: Example chromatogram from the characterization of a 19.2 cm X 75 µm capillary column packed with 1.06 µm C4 bonded superficially porous particles. This separation was performed at ambient temperature at 17,800 psi in 90/10 v/v water/acetonitrile with 0.1% TFA added. The peaks are, in order of elution, ascorbic acid, hydroquinone, resorcinol, catechol, and 4-methyl catechol. Plate counts for this separation are 72,000 for hydroquinone, 63,000 for resorcinol, 65,000 for catechol, and 59,000 for 4-methyl catechol.
Figure 3.26: h-ν plot for the characterization of 3 capillary columns of 75 µm ID packed with 1.06 µm diameter C4 bonded superficially porous particles. The columns were characterized at ambient temperature in 90/10 v/v water/acetonitrile with 0.1% TFA added. The columns were, in name order, 18.7, 19.2, and 17.1 cm long, respectively. Efficiencies are shown for hydroquinone, k’~0.15. The parameters shown for each of the columns are the result of fitting the experimental data to the reduced van Deemter equation.
Figure 3.27: Loading comparison using the 1.06 μm C8 bonded superficially porous particles. The separation was accomplished at 40 °C with a 16.5 μL 0.5-40% acetonitrile gradient. The sample was a tryptic digest of alcohol dehydrogenase loaded at either 60 fmol (A) or 20 fmol (B) on column. The notated ions are the peaks used to mark the beginning and the end of the elution window for peak capacity calculation.
Figure 3.28: Retention time comparison for the separation of a tryptic digest of alcohol dehydrogenase on a column packed with C18 bonded Cortecs particles (60 fmol, 33.0 µL gradient) and a column packed with in-house synthesized C8 bonded particles (20 fmol, 32.5 µL gradient). The equation of the linear regression is shown above. Error bars are ± 1 standard deviation.
Figure 3.29: Peak capacity as a function of gradient length on in-house synthesized superficially porous particles. All separations were carried out at 40 °C with a 20 fmol sample of alcohol dehydrogenase tryptic digest standard. Error bars are ± 1 standard deviation.
Figure 3.30: Separation of *E. coli* whole cell lysate on a 19.1 cm X 75 µm column packed with 1.06 µm C8 bonded superficially porous particles. All separations performed at 40 °C with a 25 µL 0.5-60% acetonitrile gradient. (A) has 0.2% formic acid in the mobile phase with 250 ng injected onto the column, (B) has 0.3% formic acid and 0.025% trifluoroacetic acid in the mobile phase with 250 ng injected onto the column, and (C) has 0.3% formic acid and 0.025% trifluoroacetic acid in the mobile phase with 100 ng injected onto the column.
Figure 3.31: Select deconvoluted masses from the separations of *E coli* on the 19.1 cm x 75 µm column packed with 1.06 µm C8 bonded superficially porous particles. Separation was performed using a 25 µL gradient from 0.5-60% acetonitrile with 0.3% formic acid and 0.025% trifluoroacetic acid in the mobile phase. These masses cover a range of molecular weights and retention times. The top trace (A) is the 250 ng injection on column, while the bottom trace (B) is the 100 ng injection on column. The leading edge of the peaks is very steep, so that the protein trace often doesn’t start until appreciably far up the peak. The deconvoluted masses of the peaks are written above the respective peaks, retention order is the same on both columns.
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Figure 3.33: Summed mass spectra from the ribonuclease a peak. (A) is summed over the entire peak, and (B) is summed over just the leading edge of the peak. The minority component peak is clearly present in the spectrum summed over just the leading edge of the peak.
Figure 3.34: MaxEnt deconvoluted masses of the spectra seen in the previous figure. (A) is deconvoluted from the entire peak, while (B) only represents the leading edge of the peak.
Figure 3.35: Reconstructed chromatogram for the different masses from Figure 3.34 from the ribonuclease A peaks. The plotted data was taken from the autoME output. The black trace is 13682 Da, red trace is 13698 Da, green trace is 13714 Da, and blue trace is 13730 Da.
Figure 3.36: Comparison between the two peaks. (A) is the total ion current from the ribonuclease A peak, while (B) is the m/z 1918-1919 extracted ions, showing the narrow early eluting peak.
3.7 References


CHAPTER 4: HIGHLY EFFICIENT CAPILLARY COLUMNS PACKED WITH COMMERCIAL PROTOTYPE SUPERFICially POROUS PARTICLES

4.1 Introduction

Aside from the superficially porous particles produced in-house, another project that was pursued was packing capillary columns with prototype reversed-phase bonded superficially porous silica particles from Waters Corporation.

4.1.1 Packing Capillary Columns

Packing capillary columns has been already mentioned in Chapters 2 and 3, but for the current chapter a more thorough introduction is warranted. Liquid chromatography column packing is only partially understood, with so many different variables at work that it can be very difficult to understand in a systematic manner. Bed structure has a strong impact on column performance, as has been seen with packed capillary bed imaging[1-3]. The radial column morphology heterogeneity can influence the performance of packed beds at both the millimeter-bore scale and at the capillary scale[2, 4, 5]. In packed beds, the region near the column wall often has a different packing structure than the region towards the center of the column, leading to a radial velocity gradient across the column. This can lead to a velocity-dependent trans-column eddy dispersion term for all but the highest aspect ratio capillary columns[6]. It has also been found that the particle morphology can impact the morphology of the bed, with superficially porous particles being found to have different and often inferior radial heterogeneity profiles as compared with fully porous particles in capillary columns near the column wall[1]. This observation is in line with published work at the millimeter-bore scale showing that
superficially porous particles often show improved performance in larger internal diameter (ID) columns (4.6mm) as compared to smaller ID columns (2.1mm)[7].

In column packing, there have been many approaches to improving the bed structure of capillary columns. These often have to do with the influence of slurry solvent, which can have an effect upon the degree of particle aggregation in solution before packing[8-10]. In some reports aggregating solvents have been found to produce the best capillary columns. Another approach to improving the performance of capillary columns has been increasing the slurry concentration, for both fully porous and superficially porous particles[3, 9, 11]. For fully porous particles, on-column imaging has shown that a column packed at a higher slurry concentration had more homogenous bed structure than a column packed at a lower slurry concentration[3]. Such on-column imaging of slurry concentration effects has also been extended to the packing of superficially porous particles into capillary columns. A capillary column packed with superficially porous particles at higher slurry concentration showed a less pronounced wall region and thus reduced radial heterogeneity than a column packed at a lower slurry concentration[3]. With all of this in mind, the most straightforward approach to creating capillary columns with a new particle type is still often empirical experimentation.

4.1.2 Packing Capillary Columns with Superficially Porous Particles

As recently reported in a review by Hayes et al [12], there have been few studies published on capillary columns packed with superficially porous particles. This is not surprising as there have been several reports of these particles being difficult to pack into analytical bore columns[7, 13]. One report by Fanali and co-workers compares the application of the columns in capillary electrochromatography (CEC) and capillary liquid chromatography [14]. Studies by Zhang et al and Roth et al reported gradient separations of intact protein mixtures using 5 μm
superficially porous particles in capillary columns [15, 16]. In another report by Fanali, Rocchi, and Chankvetadze, 2.6 μm particles packed into capillaries achieved minimum reduced plate heights of 2.5, 2.8, and 4.0 in a 25, 50, and 75 μm ID capillaries respectively [17]. Other reports by Bruns et al have shown a best $h_{\text{min}}$ value of 2.2 for 2.5 μm particles packed into 100 μm ID capillaries and an $h_{\text{min}}$ of 2.4 for a 50 μm ID column packed with 1.9 μm particles [1, 3]. As stated in a 2012 paper by Bruns et al, “Thus it seems unsurprising that we failed to pack a highly efficient column with core-shell particles up to this point; also, we are unaware of any work that claims this achievement” [1]. Recently published work from Blue and Jorgenson demonstrated a reduced minimum plate height around 2.4 for 1.1 μm in-house superficially porous particles and below 1.9 for 2.7 μm commercial superficially porous particles, both packed into 30 μm ID capillaries [9].

The following work presents the creation of highly efficient capillary columns packed with superficially porous particles. The motivation for this work is to extend the efficiency advantages seen with superficially porous particles packed into 4.6 mm-bore columns to columns of capillary dimension. To determine the best packing conditions for the columns, differing slurry concentrations and capillary internal diameters were investigated. Previous work has shown that changing slurry concentration [3, 9] and capillary column internal diameter [2, 17-19] can have an impact on column performance.

4.1.3 Packing Progress Plots and Modeling

When trying to understand the behavior of capillary columns packing at constant pressure, it would be useful to understand how to theoretically model this process, specifically looking at expected column length as a function of packing time. Column length as a function of time is the most readily measured attribute of a packing capillary column. The most
A straightforward system to model is a capillary column starting at zero length and packing under constant pressure, assuming a constant slurry concentration over the packing time. The flow of slurry to the bed is regulated by the flow rate of slurry liquid through the growing column, decreasing over time as the packed bed elongates. The goal of this derivation is to determine a relation between column length \( L \) as a function of packing time \( t \). The derivation begins with the expression for the interparticle linear velocity of a fluid through a packed bed known as the Kozeny-Carman Equation, Equation 4.1\[20\]:

\[
v_i = \frac{\Delta P \cdot d_p^2 \cdot \varepsilon_i^2}{180 \cdot \eta \cdot L \cdot (1 - \varepsilon_i)^2}
\]

where \( v_i \) is the interstitial velocity, \( \Delta P \) is the pressure drop across the column, \( d_p \) is the particle diameter, \( \varepsilon_i \) is the interparticle porosity, and \( \eta \) is the viscosity of the fluid. Using Equation 4.1, the volumetric flowrate for the slurry towards the packed bed, \( F_s \), is given by Equation 4.2, assuming that flow resistance from the packed bed dominates total flow resistance:

\[
F_s = v_i \cdot \pi \cdot r_0^2 \cdot \varepsilon_i
\]

where \( r_0 \) is the inner diameter of the column being packed. It would also be useful to determine the volumetric flowrate of packing material toward the column. Using the volumetric flowrate of the slurry, a ‘particle volumetric flowrate,’ \( F_p \), can be defined by Equation 4.3

\[
F_p = \frac{F_s \cdot m_{p,s}}{\rho_p \cdot V_{slurry}}
\]

where \( m_{p,s} \) is the mass of particles in the slurry added to the packing reservoir, \( \rho_p \) is the overall density of the particle (including pores), and \( V_{slurry} \) is the volume of the slurry reservoir. Using this ‘particle volumetric flowrate,’ the packing linear velocity, \( v_p \), or instantaneous rate of bed elongation can be defined by Equation 4.4.
\[ v_p = \frac{F_p}{\pi \cdot r_i^2 \cdot (1 - \varepsilon_i)} \]  
Equation 4.4

Substituting Equation 4.1 into Equation 4.2, then Equation 4.2 into Equation 4.3, then Equation 4.3 into Equation 4.4, then simplifying, the following expression (Equation 4.5) is obtained for packing linear velocity.

\[ v_p = \frac{\Delta P \cdot d_p^2 \cdot \varepsilon_i^3 \cdot m_{p,s}}{180 \cdot \eta \cdot L \cdot \rho_p \cdot (1 - \varepsilon_i)^3 \cdot V_{slurry}} \]  
Equation 4.5

Realizing that packing linear velocity is defined as the instantaneous time rate of change in bed length, Equation 4.6 can be written, which is a first order differential equation:

\[ \frac{dL}{dt} = \frac{\Delta P \cdot d_p^2 \cdot \varepsilon_i^3 \cdot m_{p,s}}{180 \cdot \eta \cdot L \cdot \rho_p \cdot (1 - \varepsilon_i)^3 \cdot V_{slurry}} \]  
Equation 4.6

By separating variables and integrating both sides, the following relation is found, Equation 4.7:

\[ \frac{L^2}{2} = \frac{\Delta P \cdot d_p^2 \cdot \varepsilon_i^3 \cdot m_{p,s}}{180 \cdot \eta \cdot \rho_p \cdot (1 - \varepsilon_i)^3 \cdot V_{slurry}} \cdot t + c \]  
Equation 4.7

where \( c \) is a constant of integration. Applying the boundary condition that \( L=0 \) when \( t=0 \), the constant of integration must also be zero. Taking this into account and simplifying the equation leads to the final result, Equation 4.8, which should predict column length \( L \) at any time \( t \) after reaching packing pressure:

\[ L = \sqrt{\frac{\Delta P \cdot d_p^2 \cdot \varepsilon_i^3 \cdot m_{p,s}}{90 \cdot \eta \cdot \rho_p \cdot (1 - \varepsilon_i)^3 \cdot V_{slurry}}} \cdot \sqrt{t} \]  
Equation 4.8

The result suggests that column length should have a square root dependence on packing time.

This relation can be used to empirically fit experimental data using Equation 4.9:

\[ L = \alpha \cdot \sqrt{t} + \beta \]  
Equation 4.9
The physical meaning of the β term from Equation 4.9 is the column length packed before reaching maximum pressure. This deviation from the theoretical boundary condition stated above is due to the experimental necessity of packing a certain amount of column bed before reaching final pressure to ensure that the outlet frit holds. The β fits reported later in this chapter are reported by empirical fits to the experimental data. The α term from Equation 4.9 is related to the speed of packing by Equation 4.10, though in practice it is obtained through an empirical fit to the data.

\[ \alpha = \sqrt[3]{\frac{\Delta P \cdot d^2 \cdot \epsilon_i \cdot m_{p,s}}{90 \cdot \eta \cdot \rho_p \cdot (1 - \epsilon_i)^3 \cdot V_{slurry}}} \]  

Equation 4.10

Though it is difficult to use this relation to predict exact values due to the large number of variables involved, it is useful to see the expected interplay of the differing variables at work during column packing.

To show the applicability of Equation 4.9 to model column packing, Figure 4.1 shows experimental data for a 50 µm ID capillary being packed at around 30,000 psi using a 50 mg/mL slurry of prototype superficially porous particles. The experimental data is fit to Equation 4.9 with the fit parameters reported in the figure. It is clear to see that the fit line overlays the experimental data points and was therefore judged to be representative of the data. To show the applicability of Equation 4.9 to modeling column packing another way, Figure 4.2 shows the same data as shown in Figure 4.1 but with the time axis being expressed as the square root of time and fit with a simple linear regression. The good agreement between the data and the linear fit in Figure 4.2 indicates that Equation 4.9 is a good model for a packing capillary column.
4.2 Materials and Methods

4.2.1 Materials Employed

For this study, prototype superficially porous particles bonded with C18 were provided by Waters Corporation (Milford, MA). These particles had a Coulter average (50%, volume) diameter of 1.45 μm, specific surface area of 88 m²/g (unbonded), 84 Å pores (unbonded), and a core diameter to particle diameter ratio of 0.76 (data courtesy Waters Corporation). The particles were used as provided. In-house particle size characterization was done using a Hitachi S-4700 cold cathode field emission scanning electron microscope (SEM) (Tokyo, Japan) as described in Chapter 2. This yielded a number average particle diameter of 1.54 μm (4.2% RSD, n=108), which was used for calculating reduced parameters. A representative SEM image of these particles is shown in Figure 4.3.

Acetone (HPLC Grade, submicron filtered), acetonitrile (HPLC Grade), and L-ascorbic acid were purchased from Fisher Scientific (Fair Lawn, NJ). Trifluoroacetic acid (TFA), hydroquinone, resorcinol, catechol, 4-methyl catechol, and formamide were purchased from Sigma-Aldrich (St. Louis, MO). A Barnstead NANOpure system from Thermo Scientific (Waltham, MA) was used to purify water. 360 μm outer diameter (OD) fused silica capillary was purchased from Polymicro Technologies (Phoenix, AZ) with nominal internal diameters of 30, 50, and 75 μm. Potassium silicate (Kasil) was purchased from PQ Corporation (Valley Forge, PA). Glass fiber filters were purchased from Whatman (Pittsburgh, PA).

4.2.2 Capillary Column Preparation and Characterization

For this study, capillary columns were prepared with internal diameters (ID) of 30, 50, and 75 μm as introduced in Chapters 2 and 3, generally following the previously described methodology [3]. These processes are described here for clarity since this chapter has an
emphasis on packing methodology. For the 30 μm ID columns, the outlet frit was created by pushing a 50-100 μm plug of 2.5 μm nonporous silica particles (Bangs Laboratories, Fishers, IN) 100-200 μm into the column and sintering them in place using an electric arcing device [21] to allow for negligible postcolumn volume for electrochemical detection with a 100-200 μm long carbon fiber microelectrode [19]. For the 50 and 75 μm ID columns, the column outlet was fritted by pushing the outlet repeatedly against a glass microfiber filter wetted with 1:1 v/v potassium silicate/formamide and then heating the column at 50 °C overnight [22]. The detection microelectrode is pushed up to the outlet frits on the 50 and 75 μm ID capillaries to ensure negligible post column band broadening.

Column packing was similar to the high pressure slurry packing of capillary columns that has been described previously [3, 18, 23] and follows the general methodology laid out in Chapter 3. Briefly, particles were slurried in 0.2 μm filtered acetone and were dispersed using an Elma P30H sonicator (Elma Schmidbauer, GmbH, Singen, Germany) at 80 kHz frequency and 100% power in pulse mode for 5-8 min. The suspended slurry was added to the high pressure packing reservoir (~0.5 mL) and a fritted empty capillary was attached to the reservoir using a high pressure fitting. The slurry was stirred continuously in the packing reservoir using a small stirbar and a magnetic stirplate (Fisher Scientific, Fair Lawn, NJ). The bed was formed by applying pressure from a DSHF-300 pneumatic amplifier pump (Haskel, Burbank, CA) with the pushing solvent being acetone. Packing was initiated at low pressure then quickly ramped to 30,000 psi. Pump liquid pressure was calculated using the applied air pressure multiplied by the pump’s amplification ratio. Column packing progress was followed by use of back illumination with a white LED light to illuminate the bed as it formed. After attaining the desired length, the pressure was removed and the column was vented to ambient pressure.
One difference between the columns prepared in this chapter and those prepared in previous chapters was the use of sequential column packing in this chapter, which is described here. For this packing methodology, one column was packed as stated above. After that column (the first column) had finished packing, that column was then removed and within a few minutes a new unpacked, fritted capillary was attached to the reservoir. The slurry was not manipulated in any way and was stirred continuously throughout this process. Packing was then performed with this and subsequent capillaries in the same manner. This method was first employed as a convenience in lab, as a way to generate several capillary columns rather quickly. From a practical standpoint, this methodology also helps to conserve particles since multiple columns are created from the same slurry.

After packing, the columns were flushed using the same high pressure setup described in Chapter 3. The columns were usually flushed with 60/40 v/v water/acetonitrile with 0.1% trifluoroacetic acid added. For column flushing the pressure was gradually increased to just over 40,000 psi and held for 1 hour. The column was then allowed to slowly depressurize for 1 hour, after which time pressure was increased back to 10,000 psi and a temporary frit was created using a heated wire stripper (Teledyne Interconnect Devices, San Diego, CA). After fully depressurizing, the column was removed. The temporary heat frit was then cut off and a glass fiber/potassium silicate/formamide frit was applied, being set in place using an electric arcing device [21].

Isocratic chromatographic characterization was performed as described in Chapter 3. All columns were characterized in 60/40 water/acetonitrile with 0.1% trifluoroacetic acid added. Most columns in this study were between 26 and 33 cm long when characterized. For this study, the columns were not necessarily evaluated in the order in which they were packed.
4.3 Results and Discussion

4.3.1 Sequential Column Packing

Sequential column packing was first attempted using 30 µm ID capillaries and a 30 mg/mL particle slurry. Looking at the packing progress plots for these columns in Figure 4.4, it can be seen that the packing traces for the four columns overlay each other very well. This was not surprising since the same packing parameters were being repeated, only with the slurry aging. For a full table of packing and characterization data, see Table 4.1. When the study at 30 mg/mL in a 30 µm ID capillary was repeated, the first column stopped packing early and could not be restarted, but the next three columns appeared to pack in a manner similar to the first set and can be seen in Figure 4.5. For the 50 µm ID columns packed using a 30 mg/mL slurry, it was observed that packing rate decreased with each subsequent column, shown in Figure 4.6. This is not surprising as more of the particles are required to fill a 50 µm ID column than a smaller 30 µm ID column. Thus slurry dilution was the suspected cause of packing slowdown with each subsequent column. When the slurry concentration was increased to an initial concentration of 50 mg/mL, the 50 µm columns predictably packed faster and appeared to have less decrease in packing speed with each subsequent column than was seen at 30 mg/mL. These results are shown in Figure 4.7. To assess the impact of moving to an even larger capillary ID, 75 µm capillaries were packed. For 75 µm ID columns packed using a 30 mg/mL slurry, there is a pronounced decrease in packing velocity with each subsequent column, likely due to slurry dilution as seen for the 50 µm ID capillaries. The packing progress plot for this set of packed columns is shown in Figure 4.8. Throughout the packing portion of this work, it was found that it was relatively easy to pack many columns in a short amount of time, which is a benefit in and of
itself as compared to only packing one column per slurry. As most of the columns packed in around 25 to 50 minutes, a batch of 4 columns could be packed in an afternoon.

To try to understand the slowdown in column packing with subsequent columns in a packing set, it was useful to think about the mass of particles required to pack a column. The mass of particles \( m_{p,c,r} \) required to pack a column of length \( L \) and internal radius \( r_0 \) is shown in Equation 4.11.

\[
m_{p,c,r} = \pi \cdot r_0^2 \cdot L \cdot (1 - \varepsilon_i) \cdot \rho_p
\]

Equation 4.11

Assuming a packed bed length of 35 cm, interparticle porosity of 0.4, particle density (including pores) of 1.55 g/mL, and considering capillaries with internal diameters of 30, 50 and 75 μm, the expected masses of particles required to pack the columns is given in Table 4.2. From Equation 4.10 it can be seen that the packing parameter \( \alpha \) should vary linearly with the square root of the mass of the particles in the slurry \( m_{p,s} \), expressed as Equation 4.12:

\[
\alpha \propto \sqrt{m_{p,s}}
\]

Equation 4.12

Though as stated earlier it is difficult to calculate an accurate value for \( \alpha \) just using Equation 4.10, if it is assumed that all parameters remain constant except the slurry concentration, the relation set forth in Equation 4.13 should be valid for columns 1 and 2 packed with respective particle masses in the same volume packing vessel:

\[
\frac{\alpha_1}{\alpha_2} = \sqrt{\frac{m_{p,s,1}}{m_{p,s,2}}}
\]

Equation 4.13

In the sequential column packing investigation, the slurry concentration should be the only variable that is changing between columns. From the data shown in Table 4.1, experimental \( \alpha \) values for all of the columns in this study are known. Using the assumption that 500 μL of slurry is initially placed in the packing reservoir, Equation 4.14 can be used to predict the mass
of particles in the packing reservoir at the beginning of packing the jth column (starting at 1),
\( m_{p,s,j,\text{calc}} \), based on the initial mass of particles in the slurry, \( m_{p,s,0} \) and the expected mass of
particles required to pack a capillary of certain internal radius, \( m_{p,c,r} \), from Table 4.2.
\[
m_{p,s,j,\text{calc}} = m_{p,s,0} - (j - 1) \cdot m_{p,c,r}
\]
Equation 4.14
To test the relation shown in Equation 4.13, the experimentally observed proportional change in
\( \alpha \) between columns was compared to the expected proportional change in \( \alpha \). The experimentally
observed proportional change in \( \alpha \) from earlier packed column \( j_1 \) to later packed column \( j_2 \),
\( \Delta \alpha_{\text{prop},j_1,j_2,\text{obs}} \) was determined from Equation 4.15.
\[
\Delta \alpha_{\text{prop},j_1,j_2,\text{obs}} = \frac{\alpha_{j_2}}{\alpha_{j_1}}
\]
Equation 4.15
The expected proportional change in \( \alpha \) from earlier packed column \( n_1 \) to later packed column \( n_2 \)
as determined from the calculated particle mass from Equation 4.14 in the reservoir when
beginning column packing, \( \Delta \alpha_{\text{prop},n_1,n_2,\text{calc}} \), was determined from Equation 4.16 below, a rewriting
of Equation 4.13
\[
\Delta \alpha_{\text{prop},j_1,j_2,\text{calc}} = \sqrt{\frac{m_{p,s,j_2,\text{calc}}}{m_{p,s,j_1,\text{calc}}}}
\]
Equation 4.16
To allow a fair comparison between the different column packing sets, the proportional
difference in \( \alpha \) was compared between the second packed (\( j_1=2 \)) and the fourth packed (\( j_2=4 \))
columns in each case. This allowed for some data averaging while not biasing against the
second batch of 30 µm ID capillaries packed at 30 mg/mL, where the first column stopped
packing before packing characterization could be obtained. The results of this comparison are
shown in Table 4.3. From this comparison, it was seen that the expected and observed
proportional changes in packing rate agreed reasonably well. The \( \alpha \) values remained almost
unchanged for the 30 µm diameter columns, as expected since so little material is packed per column (one of the experimental series actually showed a slight increase in α). For the larger bore columns, it was seen that there was a greater slowdown in packing rate than was expected due solely to the preceding slurry dilution through column packing mechanism. This was probably due to other factors working to dilute the slurry during the packing and column replacement processes besides particle loss into the column.

4.3.2 Chromatographic Characterization of Sequentially Packed Columns

This work was motivated by the impressive efficiency results seen for the first sequential packing of 30 µm ID capillaries with a 30 mg/mL slurry. As can be seen in Figure 4.9, the most efficient column (Column 3) from this set had an $h_{\text{min}}$ of 1.44. Significant variability was seen column-to-column however, with the least efficient column (Column 2) having an $h_{\text{min}}$ of 2.53. The promising results informed a repetition of the packing sequence. The overlaid efficiency of the second set of 30 µm ID columns packed with 30 mg/mL slurry can be seen in Figure 4.10. For this set, the most efficient column (Column 3) had an $h_{\text{min}}$ of 1.24 for the first retained analyte, hydroquinone, which for the 31.7 cm long column comes to around 166,000 plates or over 520,000 plates/m. An example chromatogram from the most efficient run can be seen in Figure 4.11, operated at 16,000 psi at ambient temperature. The second most efficient column in this packing series (Column 4), was similar to the most efficient column in the first set of sequentially packed columns, with an $h_{\text{min}}$ of 1.42. The worst performing column in this set (Column 1) had an $h_{\text{min}}$ of 1.90. This set of columns confirmed that very high efficiency columns can be packed in 30 µm ID capillaries. The next thrust of the work was to test this approach with larger ID capillaries.
The efficiencies obtained for the first set of sequentially packed 50 μm ID columns, packed at 30 mg/mL, can be seen in Figure 4.12. The most efficient column from this set (Column 2) with an $h_{\text{min}}$ of 1.60 was an excellently performing column. Even the most poorly performing column out of this set (Column 4) had an $h_{\text{min}}$ of 1.91, still a highly efficient column. The next set of 50 μm ID columns, packed at 50 mg/mL, was also chromatographically evaluated. When compared with the 50 μm ID capillaries packed at 30 mg/mL, the efficiencies seen in Figure 4.13 were not systematically improved. The most efficient column (Column 4) had an $h_{\text{min}}$ of 1.63, while the least efficient column (Column 3) was seen to have an $h_{\text{min}}$ of 2.50. For this set of columns, increasing the slurry concentration did not appear to improve column performance.

The set of 75 μm ID capillaries packed at 30 mg/mL were next to be evaluated, shown in Figure 4.14. The best performing column (Column 4) had an $h_{\text{min}}$ of 1.77 while the least efficient column (Column 1) had an $h_{\text{min}}$ of 3.58. This set of columns showed increased column-to-column variability as compared to the 50 μm ID columns. Additionally, more of these 75 μm ID columns (3) had $h_{\text{min}}$ values above 2 than any of the other column sets.

When assessing the performance data for the five sequential packing sets, it is difficult to establish many clear trends. One trend is that lowest observed $h_{\text{min}}$ for each column diameter decreases with decreasing column diameter, in line with previous reports that efficiency increases with decreasing column diameter [2, 17-19]. Another observation is that the first packed column never proved to be the most efficient, actually always performing worst or second to worst. One possible explanation for this behavior could be beneficial slurry aging. As time passes with each subsequent column being packed, the slurry may be aggregating more, which has been suggested to in fact improve the resulting column’s performance [8, 9, 11].
Pressure cycling between packing subsequent columns as well as time since sonication may both influence slurry aging and aggregation.

4.4 Conclusions

The findings in this chapter establish the fact that superficially porous particles can be used to create highly efficient capillary columns. The best columns created for each column ID using sequential column packing are the most efficient capillary columns packed with superficially porous particles reported to date. In fact, the 520,000 plates/m generated with the most efficient column created in this study is comparable to the highest reported plates/m count to date with superficially porous particles, just over 500,000 plates/m for a 50 x 2.1mm column packed with 1.3 μm superficially porous particles [24]. Also through this study it was seen that serial column packing was an effective method for creating efficient columns. This packing method allows for the faster creation of multiple columns than is possible by packing only one column per slurry. In accordance with previous work, the smallest ID capillaries produced the most efficient columns [2, 17-19]. The larger ID capillaries, however, still showed high efficiency with at least one column of each ID having $h_{\text{min}}$ less than 2 using sequential column packing. Similar studies could be carried out with other particle types or columns of differing final length to ascertain if sequential column packing could be a more generally beneficial technique for capillary column creation.
### 4.5 Tables

<table>
<thead>
<tr>
<th>Column Batch</th>
<th>Packing Order</th>
<th>Column Length (cm)</th>
<th>$h_{\min}$</th>
<th>$\alpha$ (cm/s(^{1/2}))</th>
<th>$\beta$ (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>30 μm ID</strong></td>
<td>1</td>
<td>30.0</td>
<td>1.96</td>
<td>5.8 ± 0.2</td>
<td>1.7 ± 0.9</td>
</tr>
<tr>
<td><strong>30 mg/mL – 1</strong></td>
<td>2</td>
<td>31.0</td>
<td>2.53</td>
<td>5.9 ± 0.1</td>
<td>1.5 ± 0.4</td>
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<td></td>
<td>3</td>
<td>30.35</td>
<td>1.44</td>
<td>6.0 ± 0.1</td>
<td>0.7 ± 0.5</td>
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<tr>
<td></td>
<td>4</td>
<td>30.25</td>
<td>1.68</td>
<td>6.0 ± 0.1</td>
<td>0.5 ± 0.6</td>
</tr>
<tr>
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<td>1</td>
<td>31.0</td>
<td>1.90</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>30 mg/mL – 2</strong></td>
<td>2</td>
<td>28.9</td>
<td>1.42</td>
<td>6.4 ± 0.1</td>
<td>0.5 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>31.7</td>
<td>1.24</td>
<td>6.1 ± 0.1</td>
<td>1.3 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>29.1</td>
<td>1.66</td>
<td>6.2 ± 0.1</td>
<td>0.5 ± 0.3</td>
</tr>
<tr>
<td><strong>50 μm ID</strong></td>
<td>1</td>
<td>29.4</td>
<td>1.82</td>
<td>6.2 ± 0.1</td>
<td>0.8 ± 0.4</td>
</tr>
<tr>
<td><strong>30 mg/mL</strong></td>
<td>2</td>
<td>31.2</td>
<td>1.60</td>
<td>6.0 ± 0.0</td>
<td>-0.2 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>26.0</td>
<td>1.75</td>
<td>5.7 ± 0.1</td>
<td>0.1 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>30.2</td>
<td>1.91</td>
<td>5.0 ± 0.2</td>
<td>2.1 ± 0.9</td>
</tr>
<tr>
<td><strong>50 μm ID</strong></td>
<td>1</td>
<td>31.9</td>
<td>2.01</td>
<td>8.5 ± 0.1</td>
<td>0.6 ± 0.3</td>
</tr>
<tr>
<td><strong>50 mg/mL</strong></td>
<td>2</td>
<td>32.5</td>
<td>1.73</td>
<td>8.2 ± 0.0</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>29.7</td>
<td>2.50</td>
<td>8.0 ± 0.1</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>31.6</td>
<td>1.63</td>
<td>7.6 ± 0.1</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td><strong>75 μm ID</strong></td>
<td>1</td>
<td>30.6</td>
<td>3.58</td>
<td>5.7 ± 0.1</td>
<td>1.0 ± 0.5</td>
</tr>
<tr>
<td><strong>30 mg/mL</strong></td>
<td>2</td>
<td>29.6</td>
<td>2.33</td>
<td>5.5 ± 0.0</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>29.2</td>
<td>2.80</td>
<td>4.7 ± 0.1</td>
<td>1.3 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>27.2</td>
<td>1.77</td>
<td>4.7 ± 0.0</td>
<td>0.7 ± 0.2</td>
</tr>
</tbody>
</table>

Table 4.1: Packing and characterization data for the columns packed during the sequential column packing study.
<table>
<thead>
<tr>
<th>Column ID (μm)</th>
<th>Expected Mass of Particles Required to Pack Column (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>0.23</td>
</tr>
<tr>
<td>50</td>
<td>0.64</td>
</tr>
<tr>
<td>75</td>
<td>1.44</td>
</tr>
</tbody>
</table>

Table 4.2: Expected masses of the prototype superficially porous particles from this study required to pack 35 cm long columns of various internal diameters.
<table>
<thead>
<tr>
<th>Capillary Type</th>
<th>Batch</th>
<th>Observed Proportional Change in $\alpha$</th>
<th>Expected Proportional Change in $\alpha$</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 µm ID Capillary</td>
<td>1.02</td>
<td>0.98</td>
<td></td>
</tr>
<tr>
<td>30 mg/mL Batch 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 µm ID Capillary</td>
<td>0.97</td>
<td>0.98</td>
<td></td>
</tr>
<tr>
<td>30 mg/mL Batch 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 µm ID Capillary</td>
<td>0.83</td>
<td>0.95</td>
<td></td>
</tr>
<tr>
<td>30 mg/mL</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>50 µm ID Capillary</td>
<td>0.93</td>
<td>0.97</td>
<td></td>
</tr>
<tr>
<td>50 mg/mL</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>75 µm ID Capillary</td>
<td>0.85</td>
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<td></td>
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<tr>
<td>30 mg/mL</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Table 4.3: Comparison of the observed proportional change in $\alpha$ to the expected proportional change in $\alpha$ for the 5 sequential column packing events.
Figure 4.1: Example packing progress plot for a 50 μm ID column packed with a 50 mg/mL acetone slurry at 30,000 psi. Fit parameters are shown ± 1 standard deviation.
Figure 4.2: Example packing progress plot with square root of time axis for a 50 µm ID column packed with a 50 mg/mL acetone slurry at 30,000 psi. Fit parameters are shown ± 1 standard deviation.
Figure 4.3: SEM image of the prototype superficially porous particles employed in this investigation
Figure 4.4: Packing progress plot for the first set of 30 µm ID capillaries packed at 30 mg/mL. Black circles, red squares, green triangles, and blue inverted triangles are the first, second, third, and fourth packed columns respectively. All experimental data were fit to the packing modeling equation.
Figure 4.5: Packing progress plot for the second set of 30 µm ID capillaries packed at 30 mg/mL. Black circles, red squares, green triangles, and blue inverted triangles are the first, second, third, and fourth packed columns respectively. All experimental data were fit to the packing modeling equation.
Figure 4.6: Packing progress plot for the set of 50 µm ID capillaries packed at 30 mg/mL. Black circles, red squares, green triangles, and blue inverted triangles are the first, second, third, and fourth packed columns respectively. All experimental data were fit to the packing modeling equation.
Figure 4.7: Packing progress plot for the set of 50 µm ID capillaries packed at 50 mg/mL. Black circles, red squares, green triangles, and blue inverted triangles are the first, second, third, and fourth packed columns respectively. All experimental data were fit to the packing modeling equation.
Figure 4.8: Packing progress plot for the set of 75 μm ID capillaries packed at 30 mg/mL. Black circles, red squares, green triangles, and blue inverted triangles are the first, second, third, and fourth packed columns respectively. All experimental data were fit to the packing modeling equation.
Figure 4.9: h-ν plot for the characterization of the first batch of 30 µm ID columns packed at 30 mg/mL. Efficiency data is shown for hydroquinone (k’~0.2). As with the packing progress plots, black circles, red squares, green triangles, and blue inverted triangles are the first, second, third, and fourth packed columns respectively. Packing order is also indicated by the numbers on the right of the figure.
Figure 4.10: h-ν plot for the characterization of the second batch of 30 µm ID columns packed at 30 mg/mL. Efficiency data is shown for hydroquinone (k’~0.2). As with the packing progress plots, black circles, red squares, green triangles, and blue inverted triangles are the first, second, third, and fourth packed columns respectively. Packing order is also indicated by the numbers on the right of the figure.
Figure 4.11: Example chromatogram from a highly efficient separation performed on a 31.7 cm X 30 μm ID column. The separation was performed at 16,000 psi at ambient temperature in 60/40 water/acetonitrile with 0.1% trifluoroacetic acid added. The peaks are, in order of retention, ascorbic acid (dead time marker), hydroquinone, resorcinol, catechol, and 4-methyl catechol.
Figure 4.12: h-ν plot for the characterization of the 50 µm ID columns packed at 30 mg/mL. Efficiency data is shown for hydroquinone (k’~0.2). As with the packing progress plots, black circles, red squares, green triangles, and blue inverted triangles are the first, second, third, and fourth packed columns respectively. Packing order is also indicated by the numbers on the right of the figure.
Figure 4.13: h-v plot for the characterization of the 50 µm ID columns packed at 50 mg/mL. Efficiency data is shown for hydroquinone (k’~0.2). As with the packing progress plots, black circles, red squares, green triangles, and blue inverted triangles are the first, second, third, and fourth packed columns respectively. Packing order is also indicated by the numbers on the right of the figure.
Figure 4.14: h-ν plot for the characterization of the 75 µm ID columns packed at 30 mg/mL. Efficiency data is shown for hydroquinone (k’~0.2). As with the packing progress plots, black circles, red squares, green triangles, and blue inverted triangles are the first, second, third, and fourth packed columns respectively. Packing order is also indicated by the numbers on the right and top of the figure.
4.7 References


CHAPTER 5: IMPACT OF PARTICLE ARCHITECTURE AND BONDED PHASE ON CHROMATOGRAPHIC SEPARATIONS

5.1 Introduction

5.1.1 Impact of Surface Charge and Bonding

Improving the peak shape of biological analytes during separation, especially without the use of strong ion pairing agents such as trifluoroacetic acid in the mobile phase, is an area of ongoing improvement and research. One area of interest in biological separations is the utilization of charged surface particles to improve the peak shape and loading capability of ionizable analytes in reversed-phase chromatography. Recently developed materials (Charged Surface Hybrid or CSH) have been released by Waters Corporation having a low density positive surface charge placed on the particle surface before bonding and endcapping[1, 2]. The base particles are fully porous organosilica bridged-ethyl hybrid particles (BEH particles)[3], while the surface charge is imparted by bonding an amine to the particle surface at a very low density to give an overall positive charge at low pH[4]. For use in reversed-phase liquid chromatographic separations, the particles are then bonded with either C18 or phenyl functionalities. Recent literature has investigated the behavior of these particles with both neutral and basic small molecule analytes, highlighting that the surface charge at low pH does reduce the interaction of basic analytes with unreacted surface silanol groups through electrostatic repulsion of cations[4-8]. One issue that was seen in these works, however, was decreased retention of basic analytes when using charged particles, due to the electrostatic repulsion between the particles’ surface charge and the charged analytes.
The promising results with basic small molecule analytes in the aforementioned reports suggest the utility of CSH particles for peptide separations. At the low pH conditions often employed for reversed-phase separations, peptides are ionized in solution, thus leaving them vulnerable to the unwanted secondary interactions with unbonded surface silanols described previously. One study was performed comparing the LC-MS analysis of peptides on BEH-C18 particles and with CSH-C18 particles[9]. This study found that peak shape and sample loading capacity was markedly improved using the charged surface particles, especially when employing a mobile phase gradient containing 0.1% formic acid. Avoiding the use of trifluoroacetic is beneficial for mass spectrometric analysis due to its tendency for ion suppression, as this same study showed over a 10-fold decrease in mass spectral signal when going from 0.1% formic acid to 0.1% trifluoroacetic acid in the mobile phase. Many of the other attributes previously described for small molecules on these CSH particles were seen for peptides as well, namely a decrease in retention on the CSH material and differences in selectivity. The retention time decrease is not surprising due to the positive charge usually carried by peptides in acidic solution.

Moving forward past peptide separations, these CSH particles have also been employed for protein separations[10]. In this study, BEH particles with increasing charge densities (including uncharged particles) were evaluated to see the impact of separating proteins, both at analytical loading as well as purposefully overloading the column. This study found that the positive charge on the particle’s surface adversely impacted the peak profiles of intact proteins, especially with the use of mobile phase additives other than trifluoroacetic acid. As with the peptide separations, it was seen that the positively charged proteins eluted earlier with the charged surface particles than with particles with less or no positive surface charge. The
presence of positive surface charge was also found to adversely impact the peak shapes of intact proteins at high loading, perhaps adding to donnan exclusion from pores at high loading when the analyte itself is already adding to the positive charge within the particle’s pores. Thus it appears that the CSH particles show their greatest utility for peptides and small molecules.

5.1.2 Motivations for the Study

With the rapid advances in particle technology in recent years, it is important to continuously evaluate new particles for their applicability to laboratory analyses. There has been a sustained interest in the Jorgenson lab in the separation of biological molecules in proteomic analyses [11-17]. Much of the work in the lab has been carried out with either nonporous silica particles or fully porous BEH particles, both of which have been available for over a decade. The lab has also found that learning how to pack particles into capillaries to create efficient columns is a non-trivial task, with specific conditions being required for each type of particle[18-23]. Therefore finding an advantageous particle to invest the effort of learning how to pack efficient capillaries is a worthwhile task. One way to try to evaluate novel packing materials is to look to the literature.

When looking to the literature, comprehensive comparisons evaluating both particle type and particle charge characteristics are lacking. It would also be useful to compare these characteristics as offered by a single manufacturer, as their bonding strategies may be more similar than particles offered by different vendors. Waters Corporation has recently released their Cortecs C18+ particles, which are silica based superficially porous particles with a charged surface bonding similar to the CSH particles described above. While the work detailed in the preceding section concerned surface charge modified fully porous BEH particles, analogous work does not exist for Cortecs C18+ particles or any equivalent particles from alternate
manufacturers. Work has been published on the silica based superficially porous particles known as Cortecs offered by Waters Corporation with both traditional C18 reversed phase bonding[24-27] as well as unbonded Cortecs particles for HILIC separations[28], mostly focusing on separations of small molecules. In these analyses, the uncharged Cortecs particles are often compared to uncharged C18 bonded BEH particles, which is a fair comparison. They are not, however, compared to any charged surface particles.

It would be useful to carry out a systematic comparison of these four particles: Cortecs C18, Cortecs C18+, BEH C18, and CSH C18 (referred to hereafter as BEH C18+ for simplicity of terminology). This may allow for a more careful understanding of the relative merits of the particle surface functionalization, since two differing particle substrates are being used. This may also allow for a more careful understanding of the relative merits of the differing particle morphologies, since each would be tested with two differing bonding regimes. Thus insight can be gained into a particle morphology for utilization within the lab. It would also be advantageous to apply these particles to differing analyte classes to understand differences between the particles.

5.2 Materials and Methods

5.2.1 Particle Physical Characteristics and Column Creation

Cortecs C18 and C18+ particles were analyzed by SEM imaging as previously described. Particles were then sized using ImageJ software (National Institutes of Health, Bethesda, MD) to ascertain an average particle diameter. The Cortecs particles are listed as having 100 m²/g specific surface area and 90 Å pores by the manufacturer. The 1.9 µm BEH C18 particles employed in this study were those previously employed in the lab[18], which were received from Waters Corporation (Milford, MA). The BEH C18+ particles, listed as the same nominal
diameter as the BEH C18 particles, were also received from Waters Corporation (Milford, MA). The BEH particles are listed as having a 185 m$^2$/g specific surface area and 130 Å pores by the manufacturer.

The columns employed in this study were created in a fashion similar to that detailed previously in Chapter 3. Particles were suspended in acetone at either 15 or 30 mg/mL before packing. Particle dispersion was effected by the use of sonication with the P30H sonicator (Elma Schmidbauer, GmbH, Singen, Germany) for 5 minutes at 100% power, 80kHz frequency, pulse mode. Hand agitation was employed as well to ensure suspension. The particles were packed using the same high pressure packing setup Described in Chapter 3. The particles were packed into 75 µm ID 360 µm OD fused silica capillaries (Polymicro Technologies, Phoenix, AZ) with an outlet frit prepared as stated in Chapter 3. The columns were packed at 30 kpsi to around 50 cm in length. This length was chosen to produce a column long enough to show its utility for proteomic separations but still short enough for improved ease of use and shorter analysis time than possible with meter-long columns, which are commonly employed in the lab. After packing, the columns were flushed up to 50 kpsi using the flushing setup described in Chapter 3 in the anticipated isocratic characterization mobile phase. The fully porous BEH particles were flushed in 50/50 v/v water/acetonitrile with 0.1% trifluoroacetic acid added while the superficially porous Cortecs particles were flushed in 60/40 v/v water/acetonitrile with 0.1% trifluoroacetic acid added. The water was purified on a Barnstead NANOpure system (Thermo Scientific, Waltham, MA). The acetonitrile and trifluoroacetic acid was purchased from Fisher Scientific (Pittsburgh, PA). After flushing was complete, the columns were fritted successively with temporary and permanent frits as described in Chapter 3.
5.2.2 Isocratic Characterization

The isocratic characterization of the columns was carried out as described in Chapter 3. The BEH particles were characterized in 50/50 v/v water/acetonitrile with 0.1% trifluoroacetic acid added, while the Cortecs particles were characterized in 60/40 v/v water/acetonitrile with 0.1% trifluoroacetic acid added. The difference in mobile phase was to make up for differences in specific surface area between the particles and therefore differences in retention, allowing the analytes to have similar retention factors on the different columns. The isocratic efficiency data was used to create h-ν plots as described in Chapter 2. Retention factor was calculated by obtaining the retention factor at the various pressures used during column characterization and extrapolating to zero pressure, so as to make the retention factor comparison fair between the particles. For the isocratic column characterization, all of the columns used were between 49 and 51 cm in length, with 75 µm ID.

5.2.3 LC-MS Gradient Characterization – Initial Comparison

After isocratic characterization with small molecules, gradient separations of peptides were investigated. For the first column comparison, three columns were compared, each with one of the three particles types on hand in the laboratory at that time: Cortecs C18, Cortecs C18+, and BEH C18 (all from Waters Corporation, Milford, MA). They were 50.1, 49.1, and 50.6 cm long respectively, all with a 75 µm ID and a 360 µm OD. For this study, alcohol dehydrogenase tryptic digest standard (Waters Corporation, Milford, MA) was used as the analyte, prepared at 100 fmol/µL and 10 fmol/µL in 97/3 v/v water/acetonitrile with 0.1% formic acid.

The in-house modified ultrahigh pressure gradient system from Chapter 3 and described in detail elsewhere[15, 17, 19] was employed. In the following study, the gradient system was
configured with a gradient storage loop consisting of around 31 meters of 250 μm ID stainless steel tubing attached to approximately six meters of 50 μm ID fused silica capillary closest to the column tee. High pressure was provided by a DSXHF-903 pneumatic amplifier pump (Haksel, Burbank, CA) with a listed pressure amplification ratio of 1038:1.

The mobile phase employed in this study consisted of water and acetonitrile (Fisher Scientific, Pittsburgh, PA), with 0.1% formic acid added (mass spectrometry grade, Sigma-Aldrich, St. Louis, MO). For this study, a 2 μL full loop direct injection was used to introduce the sample to the gradient storage loop, with a 99.5% water push to position it for introduction to the column. Between the sample and the gradient, a 1.5 μL pre-gradient hold at the gradient start condition (4% acetonitrile) was used to help concentrate the sample at the head of the column. The gradient employed for all of the columns was a 19 μL 4-40% acetonitrile gradient. After reaching 40% acetonitrile, a 2 μL wash at 85% acetonitrile was employed. A graphical representation of the gradient and sample can be found in Figure 5.1.

During acquisition, the column was maintained at 40 °C using a Waters (Milford, MA) Temperature Control Module II. Pressure was adjusted to give a measured volumetric flowrate of around 300 nL/min, as measured using an isocratic 80/20 water/acetonitrile mixture. The column was connected to a 20-25 cm length of 20 μm ID fused silica capillary (the pigtail) using an PicoClear zero dead volume connector from New Objective (Woburn, MA). This pigtail was then connected to a 20 μm ID capillary pulled to a 10 μm ID electrospray tip (New Objective, Woburn, MA). Electrospray was performed at 3 kV spray voltage. Data was obtained in MS² mode, a data independent acquisition methodology[29], with 0.6 second scans. Low collision energy was 5 volts while the high collision energy was ramped from 15 to 40 volts. A lockspray solution of 70/30 v/v water/acetonitrile with 0.1% formic acid added and containing [Glu¹]-
fibrinopeptide B and leucine enkephalin acetate hydrate (both from Sigma Aldrich, St Louis, MO) was infused through the reference spray needle and used for mass correction.

Data acquisition was performed using MassLynx version 4.1 software from Waters Corporation (Milford, MA). Data acquisition began shortly (2-3 minutes) after engaging the high pressure pump for gradient elution. Chromatographic full width half max peak height (FWHM) data were obtained from the Apex peak fitting algorithm output generated using the ProteinLynx data processing software used to process the data (Waters Corporation, Milford, MA). All acquisitions were performed in duplicate and the arithmetic mean of the FWHM measurements for the two runs was used for peak characterization. The 4σ peak widths were calculated by multiplying the FWHM values by 1.7 (approximation of 4/2.35 (1.7021), the expected ratio of standard deviations present at FWHM and 4σ peak width for a Gaussian peak[9]). Peak identities were assigned based on low energy m/z values and peak intensities. Table 5.1 shows the 16 mass ions that were used to determine peak capacity for all separations except for Cortecs C18+ for which the first peak was not eluted with the gradient (low retention) and was therefore omitted. Elution window was calculated by the retention times of the first (m/z 474.24 for BEH C18 and Cortecs C18, m/z 817.41 for Cortecs C18+) and last (m/z 950.48) sharp peak found in the chromatogram. The preceding m/z values refer to the m/z values obtained during the low energy portion of the scan. Figure 5.2 shows example alcohol dehydrogenase tryptic digest separations (low collision energy) with BEH C18 and Cortecs C18+ particles with relevant peaks labeled. Peak capacity, n_c, was calculated using Equation 5.1, where t_g is the gradient time and w_p is the average peak width (4σ) [30].

\[ n_c = \frac{t_g}{w_p} + 1 \]  

Equation 5.1
The elution window was used in place of gradient length $t_g$ since it could be accurately measured, though the true gradient length was always longer than the elution window. The peak width for a given separation condition, $w_p$, was the arithmetic mean of the calculated $4\sigma$ peak widths for the ions shown in Table 5.1.

5.2.4 LC-MS Gradient Characterization – Second Comparison

For the final column comparison, four columns were compared, each with one of the four particle types: Cortecs C18, Cortecs C18+, BEH C18, and BEH C18+ (Waters Corporation, Milford, MA). The columns were 48.6, 50.5, 50.6, and 50.7 cm long respectively, all with a 75 μm ID and a 360 μm OD. For this portion of the work, alcohol dehydrogenase tryptic digest standard (Waters Corporation, Milford, MA) was used, prepared at 10, 30, and 90 fmol/μL in 100% water with 0.1% formic acid added. Samples were created from a 1000 fmol/μL stock solution of water with 0.1% formic acid added. Stock solution was stored at -80 °C when not in use. Samples were maintained at 10 °C in the instrument before analysis.

The same in-house modified ultrahigh pressure system used for the initial column study was employed in the final column comparison, with one modification. For this final comparison, the gradient storage loop was around 50 meters of 50 μm ID 360 μm OD fused silica capillary instead of the previous stainless steel and fused silica gradient storage loop. Sample injections were 2 μL full loop direct injections. The same gradients were used to characterize all four of the columns in the final study, and as with the previous study the mobile phase components were water and acetonitrile, both with 0.1% formic acid added. The gradient profile consisted of a 1.5 μL pre-gradient hold at 0.5% acetonitrile, followed by a gradient from 0.5 to 40% acetonitrile, then a 2 μL wash at 85% acetonitrile, followed by column re-equilibration at 0.5% water (more is added than is played back through the column to allow for ease of method development). To
assess the impact of gradient length on the separations, gradients of 16.5, 33.0, and 66.5 μL were employed. Figure 5.3 shows the gradient profile employed for the 33.0 μL gradient. All other operating conditions and setups were the same as what was employed for the first gradient column comparison.

For the second gradient comparison, mass spectrometric detection was employed. All data was acquired on a Waters Q-TOF Premier instrument using continuum mode (constant 5 eV collision energy) with either 0.9, 0.53, or 0.36 second scans with 0.02 second interscan delays to yield acquisition rates of 1.1, 1.8, and 2.6 Hz respectively. The differing scan rates were matched with the differing gradient lengths to ensure an adequate number of points across each peak were recorded. As with the first comparison, a 3 kV spray voltage was employed to provide ionization, with the same lockspray reference solution employed. The same 16 m/z ions were used to calculate peak capacity for all separations. Separations were performed in duplicate. Peak widths were determined as previously described. By employing the weaker starting mobile phase in these separations (0.5% acetonitrile), the same 16 m/z ion peaks employed previously were used to calculate peak widths for all four of the columns. The elution window was defined as the time between the early eluting m/z 474.24 peak and the late eluting m/z 950.48 peak for all of the columns packed with the differing particle types. A change in the acquisition method for these separations resulted in the data starting to be collected a few minutes before high pressure was engaged. Thus, the retentions times shown for this study were longer than those for the previous studies within this chapter.

5.2.5 Gradient Separations of Intact Proteins

After assessing the performance of the columns with model peptide mixtures, it was decided to determine if any of the particle types investigated would be useful for intact protein
separations. The intact protein sample used was a soluble *Escherichia coli* whole cell lysate, the same sample used in Chapter 3. The sample was diluted using water with the same acid content (0.2% formic acid) used in the analysis of *E. coli* whole cell lysate described in Chapter 3. The protein content of the samples was determined via a Bradford colorometric assay, with either 100 or 250 ng of protein injected onto the column depending on which sample was injected.

For the chromatographic separation of the proteins, BEH C18, BEH C18+, Cortecs C18, and Cortecs C18+ 75 μm ID columns were employed (50.2 cm, 49.9 cm, 47.6 cm, and 50.5 cm long, respectively). For this investigation, the column was held at 40 °C. Flowrate was maintained around 300 nL/min as previously. For all columns employed, a 0.5 to 60% acetonitrile gradient was employed, with the entire gradient being either 25 or 50 μL in volume. At the end of each gradient there was a 2 μL ramp up to 85% acetonitrile, then 2 μL of 85% acetonitrile, then a 2 μL transition back to 0.5% acetonitrile and subsequent column re-equilibration to starting conditions before the next separation. Figure 5.4 shows the gradient profile for the 25 μL gradient. Both mobile phases had 0.2% formic acid added, following previous work[11, 31].

The proteins were detected using the same Waters Q-TOF Premier mass spectrometer used for the rest of this work. Electrospray was carried out at 3.0 kV. Data was acquired at 1.9 Hz for the 25 μL gradients and at 1.0 Hz for the 50 μL gradients in continuum mode at a constant low collision energy (5 eV). Since the analytes of interest for this separation were proteins, only m/z 400-1990 were recorded. Most protein envelopes do not extent below m/z 400 and this allows for longer separations to be recorded without exceeding the maximum data file size. For this study, obtained masses were not corrected with the use a lockspray reference. Peak analysis was performed by using the autoME automated deconvolution program as
described in Chapter 3. Protein intact masses were calculated using the MaxEnt 1 maximum entropy deconvolution algorithm available through Waters MassLynx software. The mass, time, and intensity data from the autoME program were used to extract deconvoluted masses over time and thus obtain chromatographic protein peak profiles at given masses in the same way as was done in Chapter 3.

One approach to interpreting the peaks from the autoME program was to fit them with an exponentially modified Gaussian (EMG) equation using Igor Pro 6.37 (Wavemetrics, Inc., Lake Oswego, OR), yielding $\sigma$ and $\tau$ values relating to the Gaussian and exponential parts of the fit, respectively[32]. Peak variance is defined by Equation 5.2, with variance defined as the second central moment of the EMG distribution, $M_2$:

$$M_2 = \sigma^2 + \tau^2$$  
Equation 5.2

For peak capacity, peak width was defined as 4 times the square root of the variance, functionally analogous to that used with the $4\sigma$ peak width computed peak capacity using the Gaussian fits used previously in this chapter. Peak capacity was calculated using Equation 5.1.

5 protein masses (6856, 8325, 11185, 11673, and 21135 Da) were used for peak width determination due to their strong signal and reasonable peak shape across all of the columns, along with an additional peak (32904 Da) to mark the end of the gradient window, though it was not used for peak width analysis. An example chromatogram including the protein masses used is shown in Figure 5.5. All samples were analyzed in duplicate.

Another approach to interpreting the deconvoluted protein data from the autoME program was to create a deconvoluted protein mass base peak intensity plot[11]. This investigation used the same data that was analyzed using the EMG analysis above. For this approach, only the intensity of the most intense deconvoluted mass for each time point was
plotted as a function of time. This allows the profiles of the intense peaks, which can be investigated well by autoME, to be discerned apart from background signal. This is analogous to the base peak intensity plots routinely employed when displaying peptide separations.

After the deconvoluted protein mass base peak intensity plots were created, the peak capacity was computed based upon the magnitude-concavity method described by Lan[33, 34]. In this methodology, the peak width is determined by using the magnitude of a peak apex and the second derivative (concavity) of the peak apex. This can then be used to determine the 4σ width of the peak using a Gaussian approximation for peak shape. This method is advantageous because it is less sensitive to peak overlap than measuring overall peak profiles and it is amenable to computer automation, which allows for fast, operator-unbiased measurement of peak capacity. This work was undertaken using a computer program in LabView 8.5 (National Instruments Corporation, Austin, TX) called Peak Finder.vi written by Matthew Monroe[35]. This program employs the magnitude-concavity method described previously to determine peak widths. The width used by the program to evaluate peaks was set to 5 seconds for this study. The program sets the time axis based upon an input sampling frequency starting at time 0 rather than the actual input time values. For this data, the average sampling frequency was computed from the experimental data (only a few deviations are present), which gave reasonable agreement to the experimentally recorded time. Starting at time 0 has no impact on the computed peak capacity. No baseline correction was employed for the analysis. The peak capacity for this study was computed using the relation shown in Equation 5.3, where $t_{\text{max}}$ is the retention time of the last retained peak, $t_{\text{min}}$ is the retention time of the first retained peak, and $w_{\text{med}}$ is the median peak width for the separation.
\[ n_c = \frac{t_{\text{max}} - t_{\text{min}}}{w_{\text{med}}} \]

Equation 5.3

This relation is similar to Equation 5.1. The median peak width was used to calculate peak capacity since there is a large degree of scatter in the peak widths across the separation space and the median is rather insensitive to the fitting errors (very large or small) returned by the fitting program. The program also outputs the number of peaks found in the chromatogram. The program sometimes misses a peak or inserts more peaks than are actually present, but for the most part it gives acceptable peak identification. To reduce the impact of peaks being fitted to noise in the baseline, only peaks above a threshold of 6000 intensity were considered. For an initial comparison, the entire chromatogram was investigated. To obtain a more fair comparison, the program was set to only evaluate the chromatogram between two masses common to all of the separations (12518 Da first peak, 32903 Da last peak). These windows were manually set using the mass spectral data available since the Peak Finder program does not take mass into consideration when analyzing a chromatogram.

5.3 Results and Discussion

5.3.1 Particle Physical Characterization and Column Creation

The particle size characterization of the two batches of the Cortecs material yielded very similar results. The Cortecs C18 particles were found to have a number average particle diameter of 1.77 µm with an RSD of 2.0% (n=142). The Cortecs C18+ particles were found to have a number average particle diameter of 1.77 µm with an RSD of 2.0% (n=117). Graphical depictions of the particle size distributions are shown in Figure 5.6. While this result is not surprising, since the particles should only differ in bonded phase, it is reassuring to get this confirmation. Some small multiplets were seen in the material, as has been already reported for commercial material[36], but they were not at a high concentration and thus were not of concern.
Column packing proceeded smoothly for both the Cortecs and the BEH particles. The columns packed well, usually generating a packed bed of sufficient length within 30 to 70 minutes of reaching packing pressure.

5.3.2 Isocratic Characterization

The isocratic characterization of these columns packed with the various particles gives a first indication of the differences between them. Example chromatograms for the differing columns are shown in Figure 5.7. It can be seen that all four of the columns exhibit good peak shape, with slight differences in selectivity being seen between the two particle types. This may be due to either the difference in particle or the difference in the organic content of the two different mobile phases used for isocratic characterization. To look more closely at the performance of the packed columns, h-v plots for the Cortecs and BEH columns can be seen in Figure 5.8 and Figure 5.9, respectively. A list of the reduced minimum plate heights obtained for hydroquinone for all of the columns evaluated is found in Table 5.2. It can be seen that the BEH particles generally pack into more efficient columns than the Cortecs particles. The packing methodology employed in this study is derived from that used to routinely pack BEH particles, so perhaps the Cortecs particles would pack better under different conditions. It is of note that the best behaving Cortecs column was packed at the lowest slurry concentration, 15 mg/mL. This may be a route of future improvement in the packing of Cortecs particles, but was not pursued further during this study. At the time of characterization, all of the columns appeared to be performing reasonably well, so a more in-depth packing study was not undertaken at that time. In terms of performance, for both particles there appeared to be little difference in terms of performance between the two bonding types. Most importantly for this study, there did not appear to be drastic systematic differences in performance between the two bondings.
Another area of comparison between the particles was retention factor. The retention factors for the first eluting (hydroquinone) and last eluting (4-methyl catechol) components were listed in Table 5.3 for both the BEH and Cortecs materials with the two bondings. It should be noted that the two particle morphologies were evaluated using different mobile phase conditions, limiting direct comparison between the particle morphologies. Within the particle morphology, however, the bonded phases can be directly compared. It can be seen that the particles with the positive surface charge exhibit higher retention for these small molecule analytes than those without the intentional surface charge. The isocratic characterization of the columns showed that capillary columns packed with these particles show acceptable efficiency and that retention differences can be seen between the two types of bonding for both particle architectures.

5.3.3 Initial Gradient Column Comparison

The comparison between the BEH C18, Cortecs C18, and Cortecs C18+ was undertaken to assess their relative merits for peptide separations. Two main areas were assessed as they impacted calculated peak capacity: retention of poorly retained analytes and differences in peak capacity related to column loading. Both of these attributes are important when doing highly complex proteomic peptide separations, where wide variations in peptide loading are expected and at least some of the peptides will be lightly retained.

5.3.3.1 Retention Differences

One difference which was easily observed between the three columns was the difference in retention. This can be seen in Figure 5.10, with all of the separations being done with identical gradients and the 100 fmol/μL alcohol dehydrogenase tryptic digest standard. As expected by the specific surface areas laid out earlier, for the same bonding (C18) the higher surface area BEH have slightly higher retention. Most strikingly, however, the C18+ bonding
was shown to appreciably decrease retention relative to the C18 bonding. This is in accordance with the reports from literature that charged surface particles show decreased analyte retention. This loss of retention with Cortecs C18+ is large enough to impact the peak capacity calculation since some of the peaks retained on other columns were not retained on the Cortecs C18+ column. This negatively impacts calculated peak capacity for the Cortecs C18+ column as seen in Table 5.4, where the Cortecs C18+ column has a much shorter elution window than the other columns. This effect would also have the practical impact that when analyzing a proteomic sample, many of the peptides may not be adequately retained on the column. This would adversely impact protein identifications.

5.3.3.2 Column Loading

Another difference that can be seen between the columns is the impact of sample loading on peak width and therefore calculated peak capacity. For this study, the 10 and 100 fmol/μL samples of alcohol dehydrogenase tryptic digest standard were employed to look at relative loss in retention between the low and high loading levels. Figure 5.11 shows the most extreme case of peak broadening increasing with increasing sample loading, with the Cortecs C18 column. It should be noted that the peak widths have increased and the peaks have become more tailed upon going to the higher sample loading level. Table 5.5 shows the increase in peak width between the low and high loading levels for the three columns investigated. As expected, for the two particle architectures bonded with C18, the higher specific surface area BEH shows less of a relative decrease in peak capacity than the lower specific surface area Cortecs. Most strikingly, however, the Cortecs C18+ showed very little increase in peak width between the low and high loading levels. This is in accordance with previous reports, and is assumed to be due to the light positive surface charge on the Cortecs C18+ particles exerting a repulsion on the positively charged
analytes. The practical impact of the wider peaks at higher loading on peak capacity can be seen in Table 5.6.

Looking at both the retention and loading comparisons between the three columns, a number of trends become obvious. The BEH particles, which have been used extensively in the lab for proteomic work, appear to be well suited to this kind of separation. Their high specific surface area and C18 bonding allow for good retention of analytes and only moderate peak broadening with an increase in sample loading. For this study, the Cortecs C18 particles with their lower surface area show somewhat lower performance when compared to the BEH C18 particles. The most interesting findings were for the Cortecs C18+. The C18+ bonding shows markedly decreased retention relative to the C18 bonding, but the C18+ bonding improved loadability out of proportion to the particle’s surface area; the Cortecs C18+ showed even less peak broadening at higher loading than the BEH C18 particles. Cortecs C18+ also appear to have the thinnest peaks. Clearly more investigation was required to determine beneficial acquisition parameters for Cortecs C18+ to take advantage of their promising peak widths and loadability.

5.3.4 LC-MS Experimental Improvements

From the results of the initial gradient column comparison, it was obvious that experimental parameters would need to be improved to allow for a fair comparison of the particle types. As stated previously, reduced retention was seen on the Cortecs C18+ column using the experimental conditions laid out above. This lack of retention for early eluting analytes was reducing the length of the elution window and thereby reducing the calculated peak capacity of the Cortecs C18+ column relative to the other two C18 columns. To allow for a fair
comparison between the different particle types, the retention on the C18+ column would need to be improved.

5.3.4.1 Sample Diluent

One area of improvement was the composition of the sample diluent. The preceding work was done with a 97/3 v/v water/acetonitrile diluent with 0.1% formic acid added. It was hypothesized that this might be too strong of a sample diluent and would not allow lightly retained peptides to be retained at the column head. This is especially important since with this injection system, the injection volume of 2 \( \mu \text{L} \) was always greater than the column volume itself, allowing lightly retained analytes to elute undetected in wide bands. To probe the impact of sample diluent on sample retention, samples of alcohol dehydrogenase tryptic digest standard were prepared at 100 fmol/\( \mu \text{L} \) in 3%, 0.5%, and 0% acetonitrile in water with 0.1% formic acid added. These samples were run on the Cortecs C18+ column used in the preceding study. All of these samples were run with the 4-40% acetonitrile gradient employed in the previous study.

Example chromatograms are shown in Figure 5.12. It can be seen that a relatively high concentration of acetonitrile in the diluent (3%), lightly retained peaks are either unretained or lightly retained. Decreasing the concentration of acetonitrile to 0.5% and 0% improved the retention of these early eluting peaks. Making up standards in 100% water should also be more facile and reproducible than samples made up with a very small fraction of acetonitrile. Moving forward, samples were made up in 100% water with 0.1% formic acid added.

5.3.4.2 Preconcentration and Gradient Starting Condition

Another area of improvement was in the gradient profile itself. As stated in the previous section, the original gradient incorporated a 1.5 \( \mu \text{L} \) preconcentration hold at 4% acetonitrile followed by a gradient from 4-40% acetonitrile. This gradient had been previously shown to
work well with BEH C18 in the lab but perhaps it employed too strong of a starting solvent condition for the C18+ bonded phase. To assess the impact of gradient starting condition on the separation, the pre-gradient hold and gradient start was modified to be either 4%, 0.5%, or 0% acetonitrile. The total gradient volume was modified to keep the percentage change in acetonitrile across the column constant across the conditions, with all gradients ending at 40% acetonitrile. The same Cortecs C18+ column used for the preceding sample diluent study was used to assess performance with the differing gradient start conditions. A 100 fmol/μL sample of alcohol dehydrogenase tryptic digest standard with 0% acetonitrile (100% water) with 0.1% formic acid was used, as was previously seen to give good retention.

Example chromatograms from these separations are shown in Figure 5.13. As shown, moving from 4% acetonitrile at the beginning of the gradient (both preconcentration and gradient start) to 0.5% acetonitrile at the beginning of the gradient greatly increases peak retention at the beginning of the chromatogram. Moving from 0.5% to 0% for preconcentration and gradient beginning was not seen to make any noticeable improvement and some very early peaks in fact appeared to exhibit inferior peak shape with 0% acetonitrile. Thus moving, a 0.5-40% acetonitrile gradient was employed for gradient separations of peptides.

It is apparent from these studies that both the sample diluent and the gradient start condition have a considerable impact on sample retention for Cortecs C18+ particles. This issue has become salient for these particles in particular due to their relatively lower specific surface area than fully porous BEH particles and the lower retention associated with the positive surface charge imparted to these particles. By lowering solvent strength for both the sample diluent and the mobile phase at the beginning of the separation, improved retention was seen on the Cortecs C18+ column. It was found that loading the sample in 100% water and starting the gradient and
pre-gradient hold at 0.5% acetonitrile gave adequate retention of lightly retained peaks in this separation. While these conditions are advantageous for separations on Cortecs C18+, they should also be amenable to use with the fully porous BEH C18 particles more traditionally used in our lab.

5.3.4.3 Electrospray Tip Connection

Beyond the chromatographic conditions employed, it was found that the spray tip connection itself was important for maintaining chromatographic peak shape. On the nanospray source which was employed in this study, the connection between the column pigtail and the nanospray tip is created by butting the two capillaries directly up to one another and securing them with PEEK finger-tight fittings, as shown in Figure 5.14. This means that the faces of both the pigtail and the spray tip should be as flat as possible to ensure a good fit.

Initially, the pigtail coming from the column was a length of 20 μm ID, 360 μm OD fused silica capillary which had been cleaved using a ceramic capillary cutter. Spray tips inlets were often also cut with the ceramic cutter if the tip inlet had crushed or clogged. This methodology generally allows for adequate spray tip connections, electrospray and the acquisition of data. However, it is often seen with such connections that all of the peaks tail slightly to moderately. This behavior would be consistent with a sub-optimal pigtail-spraytip connection which would allow for extracolumn band broadening due to dead volume. This hypothesis is supported by the fact that upon visual inspection of ceramic cut capillary under magnification there is often roughness or spurs left over from the cutting process.

It was found that using well-cut capillary provided the best pigtail-spraytip connection. Well-cut capillary is most easily obtained by purchasing commercially cleaved 25-cm lengths of 20 μm ID, 360 μm OD fused silica capillary from New Objective (Woburn, MA) for the column-
to-spraytip pigtail and using fresh, pre-cleaved fused silica spraytips, also obtained from New Objective (Woburn, MA). Under visual inspection these capillary cuts are very good, often showing a flat, reflective face. Employing these components has been shown to improve peak shape, as shown in Figure 5.15. This figure shows two consecutive, identical separations (11 μL 0.5-40% acetonitrile gradient, 2 μL 30 fmol/μL alcohol dehydrogenase tryptic digest sample) only differing in the makeup of the pigtail and spraytip. As can be seen by this close-up image, the peaks with the in-house cut connections show far more tailing than those seen with commercially made connections. Since this study, the commercially cut components have been used repeatedly, with a very high success rate in terms of desired peak shape. Moving forward, the combination of commercial pigtail and commercial spraytip was used when possible to minimize extracolumn band broadening due to the spraytip connection.

5.3.4.4 Mass Spectrometry Acquisition Rate

An area which needed to be addressed for the accurate acquisition of data was the sampling rate employed. As mentioned previously, data acquisition for the preceding studies was done in MS^E mode with 0.6 second scans. MS^E operates by acquiring data at low collision energy and then ramping at high collision energy, with 0.1 second interscan delay after both the high and low energy portions of the scans where data is collected[29]. Thus ‘0.6 second’ MS^E acquisition results in a 1.4 second point-to-point time, or practical data acquisition at around 0.7 Hz. To get a fair comparison between columns which may have differing peak widths, it was important to understand at what point undersampling would practically impact measured peak capacity.

To assess the impact of sampling rate on peak capacity, separations were performed on the same Cortecs C18+ column using an 11 μL 0.5-40% acetonitrile gradient with a 2 μL full
loop injection of 30 fmol/μL alcohol dehydrogenase tryptic digest standard prepared in 100% water with 0.1% formic acid added. As usual, the column was run at around 300 nL/min. Mass spectrometric acquisition was performed using either MS^E mode at 0.7, 1.0, or 1.3 Hz or continuum mode (only low energy scans) at 2.0 and 3.1 Hz on one day. After assessing that data, it was determined that faster acquisition should be investigated and continuum acquisitions at 2.4, 3.1, 4.5, and 5.9 Hz were performed. All data were acquired in duplicate for each day.

By plotting the calculated peak capacity as a function of acquisition rate in Figure 5.16, it could be seen that measured peak capacity was influenced by acquisition rate. Additionally, the overlap of the data between the two days and the low observed standard deviation speaks to the repeatability of the measurements taken. It can be seen that low acquisition rates adversely impact the measured peak capacity up to a point (for this separation around 2.4 Hz) after which point faster acquisition does not improve calculated peak capacity. An important observation from this study is that the MS^E acquisition rates (0.7, 1.0, and 1.3 Hz) were too low to show the full performance of this column using the given gradient conditions.

Following from these observations, it would be useful to generalize the findings to understand what the practical minimum sampling frequency would be to allow accurate characterization of a separation. This was accomplished by determining the average 4σ peak width for this separation from the higher frequency acquisition data, which was 4.7 seconds. This width was used as a normalization factor to convert sampling frequency to calculated points per 4σ peak width. Figure 5.17 shows peak capacity as a function of calculated points per 4σ peak width. This plot shows that peak capacity plateaus at around 12 points per 4σ peak width. This will allow these findings to be generalized to separations with differing characteristic peak widths (such as those with differing gradient steepnesses). This finding also suggests that using
the standard 0.6 second scan MS\textsuperscript{E} acquisition may result in noticeable artificial peak broadening for peaks narrower than 17 seconds (0.28 minutes).

5.3.5 Second Gradient Column Comparison

After implementing the improved experimental parameters outlined above, it was determined that another, more thorough comparison between the differing particle architectures and bondings should be carried out. The improvements in experimental setup would hopefully allow for a more careful comparison of the columns themselves, without undue impact from experimental biases. This work was also motivated by the acquisition of BEH C18+ particles, which allows a comparison between all of the possible particle architecture/bonding combinations: BEH C18, BEH C18+, Cortecs C18, and Cortecs C18+.

5.3.5.1 Retention Differences

As with the preceding study, one striking impact of the particle types were the differences in retention. An example of this can be seen in Figure 5.18, where for the same gradient length (33 \textmu L) and sample loading (60 fmol) retention differences are apparent. Within each particle architecture, the C18 bonding showed greater retention than the C18+ bonding. Additionally, it can be seen that retention is similar for a specific bonding between the different particle architectures. For all the phases however, there was adequate preconcentration of the analytes of interest. It can also be seen that the peaks show reasonably good peak shape as compared to the previous column comparison study, due to the improvements listed in the preceding section.

To look at the retention relationships more closely, it was found useful to plot the retention times of the monitored analytes on two differing phases against each other. An example of this can be seen in Figure 5.19, where the analytes’ retention times on BEH C18+ are plotted against those on BEH C18. This was done for separations of 60 fmol alcohol
dehydrogenase tryptic digest standard using a 33 µL 0.5-40% acetonitrile gradient at 40 °C. It can be seen that the two phases show similar retention characteristics (regression line slope near 1) but the C18+ bonding shows systematically lower retention (-5.6 min y-intercept). This is in line with the previous retention observation and external investigations previous findings that peptides have overall lower retention on the charged phase. Additionally, though the overall elution order is similar, it can be seen from Figure 5.19 that some closely eluting peaks show differences in selectivity between the two phases. While neither phase shows itself to be systemically superior, the slight difference in selectivity is worthy of note.

To expand this comparison to encompass all of the columns used in this study, Figure 5.20 shows the analytes’ retention on several different columns plotted against their retention on BEH C18. This was again done for separations of 60 fmol alcohol dehydrogenase tryptic digest standard using a 33 µL 0.5-40% acetonitrile gradient at 40 °C. It can be seen that selectivity appears to be associated with bonding and not particle architecture, as the plots for the C18+ particles show a great deal of similarity and the plot for Cortecs C18 shows very strong agreement (slope of 1.01, y-intercept of -0.9 minutes, most points falling close to the linear regression line) with the retention seen on BEH C18. Also it is seen that the overall retention of the C18+ bonding is lower than that for C18 bonding, with the BEH and Cortecs C18+ particles having y-intercepts of -5.6 and -6.1 minutes respectively. Overall, retention between the four columns is similar, but some differences are noticeable.

5.3.5.2 Impact of Gradient Length

Another aspect of these columns’ separation performance that was assessed in this study was the dependency of peak capacity on gradient length. It has been shown previously for BEH C18 particles that peak capacity increases when going to longer, shallower gradients. It was of
interest to compare the performance of all of these columns to see if either particle architecture or bonding had an impact on this behavior. Overall, it can be seen that all of the particle types exhibit very similar behavior in terms of increase in peak capacity with respect to increasing gradient length. In fact none of the lines ever cross, suggesting that the relative efficacy between the particles does not depend upon the length of gradient employed, so long as it is kept constant.

With all of the efficiency data laid out in Figure 5.21 it is a good opportunity to assess the relative peak capacities between the various particle types. First looking at the impact of bonded phase, it can be seen for both the BEH and the Cortecs particles that the C18+ bonding resulted in higher measured peak capacity. This is perhaps due to the action of the surface charge to decrease unwanted secondary interactions between the analyte and the particle surface. This increase in peak capacity was due to a decrease in peak width with the use of this bonding. As seen in Figure 5.21, the retention windows for all of the columns were very similar so differences in peak capacity reflect differences in peak width. Looking at the impact of particle architecture, the fully porous BEH particles outperform the superficially porous Cortecs particles, seen for both bonding types.

5.3.5.3 Impact of Sample Loading

When assessing the differences between the particles for peptide separations, the impact of sample loading on peak capacity is of interest. For real-world samples, peptides are often present at a wide range of concentrations, leading often to high loadings for at least some analytes. Building on the loading results from the first study, three loading levels were studied in this experiment. The results are shown in Figure 5.22 where peak capacity is plotted as a function of sample loading. At low sample loading, 20 fmol alcohol dehydrogenase tryptic digest standard, for each particle architecture there is good agreement between the peak
capacities measured for each bonding of that specific particle architecture. There remains a difference between the particle architectures at low loading, with the BEH particles providing higher peak capacity, suggesting that there is some property inherent to the fully porous BEH architecture that provides higher peak capacity than the Cortecs superficially porous architecture. Moving to higher sample loading shows a marked decrease in observed peak capacity for the C18 bonded particles of both architectures but only small decreases in peak capacity for the C18+ bonded particles. This impact is so significant that at the highest loading level the Cortecs C18+ particles noticeably outperform the BEH C18 particles, an inversion of performance order from the low loading level. This suggests that at high loading the bonding on the particle has a greater impact on the peak capacity than does the particle architecture itself for the investigated particles. It is also overall striking to see the similarity in the response of either bonding to increased sample loading between the two architectures, lending further support to the observation that bonding is the more important parameter in terms of column loadability.

5.3.5.4 Impact of Pore Size

When determining the utility of these differing particles to the separation of peptides, it was important to take pore size into consideration. Many particles specifically tailored to peptide separation exist, often with pore sizes greater than those employed in this study. One interest in the current study was to investigate if there is a detrimental impact on the separations based upon the difference in reported pore size for the two materials (90 and 130 Å for the Cortecs and BEH material, respectively). To look into this, the ratio of peak width between the two particle architectures was plotted against the molecular weight (MH+, shown in Table 5.1) of the analyte reported by MassLynx. This data was obtained from duplicate separations of 60 fmol alcohol dehydrogenase tryptic digest standard employing a 33 µL 0.5-40% acetonitrile
gradient at 40 °C. If pore size were playing a role in peak broadening through restricted diffusion, the ratio of Cortecs to BEH peak width should increase as a function of increasing molecular weight of the analyte. The data is shown in Figure 5.23 for the C18 bonded particles, and in Figure 5.24 for the C18+ particles. As can be seen from these plots, there is no strong correlation between analyte molecular weight and differential broadening between the two particle architectures. This surprisingly suggests that for these separations the difference in pore size between the particles is not meaningfully impacting peak width. It should be noted that these findings are over a relatively narrow window of molecular weights, but nonetheless cover a useful range for peptides.

5.3.6 Gradient Separation of Intact Proteins

The next analyte class of consideration was intact proteins, here illustrated by the separation of an *E coli* whole cell lysate on columns created from each of the four particles types. Example chromatograms from the separation of 250 ng of protein on column with a 25 µL gradient from 0.5-60% acetonitrile at 40 °C on each of the different columns is shown in Figure 5.25. Two main differences were apparent within this set of chromatograms, retention and peak sharpness. In terms of retention, the C18+ bonded particles of both particle morphologies appear to show earlier elution than do the C18 bonded particles. The retention within each phase between the particle morphologies appears to be similar. In terms of peak sharpness, the Cortecs material appears to show sharper peaks than does the BEH material. To get a more quantitative look at how different parameters affect the separation of intact *E coli* proteins, peak capacity measurements were carried out. The first analysis was performed using the separations carried out on the BEH C18 material at different loading and gradient conditions. The results are shown in Table 5.7. It can be seen that the longer gradient time increased the observed peak capacity.
for the separation at both loading conditions. Lower sample loading also had an impact on peak capacity, with the lower loading always showing a higher peak capacity than the higher loading. This is unsurprising and agrees with the trend seen for the particles with peptides. The next comparison assessed the peak capacities obtained when doing the same separation on different columns. All of the columns had 250 ng of sample injected and were separated using a 25 µL 0.5-60% acetonitrile gradient with 0.2% formic acid added. The column was maintained at 40 °C for all separations. These are the same gradient conditions shown for the example chromatograms in Figure 5.25. The results of the peak capacity comparison are shown in Table 5.8. It should be noted that the EMG fits appeared to model the BEH peaks better than the Cortecs peaks. The EMG fitting results suggested that peak capacity stayed consistent across the different columns. This contradicts the observation that the Cortecs peaks appear sharper by visual inspection.

To try to better understand the differences between separations of intact proteins performed using capillary columns packed with the different particle types, another method was employed. The same raw data used for the EMG investigation was analyzed in this study. This method involved the utilization of deconvoluted protein mass base peak intensity chromatograms for each of the separations using the autoME data. Example chromatograms for each of the particle types employed are shown in Figure 5.26. Due to computational limitations, only pieces of each of the chromatograms were processed using the autoME program (those judged to hold the majority of the protein peaks) and this is seen in the figure. It is apparent that the peak profiles for the intense peaks are more easily discernible using this presentation format than when showing the TIC as in Figure 5.25. It should be noted that the Cortecs C18 column had more issues with spray stability than the other columns, seen by jagged features on some peaks,
which sometimes resulted in multiple thinner peaks being fit by the program to a single broader peak. This was not manually corrected for, and should not have a substantial impact upon the median peak width, though it increases the reported number of peaks identified by the program for this column. An initial comparison of peak capacity, separation window, and number of peaks found using 2 replicate analyses for each column is shown in Table 5.9, using all peaks with intensity greater than 6000. It can be seen that substantially larger peak capacities were obtained using the capillary columns packed with Cortecs superficially porous particles then with the columns packed with BEH fully porous particles. This would be in keeping with the expected efficiency improvements for the separation of large molecular weight analytes using superficially porous particles. It was also seen that the C18+ bonding appeared to improve the peak capacities observed relative to the C18 bonded particles of either type. It is also seen that many more peaks are fit by the program for the separations using the Cortecs particles than the BEH particles.

A further refinement of the methodology was performed to standardize the peak window, similar to what was performed previously for the peptide separation work. The 12518 Da peak was defined as the first peak, and the 32903 Da peak was defined as the last peak for all of the separations. These peaks are indicated in Figure 5.26. From this figure it can be seen that for all the particle types except the Cortecs C18, this definition of the separation window resulted in the exclusion of the first few peaks. Further analysis of the chromatograms justifies this treatment, since the defined window is when most of the proteins actually elute. To allow for more straightforward visual comparison between the separations, the chromatograms from Figure 5.26 were horizontally translated to align all of the first peaks as defined by this method approximately line up, shown in Figure 5.27. From this it appears that the Cortecs particles
thinner peaks and that at least the separations using Cortecs C18+ particles has many more peaks eluting over the chromatogram than any of the other columns do. Using this defined window approach, a second comparison of peak capacity, separation window, and peaks identified was performed using the automated maximum-concavity program. The results are shown in Table 5.10. It can be seen that using this more careful method, the Cortecs particles show a noticeable advantage over the BEH material for the separation of intact proteins. The influence of bonded phase appears to be less pronounced than the influence of particle architecture for the separation of intact proteins, with the charged surface bonding showing a slight advantage. Separations using the Cortecs particles also appear to show a greater number of fit peaks than separations using the BEH material. This phenomenon is supported by visual inspection for at least the separation employing Cortecs C18+ particles in Figure 5.27. From this comparison between the particle types for the separation of intact proteins, the columns packed with Cortecs particles, and especially the column packed with Cortecs C18+ particles, showed advantages over columns packed with the other types of particles.

5.4 Conclusions

In conclusion, both the different particle morphologies and bonding types played a role in determining the separation characteristics of the particles. From a column creation standpoint, the particles appear to pack in a similar way, or at least the standard acetone packing procedure worked well for all particle types. This suggests that a packing study to improve the packing of any one of the particles would be feasible. In terms of the isocratic separation of small molecules, it would be interesting to compare the separation characteristics of the differing particle types to basic small molecule analytes, which may highlight the characteristics of the bonding more than was seen in this study.
The separation of peptides was where many of the differences between the particles were seen. It is currently unknown why the BEH particles outperformed the Cortecs particles when stationary phase was analogous. It perhaps is due to the increased surface area of these particles and better sample preconcentration, as more than a column volume of sample is injected onto the column. The other major takeaway from this study was that the charged surface bonding increased the loadability of the columns, and the effect was very similar for both of the particle types. Using this bonding would be especially advantageous for peptide proteomic studies where many analytes will be inevitably overloaded in order to improve sequence coverage and protein identifications. The peptide results also indicate that the influence of particle morphology and particle bonding are qualitatively additive, suggesting that BEH C18+ particles (CSH particles) may be the best for use in proteomic studies. For high loading peptide samples, it appears that the second best column may be the Cortecs C18+ column due to its improved loadability due to its charged surface.

The initial protein results on BEH C18 particles using EMG fitting indicate that gradient length and sample size have an impact on the separation of E. coli intact proteins. This result is unsurprising. Using the maximum-concavity analysis of the deconvoluted protein mass base peak chromatograms, it was seen that differences existed between the columns packed with the differing particle types. The superficially porous Cortecs particles gave better protein separations than the fully porous BEH particles did, which fits with the theoretical expectation that superficially porous particles show their greatest utility for large molecular weight analyte separations. It was also of note that in some cases the charged surface C18 bonding showed improved peak capacity for the protein separations relative to the uncharged C18 bonding. Overall, these protein results were somewhat surprising because both of the particle
morphologies have pores smaller than those typically used for the separation of intact proteins, but still gave acceptable separations of intact proteins. This may be due to the high quality commercial bonding used for these particles. Bonding differences may explain the differences seen between the separation of the intact *E. coli* whole cell lysate on the superficially porous particles prepared in-house in Chapter 3 and the particles employed in this chapter.

Investigations of materials with wide pore diameter but with high quality commercial bonding may result in even higher peak capacities for intact proteins than those seen in this investigation.
### 5.5 Tables

<table>
<thead>
<tr>
<th>Observed m/z</th>
<th>Molecular Weight (MH+) (Da)</th>
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<tbody>
<tr>
<td>549.29</td>
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</tr>
<tr>
<td>844.45</td>
<td>844.45</td>
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<td>567.29</td>
<td>1133.58</td>
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<td>1197.58</td>
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<td>723.38</td>
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<td>675.35</td>
<td>675.36</td>
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<td>507.30</td>
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<td>598.33</td>
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<td>484.74</td>
<td>968.48</td>
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<td>724.41</td>
<td>1447.80</td>
</tr>
<tr>
<td>950.48</td>
<td>950.48</td>
</tr>
</tbody>
</table>

Table 5.1: The 16 ions used for peak capacity calculations in this study in elution order on BEH C18.
<table>
<thead>
<tr>
<th>Column Identifier</th>
<th>( h_{\text{min}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortecs C18 – 1</td>
<td>1.89</td>
</tr>
<tr>
<td>Cortecs C18 – 2</td>
<td>2.40</td>
</tr>
<tr>
<td>Cortecs C18+ – 1</td>
<td>2.46</td>
</tr>
<tr>
<td>Cortecs C18+ – 2</td>
<td>2.20</td>
</tr>
<tr>
<td>Cortecs C18+ – 3</td>
<td>2.14</td>
</tr>
<tr>
<td>Cortecs C18+ – 4</td>
<td>2.25</td>
</tr>
<tr>
<td>BEH C18 – 1</td>
<td>1.74</td>
</tr>
<tr>
<td>BEH C18 – 2</td>
<td>1.81</td>
</tr>
<tr>
<td>BEH C18 – 3</td>
<td>1.35</td>
</tr>
<tr>
<td>BEH C18+ – 1</td>
<td>1.57</td>
</tr>
</tbody>
</table>

Table 5.2: Minimum reduced plate height values for hydroquinone for all of the investigated columns. The superficially porous Cortecs particles were evaluated using a 60/40 v/v water/acetonitrile mobile phase while the fully porous BEH particles were evaluated using a 50/50 v/v water/acetonitrile mobile phase, both with 0.1% trifluoroacetic acid added. All columns were between 49 and 51 cm long and were operated at ambient temperature.
<table>
<thead>
<tr>
<th>Column Identifier</th>
<th>Hydroquinone k’</th>
<th>4-methyl Catechol k’</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortecs C18 – 1</td>
<td>0.22</td>
<td>0.70</td>
</tr>
<tr>
<td>Cortecs C18 – 2</td>
<td>0.21</td>
<td>0.69</td>
</tr>
<tr>
<td>Cortecs C18+ – 1</td>
<td>0.24</td>
<td>0.78</td>
</tr>
<tr>
<td>Cortecs C18+ – 2</td>
<td>0.25</td>
<td>0.79</td>
</tr>
<tr>
<td>Cortecs C18+ – 3</td>
<td>0.25</td>
<td>0.80</td>
</tr>
<tr>
<td>Cortecs C18+ – 4</td>
<td>0.24</td>
<td>0.77</td>
</tr>
<tr>
<td>BEH C18 – 1</td>
<td>0.22</td>
<td>0.55</td>
</tr>
<tr>
<td>BEH C18 – 2</td>
<td>0.21</td>
<td>0.55</td>
</tr>
<tr>
<td>BEH C18 – 3</td>
<td>0.21</td>
<td>0.54</td>
</tr>
<tr>
<td>BEH C18+ – 1</td>
<td>0.23</td>
<td>0.59</td>
</tr>
</tbody>
</table>

Table 5.3: Comparison of retention factor between the four studied particle types. The superficially porous Cortecs particles were evaluated using a 60/40 v/v water/acetonitrile mobile phase while the fully porous BEH particles were evaluated using a 50/50 v/v water/acetonitrile mobile phase, both with 0.1% trifluoroacetic acid added. Retention factors were obtained by plotting retention factor as a function of pressure and extrapolating to retention factor at atmospheric pressure. Retention factors are given for the first eluting (hydroquinone) and last eluting (4-methyl catechol) analytes in the separations.
<table>
<thead>
<tr>
<th>Particle Type</th>
<th>Peak Width (min)</th>
<th>Separation Window (min)</th>
<th>Peak Capacity</th>
</tr>
</thead>
<tbody>
<tr>
<td>BEH C18</td>
<td>0.209 ± 0.003</td>
<td>34.8 ± 0.0</td>
<td>168 ± 2</td>
</tr>
<tr>
<td>Cortecs C18</td>
<td>0.237 ± 0.000</td>
<td>35.5 ± 1.2</td>
<td>151 ± 5</td>
</tr>
<tr>
<td>Cortecs C18+</td>
<td>0.174 ± 0.011</td>
<td>29.9 ± 0.6</td>
<td>174 ± 7</td>
</tr>
</tbody>
</table>

Table 5.4: Peak width, elution window, and peak capacity for the three columns. The peak widths represent the arithmetic mean of the ions used to calculate peak capacity. All separations were performed with a 19 µL 4-40% acetonitrile gradient at 40 °C. The sample was 200 fmol alcohol dehydrogenase tryptic digest standard injected on column.
<table>
<thead>
<tr>
<th>Particle Type</th>
<th>Peak Width (min) 20 fmol on Column</th>
<th>Peak Width (min) 200 fmol on Column</th>
<th>Percent Increase in Peak Width</th>
</tr>
</thead>
<tbody>
<tr>
<td>BEH C18</td>
<td>0.171 ± 0.006</td>
<td>0.209 ± 0.003</td>
<td>22 %</td>
</tr>
<tr>
<td>Cortecs C18</td>
<td>0.168 ± 0.006</td>
<td>0.237 ± 0.000</td>
<td>41 %</td>
</tr>
<tr>
<td>Cortecs C18+</td>
<td>0.155 ± 0.005</td>
<td>0.174 ± 0.011</td>
<td>12 %</td>
</tr>
</tbody>
</table>

Table 5.5: Calculated 4σ peak width comparison between the high (200 fmol) and low (20 fmol) loading of alcohol dehydrogenase tryptic digest standard on the three columns. All separations were performed using a 19 µL 4-40% acetonitrile gradient at 40 °C. The peak widths represent the arithmetic mean of the ions used to calculate peak capacity. The last column in the table shows the percent increase in calculated peak width between the two loading levels.
<table>
<thead>
<tr>
<th>Particle Type</th>
<th>Peak Capacity 20 fmol on Column</th>
<th>Peak Capacity 200 fmol on column</th>
<th>Percent Decrease in Peak Capacity</th>
</tr>
</thead>
<tbody>
<tr>
<td>BEH C18</td>
<td>205 ± 8</td>
<td>168 ± 2</td>
<td>18 %</td>
</tr>
<tr>
<td>Cortecs C18</td>
<td>207 ± 9</td>
<td>151 ± 5</td>
<td>27 %</td>
</tr>
<tr>
<td>Cortecs C18+</td>
<td>189 ± 5</td>
<td>174 ± 7</td>
<td>8 %</td>
</tr>
</tbody>
</table>

Table 5.6: Calculated peak capacity comparison between the high (200 fmol) and low (20 fmol) loading of alcohol dehydrogenase tryptic digest on the three columns. All separations were performed using a 19 µL 4-40% acetonitrile gradient at 40 °C. The last column in the table shows the percent decrease in peak capacity between the two loading levels.
Table 5.7: Calculated peak capacity for separations of *E. coli* whole cell lysate on the 50.2 cm X 75 µm BEH C18 column at differing sample loading and gradient conditions. All gradients were 0.5-60% acetonitrile with 0.2% formic acid in the mobile phase, and conducted at 40 °C. All separations were done in duplicate.
<table>
<thead>
<tr>
<th>Particle Type</th>
<th>Peak Capacity</th>
</tr>
</thead>
<tbody>
<tr>
<td>BEH C18</td>
<td>80 ± 3</td>
</tr>
<tr>
<td>BEH C18+</td>
<td>80 ± 2</td>
</tr>
<tr>
<td>Cortecs C18</td>
<td>83 ± 3</td>
</tr>
<tr>
<td>Cortecs C18+</td>
<td>80 ± 10</td>
</tr>
</tbody>
</table>

Table 5.8: Peak capacities for separations of *E. coli* whole cell lysate on capillary columns packed with the differing particle types using the EMG fitting method. In this comparison, 250 ng of protein was injected onto each column and was separated with 25 µL 0.5-60% acetonitrile gradient, with 0.2% formic acid in the mobile phase. All separations were carried out in duplicate at 40 °C.
<table>
<thead>
<tr>
<th>Particle Type</th>
<th>Peak Capacity</th>
<th>Median Peak Width (s)</th>
<th>Separation Window (min)</th>
<th>Number of Peaks</th>
</tr>
</thead>
<tbody>
<tr>
<td>BEH C18</td>
<td>144 ± 6</td>
<td>16.7 ± 1.2</td>
<td>39.7 ± 1.0</td>
<td>47 ± 2</td>
</tr>
<tr>
<td>BEH C18+</td>
<td>161 ± 2</td>
<td>14.4 ± 0.6</td>
<td>38.5 ± 1.2</td>
<td>58 ± 3</td>
</tr>
<tr>
<td>Cortecs C18</td>
<td>193 ± 12</td>
<td>12.3 ± 1.0</td>
<td>39.4 ± 0.9</td>
<td>65 ± 1</td>
</tr>
<tr>
<td>Cortecs C18+</td>
<td>239 ± 21</td>
<td>11.2 ± 0.5</td>
<td>44.6 ± 5.7</td>
<td>86 ± 3</td>
</tr>
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</table>

Table 5.9: Initial comparison between the separation of 250 ng of intact protein from *E. coli* whole cell lysate using the automated maximum-concavity method on deconvoluted protein mass base peak intensity chromatograms for capillary columns packed with different particles. The separation was performed using a 25 µL 0.5-60% acetonitrile gradient with 0.2% formic acid added. The separation was performed in duplicate at 40 °C.
<table>
<thead>
<tr>
<th>Particle Type</th>
<th>Peak Capacity</th>
<th>Median Peak Width (s)</th>
<th>Separation Window (min)</th>
<th>Number of Peaks</th>
</tr>
</thead>
<tbody>
<tr>
<td>BEH C18</td>
<td>126 ± 9</td>
<td>16.9 ± 1.4</td>
<td>35.4 ± 0.5</td>
<td>45 ± 1</td>
</tr>
<tr>
<td>BEH C18+</td>
<td>138 ± 1</td>
<td>14.6 ± 0.2</td>
<td>33.6 ± 0.8</td>
<td>57 ± 2</td>
</tr>
<tr>
<td>Cortecs C18</td>
<td>193± 12</td>
<td>12.3 ± 1.0</td>
<td>39.4 ± 0.9</td>
<td>65 ± 1</td>
</tr>
<tr>
<td>Cortecs C18+</td>
<td>195 ± 18</td>
<td>11.7 ± 0.0</td>
<td>38.1 ± 3.6</td>
<td>81 ± 1</td>
</tr>
</tbody>
</table>

Table 5.10: Comparison between the separations of 250 ng of intact protein from *E. coli* whole cell lysate using the separation window defined at the beginning by the 12518 Da peak and at the end by the 32903 Da peak. The data analysis was done using the automated maximum-concavity method on deconvoluted protein mass base peak intensity chromatograms. The separation was performed using a 25 µL 0.5-60% acetonitrile gradient with 0.2% formic acid added. The separations were performed in duplicate at 40 °C.
5.6 Figures

Figure 5.1: Gradient profile employed for the first column comparison. Note that the gradient is loaded from right to left, for sample elution from the column the gradient is played back from left to right.
Figure 5.2: Example chromatograms from equivalent alcohol dehydrogenase tryptic digest separations on (A) BEH C18 and (B) Cortecs C18+ showing the peaks used to calculate separation window. Data shown are for the low energy scans.
Figure 5.3: Sample gradient profile employed for the second column comparison. This gradient is the intermediate length gradient employed, 33.0 μL. Note that the gradient is loaded from right to left, for sample elution from the column the gradient is played back from left to right.
Figure 5.4: Sample gradient profile employed for the separation of intact proteins, in this case the 25 μL gradient. Note that the gradient is loaded from right to left, for sample elution from the column the gradient is played back from left to right.
Figure 5.5: Example chromatogram showing the separation of intact proteins from *E. coli* whole cell lysate on a 50.2 cm X 75 µm ID column packed with 1.9 µm BEH C18 particles. For this separation, 250 ng of sample was placed on the column and was then separated using a 25 µL 0.5 to 60% acetonitrile gradient, with 0.2% formic acid in the mobile phase. Column was held at 40 °C during the separation. Deconvoluted masses used for separation window determination (11185 and 32904 Da) and peak width determination (6856, 8325, 11185, 11673, and 21135 Da) are indicated.
Figure 5.6: Particle size distributions as imaged by SEM for the Cortecs C18 (A, n=142) and for the Cortecs C18+ (B, n=117) particles.
Figure 5.7: Example chromatograms obtained from the four particle types: (A) BEH C18, (B) BEH C18+, (C) Cortecs C18, and (D) Cortecs C18+. For all the separations, the peaks are, in ascending elution order: ascorbic acid (dead time marker), hydroquinone, resorcinol, catechol, and 4-methyl catechol. Note that the x and y axes change to account for differences in the specific separations. Separations on the fully porous BEH particles were performed in 50/50 water/acetonitrile while separations performed on the superficially porous Cortecs particles were performed in 60/40 water/acetonitrile, with both mobile phases having 0.1% trifluoroacetic acid added. Separations were performed at ambient temperature.
Figure 5.8: Isocratic performance comparison between capillary columns packed with the superficially porous Cortecs particles showing fits to the reduced van Deemter equation. The columns packed with C18 particles are shown as the solid markers and lines while the particles functionalized with the C18+ bonding are shown with the open markers and dotted lines. Performance is shown for the first retained compound, hydroquinone. All of the columns were evaluated at ambient temperature using a 60/40 v/v water/acetonitrile mobile phase with 0.1% trifluoroacetic acid added.
Figure 5.9: Isocratic performance comparison between capillary columns packed with fully porous BEH particles showing fits to the reduced van Deemter equation. The columns packed with the C18 functionalized particles are shown with the solid markers and lines while the columns packed with particles functionalized with the C18+ bonding are represented by the open markers and dotted lines. Performance is shown for the first retained compound, hydroquinone. All of these columns were evaluated at ambient temperature using a 50/50 v/v water/acetonitrile mobile phase with 0.1% trifluoroacetic acid added.
Figure 5.10: Separation comparison between the three columns employed. Separations are shown on (A) a BEH C18 column, (B) a Cortecs C18 column, and (C) a Cortecs C18+ column. All separations were performed with a 19 µL 4-40% acetonitrile gradient at 40 °C with a 200 fmol sample of alcohol dehydrogenase tryptic digest standard. The m/z 817 peak is noted in each chromatogram to illustrate retention differences between the columns. Data is shown from the low energy scans.
Figure 5.11: Separation comparison between the (A) low (20 fmol) and (B) high (200 fmol) loadings of alcohol dehydrogenase tryptic digest on the Cortecs C18 column. All separations were performed with a 19 μL 4-40% acetonitrile gradient at 40 °C. Data shown is from the low energy scans.
Figure 5.12: Impact of sample diluent on retention of early eluting peaks with a column packed with Cortecs C18+ particles. All separations performed with a 4-40% acetonitrile with 0.1% formic acid added. The 200 fmol alcohol dehydrogenase tryptic digest standard was prepared in either (A) 3% acetonitrile, (B) 0.5% acetonitrile, or (C) 0% acetonitrile with 0.1% formic acid added. Data is shown for the low energy scans.
Figure 5.13: Impact of gradient initial conditions on peak retention with a column packed with Cortecs C18+ particles. All separations were performed with 200 fmol alcohol dehydrogenase tryptic digest standard prepared in 100% water with 0.1% formic acid added. Initial gradient conditions were varied between (A) 3%, (B) 0.5%, and (C) 0% acetonitrile with the balance made up with water, with 0.1% formic acid added. All gradients ended at 40% acetonitrile. Data is shown for the low energy scans.
Figure 5.14: Schematic cutaway drawing of the pigtail-electrospray tip connection for the Waters QTOF Premier instrument nanospray source. (A) shows the way the connection is made, with a 20 μm ID capillary ‘pigtail’ coming from the column (at left) and the 20 μm ID capillary electrospray tip (at right) both coming through PEEK fingertight nuts and forming a connection within the metal union, shown as a dark square. Panel B) depicts a properly made connection, with both capillaries connected end-to-end within the metal union with zero dead volume.
Figure 5.15: Example chromatograms from consecutive, identical runs. These are the same set of peaks for each separation. With the x-axis slightly shifted for (A) to better line up the peaks. (A) shows a separation with ceramic-cut capillaries used for the pigtail-spraytip connection. (B) shows the identical separation with commercially cut components used for the pigtail-spraytip connection.
Figure 5.16: Impact of data acquisition rate on calculated peak capacity. Data from the first day are shown with open circles, data from the second day shown with closed circles. All points were taken in duplicate, error bars are ± 1 standard deviation.
Figure 5.17: Impact of calculated points per 4σ peak width on calculated peak capacity. This assumes a 4.7 4σ peak width, consistent with the observed data. Data from the first day are shown with open circles, data from the second day shown with closed circles. All points were taken in duplicate, error bars are ± 1 standard deviation.
Figure 5.18: Comparison between the separations on the four columns employed running 33.0 μL 0.5-40% acetonitrile gradients, 60 fmol alcohol dehydrogenase tryptic digest standard on column. The separation is shown on a (A) BEH C18 column, (B) BEH C18+ column, (C) Cortecs C18 column, and (D) Cortecs C18+ column. First 30 minutes removed to allow emphasis on actual separation space.
Figure 5.19: Comparison between the retention times of the monitored analytes on the BEH C18+ column and the BEH C18 column. All points were taken in duplicate, error bars are ± 1 standard deviation. No alteration has been made to the data to compensate for slight variations run-to-run in flow rate, etc. The sample was 60 fmol alcohol dehydrogenase tryptic digest standard, and it was separated using a 33 µL 0.5-40% acetonitrile gradient at 40 °C.
Figure 5.20: Comparison between retention times of the monitored analytes on the BEH C18+ column (red squares), Cortecs C18 column (green triangles), and Cortecs C18+ (blue inverted triangles) compared to their retention times on the BEH C18 column. All points were taken in duplicate, error bars are ± 1 standard deviation. No alteration has been made to the data to compensate for slight variations run-to-run in flow rate, etc. The sample was 60 fmol alcohol dehydrogenase tryptic digest standard, and it was separated using a 33 µL 0.5-40% acetonitrile gradient at 40 °C.
Figure 5.21: Relation between measured peak capacity and measured elution window for the four column types: BEH C18 (black circles), BEH C18+ (red squares), Cortecs C18 (green triangles), and Cortecs C18+ (blue inverted triangles). The three elution windows are created by 16.5, 33.0, and 66.5 μL gradients of 0.5-40% acetonitrile, all with 60 fmol injections of alcohol dehydrogenase tryptic digest standard. All points were taken in duplicate, error bars are ± 1 standard deviation.
Figure 5.22: Relation between measured peak capacity and sample loading for the four column types: BEH C18 (black circles), BEH C18+ (red squares), Cortecs C18 (green triangles), and Cortecs C18+ (blue inverted triangles). Either 20, 60, or 180 fmol of alcohol dehydrogenase tryptic digest standard was injected on column using a 2 μL injection. All separations were carried out using a 33.0 μL 0.5-40% acetonitrile gradient. All points were taken in duplicate, peak capacity error bars are ± 1 standard deviation.
Figure 5.23: Ratio of peak widths of selected analytes between the Cortecs and BEH material as a function of molecular weight (MH+) of the analyte on the C18 stationary phase. This work used separations of 60 fmol of alcohol dehydrogenase tryptic digest standard separated using a 33 µL 0.5-40% acetonitrile gradient at 40 °C.
Figure 5.24: Ratio of peak widths of selected analytes between the Cortecs and BEH material as a function of molecular weight (MH+) of the analyte on the C18+ stationary phase. This work used separations of 60 fmol of alcohol dehydrogenase tryptic digest standard separated using a 33 µL 0.5-40% acetonitrile gradient at 40 °C.
Figure 5.25: Example chromatograms from the separation of intact proteins from *E. coli* whole cell lysate (250 ng) on each of the column types using a 25 µL 0.5-60% acetonitrile gradient with 0.2% formic acid in the mobile phase at 40 °C. The columns were, in order from top to bottom, (A) BEH C18, (B) BEH C18+, (C) Cortecs C18, and (D) Cortecs C18+. All of the chromatograms except the Cortecs C18+ column have been vertically offset to provide ease of viewing.
Figure 5.26: Deconvoluted protein mass base peak intensity chromatograms for separations using capillary columns packed with the four particle types employed. These were created using the same separations shown in Figure 5.26. The first and last peaks used for the set analysis window are indicated by arrows. All show the separation of intact proteins (250 ng) from E. coli whole cell lysate using a 25 µL 0.5-60% acetonitrile gradient with 0.2% formic acid in the mobile phase at 40 °C. The columns were, in order from top to bottom, (A) BEH C18, (B) BEH C18+, (C) Cortecs C18, and (D) Cortecs C18+. All chromatograms except for the Cortecs C18+ have been vertically offset for ease of viewing.
Figure 5.27: Deconvoluted protein mass base peak intensity chromatograms for separations using capillary columns packed with the four particle types employed. These are the same chromatograms shown in Figure 5.27 but translated to approximately match the retention of the first peak used (12518 Da) for the second peak capacity comparison. This allows easier visual comparisons of the separations. The chromatograms have been horizontally translated but not stretched or shrunk in either direction.
5.7 References


