Published in final edited form as:

Electrophoresis. 2008 September ; 29(18): 3842–3849. doi:10.1002/elps.200800142.

Retention of Proteins and Metalloproteins in Open Tubular Capillary Electrochromatography with Etched Chemically Modified Columns

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Abstract

Etched chemically modified capillaries with two different bonded groups (pentyl and octadecyl) are compared for their migration behavior of several common proteins and metalloproteins as well as metalloproteinases. Migration times, efficiency and peak shape are evaluated over the pH range of 2.1 to 8.1 to determine any effects of the bonded group on the electrochromatographic behavior of these compounds. One goal was to determine if the relative hydrophobicity of the stationary phase has a significant effect on proteins in the open tubular format of capillary electrochromatography as it does in HPLC. Reproducibility of the migration times is also investigated.

Keywords

Open tubular CEC; C5 bonded phase; metalloproteins

Introduction

Capillary electrochromatography (CEC) is an electrophoretic method that utilizes features of both electrophoresis and liquid chromatography [1,2]. Solutes are moved through the column by application of high voltage to buffer reservoirs at the two ends of the filled capillary as in HPCE. Chromatographic interactions, such as those found in HPLC, are produced due to a stationary phase within the column. In this investigation, the stationary phase is attached to the inner wall of column and thus this particular mode is referred to as open tubular capillary electrochromatography (OTCEC). A significant disadvantage of this approach is the low phase ratio of the bonded material because of the small surface area available for attaching the stationary phase. Both low loadability of the sample and insufficient chromatographic effects (low k' values) are often encountered in OTCEC because of the small amount of stationary phase and the relatively long distance the solute must travel in order to interact with the bonded material. In addition, efficiencies (N) are typically lower than found in CE but higher than in HPLC.

An alternate OTCEC separation medium has been developed by etching the inner wall of a fused silica capillary at a temperature of 300 or 400 °C in the presence of ammonium hydrogen difluoride (NH_4HF_2) for three to four hours. Under these conditions, the surface area of the inner wall can be increased by a factor of 1000 or more and radial extensions of up to 5 μm in length can also be created [3,4]. This process can partially alleviate the low capacity of the bare capillary and shorten the distance solutes must travel to interact with a stationary phase attached to the etched surface. In addition, elements from the etching reagent, nitrogen and fluoride, are also incorporated into the new surface matrix [5]. Their presence decreases the strong adsorptive properties of the silanols thus making the new surface more biocompatible. Nitrogen also has a significant effect on the electrophoretic

behavior of the capillary. At low pH the nitrogen functional groups in the surface matrix become protonated giving an overall positive charge to the inner wall of the capillary. This results in reverse electroosmotic flow (EOF) under these conditions. As the pH is raised to less acidic conditions, the EOF passes through zero and then becomes cathodic due to the deprotonation of the nitrogens, ionization of the remaining silanols and the negatively charged fluoride species [5,6]. The presence of these additional elements in the surface matrix does not interfere with the subsequent chemical modification. Further enhancements in capillary performance can be obtained from the bonding procedure used to modify the etched surface. In order to substantially eliminate the effects of residual silanols, a silanization reaction is used to create a new surface composed primarily of hydride moieties [7,8]. In order to attach an organic group to the surface that will determine some of the selectivity of the capillary, a hydrosilation reaction is used [8,9].

The migration behavior of solutes on the etched chemically modified capillaries is a function of both chromatographic and electrophoretic effects. A number of experimental variables can be used to optimize the separation capabilities of the capillaries. These variables include the bonded phase moiety, pH, buffer components, amount and type of organic modifier in the buffer, and temperature [10–16]. Another essential feature of these capillaries is their durability and reproducibility. A number of studies have shown that the column lifetime is at least several hundred injections with many capillaries performing well after 300 – 400 analyses [13,17,18]. With respect to reproducibility, consecutive runs of a particular analyte show %RSD values less than one and less than two when an initial injection is compared to a result taken after one hundred or more subsequent analyses. Capillary to capillary reproducibility is also good with variations in the relative migration times (t_{R2}/t_{R1}) of two analytes on the order of one percent. These data suggest that all of the factors involved in the fabrication of the capillaries are reproducible and that the organic moiety bonded to the surface is stable as well.

The analysis of proteins and peptides by CEC has been studied extensively in recent years [19–23]. In this investigation a number of proteins, particularly metalloproteins, are tested on two types of etched chemically modified capillaries. These solutes were selected since a number of other types of proteins and peptides have been previously studied on these types of columns [4,6,10–18,24]. A standard commercially available etched C18 column is compared to a newly developed C5 column in order to determine if hydrophobic effects are as significant in this OTCEC format as is often encountered in the retention of proteins in HPLC.

Experimental

Materials

The fused silica capillaries used for making the C5 column had a 380 μm o.d. with a 50 μm i.d. inner channel and were obtained from Polymicro Technologies, Phoenix, Az, USA. The laboratory fabricated etched capillary with C5 modification had a total length (L) of 49.5 cm and an effective length (l) of 41.0 cm. The etched chemically modified capillaries were prepared as described previously [4,5]. Briefly the fused silica surface was etched with a 5% ammonium bifluoride/methanol solution for a total of 4 hours at elevated temperature (300–400 °C) in a gas chromatographic oven. A silica hydride layer was then covalently attached by reacting the etched surface with ~ 1 M triethoxysilane solution in dioxane with a hydrochloric acid catalyst (115 μmoles). The C5 (1-pentyne) was attached to the hydride via hydrosilation using hexachloroplatinic acid as a catalyst. 1-Pentyne was added to 2.0 mL of toluene and 100 μL of catalyst and heated to 70° for 1 hour. The solution was then passed through the capillary which was heated at 100°C for 24 hours. Additional solution was passed through the capillary each day for 4 days with the column being kept in the GC oven

at 100°C. At the end of the process the capillary was rinsed with toluene and methanol. Capillary dimensions for the etched C18 modified capillary (Microsolv Technology Corp., Eatontown, NJ, USA) were i.d. = 50 µm, L = 58.5.0 cm and l = 50.0 cm.

Deionized water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Ammonium bifluoride, the etching agent, was purchased from Sigma-Aldrich (St. Louis, MO/Milwaukee, WI, USA). Triethoxysilane (Gelest, Morrisville, PA, USA), 1-pentyne (GFS Chemicals, Columbus, OH, USA) and hexachloroplatinic acid (Sigma-Aldrich) were used for the modification of the inner walls of the capillary. The buffer materials were as follows: TRIS [tris [hydroxymethyl] amino methane], MES [(2-[N-morpholino] ethane sulfonic acid)], MOPS [(2-[N-morpholino] propane-sulfonic acid), L-histidine, GABA (gamma-amino butyric acid) and citric acid were purchased from Sigma-Aldrich; boric acid (J.T. Baker, Phillipsburg, NJ) and glacial acetic acid (Mallinckrodt, St. Louis, MO) were obtained in analytical reagent grade; and imidazole from Calbiochem (San Diego, CA). The solutes myoglobin, transferrin, human serum albumin, carbonic anhydrase, human IgG were purchased from Sigma-Aldrich and a series of recombinant matrix metalloproteases (MMPs) were obtained from BIOMOL International (Plymouth Meeting, PA, USA).

1.2 Instrumentation

The HPCE instrument used was an Agilent (Waldbronn, Germany) 3D Capillary electrophoresis instrument having a UV detector. The oven used for etching of capillaries was part of a Hewlett-Packard Model 5890 gas chromatograph. The GC oven was used for the control of the etching temperature and was modified so that multiple capillaries could be accommodated.

2.3 CEC Experiments

Buffers reagents were selected to produce low conductance. The following buffer compositions (diluted 1:10) and pH values were used in this study: pH 2.14, 0.3 mol/L H₃PO₄ and 0.19 mol/L TRIS; pH 3.00, 0.3mol/L citric acid and 0.25 mol/L β-alanine; pH 4.38, 0.3 mol/L acetic acid and 0.375 mol/L γ-amino butyric acid; pH 6.00, 0.3 mol/L MES and 0.21 mol/L l-histidine [l-α-amino-β-imidazole propionic acid]; pH 7.06, 0.3 mol/L MOPS] and 0.215 mol/L imidazole; pH 8.14, 0.1 mol/L TRIS and 0.15 mol/L boric acid. Injection was done by pressure at 50 mbar. Solute concentrations were approximately 1 mg/mL for all proteins.

Results and Discussion

OTCEC with etched chemically modified capillaries can be used for molecules ranging in size from small organic compounds up to large biomolecules such as proteins and peptides [25]. However only a limited number of proteins have been studied to date with this format and since CE has been proven to be a valuable technique for their analysis, further investigations to better understand the migration behavior of biomolecules with etched chemically modified columns are needed.

A major focus of this investigation was to determine if the length of the bonded alkyl chain on the etched capillary wall had any impact on either the migration behavior or peak shape of proteins. In order to examine this effect, capillaries modified with a standard octadecyl (C18) group were compared to those that had a pentyl (C5) moiety attached to the surface. It has already been demonstrated that etched capillaries have anodic EOF at low pH (< 4.5) and cathodic EOF as the pH is raised above this point [5]. One comparison between the two types of capillaries is shown in Figure 1 for the iron containing protein transferrin at two pH

values. In Figure 1A the electrochromatogram of the protein is shown on a C5 capillary under acidic (pH = 4.38) conditions. The peak shape is symmetrical ($A_S \cong 1.0$) with very high efficiency ($N \cong 1000000$ plates/m). As shown in Figure 1B increasing the pH to slightly basic conditions (8.10) maintains both the good peak shape ($A_S \cong 1$) and almost as high efficiency ($N \cong 300000$ plates/m) observed under acidic conditions but the migration time becomes longer (T_M 10.94 min at pH 8.10 vs. 3.12 min at pH 4.38). The migration time is dependent on three different factors: the electroosmotic flow, the electrophoretic mobility and the chromatographic interactions between the transferrin and the bonded C5 moiety. In this case increasing the pH should increase the electroosmotic flow due to higher negative charge on the surface but decrease the electrophoretic mobility since the protein will have less positive charge. The lower positive charge on the protein could also increase the k' value between the bonded moiety and transferrin and thus increase the migration time as indicated by the slightly lower efficiency presumably due to mass transfer effects. The presence of much fewer residuals silanols than on the bare capillary is evident by this increase in migration time since on a bare capillary having many more Si-OH moieties actually results in a shorter migration time for transferrin at higher pH (3.12 min at pH 4.38 vs 1.92 min at pH 8.14). The small alkyl group bonded to the surface (weaker hydrophobic interactions) and the lack of silanols results in the good peak shape and high efficiency observed on the C5 bonded capillary. Detection is shown at two different wavelengths but as expected higher sensitivity is achieved when 210 nm is used instead of 254 nm.

These results can be compared to those obtained for transferrin on a C18 etched capillary. At low pH (Fig. 1C) the migration time is shorter when compared to that obtained at higher pH (Fig. 1D). However, in contrast to the results on the C5 capillary, the peak shape is no longer symmetric and the efficiency has deteriorated considerably. The migration times for transferrin increase when decreasing the pH from 4.0 to 2.14. At the lower pH value the C18 capillary has an anodic EOF like all etched capillaries in contrast to the approximately zero EOF obtained around pH 4.0 [5]. This same effect is observed on the C5 capillary with a longer migration time at pH 2.14 (3.96 min) than pH 4.38 (3.12 min). The anodic EOF is due to the presence of nitrogen in the surface matrix that becomes positively charged at low pH. The other factors on the C18 capillary that produce a considerably longer migration time are due to the longer effective length and the interaction between transferrin and the bonded C18 moiety. The latter is evident by the lower efficiency and the nonsymmetrical peak shape. If the pH is raised to basic conditions (pH 8.14) the migration time increases over the lower pH as shown in Figure 1D. Interaction between the bonded moiety and transferrin is evident by the low efficiency and asymmetric peak.

A similar result for the metalloenzyme carbonic anhydrase is also obtained. Figure 2A shows the electrochromatogram for carbonic anhydrase under acidic conditions obtained on the C5 capillary. Excellent peak shape ($A_S \cong 1$) and high efficiency ($N > 1000000$ plates/m) are achieved under these conditions. These results can be contrasted to the electrochromatogram for carbonic anhydrase using the C18 modified etched capillary (Fig. 2B). Lower efficiency and poorer peak shape indicate that the solute has significant hydrophobic interactions with the octadecyl bonded moiety. The longer migration time on the C5 column is due to its higher anodic EOF. Similar peak shapes and symmetries shown in Figure 2 are obtained on the two capillaries over the pH range of 2.14 to 6.0. Comparable results are also obtained for human serum albumin and human IgG, i.e. better peak shape and higher efficiencies are achieved on the etched C5 capillary than on the etched C18 column. The separation of these two proteins on the C5 column is shown in Figure 3. In addition the migration time for the two proteins on both columns decreases from pH 2.1 to 4.38 and then increases as the pH is raised up to 8.10 as observed for both transferrin and carbonic anhydrase. The initial decrease in migration time at low pH is due to the decrease

in anodic EOF as the pH is increased and the increase in migration time above pH 4.38 is due to the decrease in protein charge and greater interaction with the bonded moiety.

One interesting difference is observed for the heme containing protein myoglobin as shown in Figure 4. At low pH myoglobin has relatively good efficiency on the C5 capillary (Fig. 4A) but peak symmetry is not as good as observed for the other proteins tested. On the C18 column (Fig 4B) under acidic conditions, myoglobin has somewhat better peak shape and efficiency than the other proteins tested but not as good as on the C5 capillary. However under slightly basic conditions (pH 8.10), peak shape and efficiency for myoglobin on C5 are comparable to what is observed in Fig. 4A but improve significantly ($N = 20,000$ plates/m at pH 3.0 vs 50,000 plates/m at pH 8.14) on the C18 (Fig. 4C) and two minor components (impurities) are detected. The migration time is still longer at pH 8.14 than at the point of zero EOF (pH 4) indicating that interactions between the protein and the bonded moiety are still occurring. It is not clear why this change occurs only for the C18 bonded group but it may be a combination of charge or conformational effects resulting in improved mass transfer for this column. Further experiments are underway to investigate this effect in more detail. In many cases direct comparisons between different capillaries is not straightforward and determining the cause of differences in migration behavior is complex due to the fact that protein variables (pI, conformational changes, extent of hydrophobic and hydrophilic domains) as well as column variables (chain length of bonded moiety, degree of surface coverage and variations in EOF) can all contribute to the mobility of the solute [26]

Further comparisons of the two columns were done using several matrix metalloproteinases. These are extracellular proteases with zinc at the active site that cleave a wide variety of substrates and have been implicated in a number of disease processes including cancer, arthritis and emphysema [27–29]. The designation, molecular weight and class of these metalloenzymes are shown in Table 1. Figure 5 shows the comparison of a collagenase run on the two capillaries under identical conditions. In this case the two columns give reasonably comparable results, i.e. efficiencies and peak symmetry are both good. The migration time is longer on the C18 etched capillary and the efficiency is slightly lower. The results of the other compounds in this particular class of proteins are generally very similar to those shown in Figure 5. For the gelatinase (MMP-9, Figure 6A) and matrilysin (MMP-7, Figure 6B) the peaks were slightly broader than the other metalloproteinases but as can be seen still retained very good symmetry. In only one case (MMP-3) did a metalloproteinase exhibit any significant asymmetry (slight fronting) on the C18 column. Thus it appears that the metalloproteinases do not have the same degree of strong hydrophobic interactions as most of the other proteins described above and either column could provide adequate analyses. There is some variation in efficiencies over the pH range investigated (2.1 – 8.1) for each of the metalloproteinases on both of the columns so for optimum performance evaluation of experimental conditions is necessary.

The migration behavior of the metalloproteinases as a function of pH can also be used to characterize the two columns. A plot of migration time vs. pH for the etched C18 column is shown in Figure 7A. The trend is quite similar to what was described above for the other proteins tested. That is there is a general decrease in the migration time going from the lowest pH (2.1) until the point where the anodic EOF decreases to zero and then an increase in migration from this point up to a pH of approximately 7.0. Over this range it would appear that the combination of stronger interaction with the bonded moiety and reduced charge on the protein combine to increase the migration time even though there is an increase in cathodic EOF. A difference occurs above pH 7 for the metalloproteinases where the migration time decreases in comparison to the other proteins where it continues to increase to the highest value (8.14) investigated. This could be due to the fact that over this pH the charge on these proteins is not decreasing as much or the interaction with the

bonded material is not as strong and the increased EOF is more dominant. The same plot for these metalloproteinases using the etched C5 capillary is shown in Figure 7B. The same general trends of migration time with respect to pH are observed for the C5 column as on the C18 capillary although the absolute magnitude and relative differences in migration times among the proteins is different. Many factors could contribute to the variations observed. Among these would be the hydrophobicity of the surface as determined by the chain length of bonded moiety and the surface coverage thus controlling the extent of interaction between the analyte and the stationary phase. These surface variables are likely to have an effect on the zeta potential and therefore affect the EOF. In order to demonstrate that the bonded moiety has an influence on the migration behavior of the metalloproteinases, these same compounds were also run on a bare capillary over the same pH range. The plot of migration time vs. pH is shown in Figure 7C. In this case there are only silanols on the surface so there is a continuous decrease in the migration time from the lowest pH (2.1) until about pH 4.2. At this point the migration time either becomes constant or increases slightly up to pH 6.0. As the pH is increased above 6.0 the migration time decreases further. The overall shape of this curve is dominated by the EOF which is expected to increase over this pH. There is no substantial increase in migration time between pH 4.0 and 7.0 for any of the metalloproteinases as is observed on the etched C5 and C18 capillaries. These results strongly indicate that both the bonded C5 and C18 moieties have a measurable influence on protein migration in the etched chemically bonded capillaries, most likely through solute/bonded phase interactions.

A final consideration is the reproducibility of these etched chemically modified capillaries for metalloprotein analysis. A series of 10 runs for several of the proteins and metalloproteinases was done on the etched C5 capillary. The results are shown in Table 2. The run-to-run reproducibility is excellent (% RSD = 1.5%) and both efficiency ($N > 150000$ plates/m) as well as peak symmetry ($A_S < 1.3$) are maintained over the consecutive analyses. This data is comparable to other reproducibility studies done for a variety of other solutes on various etched chemically modified capillaries [20]. Both the C5 and C18 columns were used for more than 300 injections and were still operable at the end of the study.

Conclusions

Etched chemically modified capillaries have been shown to be a viable separation medium for the analysis of proteins and metalloproteins. The degree of hydrophobicity of the bonded moiety has an effect on the migration behavior, efficiency and peak symmetry of these analytes. In some cases the more hydrophobic C18 displays stronger interactions with some of the proteins resulting in longer migration times, broader peaks and in some cases greater asymmetry. The etched capillaries with bonded alkyl moieties display a different profile than a bare capillary for migration time as a function of pH that further substantiates the influence of the chemical modification on the elution behavior of the proteins investigated. Run-to-run reproducibility is excellent demonstrating the analytical capabilities of these types of electrophoretic separation materials.

Acknowledgments

The financial support of the National Institutes of Health (Grant GM079741-01) is gratefully acknowledged. Dr. Viorica Lopez-Avilla of Agilent Technologies donated the solutes used in this study. JJP would like to acknowledge the support of the Camille and Henry Dreyfus Foundation through a Scholar Award.

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Figure 1.

Electrochromatograms of transferrin on the etched modified capillaries. A) C5 column at pH 4.38; B) C5 column at pH 8.14; C) C18 column at pH 2.14; and D) C18 column at pH 8.14. Detection at 210 nm. $V = 25$ kV. Injection hydrodynamic at 50 mbar for 8 s.

Figure 2. Electrochromatograms of carbonic anhydrase on A) etched C5 modified capillary at pH 3.0 and B) etched C18 modified capillary at pH 2.14. Other conditions same as Fig. 2.

Figure 3.
Separation of human IgG and human serum albumin on etched C5 capillary at pH 3.0. Other Conditions same as Fig. 2.

Figure 4. Electrochromatograms of myoglobin on A) etched C5 modified capillary at pH 3.0; B) etched C18 modified capillary at pH 3.0; and C) etched C18 modified capillary at pH 8.14. Other conditions same as Fig. 2.

Figure 5. Electrochromatograms of a collagenase (MMP-1) on A) etched C5 modified capillary at pH 3.0 and B) etched C18 modified capillary at pH 3.0. Other conditions same as Fig. 2.

Figure 6. Electrochromatograms on etched C18 modified capillary for A) gelatinase (MMP-9) at pH 2.14 and B) matrilysin (MMP-7) at pH 2.14. Other conditions same as Fig. 2.

Figure 7. Plots of migration time vs. pH for various metalloproteases on A) etched C18 modified capillary; B) etched C5 modified capillary; and C) bare capillary. Other conditions same as Fig. 2. Solute identification shown in Table 1.

Table 1

Properties of matrix metalloproteinases used in this study

Designation	Mol. Wt. (kDa)	Enzyme Type
MMP-1	19.9	collagenase
MMP-3	19.5	stromelysin
MMP-7	20.4	matrilysin
MMP-8	20.3	collagenase
MMP-9	39.0	gelatinase
MMP-10	19.4	stromelysin
MMP-11	19.3	stromelysin
MMP-12	20.3	metalloelastase
MMP-13	20.4	collagenase

Table 2

Representative results for C5 capillary

Protein	pH	Migration time ^I (min)	Efficiency (plates/m)	Symmetry (A _S)
Transferrin	4.38	3.12 (1.3)	1000000	1.03
Human IgG	4.38	8.36 (1.2)	800000	0.95
Carbonic Anhydrase	3.00	10.68 (1.5)	750000	1.00
Myoglobin	3.00	10.54 (1.2)	500000	1.25
MMP-1	6.00	8.21 (0.5)	1000000	1.00
MMP-3	2.14	7.12 (0.7)	1000000	1.00
MMP-7	3.00	6.42 (0.7)	400000	1.10
MMP-9	7.06	5.95 (0.6)	300000	1.15
MMP-11	8.14	3.92 (0.7)	150000	1.20
MMP-13	7.06	7.14 (0.5)	650000	1.15

^I Value in parentheses is %RSD for ten consecutive measurements