Published in final edited form as:

J Chromatogr A. 2013 August 2; 1301: 156–161. doi:10.1016/j.chroma.2013.05.076.

Polyacrylamide brush layer for Hydrophilic Interaction Liquid Chromatography of intact glycoproteins

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Abstract

A chromatographic column of nonporous silica particles with a bonded phase of linear polyacrylamide chains is evaluated for hydrophilic interaction liquid chromatography (HILIC) of intact glycoproteins. The column is shown to retain glycoproteins significantly more strongly than non-glycoproteins. A particle diameter of 700 nm gives two-fold higher resolution than does a 1.4 μm particle diameter, and the column efficiency is found to be mostly limited by packing heterogeneity. LCMS is able to resolve the five glycoforms of ribonuclease B and give high quality mass spectra, but there is loss of resolution of the isomers of glycoforms due to the lower amount of TFA. Compared to two leading commercial HILIC columns operated at 60 °C, the polyacrylamide column operated at 30 °C provided at least two-fold higher resolution for intact ribonuclease B, and showed peaks for glycoforms of prostate specific antigen, although not resolved.

1. Introduction

Glycosylation is the most common post-translational modification occurring in living biological systems [1]. Indeed, more than half of all proteins are glycosylated [2]. Glycosylation has the potential to affect nearly all protein activities, including folding, delivery to the cell surface, binding affinity with other molecules, degradation and turnover [1, 3]. Considerable variation of structure exists in the branches of the carbohydrate moieties (glycans) regardless the glycosylation site, and these variations have the potential as markers in disease diagnosis [4-5].

High-resolution separation of intact glycoproteins is required to reduce the sample complexity for top-down proteomics, which is a powerful technique to characterize the glycosylation site and sequence simultaneously [6-7]. Hydrophilic interaction liquid chromatography (HILIC) is useful in glycosylation analysis due to the hydrophilicities of the sugar groups. HILIC was first used for carbohydrate analysis in 1975 [8] and then defined by Alpert in 1990 [9] as a variant of normal phase chromatography, where water is a component of the mobile phase. HILIC has become a standard separation technique for cleaved glycans [10] in glycomics analysis and is used for separation of glycopeptides [11-12]. The ability to analyze intact glycoproteins would save the time required for digestion into glycopeptides or glycans, providing another tool for top-down proteomics. HILIC separation of intact proteins is not common, perhaps in part because reverse phase

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liquid chromatography (RPLC) is already well developed very well for separation of intact proteins [13-15]. The poor solubility of proteins in organic solvents [16] is another factor. Although one might expect that HILIC would be widely applied for intact glycoprotein analysis, the first attempt has not been made until very recently: Tetaz [17] demonstrated a separation of three proteins, human apoA-I, recombinant human apoM and cytochrome c. Five different commercial HILIC columns were compared, and among these, TSKgel Amide-80 column was found the best for glyco-profiling of apoM. Nonetheless, poor resolution was obtained, possibly due to the pores being too small for protein separations [17]. Another possibility could be insufficient selectivity of the stationary phase, which is a blend of amide and carbamoyl groups, according to the manufacturer's information.

Polyacrylamide is a hydrophilic material that forms a homogenous surface when grown using atom-transfer polymerization [18]. Polyacrylamide has not been used for HILIC separations of intact proteins. Recently, our group showed that a brush layer of polyacrylamide (PAAm) grown on 700 nm silica particles swells from 1 nm to 17 nm in thickness when the solvent is changed from toluene to water [19]. This indicates that the hydrated brush layer is overwhelmingly aqueous. The brush layer had been designed to be closely spaced to sterically exclude proteins, hence protein-silanol interactions are avoided [20]. These characteristics could make the PAAm brush layer effective for HILIC by forming a highly aqueous layer that allows the carbohydrate moiety to enter, but not the protein. The concept is depicted in Figure 1, where parent proteins would be separated by virtue of differential adsorption just at the PAAm surface, whereas carbohydrates might be separated by more subtle differences in structure by virtue of differential partitioning into the hydrophilic layer. Thin-layer chromatography (TLC) of proteins, albeit nonglycosylated, show that HILIC can be carried out with this bonded phase [21]. The purpose of this paper to investigate HILIC using the PAAm brush layer for Ultrahigh Performance Liquid Chromatography (UHPLC) of intact glycoproteins.

2. Materials and Methods

2.1 Reagents and Materials

Silica particles (700 nm and 1.4 μm in diameter) were purchased from Fiber Optic Center, Inc. (New Bedford, MA), and then calcined at 600 $^{\circ}\text{C}$ for 12 hours. Empty stainless steel columns (2.1 mm I.D., including both 3 cm and 5 cm lengths) were purchased from Isolation Technologies (Middleboro, MA). Stainless steel tubing, ferrules and internal nuts were all purchased from Valco Instruments Co. Inc. (Houston, TX). Rheodyne Rheflex stainless steel fittings were purchased from IDEX Health&Science LLC (Oak Harbor, WA).

Trichloromethylsilane (Gelest, Inc., Morrisville, PA), (chloromethyl)phenylethyl-trichlorosilane (Gelest, Inc., Morrisville, PA), acrylamide (Sigma-Aldrich, St. Louis, MO), CuCl (99.999%; Alfa Aesar, Ward Hill, MA), CuCl₂ (99%; Acros Organics, Morris Plains, NJ) were used as received. Acetonitrile, formic acid and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (St. Louis, MO).

Proteins including ribonuclease B, ribonuclease A, lysozyme, cytochrome c, carbonic anhydrase and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO). Prostate-specific antigen (PSA) was purchased from Lee Biosolutions (St. Louis, MO). All proteins were prepared in 50% isopropanol alcohol, 30% acetonitrile and 20% H₂O to desired concentration.

2.2 Instruments

UHPLC analysis was performed using Thermo Accela UHPLC system with either absorbance detection or with mass spectrometry using a Thermo LTQ Velos mass

spectrometer (Thermo-Scientific, Waltham, MA, USA). Lab-made HILIC columns (2.1×30 mm, 700 nm or $1.4 \mu\text{m}$ nonporous silica particles coated with polyacrylamide) were used as the analytical columns. 2.1×50 mm columns with $1.4 \mu\text{m}$ particles were also packed in lab for comparison. For comparison, a commercial column, BEH amide column (2.1×30 mm, $1.7 \mu\text{m}$), from Waters (Milford, MA) and TSKgel Amide-80 column (2×50 mm, $3 \mu\text{m}$) from Tosho Bioscience (King of Prussia, PA) were used. For protein separations with absorbance detection (absorbance=214 nm), a gradient of solvent A, acetonitrile with 0.1% TFA, and solvent B, water with 0.1% TFA, was used. In the case of MS detection, 0.5% formic acid and 0.05% TFA were used.

2.3 Stationary Phase Modification and UHPLC Column Packing

The silica particles were modified as described earlier [21-22]. Freshly rehydrolyzed silica particles were suspended in a dry toluene solution containing 2% (v/v) of (chloromethyl)phenylethyl-trichlorosilane and trichloromethylsilane at a volume ratio of 1:20. The solution was stirred under nitrogen overnight. The reacted particles were then rinsed with dry toluene and allowed to dry in oven at 120°C for 2 h. A deoxygenated solution containing acrylamide, CuCl , CuCl_2 and Tris (2-dimethylaminoethyl) amine in N,N-dimethylformamide (DMF) was prepared as described earlier [22]. A dense layer of low-polydispersity chains was synthesized on the silica surface via this method [18, 20, 23], which is a brush layer of polyacrylamide that was characterized in our previous work [22]. The modified particles were then rinsed with DMF, EDTA solution, water and ethanol, and stored in a vacuum desiccator. Later, the PAAm modified particles were suspended in water at a concentration of 100 mg/mL and packed into 2.1 mm I.D. stainless steel column using a LabAlliance Series 1500 HPLC Pump (Laboratory Alliance of Central New York, LLC, Syracuse, NY).

3. Results and discussions

Figure 2A shows a HILIC separation of a mixture of a nonglycoprotein, ribonuclease A, and its glycosylated version, ribonuclease B, which has a high mannose, N-linked glycan varying from five to nine mannose groups. Ribonuclease A is shown to elute much earlier than the first eluting glycoform of ribonuclease B. The chromatogram is consistent with the notion depicted in Figure 1: the carbohydrate group interacts strongly with the stationary phase to delay the elution of the glycosylated form of the protein. The five glycoforms of ribonuclease B are well resolved in the chromatogram of Figure 2A, which also shows the structures of the five glycans. These glycans each differ from one another by a single mannose group, indicating that the PAAm phase is promising for HILIC separations of glycoproteins. Figure 2B shows the separation of ribonuclease B and a mixture of nonglycosylated proteins. Again, ribonuclease B elutes last, appearing later than bovine serum albumin, which is a considerably larger protein. Except for bovine serum albumin, which is beginning to separate into at least two peaks, the full width at half maximum (FWHM) are 0.2 min, which is as narrow as UHPLC peaks in reversed phase liquid chromatography. The non-Gaussian peak shapes are attributed to protein heterogeneity. Unlike for small molecules, one cannot purchase pure proteins. The brush layer of PAAm thus has good adsorptivity for carbohydrates, and it provides reasonable efficiency.

To optimize the gradient for the HILIC separation of ribonuclease B, Figure 3 shows the HILIC separation of ribonuclease B on three different PAAm columns. Figure 3A is for a column made using $1.4 \mu\text{m}$ nonporous silica, Figure 3B for 700 nm particles, and Figure 3C for 700 nm particles but with the PAAm layer that was half as thick, as determined by FTIR. Each panel denotes the resolution, R_s , between the first and second glycoforms. The column with 700 nm particles gave nearly a 50% higher resolution than that with $1.4 \mu\text{m}$ particles. More peaks are resolved for the 700 nm particles compared to the $1.4 \mu\text{m}$ particles, and

these are due to isomers of the glycans [24], revealing about a dozen peaks in total. The two-fold thinner PAAm layer gave poorer resolution. The selectivity was similar, based on the spacing of the peaks, but the peaks are significantly broader, perhaps due to the thinner film allowing interactions with substrate. The PAAm column of Figure 3B gave FWHM of only 8 s wide for the mobile phase conditions used in this separation. This shows that the medium has high peak capacity: 30 peaks per 8 min. Under the optimized gradient conditions, more than 5 peaks could be observed for ribonuclease b due to protein impurities and numbers of modifications. The column used for Figure 3B is used for the remainder of this work.

The ability to couple LC of intact glycoproteins to mass spectrometry would be valuable for glycoproteomics, but the challenge is that TFA suppresses ionization. To determine whether liquid chromatography-mass spectrometry (LCMS) is feasible with the PAAm column, 0.5% formic acid was included in the mobile phase, and the amount of TFA was reduced until acceptable mass spectra were obtained. It was found that a small amount of TFA improved separation efficiency compared to formic acid alone, without diminishing the signal in mass spectrometry. Figure 4A shows the resulting chromatogram of ribonuclease B, which still resolves the main glycoforms with a respectable resolution of 2.2, but the isomers are no longer resolved. Figure 4B shows the mass spectrum for the first glycoform over a wide range of m/z values, demonstrating that it is cleanly detected and it correctly gives the 14.9 kDa molecular weight of this glycoform. In Figure 4, the mass spectra for the $z=8$ ions in panels C-F show the progressive shifts in the mass of subsequently eluting glycoforms. The results show that in-line LCMS is thus feasible, albeit with a 2.2-fold loss of resolution compared with UV detection.

The PAAm column is compared with two commercial HILIC columns for the separation of the glycoforms of ribonuclease B. The commercial columns were both run at 60 °C which is typically used for protein separations to enhance intraparticle diffusion. This temperature gave higher resolution than did 30 °C (not shown). The PAAm column was found to be affected negligibly by temperature, owing to its nonporous particles, therefore it was operated at 30 °C. The chromatogram of Figure 5A shows that the Waters column gave baseline resolution of all five glycoforms of ribonuclease B with a resolution of 2.7 between the first two glycoforms. Figure 5B shows that the TOSOH column gave a resolution of only 0.8. The TOSOH column has both lower selectivity and lower efficiency, hence the much lower resolution. Figure 5C shows that the PAAm column has the highest resolution of the three columns: 4.9. The selectivities of the PAAm and Waters columns are similar: the time span between the first and last peaks is 4 min in each case. The peaks are narrower for the PAAm column, accounting for the higher resolution of the latter.

To understand what controls the efficiency of the PAAm column, a van Deemter plot is given in Figure 6A. The data were obtained for the first eluting glycoform of ribonuclease B under isocratic conditions. The PAAm column has a large A term, and negligible B and C terms giving a constant value of $20.7 \pm 2 \mu\text{m}$. B is small because diffusion is obstructed for proteins and this range of linear velocities is relatively high. There can be no contribution from intraparticle diffusion because the particles are nonporous, and the small interstitial dimension of the 700 nm particles ought to make the C term from the Poiseuille flow profile much smaller, consistent with the van Deemter plot. The A term is typically due to packing heterogeneity, but extracolumn broadening from a wide injection length would also appear as a velocity independent term. Injection length was investigated by varying the amount injected, and a plot of peak FWHM vs. injected amount is shown in Figure 6B. The peak width greatly increased with injected width. The data fit well to a Langmuir adsorption isotherm, and the fitting parameters show that concentration at half of the saturated coverage is $5.5 \mu\text{g}$ of protein. Given that all of the protein is adsorbed upon injection, the fit implies a capacity of $11 \mu\text{g}$ of proteins. This capacity agrees with what one would estimate from the

surface area of the particles if a 4 nm^2 protein footprint is assumed, which is reasonable. The chromatograms for the van Deemter plot were obtained using $0.15 \text{ }\mu\text{g}$ of injected protein, which is close to intercept of Figure 6B, as is evident in the inset. This allows the prediction that a concentration approaching the limit at the intercept would decrease the A term from 20 to $12 \text{ }\mu\text{m}$. Using either a wider or a longer column to increase the column capacity would be another way to reduce this contribution.

The fact that the intercept in Figure 6B is non-zero, i.e., 0.13 min reveals that there is at least another factor at work that contributes $12 \text{ }\mu\text{m}$ to the A term when the injection width is negligible. The other extra-column broadening factor that could contribute a velocity-independent term is the detection width. The absorbance detector has a flow cell volume of $2 \text{ }\mu\text{L}$ with a 1-cm path length, and a 40% porosity for the column from random packing, the contribution to the plate height from the detector is $5 \text{ }\mu\text{m}$. This calculation suggests that packing heterogeneity contributes the remaining $7 \text{ }\mu\text{m}$ to the plate height. An optimization of the packing procedure was not performed. Choice of solvent for the slurry is likely to be important because extended polymer chains would impede dense packing. With virtually no C term, packing optimization is an opportunity for a significant advance in HILIC with the PAAm column.

One application where glycoform separations are needed is biomarker discovery. The glycosylation of prostate-specific antigen (PSA) is thought to be related to different cancer stages [4, 25], suggesting that the separation of the glycoforms could facilitate biomarker discovery. PSA has about 40 glycoforms [26], therefore, one cannot expect the baseline resolution exhibited by ribonuclease B. Nonetheless, it is instructive to see how far the column is from separating the glycoforms. Figure 7 shows a separation of PSA glycoforms by the PAAm column and the two commercial HILIC columns. The PAAm column shows evidence of many peaks in the envelope. In the vicinity of 14 min, expanded in the inset, the resolution has to be on the order of 0.5 to see peaks rather than a continuous envelope. To look at this another way, given the peaks spacings in the inset, if the peak widths were comparable to those of the ribonuclease B glycoforms, the resolution would be about 0.5. This means a factor of four decrease in plate height would significantly improve the ability to resolve peaks in the PSA chromatogram. Increasing the capacity and the packing homogeneity for the PAAm column is thus promising for studying this complex glycoprotein. Figures 7B and C show that the Waters and TOSOH columns exhibit little structure in the broad envelopes. The results thus indicate that a larger protein, such as PSA, is amenable to HILIC separation, but a quantitative assessment of the effect of protein size will require comparing glycoproteins having a similar number of glycoforms.

4. Conclusions

A PAAm brush layer grown on nonporous silica nanoparticles of 700 nm in diameter is promising for separating intact glycoproteins by HILIC. Glycoproteins are adsorbed more strongly than nonglycoproteins, indicating that the carbohydrate moiety of the glycoprotein is what interacts most with the hydrophilic stationary phase. The higher surface area of the submicrometer particles improves selectivity, giving higher resolution than commercial HILIC columns. Glycoforms differing by single mannose groups are easily resolved, even with LCMS, whereas isomers of glycans are resolved when using 0.1% TFA and absorbance detection. Improvements in the homogeneity of the packing could significantly impact the HILIC resolution of intact glycoproteins.

Acknowledgments

This work was supported by NIH under grant GM101464.

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Highlights

- A polyacrylamide brush layer on silica particles adsorbs the carbohydrate moiety of a glycoprotein.
- For the high-mannose glycoprotein, ribonuclease B, peaks are well resolved for differences of single mannose groups.
- Isomers of glycoforms of ribonuclease B are also resolved.
- The dominant broadening mechanism is heterogeneous packing of the polymer coated particles.

Figure 1.

Depiction of PAAm brush layer and postulated mechanism of HILIC separation of intact glycoproteins. The polymer segments are drawn in black and surrounded by water, and the mobile phase is a mixture of water and acetonitrile. Protein structure of ribonuclease B is taken from Wormald and Dwek.[27]

Figure 2.

Gradient elution separations of ribonuclease B and nonglycoproteins. A) Mixture of nonglycoprotein, ribonuclease A, and glycoprotein, ribonuclease B. Gradient: 75-60% A in 30 min with a flow rate of 150 $\mu\text{L}/\text{min}$ at 30 $^{\circ}\text{C}$. B) Mixture of nonglycoproteins (lysozyme, cytochrome c, carbonic anhydrase and bovine serum albumin) and glycoprotein, ribonuclease B. Gradient: 78-60% A in 36 min with a flow rate of 150 $\mu\text{L}/\text{min}$ at 30 $^{\circ}\text{C}$

Figure 3.

Comparison of columns for HILIC separation of ribonuclease B. A) 1.4 μm nonporous particles with PAAm brush layer, gradient: 75-65% A in 20 min under 500 $\mu\text{l}/\text{min}$ at 30 $^{\circ}\text{C}$; B) 700nm nonporous particles with PAAm brush layer, gradient: 72-62% A in 20 min under 270 $\mu\text{L}/\text{min}$ at 30 $^{\circ}\text{C}$; C) 700nm nonporous particles with half thinner PAAm brush layer, gradient: 76-66% A in 20 min at 360 $\mu\text{l}/\text{min}$ at 30 $^{\circ}\text{C}$.

Figure 4.

LCMS of ribonuclease B using the PAAm column. A) Chromatogram for a gradient of 70-60% ACN with 0.5% formic acid and 0.02% TFA over 30 min at 30°C, with a flow rate of 150 μ l/min. B) Mass spectrum of first eluting glycoform over the range of 1000-2000 m/z, showing ions for $z=8$ through 12, corresponding to the correct mass of 14.9 kDa. C-F) Mass spectra of the subsequently eluting glycoforms in order of increasing number of mannose groups, with only the spectra for $z=8$ shown for conciseness, detailing the addition of successive mannose groups to increase m/z.

Figure 5. Chromatograms for three different columns separating the glycoforms of ribonuclease B. A) Waters BEH Amide, gradient: 71.5-61.5% A in 20 min at 250 $\mu\text{L}/\text{min}$ at 60 $^{\circ}\text{C}$; B) TSKgel Amide-80, gradient: 68-58% A in 20 min at 500 $\mu\text{L}/\text{min}$ at 60 $^{\circ}\text{C}$, C) PAAm column, gradient: 72-62% A in 20 min at 270 $\mu\text{L}/\text{min}$ at 30 $^{\circ}\text{C}$.

Figure 6.

Factors controlling column efficiency. A) Van Deemter plot for the PAAm column, obtained isocratically using a mobile phase of 70% acetonitrile with 0.1% TFA. The amount injected was μg . B) Plot of peak FWHM vs. the amount injected, for the same PAAm column. Solid line is fit to a Langmuir adsorption isotherm. Inset shows initial points on an expanded scale.

Figure 7.

HILIC chromatograms for PSA. A) PAAm column, gradient: 77-60% A in 34 min at 100 $\mu\text{L}/\text{min}$ and 30 $^{\circ}\text{C}$, B) Waters BEH amide column, gradient: 74-57% A in 34 min at 300 $\mu\text{L}/\text{min}$ and 60 $^{\circ}\text{C}$, and C) Tosoh TSKgel Amide-80 column, gradient: 80-60% A in 40 min at 500 $\mu\text{L}/\text{min}$ and 60 $^{\circ}\text{C}$.