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Weak anion exchange chromatographic profiling of glycoprotein isoforms on a polymer monolithic capillary

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ABSTRACT

High resolution separation of intact glycoproteins, which is essential for many aspects such as finger-print profiling, represents a great challenge because one glycoprotein can exhibit many isoforms with close physicochemical properties. Monolithic columns are important separation media for the separation of intact proteins due to its significant advantages such as easy preparation, high column efficiency and high permeability. However, there are few reports on high resolution profiling of intact glycoproteins. Herein, we presented a polymeric weak anion exchange (WAX) monolithic capillary for high resolution separation of glycoprotein isoforms. A base monolith was first prepared through ring-opening polymerization between tris(2,3-epoxypropyl)isocyanurate and tri(2-aminoethyl), and then modified through reacting with ammonia aqueous solution to convert the unreacted epoxide moieties into primary amino groups. The prepared monolithic capillary was characterized in terms of morphology, pore size, hydrophilicity and reproducibility. The obtained WAX monolithic capillary exhibited desired through-pores and mesopore size, stable skeleton and hydrophilic nature. The performance of the capillary was evaluated using several typical glycoproteins such as α_1-acid glycoprotein (AGP) as mode analytes. Effects of the experimental parameters on the glycoform resolution were investigated. Under the optimized separation conditions, the tested glycoproteins were all resolved into distinct glycoforms. A comparative investigation with capillary zone electrophoresis (CZE) revealed that this WAX column provided better selectivity as more isoforms were observed, although the resolution of some glycoprotein isoforms decreased.

1. Introduction

Glycosylation of protein, as an important post-translational modification, determines the biological activity of proteins, such as the solubility [1], the affinity to other biomolecules [1,2], inter- and intra-cell signaling [3,4], and so on. A variety of glycoproteins such as α-fetoprotein (AFP), carcino-embryonic antigen (CEA), and prostate specific antigen (PSA) have been used as disease biomarkers for routine clinic cancer screening. An increasing number of glycoproteins are being suggested as biomarker candidates for diseases such as cancer [5,6], Alzheimer's disease [7], rheumatoid arthritis [8] and chronic obstructive pulmonary disease [9]. On the other hand, glycoproteins are important biopharmaceutical drugs for disease treatment [10]. Since different glycoprotein isoforms may exhibit different biological activities, high-resolution profiling of glycoprotein isoforms is essential for quality control. Besides, glycoform profiling is a critical means to differentiate endogenous and exogenous erythropoietin (EPO) to determine the abuse of EPO in sports [11]. However, the glycoform profiling of a glycoprotein is often greatly challenging, because the total number of the isoforms may be rather large meanwhile the isoforms may differ slightly in their physicochemical properties. The workhorse tools for high-resolution profiling of glycoprotein isoforms have relied on electrophoresis-based platforms including isoelectric focusing (IEF) [12], two-dimensional gel electrophoresis (2DGE) [13,14], capillary zone electrophoresis (CZE) [15–17], and capillary isoelectric focusing (CIEF) [18]. However, these methods are associated with apparent disadvantages, such as time-consuming, poor reproducibility, and the limited pH range of carrier ampholytes. Therefore, new alternatives to overcome these issues are highly desirable.

Monolithic columns, generally recognized as the forth-generation separation media, have significantly changed the LC field [19–25]. Monolithic column particularly monolithic capillary [26] provides several significant advantages, including rapid convectional mass transport, ease in preparation, and good compatibility with MS for high sensitivity. Thus, monolithic capillaries have gained increasing applications in the separation of proteins and peptides, among which ion-exchange monolithic columns are of particular use for separating intact proteins. Lee et al. [27,28] reported strong cation-exchange (SCX) monolithic columns suitable for separation of both peptides and proteins. Li et al. [29]

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represented a successful attempt to prepare polymeric monoliths via a single step polymerization of monomers with amine functionalities as weak or strong anion exchanger. Separation of standard proteins was achieved under gradient elution conditions using these monolithic columns within 30 min. Direct copolymerization of functional monomers undoubtedly provided the simplest method to prepare functionalized monoliths. Legido-Quigley et al. [30] developed a styrene-based weak cation-exchange (WCX) capillary monolithic column for protein separation. By post modified using *N*, *N*-dimethylbutylamine, a quaternary ammonium group and a butyl chain were introduced onto the chromatographic surface of the monolith. Ding et al. [31] prepared a novel short cation-exchange monolithic capillary and applied for rapid separating proteins. The cation exchanger was sulfonated using 1 mol/L Na_2SO_3 based on a ring opening of epoxides. Four proteins were baseline separated within 6 min. Tanaka et al. [32] reported monolithic silica columns post-modified with acrylate and methacrylate polymers that possessed anion-exchange functionalities. As compared with conventional particle-packed anion-exchange column, better separation efficiency for proteins was obtained. However, there were few reports of high resolution profiling isoforms of intact glycoproteins on monolithic columns.

In this work, we reported weak anion exchange (WAX) chromatographic profiling of glycoprotein isoforms on an epoxy polymer monolithic capillary column. Epoxy-based polymer monolith was selected for this application due to its hydrophilicity [33,34] and easy post-modification. The monolithic column was prepared by thermally initiated ring-opening polymerization. Post modification with ammonia aqueous solution was used to generate primary amino groups for weak anion exchange. The feasibility of isoform profiling using the monolithic column in WAX mode was examined through the separation of several intact glycoproteins. Effects of the experimental parameters on the glycoform resolution were investigated. Under the optimized conditions, the glycoproteins under test were all resolved into distinct glycoforms. A comparative investigation with capillary zone electrophoresis (CZE) revealed that this WAX column provided better selectivity as more isoforms were observed.

2. Experimental

2.1. Chemicals and materials

Adenosine monophosphate (AMP), thymidine, α_1-acid glycoprotein (AGP) from bovine serum, myoglobin from horse heart, tris(2,3-epoxypropyl)isocyanurate (TEPIC), and thiourea were purchased from Sigma–Aldrich (St. Louis, MO, USA). Adenosine-5′-monophosphoric acid, (3-aminopropyl)trimethoxysilane, and tri(2-aminoethyl)amine (TAEA) were purchased from Alfa-Aesar (Ward Hill, MA, USA). Acrylamide and tris(hydroxymethyl)aminomethane (Tris) were obtained from Bio-Rad (Mannheim, Germany). α-Fetal protein (AFP) was purchased from Shuangliu Zhenglong Biochemical Product Lab. (Sichuan, China). Human glycated hemoglobin HbA1c reference standard was from Exocell (Philadelphia, USA). Bovine serum albumin (BSA) and ovalbumin were from Sunshine Biotechnology (Nanjing, China). Methanol, acetonitrile (ACN), tetrahydrofunan (THF), sodium hydroxide, hydrochloric acid, poly(ethylene glycol)200 (PEG 200), ammonium hydroxide ($NH_3{\cdot}H_2O$), sodium acetate (NaAc), sodium chloride (NaCl), ammonium formate, formic acid, and toluene were purchased from Nanjing Chemical Reagent Company (Nanjing, China). Ultrapure water used in all experiments was purified by a Milli-Q system (Millipore, Milford, MA, USA). Fused-silica capillaries of 150 μm I.D. and 375 μm O.D. were purchased from Yongnian Optic Plant (Hebei, China).

2.2. Conditions for the polymerization and post modification reactions

The capillary was pretreated with an amino silanizing agent in order to covalently anchor the polymer to the inner wall. First, the capillary was rinsed with methanol and water. Second, the capillary was rinsed with 1 M sodium hydroxide aqueous solution for 1 h, followed by washing with water until the pH value of the outlet solution reached 7.0. Third, the capillary was rinsed with 1 M hydrochloric acid aqueous solution for 1 h, followed by washing with water until the pH value of the outlet solution reached 7.0. After washed with methanol, the capillary was dried with the passage of nitrogen gas overnight. Then a (3-aminopropyl)-trimethoxysilane/THF (1:1 v/v) solution was injected into the capillary and kept at 80 °C for 12 h. After the reaction, the modified capillary was washed with methanol to remove the residues and dried with the passage of nitrogen gas overnight.

0.32 mg TEPIC and 40 μL TAEA were completely dissolved in 1.4 g PEG 200. The polymerization mixture was sonicated for more than 30 min to degas and obtain a homogeneous solution, and then injected into the pretreated capillary. The polymerization reaction was thermally initiated at 80 °C for 12 h. The resulting capillary was washed with methanol to flush out the residues. The polymer monolith was then post-modified by reacting with continuous flowing ammonia solution ($NH_3{\cdot}H_2O/H_2O$ = 1:1 (v/v), about 7.4 mol/L) at 60 °C for 48 h, which converted the residual epoxide groups on the monolith surface into primary amino groups.

2.3. Characterization of the monolith

Scanning electron microscopy (SEM) analyses were conducted on a Hitachi FE-SEM S-4800 (Tokyo, Japan). For the pore size distribution measurement, Fourier transform infrared (FT-IR) characterization and elemental analyses of the monolith, bulk monolithic material was synthesized in a test tube using the same procedure. The bulk material was cut into small pieces and Soxhlet extracted with methanol for 12 h and then dried in vacuum at 60 °C. Nitrogen adsorption–desorption measurement was performed at 77 K on an ASAP 2020 instrument (Micromeritics, Norcross, GA, USA). FT-IR was conducted on a Bruker Tensor 27 (Bruker, German). Elemental analyses were conducted on a Vario MICRO (Elementar, German).

2.4. Chromatographic conditions

A TriSep-2100 pCEC system (Unimicro Technologies, Pleasanton, CA, USA) with an on-column UV-absorbance detector was used to perform all capillary liquid chromatographic experiments. Two micro-volume pumps were used to provide a solvent gradient delivery module. Samples were injected through an injection valve with an internal 600 nL sample loop. A three-port splitter was set before the injection valve, and the splitting ratio was set at 99:1.

Secondary retention on the WAX monolithic capillary was investigated in order to evaluate if reversed-phase mechanism and hydrophilic interaction mechanism occurred. Thiourea, toluene, and acrylamide were chosen as the test analytes. The mobile phase was prepared by mixing desired amount of ammonium formate (pH 4.10, adjusted with formic acid), ACN and water.

Exchange capacity was measured using frontal analysis. The WAX monolithic capillary was equilibrated with a loading buffer containing 10 mM Tris aqueous solution at pH 7.60 which was adjusted by 1 M hydrochloric acid. The samples containing 0.1 mg/mL thymidine and 4 mg/mL AMP or 0.1 mg/mL thymidine

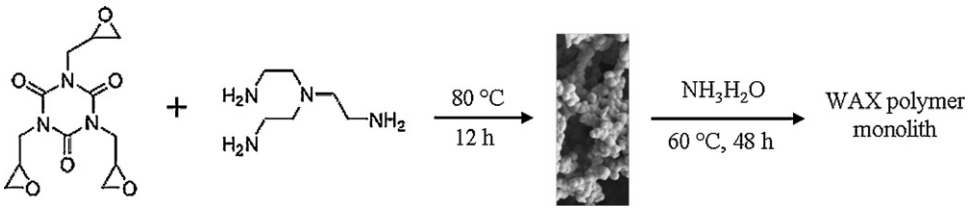

Fig. 1. Procedure of the polymerization reaction and post-modification.

and 0.5 mg/mL BSA were dissolved in the loading buffer and continuously pumped through the column. For the subsequent use, this monolithic capillary was rinsed with 10 mM NaAc aqueous solution containing 1 M NaCl at pH 4.10 to remove the analytes, followed with loading buffer to regenerate the column.

The reproducibility of the column was chromatographically checked by the separation of myoglobin which was eluted by NaCl gradient. The weak eluent was 10 mM Tris at pH 9.10 (component A), while the strong eluent was 10 mM Tris containing 400 mM NaCl at pH 9.10 (component B). The mixing proportion for gradient elution was from 20 mM NaCl to 400 mM NaCl with a gradient rate of 15% B per min.

Glycoproteins were separated with a NaCl gradient elution. The solvent A was 10 mM Tris or 10 mM NaAc, while the solvent B was 10 mM Tris containing 400 mM NaCl or 10 mM NaAc containing 400 mM NaCl. The solvents A and B were of the same pH under investigation. The mixing proportions for gradient elution were 100% A from 0 to 0.5 min, then increasing the content of B with an appropriate gradient rate.

3. Results and discussion

3.1. Optimization of the monolith preparation conditions

The schematic of the polymeric reaction is shown in Fig. 1. TEPIC and TAEA were used as monomers, while PEG 200 was used as porogen. Monoliths were prepared at four molar ratios of TEPIC/TAEA, 4:1, 3.5:1, 3:1 and 2:1. It was found that the higher the content of TAEA, the more condensed the monolith was, and the poorer the permeability was. Thus, the ratio 4:1 was chosen for the later investigations. The amount of the porogen PEG 200 was optimized among 1.20, 1.25, 1.30, 1.40 g. It was found that the content of the porogen dramatically affected the permeability of the obtained monolith. The lower the porogen content, the smaller the through-pore size and the permeability was. The optimal amount of PEG 200 was found to be 1.40 g. The reaction temperature was optimized among 70, 80, 90, 100 and 120 °C. It was found that the higher the temperature, the smaller the through-pore size was. However, too low temperature caused poor mechanical strength. The optimal temperature was found to be 80 °C.

3.2. Characterization of the monolith

3.2.1. Morphology and pore properties

The morphology of the prepared epoxy polymer was observed by SEM at different magnifications. As shown in Fig. 2, the monolith exhibited a well-controlled uniform porous integral structure. A narrow skeleton of ~1 μm in diameter and relatively large through-pores of ~3 to 6 μm were observed. The monolithic polymer attached to the inner wall of the capillary very well, without distinct disconnection. The pore properties were measured using the BET method with adsorption data in the relative pressure ranging from 0.01 to 0.99. The specific surface area was determined to be 4.1 m^2/g, and the average pore diameter was 22.3 nm. It is critical for the separation of macromolecules that the pore size must be big enough; otherwise, size exclusion mechanism may be involved in. Conventional chromatographic packing for the separation of macromolecules generally requires the pore size to be 30 nm or larger. Although not to be ideal, the mesopores of the monolithic capillary prepared in this study was large enough.

3.2.2. Characterization of the post modification

The poly(TEPIC-co-TAEA) monolith base was chemically modified with ammonia aqueous solution to enhance the density of primary amino groups on the monolith surface. Fig. 3 compares the FT-IR spectra for polymeric monoliths modified at 60 °C for different reaction times. Compared with the FT-IR spectra for original epoxy monolithic base, new peaks at 1254, 1566 and 1646 cm^{-1} were observed when the modification reaction was carried out. The peak at 1254 cm^{-1} was attributed to the C–N vibrations, while peaks at 1566 and 1646 cm^{-1} were ascribed to the N–H vibrations. These results indicated that the modification reaction occurred as expected. According to the mechanism of the ring-opening reaction, –OH and $–NH_2$ groups were generated by the reaction process.

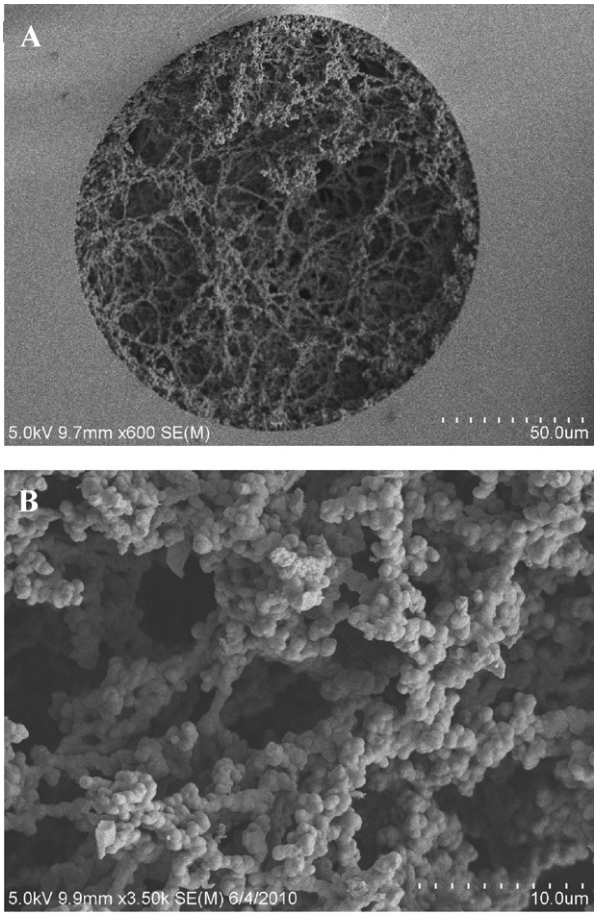

Fig. 2. Scanning-electron microscopy photographs of the cross-section of the poly(TEPIC-co-TAEA) monolithic capillary. (A) 600× (B) 3500×.

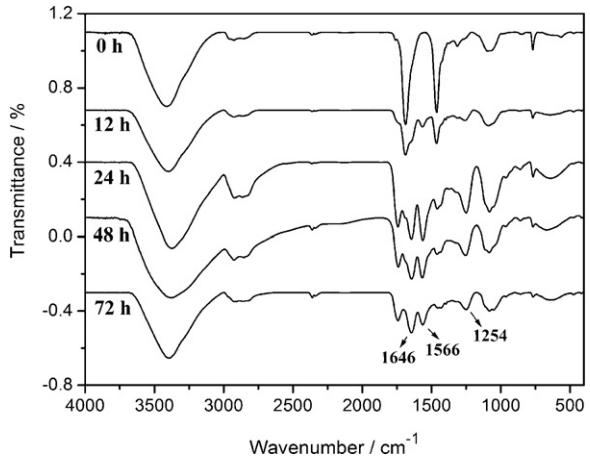

Fig. 3. FI-IR spectra for the poly(TEPIC-co-TAEA) monoliths reacted with ammonia aqueous solution at 60 °C for different reaction times.

The presence of the –OH groups could maintain the monolith hydrophilic while the $-NH_2$ groups could be used as the WAX functional groups.

The influence of the reaction time was further investigated by elemental analysis. According to the reactants, there were only four elements existing herein, including C, H, O, N. Along with the process of the post-modification reaction, the contents of N, H will change, while the contents of O, C will remain constant. Thus, the degree of the reaction can be estimated in terms of the values of the content ratios of N/O (or N/C), H/O (or H/C). If the ring-opening reaction does not occur, then the N/O and H/O ratios will remain the same. While if the ring-opening reaction occurs, the N/O and H/O ratios will increase gradually along with the process of the reaction, and then become constant when all residual epoxide groups have already been completely converted into primary amino groups. Thus, the inflection point in the plot of N/O or H/O against the reaction time can be considered as the optimal reaction time. As shown in Table 1, the experimental trends were found the same as expected, and the inflection point was found to be 48 h. Thus, the post-modification reaction was set at 60 °C for 48 h for further investigations.

3.2.3. Exchange capacity and permeability

Exchange capacity is an important parameter for ion-exchange chromatography. Frontal analysis was carried out to measure the exchange capacity of the prepared WAX monolithic capillary for both small molecule (AMP) and macromolecule (BSA). The pH of loading buffer was set at 7.60 to maintain AMP and BSA negatively charged. Thymidine, a neutral compound, was selected as the dead time marker. The exchange capacity was measured to be 32 mg/mL for AMP but only 1.3 mg/mL for BSA. The distinct difference in the exchange capacity for macromolecule and small molecule is not difficult to understand since a macromolecule could occupy more exchange sites than a small molecule. However, the exchange capacity of BSA was lower as compared with other kinds of monoliths. For instance, the exchange capacity of a WAX monolith prepared by photoinitiated copolymerization of 2-(diethylamino)ethyl methacrylate (DEAEMA) and polyethylene glycol diacrylate (PEGDA) was 24 mg/mL [29]. The lower value was most likely due to the small specific surface area of this kind of WAX column which was resulted mainly from large through-pore size. In addition, it might be partially due to the short spacer arm between the amino groups and the polymerized network.

Table 1
Elemental content at different reaction times for the post-modification reaction.

Reaction time (h)	Content (%)					
	C	H	N	O	N/O	H/O
0	44.91	6.10	15.49	33.50	0.46	0.18
12	43.36	6.81	16.85	32.98	0.51	0.21
24	43.90	7.50	18.77	29.83	0.63	0.25
48	45.57	7.94	20.42	26.07	0.78	0.30
72	45.71	8.02	20.47	25.80	0.79	0.31

The column pressure drops were measured with two solvents, i.e. water and ACN, to evaluate the mechanical stability of the column. A linear dependence of back pressure on flow rate ranging from 0.25 to 13 μL/min was obtained, which indicates that the prepared monolithic capillary promised good stability without moving and compressing of the monolithic bed under a pressure at least up to 25 MPa.

The permeability of the WAX monolithic capillary column was examined to evaluate the swelling and shrinking of the monolith when ACN and water were used as the mobile phase. According to Darcy's Law [35], the permeability was decreased from 2.87×10^{-14} m^2 to 2.36×10^{-14} m^2 (reduced by 16.7%) when the mobile phase changed from ACN to water, which is comparable to 17% for an organic-silica hybrid strong anion exchange monolithic capillary [36]. This result indicates that swelling of the monolith in aqueous buffer and shrinking in organic buffer were slight.

3.2.4. Secondary interactions

Secondary interactions between analytes and stationary phase are often detrimental to ion-exchange chromatography, which may result in irreversible adsorption and peak broadening [37]. A major secondary retention is generally reversed-phase retention when the stationary phase is hydrophobic. As the suppression of reversed-phase retention needs high-content of organic solvent while salts are not well dissolved in organic solvent, reversed-phase retention must be reduced by the greatest extent in ion-exchange chromatography through choosing appropriate stationary phase. Besides, as there are many hydrophilic groups including –OH and $-NH_2$ on the monolith, hydrophilic interactions may be involved in. Therefore, retention due to reversed-phase and hydrophilic interactions should be evaluated. Three un-charged compounds, including toluene, acrylamide and thiourea, were chosen for the evaluation since they have been used for the same purpose in literature [38,39]. The content of ACN was varied from 95% to 6%, while the concentration of ammonium formate was kept constant at 5 mM. The influence of ACN content on the retention time of this test mixture is shown in Fig. 4. Thiourea, a typical dead time marker in reversed-phase liquid chromatography, showed higher retention than acrylamide and toluene. Its retention factor decreased as the ACN content decreased from 95% to 61% while further decreasing the ACN content resulted in slight increase in retention factor.

Acrylamide, a hydrophilic compound, exhibited much less retention than thiourea. The nonpolar compound toluene had almost no retention and eluted firstly as the ACN content decreased from 95% to 27%. When the ACN content further decreased to 6%, the retention of toluene increased, and it was eluted after acrylamide. All these results indicate that the hydrophilic interaction would be involved in when the ACN content was higher than 27%, while hydrophobic interaction would be involved in when the ACN content was lower than 27%. This kind of hydrophilic/hydrophobic mechanism switch was also observed on other hydrophilic monolith materials [38,39]. However, the switching point of the ACN content in the mobile was different. The switching point was around 25 and 55% ACN for the poly(1-(3-sulphopropyl)-4-vinylpyridiniumbetaine-co-*N,N′*-methylenebisacrylamide) [38] and the poly((*N,N*-dimethyl-N-methacryloxyethyl-*N*-(3-sulphopropyl) ammonium betaine-co-ethylene dimethacrylate)

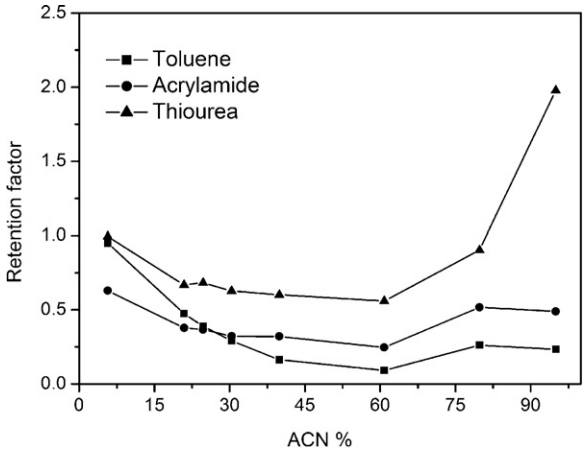

Fig. 4. Influence of ACN concentration on retention of neutral compounds. WAX column: 50 cm (45 cm effective length) × 150 μm I.D. Detection wavelength: 214 nm. Mobile phase: A, 5 mM ammonium formate (pH 4. 10); B, 96% ACN containing 5 mM ammonium formate (pH 4.10); flow rate: 0.75 μL/min. Sample: toluene, acrylamide, thiourea. Sample injection: 600 nL.

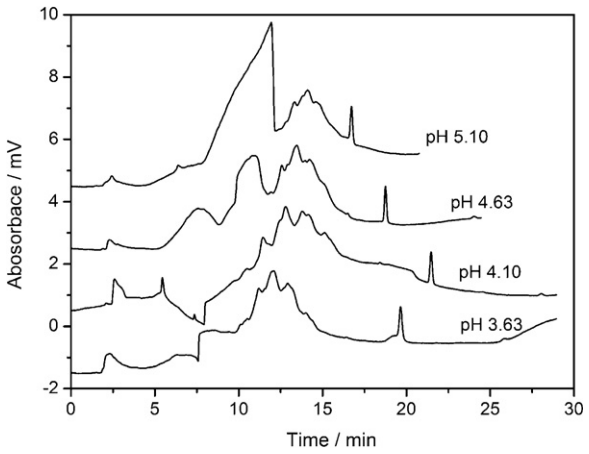

Fig. 6. Effect of the pH value of mobile phase on the WAX separation of AGP isoforms. WAX column: 45 cm (40 cm effective length) × 150 μm I.D. Sample: 0.05 mg/mL AGP. Mobile phase: A, 10 mM NaAc aqueous solution; B, 10 mM NaAc aqueous solution containing 400 mM NaCl, (A) pH = 3.63, (B) pH = 4.10, (C) pH = 4.63, (D) pH = 5.10; gradient, started from 100% A, increased by 3.5% B/min; other conditions are identical as in Fig. 4.

monolithic column [39], respectively. These data suggest that the prepared WAX monolithic capillary was highly biocompatible and non-specific adsorption was significantly suppressed.

3.2.5. Reproducibility

The reproducibility of the WAX monolithic capillary was investigated in terms of the relative standard deviation (RSD) of the retention time for two myoglobin isoforms. As shown in Fig. 5, myoglobin was separated into two isoforms with a NaCl gradient elution. The RSD value of run-to-run reproducibility for the major and minor isoforms was measured to be 1.8% and 2.2% ($n = 6$), respectively. The results suggest good stability of the monolithic capillary.

3.3. Effects of experimental conditions on the separation of glycoprotein isoforms

3.3.1. Effect of the eluent pH

The performance of this WAX polymer monolithic capillary column for complex sample was first investigated by the separation of AGP isoforms with isoelectric point (p*I*) of 2.8–3.8, for which capillary zone electrophoresis (CZE) is the only reported glycoform profiling tool [40]. As proteins carry different charge at different pHs, pH of the mobile phase is a critical parameter that affects the separation. Effect of the mobile phase pH on the resolution of AGP glycoforms was investigated within the pH range of 3.6–5.1 with an interval of 0.5 pH unit. The results are shown in Fig. 6. Using the number, the resolution of peaks and the baseline as the evaluation criteria, the optimal separation was found at pH 4.10, which was slightly higher than the average p*I* value of AGP. It is easy to understand that the charge differences were distinct for protein isoforms when the pH was slightly higher than the p*I* value, then the protein isoforms could be easily differentiated by the WAX monolith. This was used as a guideline for selection of experimental conditions in further investigations.

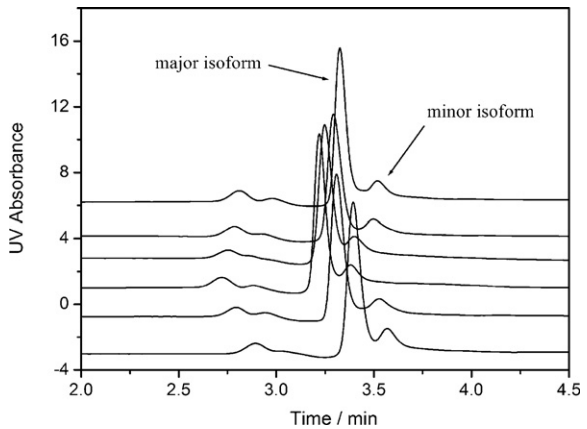

Fig. 5. Separation of myoglobin on the WAX monolithic capillary for 6 consecutive runs. WAX column: 65 cm (60 cm effective length) × 150 μm I.D. Mobile phase: A, 10 mM Tris aqueous solution (pH 9.10); B, 10 mM Tris aqueous solution containing 400 mM NaCl (pH 9.10); gradient, started from 5% B, increased by 15% B/min. Sample: 0.05 mg/mL myoglobin. Other conditions are identical as in Fig. 4.

3.3.2. Effect of the salt gradient slope

The effect of NaCl gradient rate on AGP isoform separation was investigated using 10 mM NaAc (pH 4.10) and 10 mM NaAc containing 400 mM NaCl (pH 4.10) as the solvents A and B, respectively. When the NaCl gradient rate was increased from 2.5 to 4% B/min, the retention time became shorter while the peaks for the isoforms became sharper. However, the resolution of the isoforms was nearly the same. To obtain sharp peaks, a gradient rate of 3.5% B/min was chosen for further investigations.

3.3.3. Effect of the flow rate of the eluent

Effect of the flow rate on the glycoform resolution of AGP was investigated within the range of 0.5–1.25 μL/min. The best resolution of AGP isoforms was observed at an intermediate flow rate, 0.75 μL/min (data not shown).

3.3.4. Effect of addition of urea to the eluent

As discussed in above text, hydrophilic interactions were involved in the separation mechanism on the WAX monolithic capillary. Thus, it was necessary to investigate the effect of hydrophilic interactions on the WAX separation. In order to investigate such an effect, urea was added to the eluent to suppress hydrophilic interaction between the analyte and the stationary phase. In capillary electrophoretic separation of intact glycoproteins [41,42], the presence of urea in the running buffer was found to be a critical factor for the separation. However, it was observed in this study that the presence of urea (1–2 mol/L) in the eluent and the sample matrix did not

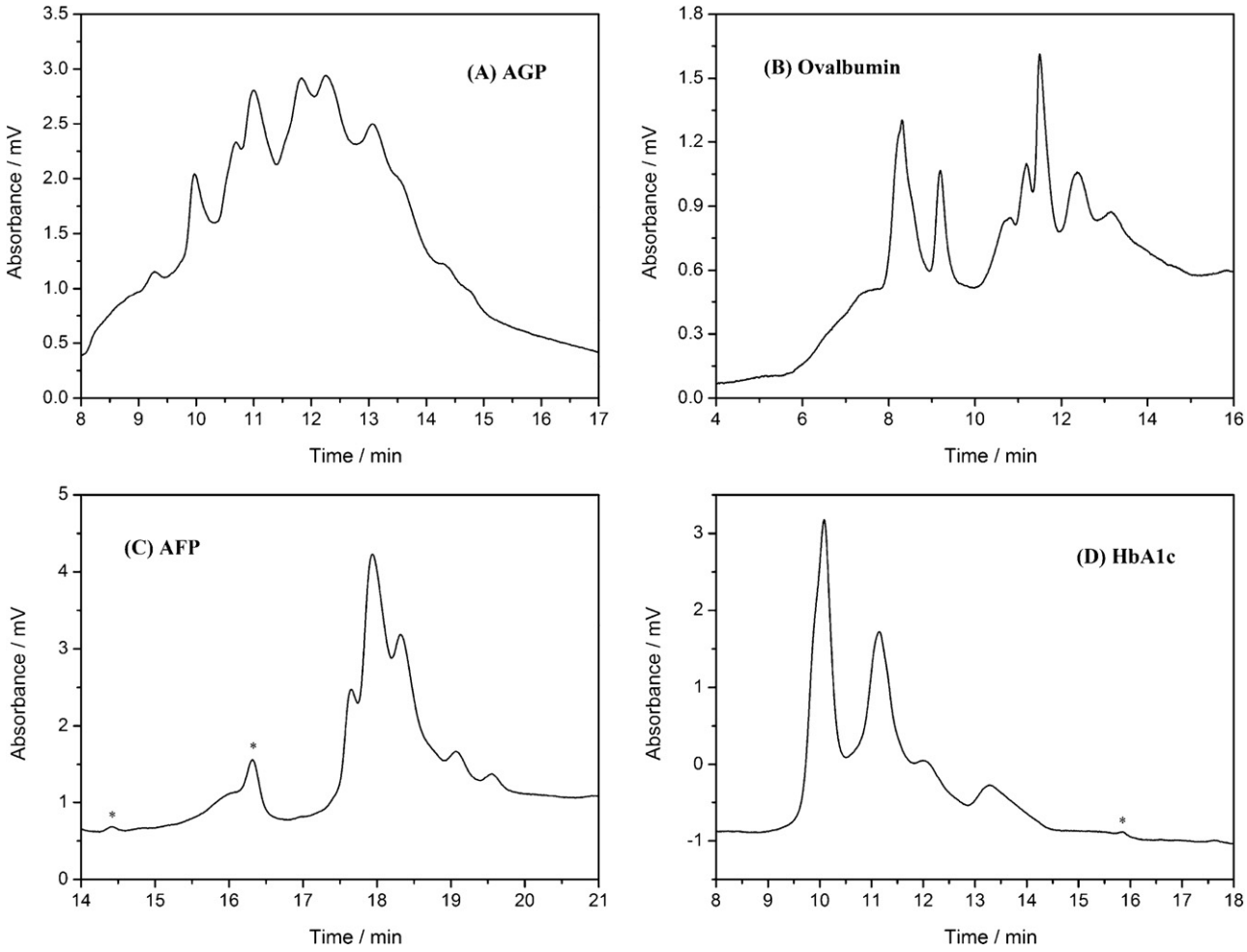

Fig. 7. Optimal separation of four intact glycoproteins on the WAX monolithic capillary column. WAX column: 150 μm I.D., (A) 50 cm (45 cm effective length); (B) and (C) 65 cm (60 cm effective length); (D) 65 cm (60 cm effective length). Sample: (A) 0.5 mg/mL AGP; (B) 0.5 mg/mL ovalbumin; (C) 0.5 mg/mL AFP; (D) 0.5 mg/mL HbA1c. Mobile phase: (A) A, 10 mM NaAc aqueous solution (pH 4.10); B, 10 mM NaAc aqueous solution containing 400 mM NaCl (pH 4.10); gradient, 100% for 0–0.5 min, then increased by 3.5% B/min; (B) and (C), A, 10 mM Tris aqueous solution (pH 7.60); B, 10 mM Tris aqueous solution containing 400 mM NaCl (pH 7.60), gradient, 100% A for 0–0.5 min, then increased by 3.5% B/min; (D) A, 10 mM Tris aqueous solution (pH 8.10); B, 10 mM Tris aqueous solution containing 400 mM NaCl (pH 8.10); gradient, 100% A for 0–0.5 min, then increased by 2.0% B/min; *Baseline disturbance; other conditions are identical as in Fig. 4.

improve the separation. It seemed that the presence of hydrophilic interactions did not result in significant negative effect.

3.4. Separation of glycoprotein isoforms under optimal conditions

According to the optimized procedure of AGP isoform separation, the ability of the prepared WAX monolithic capillary for the separation of complex system was demonstrated by four kinds of intact glycoprotein under optimal conditions, AGP (p*I* 2.8–3.8), ovalbumin (p*I* 4.6), AFP (p*I* 4.5–5.2), and HbA1c (p*I* 7.1). The WAX chromatograms of the test intact glycoproteins under optimized conditions are shown in Fig. 7. All these glycoproteins were resolved into distinct isoforms within 20 min. 10 partially resolved isoforms were observed for AGP. As compared with the CZE separation shown in Fig. 8, which was obtained by slight modification of the method in Ref. [40], more isoforms were obtained on this WAX monolithic capillary column; however, CZE provided better peak resolution. 7 isoforms were observed for ovalbumin while 5 isoforms were observed for AFP. HbA1c is a reference standard and thus one peak or at least one major peak is expected. However, two major peaks and two minor peaks were observed. All these results suggest that high resolution for the separation of glycoprotein isoforms had been achieved on the WAX monolithic capillary.

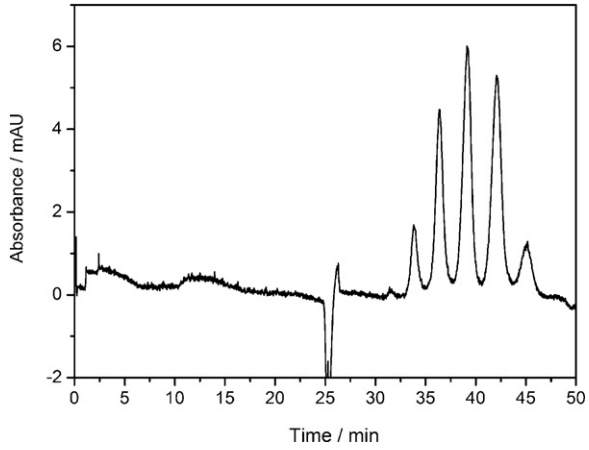

Fig. 8. CZE separation of AGP isoforms. Capillary: 60 cm (50 cm effective length) × 50 μm I.D. Detection wavelength: 214 nm; temperature: 35 °C. Background electrolyte: 10 mM NaAc containing 10 mM NaCl, 10 mM tricine, 7 M urea, and 4.5 mM putresicine, pH 4.50. Applied voltage: 15 kV. Sample: 2.5 mg/mL AGP. Injection: 0.5 psi for 20 s.

4. Conclusion

In this work, we presented high resolution separation of glycoprotein isoforms on a polymeric WAX monolithic capillary. A base monolithic capillary was first prepared by a simple thermally initiated polymerization using epoxy resin-based polymer. The epoxide groups of the column were then one-step post-modified through ring-opening reaction with ammonia solution. The prepared WAX monolithic capillary exhibited well-controlled skeletons, desired pore size distribution and limited retention due to secondary interactions, which made the WAX monolithic capillary very suitable for the separation of intact proteins. The effects of experimental conditions on the resolution of glycoproteins isoforms were systematically investigated. Separations of several protein isoforms were achieved with salt gradient elution under WAX mode, which demonstrated the high efficient separation performance of this monolith. Under optimized conditions, all test glycoproteins were resolved into distinct isoforms. More isoforms which were partially separated were observed for AGP as compared with CZE separation, while the reference standard HbA1c were resolved into 2 major peaks and 2 minor peaks. These results denoted the high resolution of the prepared monolithic capillary for the separation of glycoprotein isoforms.

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