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One-pot preparation of zwitterionic sulfoalkylbetaine monolith for rapid and efficient separation of lysozyme from egg white

Wei Gao [a], Xiao-lan Liu [a], Jia-yuan Li [a], Hong-zhen Lian [a,*], Li Mao [b,**]

[a] State Key Laboratory of Analytical Chemistry for Life Science, School of Chemistry & Chemical Engineering and Center of Materials Analysis, Nanjing University, Nanjing 210023, China
[b] MOE Key Laboratory of Modern Toxicology, School of Public Health, Nanjing Medical University, Nanjing 211166, China

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ABSTRACT

A porous zwitterionic monolithic column was prepared to rapidly and efficiently separate lysozyme from egg white. The monolith was synthesized in a stainless steel HPLC column (5 cm × 4.6 mm i.d.) by in-situ thermal initiated co-polymerization of *N*,*N*-dimethyl-*N*-methacryloxyethyl-*N*-(3-sulfopropyl) ammonium betaine (MSA) and ethylene dimethacrylate (EDMA). Due to the combination of quaternary ammonium and sulfonic groups on the monolithic matrix in one-pot process, the hydrophobic carbon chain and hydrophilic radical were obtained, which provided multiple driving forces for neutral, basic and acidic analytes, thus mix-mode chromatography mechanism contributed to the retention of different charged proteins. Properties such as composition, morphology and stability of the MSA-co-EDMA monolithic column were characterized by various analytical methods and the results showed that the monolith has large through-pores, good hydrophilicity and permeability. The effects of mobile phase pH and ionic strength on proteins were investigated, drawing the conclusion that the main adsorption and elution mechanism of lysozyme on MSA-co-EDMA monolith was electrostatic interaction, while those of other proteins included hydrophobic, hydrophilic and electrostatic interactions. Therefore, efficient separation of lysozyme and other proteins could be successfully achieved by switching the pH of mobile phase. Lysozyme can be adsorbed using 20 mmol/L phosphate buffer (pH 7.0) and eluted with 20 mmol/L phosphate buffer (pH 2.0). To prove the practicality of the monolithic column, it was also applied in the separation of lysozyme in egg white, which means the work has the potential for further development in proteome analysis of real biological samples.

1. Introduction

Porous organic polymer-based monoliths have been studied several decades since the first report by Kubin et al. in 1967 [1]. The porous polymer monolithic beads were typically prepared under the homogenous polymerization process in mold by applying different conditions such as thermal and photograft induction [2]. Because of more rapid mass transfer and larger external porosity, monolithic columns have a much larger permeability than packed columns. However, the silica monolithic columns are harder to modify various functional groups through one-pot procedure and more suitable to analyze small or medium molecular compounds. By contrast, polymer monolithic columns have been widely used to separate extremely large molecules, such as proteins and nucleic acids due to their advantages of simple synthesis, better pH tolerance and good biocompatibility [3,4].

As a typical kind of hydrophilic chromatography column, stationary phases with zwitterionic ligands bonded on polymer substrates have complex interaction mechanisms. Two approaches to synthesize the zwitterionic sulfobetaine monolith within DURAN glass columns were described by Viklund and Irgum [5]. Zwitterionic stationary phases contain both positive and negative charges, as the quaternary ammonium and sulfonate groups present in the sulfobetaine stationary phases [6–8]. The net surface charge of zwitterionic stationary phases should be neutral in theory while actually they reveal slight electronegativity over a wide pH range. Some previous studies reported that the oppositely charged groups are distributed perpendicular which contribute the negative sulfonate groups easier to get access to analytes,

* Corresponding author at: School of Chemistry & Chemical Engineering and Center of Materials Analysis, Nanjing University, 163 Xianlin Avenue, Nanjing 210023, China.
** Corresponding author at: School of Public Health, Nanjing Medical University, 101 Longmian Road, Nanjing 211166, China.
E-mail addresses: hzlian@nju.edu.cn (H.-z. Lian), maoli@njmu.edu.cn (L. Mao).

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even phosphorylcholine-type zwitterionic stationary phase with the quaternary ammonium group at the distal end of the zwitterionic ligand also shows overall negative charges [9]. Thus, most zwitterionic stationary phases could be used as weak cation exchange chromatography (WCX). Regarding the molecular structure, zwitterionic stationary phase has a hydrophilic polar dipole ion and hydrophobic non-polar long-chain alkyl. The zwitterionic sulfobetaine monolithic sorbents show many advantages for peptide separation compared to reverse-phase liquid chromatography because of the mixed mode separation mode. In the previous work of Jandera et al., they have successfully prepared an in-situ zwitterionic sulfobetaine monolith (MON-MSA-EDMA) in fused-silica capillary columns for the separation of phenolic acids, flavones, and nucleosides [10,11]. After the crosslinker in the synthesis procedure changing from EDMA to bisphenol A glycerolate dimethacrylate (BIGDMA) and dioxyethylene dimethacrylate (DiEDMA), the columns show high reproducibility and repeatability of chromatographic properties, and provide remarkably fast and highly selective separations of polar compounds [12,13]. Jiang et al. used similar approach to prepare a porous monolithic poly-(SPP-co-EDMA) column through the in-situ co-polymerization of the monomer *N,N*-dimethyl-*N*-(3-methacrylamidopropyl)-*N*-(3-sulfopropyl) ammonium betaine (SPP) and crosslinker EDMA within different i.d. fused-silica capillaries (50, 100 and 400 μm i.d.) [14–16]. Within larger-scale inner diameter columns, the monolithic columns possess more advantages such as increased sample loadability and decreased extra column effects [16]. It has been reported that the zwitterionic grafted capillary could be used for the separation of small peptides and basic proteins at high efficiency [14]. Here, we attempt to employed 4.6 mm i.d. stainless-steel as mold to prepare the zwitterionic monoliths for the separation of lysozyme from other proteins which have different surface charges.

Lysozyme is a valuable enzyme which is widely distributed in body fluid and secretions to destroy certain bacteria [17–19]. Nowadays, lysozyme has been applied in food industry as antiseptic for food preservation [20]. Moreover, because of its antibacterial and antibiotic, the lysozyme can enhance immunity and treat multiple inflammation [21,22]. Due to the commercial and medical importance of lysozyme, effective separation and purification of lysozyme from chicken egg white is an important work, thus different methods and techniques concerning liquid chromatography have been developed in recent years. As lysozyme is a highly isoelectric point enzyme (pI 11), it can directly bind negatively charged virus proteins, and ion-exchange adsorbents with negatively charged groups are conventional materials to purify lysozyme [23]. Several cation exchange monolithic stationary phases have been prepared to separate lysozyme from other proteins, as the monoliths could offer an efficient, non-denaturing and inexpensive separation. Three new calixarene-immobilized monolithic stationary phases with cation exchanger character are very promising materials for preparative protein purification [24]. A hydrophilic monolithic cation exchange column containing sulfonic acid group has been in-situ prepared with the binding capacity of 20.1 mg/mL for lysozyme [25]. A strong sulfoalkylated monolithic cryogel was used as a stationary phase for strong cation exchange chromatography of some proteins including lysozyme [26]. In addition, some molecularly imprinted and DNA aptamer materials have been prepared for the selective recognition and capture of lysozyme by chromatography [27,28]. Compared to these affinity materials, cation exchange monolithic columns have the advantages of easy to prepare and cost-saving while the selective separation still shows good performance.

In this work, a zwitterionic stainless-steel monolithic column was prepared through a one-step in-situ copolymerization of the monomer *N,N*-dimethyl-*N*-methacryloxyethyl-*N*-(3-sulfopropyl) ammonium betaine (MSA) and crosslinker ethylene glycol dimethacrylate (EDMA). The zwitterionic monolithic column prepared in 4.6 mm i.d. inner diameter column mold can increase sample loadability and decrease extra column effects. Subsequently, the zwitterionic column was used to adsorb and separate lysozyme from protein mixtures and chicken egg white. The positively charged lysozyme could be adsorbed on monoliths based on electrostatic interaction and easily eluted by changing the pH of mobile phase. Several different pI proteins are introduced to figure out the adsorption mechanism. The adsorption parameters such as pH, ionic strength and types of mobile phases were investigated.

2. Experimental

2.1. Reagents and materials

The hydrophilic monomer MSA was purchased from Aladdin Chemical (Shanghai, China). EDMA was purchased from Alfa Aesar (Tianjin, China). 2,2′-Azoisobutyronitrile (AIBN) was purchased from J&K Scientific Ltd (Beijing, China). The solvent 1,4-butanediol was purchased from Shanghai Lingfeng Chemical Reagent Co., Ltd. and 1-propanol was purchased from Sinopharm Chemical Reagent Co., Ltd. (Guangzhou, China). Lysozyme (Mw 14.3 kDa, pI 11) was purchased from GBCBIO Technologies (Guangzhou, China). Conalbumin (Mw 80 kDa, pI 6.1) and myoglobin (Mw 16.7 kDa, pI 6.99) were purchased from Sigma-Aldrich (Shanghai, China). Ovalbumin (Mw 45 kDa, pI 4.5) was purchased from Acros Organics (Shanghai, China). Cytochrome c (Mw 13.7 kDa, pI 10.7) was purchased from Aladdin Chemical (Shanghai, China). HPLC-grade acetonitrile (ACN) was purchased from Honeywell Inc (USA). Chicken eggs were purchased from a local market. Other chemicals were of analytical reagent grade or better and used without further purification. Deionized water (DIW) of 18.25 MΩ cm collected from a Milli-Q water system (Millipore, Bedford, MA, USA) was used throughout the experiment.

2.2. Preparation of poly (MSA-co-EDMA) monolithic column

The polymerization mixture for the monolithic column was prepared as follows: 20% (w/w) MSA as monomer and 15% (w/w) EDMA as cross-linker were dissolved in ternary solvent mixture, containing 25% (w/w) 1-propanol, 25% (w/w) 1,4-butanediol and 15% (w/w) water, which had been injected into a dry centrifuge tube. After the mixture solution was put under vortex movement for 60 s, the initiator AIBN was added into the homogenous mixtures (1% w/w with respect to monomers) and ultrasonicated for 20 min to dissolve. The obtained polymerization mixture was injected into stainless-steel column (5 cm ×4.6 mm i.d.) with a syringe. The both ends of the stainless-steel column were sealed with stoppers. The polymerization was performed in water bath for 20 h at 60 °C. After cooling to room temperature, the column was washed with ACN for 1 h under the flow rate of 1 mL/min to remove unreacted monomers and porogenic diluents.

2.3. Apparatus

The elemental analysis (EA) was carried out to investigate the ratio of MSA and EDMA which participate in the reaction with an Elementar Vario EL III using oxygen as the combustion gas. The scanning electron microscopy (SEM) images of the monolithic materials were obtained using a Hitachi S-3400 N II scanning electron microscope. Pore size distribution was investigated by a mercury intrusion porosimeter (Poremaster GT-60, Quantachrome, USA). An OCA30 contact angel system (Dataphysics Instruments Gmb) was used for the determination of water contact angles. ζ-potential measurements were carried out using NanoBrook 90Plus Zeta Potential Analyzer (Brookhaven Instruments Corporation). The

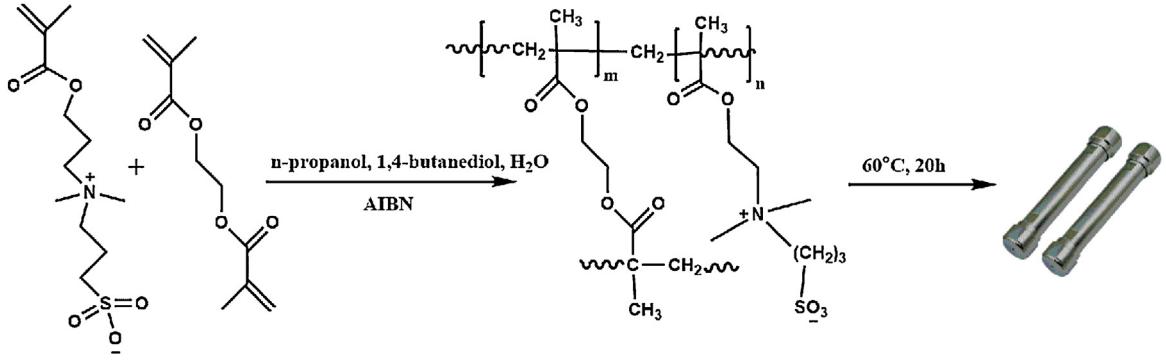

Fig. 1. The general procedure of the in-situ polymerization.

distribution of protein molecular weight was obtained by a MALDI-TOF-MS (4800 plus, Applied Biosystems, Framingham, CT, USA). All chromatographic experiments were performed on a 1200 HPLC system from Agilent Technologies (Shanghai, China). The HPLC system consists of a vacuum degasser (G1322A), a quaternary pump (G1311A), an autosampler (G1329A), and a diode array detector (DAD) (G1314A). Agilent ChemStation (Agilent, Palo Alto, CA, USA) was used and operated under Windows XP for data acquisition.

2.4. Extraction of lysozyme from chicken egg white

Chicken egg white was roughly collected from fresh eggs and diluted by 2-fold with phosphate buffer solution (20 mmol/L, pH 7.0). The diluted egg white sample was homogenized by stirred in an ice bath for 30 min and then centrifuged at 4 °C and 10,000 rpm for 15 min. The supernatant was collected and the 10-fold dilution of the collected solution was prepared as the direct injection sample solution (The overall protein concentration of the raw chicken egg white was about 111.3 mg/mL, measured by microplate reader, Thermo Fisher).

2.5. Chromatographic procedures

Lysozyme adsorption studies on the zwitterionic monolithic column were accomplished by using a continuous column system in accordance with certain parameters such as pH, concentration, flow rate, ionic strength and temperature effects. All chromatographic experiments were carried out at 30 °C (maintained by column oven) and the DAD wavelength was set up at 230 nm. All protein solutions were formulated with a concentration of 0.5 mg/mL. As to the separation experiment of lysozyme, the mobile phase A for equilibrium and sample loading was 20 mmol/L phosphate buffer (pH 7.0), and the elution solution B was 20 mmol/L phosphate buffer (pH 2.0). All executive runs were equilibrated with mobile phase A for at least 20 min prior to injection.

In order to evaluate the MSA-co-EDMA column selectivity of lysozyme, the effluent of egg white solutions from the column was collected and lyophilized for further MALDI-TOF-MS analysis. 0.5 μL of the lyophilized solution was deposited on a MALDI plate, and then another 0.5 μL of DHB or CHCA solution was introduced as a matrix to perform MALDI-TOF-MS analysis in positive ion mode.

3. Results and discussion

3.1. Preparation and characterization of zwitterionic monolithic column

The general procedure of the in-situ polymerization in the stainless column and the optical images of the formed monolith are shown in Fig. 1 and Fig. S1 respectively. According to the previous work of Jandera [12], bisphenol A glycerolate dimethacrylate (BIGDMA) as crosslinker can positively affect the solvation of the monolithic skeleton and the accessibility of the inner pores for low-molecular compounds. However, when the situation comes to the stainless column from capillary column, the high viscosity BIGDMA led to a much higher back pressure of monoliths. As a result, EDMA was chosen as crosslinker. MSA were utilized as monomer and water, 1-propanol, 1,4-butanediol were used as co-solvent [10]. From the optical photo, it can be seen that the formed column has homogeneous monolith.

Table 1
EA and EDS results of the monolithic column.

EDS	wt%	at%	EA	wt%	mole ratio
C	86.9	90.55	N	2.26	0.16
O	11.1	8.67	C	47.33	3.94
S	2.0	0.78	S	5.98	0.19
			H	8.20	8.20

Permeability, which reflects the through-pore size and porosity, could measure the ability of liquid passing the monoliths. It was calculated based on Darcy's Law:

$$K = \frac{F \times \eta \times L}{P \times \pi \times r^2}$$

Where F, η, L, P and r are the flow velocity of mobile phase ($m \cdot s^{-1}$), the viscosity of mobile phase (Pa·s) [29], the length of the monolith (m), the back pressure of the monolith (Pa) and the inner radius (m), respectively. The calculated permeability of the zwitterionic monolithic column was 1.99×10^{-14} m^2. The data indicated that the column has high permeability, which means the large numbers of pores form in monoliths.

The SEM image (Fig. 2A) shows many macropores and flow-through channels in the monolithic skeleton, which resulted in low back pressure and high permeability. The uniformly small size of the particles also offered a high surface area. The pore size distribution of the monolithic column was shown Fig. 2B, which indicated that the macropores sizes were distributed in a narrow scale. The total porosity, most probable value of the pore size, and total surface area were 29.99%, 2.785 μm, and 6.24 m^2 g^{-1}, respectively.

To measure the total percentage of functional groups in the final polymer, the EA and energy dispersive spectrometer (EDS) of the monolithic column were examined as listed in Table 1. The w/w ratio of S to N obtained from EA result was about 1.19, indicating the existence of zwitterions and sulfobetaine group was slightly more than quaternary ammonium group.

Water droplet profile (Fig. 2C) on the poly-MSA-co-EDMA substrate showed that the contact angle was 60.2° ± 0.6°, which indicated the definite hydrophilicity of the polymer surface.

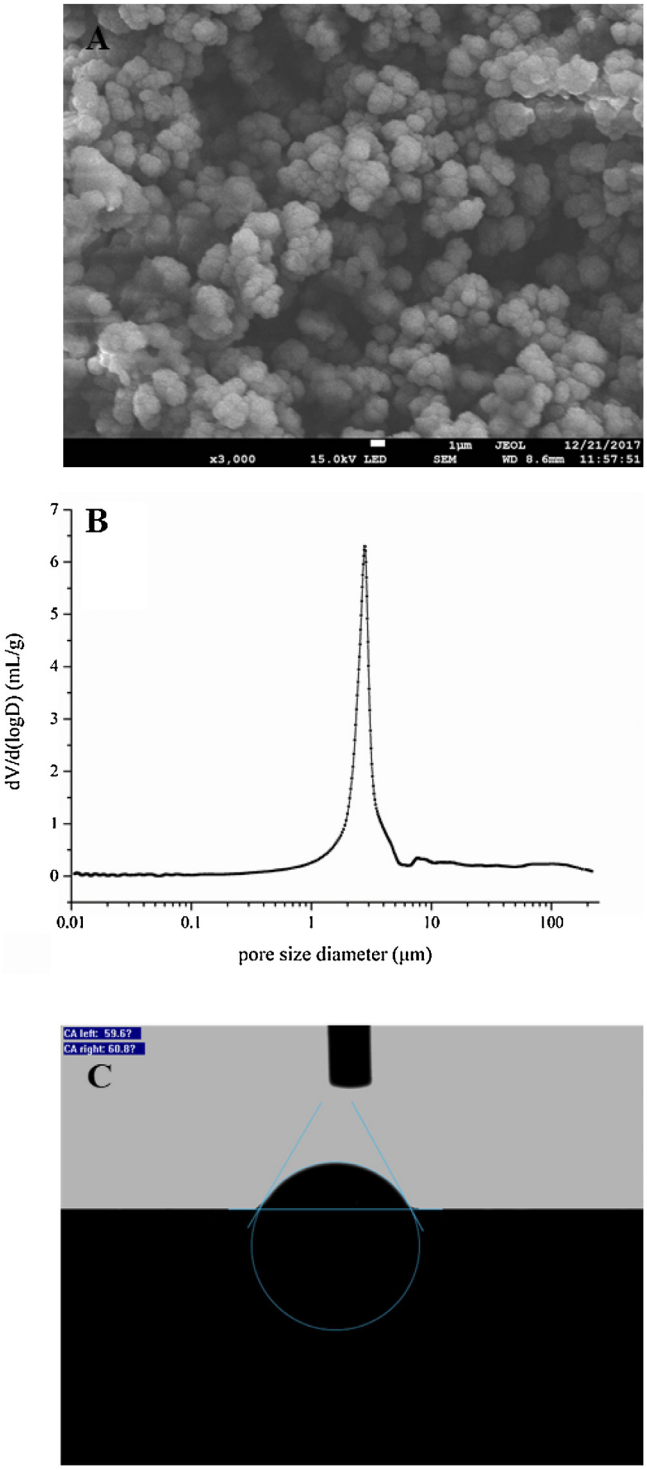

Fig. 2. A. The SEM image of the monolithic column; B. Macropore size distribution of the zwitterionic monolithic column; C. Images for the contact angle measurement.

In order to better understand the potential electrostatic interaction properties on the monolith, the ζ-potential values on the poly (MSA-co-EDMA) monolithic column was measured in 20 mmol/L ammonium phosphate buffer at eight different pH levels (pH 2.0–9.0) as shown in Fig. 3. A positive ζ-potential value was detected on the zwitterionic monolith below pH 5.0, and it turned to negative above pH 5.0. With the buffer pH increasing from 2.0 to 5.0, the absolute ζ-potential value of the monolithic material decreased, which was caused by the ionization state changing. Then, the absolute ζ-potential value increased when the buffer pH increased from 5.0 to 9.0.

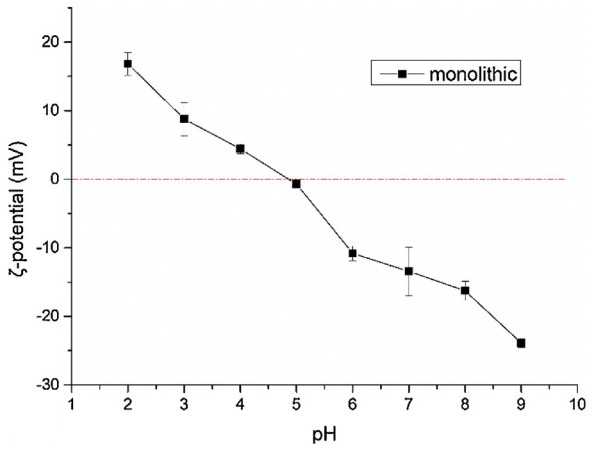

Fig. 3. ζ-potential measurement of the zwitterionic monolithic column.

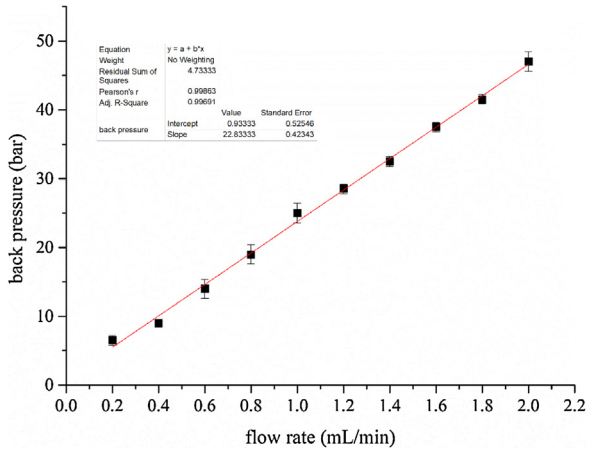

Fig. 4. Effect of water flow rate on the back pressure of zwitterionic monolithic column.

The back pressures of the monolithic column at different flow rates with water as mobile phase are important parameter to characterize the mechanical performance and permeability of the monoliths. Fig. 4 shows a back pressure plotted against the flow rates from 0.2 to 2.0 mL/min with 0.2 mL/min as an augmented interval. A good linearity (R^2 = 0.9969) indicated that the monolithic column could bear high flow rates to carry on a fast and efficient analysis.

3.2. Evaluation of the interaction behavior of MSA-co-EDMA monolithic column with proteins

To investigate the interaction behavior of the MSA-co-EDMA monolith, lysozyme and four other proteins (ovalbumin, conalbumin, cytochrome c, and myoglobin) with different pI values were selected for further evaluation. The surface of MSA-co-EDMA monolith was negatively charged above pH 5.0 and positively charged below pH 5.0, whereas five proteins have different charge values respectively. Electrostatic interactions existed between MSA surfaces and proteins, so the adsorption occurred. Therefore, mobile phase pH is critical for the separation of proteins because it influences the surface property of the monolithic column and the distribution of proteins on stationary phase.

Fig. 5 displays the adsorption behaviors of these proteins with variation in pH of the sample solution. Fig. 5A shows the adsorp-

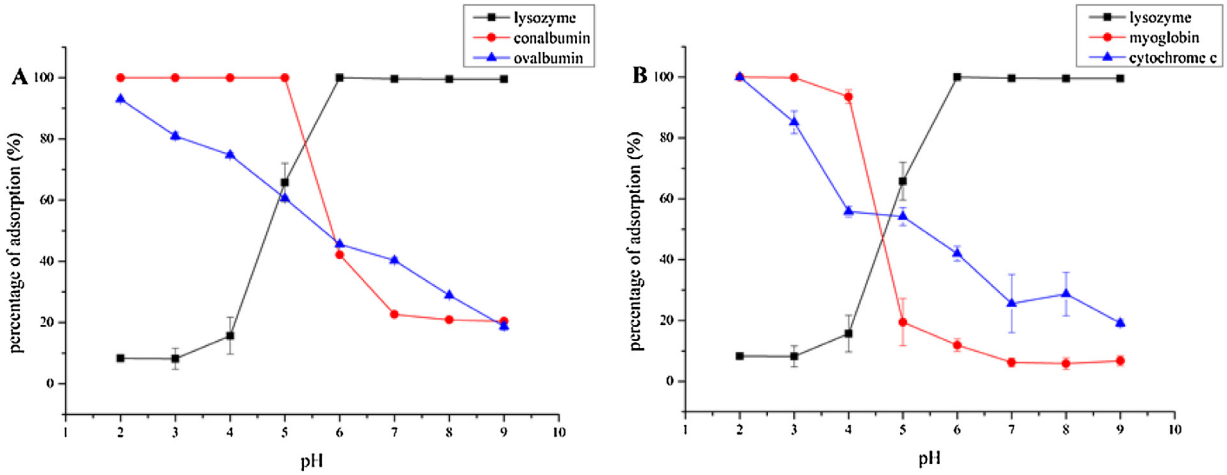

Fig. 5. Effect of pH value on adsorption of lysozyme, conalbumin and ovalbumin (A), and lysozyme, myoglobin and cytochrome c (B). Mobile phase: 20 mmol/L phosphate buffer.

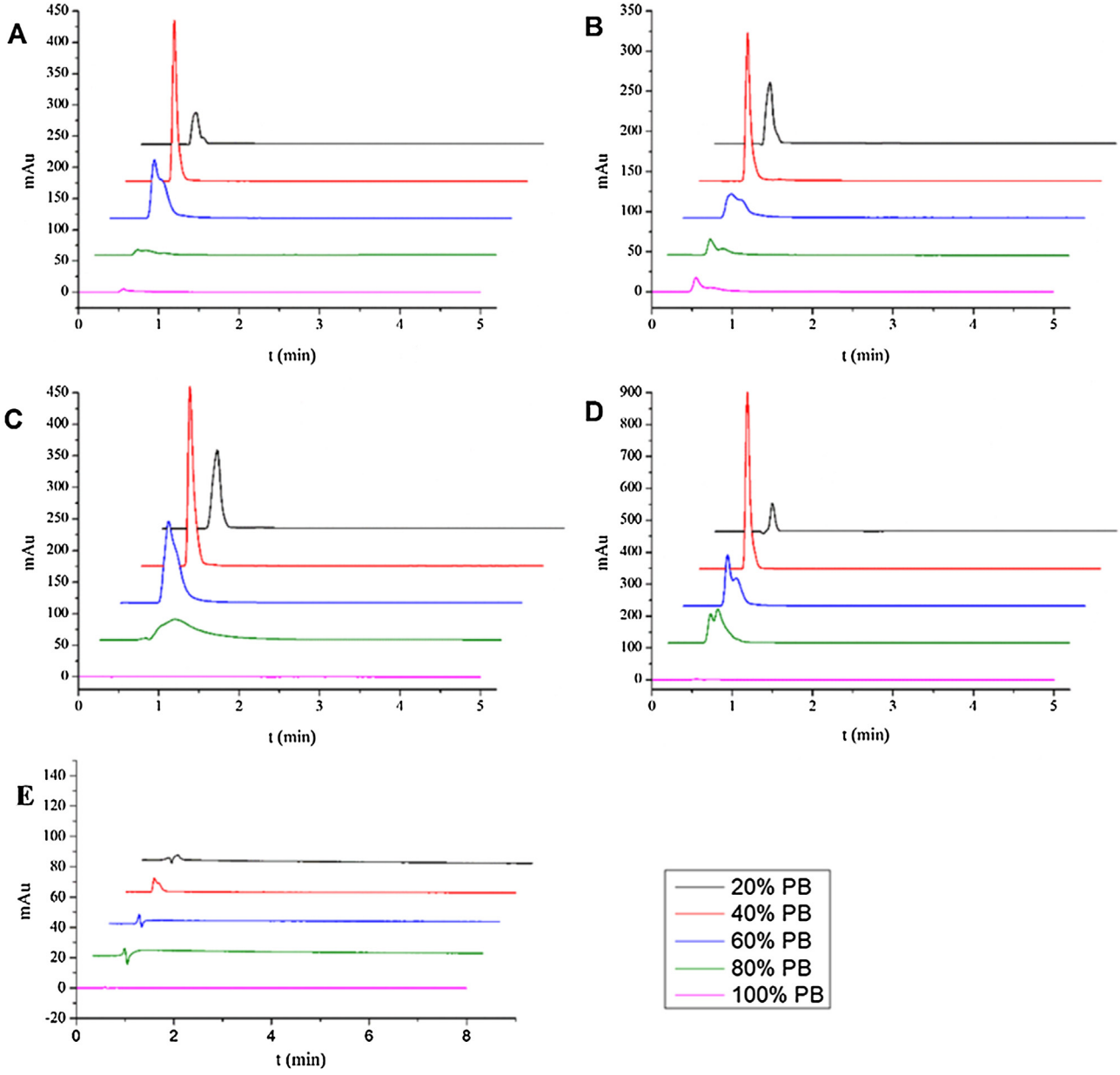

Fig. 6. The chromatograms of conalbumin (A), ovalbumin (B), myoglobin (C), cytochrome c (D) and lysozyme (E). Mobile phase: 20 mmol/L phosphate buffer in ACN (%). A, B, C and D. pH 2.0; E. pH 7.0. Flow rate: 1 mL/min. Detection wavelength: 230 nm.

Table 2
Comparison of maximum lysozyme adsorption capacities of some ion-exchange monolithic columns.

	Monolithic column	Lysozyme solution	Adsorption capacity		Ref.
			mg/mL	mg/g	
1	Calixarene-immobilized monolithic cryogels	0.5 mg/mL, pH 7.0	P-CLX-PO_4: 7.7 P-CLX-SO_3: 5.9 P-CLX-COO: 2.9	P-CLX-PO_4: 340 P-CLX-SO_3: 260 P-CLX-COO: 130	[24]
2	Silica-supported sulfonic acid group monolithic column	N/A	20.1	N/A	[25]
3	Sulfoalkylated poly (hydroxyethylmethacrylate-co-glycidylmethacrylate) monolithic cryogels	0.5 mg/mL, pH 6.4	8	N/A	[26]
4	Poly (2-carboxyethyl acrylate-co-poly (ethylene glycol) diacrylate) monolith	3.0 mg/mL, pH 6.0	72.7	N/A	[32]
5	Polymer-based strong cation-exchange monolith containing 40% sulfopropyl methacrylate	15.0 mg/mL, pH 7.0	52	N/A	[33]
6	Poly (2,3-dihydroxypropyl methacrylate-co-ethylene methacrylate) monolith	N/A	29.2	N/A	[34]
7	Zwitterionic sulfoalkylbetaine poly (MSA-co-EDMA) monolith	0.5 mg/mL, pH 7.0	22	67	This work

tion trends of three most abundant proteins in egg white, including lysozyme (pI 11), conalbumin (pI 6.1), and ovalbumin (pI 4.5). Between pH 2.0 to 9.0, lysozyme, as basic protein, showed positively charged because of the protonation while the ζ-potential value of the monolithic column turned from positive to negative at pH 5.0. As a result, the electrostatic repulsion between lysozyme and monolith was reduced and the adsorption percentage of lysozyme was enhanced. Nevertheless, the adsorption percentage experimental data and theoretical trends of other proteins on the monolithic matrix were different. In Fig. 5B, we chose other two proteins, myoglobin (pI 6.9) and cytochrome c (pI 10.7), for comparison. Even though cytochrome c has similar molecular mass and pI to lysozyme, the adsorption trends were totally opposite, which implied that except electrostatic interaction, other interaction mechanisms exist between proteins and monolithic matrix.

Organic solvent ACN was introduced into mobile phase to investigate whether the hydrophobic interaction exists between proteins and zwitterionic monolithic matrix. Fig. 6 shows the chromatograms of different proteins under different mobile phase ratios. Under the aqueous phase of 20 mmol/L PB (pH 2.0), cytochrome c and other three proteins were almost completely adsorbed on the monolithic matrix (Fig. 6A–D). With the increase of ACN concentration, these four proteins can be eluted and the peak area had a trend of first enlarging and then decreasing. In addition, small molecular compound gallic acid was chosen as target analyte to study the influence of water content on the retention of the zwitterionic monolithic column. By varying water content of the mobile phase from 10% to 70%, the plot of the logarithm of retention factor *vs* volume fraction of aqueous phase was a U-shaped curve (Fig. S2). In the range of the water content increasing from 10% to 40%, water acted as a strongest eluting solvent (typical HILIC conditions). When the water content increasing above 40%, the situation is closer to that hydrophobic retention occurs on the stationary phase surface, which is consistent with previous reports [30,31] and is a typical feature of mixed mode retention behavior. It can be seen from Fig. 6A–D that in addition to the electrostatic interaction mentioned above, there are very significant hydrophilic and hydrophobic interactions between the four proteins and the monolithic matrix. In contrast, under the aqueous phase of 20 mmol/L PB (pH 7.0), lysozyme was almost totally adsorbed on the monolithic matrix. However, despite different concentration ACN was introduced into the mobile phase, the chromatogram showed the lysozyme was not eluted (Fig. 6E). Therefore, we speculate that between lysozyme and monolithic column, the electrostatic interaction is dominant, while electrostatic interaction, hydrophobic interaction and hydrophilic interaction all exist between the other four proteins and the monolith.

The effect of ionic strength was studied by changing the concentrations of phosphate buffer in the mobile phase. As shown in Fig. 7, there was a significant reduction in the adsorption amount

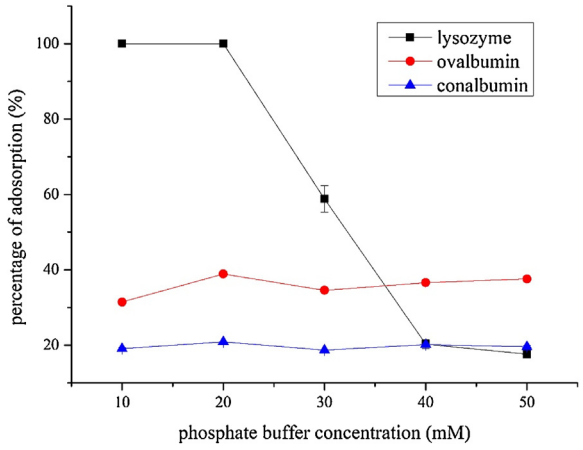

Fig. 7. Effect of ionic strength on adsorption of lysozyme, conalbumin and ovalbumin. Mobile phase: phosphate buffer (pH 7.0).

of lysozyme with the increase of ionic strength. Since electrostatic interaction is the dominant driving force for the adsorption of lysozyme on monolith, high ionic concentration can compete with positively charged lysozyme and preferentially bring a masking effect on the negatively charged zwitterionic monolith, thus leading to the reduction of adsorption amount of lysozyme. The influence of ionic strength on the adsorption of lysozyme further confirmed the speculation that electrostatic interaction is the main driving force between lysozyme and the monolith. In contrast, the adsorption amount of conalbumin and ovalbumin remained steadily stable with the increase of ionic strength, which demonstrated our conjecture that electrostatic interaction is not the only driving force for the adsorption of ovalbumin and conalbumin on monolith.

The dynamic adsorption capacity of the MSA-co-EDMA was calculated based on 5% volume of the breakthrough curve (Fig. S4), and expressed in mg/mL of the column volume. The breakthrough capacity was 22 mg/mL (67 mg/g), which was comparable with that of most of the ion-exchange monolithic columns previously reported for lysozyme as shown in Table 2 [24–26,32–34].

3.3. Separation of protein mixture

According to the above investigations, the mixtures containing lysozyme and ovalbumin (the mass ratio of lysozyme and the other protein was varied from 1:4 to 4:1) were used as samples and a suitable stepwise gradient elution procedure could be applied to carry out the separation of lysozyme from protein mixture. According to Fig. 5, lysozyme could be adsorbed on the monolithic column between pH 6.0 to 9.0 and eluted between pH 2.0 to 3.0. In the experiments, we focused on the adsorption behavior at neutral pH, and thus the adsorption and elution pH values were selected as

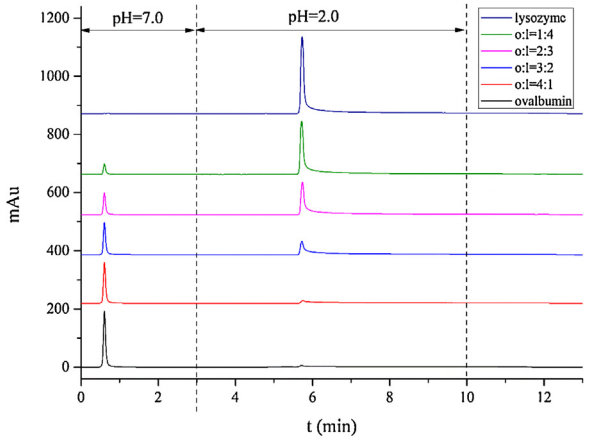

Fig. 8. The chromatograms of the mixture containing lysozyme and ovalbumin with different proportion. Mobile phase: 20 mmol/L phosphate buffer (pH 7.0) in 0–3 min and 20 mmol/L phosphate buffer (pH 2.0) in 3–10 min. Flow rate: 1 mL/min. Detection wavelength: 230 nm.

7.0 and 2.0. Thus, pH 2.0 and pH 7.0 were selected for adsorption and elution, respectively. 20 mmol/L PB (pH 7.0) was performed within 0–3 min and switched to 20 mmol/L PB (pH 2.0) after 3 min. With the stepwise gradient elution, ovalbumin was well eluted within 1 min (dead time) and lysozyme was eluted at 5–6 min (Fig. 8). The protein concentration of different mass ratios and the peak area of the chromatograms showed a good linear relationship (Fig. S3).

3.4. Separation of lysozyme in egg white

The practicability of zwitterionic monolithic column was validated by selective separation of lysozyme from fresh chicken egg white, in which the mass ratio of lysozyme, ovalbumin and conalbumin is usually 1:16:4 [35]. In this work, egg white sample was diluted 2-fold and 20-fold with 20 mmol/L PB (pH 7.0) and the stepwise gradient elution was adopted as mentioned above, 20 mmol/L PB (pH 7.0) was performed within 0–3 min and switched to 20 mmol/L PB (pH 2.0) after 3 min. By adding a spiked lysozyme of known concentration to the real sample, the peak of lysozyme could be identified. According to these results, it is clear that the MSA-co-EDMA monolith can be employed for the separation of lysozyme from fresh egg white.

As shown in Fig. 9A, other unknown proteins in egg white were directly eluted near the void time. At 3 min, the pH of mobile phase was switched from 7.0 to 2.0, lysozyme could be well eluted and its corresponding chromatographic peak was 5.73 min. The effluent near the retention time was collected for further study on MALDI-TOF-MS to identify the specific mass weight.

As the mass weight range from 4.5 kDa to 160 kDa, it was observed that only two peaks of the eluate sample appeared on the MALDI-TOF-MS spectra (Fig. 10A). The 14160.51 *m/z* peak is the protonated molecular ion peak $[M+H]^+$ of lysozyme, while the 7035.20 *m/z* peak is the double charged ion peak $[M+2H]^{2+}$. While

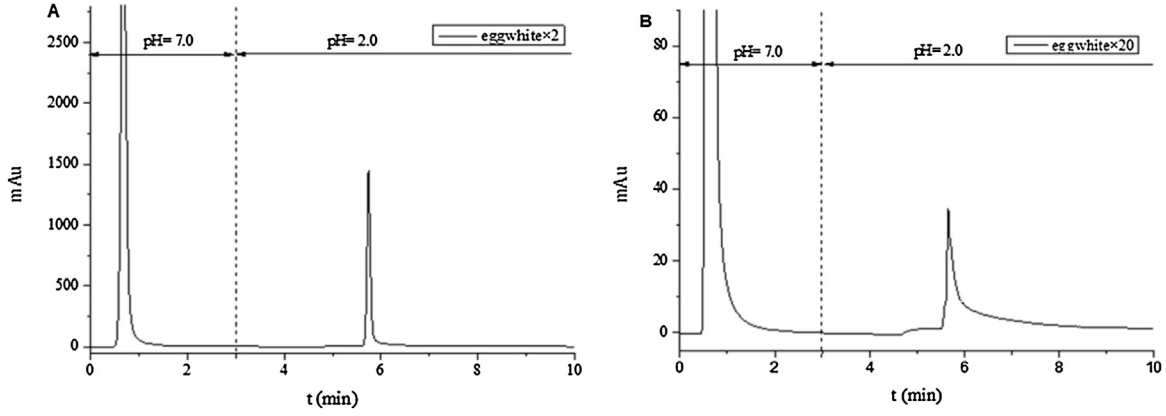

Fig. 9. The chromatograms of the 2-fold (A) and 20-fold (B) egg white sample. Mobile phase: 20 mmol/L phosphate buffer (pH 7.0) in 0–3 min and 20 mmol/L phosphate buffer (pH 2.0) in 3–10 min. Flow rate: 1 mL/min. Detection wavelength: 230 nm.

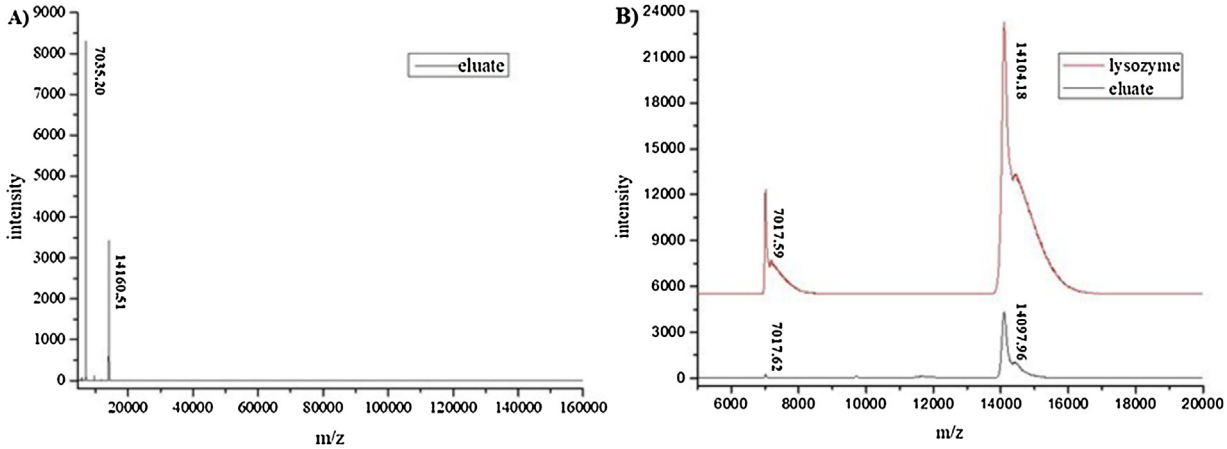

Fig. 10. The MALDI-TOF-MS spectra of the eluate of egg white sample after adsorption on the zwitterionic monolithic column (A), and the standard lysozyme and eluate of egg white sample (B). Matrix: A. CHCA; B. DHB. Mass weight range: A. 4500–160,000; B. 4500–20,000.

using DHB as matrix, the MALDI search result of the standard lysozyme was also provided in Fig. 10B for contrast, which confirmed the conclusion that the lysozyme was satisfactorily isolated and captured by the zwitterionic monolith with high selectivity. Because the *m/z* value of the peak is obtained by normal distribution, the peak mass of the eluate sample has some differences with the relative molecular mass of the standard lysozyme to some extent. It is also normal that two MALDI-TOF-MS spectra showed some differences in the intensity of the double charged ion peak, which was arisen by different matrices.

4. Conclusion

An in-situ poly (MSA-co-EDMA) zwitterionic monolithic rod was prepared in a one-pot procedure. The synthesis method was simple, facile and efficient, developing the approach from the confines of a capillary to a larger inner diameter tube. The resultant zwitterionic monolith exhibited excellent selectivity toward lysozyme, thus lysozyme can be well separated from protein mixtures under optimized chromatographic conditions. Moreover, trapping lysozyme from real chicken egg white sample was achieved with good selectivity and stability. The study shows that chromatographic retention mainly results from the electrostatic interaction between lysozyme and monoliths, while other studied proteins were adsorbed on monoliths based on mix-mode interaction mechanism. The protocol for selectively screening lysozyme suggests that the zwitterionic monolith has a promising application for proteomic analysis.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jpba.2019.07.009.

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