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Preparation of phenylboronic acid functionalized cation-exchange monolithic columns for protein separation and refolding

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ABSTRACT

In this study, we described a simple and effective modification procedure to prepare poly (methacrylate-co-ethylene glycol dimethacrylate) monolithic columns functionalized with 3-aminophenylboronic acid. The column morphology, pore size and specific surface area of the fabricated monolith were characterized by scanning electron microscopy, X-ray photoelectron spectroscopy, thermogravimetric analysis, and mercury intrusion porosimeter, respectively. The frontal analysis was carried out for dynamic loading capacity of the model protein on the modified column. The chromatographic performance of the cation-exchange monolith was evaluated through separating a mixture of five proteins such as lysozyme, cytochrome *c*, ribonuclease A, trypsin and bovine serum albumin and one-step purification of lysozyme from egg whites, and the expected results were obtained. In addition, the functionalized column was used to refold ribonuclease A and cytochrome *c*, and this procedure was monitored by circular dichroism and fluorescence spectroscopy. Compared with the conventional dilution refolding method, the ion-exchange chromatography refolding method developed here is more effective for specific bioactivity recovery.

1. Introduction

The column technology plays a crucial role in the development of new methods and technologies for separating complex biological samples. The development of monolithic stationary phases based on either silica or polymer skeleton is a relatively recent advance in the preparation of chromatographic columns [1–6]. The monolithic column has advantages over conventional particle-packed column in terms of easy preparation, versatile surface modification and higher permeability along with a good peak capacity. Moreover, the monolithic column has no need for particle-size classification, packing and frit making. Ion-exchange chromatography (IEC) has been widely applied in the biomolecule separation, which can provide near physiological environment for solutes. The ion-exchange monolithic column is also an important type of polymer-based monoliths for the analysis and separation of biomolecules. Introducing ion-exchange groups into the polymeric monolith backbone opens new avenues to the preparation of monolith with tailored surface chemistries. Different approaches have been developed to prepare ion-exchanger, including adsorption [7–10], co-polymerization [11–14] and post-modification [15–17]. Among these three methods, post-modification can significantly improve the ion-exchange capacity on the surface of macroporous monoliths, and several applications of this approach have been used to prepare ion-exchanger based on active epoxy group copolymers [18–23].

It is well known that the ion-exchange groups play important roles in the achievement of high-performance separation. Carboxylic, sulfonic and phosphonic acids are the usually used ion-exchange groups for cation-exchangers. Due to the new challenges from environmental studies, proteomics and biotechnology, the development of new and versatile separation media is desirable. Boronic acids, due to their unique chemistry properties that can reversibly form complexation with 1,2- or 1,3-cis-diol to generate five- or six-membered cyclic esters, have been used as the recognition moiety in the construction of sensors for saccharides [24–26], as nucleotide and carbohydrate transporters [27,28], and as affinity ligands for the separation of carbohydrates and glycoproteins [29–31]. In addition, the boronic acids can become cation-exchangers after being titrated to boronates at a pH above the pKa. However, their usefulness for the preparation of cation-exchanger remains unclear.

In the present work, we described a simple and effective modification procedure for the synthesis of phenylboronic acid (PBA) cation-exchange (poly (MAA-EGDMA)) monolithic column. We investigated the structure and adsorption properties of the functionalized monolithic column, such as column morphology, surface

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area, column permeability and dynamic loading capacity, respectively. The fabricated monolithic column was used to separate a mixture of five proteins, including lysozyme (Lys), cytochrome *c* (Cyt *C*), ribonuclease A (RNase A), trypsin and bovine serum albumin (BSA). We also used this column for one-step purification of Lys from egg whites as well as refolding of RNase A and Cyt *C*, and the expected results were obtained. Moreover, the refolding procedures of model proteins were also monitored by circular dichroism (CD) and fluorescence spectroscopy.

2. Experimental

2.1. Materials

3-Aminophenylboronic acid (APBA) monohydrate was purchased from Beijing Element Chem.-Tech. Company (Beijing, China). N-Hydroxysulfosuccinimide sodium salt (NHSS) was purchased from Shanghai Medpep Co., Ltd. (Shanghai, China). Ethylene glycol dimethacrylate (EGDMA) was purchased from Alfa Aesar (Tianjin, China) and used directly without further purification. Methacrylic acid (MAA), cyclohexanol, dodecanol and 2,2′-azobisisobutyronitrile (AIBN) were purchased from Tianjin Chemical Reagent Company (Tianjin, China). Lys, bovine pancreatic RNase A, trypsin, horse heart Cyt *C* and BSA were purchased from Sigma–Aldrich (St. Louis, MO, USA). Tris (hydroxymethyl) aminomethane (Tris), urea, and guanidine hydrochloride (GuHCl), sodium dodecyl sulphate (SDS), ribonucleic acid yeast, reduced (GSH) and oxidized glutathione (GSSG) were from Sigma.

All ion-exchange chromatography experiments were performed on a Shimadzu LC-20A HPLC system (Shimadzu, Kyoto, Japan), consisting of two LC-20AD pumps, a diode array detector (SPD-M20A).

2.2. Preparation of monolithic column modified by APBA

The monolithic columns were prepared by an *in situ* polymerization in stainless-steel tubes (100 mm × 4.6 mm I.D.). Functional monomer MAA (0.10 mL) and cross-linker EGDMA (0.80 mL) and initiator AIBN (20 mg) were dissolved in a mixture of cyclohexanol and dodecanol (50:50, v/v), and the ratio of monomer to porogenic solvents (25:75, v/v) was selected. The mixture was surged ultrasonically and purged with N_2 for 15 min before pouring the polymerization mixture into the stainless-steel column sealed with a dead nut at the bottom. The column was then sealed at the top and left to polymerize in a water bath at 60 °C for 12 h. After the polymerization ended, the seal was removed, the column was connected to an HPLC pump and washed exhaustively with methanol to remove the progenic solvents and unreacted monomers. Subsequently, the column was washed with water and activated by pumping NHSS (100 mM) at a flow rate of 1 mL min^{-1} for 24 h, then it was filled with 100 mM APBA at a flow rate of 1 mL min^{-1} for 24 h. The APBA-modified monolithic column was obtained after monolith was washed with water and 10 mM Tris–HCl buffer (pH 8.40).

2.3. Characterization of monolithic column

After chromatographic experiments were finished, the polymer-based monolith was washed with 2 M NaCl solutions and water to remove the proteins adsorbed on the column, then pumped out and cut into small pieces followed by drying at 50 °C for 24 h. The dried materials were characterized by X-ray photoelectron spectroscopy (XPS) using a Kratos Axis Ultra DLD multi-technique X-ray photoelectron spectroscopy. Thermogravimetric analysis (TGA) of monolith was carried out with Rigaku TG/DTA at the heating rate of 10 °C min^{-1} up to 700 °C in N_2 atmosphere. Pore size and specific surface area of monolith were measured by a mercury intrusion porosimeter (Micromeritics AutoPore IV 9500), respectively. The morphology of the polymer was obtained by scanning electron microscopy (FEI Quanta, Sweden).

2.4. Determination of protein recovery

A blank monolith which was unmodified by APBA was always connected with binary chromatographic system. Twenty microlitres of 1 mg mL^{-1} BSA, Cyt *C* and Lys were injected before and after the connection of tested monolith modified by APBA and the area of each run was recorded. The mass recoveries of the three proteins were evaluated by

$$\text{recovery} = \frac{\text{area}_{\text{blank+APBA}}}{\text{area}_{\text{blank}}} \quad (1)$$

All performances were carried out with isocratic elution by 10 mM Tris–HCl buffer (pH 7.40) containing different NaCl content.

2.5. Chemical denaturation of proteins

Denaturation experiments of proteins were carried out in the presence of three denaturants 8 M GuHCl, 8 M urea and 10 g L^{-1} SDS, respectively. The 5 mg mL^{-1} protein solutions were incubated at 30 °C for 48 h to ensure that equilibrium had been achieved.

2.6. Refolding of proteins by ion-exchange chromatography (IEC)

Refolding was conducted on monolith modified by APBA. A linear gradient from 100% buffer A containing no NaCl to buffer B containing 1 M NaCl was applied to elute protein from the column. The IEC refolding method 1 was performed using normal Tris–HCl mobile phase containing buffer A (10 mM Tris–HCl, pH 8.40) and buffer B (10 mM Tris–HCl, 1 M NaCl, pH 8.40), but redox mobile phase containing buffer A (10 mM Tris–HCl, 2 M urea, 3 mM GSH, 0.3 mM GSSG, pH 8.40) and buffer B (10 mM Tris–HCl, 2 M urea, 3 mM GSH, 0.3 mM GSSG, 1 M NaCl, pH 8.40) was employed in the IEC refolding method 2. The effluents from 5 to 15 min were collected for RNase A and from 7 to 20 min for Cyt *C*. The constant flow rate of 0.8 mL min^{-1} and detecting wavelength of 280 nm were used for all chromatographic steps.

2.7. Refolding of proteins by dilution method

To run a blank gradient elution without injection sample under the same two chromatographic conditions as above demonstrated and collect the two effluents corresponding to the peak of RNase A, the sample solutions (100 μL) containing the denatured/reduced RNase A in three denaturants were diluted with the two collected effluents and then the solutions were left for 24 h at 4 °C. The dilution refolding method 1 was performed using normal Tris–HCl mobile phase, and redox mobile phase was employed in the dilution refolding method 2.

2.8. Circular dichroism and fluorescence spectroscopy measurements

CD spectra were recorded in a Jasco J-715 spectropolarimeter. Far-UV (190–250 nm) CD spectra of native and renatured proteins were measured with a 0.1 cm path length cell. Fluorescence measurements were made using a Hitachi F-4500 spectrofluorometer. The excitation and emission bandwidths were 10 nm. The measured fluorescence data were corrected for the contribution of the buffer.

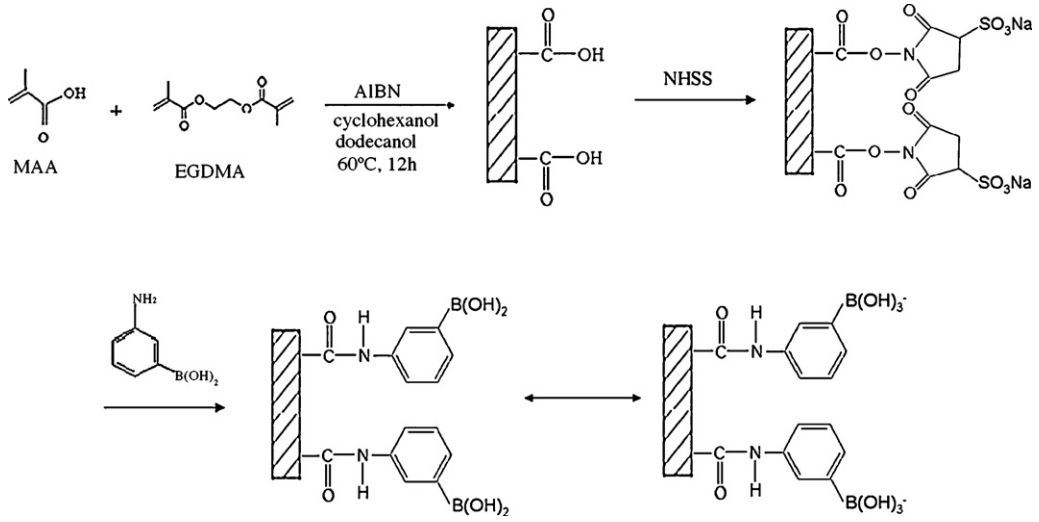

Fig. 1. The scheme of the procedure for preparation of monolithic column modified by APBA.

3. Results and discussion

3.1. Preparation and permeability of monolithic columns

The in-column reactions should be preformed under a warm condition and without formation of insoluble product during the modification. In this study, we developed a new simple and effective in-column modification method to prepare a poly (MAA-EGDMA) monolith modified by APBA. Fig. 1 shows the strategy of synthetic route for the preparation of the APBA-functionalized monolith. Initially, derivation of carboxylic acid groups on the surface of poly (MAA-EGDMA) monolith was carried out by pumping NHSS (100 mM) solution through the column for 24 h. Subsequently, APBA was covalently conjugated to the skeleton by incubating with 100 mM APBA for 24 h, and finally the APBA-modified monolith was successfully obtained.

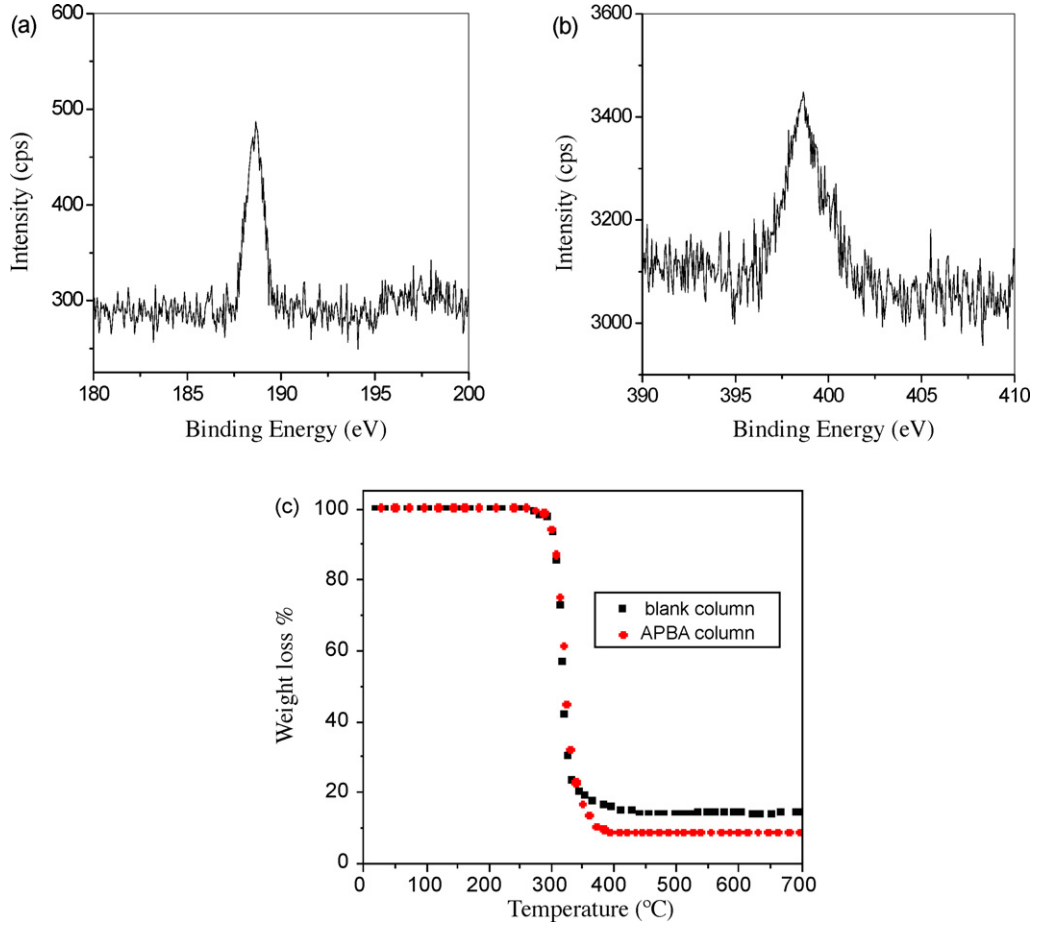

Fig. 2. XPS spectra for B (a) and N (b) element of monolithic column modified by APBA. TGA of blank column and APBA column (c).

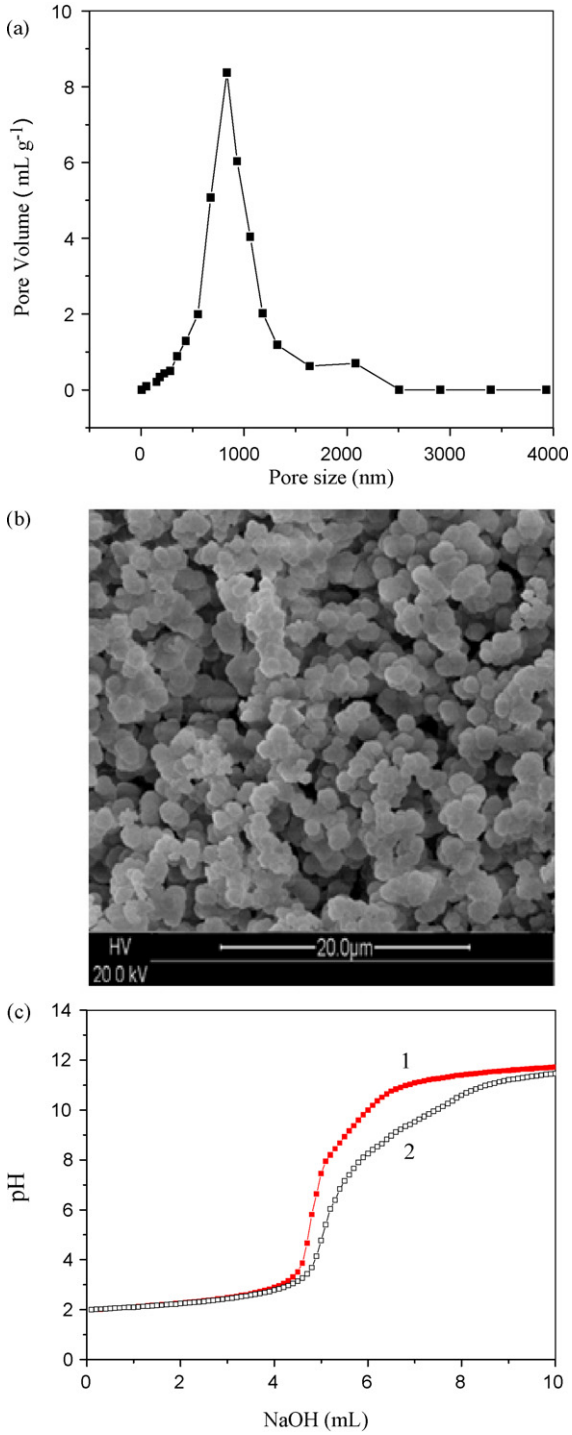

Fig. 3. Profile of the pore-size distribution (a) and SEM image (b) of the APBA column. Titration curves of the blank (1) and APBA (2) columns with 0.1 M NaOH (c).

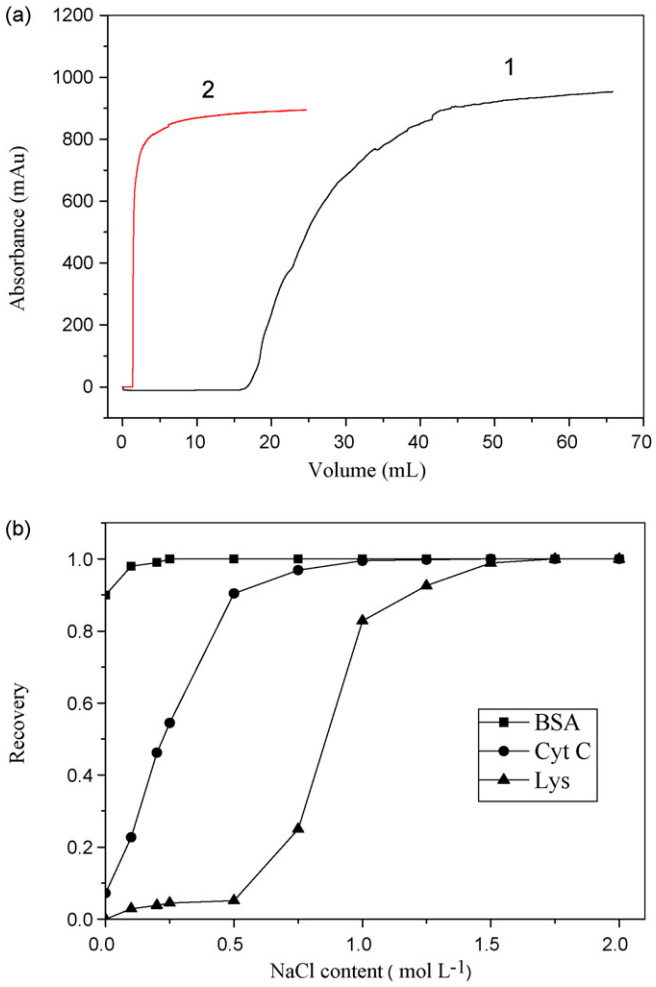

Fig. 4. The breakthrough curves on APBA column (1) and unmodified one (2) at a flow rate of 1 mL min^{-1}. 1 mg mL^{-1} Lys in 10 mM pH 8.40 Tris–HCl buffer was employed for breakthrough analysis (a). The recoveries of BSA, Cyt *C* and Lys on the APBA column *vs.* the NaCl content in 10 mM Tris–HCl buffer (pH 7.40). Twenty microlitres of 1 mg mL^{-1} protein solutions were injected into the column and eluted at 0.5 mL min^{-1} in isocratic mode (b).

In order to test whether the monolith was successfully modified by APBA, we characterized the surfaces of the APBA-modified column by XPS to obtain chemical composition and electronic structure information of the adherent layers. Fig. 2 shows that the characteristic boron peak appeared at ~189 eV for the boron element (Fig. 2a), and the nitrogen signal was observed at ~400 eV (Fig. 2b). These XPS data indicated the successful link of APBA to the surfaces of monolith. XPS analyses of the modified surfaces showed characteristic peaks of B and N elements in APBA, and the mass concentration percentage of B and N elements was ~0.90%. Furthermore, TGA was performed to evaluate the relative composition of the APBA-modified monolith. Fig. 2c shows a representative set of the mass-loss of unmodified column and APBA-modified column, respectively. The TGA curves of two samples displayed a similar mass-loss profile for the release of organic capping materials. For the organic mass-loss at approximately ~300 °C < *T* < ~400 °C, the APBA-modified column revealed a slightly higher organic mass release of ~5.7% compared with that of the unmodified one, indicating that the mass concentration percentage of immobilized APBA was 5.7% approximately, which was consistent with the result of XPS analysis.

The permeability of monolithic columns is one of the most practical factors in designing a novel type of monolithic column. The synthesized monolithic skeleton was characterized by mercury intrusion porosimeter. We showed that the specific surface area was 15.09 m^2 g^{-1}, and the average pore size was 0.773 μm. The total pore volume of the porous polymer was 2.15 mL g^{-1}, and the total porosity was 63.2%. Fig. 3a shows the pore-size distribution profile of the APBA-modified column. The pores ranged from 0.50 to 2.0 μm covering about 90% of the total pore volume, thereby being able to obtain the high permeability of the monoliths. Morphology of monolith (Fig. 3b) obtained from scanning electron microscope showed that well-proportioned flow-through

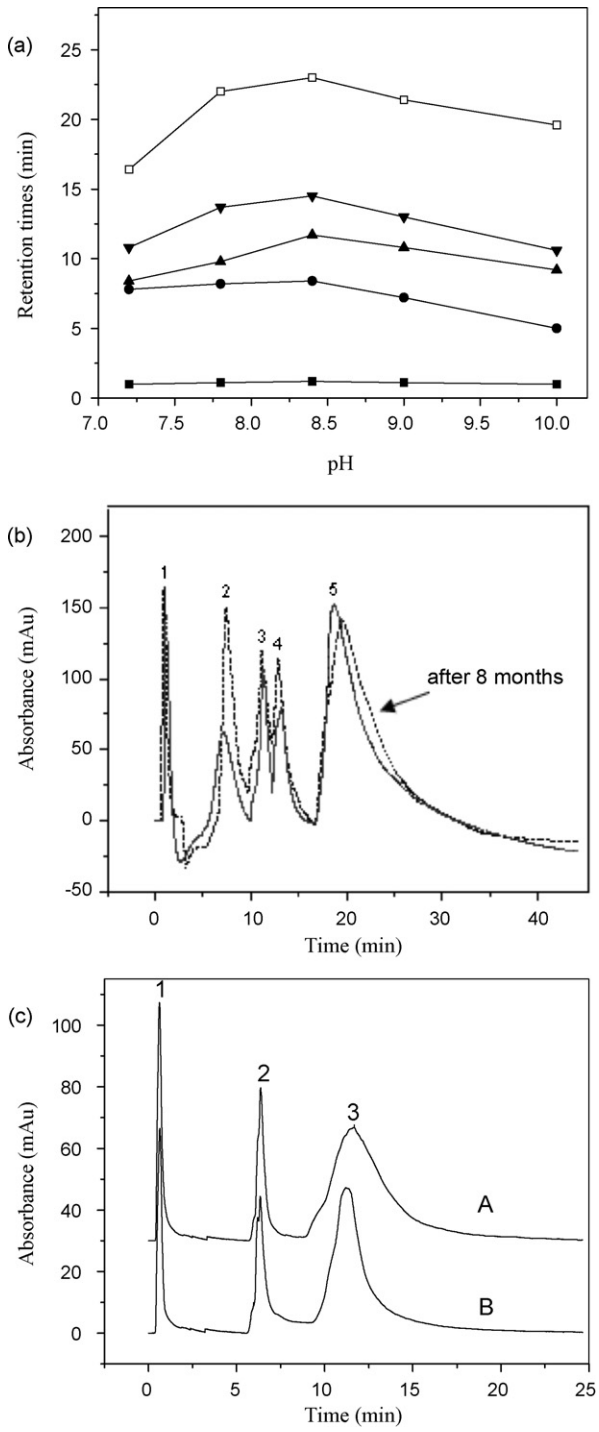

Fig. 5. Effect pH values on the retention of five proteins. Mobile phase A: 10 mM Tris–HCl and B in which 2 M NaCl in "A". Linear gradient of B from 0 to 100% in 30 min at a flow rate of 0.5 mL min^{-1} was used. Proteins: (■) BSA, (●) trypsin, (▲) RNase A, (▼) Cyt *C* and (□) Lys (a). Separation of BSA, trypsin, RNase A, Cyt *C* and Lys on the APBA column at a flow rate of 0.8 mL min^{-1}. Gradient was carried out with buffer A (10 mM Tris–HCl, pH 8.40) and B in which 2 M NaCl was added into buffer A. Linear gradient of B from 0 to 100% in 30 min was used. 1, BSA; 2, trypsin; 3, RNase A; 4, Cyt *C*; 5, Lys (b). Separation of BSA, Cyt *C* and Lys on the APBA column at a flow rate of 1 mL min^{-1}. Gradient was carried out with buffer A (10 mM Tris–HCl, pH 10.00) and B in which 2 M NaCl was added into buffer A. NaCl gradient was kept at 0 for 3 min, increased to 500 mM for 5 min, then increased to 1 M (A) and 2 M (B) for 15 min, respectively. 1, BSA; 2, Cyt *C*; 3, Lys (c).

pores were embedded in the APBA-modified monolithic column. For the APBA-modified column, a linear relation between the pressure drop and the velocity of mobile phase also proved that the porous monolith had good mechanical properties.

Fig. 3c shows the titration curves of unmodified and APBA-modified columns obtained according to a previously reported method [20]. The plot of modified monolith media departed from the unmodified media, indicating the modification of ion-exchanger by APBA. The pKa of phenylboric acid is ~8.8 [32], which suggests that the APBA-modified media can be used in a different pH range comparing to the carboxylic acid type cation-exchangers (pKa ~ 4.7).

3.2. Adsorption properties

Fig. 1 shows that the amine group of APBA could react with the carboxyl group on the surface of pores in monolithic media through the surface-functionalized procedure. In order to evaluate the effect of the grafting modification on protein adsorption of the monolith, we investigated the breakthrough curves of Lys on APBA-modified and unmodified column. As shown in Fig. 4a, the slope of the breakthrough curve from the APBA-modified column was less steep than that of the unmodified one. The dynamic binding capacity of Lys on APBA-modified column at 50% breakthrough was 24.8 mg *per* column volume, while 1.43 mg *per* column volume was for the unmodified one. These findings suggested that the grafted tentacles enhanced the multipoint adsorption of the protein, leading to the increase of protein binding capacity, while the grafting also resulted in the increase of mass transfer resistance. In addition, Wei et al. reported that the binding capacity of Lys on the carboxylic functionalized cation-exchange monolith reaches 43.6 mg *per* column volume [20], which is higher than our result (24.8 mg *per* column volume). This could be due to the lower content of ion-exchange groups on the surface of monolith, the lower specific surface area and the other basic properties of the materials used in this work.

We investigated the effect of salt concentration on the binding capabilities of BSA, Cyt *C* and Lys on APBA-modified column (Fig. 4b). BSA at a low pI value (pI 4.9) had a weak interaction with the surface of modified monolith, which could be 92% easily recovered without NaCl in buffer. Cyt *C* and Lys at a higher pI value (pI 10.6 and 11.2, respectively.) were strongly adsorbed on the stationary phase when the NaCl content in buffer was low. The ability of APBA-modified column for the adsorption of proteins with higher pI exhibited the mainly cation-exchange property of $-B(OH)_3^-$ group, which contributed anion to the retention of cationic protein.

3.3. Chromatographic properties

Fig. 5a shows the effect of pH on protein retention, and the pH of mobile phase had a significant effect on the protein retention. With the increase of pH, the retention time of five proteins, BSA (pI 4.9), trypsin (pI 8.1), RNase A (pI 9.6), Cyt *C* (pI 10.6) and Lys (pI 11.0), increased to their maximum values at around pH 8.40 (near the pKa of APBA), and then the retention time gradually decreased with the increasing pH. This phenomenon has been described by the electrostatic theory in the literature [23]. When pH increased to the pKa of ion-exchange group, more negative charges were attached onto the surface of functionalized monoliths, resulting in more interactions between the proteins and monoliths. At the pH above pKa, the positive charges of proteins decreased with the increasing pH, and the electrostatic interactions became weakened. As expected in cation-exchange mode, proteins with higher pI values should bind tighter to the column and be eluted later than the proteins with lower pI values. Fig. 5b shows that the five proteins with dif-

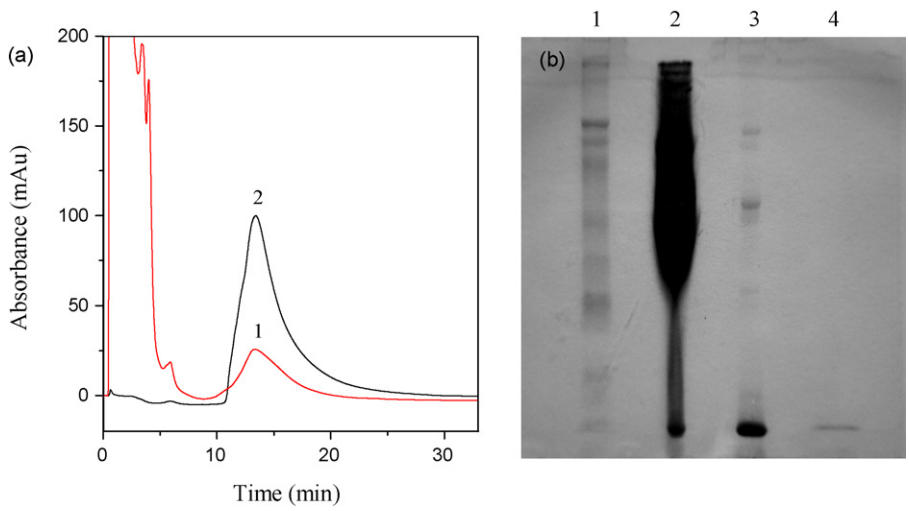

Fig. 6. Chromatograms of the egg white extract solution and standard Lys on the APBA column. Gradient was carried out with buffer A (10 mM Tris–HCl, pH 10.00) and B in which 1 M NaCl was added into buffer A. Linear gradient of B from 0 to 100% in 15 min at a flow rate of 1.0 mL min^{-1}. 1, purified Lys; 2, standard Lys (a). SDS-PAGE analysis of the purified Lys from egg white. Lane 1, protein marker; lane 2, egg white; lane 3, standard Lys; lane 4, purified Lys (b).

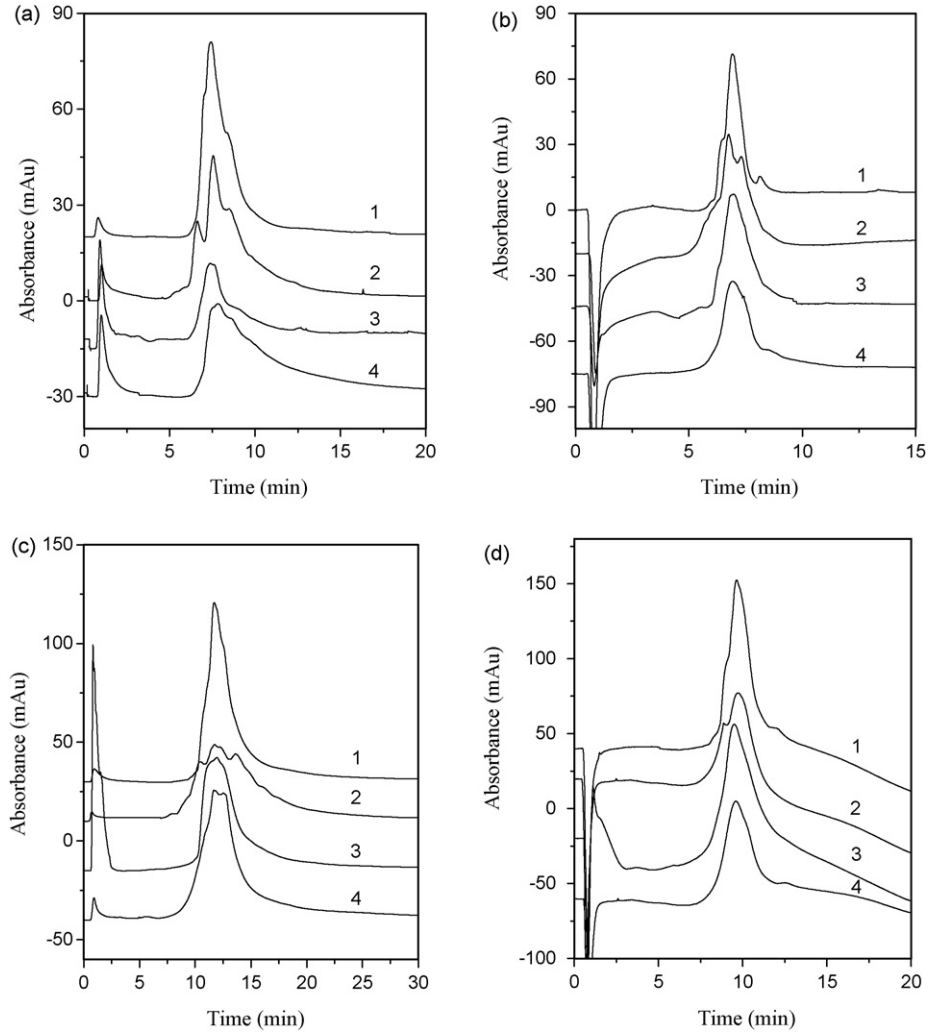

Fig. 7. Chromatograms of native RNase A (1) and refolded RNase A denatured by urea (2), GuHCl (3) and SDS (4) on the APBA column under IEC refolding method 1 (a) and IEC refolding method 2 (b). Chromatograms of native Cyt *C* (1) and refolded Cyt *C* denatured by urea (2), GuHCl (3) and SDS (4) on the APBA column under IEC method 1 (c) and IEC refolding method 2 (d).

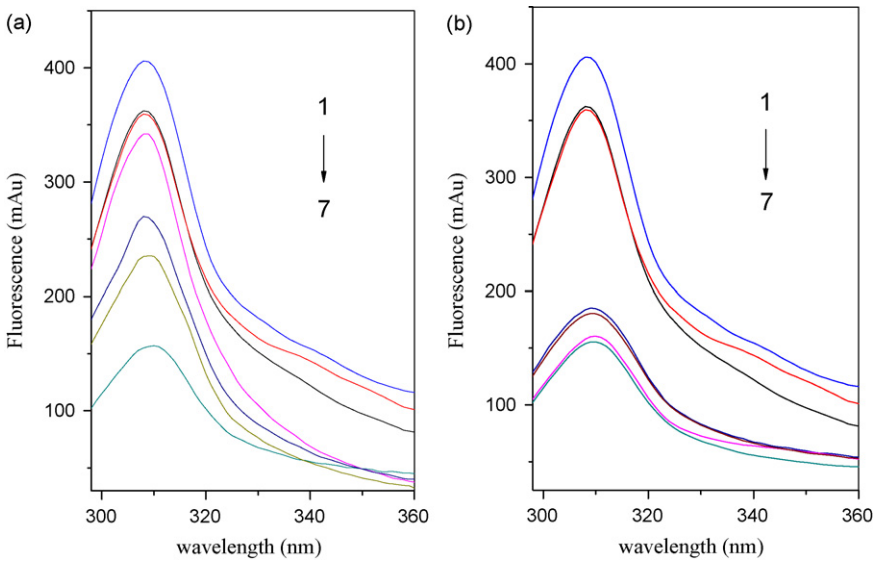

Fig. 8. Fluorescence emission spectra of RNase A denatured in the presence of SDS (1), GuHCl (2), urea (3) and fluorescence emission spectra of renatured RNase A after denatured by SDS (4), urea (5) and GuHCl (6); and native RNase A (7) under IEC refolding method 1 (a) and method 2 (b), respectively.

ferent pI values were eluted in the order predicted from the net charge concept on the modified monolith, which was also consistent with that observed in the literatures [20,23]. Due to the higher pKa (~8.8) of phenylboric acid, the obtained column in this work could be used on more basic condition than the carboxylic functionalized ion-exchanger. As expected, a separation baseline could be obtained (Fig. 5c) for separating three-protein mixture containing BSA, Cyt *C* and Lys at pH 10.00, and the peak resolution was 7.40 and 2.11.

Egg whites are major dairy products rich in proteins, in which Lys is a minor component of about 3.5% [33]. In order to investigate the ability of APBA-modified column for the separation of a mixture, we used the modified monolith to carry out one-step purification of Lys from egg whites at pH 10.00. Fig. 6a shows the chromatogram of egg white proteins on the APBA-modified column, and the flow-through fraction mostly consisted of egg white proteins. The collected fraction of Lys was analyzed by sodium dodecylsulfonate polyacrylamide gel electrophoresis (SDS-PAGE). Fig. 6b shows that the collected band (lane 4) had the same molecule weight as the standard Lys (lane 3).

3.4. Refolding performances of ion-exchange chromatography (IEC)

Chromatography methods have been successfully used for proteins refolding, which is named "protein folding liquid chromatography (PFLC)". It is a newly developed method, which purifies target proteins simultaneously during protein refolding by liquid chromatography [34]. Introduced by Creighton [35,36] in 1986, IEC method is one of the most frequently used methods for LC refolding, which has been applied to many proteins with high yields [37–39]. The pH value has a strong effect on disulfide pairing, so refolding of a disulfide-containing protein should be performed under a weakly basic condition. The as-prepared monoliths have excellent

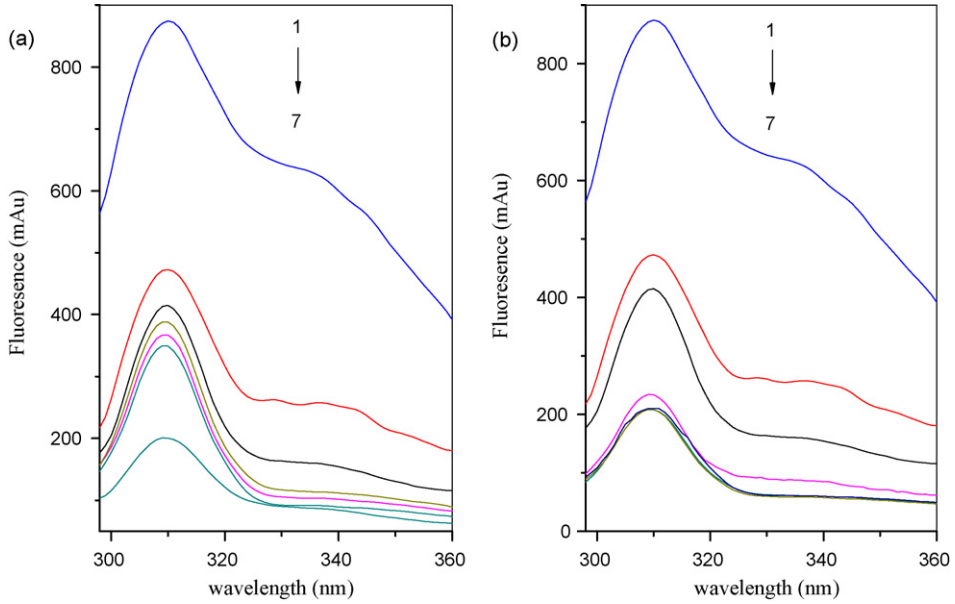

Fig. 9. Fluorescence emission spectra of Cyt *C* denatured in the presence of SDS (1), GuHCl (2), urea (3) and fluorescence emission spectra of renatured RNase A after denatured by SDS (4), urea (5) and GuHCl (6); and native RNase A (7) under IEC refolding method 1 (a) and method 2 (b), respectively.

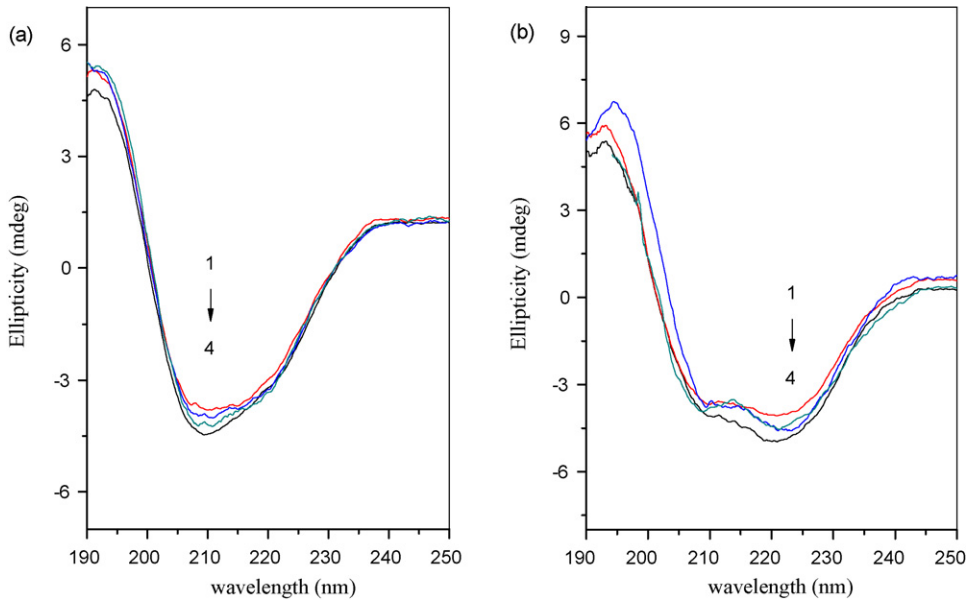

Fig. 10. Solvent-corrected far-UV CD spectra of native (4) and renatured protein after denatured in the presence of SDS (1), urea (2) and GuHCl (3) under IEC refolding method 2 for RNase A (a) and Cyt *C* (b), respectively.

Table 1
The results of bioactivity recovery of different refolding methods ($n = 3$).

	Dilution method 1	Dilution method 2	IEC refording method 1	IEC refording method 2
RNase A–urea	12.9% (2.4)	19.2% (3.6)	54.7% (2.2)	84.3% (1.5)
RNaseA–GuHCl	10.3% (1.8)	12.4% (2.1)	77.5% (3.9)	97.4% (2.1)
RNase A–SDS	9.9% (4.0)	14.1% (2.3)	48.7% (2.6)	77.9% (3.7)

chromatography performances in this pH range, so we investigated its application on protein refolding.

In this study, we selected RNase A and Cyt *C* as model proteins for refolding on the APBA-modified column. A refolding buffer containing urea (2 M) and a redox shuffling system (3 mM reduced glutathione GSH and 0.3 mM oxidized glutathione GSSG) were used. Compared with the refolding buffer, a normal Tris–HCl buffer without denaturant or redox was applied as a control. Fig. 7 shows the chromatographic results of NaCl gradient refolding process by IEC. When the normal buffer was used, "refolded" peaks had the similar retention times with native ones but with tailing, and a much less extent of peak tailing was observed when the redox mobile phase system was used. Meanwhile, the addition of urea in the refolding buffer resulted in facilitated protein elution (time shift of RNase A was from 7.43 to 6.92 min and time shift of Cyt *C* was from 11.71 to 9.46 min).

3.5. Circular dichroism and fluorescence spectrometry

Figs. 8 and 9 show the fluorescence emission spectra of the two native and six denatured proteins. The low fluorescence intensity for native RNase A and Cyt *C* indicated that a significant fraction of the aromatic residues were completely buried in a highly hydrophobic environment. After denatured by urea, GuHCl and SDS, the high increase in the fluorescence intensity was at 308 nm for RNase A, 308 and 340 nm for Cyt *C*, respectively, which might be due to that the buried aromatic residues (308 nm for tyrosine and 340 nm for tryptophan) emerged to the surface of hydrophobic pockets. The refolded proteins showed decreases in the fluorescence intensity, indicating that the emerged aromatic residues were buried in the canonical three-dimensional models once again. A suitable concentration of denaturant in mobile phase can reduce a denatured proteins chance of aggregating to a minimum, or prevent precipitates appearing altogether facilitating protein elution during PFLC [34]. The denaturant like urea used in IEC refolding method 2 is the mostly commonly denaturant added in the mobile phases, and it was easier for the reformation of disulfide bond in the redox environment, thus the IEC refolding method 2 with denaturant and redox agents had a better refolding efficiency compared with the normal buffer. In addition, the refolding efficiency of Cyt *C* with two pairs of disulfide bonds was better than that of RNase A with four pairs of disulfide bonds.

We also used CD spectra to compare the physical characteristics of refolded RNase A and Cyt *C* with native proteins. Fig. 10 shows analogous peaks of the two types of proteins in shape and signal intensity, indicating that a large part of the denatured proteins had completely refolded to the native conformation when the redox mobile phase system was used.

3.6. Comparison of the IEC and dilution refolding processes

Dilution refolding is the most widely used method for protein refolding. Table 1 shows the bioactivity recovery of RNase A by dilution refolding and IEC refolding according to the literature [40]. Compared with a low bioactivity recovery (10–20%) by dilution refolding method, a high bioactivity recovery (48–98%) of RNase A by IEC method was obtained. In particular, the IEC method 2 with redox mobile phase system had a higher bioactivity recovery (77–98%) than IEC method 1 (48–78%). In addition, we found that bioactivity recovery of GuHCl denatured RNaseA by IEC method 2 was 90.2% when a high concentration of protein (20 mg mL^{-1}) was injected.

4. Conclusion

In this study, we developed a novel cation-exchanger by functionalizing poly (MAA-EGDMA) monolith with APBA. The APBA-modified monolith provides a better permeability for protein separation and had strong retentions for cationic proteins. With this new monolith, we achieved excellent separation of five proteins and one-step purification of Lys from egg whites with NaCl gradient. In addition, we developed a column-based ion-exchange refolding procedure for RNase A and Cyt *C*. CD and fluorescence spectrometry analysis demonstrated that the artificial ion-exchange matrix could be used for the refolding of proteins containing a small number of disulfide bonds.

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