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Preparation of poly(N-isopropylacrylamide)-grafted well-controlled 3D skeletal monolith based on E-51 epoxy resin for protein separation

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ABSTRACT

A novel type of poly(N-isopropylacrylamide) grafted E-51 epoxy-based monoliths in a 100 mm × 4.6 mm I.D. stainless steel column with well-controlled three-dimensional skeletal structures has been prepared and proposed for the separation of proteins. The grafted PNIPAAm chain via surface-initiated atom transfer radical polymerization was successfully performed. The proposed method provided a new route to modify the E-51 epoxy-based monoliths for widening their applications. Meanwhile, the temperature and the salt concentration responses of the grafted monolithic columns were investigated. Under the salt gradient, six proteins were well separated in hydrophobic interaction mode. Moreover, for further confirming the application of the prepared monolith was meaningful for proteome analysis in actual system, the separation of human serum sample was performed.

1. Introduction

It is well known that monolithic materials (such as silica monolith and polymer monolith) with well-controlled three-dimensional (3D) skeletal structures have lots of advantages, such as low resistance, fast mass transfer and high performance [1]. However, compared to silica-based monolith favorable for small molecule separations, polymer-based monoliths have shown excellent performance in separation of large biomolecules due to their good biocompatibility [2], which is especially meaningful for proteome analysis. As a result, exploring different new kinds of polymer monoliths with co-continuous porous structures for biomolecule separations is necessary and significant.

As a novel type of polymer monoliths, epoxy resin-based monoliths with co-continuous porous structures, which more easily resulted in high separation efficiency than that with aggregated microglobular structures prepared by free radical polymerization [3], were firstly developed by Tanaka and co-workers [4–6] and were further studied by other researchers in recent years [7–9]. Besides the well-defined skeletal porous structures, it has been recognized that the homogeneous action cites of polymer monoliths are also very significant in their applications. However, so far, few studies about the surface modification of E-51 epoxy resin-based monoliths have been reported [9] and thus their applications have been limited [5,8,9].

Usually, the functional groups act as interaction sites anchored onto pore surfaces of the monoliths were prepared from functional monomers or through surface derivation in post-modification [10]. However, the former method was limited by monomers diversity and the latter one was tedious and often led to low productivity and heterogeneous surface chemistry. Recently, surface-initiated atom transfer radical polymerization (SI-ATRP) has emerged as a promising technique for surface modification due to its good ability in controlling the length of grafted polymer chains [11–14]. Then, many works about SI-ATRP for post-grafting of polymer monoliths have been reported [9,15–17]. However, this post-modification method has not been investigated in 3D skeletal E-51 epoxy resin-based monoliths, which might afford a new way to widen their potential applications.

Abbreviations: NIPAAm, N-isopropylacrylamide; SI-ATRP, surface-initiated atom transfer radical polymerization; 3D, three-dimensional; BACM, 4-[(4-aminocyclohexyl)methyl] cyclohexylamine; PMDETA, N,N,N′,N″,N″-pentamethyldiethylenetriamine; BiBB, 2-bromoisobutyryl bromide; Cyt c, cytochrome c; Myo, myoglobin; RNase, ribonuclease A; HSA, human serum albumin; IgG, immunoglobulin G; Thy, thyroglobulin bovine; SEM, scanning electron microscopy; BET, nitrogen adsorption–desorption; TEA, triethyleneamine; FTIR, fourier transform infrartd; HPLC, high-performance liquid chromatography; HIC, hydrophobic interaction chromatography.

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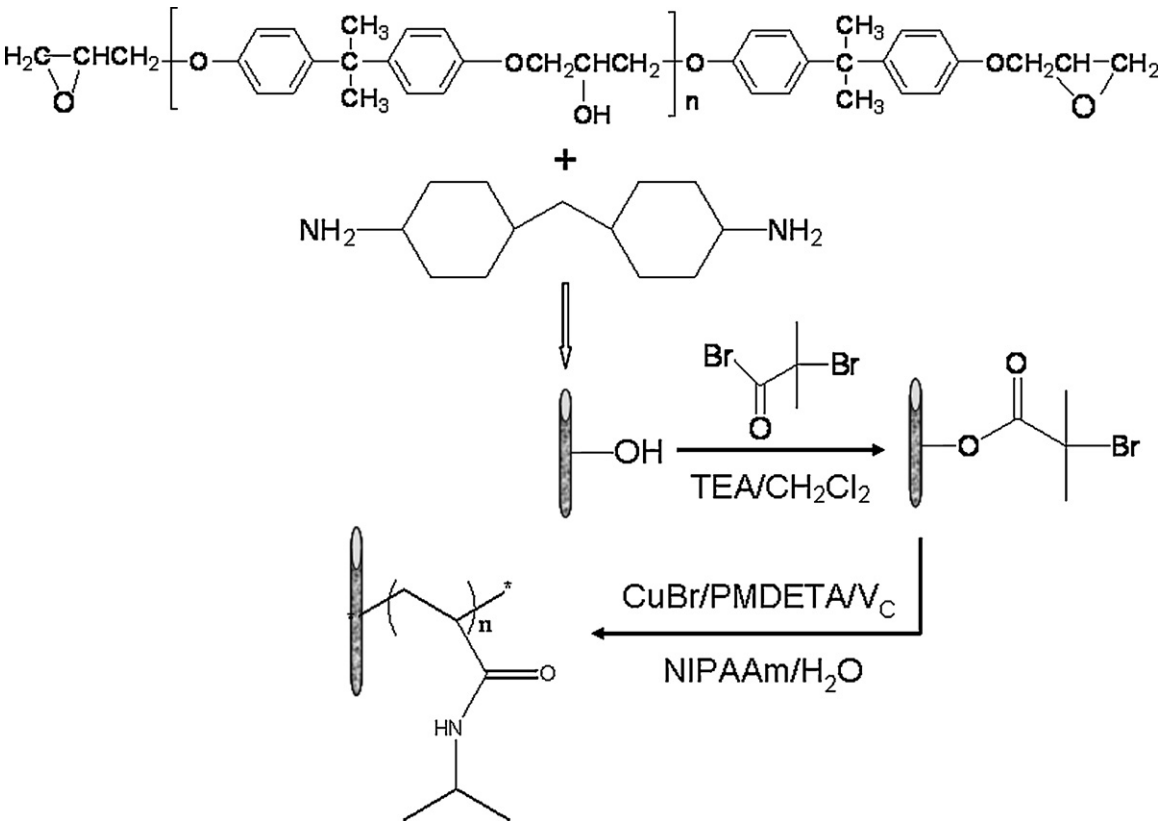

Fig. 1. Scheme for the preparation of the PNIPAAm-grafted E-51 epoxy resin-based monoliths.

In addition, poly(N-isopropylacrylamide) (PNIPAAm) which is one kind of most widely studied intelligent polymers, can undergo a reversible temperature dependent phase transition at 32 °C or salt-dependent phase transition in aqueous solutions because of its reversible hydrophobicity [18–21]. Recently, chromatographic applications of PNIPAAm based on its responsive property have been performed via grafting PNIPAAm onto various chromatography supports [22,23]. In our group, we have grafted PNIPAAm onto poly (chloromethylstyrene-divinylbenzene) monoliths as a responsive stationary phase for protein separation [17].

In this work, we have reported the PNIPAAm-grafted E-51 epoxy resin-based monolithic column with well-defined 3D porous structure, which exhibited excellent control in the grafting polyNIPAAm chains via SI-ATRP in post-modification and demonstrated the temperature and the salt concentration responsive properties of polyNIPAAm in chromatographic analysis. For the first time, the modification of E-51 epoxy resin monoliths by SI-ATRP method supplied a new route to modify this kind of monoliths. Both co-continuous porous structure and homogeneous action cites of E-51 epoxy resin-based monolith ensured its potential applications in the separation of biomolecules. Furthermore, we separated human serum samples to confirm that the PNIPAAm-grafted epoxy resin-based monolithic column could be used in actual system.

2. Materials and method

2.1. Materials

E-51 epoxy resin was purchased from Wuxi Resin Factory (Jiangsu, China) and utilized as received. 4-[(4-Aminocyclohexyl)methyl] cyclohexylamine (BACM) was from TCI (Tokyo, Japan) and utilized without purification. N-isopropylacrylamide (NIPAAm, 97.0%) from Aldrich (New Jersey, U.S.A) was purified by recrystallization from a mixture consisting of benzene and *n*-hexane (3/7, v/v) and dried at room temperature in vacuo. N,N,N′,N″,N″-pentamethyldiethylenetriamine (PMDETA) was purchased from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan). 2-Bromoisobutyryl bromide (BiBB) and CuBr were purchased from Chuang Qi Chemical Plant (Beijing, China). Ascorbic acid was obtained from Alfa Aesar (MA, USA). The steroids were purchased from Wuhan Jiuan Pharmaceutical Ltd. (China). The six test proteins, cytochrome c (Cyt c), myoglobin (Myo), ribonuclease A (RNase), human serum albumin (HSA), immunoglobulin G (IgG) and thyroglobulin bovine (Thy) were obtained from Sigma–Aldrich. The solvent as HPLC mobile phase and water obtained from a triple distilled water system were filtrated through 0.45 μm membranes before use. Other materials were from Beijing Chemical Co. Ltd. (Beijing, China).

2.2. Preparation and characterization of epoxy resin-based monoliths

E-51 (1.0 g) resin and BACM (0.25 g) were completely dissolved in 3.8 g mixture of two porogenic solvents in ultrasonic bath. This solution was then injected into a 4.6 mm i.d. × 100 mm stainless steel column by a syringe. Then the sealed column was placed in a water bath. The polymerization reaction was carried out at 80 °C for 12 h. The resulting monolithic column was connected to a HPLC pump and washed with water and methanol in sequence for the following grafting polymerization. The composition of the porogens affected the formation of co-continuous structure and will be discussed in Section 3.

For scanning electron microscopy (SEM) observation, the monolithic column was pumped out and dried in vacuum at 50 °C overnight. The dried polymer sample was snapped apart and placed on sticky copper foil, which was attached to a standard aluminum

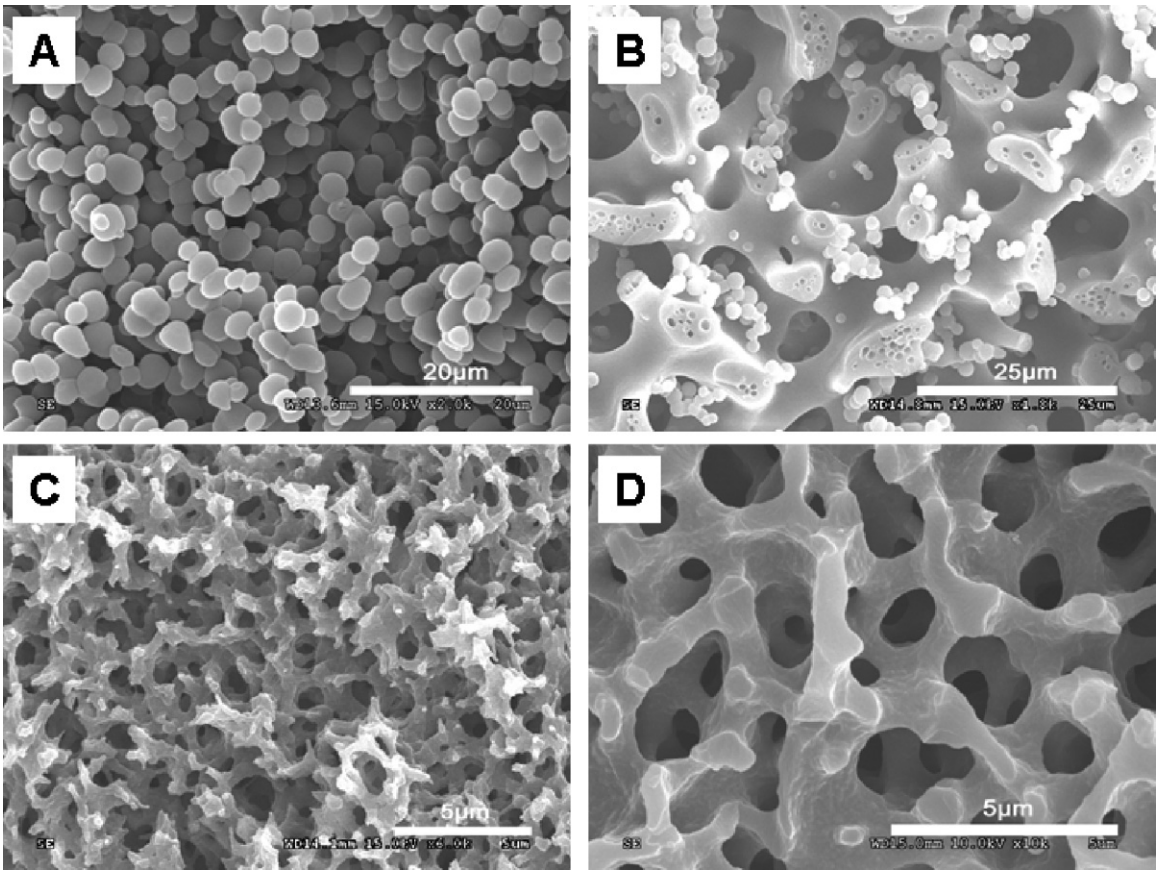

Fig. 2. SEM photographs of the porous structures obtained with different porogen compositions. (A) PEG-200 without good solvents; (B) DMSO/PEG-200 = 1/3 (w/w); (C) cyclohexanone/PEG-200 = 1/3 (w/w); (D) DMF/PEG-200 = 1/9 (w/w).

specimen stub. Then, the polymer was coated with about 10 nm of gold by Eiko IB-3 sputter coating (Eiko, Engineering Co. Ltd., Japan). Microscopic analysis was carried out with a HITACHI S-4800 SEM (Hitachi High Technologies, Japan).

The porous property of the well-defined skeletal E-51 epoxy resin-based monolithic polymers was characterized by mercury porosimetry and nitrogen sorption. Mercury porosimetry (AUTOPORE II 9220, Micromeritics, USA) was used to characterize the pore size distribution of the macroporous monoliths, whereas nitrogen adsorption–desorption (ASAP-2020, Micromeritics, USA) was employed to determine the BET surface area of these E-51 epoxy resin-based monolithic polymers.

2.3. In situ ATRP grafting from the pore surface of the prepared monolith

The grafting procedure was carried out following Fig. 1. The E-51 epoxy resin-based monolith was activated to be a macroinitiator for the following ATRP grafting reaction with BiBB at 0 °C for 12 h. The reactant solution was prepared by dissolving 0.02 mL BiBB in 10 mL dry dichloromethane containing dry TEA (2%, v/v). The reaction was carried out in situ by pumping the reactant solution through the monolithic support placed in ice bath with a syringe pump.

The grafting procedure was as follows: NIPAAm (5.0 g), CuBr (0.058 g) and PMDETA (0.065 g) in water (50 mL) were added into a flask. The flask was then sealed with rubber plug and the mixture formed a slight blue solution under ultrasound. Subsequently, the ascorbic acid (V_c) (0.281 g) aqueous solution was injected into the flask with a syringe. The resulting mixture was pumped through the activated monolith by a syringe pump at a flow rate of 0.05 mL/min and reacted for 14 h at room temperature. After the grafting polymerization was completed, 100 mL water was used to wash the grafted monolith to remove all soluble compositions in the monolith for the following chromatographic applications.

The E-51 epoxy resin-based non-grafted and grafted monolithic polymers were both characterized by Fourier transform infrartd (FTIR) spectroscopy analysis on TENSOR 27 (Bruker, Germany).

2.4. Hydrophobic interaction chromatography (HIC) of proteins

HIC of proteins was carried out with a LC-20A high-performance liquid chromatography (HPLC) system (Shimadzu, Japan) consisting of a binary LC-20AT HPLC pump and an SPD-20A UV–vis detector. Data processing was performed with a HW-2000 chromatography workstation (Nanjing Qianpu Software, China). The flow rate was 1.0 mL/min, and the detection wavelength was 280 nm. Typically, the volume of solution injected was 5.0 μL. All chromatograms were baseline subtracted to eliminate the effects of refractive index variations resulting from the use of gradient elution on UV detection. A stepwise linear gradient of salt (sodium sulfate) in pure water was used in the chromatographic evaluations. A column oven was used to keep a constant column temperature at 25 °C. Standard solutions of proteins were prepared with Cyt c, Myo, RNase, HSA, IgG and Thy in phosphate buffer at pH 7.0 (0.5 mg/mL) and stored in a refrigerator at 4 °C.

2.5. Human serum sample preparation

The human serum sample donated by the Hospital of Hebei University was obtained from healthy volunteers. Blood was collected and allowed to clot for 1 h at room temperature. Finally, the serum was separated by centrifugation at 3,000 rpm for 5 min at 5 °C and

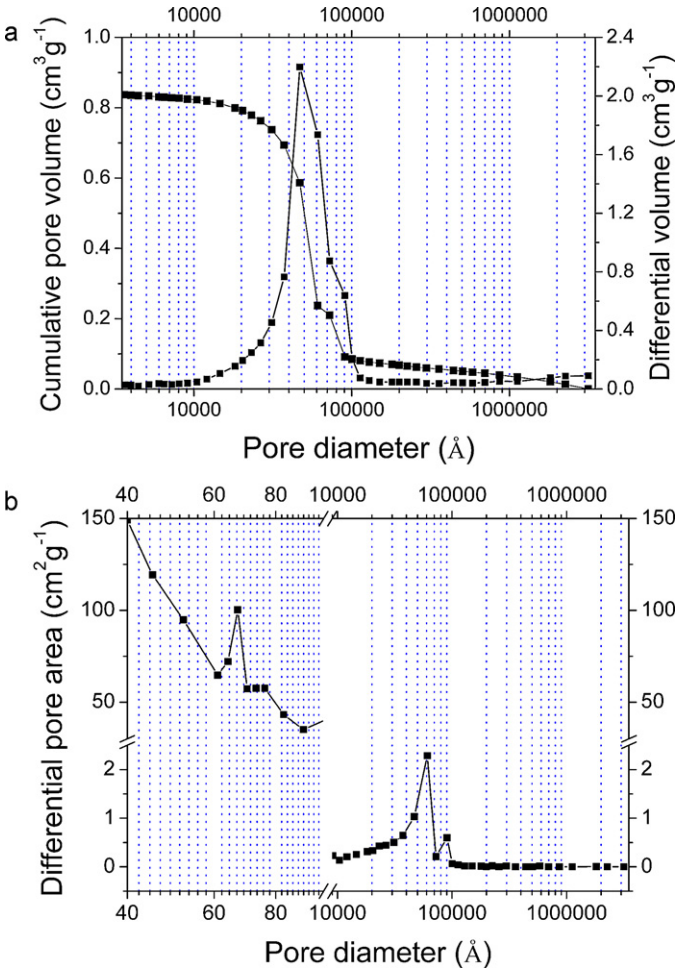

Fig. 3. Pore size distribution profile of the prepared E-51 epoxy resin-based monolith.

dispensed by 1 mL aliquots into sterile vials, used for analysis or frozen at −20 °C.

3. Results and discussion

3.1. Preparation and characterization of PNIPAAm-grafted epoxy resin-based monolith

The porous structure is the foundation of various utilizations of polymer monoliths. Generally, a good monolithic separation media should be featured with bimodal pore size distribution and well-defined 3D skeletal structure [24]. Therefore, the basis of this work was to select the E-51 epoxy resin-based monolithic support with the best porous structure for the following in situ surface modification and the final chromatographic use.

The composition of porogenic solvents was the most important factor influencing the porous morphology and the pore size distribution of monoliths, so the effect of the porogen composition was investigated at first. In order to increase pore surface area of the E-51 epoxy resin-based monolith to provide more reaction groups for the following grafting polymerization, good solvent for the polymerization system was added. Fig. 2 depicts some representative SEM results of porous structures of the E-51 epoxy resin-based monoliths obtained with different porogen compositions. It can be found that different morphologies were formed by varied composition of the porogen mixture. Notably, the structure showed in Fig. 2A was obtained without addition of good solvents, which

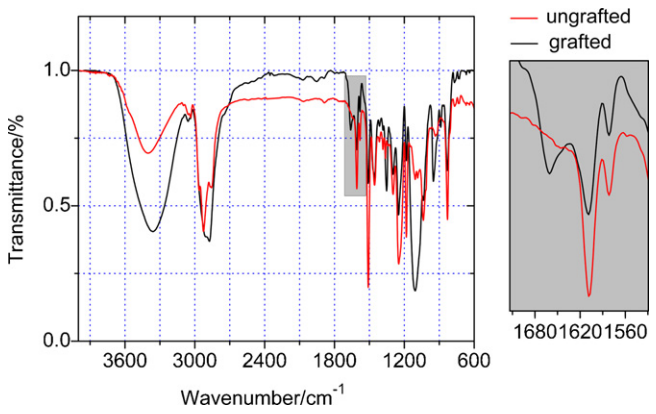

Fig. 4. FTIR spectra of grafted and non-grafted E-51 epoxy resin-based monoliths.

was an aggregated microglobular structure. The morphology in Fig. 2B obtained with DMSO/PEG-200 (1/3, w/w) presented another kind of structure featured with both 3D skeletons and aggregated microglobules. Although the E-51 epoxy resin-based monolith in Fig. 2C obtained with cyclohexanone/PEG-200 (1/3, w/w) had a good 3D skeletal structure, the pore size was a bit smaller and thus resulted in lower permeability, which was disadvantageous for high flow rates in the following modification procedure and the final chromatography application. As a result, composition used in Fig. 2D (DMF/PEG-200 = 1/9 (w/w)) was finally selected as the optimal ratio in porogen mixture for preparing the E-51 epoxy resin-based monolith because the morphology was an ideal 3D skeletal structure and the pore size was larger than that showed in Fig. 2C.

The porous property of the selected E-51 epoxy resin-based monolithic support was further investigated by mercury intrusion porosimetry and BET nitrogen adsorption as displayed in Fig. 3. The pore size covers from several nanometers to tens of micrometers, and the most probable pore diameter is at 4.6 μm. Additionally, the BET surface area of the monolithic support was 13.3 m^2/g, which was about 5 times higher than that previously reported [5]. The increased pore surface area was attributed to the addition of good solvent in polymerization system and could be related to the peak at 6.8 nm in the pore size distribution profile shown in Fig. 3b. Based on the increased surface area, this E-51 epoxy resin-based monolithic support could provide more reactive sites for the following grafting process.

Although SI-ATRP is promising for post-modification on various surfaces, ATRP is very sensitive to oxygen, since oxygen can inhibit polymerization not only by the formation of unreactive peroxy radicals but also by the irreversible oxidation of transition metal catalyst. However, it is very difficult to exclude oxygen absolutely in the in situ ATRP grafting system. Therefore, in order to overcome this problem, a reducing agent, V_C, was added into the reaction mixture to reduce Cu(II) to Cu(I) activating species as described in our previous work [17].

Then, FTIR analysis was used to characterize the prepared E-51 epoxy resin-based monoliths as showed in Fig. 4. The obvious peak located at 1670 cm^{-1} demonstrated the presence of amide groups on the grafted monolith, which suggested that the grafting method was effective and successful. In addition, the permeability of the epoxy resin-based monoliths was also investigated. From the plot of back pressure versus flow rate (Fig. 5), it can be found that the back pressure of the grafted column was much higher than that of the non-grafted one. This result provided another piece of evidence that PNIPAAm chains had been successfully grafted onto the inner pore surface.

Table 1
Retention time of steroids at different temperatures.

Temperature/°C	18	28	35	40
Hydrocortisone	1.63	1.83	1.99	2.23
Prednisolone acetate	2.11	2.70	3.66	5.22
Dexamethasone	3.04	4.17	7.05	11.9
Progesterone	5.34	8.98	17.5	34.7

3.2. The temperature responsive behavior of the grafted monolith

Once PNIPAAm was grafted onto the inner pore surface of the E-51 epoxy resin-based monolith, four steroid molecules were chosen as probes to measure the responsive behavior of the grafted PNIPAAm brushes. The retention times at various temperatures were shown in Table 1. It can be found that the retention times increased with the increasing temperature, which is different from that in reversed phase liquid chromatography. Moreover, the phenomenon is in agreement with the literature previously reported [17].

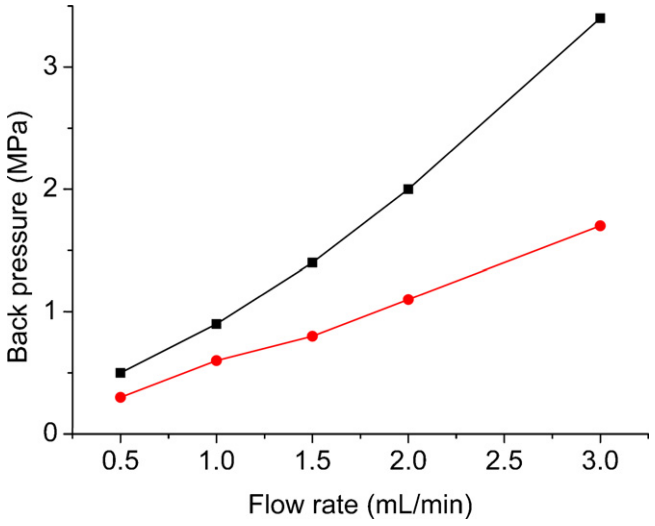

Fig. 5. Plot of back pressure versus flow rate. Conditions: mobile phase, pure water; temperature, 25 °C. (■) 4.6 mm × 100 mm i.d. polyNIPAAm-grafted E-51 epoxy resin-based monolith; (●) 4.6 mm × 100 mm i.d. ungrafted E-51 epoxy resin-based monolith.

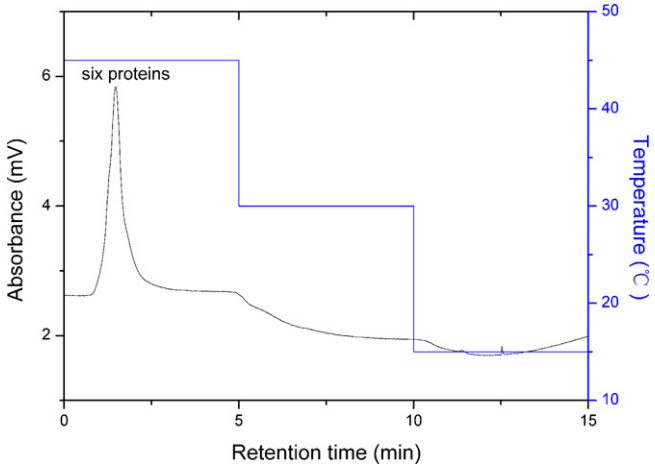

Fig. 6. Separation of a six-protein mixture under the temperature gradient. Conditions: column, 4.6 mm × 100 mm i.d. PNIPAAm grafted E-51 epoxy resin-based monolith; mobile phase, 2.0 M Na_2SO_4 aqueous solution; UV detection, 280 nm; sample, six-protein mixture.

Table 2
The effect of pH on the resolution.

pH	6.0	6.5	7.0	7.5	8.0
R_{s1}	3.6	4.0	4.4	4.9	4.9
R_{s2}	1.3	1.2	0.40	0	0
R_{s3}	2.4	2.6	1.7	2.0	2.1
R_{s4}	1.5	1.6	1.4	1.5	1.9
R_{s5}	1.4	1.7	1.3	1.4	1.8

R_{s1}: resolution between Cyt c and Myo; R_{s2}: resolution between Myo and RNase; R_{s3}: resolution between RNase and HSA; R_{s4}: resolution between HSA and IgG; R_{s5}: resolution between IgG and Thy.

Subsequently, the temperature-responsive property of the PNIPAAm-grafted monoliths was used for the separation of proteins. A temperature gradient mode was designed to gradually change the hydrophobicity of the surface grafted PNIPAAm brushes for eluting out proteins with various hydrophobicities. Although different gradient profiles were attempted, the separation was not observed (Fig. 6). This may be explained by the fact that the hydrophobicity of the grafted PNIPAAm brushes at high temperature was not enough to differentiate proteins according to their hydrophobicities. Furthermore, some proteins may be easily denatured at high temperature.

3.3. HIC separation of proteins

Although proteins could not be separated under the temperature gradient, it is well known that the hydrophobicity of PNIPAAm is also responsive to the change of salt concentrations [17,21]. Thus, we presumed that the PNIPAAm grafted E-51 epoxy resin-based monolith might be used for the hydrophobic interaction chromatography of proteins separation under a suitable salt gradient. Thereby, different salt concentration gradients were explored to separate proteins in this work. Meanwhile, several different kinds of salts (including ammonium sulfate, sodium sulfate and sodium chloride) were investigated, but sodium sulfate was finally selected for further separation because it was the most effective one to control the hydrophobicity of the grafted PNIPAAm brushes as mentioned in literatures [17,25].

Moreover, considering the hydrodynamic property of proteins could be influenced by the pH value of the mobile phases, the effect of pH on the protein separation was also studied. From Table 2, it can be found that the resolutions of most protein samples was little affected by pH in the range of 6.0–8.0, while the resolution

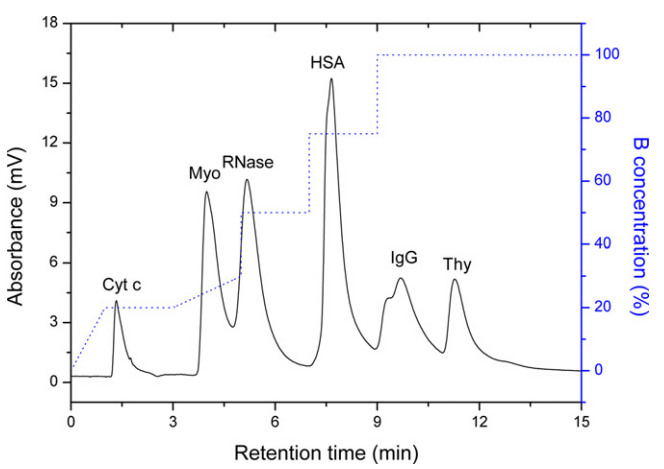

Fig. 7. Separation of a six-protein mixture using the salt gradient. Conditions: column, 4.6 mm × 100 mm i.d. polyNIPAAm-grafted E-51 epoxy resin-based monolith; mobile phase A, 2.0 M Na_2SO_4 aqueous solution; UV detection, 280 nm; sample, six-protein mixture.

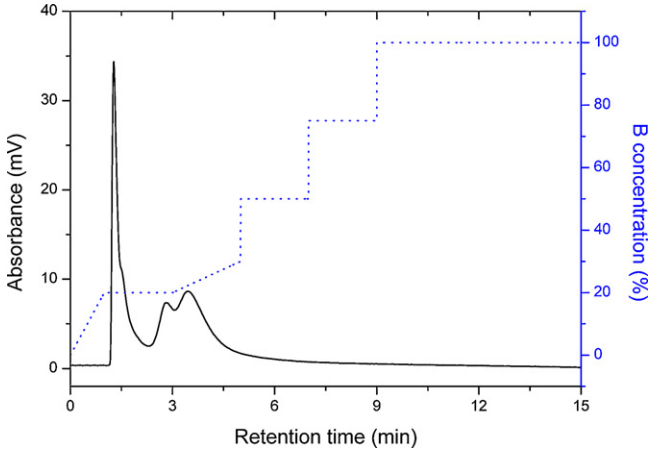

Fig. 8. Separation of a six-protein mixture using the salt gradient on the ungrafted E-51 epoxy resin-based monolith. Conditions: column, 4.6 mm × 100 mm i.d. ungrafted E-51 epoxy resin-based monolith; mobile phase A, 2.0 M Na_2SO_4 aqueous solution; UV detection, 280 nm; sample, six-protein mixture.

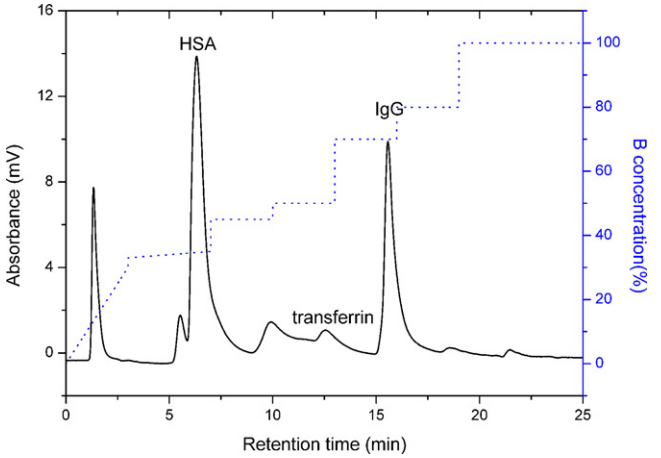

Fig. 9. Chromatogram of human serum proteins. Conditions: column, 4.6 mm × 100 mm i.d. polyNIPAAm-grafted E-51 epoxy resin-based monolith; mobile phase A, 2.0 M Na_2SO_4 aqueous solution; UV detection, 280 nm.

between Myo and RNase became worse with the increasing pH value. The reason may be that the hydrophobicity of Myo was more sensitive to pH than others. Consequently, 2.0 M sodium sulfate solution/pure water were thus finally used as the mobile phases in further chromatographic investigations.

Through the investigation on different sodium sulfate concentration gradient profiles, a well separation was achieved as displayed in Fig. 7. The resolutions for all samples were higher than 1.3. Meanwhile, the PNIPAAm grafted E-51 epoxy resin-based monolithic column showed very high efficiency for a 4.6 mm I.D. column. In addition, the tailing factors of all peaks were under 2.0 except the first one, which demonstrated that no other interaction between the stationary phase and the proteins occurred besides weak hydrophobic interaction.

To further confirm the salt concentration-responsive property of the grafted column, the separation result was compared with the ungrafted column. From Fig. 8, it can be found that poor separation was obtained under the same chromatographic conditions. The figure displayed that some proteins were weakly retained on the ungrafted column because of the hydrophobicity of the phenyl groups anchored on the monolithic support. This result could further confirm the occurrence of the grafting polymerization.

It should be mentioned that comparing with the temperature gradient, the PNIPAAm-grafted E-51 epoxy resin-based monolith was suitable for the well separation of the six proteins when the salt concentration gradient was performed. It might be attributed to the different abilities in changing the hydrophobicity of the PNIPAAm grafted surface caused by the temperature gradient or the salt concentration gradient.

3.4. HIC of human serum on the PNIPAAm-grafted monolithic column

To further confirm the application of PNIPAAm grafted E-51 epoxy resin-based monolith in biological analysis, a complex biological sample was employed to validate the performance of this column. Fig. 9 shows the high efficiency separation of natural human serum proteins: 8 peaks could be found in 25 min and 3 proteins were identified. In addition, the resolutions of these peaks were so high that the grafted monolithic column could be used for rapid and preparative scale purification of serum proteins.

4. Conclusion

A novel PNIPAAm grafted E-51 epoxy resin-based monolithic column with 3D skeletal macropores was prepared. Both temperature and salt stimuli-properties of the prepared monolith have been investigated. However, a six-protein mixture could only be well separated under the salt gradient because much higher abilities in modulation of the hydrophobicity of the prepared monolith could be caused by the salt concentration gradient than the temperature gradient. Meanwhile, the well separation of the human serum sample confirmed that the PNIPAAm grafted E-51 epoxy resin-based monolithic column is suitable for the separation and purification of biological samples.

Furthermore, PNIPAAm could be grafted onto the column by surface-initiated ATRP technique. Thus, the method can provide a new way to modify E-51 epoxy resin-based monolith and can widen the monolith applications in the future.

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