Talanta 85 (2011) 2666–2672

Contents lists available at SciVerse ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Preparation of a strong-cation exchange monolith by a novel method and its application in the separation of IgG on high performance liquid chromatography

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ARTICLE INFO

Article history:
Received 4 June 2011
Received in revised form 18 August 2011
Accepted 22 August 2011
Available online 31 August 2011

Keywords:
Strong-cation exchange
Poly(vinyl carboxylate-co-ethyleneglycol dimethacrylate) (poly(VC-co-EDMA))
Atom transfer radical polymerization (ATRP)
Human immune globulin G (IgG)
Lyszoyme (Lys)

ABSTRACT

A strong cation-exchange poly(vinyl carboxylate-co-ethyleneglycol dimethacrylate) (poly(VC-co-EDMA)) monolithic column for high performance liquid chromatography (HPLC) has been prepared firstly by atom transfer radical polymerization (ATRP) without the expensive complexing ligand, in which vinyl carboxylate was used as the monomer, ethyleneglycol dimethacrylate as the cross linking agent, carbon tetrachloride as the initiator and ferrous chloride as the catalyst. Conditions of the polymerization have been studied and optimized. Morphology of monolithic materials was studied by scanning electronic microscopy. Chemical groups of the monolith were assayed by infrared spectra method and the pore size distribution was determined by a mercury porosimeter. Moreover, the monolith was modified to bear strong-cation exchange groups and tested on the separation of human immune globulin G (IgG) from human plasma in conjunction with HPLC. Good resolution was obtained in a short time (10 min) in the separation. The effects of pH and buffer concentration on the elution of IgG have been investigated. Moreover, frontal analytical method was used to get the IgG dynamic banding capacity of the monolith that was 3.0 mg g^{-1}. Besides, the monolith was also used to separate lysozyme from egg white and separate the mixture of papain, snailase and IgG.

1. Introduction

Ion-exchange chromatography has become an indispensable technique for the separation of proteins in both research and industry due to its high capacity and ability to perform separation under non-denaturing conditions [1,2]. The ion exchanging functionalities includes many kinds of groups such as sulfonic acid groups, carboxylic acid groups and tertiary or quaternary amines. Chemical modified ion-exchange packed columns are the usual tools for protein separation and analysis. Nevertheless, separations with these columns have a number of drawbacks, such as high pressure drops, low throughput, mass transfer limitations and large recovery volumes [3–7]. The recent use of ion-exchange monoliths (silica-based and polymer-based) as stationary phases of high performance liquid chromatography (HPLC) can decrease mass transfer resistances and pressure drops. However, the drawback of the silica-based monolith is the cumbrous and time consuming process that is difficult to control.

At present, most commercial ion-exchange chromatographic columns are particle packed. As we know, the disadvantages of the packed column were described as above. The polymer-based monolith is a good choice for ion exchange column. However, the kinds of commercial ion-exchange monolithic columns are limited and most of them are prepared by sol–gel technique that is very cumbrous. Besides, the commercial ion-exchange monoliths are most coating types that have the disadvantage of instability.

The monoliths which were prepared by in situ radical polymerization have been used as the stationary phase on HPLC for several decades [8,9]. The monomer, cross linking agent, initiator and porogen were put into a stainless steel column to react at a certain temperature for a time. Then a porous monolith was obtained after removing the porogen and other solvent by eluting the polymeric monolith on HPLC system with methanol. Because there was no ecto-power in the in situ radical process, the pores were induced by the phase separation, which led a nonuniform structure with pores being created by accumulation of particles [10–12]. Moreover, the nonuniform structure leads large eddy diffusion, low permeability and high-pressure drops.

As the monomer, vinyl carboxylate is the addition product of an epoxy resin and an unsaturated carboxylic acid. There are two double bonds on the molecular of vinyl carboxylate. With mechanical and thermal characters, vinyl carboxylate has good resistance to most chemical agents. This kind of resin has superior

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0039-9140/$ – see front matter.
doi:10.1016/j.talanta.2011.08.048

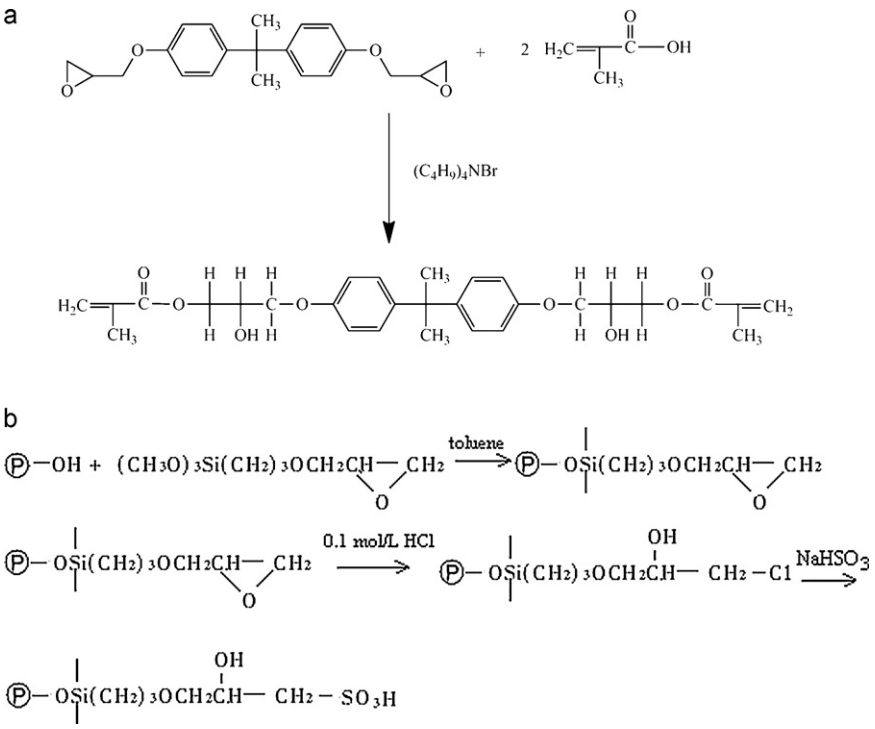

Fig. 1. Synthesis of strong-cation exchange monolith M_a. (a) The synthesis of vinyl carboxulate, (b) the process of chemical modifications with strong-cation exchanger ($-SO_3H$).

properties relative to unsaturated polyester systems [13]. Originally developed for their high corrosion resistance performance, vinyl carboxylate has been used in a wide range of applications due to their inherent physical and mechanical properties [14,15].

In our previous work [16], poly(vinyl carboxylate-co-ethyleneglycol dimethacrylate) (poly(VC-co-EDMA)) monolith had been prepared by in situ free radical polymerization and was used to separate human immune globulin (IgG) from human plasma. But nonuniformity of the structure, high-pressure drop and low resolution of the monolith were presented when it was used as HPLC stationary phase. Besides, the dynamic binding capacity of IgG on it was low (35 $\mu g\, g^{-1}$).

In order to avoid the disadvantages described above and as an alternate, atom transfer radical polymerization (ATRP) technique and chemical modification were used to prepare ion-exchange monolith in this work.

The ATRP process uses an alkyl halide as initiator and a metal in its lower oxidation state with complexing ligands [17–22]. The process involves the successive transfer of the halide from the dormant polymer chain to the ligated metal complex, thus a dynamic

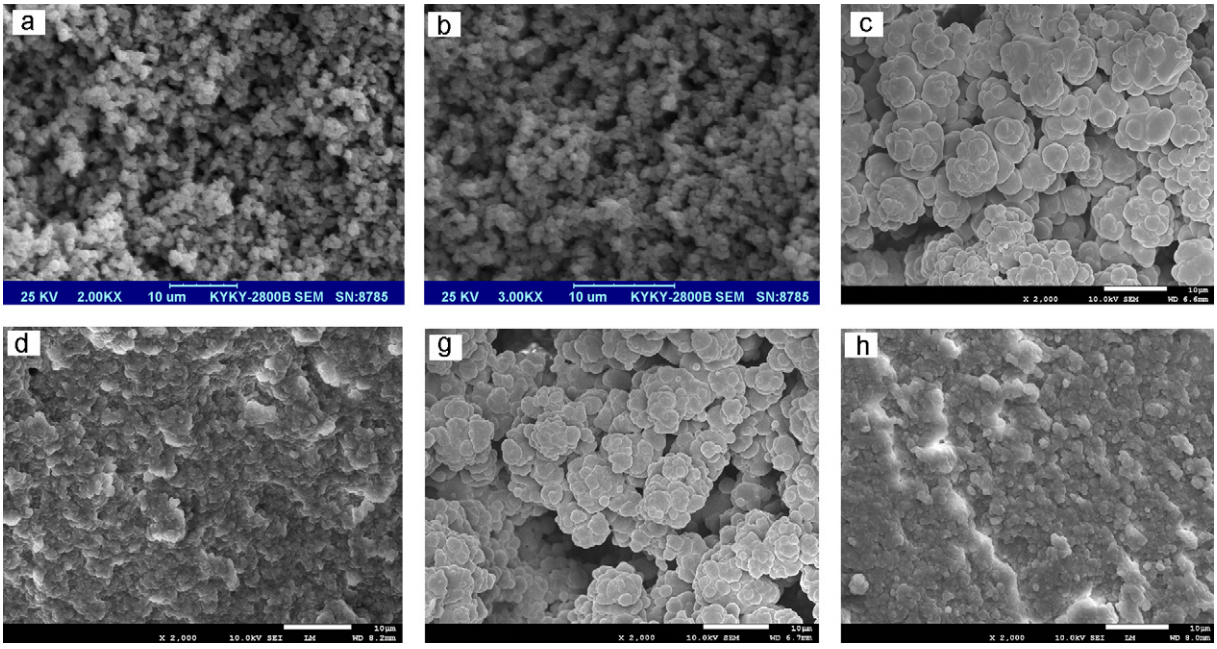

Fig. 2. SEM figures of the monoliths. SEM figures. a–d, g and h were obtained from the conditions listed in Table 1(a)–(d), (g) and (h), respectively.

equilibrium between active and dormant species was established. In ATRP, the concentration of propagating radical is low during polymerization. So the slow chain growth and negligible chain transfer are expected to form the uniform structure with narrow molecular weight distribution.

A more uniform poly(VC-co-EDMA) monolith has been prepared by ATRP without the expensive complexing ligand and applied to HPLC for separation of IgG from human plasma and lysozyme (Lys) from chicken egg white, respectively.

2. Experimental

2.1. Chemicals

IgG and Lys were obtained from RongSheng Pharmaceutical Co. Ltd. (ChengDu China). Sigma Chemical Co. (St. Louis, MO, USA). Bisphenol A diglycidyl ether (BADE) was purchased from LanXing New Materials of Resin Factory (WuXi China). Dodecyl alcohol was obtained from China Medicament Co. Ltd. (Beijing, China). EDMA was purchased from New Jersey (USA). Carbon tetrachloride (CCl_4), ferrous chloride ($FeCl_2$) and γ-glycidoxypropyltrimethoxysilane (KH560) were obtained from Yili Co. Ltd. (Beijing, China). All of these Chemicals were analytical reagent grade. Triplex distilled water was used for all experiments.

2.2. Preparations of the monoliths (M_n and M_b)

Vinyl carboxylate was prepared by BADE according to the procedure described previously [23] and the process was shown in Fig. 1(a).

The preparation of M_n was as follows: 1.0 mL of vinyl carboxylate, 1.0 mL of EDMA, 0.05 mL of CCl_4, 1.8 mL of dodecyl alcohol and 0.003 g of $FeCl_2$ were mixed in an ampul and then the mixture was resolved and degassed with an ultrasonator. The solution was not poured into a stainless-steel chromatographic tube (30 mm × 4.6 mm i.d. sealed at one end with close column head) until the solution was transparent. Then the other end of the tube was sealed with close column head and the polymerization was allowed to proceed at the temperature of 70 °C for 20 h. The monolith M_n with the steel-tube was connected to the HPLC system and was washed online by methanol to remove all of dodecyl alcohol and other soluble compounds present in the polymeric rod.

M_b was prepared by in situ radical polymerization that followed the previous technique [16].

2.3. Modification of M_n

15 mL of toluene and 4 mL of KH-560 were put into a grind-oral tube together with M_n and refluxed for 24 h at 110 °C to modify the monolith with epoxy groups. The monolith was washed on-line with toluene.

Hydrochloric acid (0.1 mol L^{-1}) and the washed monolith were put into a tube to react at 40 °C for 3 h. The monolith was washed with water until the lotion was neutral. After being washed with alcohol and acetone three times, respectively, 5 mL of sodium bisulfite (10%) solution were put into a grind-oral tube together with the monolith, and refluxed for 24 h at 80 °C. The monolith was modified with $-HSO_3$, which was the strong-cation exchanger. The processes were shown in Fig. 1(b). At last, the monolith was eluted by methanol and water online, respectively. Then the strong-cation exchange monolith (M_a) was completely prepared.

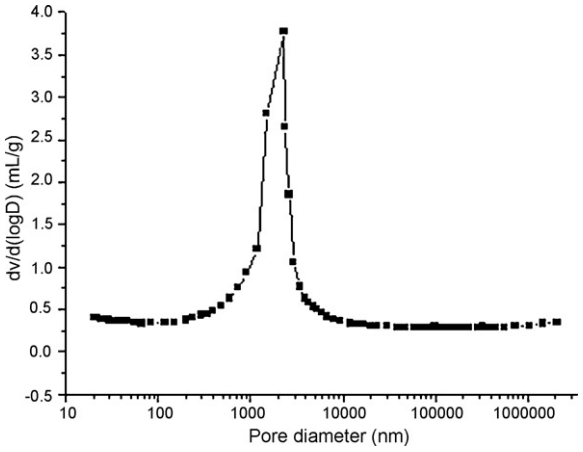

Fig. 3. Pore size distribution profiles for M_a by mercury intrusion porosimetry.

2.4. Characterizations of the monoliths

2.4.1. Morphology, pore size distribution and chemical group of the monoliths

Morphologies of M_a and M_b were shown in Fig. 2(a and b) which were observed by scanning electron microscopy (SEM), respectively. The pore size distribution of M_a was determined by mercury intrusion porosimetry and was shown in Fig. 3 (AutoPore II 9220 V3.04, Micromeritics Instrument Co., Atlanta, GA). Fourier-transfer infrared (IR) spectrum of M_a was obtained from Fourier Transform Infrared Spectrophotometer (FTIR-8400S, Shimaduzu, Japan) and was shown in Fig. 4.

2.4.2. Determination of the content of hydroxyl group on M_n

Three monoliths were prepared by following the same procedures of M_n described in Section 2.2. These monoliths were grinded and then 0.15 g of the powder was placed in a tube together with 1.0 mL of acetic anhydride and 5.0 mL of pyridina. Then the tube was put into a thermostat-controlled water-bath at 60 °C for 12 h. When the 12 h finished, 10 mL of water was added into the tube to make the excess acetic anhydride change to acetic acid. The solution was then titrated with 1.0 mol L^{-1} NaOH solution at 25 °C. Dihydroxyphthalophenone was used as the indicator. Blank assay was operated at the same time, and an average of three measures was the final content of hydroxyl group on M_a.

2.4.3. Determination of content of sulfonic groups on M_a

In order to determine the content of sulfonic group of M_a, the following experiment has been carried out [24].

Three monoliths were prepared by following the same procedures of M_a described in Section 2.3. These monoliths were grinded and then about 0.1 g of M_a was weighed accurately and put into a beaker. 20 mL of water was added into the beaker and the beaker was put into an ultrasonator to obtain a uniform mixture. The solution was then titrated with 1.0 mol L^{-1} of NaOH solution at 25 °C. Dihydroxyphthalophenone was used as the indicator. An average of three measures was the final content of sulfonic group of M_a.

2.4.4. Chromatographic characters of the monoliths

2.4.4.1. Separation of IgG from human plasma. Human blood was centrifuged at 4 °C and 12,000 rpm for 15 min. The supernatant fluid was used as an IgG source.

Chromatographic experiments were carried out with an Agilent 1100 HPLC system (quaternary pump, online vacuum degasser, autosampler with variable injection capacity from 0.1 to 100 μL and UV detector). M_a, M_b and M_n with the size of 30 mm × 4.6 mm i.d. were used as the stationary phase, respectively; the wave length

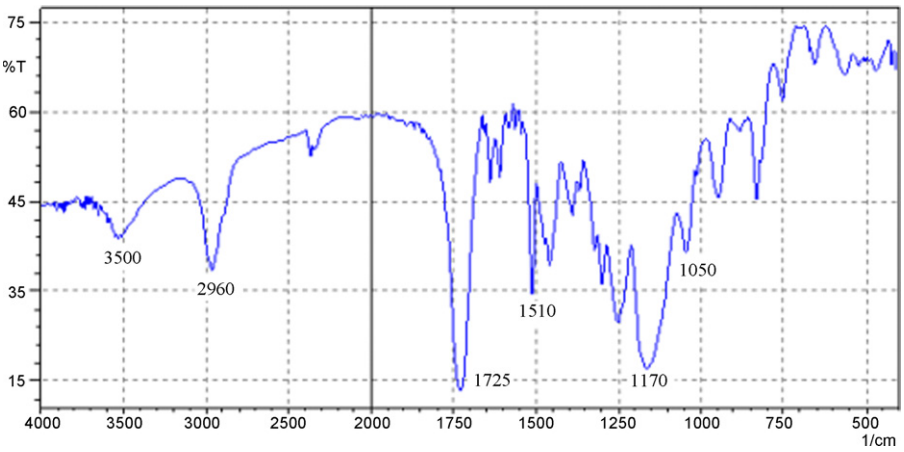

Fig. 4. The FT-IR spectrum of M_a.

was set as 280 nm; the flow rate was 1.0 mL min^{-1}; the amount of injection was 3.0 μL.

The gradient was as follows: water was used as mobile phase in the first 3 min, 0.007 mol L^{-1} of Na_2HPO_4 aqueous solution was used in the next 3 min and 0.01 of mol L^{-1} Na_2HPO_4 aqueous solution was used after 6 min. The chromatograms were shown in Fig. 5.

2.4.4.2. Separation of Lys from egg white. Chicken egg white was diluted to 50% (v/v) with phosphate buffer (0.05 mol L^{-1}, pH 7.0). The diluted egg white was homogenized in an ice-bath and centrifuged at 4 °C and 12,000 rpm for 15 min. The supernatant fluid was used as the sample.

The chromatographic conditions were the same to Section 2.4.4.1 except for the gradient of elution. The gradient was as follows: water was used as mobile phase in the first 3 min, 0.01 mol L^{-1} of Na_2HPO_4 aqueous solution was used in the next 3 min and 0.01 mol L^{-1} Na_2HPO_4 aqueous solution changed linearly to 0.1 mol L^{-1} Na_2HPO_4 aqueous solution in the last 4 min. The chromatograms were shown in Fig. 6.

Besides, M_a was used to separate the mixture of three proteins which were papain, snailase and IgG. The gradient was as follows: water was used as mobile phase in the first 2 min, Na_2HPO_4 (0.002 mol L^{-1}) together with $(NH_4)_2SO_4$ (1.0 mol^{-1}) aqueous solution was used in the next 3 min and Na_2HPO_4 (0.02 mol L^{-1}) together with $(NH_4)_2SO_4$ (1.0 mol L^{-1}) was used in the last 3 min. The chromatogram was shown in Fig. 7.

2.4.5. Determination of dynamic binding capacity of M_a for IgG

Frontal analysis of the column was carried out to determine the dynamic binding capacity of M_a for IgG. The experiment was

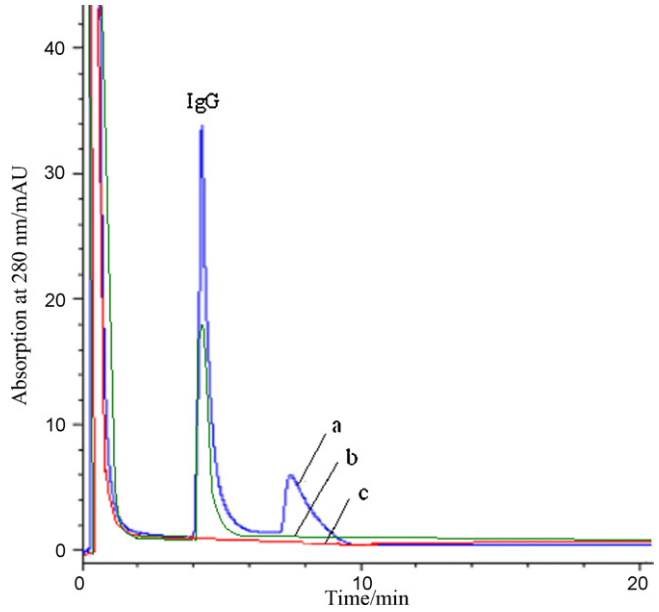

Fig. 5. Chromatogram of the separation of IgG from human plasma. (a) Chromatogram obtained by the strong-cation monolith M_a; (b) chromatogram obtained by M_b; (c) chromatogram obtained by the non-modified monolith M_n. The UV detector was set at 280 nm. The flow rate was 1.0 mL min^{-1} and the amount of injection was 3.0 μL. The gradient was as follows: water was used in the first 3 min, 0.007 mol L^{-1} Na_2HPO_4 aqueous solution was used in the next 3 min and 0.01 mol L^{-1} Na_2HPO_4 aqueous solution was used after 6 min.

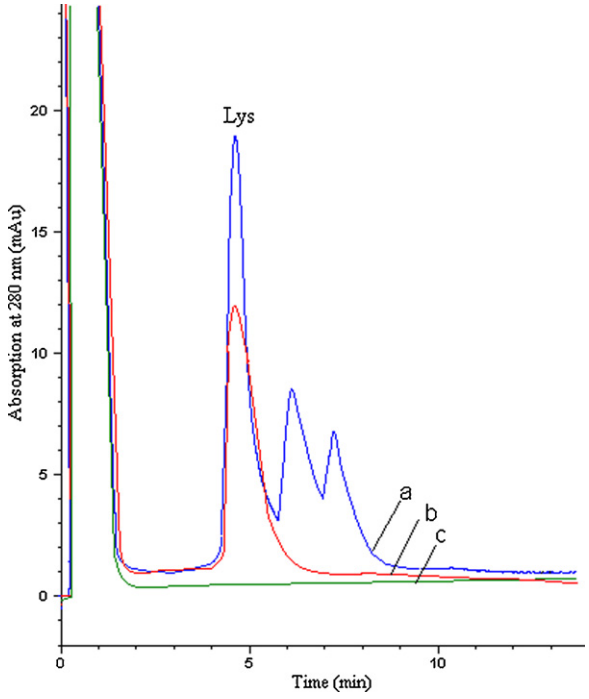

Fig. 6. Chromatogram of separation Lys from egg white. (a) Chromatogram obtained by the strong-cation monolith M_a; (b) chromatogram obtained by M_b; (c) chromatogram obtained by the non-modified monolith M_n. The UV detector was set at 280 nm. The flow rate was 1.0 mL min^{-1} and the amount of injection was 3.0 μL. The gradient was as follows: water was used as mobile phase in the first 3 min, 0.01 mol L^{-1} of Na_2HPO_4 aqueous solution was used in the next 3 min and 0.01 mol L^{-1} Na_2HPO_4 aqueous solution changed linearly to 0.1 mol L^{-1} Na_2HPO_4 aqueous solution in the last 4 min.

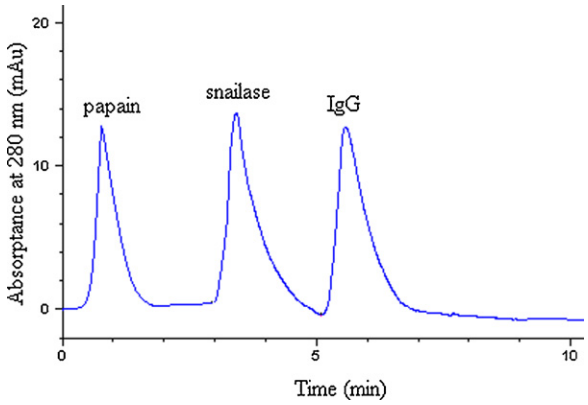

Fig. 7. Chromatogram of separation three proteins mixture by M_a. The UV detector was set at 280 nm. The flow rate was 1.0 mL min^{-1} and the amount of injection was 3.0 μL. The three peaks were papain, snailase and IgG in orders. The gradient was as follows: water was used as mobile phase in the first 2 min, Na_2HPO_4 (0.002 mol L^{-1}) together with $(NH_4)_2SO_4$ (1.0 mol L^{-1}) aqueous solution was used in the next 3 min and Na_2HPO_4 (0.02 mol L^{-1}) together with $(NH_4)_2SO_4$ (1.0 mol L^{-1}) was used in the last 3 min.

carried out with 2.0 mg mL^{-1} of IgG in the mobile phase (0.007 mol L^{-1} Na_2HPO_4 aqueous solution).

3. Results and discussion

3.1. Content of hydroxyl group of M_n

According to the experiment of Section 2.4.2, the content of hydroxyl group of M_n was 13.5% that was calculated from Eq. (1):

$$x\% = \frac{(V0 - V1)CM}{1000W} \times 100\% \quad (1)$$

V_0: the volume of NaOH that was consumed in the blank assay (mL); V_1: the volume of NaOH that was consumed in the titration of nominal sample (mL); C: the concentration of standard solution (mol L^{-1}); M: the molar mass of OH; W: the quality of sample (g).

3.2. Content of sulfonic group of M_a

According to the experiment of Section 2.4.3, the content of sulfonic group was 15.5% (ratio of mass) that was calculated from Eq. (2).

$$x\% = \frac{VCM}{1000W} \times 100\% \quad (2)$$

V: the volume of NaOH that was consumed in the titration of nominal sample (mL); C: the concentration of standard solution (mol L^{-1}); M: the molar mass of HSO_3; W: the quality of sample (g).

The results indicated that M_a was modified successfully by sulfonic groups.

3.3. Characterizations of the monoliths

3.3.1. SEM figures of monoliths

The effects of the porogen (dodecyl alcohol), initiator (CCl_4), catalyst ($FeCl_2$), monomer (vinyl carboxylate) and cross linking agent (EDMA) on the morphology of the monoliths have been studied and the conditions were listed in Table 1. The corresponding SEM figures were shown in Fig. 2.

Fig. 2(a) showed the morphology of optimized condition that listed in Table 1 ((a), M_a) and Fig. 2(b) showed the morphology of M_b (the condition was listed in Table 1 (b)). It is obviously that the morphology of M_a showed a more uniform structure than that of M_b.

There were few pores in Fig. 2(c) because of the higher ratio of porogen. Fig. 2(d) showed that lower ratio of porogen led aporate structure. Besides, the higher ratio of porogen led a more brittle structure and the lower ratio of porogen led a harder one. But the hard structure induced a bad permeability and high pressure drop. In this experiment, the proper amount of porogen was 1.8 mL.

Fig. 2(g) and (h) was the SEM figures of the conditions of (g) and (h) which were listed in Table 1, respectively. The results indicated that the ratio of monomer and cross linking agent was also an important factor for the structure of the polymer. The high ratio of monomer led nonuniform structure while high ratio of cross linking agent led aporate structure. The optimized ratio of monomer and cross linking agent was 1:1.

The effects of initiator and catalyst on the polymerization were also investigated, respectively. Neither of two conditions (Table 1(e) and (f)) led polymerized. This indicated initiator and catalyst were both indispensable in the polymerization and M_a was prepared by ATRP.

So, in order to obtain a monolith which both had high mechanical strength and good permeability, the condition that listed in Table 1(a) was selected.

3.3.2. The pore size distribution of M_a

The pore diameter distribution of M_a was characterized by mercury intrusion porosimetry that was shown in Fig. 3. The figure showed that the general pore volume, average pore diameter and interval porosity were 1.399 mL g^{-1}, 0.85 μm and 67.54%, respectively.

3.3.3. FT-IR spectrum of M_a

Fig. 4 showed that the absorption spectrum of M_a displays readily identifiable peaks at 1200 cm^{-1} and 1050 cm^{-1}, which were characteristic of $-HSO_3$ stretching vibration. This indicated that the cation-exchanger ($-HSO_3$) had been connected onto the monolith and M_a has been prepared successfully.

3.4. Chromatographic characters of the monoliths

M_a, M_b and M_n were used as the stationary phases of HPLC to separate IgG from human plasma and Lys from chicken egg white, respectively. The following parts introduced their characters on HPLC.

3.4.1. Chromatograms of separations

Fig. 5 showed the separation chromatograms of IgG with M_a, M_b and M_n being used as the HPLC stationary phases, respectively. Wherein, Fig. 5(a) was obtained by M_a, (b) was obtained by M_b and (c) was obtained by M_n. Fig. 5(a) showed there were three peaks and the second peak was IgG the third peak was IgM. Fig. 5(b) showed there were two peaks and the second peak was IgG. Fig. 5(c) showed M_n had no ability for the separation of IgG.

Fig. 6 showed the separation of Lys. Wherein, Fig. 6(a) was obtained by M_a, (b) was obtained by M_b and (c) was obtained by M_n. Fig. 6(a) showed there were four peaks and the second peak was Lys. Fig. 6(b) showed there were two peaks and the second peak was Lys. Fig. 6(c) showed M_n had no ability for the separation of Lys.

Compare to M_b, more chromatographic peaks were obtained by M_a. This was because M_a was prepared by ATRP and there were Cl groups on it that had high selectivity for the protein. Besides, the polarity of C–Cl promoted the hydrophilicity of the polymer which led high surface energy and then led a high absorption. The high absorption could avoid some protein being eluted at dead time. So, more chromatographic peaks could be separated by M_a. Moreover, the proper pore size and uniform structure of the monolith were also necessary to the successful separations.

Table 1
The conditions of polymerization.

No.	Vinyl carboxylate (mL)	AIBN (g)	EDMA (mL)	CCl_4 (mL)	Dodecyl alcohol(mL)	$FeCl_2$ (g)
a	1.0	–	1.0	0.05	1.8	0.003
b	1.2	0.09	1.2	–	3.0	–
c	1.0	–	1.0	0.05	2.5	0.003
d	1.0	–	1.0	0.05	1.0	0.003
e	1.0	–	1.0	–	1.8	0.003
f	1.0	–	1.0	0.05	1.8	–
g	1.0	–	0.5	0.05	1.8	0.003
h	0.5	–	1.0	0.05	1.8	0.003

In addition, Fig. 7 showed the separation of the three mixed proteins which were papain, snailase and IgG. The three proteins were baseline separated in 9 min and the three peaks were papain, snailase and IgG in order.

M_a revealed a good stability in the experiments. The strong-cation exchange monolith has been used to separate IgG from human plasma and Lys from chicken egg white under the given mobile phase, respectively. The RSD of retention time and peak area were 0.12% and 0.56% (n = 11), 0.14% and 0.61% (n = 11), respectively. The pressure drop and column efficiency did not change with the accumulation of the number of injections.

3.4.2. *Effects of pH and buffer concentration on the elution of IgG on M_a*

Protein retention on an ionic surface is a much more complex process and cannot be satisfactorily explained by the pI-value of a protein. Factors such as intramolecular charge asymmetry in the protein, the nature of the support surfaces and the mobile phase displacing ions also have significant impact on the retention. Nevertheless, the pI value is a very key character to the protein, and when the pH is lower or higher than pI, the protein shows different characters.

IgG is a neuter protein (pI 6.85–7.5). When pH of mobile phase is lower than pI of IgG, the protein was positive charged which lead an electrostatic attraction with the stationary phase and IgG is retained by the monolith. When pH of mobile phase is higher than pI of IgG, the protein was negative charged which lead an electrostatic repulsion with the stationary phase, and IgG is eluted by the mobile phase. When pH of mobile phase is near to pI of IgG, there is no charge with IgG and it is eluted by the mobile phase. So, in the present experiment, 0.007 mol L^{-1} Na_2HPO_4 (pH ≈ 9.0) aqueous solution was used as the mobile phase to elute IgG from M_a.

Effect of buffer concentration on the IgG elution was studied and the result was shown in Fig. 8. It showed that concentration has little effect on the IgG elution. Though the peak heights were different, the peak areas were almost same. The lower concentration (0.01 of mol L^{-1} Na_2HPO_4 aqueous solution) was selected as the mobile phase in order to protect the HPLC system.

3.4.3. *Dynamic binding capacity of the monolith*

According to the process being described in Section 2.4.5, the binding capacity (Q) was calculated by the following Eq. (3):

$$Q = \frac{(V_{HB} - V_0)c}{m} \quad (3)$$

V_{HB}: the half breakthrough volume of IgG (mL); V_0: the dead volume of the column (mL); c: the concentration of IgG in the mobile phase(mg mL^{-1}); m: the dry weight of the monolith (g).

The dynamic binding capacity of this monolithic column for IgG was found to be 3.0 mg g^{-1} that was much higher than our previous work (35 μg g^{-1}) [16].

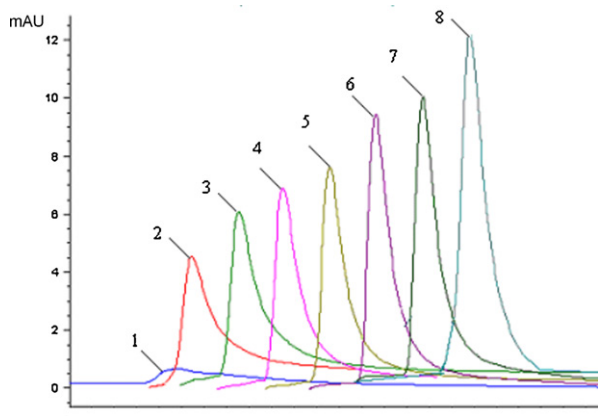

Fig. 8. Effect of buffer concentration on the elution of IgG. The mobile phases were (1) water; (2) 0.002 mol L^{-1} Na_2HPO_4; (3) 0.001 mol L^{-1} Na_2HPO_4; (4) 0.01 mol L^{-1} Na_2HPO_4; (5) 0.02 mol L^{-1} Na_2HPO_4; (6) 0.05 mol L^{-1} Na_2HPO_4; (7) 0.1 mol L^{-1} Na_2HPO_4; (8) 0.2 mol L^{-1} Na_2HPO_4.

3.4.4. *Linear velocity and back pressure drop on the column*

In order to evaluate the mechanical stability of the prepared monolith, we investigated the back pressure drop at several linear velocities. Fig. 9 showed the effect of flow rate on the back pressure when water was used as the mobile phase. Actually, the pressure drop was 0.9 MPa at 1.0 mL min^{-1}. A good linear (r = 0.9996) dependence of the column pressure on the flow rate was obtained. These results clearly showed that the monolith has a good mechanical stability.

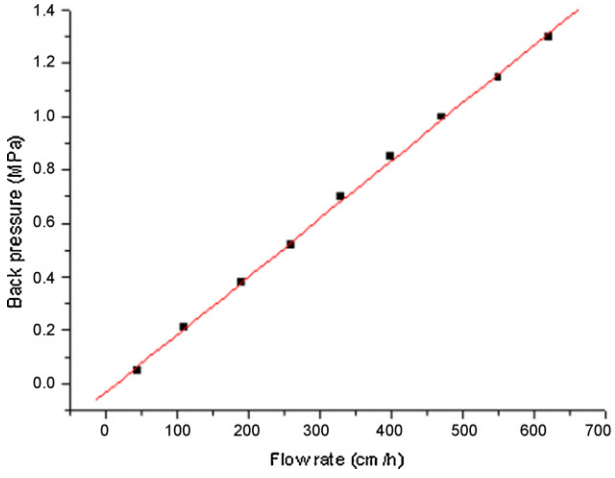

Fig. 9. Linear velocity and back pressure drop on the column. Chromatographic conditions: column size: M_a, 30 mm × 4.6 mm i.d.; mobile phase: water; temperature: 25 °C, r = 0.9996.

4. Conclusions

In this study, a novel strong-cation exchange monolithic column for HPLC has been prepared successfully by ATRP without expensive complexing ligand. Moreover, compared to our previous work, the monolith has more uniform structure, higher dynamic binding capacity and better resolution. The results suggested that such kind of monolithic column that polymerized by ATRP could be used as a simple, cheap, effective solid phase to HPLC for the separation of IgG from human plasma and Lys from chicken egg white. The monolith can be modified to other kinds of chromatographic modes to separate many other proteins.

Acknowledgements

The authors are grateful for financial support by the National Natural Science Foundation of China (Nos. 20375010 and 20675084), the Hebei Province Programs for Science and Technology Development (Nos. 06276479B and 07276407D), the funds of Education Department of Hebei Province (No. 2008416) and Natural Science Foundation of Hebei University (No. 2009-165), the National Natural Science Foundation of China (20905019, 21011140338), Natural Science Foundation of Hebei (B2010000209).

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