Talanta 85 (2011) 1193–1198

Contents lists available at ScienceDirect

Talanta

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

Investigation of temperature-responsivity and aqueous chromatographic characteristics of a thermo-responsive monolithic column

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ARTICLE INFO

Article history:
Received 14 January 2011
Received in revised form 11 May 2011
Accepted 19 May 2011
Available online 26 May 2011

Keywords:
Thermo-responsive
Cryogel
Swelling and deswelling
Steroids

ABSTRACT

A thermo-responsive and macroporous monolithic cryogel was directly prepared by in situ free-radical redox cryo-polymerization in a stainless steel chromatographic column (100 mm × 4.6 mm i.d.) using *N*-isopropylacrylamide (NIPAAm) as functional monomer and PEG-20,000 as porogen at −12 °C. The internal morphology of resulting monolithic cryogel was estimated by scanning electron microscopy (SEM). Based on that, a submicron skeleton structure was observed. Besides, the gravimetrically determined rates of swelling/deswelling for thermo-responsive monolithic cryogel were much higher than that of hydrogel adopting the same component proportion via conventional method (25 °C for 24 h). Simultaneously, a temperature-dependent resolution of steroids was also achieved using only water as a mobile phase. The theoretical plate number of every analyte was more than 2000.

1. Introduction

During the past few years, the introduction of monolithic cryogel is one of the most significant breakthroughs in polymeric monolithic stationary phase for high performance liquid chromatography (HPLC), which meets the increasingly growing needs of a fast, efficient, and environmentally friendly separation protocols [1,2]. Monolithic cryogel is a specific classification of continuous hydrogel, which was potentially formed in the presence of cryogenic treatment. Owing to excellent porosity, monolithic cryogel has recently been utilized as a promising matrix for HPLC separation of proteins and polymeric nanoparticles [3,4].

Nowadays, NIPAAm has been extensively investigated as the best-known thermo-sensitive hydrogel monomer in the intelligent polymers subdiscipline [5–10]. Poly(*N*-isopropylacrylamide) (PNIPAAm) exhibits a reversible phase transition induced by a small external temperature change below and above its unique lower critical solution temperature (LCST) at approximately 32 °C [11,12]. When the environmental temperature is below the LCST, the PNIPAAm hydrogel is hydrated and hydrophilic. Nevertheless, above the LCST, the hydrogel presents a dehydrated and hydrophobic state as a result of the disruption of the delicate hydrophilic/hydrophobic balance along with its conformation transition [13–15].

Nevertheless, conventional PNIPAAm hydrogel is often restricted in its applications, such as separation processes, due to very slow rates of swelling/deswelling in response to a temperature stimulus. Moreover, PNIPAAm was failed to be used as the stationary phase for HPLC because of the inferior mechanical strength [16]. HPLC stationary phases with PNIPAAm hydrogel-modified silica that was first reported and have been extensively investigated by Okano and Kanazawa group [17–20] and monolithic column using surface-initiated atom transfer radical polymerization (ATRP) [21–24] have been used for drugs or proteins separation with aqueous solutions as mobile phases. In addition, for the sake of accelerating the thermo-sensitive rate, either cryo-polymerization or use of polyethylene glycol (PEG) as a porogen during the polymerization reaction is one of the several successful strategies, which have been proposed in this regard [25].

In the present paper, poly(*N*-isopropylacrylamide-co-*N*,*N*′-methylenebisacrylamide) [poly(NIPAAm-co-BIS)] monolithic cryogel, as reported in our recent paper [26,27], was obtained by in situ free-radical redox cryo-polymerization (−12 °C) directly inside a stainless steel chromatographic column, using NIPAAm as functional monomer and PEG-20,000 as porogen. Then the swelling/deswelling behaviors of poly(NIPAAm-co-BIS) monolithic cryogel were investigated. In addition, a temperature-dependent separation of steroids was also further observed on these cryogels using only water as a mobile phase.

2. Experimental

2.1. Materials

N-isopropylacrylamide (NIPAAm) purchased from Tokyo Kasei Kogyo (Tokyo, Japan) was purified by recrystallization from a mix-

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doi:10.1016/j.talanta.2011.05.034

ture consisting of benzene and *n*-hexane(3:7, v/v) and dried at room temperature *in vacuo*. *N*,*N*′-Methylenebisacrylamide (BIS) was produced by Tianjin Kermel Chemical Reagent Development Center (Tianjin, China). Ammonium persulfate (APS) and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) were purchased from Shanghai Chemical Plant (Shanghai, China). PEG-20,000 was obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). The steroids were kindly provided by the National Institute for the Control of Pharmaceutical and Biological Products of China (Beijing, China). Ultrapure water was prepared from a Millipore-Q water-purification system (Taiwan, China) and solutions were filtered through a 0.45 μm membrane before use throughout.

2.2. Characterization methods

The monoliths were removed from the stainless-steel columns and cut into little pieces. Then the pieces of monoliths were dried *in vacuo* at 60 °C for 24 h. For the internal morphology studies of monoliths by SEM, the pieces of monoliths were snapped apart and placed on sticky copper foils, which were attached to a standard aluminum specimen stub. Then the attached monoliths were coated with about 20 nm of gold by an Eiko IB-3 sputter coating instrument (Eiko, Tokyo, Japan). Microscopic analysis was carried out in a Hitachi (Hitachi High Technologies, Tokyo, Japan) S-4300 SEM instrument. The HPLC measurements were performed using a PU-1580 pump and a variable-wave-length UV-1570 detector (Jasco, Japan). Furthermore, a thermostated column compartment was employed as well (Waters, America). Data processing was performed with an HW-2000 chromatography workstation (Nanjing Qianpu Software, China).

2.3. Preparation of standard solutions and calculations

Standard solutions of steroids were prepared with hydrocortisone, cortisone acetate, prednisolone acetate, fluocinolone acetonide, betamethasone 21-acetate and beclomethasone dipropionate in Millipore-Q water (0.01–0.05 mg/mL). All the standard solutions were stored in a refrigerator at 4 °C.

The % *C* (crosslinker concentration/100 mL) and % *T* (total monomer concentration/100 mL) nomenclature suggested by Hjertén [28] was used for characterization of polymerization reactant component. Chromatographic parameters such as retention factor (*k*) and resolution (*R*), symmetry factors (*S*) and theoretical plate number per meter (*N*) were calculated using the formulas according to the literature [29,30].

2.4. Synthesis of gels

The polymerization mixture for the poly(NIPAAm-co-BIS) monolithic cryogels was prepared as follows. A mixture consisting of BIS (% *C* = 40%), NIPAAm (% *T* = 26%) and PEG-20,000 (50 mg/mL) was dissolved in 1.4 mL ultrapure water, vigorously mixed, sonicated for 20 min and bubbled with nitrogen for another 5 min to remove gases. Ten microliters of a 10% (w/v) radical initiator APS solution was added and the mixture was briefly mixed, before 10 μL of a 10% (v/v) polymerization accelerator TEMED solution was finally added. Then the stainless steel chromatographic columns (100 mm × 4.6 mm i.d.) sealed at the bottom were filled with the polymerization mixture and then sealed at the top. After the polymerization was allowed to proceed at −12 °C for 24 h, the seals were removed from the chromatographic columns and the columns was provided with fittings, attached to the HPLC system and washed with water at flow rate of 1 mL/min for 60 min to remove PEG-20,000 and other unreacted compounds in the monolithic cryogels at room temperature.

For the same feed mixtures corresponding conventional poly(NIPAAm-co-BIS) hydrogels were prepared by a conventional procedure, viz. the copolymerization was conducted for 24 h at 25 °C. Then the resultant hydrogel was identically washed thoroughly with water like cryogels.

2.5. Measurement of swelling/deswelling kinetics

The dried above products having been removed from the columns and in a vacuum oven at 45 °C and then finally to constant weight were cut into discs (ca. 2 cm in length and similar in diameter). Swelling measurements were carried out on xerogel discs immersed in deionised water, using a thermostated water bath at different temperatures. The xerogel discs were swollen to equilibrium for 48 h at the temperature range from 10 °C to 60 °C. After that, the discs were removed from the swelling medium, the excess surface water was lightly surface dried with moistened filter paper, and the discs were weighed, the corresponding weight being, *Ws*. The swelling ratio (*SR*) of poly(NIPAAm-co-BIS) monolithic cryogel and conventional hydrogel is defined as $SR = (Ws - Wd)/Wd \times 100\%$, where $(Ws - Wd)$ is the weight of water in the swollen gel at a particular temperature and *Wd* is the weight of the xerogel. At least three samples of each were used to yield three values of *Ws*, the average of which was used to calculate *SR*.

Similarly, the kinetics of deswelling behavior for poly(NIPAAm-co-BIS) monolithic cryogel and conventional hydrogel were also measured gravimetrically using samples of similar dimensions. Swollen gels equilibrated first in distilled water at 25 °C were quickly transferred to another water bath with a constant temperature of 50 °C. Zero time of deswelling was thus precisely defined at the moment when the swollen gels were placed in the water bath at 50 °C. After a certain time, gels were blotted the excess surface water with moistened filter paper. The weight changes of the gels were recorded during the course of deswelling at regular time interval. Water retention (*WR*) is defined as $WR\ (\%) = (Wt - Wd)/Ws \times 100$, where *Wt* is the weight of gel at regular time interval and the other symbols are the same as defined above.

2.6. HPLC measurements

The thermo-responsive monolithic cryogels were evaluated chromatographically using the HPLC system including detector and chromatography workstation as mention in Section 2.2. Water was employed to complete all chromatographic tests as a mobile phase. The flow rate was 1.0 mL/min and 5 μL of standard solutions and a mixture of them were injected. The elution was monitored at 254 nm.

3. Results and discussion

3.1. Internal morphology studies by SEM

In order to observe the porous property of the poly(NIPAAm-co-BIS) monolithic cryogel and conventional hydrogel prepared, SEM was used to evaluate gels. Based on comparing the scanning electron micrographs shown in Fig. 1a and b, internal morphology of gels strikingly changed from a microporous one to one composed of a submicron skeleton porous structure. Two main aspects could contribute to this remarkable difference: on the one hand, the ice crystals formed after freezing acted as porogen, while the dissolved monomers and initiator were concentrated in a small fraction of a non-frozen fluid in which polymerization proceeded efficiently. After melting, a continuous porous monolithic cryogel was formed via this cryo-polymerization system [31]; on the other

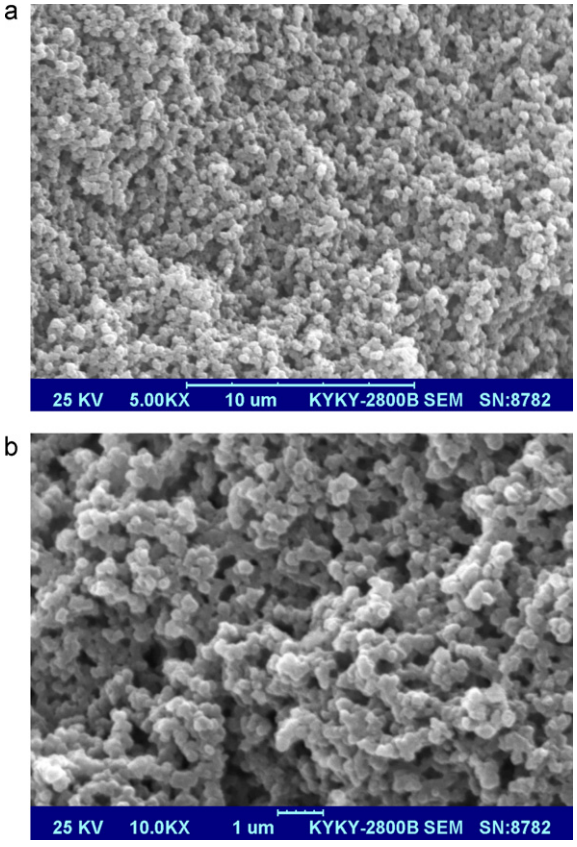

Fig. 1. Internal morphology of poly(NIPAAm-co-BIS) conventional hydrogel and monolithic cryogel: (a) hydrogel and (b) cryogel.

hand, PEG-20,000 induced lateral aggregation of polymer chains, thus contributing to the formation of more porous structures [32].

3.2. Swelling/deswelling kinetics

Fig. 2 shows the equilibrium swelling ratio (*SR*) vs. temperature which clearly illustrates the LCST behavior of poly(NIPAAm-co-BIS) monolithic cryogel and conventional hydrogel. The *SR* of monolithic cryogel was significantly larger than that of conventional hydrogel at the temperatures below the LCST. Moreover, it could be seen that when the temperature increase, the equilibrated *SR* of monolithic

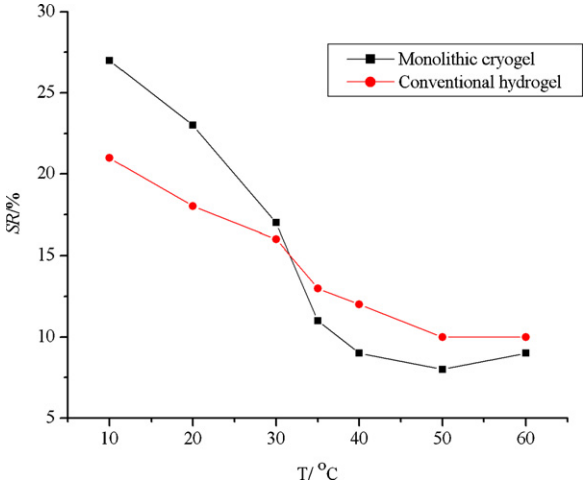

Fig. 2. Temperature dependence of the equilibrated swelling ratio of poly(NIPAAm-co-BIS) monolithic cryogel and conventional hydrogel.

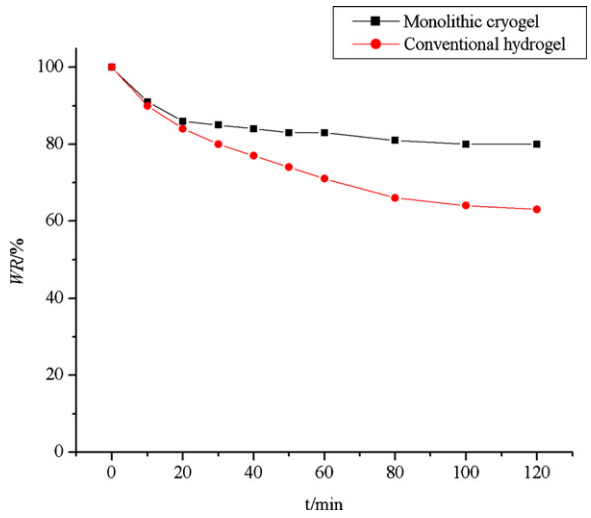

Fig. 3. Deswelling kinetics of poly(NIPAAm-co-BIS) monolithic cryogel and conventional hydrogel.

cryogel decreased quickly, and finally the *SR* was less than that of conventional hydrogel above the LCST.

The deswelling kinetics of poly(NIPAAm-co-BIS) monolithic cryogel and conventional hydrogel are shown in Fig. 3. The figure indicates that monolithic cryogel could attain deswell equilibrium much faster than conventional hydrogel. As displayed in Fig. 3, the monolithic cryogel lost approximate 14% water in only 20 min and then reached the equilibrium of water content, while the equilibrium was achieved slowly over a period of 120 min. Note that, thermo-induced water retention capacity of monolithic cryogel was found to be higher than that of conventional hydrogel. This may be owing to the basic fact that the polymeric chains in cryogel were more rigid and did not collapse to the same extent as in hydrogel [33]. High rigidity of cryogel network was proved by the phenomenon that the pores in cryogel did not collapse even in dried state according to its scanning electron micrograph shown in Fig. 1b.

The much higher swelling/deswelling rates of poly(NIPAAm-co-BIS) monolithic cryogel than conventional hydrogel could be attributed to the distinction in their pore morphology according to correlative reference [25]. Interconnectivity of pores played a crucial role in fast swelling/deswelling of cryogel as solvent molecule could move by convection across its network, while in conventional hydrogel this process is diffusion depen-

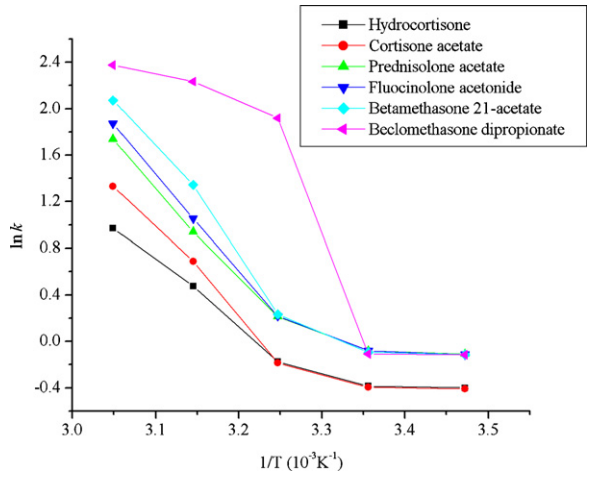

Fig. 4. Van't Hoff plots of the six steroids on the temperature-responsive cryogels.

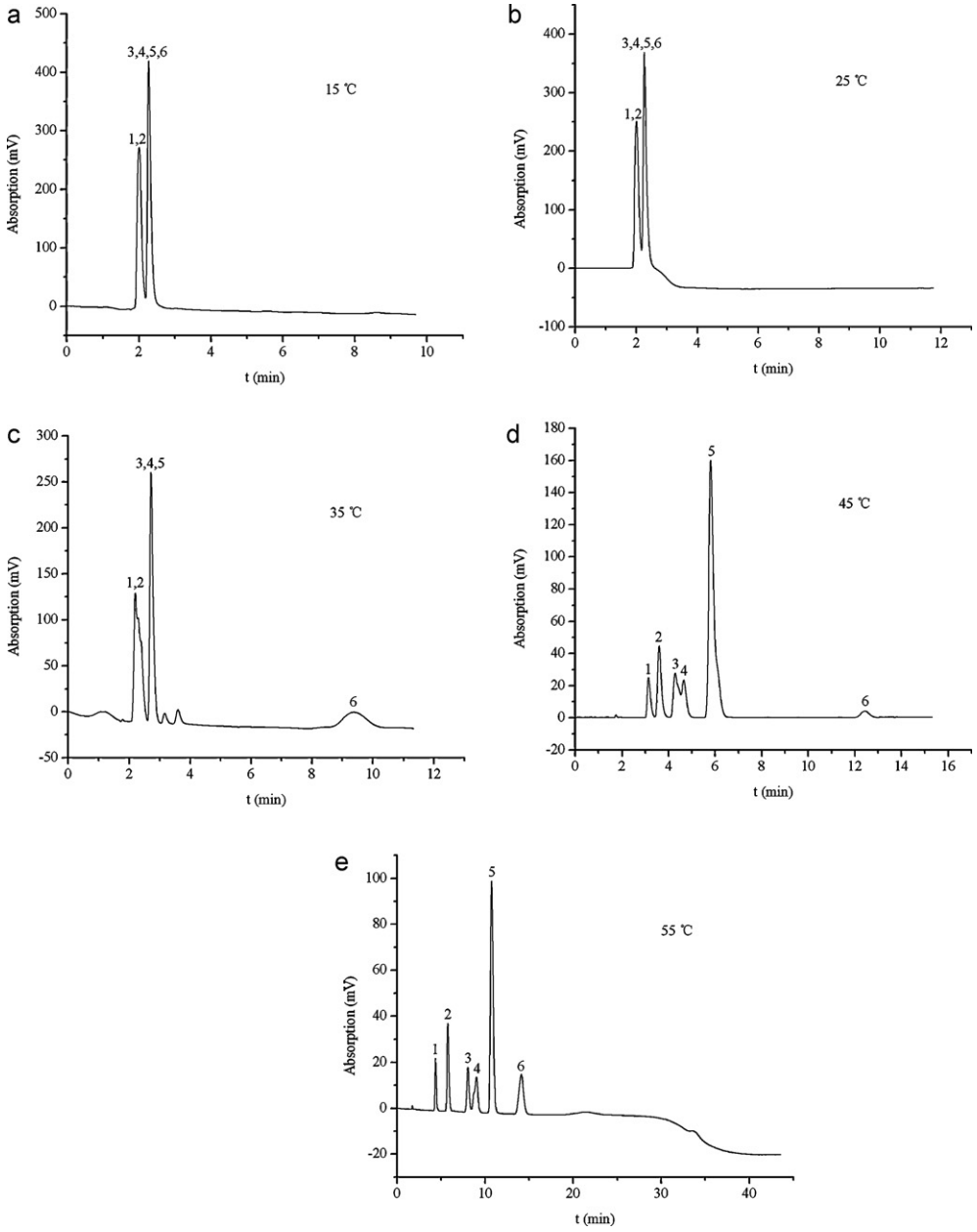

Fig. 5. Elution profiles of a mixture of steroids at different temperatures on the temperature-responsive columns. Peaks: 1, hydrocortisone; 2, cortisone acetate; 3, prednisolone acetate; 4, fluocinolone acetonide; 5, betamethasone 21-acetate; 6, beclomethasone dipropionate. HPLC conditions: mobile phase, water; flow rate, 1 mL/min; monitoring, UV at 254 nm; the injection volume, 5 μL.

dent and thus slower. The characteristic feature of resulting rapid thermo-responsive poly(NIPAAm-co-BIS) monolithic cryogel, which maintains a proper balance between micropores (which give specific surface area for the required interactions) and macropores (which allow efficient liquid mass transfer) combining with favorable mechanical strength gives vent to its succedent chromatographic application.

3.3. Thermo-responsive chromatographic separation of steroids

The temperature-responsive property of the poly(NIPAAm-co-BIS) monolithic cryogels was further investigated using HPLC with water as mobile phase at different temperatures under isocratic conditions. Six steroids with a variety of hydrophobicities that are usually defined as the logarithm of its partition coefficients in the *n*-octanol/water system (called as log*P* values), including hydrocortisone (log*P* = 1.6), cortisone acetate (log*P* = 2.1), prednisolone acetate (log*P* = 2.4), fluocinolone acetonide (log*P* = 2.5), betamethasone 21-acetate (log*P* = 2.8) and beclomethasone dipropionate (log*P* = 3.1), were used as probes to draw information about the temperature-responsive property of the poly(NIPAAm-co-BIS) monolithic cryogels.

The retention factors (*k*) of each steroid on the poly(NIPAAm-co-BIS) monolithic cryogels were determined at various temperatures over the range of 15–55 °C. Fig. 4 shows that the Van't Hoff plots for steroids on the cryogels. The *k* value in Fig. 4 is an average of six cycle measurement results derived from succedent Section 3.4 where the experimental results demonstrate a good repeatability process. Separately, benzene was employed as the mobile phase velocity marker in this experiment according to the cited reference [29]. The steroids with high value of log*P* show higher ln*k* than those with low value at the same temperature. The most hydropho-

Table 1
Chromatographic parameters of separation of the steroids.

Steroid	Retention factor	Resolution	Symmetry factor	Theoretical plate number
Hydrocortisone	2.64	5.35	1.38	3947
Cortisone acetate	3.79	1.85	1.22	3426
Prednisolone acetate	5.68	1.62	1.16	4426
Fluocinolone acetonide	6.50	2.64	0.81	2422
Betamethasone 21-acetate	7.93	2.44	1.22	5660
Beclomethasone dipropionate	10.74	0	1.00	3307

bic one (beclomethasone dipropionate) was mostly affected by temperature. This indicates that the hydrophobic interaction between the solutes and the stationary phase plays a dominating role in retaining the solutes on the cryogels. Hence, increase of $\ln k$ with temperature increase suggests that this interaction between steroids and stationary phase increase at the elevated temperature.

Note that, generally, the Van't Hoff plots should be linear for conventional chromatographic processes on commercially available reversed-phase columns under conditions where the retention mechanisms do not change, in which the slopes of the plots should be positive value. However, Fig. 4 shows a nonlinear relationship between the reciprocal temperature ($1/T$) and $\ln k$ values for each steroid, and $\ln k$ of each steroid increases with the temperature increasing. The marked discontinuities of the negative slopes in the range of 25–35 °C also can be observed due to the known configurational change of PNIPAAm at its LSCT (32 °C). Below the LCST, the extending PNIPAAm chains exhibit hydrophilic property and the steroids have relatively little retention on the surface; but above the LCST, the collapsed PNIPAAm chains show hydrophobic property as a result of relatively stronger retainment of the steroids. Therefore, these remarkable phenomena can strongly support the poly(NIPAAm-co-BIS) monolithic cryogels to be responsive to temperature change in water.

The elution profiles at five different temperatures, below and above the LCST, are given in Fig. 5a–e. Below 35 °C, the PNIPAM chains are in the expanded conformation in the aqueous mobile phase because of the strong hydration. Here the surfaces of our cryogels are hydrophilic; therefore the separation of the steroids is poor. At 15 °C and 25 °C, hydrocortisone and cortisone acetate, prednisolone acetate, fluocinolone acetonide, betamethasone 21-acetate and beclomethasone dipropionate are both being eluted together in a single peak around 2 min. Beclomethasone dipropionate is eluted circa 9 min due to the big difference in $\log P = 3.1$ compared with the other steroids in a broad peak at 35 °C. At 55 °C the steroids can be nearly baseline separated because the surface behavior of the monolithic stationary phase changes from hydrophilic into hydrophobic.

Meanwhile, some chromatographic parameters such as retention factor and resolution, symmetry factor and theoretical plate number of each steroid were calculated and the result was shown in Table 1, in which the theoretical plate number for all the analytes could be up to 2000. It can be seen that the poly(NIPAAm-co-BIS) monolithic cryogels are feasible for separation of steroids.

In the proposed thermo-responsive chromatography method, it is noteworthy that the elution of target substances could be modulated only by changing the column temperatures without any further modification of the aqueous mobile phase and the organic solvent compared with the widely used reversed-phase liquid chromatography (RPLC) method [34–36]. The ability of the developed method to separate the solutes without the use of an organic solvent as a mobile phase is advantageous from the point of view of economical cost of the mobile phases and environmental reasons. Thus, this thermo-responsive chromatography conducted under an aqueous mobile phase condition would have potential applications in the separation of biomolecules.

3.4. Repeatability and stability

A good column-to-column repeatability and column stability are important measure of the process used to prepare the columns. A good repeatability characterized by relative standard deviations (RSDs) for the retention times in the range of 2.2–6.8% was achieved on poly(NIPAAm-co-BIS) monolithic cryogels using the same process and conditions ($n = 6$). Furthermore, the chromatograms of the cryogels were obtained after numerous equilibrations and more than 100 separation runs involving the steroids mixture. The results demonstrated that the preparation process had a good repeatability and the cryogels were stable.

4. Conclusions

A thermo-responsive poly(NIPAAm-co-BIS) monolithic cryogel has been synthesized by a combination of delicate cryopolymerization (−12 °C) with PEG-20,000 porogen. The resulting porous monolithic composite with rapid swelling/deswelling kinetics has achieved the separation of six steroids utilizing the thermo-responsive alteration of surface property in an aqueous media by changing the temperature from 15 °C to 55 °C. The poly(NIPAAm-co-BIS) monolithic cryogel exhibited favorable temperature-controlled hydrophilic–hydrophobic changes. Such fast responsive monolithic cryogel has potential applications in HPLC separation system with an aqueous mobile phase only by changing the column temperature instead of changing the mobile phase composition, which will decrease load on economical cost and environmental pollution.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (Nos. 20375010, 20675084), the Hebei Province Programs for Science and Technology Development (Nos. 06276479B, 07276407D), the funds of Education Department of Hebei province (No. 2008416) and Natural Science Foundation of Hebei University (No. 2009-165).

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