



Preparation and characterization of temperature-responsive poly(*N*-isopropylacrylamide-co-*N,N'*-methylenebisacrylamide) monolith for HPLC

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ABSTRACT

A temperature-responsive poly(*N*-isopropylacrylamide-co-*N,N'*-methylenebisacrylamide) [poly(NIPAAm-co-BIS)] monolith was prepared via a free-radical polymerization technique using an aqueous redox initiator in solution at -12°C . The effect of the % *T* (total monomer concentration/100 mL) and % *C* (cross-linker concentration/100 mL) on the visual form was investigated. The effect of the porogen on the pore structure was characterized by SEM. Under the optimum condition, the monolith for HPLC was successfully prepared and its mechanical strength and permeability have been studied. Furthermore, a temperature-dependent resolution of aromatic ketones was achieved using only water as mobile phase. The increasing interaction between solutes and the monolith was observed when temperature increased. The theoretical plate number of every analyte was more than 10^4 .

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1. Introduction

High performance liquid chromatography (HPLC) technique is undoubtedly among the cornerstones of modern analytical chemistry. Nowadays, analytical chemists envisage the challenge of an increasingly growing demand for fast, efficient, and environmentally friendly separation protocols. In this situation, the introduction of monolithic materials is one of the most significant breakthroughs in analytical chemistry. Monolithic columns consist of a continuous and porous separation media that is hermetically sealed against the wall of a tube, and the stream of mobile phase is forced to flow through it absolutely [1]. In recent years, the monolithic stationary phase, has attracted increasing attention because of its, excellent mass transfer properties and versatile surface modification compared to conventional columns packed with particles [2].

Last two decades have witnessed explosive growth in the intelligent polymers sub-discipline. Intelligent polymers which are known as environmentally sensitive polymers have been diffusely used in the field of medicine and biotechnology. The characteristic feature that actually makes them “intelligent” is their ability to respond to very slight changes in the surrounding environment. The environmental trigger behind these transitions can be

either change in temperature [3] or pH shift [4], presence of certain metabolic chemicals [5] and polycation–polyanion complex formation [6]. Note that, thereinto, temperature is the most widely used stimulus in environmentally responsive polymer systems [7] and it presents great use in applications as drug delivery systems [8,9], the separation and purification of metal ions [10] and chromatography [11,12]. Temperature-responsive polymers exhibit unique lower critical solution temperature (LCST) behavior where phase transition is induced by surpassing a certain temperature threshold. Polymers of this type undergo a thermally induced, reversible phase transition along with the temperature change below and above the LCST [13].

It is also well known that poly(*N*-isopropylacrylamide) (PNIPAAm) shows above-mentioned phase transition behavior. LCST value of this polymer at which precipitation occurs is given as 32°C [14,15]. Fujimoto and Fujise have separated some uncharged compounds using PNIPAAm as the stationary phase for reversed-phase capillary electrochromatography [16]. PNIPAAm beads were prepared and packed into a glass column by Kanazawa et al. Although HPLC retention behavior of antipyretics and proteins on the column with an aqueous mobile phase was altered in response to changes of mobile phase temperature, the inferior mechanical strength and temperature-induced shrinkage of the beads sometimes produced voids and channels in the column, leading to a loss of efficiency [17]. HPLC stationary phases with PNIPAAm hydrogel-modified silica that was first reported and have been extensively investigated by Okano and Kanazawa group [18–21] and monolith using surface-

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initiated atom transfer radical polymerization (ATRP) [22–25] have been used for drugs or proteins separation with aqueous solutions as mobile phases. Nevertheless, not only the modified process was tedious, but also the amount of grafted *N*-isopropylacrylamide had the effect on the separation.

In the present study, a temperature-responsive poly(NIPAAm-co-BIS) macroporous monolithic materials was prepared via a combination of unique cryo-polymerization and using PEG-20,000 as porogen. The influence factors on the preparation of the monoliths were studied. Furthermore, the effect of external temperature on the retention behavior was investigated using aromatic ketones.

2. Experimental

2.1. Materials

N-Isopropylacrylamide (NIPAAm) purchased from Tokyo Kasei Kogyo (Tokyo, Japan) 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'*-methylene-bisacrylamide (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-10,000, 20,000 and ammonium sulfate were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). The aromatic ketones 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 scanning electron microscopy (SEM) and Fourier transform infrared spectroscopy (FT-IR) characterization. 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 FT-IR spectra were recorded on an FTIR-8400S IR apparatus in the region of 400–4000 cm^{-1} (SHIMADZU, Kyoto, Japan). Before the FT-IR spectra measurement, the desiccative piece of monolith was grinded into powder and then further dried *in vacuo* at 70 °C for 48 h. The sample of FT-IR spectra measurement was prepared by mixing 1 mg of monolith powder and 100 mg of ground KBr and pressing it in a die, forming a pellet at 10 tons and 1 min. Then a transmission spectrum with a sharp peak was obtained. Prior to the pore size distribution determination via a porosity measurement, monolithic column was Soxhlet-extracted in methanol for 24 h, to remove any soluble compounds, dried *in vacuo* at 70 °C for 5 h and subjected to a pressure cycle starting at approximately 0.57 psia, increasing to 60,000 psia in predefined steps by mercury intrusion porosimetry (AutoPore II 9220 V 3.04, Micromeritics Instrument Co., Atlanta, GA). The HPLC measurements were performed using a PU-1580 pump and a variable-wave-length UV-1570 detector (Jasco, Japan). Furthermore, a thermostatted column compartment was bestowed 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 aromatic ketones were prepared with acetophenone, propiophenone, butyrophenone in methanol (0.01–0.03 mg/mL). All the standard solutions were stored in a refrigerator at 4 °C.

The % C (cross-linker concentration/100 mL) and % T (total monomer concentration/100 mL) nomenclature suggested by Hjertén [26] was used for stationary phase characterization. Permeability, which could be used as an index for evaluating the quality of monolith, was calculated based on the Darcy's law: $K = \mu\eta L/P$, where K for permeability (m^2), μ for superficial velocity (m/s), η for mobile phase viscosity (Pa·s), L for column length (m), and P stood for back pressure (Pa) [27]. 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 [28].

2.4. Synthesis protocols

The polymerization mixture for the monolithic columns was prepared as follows: The reactants were weighted according to the desired % T and % C ratio into 2.0 mL Eppendorf vials in the following order: (1) cross-linker (BIS), (2) functional monomer (NIPAAm), and (3) PEG-10,000, 20,000 or ammonium sulfate. The mixture was dissolved in 1.4 mL ultrapure water, vigorously mixed, and sonicated for 20 min and bubbled with nitrogen for another 5 min to remove gases. Ten microliter of a 10% (w/v) APS solution was added and the mixture was briefly mixed, before 10 μL of a 10% (v/v) TEMED solution was finally added. Then the stainless-steel columns (100 mm \times 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 tubes 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-10,000, 20,000 or ammonium sulfate and other soluble compounds present in the polymer rod after the polymerization was completed. The scheme of polymerization was shown in Fig. 1.

2.5. HPLC measurements

The temperature-responsive materials 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 mobile phase. The flow rate was 1 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. Effect of the % T and % C

Like any monolithic column, the monoliths under investigation here, have to satisfy certain criteria in order to be useful as stationary phases for HPLC separations. They should be uniform over all the column length and show good mechanical and chemical stability. Moreover, they also should be porous with a favorable porosity and ideally show little tendency for swelling in any type of solvent.

Accordingly, the first step of our experiments was dedicated to the establishment of a robust synthesis approach to HPLC-compatible monolithic columns. Different compositions of polymerization mixtures were experimented, in order to acquire some information with regard to polymer properties as a function

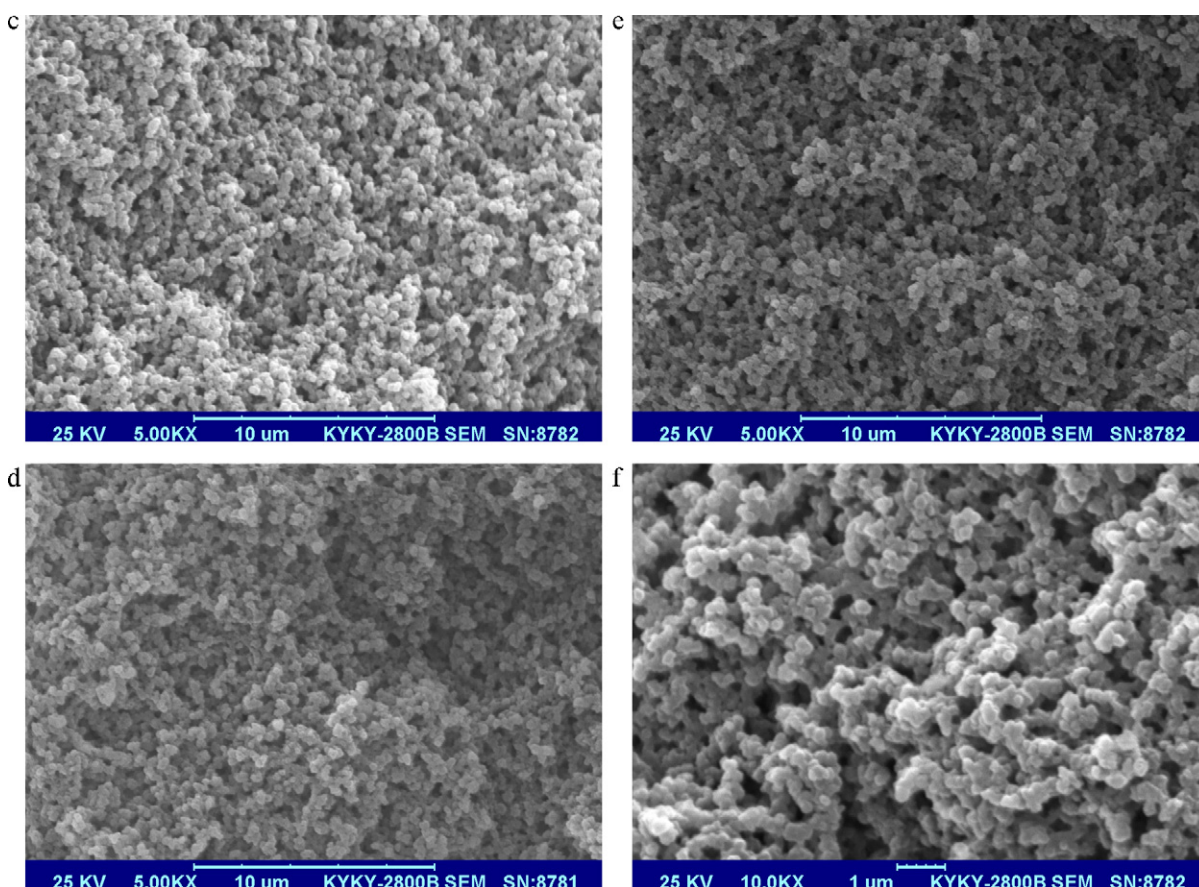


Fig. 3. Effects of varying porogen on the morphology of poly(NIPAAm-co-BIS) monolithic columns (c) no porogen, (d) ammonium sulfate, (e) PEG-10,000, (f) PEG-20,000.

monoliths are not damaged in the range of back pressure from 4 to 81 bar. Moreover, the efficiency of chromatographic separation was not affected after subjecting the high flow rates. Besides, the good permeability value of $5.59 \times 10^{-14} \text{ m}^2$ was calculated based on the Darcy's law and back pressure experiment at ambient temperature, which supported the internal morphology information of Fig. 3(f). Meanwhile, there is a significant change that the back pressures

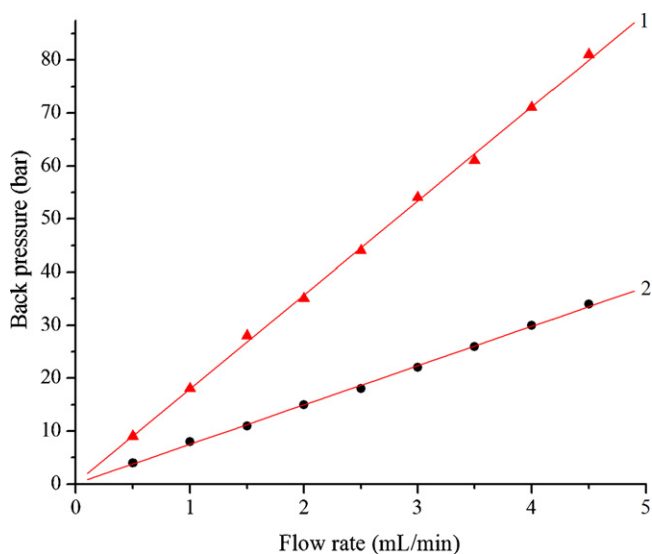


Fig. 4. Back pressures of the poly(NIPAAm-co-BIS) monoliths at different flow rates at 15 °C and 55 °C. 1: 15 °C; 2: 55 °C. Mobile phase: water.

of the monoliths become obviously lower at 55 °C than at 15 °C. Besides the effect of temperature on the mobile phase viscosity, it seemed to be related with the temperature responsive property of the poly(NIPAAm-co-BIS) monoliths. Below the LCST (32 °C) of PNIPAAm, the chains on the surface of monoliths were extending to obstruct the flow of mobile phase. Above the LCST, the chains collapsed and hydrophobic on the surface of monoliths, in this situation, mobile phase was allowed to flow through the monoliths under smaller resistance. This similar result was observed in the previous literature [31].

3.4. FT-IR study of the poly(NIPAAm-co-BIS) monolith and pore size distribution

The FT-IR spectrum of the monolith was given in Fig. 5, which was characterized by the typical absorption bands. The broad bands at $3307.69\text{--}3078.18 \text{ cm}^{-1}$ was the secondary NH amide; the spectra at $2952.03\text{--}2904.67 \text{ cm}^{-1}$ was due to the CH bands; the strong band observed at 1652.88 cm^{-1} was due to the C=O amide band I; the strong absorbance band at $1545.59\text{--}1516.23 \text{ cm}^{-1}$ was very characteristic of NH amide II band; the spectrum at 1260.00 cm^{-1} showed the presence of amide III band. Pore size distribution determines the fraction of the total pore volume accessible to molecules of a given size and shape, which is a very important property of the monolith. The pore size distribution profile by mercury intrusion porosimetry for the monolith was demonstrated in Fig. 6. Based on the figure, the total intrusion volume, average pore diameter and the porosity were 1.47 mL/g, 0.46 μm and 66.85%, respectively.

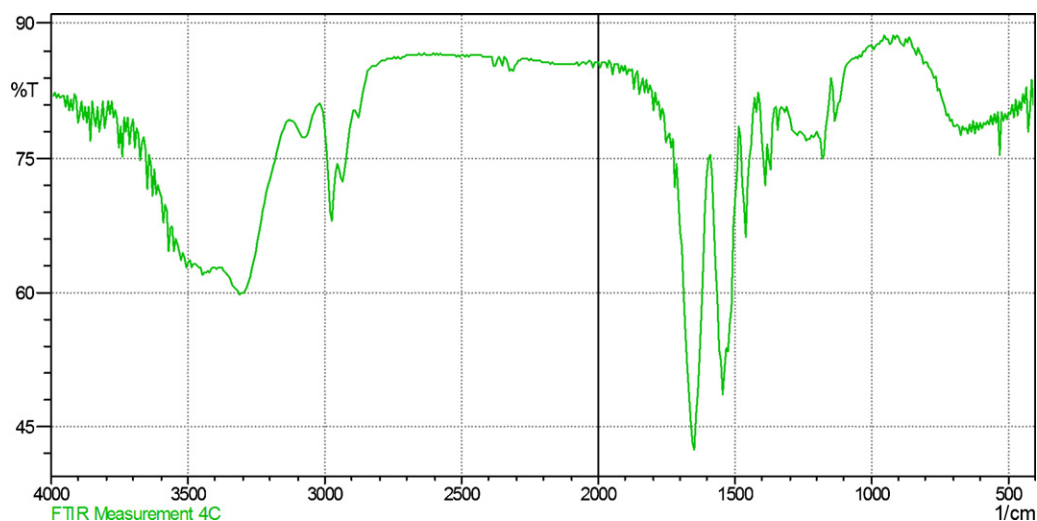


Fig. 5. The FT-IR spectrum of the poly(NIPAAm-co-BIS) monolith.

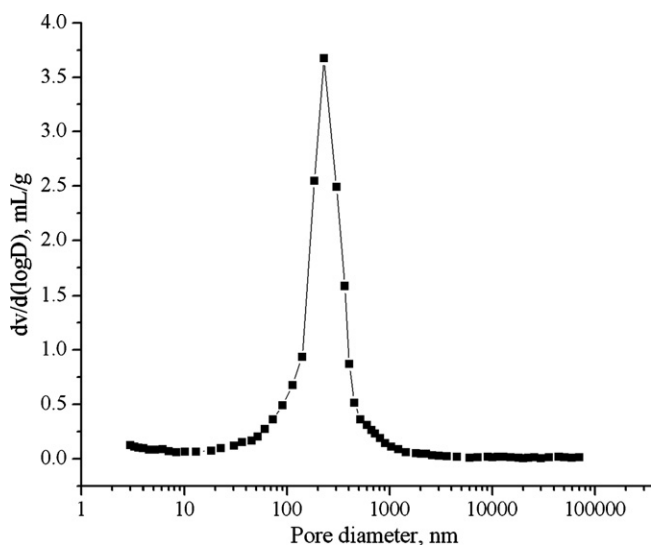


Fig. 6. Pore size distribution profile measured by mercury intrusion porosimetry.

3.5. Temperature-responsive chromatographic separation of aromatic ketones

The temperature-responsive property of poly(NIPAAm-co-BIS) monoliths was investigated using HPLC with water as mobile phase at different temperatures under isocratic conditions. Three aromatic ketones 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 acetophenone ($\log P = 1.6$), propiophenone ($\log P = 2.2$), butyrophenone ($\log P = 2.5$), were used as probes to draw information about

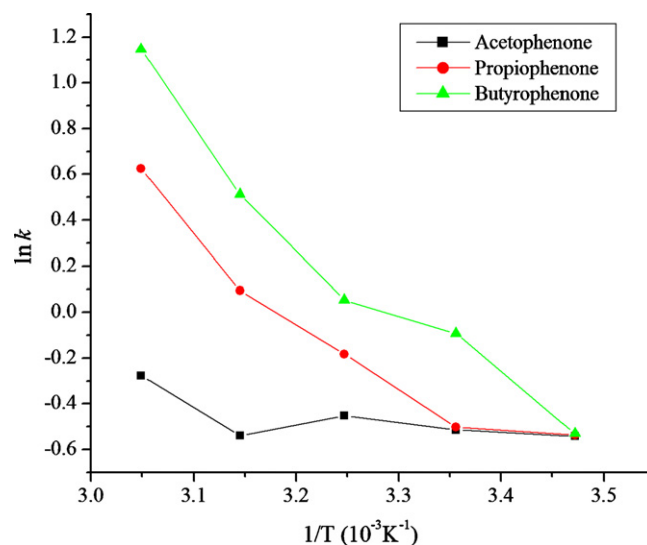


Fig. 8. Van't Hoff plots of the three aromatic ketones on the poly(NIPAAm-co-BIS) monoliths.

the temperature-responsive property of the poly(NIPAAm-co-BIS) monoliths. The chemical structures of these aromatic ketones are shown in Fig. 7.

The retention factors (k) of each aromatic ketone on the poly(NIPAAm-co-BIS) monoliths were determined at various temperatures over the range of 15–55 °C. Fig. 8 shows that the Van't Hoff plots for aromatic ketones on the monoliths. The k value in Fig. 8 is an average of six cycle measurement results derived from succedent Section 3.6 where the experimental results demonstrate a good repeatability process. Separately, benzene was employed

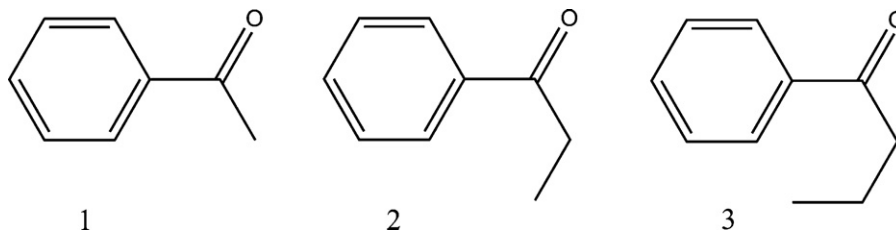


Fig. 7. The structure formula of the aromatic ketones involved in the separation process. 1: acetophenone; 2: propiophenone; 3: butyrophenone.

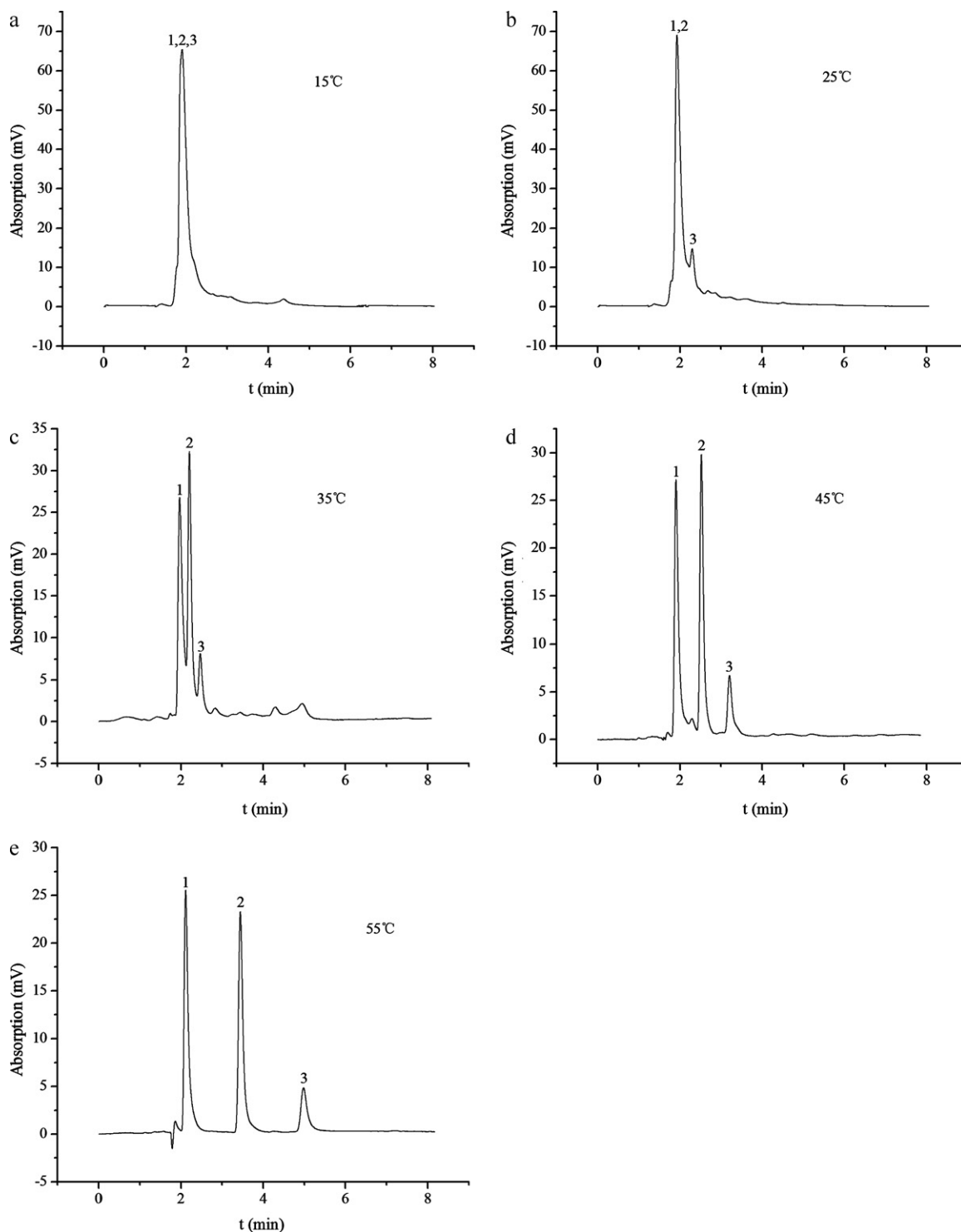


Fig. 9. Elution profiles of a mixture of aromatic ketones at different temperatures on the poly(NIPAAm-co-BIS) monoliths. 1: acetophenone; 2: propiophenone; 3: butyrophenone.

as the mobile phase velocity marker in this experiment according to the cited reference [28]. The aromatic ketones with high value of $\log P$ show higher $\ln k$ than those with low value at the same temperature. The most hydrophobic one (butyrophenone) 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 monoliths. Moreover, increasing of $\ln k$ with temperature increasing suggests that the interaction between aromatic ketones 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 change, in which the slopes of the plots should be positive value. However, Fig. 8 shows a nonlinear relationship between the reciprocal temperature ($1/T$) and $\ln k$ values for each aromatic ketone, and $\ln k$ of each aromatic ketone increases with temperature increasing. The marked discontinuities of the negative slopes

Table 2
Chromatographic parameters of separation of the aromatic ketones.

Aromatic ketone	Retention factor	Resolution	Symmetry factor	Plate number
Acetophenone	0.757	4.27	2.06	18643 ^a 1518 ^b
Propiophenone	1.866	3.22	1.68	40848 ^a 3845 ^b
Butyrophenone	3.145	0	1.71	41239 ^a 4220 ^b

Two equations have been used to calculate the plate number:

$$a: N = \frac{5.55(t_R/W_{0.5})^2}{L}$$

N is the theoretical plate number per m and t_R is the retention time of the analyte in min and $W_{0.5}$ is the peak width at half height in min and L is the length of the column [28].

$$b: N = \frac{41.7(t_R/W_{0.1})^2}{1.25+(B/A)}$$

N is the plate number of the column, t_R is the retention time of the analyte, $W_{0.1}$ is the width at 10% of the peak maximum, B is the width from the center of the peak to the tail of the peak at 10% height, and A is the width from the front of the peak to the center of the peak [32].

in the range of 25–35 °C also can be observed due to the known configurational change of PNIPAAm at its LCST (32 °C). Below the LCST, the extending PNIPAAm chains exhibit hydrophilic property and the aromatic ketones 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 aromatic ketones. Therefore, these remarkable phenomena can strongly support the poly(NIPAAm-co-BIS) monoliths to be responsive to temperature change in water.

The elution profiles at five different temperatures, below and above the LCST, are given in Fig. 9. Below 35 °C, the PNIPAAm chains are in the expanded conformation in the aqueous mobile phase because of the strong hydration. Here the surface of poly(NIPAAm-co-BIS) monoliths is hydrophilic; therefore the separation of the aromatic ketones is poor. Three aromatic ketones are eluted together in a single peak around 1.9 min at 15 °C and can be partly separated at 35 °C. At 45 °C and 55 °C, the aromatic ketones can be nearly baseline separated because the surface behavior of monolithic stationary phase changes from hydrophilic into hydrophobic.

Meanwhile, some chromatographic parameters at 55 °C such as retention factor and resolution, symmetry factor and theoretical plate number of each aromatic ketone were calculated and the result was shown in Table 2, in which the theoretical plate number for all the analytes could be up to 10⁴. It can be seen that the poly(NIPAAm-co-BIS) monoliths are excellent stationary phase for separation of aromatic ketones.

3.6. Repeatability and stability

A good monolith-to-monolith repeatability and monolith stability are important measure of the process. An excellent repeatability characterized by relative standard deviations (RSDs) for the retention times in the range of 1.4–2.9% was achieved on poly(NIPAAm-co-BIS) monoliths using the same process and conditions ($n=6$). Furthermore, the chromatograms on the poly(NIPAAm-co-BIS) monoliths were obtained after numerous equilibrations and separation runs involving the aromatic ketones mixture. The results demonstrated that the preparation process had a good repeatability and the monoliths were stable.

4. Conclusions

A temperature-responsive monolithic were prepared by free-radical redox cryo-polymerization (–12 °C) in aqueous solution. The resulting porous monolithic composite has achieved the separation of three aromatic ketones utilizing the temperature-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 columns exhibited temperature-controlled hydrophilic–hydrophobic changes. This proposed temperature-responsive chromatography system, without the use of an organic solvent as a mobile phase, is advantageous from the point of view of environmental reasons and economical cost of the mobile phase.

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