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Preparation of a novel porous poly (trimethylol propane triacrylate-co-ethylene dimethacrylate) monolithic column for highly efficient HPLC separations of small molecules

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ABSTRACT

A novel poly (trimethylol propane triacrylate-co-ethylene dimethacrylate) [poly (TMPTA-co-EDMA)] monolith was prepared by in situ free-radical polymerization in a 50 mm × 4.6 mm i.d. stainless steel column and was investigated for high performance liquid chromatography (HPLC). The porous structure of monolith was optimized by changing the conditions of polymerization. The chemical group of the monolithic column was confirmed by a Fourier transform infrared spectroscopy (FT-IR) method and the morphology of column structure was characterized by scanning electron microscopy (SEM). The mechanical strength and permeability were also studied. Finally, a series of low-molecular-weight organic compounds were utilized to evaluate the retention behaviors of the monolithic column. The result demonstrated that the prepared column exhibited an RP-chromatographic behavior and good separation performance. The method reproducibility was obtained by evaluating the run-to-run and column-to-column with relative standard deviations (RSDs) less than 0.7% ($n=6$) and 2.9% ($n=6$), respectively, which indicated that prepared monolithic columns had good reproducibility and stability.

1. Introduction

In recent years, the monolith as separation media for high performance liquid chromatography (HPLC) has undergone a rapid development in the field of sample analysis because of its excellent performance [1,2], such as cost-effective approach, fast mass transport, excellent permeability and versatile surface modification compared to conventional columns packed with particles [3–5]. Monolithic column are divided into three groups: organic polymer-based, silica-based and organic-silica hybrid monolithic columns. Silica-based monoliths can be applied for high-throughput analysis and rapid separation, but the preparation process is complicated. The silica-based hybrid monoliths are famous for their better separation efficiency, but the drawback is that the Si–O–C linkage cannot be hydrolyzed fully. In addition, the synthetic process is difficult to control and the preparation is time-consuming [6]. Organic polymer-based monolithic column including polyacrylates, polymethacrylates [7], polyacrylamids [8] and polystyrenes [9] show excellent biocompatibility, good stability of pH changes and easy surface modification. Lots of applications have been already put into effect in recent years [10–12], although organic polymer-based monolithic columns still have some disadvantages which need to be improved.

Trimethylol propane triacrylate (TMPTA) belongs to polyolacrylate that can be used as important multifunctional monomers. TMPTA could be candidate for polymer network, since it has three vinyl bonds at the end to be formed a dense network structure [13–17]. So far, in the field of monolithic stationary phase for HPLC, no significant attempts have been made with TMPTA.

In this work, a novel HPLC monolithic column was synthesized via in situ free-radical polymerization using TMPTA and ethylene dimethacrylate (EDMA) as monomer and cross linker, respectively. The influence factors on the preparation of the monoliths have been studied. Furthermore, the newly monolith was used to separate a series of small molecules.

2. Experimental

2.1. Materials

Trimethylol propane triacrylate (TMPTA) was supplied by Tianjin Tianjiao Chemical Co., Ltd. (Tianjin, China). Ethylene dimethacrylate (EDMA) was purchased from Acros (New Jersey, USA). 2,2'-azobisisobutyronitrile (AIBN) was produced by Shanghai Chemical Plant (Shanghai, China) and refined before use. Poly (ethylene glycol) (PEG, Mn=200) and methanol were obtained from Tianjin Kemiou Com (Tianjin, China). The aromatic compounds were provided by the National Institute for the Control of Pharmaceutical and Biological

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Products of China (Beijing, China). All other chemicals were of analytical grade or better. Triplex distilled water was used for all experiments. All media were filtered through a 0.45 μm membrane before use.

2.2. Instruments

All chromatographic experiments were performed on a 1100 system from Agilent Technologies (Shanghai, China). Agilent liquid chromatography system software was used and operated under Windows XP for data acquisition. The FT-IR spectra were recorded on an FTIR-8400S IR apparatus in the region of 400–4000 cm^{-1} (Shimadzu, Kyoto, Japan). Scanning electron microscopy (SEM) of the monolithic columns was carried out on a Hitachi S-4300 SEM instrument (Hitachi High Technologies, Tokyo, Japan).

2.3. Preparation of the poly (TMPTA-co-EDMA) monolithic column

The polymerization mixture for the monolithic columns was prepared as follows: 0.3 mL TMPTA, 0.3 mL EDMA, and 0.005 g AIBN were dissolved in a mixture of 0.5 g PEG and 1.4 mL methanol, which had been injected into a dry ampule. The mixture solution was sonicated for 30 min after being shook for 2 min and then bubbled with nitrogen for another 5 min to reinforce dissolve and remove gases. Then, pour the reaction solutions into a 50 mm × 4.6 mm i.d. stainless steel column that was sealed at both ends. The stainless steel was heated up to 60 °C in a water bath for 24 h. After that, the seals were removed and provided with end fittings. In order to remove all of unreacted monomers and soluble compounds, the monolith was washed by methanol online for 1 h at a flow rate of 1 mL/min. The scheme of polymerization was shown in Fig. 1.

2.4. Characterization method

A Fourier transform infrared spectroscopy (FT-IR) method was used to confirm the chemical group of the monolith. Before the measurement, a piece of monolith was grinded into powder, and then put it in a plate for drying 48 h in vacuum at 70 °C. 1 mg of the dried sample and 200 mg of KBr powder were weighed. The mixture was grounded in an agate mortar to pestle uniformly. After that, it was pressed to form a pellet, loaded it on the specimen holder, and then a transmission spectrum with a sharp peak was obtained. Morphology of the monolithic materials was studied by scanning electron microscopy (SEM). Prior to the SEM, the monolithic column should be rinsed in HPLC with methanol until a stable baseline was observed to ensure any soluble compounds were removed. Subsequently, the monolith was cut into small pieces after pushing out from stainless steel column and then dried in vacuum at 50 °C for 24 h. Then, using a small fragment of monolith sputtered with gold to carry out SEM.

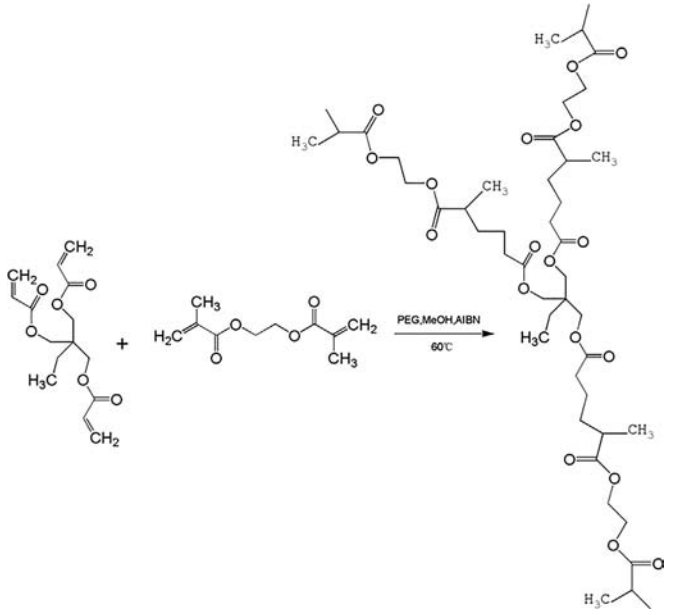

Fig. 1. Synthesis scheme of the poly (TMPTA-co-EDMA) monolithic column.

2.5. Preparation of solutions

All the solutions, including *p*-xylene, 1H-benzotriazole, phenol, α-naphthol, biphenyl, phenanthrene, and 1, 2-pheneylenediamine, 1-naphthylamine, *p*-methoxy azobenzene were dissolved in the methanol (0.1 mg/mL), which were sealed and stored at 4 °C before separated for HPLC.

2.6. HPLC conditions

The HPLC system equipped with a quaternary pump, a UV detector and an autosampler with variable injection capacity from 0.1 to 100 μL. A monolithic column was prepared with a total length of 50 mm × 4.6 mm i.d. stainless steel column. The mobile phase was the mixture of water and methanol, the UV wavelength was set at 254 nm. The room temperature was 25 °C. The sample injection volume of the autosampler was 1.0 μL.

2.7. Calculation

The ability of liquid passing the material is expressed by permeability, which reflects through-pore size and external porosity. The permeability (K) of monolithic columns was calculated by the following equation:

$$K = \frac{F \times \eta \times L}{\Delta P \times \pi \times r^2} \quad (1)$$

where F is volume flow rate of the mobile phase, η is phase dynamic viscosity of the mobile phase, L is the column length, ΔP is the column back pressure and r is the inner radius of the column [18]. In this work, methanol was used as mobile phase and its corresponding value of dynamic viscosity was 0.580×10^{-3} kg/(ms) at 25 °C [19].

The retention factor (k) of each aromatic compound on poly (TMPTA-co-EDMA) monoliths at different mobile phase for HPLC separation was determined by the equation, $k=(t_R-t_0)/t_0$, where k, t_0, t_R, stand for the retention factor, the retention time of aromatic compounds, and the retention time of void marker, respectively. The thiourea was selected as the void time marker in this experiment.

Theoretical plate number (N), one of the parameters of the chromatographic column efficiency, is a quantitative representation,

Table 1
Compositions of the mixtures used for preparation of monolithic columns and their permeability.

Column	EDMA (mL)	TMPTA (mL)	MeOH (mL)	PEG (g)	AIBN (g)	Back pressure[a] (bar)	Permeability K ($\times 10^{-14}$ m^2)
A	0.3	0.3	1.4	0.5	0.005	6	1.3388
B	0.3	0.3	1.0	0.9	0.005	7	1.1469
C	0.2	0.3	1.4	0.5	0.005	> 11	No date
D	0.4	0.3	1.4	0.5	0.005	5	1.6065
E	0.2	0.4	1.4	0.5	0.005	> 16	No date
F	0.4	0.2	1.4	0.5	0.005	4	2.0081

[a] Back pressure is obtained with methanol as the mobile phase at 1 mL/min, and the length of the stainless steel column was kept at 5 cm.

which indicates the separation efficiency. An equation has been used to calculate the plate number

$$N = \frac{5.55(Tr/W_{0.5})^2}{L} \quad (2)$$

where N is the theoretical plate number per m, Tr is the retention time of the analyte in min, $W_{0.5}$ is the peak width at half height in min and L is the length of the column [20].

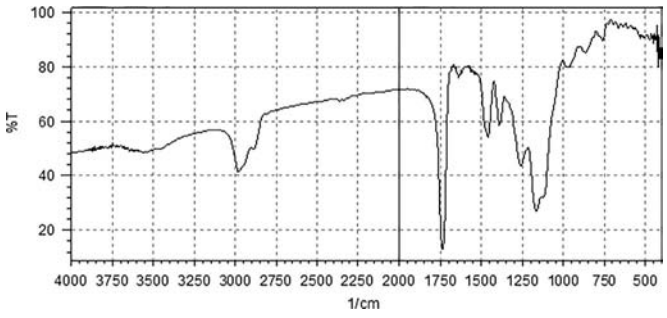

Fig. 2. The FT-IR spectrum of the poly (TMPTA-co-EDMA) monolithic column.

3. Results and discussions

3.1. Characterizations of poly (TMPTA-co-EDMA) monolith

3.1.1. IR study of the monolith

The FT-IR spectrum of the monolith was shown in Fig. 2, the apparent peaks at 2960 cm^{-1} and 2853 cm^{-1} were identified asymmetric stretching vibration and symmetric stretching vibration of the C–H bond, respectively. The existence of –COO– bond in the structure of TMPTA and EDMA was proved by a characteristic peak position: at 1749 cm^{-1} and multiple absorption peaks at 1300–1100 cm^{-1}. The process of the enlargement of core and polymerization was realized by free radical initiation using AIBN as initiator, from the FT-IR spectrum reviewed, there was absence of

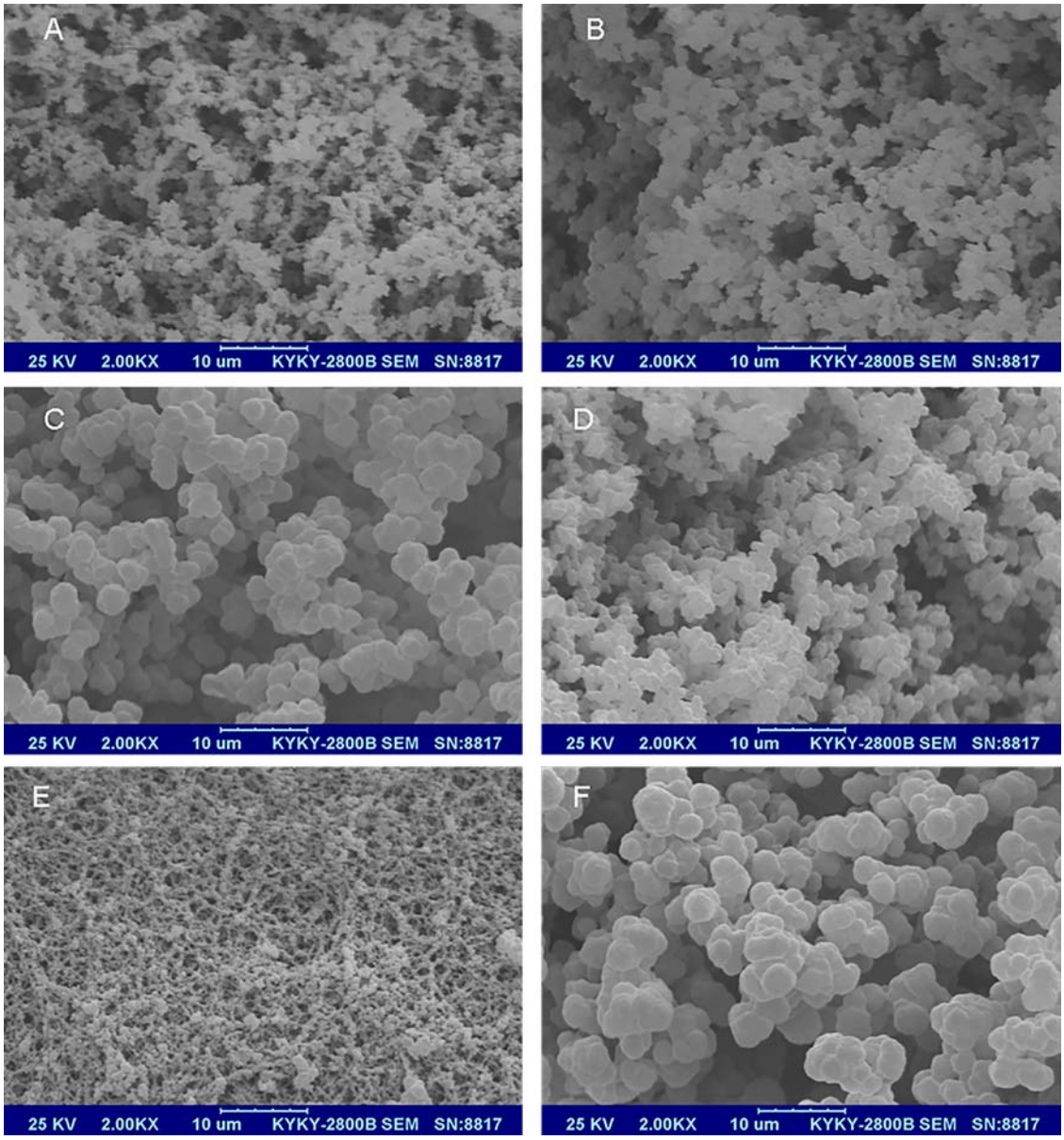

Fig. 3. Scanning electron microscopy of samples.

peak at 1638 cm^{-1} of C=C, which confirmed the polymerization conducted perfectly.

3.1.2. Morphology and chromatographic behaviors of the monolith

Because the composition of the reaction mixture has a great influence on the structure of the monolith, several parameters have been optimized in this experiment including the compositions of monomers, crosslinker, and porogen (Table 1). The corresponding micrographs studied by SEM and the results are shown in Fig. 3. From the results summarized in Table 1, columns C and E were collapsed easily so that they could not afford pressure tests. From Fig. 3, it was easily found that decreasing the EDMA concentration led to monoliths with larger size globules crowded together (Fig. 3C). When increasing the amount of TMPTA on this basis, the skeleton structure was too dense (Fig. 3E). Compared to the column A (Fig. 3A), column B (Fig. 3B) showed the pore structure much denser, column D (Fig. 3D) and column F (Fig. 3F) were relatively looser. These results were in agreement with the permeability (K) as obtained in Table 1. The K value of the poly (TMPTA-co-EDMA) monolith was calculated by Eq. (1). The data illustrated that column A has good permeability, which maybe own this to forming the large amount of pores in monoliths. Pore size distribution of column A was tested by mercury intrusion porosimetry. The average pore diameter was 0.89 μm and the porosity was 68.95%. Furthermore, the chromatographic behaviors of three investigated monoliths were examined by HPLC analysis. Fig. 4 presented the separation of 1H-benzotriazole, p-xylene and biphenyl with columns A, D and F. It could be seen that peaks on the columns D and F were much serious tailing and band broadening. The results implied that changing the amount of EDMA or TMPTA affected both the formation of monolithic skeleton and its chromatographic behaviors. Through various optimization, column A was adopted for further experiments.

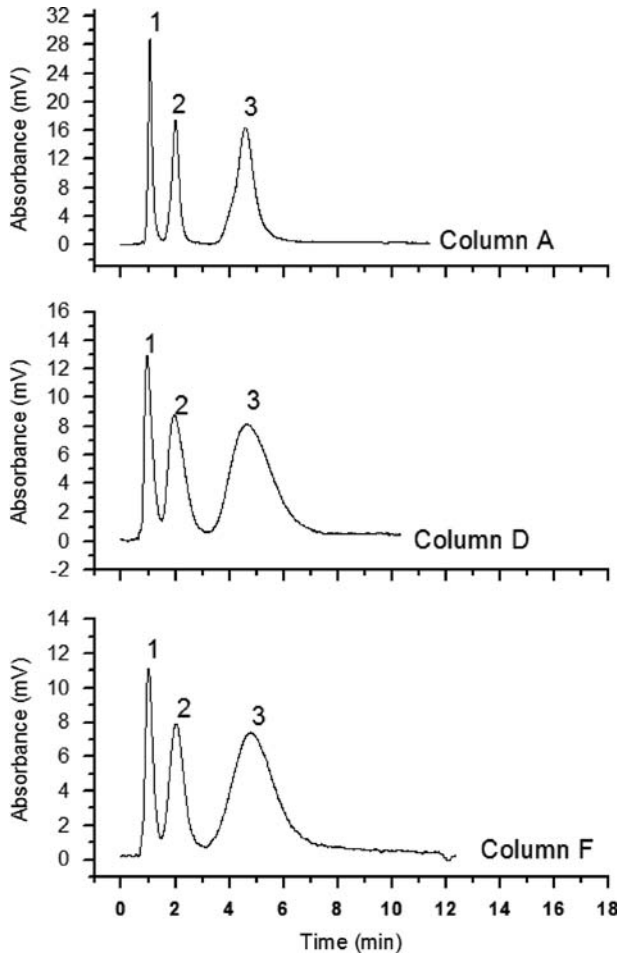

Fig. 4. Chromatographic behaviors of aromatic compounds on the monoliths with the different amounts of TMPTA and EDMA. Conditions: methanol/water: 75/25% (v/v); flow rate: 1 mL/min; detection wavelength: 254 nm; the analytes are (1) 1H-Benzotriazole; (2) p-xylene and (3) biphenyl.

3.1.3. Mechanical strength and permeability of the monolith

It is necessary that the stationary phase of HPLC should have excellent mechanical strength and permeability. Hence, in order to characterize the mechanical performance and permeability of the monoliths, the back pressures of the monoliths at different flow rates with different mobile phases were evaluated. Fig. 5 shows a back pressure plotted against the flow rates and indicated an excellent linear. Although the flow rate was raised to 7 mL/min using water as mobile phase, the maximum pressure only reached 50 bar. In view of the results, the target monolithic column could be carried on a fast and efficient analysis at higher flow rate.

3.2. Monolithic column reproducibility and stability

The reproducibility of poly (TMPTA-co-EDMA) monolithic column was characterized by measuring the relative standard deviations (RSDs) of retention times using phenol, biphenyl, and phenanthrene as test compounds to reduce numerous deleterious processes. To study column-to-column reproducibility, six columns from different batches were utilized to analyze test compounds. The RSDs for retention time were less than 2.9%. To study run-to-run repeatability of the column, six injections (1 h interval at every time) of test compounds were analyzed by HPLC. The statistic data demonstrated that the RSDs of the retention time were lower than 0.7%. In addition, the performance life and preservation time were also significant parameters to evaluate homemade monolithic column. So, a number of equilibrations and consecutive runs were carried out for months, and consistent chromatograms were obtained. These results indicated that the prepared monolithic columns had good reproducibility and stability.

3.3. Applicatin

3.3.1. Separation of neutral and acidic compounds

To investigate the retention behavior of low-molecular-weight organic compounds on the poly (TMPTA-co-EDMA) monolithic column, two weakly acidic phenolic compounds (α-naphthol and

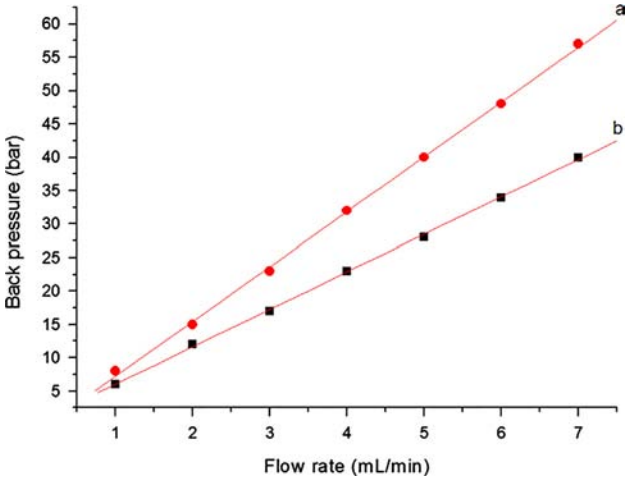

Fig. 5. Effect of mobile phase flow rate on the pressure of poly (TMPTA-co-EDMA) monolithic column. Mobile phase: (a) water and (b) methanol.

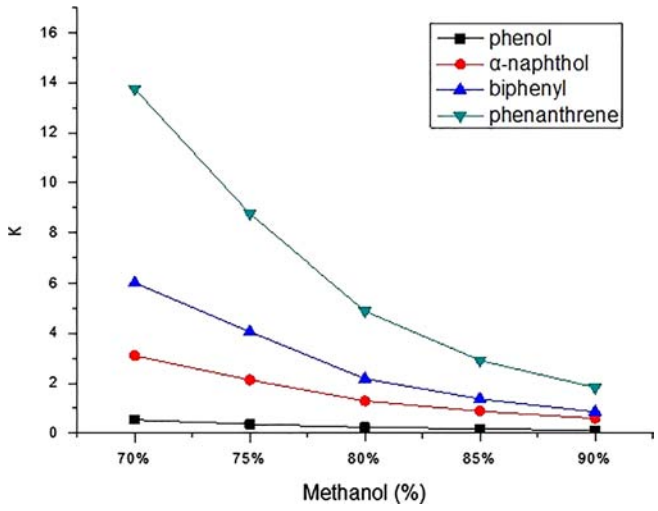

Fig. 6. Relationship between retention factor and methanol concentration of neutral and acidic compounds on the poly (TMPTA-co-EDMA) monoliths (column C).

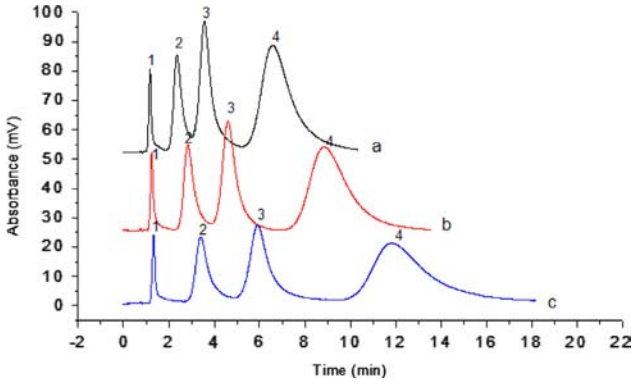

Fig. 7. Effect of methanol proportion in mobile phase on the separation of neutral and acidic compounds. Monolithic steel column, 4.6 mm i.d. × 50 mm; flow rate, 1 mL/min; injection volume, 1 μL; UV detection wavelength, 254 nm; mobile phase, methanol/water(v/v), (a) 80% v/v methanol, (b) 75% v/v methanol and (c) 70% v/v methanol; Peak identification: (1) phenol, (2) α-naphthol, (3) biphenyl, and (4) phenanthrene.

phenol) and two neutral compounds (biphenyl and phenanthrene) were used. Fig. 6 showed that the retention time of four compounds increased with the decrease of methanol content in the mobile phase from 90% to 70% (v/v), validating a typical reversed-phase HPLC retention mechanism existed in the poly(TMPTA-co-EDMA) monoliths. From Fig. 7, it was observed that phenol, α-naphthol, biphenyl and phenanthrene were eluted in order. The elution order was in accordance with their hydrophobicity (octanol–water coefficient, log P) (Table 2), confirming again a reversed-phase retention behavior. When the content of methanol was 80% (v/v), α-naphthol and biphenyl obviously could not achieve baseline separation. When the content of methanol decreased to 70% (v/v), the separation time was consumed and chromatographic peaks exhibited much serious tailing and band broadening. By comparison, when the concentration of methanol was 75% (v/v), much better peak shape could be obtained. Then on the basis of optimal concentration of methanol, the flow rate also has been optimized. The result was shown in Fig. 8. By increasing the flow rate from 1 mL/min to 1.2 mL/min, while only a modest growth, the analysis time was reduced from 13 min to less than 10 min. Meanwhile, a baseline and much higher theoretical plate number were obtained. Under this condition, the efficiencies of the four compounds orderly were 9560, 4000, 5160, and 3060 plates/m. The results showed that the poly (TMPTA-co-EDMA) monoliths could be used as RP column for separation of neutral and acidic compounds.

Table 2
The octanol–water partition coefficients (log P) of analyte.[a]

Compound	log P	Compound	log P	Compound	log P
p-Xylene	3.25	α-Naphthol	2.71	1, 2-Pheneylenediamine	0.15
1H-Benzotriazole	1.44	Phenol	1.46	p-Methoxyazobenzene	3.64
Biphenyl	4.01	Phenanthrene	4.46	1-Naphthylamine	2.25

[a] The data were obtained from RSC ChemSpider (http://www.chemspider.com/).

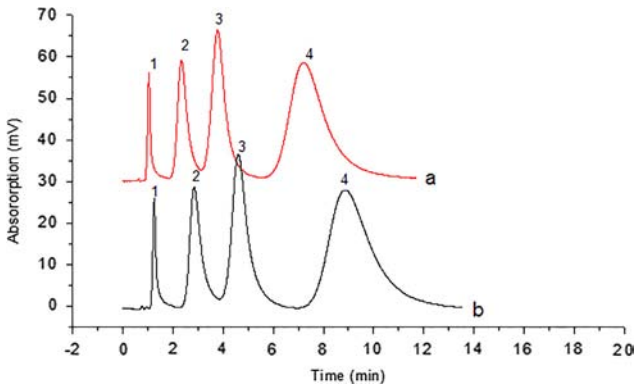

Fig. 8. The effects of flow rate on the separation of neutral and acidic compounds. Mobile phase: 75% v/v methanol; flow rate, (a) 1.2 mL/min and (b) 1.0 mL/min; other conditions were same as in Fig. 7. Peak identification: (1) phenol, (2) α-naphthol, (3) biphenyl, and (4) phenanthrene.

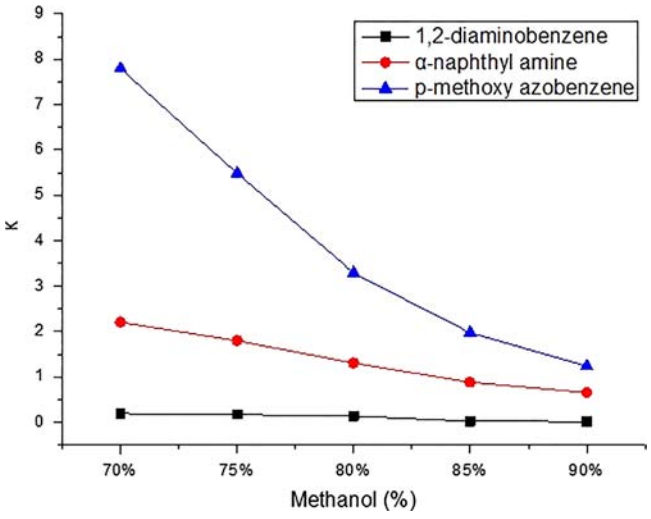

Fig. 9. Relationship between retention factor and methanol concentration of basic compounds on the poly (TMPTA-co-EDMA) monoliths (column C).

3.3.2. *Separation of basic compounds*

In order to further verify whether the target column still has a much better chromatographic separation ability of alkaline substances, three basic compounds (1, 2-pheneylenediamine, 1-naphthylamine and p-methoxyazobenzene) were selected as test compounds. Owing to the irreversible adsorption, previously reported literature attempted to use the mobile phase with buffer solution to alleviate peak broadening and peak tailing [21]. In this experiment, methanol/water was used as the mobile phase

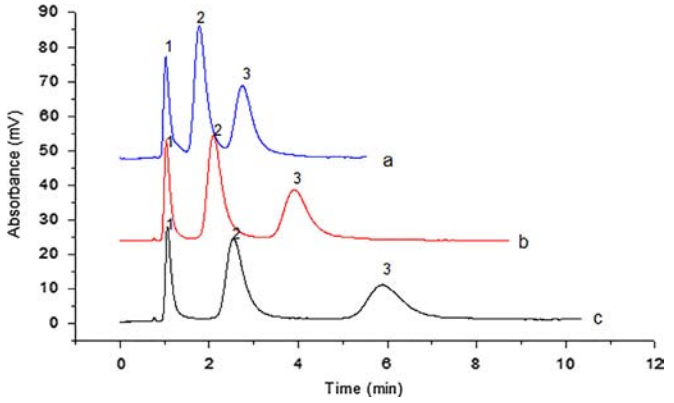

Fig. 10. Effect of methanol proportion in mobile phase on the separation of basic compounds. flow rate, 1 mL/min; mobile phase, methanol/water (v/v), (a) 85% v/v methanol; (b) 80% v/v methanol; (c) 75% v/v methanol; other conditions were same as in Fig. 7. Peak identification: (1) 1, 2-pheneylenediamine, (2) 1-naphthylamine, and (3) *p*-methoxy azobenzene.

without addition of any competing substance, which is relatively simple and inexpensive. From Fig. 9, it could be seen that the *k* values of the three test compounds decreased with increasing content of methanol. In Fig. 10, 1, 2-pheneylenediamine, 1-naphthylamine and *p*-methoxyazobenzene were eluted in order, which was in accordance with their hydrophobicity (Table 2). The peak shapes of those analytes were observed slightly trailing, possible because a little of hydrogen bonding between the carbonyl groups of the stationary phase and the amino group. But, as a whole, the three amines compounds obtained a good separation with much symmetric peaks in less than 5 min when the content of methanol was 80% (v/v).

4. Conclusions

A novel poly (TMPTA-co-EDMA) monolithic column was successfully prepared by in situ free-radical polymerization for HPLC. Character methods showed that this kind of novel monolith has much higher rigidity and mechanical stability. Moreover, the performance for the separation of neutral, acidic and basic compounds was evaluated in detail, and the obtained porous column exhibited high efficiency and fast separation of those small molecules. It appears that the stationary phase under study can provide multiple separations contributing to the analysis for further applications in HPLC.

Acknowledgments

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