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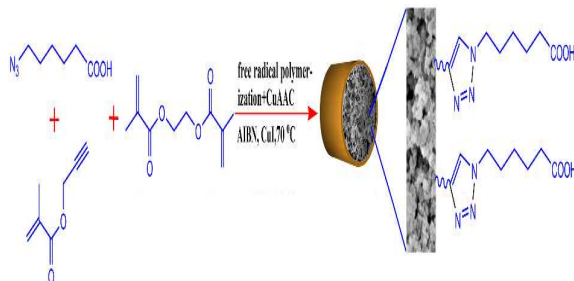
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A graphical and textual abstract for the contents pages

A facile “one-pot” method was developed for preparation of polymer monoliths by combination of free radical polymerization and CuAAC click reaction.



One-pot synthesis of polymer monolithic column by combination of free radical polymerization and azide-alkyne cycloaddition “click” reaction and its application in capillary liquid chromatography†

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Combining free radical polymerization with click chemistry via copper-mediated azide/alkyne cycloaddition (CuAAC) reaction in “one-pot” process, a facile approach was developed for the preparation of polymer monolithic column carrying 6-azidohexanoic acid (AHA), which was applied in capillary liquid chromatography (cLC) separations in the mixed-mode. The synthetic procedure was simple, time-saving and without any post modification. The morphology, permeability, and pore properties of the prepared polymer monolithic columns were characterized and the results showed the polymer monoliths have a uniform monolithic structure with high porosity. The retention behavior of the neutral and polar solutes on the polymer monoliths confirmed the successful incorporation of ligand in the monolithic matrix. A series of alkylbenzenes, phenols, and anilines were used to evaluate the chromatographic properties of the polymer monolith in terms of hydrophobic, hydrophilic and cation-exchange interactions. In addition, the poly (AHA-co-propargyl methacrylate-co-ethylene dimethacrylate) (AHA-co-PMA-co-EDMA) monolithic column could be successfully applied to the separation of polycyclic aromatic hydrocarbons (PAHs), nucleosides and nucleobases, alkaloids, peptides and proteins, respectively. The reproducibility of the monolithic column was less than 4.0% in terms of relative standard deviation (RSD) of retention factor. The results demonstrate that the proposed method was an effective alternative for preparation of polymer monolith.

1. Introduction

The growing demands for high-throughput analysis in post-genomic era, such as proteomics or metabolomics, have stimulated the development of high efficient separation techniques and tools. Polymeric monoliths, as an attractive separation medium, have gained greater acceptance as an alternative to conventional microparticle-packed columns because of the advantages of low back pressure, fast mass transfer rate, and ease of preparation.¹⁻³ Although some purely polymeric monoliths are excellent separation media, further

functionalization is of utmost importance in most situations in order to gain the desired chromatographic properties. Taking glycidylmethacrylate (GMA)-based monoliths as example, epoxy groups of the GMA-based monoliths are typically modified using standard electrophile-nucleophile displacement chemistry, or via ring-opening of a pendant reactive moiety to prepare a wide range of polymeric monoliths with a variety of separation modes.⁴⁻⁶ Compared with the single-step copolymerization, postpolymerization functionalization allows independent optimization of porous properties of the generic monolith and surface chemistry. However, the preparation procedure is rather tedious and time-consuming. Moreover, the postpolymerization functionalization sometimes has low selectivity and efficiency, which may have a significant negative impact on retention characteristics and separation mechanism.⁷ Therefore, developing a robust, reliable immobilization method with high selectivity and high yield for preparation of functionalized polymeric monoliths is highly desirable.

Click chemistry was first introduced by Sharpless in 2001.⁸ The click reactions, such as Cu(I)-catalyzed 1,3-dipolar azide-alkyne cycloaddition reaction (CuAAC) and “thiol-ene” reaction, have been widely used in polymer and dendrimer synthesis, bioconjugation and surface modification.⁹⁻¹² Thiol-ene reaction has been attracting great interest since it possesses several advantages such as simplicity, high chemoselectivity and high conversion under mild conditions. The thioether linkage formed serves as a strong and stable covalent bond, which can withstand harsh conditions. Thiol-ene reaction has been widely employed for the preparation of polymer-based monoliths and organic-inorganic hybrid monoliths.¹³⁻¹⁵ Another attractive click reaction, Cu(I)-catalyzed 1,3-dipolar azide-alkyne cycloaddition reaction (CuAAC), has been established as one of the most reliable approaches for covalent assembly of various molecules.¹⁶⁻¹⁹ This immobilization concept offers several advantages over established immobilization protocols, including mild coupling conditions, excellent coupling yields (higher yields compared to thiol-ene reaction), convenient control of the ligand loading level, and full compatibility with a broad range of functional groups.²⁰ CuAAC is a powerful tool for introduction of various functional molecules onto stationary phases to prepare different packed chromatographic columns or enzymatic reactors.²¹⁻²⁵ It is also worth to note that the adoption of CuAAC immobilization strategy for the preparation of monolithic columns is a recent achievement.²⁶⁻³⁰ For example, Guerrouache *et al.* first reported an application of CuAAC click reaction in the functionalization of macroporous organic polymer monolith.²⁶ In their work, the monolith based on nacrlyoxysuccinimide (NAS) and ethylene dimethacrylate (EDMA) was prepared firstly, then a two-step modification was carried out to graft β -cyclodextrin (β -CD) onto the monolith for chiral capillary chromatography. Similar works were reported by Fréchet’s group and Sun *et al* who applied a

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typical two-step click modification for the preparation of silica and polymer monoliths, respectively.²⁷⁻²⁹ More recently, Wu *et al.* developed a facile “one-pot” approach for fabrication of clickable organic-silica hybrid monoliths, where two clickable monomers with either azido or alkynyl moieties were adopted and the resulting hybrid monoliths were further used for the fabrication of monolith-based enzyme reactors via post-polymerization modification employing CuAAC click chemistry.³⁰ It is easy to find that the above mentioned approaches were based on two-step or multi-step post modifications, which often resulted in longer preparation time and more complicated procedures. Despite this attractive feature of CuAAC click chemistry, there are only a few studies²⁶⁻³⁰ to date on the preparation of monolithic columns using CuAAC strategy and no papers on “one-pot” preparation of polymeric monoliths by *in situ* free radical polymerization combined with CuAAC click reaction have been reported so far.

In this work, a facile “one-pot” approach based on *in situ* free radical polymerization combined with CuAAC click reaction was first developed for preparation of polymer monolithic column. When the vinyl/alkyne-organic monomers and initiator of azobisisobutyronitrile (AIBN) were mixed with azido-organic monomer in the presence of copper (I) catalyst, the resulting homogeneous mixture was introduced into a fused capillary for the subsequent click reaction and polymerization to form the polymer monolithic column. With the use of this strategy, polymer monolith with a mixed mode involving hydrophilic/hydrophobic/cation-exchange interactions was successfully prepared. Compared to the conventional multi-step post-modification method, this “one-pot” approach provides an alternative to prepare diverse polymer monoliths by using a variety of azido/alkyne-organic monomers.

2. Experimental

2.1. Materials

Propargyl methacrylate (PMA) and EDMA were purchased from Alfa Aesar (Ward Hill, MA, USA). 6-Bromohexanoic acid, copper iodide (CuI) and polycyclic aromatic hydrocarbons (PAHs) were the products of Aladdin (Shanghai, China). AIBN was obtained from Tianjin Chemistry Reagent Factory (Tianjin, China) and recrystallized with methanol (MeOH) prior to use. Alkylbenzenes, thiourea, anilines and HPLC-grade acetonitrile (ACN) were obtained from Sinopharm Chemical Reagent (Shanghai, China). Bovine serum albumin (BSA), myoglobin (Mb), ovalbumin (OVA), lysozyme (Lyz) and cytochrome C (Cyt C) were purchased from Beijing Dingguo Co. Ltd (Beijing, China). Adenine, cytosine, uracil, 6-methylaminopurine and adenosine were obtained from Sigma (St. Louis, MO, USA). Alkaloids were the products of national institute for the control of pharmaceutical and biological products (Beijing, China). Five peptides (Tyr-Gly-Gly (YGG), Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly (CYIQNCPLG), Tyr-Gly-Gly-Phe-Leu (YGGFL), Tyr-Gly-Gly-Phe-Met (YGGFM), and Phe-Gly-Phe-Gly (FGFG)) were purchased from Shanghai Apeptide Co. Ltd (Shanghai, China). All other chemicals were of analytical grade or better. Deionized water was prepared with a Milli-Q water purification system (Millipore, Milford, MA). Capillaries with 370 μm o.d. \times 100 μm i.d. were the products of Yongnian Optic Fiber Plant (Hebei, China).

2.2. Apparatus

All capillary liquid chromatography (cLC) were performed on a TriSep-2100 pressurized capillary electrochromatography (pCEC) system (Unimicro Technologies, Pleasanton, CA, USA).

A flow rate of 0.05 mL/min and the UV absorbance of 214 nm were used unless otherwise stated. Samples were injected through an injection valve with an internal 2 μL sample loop. A four-port splitter was set between the injection valve and the monolithic column to split the flow into a desirable and stable flow rate. Since the splitting ratio was set at 400:1, the actual injection volume was about 5 nanoliter (nL). The morphology of the polymer monoliths was characterized by a PhilipsXL30E Scanning Electron Microscope (SEM) (Amsterdam, Netherlands). The pore properties of the polymer monolith were measured by using physisorption analyzer (Micromeritics ASAP 2010 porosimeter, USA) at -196 $^{\circ}\text{C}$. Prior to the measurements the monolithic polymers were ground and degassed at 120 $^{\circ}\text{C}$ to a residual pressure lower than 10^{-2} Pa. Fourier transform infrared (FT-IR) spectra of the monolithic columns were recorded using the AVATAR 360 FT-IR spectrophotometer (Nicolet, Waltham, MA, USA), where 3 mg powder sample was mixed with 100 mg KBr.

2.3. “One-pot” preparation of poly (AHA-co-PMA-co-EDMA) monolithic column via CuAAC click reaction

In order to covalently anchor the polymer to the capillary wall, the capillary was pretreated with a vinyl silanizing agent according to a method reported previously.³¹

6-Azidohexanoic acid (AHA) was synthesized by using 6-bromohexanoic acid as raw material (Fig.S1, Supporting Information). For the preparation of the poly (AHA-co-PMA-co-EDMA) monolithic column, the prepolymerization mixture was prepared by dissolving various amounts of AIBN (1.0%, w/w, of monomer and crosslinker), AHA (27–45 μL), PMA (25–35 μL), EDMA (19–43 μL), and catalyst CuI (0.2 mg) in a binary porogenic solvent, which consisted of DMSO (182–218 μL) and 1-dodecanol (195–244 μL) (Table 1). The mixture was sonicated for 20 min to obtain a homogeneous solution, and then purged with nitrogen for 10 min. Subsequently, the mixture solution was filled into the pretreated capillary (50 cm) to a total length of 25 cm. The capillary was sealed at both ends with rubber stoppers and submerged into water bath at 70 $^{\circ}\text{C}$ for 24 h. After polymerization was complete, the monolithic column was connected to HPLC pump and orderly washed with methanol, 20 mM EDTA solution and water to remove the porogenic solvents, unreacted reagents and catalyst. As a control, the poly (PMA-co-EDMA) monolithic column was also prepared in the absence of AHA and CuI according to the same preparation procedure as described above.

2.4. Calculations

Column permeability (K) reflects through-pore size and external porosity, or a domain size at a constant through-pore size/skeleton size ratio. The permeability of the column was calculated using Darcy’s equation:

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

Where F , η , L , ΔP , and r stand for volume flow rate of the mobile phase, dynamic viscosity of the mobile phase, the column length, the column backpressure, and the inner radius of the column, respectively.³² In this work, MeOH was used as mobile phase and its corresponding value of dynamic viscosity was 0.580×10^{-3} kg/(m \times s) at 25 $^{\circ}\text{C}$.³³

The retention factor (k) for the analytes was obtained according to the equation, $k = (t_R - t_0) / t_0$, where t_R is the retention time of the analytes, and t_0 is the retention time of void marker, respectively.

2.5. cLC procedures

The monolithic column was placed in the instrument and equilibrated with the mobile phase until a stable baseline was obtained. Isocratic elution of a series of small molecules was performed to evaluate the retention behaviors of the polymer monolithic column in terms of hydrophobic, hydrophilic, and ion-exchange interactions. Different ratio of ACN/H₂O with or without different pH and concentration of phosphate buffer (PB) were used unless otherwise stated (PB with desired pH was adjusted with phosphoric acid or NaOH). Proteins were separated in a linear gradient elution mode.

3. Results and discussion

3.1. Preparation and characterization of poly (AHA-co-PMA-co-EDMA) monolithic column

The general scheme for “one-pot” preparation of the poly (AHA-co-PMA-co-EDMA) monolithic column based on *in situ* free radical polymerization combined with CuAAC click reaction was illustrated in Fig.1, which involved two aspects: on the one hand, the polymerization of PMA and EDMA to the formation of the poly (PMA-co-EDMA) monolithic skeleton; on the other hand, the CuAAC click reaction between AHA and alkyne-containing monolithic skeleton to the formation of the poly (AHA-co-PMA-co-EDMA) monolith. Since the composition of the reaction mixture has a great influence on the permeability, mechanical and chemical stability of the polymer monolithic column, variation of parameters such as the mass ratio of monomer/crosslinker, the amount of AHA, the choice of porogens, and polymerization temperature were further optimized as shown in Table 1.

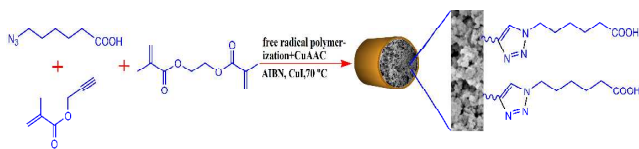


Fig.1. Scheme for synthesis of the poly (AHA-co-PMA-co-EDMA) monolithic column based on *in situ* free radical polymerization combined with CuAAC click reaction.

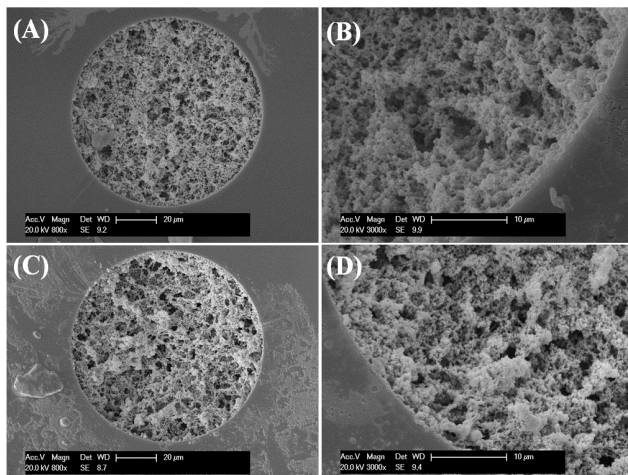


Fig.2. SEM images of poly (AHA-co-PMA-co-EDMA) monolith: (A) $\times 800$ and (B) $\times 3000$. Poly (PMA-co-EDMA) monolith: (C) $\times 800$ and (D) $\times 3000$.

Like the synthesis of other types of polymer monoliths, reaction temperature is a critical factor for the synthesis of the poly (AHA-co-PMA-co-EDMA) monolithic column. Although CuAAC click reaction can be run at room temperature, it should take several days.²⁵ Meanwhile, free radical polymerization triggered by AIBN is intensively performed at 60–75 °C. As a

result, different reaction temperatures (60 °C, 65 °C and 70 °C) were investigated and the result showed that when the reaction temperature was below 70 °C, the monolith skeleton was much slacked.

The type of porogenic solvent plays a vital role in the preparation of polymer monolith. Here a binary porogenic solvent was preferred considering the hydrophobicity of PMA and EDMA, as well as the solubility of AHA and CuI. Several porogenic solvents, such as 1-dodecanol/cyclohexanol, diethylene glycol/1,4-butanediol, DMF/ethyleneglycol, and DMSO/1-dodecanol, have been studied in detail. Of these, it was determined that a binary porogen of DMSO/1-dodecanol is best suited for the preparation of the porous poly (AHA-co-PMA-co-EDMA) monoliths. In this work, the ratio of monomers to porogens was fixed at 20:80 (wt %) (Table 1). It was observed that with an increase in weight percentage of 1-dodecanol to total porogens from 32% to 40% (Column E, B and F in Table 1), the back pressure of the polymer monoliths dramatically decreased from 23.2 MPa to 4.4 MPa with the permeability increasing from $6.63 \times 10^{-14} \text{ m}^2$ to $34.9 \times 10^{-14} \text{ m}^2$ at a constant flow rate of 5 $\mu\text{L}/\text{min}$, suggesting that 1-dodecanol acts as a macroporogenic solvent to provide good bulk flow properties.³⁴ Accordingly, DMSO decreased from 48% to 40% not only dissolved AHA and CuI well, but also favored the formation of micropores.³⁵ Therefore, DMSO/1-dodecanol with the mass ratio of 44/36 (wt %) was chosen as the optimized porogens for further studies.

The ratio of monomer to crosslinker affects not only the rigidity and permeability, but also the chromatographic selectivity and column efficiency. Since the CuAAC click reaction proceeded with chemoselectivity and equimolarity, thus the molar ratio of AHA to PMA was kept equimolar or excessive. To investigate the influence of the mass ratio of total monomer (AHA and PMA) to crosslinker (EDMA), four monoliths (Column A~D, Table 1) were prepared by varying total monomer weight percentages from 11% to 16%, in which the weight percentage of EDMA accordingly changed from 9% to 4%. Using different solvents (MeOH and water), a linear dependence of flow rate on column back pressure was observed in a series of columns (data not shown), indicating these monoliths have good mechanical stability. Furthermore, an increase in monomers or decrease in crosslinker, back pressure of the monoliths gradually increased and the corresponding permeabilities decreased as listed in Table 1. It was suggested that high ratio of monomer to crosslinker favored the formation and aggregation of smaller microglobules inside the capillary during copolymerization process, and thus resulted in high back pressure. On the other hand, although high mass ratio of total monomer to crosslinker would facilitate chromatographic selectivity, total monomer/crosslinker with the mass ratio of 14:6 (Column B, Table 1) was selected as a favorable compromise with respect to selectivity and permeability.

Fig.2(A-D) showed the SEM images of the poly (PMA-co-EDMA) monoliths incorporated with and without AHA via azide-alkyne click reaction. Overall, these monoliths showed a uniform monolithic structure with large through-pores and good attachment to the inner wall of the capillary. Compared to the poly (PMA-co-EDMA) monolith, however, a full dense and homogeneous monolithic skeleton with smaller microglobules was observed in the poly (AHA-co-PMA-co-EDMA) monolith (Fig.2(A-B)), suggesting the successful immobilization of AHA via click reaction in “one-pot” process. In addition, the pore structure of the polymer monoliths was also characterized by nitrogen adsorption-desorption measurement in the P/P_0 between 0.05 and 0.99. The specific surface area and average pore

diameter of the poly (AHA-co-PMA-co-EDMA) monolith were calculated to be 53.4 m²/g and 4.0 nm, much better than those of the poly (PMA-co-EDMA) monolith, where the corresponding

values were 13.4 m²/g and 9.3 nm, respectively. Besides, the total pore volume of the poly (AHA-co-PMA-co-EDMA) monolith was found to be 0.055 cm³/g, much higher than that of the poly

Table 1. Effects of synthesis parameters on the formation of poly(AHA-co-PMA-co-EDMA) monolith*

Column**	Monomers			Porogens		Crosslinker	Backpressure	Permeability
	AHA (wt %)	PMA (wt %)	Molar ratio of AHA/PMA	1-Dodecanol (wt %)	DMSO (wt %)	EDMA (wt %)	(MPa)	(× 10 ⁻¹⁴ m ²)
A ^a	6%	5%	1	36%	44%	9%	15.9	9.67
B ^{a,b,c,d}	8%	6%	1	36%	44%	6%	18.6	8.27
C ^a	9%	7%	1	36%	44%	4%	21.0	7.32
D ^a	10%	5%	1.5	36%	44%	5%	37.5	4.10
E ^b	8%	6%	1	32%	48%	6%	23.2	6.63
F ^b	8%	6%	1	40%	40%	6%	4.4	34.9
G ^c	0%	6%	--	36%	44%	6%	12	12.8

* Flushed with MeOH; Flow rate, 5 μL/min; ** (a) the effect of the ratio of monomer/crosslinker; (b) the effect of porogens; (c) the control monolith

(PMA-co-EDMA) monolith (0.03 cm³/g). Taking together, these results showed that the prepared poly (AHA-co-PMA-co-EDMA) monolith possessed higher specific surface area, smaller pore diameter, and larger pore volume, which made it possible to obtain satisfactory resolution and column efficiency in a relatively short analysis time.

FT-IR spectra provide a direct proof of “one-pot” synthesis of the poly (AHA-co-PMA-co-EDMA) monolith (Fig.S2, Supporting Information). The strong peak at 2090 cm⁻¹ was assigned to the N=N=N vibration of AHA (spectrum a). Characteristic bands of alkyne C-H and C≡C stretches at 3296 cm⁻¹ and 2126 cm⁻¹ in spectrum b confirmed the successful polymerization of PMA and EDMA. Compared to spectrum a and b, the peaks of 2090 cm⁻¹, 3296 cm⁻¹ and 2126 cm⁻¹ almost disappeared in spectrum c, confirming that AHA has been successfully immobilized on the surface of the poly (PMA-co-EDMA) monolithic skeleton via click reaction. In addition, ¹³C nuclear magnetic resonance (NMR) result (Fig.S3) also supported our hypothesis that the free radical polymerization and CuAAC click reaction occurred simultaneously.

3.2. Chromatographic evaluation of the poly (AHA-co-PMA-co-EDMA) monolithic column

To understand the retention behavior of the poly (AHA-co-PMA-co-EDMA) monolithic column in the mobile phase of aqueous/ACN, four test solutes including toluene, N,N-dimethylformamide (DMF), formamide and thiourea were used. The solvent (MeOH) was selected as the void time marker in this experiment. The effect of ACN content in mobile phase on retention factor (k) of the four test solutes was shown in Fig.3(a). It was clearly observed that the k value of toluene gradually decreased with the increasing ACN content from 30 % to 80%, and closed to zero as the content of ACN was over 80%. Furthermore, the hydrophobic toluene was eluted last with the ACN content in the range of 30–55%. The results indicated a typical reversed-phase retention mechanism existed in the poly (AHA-co-PMA-co-EDMA) monolith. On the contrary, the k values of hydrophilic test solutes involving DMF, formamide and thiourea increased slightly as the ACN content in mobile phase increased from 30% to 80% and then increased gradually when the ACN content further increased to 100%. At the mobile phase of 100% ACN, the four solutes were eluted out in order of toluene < DMF < formamide < thiourea according to the polarity of these compounds from low to high, suggesting a typical hydrophilic interaction liquid chromatography (HILIC) retention mechanism. Fig.3(b) showed the separation of the four solutes with the poly (AHA-co-PMA-co-EDMA) and poly (PMA-co-

EDMA) monoliths, respectively. It was observed that baseline separation of toluene, DMF, formamide and thiourea can be achieved with the poly (AHA-co-PMA-co-EDMA) monolith as the mobile phase of 100% ACN was adopted. However, the k values of the four solutes on the poly (PMA-co-EDMA) monolith were identical and an overlapping chromatographic peak was observed under the same mobile phase. The results served as another support of the successful bonding of AHA in the poly (PMA-co-EDMA) through the click reaction that changed the surface of the poly (PMA-co-EDMA) monolith from entire hydrophobicity to hydrophilicity.

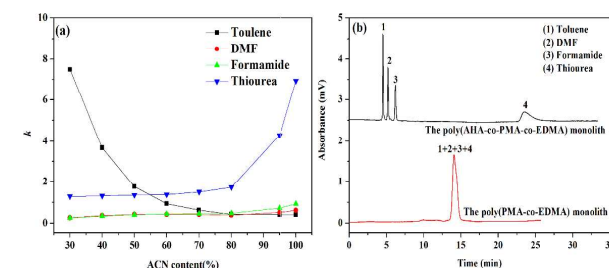


Fig.3. (a) Relationship between k and ACN concentration on the poly (AHA-co-PMA-co-EDMA) monolithic column. (b) Separation of four solutes with the poly (AHA-co-PMA-co-EDMA) and poly (PMA-co-EDMA) monolith.

Conditions: (a): ACN/water; (b): ACN/water: 100/0 (v/v %); Flow rate(actual flow rate after spilling): 0.05 mL/min (125 nL/min); Pump pressure: 3.0 MPa; Detection wavelength: 214 nm; the analytes are (1) toluene (100 ppm); (2) DMF (100 ppm); (3) formamide (100 ppm); (4) thiourea (100 ppm).

The column efficiency of the poly (AHA-co-PMA-co-EDMA) monolith was evaluated by changing the flow rate of the mobile phase in cLC. Fig.S4 (Supporting Information) showed the relationship between the flow rate and the plate height for the four test solutes, in which the lowest plate height of ~28 μm was obtained with a flow velocity of 0.265 mm/s (The flow velocity was used throughout the experiments unless otherwise stated). In this instance, a column efficiency varying from 10,000 to 35,000 plate/m was achieved for the four test solutes at the optimal flow rate. The run-to-run repeatability was assessed on a single capillary monolithic column, and the relative standard deviations (RSDs) for the k values of the four solutes were less than 1.2% (n=5). Both column-to-column and batch-to-batch repeatability for the preparation of monolithic columns were also evaluated, which were less than 2.8% (n=3) and 4.0% (n=3), respectively. These results demonstrated that the prepared poly (AHA-co-PMA-co-EDMA) monoliths had good repeatability. In addition, the life time of the prepared polymer monoliths was also examined. Fig.S5 (Supporting Information) showed consecutive runs more than two months could be anticipated according to the

current experiments, suggesting good stability of the prepared polymer monolith.

3.3. Hydrophobic interaction chromatography of the poly (AHA-co-PMA-co-EDMA) monolithic column

As mentioned above, the poly (AHA-co-PMA-co-EDMA) monolith can provide hydrophobic interaction at low ACN content and the separation ability of the monolith was tested by separating alkylbenzene analogues. A typical chromatogram was shown in Fig.4(a), which presented the baseline separation of five alkylbenzenes with the mobile phase of 45% (v/v) ACN/H₂O. Alkylbenzenes were eluted out in order of benzene < toluene < ethylbenzene < n-propylbenzene < naphthalene according to their hydrophobicity, confirming a typical reversed-phase separation mechanism. Accordingly, the column efficiencies of the five alkylbenzenes were calculated to be 23,000, 22,000, 20,000, 18,000 and 15,000 plates/m at the linear velocity of 0.265 mm/s. In addition, the effect of ACN content on the retention of the five alkylbenzenes was studied (Fig.4(b)), and the result showed that the *k* values of the five alkylbenzenes decreased with the increase of ACN content, confirming again that the reversed-phase mechanism played a dominant role in the separation of the alkylbenzenes on the poly (AHA-co-PMA-co-EDMA) monolith.

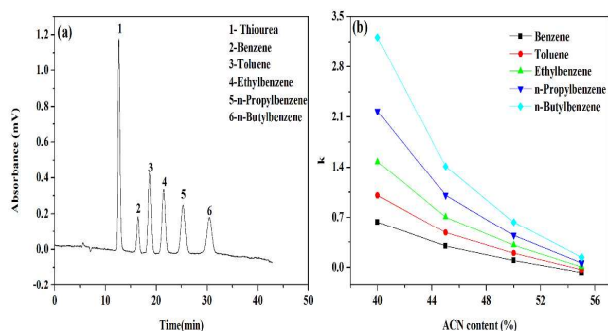


Fig.4. (a) Hydrophobic interaction chromatography for the separation of alkylbenzenes and (b) effect of ACN content on the *k* values of alkylbenzenes on the poly (AHA-co-PMA-co-EDMA) monolith.

Conditions for (a): Mobile phase: ACN/water: 45/55 (v/v %); Flow rate (actual flow rate after splitting): 0.05 mL/min (125 nL/min); Pump pressure: 6.4 MPa; Detection wavelength: 214 nm; For (b), all the conditions are same as (a) except for ACN content; (a) the analytes are (1) thiourea (100 ppm); (2) benzene (100 ppm); (3) toluene (100 ppm); (4) ethylbenzene (100 ppm); (5) n-propylbenzene (100 ppm); (6) n-butylbenzene (100 ppm).

3.4. Hydrophilic interaction chromatography of the poly (AHA-co-PMA-co-EDMA) monolithic column

As expected, the hydrophilic moieties (-COOH) of the poly (AHA-co-PMA-co-EDMA) monolith could be applied to the separation of phenols in HILIC mode (Fig.5(a)). It was observed from Fig.5(a) that the separation of four phenols was achieved with high column efficiencies of 20,000~40,000 plates/m. The retention order in the poly (AHA-co-PMA-co-EDMA) monolith was phenol < catechol < resorcinol < phloroglucinol. Besides, the corresponding *k* values increased with the increase of ACN content (Fig.5(b)). Obviously, the HILIC mechanism originated from carboxyl group of AHA can respond to the separation of the four phenols based on the above results.

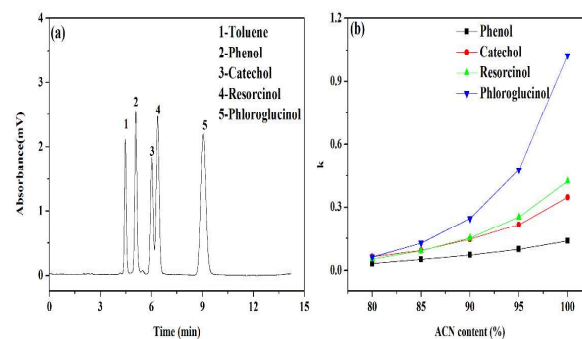


Fig.5. (a) Hydrophilic interaction chromatography for the separation of phenols and (b) effect of ACN content on the *k* values of phenols on the poly (AHA-co-PMA-co-EDMA) monolith.

Conditions for (a): Mobile phase: ACN/water: 100/0 (v/v %); Flow rate (actual flow rate after splitting): 0.05 mL/min (125 nL/min); Pump pressure: 3.0 MPa; Detection wavelength: 214 nm; For (b), all the conditions are same as (a) except for ACN content; (a) the analytes are (1) toluene (100 ppm); (2) phenol (100 ppm); (3) catechol (100 ppm); (4) resorcinol (100 ppm); (5) phloroglucinol (100 ppm).

3.5. Cation-exchange/hydrophobic interaction chromatography of the poly (AHA-co-PMA-co-EDMA) monolithic column

The poly (AHA-co-PMA-co-EDMA) monolith can offer electrostatic interaction with charged analytes due to the existence of carboxyl functionality in the monolith, which is weak acid (hexanoic acid, pKa ~4.8). To demonstrate the selectivity of the poly (AHA-co-PMA-co-EDMA) monolith to charged analytes, four anilines (phenylamine (pKa 4.6), o-nitroaniline (pKa -0.28), *a*-naphthylamine (pKa 3.92), and benzylamine (pKa 9.33)) were used for the evaluation. Retention of charged analytes in the ion exchange monolith was controlled by the pH of mobile phase that affects ionization of both the ion exchanger and the analytes. As shown in Fig.6(a), an absolute baseline separation of the four anilines was obtained with 45% ACN in 50 mmol/L PB at pH3.0. As the increasing pH, the ionization of anilines except benzylamine was suppressed and the electrostatic interaction between the analytes and stationary phase was weak. Therefore, the retention of phenylamine, o-nitroaniline, and *a*-naphthylamine kept constant. However, a tailing peak of benzylamine was observed in this case, implying the existence of strong electrostatic interaction. Apparently, the electrostatic interaction between benzylamine and AHA was enhanced, which led to the long retention time and poor tailing peak.

Additionally, the effect of salt concentration on the retention of the four anilines was investigated by varying the concentration of PB (pH5.5) from 30 to 100 mM in the mobile phase of ACN/water (45/55, v/v %), which was shown in Fig.S6 (Supporting Information). As the concentration of PB increased, the ion-exchange interaction between benzylamine and the stationary phase weakened, which resulted in decreasing retention time of benzylamine. However, the retention time of the other anilines almost kept constant. The above results demonstrated that the cation-exchange interaction played a dominant role in the separation of the four anilines.

The content of ACN in the mobile phase has significant influence on the separation of the four anilines. As shown in Fig.6(b), hydrophobic interactions were strengthened by increasing the ACN content. The retention factors of the four anilines decreased with the increase of ACN content from 40 to 55%. The result suggested that the separation of these anilines

was mainly based on hydrophobic interaction between the analytes and the monolithic stationary phase.

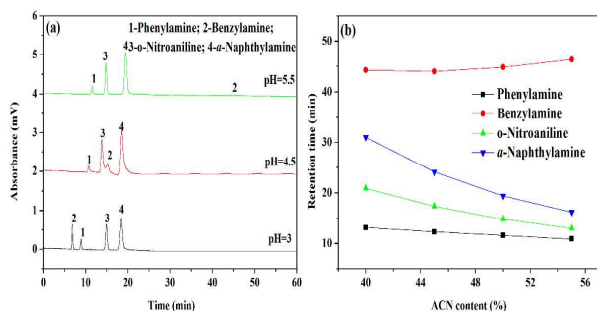


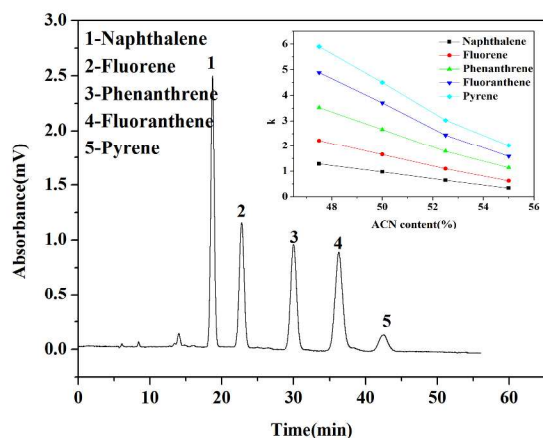
Fig.6. (a)Cation-exchange/hydrophobic interaction chromatography for the separation of anilines at varying pH and (b) effect of ACN content on retention time of anilines on the poly (AHA-co-PMA-co-EDMA) monolith.

Conditions for (a): Mobile phase: 50 mM PB containing 45% (v/v) ACN with various pH; Pump pressure: 6.4 MPa; Flow rate (actual flow rate after splitting): 0.05 mL/min (125 nL/min); Detection wavelength: 214 nm; For (b), all the conditions are same as (a) except for pH 5.5; the analytes are (1) phenylamine (100 ppm); (2) benzylamine (100 ppm); (3) o-nitroaniline (100 ppm); (4) a-naphthylamine (100 ppm).

3.6. Applications

3.6.1 Separation of polycyclic aromatic hydrocarbons (PAHs)

PAHs, legislated by the Environmental Protection Agency (EPA) as priority pollutant, were used to evaluate the applicability of the poly (AHA-co-PMA-co-EDMA) monolith. As can be seen from Fig.7, a good separation of the five PAHs (naphthalene (NAP), fluorene (FLU), phenanthrene (PHE), fluoranthene (FLT) and pyrene (PYR)) was achieved with the



monolithic column using 55% ACN in aqueous solution. Symmetrical peaks were obtained and the corresponding column efficiencies of PAHs were in the range of 21,000–28,000 plates/m. As expected, retention increased with the increase in the number of carbon within the molecules. Moreover, the structural isomers of FLT and PRY can be well separated. The retention order of the five PAHs was in accordance with their hydrophobicity. Additionally, the k values of the five PAHs decreased with increasing ACN contents from 47.5 to 55% in the mobile phase (the inset of Fig.7), indicating again that the hydrophobic interaction is responsible for the retention of the PAHs on the poly (AHA-co-PMA-co-EDMA) monolithic column.

Fig.7. Separation of five PAHs and (the inset) effect of ACN content on the k values of five PAHs on the poly(AHA-co-PMA-co-EDMA) monolith.

Conditions: Mobile phase: ACN/water: 55/45 (v/v %); Flow rate(actual flow rate after splitting): 0.05 mL/min (125 nL/min); Pump pressure: 5.6 MPa; Detection wavelength: 214 nm; the analytes are (1) naphthalene (100 ppm); (2) fluorene (100 ppm); (3) phenanthrene (100 ppm); (4) fluoranthene (100 ppm); (4) pyrene (100 ppm).

3.6.2 Separation of nucleosides, nucleobases and alkaloids

Separation of basic compounds is still a tough task in cLC with reversed-phase mode due to the multiplicity of their polar and positively charged groups. As an alternative, HILIC or HILIC/cation-exchange mode, which is capable of providing the possibility of good separation ability towards polar and charged analytes, took priority to separate the basic compounds. Fig.8(a) showed the baseline separation of nucleosides and nucleobases on the poly (AHA-co-PMA-co-EDMA) monolith with the mobile phase of 20 mM PB containing 88% (v/v) ACN at pH3.0. It was clearly observed that five nucleosides and nucleobases, including uracil (pKa 9.2), 6-methylaminopurine (pKa 4.2), adenosine (pKa 3.25), adenine (pKa 2.95) and cytosine (pKa 4.6), were well separated. It should be noted that varying the pH of the mobile phase from 3.0 to 7.0 (keeping 88% ACN/20 mM PB constant) and varying PB (pH 3.0) concentration from 5 mM to 20 mM (keeping 88% ACN constant) did not lead to the change in the elution order of the five nucleosides and nucleobases (Fig.S7, Supporting Information), suggesting that no electrostatic interaction between the nucleosides and nucleobases and the carboxyl group of AHA occurred at higher ACN content. In contrast, the k values of the five nucleosides and nucleobases gradually increased with the increase of ACN content from 72.5% to 92.5%, which was in good agreement with the mechanism described for HILIC (Fig.8(b)). Similarly, baseline separation of six alkaloids (theophylline, homoharringtonine, papaverine, N-methylephedrine, nuciferine and oxysophocarpine) was also obtained on the poly (AHA-co-PMA-co-EDMA) monolith when the same mobile phase was adopted as described above (Fig.8(c-d)). These results further confirmed that hydrophilic interaction contributed to the retention of the basic analytes on the poly (AHA-co-PMA-co-EDMA) monolith.

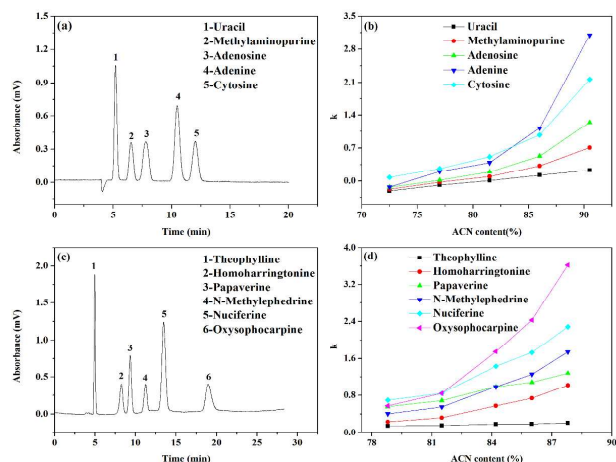


Fig.8. (a) Separation of five nucleosides and nucleobases and (b) effect of ACN content on the k values of nucleosides and nucleobases on the poly (AHA-co-PMA-co-EDMA) monolith. (c) Separation of six alkaloids and (d) effect of ACN content on the k values of alkaloids on the poly (AHA-co-PMA-co-EDMA) monolith.

Conditions for (a): Mobile phases: 20 mM PB containing 88% (v/v) ACN at pH3.0; Flow rate(actual flow rate after splitting): 0.05 mL/min (125 nL/min); Pump pressure: 4.2 MPa; Detection wavelength: 254 nm; For (b), all the conditions are same as (a) except for ACN content; the analytes are (1) uracil (100 ppm); (2) methylaminopurine (100 ppm); (3) adenosine (100 ppm); (4) adenine (100 ppm); (5) cytosine (100 ppm). For (c), mobile phase: 20 mM PB containing 88% (v/v) ACN at pH3.0; Flow rate(actual flow rate after splitting): 0.05 mL/min (125 nL/min); Pump pressure: 4.2 MPa; Detection wavelength: 214 nm; For (d), all the conditions are same as (c) except for ACN content; the analytes are (1) theophylline (100 ppm); (2)

homoharringtonine (100 ppm); (3) papverine (100 ppm); (4) N-methylephedrine (100 ppm); (5) nuciferine (100 ppm); (6) oxysophocarpine (100 ppm).

3.6.3 Separation of peptides and proteins

As mentioned above, the produced poly (AHA-co-PMA-co-EDMA) monolith exhibited adequate separation selectivity and efficiency, and then it is expected to be applied to the separation of complex samples such as peptides and proteins. Fig.9(a) showed the isocratic separation of five synthetic peptides on the poly (AHA-co-PMA-co-EDMA) monolith using reversed-phase mode. It was observed that almost baseline separation of YGG, CYIQNCPLG, FGFG, YGGFM and YGGFL was obtained with the mobile phase of 27.5% ACN in 20 mM PB (pH3.0). Moreover, the decreasing *k* values of the five peptides with the adding ACN contents (data not shown) validated a reversed-phase mode responsible for peptide separation.

The applicability of the poly (AHA-co-PMA-co-EDMA) monolith for macromolecule separation was also evaluated by gradient elution of protein mixture including Lyz, Cyt C, Mb, BSA and OVA. As presented in Fig.9(b), five proteins were well separated within 40 min by using a simple linear gradient elution with ACN in 0.1% (v/v) trifluoroacetic acid (TFA) aqueous solution. Overall, the results confirmed that the produced poly (AHA-co-PMA-co-EDMA) monolith allowed efficient separations of a large variety of compounds, ranging from small organic compounds to large biological molecules.

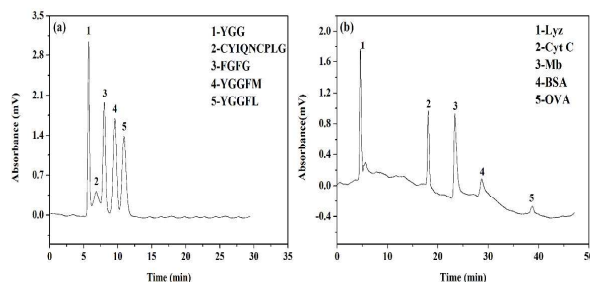


Fig.9. Separation of five peptides and five proteins on the poly (AHA-co-PMA-co-EDMA) monolith.

Conditions for (a): Mobile phase: 20mM PB containing 27.5% ACN at pH3.0; Flow rate(actual flow rate after spiltting): 0.05 mL/min (125 nL/min); Pump pressure: 7.6 MPa; Detection wavelength: 214 nm; the analytes are (1) YGG (100 ppm); (2) CYIQNCPLG (100 ppm); (3) FGFG (100 ppm); (4) YGGFM (100 ppm); (5) YGGFL (100 ppm). For (b): Mobile phase: (A) 5% ACN + 0.1%(v/v) TFA; (B) 95% ACN; Linear gradient: from 20% B to 50% B within 0–60 min; Flow rate(actual flow rate after spiltting): 0.05 mL/min (125 nL/min); Pump pressure: 6.0–7.8MPa; Detection wavelength: 214 nm; the analytes are (1) Lyz (100 ppm); (2) Cyt C (100 ppm); (3) Mb (100 ppm); (4) BSA (100 ppm); (5) OVA (100 ppm).

4. Conclusion

In summary, we developed a novel method for the preparation of polymer monoliths by the combination of free radical polymerization and CuAAC click reaction in “one-pot” process. The synthetic procedure was as simple as *in situ* polymerization of purely polymer monoliths without any special handling. On the basis of the cLC investigation of the resultant polymer monolith, it was confirmed that the poly (AHA-co-PMA-co-EDMA) monolith have the excellent chromatographic properties in terms of hydrophobicity, hydrophilicity and cation-exchange interaction. The successful separations of a series of small molecules and proteins also indicated the solid potential of this strategy for the preparation of the polymer monoliths with the desirable functional monomers. Furthermore, with the use of this approach, the limitation and difficulty of the multi-step post modifications could be easily circumvented.

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