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Short communication

A facile and efficient strategy for one-step *in situ* preparation of hydrophobic organic monolithic stationary phases by click chemistry and its application on protein separation

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ABSTRACT

A simple one-step *in situ* "click" modification strategy was developed for the preparation of hydrophobic organic monolithic columns for the first time. The column morphology and surface chemistry of the fabricated monolithic columns were characterized by scanning electron microscopy, Fourier transform infrared spectroscopy and X-ray photoelectron spectroscopy, respectively. The chromatographic performances of the C8/C18 "click" monoliths were evaluated through the separation of a mixture of five proteins such as ribonuclease A, soybean trypsin inhibitor, cytochrome c, bovine haemoglobin and bovine serum albumin. Compared with the blank column, the higher hydrophobicity stationary phases obtained from the "clicked" modification have longer retention times and higher resolution for the five proteins. The separation of five proteins mixture on click C18 monolith with gradient elution at different flow rates was also investigated, the baseline separation of five proteins could be achieved at three different flow rates.

1. Introduction

"Click chemistry" first developed by Sharpless and co-workers in 2001 [1], currently has attracted a great deal of attention across nearly all areas of modern chemistry [2–4]. Hundreds of research papers and thematic reviews related "click chemistry" were published, indicating a wealth of applications of this practical chemical approach. The exemplary click reaction copper (I) catalyzed (3 + 2) azide–alkyne cycloaddition [5], having excellent attributes in high efficiency, high selectivity, no side reactions, functional group tolerant and mild reaction conditions, was evolved into a common tool in bioconjugation [6,7], macromolecular materials [8–10], solid state phase reaction [11], surface modification [12–14], drug discovery [15,16] and others [17].

In the field of chromatographic separation, click reaction has been used to prepare stationary phases by immobilization of different functional groups onto organic [18,19] or silica beads [20–24]. Fréchet and co-workers reported the preparation of reversed-phase and affinity stationary phases based on the "click" modification of organic porous beads [18]. Liang and co-workers reported a series of applications of click chemistry for the preparation of functionalized HPLC packings based on silica beads [20–23]. The pioneering works involving in "click" functionalization of organic/inorganic particles need a time-consuming sieving and packing process for chromatographic purposes. The monolithic stationary phases firstly performed by Hjertén and co-workers [25–27], can be potentially advantageous relative to conventional particle-packed columns, due to their simplicity of preparation, versatile surface modification, higher permeability along with good peak capacity and no need for particle-size classification and packing [28–31]. Whereas the reports investigating this "click" strategy for functionalization of monolithic stationary phases were very few. Recently, Carbonnier and co-workers reported an application of click chemistry in the functionalization of macroporous organic polymer monolith [32]. In their work, the monolith based N-acryloxysuccinimide (NAS) and ethylene dimethacrylate (EGDMA) was prepared firstly, then a two-step modification was carried out to graft β-cyclodextrin (CD) onto the monolith for chiral capillary chromatography. Fréchet and co-workers gave us another example of in-column preparation of brush-type chiral stationary phase based on a silica monolith using click chemistry [33], in which a two-step modification method was employed to graft chiral selector onto a commercial silica monolith.

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In the present work, a one-step *in situ* "click" modification strategy was developed for the first time to prepare hydrophobic organic monoliths for protein separation. The column morphology and surface chemistry of the fabricated monolithic columns were characterized by scanning electron microscopy (SEM), Fourier transform infrared spectroscopy (FT-IR) and X-ray photoelectron spectroscopy (XPS), respectively. The protein separation abilities of obtained "clicked" monoliths were probed by reversed-phase high performance liquid chromatography (HPLC) and the better chromatographic performance was acquired.

2. Experimental

2.1. Materials

Propargyl methacrylate (PMA), ethylene glycol dimethacrylate (EGDMA), 1-bromooctane, 1-bromooctadecane, tetrabutylammonium iodide were purchased from Alfa Aesar (Tianjin, China) and used directly without further purification. Cyclohexanol, dodecanol and 2,2′-azobisisobutyronitrile (AIBN) were purchased from Tianjin Chemical Reagent Company (Tianjin, China). All proteins including bovine pancreatic ribonuclease A (RNase A), soybean trypsin inhibitor (SBTI), horse heart cytochrome c (Cyt C), bovine haemoglobin (BHb) and bovine serum albumin (BSA) were purchased from Sigma–Aldrich (St. Louis, MO, USA). All reagents were used as provided.

2.2. Instrumentation

NMR spectra were recorded on a Bruker AV-400 instrument as solutions in $CDCl_3$. The morphology of the polymer was obtained by a scanning electron microscopy (FEI Quanta, Sweden). FT-IR spectra were recorded on a Thermo Nicolet 6700 FT-IR spectrometer as dispersions in KBr. XPS spectra was recorded on a Kratos Axis Ultra DLD multi-technique X-ray photoelectron spectroscopy. All chromatography experiments were performed on a Shimadzu LC-20A HPLC system (Shimadzu, Kyoto, Japan), consisting of two LC-20AD pumps, a diode array detector (SPD-M20A).

2.3. Preparation of 1-azidooctane and 1-azidooctadecane

1-Azidooctane was prepared by a modification of a procedure described by Alvarez and Alvarez [34]. Sodium azide (7.15 g, 110 mmol) was dissolved in DMSO (220 mL), then 1-bromooctane (19.3 g, 100 mmol) was added. After vigorously stirring for 12 h at 25–30 °C, the reaction was quenched with water. The reacting products were extracted by Et_2O and the combined organic extracts were washed with water and brine, dried ($MgSO_4$), filtered. Finally, the product was obtained as a colorless oil (15.36 g, 99.0 mmol, 99% yield). ^{1}H NMR (400 MHz): δ 0.88 (3H), 1.28–1.39 (10H), 1.60 (2H), 3.35 (2H). ^{13}C NMR (400 MHz): δ 14.08 (CH_3), 22.65 (CH_2), 26.73 (CH_2), 28.85 (CH_2), 29.16 (CH_2), 31.78(CH_2), 51.47 (CH_2).

1-Azidooctadecane was prepared by a modification of the procedure described by Fréchet and co-workers [18]. Sodium azide (7.15 g, 110 mmol) and tetrabutylammonium iodide (20 mg, 54 mol) were dissolved in DMSO (220 mL). The next, 1-bromooctadecane (33.3 g, 100 mmol) was added, and the biphasic mixture was heated at 78–83 °C for 16 h. The reaction was quenched with water, then extracted with hexanes. The combined organic extracts were washed with water and brine, dried ($MgSO_4$), filtered, and purified by flash chromatography on silica gel, eluting with petroleum ether. The product was obtained as a colorless oil (28.96 g, 98.0 mmol, 98% yield). ^{1}H NMR (400 MHz): δ 0.88 (3H), 1.26(30H), 1.59 (2H), 3.24 (2H). ^{13}C NMR (400 MHz): δ 14.08 (CH_3), 22.71 (CH_2), 26.72 (CH_2), 28.84 (CH_2), 29.17 (CH_2), 29.38 (CH_2), 29.49 (CH_2), 29.56 (CH_2), 29.64 (CH_2), 29.67 (CH_2), 29.69 (CH_2), 29.704 (CH_2), 29.72 (CH_2), 31.94 (CH_2), 51.48 (CH_2).

2.4. Preparation and modification of monolithic columns

The organic monolith was prepared by an *in situ* polymerization in a stainless-steel tube (100 mm × 4.6 mm i.d.). Functional monomer PMA (0.60 mL) and cross-linker EGDMA (0.60 mL) and initiator AIBN (20 mg) were dissolved in a mixture of cyclohexanol and dodecanol (2:1, v/v), and the ratio of monomer to porogenic solvents (40:60, v/v) was selected. The mixture was surged ultrasonically and purged with N_2 for 15 min before pouring the polymerization mixture into the stainless-steel column sealed with a dead nut at the bottom. The column was then sealed at the top and

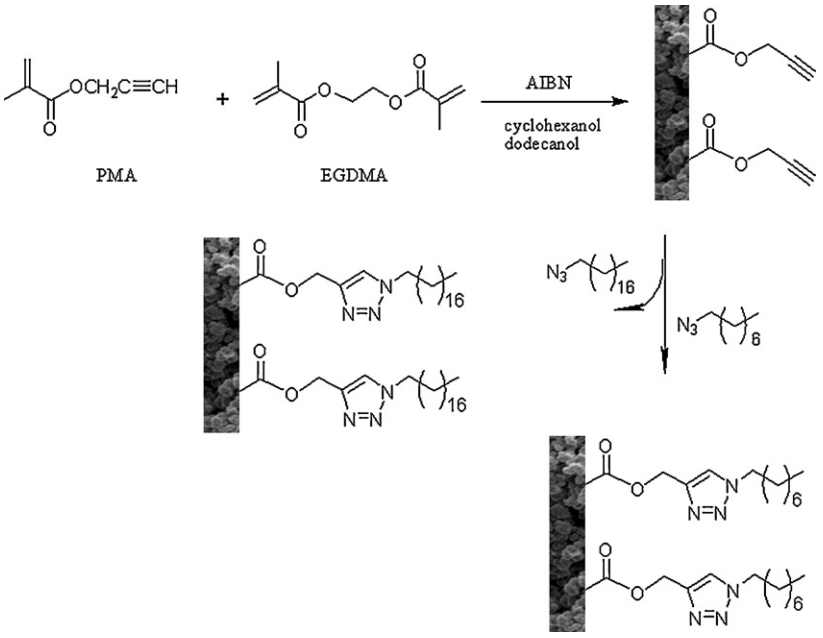

Fig. 1. The procedure for preparation of monolithic columns modified by C8 and C18.

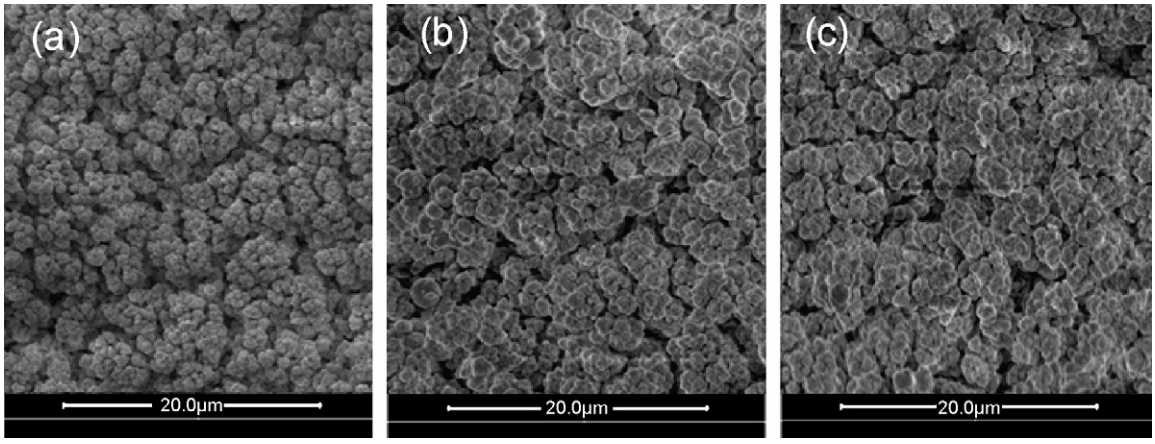

Fig. 2. SEM images of the blank (a), click C8 (b) and C18 (c) columns.

left to polymerize in a water bath at 60 °C for 12 h. After the polymerization ended, the column was connected to an HPLC pump and washed exhaustively with methanol to remove the progenic solvents and unreacted monomers.

For the click reaction step, a solution of 1-azidooctane (5 mmol) and CuI (0.2 mmol) in 50 mL acetonitrile for C8 "click" and 1-azidooctadecane (5 mmol) and CuI (0.2 mmol) in 60 mL acetonitrile/$CHCl_3$ (2:1, v/v) for C18 "click" respectively was continuously pumped through the columns for 120 h at 30 °C. The resulting monolithic columns clicked C8 and C18 were obtained after washed by acetonitrile, 50 mM disodium EDTA and water.

After chromatographic experiments for protein separation were finished, the clicked columns were washed with water and acetonitrile to remove the proteins adsorbed on the column, then pumped with methnol/H_2O (50:50, v/v) to store at room temperature.

3. Results and discussion

3.1. In-column preparation and characterizations of "clicked" monolith

The synthetic procedure for the C8/C18 functionalized monoliths can be simply divided into two parts (Fig. 1). Initially, click monomer PMA containing terminal alkyne group and cross-linker EGDMA were employed to prepare for the polymer skeleton. Subsequently, this alkyne–reactive polymer surface was one-step *in situ* "clicked" C8/C18 hydrophobic branch via the copper (I)-catalyzed 1,3-dipolar cycloaddition reaction.

The permeability of monolith is one of the most practical factors in designing a novel type of monolithic stationary phases. Morphology of the blank, click C8 and C18 monoliths was characterized by SEM. As can be seen from Fig. 2, the well-proportioned pores were embedded in the monolithic columns all along with the modification procedure.

After modification, surfaces of the blank and "clicked" monoliths were characterized by FT-IR. Attenuation of the intensity of FT-IR peak at 3309 cm^{-1} ascribing to the alkyne C–H stretch was used as a convenient analytical tool to monitor the click reaction. As shown in Fig. 3, The decrease of the intensity of ν(C≡CH) band (3300 cm^{-1}) for the click C8 and C18 columns relative to the spectrum of blank column were detected. In addition, the IR spectra of two "clicked" surfaces displayed the increase of absorptions of C–H stretching bands at 2924 cm^{-1} ($\nu_a(CH_2)$) and 2858 cm^{-1} ($\nu_\delta(CH_2)$) comparing to the starting material, and the large intensities of the C–H absorptions indicate a largely disordered structure of the hydrocarbon chains. These spectral changes are clear proofs of the reaction

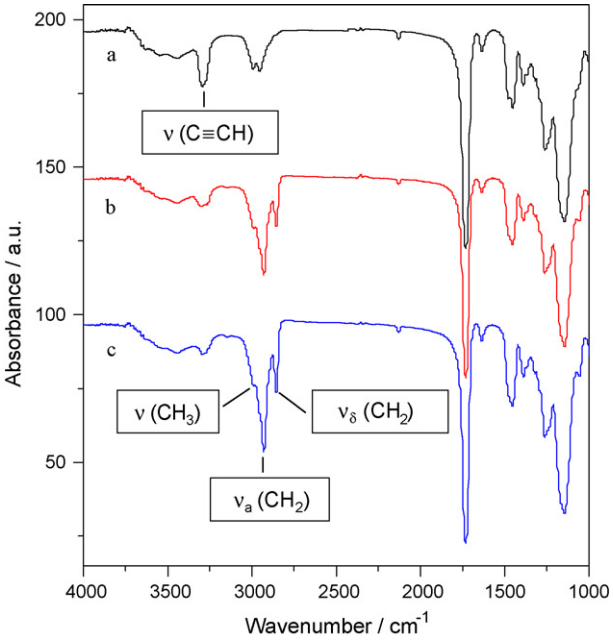

Fig. 3. FT-IR spectra of the blank (a), click C8 (b) and C18 (c) columns.

of alkyne groups with the azide groups of two long aliphatic hydrocarbon chains.

XPS spectra were used to obtain chemical composition of the "clicked" adherent layer. Fig. 4 shows the representative XPS data of click C8 and C18 monoliths. The characteristic nitrogen peak of 1, 2, 3 triazole originated from click reaction appears at ~400 eV and N contents have significant increase (there is no N element in the blank column), indicating again the successful "clicked" modification. At the same time, the mass conc % of N element ~4.47% consisting in click C8 column and ~4.04% in click C18 column were gained from the data of XPS analysis. The surface coverage of "clicked" hydrophobic branch was obtained *via* calculation based on the N content (Table 1) corresponding to 1.06 and 0.961 mmol g^{-1} for C8 and C18 group, respectively. The yield of click reaction was ~60% which was in accord with the data of FT-IR.

3.2. Chromatographic properties

Most recent studies on the hydrophobic monolithic stationary phases were focused on their development and application

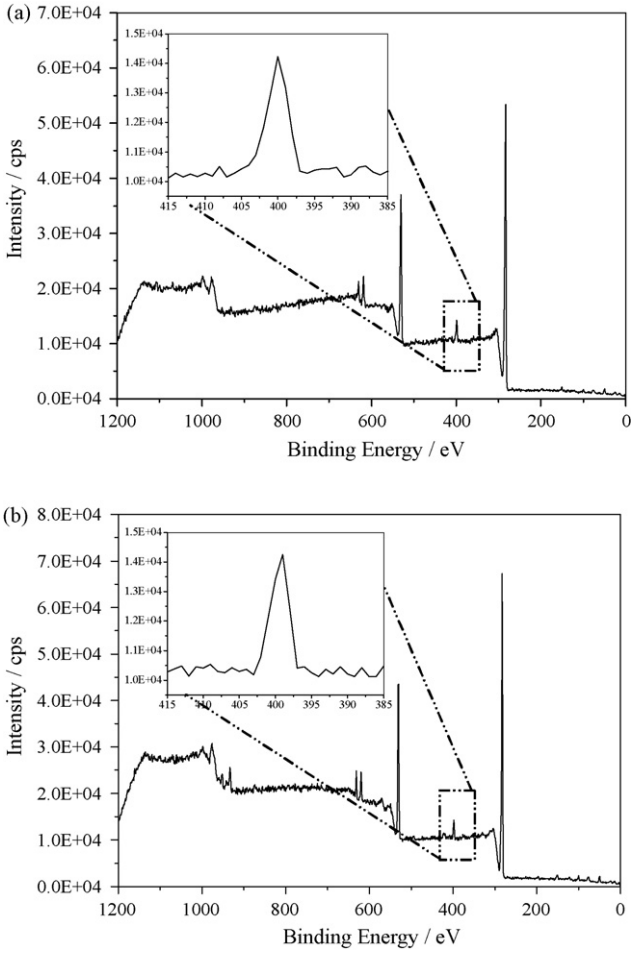

Fig. 4. XPS spectra of click C8 (a) and C18 (b) columns.

Table 1
Element contents of click C8 and C18 monolithic columns.

HPLC columns	Composition (%, w/w)	Group contents (mmol g^{-1} polymer)	
	N	C8	C18
Click C8	4.47	1.06	–
Click C18	4.04	–	0.961

in reversed-phase separation of biomacromolecules [35–37]. In this work, the porous polymer monoliths attached to two different length alkyl chains via triazole ring provide the two analogous hydrophobic monolithic stationary phases for biomacromolecules. For reversed-phase mode chromatography, the effect of surface chemistry on separation properties is clearly followed by determination of methylene selectivity. The values of three stationary phases are 1.08 for blank column, 1.24 for click C8 column and 1.46 for click C18 column calculated as the ratio of retention factors for toluene and benzene. Both nonalkylated and alkylated stationary phases were used for the reversed-phase separation of a mixture of five proteins such as RNase A, SBTI, Cyt C, BSA, BHb in the gradient elution mode, which were shown in Fig. 5a. As expected from the methylene selectivity data, the higher hydrophobicity of stationary phases obtained from the "click" modification leads to longer retention times and higher resolution for five proteins, which was also consistent with that observed in the literature [18].

The predominant advantage of the monolithic media is their ability to effect separations even at extremely high flow rates without the concomitant decrease in separation power due to the beneficial contribution of convection to the overall mass transport [38]. This means convective mass transfer between mobile phase and stationary phase can allow chromatographic performance at very fast velocity without losing resolution, which was validated by former reports [39]. The similar result was obtained in this study, Fig. 5b showed the separation of five model proteins mixture on click C18 monolith with gradient elution at different flow rates. The baseline separation of five proteins could be achieved at flow rates of 2.0 and 3.0 mL min^{-1} and the column efficiency was almost the same as that of 1.0 mL min^{-1}.

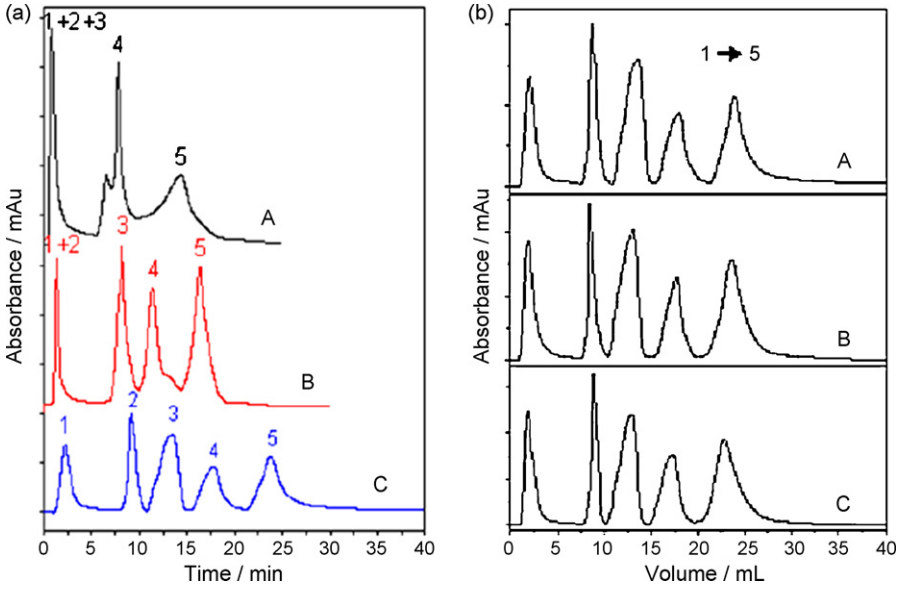

Fig. 5. Chromatograms of a five proteins mixture using blank (A), click C8 (B) and C18 (C) columns with gradient elution at a flow rate of 1 mL min^{-1} (a); separation of five proteins on click C18 column with gradient elution at the flow rate of 1.0 (A), 2.0 (B) and 3.0 (C) mL min^{-1} (b). Conditions: column, 100 mm × 4.6 mm i.d.; mobile phase 1, acetonitrile/H_2O (20:80, v/v); mobile phase 2, acetonitrile/0.1 M phosphate buffer (pH 3.00) (40:60, v/v); gradient: 0–3 mL, 100% mobile phase 1; 3–18 mL, 0 → 100% mobile phase 2; UV: 280 nm. Peaks: RNase A (1); SBTI (2); Cyt C (3); BSA (4); BHb (5).

In contrast to biomacromolecules, the separation of organic small molecules is much more sensitive to porosity and the chromatographic conditions employed for their fractionation. The lower specific surface area of the materials prepared in this work resulted in the peaks broadening and lower separation efficiencies. The study on the separation of small molecules by "clicked" hydrophobic monoliths are investigating in our lab.

4. Conclusions

In this communication, click chemistry was used as an effective strategy for coupling two aliphatic hydrocarbon chains onto the alkyne–reactive polymer monoliths. C8/C18 functionalized monolithic stationary phase was prepared by a one-step *in situ* "click" modification strategy for the first time. XPS and FT-IR data clearly indicated the successful "click" modification. Proteins separation abilities of the obtained monoliths were demonstrated by the separation of five proteins. Click chemistry is a new and promising tool for the one-step *in situ* modification of porous polymer monoliths. The *in situ* "click" method developed herein may be extended to the design of novel polymer monolithic stationary phases modified by a variety of functional compounds or biomacromolecules.

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