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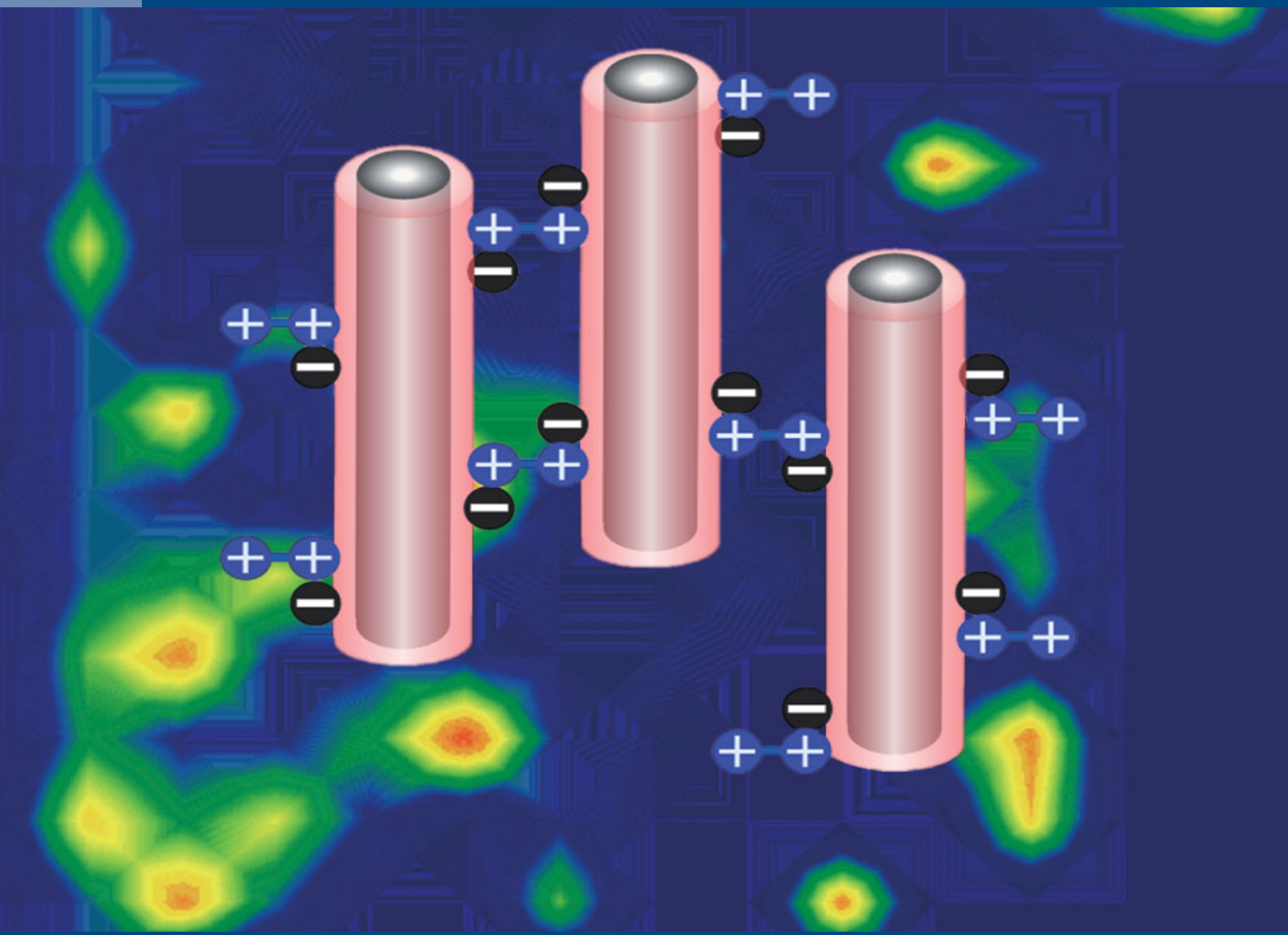

Methods
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Research Article

Synthesis of a stationary phase based on silica modified with branched octadecyl groups by Michael addition and photoinduced thiol–yne click chemistry for the separation of basic compounds

A novel silica-based stationary phase with branched octadecyl groups was prepared by the sequential employment of the Michael addition reaction and photoinduced thiol–yne click chemistry with 3-aminopropyl-functionalized silica microspheres as the initial material. The resulting stationary phase denoted as SiO_2-N(C18)$_4$ was characterized by elemental analysis, FTIR spectroscopy and Raman spectroscopy, demonstrating the existence of branched octadecyl groups in silica microspheres. The separations of benzene homologous compounds, acid compounds and amine analogues were conducted, demonstrating mixed-mode separation mechanism on SiO_2-N(C18)$_4$. Baseline separation of basic drugs mixture was acquired with the mobile phase of acetonitrile/H_2O (5%, v/v). SiO_2-N(C18)$_4$ was further applied to separate *Corydalis yanhusuo* Wang water extracts, and more baseline separation peaks were obtained for SiO_2-N(C18)$_4$ than those on Atlantis dC18 column. It can be expected that this new silica-based stationary phase will exhibit great potential in the analysis of basic compounds.

Keywords: Basic compounds / High-performance liquid chromatography / Michael addition / Stationary phases / Thiol–yne click chemistry
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1 Introduction

The analysis of basic compounds by HPLC is of great significance, as more than 70% of pharmaceuticals are basic compounds [1]. The RP mode is the most widely adopted separation mode in HPLC because it can provide versatile retention mechanism by regulating the mobile phase pH or using organic additives, and abundant knowledge and experience for analysis of a given sample conveniently. However, the separation of hydrophilic basic compounds by RPLC is not easy, as these compounds are not sufficiently retained on the hydrophobic stationary phases. Strong ion pairing agents, such as surfactants, are necessarily added in mobile phases to increase retention of the analytes [2–4]. Unfortunately, the addition of surfactants into the mobile phase makes the separation incompatible with mass spectrometric detection. In addition, surfactants tend to stick strongly to the stationary phase, which makes it difficult to recover the column [5]. Cation-exchange chromatography has also been applied for the separation of highly polar basic compounds [6–8], while it is not compatible with mass spectrometric detection because of using the mobile phase with high concentration of salt. HILIC is another approach for the analysis of basic compounds [9], but the use of a high concentration of organic solvent is not environmentally friendly, which is a detriment to dissolve the high polar basic compounds.

It should be pointed out that tailing and overloading always appear in the analysis of basic compounds by RPLC. These problems are associated with the surface structure of the RP packings. For silica-based packings, there are still silanols on the surface because the silanol groups are not completely reacted for the sake of steric hindrance even end-capped with smaller silylating agents [10]. The pK_a value of these silanol groups is around 7.1, and the metal impurities

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Abbreviations: SiO_2-N(C18)$_4$, branched octadecyl groups modified silica microspheres; **SiO_2-N($CO_2CH_2C{\equiv}CH)_2$**, branched propargyl acrylate modified silica microspheres; **SiO_2-NH_2**, 3-aminopropyl functionalized silica microspheres

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in the silica can also enhance the acidity. The pH of mobile phases affects the existence forms of silanols and the basic compounds, such as protonation and deprotonation. Therefore, the interactions between the mobile phases and the silica-based stationary phases involve hydrophobic interaction and ion-exchange interaction. Especially, the strong interaction between the deprotonated silanols and the protonated bases results in slow sorption–desorption kinetics, which is responsible for tailing [11].

RP packings with polar groups were beneficial for basic compounds separation, because polar groups could weaken the interaction of the ionized silanols and the basic analytes. For instance, the polar copolymerized stationary phase composed of *n*-octadecyl and chloropropyl had excellent performance in alkaloids separation compared to some commercial stationary phases [12]. In addition, silica-based stationary phases with ionic carboxylate groups embedded within a hydrophobic ligand (SiELC Primesep^{TM}) could also offer better peak shapes than XTerra RP-18 (with a neutral carbamate embedded group) for small solute mass at low pH [13]. As the position of ionic groups embedded within the alkyl chain (SiELC Primesep^{TM}) was quite different from that of ionized silanols buried beneath the hydrophobic ligands, the exchange kinetics of the two stationary phases were different. Thus, there might be other synergistic interaction beyond ion-exchange and hydrophobic interaction. Unfortunately, there was only one type of polar group on each strationary phase. Encouraged by these results, the stationary phase with various polar groups embedded might provide novel mechanism for basic compounds analysis.

Michael addition of primary amine to an α,β-unsaturated carbonyl compound and thiol-yne click chemistry are powerful techniques for fabricating the versatility of materials due to the diversity of monomers and mild reaction conditions. Many compounds with polar groups (tertiary amine, carboxylate esters or vicinal dithioethers) embedded were synthesized by Michael addition and thiol–yne click chemistry [14–19]. Herein, we fabricated a novel silica-based stationary phase with various polar groups embedded in branched octadecyl groups by sequential employment of Michael addition reaction and thiol–yne click chemistry (Scheme 1). The chromatographic retention mechanism and application of this stationary phase were evaluated, and excellent performance for separation of both standard basic drugs and alkaloids extracted from *Corydalis yanhusuo* Wang was obtained as compared to commercially available Atlantis dC18 column.

2 Materials and methods

2.1 Materials

(3-Aminopropyl)triethoxysilane, TFA, 1-octadecanethiol, uridine, cytosine, cytidine, *m*-trihydroxybenzene, 2,6-xylenol, *p-tert*-butylphenol, 2,4-dichlorophenol, barbital, carbamazepine, 2,4-dinitroaniline, 4-amino-biphenyl, alprenolol, pindolol and propranolol were obtained from Sigma (St. Louis, MO, USA). 2,2-Dimethoxy-2-phenylacetophenone (DMPA) was from Acros Organics (New Jersey, USA). Spherical silica (particle size, 5 μm; pore size, 20 nm) was purchased from Fuji Silysia Chemical (Aichi, Japan). A commercial Atlantis dC18 column (5 μm, 100 Å, 4.6 mm × 150 mm) was from Waters (Milford, MA, USA). Anhydrous ethanol, tetrahydrofuran (THF), acetonitrile (ACN), isopropyl alcohol, pyridine, phenol, dipotassium hydrogen phosphate, potassium phosphate monobasic, orthophosphoric acid, benzene, toluene, ethylbenzene, *n*-propylbenzene and *n*-butylbenzene were obtained from Tianjin Kermel plant of chemical reagent (Tianjin, China). Propargyl acrylate was purchased from Alfa Aesar (Ward Hill, MA, USA).

Berberine hydrochloride and palmatine hydrochloride were obtained from National Institutes for Food and Drug Control of China (Beijing, China). Methanol (MeOH, HPLC grade) was purchased from Merck (Darmstadt, Germany). Deionized water used in all experiments was purified with a Milli-Q system from Millipore (Milford, MA, USA). The actual sample of alkaloids was kindly donated by Prof. Xinmiao Liang (Dalian Institute of Chemical Physics, Chinese Academy of Science, Dalian, China), which was extracted from a kind of Chinese herbal medicine, *Corydalis yanhusuo* Wang, according to the previous reports [20,21], and the sample concentration is about 10 mg mL^{-1}.

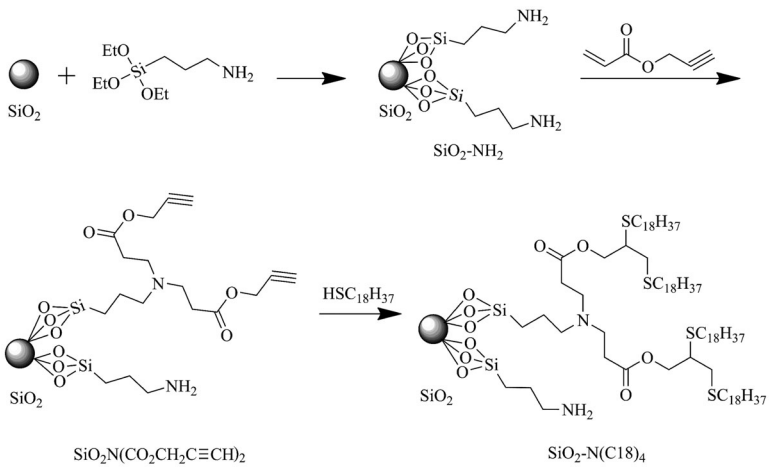
2.2 Preparation of branched octadecyl groups modified silica-based stationary phase

2.2.1 Synthesis of 3-aminopropyl functionalized silica microspheres

3-Aminopropyl functionalized SiO_2 microspheres were synthesized according to the previously reported method [22]. As seen in Scheme 1, spherical silica (4.60 g) was dispersed in a mixture of anhydrous toluene (40.0 mL) and anhydrous pyridine (2.0 mL). After ultrasonic treatment for 10 min, (3-aminopropyl)triethoxysilane (6.0 mL) was added. The resulting suspension was stirred for 48 h at reflux under argon atmosphere. Finally, the obtained product (SiO_2-NH_2) was rinsed by anhydrous ethanol for five times and dried under vacuum at 50°C for 24 h.

2.2.2 Synthesis of branched propargyl acrylate modified silica microspheres

The branched propargyl acrylate modified silica microspheres were prepared by the Michael addition reaction according to the previous literature [19]. SiO_2-NH_2 (2.40 g) was suspended in methanol (20.0 mL) by sonication for 10 min, then propargyl acrylate (1.2 mL) was added. The final reaction mixture was stirred for 24 h at 40°C. After being washed with methanol five times, the product (SiO_2-$N(CO_2CH_2C{\equiv}CH)_2$) was dried under vacuum at 40°C for 24 h.



Scheme 1. Illustration of consecutive steps for the synthesis of SiO_2-N(C18)$_4$.

2.2.3 Synthesis of branched octadecyl groups modified silica microspheres

The branched octadecyl groups modified silica microspheres were synthesized as the literature described [15]. SiO_2-$N(CO_2CH_2C{\equiv}CH)_2$ (2.30 g) was dispersed in anhydrous ethanol (80 mL), 1-octadecanethiol (0.60 g) and DMPA (0.020 g) were successively added. The resulting mixture was stirred in ice bath with a magnetic stirrer under the UV irradiation crosslinker (80 W cm^{-2}, λ_{max} = 365 nm, Shenzhen, China). Then, the thiol-yne click chemistry was photoinitiated by UV irradiation for 1 h. Finally, the product (SiO_2-N(C18)$_4$) was washed extensively with THF and ethanol, and dried under vacuum at 40°C for 24 h.

2.3 Equipment and measurements

FTIR spectra were obtained on Nicolet Fourier spectrophotometer using KBr pellets (Nicolet, Wisconsin, USA). Elemental analysis was performed on Vario EL III (Elementar, Hanau, Germany). Chemical structure of the functionalized silica was characterized by a Raman apparatus LabRAM HR from Horiba JobinYvon (Longjumeau, France) equipped with a laser emitting at 785 nm. AB Sciex 5800 MALDI-TOF/TOF mass spectrometer (AB SCIEX, USA) was equipped with a pulsed Nd/YAG laser at 355 nm in linear positive ion mode. HPLC was performed on a Hitachi Primaide HPLC system with two 5110 pumps, a 5310 column oven, a 5210 UV auto sampler and a 5410 UV detector (Minato-ku, Tokyo, Japan). Detection was performed on 214 nm.

2.4 Packing of the column

The slurry method was applied to pack the obtained SiO_2-N(C18)$_4$ into HPLC columns [23]. Briefly, a slurry of SiO_2-N(C18)$_4$ (2.30 g) was made with 30 mL of isopropyl alcohol by stirring for at least 30 min and then placed in a dynamic reservoir system. After sealing the reservoir, an empty stainless-steel column (150 × 4.6 mm id) was attached. Ethanol was used as a propelling solvent, and the system was pressured to 47 MPa using a GLK1000 air-driven fluid pump (Wuxi, China). The pressure was kept until a minimum of 200 mL of ethanol had passed through the column. The inlet pressure to the pump was shut-off, and then the system was allowed to release the pressure gradually.

2.5 Chromatographic evaluation

The SiO_2-N(C18)$_4$ column was evaluated under 20°C of column temperature and 1.0 mL/min of flow rate. The dead time was 0.937 min, which was determined by injection of 2 μL MeOH with mobile phase ACN/H_2O at 25:75 v/v.

The RP chromatographic retention of SiO_2-N(C18)$_4$ column was evaluated by separating benzene homologous compounds, slightly polar compounds, such as phenols and amine analogues. The mobile phases used for this test were ACN and H_2O. The retention of these analytes was obtained with different concentrations of ACN in the mobile phase.

The surface electrostatic properties of SiO_2-N(C18)$_4$ column were investigated by separating the mixture of aromatic acid and basic compounds. During this test, ACN and $NaHPO_4/NaH_2PO_4$ buffer were employed as mobile phases. The concentration of $NaHPO_4/NaH_2PO_4$ buffer was 10 mM, and the pH values of mobile phases were 2.7, 4.0, 5.0, 6.0, 7.0 and 7.8, respectively. The effects of concentrations of $NaHPO_4/NaH_2PO_4$ buffer on the retention of aromatic acid and basic compounds were further investigated, the concentrations of $NaHPO_4/NaH_2PO_4$ buffer were changed from 5, 10, 15 to 20 mM at pH 2.7.

2.6 Applications

The SiO_2-N(C18)$_4$ stationary phase was further used to investigate the retention of a mixture of three polar basic drugs, including pindolol, alprenolol and propranolol, and the alkaloids extracts from *Corydalis yanhusuo* Wang, respectively. At the meantime, the commercially available column Atlantis dC18 was also applied to separate both samples for comparison.

Table 1. Elemental analysis of three kinds of silica-based microspheres

Material	C (%)	H (%)	N (%)
SiO_2-NH_2	4.05	1.056	1.392
SiO_2-$N(CO_2CH_2C{\equiv}CH)_2$	5.191	1.282	1.431
SiO_2-$N(C18)_4$	8.361	1.711	1.238

3 Results and discussion

3.1 Characterization

The branched structure of SiO_2-$N(C18)_4$ was first investigated by elemental analysis (Table 1). As listed in Table 1, compared to SiO_2-NH_2, the increase of carbon and hydrogen contents in SiO_2-$N(CO_2CH_2C{\equiv}CH)_2$ showed that propargyl acrylate was successfully immobilized onto SiO_2-NH_2. The contents of carbon and hydrogen in SiO_2-$N(C18)_4$ were higher than those of SiO_2-$N(CO_2CH_2C{\equiv}CH)_2$, which also confirmed that 1-octadecanethiol was grafted. FTIR spectroscopy of products in each step also corroborated that reactions showed in the route happened successfully (Supporting Information, Fig. S1). Raman spectrum demonstrated that the bis-addition of 1-octadecanethiol to SiO_2-$N(CO_2CH_2C{\equiv}CH)_2$ was the major reaction mechanism (Supporting Information, Fig. S2). As one molecule of primary amine could react with two molecules of propargyl acrylate and one molecule of alkyne reacts with two molecules of 1-octadecanethiol [19], SiO_2-$N(C18)_4$ possesses a branched architecture.

As the surface area of this batch of spherical silica was 159.8 m^2/g [24], the surface concentrations of 3-aminopropyl, propargyl acrylate hyperbranched and octadecyl groups on the silica support were calculated to be 7.04, 0.99 and 0.92 $\mu mol/m^2$, respectively [25]. The conversion ratio of 3-aminopropyl groups in the Michael addition and propargyl acrylate in the click chemistry was calculated to be 7.04 and 46.3%, respectively [25], thus, a great many 3-aminopropyl groups and propargyl acrylate remained on the stationary phase. As the yield of homogeneous Michael addition of *n*-butylamine to the methyl 2-acetamidoacrylate was only 41% after 192 h [26]. In addition, the Michael addition in this study was heterogeneous reaction, the solubility propargyl acrylate on the silica was lower than the homogeneous reaction and the steric hindrance of the heterogeneous reaction was larger than that of homogeneous reaction. Therefore, the conversion ratio of 3-aminopropyl groups and propargyl acrylate was reasonable.

3.2 Retention properties of the column

3.2.1 Effect of flow rate on column efficiency

The relationship between flow rate of mobile phases and the stationary phase can be depicted by the plot of flow rate versus the plate height. A proper flow rate not only made the column with high column efficiency but also saved the analysis time. As there were many polar groups on the column, polar compounds, including uridine, cytosine and cytidine, were separated on this column. The plot of flow rate versus the plate height can be obtained by changing the flow rate. As can be seen in Fig. 1, the optimal resolving power was obtained with u at 0.82 mm/s corresponding to the flow rate of 0.6 mL/min. To reduce the analysis time, a value at 1.37 mm/s was chosen, which corresponded to the flow rate of 1.0 mL/min.

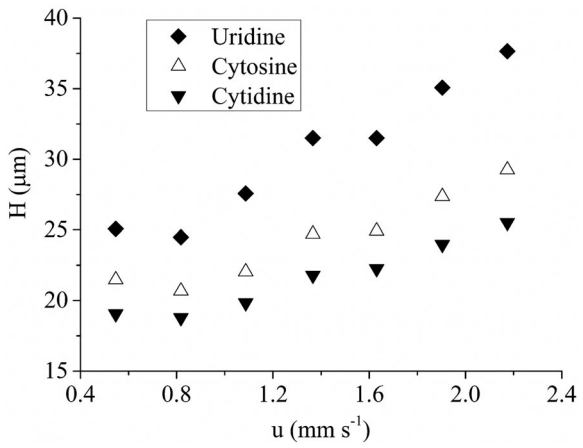


Figure 1. Plots of plate heights versus velocity *u*.

3.2.2 Effect of ACN concentration on retention

Octadecyl groups are known to have hydrophobic properties, therefore, it was expected that SiO_2-$N(C18)_4$ with octadecyl groups would exhibit hydrophobic properties. To investigate the hydrophobic property of SiO_2-$N(C18)_4$, benzene homologous compounds, such as benzene, toluene, ethylbenzene, *n*-propylbenzene and *n*-butylbenzene, were employed as test probes. The ratio of ACN in the mobile phase was changed from 10 to 30% v/v. Figure 2 showed the plots of logarithmic *k* (log *k*) versus the content of ACN in the mobile phase. The log *k* of all test probes linearly decreased with the increase of ACN content in the mobile phase, indicating a typical RP chromatographic retention mechanism. In addition, as seen in Fig. 2A, the slopes of alkylbenzenes were in accordance with the hydrophobicity of the test probes, also demonstrating RP separation mechanism on the SiO_2-$N(C18)_4$ column.

Inspired by the multiple polar groups on the SiO_2-$N(C18)_4$ column, we further evaluated the retention of phenols and amine analogues on this column. *m*-Trihydroxybenzene, 2,6-xylenol, *p-tert*-butylphenol and 2,4-dichlorophenol were separated on the SiO_2-$N(C18)_4$ column using a neutral mobile phase with ACN content at 20 or 30% v/v (Supporting Information, Fig. S3). The pK_a of these compounds was 8.45, 10.62, 10.39 and 7.89, respectively. 2,4-Dichlorophenol was slightly dissociated while others were largely undissociated in the neutral mobile

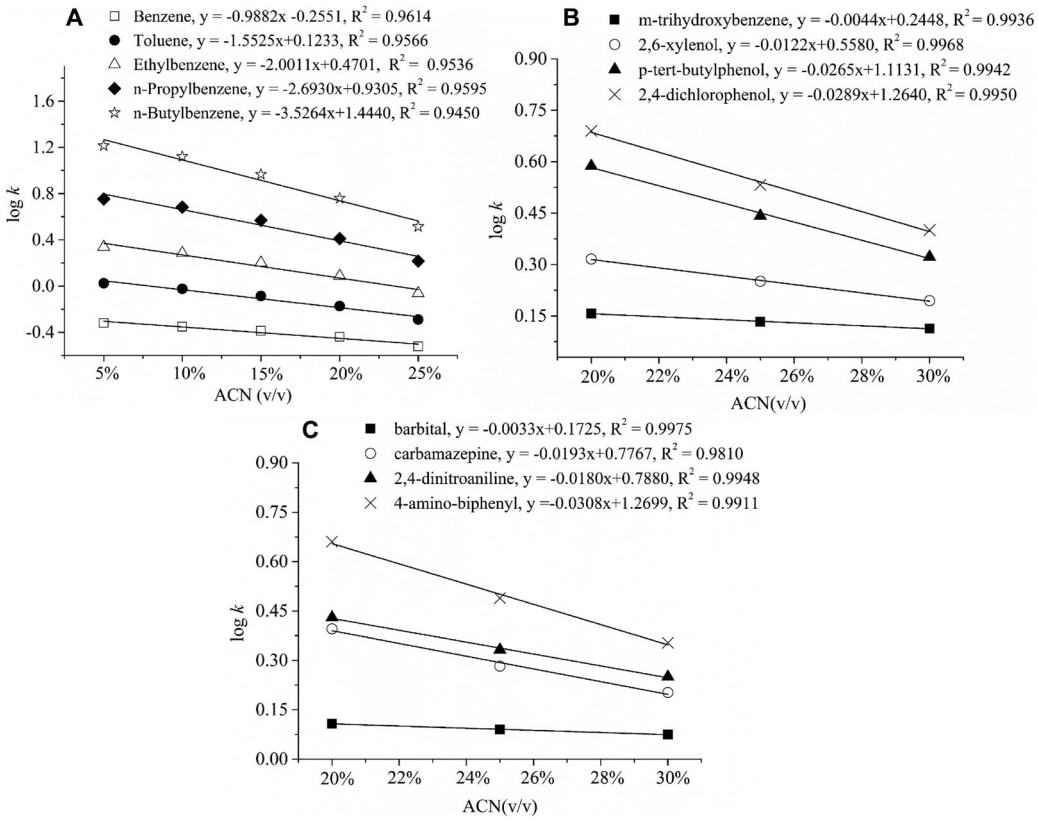


Figure 2. Plots of log *k* for (A) benzene homologous compounds, (B) phenols and (C) amino analogues versus ACN v/v in the mobile phase. Mobile phase, ACN and water. Column temperature, 20°C. Flow rate, 1.0 mL/min.

phase. The log *k* of phenols decreased linearly with the increase of ACN content in the mobile phase (R^2>0.993, Fig. 2B). This was indicative of a typical hydrophobic interaction appeared between the phenols and the stationary phase.

Amine analogues, including barbital (pK_a 8.1), carbamazepine (pK_a 15.83), 2,4-dinitroaniline (pK_a –4.25) and 4-amino-biphenyl (pK_a 4.26), were another probe mixture to evaluate the retention of the SiO_2-N(C18)$_4$ column (Supporting Information, Figures S4). Barbital and carbamazepine were slightly protonated, while 2,4-dinitroaniline and 4-amino-biphenyl were uncharged under the neutral mobile phase. The log *k* of analytes also reduced linearly under these mobile phases with an R^2>0.981 (Fig. 2C), which demonstrated that the separation of amine analogues under this mobile phase was mainly based on RP chromatographic retention mechanism. In addition, the peak shapes of these amine analogues were not peak tailing under the neutral mobile phases (Supporting Information, Fig. S4), which demonstrated that this novel stationary phase may show high potency in basic compounds separation.

3.2.3 Effect of the pH of mobile phase on retention

As mentioned above, some 3-aminopropyl groups remained on the surface of SiO_2-N(C18)$_4$ column, thus, it was deduced that the ion-exchange interaction of stationary phase would be generated. For this test, acid and basic compounds, including 1,3,5-benzenetricarboxylic acid, 4-carboxybenzaldehyde, 4,4'-methyldianiline and 4-aminobiphenyl, were selected as test probes. Meanwhile, mobile phases with different pH values (2.7, 4.0, 5.0, 6.0, 7.0 and 7.8) and sodium phosphate buffer concentrations (5, 10, 15 and 20 mM) were used to evaluate the retention of the test probes on the SiO_2-N(C18)$_4$ column. As seen in Fig. 3A, the retention factors (*k*) of 4,4'-methyldianiline and 4-aminobiphenyl slightly increased as the pH increased from 2.7 to 5.0, then tended to be stable at pH 6.0. This was related to the charge state of the analytes. The pK_a of 3-aminopropyl group, 4,4'-methyldianiline and 4-aminobiphenyl was 10.37, 5.32 and 4.35, respectively, and the protonation emerged at pH 2.7. The electrostatic repulsion between basic compounds and 3-aminopropyl groups became weak as the protonation of 4,4'-methyldianiline and 4-aminobiphenyl decreased at pH 5.0. Therefore, the retention factors of two basic compounds increased. When the pH of mobile phases was above 6.0, the basic compounds were not protonated, thus the retention factors tended to level off.

The retention factor of 4-carboxybenzaldehyde increased as the pH of mobile phase increased from 2.7 to 4.0, and then reduced with the mobile phase pH increased from 4.0 to 7.8. This phenomenon was also related to the pK_a of analytes (4-carboxybenzaldehyde, 3.77; 1,3,5-benzenetricarboxylic acid, 3.12). When the pH value of the mobile phase increased

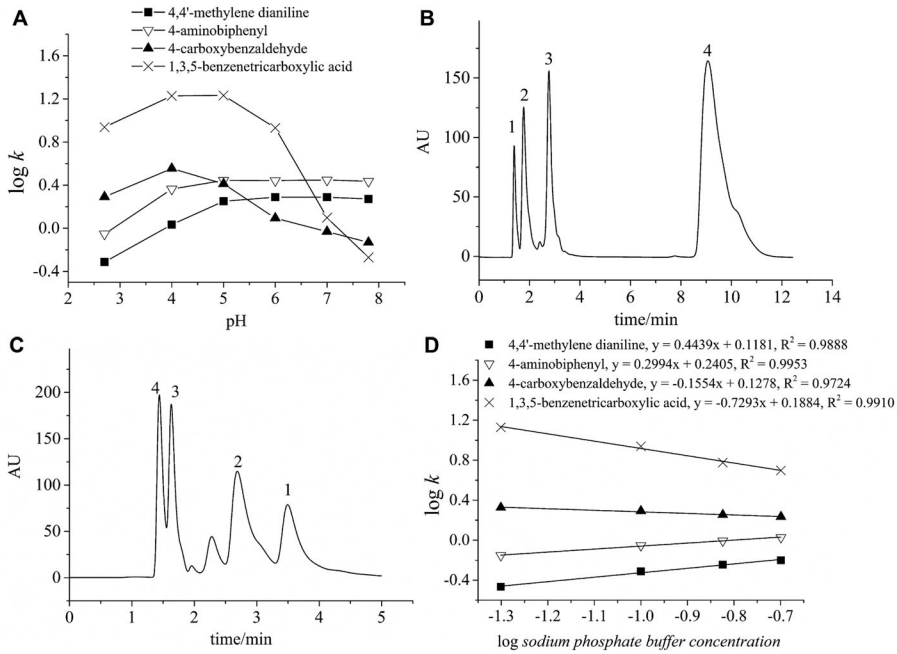


Figure 3. Plots of log *k* versus (A) pH and (D) log sodium phosphate buffer, chromatogram of a mixture of basic and acid compounds at (B) pH 2.7 and (C) pH 7.8. Mobile phases, (A) ACN and 10 mM sodium phosphate buffer, (B) ACN and 10 mM sodium phosphate buffer at pH 2.7, (C) ACN and 10 mM sodium phosphate buffer at pH 7.8 and (D) ACN and different concentrations of sodium phosphate buffer (5, 10, 15 and 20 mM) at pH 2.7. Column temperature, 20°C; flow rate, 1.0 mL/min. Peak identifications: (1) 4,4′-methyldianiline, (2) 4-aminobiphenyl, (3) 4-carboxybenzaldehyde and (4) 1,3,5-benzenetricarboxylic acid.

from 2.7 to 4.0, the dissociation of 4-carboxybenzaldehyde gradually emerged, and the electrostatic attraction of 4-carboxybenzaldehyde and 3-aminopropyl group appeared. Thus, the retention factor of 4-carboxybenzaldehyde increased. When the pH of mobile phase continued to increase, silanols on the stationary phase were gradually ionized, the ionized silanols tended to form hydrogen bond with 3-aminopropyl group [27], which weakened the interaction between 3-aminopropyl group and 4-carboxybenzaldehyde. Therefore, the retention factor of 4-carboxybenzaldehyde reduced. The variable trend of 1,3,5-benzenetricarboxylic acid was similar to that of 4-carboxybenzaldehyde, and the reason for this phenomenon was the same as that of 4-carboxybenzaldehyde. On the whole, the relationship between the retention factors of these analytes and the pH of mobile phase illustrated ion-exchange mechanism of the SiO_2-N(C18)$_4$ column. In addition, the order of the retention factors of the analytes at pH 2.7 was 1,3,5-benzenetricarboxylic acid > 4-carboxybenzaldehyde > 4-aminobiphenyl > 4,4′-methyldianiline, while the reverse order was observed at pH 7.8, which also demonstrated a typical ion-exchange mechanism of the stationary phase (Fig. 3B and C).

3.2.4 Effect of the buffer concentration of mobile phase on retention

As the pH and buffer concentration of mobile phase have an impact on the ion-exchange interaction, the influence of buffer concentration on ion-exchange mechanism could be confirmed by varying the sodium phosphate buffer concentrations (5, 10, 15 and 20 mM) of the mobile phase at a fixed pH such as pH 2.7. The effect of buffer concentrations on the retention of analytes was illustrated in Fig. 3D. It can be found that the dependence of log *k* of test probes on logarithmic buffer concentration was linear. The log *k* of 4-aminobiphenyl and 4,4′-methyldianiline linearly increased as the buffer concentration increased, while log *k* of 1,3,5-benzenetricarboxylic acid and 4-carboxybenzaldehyde linearly decreased in the same buffer concentration. 4-Aminobiphenyl (pK_a 4.35) and 4,4′-methyldianiline (pK_a 5.32) were protonated at pH 2.7, while 1,3,5-benzenetricarboxylic acid (pK_a 3.12) and 4-carboxybenzaldehyde (pK_a 3.77) were slightly dissociated. At this pH, high concentration of sodium phosphate buffer could weaken the electrostatic repulsion between the protonated basic compounds and the protonated 3-aminopropyl groups, thus, the retention of basic compounds enhanced. As the dissociated acids interacted with the protonated 3-aminopropyl groups through ionic interaction, higher concentrations of sodium phosphate buffer improved the eluting strength of the mobile phase, which led to a decrease in retention factors. In addition, the influence of the buffer concentration on the retention of the basic and acid compounds on this column was the same as that of 3-aminopropyl stationary phase [28], which also demonstrated that a great many 3-aminopropyl groups remained in this column contributed to the ion-exchange property. Most importantly, the slope of 4,4′-methyldianiline with two charges at pH 2.7 was larger than that of 4-aminobiphenyl with single charge, which further indicated the ion-exchange mechanism of the stationary phase.

3.3 Reproducibility of the column

The reproducibility of the SiO_2-N(C18)$_4$ was evaluated by analysis the RSD for the retention factor of 2,4-dinitroaniline. The run-to-run ($n = 3$) and day-to-day ($n = 4$)

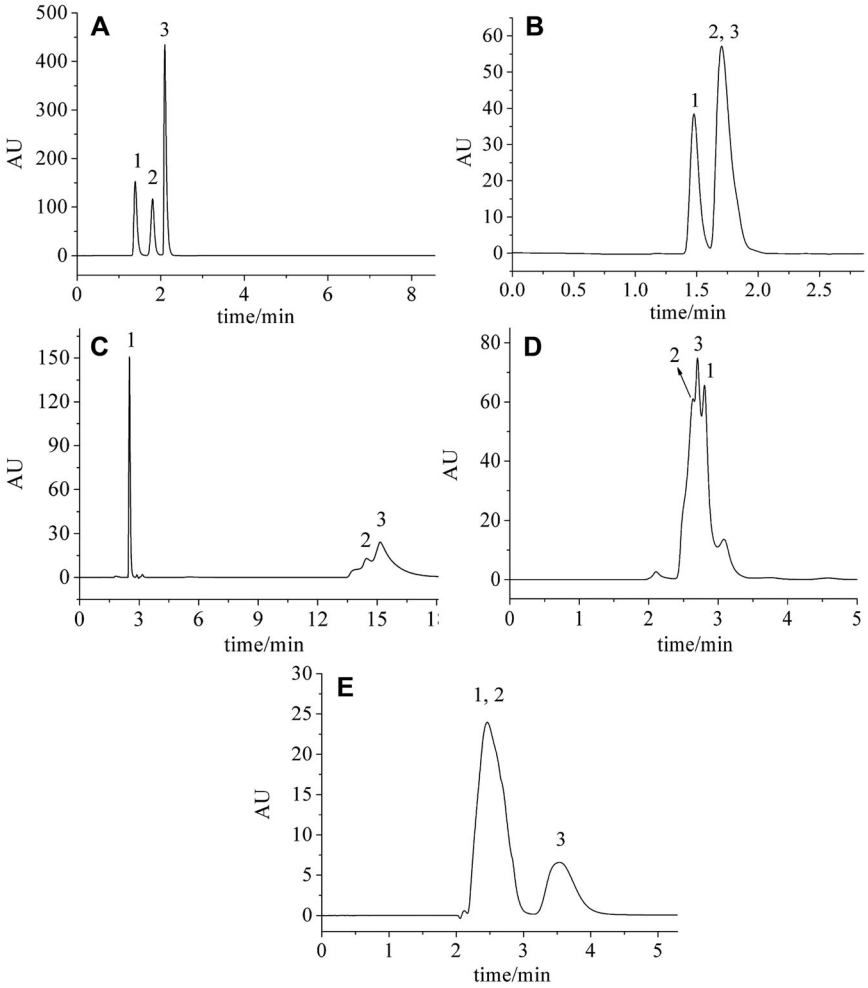


Figure 4. Chromatograms of separation of basic drugs mixture on (A) SiO_2-N(C18)$_4$, (B) and (C) Atlantis dC18 column, (D) SiO_2-NH_2 and (e) SiO_2-N($CO_2CH_2C{\equiv}CH)_2$. Mobile phases, (A) ACN/H_2O = 5:95 v/v, (B) ACN/H_2O = 50:50 v/v and (C) ACN/H_2O = 20:80 v/v, (D) ACN/H_2O = 95:5 v/v and (E) ACN/H_2O = 5:95 v/v. Column temperature, 20°C; flow rate, 0.9 mL/min. Peak identifications: (1) pindolol, (2) alpronolol and (3) propranolol.

reproducibilities were 1.96 and 2.59%, respectively. Several columns were prepared with the same conditions. The RSD value of the column-to-column ($n = 3$) and batch-to-batch reproducibilities ($n = 4$) were 2.65 and 6.40%, respectively. No obviously deterioration was observed after continuous use for 5 months, which demonstrated that the performance of column prepared by the sequential Michael addition and click chemistry was stable.

3.4 Applications

As mentioned above, the slow sorption–desorption interaction between the deprotonated silanols and the protonated bases always leads to peak tailing. The SiO_2-N(C18)$_4$ stationary phase with shielding groups (3-aminopropyl, tertiary amine, thioether and carboxylic ester) endcapped on the silica surface or embedded between the silica surface and the octadecyl group minimizes this interaction. In addition, the branched octadecyl groups made the mass transfer equilibrium can be achieved in a short time, which makes it especially suitable for analysis of basic compounds. Inspired by the no tailing peaks of amine analogues appeared in Fig. S4 (Supporting Information), a mixture of basic drugs with similar structure, such as pindolol, alprenolol and propranolol, was further separated on Atlantis dC18 column, SiO_2-NH_2, SiO_2-N($CO_2CH_2C{\equiv}CH)_2$ and SiO_2-N(C18)$_4$ stationary phases, respectively (Fig. 4). It can be seen from Fig. 4A that three basic drugs were baseline separated on SiO_2-N(C18)$_4$ phase using an isocratic mobile phase containing low content of ACN (5%). However, alprenolol and propranolol were coeluted on Atlantis dC18 column in an isocratic mobile phase with the content of ACN (50%) (Fig. 4B). As shown in Fig. 4C, reducing the content of ACN (20%) could improve the resolution and retention of these compounds on Atlantis dC18 column, however, peak tailing with high value of asymmetry factor was still occurred. As seen in Fig. 4D and E, the separation efficiency of SiO_2-NH_2 column for this mixture was inferior to that of SiO_2-N($CO_2CH_2C{\equiv}CH)_2$ under the optimized separation condition, which illustrated that Michael addition of propargyl acrylate to 3-aminopropyl groups on the SiO_2-NH_2 was beneficial for separation of basic compounds. The satisfactory separation on SiO_2-N($CO_2CH_2C{\equiv}CH)_2$ column could not be acquired even under the optimized condition, which demonstrated that the thiol-yne click chemistry was the key for success of the preparation protocol. The

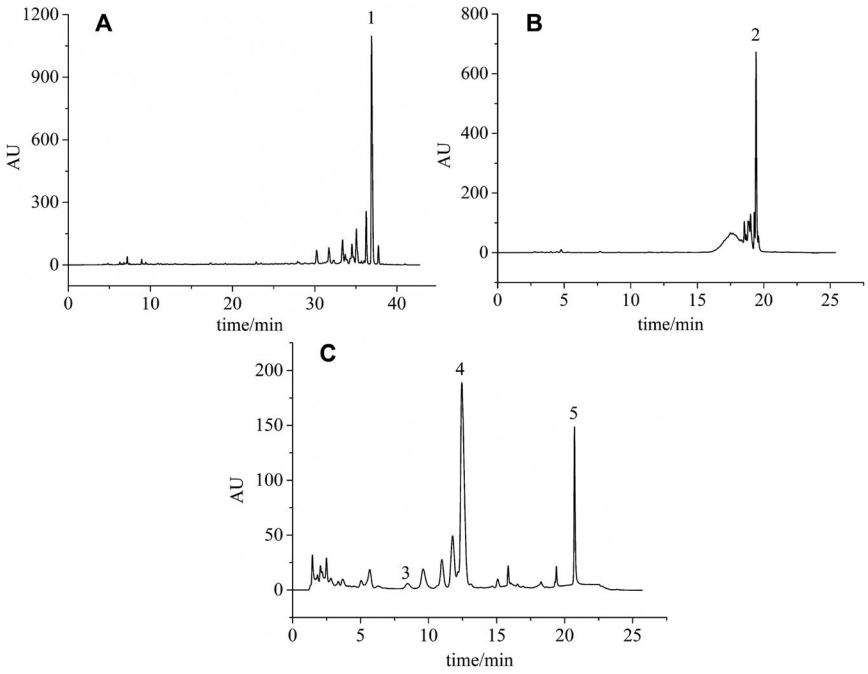


Figure 5. Chromatograms of separation of protoberberine alkaloids extracted from a traditional Chinese herbal medicine, *Corydalis yanhusuo* Wang, (A) and (B) on Atlantis dC18 column and (C) on SiO_2-N(C18)$_4$. Mobile phases, (A) 0.1% TFA water, (B) ACN; gradient for (a), 0–30 min, 0%→30% B; 30–40 min, 30%→60% B; (B) and (C), 0–5 min, 0%→3% B; 5–12 min, 3%→11% B; 12–23 min, 11%→90% B; injection volume, 15 μL; column temperature, 20°C; flow rate, 1.0 mL min^{-1}; UV detection, 254 nm. Peak identifications: (1) a mixture of dehydrocorydaline, coptisine, berberine and some protoberber alkaloids with the molecular weight of 352, (2) a mixture of palmatine, dehydrocorydaline, coptisine, berberine and some protoberber alkaloids with the molecular weight of 352, (3) berberine, (4) dehydrocorydaline and (5) palmatine.

different performance for basic compounds analysis could be attributed to the different structure of these stationary phases. At these neutral pH conditions, the silanols on the SiO_2-N(C18)$_4$ were dissociated, while the 3-aminopropyl groups (pK_a = 10.4), pindolol (pK_a = 8.8), alprenolol (pK_a = 9.6) and propranolol (pK_a = 9.7) were protonated. The positively charged 3-aminopropyl groups not only shielded the silanols from interacting with the analyte, but also repelled the analyte with same charged to be eluted in a short time. In addition, the shielding effect of the embedded polar groups included both hydrogen bonding between 3-aminopropyl groups and the residual silanols [27, 29] and π–π interaction between the polar groups (i.e. carbonyl groups of carboxylic ester and thioether) and the π active moieties on the aromatic moieties of the analytes [30, 31], which masked the undesirable effects during the separation process and improved the shape selectivity. The branched carbonyl groups on the silica were beneficial for mass transfer, which made the mass transfer equilibrium can be achieved in a short time and also reduced the analysis time.

The actual sample of water-extracts of *Corydalis yanhusuo* Wang was also separated on both SiO_2-N(C18)$_4$ and Atlantis dC18 column (Fig. 5). These extracts mainly consist of protoberberine alkaloids [32], which are always positively charged. As the a polar-copolymerized stationary phase C18HCE with positively charged surface was beneficial for the separation of alkaloids and basic macrolides [33, 34], TFA was added into the mobile phase for the protoberberine alkaloids separation. Figures 5 and C represented the chromatograms of the *Corydalis yanhusuo* Wang extracts separated on the Atlantis dC18 column and SiO_2-N(C18)$_4$ under the optimal chromatographic conditions, respectively. Although peaks shapes in Fig. 5A were symmetry, the number of baseline-separated peaks was far less than that in Fig. 5C. The highest intensity peak in Fig. 5A was further analyzed to be a mixture of dehydrocorydaline, coptisine, berberine and some protoberberine alkaloids with the molecular weight of 352 by the known standard samples and MALDI-TOF MS. However, the protoberberine alkaloids in this extract could be efficiently separated on SiO_2-N(C18)$_4$ (Fig. 5C). The separation conditions of Fig. 5B were the same as those of Fig. 5C. As shown in Fig. 5B, the alkaloids could not be eluted under the highly aqueous eluents, the main peak has also been identified to be a mixture of alkaloids by the known standard samples and MALDI-TOF MS, such as palmatine, dehydrocorydaline, coptisine, berberine and some protoberberine alkaloids with the molecular weight of 352. While this phenomenon was changed by increasing the content of organic solvent in the mobile phase. Unfortunately, the protoberberine alkaloids in this extract could not be efficiently separated on the Atlantis dC18 column. In addition, the number and shapes of baseline-separated peaks of *Corydalis yanhusuo* Wang water-extracts obtained by SiO_2-N(C18)$_4$ were superior to those of a single polar group endcapped reversed-phase C18 columns, such as C18HCE, Spursil EP C18 and TSKgel ODS-100 V [12]. These results also demonstrated that a variety of polar groups bonded C18 columns had better performance than those of a single polar group endcapped C18 columns in analysis of *Corydalis yanhusuo* Wang water-extracts.

4 Concluding remarks

A novel silica-based stationary phase with branched octadecyl groups (SiO_2-N(C18)$_4$) was successfully synthesized by Michael addition reaction and photoinduced thiol–yne click chemistry. The branched structure was beneficial for

interaction with analytes. Most importantly, this stationary phase with shielding groups (tertiary amine, thioether and ester) on the silica surface makes it especially suitable for analysis of basic compounds with mixed-mode separation mechanism. In the analysis of a mixture of three basic drugs, the SiO_2-$N(C18)_4$ stationary phase could baseline separate them with the mobile phase containing low content of ACN (5%). For the separation of *Corydalis yanhusuo* Wang water extracts, more baseline separation peaks were obtained on the SiO_2-$N(C18)_4$ stationary phase than those on Atlantis dC18 column. It is reasonable to believe that SiO_2-$N(C18)_4$ stationary phase will play an important role in the separation of other basic components, and the Michael addition combined with photoinduced thiol–yne click chemistry can also be extended to design other types of chromatographic materials.

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