Porous Organic Cage Embedded C18 Amide Silica Stationary Phase for High Performance Liquid Chromatography

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Reduced imine cage (RCC3) was first adopted for the preparation of porous organic cage embedded C18 amide silica stationary phase for high performance liquid chromatography. The prepared stationary phase was characterized by scanning electron microscope (SEM) and Fourier transformation infrared spectrum (FT-IR). Its chromatographic performance under reversed-phase mode was investigated in detail and compared with that of an ODS column. Multiple interactions, including hydrophobic interaction, π-π interactions, electrostatic interactions and hydrogen bonding, were involved due to the synergism of the C18 chain and RCC3. The column showed typical methylene selectivity and enhanced aromatic selectivity for nonpolar analytes while demonstrating high selectivity for polar analytes. In addition, the stationary phase showed the capability of separation of polar and hydrophilic compounds under per aqueous liquid chromatography mode (PALC), providing a green and economical way for the separation of polar and hydrophilic compounds. These results indicated the great application potential of the prepared stationary phase in the analysis of complex samples.

Keywords Porous organic cage, silica, stationary phase, reversed-phase liquid chromatography, per aqueous liquid chromatography

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chromatography yet to our knowledge. This may be due to the lack of proper functional groups to tether CC3 to the surface of a stationary phase and the relatively low stability of imine bonds under liquid chromatography conditions. It has been reported that the imine-linked CC3 could be converted into amine-linked RCC3 through one-step reduction reaction. Due to the high reactivity of amine, RCC3 could be conveniently and effectively converted into 12-arm organic building blocks for supramolecular chemistry through the reaction with desired acylchloride compounds, paving a way to functional derivatives of organic cage molecules.

In this study, an effort was made to explore the application of porous organic cages in liquid chromatography. RCC3 was synthesized and employed as embedded moiety for the preparation of C18 amide silica stationary phase for high performance liquid chromatography. The reversed-phase retention properties were investigated in detail using various nonpolar and polar analytes. In addition, the retention performance under per aqueous liquid chromatography mode was investigated for the separation of polar and hydrophilic compounds.

**Experimental**

**Reagents and materials**

Silica gel (with a particle size of 5 μm, a pore size of 100 Å and specific surface area of 290 m² g⁻¹) was purchased from Dalian Replete Scientific Instruments Co. Ltd. (Dalian, China). An ODS Acclaim™ 120 column (with a 5-μm average particle diameter, 15 cm×4.6 mm i.d., ThermoFisher Scientific, Waltham, MA, USA) was used as the reference column. Aniline, 3-nitroaniline, 2-nitroaniline, 1-naphthylamine, 2,4,6-trifluoroaniline, diphenylamine, deoxyxuridine, deoxyxytidine, deoxyinosine, deoxyguanosine, 9-(β-D-ribofuranosyl)guanine, cytosine, uracil, guanine, thymine and adenine were purchased from Aladdin Reagent Co., Ltd (Shanghai, China). We purchased 1,3,5-triformylbenzene (TFB) from Zhengzhou Alfachem Co., Ltd (Zhengzhou, China), while (R,R)-1,2-diaminocyclohexane, γ-chloropropyltrimethoxysilane (CPTS) and stearoyl chloride were obtained from J&K Scientific Ltd. (Beijing, China). Benzene, toluene, ethylbenzene, isopropylbenzene, n-butylbenzene, n-hexylbenzene, naphthalene, diphenyl, anthracene, fluoranthene, 1,2-benzanthracene, hydroquinone, catechol, 4-bromo-2-methylphenol, 2,5-xylene, phenol, 3-nitrophenol, 4-trifluoromethylphenol and 2-naphthol were obtained from Energy Chemical (Shanghai, China). HPLC grade methanol was used for the HPLC analysis. Ultrapure water was obtained from a MilliQ gradient ultrapure water system (Millipore Inc., Bedford, MA, USA). All other chemicals were of analytical grade and used without further treatment.

**Apparatus**

Scanning electron microscopy (SEM) analyses were performed on a Hitachi FE-SEM S-4800 (Tokyo, Japan). FT-IR spectra were recorded on a Tensor-37 FT-IR spectrometer (Bruker Optics, Ettlingen, Germany) in the wavenumber range of 600 – 4000 cm⁻¹ under ambient conditions, while an attenuated total reflection (ATR) accessory was employed for all IR spectral acquisitions. ¹H NMR spectra were recorded on a Bruker AVANCE III 400 MHz instrument (Bruker Biospin GmbH, Rheinstetten, Germany).

**Preparation of Sil-RCC3-C18 stationary phase**

RCC3 was synthesized according to the procedure described in Fig. 1A. The detailed synthesis process is described in the Supporting Information (Sect. 1) and the products were characterized by NMR (Figs. S1 and S2). The preparation of Sil-RCC3-C18 stationary phase is illustrated in Fig. 1B. Before preparation, SiO₂ was activated by hydrochloric acid/H₂O (1/1, v/v) solution with stirring for 24 h, then washed with deionized water to neutral and dried under vacuum at 120°C. The activated SiO₂ (4.00 g) was dispersed into anhydrous toluene (90 mL). To the suspension, GPTS (3.50 mL) was added under a nitrogen atmosphere. The mixture was refluxed with stirring at 110°C for 24 h, then washed with deionized water to neutral and dried under vacuum at 120°C. The activated SiO₂ (4.00 g) was dispersed into anhydrous toluene (90 mL). To the suspension, GPTS (3.50 mL) was added under a nitrogen atmosphere. The mixture was refluxed with stirring at 110°C for 24 h, then washed with deionized water to neutral and dried under vacuum at 120°C. The activated SiO₂ (4.00 g) was dispersed into anhydrous toluene (90 mL). To the suspension, GPTS (3.50 mL) was added under a nitrogen atmosphere. The mixture was refluxed with stirring at 110°C for 24 h, then washed with deionized water to neutral and dried under vacuum at 120°C. The obtained product was referred to as...
Sil-Cl (4.13 g). Subsequently, Sil-Cl (3.00 g) was mixed with chloroform (75 mL) and RCC3 (0.70 g). The mixture was refluxed with stirring for 36 h. After cooling, the mixture was filtrated. The collected silica particles, referred to as Sil-RCC3, was washed four times with chloroform and dried under vacuum at 40°C. Finally, Sil-RCC3 (2.82 g) was mixed with chloroform (80 mL) and triethylamine (1.3 mL). The mixture was placed in an ice bath with stirring. To this mixture, a solution containing stearoyl chloride (3.00 g) and chloroform (30 mL) was added dropwise and the stirring continued for a further 30 min under ice bath condition. The ice bath was then removed and the reaction continued for a further 4 h under room temperature. The temperature was then raised to 70°C and the reaction continued for a further 12 h. After cooling, the mixture was filtrated and the collected silica particles, referred to as Sil-RCC3-C18, were washed successively with chloroform and water several times and dried under vacuum at 40°C for 24 h.

Column packing
The Sil-RCC3-C18 stationary phase (2.50 g) was dispersed in carbon tetrachloride (40 mL) to make slurry by ultrasonication for 5 min. It was then packed into a stainless steel column (150 mm x 4.6 mm i.d.) under a pressure of 50 MPa for 30 min using methanol as propulsion solvent. Prior to the first separation, the columns were flushed with methanol at a flow rate of 1.0 mL min⁻¹ for 30 min.

Sample preparation and chromatographic conditions
Stock solutions (1 mg mL⁻¹) of the individual test analytes were dissolved in pure water, pure methanol or a mixture of water and methanol and stored at 4°C before use. The stock solutions were then diluted to desired concentrations with appropriate mobile phase to get the working solutions for HPLC injection.

All the chromatographic analyses were performed on an Agilent 1200 series HPLC system consisting of an Agilent Quat pump, a degasser, a Rheodyne 7725i sample injection valve equipped with a 5-μL loop, and a VWD UV detector. Chromatographic separations were performed at room temperature, at a flow rate of 1.0 mL min⁻¹. The mobile phases were filtered through a 0.22-μm membrane prior to use. The column dead time was obtained from the mobile phase signal in the UV detection.

Results and Discussion
Characterization of the Sil-RCC3-C18 stationary phase
The morphology of the Sil-RCC3-C18 stationary phase was characterized by SEM (Fig S3, see Sect. 2.1 in Supporting Information) while FT-IR analysis confirmed the successful preparation of the Sil-RCC3-C18 stationary phase (Fig. S4, see Sect. 2.2 in Supporting Information).

Reversed-phase chromatography
The reversed-phase chromatographic performance of the Sil-RCC3-C18 column was systematically evaluated by the separation of various probe analytes, including alkylbenzenes,
polycyclic aromatic hydrocarbons (PAHs), anilines and phenols, and the separation performance in terms of column efficiency (N/m), A_Pre , k , and R_Pre , were compared to that of an ODS column (Table 1).

Separation of alkylbenzenes

Alkylbenzenes are considered as model nonpolar solutes for determining the retention mode and the extent of hydrophobic interactions between the solutes and the chromatographic packing materials. As shown in Fig. 2, baseline separation of the six alkylbenzenes homologs was achieved with high selectivity on the Sil-RCC3-C18 column under methanol-water (60:40, v/v) (trace a in Fig. 2). The elution order, which followed the orders of hydrophobicity, was the same as that on the ODS column under methanol-water (90:10, v/v) (trace b in Fig. 2), implying that the separation mode on the Sil-RCC3-C18 column featured a reversed-phase mechanism. The column efficiency of the Sil-RCC3-C18 column (with N/m in the range of 5417 – 10595) was relatively lower than that of the ODS column (with N/m in the range of 35958 – 80494) and this might be due to the non-ordered distribution of C18 chains on the surface of Sil-RCC3-C18. The relationship of log k and the volume fraction of methanol in the mobile phase was studied for the six alkylbenzenes in detail. As shown in Fig. 3A, a good linear relationship (the R 2 values are greater than 0.982) was displayed for the log k of the alkylbenzenes versus the content of methanol (% v/v) in the mobile phase, which further confirmed the RP behavior of the Sil-RCC3-C18 column. Moreover, the methylene group selectivity of the Sil-RCC3-C18 column was measured by determining the relationship for log k against the carbon number according to the Martin equation:

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\log k = (\log \alpha)n_C + \log \beta
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Where log \(\alpha\) is the logarithmic methylene group selectivity, which can be calculated from the slope of the alkylbenzenes homologous series. As shown in Fig. 3B, it displayed a good linear relationship with all the R 2 values greater than 0.979 and the slopes ranged from 0.0521 to 0.1989 with the methanol content in the mobile phases (% v/v) varying from 80 to 50%. These results demonstrated that the Sil-RCC3-C18 column showed a typical RP mechanism and excellent methylene selectivity.

Separation of PAHs

Six nonpolar PAHs molecule were selected to further investigate the RP separation performance of the Sil-RCC3-C18 column. As shown in Fig. 4, baseline separation was obtained on the Sil-RCC3-C18 column under methanol-water (75:25, v/v) (trace a in Fig. 4) with symmetric peaks (A_Pre in the range of 0.91 – 1.05). In addition, naphthalene and diphenyl co-eluted on the ODS column under methanol-water (90:10, v/v) (trace b in Fig. 4), but they were efficiently separated on the Sil-
RCC3-C18 column with $R$ value of 1.13. Despite the fact that the plate count of the Sil-RCC3-C18 column was lower than that of the ODS column (Table 1), the Sil-RCC3-C18 column showed enhanced aromatic selectivity compared with the ODS column, due to the $\pi-\pi$ interactions between the PAHs and the aromatic moiety of the RCC3 cages.

Separation of anilines

Due to the presence of a polar amide bond in the stationary phase, separation of polar solutes is an important property for the Sil-RCC3-C18 column. So, anilines and phenols were selected as polar probes to test the separation performance of polar analytes. As shown in Fig. 5, six anilines were well separated with symmetric peaks (with $A_i$ in the range of 0.88 – 1.02) on the Sil-RCC3-C18 column under methanol-water (40:60, v/v) (trace a in Fig. 5) and showed different elution order from that of the ODS column under methanol-water (50:50, v/v) (trace b in Fig. 5). On the Sil-RCC3-C18 column, diphenylamine showed stronger retention than 2,4,6-trifluoroaniline, and this was mainly charged by the stronger $\pi-\pi$ interaction between diphenylamine and Sil-RCC3-C18. On the contrary, 2,4,6-trifluoroaniline showed stronger retention than diphenylamine on the ODS column and this was because the hydrophobic interaction was dominant on the ODS column. In addition, the full resolution of aniline from 3-nitroaniline ($R_s = 2.38$) was obtained on the Sil-RCC3-C18 column, while relatively poor resolution was observed on the ODS column ($R_s = 0.83$), despite the fact that the plate count of the ODS column was higher than that of the Sil-RCC3-C18 column (Table 1). And this further indicated that multiple interactions, including hydrophobic interaction, electrostatic interactions, $\pi-\pi$ interactions and hydrogen bonding might be involved in the Sil-RCC3-C18 stationary phase due to the functionalization with RCC3.

Separation of phenols

Eight polar phenols were also chromatographed on the Sil-RCC3-C18 and ODS columns to further gain insight into the retention property of polar analytes on the Sil-RCC3-C18 column. As shown in Fig. 6, baseline separation was achieved on the Sil-RCC3-C18 column under methanol-water (35:65, v/v) (trace a in Fig. 6) and the elution order was the same to that on the ODS column under methanol-water (50:50, v/v) (trace b in Fig. 6) while the Sil-RCC3-C18 column showed better peak symmetry ($A_i$ in the range of 0.65 – 1.27) than the ODS column ($A_i$ in the range of 0.54 – 1.49). It could be seen that hydroquinone and catechol, as well as 2,5-xylenol and 4-trifluoromethylphenol, could not be effectively separated on the ODS column ($R_s$ values were 0.53 and 0.37, respectively), while they were baseline separated on the Sil-RCC3-C18 column ($R_s$ values were 2.77 and 1.63, respectively). This could be attributed to the hydrogen bonding, electrostatic interactions and $\pi-\pi$ interaction provided by the RCC3 moieties. In addition, 2-naphthol and 4-bromo-2-methylphenol demonstrated split peaks on the ODS column and this might be due to the residual silanol groups which always exist in the ODS stationary phase. However, 2-naphthol and 4-bromo-2-methylphenol displayed symmetrical peaks on the Sil-RCC3-C18 column.
attributed to the polar functional groups on the surface of the Sil-RCC3-C18 stationary phase, which overcame the negative effect of residual silanol groups.

Per aqueous liquid chromatography

Per aqueous liquid chromatography (PALC) has been recognized as a green chromatographic mode, and features a polar stationary phase and water or water-rich mobile phase. It has been proven a useful alternative to hydrophilic interaction liquid chromatography (HILIC) for the separation of polar and hydrophilic compounds. The prepared Sil-RCC3-C18 stationary phase demonstrated certain polar properties, so that its chromatographic performance under PALC mode was investigated with an ODS column as reference column through the separation of a series of polar and hydrophilic analytes. As shown in Fig. 7, four deoxynucleosides were well resolved on the Sil-RCC3-C18 column within 10 min using water as the mobile phase (trace a in Fig. 7). Although deoxyuridine and deoxycytidine were not baseline separated, the peaks demonstrated high symmetry. In contrast, only two peaks corresponding to deoxyuridine and deoxycytidine appeared on the ODS column under the same conditions within even 120 min (trace b in Fig. 7). This great difference on the separation performance could be attributed to the hydrophilicity of the amide linkage of RCC3 functionalization, which endowed the Sil-RCC3-C18 stationary phase with a polar nature. As shown in Fig. S5 (Supporting Information), six nucleobases were well resolved on the Sil-RCC3-C18 column within 15 min (trace a in Fig. S5). However, only four peaks were observed on the ODS column within 60 min with coelution of cytosine and uracil (trace b in Fig. S5). These results further demonstrated the separation capability of the Sil-RCC3-C18 column under PALC mode. This may provide a green and economical way for the separation of polar and hydrophilic compounds and an opportunity for wide application of the Sil-RCC3-C18 column.

Conclusion

A novel porous organic cage embedded C18 amide silica stationary phase was prepared and characterized. Its chromatographic performance was investigated in detail. Multiple interactions, including hydrophobic interaction, π-π interactions, electrostatic interactions and hydrogen bonding, were involved due to the synergism of the C18 chain and RCC3 moieties. It showed excellent selectivity for nonpolar analytes and polar analytes. In addition, polar and hydrophilic compounds could be well resolved on the new stationary phase under per aqueous liquid chromatography mode, providing a green and economical way for the analysis of these analytes. Furthermore, axial chirality is an intrinsic property of the RCC cage, so it is foreseeable that this stationary phase can also be used as a chiral stationary phase for enantioseparation and this work is under way in our laboratory.
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Supporting Information

Synthesis and characterization of the porous organic cage and resulted stationary phase were shown in Supporting Information. This material is available free of charge on the Web at http://www.jsac.or.jp/analsci/.

References