Hyperbranched Polyglycerol Functionalized Silica Stationary Phase for Hydrophilic Interaction Liquid Chromatography

Hengye Li,* Xuemeng Zhang,* Lin Zhang,** Hui Cang,* Fenying Kong,* Dahe Fan,*† and Wei Wang*†

*School of Chemistry and Chemical Engineering, Yancheng Institute of Technology, No. 9 Yinbing Avenue, Tinghu, Yancheng 224000, China
**Yancheng Entry-Exit Inspection and Quarantine Bureau, No. 85 Kaifang Avenue, Yancheng 224000, China

Surface-initiated anionic-ring-opening multibranching polymerization was employed to prepare a hyperbranched polyglycerol (HPG) functionalized silica stationary phase for hydrophilic interaction liquid chromatography (HILIC). The obtained stationary phase was characterized by Fourier-transform infrared spectrometry (FT-IR) and thermogravimetric analysis (TGA). The chromatographic properties of the prepared stationary phase were systematically investigated. The abundance and multitude distribution of hydroxyl groups in HPG endowed the stationary phase with improved hydrophilicity and enhanced separation performance compared with the stationary phase functionalized with monolayer of hydroxyl groups. The stationary phase showed excellent retention of various polar compounds, such as nucleosides, neucleobases, phenols and sulfanilamides, indicating great potential in the separation of complex biosamples.

Keywords Hyperbranched polyglycerol, surface-initiated anionic-ring-opening multibranching polymerization, stationary phase, hydrophilic interaction liquid chromatography

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Introduction

Hydrophilic interaction liquid chromatography (HILIC) was firstly termed by Alpert in 1990 and it has been recognized as an alternative HPLC mode for the separation of polar compounds.1 Polar compounds are ubiquitous in complex biosamples, such as nucleosides in metabonomic samples, glycopeptides in proteinomic samples and saccharides in glycomics samples. HILIC is thus a promising alternative to traditional LC methods in bioanalysis.2,3

HILIC stationary phases have been developed rapidly with the growing popularity of HILIC.4-5 Silica gels modified with multitudinous polar functional groups, such as diol,6,7 amino,8 amide,9 zwitterionic moieties10,11 and saccharide,12 have been proposed as HILIC stationary phases for the separation of polar analytes13 and inorganic anions.14 Monoliths based HILIC stationary phases have also been developed.15 However, most of these stationary phases are usually modified with monolayer of functional groups with relatively poor hydrophilicity, which limits their application to complex samples.16 Compared with monolayer stationary phases, stationary phases functionalized with hydrophilic multilayer polymer have attracted more attention owing to their high hydrophilicity and large binding capacity.17,18 As for hydrophilicity modifications of stationary phases with polymers, “grafting from” polymerization has been proved to be an efficient approach. It overmatches other polymerization methods concerning the generation of polymer chains with high grafting densities from initiators on the substrate. Various approaches have been employed for “grafting from” strategy, such as surface-initiated ionic polymerization,19 atom transfer radical polymerization (ATRP),20,21 reversible-addition fragmentation chain transfer (RAFT) polymerization15 and click chemistry.22

Dendritic polymers (dendrimers and hyperbranched polymers), have highly branched structures and all bonds in dendritic polymers converge to a focal point or core.23 Theoretically, a branched polymer graft has higher hydrophilicity with the coated surface than that of its linear analogue at an equivalent grafting density.24 Compared with dendrimers, which usually require tedious multistep syntheses,25,26 hyperbranched polymers can be prepared in a one-pot synthesis, and can be a favorable alternative to dendrimers.27 Hyperbranched polymers show an advantage of an extremely high density of functional groups. Hyperbranched polymer functionalized stationary phases have been developed for HPLC applications in an affinity chromatography mode,28 a size exclusion chromatography mode29 and ion-exchange chromatography.30,31 Hyperbranched polymer was also adopted to prepare a HILIC stationary phase. Peng et al. prepared a novel polyethylenimine (PEI) functionalized HILIC stationary phase through a “graft to” method.32 The column showed excellent separation performance for polar analytes under the HILIC mode due to the hyperbranched structure of PEI. Exploring hyperbranched polymers for novel HILIC stationary phases is intriguing and significant for the development and application of HILIC. Hyperbranched polyglycerols (HPG) are highly hydrophilic hyperbranched polymers, due to these compact and globular structures with multitude distribution of hydroxyl groups down...
to the cavity levels. So, HILIC stationary phase functionalized with HPG would offer rich hydroxyl groups and a larger contact possibility with polar analytes relative to common hydroxyl stationary phases chemically bonded with small hydroxyl molecules. However, such an attempt has not yet been made.

In this work, we combined the merits of the “grafting from” strategy and HPG to prepare a HPG functionalized stationary phase for HILIC through surface-initiated anionic-ring-opening multibranching polymerization. The obtained stationary phase was well characterized, and its chromatographic characteristics under the HILIC mode was systematically investigated and compared with a control column with a monolayer of hydroxyl groups to assess the role of the HPG in the improved separation.

**Experimental**

**Reagents and materials**

Tris(hydroxymethyl)aminomethane (Tris), glycidol, potassium tert-butoxide (PTB), 3-chloropropyltrimethoxysilane (CPTMS), thymine, uracil, cytosine and guanine were purchased from J&K Scientific Ltd. (Beijing, China). SiO2 (with particle size of 5 μm, pore size of 100 Å and specific surface area of 290 m²/g) was provided by Dalian Replete Scientific Instruments Co. Ltd. (Dalian, China). 2,5-Dimethylphenol (DMP), pyrocatechol (PC) and 3-nitrophenol (NP) were obtained from Energy Chemical (Shanghai, China). Sulfadimethoxine (SDM), sulfamethoxypyridazine (SMP), sulfafurazole (SIZ) and sulfamethizole (SMZ) were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Methylthioadenosine (MA), uridine (U), adenosine (A), inosine (I), guanosine (G), thymidine (T), deoxycytidine (DU), deoxyguanosine (DA), deoxyuridine (DU), deoxycytidine (DC) and deoxyguanosine (DG) were obtained from Aladdin Reagent Co., Ltd (Shanghai, China). HPLC grade acetonitrile (ACN) was used for the HPLC analysis. Ultrapure water was prepared by reflux with sodium. All the other chemicals were of analytical grade without further treatment.

**Preparation of the SiO2@HPG stationary phase**

The target stationary phase was prepared according to the procedure shown in Fig. 1. Prior to use, SiO2 was activated in a hydrochloric acid/H2O (1/1, v/v) solution with stirring for 24 h. After the reaction, the SiO2 was washed thoroughly with deionized water to neutral pH and dried under a vacuum at 120°C for 24 h.

The activated SiO2 (6.5 g) was refluxed with CPTMS (7.0 mL) in anhydrous toluene (100 mL) with continuous stirring for 48 h under nitrogen. The product was centrifuged and washed successively with anhydrous toluene and anhydrous ethanol and dried under a vacuum at 40°C. The obtained product was termed as SiO2@Cl (6.7 g). SiO2@Cl (6.2 g) was weighed into DMF (80 mL) containing 9.0 g of Tris, and the suspension was heated at 80°C with stirring for 36 h. The resulting SiO2@Tris was collected by centrifugation and washed repeatedly with water until the aqueous supernatant was neutral. SiO2@Tris (3.2 g) was weighed into a round bottom flask equipped with a condenser and an oil bubbler. Dioxane (60 mL) containing PTB (1.5 g) was then added. The mixture was stirred and degassed with nitrogen for 30 min at 25°C. The mixture was stirred and degassed with nitrogen at 95°C. Glycidol (12 mL) was introduced dropwise under a nitrogen atmosphere and the reaction was continued for 24 h. After polymerization, the mixture was cooled down to room temperature. The SiO2@HPG was collected by centrifugation and washed repeatedly with water until the aqueous supernatant was neutral. The obtained material was dried under a vacuum at 40°C for 24 h.

**Characterization**

FT-IR spectra were obtained on a Tensor-37 FT-IR spectrometer (Bruker Optics, Ettlingen, Germany) in the wavenumber range of 600 - 4000 cm⁻¹ under ambient conditions. An attenuated total reflection (ATR) accessory was employed for all IR spectral acquisitions. Thermogravimetric analysis (TGA) was performed in a nitrogen atmosphere from room temperature to 1000°C with a heating rate of 10°C min⁻¹ (Netzsch, Selb, Germany).
Column packing
A slurry of SiO$_2$@HPG in methanol was prepared by ultrasonication for 2 min and packed into a stainless-steel column (150 × 4.6 mm) under a constant pressure of 50 MPa for 30 min with methanol as the pushing solvent. SiO$_2$@Tris packed column was prepared by the same procedure. Prior to chromatographic separation, the columns were conditioned 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 prepared in pure water, pure ACN or a mixture of water and ACN. They were separately diluted with the corresponding mobile phase to obtain working solutions for HPLC injection. All of the tested polar analytes and their chemical structures are shown in Table 1.

All of the chromatographic experiments were performed on an Agilent 1200 series HPLC system, which consisted of an Agilent Quat pump, a degasser, a Rheodyne 7725i sample injection valve equipped with a 5-$\mu$L loop, and a VWD UV detector. The flow rate was 1.0 mL min$^{-1}$. Mobile phases were filtered through a 0.22-$\mu$m membrane prior to use.

Results and Discussion
Characterization of the SiO$_2$@HPG stationary phase
FT-IR was applied to characterize the feasibility of the preparation procedure. The spectrums of activated SiO$_2$, SiO$_2$@Cl, SiO$_2$@Tris and SiO$_2$@HPG are shown in Fig. 2(A). In trace b, the peaks at 2980 and 2892 cm$^{-1}$ could be attributed to the C–H stretching vibration, while the peak at 1404 cm$^{-1}$ could be attributed to in-plane bending vibration of C–H. These results confirmed the graft of CPTMS onto the surface of SiO$_2$. In trace c, the peaks at 3672, 1388 and 649 cm$^{-1}$ could be attributed to the stretching vibration, in-plane bending vibration and out-of-plane bending vibration of O–H, respectively, while the peak at 1684 cm$^{-1}$ could be attributed to an in-plane bending vibration of N–H, confirming the attachment of Tris through nucleophilic substitution reaction. Compared with trace c, the intensities of the peaks in trace d at 3672, 2980, 2892, 1388 and 649 cm$^{-1}$ increased greatly while the intensity of the peak at 1684 cm$^{-1}$ remained almost unchanged, which was due to the production of HPG on the surface of the stationary phase. Thermogravimetric analysis (TGA) was carried out to further determine the graft of the HPG polymer on the surface of the silica particles. As shown in Fig. 2(B), the endothermic mass loss of the SiO$_2$@HPG stationary phase below 200°C was attributed to the loss of H$_2$O molecules adsorbed by SiO$_2$@HPG. From around 300°C, the SiO$_2$@HPG demonstrated a rapid mass loss, indicating that the stationary phase had good stability at as high as about 250°C. During 200 - 1000°C, 5.4 and 5.7% weight loss was observed for the SiO$_2$@Cl and SiO$_2$@Tris, respectively, while 13.2% weight loss was found for SiO$_2$@HPG, indicating that the HPG polymer was successfully grafted onto the surface of silica particles.

HILIC behavior evaluation
Cytosine and uracil were selected as test solutes to evaluate the HILIC behavior of SiO$_2$@HPG, and a comparison was made with SiO$_2$@Tris to assess the role of HPG. Uracil is less hydrophilic compared to cytosine, and the ratio $k_{\text{Cytosine}}/k_{\text{Uracil}}$ has been suggested to be a function of the ACN content as a HILIC behavioral index. As shown in Fig. 3(A), the $k_{\text{Cytosine}}$ increased greatly, while $k_{\text{Uracil}}$ increased slightly on SiO$_2$@HPG with

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<th>Functional group of analytes</th>
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increasing of ACN content. By comparison, \( k_{\text{cytosine}} \) and \( k_{\text{uracil}} \) increased marginally on SiO\(_2\)@Tris with an increase of the CAN content. In addition, the ratio \( k_{\text{cytosine}}/k_{\text{uracil}} \) for SiO\(_2\)@HPG increased much greater than that for SiO\(_2\)@Tris with the increase of the CAN content, indicating that SiO\(_2\)@HPG clearly exhibited the HILIC behavior. Figure 3(B) demonstrates that five nucleosides were well separated on SiO\(_2\)@HPG. By contrast, those nucleosides could not be effectively separated on SiO\(_2\)@Tris under typical HILIC conditions.

The effect of the ACN concentration on the retention of five...
hydrophilic nucleosides was further investigated. As shown in Fig. 4(A), the logarithmic retention factors ($k$) of the five nucleosides were plotted against the volume fraction of ACN in the mobile phase. Increasing retention factors were observed with increasing ACN content, demonstrating a typical HILIC retention characteristic.

Additionally, a quantitative HILIC model was studied based on Eq. (1),

$$\ln k = a + b \ln \phi + c \phi,$$

where $k$ is the retention factors ($a$, $b$, and $c$) are constants, and $\phi$ is the volume fraction of water in the mobile phase. As shown in Fig. 4(B), it demonstrated excellent fits (all of the regression coefficients ($r^2$) were in the range of 0.9916 - 0.9999) for the five nucleosides at five different mobile-phase compositions ($\phi$ in the range of 0.10 - 0.50). The result indicated that the retention on the SiO$_2$@HPG stationary phase was based more on a mixed-mechanism rather than a simple partitioning or adsorption process.

Chromatographic performance comparison between SiO$_2$@Tris and SiO$_2$@HPG

To further evaluate the properties of the SiO$_2$@HPG stationary phase, the performance of the SiO$_2$@Tris and SiO$_2$@HPG was compared using nucleobases, deoxynucleosides, phenols and sulfanilamides as probe analytes under the same chromatographic conditions.

Figure 5 depicts the separation of four nucleobases and a nucleoside on the SiO$_2$@Tris column and SiO$_2$@HPG column. The retention followed the polarity order, which was consistent with a typical HILIC retention mechanism, and the elution orders were the same on the two columns. The retention of the five analytes on the SiO$_2$@HPG stationary phase was based more on a mixed-mechanism rather than a simple partitioning or adsorption process.

Fig. 5 Separation of nucleobases and nucleoside on SiO$_2$@Tris column and SiO$_2$@HPG column. Mobile phase: ACN/H$_2$O (80:20, v/v), UV detection at 254 nm. Peaks: (1) thymine, (2) uracil, (3) cytosine, (4) guanine, (5) guanosine.

Fig. 6 Separation of deoxynucleosides on a SiO$_2$@Tris column and SiO$_2$@HPG column. Mobile phase: ACN/H$_2$O (80:20, v/v), UV detection at 260 nm. Peaks: (1) thymidine, (2) deoxyuridine, (3) deoxyadenosine, (4) deoxycytidine, (5) deoxyguanosine.

Fig. 7 Separation of phenols on a SiO$_2$@Tris column and SiO$_2$@HPG column. Mobile phase: ACN/H$_2$O (90:10, v/v), UV detection at 254 nm. Peaks: (1) 2,5-dimethylphenol, (2) pyrocatechol, (3) 3-nitrophenol.

Fig. 8 Separation of sulfanilamides on a SiO$_2$@Tris column and SiO$_2$@HPG column. Mobile phase: ACN/H$_2$O containing 0.1% formic acid (70:30, v/v), UV detection at 254 nm. Peaks: (1) sulfadimethoxine, (2) sulfamethoxypyridazine, (3) sulfafurazole, (4) sulfamethizole.
Chromatograms of three phenols are shown in Fig. 7: the three phenols could be well separated in the same eluting order on the two columns. On the SiO₂@Tris column, the peaks were broad and the theoretical plate numbers were 9454, 19115 and 6566 (N/m) for DMP, PC and NP, respectively. In contrast, the SiO₂@HPG column showed separation in a relatively shorter analysis time than the SiO₂@Tris column, and the theoretical plate numbers were improved to be 82455, 45613 and 152463 (N/m), respectively. In addition, the SiO₂@HPG column showed different selectivity from the SiO₂@Tris column (the selectivity factor αDMP/PC = kDMP/kPC = 1.61 on the SiO₂@HPG column versus αDMP/PC = 1.31 on the SiO₂@Tris column). The separation of four sulfanilamides on both columns is shown in Fig. 8 when the SiO₂@HPG column provided symmetrical peaks (asymmetry factor in the range of 1.01 – 1.16) and good selectivity with efficiencies of 21378 – 50016 (N/m). For comparison, the four sulfanilamides could not be separated and coeluted as one broad peak on the SiO₂@Tris column. From the above comparisons, it was obvious that the modification of HPG on the surface of SiO₂@Tris could greatly improve the separation performance, providing improved peak shape and column efficiency as well as different selectivity towards various polar analytes.

Conclusions

In summary, a hyperbranched polyglycerol (HPG) functionalized silica stationary phase was prepared for HILIC analysis in a broad and the theoretical plate numbers were 9454, 19115 and 6566 (N/m) for DMP, PC and NP, respectively. In contrast, the SiO₂@HPG column showed separation in a relatively shorter analysis time than the SiO₂@Tris column, and the theoretical plate numbers were improved to be 82455, 45613 and 152463 (N/m), respectively. In addition, the SiO₂@HPG column showed different selectivity from the SiO₂@Tris column (the selectivity factor αDMP/PC = kDMP/kPC = 1.61 on the SiO₂@HPG column versus αDMP/PC = 1.31 on the SiO₂@Tris column). The separation of four sulfanilamides on both columns is shown in Fig. 8 when the SiO₂@HPG column provided symmetrical peaks (asymmetry factor in the range of 1.01 – 1.16) and good selectivity with efficiencies of 21378 - 50016 (N/m). For comparison, the four sulfanilamides could not be separated and coeluted as one broad peak on the SiO₂@Tris column. From the above comparisons, it was obvious that the modification of HPG on the surface of SiO₂@Tris could greatly improve the separation performance, providing improved peak shape and column efficiency as well as different selectivity towards various polar analytes.

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References