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# A remarkable enhancement of selectivity towards versatile analytes by a strategically integrated H-bonding site containing phase†

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**A double β-alanylated L-glutamide-derived organic phase has been newly designed and synthesized in such a way that integrated H-bonding (interaction) sites make it very suitable for the separation of versatile analytes, including shape-constrained isomers, and non-polar, polar and basic compounds. The β-alanine residues introduced into two long-chain alkyl group moieties provide ordered polar groups through H-bonding among the amide groups.**

The fabrication of nanoscale organic structures can be obtained from the most powerful base materials, such as amino acids. Amino acid derivatives can form molecular gels through self-assembly, as our group has observed in studies with L-glutamic acid derivatives.[1] These gels have attracted interest because gelation is induced by the formation of a three-dimensional network of nanofibrillar aggregates that are based on highly ordered structures with a unique chirality.[2] For instance, dialkyl L-glutamide-derived amphiphilic lipids form nanotubes,[3] nanohelices,[4] and nanofibers[1] based on bilayer structures in water through intermolecular hydrogen bonding among the amide moieties. Another unique form of self-organization has been realized by the lipophilic derivatives of L-glutamide, even in organic solvents.[5] Previously, we reported the application of L-glutamide-derived gel-forming compound-grafted silica amphiphilic materials as stationary phases for reversed-phase liquid chromatography (RPLC).[6] We found a very high molecular-shape selectivity for the separation of shape-constrained nonpolar isomers of polycyclic aromatic hydrocarbons (PAHs), β-carotene, and tocopherols in RPLC compared to most commonly used conventional octadecylsilylated silica (ODS) and $C_{30}$ phases.[6] The shape selectivity[7] of an alkyl chain based stationary phase depends on the alkyl chain ordering, with higher-ordered alkyl chains giving a higher shape selectivity due to the higher bonding density,[7,8] longer alkyl chains,[9] and a lower column temperature for the separation of nonpolar isomers.[10] However, the separation of basic and highly polar analytes using RPLC with alkyl phases continues to challenge chromatographers due to peak tailing, phase collapse in a highly aqueous mobile phase, and poor retention and selectivity.[11]

To address one of the above-mentioned limitations (or boundaries), a number of strategies have been developed based on multi-mode or mixed-mode interactions.[12] Mixed-mode chromatography (MMC) has emerged as a possible alternative approach to the use of single-mode chromatographic columns for the separation of diverse mixtures of ionic, polar, and nonpolar solutes.[13] One of the limitations of the mixed-mode (RPLC/HILIC, hydrophilic interaction chromatography) stationary phase is that it is not able to separate shape-constrained isomers in RPLC due to a lack of shape selectivity properties.[13,14]

In this work, we have designed and synthesized a new L-glutamide-derived amphiphilic silica material by introducing two β-alanine (β-Ala) residues into two long-chain alkyl group moieties, as shown in Fig. 1. Multiple functional groups (6 amide groups/multiple interaction sites) were integrated into the organic phase and immobilized onto silica. To construct ordered functional groups on the silica surface, the introduction of β-Ala residues into two long-chain alkyl group moieties was very effective for the amphiphile because it created an environment suitable for intermolecular and intramolecular H-bonding. This newly prepared stationary phase not only showed enhanced shape selectivity towards PAHs and β-carotene (shape-constrained isomers)[7] in RPLC, instead of lower grafting density, but could also be used to separate polar nucleosides and bases, sulfa drugs, peptides, and salicylamide and its acids in HILIC mode separation with high efficiencies, irrespective of their containing long alkyl chains. Additionally, a test mixture of nonpolar, polar, and basic compounds was also successfully separated in RP mode.

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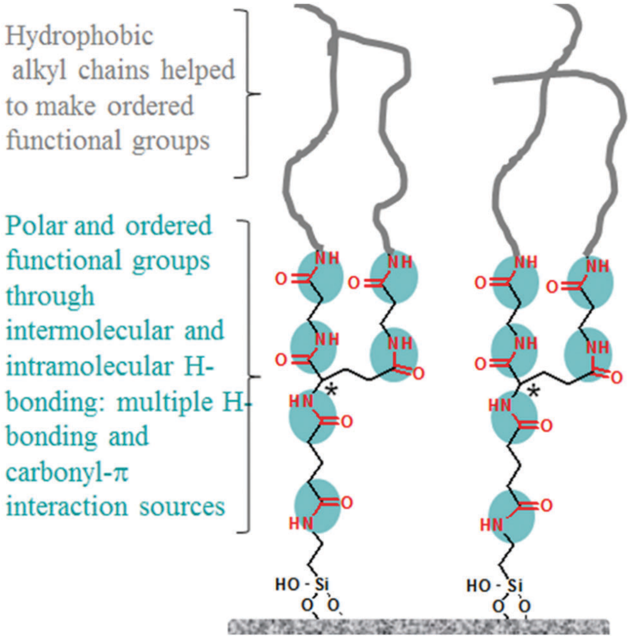

Fig. 1 Schematic illustration of the newly designed balanced polar and nonpolar functional moieties that are very suitable for mixed-mode chromatography.

The synthesis of the amphiphilic compound **6** and its immobilization onto silica to prepare the new stationary phase (Sil-Amphi**6**) are shown in Scheme S1 (ESI†). In brief, an L-glutamide-derived amphiphilic silica material was synthesized from *N*-benzyloxycarbonyl-L-glutamic acid through alkylation, debenzyloxycarbonylation, and a ring-opening reaction with glutaric anhydride, and was grafted onto aminopropyl-modified silica (Sil-APS) *via* an amide coupling reaction. The particle size of the silica used to be 5 μm. Here, we introduced β-Ala residues into two long-chain alkyl group moieties to enhance the hydrogen bonding sites to make ordered and denser functional groups in the phase (Sil-Amphi**6**). The materials were characterized by elemental analysis, thermogravimetric analysis (TGA) (Fig. S1, ESI†), diffuse reflectance infrared Fourier transform (DRIFT) (Fig. S2, ESI†), solid-state $^{13}$C CP/MAS NMR, and suspended-state $^{1}$H NMR measurements. The Tanaka column characterization protocol was also applied from a chromatographic characterization standpoint for RPLC[15] and HILIC[16] mode separations. From the elemental analysis results of Sil-APS and Sil-Amphi**6**, we obtained (%C 8.33), (%H 2.23), (%N 2.81) and (%C 12.63), (%H 2.89), (%N 2.47), respectively. The surface concentrations of Sil-APS and Sil-Amphi**6** were calculated according to the previously reported method.[7,8] The amounts of aminopropyl and amphiphilic moieties attached to the silica surface were calculated to be 7.02 and 0.22 μmol m$^{-2}$ for Sil-APS and Sil-Amphi**6**, respectively. Solid-state $^{13}$C CP/MAS NMR was carried out at variable temperature (20–50 °C).

Solid-state $^{13}$C CP/MAS NMR spectroscopy reveals that in Sil-Amphi**6**, the alkyl chains are arranged in a less ordered *gauche* conformational form, and no alteration to the ordered *trans* conformation could be observed within the temperature range 20–50 °C (Fig. S3, ESI†).[17] Suspended-state $^{1}$H NMR is another method to determine the structure and dynamics of the alkyl chains.[18] Suspended-state $^{1}$H NMR of Sil-Amphi**6** in methanol (Fig. S4, ESI†) was measured at room temperature. The intensities of the terminal methyl and methylene groups of the octadecyl moieties indicate the mobility or disorder of the alkyl chains, and the results agree with the results of solid-state $^{13}$C CP/MAS NMR spectroscopy.[17,18] After a complete characterization of the amphiphile-modified silica (Sil-Amphi**6**), it was packed into a stainless steel column for chromatographic evaluation. Aminopropyl-functionalized silica (Sil-APS) was also packed to be used as a reference column for evaluation of the interaction mechanism in HILIC mode separation.

Sil-Amphi**6** showed a RP mode retention similar to that of the ODS phase (Fig. S5, ESI†). To facilitate the assessment of shape selectivity for different chromatographic materials, Sander and Wise developed Standard Reference Material (SRM 869b), a test mixture which is composed of three shape-constrained PAHs (phenanthro[3,4-*c*]phenanthrene, PhPh; 1,2:3,4:5,6:7,8-tetrabenzonaphtalene, TBN; and benzo[*a*]pyrene, BaP). The ratio or selectivity factor ($\alpha$) of the retention factors ($k$) for TBN and BaP (*i.e.*, $\alpha_{TBN/BaP} = k_{TBN}/k_{BaP}$) provides a measure of shape selectivity that is useful for column intercomparisons, and $\alpha$ depends on the relative retention of a nonplanar solute (TBN) and a planar solute (BaP). The test provides a numerical assessment of the shape selectivity, so we can classify whether the stationary phase is monomeric (with reduced shape recognition properties or higher $\alpha$ values than 1.0), polymeric (with enhanced shape recognition properties or lower $\alpha$ values than 1.0) or intermediate (intermediate shape recognition properties).[19] At any specific temperature, more densely bonded stationary phases (*e.g.*, polymeric phases) exhibit enhanced shape recognition compared with less densely bonded phases (*e.g.*, monomeric phases).[7,8] Irrespective of its polymeric type phase and high bonding density, Sil-Amphi**6** (0.22 μmol m$^{-2}$) showed a high shape selectivity ($\alpha_{TBN/BaP}$ values 0.42 to 0.92 at 10 °C to 50 °C, respectively) and polymeric-like behaviour, while C$_{30}$ (1.66 μmol m$^{-2}$) showed a low shape selectivity ($\alpha_{TBN/BaP}$ values 0.51 to 1.80 at 10 °C to 50 °C, respectively) and a monomeric-like property at temperatures above 30 °C, as shown in Fig. 2. Fig. 3 shows a typical chromatogram for the separation of SRM869b at 15 °C on C$_{30}$ and Sil-Amphi**6**. A similar investigation was carried out on the separation of triphenylene (planar) and *o*-terphenyl (nonplanar) to characterize their shape selectivity properties. Although these two compounds possess the same number of carbon atoms and π-electrons, their molecular planarity is completely different. Therefore, the separation factor between them should be a good marker to evaluate their molecular-planarity selectivity. We observed that Sil-Amphi**6** showed a high molecular planarity recognition ability compared with ODS and C$_{30}$ as well as *o*-terphenyl isomer separation ability (Fig. S6, ESI†).

It was confirmed that the longer chain C$_{30}$ bonded phases permit excellent separation of carotenoids and tocopherols compared to ODS or C$_{18}$ sorbents.[9] We have utilized β-carotene isomers as another class of rigid, extended solutes to evaluate the selectivity of the new mixed-mode phase (Sil-Amphi**6**). The Sil-Amphi**6** phase successfully separated major isomers of β-carotene (*trans*, 13-*cis*, 9-*cis*) even better than C$_{22}$, which was designed as a shape-selective RP phase (Fig. S7, ESI†).[20] Detailed chromatographic

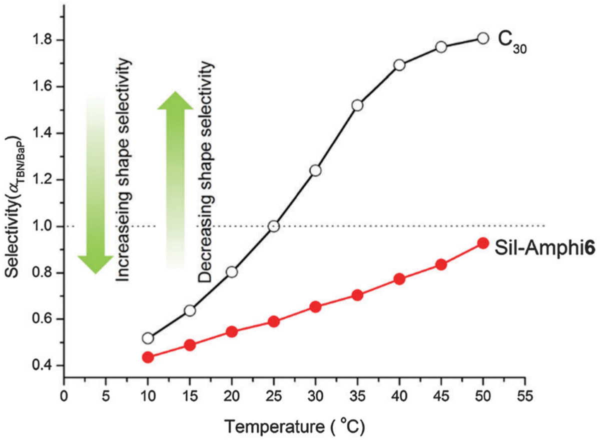

Fig. 2 Phase selectivity ($\alpha_{TBN/BaP} = k_{TBN}/k_{BaP}$) plotted as a function of temperature with $C_{30}$ and Sil-Amphi**6** columns. Mobile phase: methanol (100%); flow rate = 1 mL min$^{-1}$; detection: $\lambda$ = 254 nm.

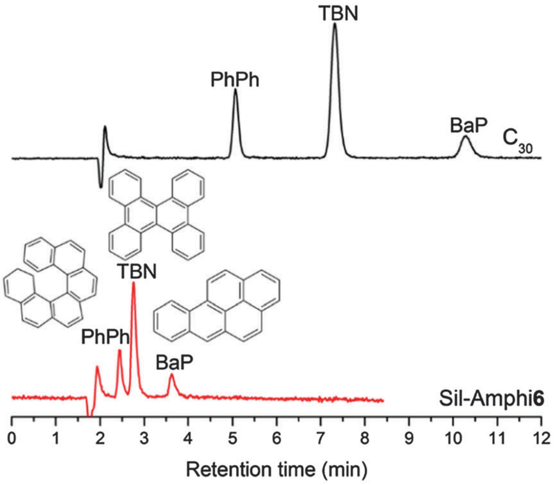

Fig. 3 Separation of SRM869b on $C_{30}$ and Sil-Amphi**6** columns. Mobile phase: methanol (100%); column temperature = 15 °C; flow rate = 1 mL min$^{-1}$; detection: $\lambda$ = 254 nm.

characterization of the Sil-Amphi**6** phase was performed in terms of its surface coverage, hydrophobic selectivity, shape selectivity, hydrogen bonding capacity, and ion-exchange capacity at pH 2.7 and 7.6 for RPLC according to the Tanaka characterization protocol and compared with ODS (Table S1, ESI†).[15]

This column can also be used to separate a test sample containing a mixture of nonpolar, polar, and basic compounds (uracil, propranolol, butyl paraben, dipropyl phthalate, naphthalene, amitriptyline, and acenaphthene) in the RP mode (Fig S8, ESI†).

The amphiphile**6**-grafted silica phase (Sil-Amphi**6**) contains six polar (amide) groups, making it a highly polar head group. This knowledge leads us to apply the stationary phase in HILIC mode separation. Nucleic acid bases and nucleosides are commonly used as test probes in HILIC mode separations.[21] The first investigation was carried out with the polar compounds (bases and nucleosides) (Fig. S9, ESI†) to evaluate the selectivity and separation efficiency of the Sil-Amphi**6** separation material (Fig. 4). The eight bases and nucleosides (small compounds) were successfully separated with high column efficiency. Detailed chromatographic parameters are given in Table S2, ESI.† The chromatographic results were compared with the reference columns (Sil-APS, HILIC-Amide, and HILIC-Diol). The Sil-APS column was not able to separate 8 nucleobases, and the elution orders were completely different, indicating a dissimilar interaction mechanism with the Sil-Amphi**6** phase. To evaluate the HILIC mode separation properties of Sil-Amphi**6** in detail, the content of acetonitrile was varied from 80 to 95% while keeping the pH and concentration of ammonium acetate buffer solution constant, 6.76 and 10 mM, respectively.

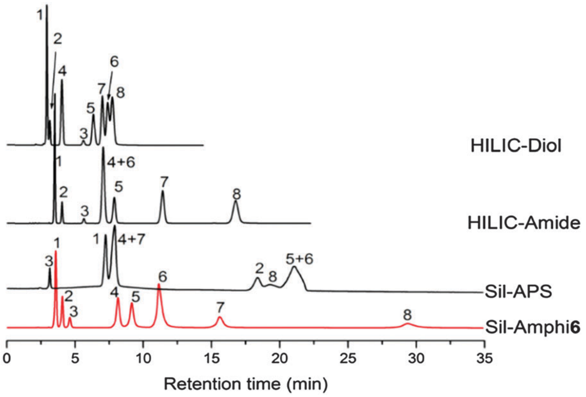

Fig. 4 Separation of eight bases and nucleosides on Sil-Amphi**6**, Sil-APS, HILIC-Amide, and HILIC-Diol. Mobile phases: 10 mmol L$^{-1}$ ammonium acetate/acetonitrile (5 : 95, v/v) for Sil-Amphi**6** and (10 : 90, v/v) for Sil-APS, HILIC-Amide, and HILIC-Diol, respectively; column temperature = 25 °C; flow rate = 1 mL min$^{-1}$; detection: $\lambda$ = 254 nm.

Fig. S10 (ESI†) shows the effect of the acetonitrile contents on the retention behavior of the investigated analytes and reveals that the retention factors of all selected nucleosides and nucleobases increase with an increase of the acetonitrile content. This trend is similar to that of typical HILIC retention characteristics.[22] The successful separation of the set of sulfa drugs,[23] pentapeptides[24] and a group of small polar acidic compounds[25] was achieved using the Sil-Amphi**6** phase (Fig. S11–S13, ESI†). Furthermore, characterization of Sil-Amphi**6** was carried out in terms of the degree of hydrophilicity, the selectivity for hydrophilic–hydrophobic substituents, the regioselectivity and selectivity for configurational differences in hydrophilic substituents, the selectivity for molecular shapes, the evaluation of electrostatic interactions, and the evaluation of the acidic–basic nature of the stationary phases using nucleoside derivatives, phenyl glucoside derivatives, xanthine derivatives, sodium *p*-toluenesulfonate, and trimethylphenylammonium chloride as a set of test samples (Fig. S14; Tables S3–S8, ESI†).[16]

As was discussed about the high separation ability of the Sil-Amphi**6** phase in both RPLC and HILIC mode separations, we cannot say the interaction mechanism is similar to that for the conventional RPLC[6–8] or HILIC[22] phases because to show a high shape selectivity or to separate shape-constrained isomers, it is necessary to have high concentration of alkyl chains or longer alkyl chains and also a low column temperature in RPLC.[7–10] However, the surface coverage of Sil-Amphi**6** is very low

(0.22 μmol m$^{-2}$) and has disordered alkyl chains, while the surface coverage of $C_{30}$ was calculated to be 1.66 μmol m$^{-2}$. On the other hand, HILIC phases do not usually contain hydrophobic long alkyl chains,[22] while Sil-Amphi**6** does contain long alkyl chain moiety. Therefore, the design of the stationary phase was important. The introduction of β-Ala residues into two long-chain alkyl group moieties was very effective for the amphiphile to make ordered functional groups through H-bonding. As shown in Fig. 1, Sil-Amphi**6** contains balanced hydrophilic and hydrophobic moieties, multiple H-bonding sources,[26] and multiple carbonyl-π interaction sources.[6] Although the alkyl chains were disordered, the ordered functional groups facilitated multiple carbonyl-π interactions with the solutes. Six functional groups (amide) played a key role in the separation of nonpolar and shape-constrained isomers in RPLC and polar compounds in HILIC. Previously developed mixed-mode phases did not show such high shape selectivity in RPLC due to the lack of multiple interacting functional groups.[13,14] In HILIC, the primary mechanism of retention is postulated to be partitioning of the analyte into the water-rich organic layer and the moving organic rich-eluent.[22] However, adsorption, ion-exchange, dipole–dipole interaction, hydrogen bonding,[27] π–π, and n–π interactions[28] also contribute to retention on the HILIC stationary phases. In Sil-Amphi**6**, because the surface coverage was very low, many aminopropyl functionalities remained unreacted. Thus, ion-exchange interactions were supposed to be the major interaction. However, Sil-APS a showed reversed elution order with the Sil-Amphi**6** for the separation of ionic compounds (Table S7, ESI†). Mixed interactions (adsorption, dipole–dipole interaction, hydrogen bonding, carbonyl-π and n–π) contributed on Sil-Amphi**6** due to the presence of polar multiple amide groups in the stationary phase. Interactions from multiple amide groups as well as aminopropyl functionalities made the Sil-Amphi**6** phase very suitable for mixed-mode liquid chromatography.

In summary, we have demonstrated the synthesis, characterization, and applications of an octadecyl-β-alanyl double-alkylated L-glutamide-derived stationary phase in both RPLC and HILIC mode (mixed-mode) separation. In RPLC, the new phase showed very high shape selectivity towards shape-constrained isomers. In HILIC, it also showed a high separation ability with high efficiency. This improved performance is based on the presence of highly hydrophobic moieties and highly hydrophilic moieties (interaction sites) in the same phase, which is induced by hydrogen bonding between amide groups. Therefore, the versatile performance of the new phase leads to a breakthrough in the field of analytical chemistry and materials science dealing with separation.

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