Molecular Shape Selectivity through Multiple Carbonyl–π Interactions with Noncrystalline Solid Phase for RP-HPLC

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A new approach for the synthesis of double-alkylated L-glutamide-derived stationary phases to use in RP-HPLC is described. TEM observation of lipid distearylglutamide (DSG) showed the formation of fibrous aggregates in methanol or in chloroform through intermolecular hydrogen bonding among the amide moieties while dibutylglutamide (DBG) cannot aggregate in aqueous or organic media due to its lower order of short alkyl chain. DSG and DBG were covalently bonded to silica via amino–propyl linkages. Lipid membrane analogues (e.g., DSG) attached to the silica surface have been found in noncrystalline and solid states and can form supramolecular assemblies with specific properties based on their highly ordered structures in aqueous and organic media. 13C CP/MAS NMR and suspension (in methanol)-state 1H NMR, elemental analysis, and DSC measurements were used to characterize Sil-DSG and were compared with the three other octadecyl phases, i.e., monomeric C18, polymeric C18, and silica grafted poly(octadecyl acrylate) Sil-ODA25. The chromatographic behavior of the new RP material was investigated using detailed retention studies of planar and nonplanar polyaromatic hydrocarbons (PAHs) and nonpolar aromatic positional isomers. Aspects of shape selectivity were also evaluated with Standard Reference Materials 869a, Column Selectivity Test Mixture for Liquid Chromatography. Detailed chromatographic study revealed that Sil-DSG showed extremely enhanced molecular shape selectivity compared with the other phases investigated. The higher molecular shape selectivity obtained by Sil-DSG can be explained by a carbonyl π (present in lipid-grafted stationary phases)—benzene π (present in guest PAHs) interaction mechanism, and these interactions are more effective for ordered carbonyl groups.

The evolution of liquid chromatography as a modern analytical technique is due in part to advances made in column technology. Chemical modification of silica packing materials remains a popular approach for achieving novel solute selectivity in high-performance liquid chromatography (HPLC).1−4 Most progress in reversed-phase (RP)-HPLC separation has been achieved due to the introduction of numerous silica-based stationary-phase materials. However, there is still no universal stationary-phase material suited to the specific properties of all possible solutes. Alkylamide phases show interesting chromatographic properties,5−8 which are ascribed to the participation in the separation process of different interaction sites, e.g., residual, unreacted silanols, and unreacted amine groups as well as hydrophobic chains with amine groups. In addition, the affinity of these phases to solute molecules differs significantly from that observed for conventional alkyl phases. These advantages have special importance under hydro-organic conditions in binary or ternary mobile phases. Therefore, intermolecular interactions determining elution of solutes of different character on alkylamide phases are interesting from both practical and theoretical points of view. On the other hand, we have proposed that self-assembled systems such as lipid membrane aggregates can provide a highly ordered microenvironment leading to unique host–guest chemistry exceeding the functions of the original lipid. Earlier we reported about the use of poly(octadecyl acrylate)-grafted silica (Sil-ODA), a lipid membrane analogue, as stationary phase for RP-HPLC.9–11 Sil-ODA showed unique separation behaviors with ordered-to-disordered phase transitions of long alkyl chains. In particular, extremely high selectivity toward polycyclic aromatic hydrocarbons (PAHs) was observed in the ordered (crystalline) state,12−15 and the combination of chromophoric diasteriomeric reagents yielded better...
selectivity for enantiomers than conventional C18 phases. In general, better separation of PAH isomers can usually be achieved with stationary phases prepared by polymeric surface modification chemistries (compared with monomeric surface modification). Other factors have been shown to influence shape recognition in LC, including stationary-phase bonding density, alkyl-phase chain length, and column temperature, and increased shape recognition is due to increased phase order brought on by higher densities, lower temperatures, and longer alkyl-phase chain length. Our detailed investigations showed that the highly ordered structure in Sil-ODA\textsubscript{25} induced the orientation of carboxyl groups that work as a π-π interaction source with solute molecules. We have also found that the aligned carbonyl groups are effective for recognition of length and planarity of PAHs through multiple π-π interactions. In this regard, we focused on amino acid derivatives, especially L-glutamic acid-derived lipid membrane analogues. It is well known that the lipophilic L-glutamic acid-derived systems exhibit specific interactions for various fields such as catalysis, sensor technology, materials science, and separation science. We had an attempt to utilize these special amphiphiles in separation science especially in HPLC stationary phases. Considering all these facts, we synthesized two silica-based organic phases (one with short and the other with long alkyl chains) as lipid membrane analogues.

Dialkyl L-glutamide-derived amphiphilic lipids form nanotubes and nanohelices based on bilayer structures in water and on the fact that intermolecular hydrogen bonding among the amide moieties contributes to self-assembly. Similar self-organization has been realized by lipophilic derivatives of L-glutamide even in organic solvents. The unique properties exhibited by these self-assemblies can be explained by ordered-to-disordered phase transition, phase separation behavior, and enhancement of the chirality. This paper reports a new approach for the synthesis of a new stationary phase from an L-glutamide-derived lipid membrane analogue based on covalent immobilization. The detailed investigation of chromatographic behavior toward PAHs and the characterization of this newly developed phase will be discussed in this paper.

### EXPERIMENTAL SECTION

**Materials.** The silica-supported L-glutamide-derived stationary phases diestyrylglutamide (Sil-DSG) and dibutylglutamide (Sil-DBG) were synthesized, characterized, and packed into stainless steel column (250 x 4.6 mm i.d.). A YMC silica (YMC SIL-120-S5 having diameter 4.4 \( \mu \)m, pore size 12.4 nm, and surface area 339 m\(^2\) g\(^{-1}\) (YMC-gel, Kyoto, Japan) was used in both cases. Poly(octadecyl acrylate) (Sil-ODA\textsubscript{25}) uses YMC silica gel, 5\( \mu \)m diameter, pore size 12.0 nm, surface area 300 m\(^2\) g\(^{-1}\) containing 15.7% C in the bonded ligand, was prepared and characterized. For spectral (NMR) analysis, we have synthesized both monomeric (13.8% C, 2.7% H, surface coverage by octadecyl moiety was found to be 2.5 \( \mu \)mol m\(^{-2}\) or 638 \( \mu \)mol g\(^{-1}\)) and polymeric (23.3% C, 4.3% H with surface coverage 4.9 \( \mu \)mol m\(^{-2}\) or 1078 \( \mu \)mol g\(^{-1}\)) C\textsubscript{18} grafted silica phases using YMC gel. In contrast, we have used two commercial monomeric and polymeric C\textsubscript{18} columns for chromatographic analysis. The monomeric C\textsubscript{18} column (Inertsil, ODS 3, column size 250 mm x 4.6 i.d. with particle size 5.5 \( \mu \)m, pore size 10 nm, and surface area of silica particles 450 m\(^2\) g\(^{-1}\)) was purchased from G. L. Science (Tokyo, Japan). This contains 13.8% C in the bonded octadecyl phase. The polymeric C\textsubscript{18} column (Shodex, C18 P, particle size 5 \( \mu \)m, pore size 10 nm, surface area 300 m\(^2\) g\(^{-1}\) with end cap of the unreacted silanol group) containing 17.5% C was obtained from Shodex (Tokyo, Japan).

**Preparation of L-Glutamide-Derived Lipid Grafted Silica.** The synthesis scheme of lipid diestyrylglutamide or DSG and the immobilization process of the lipid membrane analogue on to silica is shown in Figure 1. The chemical structures of these compounds were identified by melting point measurements, FT-IR, \(^1\)H NMR, and elemental analysis.

**N,N'-Diocetyl-N-benzoyloxy carbonyl-L-glutamide (2).** N-Benzoyloxy carbonyl-L-glutamic acid (L-Glu (Z)) or 1 (4.0 g, 1.42 x 10\(^{-2}\) mol), stearylamine (8.6 g, 2.99 x 10\(^{-2}\) mol), and triethylamine (4.4 g, 4.3 x 10\(^{-2}\) mol) were dissolved in THF (400 cm\(^3\)). The solution was cooled to 0 °C, diethylphosphorocyanidate (DEPC) (5.8 g, 3.3 x 10\(^{-2}\) mol) was added to the solution, and the resultant mixture was stirred for 1 h at this temperature. After being stirred for 1 day at room temperature, the solution was concentrated in vacuo, and the residue was dissolved in 350 cm\(^3\) of chloroform. The solution was washed with 10% NaHCO\textsubscript{3}, 0.1 M HCl, and water. The solution was dried over Na\textsubscript{2}SO\textsubscript{4}, concentrated in vacuo, and finally recrystallized from ethanol, which gave white solid powder: yield 10.69 g (96%); mp 133–135 °C; \( \nu_{\text{max}} \) (KBr)/cm\(^{-1}\) 3296, 3094, 1690, 1644, 1539; \(^1\)H NMR (CDCl\textsubscript{3}) \( \delta \) 0.85–0.90 (t, 6H, CH\textsubscript{3}(2)), 1.2–1.6 (m, 6H, CH\textsubscript{2}(CH\textsubscript{3})(2)), 1.85–2.20 (2H, \( \text{CH}_{2}\text{CH}_{2}\text{CH}_{2}\text{CO} \)), 2.30–2.45 (2H, \( \text{CH}_{2}\text{CH}_{2}\text{CO(O)NH} \)), 3.20–3.40 (4H, \( \text{CH}_{2}\text{NHC(O)CH}_{2} \)), 4.00–4.40 (2H, \( \text{CH}_{2}\text{NHC(O)CH}_{2} \)), 4.90–5.20 (2H, \( \text{CH}_{2}\text{NHC(O)CH}_{2} \)).

Figure 1. Reaction schemes for the synthesis of (a) dioctadecyl L-glutamide (DSG) lipid membrane analogue, (b) immobilization of lipid on to silica gel, and (c) and (d) structure of Sil-DBG and Sil-ODAₙ.

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**Immiscibility of Lipid Membrane Analogue (DSG) on to Silica.** (3-Aminopropyl)trimethoxysilane (APS) grafted silica (Sil-APS) was prepared by refluxing porous silica gel (3.0 g) and 1.5 cm³ of APS in toluene for 20 h. After successive washing with toluene, ethanol, and diethyl ether, the particles were dried in vacuo. The dried particles were characterized by elemental analysis (H, 2.2; C, 8.4; N, 2.6%). Sil-APS was then coupled with lipid 4. Sil-APS or 5 (3.0 g) and lipid 4 (3.0 g, 3.92 × 10⁻³ mol) were taken in 100 cm³ of dry THF and stirred. DEPC (1.5 g, 9.6 × 10⁻³ mol) and TEA (1.1 g, 10.6 × 10⁻³ mol) were added to the solution and stirred at 60 °C. After being stirred for 1 day, the grafted particles were washed with hot THF and hot chloroform several times to remove the unreacted lipid molecule and dried in vacuo. The phase transition behavior of lipid (solid or in suspension with methanol or ethanol) and the stationary phases (both as solid powder and in suspension of methanol and ethanol) were done by differential scanning calorimetry (DSC) measurement.

**Transmission Electron Microscopy (TEM).** Transmission electron micrographs were recorded by using a JEOL 2000FX. The samples were spotted on carbon-coated copper grids (200 Å). After excess of the samples was removed by a tissue paper and air-dried, they were stained with 2 wt % molybdate.

**Differential Scanning Calorimetry (DSC).** DSC was carried out using EXTRA 6000 with DSC6200 from Seiko Instruments Inc.

**Spectroscopic Measurements.** Conformational structures and the mobility of alkyl chain of the stationary phase were determined by measuring suspension-state ¹H NMR and solid ¹³C CP/MAS NMR spectra. NMR spectra was measured by Varian UnityInova AS400 at a static magnetic field of 9.4 T using nanoprobe GHX for suspension-state NMR and solid probe for CP/MAS NMR as spin rate of 2000–3500 Hz for suspension-state NMR and 4000–
RESULTS AND DISCUSSION

Brief Description on Dialkyl-l-glutamide-Derived Lipid Bonded Stationary-Phase Synthesis. Double-alkylated l-glutamidine-derived derivatives DSG and DBG were synthesized from N-benzoxycarbonyl-l-glutamic acid through alkylation, debenzyl-oxycarbonylation, and ring-opening reaction with glutaric anhydride. The lipid DSG containing three amide groups per molecule and THF), which exhibited a thermoreversible gel-to-sol phase transition behavior with concentration as low as 2 mmol L⁻¹. In our previous studies, we have reported that dialkyl l-glutamide-derived lipid with long alkyl chain formed highly oriented structures with chiral arrangements below T<sub>c</sub> and that the molecular assembly is brought about by complementary hydrogen bonds between the neighboring molecules in the lipid. On the other hand, DBG cannot form gel in aqueous or in organic media even at higher concentrations (100 mmol L⁻¹) due to the lower ordering ability of the short alkyl chain. TEM observation of lipid DSG and DBG in methanol, chloroform, and toluene showed that DSG forms fibrous aggregates whereas DBG cannot form aggregates in aqueous or in organic solvents. The aggregation of the lipid molecule in organic solvents can make the carbonyl group in a highly ordered state by forming hydrogen bonding between the amide moieties.

The lipid molecules were immobilized onto APS grafted silica (SIL-APS) by covalent linkages (amide bond via aminopropylsilica as shown in Figure 1) using DEPC. The elemental analysis results for SIL-APS, Sil-DSG, and SilDBG are shown in Table 1. The C/N value of Sil-APS is 3.11, which indicates that almost all of the methoxy groups of APS were consumed for silanation to silica, for cross-linking, or for both. The surface coverage with APS was calculated from the carbon percentage (C%) of Sil-APS to be 7.84 μmol m⁻². The percentage of carbon in Sil-DSG was found to increase from 8.41 to 20.1 after the grafting process. Surface coverage and alkyl chain densities of different stationary phases were calculated by using the equations given below, these equations were also mentioned in our recent paper.

The molar amount of organic phase per 1 g silica (M) can be calculated as

\[ M (\mu \text{mol g}^{-1}) = 10^6 (P_c/100)/12n \quad (1) \]

where \( P_c \) is the percentage of carbon element according to elemental analysis and \( n \) is the number of carbons present in the grafted organic phases.

The weight percentage of the grafted phase (\( P_w \)) in each case can be calculated as

\[ P_w = m \times 10^{-4}M (n/n_1) \quad (2) \]

where \( m \) is the molecular mass and \( n_1 \) is the number of carbon in each molecule of the organic phases grafted onto silica surface.

\[ \text{Table 1. Elemental Analysis Data of Sil-APS and Lipid-Grafted Stationary Phases} \]

<table>
<thead>
<tr>
<th></th>
<th>% C</th>
<th>% H</th>
<th>% N</th>
<th>C/N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sil-APS</td>
<td>8.40</td>
<td>2.20</td>
<td>2.70</td>
<td>3.11</td>
</tr>
<tr>
<td>Sil-DSG</td>
<td>20.1</td>
<td>3.10</td>
<td>2.98</td>
<td>6.74</td>
</tr>
<tr>
<td>Sil-DBG</td>
<td>13.5</td>
<td>2.58</td>
<td>2.69</td>
<td>5.10</td>
</tr>
</tbody>
</table>

References:


Furthermore, the surface coverage \( N \) can be calculated as

\[
N \text{ (mol m}^{-2}\text{)} = \frac{M}{S[(100 - P)_r/100]} = 10^9P_C/[12nS(100 - P_w)] \tag{3}
\]

where \( S \) is surface area of 1.00 g of nonmodified silica.

The alkyl chain density can be calculated as

\[
D \text{ (mol m}^{-2}\text{)} = Nf \tag{4}
\]

where the factor \( f \) indicate the number of alkyl chain present in

the grafted molecule. For \( C_{18} \), it is considered as 1, for Sil-ODA,

\( f \) is degree of polymerization (in this case \( f = 25 \)), and for

\( \alpha \)-glutamide-derived dialkyl stationary phase \( f = 2 \).

It is obvious that only monomeric \( C_{18} \) (1.72\, \text{mol m}^{-2}) showed

a lower alkyl chain density than Sil-DSG (2.13\, \text{mol m}^{-2}). The

suspension of Sil-DSG and Sil-DBG in methanol or in ethanol

showed no phase transition in thermal analysis using DSC. We

have reported previous work that significant phase transition of

poly(octadecyl acrylate) was observed even after a terminal group

was grafted onto the silica surface (Sil-ODA).\(^{13,14}\) This is due to

a small perturbation on the silica surface because only one side

of the polymer main chain is chemically bonded, allowing the

polymer side chain to remain flexible. For Sil-DSG and Sil-DBG,

the lipid molecule was directly connected to the silica surface,

and thus, the mobility is strongly restricted.

**Suspension-State \(^1H\) NMR Spectroscopic Measurements.**

Although half-height width and transverse relaxation time \( T_2 \)

are important indicators of molecular mobility of phases, these

variables were not found to be dependable in our study because

the NMR intensity of methylene groups was too small to obtain

a reliable measurement. The NMR peak is a superimposition of

several methylene groups with different relaxation times that flaws

the relation between the measured \( T_2 \) and the mobility on a

theoretical basis. Therefore, we turned to the rather new but

simple approach of determining the percentages of octadecyl

moieties in liquid-type mobility in each case. The form of

motionally averaged Hamiltonian depends very strongly on the

type and the time scale of molecular motion and, hence, on the

phase of the matter. In liquid- or suspension-state NMR, only those

molecules or parts of molecules with very fast rotational motions are

detectable. Motion must be in such a fast range that it can

average out dipolar coupling and chemical shift anisotropy until

related NMR peaks become narrow enough to be detected. The

suspension-state \(^1H\) NMR of four different stationary phases were

measured from 20 to 50 °C. Neither half-height width (line width)

of methylene groups nor spin–spin relaxation time \( T_2 \) showed

any significant change with temperature (20–50 °C). We observed

that intensity of the NMR peaks representing terminal methyl and

methylene groups of octadecyl moieties increased significantly

in Sil-ODA\(_{25}\). In monomeric \( C_{18} \), the intensity of the NMR peaks

representing terminal methyl and methylene groups increased

slightly, but in polymeric \( C_{18} \), only the peak of methylene groups

detectable when a very high vertical scale was used for

graphical presentation. On the other hand, Sil-DSG also showed

similar increases, but lower than those for monomeric \( C_{18} \).

**Figure 2.** Temperature dependencies of normalized relative \(^1H\)

NMR intensities of methylene groups of different stationary phases in

(a), suspension-state \(^1H\) NMR spectra of Sil-ODA\(_{25}\) at 20 °C in

(b), 50 °C in (c), and \(^1H\) NMR spectra of Sil-DSG at 50 °C in (d).

Intensities, to be comparable among all phases at various tem-

peratures, are weighted according to the following formula.

\[
I \text{ (mol}^{-1}\text{)} = \frac{I_m/I_D}{(4 \times 10^{-3}M)} = 250 \left[ I_m/(I_D M) \right]
\]

\( I \), the normalized intensity (relative intensity of NMR signal per

1\, \text{mol of octadecyl moiety} is as shown in Figure 2. \( I_m \) is the

underpeak area of the methylene peak, \( I_D \) is the intensity of the

few protons belonging to methanol, and \( M \) is the amount of grafted

organic molecule per 1\, g of modified silica. Normalized intensity

of \(^1H\) NMR peak of methylene groups on Sil-ODA\(_{25}\) begins to

increase distinctly at ~35 °C, but no similar trends are observed

for Sil-DSG and \( C_{18} \) phases. This corresponds to the crystalline-

to-isotropic phase transition of side alkyl chain of Sil-ODA\(_{25}\).

The mobility of methylene groups was strongly restricted in crystalline

state. The order of normalized intensity is polymeric \( C_{18} \) <

Sil-ODA\(_{25}\) < Sil-DSG < monomeric ODS at 20 °C, but above the

phase transition temperature of Sil-ODA\(_{25}\) the order changed to

polymeric \( C_{18} \) < Sil-DSG < monomeric ODS < Sil-ODA\(_{25}\).

Compared with Sil-ODA\(_{25}\), the normalized intensities of other

silla-supported polymers increased slightly and showed no

bending point in the temperature range from 20 to 50 °C. These

results indicate that the organic phases on the silica surface of

all stationary phases are in a solid state at room temperature, but

the mobility of methylene groups in organic phase is different

for each stationary phase. Since only Sil-ODA\(_{25}\) shows remarkable

enhancement of mobility, it is assumed that direct immobilization

does not allow long alkyl chain-containing molecules to exist in

liquid state.

**\(^{13}C\) CP/MAS NMR Spectroscopic Measurements.** Solid-

state NMR spectroscopy is a powerful tool for evaluation of the

chemical composition and conformational properties of chemically

modified surfaces. \(^{13}C\) CP/MAS NMR spectra were acquired for

Sil-DSG, Sil-ODA\(_{25}\), and monomeric and polymeric \( C_{18} \) phases. The

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intense signal at ~30 ppm is attributed to the methylene carbons with long alkyl chains in the bonded organic phase. The signals of aminopropyl spacer appear at 10, 17, and 41 ppm. The signal at 176 ppm results as a consequence of the peptide bonds among the glutamimoiety. To investigate the conformations of the n-alkyl units in the grafted chain, 13C CP/MAS NMR spectra were recorded. Under the conditions of magic angle spinning and dipolar coupling of protons, the chemical shift of 13C NMR spectrum depended largely on the conformation of alkyl chains. 13C CP/MAS NMR spectra of the octadecyl-containing phases used in this study are compared in Figure 3. It is well known that the 13C signal for (CH2)n carbon atoms is observed at two resonances, one at 32.6 due to trans conformation, indicating rigid and ordered chains, and the other at 30.0 ppm due to gauche conformation, characterizing mobile and amorphous regions.43-45 The 13C CP/MAS NMR spectra for Sil-DSG at room temperature showed domination of gauche (δ = 30.4 ppm) and a low-field shoulder indicating a trans (δ = 32.46 ppm) that remains almost the same even at temperatures as high as 50 °C. On the other hand, Sil-ODA25 demonstrated two well-resolved peaks, trans (δ = 32.96 ppm) and gauche (δ = 29.98 ppm) conformation. Monomeric C18 showed almost only gauche (δ = 30.4) and polymeric C18 showed predominantly trans (δ = 32.84 ppm) with an upfield shoulder indicating gauche conformation. At a temperature of 50 °C, polymeric C18 shows predominantly gauche (δ = 31.5 ppm) conformation with a low-field shoulder indicating trans. The chemical shift of gauche conformation in the case of polymeric C18 (31.5 ppm) is still considerably higher than Sil-DSG (30.16 ppm) and Sil-ODA25 (29.98 ppm). Polymeric C18 has a gauche peak 1.4 ppm higher than Sil-DSG and 1.52 ppm higher than Sil-ODA25. Interestingly, we see two peaks rather than one around the chemical shift of 12–14 ppm for polymeric C18 and Sil-ODA25. According to Pursch et al.,46 in polymeric C18, these two peaks are assigned as C1 (carbon attached to the silica surface) at resonance 14.1 ppm and C18 (carbon present in the terminal methyl group). A similar phenomenon is also observed for Sil-ODA25, while in Sil-DSG there is no peak at around 12–14 ppm. This difference may happen due to the differences in microenvironment of these two phases and also from conformational effect.

From detailed spectroscopic as well as DSC measurement, it was found that the organic phase (lipid) in Sil-DSG remained solid and in a noncrystalline state even at higher temperatures unlike Sil-ODA25 and polymeric C18 phases.

**Selectivity for Alkylbenzenes.** It is known that conventional C18 or alkyl phases can recognize the hydrophobicity of elutes and this hydrophobicity is measured by the methylene activity of the stationary phases. This reflects the possibility of the phase being able to separate two molecules that differ only in methylene groups, e.g., amylbenzene and butylbenzene or ethylbenzene and toluene. The retention mode as well as the extent of hydrophobic interaction between the elutes and the packing materials in HPLC can be determined by retention studies of alkylbenzenes as elutes.47-50 Other tests use the retention factors of chrysene,51 tolulene,52 or acenapthacene.23 Figure 4 shows the relationship between log k and log P for Sil-DSG, Sil-DBG, monomeric C18, and Sil-ODA25 phases. It is also observed that the retention mode of Sil-DSG and Sil-DBG showed a reversed-phase mode to that of conventional C18 phase. As shown in Figure 4, Sil-DSG showed extremely lowered retention of alkylbenzenes as compared with C18 and even lower than that of Sil-ODA25. All the samples (both alkylbenzene and PAHs) showed least retention on Sil-DBG although a higher amount of water was used (30%) in the mobile phase than others (10%). It was also observed that log k and log P plots of alkylbenzenes and PAHs in C18 were parallel and almost coincided with each other. This happened because monomeric C18 can recognize only the hydrophobicity of elutes. It has been found that Sil-DSG showed higher retention for PAHs compared to its values for alkylbenzenes. For instance, the log P of naphthacene (5.71) is much smaller than dodecylbenzene (8.43), but the log k value of naphthacene (0.86) is higher than dodecylbenzene (0.74). The increase of log k for PAHs was accompanied by selectivity enhancement. For example, oNaphthacene/}

\[
\frac{\text{oNaphthacene}}{\text{Triphenylene}} = 3.36 \text{ for Sil-DSG whereas monomeric C18 yielded } \frac{\text{oNaphthacene}}{\text{Triphenylene}} = 1.22. \text{ These results indicate that the Sil-DSG phase provides specific interactive sites for PAHs like Sil-ODA25 which recognizes aromaticity through carbonyl–π interaction.16,25 The detailed chromatographic behavior with separation mechanism will be discussed in the following section.}

**Selectivity toward PAHs.** Several size and shape parameters for PAHs were introduced for systematic investigations on

retention behavior. The $F$ number is a molecular size descriptor proposed by Hurtubise et al., which is defined as follows: $F = (number\ of\ double\ bonds) + (number\ of\ primary\ and\ secondary\ carbons) - 0.5(number\ of\ nonaromatic\ rings)$. The selectivity for the two-dimensional shape has been further studied with a molecular shape descriptor, length-to-breadth ($L/B$) ratio. The parameter was proposed by Wise et al. and Kaliszan et al. and has been defined as the length-to-breadth ratio of the two-dimensional shape of a molecule, and it quantitatively classifies “rodlike” molecules and “squarelike” molecules. The combination of these size and shape parameters, $F$ and $L/B$, has been successfully introduced for the characterization of C$_{18}$ phases.

The detailed retention data of PAHs on different stationary phases is given in Table 3. To evaluate the planarity recognition capability of C$_{18}$ phases, Tanaka et al. and Jinno et al. introduced the selectivity for two solutes, o-terphenyl ($F = 9, L/B = 1.11$) and triphenylene ($F = 9, L/B = 1.12$). Other sample sets (phenanthrene/cis-stilbene, fluorene/diphenylmethane) were also used to assess the planarity recognition ability of unknown stationary phases. We observed that Sil-DSG (triphenylene/o-terphenyl = 5.70) showed extremely enhanced molecular planarity recognition ability compared to other phases studied; for instance, monomeric C$_{18}$ demonstrated $\sigma_{triphenylene/o-terphenyl} = 1.5$, polymeric C$_{18}$ $\sigma_{triphenylene/o-terphenyl} = 3.0$, Sil-ODA$_{25}$ $\sigma_{triphenylene/o-terphenyl} = 4.5$, and Sil-DBG $\sigma_{triphenylene/o-terphenyl} = 2.6$. Planar and nonplanar solutes retention have also been investigated for PAHs with similar L/B values but dissimilar planarity. In this model, a stationary phase is represented as consisting of a number of slots into which a solute molecule can penetrate during retention. For slots of finite size, planar solutes penetrate more slots and will be retained in preference to nonplanar solutes, and long narrow molecules with higher L/B ratio will be retained in preference to square molecules. Naphthacene (L/B = 1.89) long narrow molecules with large length eluted later in Sil-DSG than pyrene (L/B = 1.27) square-shaped molecules. Retention of coronene (a planar PAH with L/B = 1.00) and phenanthro[34-c]phenanthrene (PhPh) a nonplanar PAH with L/B = 1.07 was also studied. The $k$ ratio for coronene to PhPh is 17.27 for Sil-DSG, indicating that significantly greater planar/nonplanar shape discrimination is possible by Sil-DSG. This distinction is also valid for linear and nonlinear molecules, i.e., naphthacene and chrysene in Sil-DSG. We have

Table 2. Alkyl Chain Densities of Different Phases

<table>
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<tr>
<th>Phase</th>
<th>Surface Area</th>
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<th>Alkyl Chain Density</th>
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<td>C$_{18}$, monomeric</td>
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<td>13.8</td>
<td>1.72</td>
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<tr>
<td>C$_{18}$, monomeric</td>
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<td>C$_{18}$, polymeric</td>
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<td>17.5</td>
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<td>C$_{18}$, polymeric</td>
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<td>4.90</td>
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<tr>
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<td>2.61</td>
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<td>Sil-DBG</td>
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<td></td>
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<tr>
<td>Sil-DSG</td>
<td>339</td>
<td>11.7</td>
<td>2.13</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(a) C$_{18}$ phases used for NMR measurements.

Figure 4. Relationships between log $k$ and log $P$ of different stationary phases. Mobile phase: methanol–water (90:10 v/v) for monomeric C$_{18}$, Sil-ODA$_{25}$, and for Sil-DSG, methanol–water (70:30 v/v) was used for Sil-DBG. Column temperature: 30 °C. Elutes: 1, benzene; 2, naphthalene; 3, anthracene; 4, naphthacene; 5, toluene; 6–11, ethyl-, butyl-, hexyl-, octyl-, decyl-, and dodecylbenzene.
observed that the retention of isomeric PAHs (i.e., phenanthrene and anthracene, triphenylene, benz[a]anthracene, chrysene, and napthacene, or benzo[ghi]pyrene, perylene, and benzo[a]pyrene, or dibenz[a,c]anthracene, dibenz[a,h]anthracene, and pentacene) on Sil-DSG increases with increasing L/B ratio as a similar phenomenon was observed for other columns with lower selectivity. The elution of PAHs isomers in Sil-DSG and Sil-ODA25 followed the retention ordered in polymeric C18 phase while Sil-DBG followed the monomeric C18 one. The elution of indeno[1,2,3-cd]pyrene followed benzo[ghi]perylene in monomeric C18 column, and the lack of resolution of acenaphthane and fluorene, and dibenzo[a,h]anthracene and dibenzo[a,c]anthracene are also a common phenomenon. A similar pattern was also observed for Sil-DBG. While the elution of benzo[ghi]perylene followed by indeno[1,2,3-cd]pyrene occurred in Sil-DSG and Sil-ODA25 as well as polymeric C18 column, better resolutions were also obtained for acenaphthane and fluorene, dibenzo[a,h]anthracene, and dibenzo[a,c]anthracene by Sil-DSG than all other stationary phases. The chromatograms for four-ring PAHs by different phases are shown in Figure 5. It is observed that Sil-DSG (R<sub>Naphthacene/Chrysene</sub> 2.3) can recognize molecular linearity or slenderness better than both Sil-ODA25 (R<sub>Naphthacene/Chrysene</sub> 1.85) and polymeric C18 (R<sub>Naphthacene/Chrysene</sub> 1.4).

Selectivity for SRM869a Test Mixture. The shape selectivity performance of Sil-DSG and Sil-DBG was also assessed by SRM869a, the column selectivity test mixture for liquid chromatography. The test was originally developed to facilitate the classification of C18 column in terms of stationary-phase bonding chemistry (i.e., monomeric vs polymeric surface modification) and to evaluate the selectivity for shape-constrained solutes. In recent studies, SRM 869a has also been used to characterize column selectivity for longer alkyl chain length stationary phases. The elution order of the probe compounds is indicative of column selectivity toward a variety of classes of shape-constrained solutes such as PAHs, PCBs, and carotenoids. In general, late elution of BaP relative to TBN indicates enhanced column selectivity toward PAH isomers. Early elution of BaP indicates reduced shape selectivity, and the elution order is typical of most commercial monomeric C18 columns. Figure 6 shows the chromatogram of SRM869a test mixture on Sil-DSG and Sil-DBG phase. It was found that Sil-DSG (R<sub>TBN/BaP</sub> 0.26) showed polymer-like retention behavior with extremely enhanced shape selectivity compared to other phases investigated, followed by Sil-ODA25 (R<sub>TBN/BaP</sub> 0.39).

Table 3. Retention and Separation Factors of PAHs for Different Stationary Phases

<table>
<thead>
<tr>
<th>MW</th>
<th>L/B</th>
<th>k&lt;sub&gt;C18(mon)&lt;/sub&gt; α</th>
<th>k&lt;sub&gt;C18(poly)&lt;/sub&gt; α</th>
<th>k&lt;sub&gt;Sil-ODA25&lt;/sub&gt; α</th>
<th>k&lt;sub&gt;Sil-DBG&lt;/sub&gt; α</th>
<th>k&lt;sub&gt;Sil-DSG&lt;/sub&gt; α</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Benzene</td>
<td>78</td>
<td>1.099</td>
<td>1.12</td>
<td>0.56</td>
<td>0.21</td>
<td>0.10</td>
</tr>
<tr>
<td>1-Naphthacene</td>
<td>128</td>
<td>1.238</td>
<td>1.80</td>
<td>2.0</td>
<td>2.14</td>
<td>4.3</td>
</tr>
<tr>
<td>1-Diphenylenemethane</td>
<td>166</td>
<td>1.520</td>
<td>1.12</td>
<td>0.38</td>
<td>1.90</td>
<td>1.06</td>
</tr>
<tr>
<td>1-Fluorene</td>
<td>166</td>
<td>1.520</td>
<td>2.19</td>
<td>1.96</td>
<td>0.72</td>
<td>1.50</td>
</tr>
<tr>
<td>1-Benz[a]anthracene</td>
<td>178</td>
<td>1.463</td>
<td>2.79</td>
<td>2.52</td>
<td>1.00</td>
<td>0.89</td>
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<tr>
<td>1-Anthracene</td>
<td>178</td>
<td>1.566</td>
<td>3.00</td>
<td>3.00</td>
<td>1.2</td>
<td>1.22</td>
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<tr>
<td>1-Pyrene</td>
<td>202</td>
<td>1.257</td>
<td>4.42</td>
<td>4.56</td>
<td>1.29</td>
<td>1.90</td>
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<td>1-Triphenylene</td>
<td>228</td>
<td>1.119</td>
<td>5.43</td>
<td>5.89</td>
<td>2.80</td>
<td>1.92</td>
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<td>1-Benzo[a]-anthracene</td>
<td>228</td>
<td>1.599</td>
<td>5.84</td>
<td>6.72</td>
<td>3.15</td>
<td>2.13</td>
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<tr>
<td>1-Chrysene</td>
<td>228</td>
<td>1.734</td>
<td>5.84</td>
<td>9.37</td>
<td>1.17</td>
<td>4.00</td>
</tr>
<tr>
<td>1-Naphthacene</td>
<td>228</td>
<td>1.896</td>
<td>6.90</td>
<td>9.37</td>
<td>1.60</td>
<td>7.30</td>
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<tr>
<td>2-Benz[a]acenaphthylene</td>
<td>252</td>
<td>1.387</td>
<td>0.90</td>
<td>1.02</td>
<td>1.11</td>
<td>1.13</td>
</tr>
<tr>
<td>2-Benz[k]fluoranthene</td>
<td>252</td>
<td>1.474</td>
<td>0.88</td>
<td>1.02</td>
<td>1.20</td>
<td>1.13</td>
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<tr>
<td>2-Benzo-e-pyrene</td>
<td>252</td>
<td>1.118</td>
<td>0.71</td>
<td>1.24</td>
<td>1.07</td>
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<tr>
<td>2-Benzo-a-pyrene</td>
<td>252</td>
<td>1.276</td>
<td>0.76</td>
<td>1.07</td>
<td>1.32</td>
<td>1.26</td>
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<tr>
<td>2-Dibenzo[a,c]anthracene</td>
<td>278</td>
<td>1.238</td>
<td>0.72</td>
<td>1.04</td>
<td>1.31</td>
<td>0.57</td>
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<tr>
<td>2-Dibenzo[a,h]anthracene</td>
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<td>1.822</td>
<td>0.75</td>
<td>1.04</td>
<td>1.31</td>
<td>0.75</td>
</tr>
<tr>
<td>2-Pentacene</td>
<td>278</td>
<td>2.228</td>
<td>1.35</td>
<td>1.88</td>
<td>4.95</td>
<td>1.60</td>
</tr>
<tr>
<td>2-bPh</td>
<td>328</td>
<td>1.074</td>
<td>1.07</td>
<td>0.87</td>
<td>1.02</td>
<td>1.13</td>
</tr>
<tr>
<td>2-TBN</td>
<td>328</td>
<td>1.095</td>
<td>1.09</td>
<td>1.02</td>
<td>1.34</td>
<td>0.54</td>
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<tr>
<td>2-Coronen</td>
<td>300</td>
<td>1.002</td>
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<td>0.93</td>
<td>3.97</td>
<td>5.22</td>
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<tr>
<td>2-Benzo[ghi]perylene</td>
<td>276</td>
<td>1.124</td>
<td>1.35</td>
<td>0.91</td>
<td>1.88</td>
<td>4.09</td>
</tr>
<tr>
<td>2-Indeno[1,2,3-cd]pyrene</td>
<td>276</td>
<td>1.388</td>
<td>1.23</td>
<td>2.10</td>
<td>1.11</td>
<td>2.52</td>
</tr>
</tbody>
</table>

* Mobile phase: methanol/water (90:10) for PAHs (1) and ethanol (100%) for PAHs (2) and for stationary phases (x). Mobile phase: methanol/water (65:35) for PAHs (1-2) for stationary phase (y). Column temperature: 20 °C. Flow rate: 1.00 mL/min.


and polymeric C₁₈ phases (α_{RTBN/BaP} = 0.86). On the other hand, Sil-DBG (α_{RTBN/BaP} = 1.63) yielded a monomeric-like (α_{RTBN/BaP} = 1.64) retention for SRM 869a. Further examination of selectivity was carried out using phenanthrene and anthracene as test solutes. Similar solute pairs have been used previously to assess resolution obtainable on other columns employed for PAH separations. The relatively higher (α_{Anthracene/Phenanthrene} = 1.38) selectivity obtained by Sil-DSG column is related to the homogeneous distribution of DSG molecules on the silica surface. One of the most difficult PAHs pairs to separate with nonshape selective phase is benzo[a]anthracene/chrysene. But this critical pair can be separated by Sil-DSG (α_{Chrysene/Benzo[a]Anthracene} = 1.23) while polymeric C₁₈ showed less selectivity for this set of PAHs (α_{Chrysene/Benzo[a]Anthracene} = 1.03).

Separation Mechanism for Lipid Grafted Noncrystalline Stationary Phase. Generally, the molecular shape selectivity in C₁₈ phase increases with increasing carbon loading; i.e., higher alkyl chain density exhibits higher molecular shape selectivity. However, Sil-DSG showed extremely enhanced selectivity toward shape-selective compounds compared to other columns investigated regardless of the fact that Sil-DSG had a lower surface coverage and lower alkyl chain density than polymeric C₁₈ and Sil-ODA. It was also observed that monomeric C₁₈ phase showed higher alkyl chain density than Sil-DBG but showed lower planarity recognition for planar and nonplanar PAHs. The unusually enhanced molecular shape selectivity yielded by Sil-DSG cannot be explained by common phenomena alone, as shape selectivity is high with higher bonding density and alkyl phase chain length.

To explain the unique selectivity of the Sil-DSG phase, we applied a multiple carbonyl interaction mechanism. We have reported previously that carbonyl groups in Sil-ODA are polarized to + (carbon) and - (oxygen). These polarized atoms can work as an electrostatic source of interaction in which carbon atoms act as electron donors and (68) Pursch, M.; Sander, L. C.; Egelhaaf, H. J.; Raitza, M.; Wise, S. A.; Oelkrug, D.; Albert, K. J. Am. Chem. Soc. 1999, 121, 3201–13.

Figure 5. Chromatogram for four-ring PAHs on different columns. Mobile phase: methanol–water (90/10 v/v) for all columns except Sil-DBG as methanol–water (55/45 v/v) for Sil-DBG at 1.00 mL min⁻¹ flow rate. Column temperature: 25 °C. Detection: UV at 254 nm. Injection volume: 5 μL.

Figure 6. Separation of SRM869a, Column Selectivity Test Mixture for Liquid Chromatography, on (a) Sil-DSG and (b) Sil-DBG columns. Mobile phase: methanol for Sil-DSG and methanol–water (50/50 v/v) for Sil-DBG at 1.00 mL min⁻¹ flow rate. Column temperature: 20 °C. Detection: UV at 254 nm. Injection volume: 5 μL.

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interact with \( \pi \) electrons containing guest molecules. The carbonyl groups in stationary phases interact with aromatic elutes through \( \pi-\pi \) interactions that are stronger (1.87 kcal mol\(^{-1}\) in HCHO-benzene) than those of both CH-\( \pi \) (0.57 kcal mol\(^{-1}\) in CH\(_3\)-benzene)\(^{22}\) and benzene \( \pi-\pi \) interactions (0.49 kcal mol\(^{-1}\) in the plane-to-plane stacking), and the aligned carbonyl groups in Sil-ODA\(_{25}\) are effective for enhancing higher selectivity toward PAHs. Based on that idea, we have clarified those carbonyl groups in the lipid main chain work as a \( \pi \)-electron interaction source for Sil-DSG and Sil-DBG as these contained three carbonyl groups in the glutamide moiety. We have already described that the lipid DSG can aggregate by self-assembly in various organic solvents while DBG cannot aggregate. The electrostatic carbonyl-\( \pi \) and benzene-\( \pi \) interactions worked more effectively in Sil-DSG than Sil-DBG. The carbonyl groups in Sil-DSG form a two-dimensionally condensed layer by forming hydrogen bonding among the glutamide moieties, which makes the carbonyl groups in an ordered form favorable for multiple \( \pi-\pi \) interactions with the guest PAH molecules. This phenomenon was also supported by \(^{13}\)C CP/MAS NMR spectral results, in which the alkyl chain does not form an ordered trans conformation even at lower temperature unlike the polymeric C\(_{18}\) phase. It is known that higher shape selectivity can be obtained by the polymeric C\(_{18}\) phase and that highly ordered octadecyl chains enhance the selectivity. This also strongly supports our hypothesis of a \( \pi-\pi \) interaction mechanism. Furthermore, since the separation factor (\( \alpha \)) values remained unchanged in Sil-DSG and Sil-DBG after 0.01% trifluoroacetic acid (TFA) was added into the mobile phase, the \( \pi-\pi \) interactions with the guest PAH are effective for enhancing higher selectivity in LC.\(^{75,76}\) To understand the temperature dependencies on selectivity of Sil-DSG, Sil-DBG, and Sil-ODA\(_{25}\), the separation factors in temperatures ranging from 10 to 60 °C were examined using naphthacene and chrysene as elutes, and the results are shown in Figure 7. These two PAHs have the same number of carbon atoms and \( \pi \) electrons but different molecular shapes (L/B ratio). Figure 7 shows that the effect of temperature on the selectivity is very low for monomeric C\(_{18}\) (\( \alpha = 1.2-1.1 \)) as well as for polymeric C\(_{18}\) (\( \alpha = 1.5-1.2 \)). Sil-ODA\(_{25}\) showed remarkable temperature dependence with high selectivity, especially at a low temperature (\( \alpha = 2.1 \) at 10 °C) and a distinct bending is attributed to the phase transition between ordered and disordered state of the ODA\(_{25}\) moiety. Similar bending

**Table 4. Effect of Addition of Acetone to the Mobile Phase on Planarity and Linearity Selectivity of PAHs by Different Phases**

<table>
<thead>
<tr>
<th></th>
<th>( \alpha ), naphthacene/chrysene</th>
<th>( \alpha ), triphenylene-terphenyl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MeOH/H(_2)O (90/10)</td>
<td>MeOH/H(_2)O (90/10)</td>
</tr>
<tr>
<td>C(_{18}) (mon)</td>
<td>1.72</td>
<td>1.63</td>
</tr>
<tr>
<td>C(_{18}) (polym)</td>
<td>3.40</td>
<td>0.86</td>
</tr>
<tr>
<td>Sil-ODA(_{25})</td>
<td>2.63</td>
<td>0.39</td>
</tr>
<tr>
<td>Sil-DBG</td>
<td>1.41</td>
<td>1.64</td>
</tr>
<tr>
<td>Sil-DSG</td>
<td>2.13</td>
<td>0.26</td>
</tr>
</tbody>
</table>

\(^{a}\) Column temperature: 10 °C. Flow rate: 1.00 mL/min.

**Figure 7.** Temperature dependencies on the selectivity of naphthacene and chrysene on different stationary phases (other conditions as described in Figure 5).

---

was observed in trans-stilbene, cis-stilbene, triphenylene, and o-terphenyl. DSC measurement of Sil-ODA<sub>25</sub> showed an endothermic peak in the range of 30–47 °C, with a peak top at 42 °C in a methanol–water (45:55) suspension. This temperature range is close to the bending temperature in Figure 7. Therefore, the selectivity enhancement of Sil-ODA<sub>25</sub> was brought about by ordering of the organic phase. On the other hand, Sil-DSG, Sil-DBG, and C<sub>18</sub> phase did not show any phase transition in the temperature ranges from 10 to 60 °C, and no such bending or sudden decrease of selectivity was observed for these phases. The thermal effects on the elution of the analytes can be explained in two ways. First, at higher column temperatures, the thermal mobility of the bonded-phase chain (Sil-DSG) becomes greatly enhanced like C<sub>18</sub>, and this reduces the hydrophobic interaction as well as π–π interaction between the stationary phases and the analytes, resulting in increased distribution of the analytes into the mobile phase. Second, as the temperature of the mobile phase is increased, the solubility of the analytes is also increased, which facilitates the distribution of analytes into the mobile phase, leading to reduced retention times.

**Selectivity toward Geometrical Isomers.** A series of phenyl-substituted isomers were used to examine the isomeric selectivity of Sil-DSG and Sil-DBG. The separation factors of different geometrical isomer sets are shown in Table 5. As shown in the table, Sil-DSG showed enormously higher selectivity for geometrical isomers, especially for the long narrow molecules (para and trans isomers). The selectivity of geometrical isomers on Sil-ODA<sub>n</sub> is enhanced by the carbonyl–π interaction, and this is more pronounced for para and trans isomers. Multiple interactions are an advantage in recognition of isomers and are especially effective in case of planar-to-planar and rigid-to-rigid structures.

**CONCLUSION**

In this work, we have discussed the new application of L-glutamide derivatives in separation science, especially as stationary phases in high-performance liquid chromatography. The alkyl moiety in Sil-DSG exists in a solid and noncrystalline state after immobilization on to the silica surface and no conformational change of alkyl chain occurs. Spectroscopic results of Sil-DSG indicated that the carbonyl groups are rigidly immobilized on the silica surface and might form condensing layers. Inter- or intra-molecular hydrogen bonding most likely promotes this assembly and can be a driving force for multiple π–π interactions. The enhancement of selectivity in this phase is due to multiple π–π interactions, and it was endorsed by orientating the carbonyl groups of the glutamide moieties that assembled themselves in DSG. In Sil-ODA<sub>n</sub>, the carbonyl groups can be oriented by forming a highly ordered structure of the side chains, and a thermally induced disordered-to-ordered phase transition was also observed. On the other hand, Sil-DSG and Sil-DBG did not show any phase transition behavior in DSC, suspension-state <sup>1</sup>H NMR, and also in HPLC measurement. This idea of π–π interaction between the stationary phases might be helpful for further development of stationary phases in liquid chromatographic science. Finally, from our detailed investigation, we hope this novel HPLC packing material (Sil-DSG) can play a significant role for the separation of PAHs and aromatic positional isomers.

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| Table 5. Separation Factors (α) of Geometrical Isomers for Various Stationary Phases<sup>a</sup> |
|-----------------------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| trans/cis-stilbene | m-α-terphenyl | p-α-terphenyl | m-α-phenyltoluene | p-α-phenyltoluene | γ-α-terpine |
| C<sub>18</sub>(mon) | 1.08 | 1.52 | 1.03 | 1.10 | 1.00 | 1.02 |
| C<sub>18</sub>(polym) | 1.33 | 1.70 | 1.26 | 1.23 | 1.09 | 1.10 |
| Sil-ODA<sub>25</sub> | 2.15 | 1.86 | 1.90 | 1.34 | 1.24 | 1.10 |
| Sil-DBG | 1.44 | 1.75 | 1.05 | 1.20 | 1.04 | 1.07 |
| Sil-DSG | 3.04 | 1.95 | 4.29 | 1.35 | 1.35 | 1.12 |

<sup>a</sup> Mobile phase: methanol/water 70/30. Column temperature: 20 °C. Flow rate: 1.00 mL/min.