Self-Assembled Organic Phase for Reversed-phase HPLC

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INTRODUCTION

Reversed-phase liquid chromatography (RP-HPLC) is the most popular method for analytical separation. Particularly, octadecylated silica (ODS) has been widely used for this method and represents an extremely high share of commercial HPLC because of its wide applicability with only slight modification of mobile-phase systems. Most kinds of ODS fall into one of two groups: monomeric or polymeric. Polymeric ODS provides specific separation behaviors, especially for polycyclic aromatic hydrocarbons (PAHs), while monomeric ODS is generally used for routine analysis. This difference derives from the highly dense packing of octadecyl groups on silica in polymeric ODS.

SELF-ASSEMBLED PHASES

Further specificity has been desirable in RP-HPLC with the development of biotechnology and combinatorial chemistry. One simple solution may be to develop new organic phases. For this purpose, many special ligands have been immobilized onto silicas.[1–6] Typical examples are seen in fullerene- and porphyrin-immobilized silicas, for which unique selectivity has been realized. However, it is certain that their separation modes are not only from simple reversed-phase mode, but also exceed the functions of the original ligands.

On the other hand, self-assembling systems are very attractive, as they yield supramolecular functions beyond those of unit segment molecules in a solution state. The most successful examples can be seen in bio-organic systems, such as lipid membranes, proteins, and nucleic acids. In this article, we introduce self-assembled organic systems composed of simple hydrocarbons, which provide unique functions as organic phases that exceed RP-HPLC with ODS.

MOLECULAR DESIGN FOR SELF-ASSEMBLED ORGANIC PHASE

Lipid bilayer membrane systems, having gel (solvated crystalline state)-to-liquid crystalline phase transition, are attractive models for realization of self-assembled systems for separation chemistry. The first approach for this purpose was direct immobilization of a phosphatidylcholine lipid onto silica.[7] This modified silica showed unique selectivity against amino acids, but the separation mode was too complicated due to the zwitterionic property of the immobilized molecule. In addition, no lipid membrane function was realized on the silica because of the direct immobilization with covalent bonding, which prohibits lateral diffusion of lipids from forming the highly ordered structures that lead to supramolecular functions of lipid membrane systems.

However, it is essential to immobilize lipid membrane structures for use in column chromatographic systems. We must overcome this simple dilemma to achieve both stability and flexibility. Polymerization of lipids (Fig. 1a)[8–10] is one of the most useful ways for stabilization of lipid membrane systems. While the polyion complex method (Fig. 1b)[11,12] is also effective for biomimetic sensor systems, elution of lipids cannot be completely avoided with this method. Furthermore, complicated ionic systems should be avoided to achieve a simple separation mode. On the basis of these facts, we have designed and synthesized the comb-shaped polymer, ODA_n[13] (Fig. 2) as a lipid membrane analogous organic phase. This polymer cannot form bilayer structures in water but forms ''nanogels'', which undergo temperature-responsive phase transition between ordered and disordered structures like aqueous lipid membrane systems.

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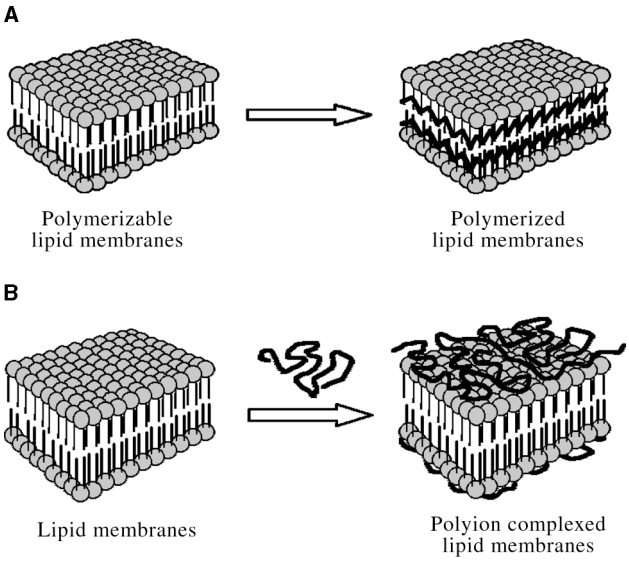

Fig. 1 Stabilization of lipid membranes by polymerization (A) and complexation with polyions (B).

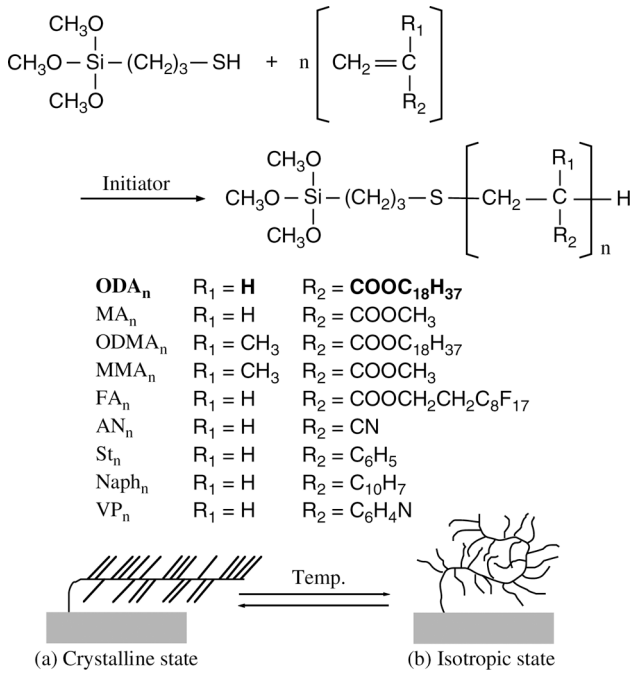

Fig. 2 The chemical structures of novel polymeric organic phases prepared by one-step telomerization. These polymers can be readily immobilized onto silica through the terminal methoxysilyl group. In the case of ODA$_n$ and ODMA$_n$, the resultant organic phase undergoes thermally induced phase transition between crystalline (a) and isotropic (b) states.

Another advantage of this polymer is due to its having a reactive terminal group at one side of the polymer main chain. Therefore, even after immobilization of the polymer with the terminal group, the bonded organic phase can maintain flexibility without being constrained by silica.

FUNDAMENTAL FEATURES OF COMB-SHAPED POLYMER

The comb-shaped polymer, ODA$_n$, can be synthesized by a one-step telomerization of octadecyl acrylate (ODA) with 3-mercaptopropyltrimethoxysilane (MPS). The average degree (n) of polymerization can be readily controlled by the initial molar ratio of ODA to MPS because of the good chain transfer constant of the mercapto group. The degree of n was estimated by the proton ratio, based on the terminal methoxy group, with ^{1}H-NMR spectroscopy, and the value of M_w/M_n was determined by SEC to be approximately 1.4–1.6.

Immobilization of ODA$_n$ is carried out by mixing with silica at 60–80°C for 1–2 days. Toluene and tetrachloromethane are good solvents for this procedure. If porous silicas with diameter 5 μm, pore size 146 Å, and specific surface area 330 cm^2 g^{-1} (Fuji Silysia 100A-5D) are used, the final immobilization reaches a maximum of 22–25 wt%.

The uniqueness of ODA$_n$ is shown by differential scanning calorimetry (DSC).[14] The DSC thermogram of ODA$_n$ shows a sharp endothermic peak in both the heating and cooling processes. For example, the temperature of ODA$_{27}$ (n = 27) peaks at around 49°C (T_{c2}) with a shoulder (T_{c1}) at 42–47°C in the heating process. Polarization microscopic observation indicates that T_{c1} and T_{c2} can be assigned to crystalline-to-liquid crystalline and liquid crystalline-to-isotropic phase transitions, respectively. The liquid crystalline state contains a Shlieren issue which belongs to a nematic phase and is observed at a temperature range of 42–47°C. Similar phase transitions are also observed in methanol (or methanol–water) dispersions as mobile phases, accompanied by a slight decrease in temperature of about 2–10°C. This indicates that ODA$_{27}$ can form highly ordered structures (such as crystalline state) even in the presence of organic solvents used in the column chromatography process. Phase transitions are observable in ODA$_n$ with n = 9–60, although T_{c2} is somewhat dependent on n [for example, T_{c2} = 41°C (n = 9), 44°C (n = 14), 49°C (n = 33), and 49°C (n = 60)]. The silica-supported ODA$_n$ (Sil-ODA$_n$) also show endothermic peaks in their DSC measurements. In the case of ODA$_{27}$ in a methanol–water (7 : 3) mixture, the DSC thermogram shows about 8°C lowering of peak-top temperature (T_{c2}) compared with the original T_{c2}. This indicates that silica slightly influences the orientation of bound ODA$_{27}$, but the bonded phase can maintain oriented structures and undergo crystalline-to-isotropic phase transition on silica (Fig. 2).

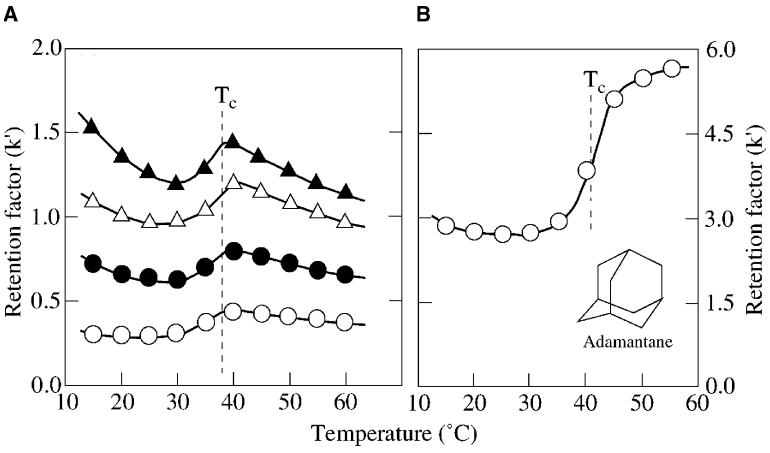

Fig. 3 Temperature dependencies of retention factors with Sil-ODA$_n$. Samples: (A) open circles, naphthalene; solid circles, anthracene; open triangles, pyrene; solid triangles, triphenylene; (B) adamantine. Mobile phase: (A) methanol–water, 9 : 1; (B) methanol.

HPLC WITH COMB-SHAPED POLYMER-IMMOBILIZED SILICA

Temperature Dependence of Retention Factor

Sil-ODA$_n$ performs complete separation for mixtures of various alkylbenzenes and aromatic hydrocarbons containing naphthalene, anthracene, pyrene, and triphenylene in methanol–water as a mobile phase. Their elution orders are the same as those that can be observed with ODS. It is also confirmed that the retention factor (k') increases with an increase in mobile-phase polarity. These results indicate that Sil-ODA$_n$ has a retention mode similar to RP-HPLC.

The performance of Sil-ODA$_n$ is unique in that it is characterized by temperature dependency. As shown in Fig. 3a, Sil-ODA$_n$ shows distinct bending in the plots of temperature vs. k' for naphthalene, anthracene, pyrene, and triphenylene. No similar temperature dependence is observed with ODS. As shown by the dotted lines of Fig. 3a, T_{c2} of Sil-ODA$_n$ observed in DSC (in a methanol dispersion) almost completely agrees with the temperature of the bending point. This strongly suggests that the unusual temperature dependencies in Sil-ODA$_n$ occur at the crystalline-to-isotropic phase transition temperature of the bonded phase.

Molecular Shape-Selective HPLC

The specificity of Sil-ODA$_n$ can be emphasized by testing retention behaviors for samples with different molecular shapes such as triphenylene and *o*-terphenyl. These compounds have the same carbon number per molecule and, thus, their molecular hydrophobicities are similar. Sil-ODA$_n$ shows complete separation for this mixture as shown in Fig. 4a. The separation factor (α) at 20°C is 4.3 in Sil-ODA$_n$ but 1.4 in ODS. Sil-ODA$_n$ also shows remarkable temperature dependence. As shown in Fig. 5a, the separation factor is much higher at the crystalline temperature of Sil-ODA$_n$ than at the isotropic temperature. On the other hand, ODS shows no significant temperature dependence. Therefore, the enhanced selectivity at the crystalline temperature cannot be explained by only hydrophobicity recognition. It is known that planar compounds, such as triphenylene, are more strongly incorporated into an oriented medium than nonplanar compounds, such as *o*-terphenyl. For example, we have previously reported that α-helical poly(γ-methyl-L-glutamate) produces highly oriented bundles and shows unexpected higher retention capacity for planar

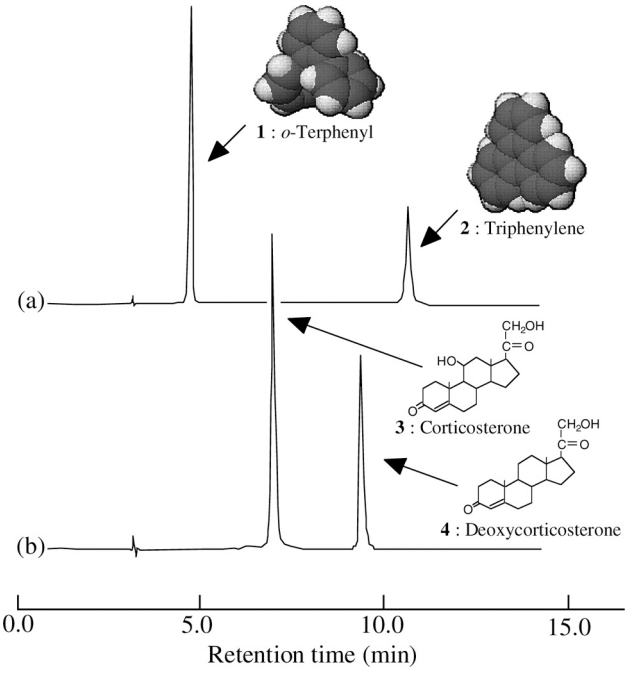

Fig. 4 Typical chromatograms with silica-supported ODA$_n$ (Sil-ODA$_n$). Mobile phase: methanol, 20°C.

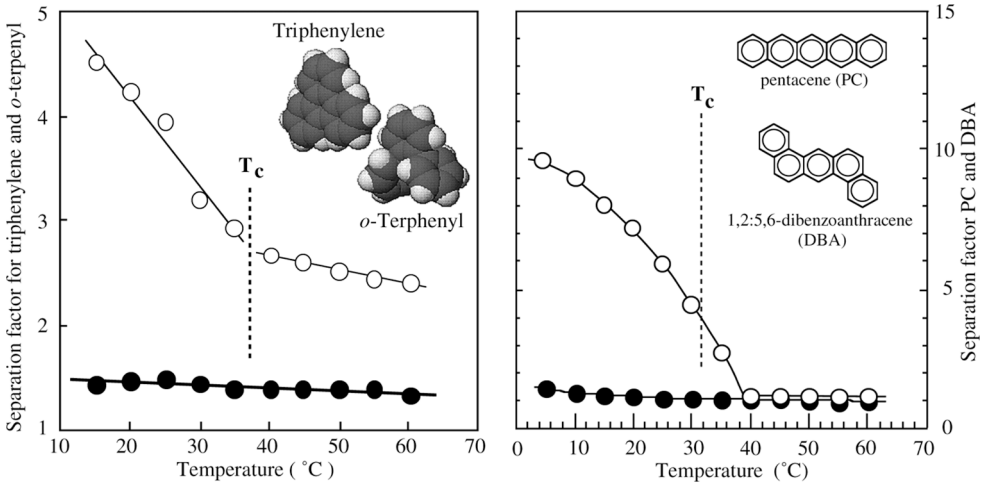

Fig. 5 Temperature dependencies on the separation factor. Columns: open circles, Sil-ODA$_n$; solid circles, ODS. Mobile phases: (a) methanol; (b) ethanol.

aromatics. This is understandable on the basis of the molecular slit model.[15]

To clarify the separation mechanism of Sil-ODA$_n$, we investigated the selectivity with geometrical isomers from various substituted azobenzene compounds.[16] As a result, it was found that the separation factor between the trans- and cis-isomers was remarkably dependent on the electron-donating property of the substituted group. This indicates that bonded ODA$_n$ works as an electron acceptor. Therefore, we focused on the role of carbonyl groups in ODA$_n$. Fig. 6 shows temperature dependencies of the separation factors with respect to geometrical isomers of stilbene. Expectedly, Sil-ODA$_n$ shows higher selectivity at its crystalline temperature than ODS. However, further significant information is observed at its isotropic temperature: The selectivity is still higher than in ODS. This must be due, in part, to effects of carbonyl groups because Sil-MA$_n$,[17] which can be prepared by methyl acrylate without long-chain alkyl groups, shows selectivity similar to that of Sil-ODA$_n$ which is in an isotropic state (Fig. 6).

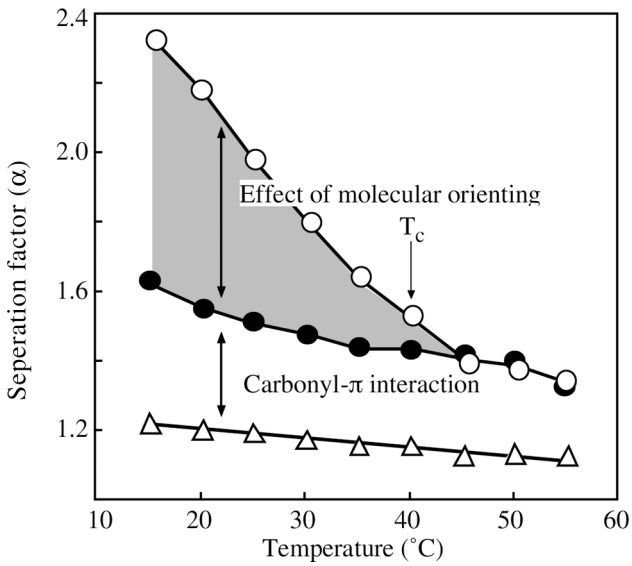

Fig. 6 Temperature dependencies of the separation factor (α) between *cis*- and *trans*-stilbenes with Sil-ODA$_n$ (open circles), Sil-MA$_n$ (solid circles), and ODS (open triangles) columns. Mobile phase: methanol–water (7:3). Sil-MA$_n$: poly(methyl acrylate)-grafted silica.

Additional supporting data can be obtained by calculation.[18–20] A carbonyl π–benzene π complex was evaluated against a formaldehyde–benzene complex model (Fig. 7) by ab initio MO/MP2 calculations performed with the Gaussian 94 package. The binding energy was calculated as a function of distance, R, between the carbon atom of formaldehyde and benzene plane, in which formaldehyde was moved perpendicular to the benzene plane (plane-to-plane interaction) with the orientation fixed to that of the optimized geometry. According to our calculated results, a formaldehyde–benzene interaction ($1.83\,\mathrm{kcal\,mol^{-1}}$) is more effective than CH_4–benzene[18] ($0.57\,\mathrm{kcal\,mol^{-1}}$) and benzene–benzene[18] (0.49 and $1.78\,\mathrm{kcal\,mol^{-1}}$ in the parallel and perpendicular interactions) complexes. Therefore, we conclude that the higher selectivity of Sil-ODA$_n$ is brought about by a carbonyl π–benzene π interaction, especially on a highly oriented state.

Specificity of Sil-ODA$_n$ is often found with PAHs. Fig. 5b shows temperature dependencies for the separation factors between structural isomers of five-membered ring compounds. Since both samples used are planar with no difference of π-electrons, the selectivity may be related to their molecular shapes.

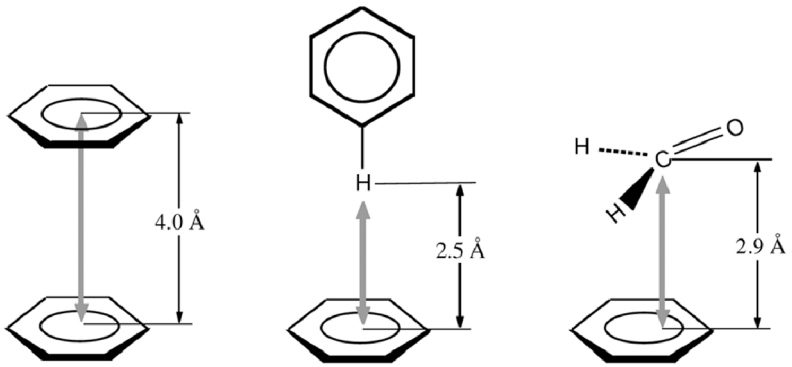

Fig. 7 Proposed structures and energies of benzene–benzene (a and b) and formaldehyde–benzene[20] (c) complexes estimated by ab initio study.

However, both samples are equally thin and, thus, the conventional molecular slit model can hardly be applied for understanding the high selectivity of Sil-ODA$_n$. A surface adsorption mechanism is more likely because the bonded phase is rigid in a crystalline state and does not allow partition of solutes into the bonded phase, even if the solute molecule is thin and planar. Based on these observations, we propose the modified mechanism schematically illustrated in Fig. 8: A carbonyl π–benzene π interaction as an essential driving force for bonded phase–solute interaction. This estimation is supported both experimentally and theoretically. For multiple carbonyl π–solute π interactions, as illustrated in Figs. 8 and 8b, it is certain that a crystalline bonded phase can interact more effectively with linear and planar compounds (Fig. 8b) than with disk-like or bending compounds (Fig. 8a). Therefore, we conclude that solute molecules are not incorporated into the crystalline phase, but rather adsorbed on the aligned carbonyl groups. On the other hand, the isotropic bonded phase (Fig. 8c) does not have such specificity. The solutes can partition into the bonded phase and, thus, the separation mode is similar to that of ODS.

Recognition of Molecular Bulkiness

Here, it is demonstrated that Sil-ODA$_n$ is sensitive to molecular bulkiness. Fig. 3a shows the temperature dependence of the retention factor for adamantane as a sterically bulky compound. The plots include a remarkable k' jump at temperatures around T_{c2}. Particularly, it should be noted that the retention factor is much smaller at the crystalline temperature than at the isotropic temperature. This indicates that adamantane has no π-electron and that a sterically bulky compound can hardly be incorporated into the highly oriented bonded phase.[14]

Retention Behavior for Rigid Hydrocarbons and Steroids

It is known that highly oriented aggregates, such as lipid bilayer membranes, specifically incorporate rigid and hydrophobic compounds. For example, cholesterol can be easily incorporated into lipid bilayer membranes. Therefore, the retention behavior for cholesterol was examined using Sil-ODA$_n$.[14] The temperature dependence on the k' of cholesterol showed a

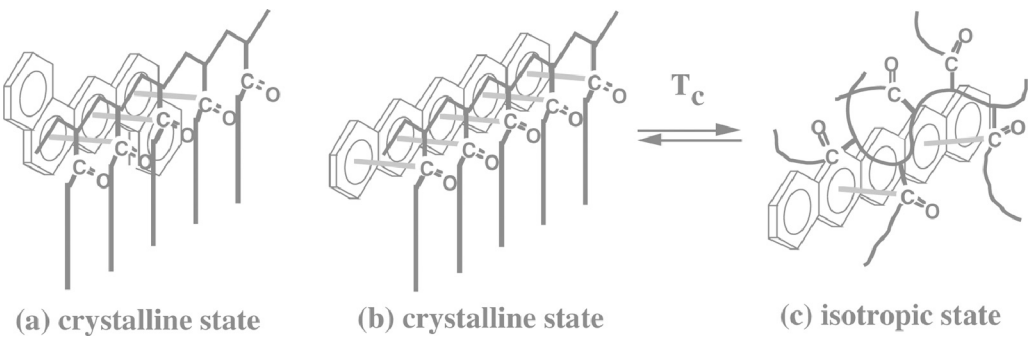

Fig. 8 Schematic illustration to explain the temperature dependence of molecular-shape selectivity through carbonyl–π interaction. If the carbonyl groups are linearly aligned on highly oriented structure (a), multiple interaction effect will be expected for a linear and planar substance such as pentacene (a) than 1,2 : 5,6-dibenzoanthracene (b). However, no such effect occurs when ODA$_n$ is disordered (c).

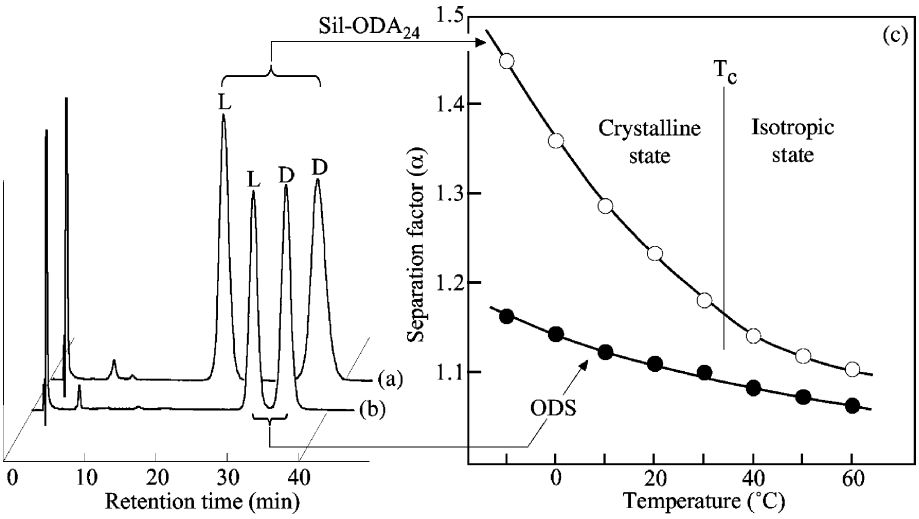

Fig. 9 Chromatograms (a and b) at –10°C and temperature dependencies of the separation factor for diasteromized DL-phenylalanine methyl ester with *N*-dansyl-L-proline. Columns: Sil-ODA$_n$ (open circles); ODS (solid circles). Mobile phase: methanol–water, 6 : 4. Detection wavelength: 340 nm.

distinct bending point around T_{c2} and a remarkable increase at temperatures below T_{c2}: k' (in methanol) = 1.8 (60°C), 2.0 (50°C), 2.4 (40°C), 2.5 (30°C), 3.2 (20°C), and 4.3 (15°C). These results indicate that Sil-ODA$_n$ incorporates cholesterol specifically at temperatures below 30°C. Fig. 4b shows a chromatogram of a mixture of steroid hormones, corticosterone and deoxycorticosterone. Complete separation (α = 1.6) was obtained in spite of the small structural difference. In the case of ODS, the α was only 1.1.

Diastereomer Selectivity

Diastereomerization of enantiomeric isomers is useful for chiral discrimination. Although not suitable for fractionation, when this method is used with RP-HPLC, it yields a very convenient and quick analysis of enantiomer mixtures. Therefore, many diastereomerizing reagents have been developed: for example, *o*-phthalaldehyde-*N*-acetyl-L-cystein, (+)-1-(9-fluor enyl)ethyl chloroformate, (a2)-2′-methyoxy-1, 1′-binaphthyl-2-carboxylate, (*S*)-(–)(2,3-naphthalenedicarboximidyl) propionyl fluoride and chiral monohalo-*s*-triazines, and 4-nitro-7-(3-aminopyrrolidin-l-yl) -2,1,3-benzoxadiazole and *O*-(4-nitrobenzyl)tyrosine methyl ester for racemic amines and/or amino acids. These diastereomerizing reagents are characterized by the fact that chromophoric groups are included for sensitive detection, and chiral separation is realized by discriminating the hydrophobicity (or polarity) difference between the resulting diastereomers. Therefore, ODS has been

Table 1 Comparison of the separation factor with various diastereomerizers with Sil-ODA$_n$ and ODS

			Separation factor (α)	
Enantiomers	**Diastereomerizer**	**Mobile phase**	**Sil-ODA$_n$**	**ODS**
RS-Phenylethylamine	Nip-F	A	1.16	1.09
N-Ac-DL-phenylalanine	NBD	B	1.35	1.23
N-Ac-DL-leucine	NBD	C	1.25	1.02
N-Ac-DL-valine	NBD	D	1.22	1.03
N-BOC-DL-phenylalanine	NBD	B	1.22	1.04
DL-Alanine(OMe)	DLP	E	1.31	1.20
DL-Valine(OMe)	DLP	E	1.57	1.33
DL-Leucine(OMe)	DLP	E	1.46	1.28
DL-Phenylalanine(OMe)	DLP	E	1.45	1.16
DL-Phenylalanine(OMe)	DLA	E	1.26	1.18
DL-Phenylalanine(OMe)	DLV	E	1.29	1.21
DL-Phenylalanine(OMe)	DLL	E	1.35	1.28

Nip-F: (S)-(–)-(2,3-naphthalenedicarboximidyl)propionyl fluoride, NBD: 4-nitro-7-(3-aminopyrrolidin-1-yl)-2,1,3-benzoxadiazole, DLP: *N*-dansyl-L-proline, DLA: *N*-dansyl-L-alanine; DLV: *N*-dansyl-L-valine; DLL: *N*-dansyl-L-leucine.
Mobile phase: (A) methanol–water (45 : 55) at 0°C; (B) methanol–water–TFA (7 : 3 : 1/1000) at 5°C; (C) methanol–water–TFA (6 : 4 : 1/1000) at 5°C; (D) methanol–water–TFA (5 : 5 : 1/1000) at 5°C; (E) methanol–water (60 : 40) at –10°C.

mainly used for organic stationary phases in RP-HPLC. However, it is fairly certain that ODS does not show high selectivity in this method because the difference in hydrophobicities of resultant diastereomers is rather small. Here, Sil-ODA$_n$ was used instead of ODS in order to increase the diastereomer selectivity. This attempt is reasonable because almost all diastereomer reagents are π-electron-rich compounds that can be used as π−π interaction sources. As a result, we reallized that the combination of (*S*)-(–)(2,3-naphthalenedicarboximidyl)propionyl fluoride,[19,20] 4-nitro-7-(3-amino-pyrrolidine-1-yl)-2,1,3-benzoxadiazole[21] and *N*-dansyl amino acids[22] showed better selectivity for racemic samples than ODS. A typical chromatogram is shown in Fig. 9. Table 1 summarizes the separation factors.

Fig. 9c shows the temperature dependencies of the selectivity for diastereomerized DL-phenylalanine derivatives with *N*-dansyl-L-proline. It is clearly seen that the selectivity of Sil-ODA$_n$ is remarkably temperature dependent. A similar increase in selectivity with decreasing temperature was observed for methyl esters of DL-alanine, valine, and leucine. As mentioned earlier, ODA$_n$, which is in a crystalline state, shows distinct molecular-shape recognition with respect to polyaromatic hydrocarbons, and this ability is due to a carbonyl–π interaction. Supporting this, it was observed that addition of 20% (v/v) acetone as an inhibitor against π–π interaction to the mobile phase reduced the selectivity by ca. 20%, but 2-propanol did not show any decrease. Similar decreases of selectivity have been reported for polyaromatic hydrocarbons. Therefore, it is estimated that a π–π interaction allows for diastereomer selectivity.

CONCLUSIONS

1. A special polymeric bonded phase for RP-HPLC can be synthesized by one-step telomerization of ODA using MPS. The degree of polymerization is readily controlled by adjusting the initial molar ratio in the telomerization process. This method provides increased applicability through the greater possible selection of monomers (Fig. 2). For example, perfluoroalkylated polymer- or poly(vinylpyridine)-immobilized silicas[23,24] have been developed.
2. The polymer (ODA$_n$) was readily immobilized onto silica with a reactive terminal group. Therefore, the polymer maintains flexibility even after grafting.
3. The immobilized polymer (Sil-ODA$_n$) undergoes crystalline-to-isotropic phase transition on silica. The elution order for hydrophobic compounds with Sil-ODA$_n$ usually agrees with the order expected from the RPLC mode. However, Sil-ODA$_n$ at crystalline state temperature provides exceptionally specific selectivity and recognition for molecular planarity, slenderness, and bulkiness. The driving force is clearly related to the highly oriented structures. The π-electron due to the carbonyl group also plays an important role for molecular recognition.

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