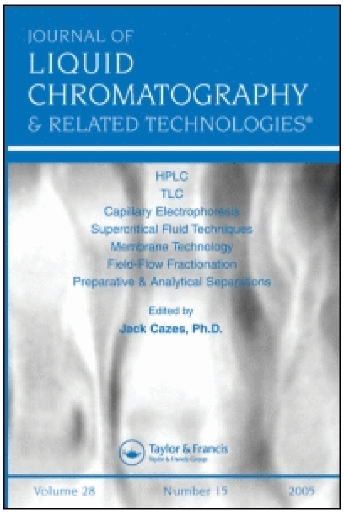

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Hirotaka Ihara[a]; Shoji Nagaoka[b]; Hideaki Tanaka[a]; Sigeyoshi Sakaki[a]; Chuichi Hirayama[a]
[a] Department of Applied Chemistry & Biochemistry, Kumamoto University Kurokami, Kumamoto, Japan [b] Kumamoto Industrial Research, Institute Higashimachi, Kumamoto, Japan

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LIPID MEMBRANE ANALOGUE-IMMOBILIZED SILICA GELS FOR SEPARATION WITH MOLECULAR RECOGNITION

Hirotaka Ihara,[1] Shoji Nagaoka,[2] Hideaki Tanaka,[1]
Sigeyoshi Sakaki,[1] Chuichi Hirayama[1]

[1]Department of Applied Chemistry & Biochemistry
Kumamoto University
Kurokami
Kumamoto 860, Japan

[2]Kumamoto Industrial Research Institute
Higashimachi
Kumamoto 862, Japan

ABSTRACT

The comb-shaped polymer (ODA_n) composed of a reactive terminal group and highly-orienting side-chain groups was prepared by telomerization using 3-mercaptopropyl trimethoxysilane and octadecylacrylate. The polymer was readily immobilized onto porous silica gels through a terminal reactive group. DSC indicated that the silica-supported polymer ($Sil-ODA_n$) underwent crystal-to-isotropic phase transition on silica gels at a temperature range of 28 - 47°C (in the case of n = 27) in methanol. Polarity microscopic observation of the polymer showed the phase transition included a nematic liquid crystalline state. The packed column showed a remarkably higher separation factor for mixtures of planar aromatics (e.g. triphenylene and *trans*-stilbene) and non-planar aromatics (e.g. *o*-terphenyl or *cis*-stilbene) at room temperatures than did the conventional hydrophobic stationary phases, i.e. octadecylated silica gels. In addition, the Sil-ODA$_n$ column showed remarkable temperature dependence on both retention capacity (k') and separation factor (α). The k'-temperature and α-temperature plots showed distinct bending at temperatures around the phase transition temperature of immobilized ODA$_n$. These results indicate that the selective retention for planar compounds is related to highly-orienting structure formed from long-chain alkyl groups. This paper discusses the molecular recognition mechanism using additional chromatographic behaviors and MOPAC calculation.

INTRODUCTION

Lipid membranes act as most important intermediaries for producing biofunctions including various chemical reactions and selective transportation. Immobilization of their functions on artificial carriers expands their possible applications, especially for separation chemistry purposes such as column chromatography. Therefore, various methods for stabilization and immobilization of lipid membranes have been developed, because lipid membranes are just aggregates which can be easily dissolved in organic media.

The polymerization of lipids[1-7] is a good technique for stabilizing lipid membranes. This polymerization is usually performed through introduction of polymerizable groups into either hydrophilic or hydrophobic moieties. In this technique, the molecular orientation of lipids can be maintained after polymerization, but causes remarkable suppression of molecular fluidity. Since membrane fluidity is an essential property for producing membrane functions, this loss is highly disadvantageous.

To alleviate this problem, Kunitake et al.[8] and Regen et al.[9,10] have developed lipid membrane systems containing polyions as counter ions. In this technique, the membrane stability was increased without damaging either molecular orienting or fluidity. Okahata et al. found that quartz-crystal microbalances covered with polyion-complexed lipid membranes could be used as sensing materials.[11,12] However, this stabilization technique is still not satisfactory for use as immobilized stationary phases for column and membrane separation processes because 1) it is impossible to prevent the elution of lipids to mobile phases and 2) the polyion complex system provides many complicated electrostatic interactions between immobilized phases and solutes, which lead to peak-broadening and abnormal adsorption in liquid chromatography.

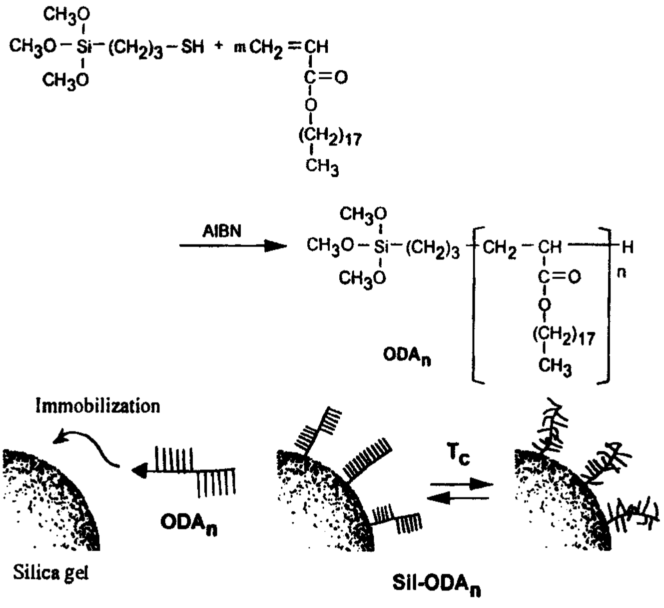

Figure 1. Synthetic scheme of lipid membrane analogue (ODA_n) and illustration of the phase transition of immobilized ODA_n on silica gels.

On the other hand, direct immobilization of lipids onto silica gels was reported by Pidgeon and Venkataram.[13] This was obtained by amidation of aminated silica gels with a dimyristoyl phosphatidylcholine derivative containing a terminal carboxylic group. Such chemically bonded lipids are more stable than polyion-complexed lipid membranes, but it may be impossible to reproduce molecular orientation and lateral diffusion of lipids on carrier particles.

Therefore, in order to introduce lipid membrane functions as silica-based stationary phases, it is necessary to develop a new approach for immobilization of lipid membranes. For this purpose, we have synthesized comb-shaped polymer ODA_n (Fig. 1).[14-16] This polymer can be readily immobilized onto silica gels through a reactive terminal group, and the silica-supported polymer (Sil-ODA_n) shows several essential functions (highly-orienting structure, hydrophobicity and phase transition behavior) like those of lipid membranes. This is because the polymer is immobilized at the one side of terminal groups and still maintains molecular fluidity after immobilization. In this paper, we demonstrate that the immobilized polymer does not form bilayer membrane structures in water, but shows unique retention behaviors for various aromatic compounds and steroids analogous to those of aqueous lipid membranes. In addition, the molecular recognition mechanism was discussed.

EXPERIMENTAL

Materials

The comb-shaped polymer (ODA_n, where n is the average polymerization degree) was prepared by telomerization of octadecylacrylate and 3-mercaptopropyltrimethoxysilane in ethanol. The synthetic procedure of ODA_{28} is as follows: octadecylacrylate and 3-mercaptopropyltrimethoxysilane (30 : 1 in the molar ratio) were dissolved in ethanol. Azobisisobutyronitrile (0.1 wt% for the monomer) was added to the solution at 80°C. The mixture was stirred for 6 h at 80°C under N_2 gas atmosphere. The white precipitates obtained were gathered by filtration, washed successively with methanol and acetone and dried in vacuo. The structure and polymerization degree of the polymer were determined by NMR spectroscopy: ^1H-NMR chemical shifts of ODA_n in $CDCl_3$ were follows: $\delta = 0.75$ ppm ($SiCH_2$), $\delta = 2.28$ ppm (CHCO), $\delta = 3.58$ ppm ($SiOCH_3$), and $\delta = 4.15$ ppm ($C(=O)OCH_2$).

ODA_n was readily introduced onto porous silica gels by mixing in tetrachloromethane at reflux temperature (12 h). YMC 120-S5 (diameter 5 μm, pore size 120 Å) were used as porous silica gels. Successive washing of the resulting gels with chloroform showed no change in weight. The amount of ODA_n introduced was determined by elemental analysis.

Measurements

Silica-supported ODA_n (Sil-ODA_n) was packed into a stainless steel column (4.6 mm I.D. x 250 mm) using a hexanol-chloroform (1 : 1) mixture and the liquid chromatographic property was examined using methanol or methanol-water as mobile phases. The chromatograph included a JASCO 880 PU pump, a Shimadzu UV-visible photodiode array SPD-M6A and a Shodex reflactomonitor SE-51. Five μL of the sample dissolved in methanol was injected through a Reodyne Model 7125 injector. Chromatography was carried out at flow-rate 0.5 mL min^{-1}. The retention capacity (k') was determined by (t_e

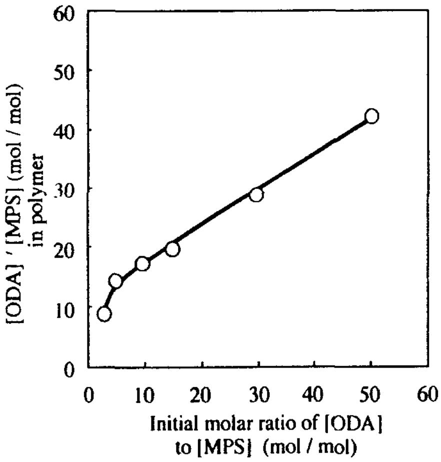

Figure 2. Relationship between the molar ratio ([ODA]/[MPS]) in the telomerization process and the polymer obtained. AOD: octadecylacrylate, MPS: 3-mercaptopropyltrimethoxysilane.

- t_o) / t_o where t_e and t_o are retention time of samples and glycerol, respectively. The separation factor (α) was given by the ratio of retention capacity. DSC thermograms of ODA_n and Sil-ODA_n were obtained using a heating rate of 1 °C min^{-1} with Seiko I & E SSC-580 with a DSC-10 instrument.

Calculations

The structures of ODA_n and aromatic compounds were estimated using Sony-Tektronix CAChe-mechanics (the optimization with MM2 parameters continued until the energy change was less than the 0.001 kcal mol^{-1}) or CAChe-MOPAC (Ver. 6, PM3 option) . The energy level of HOMO and log P were also estimated by CAChe-MOPAC with the PM3 and AM1 options, respectively.

RESULTS AND DISCUSSION

Polymerization Degree of ODA_n

The polymerization degree (n) of ODA_n was determined by ^{1}H-NMR spectroscopy ($SiOCH_3$: $\delta = 3.58$ ppm, $C(=O)OCH_2$: $\delta = 4.15$ ppm). The

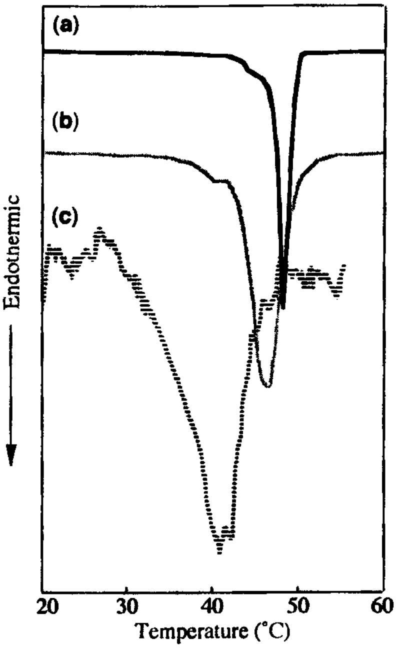

Figure 3. DSC thermograms of ODA_{27} (**a** and **b**) and Sil-ODA_{27}. The thermogram **a** was observed in the absence of solvent. The thermograms **b** and **c** were obtained in the presence of methanol and methanol-water (7 : 3), respectively.

molecular weights of ODA_n determined by ^{1}H-NMR corresponded to the values estimated by size exclusion chromatography (SEC) using a Shodex KF-803 column. For example, the molecular weight of ODA_{28} was 9.3×10^3 as observed by NMR spectroscopy. SEC analysis of ODA_{28} showed that $M_w = 1.3 \times 10^4$ and $M_n = 7.4 \times 10^3$. The small value of $M_w/M_n = 1.76$ indicates that the distribution of the polymerization degree in ODA_n is relatively narrow in spite of the radical polymerization. This may be related to the fact that an alkyl mercaptan is a good chain-transfer agent.

Supporting this, the plots of the polymerization degree (n) and the initial molar ratio show a good linearity (Fig. 2). In this study, ODA_ns with n = 9, 12, 14, 17, 22, 27, 28, 33 and 60 were synthesized by adjusting the initial molar ratio in the telomerization process.

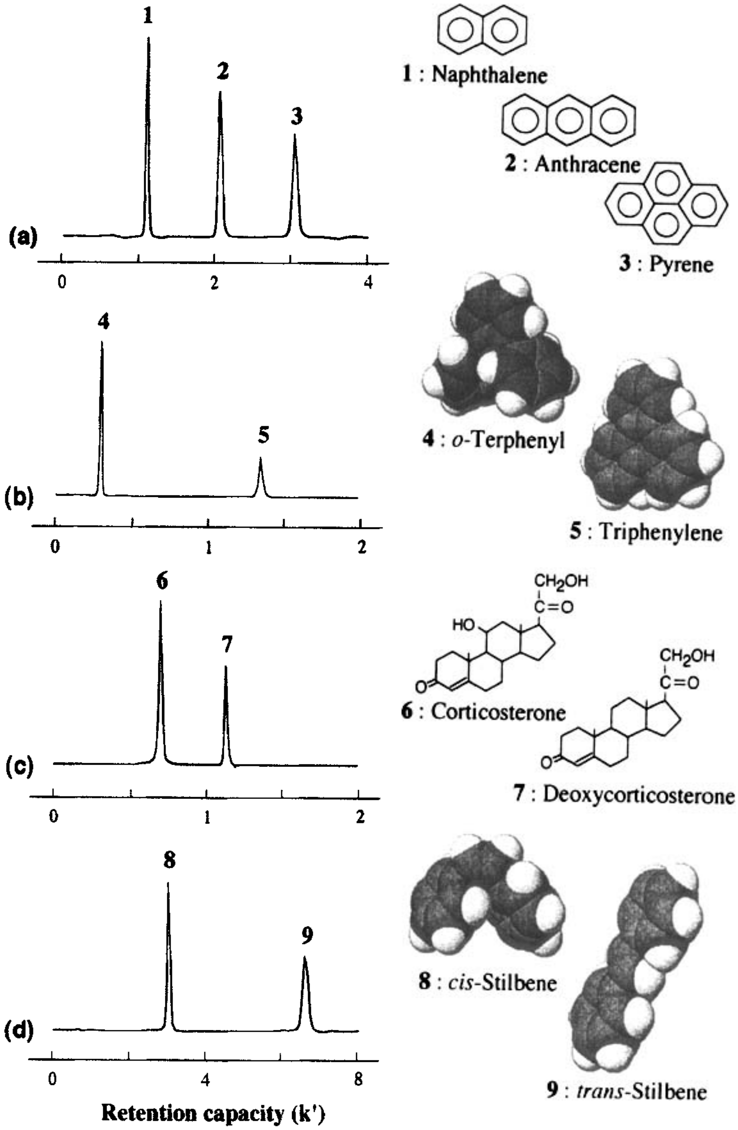

Figure 4. Typical chromatograms with Sil-ODA$_n$ columns (n = 27 in **a**, **b** and **d**; n = 17 in **c**) at 20°C. Mobile phases: methanol (**a** and **b**); methanol-2ater (7 : 3, **c** and **d**).

Phase Transition of ODA_n

The DSC thermograms of ODA_n showed a sharp endothermic peak in both heating and cooling processes. For example, the peak-top temperature of ODA_{27} was 49°C (T_{C2}) with a shoulder (T_{C1}) at 42 - 47°C in the heating process as shown in Fig. 3-a. Polarity microscopic observation indicated that T_{C1} and T_{C2} were assigned to crystal-to-liquid crystal and the liquid crystal-to-isotropic phase transition, respectively. This liquid crystalline state contains Shlieren issue which belonged to a nematic phase and was observed at a temperature range of 42 - 47°C. Similar phase transitions were also observed in methanol (or methanol-water) dispersions, accompanied by a slight decrease in temperature by about 2 - 4°C. This indicates that ODA_n can form highly-ordered structures such as crystal states even in the presence of organic eluents used in the column chromatography process. Phase transitions were also observed in ODA_n with n = 9 - 60, although T_{C2} depended on n (for example, T_{C2} = 41°C (n = 9), 44°C (n = 14), 45°C (n = 20), 49°C (n = 27), 49°C (n = 33) and 49°C (n = 60).

The ODA_n was readily immobilized on silica gels. Successive washing of resulting gels with chloroform showed no change in weight: chloroform is a good solvent for ODA_n. In the case of ODA_{27}, the elemental analysis showed that 20.6 wt% (0.6 unit-mmol g^{-1}) of ODA_{27} was introduced on the silica gels (YMC SIL-120). The amount of ODA_n introduced was dependent on the type of silica gel as well as on the reaction time and the concentration of ODA_n in the immobilization process (detailed results will be reported elsewhere). The immobilized ODA_n (Sil-ODA_n) also showed an endothermic peak in their DSC measurements. Fig. 3 includes a DSC thermogram of Sil-ODA_{27} in methanol-water (7 : 3). The thermogram shows about 8°C lowering of peak-top temperature (T_C) compared with T_{C2} of original ODA_{27}. This indicates that silica gels influence the orientation of bound ODA_{27}, but the bonded phase can maintain oriented structures and undergo crystal-to-isotropic phase transition on silica gels (Fig. 1). Similar phase transition behavior was also observed in Sil-ODA_n with n = 17, 28 and 33.

Retention Behavior for Polyaromatics

Fig. 4-a shows that the column packed with Sil-ODA_{27} performs complete separation for a mixture of naphthalene, anthracene and pyrene. The theoretical plate number and the separation factor (α) at 20°C were about 18000 in pyrene and 3.4 for naphthalene and pyrene, respectively. The elution order was the same as those that were observed in conventional reversed phase

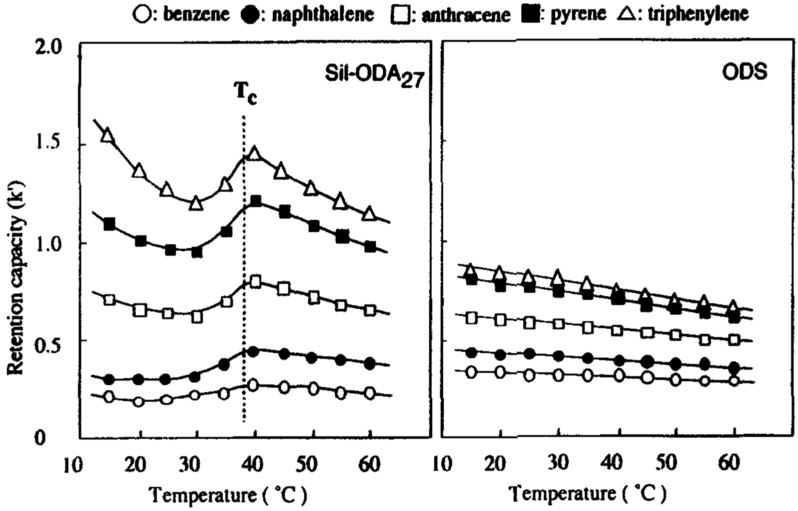

Figure 5. Temperature dependencies on retention capacity for polyaromatics with Sil-ODA_{27} and ODS columns. Mobile phase: methnol, Tc was determined in a methanol dispersion.

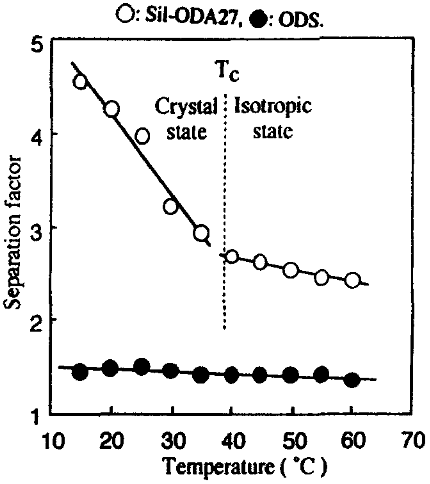

Figure 6. Temperature dependencies on separation factor (the ratio of retnetion capacity for triphenylene to *o*-terphenyl). Tc was determined in a methanol dispersion. Mobile phase: methanol .

liquid chromatography (RPLC) packings, i.e. octadecylated silica gels (ODS: Inertsil ODS, 4.6 I.D. x 300 mm, GL Science Co., Ltd.). It was also confirmed that the retention capacity (k') increased with an increase in mobile phase polarity. These results indicate that the Sil-ODA$_{27}$ has a retention mode similar to RPLC and recognizes molecular hydrophobicity: log P values determined by MOPAC calculation were 3.47, 4.43 and 4.64 in naphthalene, anthracene and pyrene, respectively.

The performance of the Sil-ODA$_{27}$ column was unique in that it was characterized by temperature dependency. As shown in Fig. 5-a, the Sil-ODA$_{27}$ column showed distinct bending in the plots of temperature vs. k'. On the other hand, no similar temperature dependence was observed in ODS, but it must also be taken into account that the separation factor was much smaller in ODS (α = 1.8 at 20°C) than in Sil-ODA$_{27}$ (α = 3.4 at 20°C). As shown in the dotted line of Fig. 5-a, the T_C of Sil-ODA$_{27}$ observed in the DSC (in a methanol dispersion) measurement almost agrees with the temperature of the bending point. This strongly suggests that the unusual temperature dependencies in Sil-ODA$_{27}$ occur at the crystal-to-isotropic phase transition temperature of the bonded phase.

Recognition of Molecular Planarity

The specificity of Sil-ODA$_n$ was emphasized by checking retention behaviors for triphenylene and *o*-terphenyl. These compounds have the same carbon number per molecule and thus the molecular hydrophobicity is similar.

The Sil-ODA$_{27}$ column showed complete separation for triphenylene and *o*-terphenyl as shown in Fig. 5-b. The separation factor (α) at 20°C was 4.2 in Sil-ODA$_{27}$ but 1.5 in ODS. The Sil-ODA$_{27}$ column provided remarkable temperature dependencies with respect to the retention capacity (k') for both samples. The k'-temperature plots show a distinct jump at 30 - 40°C similar to those of anthracene and pyrene. Remarkable temperature dependencies were also observed for the separation factor (Fig. 6). According to Fig. 6, the separation factor is much higher at the crystal state temperature than the isotropic state temperature: 4.7 at 15°C and 2.6 at 45°C. On the other hand, the ODS column showed almost no temperature dependence for the separation factor. Therefore, the enhancement of separation factor at the crystal state temperature cannot be explained only by hydrophobicity recognition. It is known that planar compounds such as triphenylene are more strongly incorporated to an oriented medium than non-planar compounds such as *o*-terphenyl.[17-19] For example, we have previously reported that poly (γ-methyl L-

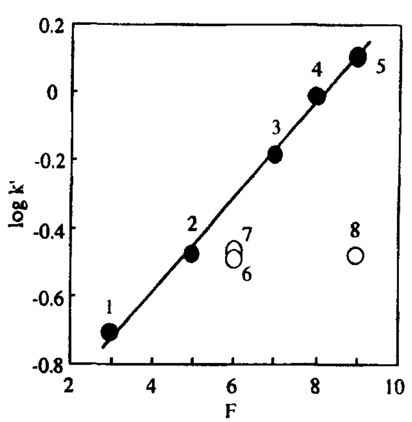

Figure 7. Relationship between retention capacity and correlation factor F with a Sil-ODA_{27} column. mobile phase: methanol. Solutes: 1, benzene; 2, naphthalene; 3, anthracene; 4, pyrene; 5, triphenylene; 6, diphenyl; 7, fluorene; 8, *o*-terphenyl. F = number of double bonds + the number of primary and secondary carbons - 0.5 for a nonaromatic ring.[20]

glutamate) whose main chain is α-helical produces highly-oriented structures and shows unexpectable higher retention capacity for planar aromatics than for non-planar aromatics.[18,19] Therefore, it is presumed that Sil-ODA_{27} also recognizes molecular planarity as well as molecular hydrophobicity. To support this assumption, when the retention capacity was plotted against the correlation factor F proposed by Schabron et al.,[20] whose parameter is related to molecular planarity, the experimental data point in planar aromatics such as benzene, naphthalene, anthracene, pyrene and triphenylene, are approximately on a straight line, but are not lined up in non-planar aromatics such as diphenyl, fluorene or *o*-terphenyl (Fig. 7).

Recognition of Molecular Bulkiness

It is shown that Sil-ODA_n is sensitive to molecular bulkiness. Fig. 8 shows the temperature dependence of retention capacity for adamantane as a sterically bulky compound. The plots include a remarkable increase of retention capacity at temperatures around T_C. Especially, it should be noted that the retention capacity is much smaller at the crystal state temperature than at the isotropic state temperature. This indicates that a sterically bulky compound is hardly incorporated into the highly-oriented bonded phase.

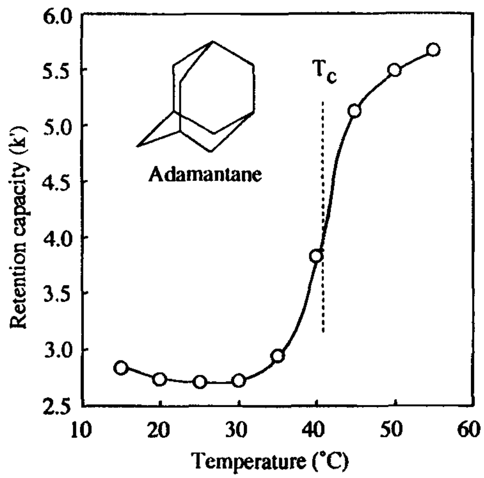

Figure 8. Temperature dependence on retention capacity for adamantane with a Sil-ODA$_{27}$ column. T_C was determined in a methanol-water (7 : 3) dispersion. Mobile phase: methanol-water (7 : 3).

Retention Behavior for Rigid Hydrocarbons, Steroids

It is well-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 the Sil-ODA$_{27}$ column. The temperature dependence on the k' of cholesterol showed a critical bending point around T_C and remarkable increase at temperatures below T_C: 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$_{27}$ incorporates cholesterol specifically at temperatures below 30°C. Fig. 4-c shows a chromatogram of a mixture of steroid hormones, corticosterone and deoxycorticosterone. Complete separation (α = 1.6) was obtained in spite of small structural differences. In the case of ODS, the α was only 1.1.

Discussion on Recognition Mechanisms for Geometrical Isomers

Fig. 4-d shows complete separation for a mixture of *trans*- and *cis*-isomers of stilbene with the Sil-ODA$_{27}$ column. The separation factor was 2.2 at 20°C, although ODS provided a very small separation factor (α = 1.1 at 20°C). The

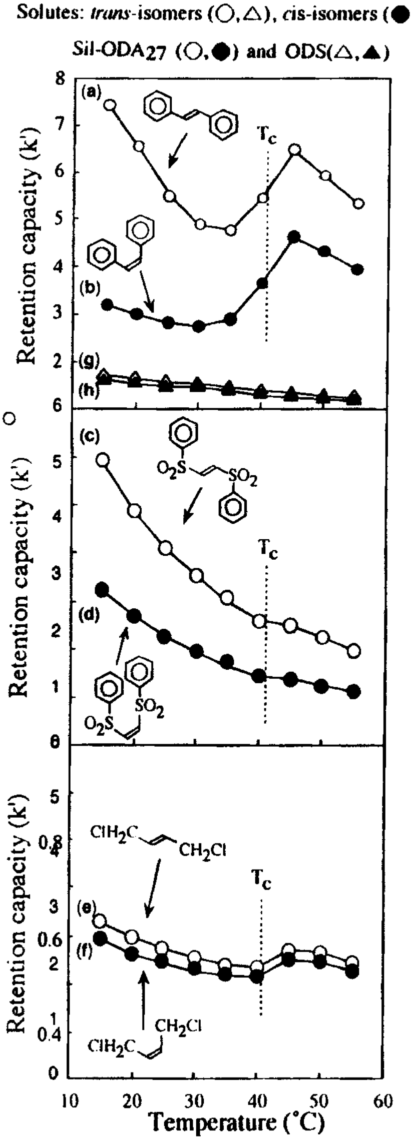

Figure 9. Temperature dependencies on retention capacities for geometrical isomers of stilbene (**a**, **b**, **g** and **h**), 1,2-bis(sulphonyl)ethylene (**c** and **d**) and 1,4-dichloro-2-butene (**e** and **f**). Tc was determined in a methanol-water (7 : 3) dispersion. Mobile phases: methanol-water = 7 : 3 (**a** and **b**), = 5 : 5 (**c** ~ **f**), 9 : 1 (**g** and **h**).

specificity of Sil-ODA$_{27}$ is emphasized by comparing the temperature dependence of Sil-ODA$_{27}$ to ODS. As shown in Fig. 9-g and -h, in the case of ODS. the k' values decreased with increase of temperature. Such negative slopes are commonly observed in usual RPLC packings.[21,22] These can be explained by increase of the solubility of solutes from stationary to mobile phases with increase of temperature.[21,22] Therefore, in the case of ODS, the separation factor should be independent of temperature, and is small and constant (1.04 - 1.07 at 15 - 55°C). This can be also expected by the fact that hydrophobicity of *cis*-stilbene (log P = 4.92) is very similar to that of *trans*-stilbene (log P = 4.88). On the other hand, Sil-ODA$_{27}$ provided critical bending points and positive slopes at temperatures between 35 and 45°C in their plots (Fig. 9-a and -b). These unusual temperature dependencies also include remarkable selectivity changes of 2.33 (15°C), 1.97 (25°C), 1.64 (35°C), 1.55 (40°C), 1.40 (45°C), 1.38 (50°C) and 1.35 (55°C). However, it is believed that the positive slope in the k'-temperature plots at 30 - 45°C and selectivity change are not due to the change of the retention mechanism. To explain this unusual phenomenon, we hypothesized the following: immobilized ODA$_{27}$ forms a mixed phase containing both crystalline and isotropic phases and the ratio of isotropic ODA$_{27}$ to crystalline ODA$_{27}$ increases with increase of temperature between 30 - 45°C. The retention capacity (k') also increases with increase of the ratio, because k' is larger in the isotropic state than in the crystalline state.

On the other hand, we also encountered retention phenomena which cannot be explained only by the above-mentioned molecular recognition mechanism. For example, Sil-ODA$_{27}$ showed geometrically selective retention for 1,2-bis(phenylsulphonyl)-ethylene isomers (α = 1.79 at 20°C, Fig. 9-c and -d), but showed small selectivity for geometrical isomers of 1,4-dichloro-2-butene (Fig. 9-e and -f), 2-hexenol and 1,2-dimethylcyclohexane. The selectivities were only 1.11, 1.09 and 1.05 at 20°C, respectively. Interestingly, however, the k'-temperature plots in all samples showed distinct jumps at temperatures around T_C. This indicates that the difference of bulkiness between *cis*- and *trans*-isomers is not always reflected in geometric selectivity.

On the basis of these results, our proposed mechanism for molecular recognition should be modified and developed as follows: (1) immobilized ODA$_{27}$ forms a highly-oriented structure at its crystal state temperature. A planar compound such as *trans*-stilbene can be more easily incorporated into this oriented polymer than a bulky non-planar compound such as *cis*-stilbene. This mechanism is analogous to the fact that cholesterol as a rigid and planar compound is readily incorporated into lipid bilayer membranes which form highly oriented structures. (2) Immobilized ODA$_{27}$ can interact with double

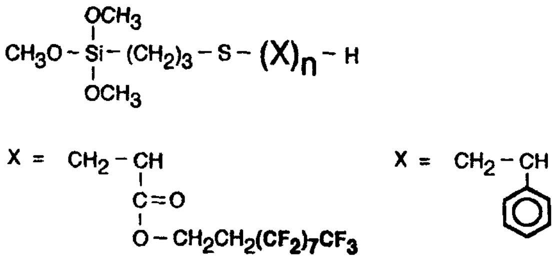

Figure 10. Poly(perfluoroalkylacrylate)[25] and poly(styrene)[26] with reactive terminal groups. Both polymers can be readily immobilized onto silica gels and show specific chromatographic separations.[25,26]

bonds of solutes with recognizing their π electron density. Perhaps this interaction is derived from the carbonyl π electrons of acrylate moieties which can work as electron-accepting groups. This assumption is supported by following facts: (i) it was confirmed that the selectivity with crosslinked poly(methyl acrylate) polymer (MA) particles as a reference showed higher selectivity for various isomeric aromatics was smaller than with Sil-ODA$_n$ but significantly higher than with ODS, although these MA particles provided comparably small k' values because there were no long-chain alkyl groups. This result indicates that the carbonyl groups of MA moiety play an important role for molecular recognition. The detailed date for MA particles were briefly reported.[23] (ii) Judging from the calculation of the HOMO (Highest Occupied Molecular Orbital) energy level in stilbenes, *trans*-isomers are more electron-donating (-8.63 eV) than *cis*-isomers (-9.06 eV). Bredás and Street have reported using computer calculations that similar interactions occur between benzene and formic acid.[24] On the other hand, geometrical isomerism for 1,4-dichloro-2-butene (-9.63 and -9.86 eV in *trans*- and *cis*-isomers) and 2-hexenol (-10.28 and -10.13 eV in *trans*- and *cis*-isomers) having non-conjugating substituent groups provides no significant difference in energy level of HOMO. In these cases, Sil-ODA$_{27}$ shows a small selectivity for retention of these isomers.

CONCLUSIONS

(1) A special polymer-bonded phase on silica gels was synthesized by one-step telomerization of octadecylacrylate using 3-mercaptopropyltrimethoxysilane. The degree of polymerization was readily controlled by adjusting the initial molar ratio in the telomerization process.

(2) By selecting monomers, various polymer-bonded phases can be prepared (Fig. 10) and show unique separations. Detailed results have been reported elsewhere.[25-27]

(3) The polymer (ODA_n) was readily immobilized onto silica gels with a reactive terminal group. Therefore, the polymer still maintains flexibility after immobilization.

(4) The immobilized polymer ($Sil\text{-}ODA_n$) underwent crystal-to-isotropic phase transition on silica gels. The elution order for hydrophobic compounds in the packed column with $Sil\text{-}ODA_n$ usually agreed with the order expected as RPLC mode regardless of temperature. However, $Sil\text{-}ODA_n$ at crystal state temperature provided exceptionally specific selectivity, recognizing for molecular planarity 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.

(5) The specificity of $Sil\text{-}ODA_n$ was drastically appeared by examining the temperature dependency.

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