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Enantiomer separation by reversed-phase liquid chromatography with novel hydrophobic phases composed of chiral cationic surfactants

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Abstract

This paper describes enantiomer separation using four kinds of chiral stationary phases (CSPs) where quaternary ammonium surfactants containing L-valine diamide moieties into long alkyl chains were bound to silicagel supports by reversed phase liquid chromatography. Our aim was to examine hydrogen bonding association of the chiral moiety in hydrophobic phase brought about by aggregation of the micelle-forming surfactants on the surface. The following CSPs were thus derived from the vinyl-terminated chiral surfactants via hydrosilylation: CSP **1** from *N*-[3-(10-undecenoyl-L-valylamino)propyl]-*N*,*N*,*N*-trimethylammonium bromide, CSP **2** from *N*-[6-(10-undecenoyl-L-valylamino)hexyl]-*N*,*N*,*N*-trimethyl-ammonium bromide, CSP **3** from *N*-[3-(10-undecenoyl-L-valylamino)propyl]-*N*-octadecanyl-*N*,*N*-dimethyl-ammonium bromide and CSP **4** from *N*-[6-(10-undecenoyl-L-valylamino)hexyl]-*N*-octadecanyl-*N*,*N*-dimethylammonium bromide. The degree of hydrophobicity in the interfacial phase was observed by measuring pyrene fluorescence in aqueous media including an organic modifier. Retention of racemic *N*-acylleucine isopropyl esters was highest in CSP **4**, followed by **3**, **2**, and **1**. Largest α values toward enantiomer separation were observed for CSP **4** where the chiral moieties were kept through a hexamethylene unit apart from the polar head groups and to which another long alkyl chain was attached, as compared with those for CSP **4**. In CSP **4**, the chiral moiety to interact with enantiomeric solutes should be buried into the interfacial phase deeply in more extent than CSP **3**. In a similar manner, CSP **2** has more effective for enantiomer separation than CSP **1**. The interfacial phase of these CSPs was easily exposed to the bulk phase because of the affinity between the bulk phase and the polar head groups as well as their electrostatic repulsion. However, degree of the enantiomer separation can be controlled by the depth of the chiral moiety in the hydrophobic interfacial phase.

Keywords: Enantiomer separation using aqueous media; Reversed phase liquid chromatography; Cationic surfactant; Half lipid structure

1. Introduction

Liquid chromatography (LC) using chiral stationary phases (CSPs) is one of the most sophisticated means for detecting chiral recognition ability of transient diastereomeric intermolecular association. This can be recognized as enantiomer separation when the chiral molecule is bound to solid support and the enantiomer is sorbed to the support from the mobile phase [1,2]. Hydrogen bonding is one of the most significant contributors to form diastereomeric associations between the enantiomers [3]. Through the preliminary study, it was revealed that hydrogen-bond associations are weakened in reversed phase liquid chromatography with aqueous media because of their strong polarities to prevent interactions between CSP and the enantiomeric solutes. Using acetyl-L-valine *tert*-butylamide as a model compound for the chiral selection with silicagel modified with (10-undecenoyl)-L-valine *tert*-butylamide via hydrosilylation (CSP **5**), the hydrogen bonding associations between this region and 4-nitrobenzoyl(NB)-L-leucine isopropyl ester were measured using nuclear magnetic resonance (NMR) techniques [3]. Hydrogen bonds at the two amide sites of the chiral diamide moiety confirmed enantioselectivity in both the normal and reversed phases. CSP **5** showed enantioseparability and proved the effectiveness of hydrogen-bond association in aqueous media [4]. The chiral separation of enantiomers should thus occur when such hydrogen bond-

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ings are formed in a hydrophobic environment, shielded from the bulk aqueous phase. This was also demonstrated by the fact that chiral micelles formed by *N*-[3-(dodecanoyl-L-valylamino)propyl]-*N*,*N*,*N*-trimetylammonium bromide (surfactant **1a**) and *N*-[6-(nonanoyl-L-valylamino)hexyl]-*N*,*N*,*N*-trimetylammonium bromide (surfactant **2a**) were capable of separating enantiomers in aqueous media by electrokinetic chromatography (EKC) [5,6]. In EKC, these micelles, formed in dynamic association-dissociation equilibrium with monomeric surfactants in water [7,8], functioned as a transient stationary phase entrapping enantiomers by hydrophobic interactions. Micellar EKC (MEKC) was known as method of chiral purity determination in micro-scale.

Immobilization of micellar structures on solid supports is thus thought to be an effective method to separate substrates using a combination of hydrophobic interaction and hydrogenassociation in LC with aqueous media. Aqueous LC using phosphatidylcholine-bonded silicagel has been found to selectively separate small hydrophobic peptides both with and without cysteine residues [9,10]. This chromatographic technique, in which half of the lipid bilayer is immobilized on the solid support, is based on the hydrophobic effects of the hydrocarbon chain in phosphatidylcholine and the additional electrostatic and steric interactions of its polar head group. In this study, *N*-[3-[(10-undecenoyl)-L-valylamino]propyl]-*N*,*N*,*N*-trimethylammoniumbromide (surfactant **1b**) and *N*-[6-(10-undecenoyl-L-valylamino)hexyl]-*N*,*N*,*N*-trimethylammonium bromide (surfactant **2b**), which are congeners of **1a** and **2a**, respectively, were chemically bound to silicagel in order to produce a hydrophobic interfacial phase composed of the local aggregation of these surfactants on the silicagel surface. These stationary phases were CSP **1** and **2**, respectively. The surfactants grafted onto the surface are expected to stretch due to the associations between the polar head group and the bulk water. On the other hand, at least in part, the bonded surfactant molecules should be folded and/or collapsed on the surface, as has been cited as random walk of surfactants into micelles; thus leading to make up hydrophobic interfacial phases based on their aggregation under aqueous media. Under these conditions, the difference in the depth with which the valine diamide moiety is located in the interfacial phases of CSP **1** and **2** should be observed in the enantiomer separation with aqueous mobile phase solvents.

N-[3-(10-Undecenoyl-L-valylamino)propyl]-*N*-octadecanyl-*N*,*N*-dimethylammonium bromide (surfactant **3a**) and *N*-[6-(10-undecenoyl-L-valylamino)hexyl]-*N*-octadecanyl-*N*,*N*-dimethylammonium bromide (surfactant **4a**), in which a long hydrocarbon chain was added to surfactants **1b** and **2b**, respectively, were considered to provide vesicle structures and be more hydrophobic around the valine diamide moieties compared to surfactants **1b** and **2b**, from which they were derived. These surfactants were thus chemically bound to silicagel to obtain CSP **3** and **4**, which have interface structures similar to half of the lipid bilayer structure.

The hydrophobic environment brought about by aggregation of surfactants bonded on silicagel surface was evaluated with pyrene whether it has enough hydrophobicity and volume to form hydrogen-bond association between valine diamide moieties and solutes in aqueous media. The chiral selectivity of CSPs **1**–**4** was evaluated using benzoyl, 3,5-dinitrobenzoyl (DNB), and 4-nitrobenzoyl (NB) amino isopropyl esters as model enantiomers, separated using mobile phases composed of water–organic solvent mixtures.

2. Experimental

2.1. Preparation of chiral cationic surfactants and surfactant-bonded silicagels

Three chiral cationic surfactants capable of forming micelles, surfactants **1a**, **1b** and surfactant **2a**, and CSP **5** were prepared as previously reported [6].

*2.2. CSP **1** from N-[3-(10-undecenoyl-L-valylamino) propyl]-N,N,N-trimethylammonium bromide (surfactant **1b**)*

CSP **1** was prepared by binding surfactant **1b** to silicagel by hydrosilylation, according to the procedure previously reported [6].

*2.2.1. N-[3-[(11-chlorodimethylsilylundecanoyl) valylamino]propyl]-N,N,N-trimethyl-ammonium bromide (**1c**)*

Surfactant **1b** (778 mg) and a catalytic amount of chloroplatinic acid (5 mg) were dissolved in 5 mL of dry chloroform. To the solution was added 3 mL dimethylchlorosilane, after which the mixture was refluxed 60 °C under an argon atmosphere. After 20 h, a 0.5 mL portion of the mixture was extracted, evaporated to dryness using a vaccum and the residue was redissolved in 0.5 mL of deuteriochloroform. The completion of the hydrosilylation was confirmed using ^{1}H NMR to monitor the disappearance of the vinyl protons signal in surfactant **1b**. The solvent was then removed under reduced pressure from the original solution, the residue was redissolved in dry chloroform, and the solvent was subsequently evaporated. This procedure was repeated twice in order to completely remove the excess silane reagent. The residue was used in the following step without further purification.

*2.2.2. CSP **1***

Silicagel [1.25 g; Nucleosil 100-5, 5 μm (specific surface area, 350 m^2/g), Macherey-Nagel, Dueren, Germany] was dried at 180 °C for 20 h under reduced pressure and then cooled to room temperature. The silicagel was suspended in 3 mL of freshly distilled anhydrous pyridine. The silylated surfactant **1c** was dissolved in 5 mL of anhydrous pyridine, and subsequently added to the suspension. The mixture was gently stirred at room temperature for 20 h. The

modified silica gel was filtered and consecutively washed with 30 mL of each; methanol, chloroform and acetone. IR (diffuse reflectance) (KBr, cm^{-1}): 3281, 2927, 2855, 1657, 1531 and 1468; Anal. Found: C, 12.70; N, 1.83; surface coverage calculated from the nitrogen content, 0.57 mmol/g (1.62 μmol/m^2).

2.3. CSP **2** from N-[6-(10-undecenoyl-L-valylamino) hexyl]-N,N,N-trimethylammonium bromide (surfactant **2b**)

Surfactant **2b** was obtained from benzyloxycarbonyl(Z)-L-valine 6-bromohexyl-amide according to a procedure similar to that reported previously [6]. An outline for the procedure is as follows.

2.3.1. N-(10-Undecenoyl)-L-valine 6-bromohexylamide (**2c**)

Z-L-Valine 6-bromohexylamide was treated with 25% HBr-AcOH solution in order to remove a Z group, and subsequently reacted with 10-undecenoyl chloride in a catalytic amount of 4-(*N*,*N*-dimethylamino)pyridine in $NaHCO_3$ solution in order to obtain the desired compound: mp 110.0–111.7 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.94 (d, 3H, *J* = 7 Hz), 0.95 (d, 3H, *J* = 7 Hz), 1.25–1.65 (brd, 18H, $NHCH(CH_2)_3$, $(CH)_6CH_2CO$), 1.85 (tt, 2H, *J* = 7 Hz), 2.00–2.15 (m, 1H), 2.05 (m, 2H), 2.21 (t, 2H, *J* = 7 Hz), 3.14–3.35 (m, 2H), 3.39 (t, 2H, *J* = 7 Hz), 4.15 (dd, 1H, *J* = 8, 9 Hz), 4.90–5.02 (m, 2H, CH_2=$CHCH_2$), 5.73–5.88 (m, 1H, CH_2=$CHCH_2$), 6.00–6.10 (brd, 2H, CONHCH, $CONHCH_2$); Anal. Calcd for $C_{22}H_{41}N_2O_2Br$: C, 59.32; H, 9.28; N, 6.29. Found: C, 59.37; H, 9.24; N, 6.15.

2.3.2. Surfactant **2b**

The derivative **2c** was reacted with trimethylamine in 2-propanol to obtain surfactant **2b**: mp 144.5–145.5 °C; Anal. calcd for $C_{25}H_{50}N_3O_2Br$: C, 59.51; H, 9.99; N, 8.33. Found: C, 58.56; H, 9.86; N, 8.26.

2.3.3. CSP **2**

CSP **2** was prepared from surfactant **2b** and silicagel via hydrosilylation, as described in above section: IR (diffuse reflectance)(KBr, cm^{-1}): 3281, 2925, 2855, 1657, 1531, 1468; Anal. Found; C, 14.02; N, 1.79; surface coverage calculated from the nitrogen content, 0.57 mmol/g (1.62 μmol/m^2).

2.4. CSP **3** from N-[3-(10-undecenoyl-L-valylamino) propyl]-N-octadecanyl-N,N-dimethylammonium bromide (surfactant **3a**)

Surfactant **3a** was prepared from *N*-(10-undecenoyl)-L-valine 3-bromopropyl-amide, which has been reported for preparation of surfactant **1a** [6].

2.4.1. N,N-Dimethyloctadecanamide (**3b**)

Octadecanoyl chloride (12.1 g), prepared from octadecanoic acid and thionyl chloride in DMF, was dissolved in 20 mL of benzene and to the solution was bubbled gaseous dimethylamine for 1 h under stirring. The mixture was adjusted to pH 4.0 with 1 M HCl, extracted with 30 mL of ethyl acetate three times and washed with a saturated NaCl solution. The residue was purified by silicagel chromatography [hexane:ethyl acetate, 3:1 (v/v)] and crystallized with diisopropyl ether to obtain 10.8 g (yield 87%) of the desired colorless compound: mp 49.0–50.0 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.87 (t, 3H, *J* = 6 Hz), 1.20–1.30 (brs, 30H), 2.29 (t, 2H, *J* = 8 Hz), 2.94 (s, 6H).

2.4.2. N,N-Dimethyloctadecanylamine (**3c**)

N,*N*-Dimethyloctadecanamide (9.54 g) was dissolved in 13 mL of diethyl ether. This solution was added dropwise to a suspension of lithium aluminum hydride (1.74 g) in 50 mL of diethyl ether under stirring at 50 °C. After refluxing for 17 h, saturated Na_2SO_4 solution was added and the precipitate was removed by filtration. The precipitate was washed with 30 mL of diethyl ether and the combined filtrate was evaporated to dryness to result in a pale yellow oil (8.86 g, yield 97.4 %): ^{1}H NMR (300 MHz, C^2HCl_3) δ 0.88 (t, 3H, *J* = 6 Hz), 1.25–1.35 (brs, 30H), 1.42–1.52 (brs, 2H), 2.25 (s, 6H).

2.4.3. Surfactant **3a**

N,*N*-Dimethyloctadecanylamine (**3b**) was reacted with *N*-(10-undecenoyl)-L-valine 3-bromopropylamide [6] to form surfactant **3a**: mp 94.5–98.0 °C; $[\alpha]^{25}D = -8.90°$ (*c* = 1.00, chloroform); ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.87 (t, 3H, *J* = 7 Hz), 0.97 (d, 6H, *J* = 7 Hz), 1.25–1.75 (brd, 46H, $NHCH_2CH_2$, $(CH_2)_6CH_2CO$, $NCH_2(CH_2)_{16}$), 2.02 (dt, 2H, *J* = 8 Hz, CH_2=$CHCH_2$), 2.00–2.15 (brd, 1H), 2.35–2.50 (m, 2H), 3.20 (s, 6H), 3.27–3.37 (brd, 4H), 3.75–3.85 (brd, 2H), 4.29 (dd, 1H, *J* = 6, 9 Hz), 4.89–5.02 (m, 2H, CH_2=$CHCH_2$), 5.73–5.88 (m, 1H, CH_2=$CHCH_2$), 7.28–7.33 (brd, 1H), 8.15–8.20 (brd, 1H); Anal. Calcd for $C_{40}H_{81}N_3O_2Br$: C, 66.83; H, 11.22; N, 5.59. Found: C, 66.80; H, 11.17; N, 5.92.

2.4.4. CSP **3**

CSP **3** was then prepared from surfactant **3a** and silicagel, as described above: IR (diffuse reflection)(KBr, cm^{-1}): 3283, 2925, 2855, 1657, 1531, 1468; Anal. Found: C, 18.25; N, 1.41; surface coverage calculated from the nitrogen content, 0.50 mmol/g (1.42 μmol/m^2).

2.5. CSP **4** from N-[6-(10-undecenoy)-L-valylamino) hexyl]-N-octadecanyl-N,N-dimethylammonium bromide) (surfactant **4a**)

2.5.1. Surfactant **4a**

N-(10-Undecenoyl)-L-valine 6-bromohexylamide (**2c**) (4.29 g) and *N*,*N*-dimethyloctadecanylamine (**3c**) (3.18 g) were dried and dissolved in 5 mL of benzene. After reflux for 70 h, the solution was evaporated to dryness. The

residue was applied to gel permeation chromatography (LH-20, MeOH) to obtain 6.31 g of amorphous crystals (yield 88%): mp 86.5–89.0 °C; $[\alpha]^{26}D = -3.90°$ (c = 1. 00, $CHCl_3$); ^1H NMR (300 MHz, $CDCl_3$) δ 0.87 (t, 3H, J = 7 Hz), 0.98 (dd, 6H, J = 7, 7 Hz), 1.20–1.80 (brd, 52H, $NHCH_2(CH_2)_4$, $(CH2)_6CH_2CO$, $NCH_2(CH_2)_{16}$), 2.02 (dt, 2H, J = 7, 8 Hz, CH_2=CHCH_2), 2.25–2.40 (m, 1H), 2.40–2.50 (brd, 2H), 3.30 (s, 6H), 3.35–3.42 (brd, 4H), 3.60–3.75 (brd, 2H), 4.47 (dd, 1H, J = 8 Hz), 4.89–5.02 (m, 2H, CH_2=CHCH$_2$), 5.73–5.86 (m, 1H, CH_2=CHCH$_2$), 7.96–8.00 (brd, 1H), 8.55–8.65 (brd, 1H); Anal. Calcd. for $C_{43}H_{87}N_3O_2Br$: C, 67.89; H, 13.19; N, 5.66. Found: C, 67.73; H, 11.42; N, 5.53.

2.5.2. CSP 5

CSP **4** was prepared from surfactant **4a**, as described above: IR (diffuse reflection)(KBr, cm^{-1}): 3278, 2925, 2855, 1646, 1537, 1468; Anal. Found: C, 18.64; N, 1.68; surface coverage calculated from the nitrogen content, 0.48 mmol/g (1.37 mmol/m^2).

2.6. Measurement of microenvironment hydrophobicity in interfacial phases of CSPs

Observation of the microenvironment polarity of CSPs **1**–**4** using pyrene as a fluorescence probe [11,12] was conducted under the same conditions as previously described [6].

2.7. Determination of surfactant aggregation numbers

Subsequent calculations of surfactant aggregation numbers were conducted using a DLS-700 laser light scattering spectrophotometer (21 mm cell length) and a DRM 1020 reflective index detector (Otsuka Electronics, Osaka, Japan).

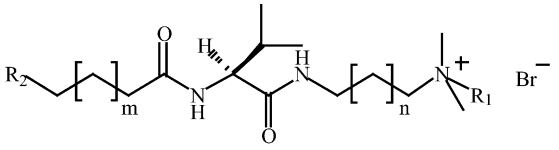
2.8. Liquid chromatography using CSPs

Liquid chromatography was carried out using an LC-5A pump (Shimadzu, Tokyo, Japan), an SPD-2AM UV detector (Shimadzu, Kyoto, Japan) equipped with a 0.5 μL flow cell, or an SPD-2A UV detector (Shimadzu, Kyoto, Japan) equipped with a 12 μL flow cell at 25 °C. Solute elution was detected at 254 nm. CSP packing was performed at GL Science, Tokyo, and columns were stainless-steel tubings of 150 mm × 4.6 mm I.D. Theoretical plate hights for all CSP columns were measured with naphthalene as a solute and a mixture of acetnitrile and water [1:1, v/v] as a eluent: 6300 for CSP **1**; 12 700 for CSP **2**; 9000 for CSP **3**; 8300 for CSP **4**.

3. Results and discussion

3.1. Surfactant aggregation number measurement of trimethylammonium-terminated surfactant *1a* and *2a*

Self-organization nature of surfactants **1a** and **2a** were revealed to be different in the previous paper [6] (see Fig. 1). Surfactant **2a** exhibited a higher critical micelle concentration (CMC) and hydrophobicity of miceller interior than surfactant **1a** (CMC measured with pyrene in water: **1a**, 5.5 mM; **2a**, 35 mM; CMC measured with pyrene in 0.1 M Tris-HCl: **1a**, 1.8 mM; **2a**, 25 mM [6]). The structural difference between surfactants **1a** and **2a** was the location of the valine moieties; the valine moieties in surfactant **2a** were located further from the polar head group than in surfactant **1a**. We examined the average molecular weight of these micelles using light scattering analysis, the aggregation number of surfactant **1a** was found to be about 61 in 0.1 M Tris-HCl buffer. Surfactant **1a** thus formed spherical micelles of comparable size to the general cationic surfactant, dodecyl trimethylammonium bromide (DTMAB); its aggregation number was about 50 in water [7]. On the other hand, the aggregation number of surfactant **2a** in 0.1 M Tris-HCl buffer was very high (around 725) and it did not form spherical micelles but rather formed rod-shaped micelles, as shown by the results of light scattering analysis.



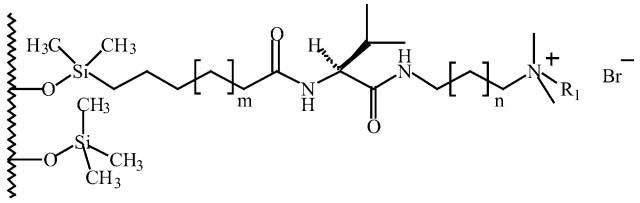
surfactant	m	n	R_1	R_2
1a	6	1	CH_3	CH_2CH_3
1b	6	1	CH_3	$CH=CH_2$
2a	4	4	CH_3	CH_2CH_3
2b	6	4	CH_3	$CH=CH_2$
3a	6	1	$(CH_2)_{17}CH_3$	$CH=CH_2$
4a	6	4	$(CH_2)_{17}CH_3$	$CH=CH_2$

Fig. 1. Structure and abbreviations of the cationic surfactants **1**–**4**, consisting of L-valine dimaide moieties.

The change in micelle shape and the higher CMC of surfactant **2a** were due to the position of the valine diamide moieties, which have intermediate polarity, interfering with the hydrophilicity-hydrophobicity balance between the polar head group and the long hydrocarbon chain. Surfactants **1a** and **2a** did not, however, exhibit marked differences in their chirality recognition during MEKC [6]. This probably indicates that a middle position of the valine diamide moiety in one surfactant **2a** is not reflected to that in self-organization of surfactant **2a** so as to make the chiral moiety in the hydrocarbon chain deeper in its position than surfactant **1a**. Immobilization of surfactants **1b** and **2b** (analogues of **1a** and **2a**) on silicagel surface should generate the ideal position in their bonded layer because of local dissociation of the terminal quaternary ammonium group in bulk water (see Fig. 2).

3.2. Observation of microenvironment polarity of CSPs *1–4*

The intensity ratio of third absorption band at 383 nm relative to first band at 373 nm in pyrene fine vibronic bands (III/I



CSP	m	n	R_1
1	6	1	CH_3
2	6	4	CH_3
3	6	1	$(CH_2)_{17}CH_3$
4	6	4	$(CH_2)_{17}CH_3$

Fig. 2. Structure and abbreviations of chiral stationary phases (CSPs) **1**–**4**, containing the cationic surfactants **1b**–**4b**, consisting of L-valine dimaide.

ratio) indicates microenvironment polarity around pyrene and ranges from 0.5 in water to 1.6 in hexane [12]. Fig. 3 shows the III/I ratio of pyrene sorbed on the surface of CSPs in water–methanol mixtures. Methanol concentration ranged from 0.8 to 42.8% (v/v). When using 0.8% methanol mixture, CSP **3** gave the maximum III/I ratio of 0.96 and CSP **2** gave the minimum ratio of 0.85. This indicates that the surfactant-like residues on CSPs **1**–**4** were hydrophobically aggregated each other to form the interfacial phase in aqueous media. This formation of interfacial phase is indispensable to the molecular recognition based on a hydrogen-bond association in reversed-phase LC with aqueous media. CSP **5** showed higher III/I ratio (1.14) than CSPs **1**–**4**, which is probably due to water penetration induced by the electrostatic repulsion of the polar head groups and their solvation by the bulk water. Into DTMAB micelles, water penetrates at least the first five carbon atoms of the chain from the polar head group [8]. Similar water penetration is thought to occur with CSPs **1**–**4**. With increasing methanol concentration, the III/I ratio for CSP **1**–**4** showed a weak but apparent increase. In the same measurements with octadecylsilylated silicagel (ODS) at low concentrations of methanol, pyrene was adsorbed to ODS interfacial phase and partially exposed to the surrounding solvent as the amounts of intercalated methanol and the interfacial phase volume were increased [13]. The interfacial phases of CSPs **1**–**4** showed the same hydrophobic nature as that of ODS. Polar head groups of CSPs **1**–**4** however induced bulk water penetration compared with octadecyl groups of ODS, III/I ratio increase brought about strong hydrophobic interaction with long alkyl chains was repressed.

Continually, acetonitrile or THF which is easy to distribute to the interfacial phase [14–16] was added to aqueous media, hydrophobic nature of interfacial phase found onto the surface of silicagel was predicted. III/I ratio of CSPs **1**–**4** suspension was slight decreased with increase of acetonitrile or THF concentration as shown in Fig. 4. Especially, effect of organic modifiers on CSP **3** and **4** was little, those hydrophobic interfacial phase were not affected by the polarity of bulk phase. Polar head of CSPs **1**–**4** not only brought about the penetration of water molecules from bulk phase but also kept residues of silicagel surface an extended posture to prevent penetration of organic modifier from bulk phase.

CSPs **3** and **4**, which possess *N*-octadecyl groups along with the decamethylene spacer to support the valine diamide moiety, provided hydrophobicity higher than CSPs **1** and **2**, which only possess the decamethylene spacer. The coverage of residues bonded on the silicagel (mmol/g) are as follows: CSP **1**, 0.57; **2**, 0.57; **3**, 0.50; **4**, 0.48. The bonded residues of CSPs **3** and **4** have lower coverage than those of CSPs **1** and **2**, but the former CSPs still exhibit higher hydrophobicity. Biological bilayer membranes are generally known to have a density of 67–77 A^2/molecule [9], while CSPs **3** and **4** formed the bonded layer at a density of about 100 A^2/molecule, which is similar to that of biological membranes. CSPs **3** and **4** would be thus considered to have a half structure of the lipid bilayer where surfactants having both two long hydrocarbon

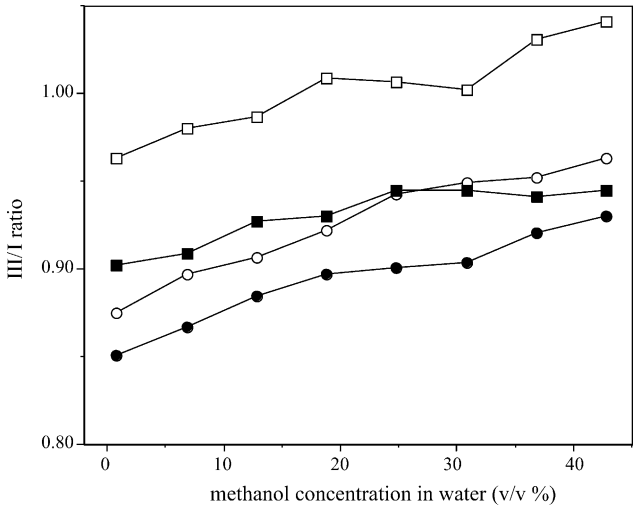


Fig. 3. The III/I ratios of pyrene sorbed onto CSPs **1**–**4** as a function of the methanol concentration in water: (a) CSP **1**, open circle; **2**, filled circle; **3**, open square and **4**, filled square.

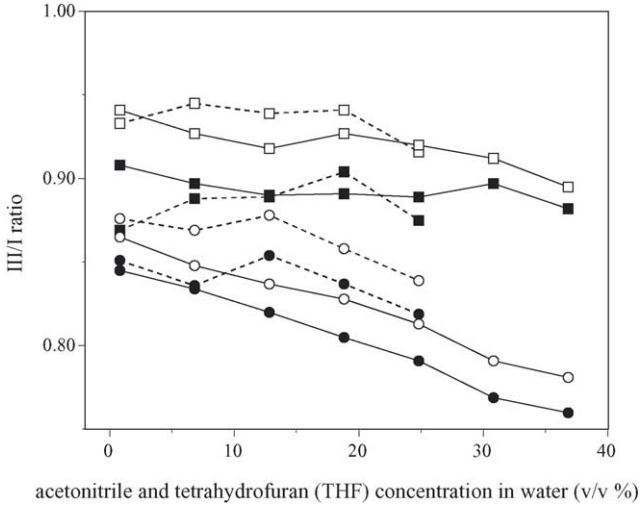


Fig. 4. The III/I ratios of pyrene sorbed onto CSPs **1**–**4** as a function of the acetonitrile and tetrahydrofuran (THF) concentration in water: (a) CSP **1**, open circle; **2**, filled circle; **3**, open square; **4**, filled square; acetonitrile, solid line; THF, broken line.

Table 1
Enantiomer separation of three types of racemic *N*-acylleucine isopropyl esters on CSPs **1**–**4** using a 50% (v/v) water–methanol mixture as an eluent[a]

CSP	3,5-Dinitrobenzoyl (DNB)		4-Nitrobenzoyl (NB)		Benzoyl	
	k'_D[b]	α	k'_D[b]	α	k'_D[b]	α
1	4.80	1.08	3.17	1.04	2.44	1.06
2	5.33	1.15	3.88	1.09	2.89	1.11
3	11.25	1.07	5.30	1.00	4.15	1.05
4	14.66	1.19	7.81	1.12	5.38	1.13

[a] Other chromatographic conditions are as described in Section 2.
[b] Retention factor (k') of the first eluted D-enantiomer.

chains and a polar head group are densely arranged so as to contact these chains each other [10,17].

3.3. Separation of racemic amino acid derivatives using CSPs *1*–*4* in aqueous media

Table 1 shows enantiomer separation of three different types of acylated leucine isopropyl esters on CSPs **1**–**4** using 50%(v/v) water–methanol mixtures as an eluent. DNB derivatives gave the highest separation factor (α) for all CSPs, while for NB derivatives their separations were smaller than those for DNB derivatives, particularly on CSP **3**, NB derivative was not separated at all. For the derivatives resolved, all CSPs retained the L-enantiomer more strongly than the D-enantiomer.

This result thus confirmed that the diastereomeric complex was formed by hydrogen bondings between the enantiomer and valine diamide moieties within the bonded layers of CSPs **1**–**4**. These CSPs gave separation and retention factors less than those observed for CSP **5** (solute; NB-leucine isopropyl ester, k': 9.75, α: 1.33). The decrease in retention factor is probably ascribed to the hydrophobic interactions of the solutes to these CSPs, which was lessened with water penetration to their bonded layers. This penetrated water can competitively form hydrogen bonds at the valine diamide moiety into the bonded layers to reduce separation factors between the enantiomers. The high α value of DNB-leucine isopropyl ester (1.34) and NB-leucine isopropyl ester (1.24) obtained with MEKC using surfactant **1b** micelle were suggested that *tert*-butyl group of CSP **5** prevented invasion of water molecules into interfacial face on the silicagel rater than contributed to enantioselectivity. The residues of CSP **1** and **2** could not form hydrophobic interfacial face on its surface of silicagel enough to recognize enantiomer with hydrogen bonding association in the aqueous media.

3.4. Comparison of α and k' of N-acylleucine isopropyl esters between CSPs modified with micelle-forming surfactants (*1* and *2*) and CSPs with vesicle-forming surfactants (*3* and *4*)

When the surfactant-like residues bonded on the support take place the extended posture by the local dissolution of their quaternary ammonium head groups, the valine diamide moiety of CSP **2** should be buried into the hydrophobic interfacial phase more deeply than that of CSP **1** because of its longer spacer length between the diamide moiety and the polar head group. This should make α and k' values observed for CSP **2** larger than those for CSP **1**. Above hypothesis, which has already been constructed in EKC separation using chiral micelles formed with surfactants **1b** and **2b**, was at first demonstrated by LC separation with CSPs **1** and **2**. CSP **2** exhibited however a lower III/I ratio than CSP **1**. This may be explained by the reason that pyrene is sorbed around the valine diamide site and thus influenced by its dipole moment.

Substitution of *N*-methyl group in the ammonium head group of CSP **2** to *N*-octadecyl group gave CSP **4**, which should make the bonded layer more hydrophobic than that of CSP **2**. Schematic representation of the interfacial phase of CSP **4** is presented in Fig. 5. CSP **4** gave, as expected, α and k' values higher than those for CSP **2**: α and k' for the D-enantiomer of the DNB derivative increased from 1.15 and 5.33 on CSP **2** to 1.19 and 14.66 on CSP **4**, respectively. CSP **3** showed k' values higher but α values lower than those for CSP **1**. Because the valine diamide moiety in both CSP **1** and **3** is located near the ammonium head groups, it is exposed to the water penetrating into the bonded layer in greater extent than CSPs **2** and **4**. CSP **3** provided the bonded layer more hydrophobic than CSP **1** due to the additional octadecyl group. However, the penetrated water probably induces local hydrophobic interactions between the octadecyl group and the valine diamide moiety; the chiral moiety is thus shielded for approach of the enatiomeric solutes to lessen their recognizing ability. Above explanation is, at least in part, supported by measurement of the pyrene fluorescence from the CSP surface.

3.5. Effects of water concentration on enantiomer separation of DNB-amino acid isopropyl esters

Table 2 shows the enantiomer separation of DNB-amino acid isopropyl esters using CSP **4** using various methanol concentrations in water as an eluent. Fig. 6 illustrates a typical separation of racemic DNBphenylalanine isopropyl ester using 50% (v/v) water–methanol mixture. Increasing the water concentration prolonged the retention factors for all amino acid derivatives. This tendency was, as generally expected for reversed phase LC, stronger with an increase in the carbon number constructing side chains as follows: alanine, valine, leucine, and phenylalanine derivatives. This clearly indicates

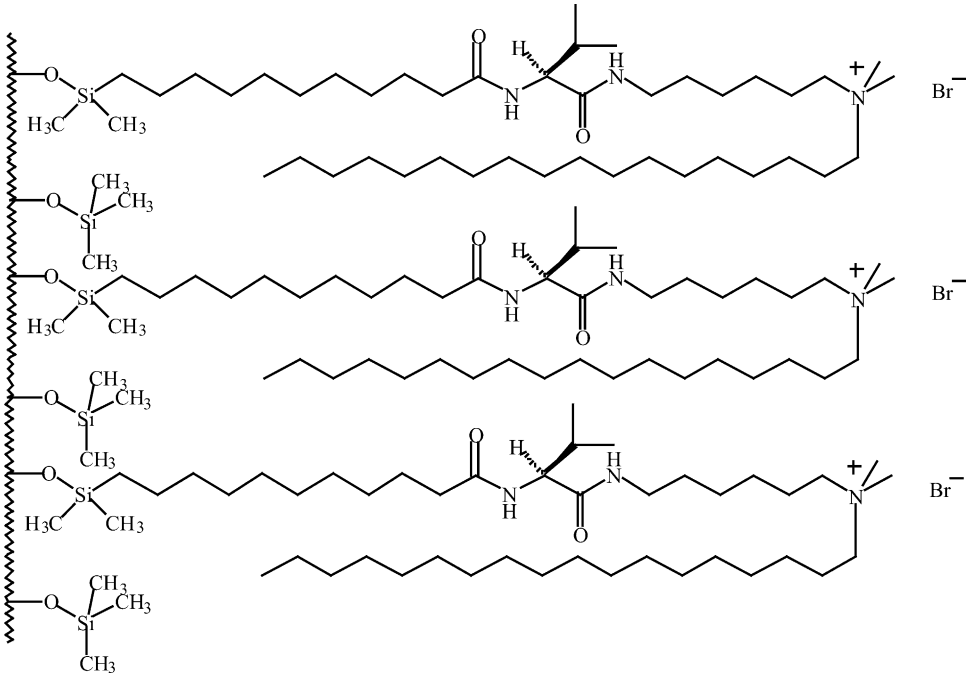


Fig. 5. Schematic representation of the interfacial phase of CSP **4** illustrating how to bury the chiral moiety into the double chain-type surfactants in the bonded residues.

Table 2
Enantiomer separation of racemic DNB-amino isopropyl esters on CSP **4**

Water concentration (%) (v/v)	Alanine		Valine		Leucine		Phenylalanine	
	k'_D [a]	α	k'_D [a]	α	k'_D [a]	α	k'_D [a]	α
30	0.73	1.07	1.09	1.08	1.44	1.13	1.70	1.11
40	1.67	1.09	2.96	1.10	4.63	1.17	5.55	1.13
50	3.84	1.09	8.23	1.11	14.66	1.19	18.07	1.14
60	7.31	1.10	17.24	1.11	35.90	1.21	46.15	1.15

[a] Retention factor (k') of the first eluted D-enantiomer.

that the solute retention is controlled by hydrophobic interactions between the solutes and the bonded layers of CSPs in aqueous media.

LC experiments using CSPs were carried out by using 30–60% (v/v) water in methanol as eluents. On the other hand, pyrene fluorescence on their bonded layers was measured at volume ratio of methanol to water up to 42.8:100, which was equivalent to about 70% (v/v) water. Fluorescence measurement was not conducted at the conditions that water concentration under 70%, because non-quenching fluorescence intensity of pyrene in the bulk phase was quite high. Above this water concentration, with increase of water concentration the retentions of amino acid derivatives were decreased at LC measurement, decrease of hydrophobicity of interfacial phase on the surface of the CSPs was caused by distribution of methanol into the interfacial face, III/I ratio would be decreased.

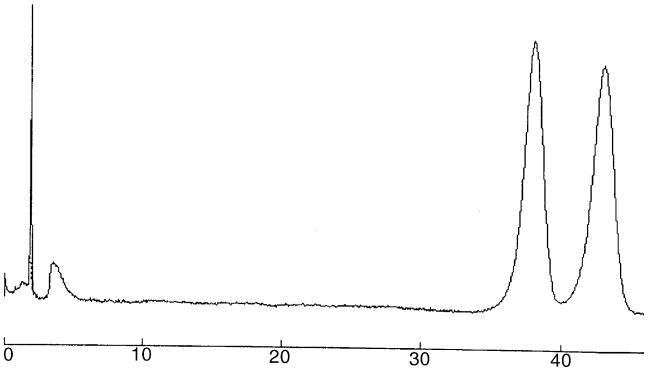


Fig. 6. Enantiomeric separation of racemic DNB-phenylalanine isopropyl ester on CSP **4** eluted with a 50% (v/v) water–methanol mixture: Conditions: flow rate, 0.8 mL/min; other chromatographic conditions are as described in Section 2.

3.6. Effects of organic solvents on enantiomer separation of DNB-leucine isopropyl ester

Table 3 shows changes in k' and α of DNB-leucine isopropyl ester on CSP **3** with different concentrations of organic solvents as the eluent. When using THF, the leucine derivative was not separated regardless of its concentration and the k' was smaller than that obtained using methanol. CSP **3**

Table 3
Enantiomer separation of racemic DNB-leucine isopropyl ester on CSP **3** with three different water–organic solvent mixtures

Water concentration (%) (v/v)	Methanol		Acetonitrile		Tetrahydrofuran	
	k'_D [a]	α	k'_D [a]	α	k'_D [a]	α
40	3.29	1.05	0.38	1.00	0.64	1.00
50	11.25	1.07	1.54	1.00	0.90	1.00
60	33.42	1.08	5.18	1.03	1.12	1.00

[a] Retention factor (k') of the first eluted D-enantiomer.

Table 4
Enantiomeric separation of DNB-amino isopropyl esters on CSP **4** with either 40% (v/v) water–methanol mixture or that containing 1 mM concentration of the surfactants

Surfactant	Alanine		Valine		Leucine		Phenylalanine	
	k'_D [a]	α	k'_D [a]	α	k'_D [a]	α	k'_D [a]	α
No addition	1.89	1.08	2.98	1.09	5.49	1.17	5.72	1.13
Surfactant **2a**	2.16	1.08	4.17	1.10	6.95	1.17	9.25	1.13
Surfactant **5a**	1.31	1.07	1.71	1.08	2.50	1.15	3.04	1.12

[a] Retention factor (k') of the first eluted D-enantiomer.

gave small k' values at water concentrations of 50–60% (v/v) in acetonitrile and these k' values were intermediate when compared to those using methanol and THF. THF possessing lower polarity permeated into the bonded layer more readily than the others and decreased its hydrophobicity on CSP with increasing THF concentration. Although this tendency was already confirmed by measurement of pyrene fluorescence, it was never larger than that observed on CSP **5**. When using water concentrations more than 30%, as observed for LC experiments, hydration of the polar head groups followed by water penetration into the inner space would be lessened and thus similar results as for CSP **5** were observed.

*3.7. Effects of addition of surfactant to aqueous eluents using CSP **4***

CSPs **3** and **4** are considered to form the bonded layer which is similar to a half-structure in lipid bilayer of vesicles. Of the two, CSP **4** provided hydrophobic microenvironment where the amino acid derivatives interacted with the chiral moiety, effectively more than CSP **3**, so as to be separated between their enantiomeric pairs. Hydrophobicity on CSP **4** should be enhanced by intercalating *N*-[6-(nonanoyl-L-valylamino)hexyl]-*N*,*N*,*N*-trimetylammonium bromide (surfactant **2a**) which has the similar structure to the residue bonded on the support surface. Addition of 1 mM surfactant **2a** to a 40% water–methanol solution prolonged retention of DNB-amino acid isopropyl esters as shown in Table 4. For the phenylalanine derivative, k' of the D enantiomer increased from 5.72 to 9.25 but little change was observed in α. Because surfactant **2a** make no micelles under this eluent composition (its CMC is 35 mmol in water), this prolonged retention should be ascribed to modification of the interfacial phase of CSP **4**.

Chiral surfactants introduced into CSPs **1**–**4** formed hydrophobic interfacial phases but inhibited local cohesion due to repulsion of their polar head groups. The deeper in the interfacial phase that the valine diamide moiety was located, the greater the enantioselectivity of CSPs. Addition of a long hydrocarbon chain to the polar head groups of quaternary ammonium salts also improved enantioselectivity of CSPs due to the increased hydrophobicity in the interfacial phase of CSPs.

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