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Enantiomeric separation using temperature-responsive chiral polymers composed of L-valine diamide derivatives in aqueous liquid chromatography

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Abstract

This paper describes enantiomer separation by aqueous liquid chromatography using chiral stationary phases (CSPs) in which temperature-responsive polymers derived from acryloyl-L-valine *N*-methylamide (**1**) and its *N*,*N*-dimethylamide analogue (**2**) were bound on silica gel supports. The linear polymers composed of monomer **1** and monomer **2** are temperature-responsive in solution and their aggregation and extension states related to water solubility are reversible at particular critical temperatures. During chromatography, enantioselectivity and retentivity for solute enantiomers were controlled by column temperature, which changes the aggregation and extension states of the chiral polymers depending upon their interior hydrophobic nature. Two different types of CSPs were made: a temperature-responsive linear polymer derived from 3-mercaptopropyl silica gel, and another polymer cross-linked with ethylene dimethacrylate from 3-methacryloyloxypropyl silica gel. The former CSP could separate racemic *N*-(3,5-dinitrobenzoyl(DNB))amino acid isopropyl esters. Retention of the amino acid derivatives was prolonged with an increase in column temperature. Enantioselectivity was also enhanced with temperature increase until the particular critical temperature. The latter, cross-linked CSP could not provide enantioselectivity for the amino acid derivatives in aqueous media, although the chiral valine diamide moieties were effective for enantiomer separation in non-aqueous media. The degree of hydrophobicity and volume of the bonded phase formed by the polymers on the support surface was determined by measuring the fluorescence of pyrene.

Keywords: Enantiomer separation; Amino acid diamide polymer; Amino acids; Acryloylvaline methylamide; Acryloylvaline dimethylamide

1. Introduction

Liquid chromatography using chiral stationary phases (CSPs) is one of the most sophisticated means of separating enantiomers and determining their composition [1,2]. Among CSPs that form diastereomeric associations between enantiomers, hydrogen bonding is the most significant contributor to the associations [3]. Silica gel modified with (10-undecenoyl)-L-valine *N*-*tert*-butylamide via hydrosilylation separated enantiomers of amino acid derivatives [4]. Hydrogen bonds at the two amide sites of the chiral diamide moiety provided enantioselectivity in both normal and reversed phases. The chiral separation of enantiomers should thus occur when hydro-

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gen bonds are incorporated with the hydrophobic environment shielded from bulk aqueous phase. This was demonstrated by the ability of chiral micelles formed by surfactants containing the amino acid diamide moiety to separate the enantiomers in aqueous media by electrokinetic chromatography [5,6]. In reversed-phase liquid chromatography, the degree of hydrogen bonding affinity and that of the formation of a hydrophobic environment are controlled by the composition of the organic solvents of the eluent. In the present study, our aim was focused on controlling these two factors using the influence of column temperature on temperature-responsive polymers whose hydrophobic nature can be altered by temperature and the chiral amino acid diamide selectors constructed into their polymer chains. This will lead to novel chromatographic enantiomer separation techniques involving chiral temperature-responsive polymers in aqueous media.

Poly(*N*-isopropylacrylamide) (PNIPAAm) is well-known as a temperature-responsive polymer that undergoes reversible changes between water-soluble and -insoluble states at 32 °C [7]. The aggregation of the polymer brought about by dehydration above this critical temperature causes it to form an exclusive hydrophobic environment in water. When PNIPAAm is bound to silica gel, the support permits temperature-responsive control of solute retention during aqueous liquid chromatography [8–10]. Increasing column temperature causes a delay in solute retention.

When acryloyl-L-valine *N*-methylamide (**1**) and acryloyl-L-valine *N*,*N*-dimethylamide (**2**) are polymerized along with 3-mercaptopropionic acid in the presence of a radical initiator, linear polymers with terminal carboxylic acids were obtained. These polymers exhibited a solubility transition similar to that of PNIPAAm [11]. The linear polymer derived from acryloyl-L-valine *N*-methylamide (polymer **1**) was soluble in water below 18 °C and insoluble above 18 °C. This reversible solubility change occurs when intermolecular hydrogen bonding between the amide groups in the polymer and water are cleaved and replaced with intramolecular amide and amide interactions between the residues in the polymer at temperatures exceeding 18 °C. Dehydration of the polymer gives rise to intramolecular aggregation of the polymer followed by intermolecular aggregation and the polymer precipitates out of the water. The polymer from acryloyl-L-valine *N*,*N*-dimethylamide (polymer **2**) had a solubility transition at 14 °C.

If the aggregation of these polymers can provide the hydrophobic environment that allows hydrogen bonds to form between the chiral diamide residues of the polymer and amino acid derivatives as solutes, separation of these enantiomers should be brought about with increasing column temperature. Two different types of CSPs were thus made by the following method, one consisting of temperature-responsive linear polymers derived from 3-mercaptopropyl silica gel and another cross-linked with ethylene dimethacrylate from 3-methacryloyloxypropyl silica gel. These polymer-bonded supports were tested for the ability to separate the enantiomers of amino acid derivatives during aqueous liquid chromatography.

2. Experimental

2.1. Preparation of linear and cross-linked polymer-bonded silica gels

2.1.1. Procedure 1. Coupling of chiral linear polymers to 3-aminopropyl silica gel

Chiral linear polymers (polymer **1**) derived from acryloyl-L-valine *N*-methylamide (**1**) were prepared as described previously [11]. The terminal carboxylic acid of polymer **1**, from 3-mercaptopropionic acid, was activated using a mixed acid anhydride followed by the coupling of 3-aminopropyl silica gel.

A 532.2-mg amount of 3-aminopropyl silica gel (Nucleosil $5NH_2$, 5 μm, surface coverage of 0.72 mmol/g; Macherey–Nagel, Düren, Germany) was dried under reduced pressure at 80 °C and suspended in 4 ml of *N*,*N*-dimethylformamide (DMF) after cooling to room temperature. *N*-Methylmorpholine (0.055 ml) was then added to the suspension and the mixture cooled to −15 °C. A 538.4-mg amount of of polymer **1** (average molecular mass of 6800 estimated by gel permeation chromatography [11]) was dissolved in 4 ml DMF. To the solution cooled at −15 °C were added *N*-methylmorpholine (0.1 ml) and isobutyl chloroformate (0.11 ml). The resulting solution was added to the suspension and the mixture

gently stirred at −15 °C for 2 h and then kept at room temperature for 20 h. The modified silica gel was filtered and washed consecutively with 500 ml each of methanol, water, and acetone. After drying under reduced pressure, 538.4 mg of the desired material (CSP **1a**) was obtained: IR (KBr, cm^{-1}): 1543, 1684; Anal. Found: C, 5.84; H, 1.60; N, 1.20.

Another CSP was derived from poly(acryloyl-L-valine *N*,*N*-dimethylamide) (polymer **2**; average molecular mass, 30 300): Anal. Found: C, 6.58; H, 1.61; N, 1.07.

*2.1.2. Procedure 2. In situ telomerization of monomer **1** from 3-mercaptopropyl silica gel*

A 2.30-g amount of silica gel (Nucleosil 100-5, 5 μm; Macherey–Nagel, Düren, Germany) were dried at 200 °C for 18 h under reduced pressure and then cooled to room temperature. To the silica gel were added 15 ml of toluene and 7.5 ml of 3-mercaptopropyltrimethoxysilane. The mixture was gently stirred at 130 °C for 22 h. The modified silica gel was filtered and washed consecutively with 70 ml each of methanol and acetone. A 2.41-g amount of the desired material was obtained: IR (KBr, cm^{-1}): 2933; Anal. Found: C, 3.65; H, 1.25; surface coverage calculated from the carbon content, 1.03 mmol/g.

3-Mercaptopropyl silica gel (0.7 g) was suspended in a solution of 200 ml DMF containing 9.86 g of **1**. After the mixture was degassed by bubbling with argon for 20 min, a catalytic amount of 2,2′-azobis(isobutyronitrile) (AIBN) was added to the mixture as a radical initiator. The resulting mixture was heated at 80 °C with gentle stirring for 17 h under an argon atmosphere. The modified silica gel was filtered and washed consecutively with 100 ml each of methanol and acetone to yield 0.78 g of the desired material (CSP **1b**). The polymerization was carried out in an approximate 60:1 molar ratio of monomer **1** to the 3-mercaptopropyl group on silica gel. IR (KBr, cm^{-1}): 2967, 1543, 1654; Anal. Found: C, 12.67; H, 2.48; N, 2.39.

Acryloyl-L-valine *N*,*N*-dimethylamide (monomer **2**) was polymerized on 3-mercaptopropyl silica gel (surface coverage, 1.15 mmol/g) using the procedure described above (CSP **2b**). IR (KBr, cm^{-1}): 2967, 1542, 1637; Anal. Found: C, 16.94; H, 3.15; N, 3.27.

*2.1.3. Procedure 3. Polymerization of monomer **1** with ethylene dimethacrylate as a cross-linking agent on 3-methacryloyloxypropyl silica gel*

3-Methacryloyloxypropyl silica gel was prepared from 2.12 g silica gel (Nucleosil 100-5) and 5 ml 3-(trimethoxysilyl)propyl methacrylate in 10 ml of toluene using a procedure similar to that used for 3-mercaptopropyl silica gel. A 2.08-g amount of the desired material was obtained. IR (KBr, cm^{-1}): 2956, 1710; Anal. Found: C, 7.90; H, 1.63; surface coverage calculated with the carbon content, 0.94 mmol/g.

3-Methacryloyloxypropyl silica gel (1.0 g) and ethylene dimethacrylate (EDMA) (0.05 ml) were added to a solution of monomer **1** (5.20 g) in 200 ml DMF. After the mixture was degassed by bubbling with argon for 20 min, a catalytic amount of AIBN was added. The resulting mixture was heated at 80 °C with gentle stirring for 18 h under an argon atmosphere. The modified silica gel was treated in the same manner as CSP **1b** to yield 1.03 g of the desired material (CSP **3**). IR (KBr, cm^{-1}): 2970, 1654, 1718; Anal. Found: C, 12.56; H, 2.46; N, 1.47. The polymerization was carried out in an approximate 100:1 molar ratio of **1** to EDMA on 3-methacryloyloxypropyl silica gel.

CSP covered by a cross-linked polymer of monomer **2** was also derived from 3-methacroyloxypropyl silica gel (surface coverage, 0.77 mmol/g) using the same procedures as described above to yield CSP **4**. IR (KBr, cm^{-1}) 2970, 1654, 1718; Anal. Found: C, 11.79; H, 2.34; N, 1.42.

2.2. III/I ratio for determination of hydrophobicity on the surface of the polymer-bonded silica gels

The hydrophobic microenvironment formed by aggregation of the polymer chains bonded on the silica gel was demonstrated by the fluorescence of pyrene sorbed onto the surface using a Jasco FP-777 fluorometer (Japan Spectroscopic, Tokyo, Japan) with a slit width of 3 nm for the emission and 1.5 nm for the excitation light and equipped with a cell holder (Jasco ECT-271) temperature-controlled by the Peltier effect. A 10.0-mg amount of the modified silica gel was suspended in a mixture of 4.8 ml 0.025 *M* $CuSO_4$, 0.2 ml 0.042 *M* sodium dodecylsulfate (SDS) solution, and 40 ml methanol containing 2.5×

10^{-4} *M* pyrene. Then 2.5 ml of the suspension was added to a quartz cell (10×10 mm) along with a small stirrer. The fluorescence intensity of pyrene at 373 and 383 nm, which correspond to the first and third bands of its fine vibronic bands, was measured at temperatures ranging from 0 to 60 °C under gentle stirring. The suspension was centrifuged and fluorescence of the supernatant was measured as described above. The two intensities in the non-sorbed pyrene were subtracted from those observed for the suspension at each temperature. The ratio of the intensity of the third to that of the first band, which is abbreviated III/I ratio, was used as the measure of hydrophobicity.

*2.3. Trimethylsilylation of CSP **1b***

CSP **1b** (454.6 mg) was dried for 8 h at 80 °C under vacuum. After the support was cooled at room temperature and then suspended in 2 ml chloroform, trimethylsilylimidazole (0.28 ml) was added. The mixture was heated at 65 °C for 7.5 h under gentle stirring. The modified support was filtered and then washed in turn with chloroform and methanol. A 463.5-mg amount of the desired material was obtained. IR (KBr, cm^{-1}): 1550, 1646; Anal. Found: C, 13.77; H, 2.79; N, 2.22.

2.4. Aqueous liquid chromatography using the polymer-bonded silica gels

The polymer-bonded silica gels were packed into stainless-steel columns (150 mm×1.5 mm I.D., GL Science, Tokyo) as follows. The polymer-bonded silica gel (230 mg) was suspended in 1.5 ml 50% (v/v) 2-propanol–chloroform mixture and sonicated for 2 min. The suspension was added to a 2.5 ml volume of stainless-steel packer connected to the column and pressurized into the column with chloroform at a constant flow-rate of 0.5 ml/min. After the pressure reached 300 kg/cm^2, 10-ml portions of chloroform, methanol, and water were successively passed through the column at a constant pressure of 300 kg/cm^2.

Liquid chromatography was carried out with a Jasco Familic 300 pump (Japan Spectroscopic, Tokyo, Japan), a SPD-2AM UV detector (Shimadzu) equipped with a 1.5-μl flow cell, an LC-300 column oven (Chromato Science, Osaka) and a CR-3A data processor (Shimadzu, Kyoto). Solute elution was detected at 254 nm. A 1% (v/v) methanol–water mixture was used as an eluent at a flow-rate of 50 μl/min for all aqueous chromatographic studies. Oven temperature was controlled within the range of 0–70 °C. Non-aqueous chromatography was performed with hexane containing either 2.5 or 7.5% (v/v) 2-propanol at a flow-rate of 100 μl/min at 23 °C.

2.5. Calculation of log P values

The log *P* value, which is the logarithm of the partition coefficient of the compound in octanol–water systems, was predicted using Pallas PrologP 3.0 software (CompuDrug International, South San Francisco, CA, USA) based on the structural formulas of the compounds.

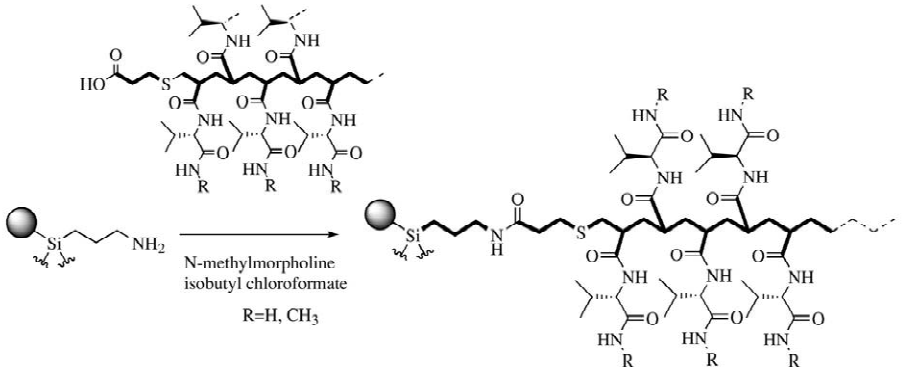
3. Results and discussion

3.1. Preparation of linear and cross-linked polymer-bonded supports and their ability to retain the amino acid derivatives

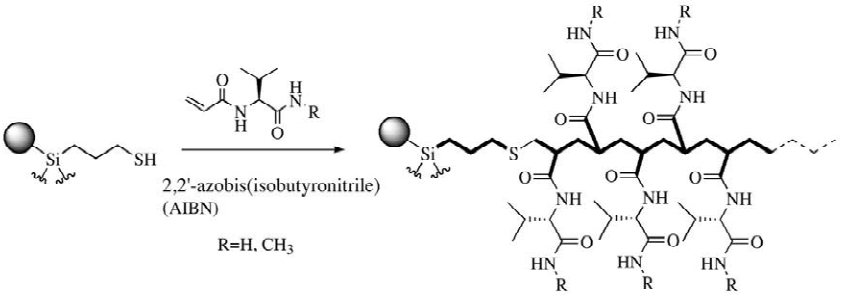
The three procedures used to prepare the chiral polymer-bonded silica gels are illustrated in Fig. 1. For each procedure, monomers **1** and **2** were used as the functional monomers to obtain the temperature-responsive polymers. When these temperature-responsive polymers are prepared on the silica gel surface, increasing column temperature can enhance the internal hydrophobicity of the bonded phases and, thus, in the interior region, hydrogen bonds between the valine diamide moieties of the polymers and the enantiomeric solutes can become effective. Such internal hydrophobicity and enantioselectivity should be thus controlled by the *N*-methylation of the C-terminal amide of monomer **1**.

CSPs **1a** and **2a** were derived from poly(acryloyl-L-valine *N*-methylamide) (polymer **1**) and poly-(acryloyl-L-valine *N*,*N*-dimethylamide) (polymer **2**), respectively, with procedure 1 using 3-aminopropyl silica gel as a starting material. Surface coverage of the polymers on CSP **1a** and CSP **2a** calculated from carbon content were approximately 5.1 and 1.4

Procedure 1. Coupling of chiral linear polymers to 3-aminopropyl silica gel



Procedure 2. In situ telomerization of functional monomers from 3-mercaptopropyl silica gel



Procedure 3. Polymerization of functional monomers with ethylene dimethacrylate as a cross linking agent on 3-methacryloyloxypropyl silica gel

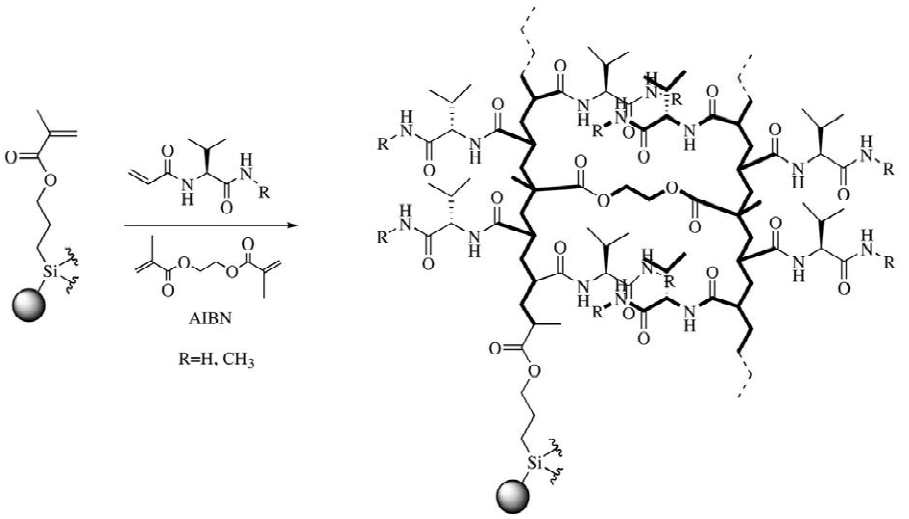


Fig. 1. Preparation of temperature-responsive chiral polymers bonded to silica gel.

μmol/g, respectively. When using 1% (v/v) methanol–water as an eluent, and racemic *N*-(3,5-dinitrobenzoyl (DNB))amino acid isopropyl esters as solutes, both CSP **1a** and **2a** exhibited a slight increase in retention factor (k') with increasing column temperature, but did not achieve enantiomeric separation.

Condensation of the terminal carboxyl groups of the linear polymers to 3-aminopropyl silica gel (procedure 1 in Fig. 1) provided only low surface coverage of the polymers. This is probably due to large size of polymers **1** and **2**, whose average molecular masses are 6800 and 30 300, respectively. Therefore, two different procedures were examined for preparation of the chiral polymer-bonded supports. One procedure provided the linear polymers by telomerization of the chiral monomer from 3-mercaptopropyl silica gel. CSP **1b** was derived from monomer **1** and CSP **2b** from monomer **2**, as illustrated in Fig. 1. Another procedure yielded the polymers cross-linked with ethylene dimethacrylate from 3-methacryloyloxypropyl silica gel. CSP **3** was derived from monomer **1** and CSP **4** from monomer **2**. These procedures provided a greater surface coverage than those obtained using procedure 1. CSPs obtained using procedure 2 could, as expected, perform the temperature-responsive chiral separation of the enantiomers.

*3.2. Chiral separation of the amino acid derivatives using CSP **1b** and **2b** in aqueous media*

Table 1 shows enantiomer separation of racemic *N*-DNBamino acid isopropyl esters obtained with column temperatures ranging from 5 to 70 °C using CSP **1b** and **2b**. With CSP **1b**, retention factors of the D-enantiomers (k'_D) became larger with increasing temperature and reached a maximum at 50 °C. The amino acid derivatives were retained on CSP **2b** more strongly than on CSP **1b**. CSP **2b** showed an increase in retention factor with temperature until 70 °C, to a greater extent than that observed for CSP **1b**. This indicates that the bonded phase of CSP **2b** became more hydrophobic than that of CSP **1b** when the column temperature was increased.

With both CSP **1b** and **2b**, all the amino acid derivatives were separated at temperatures ranging from 25 to 70 °C, but not at 5 °C, as shown in Table 1. Fig. 2 illustrates a typical separation of racemic DNBleucine isopropyl ester on CSP **1b** in which the L-enantiomer was retained more strongly than the D-enantiomer. The separation factors (α values) for the leucine derivative reached a maximum at 25 °C and decreased with increasing column temperature from this critical point. Maximum separation factors were found for all the chromatographic experiments using CSP **1b** and **2b**. For all chromatographic

Table 1
Chiral separation of DNBamino acid isopropyl esters on CSP **1b** and **2b** with column temperatures ranging from 5 to 70 °C

CSP	Temperature (°C)	Amino acid					
		Alanine		Valine		Leucine	
		k'_D [a]	α [b]	k'_D [a]	α [b]	k'_D [a]	α [b]
1b	5	2.18	1.00	3.80	1.00	5.95	1.00
	25	2.35	1.08	4.74	1.07	8.11	1.12
	35	2.35	1.09	5.04	1.07	8.89	1.11
	50	2.38	1.07	5.15	1.06	14.43	1.06
	70	2.22	1.05	4.93	1.05	8.90	1.07
2b	5	2.37	1.00	4.75	1.00	– [c]	–
	25	5.28	1.09	10.08	1.11	–	–
	35	5.19	1.11	13.23	1.10	–	–
	50	6.12	1.06	17.05	1.08	–	–
	70	6.83	1.04	18.71	1.05	–	–

[a] Retention factor for the D-enantiomer.
[b] $\alpha = k'_L/k'_D$.
[c] The solute could not be eluted within a reasonable time.

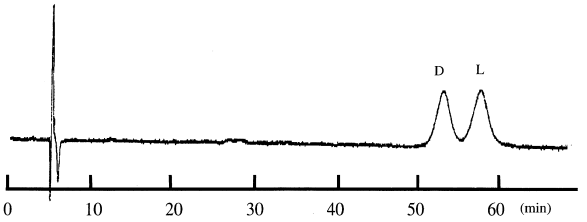


Fig. 2. Separation of racemic DNBleucine isopropyl ester on CSP **1b**. Conditions: eluent, 1% (v/v) methanol in water; column temperatures, 50 °C; flow-rate, 50 μl/min; detection, UV at 254 nm.

studies, the phenylalanine derivative could not be eluted within a reasonable time due to its relatively high hydrophobicity.

The amino acid derivatives should enter into a hydrophobic interfacial phase formed by aggregation of the temperature-responsive polymers and interact with interior chiral valine diamide moieties to form diastereomeric associations. The enantiomeric separation obtained demonstrates that this association is due to hydrogen bonding. Therefore, solute retention should be affected by hydrogen bonding as well as hydrophobic interactions within the aggregated polymers. Hydrogen bonding between the chiral diamide moieties within one linear polymer, that is, intra-residual hydrogen bonding, generates a hydrophobic microenvironment that entraps the solutes. The chiral residue contained in CSP **1b** has two CONH sites, which form stronger inter-residual hydrogen bonds than those formed with CSP **2b** having one CONH and another $CON(CH_3)_2$. Therefore, in CSP **1b**, an increase in hydrophobicity of the interfacial phase with temperature caused a smaller increase in retention factor than in CSP **2b**. CSP **2b** has only one amide NH and thus weak hydrogen bonding between the chiral residues of the polymers and strong hydrophobicity formed by additional *N*-methyl group. The difference in the hydrogen bonding nature of the valine diamide moieties between CSP **1b** and **2b** has been studied by examining the infrared spectra of polymer **1** and **2** [11].

Trimethylsilylation of CSP **1b** enhanced retention and separation factors for the solutes but these factors simply decreased with an increase in column temperatures. The retention and separation factors observed for racemic DNBAlanine isopropyl ester were 31.1 and 1.20 at 5 °C. This retention behavior different from the untreated CSP **1b** may indicate that additional hydrophobicity brought about by the trimethylsilyl groups, neighboring around the polymers, lessens intramolecular residual-to-residual interaction leading to temperature sensitivity of the polymer because its aggregation is induced by such hydrophobic interaction.

3.3. Assessment of hydrophobicity at the surface of the bonded silica gel

Fig. 3 illustrates dependence of the ratio of fluorescence of pyrene at 373 and 383 nm on temperature from 0 to 60 °C. This III/I ratio indicates the hydrophobicity of the microenvironment where pyrene is sorbed, and increases with hydrophobicity [12,13]. Aggregation of the polymer chains on CSP **1b** and **2b** should create a hydrophobic interfacial phase that excludes water when column temperature exceeds the critical temperature. Solutes

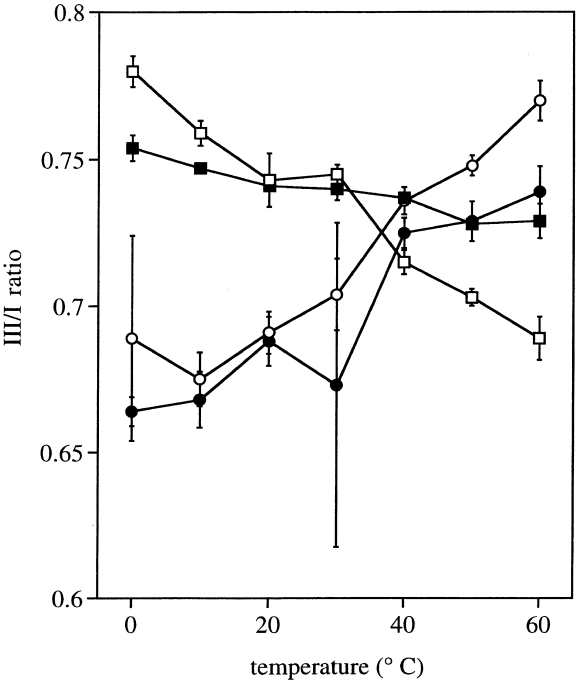


Fig. 3. Temperature dependence of III/I ratios of pyrene sorbed on bonded silica gels: CSP **1b**, open square; CSP **2b**, filled square; CSP **3**, open circle; CSP **4**, filled circle.

to be separated become sorbed into this phase, at least in part, by hydrophobic interactions.

On CSP **1b** and **2b**, the III/I ratio decreased with an increase in column temperature. The decrease of the ratio observed for CSP **1b** from 0 to 60 °C was greater than that for CSP **2b**. Although aqueous solutions of polymers **1** and **2** underwent a phase transition between water-soluble and -insoluble state at 18 and 14 °C, respectively [11], reduction of the III/I ratios had no discontinuous change related to those critical temperatures. This microscopic observation indicates pyrene becomes excluded from the interior region of the polymers as aggregation of the polymer on the supports is enhanced by increasing temperature in the range of 0–60 °C.

The III/I ratio of CSP **1b** increased rather than decreased upon addition of methanol when using higher temperatures at which the polymer chains should be aggregated, as illustrated in Fig. 4. The ratio also increased with methanol concentration due to intercalation of methanol in the interfacial phase. Thus, aggregation of the bonded polymers on exposing pyrene to the bulk water would appear to account for the reduction in the III/I ratio at high temperatures.

Addition of methanol to a suspension of octadecyl silica gel in water caused the III/I ratio to increase and reach a maximum at 50% (v/v) methanol, followed by a decrease when the methanol concentration exceeded 50% [14]. This implies that the octadecyl-bonded phase is collapsed by hydrophobic interactions in water. Thus, pyrene allowed into the bonded phase is not completely shielded from the bulk water and, at least in part, is exposed to the water.

Aggregation of the polymer chains on CSP **1b** and **2b** should reduce the volume sufficiently to adequately shield pyrene from the bulk water, in a manner similar to that observed with octadecyl silica gel. Hydrophobicity of the interfacial phase on the CSPs would however increase with increasing column temperature. Of the two, the decrease in III/I ratio of CSP **1b** was steeper than that of CSP **2b** because the former would collapse to a greater extent than the latter.

The III/I ratios for CSP **3** and **4** increased with

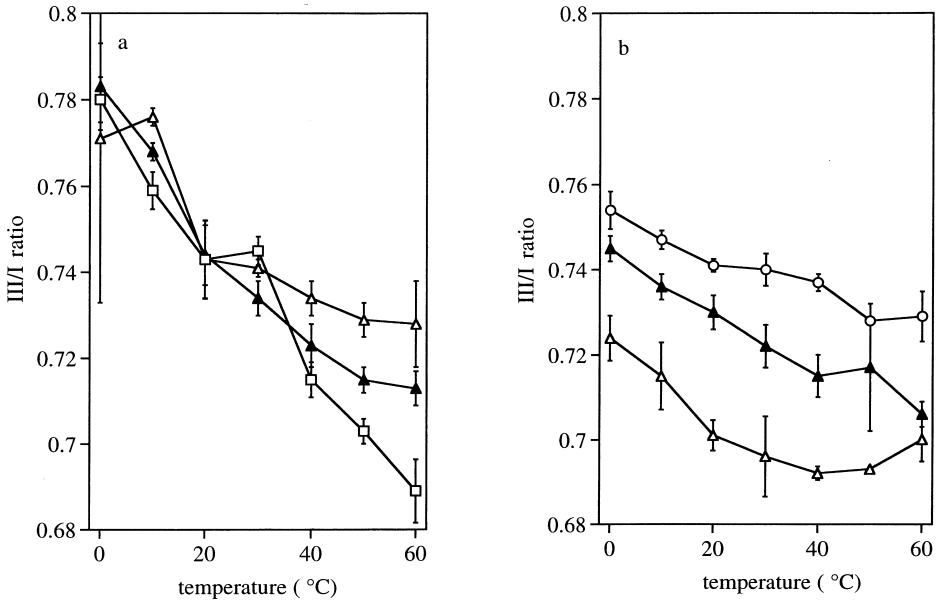


Fig. 4. Changes in the temperature dependence of III/I ratios by addition of methanol on CSP **1b** (a) and **2b** (b). CSP **1b**, open square (a); CSP **2b**, open circle (b); addition of 1% (v/v) methanol to the CSP suspensions, filled triangle; addition of 10% (v/v) methanol, open triangle.

temperature in contrast to the behavior observed for CSP **1b** and **2b**. These ratios increased slightly with methanol concentration, in a manner similar to that observed for CSP **2b**. This is because the aggregation of the cross-linked polymers upon dehydration occurs to a lesser extent than aggregation of the linear polymers, or, in other words, hydrogen bonding between chiral diamide moieties is restricted by the cross-linked network to make aggregation less dense in the cross-linked than in the linear polymers.

3.4. Temperature-responsive chromatography for benzene and steroids

In order to estimate the volume of the hydrophobic interfacial phase that can entrap enantiomers, temperature dependence of retention on CSP **1b** and **2b** was examined with benzene and five steroids: dexamethazone, predonisolone, cortisone, hydrocortisone and methylpredonisolone, in a manner similar to that described for the amino acid derivatives. Retention of benzene increased with column temperature in both CSPs, as illustrated in Fig. 5. The tendency for an increase in retention for these solutes was observed for both CSPs, but was lower for CSP **1b**. Retention on CSP **1b**, following a slight increase from 5 to 25 °C, gradually decreased with increasing temperature. CSP **2b** showed a greater increase in retention than CSP **1b** and reached maximum retention at 50 °C. The solute steroids eluted in the following order: dexamethazone, predonisolone, cortisone, hydrocortisone, and methyl-predonisolone. In order to estimate hydrophobicity of these steroids, which also estimates their retention to CSPs, the logarithm of the partition coefficient for each compound in octanol/water systems ($\log P$) was predicted, using Pallas PrologP software, as 1.16 for dexamethazone, 1.44 for predonisolone, 1.20 for cortisone, 1.66 for hydrocortisone, and 1.66 for methylpredonisolone. On CSP **2b**, the retention time of the steroids tended to increase with hydrophobicity and showed a remarkable change with increas-

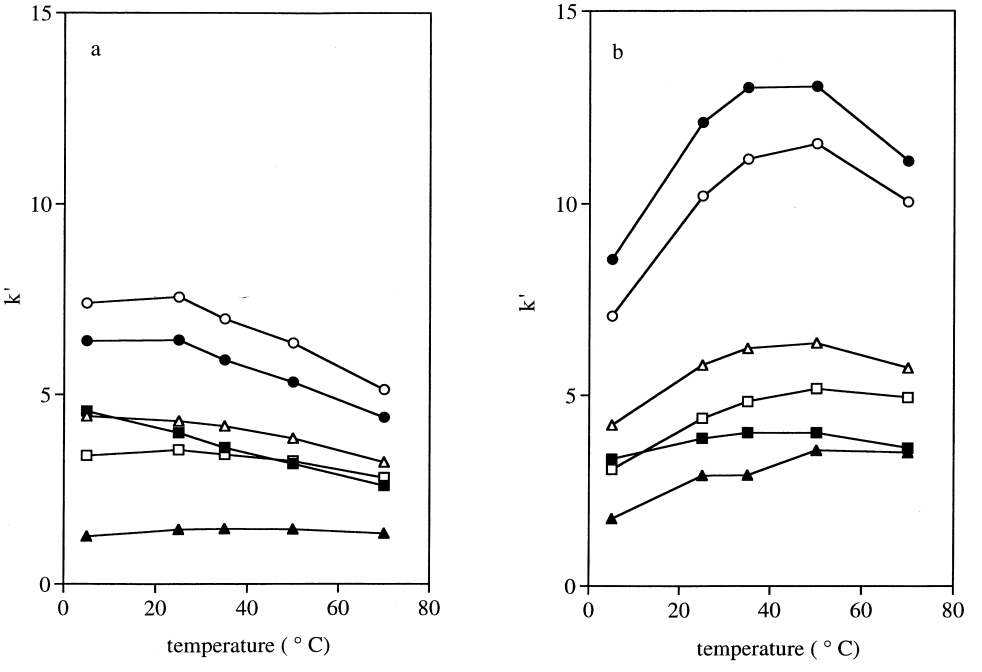


Fig. 5. Temperature dependence of chromatographic retention of benzene and five steroids on CSP **1b** (a) and **2b** (b): benzene, filled triangle; dexamethazone, filled square; predonisolone, open square; cortisone, open triangle; hydrocortisone, open circle; methyl predonisolone, filled circle. Conditions are as described in Fig. 2 except column temperatures.

ing temperature. The volume of interfacial phase of CSP **2b** thus should be sufficient to accommodate both the small benzene molecule and the large steroid molecules in contrast to that of CSP **1b**. This prediction was supported by pyrene fluorescence measurement.

Changes in the III/I ratio of CSP **2b** with increasing temperature were smaller than those of CSP **1b** and CSP **2b** thus tends to maintain a particular interfacial phase volume to entrap large hydrophobic molecules such as the steroids. In a comparison between CSP **1b** and **2b**, the residue of CSP **2b** is more hydrophobic than that of CSP **1b** and gives rise to weaker hydrogen bonds between the residues than that of CSP **1b**, as described in the previous section. These factors should make CSP **2b** more hydrophobic and more effective for controlling solute retention using column temperature.

3.5. Chiral separation with the cross-linked polymers

In spite of addition of the cross-linking agent to the monomer at a ratio of 1:100, cross-linked polymers formed on the silica gel support lost temperature sensitivity for aggregation as observed by pyrene fluorescence measurement and enantioselectivity for amino acid derivatives in aqueous media. CSP **3** and **4** provided ordinal retentivity, that is, larger retention factors when the column temperature was high (the alanine derivative gave 14.3 at 35 °C and 9.78 at 50 °C for CSP **3**, and 11.2 at 35 °C and 8.09 at 50 °C for CSP **4**). This is due to the cross-linking of the polymers, which inhibits its flexibility to aggregate densely and prevents formation of the hydrophobic microenvironment as discussed in the previous section. Enantiomeric separation of the amino acid derivatives was regained using 2-propanol-hexane mixtures instead of methanol–water mixtures as an eluent. The elution order was the same as that observed for CSP **1b** and **2b** in the aqueous eluents: the L-enantiomer was retained more strongly than the D-enantiomer.

3.6. Conclusion

Radical telomerization of acryloyl-L-valine *N*-methylamide (monomer **1**) from 3-mercaptopropyl silica gel provided higher coverage of polymer chains onto silica gel than did condensation of the terminal carboxyl groups of the corresponding linear polymer with 3-aminopropyl silica gel. The former support (CSP **1b**) allowed temperature-responsive chiral separation of racemic *N*-(3,5-dinitrobenzoyl(DNB))amino acid isopropyl esters. Chiral separation of enantiomeric amino acid derivatives was improved with an increase in column temperature that made the aggregation state of the polymers more hydrophobic. Their retentivity was also controlled by temperature, and increasing temperature imparted higher retention factors (*k*'s) to all solutes separated. An analogous CSP **2b**, derived from acryloyl-L-valine *N*,*N*-dimethylamide (**2**), exhibited solute retentivity capable of being controlled by temperature more strongly than that observed for CSP **1b**. This is probably due to the volume of hydrophobic interfacial phase on CSP **2b** being larger than that on CSP **1b**. Separation of the amino acid derivatives should be made possible by hydrogen bond formation between the chiral valine diamide moieties in the aggregated polymers and the enantiomers.

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