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Effect of polymer containing a naphthyl-alanine derivative on the separation selectivity for aromatic compounds in temperature-responsive chromatography

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ABSTRACT

A novel polymer-grafted stationary phase of high-performance liquid chromatography (HPLC) was developed, utilizing a temperature-responsive polymer containing an aromatic moiety. Firstly, we synthesized novel functional polymer poly(*N*-isopropylacrylamide-*co*-*N*-acryloyl-3-(2-naphthyl)-L-alanine methyl ester) [poly(NIPAAm-*co*-Nap)], which has temperature-responsiveness and selective retention of aromatic compounds by an intermolecular π–π interaction. The polymer exhibited a significant reversible phase transition from hydrophilic to hydrophobic in the vicinity of its lower critical solution temperature. Employing the developed polymer-grafted silica column, temperature-responsive chromatography was conducted using water as a sole mobile phase. A comparison with a conventional ODS column or a homogeneous PNIPAAm-grafted silica column showed that the retention of aromatic compounds was dramatically increased on the poly(NIPAAm-*co*-Nap)-grafted stationary phase. Introducing the naphthyl-alanine derivative caused a significant effect on the retention selectivity for aromatic compounds.

1. Introduction

As is well known, the molecular interaction between π systems (π–π interaction) plays a crucial role in biological systems. In particular, arene–arene interactions have been widely researched, and found to play important roles in enzyme–substrate recognition [1], the drug–receptor interaction [2], DNA/RNA base-stacking [3], protein three-dimensional structures and their function [4]. Furthermore, significant experimental and computational investigations for clearly understanding of these interactions have been conducted in many research areas [5,6]. In analytical sciences, the aromatic interaction has been used as one of the separation modes. In fact, the useful HPLC-column containing phenyl-stationary phase is available [7,8]. The main advantage of using phenyl-stationary phases is an ability to resolve compounds using the π–π interaction, commonly referred to as aromatic selectivity. Phenyl-stationary phases are often used to provide alternate selectivity to an ODS column, but they also offer an effective way of resolving drug substances and impurities, which usually contain an aromatic ring or conjugated bonds, and often differ by levels of unsaturation or electron-withdrawing substituents. Additionally, Ihara et al. developed novel packing materials composed of poly(*N*-alanine)-grafted porous silica or poly(octadecylacrylate-*alt*-*N*-octadecylmaleimide)-grafted silica, and explained the separation mechanism by π–π interaction and (or) shape-selectivity [9–11].

Recently, many polymers that respond to external stimuli have been developed, and these change their functions and structure reversibly, in response to the surrounding conditions, such as light, pH, temperature, electric field and chemical substances. One of them is poly(*N*-isopropylacrylamide) (PNIPAAm), which exhibits a thermally reversible soluble-insoluble change in response to a temperature change across a lower critical solution temperature (LCST) at 32 °C in aqueous solution [12]. PNIPAAm undergoes a sharp phase separation and coil globule transition of the polymer chain structure in the vicinity of its LCST. In aqueous solution, PNIPAAm exhibits an expanded conformation below the LCST due to strong hydration, and a change to a compact form above the LCST by sudden dehydration. Based on its unique property, PNIPAAm has been used for drug delivery systems [13], cell culture dishes [14] and bio-imaging [15].

In the context of these studies, we have been investigating a novel method of HPLC using packing materials modified with PNIPAAm [16–23]. The properties and function of the stationary phase can be regulated by the external temperature. In chromatographic system utilizing the PNIPAAm-grafted stationary phase, the retention times of analytes are controlled by a small change in the column temperature by only using pure water as a mobile phase. In addition, PNIPAAm is able to provide many functions by introducing of various kinds of monomers into the polymer unit. For example, by introducing of butylmethacrylate as a hydrophobic comonomer leads to a lower LCST [17],

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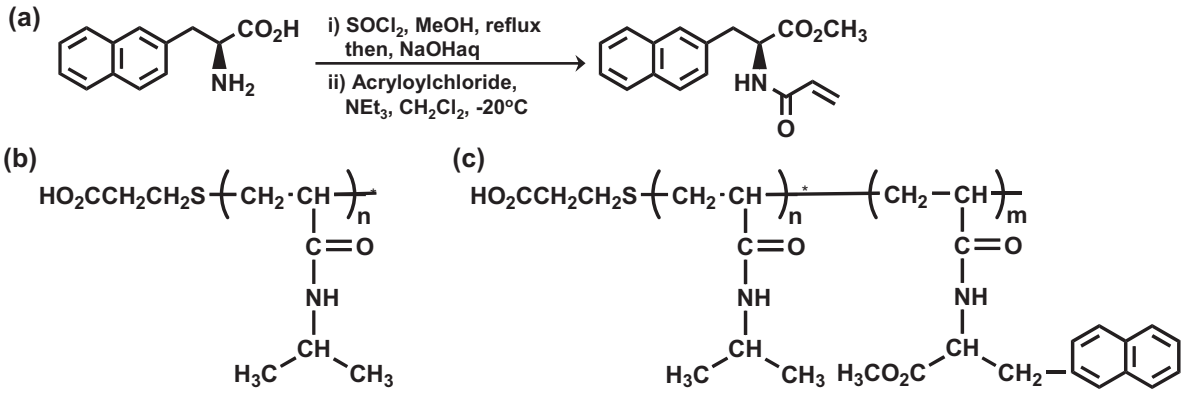

Fig. 1. The synthetic route for the preparation of *N*-acryloyl-3-(2-naphthyl)-L-alanine methyl ester (a), and chemical structures of PNIPAAm (b) and poly(NIPAAm-*co*-Nap) (c).

and a hydrophilic comonomer increases LCST. Additionally, the introduction of an ionic comonomer, such as acrylic acid or 2-(dimethylamino)ethyl methacrylate, leads to the production of a dual pH- and temperature-responsive polymer [19,20]. Furthermore, the introduction of proline, a kind of amino acid leads to the production of a molecular-recognition type polymer [21].

In present study, the purpose of creating a novel chromatographic system, we designed and synthesized a temperature-responsive polymer carrying aromatic moiety for π–π interaction with the aromatic compounds. Furthermore, property of developed polymer-grafted stationary phase was compared with conventional ODS, phenyl–hexyl stationary phase and PNIPAAm homo-polymer grafted stationary phase in terms of temperature-responsibility and inter molecular interaction with solute.

2. Experimental

2.1. Materials and chemicals

N-Isopropylacrylamide (NIPAAm) was kindly provided by KOHJIN (Tokyo, Japan), and was purified by recrystallization from *n*-hexane and dried at 25 °C *in vacuo*. Thionyl chloride, acryloyl chloride and 3-(2-naphthyl)-L-alanine was purchased from Tokyo Kasei Kogyo (Tokyo). Dichloromethane, 2,2′-azobisisobutyronitrile (AIBN), 3-mercaptopropionic acid (MPA), *N*,*N*-dimethylformamide (DMF), *N*,*N*′-dicyclohexylcarbodiimide (DCC) and *N*-hydroxysuccinimide (NHS) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Aminopropyl silica (average diameter of 3 μm) was purchased from Nishio Kogyo (Tokyo). Uracil, naphthalene, 1,5-dinitronaphthalene,

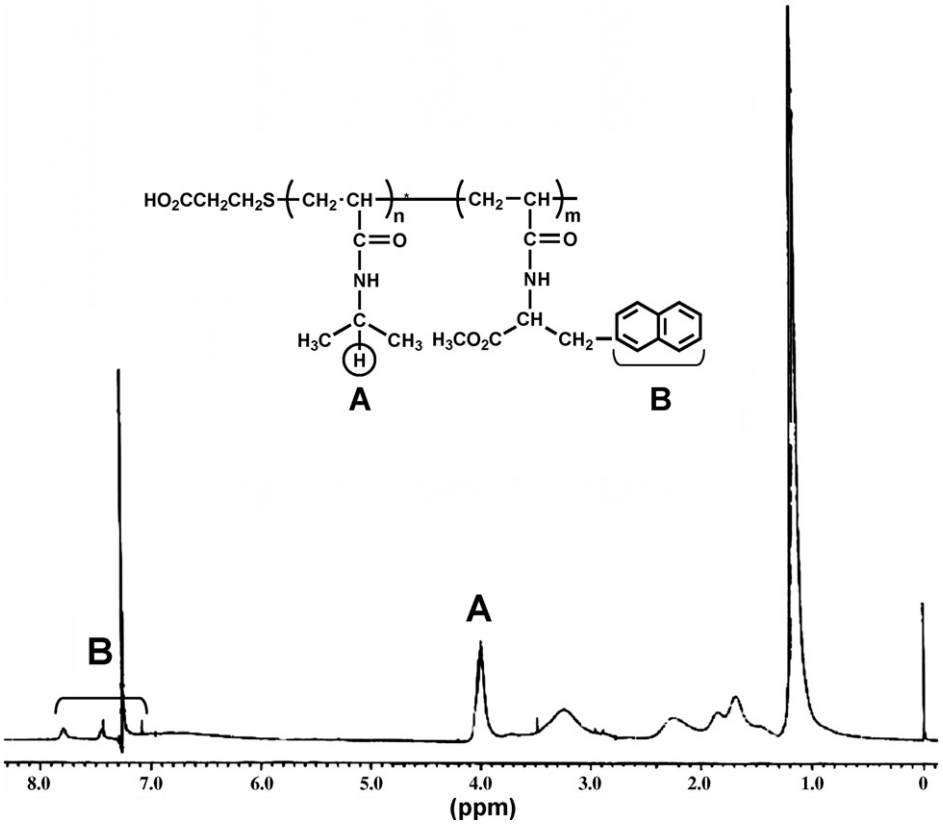

Fig. 2. The structure and the ^{1}H NMR spectrum of poly(NIPAAm-*co*-Nap). Methine proton from isopropyl group of NIPAAm (A) and the aromatic protons arising from naphthyl-alanine derivative (B).

Table 1
Properties of temperature-responsive polymers.

	Mole fraction of naphthyl-alanine deriv. (mol%)		Molecular weight[b]			LCST[c] (°C)
	In feed[a]	Obsd[a]	M_n	M_w	M_w/M_n	
PNIPAAm.	0	0	20,100	38,700	1.9	32.1
Poly(NIPAAm-*co*-Nap).	3.0	2.7	17,200	35,200	2.9	25.8

[a] Introduction rate of naphthyl-alanine derivative was determined by ^{1}H NMR analysis.
[b] M_n and M_w were determined by GPC analysis.
[c] LCST was determined by transmittance measurement at 500 nm.

hydrocortisone, prednisolone, dexamethasone, hydrocortisone acetate, testosterone, estradiol and ethynylestradiol were purchased from Wako Pure Chemical Industries. Norethisterone and norgestrel were purchased from Tokyo Kasei Kogyo. Water was distilled and passed through a Milli-Q purification system (Millipore, Bedford, MA, USA). All other reagents and solvent were of analytical grade.

2.2. Apparatus

HPLC analysis was carried out using a Hitachi D-7000 controller equipped with L-7100 pump, L-7405 UV detector (wavelength; 254 nm). The column oven was an Aqua Way Gradienter product of CellSeed (Tokyo, Japan). The ^{1}H NMR spectra were obtained using a JEOL JNM-ECP600 spectrometer (600 MHz, Tokyo, Japan) and tetramethylsilane was used as the internal standard. A Inertsil ODS-3 column (3 μm, 2.1 mm i.d. × 100 mm; GL Sciences Inc., Tokyo) and Luna 3u Phenyl–Hexyl column (3 μm, 2.1 mm i.d. × 150 mm; Phenomenex, Torrance, CA, USA) were used as conventional HPLC methods.

2.3. Synthesis of N-acryloyl-3-(2-naphthyl)-L-alanine methyl ester

N-Acryloyl-3-(2-naphthyl)-L-alanine methyl ester was synthesized by the reaction of 3-(2-naphthyl)-L-alanine methyl ester with acryloyl chloride. Thionyl chloride (2.0 mL, 0.03 mmol) was dropped into a MeOH solution of 3-(2-naphthyl)-L-alanine (5.0 g, 23.0 mmol) at 0 °C. After the reaction solution was refluxed for 5 h, the solvent was evaporated. Methyl ester hydrochloride salt was neutralized by a NaOH aqueous solution and extracted by chloroform. The organic layer was dried over Na_2SO_4, followed by concentration to give the slightly yellow oil [3-(2-naphthyl)-L-alanine methyl ester].

3-(2-Naphthyl)-L-alanine methyl ester (2.4 g, 10.5 mmol) was dissolved in CH_2Cl_2 (100 mL). Then, acryloyl chloride (0.9 mL,

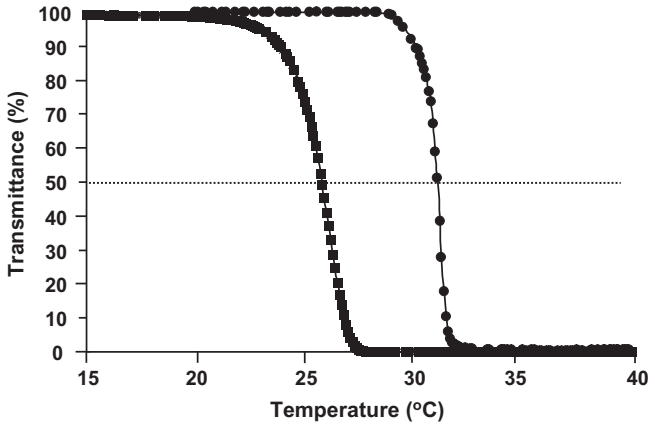

Fig. 3. Temperature dependent optical transmittance changes for polymer solutions at 500 nm: PNIPAAm (closed circle); poly(NIPAAm-*co*-Nap) (closed square).

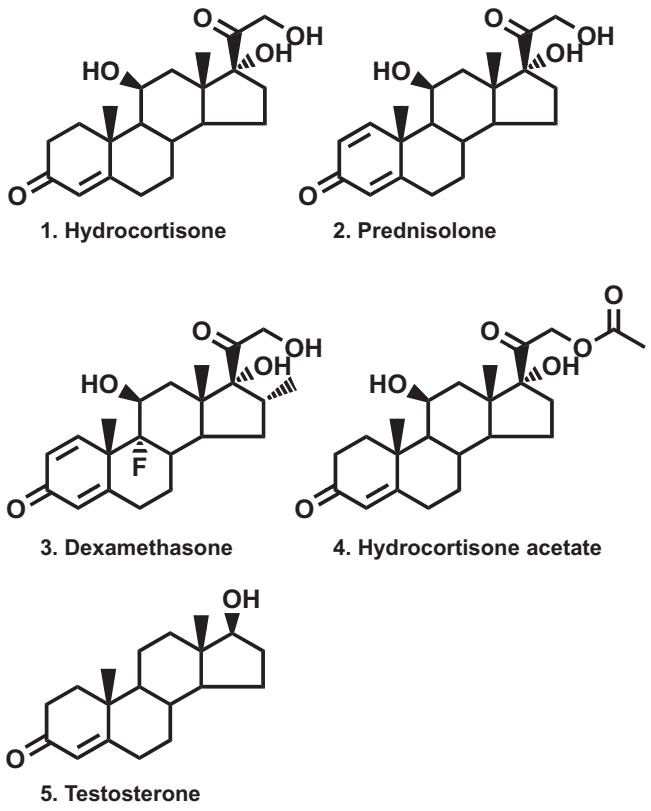

Fig. 4. Chemical structures of steroids using temperature-responsive chromatography.

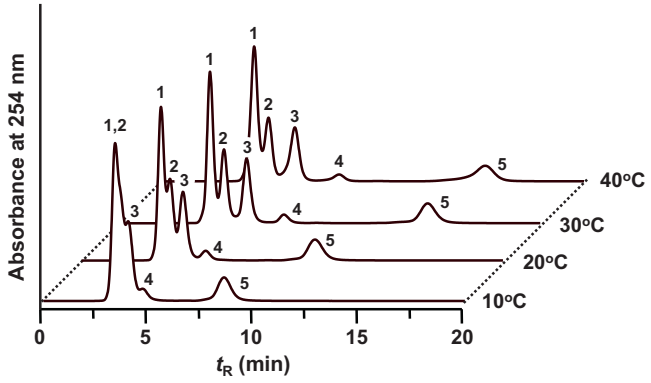

Fig. 5. Chromatograms of a mixture of five steroids with Milli-Q water as a mobile phase at from 10 °C to 40 °C. Peaks: 1, hydrocortisone; 2, prednisolone; 3, dexamethasone; 4, hydrocortisone acetate; 5, testosterone. Column; poly(NIPAAm-*co*-Nap)-modified silica column (2.1 mm i.d. × 100 mm). Detection wavelength: 254 nm.

Table 2
Comparison of the retention times of naphthalene and 1,5-dinitronaphthalene obtained by HPLC analysis using different four stationary phases.

Column	t_R (min)[b]	
Stationary phase[a]	Naphthalene	1,5-Dinitronaphthalene
ODS[c]	16.8 ± 0.09	12.0 ± 0.10
Phenyl–hexyl silica[c]	12.6 ± 0.05	17.2 ± 0.09
PNIPAAm-modified silica[d]	5.8 ± 0.11	4.7 ± 0.14
Poly(NIPAAm-*co*-Nap)-modified silica[d]	5.5 ± 0.10	7.6 ± 0.15

[a] Column temperature was set at 20 °C.
[b] Mean ± standard deviation (n = 3).
[c] Mobile phase: MeOH–H_2O (7:3, v/v).
[d] Mobile phase: Milli-Q water.

11.6 mmol) and triethylamine (0.9 mL, 12.6 mmol) were added to the reaction mixture with stirring below −20 °C. After the reaction proceeded for 15 min, the reaction mixture was washed with water. The organic layer was evaporated and the residue was chromatographed on silica gel [column; 120 × 12 mm i.d., mobile phase; ethyl acetate–hexane (1:2, v/v)] to give a slightly yellow amorphous [*N*-acryloyl-3-(2-naphthyl)-L-alanine methyl ester] (2.0 g). ^{1}H NMR in chloroform-d (ppm), 2.04 (q, J = 6 Hz, 2H), 3.74 (s, 3H), 4.12 (m, 1H), 5.10 (brs, 1H), 5.66 (d, J = 4 Hz, 1H), 6.07 (m, 1H), 6.30 (d, J = 6 Hz, 1H), 7.25 (m, 1H), 7.46 (s, 2H), 7.57 (s, 1H), 7.77 (m, 3H).

2.4. Synthesis of polymer

Poly(*N*-isopropylacrylamide-*co*-*N*-acryloyl-3-(2-naphthyl)-L-alanine methyl ester), poly(NIPAAm-*co*-Nap), was prepared using radical polymerization. After NIPAAm (9.28 g, 81.7 mmol) and *N*-acryloyl-3-(2-naphthyl)-L-alanine methyl ester (0.72 g, 2.54 mmol) were dissolved in DMF (25 mL), AIBN (58 mg, 0.35 mmol) and MPA (263 mg, 2.48 mmol), which act as a radical initiator and a chain transfer agent, were added to the mixed solution. The reaction mixture was degassed by subjecting to freeze–thaw cycles and then heated at 70 °C. After polymerization for 5 h, the reaction solution was poured into 2 L of diethyl ether to precipitate the crude polymer. The obtained product was further purified by repeated precipitation from 55 mL of acetone into 2 L of diethyl ether and then dried to give a white solid [poly(NIPAAm-*co*-Nap)] (7.08 g).

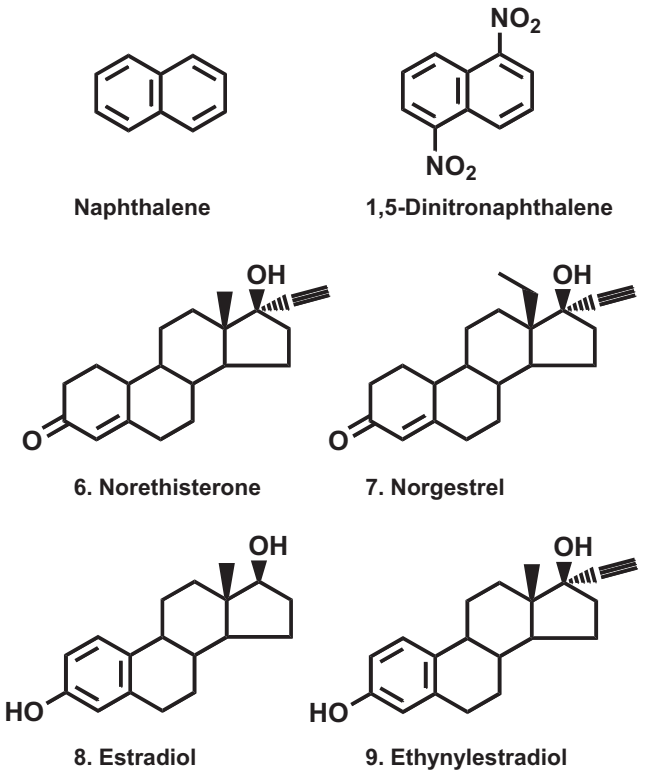

Fig. 6. Chemical structures of naphthalene, 1,5-dinitronaphthalene and steroids.

2.5. Characterization of polymer

The lower critical solution temperatures (LCST) of the polymer were determined by measuring the optical transmittance of its aqueous solution (0.5%, w/v). The optical transmittance of the polymer solution was measured at 500 nm at various temperatures using a UV–VIS spectrophotometer (V-630, JASCO, Tokyo, Japan). The temperature was controlled by an ETC-717 controller (JASCO) and a PT-31 peltier system (KRÜSS, Hamburg, Germany); the heating rate was 0.1 °C/min. The LCST was determined by the temperature at 50% of the optical transmittance of the polymer solution.

The number-averaged molecular weight (M_n) and weight-averaged molecular weight (M_w) were determined by gel permeation chromatography (GPC). GPC was conducted on a TOSHO GPC-8020 system equipped with a differential refractive-index detector, TSK guard column and two TSK GEL α-M columns (7.8 mm i.d. × 300 mm); 10 mM LiCl in DMF at 40 °C was used as the mobile phase (flow rate, 1.0 mL/min). Calibration was performed using near-monodisperse poly(ethylene glycol) standards from TOSHO (Tokyo, Japan).

2.6. Temperature-responsive chromatography

Modification of aminopropyl silica with synthetic polymer was carried out by activated ester–amine coupling utilizing DCC and NHS as previously reported [16]. A polymer-grafted silica support was packed into a stainless-steel column (100 mm × 2.1 mm i.d.). The temperature-responsive column was used at a flow rate of 0.2 mL/min and Milli-Q water was used as the mobile phase. Standard solutions of all analytes were prepared at a concentration of 0.1 mg/mL. The retention factors of the analytes were calculated from the following equation: retention factor = $(t_R - t_0)/t_0$, where t_0 and t_R express the retention time of uracil and target analyte, respectively.

3. Results and discussion

3.1. Temperature-responsive polymer carrying aromatic moiety

The naphthalene moiety was introduced into a temperature-responsive polymer for the purpose of developing a novel separation mode utilizing a π–π interaction between the stationary phase and the solute. 3-(2-Naphthyl)-L-alanine was selected as an aromatic component in the polymer, and was converted to *N*-acryloyl derivative via several steps for the copolymerization

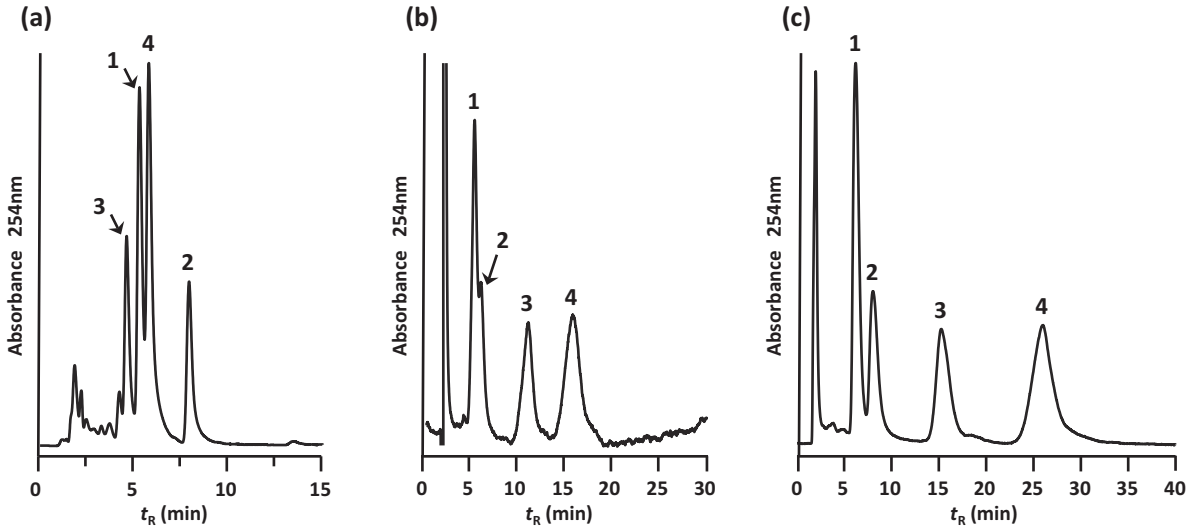

Fig. 7. Chromatograms of a mixture of four steroids utilizing conventional ODS column (a), PNIPAAm-modified silica column (b) and poly(NIPAAm-*co*-Nap)-modified silica column (c). Column size: 2.1 mm i.d. × 100 mm. Column temperature; 20 °C. Detection wavelength: 254 nm. Mobile phase: (a) CH_3CN–H_2O (1:1, v/v), (b) and (c) Milli-Q water, Flow rate: 0.2 mL/min. Peaks: 6, norethisterone; 7, norgestrel; 8, estradiol; 9, ethynylestradiol.

of NIPAAm, which is a monomer of PNIPAAm (Fig. 1). Poly(NIPAAm-*co*-Nap) with the feed ratio of the NIPAAm/naphthyl-alanine derivative molar ratio be fixed to 97:3 was synthesized by radical telomerization using MPA as a chain-transfer agent (telogen) at 70 °C in DMF. Fig. 2 shows the structure of poly (NIPAAm-*co*-Nap); its composition was determined by the ^{1}H NMR spectra in a $CDCl_3$ solution. The mole fraction of *N*-acryloyl 3-(2-naphthyl)-L-alanine methyl ester in the polymer was calculated by comparing the peak area arising from the aromatic group of the naphthyl-alanine derivative with the area of the singlet peak at 4.01 ppm, attributed to the resonance of the methine proton from the NIPAAm moiety. The introduction rate of the naphthyl-alanine derivative was calculated to be 2.7%, which is comparable to the theoretical value. Table 1 summarizes the characterization data of the synthetic copolymer. Fig. 3 shows the temperature-dependent optical transmittance change for the polymers at 0.5 w/v % aqueous solution. Poly(NIPAAm-*co*-Nap) showed a lower LCST (25.8 °C) than that for homogeneous PNIPAAm. In general, the incorporation of a hydrophobic comonomer leads to a lower LCST, and a hydrophilic comonomer leads to a higher LCST. Therefore, the introduction of the naphthyl-alanine moiety which works as a hydrophobic comonomer into PNIPAAm, enhances the hydrophobicity of the copolymer and shifts the LCST to a lower temperature than that of PNIPAAm homopolymer [23]. Poly(NIPAAm-*co*-Nap) undergoes a reversible phase transition from the water-soluble form into aggregates similar to PNIPAAm.

3.2. Temperature-responsive chromatography for separation of steroids

For preparing temperature-responsive packing, the synthetic polymer was conjugated to the aminopropyl silica beads using reactive ester coupling, followed by packing into stainless-steel HPLC columns. The effect of the column temperature was examined on a poly(NIPAAm-*co*-Nap) terminally modified surface using steroids. Using Milli-Q water as the sole mobile phase, the separation of steroids was carried out by changing the column temperature at from 10 °C to 40 °C. As shown in Figs. 4 and 5, a temperature-dependent resolution of steroids was achieved. The retention of steroids exhibited a linear relationship with the log *P* values (the partition coefficients in the 1-octanol/water system). Although the peaks of steroids are not properly resolved at lower temperature than the LCST, they were well resolved with increasing column temperature using only water as a mobile phase. With increasing

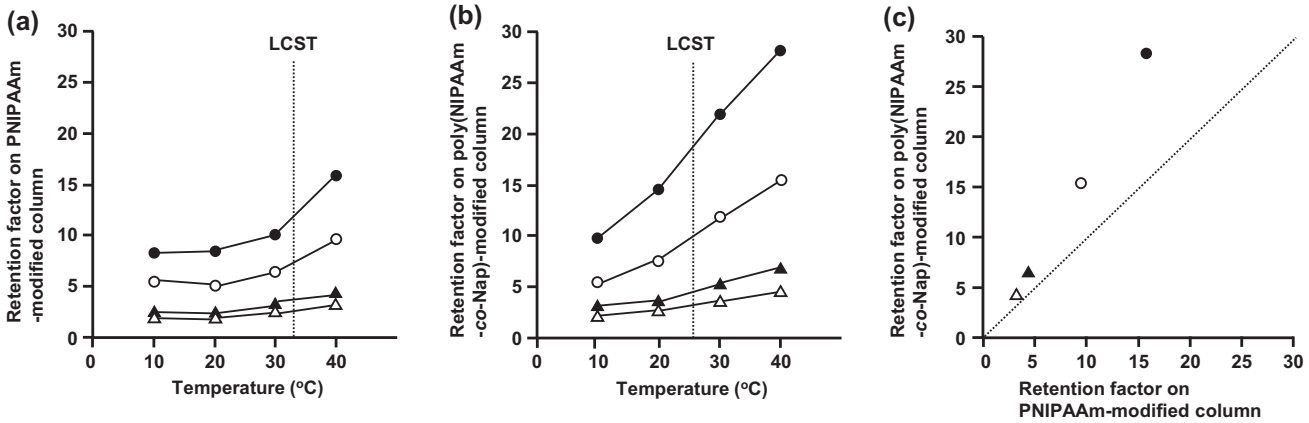

Fig. 8. Effect of naphthyl-alanine derivative introduction on the retention factors of aromatic- and non-aromatic steroids on a PNIPAAm-modified silica column (a) and poly(NIPAAm-*co*-Nap)-modified silica column (b). Comparison of retention factors of steroids on both columns at 40 °C (c). Steroids: estradiol (open circle), ethynylestradiol (closed circle), norethisterone (open triangle) and norgestrel (closed triangle).

the temperature, increased interactions between the steroids and the poly(NIPAAm-*co*-Nap)-grafted surface of the stationary phase were observed. Previously, this phenomenon demonstrated that a hydrophobic interaction between steroids and a homogeneous PNIPAAm-grafted surface could be controlled by the column temperature [13,14].

3.3. Evaluation of molecular interaction between solute and stationary phase

We investigated whether poly(NIPAAm-*co*-Nap) induces the π–π interaction between the solute molecule containing an aromatic group. We chose naphthalene and 1,5-dinitronaphthalene as analytes (Fig. 6). The π-electron density of an aromatic ring of 1,5-dinitronaphthalene is lower than the naphthalene because of the strong electron-withdrawing property of the nitro group. Concerning the hydrophobicity, naphthalene (log*P* 3.3) is more hydrophobic than 1,5-dinitronaphthalene (log *P* 2.6). Table 2 gives the results of a comparative experiment using a conventional ODS column and a phenyl–hexyl column. As is well known, a hydrophobic interaction occurs predominantly between the solute and the ODS stationary phase. For this reason, the retention time of naphthalene is longer than that of 1,5-dinitronaphthalene on the ODS column. In contrast, on the phenyl–hexyl column, the π–π interaction plays a crucial role in the separation mechanism for the aromatic solute. Tsuzuki et al. demonstrated that the calculated interaction energy of the nitrobenzene–benzene complex is significantly larger than that of the benzene dimmer [8]. As a result, the reversed elution order was observed on a phenyl–hexyl column. We then evaluated the property of the temperature-responsive polymer-grafted stationary phase. The homogeneous PNIPAAm-grafted surface retained naphthalene longer than 1,5-dinitronaphthalene. The result of this elution order was confirmed with that of the ODS column. On the other hand, the poly(NIPAAm-*co*-Nap)-grafted column showed the same elution order as did the phenyl–hexyl column. These results indicated that the π–π interaction dominates in the separation mode of chromatography utilizing the poly(NIPAAm-*co*-Nap)-grafted stationary phase. Given the introduction rate of the naphthyl-alanine derivative (2.7%), it is an intriguing possibility that a small quantity of material induces a large influence on the function of PNIPAAm [21].

3.4. Comparison of the structural selectivity of the polymer-grafted stationary phase

Based on the finding in the previous section, a further investigation of functionality on a poly(NIPAAm-*co*-Nap)-grafted silica column were conducted. We chose aromatic steroids (estradiol and ethynylestradiol), and non-aromatic steroids (norethisterone and norgestrel) as the analytes, and then analyzed them using a conventional ODS column, a homogeneous PNIPAAm-grafted silica column and a poly(NIPAAm-*co*-Nap)-grafted silica column (Figs. 7 and 8). Fig. 7 shows typical chromatograms of a mixture of steroids obtained by using three different stationary phases. The elution orders were clearly different between the ODS column and the polymer-grafted silica column. In comparing the ODS column with PNIPAAm-grafted silica column, the resolution between norethisterone and ethinylestradiol is higher in the latter than the former. These result shows that differences exist in the solute-stationary phase interaction mechanism between ODS and PNIPAAm-based polymers. It may be suggested that polymer-modified stationary phase more or less has the selectivity for the aromatic moiety. Further research about the detail of this phenomenon is now progress in our laboratory. In addition, comparing with on a PNIPAAm-grafted silica column, the good peak resolution of each steroid was obtained on a poly(NIPAAm-*co*-Nap)-grafted silica column. The significant increases in the retention time of an aromatic steroid were observed when using a poly(NIPAAm-*co*-Nap)-grafted silica column.

Finally, a comparison of the separation selectivity of steroids between PNIPAAm- and the poly(NIPAAm-*co*-Nap)-grafted stationary phase was carried out. Fig. 8(a) and (b) shows that the retention factors of aromatic steroids were dramatically increased on the poly(NIPAAm-*co*-Nap)-grafted column in contrast to that on a PNIPAAm-grafted column. In Fig. 8(c), poly(NIPAAm-*co*-Nap) showed greater selectivity for aromatic steroids than the PNIPAAm. These observations would be due to the π–π interaction between the aromatic component of both analytes and the polymer.

4. Conclusion

We developed a novel functional polymer by incorporating the naphthyl-alanine moiety, which has temperature-responsiveness and a selective retention of aromatic compounds by the intermolecular π–π interaction. The Poly(NIPAAm-*co*-Nap) has its LCST at 25.8 °C, and changes from the hydrophilic to the hydrophobic phase transition reversely in the vicinity of LCST. In temperature-responsive chromatography using poly(NIPAAm-*co*-Nap)-grafted silica as the HPLC stationary phase, we succeeded to demonstrate π–π interaction chromatography-like separation selectivity of the aromatic steroids by simply changing the column temperature with pure water as a sole mobile phase. The incorporation of the naphthyl-alanine derivative causes a significant effect on the retention selectivity for aromatic compounds. We are currently performing studies to develop a temperature-responsive polymer carrying another aromatic moiety for the multi-functional property, and applying chromatographic research.

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