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Synthesis of monodispersed molecularly imprinted polymer particles for high-performance liquid chromatographic separation of cholesterol using templating polymerization in porous silica gel bound with cholesterol molecules on its surface

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ABSTRACT

Monodispersed molecularly imprinted polymer particles selective for cholesterol were prepared by the copolymerization of styrene and divinylbenzene in the presence of template silica gel particles (particle size: 5 μm; pore size: 10 nm) functionalized with cholesterol on the surface, followed by dissolution of the cholesterol-bonded silica gel with a NaOH aqueous solution. Transmission and scanning electron micrographs of the molecularly imprinted polymer (MIP) particles revealed good monodispersity and porous structure. The MIP particles were packed into a high performance liquid chromatographic column, and its recognition ability of cholesterol was evaluated using cholesterol, cholesterol esters and fatty acid methyl esters by comparison with the non-imprinted polymer (NIP) particles prepared from styrene and divinylbenzene without cholesterol. The MIP particles showed a high affinity for cholesterol and cholesterol esters ($K'_{MIP}/K'_{NIP} > 5.7$).

1. Introduction

Molecularly imprinted polymers (MIPs) have attracted considerable attention as separation media in liquid chromatography [1–3] and solid-phase extraction [4–6], due to their selective molecular recognition properties. In most cases, MIPs have been prepared by bulk polymerization followed by grinding and sieving into fine particles [1–3,6]. Therefore, the obtained polymer particles have a broad particle size distribution and irregular shapes, which lower the resolution efficiency of a chromatographic column. In order to avoid such nonuniformity in size of these MIP particles, a few methods such as a multistep swelling and polymerization method [7,8], and a core–shell emulsion polymerization method [9,10] have been reported.

The templating polymerization for the preparation of monodispersed porous polymer particles has also been reported: the polymerization of a monomer was performed in a porous matrix such as silica gel and then the matrix was dissolved using an alkaline solution [11–13]. We have recently reported the preparation of monodispersed porous polymer resins by the templating polymerization method with template silica gel particles [14]. The resins were converted to the quaternary ammonium salt by chloromethylation, followed by a reaction with *N*,*N*,*N*′,*N*′-tetramethyl-1,6-diaminohexane. The monodispersed anion-exchange polymer resins were then applied to the stationary phase for high-performance liquid chromatographic separation of carbohydrates. With these resins, thirteen kinds of mono- and oligosaccharides were separated with good spacing.

These results prompted us to explore the possibility of using template silica gel functionalized with specific recognition molecules for the preparation of MIPs. There are two types of preparation methods for monodispersed and spherical MIP particles using silica gel: bulk polymerization using the pore of silica gel and polymerization with template-immobilized silica gel. The former method is that silica gel is used as micro-vessels and bulk polymerization is carried out in the pore of silica gel [15,16]. In the latter method polymerization is carried out in the pore of template-immobilized silica gel. A few examples using the template-immobilized silica gel have been reported: Mosbach et al. have reported the preparation method of the MIPs for theophylline from trifluoromethylacrylic acid and divinylbenzene using 7 μm-derivertized silica gel [17], and the methacrylate-based MIPs for adenine [18] and peptide [19] were also prepared. The homogeneity in cavities for the recognition in MIPs prepared with template-immobilized silica gel would be higher than that in MIPs prepared with silica gel.

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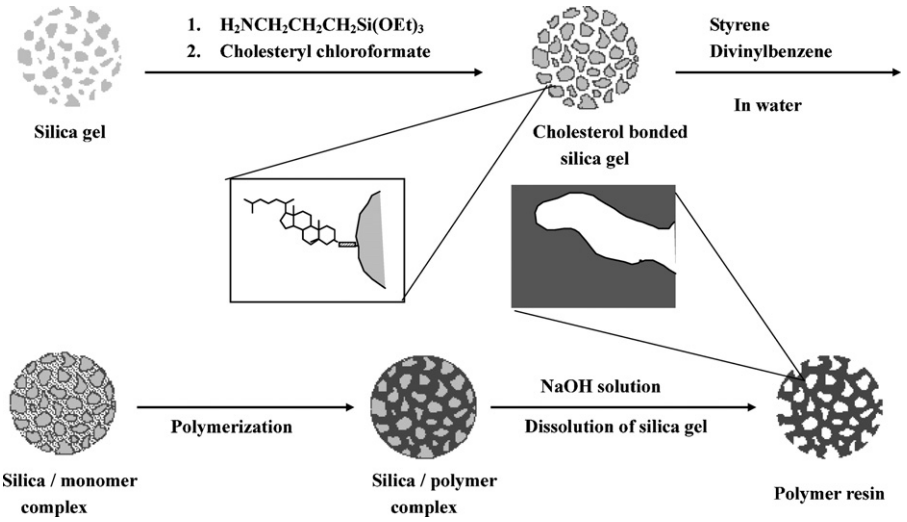

Fig. 1. Preparation of monodispersed molecularly imprinted polymer resins for separating cholesterol.

In this study, cholesterol was chosen as the target molecule due to its biological significance: cholesterol is an intermediate in the biogenesis of biologically important steroids, such as bile acids, adrenal cortical hormones, sex hormones and cardiac glycosides. Moreover, cholesterol has rigid fused ring structures which enable the construction of a rigid cavity fitting to template molecule. A few studies concerning the preparation of MIPs as HPLC column packings for cholesterol have been examined: the bulk polymerization without the use of silica gel [3], and two-stage aqueous emulsion polymerization methods of the monodispersed submicrometer particles for batch binding separation [9,10] are reported.

Herein, we describe a preparation of monodispersed MIPs for cholesterol via the templating polymerization method using cholesterol-immobilized silica gel (Fig. 1), and the usefulness of these MIPs for the recognition of cholesterol.

2. Materials and methods

2.1. Materials

Nucleosil Silica 100-5 (particle size 5 μm, pore size 10 nm) was purchased from Macherey-Nagel (Duren, Germany). Cholesterol, cholesterol esters, 5β-cholestan-3α-ol and fatty acid methyl esters were from Sigma (St. Louis, MO, USA). 5α-Cholestan-3α-ol was from Steraloids (Newport, RI, USA). 3-Aminopropyltriethoxysilane, styrene, cholesteryl chloroformate were from Tokyo Kasei (Tokyo, Japan). Divinylbenzene (80% grade) and inhibitor remover were from Aldrich (Milwaukee, WI, USA). Two poly (vinyl alcohol) powders, average degree of polymerization of 1000 (86–90% hydrolyzed) and 1500 (100% hydrolyzed), were from WAKO Pure Chemical Industries, Ltd. (Osaka, Japan), and were mixed for use in the ratio of 1:25. Toluene and 1,4-dioxane were dried by refluxing over sodium and then distilled before use. All other chemicals were of analytical grade and used without further purification.

2.2. Microscopy

A JEM-1200EX transmission electron microscope from JEOL (Tokyo, Japan) was employed to observe the morphology of the resultant polymer particles. Also, the scanning electron micrograph of MIP particles was taken by a S-2300 scanning electron microscope from Hitachi (Tokyo, Japan).

2.3. High-performance liquid chromatography

The HPLC analysis was performed using a JASCO system (Tokyo, Japan) consisting of a TRIROTAR-III pump, a VL-613 injector, an 875-UV detector, and a differential refractive index detector. A Chromatocorder 12 from SIC (Tokyo, Japan) was employed for the data analysis.

For the chromatographic analysis, cholesterol, cholesterol esters and fatty acid methyl esters were dissolved in heptane–chloroform (9:1, v/v) to 0.1% (w/v) solutions. These samples were eluted with acetonitrile–diethyl ether (7:3, v/v) at a flow rate of 1.0 mL/min and were detected at 210 nm. For 5α-cholestan-3α-ol and 5β-cholestan-3α-ol, acetonitrile–diethyl ether (7:3, v/v) were used as an eluent, and a differential refractive index detector was used. The solvent peak was used to determine the holdup time (t_0). The theoretical plate number was calculated based on the formula: $N = 5.54 \times (t_R/W_{1/2})$, where t_R and $W_{1/2}$ are retention time and the peak width in time at half peak height, respectively.

2.4. Preparation of molecularly imprinted polymer particles

An outline of the preparation method for the MIP particles is shown in Fig. 1. Silica gel was silanized with 3-aminopropyltriethoxysilane, and then cholesterol was connected to the silica gel through carbamate linkage by the reaction of cholesteryl chloroformate with the amino groups of the silanized silica gel (Fig. S1, Supporting Material). A mixture of styrene, divinylbenzene and benzoyl peroxide in water was vigorously stirred in the presence of the cholesterol-bonded silica gel in order to intrude the monomers into the pores of the silica gel, and then the mixture was heated for polymerization. The cholesterol-bonded silica gel was removed by dissolution with an alkaline aqueous solution. As a control experiment, the NIP particles were prepared in the same way using silica gel silanized with butyldimethylchlorosilane.

2.4.1. Preparation of cholesterol-bonded silica gel

The synthesis of the cholesterol-bonded silica gel is shown in Fig. S1. The silica gel was dried in a flask under vacuum at 150 °C for 12 h. Dry silica gel (9.87 g), dry toluene (50 mL) and 25 g of aminopropyltriethoxysilane were added to the flask, and the mixture was gently refluxed for 30 h under a nitrogen atmosphere. The silanized silica gel was filtered through a sintered-glass filter and washed with toluene (200 mL) followed by tetrahydrofuran (200 mL) and

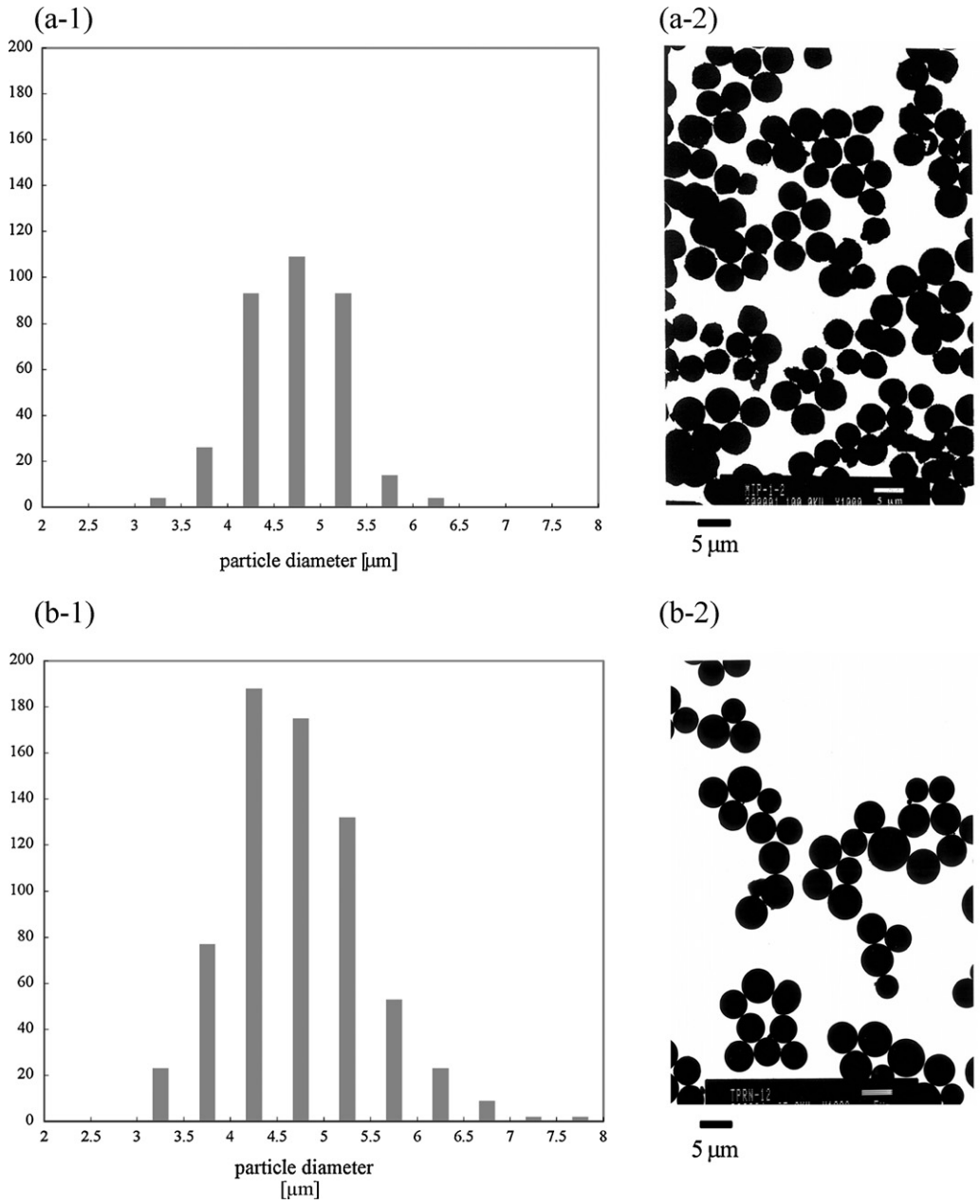

Fig. 2. Size distributions of MIP particles (a-1), NIP particles (b-1), and TEM images of MIP particles (a-2), NIP particles (b-2). Mean particle diameters: 4.72 μm (a), 4.83 μm (b), and total number of particles: 343 (a), 486 (b).

methanol (200 mL), then dried under vacuum overnight. The yield of the silanized silica gel (aminopropyl-bonded silica gel) was 11.06 g, and the N% was evaluated to be 1.11% by elemental analysis.

To the aminopropyl-bonded silica gel (4.10 g), cholesteryl chloroformate (4.50 g) and dry dioxane (35 mL) were added, and the mixture was refluxed with stirring under nitrogen for 4 h. The reaction product was filtered and then washed with dioxane, dichloromethane, tetrahydrofuran and methanol. After drying, the cholesterol-bonded silica gel (5.07 g) was obtained. From the increase in weight, the mole content of the cholesterol group per 1 g of cholesterol-bonded silica gel was estimated to be 0.46 mmol.

2.4.2. Preparation of molecularly imprinted polymer particles

Cholesterol-bonded silica gel (4.94 g) and ultrapure water (50 mL) were added to a three-necked flask, and the mixture was aerated with argon gas for 30 min with gentle stirring. The mixture (2.71 g) containing styrene, 80%-divinylbenzene and benzoyl peroxide (molar ratio, 1:1:0.07), freed from the polymerization inhibitor by passing through the inhibitor remover column, was added to the flask, followed by 30 mL of 0.35 wt.% aqueous poly (vinyl alcohol) solution. The mixture was stirred at 700 rpm under an argon atmosphere for 24 h at room temperature and then stirred at 90 °C for 24 h. The reaction mixture was then cooled to room temperature, and the precipitate was filtered through a sintered-glass filter and washed with water (200 mL) and methanol (100 mL). After drying, the precipitate (7.02 g) was added to a mixture of a 5 M NaOH aqueous solution (120 mL) and methanol (80 mL). The mixture was stirred for 24 h at room temperature to dissolve the template cholesterol-bonded silica gel. The product polymer particles were filtered and washed with water until the filtrate was neutral, followed by 100 mL each of methanol, dichloromethane, tetrahydrofuran, acetone and methanol, then dried under vacuum

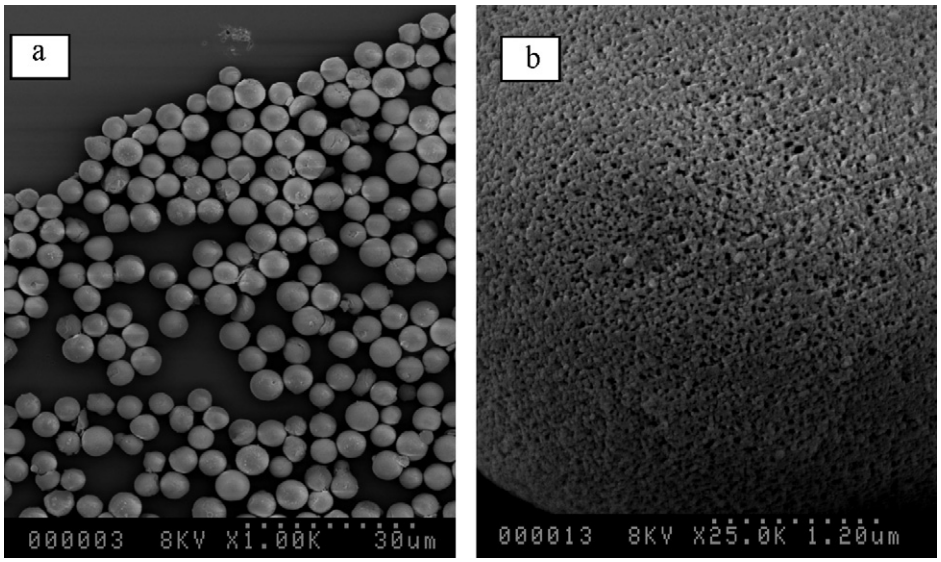

Fig. 3. SEM images of MIP particles at magnification of (a) 1000× and (b) 25,000×.

at room temperature. The yield of the MIP particles was 1.97 g (73%). The morphology was examined using a transmission electron microscope and a scanning electron microscope.

As a control experiment, the NIP particles were prepared according to the method described in the literature [14]. From the template silica gel (3.74 g) silanized with butyldimethylchlorosilane, and the mixture (1.93 g) containing styrene, 80%-divinylbenzene and benzoyl peroxide (molar ratio, 1:1:0.07), the NIP particles (1.58 g, 82%) were synthesized.

2.5. Column packing

The MIP particles were packed into a stainless-steel column (4.6 mm I.D. × 250 mm) by a conventional slurry packing method using acetonitrile–water (8:2, v/v) as an eluent at a constant pressure of 20 MPa. The NIP particles were packed into a column in the same manner.

3. Results and discussion

3.1. Preparation of molecularly imprinted polymer particles

The monodispersed MIP particles for separation of cholesterol were synthesized (Fig. 1). The copolymer of styrene and divinylbenzene was prepared in the pores of cholesterol-bonded silica gel which, prepared from the silanization of the silica gel with aminopropyltriethoxysilane followed by the reaction with cholesteryl chloroformate. Then, the cholesterol-bonded silica gel was dissolved using a NaOH aqueous solution. The NIP particles were also synthesized using the template silica gel silanized with butyldimethylchlorosilane. Yields of MIP and NIP particles were around 80%. The elemental analysis of these MIP particles gave C 86.92%, H 8.15% and ash 2.42%. The amount of ash shows that template silica gel was mostly removed by the dissolution with a NaOH aqueous solution.

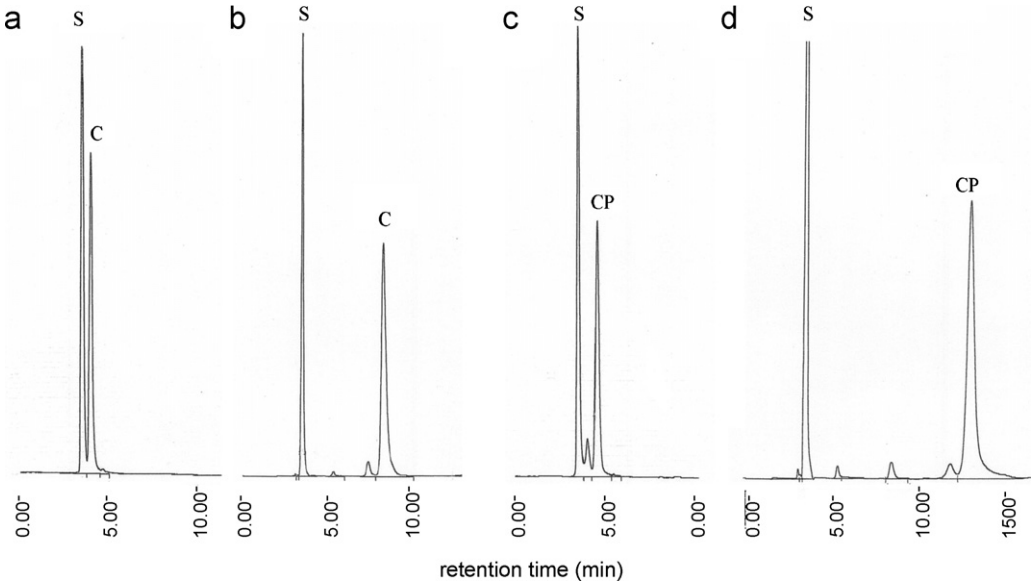

Fig. 4. Chromatograms of cholesterol and cholesteryl palmitate: (a, c) on the NIP-column and (b, d) on the MIP-column. Peak identification: S, solvent peak; C, cholesterol; CP, cholesteryl palmitate. HPLC conditions: column size, 250 mm × 4.6 mm I.D.; mobile phase, acetonitrile–diethyl ether (7:3, v/v) at flow rate 1.0 mL/min; detection, UV at 210 nm; sample concentration, 0.1% (w/v) in heptane–chloroform.

Table 1
Pore properties of the MIP, NIP and template silica gel.

	Pore volume[a] (cm^3/g)	Surface area[b] (m^2/g)	Pore size[b] (nm)	Particle size[c] (μm)
MIP	0.62	302	8.3	4.7
NIP	0.81	420	7.6	4.8
Silica gel[d]	1	350	10	5.0

[a] By the BET nitrogen adsorption method.
[b] By the BJH desorption.
[c] From TEM images.
[d] From the specification of Nucleosil silica 100-5.

The transmission electron micrographs and the size distributions of the MIP particles and the NIP particles are shown in Fig. 2. Both the MIP and NIP particles showed a good monodispersity (mean particle size: 4.72 μm for MIPs and 4.83 μm for NIPs). The pore properties of these particles were examined by BET nitrogen adsorption analysis, and the data of Table 1 showed the porous structure of these particles. In addition, the scanning electron micrograph of the MIP particles also clearly revealed the porous structure (Fig. 3). Therefore, unlike the bulk-polymerization technique, this method allows facile preparation of the MIP particles with high monodispersity.

3.2. Separation of cholesterol, cholesterol esters and fatty acid methyl esters

The monodispersed MIP products were packed into an HPLC column and the selective affinity for cholesterol was evaluated by comparison with the separation by the NIP particles using CH_3CN–Et_2O as a mobile phase. The chemical structures of the analytes are depicted in Fig. S2 (Supporting Material). The representative chromatograms of cholesterol and cholesterol palmitate on MIP and NIP columns are shown in Fig. 4. The comparatively narrow peaks in the separation of analytes using these copolymer resins are observed. Theoretical plate numbers of our MIP column for peaks of cholesterol and cholesterol palmitate were 4500 and 3700, respectively.

MIPs particles prepared by bulk polymerization usually lead the peak broadening and tailing because of heterogeneity in binding site due to irregularities in both particles size and shape [8]. Hwang and Lee [20] prepared MIPs for cholesterol by bulk polymerization of cholesteryl (4-vinyl)phenyl carbonate and ethylene glycol dimethacrylate followed by the removal of cholesterol moiety with NaOH-methanol solution. They reported that the theoretical plate number (in a 25 cm × 0.46 cm I.D. column) of this MIPs for the elution peak of cholesterol was 1240. Although this value seems to be relatively high, peak boarding and tailing were still observed.

By our MIPs consisting of hydrophobic copolymer of styrene and divinylbenzene, cholesterol is eluted faster than cholesterol esters as shown in Table 2 and Fig. 4. These results indicate that the reversed phase separation mode is working in our HPLC conditions. Our relatively high theoretical plate numbers observed for MIPs would be attributed to the homogeneity in cavities for cholesterol, and the weak hydrophobic interaction between MIPs and analytes.

The retention factors (k') and the imprinting factors (K'_{MIP}/K'_{NIP}) of the analytes are shown in Table 2. The MIP column possessed a clear retention for cholesterol ($k' = 1.61$), while the NIP column showed little retention ($k' = 0.21$). The imprinting factor for cholesterol was 7.7. Similar results were observed for the cholesterol esters. On the other hand, the retention factors for the fatty acid methyl esters with the MIP column showed very weak retention ($k' = 0.09$–0.23). These results indicate that the MIP particles recognize the cholesterol moiety and have a pronounced retention for the cholesterol and cholesterol esters. The separation of cholesterol analogues, 5α- and 5β-cholestan-3α-ol, was also examined using the MIP column (Table 2). The imprinting factor for 5α-cholestan-3α-ol (6.8) was similar to that for cholesterol, whereas 5β-cholestan-3α-ol showed a lower value (2.2) than cholesterol. Both 5α-cholestan-3α-ol and cholesterol have *trans*-fused A/B rings, whereas 5β-cholestan-3α-ol has *cis*-fused A/B rings as shown in Fig. S2. The high affinity for the 5α-analogue clearly indicates that the MIP particles recognize the difference in the shape of the steroidal skeleton (Fig. S2).

Table 2
Separation of cholesterol and cholesterol esters on MIP and NIP columns.[a]

Compounds	Retention		Imprinting factor
	MIP	NIP	K'_{MIP}/K'_{NIP}
Cholesterol (1)	1.61	0.21	7.7
Cholesteryl palmitate (2)	3.14	0.39	8.1
Cholesteryl palmitoleate (3)	2.44	0.43	5.7
Cholesteryl stearate (4)	3.63	0.37	9.8
Cholesteryl oleate (5)	2.85	0.40	7.1
Methyl palmitate (6)	0.17	–[b]	–
Methyl palmitoleate (7)	0.09	–[b]	–
Methyl stearate (8)	0.23	–[b]	–
Methyl oleate (9)	0.14	–[b]	–
5β-Cholestan-3α-ol (10)	0.94	0.42	2.2
5α-Cholestan-3α-ol (11)	1.29	0.19	6.8

[a] HPLC conditions: column, 4.6 mm I.D. × 25 cm; mobile phase, CH_3CN–$(CH_3CH_2)_2O$ (7:3, v/v) at flow rate 1.0 mL/min; detection, UV at 210 nm for (1)–(9) and RI for (10)–(11).
[b] Almost unretained.

4. Conclusion

Monodispersed MIP particles for the HPLC separation of cholesterol were synthesized by templating copolymerization in porous silica gel particles bound with cholesterol on the surface. The polymer particles showed a high affinity for cholesterol and cholesterol esters, but showed no affinity for fatty acid methyl esters. These results indicate that the MIP column recognizes the cholesterol moiety. In the bulk polymerization technique, the recognition cavities may have various shapes with regard to their direction and depth. On the other hand, the polymer particles prepared by the present method would probably have the homogeneity of recognition cavities. Therefore, the templating polymerization method in the porous silica gel particles will be useful for preparing monodispersed MIP particles.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2010.09.041.

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