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Chromatographic characteristics of cholesterol-imprinted polymers prepared by covalent and non-covalent imprinting methods

Ching-Chiang Hwang, Wen-Chien Lee*

Department of Chemical Engineering, National Chung Cheng University, Chiayi 621, Taiwan

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Abstract

Cholesterol-imprinted polymers were prepared in bulk polymerization by the methods of covalent and non-covalent imprinting. The former involved the use of a template-containing monomer, cholesteryl (4-vinyl)phenyl carbonate, while the latter used the complexes of template and functional monomer, methacrylic acid or 4-vinylpyridine prior to polymerization. Columns packed with these molecularly imprinted polymers (MIPs) were all able to separate cholesterol from other steroids. For different combinations of cholesterol and β-estradiol concentrations in a total of 1 g/l, the peak retention times for both compounds were nearly constant. The adsorption capacity for cholesterol onto the MIPs was found to significantly depend on the use of functional monomers, but the selectivity factors were only slightly different from each other at 2.9 to 3.2 since the separation was all based on the specific binding of cholesterol to recognition sites formed on the imprinted polymers. The capacity factors for cholesterol were determined to be 3.5, 4.0 and 3.1, respectively, for covalently imprinted, 4-vinylpyridine-based, and methacrylic acid-based non-covalently imprinted polymers. However, the covalently imprinted polymer was found to have a higher adsorption capacity for cholesterol and about fivefold higher chromatographic efficiency for cholesterol separation, in comparison with non-covalently imprinted polymers. The use of covalent imprinting significantly reduced the peak broadening and tailing. This advantage along with constant retention suggests that the covalently imprinted polymer has potential for quantitative analysis.

Keywords: Covalent imprinting; Non-covalent imprinting; Stationary phases, LC; Molecular imprinting; Molecularly imprinted polymers; Cholesterol

1. Introduction

Molecularly imprinted polymers (MIPs) have become one of the important sources of stationary phases for the chromatography of drugs and other biological compounds. Their widespread application and preparation methods have gained much attention as shown in many reviews [1–4]. The technique for preparing these polymers involves polymerization of functional monomers and a crosslinking monomer around a template. Prior to polymerization, a complex is formed by the interactions between functional monomers and the template. After removal of template molecules, specific recognition sites are created in the imprinted polymer for the adsorption of template. The formation of complex can be in either

*Corresponding author. Present address (until July 2002): 120 Olin Hall, School of Chemical and Biomolecular Engineering, Cornell University, Ithaca, NY 14853-5201, USA. Tel.: +1-607-255-4616; fax: +1-607-255-9166.

E-mail address: chmwcl@ccunix.ccu.edu.tw (W.-C. Lee).

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covalent or non-covalent manner. The covalent imprinting is superior in preventing leakage of template molecules during polymerization, because of the formation of labile covalent bonds between functional groups of the template and those of monomer. In order to remove the template, the covalent bonds connecting the template to the polymer should be cleaved. On the other hand, non-covalent imprinting has been reported to be a more direct and flexible approach because of its use of a larger range of compounds including chiral molecules that can be imprinted [5]. The removal of print molecules is more straightforward, in comparison with covalent imprinting.

For non-covalent imprinting, methacrylic acid and vinylpyridine have been commonly used as the functional monomers, which provide the function groups interacting with the template. The drugs for print include β-blockers, derivatives of amino acids, peptides, nucleic acids, and diazepam derivatives [6–12]. Covalent imprinting was first suggested by Wulff et al. [13,14]. They used vinyl derivatives as the functional monomers, which were bound with print molecule via covalent binding, and then co-polymerized with crosslinking monomer in an inert solution. The resultant co-polymer particles have an accessibility to print molecules. When the particles were used as the chromatographic support, print molecules could selectively adsorb on the support and be separated from the mixture. Whitcombe and co-workers [15–17] employed the covalent imprinting method to prepare MIPs using cholesterol and other steroids as the templates. Based on their method, the imprinting of cholesterol, a compound with a single hydroxyl group, is performed by relying on the use of a 4-vinylphenyl carbonate ester. The latter functions as a covalently template-bound monomer, which is efficiently cleaved hydrolytically, with the loss of carbon dioxide, resulting in a recognition site that interacts with the print molecule through hydrogen bonding. Non-covalent imprinting of cholesterol was also promising in the polymer of acrylic acid, 2-hydroxyethylmethacrylate, or β-cyclodextrin, or even copolymers of these compounds [18–23]. Most of the cholesterol-imprinted polymers prepared by the non-covalent method involved polymerization initiated with gamma rays [19–22].

The present paper describes the use of both covalent and non-covalent methods for the imprinting of cholesterol and the discovery of chromatographic characteristics using the resultant imprinted polymeric particles. By covalent imprinting, the bulk polymerization of the complex formed from cholesterol-bound functional monomer and crosslinker (crosslinking monomer). While when non-covalent imprinting was used, the bulk polymerization was carried out simply on the mixture of cholesterol, functional monomer and crosslinker. Non-covalent interactions were present and to stabilize the complex of template and monomer during polymerization. Two different functional monomers were used for the non-covalent approach.

2. Materials and methods

2.1. Materials

Methacrylic acid (99%), ethylene glycol dimethacrylate (EGDMA, 98%), acetic acid and glacial acetic acid were obtained from Merck (Germany) and used as received. 2,2′-Azobisisobutyronitrile (AIBN) was obtained from TCI (Tokyo, Japan). Potassium hydroxide was from Hayashi Chemical Industry Co. (Japan). Acetone and acetonitrile were from J.T. Baker (USA) and all of HPLC grade. Chloroform and methanol were from Mallinckrodt (USA) and of GC grade. Triethylamine and *p*-acetoxystyrene were obtained from Aldrich (USA) and dichloromethane from Riedel-de Haen (Germany). Cholesterol (95%), 4-vinylpyridine, tetrahydrofuran (THF), 2,6-di-tert.-butyl-4-methylphenol and cholesteryl chloroformate (97%) were purchased from Acros (USA). β-Estradiol and *o*-phthalaldehyde were obtained from Sigma (MO, USA). All chemicals were used without further purification.

2.2. Covalent molecular imprinting

The template-containing monomer, cholesteryl (4-vinyl)phenyl carbonate, was prepared by the method of Whitcombe et al. [15]. Briefly, to a cooled solution (in an ice-bath) of 4-vinylphenol (2 g, 16.6 mmol) in THF (60 ml) and triethylamine (4 ml) containing a trace of 2,6-di-tert.-butyl-4-methylphenol was added dropwise a solution of

cholesteryl chloroformate (7.5 g, 16.6 mmol) in THF (40 ml), and the mixture was stirred overnight at room temperature. After the removal of solvent by filtration, the product was washed with water, dried and evaporated. 4-Vinylphenol was prepared according to the method of Corson et al. [24]. Briefly, a mixture of 16.2 g (0.1 mol) *p*-acetoxystyrene and 13.8 g (0.25 mol) potassium hydroxide in 140 ml of water was stirred at 0–5 °C. Gaseous carbon dioxide was passed into the stirred solution to pH 8 to produce 4-vinylphenol.

For the preparation of MIP by the covalent imprinting method, the prepared cholesteryl (4-vinyl)phenyl carbonate (0.62 g) was mixed with EGDMA (4.38 g), AIBN (74.5 mg) in the solvent hexane and allowed bulk polymerization at 65 °C in the water bath for 24 h. The resultant polymer was dried and ground and sieved. Powders with a size ranging from 25 to 44 μm were collected for batch adsorption and packing chromatographic columns. To remove the template molecule, polymer particles were suspended and refluxed with NaOH in methanol. After reflux for 6 h, carbon dioxide was passed into the suspension to reduce the pH to 7. The particles were then extensively washed with water and methanol until no more cholesterol was released.

2.3. Non-covalent molecular imprinting

The MIP stationary phase was prepared by the method of bulk polymerization at low temperature. Methacrylic acid and 4-vinylpyridine were, respectively, used as the functional monomer to prepare the MIP by the non-covalent imprinting method. Briefly, cholesterol (0.387 g), methacrylic acid (0.682 ml), EGDMA (4.72 ml), and AIBN (0.05 g) were dissolved in 7.5 ml of chloroform in a conical Erlenmeyer flask. After degassing and nitrogen purging, the flask was sealed and allowed to polymerize at 4 °C for 6 h under UV (365 nm, 100 W lamp) irradiation. When 4-vinylpyridine was used as the functional monomer, the recipe was cholesterol (0.387 g), 4-vinylpyridine (0.112 ml), EGDMA (2.52 ml) and AIBN (0.02 g); all were dissolved in 7.5 ml of chloroform. EGDMA was used here as the crosslinking monomer and AIBN as the free radical initiator. After the polymerization, the chloroform was removed and the polymer product was dried in a vacuum oven for 12 h at room temperature. Finally, the bulk MIPs resulting from two different recipes were separately ground and sieved. For each MIP, the fraction of powders having a particle size ranging from 25 to 44 μm was collected for packing a chromatographic column. Template molecules were removed from the particles after packing into columns by continuously washing with acetonitrile until a stable baseline was reached.

2.4. Liquid chromatography

Cholesterol-imprinted particles were suspended in methanol by sonication and then slurry packed into 25 cm×0.46 cm I.D. stainless-steel columns using an air-driven fluid pump with acetone as the solvent. The backpressure for packing was 300 bar. For the HPLC analysis, a 10-μl sample solution was injected and eluted isocratically at a flow-rate of 0.5 ml/min, using a mixture of water and acetonitrile (1:19) as the mobile phase. The temperature was kept at 25 °C. Measuring the absorbance at 210 nm constantly monitored the effluent solution. Toluene was used as the non-retained component for the determination of the void fraction for each column. Capacity factors (k') were calculated according to standard chromatographic theory as $k' = (t_R - t_0)/t_0$, where t_R is the retention time of cholesterol or β-estradiol, and the retention time of toluene was used as the retention time of the non-retained component, t_0. The separation factor (α) was defined as the ratio of capacity factor of cholesterol to that of β-estradiol. The plate number was calculated based on the formula: $N = 5.54(t_R/W_{0.5h})^2$, where $W_{0.5h}$ is the peak width at half-height.

2.5. Batch adsorption of cholesterol

For the batch adsorption, a specific amount (20 mg) of imprinted polymer particles was incubated with 3 ml of cholesterol solution in glacial acetic acid with different concentrations at 25 °C for 3 h. The amount of cholesterol adsorbed on the MIPs was estimated by determining the loss of cholesterol in the solution. Adsorption isotherms were obtained by plotting the amount of cholesterol adsorbed onto particles vs. the concentration of cholesterol in the final solution. The concentration of cholesterol was

assayed by the method using an *o*-phthalaldehyde reagent [25]. The *o*-phthalaldehyde reagent was prepared by dissolving 25 mg of *o*-phthalaldehyde in 50 ml of glacial acetic acid. A small volume (0.1 ml) of cholesterol sample or each standard prepared by dissolving cholesterol in glacial acetic acid was well mixed with 2 ml of *o*-phthalaldehyde reagent, and then with 1 ml of concentrated sulfuric acid thoroughly. After 10 min, the absorbance of the color formed was measured against a reagent blank at 550 nm. The calibration curve obtained from a series of standards was used for the determination of cholesterol concentration in the sample.

3. Results and discussion

3.1. Chromatography of cholesterol on molecularly imprinted polymers

All the prepared MIPs were cross-linked polymers and successfully introduced recognition sites for cholesterol. They were ground into powders and the fraction having a particle size ranging from 25 to 44 μm was selected for packing of columns. There was apparently no difference among these MIPs using cholesterol as the template (print molecule), all were white powders after grinding. Fig. 1 shows the hypothetical formation of these cholesterol-imprinted polymers resulting from the free-radical polymerization in the bulk mode. After the removal of the template molecules, all the cholesterol-imprinted polymers were able to selectively adsorb cholesterol from the solution. However, the chromatographic behaviors were different using the polymers as stationary phases. Results from the liquid chromatography of cholesterol on imprinted polymers prepared by covalent method using cholesteryl (4-vinyl)phenyl carbonate as the covalently template-bound monomer and non-covalent method using either methacrylic acid or 4-vinylpyridine as the functional monomer are shown in Figs. 2–5. Fig. 2 shows typical peak profiles from the chromatographic separation of cholesterol and β-estradiol using these MIPs. As shown in Fig. 3, every component has the same retention time both when eluted in mixture and alone. For either cholesterol or β-estradiol, the peak area eluted alone was found to be

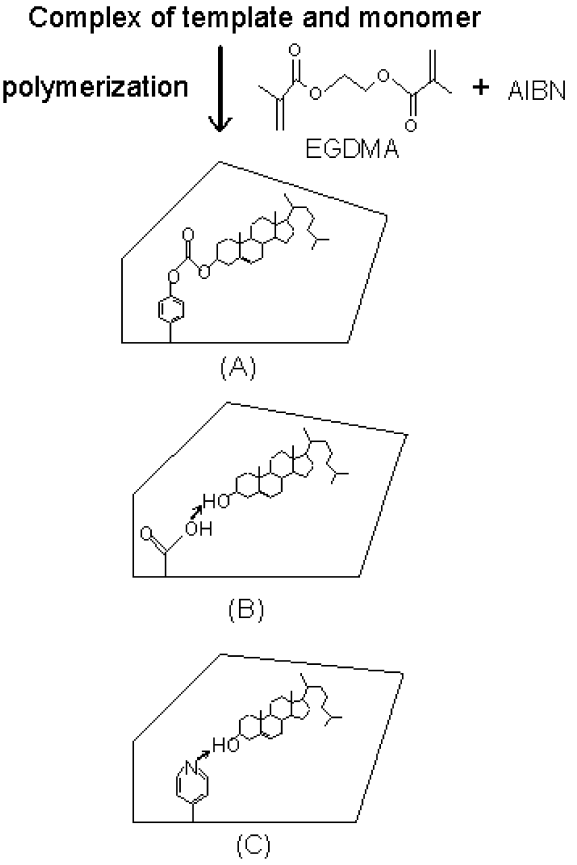

Fig. 1. Schematic representation of hypothetical formation of cholesterol-imprinted polymers prepared by the methods of covalent (A) and non-covalent imprinting using methacrylic acid (B) or 4-vinylpyridine (C) as the functional monomer. Arrows represent potential hydrogen-bonding interactions.

almost double that eluted in mixture, in concordance with the fact that the amount of each component in the mixture sample is half that in the pure sample. Samples with different combinations of cholesterol and β-estradiol concentrations in a total of 1 g/l were applied to columns packed with different MIPs. The retention time and capacity factor for each component were collected and plotted in Figs. 4 and 5. The chromatographic separation using these MIPs was reproducible and each data point reported in these figures was an average taken from 2 to 4 runs for each combination of solute concentrations. Fig. 5 also shows the selectivity of cholesterol and β-estradiol resulting from each column. Table 1 summarizes the results of chromatographic separation

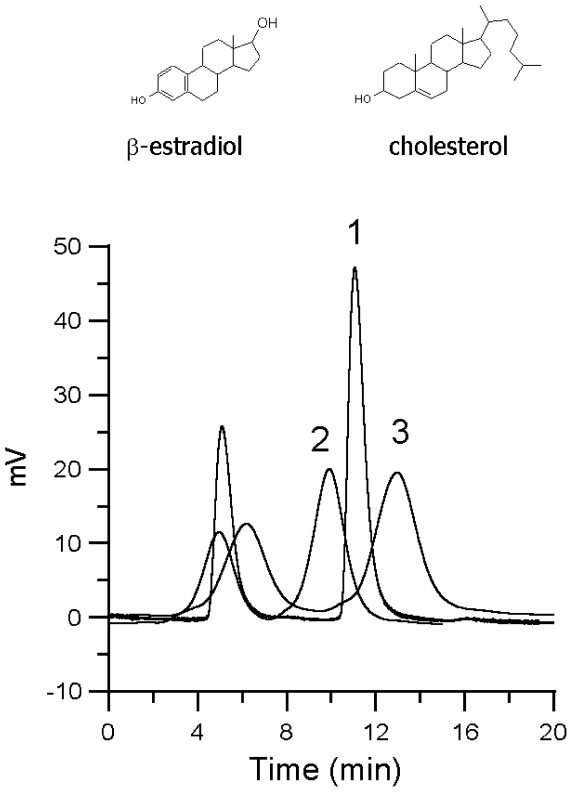

Fig. 2. Chromatography of cholesterol and β-estradiol on cholesterol-imprinted polymers prepared by the methods of covalent (1) and non-covalent imprinting using methacrylic acid (2) or 4-vinylpyridine (3) as the functional monomer. Samples of a mixture of cholesterol (0.7 g/l) and β-estradiol (0.3 g/l) were injected to columns packed with different MIPs.

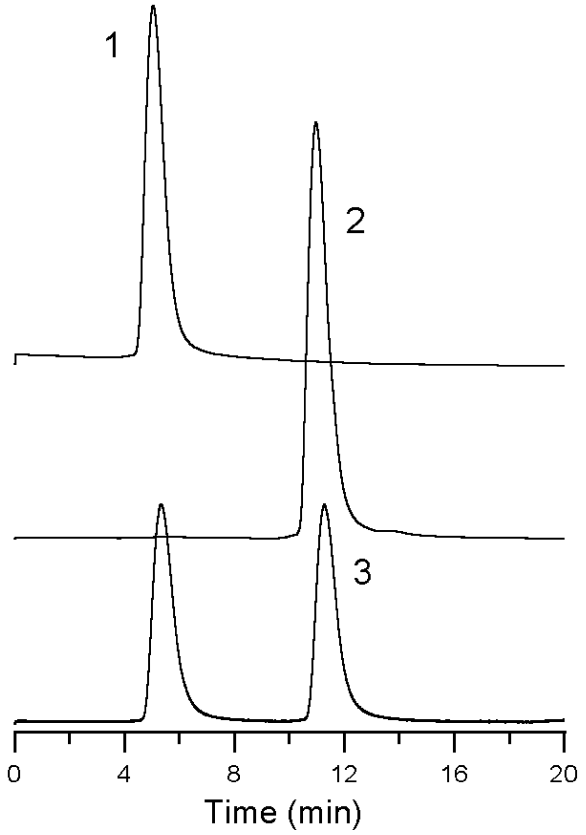

Fig. 3. Chromatography of cholesterol and β-estradiol on cholesterol-imprinted polymer prepared by the methods of covalent imprinting. Samples are 1.0 g/l β-estradiol (1), 1.0 g/l cholesterol (2), and a mixture of 0.5 g/l cholesterol and 0.5 g/l β-estradiol (3). The chromatographic signals are plotted at the same scale.

between cholesterol and β-estradiol using these three types of MIPs.

For different combinations of β-estradiol and cholesterol concentrations with a total of 1 g/l, the peak retention times for both compounds were nearly constant, although the peak height and peak area for each compound increased with concentration in the sample. With the change in sample concentration, the plate number also remained unchanged for all these columns packed with different cholesterol-imprinted polymers. As shown in Fig. 4, β-estradiol and cholesterol had the longest retention times in the column packed with 4-vinylpyridine-based non-covalently imprinted polymer and shortest retention times in the column with methacrylic acid-based imprinted polymer. These two compounds had a medium retention but much sharper peaks when they were chromatographized on the covalently imprinted polymer.

Cholesterol is a 27-carbon alcohol and the major sterol in the human and animal body. Cholesterol has a hydroxyl group at C3 position and an eight-carbon branched aliphatic group attached to C17. β-Estradiol has also a hydroxyl group connected to C3 but differs from cholesterol by substituting the branched aliphatic group at C17 position with a hydroxyl group (17-β-hydroxyl) and lacking a methyl group at C19 position. Therefore, β-estradiol could stay in the MIPs column for a longer time than toluene (as a non-retained compound) due to the hydrogen bonding between the hydroxyl group on its

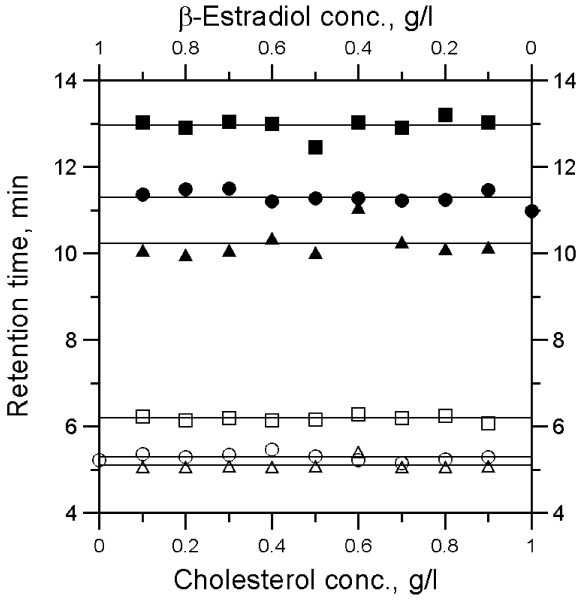

Fig. 4. Retention times of cholesterol (solid symbols) and β-estradiol (open symbols) vary with the concentration of each compound in the samples, which were applied to the chromatographic columns packed with different cholesterol-imprinted polymers. MIPs were prepared by the methods of covalent (spherical symbols) and non-covalent imprinting using methacrylic acid (triangles) or 4-vinylpyridine (squares) as the functional monomer.

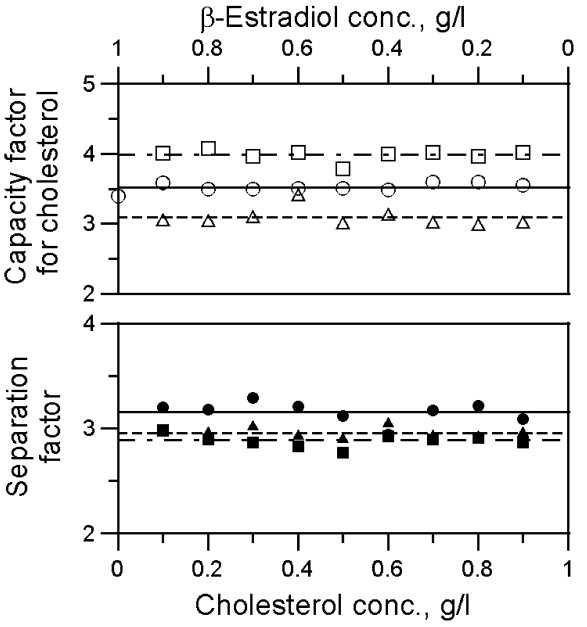

Fig. 5. Separation factor for cholesterol and β-estradiol (solid symbols) and capacity factor for cholesterol (open symbols) vary with the concentration of each compound in the samples, which were applied to the chromatographic columns packed with different cholesterol-imprinted polymers. MIPs were prepared by the methods of covalent (spherical symbols) and non-covalent imprinting using methacrylic acid (triangles) or 4-vinylpyridine (squares) as the functional monomer.

C3 position and immobilized acid or pyridine group. As shown in Table 1, the interactions of β-estradiol to carboxylic groups of immobilized methacrylic acid and phenolic groups of the covalently imprinted polymer contributed capacity factors of 1.0 and 1.1, respectively. While the interaction between β-estradiol and 4-vinylpyridine contributed a capacity factor of 1.4. Since the volume phase ratios for these MIPs columns are unknown, that the latter interaction is stronger than the other two types of weak interactions is possible but could not be asserted. In comparison with cholesterol, the β-estradiol molecule is smaller and more hydrophilic. Due to this hydrophilic property, the stay of β-estradiol in those columns could be changed by the composition of mobile phase consisting of acetonitrile and water. As the water content in the mobile phase increased, the retention of cholesterol did not change too much, but the retention of β-estradiol increased significantly (data not shown).

As shown in Table 1 and Fig. 5, capacity factors for cholesterol from the columns packed with covalently imprinted polymer and 4-vinylpyridine-based non-covalently imprinted polymer, were 3.5 and 4.0, respectively, which were higher than that obtained from the column with methacrylic acid-based non-covalently imprinted polymer (3.1). The average separation factors for resolution of β-estradiol and cholesterol, however, were 3.2, 3.0 and 2.9, respectively, resulting from the columns with MIPs made by covalent and non-covalent imprinting using methacrylic acid and 4-vinylpyridine. These separation factors are all higher and not too much different from each other, suggesting that selection mechanisms involving the binding of β-estradiol and cholesterol to the recognition sites are very similar for these three MIPs. The successful separation is believed to be dependent on the biospecific adsorption of cholesterol to the recognition sites left by the removed templates due to the shape complemen-

Table 1
Chromatographic separation of cholesterol and β-estradiol using MIPs prepared by covalent and non-covalent imprinting of cholesterol[a]

MIP	Retention time, min (capacity factor[b])		Plate number N	α $(=k'_C/k'_{\beta E})$
	β-estradiol ($k'_{\beta E}$)	Cholesterol (k'_C)		
Covalent imprinting	5.3 (1.1)	11.3 (3.5)	1240	3.2
MAA-based non-covalent	5.1 (1.0)	10.2 (3.1)	220	3.0
4-Vinylpyridine-based non-covalent	6.2 (1.4)	13.0 (4.0)	260	2.9

[a] The reported were average values calculated from the data present in Figs. 4 and 5. Concentrations of cholesterol and β-estradiol were totally 1 g/l in each sample.

[b] Calculated using the average retention times for the non-retained compound (toluene) which was determined to be 2.5, 2.5 and 2.6 min, respectively, from the columns packed with these three cholesterol-imprinted polymers.

tary. The reproducibility of column packing was also good. The alternative column packed with MIPs prepared by non-covalent imprinting using either methacrylic acid or 4-vinylpyridine resulted in almost the same peak retention and column efficiency as those in Table 1.

3.2. Comparison of imprinting methods

The covalent and non-covalent imprinting methods are different from each other in the formation of specific recognition sites in highly cross-linked porous polymers. The covalent imprinting involves the use of covalently template-bound monomer, in this study cholesteryl (4-vinyl)phenyl carbonate, which becomes fixed in their spatial arrangement by co-polymerization with crosslinking monomer. In the non-covalent imprinting, molecules of functional monomer assemble around the template (cholesterol) by non-covalent interactions to form the recognition site after co-polymerization with crosslinker. Removal of the template from the crosslinked polymers yields cavities that are shaped complimentary to cholesterol. This could be done by cleavage of covalent bonds and release of carbon dioxides from the polymer prepared by the covalent imprinting. The release of template from the polymers prepared by non-covalent imprinting was simply done by extraction with the solvent. However, the re-adsorption of cholesterol to the imprinted polymers prepared by either covalent or non-covalent imprinting method is always in a non-covalent manner.

For the preparation of cholesterol-imprinting polymer by covalent method, cholesteryl (4-vinyl)phenyl carbonate should be prepared prior to polymerization. In the present study, this template-containing monomer was prepared by reacting 4-vinylphenol to cholesteryl chloroformate, where 4-vinylphenol was synthesized from *p*-acetoxystyrene and carbon dioxide under alkaline conditions. A 99.5% yield was obtained with an m.p. of 68.2 °C for the resultant 4-vinylphenol. This m.p. for 4-vinylphenol is within the range of reported values of 68–69 °C [24]. The yield for cholesteryl (4-vinyl)phenyl carbonate synthesis was 47% and the product was a light-khaki-colored solid with an m.p. of 146.3 °C, which is also within the range of reported values (146–147 °C) [15].

Although polymers were all prepared in bulk polymerization with free radicals initiated by an azo-initiator, the polymerization proceeded at different temperatures. For covalent imprinting the template-bound monomer and crosslinker (EGDMA) co-polymerized at 65 °C. But for non-covalent imprinting, the functional monomer (methacrylic acid or 4-vinylpyridine) and EGDMA were allowed for polymerization at 4 °C. This low temperature was necessary to maintain the template in the positions by non-covalent interactions during the polymerization. The low temperature polymerization method was previously used to imprint a large range of compounds including chiral molecules [26–28].

A significant advantage of using covalent imprinting was found to be that it resulted in an imprinting polymer with higher chromatographic efficiency as shown in Fig. 2. The average plate number for the elution peak of cholesterol from the column packed with covalently imprinted polymer was determined to be 1240 (Table 1). In comparison with results from columns packed with non-covalently imprinted polymers using MAA and 4-vinylpyridine, 220 and 260, respectively, the plate number is about fivefold

larger. A higher plate number represents a better column efficiency. These results suggest that the formation mechanism and the resultant distribution of recognition sites on the imprinted polymers are different between imprinting methods. In the covalent imprinting, the template molecule is covalently bound to the monomer and after polymerization the template occupies exactly the position of the recognition site. Each recognition site formed after the release of template was shown to bind one molecule of cholesterol through hydrogen bonding. As reported in a previous study, the covalently imprinted polymer was shown capable of interacting with cholesterol with a single dissociation [15]. On the contrary, the complexes in the non-covalent imprinting were formed from template and functional monomer in a somewhat loose manner. Cholesterol has only one hydroxyl group that could interact with the functional monomer to form a complex. Since the complexes were formed simply by just mixing these two together and stabilized by weak interactions like hydrogen bonding during the polymerization, the construct of future recognition sites was looser in comparison with covalent imprinting and even contained aggregates of two or more of the template molecules. This resulted in a heterogeneous distribution of recognition sites with respect to the affinity for cholesterol in the imprinted polymer, and finally a peak broadening and tailing when the polymer was used in liquid chromatography. The disadvantage of peak broadening and tailing is usually associated with imprinted polymers in use as the chromatographic supports [29]. Factors claimed to contribute to peak broadening are mass transfer limitations, heterogeneous distribution of sites from high to low affinity for the print molecule, and variable association and dissociation kinetics [30,31]. This study suggests that the use of covalent imprinting could yield an imprinted polymer having less heterogeneous distribution in recognition sites that led to a higher chromatographic efficiency. The peak broadening could significantly be reduced, as shown in Fig. 2 (peak 1) and Fig. 3. The slight peak broadening and tailing remaining could be due to the fact that MIPs are prepared as bulk polymers, and then ground and sized into irregular particles of 25 to 44 μm before packing into columns. The higher plate number and conservation in retention as shown in Fig. 3 suggest that the covalently imprinted polymer has potential for quantitative analysis of the print compound.

3.3. Comparison of functional monomers

Although the formation of complexes from template and monomer was different for covalent and non-covalent imprinting, resultant imprinted polymers all had non-covalent recognition sites. However, different immobilized groups on the recognition sites were involved in the adsorption of cholesterol to those polymers. The immobilized group along the recognition sites were inherited from the monomer employed for cholesterol imprinting. After removal of template, the covalently imprinted polymer had phenolic groups that could interact with the hydroxyl groups of cholesterol and β-estradiol. The immobilized phenolic groups behaved similar to the carboxylic group in methacrylic acid-based MIP; these two cholesterol-imprinted polymers had the same retention time for toluene (2.5 min at a flow-rate of 0.5 ml/min). Also, the retention times of β-estradiol on these two polymers were very close (5.3 and 5.1 min, respectively). The polymer prepared with 4-vinylpyridine however had longer retention times of toluene, β-estradiol, and cholesterol (Table 1).

For the re-adsorption of cholesterol (print molecule) onto the MIPs, the adsorption capacity was found to significantly depend on the use of functional monomer. The binding capacity sometimes represents the density of effective recognition sites on the polymer. Batch adsorption of cholesterol as shown in Fig. 6 revealed that the binding capacity of the methacrylic acid-based polymer was the smallest one among these three MIPs. The equilibrium adsorption data for these cholesterol-imprinted polymers could fit to the Langmuir isotherms with a coefficient of determination (R^2) in the range of 0.870–0.887. The best fittings (dashed curve in the linear plot of Fig. 6) yield the maximum binding capacities of 95, 73, and 65 μmol/g polymer particle, respectively, for cholesterol-imprinted polymers prepared by the methods of covalent and non-covalent imprinting using 4-vinylpyridine and methacrylic acid as the functional monomers. However, as shown in Fig. 6, the data

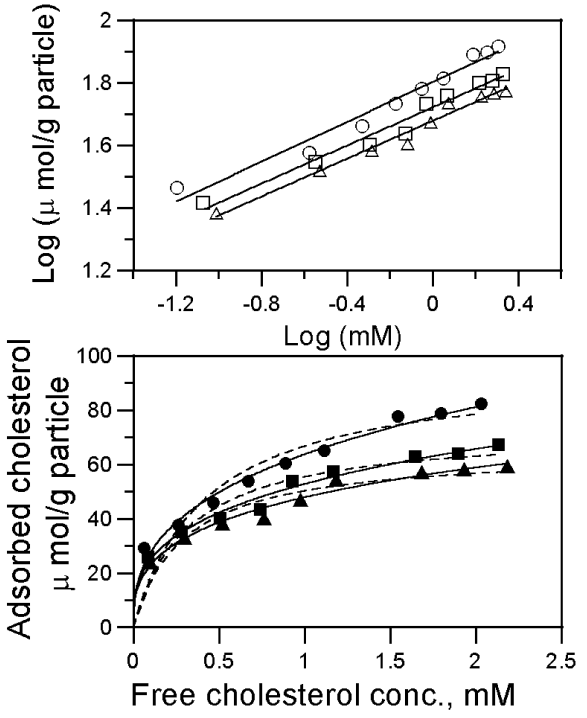

Fig. 6. Adsorption isotherm for cholesterol-imprinted polymers prepared by covalent imprinting (spherical symbols) and non-covalent imprinting using methacrylic acid (triangles) or 4-vinylpyridine (squares) as the functional monomer. Solid curves in the linear plot (lower figure) represent the best fit to the Langmuir–Freundlich isotherm, while dashed curves are best fits to the Langmuir isotherm. Solid lines in the log plot (upper figure) represent fitted Freundlich isotherms.

fitted well to the Langmuir–Freundlich ($R^2 = 0.971$–0.978, solid curves in linear plot) and Freundlich isotherm ($R^2 = 0.967$–0.978, solid lines in log plot). The good fit of MIPs adsorption to the Langmuir–Freundlich isotherm suggests that a unimodal heterogeneous distribution of binding sites was present in the MIPs [32]. The covalently imprinted polymer was found to have the lowest degree of binding site heterogeneity with the highest heterogeneity index. Since the heterogeneity has been claimed as a main contributor to peak broadening and asymmetry, the MIPs obtained from covalent imprinting led to less peak broadening as shown in Fig. 2.

The result obtained in this study is close to that reported by Whitcombe et al. [15]. The covalent cholesterol-imprinted polymer they made has a maximum capacity of 114 ± 6 μmol/g, for cholesterol binding. In another experiment on the adsorption of 100 mg particles in 10 ml of cholesterol solution (1 g/l), the covalently imprinted polymer prepared in the present study could adsorb 58 ± 9 μmol/g, while the non-covalently imprinted polymers with 4-vinylpyridine and methacrylic acid as the monomers could adsorb 52 ± 11 and 20 ± 2 μmol/g of cholesterol, respectively. These results suggest that the adsorption capacity of the MIPs was dependent on the functional monomer used to form a complex with template, and the immobilized group in the polymer along the recognition sites significantly influenced the adsorption of cholesterol. Under the same adsorption conditions, the cholesterol-printed poly(2-hydroxyethylmethacrylate) adsorbed 3.92 mg/g (= 10.1 μmol/g) cholesterol [19]. In other similar adsorption experiments, Sreenivasan reported that the MIPs made by using 2-hydroxyethylmethacrylate and *N*-vinyl pyrrolidone as the monomer adsorbed 4.86 mg/g (= 12.6 μmol/g) and 3.06 mg/g (= 7.9 μmol/g) cholesterol, respectively [20].

In this study, two different functional monomers were used for forming a template–monomer complex prior to polymerization in the non-covalent imprinting method. Although methacrylic acid is the commonly used monomer for non-covalent imprinting, the use of other electrophilic group-containing monomers like 4-vinylpyridine might be also useful. The low-temperature synthesized polymer using 4-vinylpyridine was a slightly yellow bulk, but it turned to white powders after drying and grinding. After the removal of the template, the bound pyridine on the 4-vinylpyridine-based MIP seemed to have a stronger interaction to hydroxyl groups of sterols, reflecting the increase of capacity factors for β-estradiol and cholesterol. Also the bound pyridine groups were able to interact with hydrophobic compounds. Toluene thus could stay a longer time in the column under the same mobile phase conditions. However, the separation factors were higher and almost the same for these two non-covalently imprinted columns. This suggests that the selectivity in the chromatography was mainly based on the affinity of shape match to the cavity created by template printing.

4. Conclusion

In this study, we have demonstrated chromatographic behaviors using molecularly imprinted polymers as stationary phases, prepared by different approaches. Three types of cholesterol-imprinted polymers were prepared and, after removal of template, used as the stationary phase for the chromatographic separation of cholesterol from other steroids. One of the molecularly imprinted polymers (MIPs) was obtained by covalent imprinting involving the use of cholesteryl (4-vinyl)phenyl carbonate as the covalently template-bound monomer, while the other two MIPs were obtained by non-covalent imprinting method. Although the non-covalent imprinting was much easier to practice, the formation of complex by simply mixing template and monomer in solution prior to polymerization could lead to a higher heterogeneity of recognition sites on the resultant imprinted polymers and result in peak broadening for the cholesterol chromatography. On the contrary, the covalently imprinted polymer resulted in a much higher chromatographic efficiency, which shows potential for quantitative analysis. The adsorption capacity of the MIPs was found to depend on the functional monomer used to form a complex with the template, and the immobilized group in the polymer along the recognition site significantly influenced the adsorption of cholesterol. The MIP prepared by covalent imprinting was found to bind more cholesterol. However, among these imprinted polymers the difference in the selectivity for chromatographic separation of cholesterol and β-estradiol was insignificant, suggesting that the separation was based on the specific binding of cholesterol to recognition sites. The study also reveals some important properties of MIP when applied for chromatographic separation.

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