Molecularly Imprinted Polymer Microspheres Prepared by Precipitation Polymerization Using a Sacrificial Covalent Bond

Somchai Boonpangrak,[1,2] Virapong Prachayasittikul,[2] Leif Bülow,[1] Lei Ye[1]

[1]*Pure and Applied Biochemistry, Chemical Center, Lund University, 221 00 Lund, Sweden*
[2]*Department of Clinical Microbiology, Faculty of Medical Technology, Mahidol University, Bangkok, Thailand*

Received 23 March 2005; accepted 24 June 2005
DOI 10.1002/app.22519
Published online in Wiley InterScience (www.interscience.wiley.com).

ABSTRACT: Molecularly imprinted polymer microspheres were prepared by precipitation polymerization using a sacrificial covalent bond. In the present model, cholesteryl (4-vinyl)phenyl carbonate was used as a template monomer. The imprinted microspheres were prepared using ethylene glycol dimethacrylate (EDMA) and divinylbenzene (DVB) as crosslinker. The base-labile carbonate ester bond was easily hydrolyzed to leave imprinted cavities in the resulting polymers. Radioligand binding analysis, elemental analysis, and scanning electron microscopy were used to characterize the imprinted materials. Imprinted microspheres prepared from DVB crosslinker had larger and more defined spherical shape, and displayed better imprinting effect than did the EDMA-based microparticles. For comparison, imprinted bulk polymers were also prepared in the same reaction solvent as that used in precipitation polymerization. Elemental analysis results indicated that imprinted microspheres contained more template monomer units than bulk materials. The efficiency of template removal by hydrolysis treatment for microspheres was also higher than that for bulk polymers. For DVB-based polymers, imprinted microspheres displayed higher specific cholesterol uptake than did the corresponding bulk polymer. © 2005 Wiley Periodicals, Inc. J Appl Polym Sci 99: 1390–1398, 2006

Key words: molecular imprinting; precipitation polymerization; microspheres; cholesterol

INTRODUCTION

Molecular imprinting has attracted a broad research interest in recent years. The simplicity of creating tailored recognition sites in synthetic materials, as compared with that of complicated multi-step organic synthesis, is very attractive from an application's point of view, although certain limitations with molecularly imprinted polymers (MIPs) still need to be addressed, such as slow binding kinetics, aqueous compatibility, and heterogeneity of binding site distribution. In general, two different approaches have been followed to prepare MIPs: noncovalent and covalent imprinting, depending on the molecular interactions utilized between the template and functional monomer during the free radical polymerization.[1–3] Because of the easy access to a broad range of functional monomers from commercial sources, the noncovalent imprinting method has been used by most research groups, which resulted in a large number of noncovalent MIPs displaying favorable molecular recognition properties.

Previously, we have demonstrated that noncovalent MIPs in microbead format can be easily prepared using precipitation polymerization.[4,5] In this study, we intended to determine if the same synthetic methodology could be extended to the preparation of covalent MIP microspheres. In addition, the small particle size of microspheric MIPs should allow easy template removal using an appropriate chemical cleavage. Instead of using covalent interaction for MIPs to bind the target analyte, we select to follow the "semicovalent" or "sacrificial bond" strategy introduced by Whitcombe et al.,[6] in which analyte binding was accomplished by noncovalent interaction inside the covalently imprinted cavities. We were also interested in investigating whether the covalently imprinted polymers have a homogeneous binding site distribution as previously expected, or if binding site heterogeneity is rather an intrinsic character for MIPs prepared by free radical addition polymerization. For these purposes, we had to study analyte binding covering a broad concentration range, to obtain a global binding isotherm. This was achieved using homologous radioligand binding experiments, where radioisotope-labeled and unlabeled analyte have the same chemical identity, and thus, interact with imprinted sites with the same mechanism.

Correspondence to: L. Ye (lei.ye@tbiokem.lth.se).
Contract grant sponsor: Swedish Strategic Research Foundation (SSF).

TABLE I
Preparation and Characterization of Molecularly Imprinted Polymers

Polymer	Template monomer		Crosslinker		Solvent[a] (mL)	O content[b] (wt %)	Polymerized template (mol %)[c]	Template removal (mol %)[c]	Cholesterol uptake[d] (%)
	Chol (mmol)	Ace (mmol)	DVB (mmol)	EDMA (mmol)					
Chol-M1	0.29	0	0	5.51	60	n.d.	n.d.	n.d.	52
Chol-M1H	0.29	0	0	5.51	60	n.d.	n.d.	n.d.	67
M1H	0	0	0	5.80	60	n.d.	0	0	58
Chol-M2	0.29	0	5.51	0	60	3.3	12	0	13
Chol-M2H	0.29	0	5.51	0	60	2.5	12	50	38
M2H	0	0	5.80	0	60	0	0	0	12
Ace-M2	0	0.29	5.51	0	60	1.2	5	0	n.d.
Ace-M2H	0	0.29	5.51	0	60	0.9	5	43	12
Chol-B2	0.29	0	5.51	0	0.88	2.3	8	0	14
Chol-B2H	0.29	0	5.51	0	0.88	1.9	8	31	35
B2H	0	0	5.80	0	0.88	0	0	0	16

Chol, cholesteryl(4-vinylphenyl) carbonate; Ace, 4-acetoxystyrene; n.d., not determined.
[a] Acetonitrile: toluene (2:1, v/v).
[b] Results from elemental analysis.
[c] Calculated from O content.
[d] Cholesterol binding to 25 mg polymer in 1 mL of hexane. Initial cholesterol concentration was 1.4 n*M*.

EXPERIMENTAL

Chemicals and methods

Methacrylic acid (MAA), ethylene glycol dimethacrylate (EDMA), and azobis-isobutyronitrile (AIBN) were purchased from Merck (Darmstadt, Germany) and used without purification. Divinylbenzene (DVB; technical, mixture of isomers, 80%) from Aldrich was passed through an aluminum oxide column to remove the stabilizer 4-*tert*-butylcatechol before use. 4-Acetoxystyrene was obtained from Aldrich and used as received. (*S*)-Propranolol hydrochloride was purchased from Fluka and converted into free base form before use. [1α,2α-^{3}H(N)]Cholesterol (specific activity 41.3 Ci mmol^{-1}) was supplied by Sigma. [2,4,6,7-^{3}H(N)]Estradiol (specific activity 72.0 Ci mmol^{-1}) and (*S*)-[4-^{3}H]-propranolol (specific activity 15.0 Ci mmol^{-1}) were purchased from NEN (Boston, MA). Cholesteryl (4-vinyl)phenyl carbonate was synthesized according to a literature protocol.[6] Solvents and other reagents were of analytical grade unless otherwise stated. Elemental analysis for oxygen content was carried out at MikroKemi AB, Uppsala, Sweden. Scanning electron microscopy (SEM) images were obtained with a JEOL JSM-840A microscope at the Department of Materials Chemistry, Chemical Center, Lund University.

Polymer synthesis

Molecularly imprinted polymers, unhydrolyzed (Chol-M1, Chol-M2, Ace-M2, and Chol-B2)

Imprinted polymer microspheres (Chol-M1, Chol-M2, and Ace-M2) were synthesized using the precipitation polymerization method described previously.[4,5] Imprinted bulk polymer (Chol-B2) was synthesized from a concentrated monomer solution. Reagent feedings are detailed in Table I. Briefly, the functional monomer, crosslinker, and AIBN (17.5 mg, 0.106 mmol) were dissolved in a mixture of acetonitrile and toluene. The solution was gently flushed with argon for 5 min and sealed under argon. Polymerization was started at 60°C and continued for 24 h. After polymerization, the imprinted microspheres were collected by centrifugation. The imprinted bulk monolith was broken and fragmented with a mechanical mortar. The polymer particles were washed with methanol (2 × 20 mL), hexane (2 × 20 mL), and dried in vacuum.

Molecularly imprinted polymers, hydrolyzed (Chol-M1H, Chol-M2H, Ace-M2H, and Chol-B2H)

Each of the imprinted polymers (Chol-M1, Chol-M2, Ace-M2, and Chol-B2) was suspended in 20 mL of 1*M* NaOH solution in methanol and refluxed for 6 h. Upon returning to ambient temperature, the suspension was neutralized to pH 7 by adding 1*M* HCl. Polymer particles were collected by centrifugation, washed with methanol (2 × 20 mL), hexane (2 × 20 mL), and dried in vacuum.

Nonimprinted polymers, hydrolyzed (M1H, M2H, and B2H)

Nonimprinted polymers (M1H, M2H, and B2H) were synthesized and hydrolyzed under the same conditions as that used to prepare polymers Chol-M1H, Chol-M2H, and Chol-B2H, respectively, except that

the functional monomer was omitted during the polymerization.

Radioligand binding analysis

Polymer particles were incubated in 1 mL of radioisotope-labeled analyte solution (1.4 n*M*) at 20°C for 16 h. In competitive binding experiments, different unlabeled analyte was added in the same solution. A rocking table was used to provide gentle mixing. After the incubation, samples were centrifuged to separate the labeled analyte bound on the solid particles. Supernatant (200 μL) was taken and mixed with scintillation liquid Ecosint A (10 mL), and counted for 1 min using a Rackbeta 2119 liquid scintillation counter (LKB Wallac, Sollentuna, SE). The liquid counting results were used to calculate the percentage of radioligand that bound to polymer particles.

RESULTS AND DISCUSSION

Experimental design: Investigation of covalently imprinted cavities with different molecular probes

Because of the high crosslinking density used for polymer preparation, complete removal of template from covalently imprinted polymers by hydrolytic cleavage is often difficult to achieve. The situation becomes even more complicated when a template is covalently linked to polymer matrix via multiple chemical bonds. In this study, we selected to use the single carbonate sacrificial linkage first introduced by Whitcombe et al.[6] for preparation of imprinted polymer microspheres [Fig. 1(a)]. After template removal, the free cavities were expected to bind cholesterol via noncovalent (hydrogen bond) interaction in nonpolar organic solvent. Since highly specific molecular recognition (e.g., for chiral resolution of racemate mixtures) often requires multiple interaction points, we expected that the cholesterol-imprinted sites would show certain cross-recognition toward molecules that have similar size and functional group distribution.

To test the cross-recognition of imprinted sites, we decided to use radioisotope-labeled cholesterol, (*S*)-propranolol and 17β-estradiol [Fig. 1(a)], to probe the imprinted binding sites. Imprinted but un-hydrolyzed polymers, as well as nonimprinted but hydrolyzed polymers were used as two reference materials to estimate nonspecific adsorption. In addition, a polymer containing a smaller binding site was prepared as another control [Fig. 1(b)]. This polymer, because of its limited cavity size, would not allow cholesterol to enter the specific sites, and the uptake of cholesterol can only be explained by nonspecific adsorption.

Effect of crosslinker on physical morphology of polymer particles

It is now generally accepted that the binding performance of MIPs can be largely influenced by the reaction solvent used during polymer preparation. We expected that MIP microspheres prepared using the "semicovalent" approach under precipitation polymerization condition may have different binding performance, as compared with the bulk MIPs and MIP beads obtained previously.[6–8] In the present work, two different crosslinkers, EDMA and DVB, were used to prepare molecularly imprinted microspheres in a large volume of a mixture of acetonitrile and toluene. The choice of acetonitrile as reaction solvent was on the basis of the previous findings that it resulted in regularly shaped microspheres when the two crosslinkers were employed. The use of toluene was due to the fact that the template, cholesteryl (4-vinylphenyl) carbonate had poor solubility in pure acetonitrile. Polymer particles obtained from the

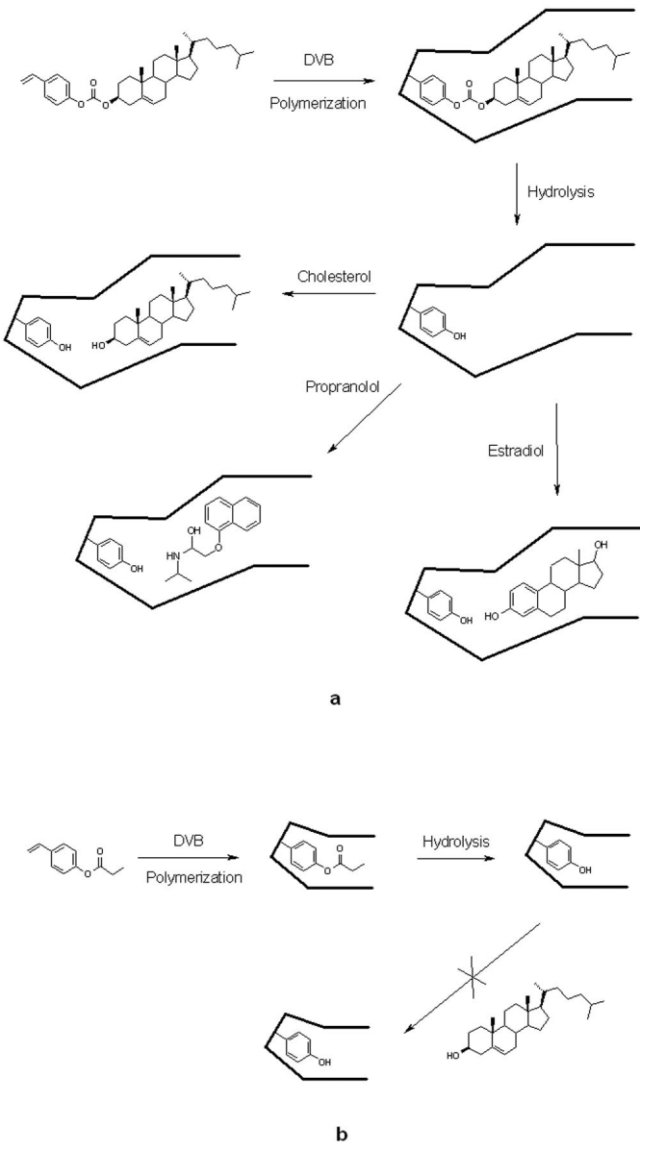

Figure 1 Schematic representation of the sacrificial approach used in the present study. (a) Cholesterol-imprinted cavities bind the template and related molecules. (b) Smaller cavities can not take up large cholesterol molecule.

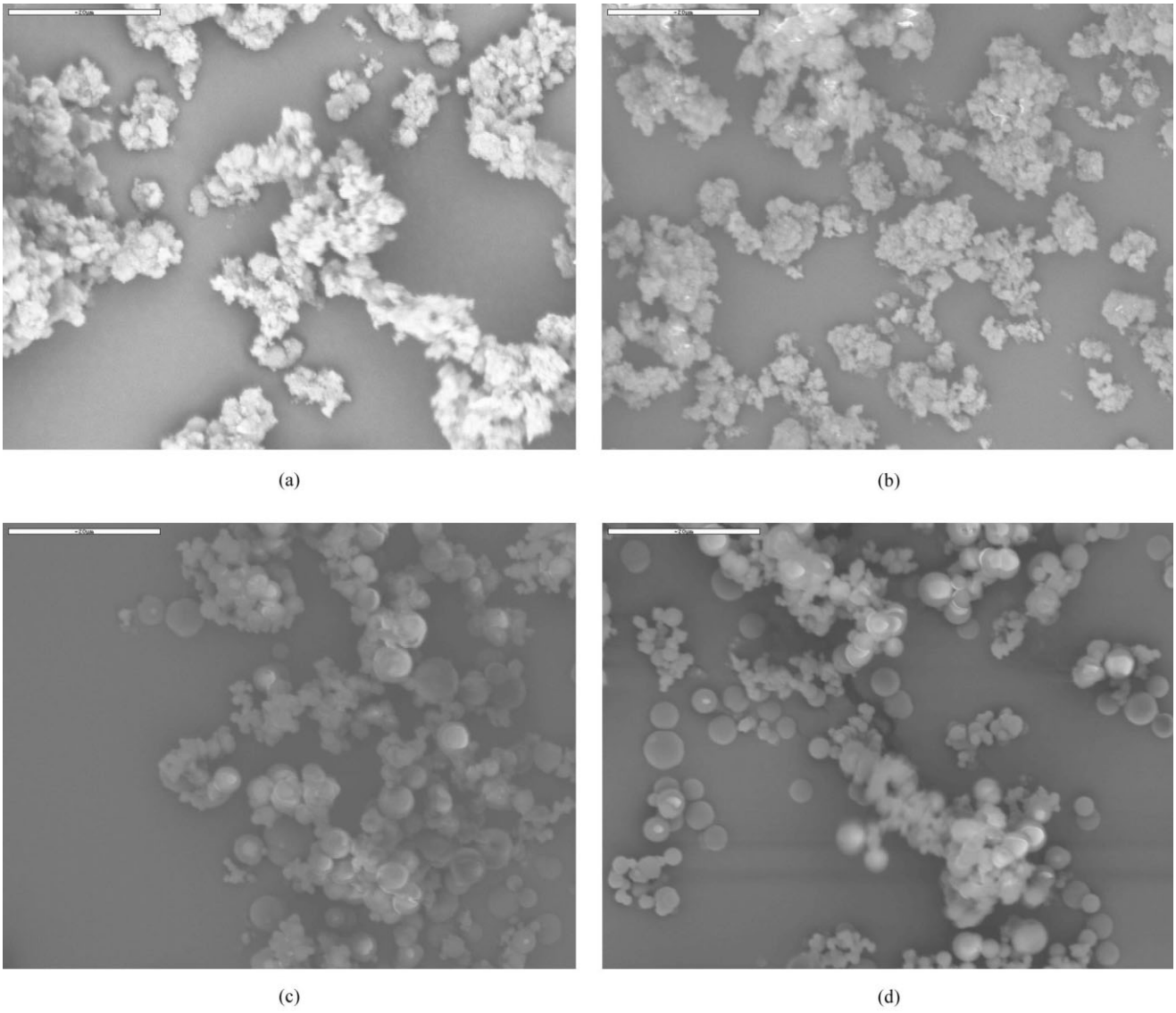

Figure 2 SEM image of Chol-M1 (a), Chol-M1H (b), Chol-M2 (c), and Chol-M2H (d). The scale bar represents 20 μm.

precipitation polymerization had quite different morphologies when the two different crosslinkers were used. The EDMA-based MIP formed particle agglomerates that were composed of smaller nuclei (of diameter smaller than 0.4 μm) [Fig. 2(a)]. The DVB-based MIP existed as more defined microspheres, although with a rather large size distribution between 0.3 and 2.5 μm [Fig. 2(c)]. The less ideal particle morphology may be due to the use of the present solvent mixture, which deviated from the optimal composition used in previous precipitation polymerization reactions.[4,9] It should be mentioned that particle size distribution of imprinted microspheres can be affected by many factors including template loading, the type of crosslinker used, and the composition of the imprinting solvent. For DVB-based polymers, appropriate agitation may also be required to assist narrowing particle size distribution.

The two imprinted polymers were subjected to the same hydrolysis treatment to remove the cholesterol template. It has been observed earlier that the treatment with NaOH in methanol, although under optimized condition, still caused the backbone of EDMA-based polymers to be partially hydrolyzed.[7] The additional carboxyl groups generated by backbone hydrolysis may increase nonspecific cholesterol binding. For DVB-based polymers, treatment with NaOH in methanol can not change the crosslinked structure. Despite the different behaviors of the EDMA and DVB polymers, scanning electron microscopy images [Figs. 2(b) and 2(d)] indicated that the physical appearance of the polymers was not affected by the hydrolysis treatment.

Incorporation of templated sites and efficiency of hydrolytic cleavage for DVB-based microspheres

For the DVB-based microspheres, elemental analysis for oxygen content could be used to calculate the number of template units introduced into polymer

matrix, as well as the hydrolysis-generated empty sites (Table I). As an example, the calculation for polymer Chol-M2 and Chol-M2H is described in detail. For polymer Chol-M2, supposing the molar fraction of template monomer and crosslinker are x and y, respectively, the following equations are established:

$$x + y = 1 \quad (1)$$

$$532 \times 9.01\% \times x/(532 \times x + 130 \times y) = 3.3/100 \quad (2)$$

Where the following constants are used:

Molecular weight of cholesteryl (4-vinyl)phenyl carbonate: 532; oxygen content of cholesteryl (4-vinylphenyl) carbonate: 9.01%; molecular weight of DVB: 130; oxygen content of polymer Chol-M1: 3.3%.

Solution of eqs. (1) and (2) gives:

$$x = 0.12$$

$$y = 0.88$$

Therefore, polymer Chol-M2 contains 12% (mol/mol) of template monomer unit. Now, supposing the molar fraction of phenol and carbonate units in polymer Chol-M2H are x' and y', respectively, the following equations are established:

$$x' + y' = 0.12 \quad (3)$$

$$(120 \times 13.32\% \times x' + 532 \times 9.01\% \times y')/(120 \times x' + 532 \times y' + 0.88 \times 130) = 2.5/100 \quad (4)$$

Where the following constants are used:

Molecular weight of cholesteryl (4-vinyl)phenyl carbonate: 532; oxygen content of cholesteryl (4-vinylphenyl) carbonate: 9.01%; molecular weight of 4-vinylphenol: 120; oxygen content of 4-vinylphenol: 13.32%; molecular weight of DVB: 130; molar fraction of DVB: 0.88; combined molar fraction of 4-vinylphenol and cholesteryl (4-vinylphenyl) carbonate: 0.12; oxygen content of polymer Chol-M1H: 2.5%.

Solution of eqs. (3) and (4) gives:

$$x' = 0.06$$

$$y' = 0.06$$

Therefore, the efficiency of template removal, i.e., loss of cholesteryl carbonate, for polymer Chol-M2H is 50%. Based on the above value, the maximum number of binding sites in polymer Chol-M2H can be calculated as:

$$0.06/(120 \times 0.06 + 532 \times 0.06 + 0.88 \times 130) = 3.9 \times 10^{-6}\,\mathrm{mol\,g^{-1}}$$

Using similar calculation, we obtained the amount of polymerized template and the level of template removal obtained by hydrolysis for other polymers, as listed in Table I.

While microspheres Ace-M2 contained 5% (molar fraction) of acetoxy unit, which was identical to the feeding composition in the prepolymerization solution, the molar fraction of cholesterol template in Chol-M2 and Chol-B2 were found to be 12 and 8%, respectively. The seemingly high reactivity of cholesteryl (4-vinylphenyl) carbonate may be explained by possible cholesterol–cholesterol interaction in acetonitrile: toluene mixture.[10,11] Formation of self-associated template clusters during imprinting reaction has been discussed in several papers from other research groups.[12–14] In fact, use of plausible cholesterol–cholesterol interaction for preparation of noncovalent MIPs in polar solvent has been reported.[15,16] In a dilute monomer solution used to synthesize Chol-M2, the cholesterol–cholesterol interaction may become more important in raising a local concentration of template monomer in vicinity of reactive radicals. As a result, the number of cholesterol units incorporated into a growing polymer chain can be increased even more.

The hydrolysis condition has been optimized by Whitcombe et al. for removing cholesterol template from EDMA-based bulk polymers.[6] For the EDMA-based microparticles (Chol-M1 and Chol-M1H), we were not able to calculate the level of template incorporation or template removal by elemental analysis. However, because of the much reduced particle size, it is reasonable to assume that template removal from polymer Chol-M1H is more efficient than from bulk polymers as well as from the DVB-based polymer Chol-M2H (Table I). In fact, it was more difficult to hydrolyze away cholesterol template from the present DVB-based bulk polymer (31%) than from the EDMA-based bulk polymer prepared by Whitcombe et al.[6]

Radioligand binding analysis

The two imprinted polymer microparticles prepared using EDMA and DVB as crosslinker were tested in hexane to bind cholesterol. Incubation with radioligand was continued for 16 h to ensure that binding equilibrium was reached for all samples. When EDMA was used as crosslinker, cholesterol uptake by Chol-M1H (67%) was only slightly higher than by the two control polymers, i.e., Chol-M1 (52%) and M1H (58%) (Table I, last column). For the DVB-based polymer microspheres, Chol-M2H displayed cholesterol uptake more than two times higher (38%) than either

Chol-M2 (13%) or M2H (12%). It is worth a mention that the above control polymers did not carry any free hydroxyl group, therefore, uptake of cholesterol should be explained as a result of nonspecific interaction with the polymer backbone. The high level of cholesterol uptake on EDMA-based polymers (Chol-M1, Chol-M1H, and M1H) is presumably caused by hydrogen bond interaction between cholesterol and the ester functional groups on the polymer backbone. To verify that the specific cholesterol binding took place in template-generated cavities in polymer Chol-M2H, we synthesized another imprinted and hydrolyzed polymer Ace-M2H. Because of the smaller acetoxy templating moiety, Ace-M2H has reduced cavity size that does not allow cholesterol to form hydrogen bond interaction with the in-cavity hydroxyl groups (size exclusion). In fact, cholesterol uptake on Ace-M2H was almost equivalent to that obtained on the other two control polymers (Chol-M2 and M2H, Table I, last column).

The present DVB-based bulk polymer also displayed interesting binding performance: Chol-B2H bound two times more cholesterol (35%) than either Chol-B2 (14%) or B2H (16%). The improvement for DVB-based polymer is most probably caused by the new reaction solvent used in the present study. The poorer cholesterol recognition by the EDMA-based polymer Chol-M1H, as compared to previous results,[6] may also be explained by the new solvent composition used for polymer synthesis. Although EDMA crosslinker in general gives better imprinting efficacy than DVB, this is true only if the crosslinker does not participate in molecular interaction with the template, either during the imprinting reaction or in rebinding experiment. When crosslinkers are involved in template binding, the situation may become different. For example, we found previously that replacing an acrylate-based crosslinker trimethylolpropane trimethacrylate (TRIM) with DVB could greatly improve template recognition for propranolol-imprinted microspheres. In that case, the improved binding selectivity was attributed to the additional solvophobic effect or π–π interaction between DVB and the template molecules, both during the imprinting reaction and in the binding experiments.[5,17] In the present system, all the polymers were prepared in acetonitrile: toluene mixture, neither EDMA nor DVB could interact with the cholesterol template to enhance imprinting efficiency. On the contrary, when the binding experiments were carried out in hexane, the ester functional groups in EDMA-based polymers actually caused higher nonspecific cholesterol absorption.

Binding isotherm measured by homologous competitive assay

Using tritium-labeled cholesterol, we carried out homologous competitive binding experiments: The labeled template was allowed to compete with increasing amount of unlabeled cholesterol to bind to a limited number of imprinted sites. As the labeled cholesterol has the same chemical structure as that of the unlabeled compound, it is reasonable to assume that they have the same binding characteristics when exposed to the same imprinted polymers.[18,19] Therefore, the fraction of bound labeled cholesterol should be equal to the fraction of bound cholesterol in total (eq. 5). This allowed us to establish binding isotherm for cholesterol in a broad concentration range.

$$[Chol^*]_{bound}/[Chol^*]_{total} = ([Chol^*]_{bound} + [Chol]_{bound})/([Chol^*]_{total} + [Chol]_{total}) \quad (5)$$

Where [Chol*] is the concentration of labeled cholesterol, [Chol] the concentration of un-labeled cholesterol.

When DVB was used as crosslinker, the amount of cholesterol bound to imprinted and hydrolyzed microspheres (Chol-M2H) was much higher than that bound to the two control polymers (imprinted microspheres before hydrolysis (Chol-M2) and nonimprinted microspheres after hydrolysis (M2H)) (Table I). The in-cavity binding was mainly mediated by hydrogen bond interaction, because replacement of hexane with a polar solvent (acetonitrile:toluene = 2:1, v/v) drastically reduced cholesterol uptake to below 5% (data not shown).

Despite the favorable imprinting effect, the binding isotherm observed in Figure 3(a) indicated a heterogeneous site distribution for the present covalent imprinting system. This somewhat surprising result can be more clearly demonstrated by presenting the binding data in a Scatchard plot (Fig. 3(b)), which shows the apparent two types of binding sites with very different affinities for cholesterol. The number of high affinity sites was however very limited. A simple linear curve fit in the high affinity range ([Bound] = 40 p*M*–350 n*M*) was used to get an approximate apparent dissociation constant (K_D) of (5.2 $\pm$ 0.3) $\times$ 10^{-6} *M*, with a corresponding site population (B_{max}) of 85 $\pm$ 5 nmol g^{-1}. Similarly, for the low affinity sites ([Bound] = 350 n*M*–2.5 m*M*), the parameters were calculated to be K_D = (1.9 $\pm$ 0.1) $\times$ 10^{-2} *M* and B_{max} = 258 $\pm$ 13 μmol g^{-1}. While the high affinity sites accounted for less than 0.02% of the hydrolysis-generated cavities (390 μmol g^{-1}, calculated from oxygen content value), the portion of low affinity binding sites were ~66%. Based on the present result, we suggest that the covalent molecular imprinting technique does not necessarily generate homogeneous binding sites. This is true at least for the present DVB-based microspheres that are prepared in acetonitrile: toluene mixture. Several factors during the imprinting reaction, for example, the relative reactivity of functional monomer and

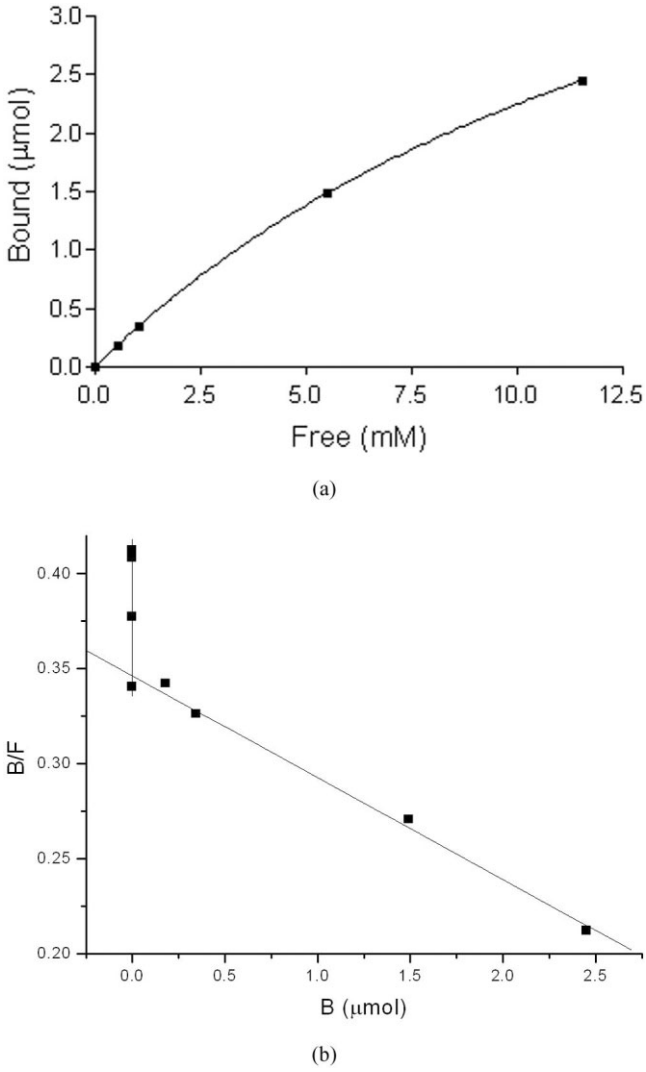

Figure 3 (a) Binding isotherm for cholesterol on 25 mg of polymer Chol-M2H. (b) Scatchard plot for cholesterol binding to polymer Chol-M2H.

crosslinker and the nonideal packing of polymer backbone at gelation point, may hamper the formation of identically defined, three dimensional binding sites in crosslinked polymer matrix. In the present system, an additional impact might come from the possible cholesterol–cholesterol interaction that led to formation of local template clusters during the crosslinking reaction.

Cross-reactivity of cholesterol-imprinted microspheres

Because of the fact that only a single functional group (phenol) was introduced into each binding site, we expected that cholesterol-imprinted polymer microspheres could exhibit certain cross-reactivity toward molecules that have size and hydrogen bond capability similar to cholesterol. The imprinted microspheres were, therefore, challenged with the same concentration of radioisotope labeled 17β-estradiol and (*S*)-propranolol in hexane. Although total binding of these compounds by the imprinted microspheres was much higher than that of cholesterol, the specific part, as reflected by the difference between hydrolyzed and unhydrolyzed polymers, was almost identical (~20%, Fig. 4). More interestingly, the majority of (*S*)-propranolol bound to polymer Chol-M2H was within the imprinted cavities (Fig. 4).

To further confirm that propranolol uptake by cholesterol-imprinted polymer was caused by in-cavity hydrogen bond interaction, we attempted to saturate the limited number of binding sites with increasing amount of (*S*)-propranolol. This was simply achieved using the same homologous competition experiment as used for measuring cholesterol binding. A saturation curve for propranolol binding was obtained (Fig. 5(a)). Using the same binding data, the Scatchard plot (Fig. 5(b)) indicated that only one type of binding site could be probed by propranolol molecule.

The binding curve for propranolol shown in Figure 5(a) could be fitted with a Langmuir isotherm using eq. 6:

$$B = B_{\max}F/(K_D + F) \quad (6)$$

This gives propranolol the following apparent dissociation constant and site population: $K_D = (6.2 \pm 0.4) \times 10^{-4}M$, and $B_{\max} = (66.4 \pm 0.9)\ \mu\text{mol g}^{-1}$. Thus, the number of binding sites for (*S*)-propranolol only accounted for 17% of the hydrolysis-generated cavities in polymer Chol-M2H. These cholesterol-imprinted

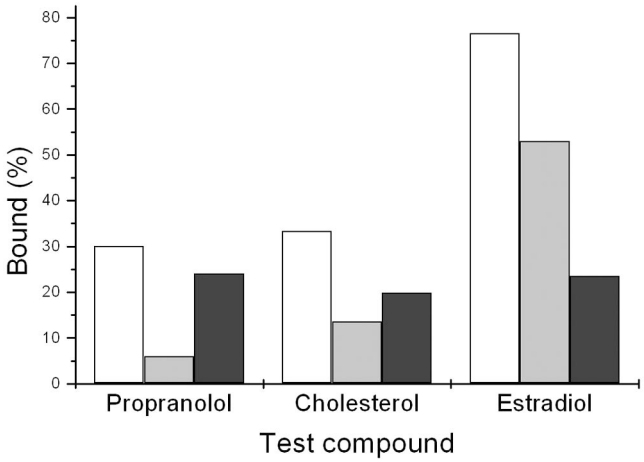

Figure 4 Uptake of different test compounds by polymer Chol-M2H (empty column) and Chol-M2 (gray column). The black column indicates the difference of analyte binding to the two polymers. For propranolol and estradiol, 5 mg polymer was used. For cholesterol, 25 mg polymer was used.

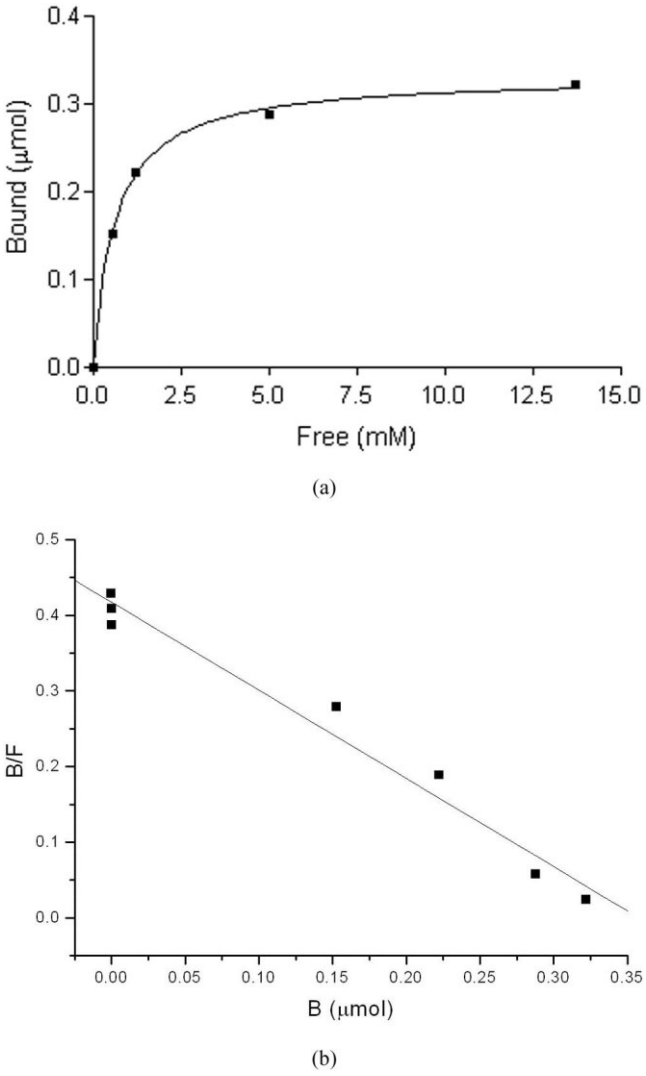

Figure 5 (a) Binding isotherm for propranolol on 5 mg of polymer Chol-M2H. (b) Scatchard plot for propranolol binding to polymer Chol-M2H.

binding sites were, however, homogeneous and displayed higher affinity for (*S*)-propranolol.

If (*S*)-propranolol binding was mediated by hydrogen bond interaction with the phenol groups located within the imprinted cavity, it should be possible to displace the bound propranolol molecules with a large excess of cholesterol. This was tested by incubating 5 mg of Chol-M2H with labeled (*S*)-propranolol (1.4 n*M*) and unlabeled cholesterol (14 m*M*) until equilibrium. The added cholesterol could displace up to 34% of (*S*)-propranolol in hexane, indicating that the competing molecules were binding to the same cavities. When smaller compounds (isopropanol and 3-methylindole) were tested in the competition experiment, they were not able to show the same competing effect as that obtained with cholesterol. Therefore, the cross-recognition of cholesterol-imprinted sites had certain selectivity: part of the imprinted cavities could take up compounds that have molecular size and functionality similar to the original template. The high affinity of Chol-M2H for (*S*)-propranolol may be attributed to a favorable hydrogen bond interaction between the in-cavity phenol and the amine group of (*S*)-propranolol, rather than the weaker phenol–alcohol interaction (Fig. 1(a)).

CONCLUSIONS

Under a high dilution condition, imprinting reaction (free radical polymerization) proceeds more slowly than under the conventional condition (high monomer concentration) used for bulk polymer synthesis. This may affect incorporation of functional monomer units into a growing polymer chain, if any template–template interaction (template clustering) exists under a particular reaction condition. The effect of template clustering may be more important in covalent imprinting systems. In noncovalent imprinting, functional monomer is often used in a large excess compared with template molecules, the possibility of forming template cluster would be relatively low.

The present study demonstrated that precipitation polymerization method can be used to prepare covalently imprinted polymer microspheres. When DVB was used as crosslinker, cholesterol-imprinted microspheres bound the original template by means of hydrogen bond interaction in nonpolar solvent. Because a single sacrificial covalent bond was used, the imprinted microspheres also displayed certain cross-recognition toward (*S*)-propranolol and 17β-estradiol, which have molecular size and hydrogen bond potential similar to cholesterol. The number of (*S*)-propranolol binding sites is, however, only a small fraction of the hydrolysis-generated cavities, indicating that the present covalently imprinted cavities have certain heterogeneity. When evaluated by cholesterol uptake, the binding performance of imprinted microspheres is similar to that of bulk polymers. However, because of the small particle size, template removal by hydrolysis can be more easily achieved with imprinted polymer microspheres.

S.B. is a recipient of the Royal Golden Jubilee Ph.D. scholarship from the Thailand Research Fund.

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