Molecular Imprinting of Biotin Derivatives and Its Application to Competitive Binding Assay Using Nonisotopic Labeled Ligands

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Synthetic biotin-binding polymers were prepared by molecular imprinting. Methacrylic acid (MAA) was copolymerized with ethylene glycol dimethacrylate in the presence of biotin methyl ester (B-Me) in chloroform. Hydrogen-bonding-based complexation of B-Me with MAA generates the binding sites complementary to B-Me after extracting B-Me from the resulting copolymers. Data from NMR titration suggest a one-to-one prepolymerization complex formation of B-Me with MAA in chloroform. A possible complex structure was estimated by docking of the most stable conformers by intermolecular Monte Carlo conformational search under the assumption of a one-to-one association. The selectivity of the imprinted polymers was investigated and an imprinted polymer-based competitive binding assay for B-Me was demonstrated using biotin p-nitrophenyl ester as a nonisotopic-labeled ligand.

Biotin is well known as a strong binder to avidin, and many detection techniques have been developed using avidin—biotin systems in conjunction with enzymes, antibodies, DNA, and other biologically active compounds. Although the avidin—biotin system is recognized as a useful tool for highly sensitive detection, it works only in aqueous solution. Therefore, the development of stable avidin-like materials with binding properties for biotin derivatives in organic solvents may enable the development of new avidin—biotin applications.

Here, we describe synthetic biotin-binding polymers prepared by molecular imprinting. This template polymerization technique involves three steps: (1) formation of the target molecule (template)—functional monomer complexes using covalent and/or noncovalent binding, (2) polymerization with the cross-linking reagent(s) and porogen(s), and (3) removal of the template yielding complementary binding sites for the template.²⁻⁶

In this study, we employed hydrogen-bonding-based molecular imprinting techniques, using two functional monomers with dissimilar pKₐ, methacrylic acid (MAA) and 2-(trifluoromethyl)acrylic acid (TFMAA). The possible prepolymerization complex structure of MAA and the template biotin methyl ester (B-Me) and the recognition ability in both less polar (chloroform) and more polar (acetonitrile) solvents were investigated. In addition, as an application example we present an imprinted polymer-based competitive binding assay using a nonisotopic-labeled ligand.

**EXPERIMENTAL SECTION**

**Reagents.** B-Me and biotin p-nitrophenyl ester (B-pNP) were purchased from Sigma (St. Louis, MO). p-Nitrophenol (pNP) was obtained from Wako Pure Chemicals (Osaka, Japan), and TFMAA was from Tokyo Chemical Industry (Tokyo, Japan). MAA, ethylene glycol dimethacrylate (EGDMA), 2,2-azobisisobutyronitrile (AIBN), chloroform, acetonitrile, and other chemicals were obtained from Katayama Chemicals (Osaka, Japan).

**Procedure for Bimolecular Complex Modeling.** Configurational and conformational searches of B-Me–MAA complexes were carried out with a Monte Carlo simulation technique, with possibilities of rotating and translating MAA toward B-Me. The docked bimolecular complexes were set by a random rotation around all three axes at its center of mass and translational movement in three-dimensional space. B-Me was allowed to be flexible and rotate randomly on the tetramethylene linker between the methoxycarbonyl and bicyclic moieties. All calculations were performed with the molecular modeling software Macromodel 4.5 (Department of Chemistry, Columbia University, New York) using MM2 as an empirical force field.

**NMR Titrations.** For the proton NMR titration of B-Me with MAA, various concentrations of MAA in CDCl₃ (from 1 to 96 mM, 350 μL) were added to 350 μL of 8 mM B-Me in CDCl₃ and the chemical shifts of two NH protons of the cyclic urea of B-Me were measured. Assuming that the chemical shift changes reflect only the one-to-one complexation process between B-Me and MAA, their binding constants and limiting chemical shifts for the

RESULTS AND DISCUSSION

Chemicals at a given temperature were estimated by using the following equation and iterative two-parameter fit:

\[
\frac{\delta - \delta_{\text{free}}}{\delta_{\text{assoc}} - \delta_{\text{free}}} = (1 + (C_a - C_s)K - \\
((C_a - C_s)^2K^2 + 2(C_a + C_s)K + 1)^{1/2})/2KC_s
\]

where \(\delta\) is an observed chemical shift of NH protons of B-Me, \(\delta_{\text{free}}\) is a chemical shift without addition of MAA (4.59 and 4.80 ppm observed at 4 mM B-Me in CDCl\(_3\) were used as the constants), \(\delta_{\text{assoc}}\) is a limiting chemical shift of the complex, \(C_s\) is a concentration of MAA, \(C_a\) is a concentration of B-Me (4 mM), and \(K\) is a binding constant for the complex. This fitting was performed with a least-squares regression software proFit 5.0.1 (Cherwell Scientific Publishing, Palo Alto, CA).

To generate data for the Job’s plots, 20 mM B-Me and MAA dissolved in CDCl\(_3\) were mixed to yield appropriate mole fractions. Chemical shifts of NH protons of B-Me were used to calculate B-Me–MAA complex concentrations. All experiments were conducted with a JEOL FT-NMR model JNM-LA300 (Tokyo, Japan).

Polymer Preparation. B-Me (1.94 mmol), a functional monomer (MAA or TFMA, 7.74 mmol), EGDMA (15.4 g), and AIBN (an initiator, 0.20 g) were dissolved in chloroform (30 mL), and the polymerization was initiated by UV light irradiation under nitrogen atmosphere. After 17 h, the resulting polymer was crushed and sieved to yield 30–60 µm particles.

Polymer Evaluation. Evaluation of the polymers was conducted batchwise. The B-Me imprinted polymer (5 mg) was put into 1.5-mL polypropylene tubes (for acetonitrile solution) or 1.5-mL glass vials with silicon caps (for chloroform solution). Sample solutions (1 mL) were added and incubated overnight with rotation at room temperature. After centrifugation, unbound sample in the supernatants was determined with a flow injection system consisting of an L-6200 pump (flow rate, 0.8 mL/min; Hitachi), a model 234-401 autosample injector (sample size, 20 µL; Gilson), a Unipoint data processing system (Gilson), and either a model 875-UV detector (detection wavelength, 270 nm for B-Me) or a Sedex 55 evaporative light-scattering detector (for MAA and B-Me).

Competitive Binding Assays. The B-Me imprinted polymer (5 mg) was put into 1.5-mL polypropylene tubes and either 400 or 20 µL B-pNP in acetonitrile (500 µL) and various concentrations of B-Me in acetonitrile (500 µL) were added. After overnight incubation with rotation at room temperature, concentrations of B-pNP in the supernatants were determined with the same flow injection system as described above. The amount of B-Me bound without B-pNP is termed 100% bound, and each amount bound (%) was calculated.

RESULTS AND DISCUSSION

Interaction between B-Me and MAA in the Prepolymerization Stage. The polymerizing monomer MAA binds effectively to B-Me in solution. B-Me as a cyclic urea derivative presents two potential hydrogen-bonding sites to MAA. This was indicated by large downfield shifts of the two NH resonances of B-Me when the concentration of MAA in CDCl3 solutions was increased. These NH resonances were shifted from 4.59 (4 mM B-Me alone) to 6.00 ppm (4 mM B-Me in the presence of 48 mM MAA) and 4.80 to 6.22 ppm, respectively. Molecular modeling studies suggested that the single molecule might bind to one side of B-Me to form bidentate hydrogen bonds, as illustrated in Figure 1. This modeling shows the most stable complex structure between MAA and B-Me, obtained with the Monte Carlo technique. As tighter complex formation between MAA and B-Me prior to copolymerization with EGDMA is a prerequisite for constructing more effective cavities to entrap the template, an optimized structure for the complex of MAA and B-Me illustrated here may show an ideal orientation of MAA situated on the polymer cavities to B-Me.

NMR experiments in the mixtures of MAA and B-Me in CDCl3 supported this modeling. A continuous variance method (Job’s plot) revealed one-to-one stoichiometry for the complex formation (Figure 2). Chemical shifts for two NH proton resonances showed the different downfield shifts when the concentration of MAA was increased. A Monte Carlo conformational search of B-Me suggested that its terminal methoxycarbonyl group is linked via an intramolecular hydrogen bond to the NH group. This intramolecular hydrogen bonding may be supported by the aforementioned chemical shift difference in the two NH protons of B-Me. The NH proton revealing the smaller deviation of chemical shifts can be assigned as the inner NH proton since this hydrogen bond can induce chemical shift changes in the inner NH proton near the methoxycarbonyl group to lesser extent than that in the outer NH proton. MAA can therefore bind to both sides of B-Me and may probably be held by multiple interactions in both the inner NH side and the methoxycarbonyl group, as shown by the bimolecular modeling. The bimolecular association constant calculated by using the chemical shift changes of the outer NH proton, was 1.55 × 10^2 M^-1 (standard deviation (SD), 13.3) and

Figure 1. The most stable complex between B-Me and MAA proposed by a Monte Carlo simulation. This complex shows the most favorable interaction but one snapshot of many possible interactions between these molecules under the condition of a time-averaged event.
mainly by entropy-induced factors.

The apparent association constant with the inner NH proton was calculated by the chemical shifts of the outer NH of B-Me; the limiting chemical shift of the complex 6.43 ppm (SD, 0.34).

### Table 1. Selectivity of the B-Me Imprinted Polymer

<table>
<thead>
<tr>
<th>solvent</th>
<th>B-M e</th>
<th>B-pNP</th>
<th>pNP</th>
</tr>
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<tbody>
<tr>
<td>chloroform</td>
<td>6.81</td>
<td>5.61</td>
<td>1.89</td>
</tr>
<tr>
<td>acetonitrile</td>
<td>3.86</td>
<td>2.53</td>
<td>0.64</td>
</tr>
</tbody>
</table>

a 5 mg in 1 mL of chloroform or acetonitrile. b 50 μmol.

the limiting chemical shift of the complex 6.43 ppm (SD, 0.34). The apparent association constant with the inner NH proton was 1.49 × 10⁵ M⁻¹ (SD, 12.6), similar to that obtained with the outer NH proton, though the effect of intramolecular hydrogen bonding in B-Me onto chemical shift changes cannot be excluded from the intermolecular hydrogen bonding of MAA (the limiting chemical shift obtained along with the binding constant was 6.23 ppm (SD, 0.34)).

### Binding Properties of MAA- and TFMAA-Based B-Me Imprinted Polymers.

The MAA-based imprinted polymer bound B-Me (6.81 μmol/g of polymer) more strongly than the blank polymer (1.39 μmol/g of polymer) prepared without B-Me. This enhanced affinity is most probably due to the imprint effect induced during the polymerization in the presence of B-Me. The selectivity was examined by the comparison of the affinity for B-Me, B-pNP, and pNP, respectively (Table 1). The strongest binding observed was to B-Me, with the affinity being 1.4 times as high as that of B-pNP and 4 times as high as that of pNP. Very possibly the imprinted polymer recognizes the cyclic urea structure, not the p-nitrophenyl structure.

According to a Scatchard analysis of B-Me saturation experiments, the association constant and number of binding sites in the lower concentration range are estimated to be 2.5 × 10⁵ M⁻¹ and 7.7 μmol/g of polymer, respectively (Figure 3). Those in the higher concentration range are estimated to be 6.3 × 10⁴ M⁻¹ and 65 μmol/g of polymer, respectively (Figure 3). The estimated dissociation constant of the higher affinity sites is ~1700 times as high as that of the 1:1 complex of B-Me and MAA in the prepolymerization stage, suggesting that the imprinting process would contribute to the stabilization of B-Me–MAA complexes mainly by entropy-induced factors.

When TFMAA was used instead of MAA, the affinity to B-Me in the imprinted polymer decreased to half (3.57 μmol/g of polymer) although TFMAA is a stronger acid and is expected to form stronger hydrogen bonding due to its hydrogen donation property. This discrepancy could be explained by the property of MAA, i.e., MAA being a weak acid can function both as hydrogen donor and acceptor and is bound both to the oxygen atom and one of the protons of the ureide structure of biotin. Compared to MAA, TFMAA is a better hydrogen donor, but a weaker hydrogen acceptor. As a result, TFMAA can interact mainly with the oxygen atom of the biotin–ureide structure whereas MAA may be able to form double hydrogen bonds with the ureide structure of B-Me, so that such weak affinity in the TFMAA-based imprinted polymer was observed. Consequently, it appears that MAA is a better functional monomer for imprinting the biotin structure.

For the development of high affinity and selectivity in the hydrogen-bonding-based imprinted polymers, less polar solvents would be favorable. To verify the B-Me binding mechanism, the binding characteristics in a polar solvent were examined (Table 1). When acetonitrile was used, the affinity was decreased, and according to a Scatchard analysis of B-Me in acetonitrile saturation experiments, the association constant and number of binding sites in the lower concentration range were estimated to be 5.0 × 10⁴ M⁻¹ and 24 μmol/g of polymer, respectively. Those in the high concentration range were estimated to be 2.0 × 10³ M⁻¹ and 45 μmol/g of polymer, respectively (Figure 4).

From these results, the observed affinities were found to be mainly due to hydrogen bond formation. Higher affinity binding sites available in chloroform seem not to function in acetonitrile, although the selectivity remained. The template retentivity depends on degrees of hydrogen bonding between the carboxylic acid moiety on the cavity and the template. Since the more polar acetonitrile breaks the hydrogen bonds more effectively than the less polar chloroform, the binding ability to the biotin should be decreased in the more polar acetonitrile.

### Imprinted Polymer-Based Binding Assay Using Nonisotopic-Labeled Ligands.

Imprinted polymer-based competitive binding assays were first reported by Mosbach and co-workers.
where the assays for theophylline and diazepam were conducted using radiolabeled ligands. Since this work, several imprinted polymer-based binding assays have been demonstrated for various compounds. Recently, nonisotopic assays have been reported in which a fluorescent template compound analogue was used as the labeled ligand. As many target compounds and their analogues do not always have strong fluorescence or UV/visible absorption characteristics, fluorophore or chromophore labeling will be inevitable for further development of this technique. However, no imprinted polymer-based assay using nonisotopic-labeled ligands has been reported so far. Therefore, the use of labeled biotin was attempted to use in imprinted polymer-based competitive binding assays.

As B-pNP absorbs UV light at ~270 nm, it can be used as a chromophore-labeled biotin. B-Me, which has a weak UV absorption property, can be determined by measuring unbound labeled ligand B-pNP after competitive binding between a fixed amount of B-pNP and the unlabeled ligand, B-Me. As shown in Figure 5, the amount of B-pNP bound was decreased with the increase of B-Me added, and a displacement curve was obtained. When 10 μM B-pNP was used, the amount of B-pNP bound was 4.4 nmol, 44% of the initial amount of B-pNP added, and this amount was termed 100% bound in the displacement curve. It was replaced to be 0.3 nmol (3% of the initial amount of B-pNP added) by large excess of B-Me (10 mM), indicating that almost no nonspecific binding of B-pNP was observed under these conditions. When 20 times higher concentration of B-pNP (200 μM) was used, the displacement curve was shifted to a high concentration range (Figure 5). In this case, the replacement was not completed due to the solubility of B-Me in acetonitrile. However, if the observed linear decrease from approximately 1–10 mM B-Me added is extrapolated, it seems that most of bound B-pNP could be replaced by a large excess of B-Me. These results suggest that imprinted polymers can be used to establish nonisotopic competitive binding assays. The determination range could be controlled by changing the amount of B-pNP added and/or modifying the content of polar and nonpolar solvents such as chloroform and acetonitrile.

CONCLUSIONS

Synthetic biotin-binding polymers can be prepared by molecular imprinting and it could be shown that they can be used in nonisotopic binding assay of biotin derivatives. Many commercially available labeled biotins other than B-pNP could be also applied to imprinted polymer-based nonisotopic synthetic receptor assays. Unlike natural binders such as receptors, antibodies, and other binding proteins, nonspecific binding of the labeled ligand was not observed, suggesting that precise assays could be performed using the proposed method. Since imprinted polymers using dummy templates analogous to target molecules were successfully prepared and showed comparable selectivity to the imprints prepared with the original templates, imprinted polymers prepared with designed templates that make the binding sites more reliable for the target and labeled ligands should also be considered to develop specific and feasible binding assays. Furthermore, functional molecules conjugated with biotin may be immobilized on this biotin-binding polymer. This immobilization technique could be used to develop many applications with the addition of functionalities to the biotin-binding polymers, as chiral stationary phase, sensing materials, specific adsorbents, and so on. In this study, only MAA and TFMMA were examined; however, by using a recently developed combinatorial approach in the selection of functional monomers and the optimization of imprinting conditions, biotin-imprinted polymers with superior perfor-

mance and their applications could be developed and such efforts are ongoing.

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