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Molecularly Imprinted Tunable Binding Sites Based on Conjugated Prosthetic Groups and Ion-Paired Cofactors

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Abstract: A molecular imprinting technique was applied to the construction of prosthetic group-coupled tunable binding cavities for bisphenol A (BPA). A novel template molecule, with a structure consisting of BPA covalently conjugated with two allyl(4-carboxyphenyl)disulfides through ester bonds (BPA-D), was designed. After copolymerization of BPA-D with styrene and divinylbenzene, the BPA di(4-mercaptobenzoate) moieties were removed by reductive cleavage of the disulfide bonds, resulting in apo-type molecularly imprinted cavities bearing two thiol residues. 4-Mercaptobenzoic acid was introduced into the apo-type cavities as a prosthetic group through a disulfide bond by addition of 4,4′-dithiodibenzoic acid, which transformed the apo-type cavities into holo-type cavities with two carboxylic acid residues for binding BPA. When pyridyl prosthetic groups were introduced instead of 4-mercaptobenzoic acid by using 4,4′-dithiodipyridine, BPA recognition ability was maintained but with improved selectivity. The binding affinity was successfully altered several times by attaching and detaching these prosthetic groups, which showed that the apo-type scaffold could be reused. Furthermore, noncovalent-type ion-paired cofactors could be introduced, when the two thiol groups in the apo-type cavities were oxidized to sulfonic acid groups. When 1,2-diaminoethane (DAE) was added to the oxidized apo-type scaffold as a noncovalent-type cofactor, the binding activity was regulated successively, depending upon the concentrations of DAE added. By using various prosthetic groups and cofactors, the binding properties of the holo-type cavities could be tuned in a similar way to those found in biological systems.

Introduction

Conjugated proteins such as hemoproteins[1]and lipoproteins,[2] are known to have prosthetic groups in their structures which are conjugated by covalent bonds. Without these prosthetic groups the molecules have no biological activity, and the integration of such groups into conjugated proteins allows the development of sophisticated molecular recognition-based functions in biological systems. Thus, such prosthetic groups are considered to function as biological switching systems, in which bioinformation is transferred by attachment and detachment of the groups. If such systems can be mimicked using synthetic polymer-based artificial receptors with prosthetic groups conjugated at the binding sites, this may lead to the development of unique bioinspired materials. A diverse range of artificial receptors have been reported;[3] however, polymer-based molecular recognition cavities bearing prosthetic groups have not yet been synthesized. In this work, we propose artificial polymer receptors bearing prosthetic group- or cofactor-coupling apo-type cavities prepared by molecular imprinting, which show predetermined molecular recognition ability through covalent conjugation of designed prosthetic groups with apo-type binding sites inside the imprinted cavities. The apo-type binding sites are precursors that require the presence of the corresponding prosthetic groups or cofactors to form the complete binding sites, which are the functioning binding sites (holo-type binding sites). In addition, noncovalently coupled cofactors for apo-type binding sites were also investigated. It was expected that the binding affinity of such conjugated sites could be tuned by replacing the prosthetic groups or cofactors bearing alternative groups that have different binding mode or affinity with the target molecule.

Molecular imprinting has a reputation as a promising strategy for the preparation of tailor-made receptors for target molecules.[4] Molecular templates—often target molecules or their derivatives—are used to assemble the functional monomers surrounding them into complementary orientations, and are then subjected to coimmobilization using cross-linkers. After removal of the templates, tailor-made binding sites for the target molecules are left within the synthetic polymer matrix. Thus, the binding cavities of imprinted polymers are constructed by self-assembly on the basis of the shapes and

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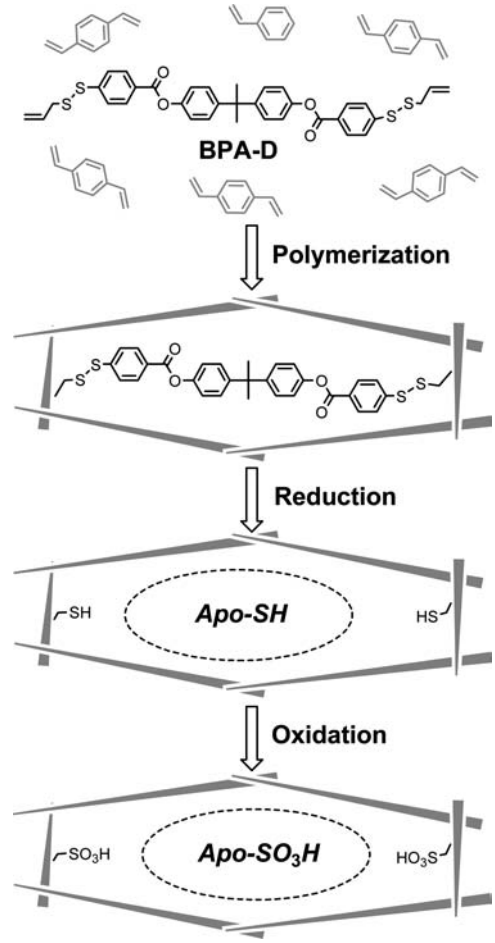
chemical properties of template molecules used. In the present work, we used this template polymerization technique to prepare switchable binding sites coupled with covalently conjugating prosthetic groups or noncovalently ion-pairing cofactors, featuring tunable affinity and selectivity for a target molecule. Bisphenol A (BPA), an endocrine disruptor, was used as a model target molecule, since BPA-imprinted polymers have been the subject of intensive investigation, including noncovalent[5] and covalent[6] imprinting.

Results and Discussion

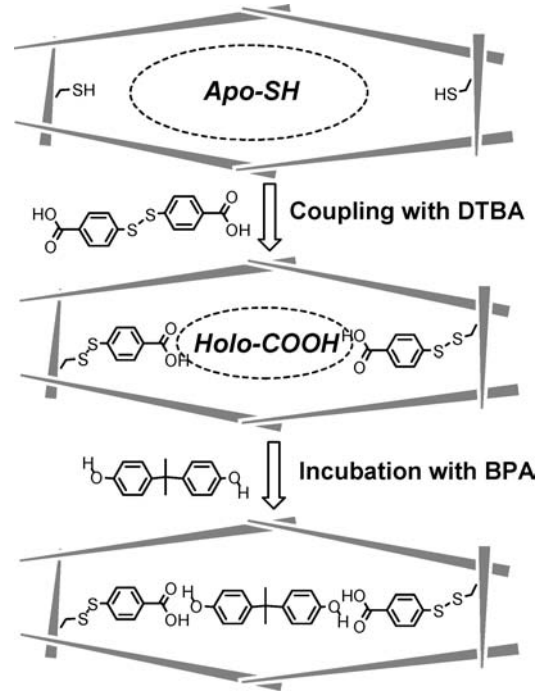
Preparation of Apo-Type Scaffolds Apo-SH and Apo-SO_3H. The synthetic strategy for the construction of molecular recognition cavities, which were integrated with covalently conjugating prosthetic groups or noncovalently ion-pairing cofactors, is shown in Schemes 1−4. A covalent molecular imprinting technique involving postreduction and postoxidation[7] was employed to afford apo-type binding sites capable of prosthetic group or cofactor integration (Apo-SH and Apo-SO_3H,) as shown in Scheme 1. A novel template molecule (BPA-D, Scheme 1), with a structure consisting of bisphenol A covalently conjugated with two allyl(4-carboxyphenyl)-disulfides by ester bonds, was designed. Polymerization of BPA-D was carried out using styrene as a comonomer and divinylbenzene as a cross-linker. BPA-D was then removed by reducing the disulfide bonds with $LiAlH_4$ to yield the imprinted polymer bearing apo-type binding sites (Apo-SH). Elemental analysis showed that the amount of sulfur contained in Apo-SH was 0.97%, which corresponds to copolymerization of about 60% of the initial amount of BPA-D. HPLC determination of BPA released from the polymer showed a consistent amount (66% of the initial amount in the prepolymerization mixture). These results showed that BPA-D underwent successful copolymerization and that apo-type binding sites containing thiol groups were generated by the removal of almost all of the bisphenol A di(4-mercaptobenzoate) moiety introduced during the polymerization process.

Holo-Type Acidic Binding Sites Prepared Using DTBA (Holo-COOH). The prosthetic group, namely, 4-mercaptobenzoic acid (MBA), can be covalently introduced into the apo-type binding sites by disulfide exchange reaction with 4,4′-dithiodibenzoic acid (DTBA), transforming the binding sites to holo-type sites (Holo-COOH) as shown in Scheme 2. In order to confirm the insertion of the prosthetic MBA group, ^{13}C CP/MAS NMR measurements were carried out for Holo-COOH. A new peak at 173 ppm was observed (see Supporting

Scheme 1. Preparation of Apo-SH and Apo-SO_3H



Scheme 2. Preparation of Holo-COOH



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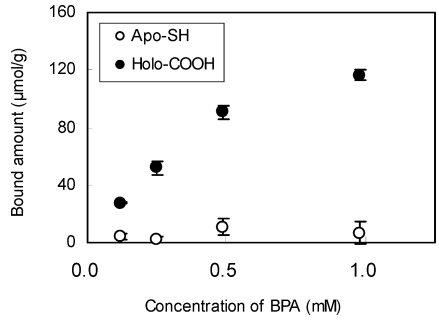
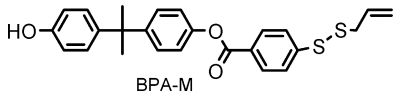


Figure 1. Binding isotherms of Holo-COOH and Apo-SH.

Information), which was assigned as the carbonyl group of the MBA moiety. This suggests that holo-type binding sites are formed in the disulfide exchange reaction.

The binding property of Holo-COOH was examined by drawing a binding isotherm of BPA. As a reference, Apo-SH was also examined. Holo-COOH gave a saturation binding curve, which means that a finite number of binding cavities can be created by the imprinting process followed by the prosthetic group conjugation process (Figure 1). An association constant of BPA with Holo-COOH was estimated from a Langmuir plot to be $K_a = 3.3 \times 10^3\ M^{-1}$ (See Supporting Information). In contrast, Apo-SH showed much lower affinity for BPA. These results clearly show that binding is switchable, depending on prosthetic group conjugated at the apo-type binding sites.

A reference polymer was prepared by using a monoester type template molecule, bisphenol A mono[allyl(4-carboxyphenyl) disulfide] (BPA-M), in which the binding cavities have only



BPA-M

one MBA moiety. Binding experiments of the holo-type reference polymer (Holo-COOH-M) were performed, and from a Langmuir plot, an association constant was estimated to be $5.3 \times 10^2\ M^{-1}$ (see Supporting Information). This lower binding activity of Holo-COOH-M suggests that the two carboxyl groups in the cavity of Holo-COOH could take part in the binding of BPA, where two interactive sites of BPA are captured through cooperative interaction with the two MBA moieties as prosthetic groups.

Figure 2 illustrates the selectivity of Holo-COOH for various phenolic compounds. The target BPA was bound more strongly than the structurally related bisphenol B (BPB) and hexestrol (HEX). A smaller compound, resorcinol (RES), showed weaker affinity toward Holo-COOH. These results reveal that the holo-type cavity has a shape selectivity for BPA. A methoxy phenyl compound, 4,4′-dimethoxybenzophenone (DMB), showed low affinity; thus, the primary binding interaction is considered to be based on hydrogen bonding between the hydroxy group of BPA and the carboxyl group of the prosthetic MBA moiety. The similar selectivity was observed for the conventional covalent molecular imprinting of BPA prepared using BPA dimethacrylate as a template molecule and ethylene glycol dimethacrylate as a cross-linker (see Supporting Information), confirming that the observed selectivity could be induced by the imprinting process in the polymerization with BPA-D.

The binding activity of an aminophenyl compound, 4,4′-diaminodiphenylmethane (DAD), which has two amino

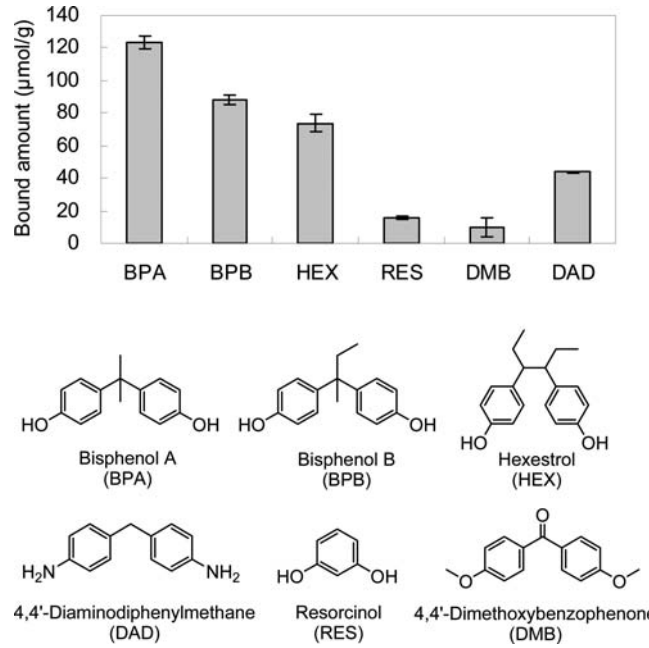


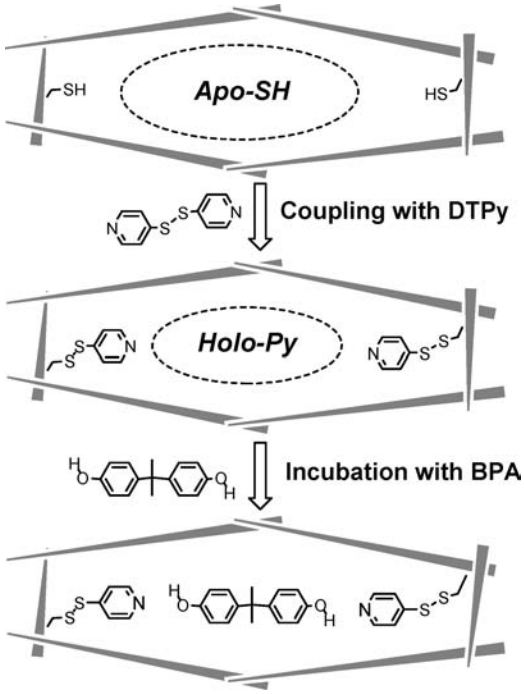
Figure 2. Selectivity of Holo-COOH for structurally related compounds.

groups instead of hydroxyl groups, showed high affinity to Holo-COOH. This may be due to the location of the two basic amino groups of DAD at a suitable position for interaction with the carboxyl groups of the conjugated prosthetic groups at the holo-type binding sites. Conventional BPA-imprinted polymers previously reported also showed nonspecific binding of DAD (see Supporting Information); thus, this is an intrinsic property of BPA-imprinted polymers prepared using acidic functional monomers. Although the basicity of DAD is higher than that of BPA, the binding activity of Holo-COOH toward DAD is lower than BPA, meaning that the binding event is not dependent on a simple ion-exchange mechanism and the selectivity can be developed by the imprinting effects.

Holo-Type Basic Binding Sites Prepared Using DTPy (Holo-Py). In order to tune the chemical properties of the BPA-imprinted binding cavities to reduce nonspecific binding of basic compounds such as DAD, 4,4′-dithiodipyridine (DTPy) was introduced as an alternative prosthetic group instead of DTBA (Scheme 3). New holo-type binding sites were constructed in the same manner as the Holo-COOH preparation: DTPy was conjugated with the apo-type binding sites of Apo-SH by disulfide exchange reaction to obtain Holo-Py bearing pyridine-based binding cavities. The binding isotherm showed a similar saturation curve to that of Holo-COOH, and the binding constant was estimated to be $7.2 \times 10^2\ M^{-1}$ from a Langmuir plot (see Supporting Information).

As shown in Figure 3, Holo-Py showed similar selectivity to Holo-COOH toward BPA, BPB, HEX and DMB, which implies that the 4,4′-dithiodipyridine moieties were successfully introduced into the apo-type binding sites of Apo-SH and binding cavities suitable for BPA were reconstructed, as occurred in the case of Holo-COOH. This selectivity enhancement could be due to the basicity of the conjugated pyridyl group, which may have a lesser tendency to interact with the amino groups of DAD. The reduced binding activity was observed since the pyridyl group works only as a hydrogen acceptor for the hydroxyl groups of BPA, while

Scheme 3. Preparation of Holo-Py



carboxyl group shows both hydrogen donation and acceptation abilities, resulting in the difference of binding activity. Interestingly, there was less nonspecific binding of DAD to Holo-Py (8% of the BPA binding) than to Holo-COOH (36% of the BPA binding).

It appears that the selectivity of the conjugated holo-type imprinted polymers can easily be tuned by replacing the prosthetic groups in the binding cavities. In this case, changing from a benzoic acid moiety to a pyridyl moiety resulted in a decrease in nonspecific binding, which enhanced selectivity for BPA. Consequently, the tunable binding sites coupled with the prosthetic groups can gave a resolution to drastically improve the intrinsic properties of originally prepared polymers without going back to the monomer design and polymerization steps.

Repetitive Reconstruction of Holo-COOH and Holo-Py. The binding of BPA can be regulated repeatedly by attaching and detaching the prosthetic groups in Holo-COOH and Holo-Py. The affinity was switched by carrying out the thiol-exchange reaction to prepare holo-type binding sites, followed by the reduction to remove the prosthetic groups, yielding Apo-SH (Figure 4). The recovered holo-type binding cavities showed the same binding activity toward BPA, even after several repetitions. These results reveal that the prosthetic group conjugation process is fully reversible and Apo-SH is reusable.

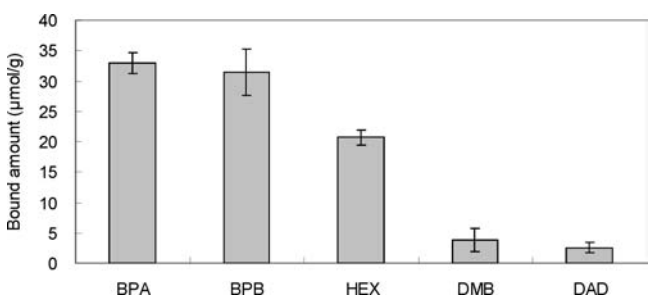


Figure 3. Selectivity of Holo-Py for structurally related compounds.

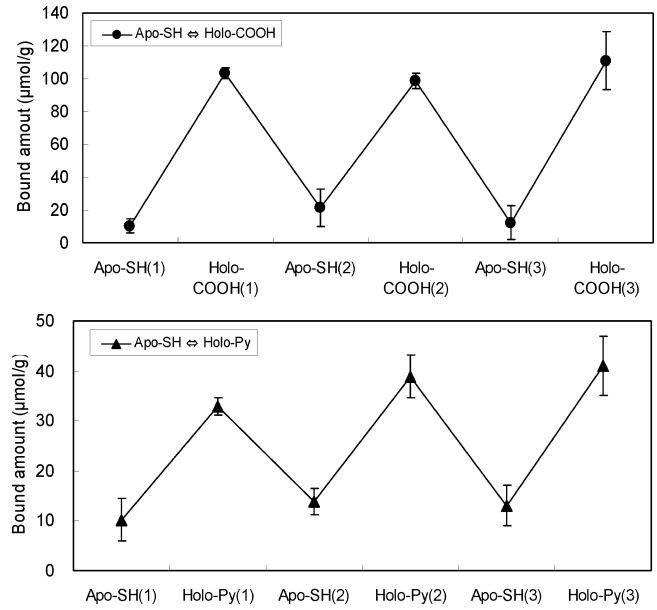
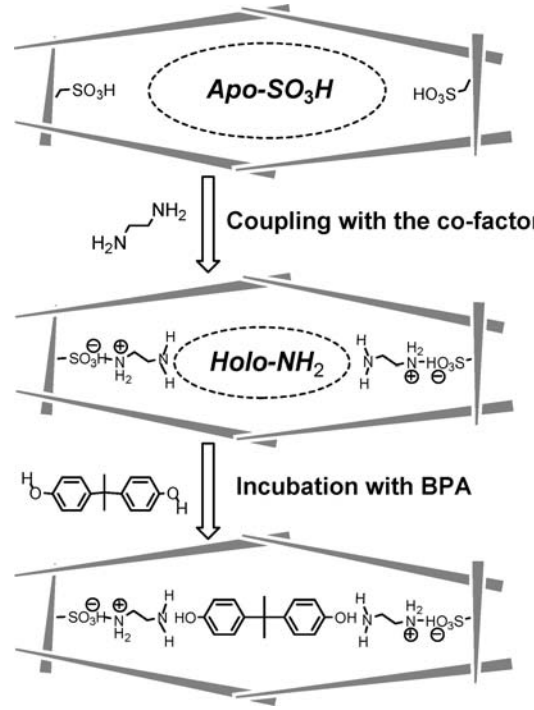


Figure 4. Repetitive binding behaviors of the apo-type and the holo-type binding sites by the consecutive introduction and removal of the prosthetic groups.

Scheme 4. Preparation of Holo-NH_2



Therefore, the apo-type molecularly imprinted binding cavities can act as a scaffold for robust artificial polymer receptors.

Holo-Type Binding Sites Ion-Paired with DAE (Holo-NH_2). The other apo-type polymer, Apo-SO_3H, was transformed into the corresponding holo-type polymer by adding 1,2-diaminoethane (DAE) as a noncovalent-type cofactor, which can electrostatically interact with SO_3H residues in the cavities of Apo-SO_3H to form Holo-NH_2, ion-paired holo-type binding sites for BPA, (Scheme 4). When DAE was added to Apo-SO_3H, the binding affinity for BPA was increased, and after 1 mM, the affinity was decreased, suggesting that the Apo-SO_3H was successfully converted

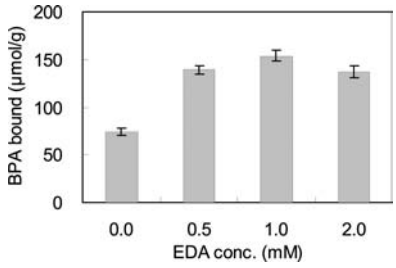


Figure 5. Effect of DAE concentrations on the binding activity of Holo-NH_2.

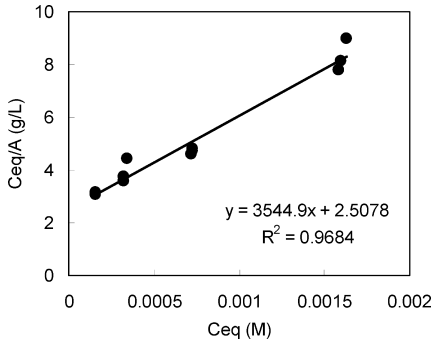


Figure 6. Langmuir plot for the binding of BPA toward Holo-NH_2 (1 mM).

to Holo-NH_2 by the addition of DAE as expected (Figure 5). However, excess of DAE interfered with the binding of BPA to Holo-NH_2. Since an association constant of BPA with free DAE in toluene was estimated to be 1.6×10^2 M^{-1} by isothermal titration calorimetry (see Supporting Information), free DAE could be ion-paired with free BPA in the binding solution, resulting in the decrease of free DEA that could bind to Apo-SO_3H. These results reveal that the binding affinity of Holo-NH_2 would be continuously tunable, in accordance with the amount of DAE added.

The BPA binding isotherm for Holo-NH_2 was saturated (see Supporting Information), and an association constant was estimated from a Langmuir plot to be 1.4×10^3 M^{-1} (Figure 6). Because a linear plot was observed, the ion-paired holo-type binding sites formed could be fairly homogeneous, where BPA may be bound to the holo-type binding cavity with 1-to-1 stoichiometry. An association constant of BPA with free DAE in toluene was lower than that with Holo-NH_2, so that the Holo-NH_2 is composed of DAE, where DAE works as a binding site for BPA inside the imprinted cavity, resulting in the enhancement of affinity.

The selectivity of Holo-NH_2 was examined by using structurally related phenolic compounds (Figure 7). Since DMP showed less binding, it appears that the phenolic hydroxy groups of BPA are involved in the binding to Holo-NH_2 as is in the case of Holo-COOH and Holo-Py. Compared with Holo-COOH and Holo-Py, in which the prosthetic groups were covalently attached, Holo-NH_2 showed less selectivity among BPA, BPB, and HEX. This may be due to the flexibility of the ethylene chain of DAE. A basic compound, DAD also showed lower affinity than BPA, confirming that most of SO_3H-based binding sites in Apo-SO_3H are ion-paired with DAE, i.e. Apo-SO_3H is successfully transformed to be Holo-NH_2 by the addition of DAE under the conditions employed.

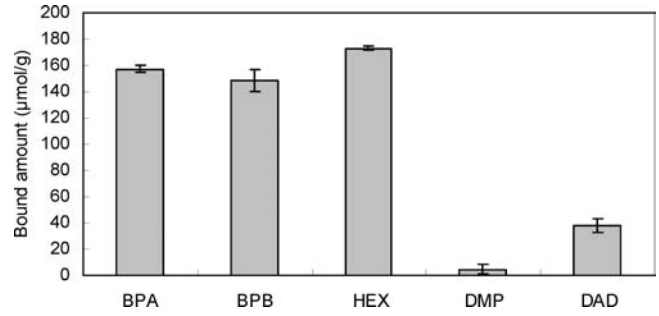


Figure 7. Selectivity of Holo-NH_2 (1 mM DAE) for structurally related compounds.

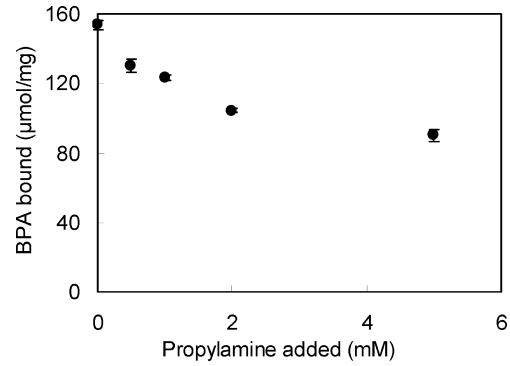


Figure 8. Effect of *n*-propylamine concentrations on the binding activity of Holo-NH_2 (1 mM DAE).

Addition of *n*-propylamine (PA) as a competitor for DAE affected the binding activity of Holo-NH_2 as shown in Figure 8. The binding activity was decreased with the increase of *n*-propylamine concentrations, meaning that the bound DAE in the cavity was replaced by *n*-propylamine and the alkyl chain of PA bound interfered with the binding of BPA. This is evident that the binding sites in Holo-NH_2 are reversibly constructed by the ion-paired DAE with SO_3H residues in the cavity of Apo-SO_3H, and the affinity can be tuned by adding a competitor toward the SO_3H residues in Apo-SO_3H.

Conclusion

The covalent imprinting method was employed to prepare BPA-imprinted apo-type binding cavities, which can be conjugated with prosthetic groups or cofactors to yield holo-type binding cavities for BPA. Without the conjugation to yield the holo-type binding sites, less binding activity was observed. Tuning of the activity of the holo-type binding cavity was demonstrated by addition of the covalently conjugating prosthetic groups and the noncovalently ion-pairing cofactor. As long as the three-dimensional fit for the shape of the BPA molecule was retained when the prosthetic groups and cofactors were replaced, the molecular recognition ability appeared to be preserved. This implies that functional groups can be screened to optimize selectivity.

As the properties of the binding sites were switched dramatically by introducing and removing the prosthetic groups in Holo-COOH and Holo-Py, they may be considered to be stimuli-responsive materials, with oxidation and reduction conditions acting as chemical stimuli. In the case of Holo-NH_2, the binding activity can be controlled *in situ* by adding the cofactor, DAE. Compounds which interact with the binding sites of Apo-SO_3H can work as inhibitors of the

transformation into Holo-NH_2, regulating the binding activity. By using such noncovalently coupling cofactors, not only could the binding activity be tuned successively, but also the alteration of functionalities in the holo-type binding cavities could be easily achieved. When binding activity decreases due to aging, prosthetic groups and cofactors may be changed to obtain new binding sites. These molecularly imprinted tunable binding cavities coupled with prosthetic groups and cofactors could open the way to the development of a new class of synthetic polymeric receptors with bioinspired functions.

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Supporting Information Available: Experimental details, Langmuir plot of Holo-COOH and Holo-COOH-M, binding isotherm and Langmuir plot of Holo-Py, binding isotherm of Holo-NH_2, ^{13}C CP/MAS NMR spectrum of Holo-COOH, ^{1}H- and ^{13}C NMR spectra of the synthesized compounds, isothermal titration calorimetry of BPA with DAE. This material is available free of charge via the Internet at http://pubs.acs.org.

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