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DUMMY TEMPLATE-IMPRINTED POLYMERS FOR BISPHENOL A PREPARED USING A SCHIFF BASE-TYPE TEMPLATE MOLECULE WITH POST-IMPRINTING OXIDATION

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A new dummy template molecule, N,N′-bis(3-vinylbenzylidene)-4,4′-diaminodiphenylmethane (VB-DADPM) was designed to synthesize dummy template-imprinted polymer (DIP) for Bisphenol A (BPA). Since the Schiff-base part of VB-DADPM is easily cleaved by a weak acid treatment, the DADPM moiety can be removed after the co-polymerization with cross-linkers. After the post-imprinting oxidation was carried out to transform the residual aldehyde to carboxylic acid, binding sites toward BPA were generated only inside the binding cavity, which had an estimated binding constant of $1.3 \times 10^6\ M^{-1}$, and could recognize the difference between similar molecules BPA and BPB, the latter of which has an ethyl group instead of a methyl group at the central part of BPA.

Keywords: Bisphenol A; Dummy template; Molecularly imprinted polymer; Post-imprinting; Schiff base

INTRODUCTION

Bisphenol A (BPA) has been used for the production of polycarbonate plastics and epoxy resins, and is sometimes eluted from these products by washing with acidic/basic solutions and strong surfactants. BPA is a suspected endocrine disruptor, which has been reported to show estrogen-like activities. Recent studies seem to indicate that the risks associated with BPA are somewhat lower than previously estimated, but there still exists a major concern regarding BPA exposure for fetuses, infants, and young children. Thus, it remains vital that levels of BPA in our environment are monitored and assessed accurately (Kuiper et al. 1997; Kuiper et al. 1998; Okada et al. 2008; Prins et al. 2008). For the detection of BPA, immunoassays and related techniques have been frequently utilized; however, because BPA is a relatively small molecule, it is challenging to obtain highly selective antibodies, and so

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such immuno-based assays are somewhat problematic. Furthermore, BPA is poorly soluble in water; thus organic solvent-based assays are more desirable.

As tailor-made synthetic materials capable of molecular recognition in organic solvents, molecularly imprinted polymers (MIPs) have been applied to separation media and sensor materials (Haupt and Mosbach 2000; Sellergren 2001; Wulff 2002; Komiyama et al. 2003; Zimmerman and Lemcoff 2004; Turner et al. 2006; Wei, Jakush, and Mizaikoff 2006). The MIP assembly was achieved through co-polymerization carried out with template molecule-functional monomer complexes and cross-linkers, where the template molecule was either a target molecule or a structurally related derivative. After the removal of the template, specific binding cavities toward the target molecules were left in the resulting polymers.

The BPA-imprinted polymers have been previously prepared by using template molecules that were linked with functional monomers covalently (Joshi, Kulkarni, and Mashelkar 2000; Ikegami et al. 2004a,b; Ikegami, Mukawa et al. 2004; Takeda and Kobayashi 2006; Wang et al. 2011) and non-covalently (Haginaka and Sanbe 1999; Hernández, Martínez, and Gonzalo 2009; Zu et al. 2010; Apodaca et al. 2011). The MIPs prepared by a covalent imprinting system may have more homogeneous recognition sites in their polymer matrices (Wulff et al. 1986; Hart and Shea 2001).Our group has reported BPA-imprinted polymers prepared using template molecules covalently linked with functional monomers, that is, BPA-dimethacrylate and BPA-diacrylate (Ikegami et al. 2004a,b; Ikegami, Mukawa et al. 2004; Ikegami et al. 2004). Recently, controlled/living radical polymerization (CLRP) such as atom transfer radical polymerization (ATRP) was applied to BPA imprinting (Sasaki, Ooya, and Takeuchi 2010), and the ATRP-based MIP showed an enhanced selectivity. Although selective binding behavior toward BPA and related compounds was observed, the removal of the BPA moiety from the resulting polymers was not easy, since the phenol ester inside the polymer matrix was not completely hydrolyzed by aqueous alkaline solution, leading to the decrease of the number of binding cavities generated during the polymerization step and possible leaching of BPA during the detection stages that may cause high background and/or error in quantitative analysis. Therefore, for practical applications of MIPs, a design for template molecules should include consideration toward the ease of cleavability and the potential use of structurally-related dummy template molecules instead of the target molecule itself.

In this study, a new dummy template molecule, N,N′-bis(3-vinylbenzylidene)-4,4′-diaminodiphenylmethane (VB-DADPM) was designed for BPA-dummy imprinted polymer (DIP). Figure 1 shows the molecular structure of the template and polymerization step of the DIP. The template has two imine bonds connected between diphenylmethane and styrene moieties in its structure. After the polymerization, the diaminodiphenylmethane moiety can be easily removed under mild acidic conditions due to the hydrolysis of the Schiff base parts. Here, dummy imprinted polymers for the recognition of BPA and related compounds were prepared by co-polymerizing VB-DADPM with styrene/divinylbenzene, followed by post-imprinting oxidation to transform the residual aldehyde to carboxylic acid. Binding characteristics of the dummy imprinted polymers were investigated in batch rebinding experiments.

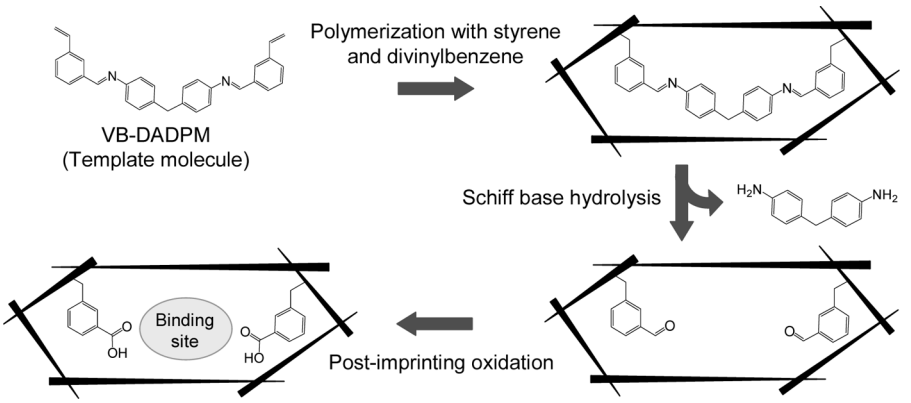

Figure 1. Structure of the designed BPA-dummy template molecule (VB- DADPM) and the BPA-selective binding cavity generation by dummy molecular imprinting followed by the post-imprinting oxidation.

EXPERIMENTAL

Materials

Bisphenol A (BPA), 17β-estradiol, styrene, hydrochloric acid (HCl), 30% hydrogen peroxide (H_2O_2), dichloromethane, hexane, chloroform, toluene were purchased from Nacalai Tesque (Kyoto, Japan). 4,4′-Diaminodiphenylmethane (DADPM), 4-vinylbenzoic acid, and Bisphenol B (BPB) were purchased from Tokyo Chemical Industry CO., LTD (Tokyo, Japan). Divinylbenzene, 2,2′-azobis(4-methoxy-2,4-dimethylvaleronitrile) (V-70), hexestrol ware purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 3-Vinylbenzaldehyde was purchased from Sigma Aldrich Corp. (St. Louis., USA). Styrene, divinylbenzene, dichloromethane, chloroform, and toluene were purified by distillation prior to use. Other reagents and solvents were used without further purification.

Synthesis of N,N′-bis(3-vinylbenzylidene)-4,4′-diaminodiphenylmethane (Template Molecule, VB-DADPM)

DADPM (198.26 mg, 1 mmol) was dissolved in dry dichloromethane (8 mL), and added to a solution containing 3-vinylbenzaldehyde (0.508 mL, 4 mmol) in dry dichloromethane (24 mL). The suspension was stirred for 24 h at room temperature. The solvent was evaporated, and then hexane was added to make a precipitate. The precipitate was filtered and the residue was washed with hexane. The obtained product was dried in vacuo. The resulting product was given as a pale yellow solid (379.07 mg).

Yield: 89% ^{1}HNMR(300 MHz,$CDCl_3$) δ(ppm) = 4.03(s, CH_2, 2H), 5.34(d, $H_{(a)}H_{(b)} = CH_{(c)}$-, 2H), 5.88(d, $H_{(a)}H_{(b)} = CH_{(c)}$-, 2H), 6.83(q, $H_{(a)}H_{(b)} = CH_{(c)}$-, 2H), 7.26(d, benzene, 4H), 7.45(t, benzene, 2H), 7.54(d, benzene, 2H), 7.77(s, benzene, 2H), 8.47(s, CH, 2H).

Hydrolysis of VB-DADPM

The UV/VIS spectra were measured with a JASCO V-560 UV/VIS spectrophotometer. VB-DADPM (1 mM) was incubated in 1 mM HCl methanolic solution containing 0.1% water at room temperature, and the absorbance was measured after the incubation for 0, 3, and 60 min.

Preparation of BPA-Dummy Imprinted Polymer (DIP)

VB-DADPM (356.17 mg, 0.835 mmol), styrene (614.49 mg, 5.9 mmol), divinylbenzene (2304.54 mg, 17.7 mmol), and V-70 (154.21 mg 0.5 mmol) were dissolved in dry chloroform in a Schlenk flask. After the solution was degassed in vacuo under nitrogen atmosphere, polymerization was carried out in water bath at 30°C for 18 h. The obtained polymer was crushed via mortar and pestle, washed with methanol and dried in vacuo. In order to remove the template, the polymer (0.5 g) was suspended in 25 mM methanolic HCl solution (water/methanol, 1:1, v/v) and stirred for 18 h at room temperature. The suspension was separated by filtration to collect the imprinted polymer. The DADPM released form the polymer to the filtrate was determined by LC-MS/MS consisting of UPLC (Waters, USA, Column: BEH-C18, 2.1×50 mm, 1.7 μm; eluent: methanol containing 5 mM ammonium acetate at a flow rate of 0.2 mL min^{-1}), and API-2000 mass spectrometer (Applied Biosystems, USA). Positive ion mode was used for both Q1 scan and product ion scan with Ion-Spray voltage of 5500 V.

The extracted polymer was washed with water and methanol, and then dispersed in water containing 0.042 M HCl and 3.75% H_2O_2. The suspension was stirred for 18 h at room temperature for oxidizing an aldehyde to a carboxylic acid, resulting in the creation of the binding sites for BPA. The polymer was dried in vacuo after washing with 1 M HCl, water, and methanol successively. A non-imprinted polymer (NIP) was prepared in the same manner with the use of 4-vinyl benzoic acid (247.44 mg, 1.67 mmol), instead of VB-DADPM.

Fourier Transform Infrared (FTIR) Measurement

FTIR measurements were performed with a VARIAN 660 KU-RI FT-IR spectrometer. The obtained polymers were ground with KBr and the spectra were scanned from 4000 to 400 cm^{-1} with a resolution of 4 cm^{-1}.

Batch Rebinding Experiments

The DIP (3 mg) was incubated with BPA in toluene (1 mL) at 25°C for 18 h. Concentrations of BPA in solution were varied from 0 to 1 μM. After the incubation, the suspensions were filtered to remove the DIP, and the concentrations of BPA in the filtrates were determined by LC-MS/MS system (Negative ion mode, Ion Spray voltage was −4500 V). Each sample was determined three times. Curve fitting was performed with Deltagraph (DeltaPoint, Monterrey, CA) to determine the binding constant. All experiments were conducted in triplicate.

Selectivity Evaluation

Selectivity tests of DIP were conducted with using BPA, BPB and 17β-estradiol. The BPB was chosen to confirm whether imprinted polymer could recognize the difference in target by not only the recognition with the functional group but also the form of a cavity. This is because the BPB was notably similar to BPA in bisphenol group and the difference is only one carbon number. In addition, BPA shows estrogen-like activities, 17β-estradiol which is one of estrogen was added to selectivity test. The DIP (3 mg) was incubated with 1 mL of a 500 nM toluene solution of each template at 25°C for 18 h. The procedure used was the same to that described in the previous section.

RESULTS AND DISCUSSION

Characterization of the Imprinted Polymer

First, we examined the stability of VB-DADPM, which has ambilateral imine parts connected with styrene moiety, under the polymerization conditions. After the incubation in $CDCl_3$ for 18 h at 30°C, NMR spectra of the VB-DADPM showed that 80% of the original molecule remained unaltered, suggesting that most of the VB-DADPM can be co-polymerized with the cross-linker with the original structure and, thus, work as the template molecule during the imprinting process.

Hydrolysis of the imine bond under acidic conditions was also investigated. When VB-DADPM (1 mM) was incubated in methanol containing 1 mM HCl at room temperature, the cleavage rapidly proceeded and VB-DADPM was completely hydrolyzed into 4′-diaminodiphenylmethane (DADPM) within 60 min, meaning that the DADPM moiety may be easily removed from the resultant polymer under acidic conditions (Fig. 2). In order to estimate the post-imprinting oxidation process, a model compound, 3-vinylbenzaldehyde was oxidized under the post-imprinting treatment condition, and about 85% of 3-vinylbenzaldehyde was transformed to

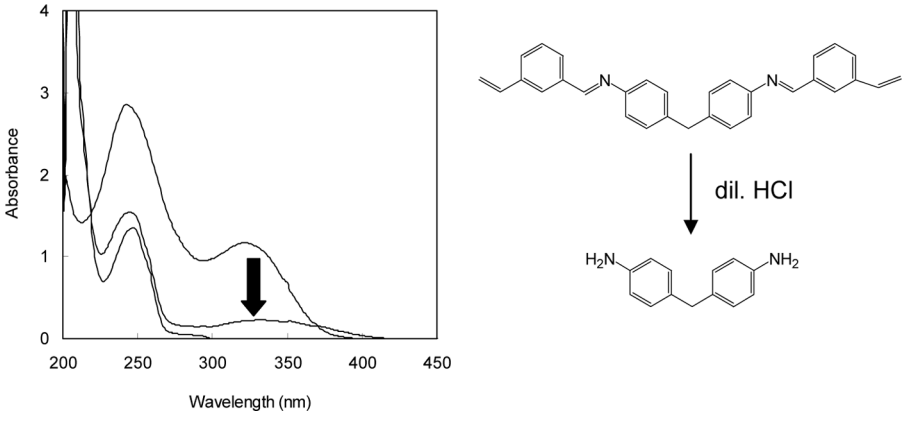

Figure 2. Absorption change by the hydrolysis of VB-DADPM. VB-DADPM (1 mM) was incubated in 1 mM HCl methanolic solution containing 0.1% water at room temperature. The absorbance was measured after the incubation for 0, 3, and 60 min.

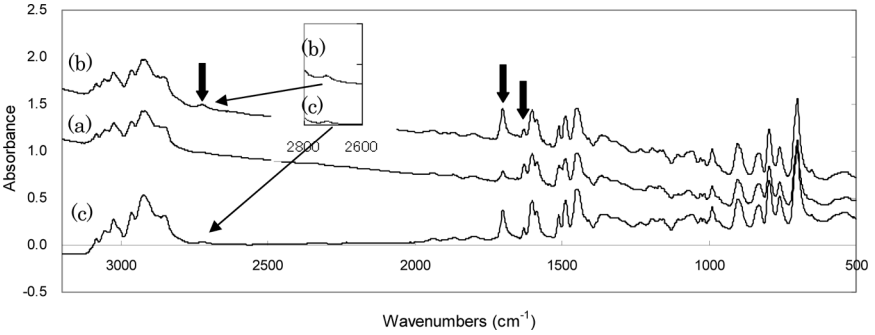

Figure 3. IR spectra of DIP before (a) and after HCl treatment (b) and after the post-imprinting oxidation with H_2O_2(c).

3-vinylbenzoic acid, which was estimated by measuring the decrease of a proton NMR signal corresponding to the aldehyde (data not shown).

The dummy imprinted polymer (DIP) was prepared by cross-linking VB-DADPM with styrene/divinylbenzene. Styrene and divinylbenzene were employed due to their stability under the post-imprinting oxidation, enabling the polymer matrix to maintain its structure. As described, the Schiff base can be cleaved by a weak acid treatment, allowing the DADPM moiety to be easily removed from the resulting polymer, yielding the binding cavity. After the 18 h-Schiff base hydrolysis, the amount of DADPM released from the polymer was determined by LC-MS/MS, and it appeared that approximately 90% of the initial DADPM was extracted from the obtained DIP. The increase of absorption at 1701 cm^{-1} and the appearance of a new peak at 2722 cm^{-1}, corresponding to the stretching vibrations of the carbonyl (C = O) and aldehydic C-H, respectively, in the infrared spectrum of the HCl-treated DIP compared with that of the untreated DIP, confirmed the generation of aldehyde residues during the hydrolytic step. Furthermore, a significant decrease of the C = N stretching frequency at 1630 cm^{-1}, allotted to the imine bonds, is observed after the HCL treatment (Fig. 3). Therefore, although the hydrolysis condition was gentler, the removal ratio was much higher than the BPA-dimethacrylate or BPA-diacrylate - based MIPs previously reported (ca. 60%), where the phenol esters were hydrolyzed by a concentrated sodium hydroxide solution (Ikegami et al. 2004a,b; Ikegami, Mukawa et al. 2004; Ikegami et al. 2004).

The post-imprinting oxidation was carried out to transform the aldehyde residues to carboxylic acids. This technique has been previously proposed by our group provide specific binding sites only inside the imprinted cavities in MIPs (Mukawa et al. 2002; Takeda et al. 2009; Takeuchi et al. 2006; Sunayama, Ooya, and Takeuchi 2010). After H_2O_2 treatment for 18 h, a peak corresponding to the aldehyde group decreased.

Analysis of Binding Properties and Selectivity

In order to investigate the adsorption activity of the DIP, a binding isotherm of BPA toward DIP was drawn to calculate binding constants (Fig. 4). The binding

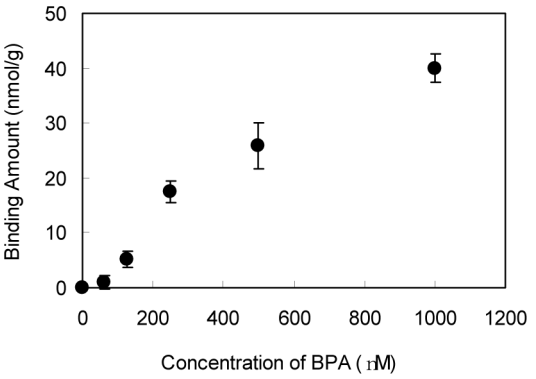

Figure 4. Binding isotherm of BPA for DIP. The DIP was incubated with various concentrations of BPA in Toluene at 25°C for 18 h. Error bars represent the standard deviations ($n = 3$).

constant was estimated to be $1.3 \times 10^6\,M^{-1}$ by a curve-fitting software (Deltagraph) using a nonlinear least squares algorithm with one to one complex formation basis, revealing that high binding activity was generated via the imprinting process.

Selectivity tests of DIP were conducted using BPA, BPB, and 17β-estradiol, where BPB is a structurally related compound with a slightly different molecular size, and 17β-estradiol was chosen due to a natural ligand toward estrogen receptors. The BPA could interact with the receptors, resulting in estrogen-like activities. Figure 5 shows the selectivity of DIP and NIP for BPA, BPB, and 17β-estradiol. The relative binding activity, where the amount of BPA bound in the polymer was set as unity, was used to examine the selective binding activity. As can be seen, BPA bound to the DIP more strongly than BPB, suggesting that only one carbon

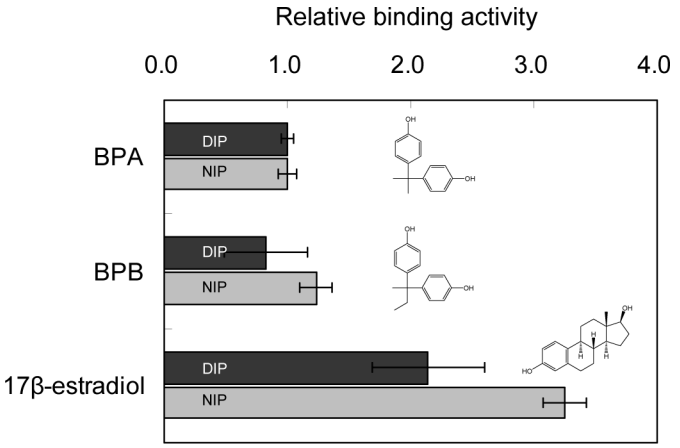

Figure 5. Relative binding activity of DIP and NIP for BPA, BPB, and 17β-estradiol for DIP (black) and NIP (gray). Polymer (3 mg) was incubated with 1 mL of each sample (500 nM) in toluene. Relative binding activity was calculated by dividing the amount of bound sample by the amount of BPA bound. Error bars represent the standard deviations ($n = 3$).

number difference can be recognized by the imprinted binding cavity. These results selectivity was induced by the imprinting effect. Furthermore, the NIP showed no selectivity on BPA; that is, it bound BPB stronger than BPA. It should be noted that the number of benzoic acid moieties in NIP may be greater than that in DIP, since NIP was prepared with the equivalent theoretical amount of 4-vinyl benzoic acid, while the amount of benzoic acid groups in DIP may change according to the removal ratio of DADPM and the yield following oxidation for the generation of carboxylic acid from aldehyde, thus impeding accurate calculation. Concerning 17β-estradiol, strong non-specific binding was observed for both DIP and NIP. The mechanism remains unclear.

Calculated log P values (octanol-water partition coefficient) of BPA, BPB, and 17β-estradiol were 3.76, 3.89, and 4.27, respectively, which were given by PALLAS 3.0 (CompuDrug Chemistry Ltd.). The order of log P values is inconsistent with the order of specific binding activity; consequently, the observed binding may not be attributed to hydrophobic interaction as a main force. It can be considered that a carboxyl group is a better hydrogen donating group than alcoholic and phenolic OH groups, while an alcoholic OH is also a better hydrogen accepting group than phenolic OH. Therefore, a combination of carboxyl group with alcoholic OH group may result in somewhat stronger hydrogen bonding formation than that with the phenolic OH group, leading to the observed non-specific binding. Because of the imprinting effect, the non-specific binding of 17β-estradiol appeared to be suppressed in DIP.

CONCLUSION

The VB-DADPM was designed in order to synthesize the DIP capable of BPA recognition. In ordinary MIPs prepared using target molecules covalently linked with functional monomers, the removal rate of co-polymerized template molecules is not always high. In this study, an easily cleavable imine bond was employed to connect the dummy template molecule, which was structurally related to BPA, with the functional monomer. After the easy removal of the dummy template moiety, post-oxidation was carried out to generate binding sites for BPA located only inside the imprinted cavity. The imprinted cavities could recognize the difference between similar molecules BPA and BPB, the latter of which has an ethyl group instead of a methyl group at the central part of BPA. A combination of the proposed method with controlled/living radical polymerization techniques should provide further enhancement of usability and reliability of MIPs/DIPs in practical use.

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