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Removal of Estrogenic Pollutants from Contaminated Water Using Molecularly Imprinted Polymers

ZIHUI MENG,† WILFRED CHEN, AND ASHOK MULCHANDANI*

Department of Chemical and Environmental Engineering, University of California, Riverside, California 92521

A synthetic molecularly imprinted polymer (MIP) sorbent for estrogenic compounds was prepared using a noncovalent imprinting technique. MIP microspheres sized from 1 to 2 μm were synthesized in acetonitrile by using α-estradiol as the template, acrylamide as the functional monomer, and trimethylpropanol trimethacrylate as the cross-linker. When compared with the nonimprinted polymer (NIP), the MIP showed outstanding affinity toward α-estradiol in aqueous solution with a binding site capacity (B_{max}) of 380 nmol mg^{-1} MIP, imprinting effect of 35, and a dissociation constant (K_d) of 38 μM. The MIP exhibited significant binding affinity toward other related estrogenic compounds such as β-estradiol, diethylstilbestrol, estriol, and estrone, suggesting that this material may be appropriate for treating a complex mixture of estrogenic pollutants. The feasibility of removing estrogenic compounds from environmental water by the MIP was demonstrated using lake water spiked with α-estradiol. In addition, the MIP reusability without any deterioration in performance was demonstrated for at least five repeated cycles.

Introduction

During recent years, estrogenic compounds such as nonylphenol, octylphenol, nonylphenol polyethoxylates, dihydrofolliculin (β-estradiol), estrone, estriol, and ethynylestradiol (α-estradiol) have increasingly been found in treated domestic wastewater effluents (*1*−*8*). These estrogenic compounds are of great concern because of their potential in altering the normal endocrine function and physiological status of animals and humans (*9*−*12*). Reports of an apparent increase in hormone-dependent cancers and a corresponding decrease in sperm quantity and quality in humans have raised questions about the role of natural and synthetic estrogenic compounds in these trends (*13*−*15*). Natural estrogens are excreted by both humans and animals, and an estimated 10 million cows and 43 million swine excrete a daily mix of 10−30 kg of β-estradiol and 80 kg of α-estradiol in the U.S. (*16*). Increasingly, conjugated estrogens used in the treatment of cancer, hormonal imbalance, osteoporosis, and other ailments are also contributing to the increasing source of estrogen pollution (*17*, *18*). Many of these known estrogenic compounds end up in the aquatic environment via sewage, the discharge of municipal and/or industrial effluents, and agricultural runoff. The Safe Drinking Water Act Amendments of 1995 (Bill No. S.1316) and the Food Quality Protection Act of 1996 (Bill No. P.L. 104-170), which mandate comprehensive screening for estrogenic and antiestrogenic chemicals, are examples of the increasing public concern regarding estrogenic pollutants.

Many municipal wastewater treatment plants can reduce estrogenic compounds to some extent; however, the levels are above the known effective concentrations for fish (*19*−*21*). Ozonation, UV radiation, membrane filtration, reverse osmosis, and activated carbon adsorption are potential treatments that might improve the effectiveness of estrogen removal in municipal wastewater treatment plants (*22*−*25*). However, implementation of these techniques would increase the cost of wastewater treatment. Additionally, these methods have low efficiency for removing α-estradiol. For these reasons, the search for low-cost and highly selective removal methods is still warranted.

Molecular imprinting is a useful technique for the preparation of polymeric materials as specific molecular recognition receptors (*26*, *27*). Molecularly imprinted polymers (MIPs) are prepared by copolymerization of a cross-linking agent with the complex formed from a template and polymerizable monomers that have functional groups specifically interacting with the template through covalent or noncovalent bonds. After the template is removed from the resulting polymer matrixes, binding sites having the size and shape complementary to the template are generated. These molecularly imprinted polymers (MIPs) are synthesized with "tailor-made" binding sites for a template and strongly interact with the template. Therefore, MIPs have been utilized in a variety of separation purposes. The specific binding sites in MIPs have proven to be valuable for a variety of separation purposes, enabling selective removal of the templates from a mixture of closely related compounds, in many instances with binding affinities approaching those demonstrated by antigen−antibody systems.

In this research we describe a simple approach to prepare synthetic receptors for estrogenic compounds using a noncovalent molecular imprinting technique. α-Estradiol, a widely used synthetic estrogen that is generally more stable in water and has greater potency, was used as a model (*8*, *21*, *28*, *29*). The resulting MIPs for α-estradiol were evaluated for the repeated removal of estrogenic pollutants from spiked lake water simulating contaminated environmental water.

Experimental Section

Materials. Acrylamide (AM), α-estradiol, estriol, estrone, diethylstilbestrol, dihydrofolliculin (β-estradiol), 2-hydroxyethyl methacrylic acid (HEMA), itaconic acid (IA), methacrylic acid (MAA), 4-vinylbenzene boronic acid (VBA), and 2,2′-azodi-(2,4-dimethylvaleronitrile) (ABDV) were purchased from Sigma, St. Louis, MO. Trimethylpropanol trimethacrylate (TRIM) was purchased from TCI, Tokyo, Japan. All the other chemicals were of reagent grade.

Preparation of MIPs. Table 1 lists the compositions of prepolymer mixtures for the different MIPs synthesized in this work. For a typical run, 0.5 mmol of template was mixed with 3 mmol of monomer in 40 mL of solvent, followed by the addition of 3 mmol of cross-linker (TRIM) and 0.035 mmol of initiator (ABDV). The prepolymerization mixture was thoroughly purged with nitrogen for 5 min and then polymerized at 4 °C under 360 nm UV irradiation for 15 h. The resulting microspheres were collected by centrifugation, washed extensively with 30 mL of methanol/acetic acid (3/1, v/v) overnight continuously, followed by five washings with

* Corresponding author phone: (951)827-6419; fax: (951)827-5696; e-mail: adani@engr.ucr.edu.

† Current address: Department of Chemical and Environmental Engineering, Beijing Institute of Technology, Beijing, 100081, P.R. China.

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TABLE 1. Compositions of the MIPs for Estrogenic Compounds

MIPs	template (mmol)	monomer (mmol)	cross-linker (mmol)	solvent (mL)
1A	diethylstilbestrol, 0.5	IA, 3	TRIM, 3	2-propanol/glycerol (3/1, v/v), 40
1B	diethylstilbestrol, 0.5	AM, 3	TRIM, 3	MeCN, 40
2	α-estradiol, 0.5	AM, 3	TRIM, 3	MeCN, 40

15 mL of methanol for 1 h, and dried at 45 °C. The corresponding nonimprinted polymers (NIPs) were prepared in the same manner except in the absence of template.

Binding of Estrogenic Compounds. Binding studies were carried out in the deionized water. A preweighed amount of MIP-2 suspended in 0.1 mL of water (containing 1% Triton-100, v/v) was mixed with 0.9 mL of 0.1 mM estrogenic compound solution and incubated at 30 °C while stirring at 300 rpm for 60 min. The polymers were then removed by centrifugation at 13 200*g* for 3 min, and 0.5 mL of supernatant was removed for analysis by HPLC. The binding site capacity (B_{max}) and dissociation constant K_d were determined by fitting the equation $y = B_{max}C_f/(K_d + C_f)$ (where y is the nmoles of target adsorbed per mg of polymer and C_f is the concentration of free target in μM, both at equilibrium) to the binding isotherm data at 25 °C using Prism 4 program from GraphPad Software Inc.

Removal of α-Estradiol from Spiked Lake Water. Lake water collected from the Lake Elsinore, CA was spiked with 0.095 mM α-estradiol. A preweighed amount of MIP-2 suspended in 0.1 mL of water (containing 1% Triton-100, v/v) was mixed with 0.9 mL of contaminated lake water and incubated at 30 °C while stirring at 300 rpm for 1 h. The MIP was then removed by centrifugation at 13 200*g* for 3 min, and 0.5 mL of supernatant was removed for analysis by HPLC.

Regeneration of MIP. A volume of 0.1 mL of 40 mg mL^{-1} MIP-2 (containing 1% Triton-100, v/v) was mixed with 0.9 mL of lake water spiked with 0.075 mM α-estradiol and incubated at 30 °C while stirring at 300 rpm for 30 min followed by centrifugation at 13 200*g* for 3 min to remove the MIP-2. Then, 0.5 mL of supernatant was removed and analyzed by HPLC. The recovered MIP-2 pellet was washed with 1 mL of methanol/acetic acid (9/1, v/v) followed by 4 × 1 mL of methanol, dried in vacuum, and reused for adsorption of α-estradiol.

Detection of Estrogenic Compounds. The concentrations of estrogenic compounds were determined using an HP1100 (Agilent) HPLC with a UV−vis detector and a ZORBAX XDB-C8 column (4.6 × 150 mm). Methanol/H_2O (70/30, v/v) was used as the mobile phase at 1 mL min^{-1} flow rate. The sample size injected was 20 μL, and the detection wavelength was 280 nm.

Result and Discussions

MIP Synthesis. A noncovalent molecular imprinting method was adopted in this research to prepare MIPs for estrogenic compounds. Theoretically, the affinity and imprinting effect of a MIP toward its template depend on the interaction between the template and the functional monomer. Thus, the selection of the proper monomer for a template is crucial for a successful imprinting procedure.

Due to the high toxicity of estrogenic compounds, we initially optimized the functional monomers for the imprinting of estrogenic compounds by using an estrogen analogue, diethylstilbestrol, as the template. Four different monomers, MAA, 2-HEMA, IA, and VBA, were tested using 2-propanol/glycerol (3/1, v/v) as a solvent which can dissolve all the monomers. Independent of the monomer employed, the affinity of all the MIPs was very low (<5% of the total amount of template added was adsorbed) in organic solvent (2-propanol/glycerol, 3/1, v/v). Only MIP-1A using IA as the functional monomer showed a clear imprinting effect in deionized water with a slightly higher level of diethylstilbestrol binding than that of the NIP-1A (Figure 1A), while the MIPs utilizing other monomers did not show an imprinting effect (data not shown). The overall imprinting effect B_{max}(MIP)/B_{max}(NIP) of 1.6 for MIP-1A (B_{max} is the binding site capacity in nmoles mg^{-1} of polymer) was not improved compared with the α-estradiol-imprinted polymer reported by Dong et al. (*30*). The poor imprinting efficiency can be attributed to (1) the use of alcohol as a solvent, which while ideal for monomer solubility, may interfere with the formation of hydrogen bonds involved in noncovalent imprinting between the template and monomers or/and (2) the nonspecific binding of diethylstilbestrol due to hydrogen bond formation with the two −COOH groups of excessive IA.

As a first step to improve the imprinting effect forward, we replaced the 2-propanol/glycerol with acetonitrile, which is an inert and more convenient solvent for noncovalent imprinting. Although the solubility of IA in acetonitrile was poor, the new MIP exhibited slightly improved affinity as it adsorbed 80% of the added diethylstilbestrol within 30 min. However, the imprinting effect was still low (data not shown) as the corresponding NIP also adsorbed a significant amount of diethylstilbestrol (73%). We next turned to replacing the IA with a moderate monomer, AM, which has only one $-CONH_2$ group to form hydrogen bonds with the template. As shown in Figure 1B, nonspecific binding of diethylstilbestrol by NIP-1B was very low (<10%) with a B_{max} value of 59 nmol mg^{-1}, while MIP-1B adsorbed a significant amount of diethylstilbestrol (>80%) with a B_{max} value of 260 nmol mg^{-1}, corresponding to an imprinting effect of 4.4.

MIP-2 Binding Isotherm for α-Estradiol from Deionized Water. With the use of the optimized conditions established

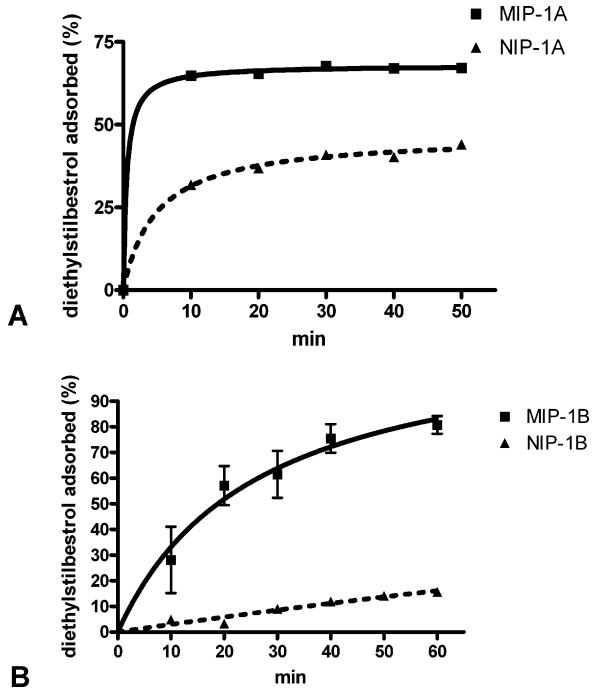

FIGURE 1. Time profiles of diethylstilbestrol (0.1 mM) binding by 1 mg mL^{-1} polymer from deionized water: (A) MIP-1A (■) and NIP-1A (▲); (B) MIP-1B (■) and NIP-1B (▲).

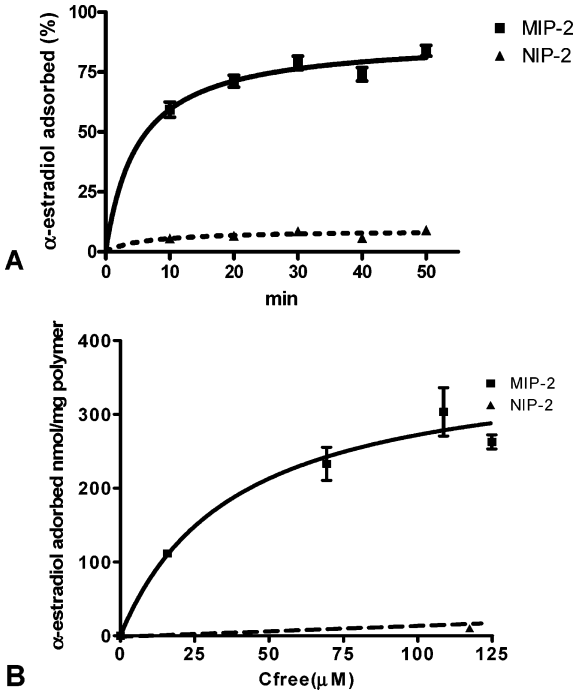

FIGURE 2. (A) Time profiles of α-estradiol (0.1 mM) binding by 1 mg mL^{-1} MIP-2 (■) and NIP-2 (▲) from deionized water. (B) Binding isotherm of MIP-2 (■) and NIP-2 (▲) for α-estradiol from deionized water.

for the preparation of MIP for diethylstilbestrol, MIP for α-estradiol was prepared using AM as the functional monomer and acetonitrile as the solvent. This new MIP (MIP-2) had high affinity toward α-estradiol in deionized water, adsorbing more than 80% of the α-estradiol within 50 min (Figure 2A). In comparison, NIP-2 adsorbed less than 10% of the α-estradiol in the same time period. A B_{max} value of 380 nmol mg^{-1} for α-estradiol was obtained for MIP-2 (Figure 2B), while the B_{max} value of NIP-2 was 11 nmol mg^{-1}. This result demonstrated the outstanding imprinting effect of ~35 representing a negligible amount of nonspecific binding sites on MIP-2. In addition to the good binding capability, a dissociation constant K_d of 38 μM demonstrated the strong affinity between MIP-2 and α-estradiol. When compared to MIPs for estrogenic compounds reported in the literature, the MIP-2 has 300- to 600-fold higher binding capacity and 31- to 88-fold better imprinting effect even in aqueous medium as opposed to adsorption from organic or aqueous−organic mixtures (*30*−*36*). In addition, unlike the typical amorphous MIP particles obtained from the grinding of bulk polymers, the MIPs obtained by our method are microspheres of 1−2 μm diameter with a narrow size distribution providing excellent surface areas for binding and easy handling. Moreover, the MIP microspheres in our method could be directly synthesized without further processing in contrast to a complicated multistep procedure of swelling and polymerization reported previously to synthesize 2−4.7 μm diameter spherical MIPs for estrogenic compounds (*37*, *38*).

MIP-2 Cross-Reactivity. The binding of MIP-2 for α-estradiol was compared to several natural and synthetic related phenolic steroids, whose structures are depicted in Figure 3, which have the same general structure but differ in their functionality in the 3- and 17-positions. Because of our main interest in applying the resulting MIPs to water remediation applications, we evaluated the selectivity of MIP-2 to other estrogenic compounds in an aqueous environment as opposed to nonaqueous or aqueous−organic mixtures conventionally used in the literature for selectivity evaluation by a MIP-packed HPLC column (*31*, *35*−*38*). As shown in Figure 3, besides binding α-estradiol, the MIP-2 also adsorbed other estrogenic compounds. A comparison of the MIP binding to NIP suggests that while diethylstilbestrol and estriol binding is specific to the template created by α-estradiol, β-estradiol and estrone binding is to the polymer matrix and hence nonspecific. The latter is due to the hydrophobic interaction between the compound and the polymer. While the compromised selectivity may be undesirable for the application of the MIP-2 for sensors, this could be actually an advantage in water treatment because different kinds of estrogenic pollutants can also be removed efficiently.

MIP-2 Binding Isotherm for α-Estradiol from Lake Waters. The feasibility of applying MIP-2 for removing estrogenic pollutants from polluted environmental water was evaluated by comparing the adsorption isotherm for removal of α-estradiol spiked in water from Lake Elsinore, CA to that from deionized water. The B_{max} and K_d for the adsorption of α-estradiol by MIP-2 were 76 nmol mg^{-1} and 4.4 μM, respectively, which were 5- and 9-fold lower when compared to adsorption from deionized water. The decline of the MIP-2 binding affinity for α-estradiol in untreated water compared to that of deionized water may be attributed to the higher pH (9 in comparison to the 5.5) and high content of organics such as chlorophyll (*39*). The MIP-2 binding characteristics, however, were still far superior to the many MIPs for EDCs reported in the literature (*30*−*36*) and should be sufficient for removing EDCs present at several nanograms per liter from untreated environmental waters (*1*).

MIP-2 Regeneration/Reuse. Experiments were performed to determine if the estrogenic compounds bound to the MIP can be desorbed/released and whether the MIP can be reused in a new estrogenic compound removal experiment. As shown in Figure 4, the MIP-2 can be regenerated after having the bound α-estradiol removed by washing and retained their

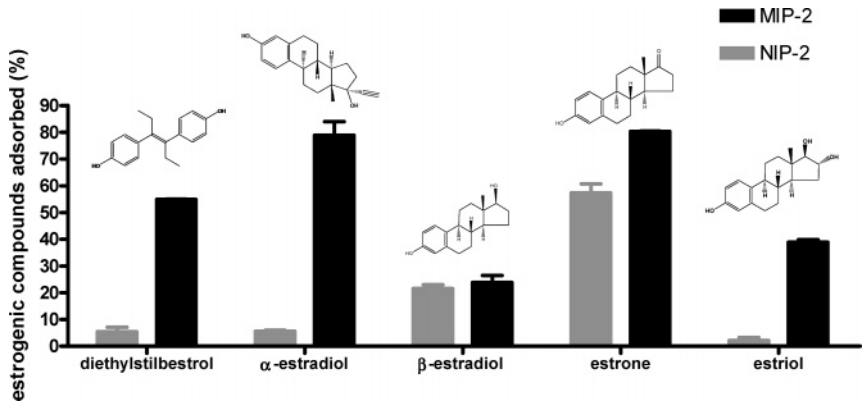

FIGURE 3. MIP-2 selectivity. Estrogenic compound (%) adsorbed by 1 mg mL^{-1} MIP-2 and NIP-2 from a 0.1 mM solution of estrogenic compound in deionized water in 30 min.

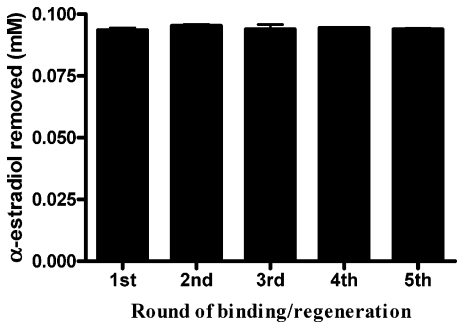

FIGURE 4. MIP-2 regeneration cycles in spiked Lake Elsinore water.

removal efficiency for at least five binding/removal (regeneration) cycles upon treatment with methanol/acetic acid (9/1, v/v) followed by methanol. The demonstrated reusability of the MIP over several adsorption/desorption cycles is an advantage over single-use activated carbon.

In conclusion, we have shown, for the first time, the possibility of generating MIPs for the removal of estrogenic pollutants from wastewaters. The MIPs developed in this research exhibited outstanding affinity and can be reused for at least five rounds without any loss of performance. Besides water remediation applications, the MIPs developed in this research can also be applied for solid-phase extraction to improve the detection limit of estrogenic pollutants or as selective coatings for quartz crystal microbalance (QCM) sensors to monitor trace amounts of estrogenic compounds in pharmaceuticals, body fluids, and environmental samples. These applications are currently under investigation.

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