DOI:10.1021/jf900505k

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

# Development of a Biomimetic Enzyme-Linked Immunosorbent Assay Method for the Determination of Estrone in Environmental Water using Novel Molecularly Imprinted Films of Controlled Thickness as Artificial Antibodies

Shuo Wang,*,† Zhixiang Xu,†,§ Guozhen Fang,† Yan Zhang,† Bing Liu,† and Huaping Zhu†

†Key Laboratory of Food Nutrition and Safety, Ministry of Education of China, Tianjin University of Science and Technology, Tianjin 300457, China, and §College of Food Science and Engineering, Shandong Agricultural University, Taian 271018, China

We developed a fast and new direct competitive biomimetic enzyme-linked immunosorbent assay (BELISA) method for the determination of estrone in environmental water based on a novel molecularly imprinted film of controlled thickness as an artificial antibody. The imprinted film was directly synthesized on the well surface of MaxiSorp polystyrene 96-well plate by a room temperature ionic liquid-mediated chemical oxidative polymerization in conjunction with molecular imprinting technology. This novel film was characterized, and results showed that it exhibited an antibody-like binding ability, rapid adsorption speed, high stability, and hydrophilicity, which was particularly advantageous and suitable for BELISA development. This BELISA method had a higher selectivity for estrone than for the structurally related compounds, and competitive binding studies demonstrated various degrees of cross-reactivity with five estrogenic compounds ranging from 30 to 47%. Eighty minutes of analysis time was reduced when compared to that of traditional ELISA, while the imprinted film was able to be reused for more than 50 times without loss of sensitivity. The $IC_{50}$ (calculated as the concentration giving 50% inhibition of color development) and the detection limit values under optimized experimental conditions were 200 ± 40 $\mu$g $L^{-1}$ and 8.0 ± 0.2 $\mu$g $L^{-1}$, respectively. This developed method was applied to the determination of estrone in spiked environmental water samples with excellent recoveries ranging from 80 to 95%, and the results correlated well with that obtained using the high performance liquid chromatography method.

**KEYWORDS: Imprinted polymer film; artificial receptor; biomimetic enzyme-linked immunosorbent assay; controlled thickness**

## INTRODUCTION

Endocrine-disrupting compounds (EDCs) are environmental contaminants that can disturb normal endocrine function even at low levels (1−10 ng $L^{-1}$ for estradiol) and have become a new concerned area of environmental science (*1*). EDCs mainly include natural and synthetic hormones, phytoestrogens, estrogen mimics, and a wide variety of organic pollutants. Estrone (**Figure 1**) as one of the natural estrogenic hormones can be toxic and carcinogenic and has been identified as the major contributors to the endocrine disrupting activity observed in environmental water samples (*2*). In order to prevent the uncontrolled effects on human health and the deleterious effects on the aquatic environment, trace estrone needs to be monitored; therefore, an accurate and reliable analytical method for the determination of estrone in the environment is required.

Many efforts have been devoted to the development of analytical methodologies for the detection of the estrogenic compounds in environmental samples in the last few decades (*3*, *4*). The analytical protocols based on gas chromatography, high performance liquid chromatography or coupled to mass spectrometry, and enzyme-linked immunosorbent assay (ELISA) have been reported (*5*−*8*). Among these methods, ELISA is one of the most commonly used methods as it is sensitive and selective, can run many analyses simultaneously, and does not require skilled workers (*9*). However, the high cost and difficulties associated with antibody production, together with the need to use laboratory animals, are often considered problems. In addition, the production of antibodies for toxic compounds or immunosuppressants is particularly difficult because of their adverse action on metabolism and the immune system (*10*).

Some studies have attempted to replace antibodies with more stable counterparts and have been searching for ways to design and synthesize artificial antibody-like receptors. One of the most promising approaches is using the molecular imprinting technique to prepare such mimics. The resulting molecularly imprinted polymers (MIPs) not only possess binding properties that often rival those of antibodies and display good selectivity and specificity

*Corresponding author. Tel: (86 22) 6060 1456. Fax: (86 22) 6060 1332. E-mail: s.wang@tust.edu.cn.

 Published on Web 05/18/2009 © 2009 American Chemical Society

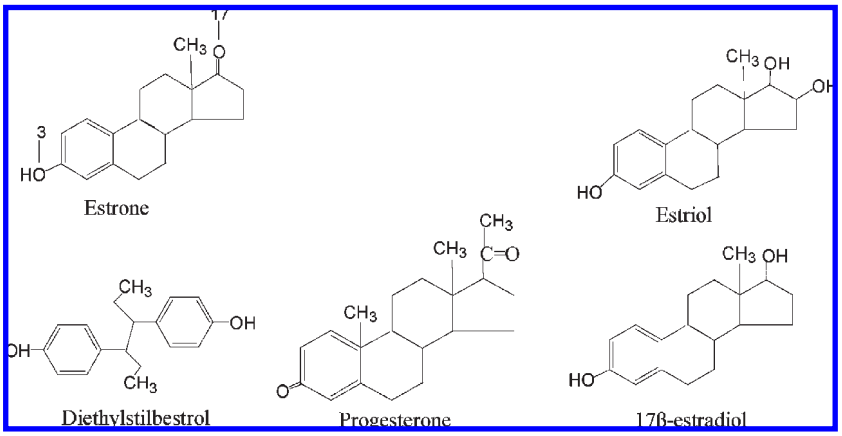


**Figure 1.** Chemical structures of estrone, estriol, diethylstilbestrol, progesterone, and 17$\beta$-estradiol.

for various analytes of medical, environmental and industrial interest (*11*−*13*) but also exhibit far greater physiochemical stability and applicability in harsh chemical media (*14*), making them suitable for use in immunoassay-like analysis as an artificial antibody.

During the past few years, interest in MIPs and their use as biological receptor mimics has seen a considerable increase. The first molecularly imprinted immunoassay was based on a competitive radioligand-binding measurement (*15*). This format is analogous to the solid-phase radioimmunoassay, except that the immobilized antibody is replaced with a MIP. Other assays were later developed using the same principle (*10*, *16*−*20*). However, in comparison with the immunoassay using biological antibodies as selective recognition elements, MIP-based ELISA has several shortcomings and seems to be less practical in real analysis (*21*−*24*). First, enzymes often only work in aqueous buffers, whereas because MIPs are traditionally prepared using an organic-polymer-based system, the synthesis and use of many imprinted polymers is restricted to organic solvents. Second, because most of the used MIPs were polymer particles or microspheres, the rather hydrophobic nature and highly cross-linked structure of the MIPs limit the access of large protein molecules such as enzymes to the imprinted binding sites. Third, in the reported MIP-based immunoassay, the MIP was removed by centrifugation after incubating, and the supernatant was withdrawn for colorimetric measurements, which are intricate, and it is difficult to eliminate the interference of nonconjugated enzyme. Thus, the further development of ELISA using MIP as the antibody has remained challenging.

For application in ELISA, a large surface area, appropriate wetting properties, and easily accessible binding sites are very important for MIPs. This usually requires a MIP that has been prepared in a film format, and it is often necessary to control the thickness of the film in order to optimize the sensitivity of the MIP-based immunoassay (*25*). Several approaches relying on bulk-polymerization techniques have been proposed to in situ generate MIP films, resulting in the poor control of film thickness and uniformity (*26*). Recently, there has been a growing interest in synthesizing MIPs in the form of thin films on surfaces (*17*, *18*, *27*), offering the potential to be used for immunoassay.

However, the traditional MIP may shrink or swell when exposed to different mobile phases, and the various degrees of swelling in different solvents may considerably change the morphology of the polymer network and the relative positions of the functional groups that are essential for recognition. Room temperature ionic liquids (RTILs) are interesting solvents with unique characteristics. The low vapor pressure of RTILs could assist in reducing the problem of MIP bed shrinkage and can also act as the pore template in polymerization reactions (*28*, *29*). RTILs have been shown to accelerate the synthesis process and improve the selectivity and adsorption of *trans*-asconitic acid imprinted organic polymers (*30*, *31*).

The objective of this work was to develop a fast and new direct competitive biomimetic enzyme-linked immunosorbent assay (BELISA) method using a novel molecularly imprinted film of controlled thickness as the artificial antibody. This novel film was directly synthesized on the well surface of a MaxiSorp polystyrene 96-well plate by a room temperature ionic liquid-mediated, chemical oxidative polymerization in conjunction with molecular imprinting technology in order to overcome the drawbacks previously described. The parameters affecting the performance of this BELISA method were discussed in detail. This developed method was applied to the analysis of estrone in environmental water samples, and the results were validated by the HPLC method.

## MATERIALS AND METHODS

**Materials and Reagents.** Estrone, estriol, and carboxymethoxylamine hemihydrochloride (CMHD) were purchased from Fluka (Sigma-Aldrich, USA). 1-Butyl-3-methylimidazolium hexafluorophosphate ($BMIM^+PF_6^-$) was obtained from Henan Lihua Pharmaceutical Co., Ltd. (Henan, China). Diethylstilbestrol, progesterone, 17$\beta$-estradiol, 3-aminophenylboronic acid (APBA), acrylamide, and ethyleneglycol dimethacrylate were purchased from Sigma (Sigma-Aldrich, USA). Ammonium persulfate ($(NH_4)_2S_2O_8$), dimethyl sulfoxide (DMSO), toluene, methanol, ethanol, sodium bicarbonate ($NaHCO_3$), potassium hydroxide (KOH), and ethyl acetate were purchased from Tianjin Chemical Reagent Factory (Tianjin, China).

HPLC grade ethanol was purchased from Merck (Darmstadt, Germany). Horseradish peroxidase (HRP) was purchased from Boehringer-Mannheim (Germany). Reagent grade hydrogen peroxide and Tween 20 were purchased from Sigma (Sigma-Aldrich, USA). Maxisorp polystyrene 96-well plates were obtained from Nunc (Roskilde, Denmark). Dimethylformamide (DMF), *N*, *N*-dicyclohexylcarbodiimide (DCC), *N*-hydroxysuccinate, and other organic chemicals used for hapten synthesis were purchased from Fluka. All reagents were of the highest available purity and at least of analytical grade. The thin layer chromatography (TLC) plate was purchased from Merck (Darmstadt, Germany). Doubly deionized water (DDW, 18 M$\Omega$ $cm^{-1}$) obtained from a Water Pro. water system (Labconco Corp, Kansas City, MO, USA) was used throughout the experiments.

**Solutions.** Phosphate-buffered saline (PBS; 10 mmol L-1 sodium phosphate, 137 mmol $L^{-1}$ NaCl, and 2.7 mmol $L^{-1}$ KCl, pH 7.5), phosphate-buffered saline with 0.05% Tween 20 (PBS/T), and substrate solution (1.25 mmol $L^{-1}$ 3,3′,5,5′-tetramethylbenzidine-1.6 mmol $L^{-1}$ hydrogen peroxide in acetate buffer, pH 5.0) were used.

**Instrumentation.** The high performance liquid chromatographic system consisted of two LC-10ATVP pumps and a Shimadzu SPD-10AVP ultraviolet detector (Shimadzu, Kyoto, Japan). All separations were achieved on an analytical reversed-phase Thermo $C_{18}$ column

(4.6 mm × 250 mm, USA). Class-vp software was used to acquire and process spectral and chromatographic data. Analysis of mass spectra (MS) was carried out on ESI mode on a Survegor-Lcce Advantage Max 10 mass spectrometer (Thermofinnigan, USA).

Immunoassay absorbance was read in dual-wavelength mode (450−650 nm) with a Labsystems 96-well plate reader (Helsinki, Finland). FT-IR spectra (4000−400 $cm^{-1}$) in KBr were recorded using a Vector 22 spectrometer (Bruker). For determination of the adsorption capacity of the novel imprinted and nonimprinted films, a Cary 50-Bio UV spectrometer (Victoria, Australia) was used. A 20.0 KV on a SS-550 scanning electron microscope (Shimadzu, Japan) and a US-501 horizontal shaker (Beijing, China) were used in this study.

**Synthesis of the Novel Molecularly Imprinted Film.** The novel imprinted film was directly polymerized on the 96-well plate wells as follows: 0.135 g of estrone (0.5 mmol) was dissolved in 6.1 mL of DMSO and 1.0 mL of toluene solution, and then mixed with 0.31 g (1.5 mmol) of 3-aminophenylboronic acid and 0.57 mL of $BMIM^+PF_6^-$. After magnetic stirring for 30 min, 100 μL of the mixture was placed in the wells of a 96-well plate and mixed with 100 μL of ($(NH_4)_2S_2O_8$) solution (0.17 M), and the reaction was carried out with shaking (100 times/min) for 20 min at room temperature by a horizontal shaker. After polymerization ended, the 96-well plates were washed three times with deionized water to remove the unreacted reagents and template molecules. The novel imprinted film was first extracted with 300 mL of methanol/acetic acid (9: 1, v/v) for 48 h by an ultrasonic cleaner, followed by 300 mL of methanol for 12 h to be free of estrone, which was verified by detection of the methanol eluent using HPLC. Finally, the product was dried at 40 °C for 2 h. For comparison, the novel nonimprinted film was prepared in the same manner in the absence of template molecule. An imprinted film (film b) was also prepared following the same procedure but without the addition of $BMIM^+PF_6^-$, and a traditional imprinted polymer (film c) was synthesized on the plate wells by a bulk polymerization process using acrylamide as the functional monomers and ethyleneglycol dimethacrylate as the cross-linker.

**Characterization of the Novel Imprinted Film.** To measure the adsorption capacity, the novel imprinted or nonimprinted wells of the 96-well plate were equilibrated with 200 μL of 10% methanol in water solution containing estrone at various concentrations (10−120 mg $L^{-1}$). After the 96-well plates were mechanically shaken (50 times/min) for 60 min at room temperature. The supernatants were measured for the unextracted estrone by a UV spectrometer at 280 nm, and the adsorption capacity ($Q$) was calculated. The adsorption capacity of film b at 100 mg $L^{-1}$ concentration was also determined.

Uptake kinetics of the novel imprinted films by 50 mg $L^{-1}$ estrone was evaluated. After shaking for 5, 10, 20, 30, 40, 60, and 80 min at room temperature, the final concentration of supernatants was determined by UV spectrometry at 280 nm.

To investigate the structure of the novel imprinted film, synthesized with the addition of $BMIM^+PF_6^-$, the bottom of the wells of the 96-well plate coated with three kinds of imprinted polymers were cut from the 96-well plate, and the scanning electron microscopy (SEM) images were observed by a scanning electron microscope.

**Synthesis of Estrone-17-(*O*-carboxymethyl) Oxime.** The method for estrone-17-(*O*-carboxymethyl) oxime synthesis followed that of Li et al. (*32*) and Dean et al. (*33*) (**Figure 2**). Briefly, 0.135 g of estrone and 0.1547 g of carboxymethoxylamine hemihydrochloride were dissolved in 20 mL of freshly distilled ethanol, and then 3 mL of 1.0 mol $L^{-1}$ KOH was added. Following stirring for 8.0 h at 90 °C, the mixture was dried under reduced pressure. The resulting residue was redissolved in water, and the pH of the soultion was adjusted to 8−9 by the addition of $NaHCO_3$ saturation solution. After extraction by ethyl acetate (3 × 30 mL), all of the organic fractions were combined and evaporated to dryness under reduced pressure. The crude product was then recrystallizated by acetone. $R_f$ = 0.70 (methanol/dichloromethane, 1:40). MS, ESI: [M−H] 343 (C20H25O4N, M 344).

**Enzyme Conjugate Preparation.** The method for the synthesis of the active ester was the same as that of estrone-3-hemisuccinate (*8*) (**Figure 2**). To prepare the enzyme conjugates, 12.4 mg of DCC (0.06 mmol) and 3.4 mg of *N*-hydroxysuccinate (0.03 mmol) were added to the active ester solution (1.38 mg of active ester dissolved in 138 μL of dry DMF), and the mixture was stirred for 4 h at room temperature. After centrifugation, 30 μL of supernatant was added slowly to 10 mg of HRP in 2 mL of

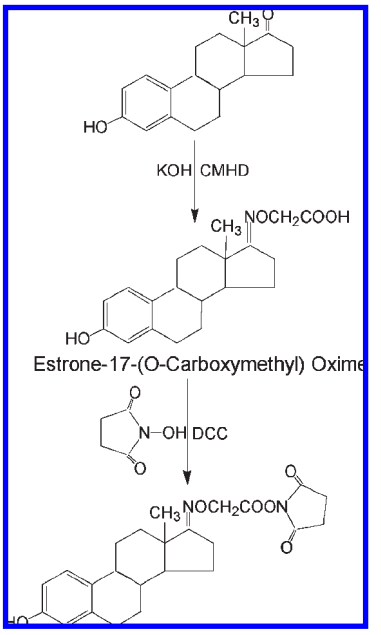


**Figure 2.** Reaction scheme for the synthesis of estrone hapten.

50 mmol $L^{-1}$ $K_2HPO_4$ at 4 °C. The reaction solution was gently mixed by hand and then left at 4 °C overnight. The enzyme conjugated solution was then dialyzed against phosphate buffered saline (pH 7.0) and stored at 4 °C before use.

**Standard Solution Preparation.** For the construction of the calibration curve, six standard solutions containing estrone in 10% methanol within the range of 0.5 μg $L^{-1}$ to 50 mg $L^{-1}$ were prepared freshly in glass tubes. The stock solution (1000 mg $L^{-1}$ in methanol) of estrone was diluted to 1/20, and the solution of 50000 μg $L^{-1}$ (in 10% methanol) was sequentially diluted to give 5000, 500, 50, 5, and 0.5 μg $L^{-1}$.

**Direct Competitive BELISA Procedure.** First, 100 μL of 10% methanol in water was added to the control and blank wells, and 100 μL of standard solution or sample extracts was applied to the allocated wells. Then 100 μL of enzyme conjugate was immediately added to each well, except for the blank wells, and the mixture was incubated for 1.0 h. Following the washing with PBS/T solution five times, 150 μL of substrate solution was added to each well. The reaction was stopped after 30 min by adding 50 μL of 1.25 mol $L^{-1}$ $H_2SO_4$, and absorbances were recorded. Finally, the novel imprinted film was extracted with 300 mL of methanol/acetic acid (9:1, v/v) for 2.0 h by an ultrasonic cleaner, followed with 300 mL of methanol for 1.0 h, for the next BELISA procedure.

**Preparation of Samples.** The lake and river water samples from Dongli Lake and Haihe of Tianjin, China, were taken in February, 2008. The samples were filtered by 0.45 μm filters without any other pretreatment and stored in the precleaned glass bottles at 4 °C prior to ELISA or high performance liquid chromatography (HPLC) analysis. To check the accuracy of the developed method, the samples of lake and river water for spiking were determined to be free of estrone with HPLC. Briefly, 10, 20, or 40 μL of the standard estrone solution (100 mg $L^{-1}$) was added to 10 mL of 10% methanol in lake water solution.

**Selectivity Studies.** Cross-reactivity (CR) studies were carried out by measuring the competitive curves and selectivity properties for other chemically related compounds under the optimized conditions. Cross-reactivity was calculated as the percentage between the $IC_{50}$ value (calculated as the concentration giving 50% inhibition of color development) for estrone and the $IC_{50}$ value for the interfering compound with the following equation:

$$\%CR = \{IC_{50}(\text{estrone})/IC_{50}(\text{cross} - \text{reacting compound})\} \times 100$$

**Sample Analysis and HPLC Validation.** The samples were determined using a HPLC at a mobile flow rate of 1.0 mL $min^{-1}$ under isocratic conditions, and a mixture of methanol/water (61:39, v/v) was used as the mobile phase. Class-vp software was used to acquire spectral and chromatographic data. The injected sample size was 20 μL, and the

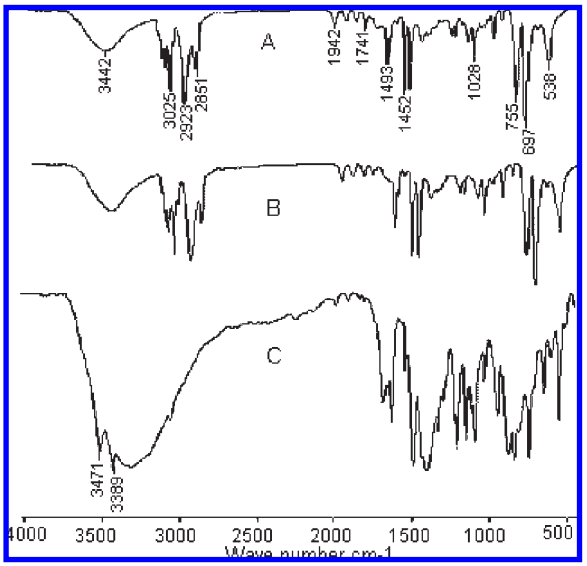


**Figure 3.** FT-IR spectra of 3-aminophenylboronic acid (**A**), imprinted film synthesized without the addition of $BMIM^+PF_6^-$ (**B**), and novel imprinted film synthesized with the addition of $BMIM^+PF_6^-$ (**C**).

UV detection wavelength was 280 nm. All of the analyses were carried out in triplicate.

## RESULTS AND DISCUSSION

**FT-IR Spectra of Imprinted Films and APBA.** To ascertain the presence of APBA in the novel imprinted film, FT-IR spectra of the novel imprinted films that have removal of the template and APBA were compared in **Figure 3**. The observed features around 3389 and 3471 $cm^{-1}$ in FT-IR spectra (**Figure 3A**) indicated $-NH_2$ vibrations of APBA, and that around 3439 $cm^{-1}$ of novel imprinted films (spectra in **Figure 3B**) indicated −NH vibrations. These results demonstrated that the amine groups of APBA had converged to an imine group (*17*), APBA had been reacted with estrone, and the novel imprinted film was successfully synthesized.

These data also suggested that the $BMIM^+PF_6^-$ used did not interfere with the synthesis and recognition of the novel imprinted film and was completely removed from the novel imprinted film after extraction because the main peaks of the $BMIM^+PF_6^-$ were absent (**Figure 3C**), and the FT-IR spectra (**Figure 3C**) and (**Figure 3B**) showed similar locations and appearances of the major bands.

**Characterization of the Novel Imprinted Film.** The isothermal adsorptions of novel imprinted and nonimprinted films are plotted in **Figure 4A**. It was shown that the amounts of the template molecules extracted by the novel imprinted or nonimprinted film increased with increasing estrone initial concentrations. However, the novel imprinted film exhibited a higher adsorption capacity for estrone than the nonimprinted film, and the adsorption capacity of the novel imprinted film (1.3 mg/well) was more than 2-fold that of the nonimprinted film (0.5 mg/well) at a 100 mg $L^{-1}$ concentration. The adsorption capacity of film b was 1.2 mg/well at the same concentration.

Uptake kinetics of estrone by the novel imprinted film was also examined at 50 mg $L^{-1}$ concentration (**Figure 4B**). Results indicated that the prepared novel imprinted film had fast uptake kinetics, 71% of binding was obtained within a short period of 5 min, and the adsorption equilibrium was almost reached within 40 min. If the concentration of estrone was lower, the time to saturation would become shorter. The rapid adsorption kinetics of the novel imprinted film is an obvious advantage for its application in the BELISA.

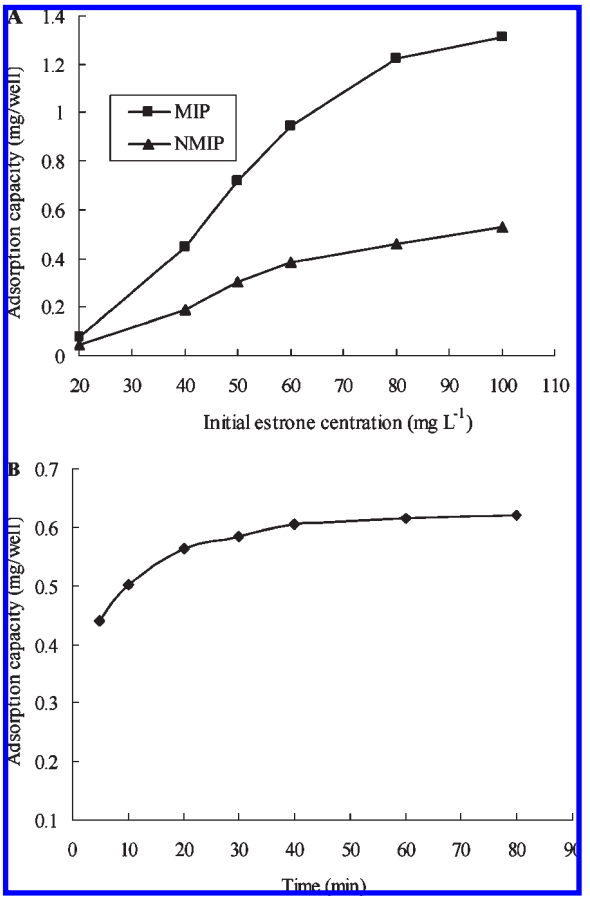


**Figure 4.** Adsorption isotherms of the novel imprinted and nonimprinted films (**A**) and kinetic uptake plot of the novel imprinted film (**B**).

The structure of the imprinted polymer films on the well surface of 96-well plate was visualized using SEM (**Figure 5**). The SEM images revealed that the novel imprinted film and film b synthesized by the chemical oxidative polymerization method exhibited almost identical, relatively smooth and reproducible surfaces (**Figure 5A**,**B**); however, film c synthesized by the bulk polymerization process (**Figure 5C**) was formed as irregular particles. The novel imprinted film was provided with many relatively small through pores (**Figure 5A**) in comparison with that of film b synthesized without the addition of $BMIM^+PF_6^-$ (**Figure 5B**), which may be attributed to the involvement of RTILs in the imprinting process (*30*).

This novel imprinted film was transparent and homogeneous with the binding sites situated at the surface, which may allow easy access for the enzyme conjugate. In the polymerization process, hydrophobic RTILs of $BMIM^+PF_6^-$ were used to reduce the cracking and shrinking of the novel imprinted film and to act as a pore template. Interestingly, it was directly synthesized on the 96-well plate wells, which simplified the operation procedure and was particularly useful for BELISA, and the thickness of novel film can be controlled by polymerization time. Thus, the novel imprinted film possessing good merits and high stability was more suitable as an attractive alternative to the conventional antibody or receptor in ELISA than the particles obtained by grinding a bulk polymer monolith.

**Hapten Selection and Synthesis.** As previous studies have shown, the spatial arrangement, nature, and size of the enzyme conjugate must be tailored to the template structure to achieve efficient competition for the MIP binding sites. The labeled conjugate showing the best performance in a MIP-based

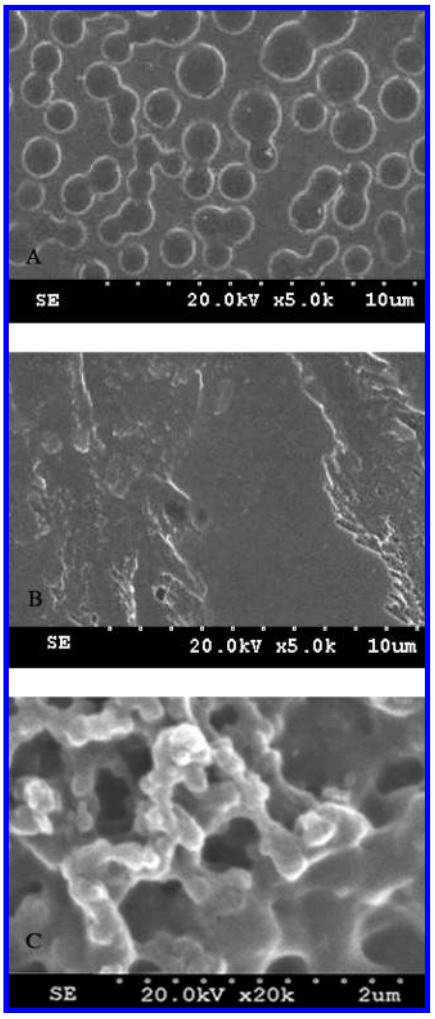


**Figure 5.** SEM images of novel imprinted film synthesized with the addition of $BMIM^+PF_6^-$ using a chemical oxidative polymerization process (**A**) (5000×), imprinted film (film b) synthesized without the addition of $BMIM^+PF_6^-$ using a chemical oxidative polymerization process (**B**) (5000×), and imprinted film (film c) synthesized without the addition of $BMIM^+PF_6^-$ by a bulk polymerization process, using acrylamide as functional monomers and ethyleneglycol dimethacrylate as the cross-linker (**C**) (20000×).

assay was also the one providing the highest sensitivity in an immunoassay based on the same type of measurements.

A significantly different binding between a HRP-labeled conjugate and HRP to the novel imprinted film clearly indicated that hapten played an essential role in the specific recognition of MIP in the BELISA. Estrone (**Figure 1**) contains one reactive phenolic group, ideal for chemical modification to produce a hemisuccinate derivative with a terminal carboxyl group for conjugating to the enzyme. However, the binding affinity and recognition ability of the template molecule by the imprinted polymer relied on not only the imprinted cavities but also the template-specific binding sites, and the phenolic group of estrone has an important effect on the specificity of the assay as it reacted with the monomer in the molecularly imprinted polymerization process.

In order to obtain higher sensitivity for the developed BELISA, the hapten was synthesized by reaction of the ketyl group of estrogen with the carboxymethoxylamine hemihydrochloride (**Figure 2**). The carboxylic acid hapten was converted to the active ester for the coupling of hapten to enzyme. The estrone-17-(*O*-carboxymethyl) oxime ester obtained was compatible with enzymes, making an HRP-labeled conjugate possible.

**Table 1.** $IC_{50}$ and Cross-Reactivity Values for Five Estrogenic Compounds with the Developed BELISA Method (Mean ± S.D., $n = 3$)

| compounds | $IC_{50}$ ($\mu g\ L^{-1}$) | CR (%) |
|---|---|---|
| estrone | 200 ± 40 | 100 |
| 17$\beta$-estradiol | 430 ± 50 | 47 |
| estriol | 470 ± 40 | 43 |
| progesterone | 670 ± 70 | 30 |
| diethylstilbestrol | 500 ± 90 | 40 |

**Condition Optimization.** The applicability of the developed BELISA method using the novel imprinted film as an artificial antibody for the determination of estrone was evaluated. The preparing solvent and thickness of the novel imprinted film were optimized to achieve good sensitivity and precision.

The solvent used in the preparation of standard solutions and samples can affect the BELISA. In order to investigate the influence on assay performance, the methanol solutions at 5, 10, and 20% in water were tested for their effects by comparing the standard curves. Methanol at 10% had higher assay sensitivity, and the sensitivity was reduced when the concentration of methanol was more than 10%. Thus, 10% methanol aqueous solution was chosen as the preparing solvent for the routine analysis of estrone in water samples.

As previously reported for the immunoassay, different concentrations of immobilized antibodies in the 96-well plate well may have a direct effect on the sensitivity of the direct competitive ELISA (*8*). The effect of the novel imprinted film thickness and polymerization time on the assay sensitivity was also investigated in the present study. Results indicated that lower $IC_{50}$ values ($200 \pm 40\ \mu g\ L^{-1}$) and optimized competitive binding between estrone and the HRP conjugate were observed when the 96-well plate was coated with this novel imprinted film for 20 min.

**Cross-Reactivity.** The specificity of the developed BELISA was evaluated in the presence of other structurally related compounds. Cross-reactivity was calculated as the percentage between the $IC_{50}$ value for estrone and the $IC_{50}$ value for the interfering compound, and the results are depicted in **Table 1**.

The novel imprinted film had a higher selectivity for estrone than other related estrogenic compounds, and different cross-reactivities were obtained with 17$\beta$-estradiol (47%), estriol (43%), diethylstilbestrol (40%), and progesterone (30%). This may have resulted from the imprinting effect, the difference of the molecular interactions, and their structures. During the preparation of the novel imprinted film, the boronic acid moiety of APBA reacted with phenolic groups of estrone and the polyaniline complex formed. After removal of the estrone, the imprinted cavities and specific binding sites for the phenolic groups in a predetermined orientation were generated.

However, compared with the biological antibody, relatively high cross-reactivities for all the tested analogues, especially 17$\beta$-estradiol, estriol, and diethylstilbestrol, were observed in this study, which resulted from the nonspecific binding of the novel imprinted film.

We also observed that the novel imprinted film had higher selectivity for 17$\beta$-estradiol than for estriol, diethylstilbestrol, and progesterone as the structure of 17$\beta$-estradiol was more closely related to the structure of estrone than the other three estrogenic compounds (**Figure 1**). Hence, the structure and the binding sites of the template molecules all play important roles in the recognition of the polymer and the competition reaction of BELISA.

**Analytical Characterization.** The normalized competition curves obtained using the novel imprinted film and nonimprinted film as the artificial antibody for estrone standards at concentrations from 50000 $\mu g\ L^{-1}$ to 0.5 $\mu g\ L^{-1}$ in 10% methanol aqueous

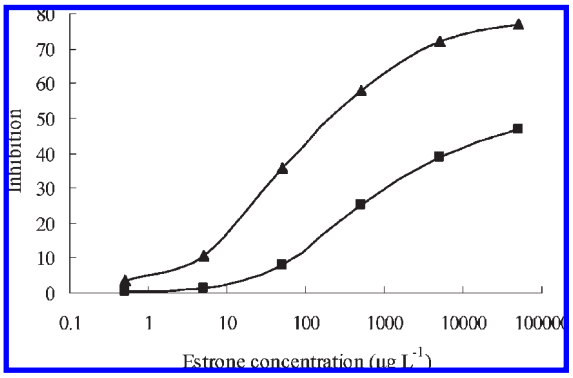


**Figure 6.** Estrone BELISA standard curves using the novel imprinted film as the antibody (▲) and the novel nonimprinted film as the antibody (■).

solution can be seen in **Figure 6**. In the competitive assay format, free estrone effectively competed with the HRP conjugate for the binding sites in the novel imprinted film surface (78% inhibitor), whereas only 46% inhibitor was obtained with the nonimprinted film due to nonspecific adsorption, and 74.9% inhibitor was achieved when using film b for the substitute receptor. Thus, a better BELISA result was achieved by using the novel imprinted film as the artificial antibody.

Under optimized experimental conditions, the $IC_{50}$ of estrone standard curves was 200 ± 40 $\mu g\ L^{-1}$, and the limit of detection (LOD), calculated as the concentration of standard solution causing 15% inhibition of color development, was 8.0 ± 0.2 $\mu g\ L^{-1}$. These values were much lower than those obtained using the same labels and methacrylic acid- or 4-vinylpyridine-based MIP as an artificial antibody (*20*). This method is relatively insensitive, and the LOD for the analysis of estrone was only 100 times higher than that obtained using polyclonal antibodies against estrone as the recognition element (*8*, *34*). Because there was no antibody coating and BSA/PBS blocking procedure, 80 min of analysis time was reduced in this method compared with that in traditional ELISA. Furthermore, the working life of the novel imprinted film had a reusability of more than 50 times of cycles without the loss of sensitivity, where the $IC_{50}$ was 230 ± 90 $\mu g\ L^{-1}$ and LOD was 8.1 ± 0.6 $\mu g\ L^{-1}$. Thus, the cost per analysis of the BELISA method was drastically reduced, and it was more suitable for rapid detection.

**Sample Analysis and Accuracy of the BELISA.** In an attempt to evaluate the suitability and applicability of the optimized assay for the measurement of real samples, the lake and river water spiked at 100, 200, and 400 $\mu g\ L^{-1}$ were determined (with three replicates for each concentration). The analytical data are shown in **Table 2**. A good correlation of results was observed with recoveries ranging from 80 to 95%, indicating that there was little matrix effect in the analysis of environmental sample using the developed BELISA without any other sample treatment procedure except filtration.

The accuracy of this BELISA method was validated by comparative analysis of the spiked samples with HPLC (**Table 2**), and no significant differences were observed between the results obtained by both methods.

**Conclusions.** We developed a fast, direct competitive BELISA method using a novel imprinted film of controlled thickness as the artificial antibody. This BELISA method exhibited excellent performance in real sample analysis with a simple matrix dilution step, which has potential for being applied in the quantitative determination of estrone in environment. We also presented a methodology for the application of MIPs in routine immunoassays, although its sensitivity is lower. With the development of molecular imprinting technology, the sensitivity and accuracy of MIP-based immunoassays would be improved, and they can provide an important analysis platform in the future.

**Table 2.** Analysis Results of Spiked Estrone in Lake and River Water Samples by the Developed BELISA and HPLC Methods (Mean ± S.D., $n = 3$)

| sample | spiked level ($\mu g\ L^{-1}$) | HPLC ($\mu g\ L^{-1}$) | BELISA ($\mu g\ L^{-1}$) |
|---|---|---|---|
| lake water | 100 | 89 ± 1 | 82 ± 3 |
| | 200 | 176 ± 1 | 190 ± 4 |
| | 400 | 373 ± 5 | 340 ± 14 |
| river water | 100 | 91 ± 2 | 81 ± 3 |
| | 200 | 180 ± 4 | 188 ± 13 |
| | 400 | 373 ± 3 | 345 ± 21 |

## ABBREVIATIONS USED

BELISA, biomimetic enzyme-linked immunosorbent assay; EDCs, Endocrine-disrupting compounds; ELISA, enzyme-linked immunosorbent assay; MIPs, molecularly imprinted polymers; RTILs, room temperature ionic liquids; CMHD, carboxymethoxylamine hemihydrochloride; $BMIM^+PF_6^-$, 1-butyl-3-methylimidazolium hexafluorophosphate; APBA, 3-aminophenylboronic acid; DMSO, dimethyl sulfoxide; HRP, horseradish peroxidase; DMF, dimethylformamide; TLC, thin layer chromatography; DDW, doubly deionized water; PBS, phosphate-buffered saline; PBS/T, phosphate-buffered saline with 0.05% Tween 20; MS, mass spectra; *Q*, adsorption capacity; SEM, scanning electron microscopy; HPLC, high performance liquid chromatography; CR, cross-reactivity.

## LITERATURE CITED

(1) Salste, L.; Leskinen, P.; Virta, M.; Kronberg, L. Determination of estrogens andestrogenic activity in waste water effluent by chemical analysis and the bioluminescent yeast assay. *Sci. Total Environ*. **2007**, *378*, 343–351.

(2) Sole, M.; de Alda, M. J. L.; Castillo, M.; Porte, C.; Ladegaars-Pedersen, K.; Barcelo, D. Estrogenicity determination in sewage treatment plants and surface waters from the Catalonian area (NE Spain). *Environ. Sci. Technol*. **2000**, *34*, 5076–5083.

(3) Meng, Z. H.; Chen, W.; Mulichandani, A. Removal of estrogenic pollutants from contaminated water using molecularly imprinted polymers. *Environ. Sci. Technol*. **2005**, *39*, 8958–8962.

(4) Wang, S.; Xu, Z. X.; Fang, G. Z.; Zhang, Y.; He, J. X. Separation and determination of estrone in environmental and drinking water by molecularly imprinted cartridge coupled with HPLC. *J. Sep. Sci*. **2008**, *31*, 1181–1188.

(5) Stafiej, A.; Pyrzynska, K.; Regan, F. Determination of anti-inflammatory drugs and estrogens in water by HPLC with UV detection. *J. Sep. Sci*. **2007**, *30*, 985–991.

(6) Lierre, L.; Hill, E. M. Analysis of estrogens in river sediments by liquid chromatography-electrospray ionization mass spectrometry comparison of tandem mass spectrometry and time-of-flight mass spectrometry. *J. Chromatogr., A* **2007**, *1141*, 174–181.

(7) Farré, M.; Kuster, M.; Brix, R.; Rubio, F.; López, M.; Barceló, D. Comparative study of an estradiol enzyme-linked immunosorbent assay kit, liquid chromatography-tandem mass spectrometry, and ultra performance liquid chromatography-quadrupole time of flight mass spectrometry for part-per-trillion analysis of estrogens in water samples. *J. Chromatogr., A* **2007**, *1160*, 166–175.

(8) Li, Z. L.; Wang, S.; Alice Lee, N.; Allan, R. D.; Kennedy, I. R. Development of a solid-phase extraction-enzyme-linked immunosorbent assay method for the determination of estrone in water. *Anal. Chim. Acta* **2006**, *569*, 50–57.

(9) Hage, D. S. Immunoassays. *Anal. Chem*. **1995**, *67*, R455–R462.

(10) Urraca, J. L.; Moreno-Bondi, M. C.; Orellana, G.; Sellergren, B.; Hall, A. J. Molecularly imprinted polymers as antibody mimics in automated on-line fluorescent competitive assays. *Anal. Chem*. **2007**, *79*, 4915–4923.

(11) Wulff, G. Molecular imprinting in cross-linked materials with the aid of molecular templates-away towards artificial antibodies. *Angew. Chem., Int. Ed. Engl*. **1995**, *34*, 1812–1832.
(12) Sellergren, B. Noncovalent molecular imprinting: antibody-like molecular recognition in polymeric network materials. *Anal. Chem*. **1997**, *16*, 310–320.
(13) Haupt, K.; Mosbach, K. Plastic antibodies: developments and applications. *Trends Biotechnol*. **1998**, *16*, 468–475.
(14) Alexander, C.; Andersson, H. S.; Andersson, L. I.; Ansell, R. J.; Kirsch, N.; Nicholls, I. A.; Mahony, J. O.; Whitcombe, M. Molecular imprinting science and technology: a survey of the literature for the years up to and including 2003. *J. Mol. Recognit*. **2006**, *19*, 106–180.
(15) Vlatakis, G.; Andersson, L. I.; Muller, R.; Mosbach, K. Drug assay using antibody mimics made by molecular imprinting. *Nature* **1993**, *361*, 645–647.
(16) Surugiu, I.; Svitel, J.; Haupt, K.; Danielsson, B. Development of a flow injection capillary chemiluminescent ELISA using an imprinted polymer instead of the antibody. *Anal. Chem*. **2001**, *73*, 4388–4392.
(17) Piletsky, S. A.; Piletska, E. V.; Chen, B. N.; Karim, K.; Weston, D.; Barrett, G.; Lowe, P.; Turner, A. P. F. Chemical grafting of molecularly imprinted homopolymers to the surface of microplates. Application of artificial adrenergic receptor in enzyme-linked assay for $\beta$-agonists determination. *Anal. Chem*. **2000**, *72*, 4381–4385.
(18) Surugiu, I.; Danielsson, B.; Ye, L.; Mosbach, K.; Haupt, K. Chemiluminescence imaging ELISA using an imprinted polymer as the recognition element instead of an antibody. *Anal. Chem*. **2001**, *73*, 487–491.
(19) Surugiu, I.; Ye, L.; Ecevit, Y.; Dzgoev, A.; Danielsson, B.; Mosbach, K.; Haupt, K. An enzyme-linked molecularly imprinted sorbent assay. *Analyst* **2000**, *125*, 13–16.
(20) Piletsky, S. A.; Piletska, E. V.; Bossi, A.; Karim, K.; Lowe, P.; Turner, A. P. F. Substitution of antibody and receptors with molecularly imprinted polymers in enzyme-linked and fluorescent assays. *Biosens. Bioelectron*. **2001**, *16*, 701–707.
(21) Bengtsson, H.; Roos, U.; Andersson, L. I. N-Methylidene[bis (trimethylsilyl)methyl]amine: the first isolable and stable monomeric methanimine allowing thermal [2 + 2] cycloadditions with ketenes. *Anal. Commun*. **1997**, *34*, 233–234.
(22) Andersson, L.; Muller, R.; Vlatakis, G.; Mosbach, K. Mimics of the binding sites of opioid receptors obtained by molecular imprinting of enkephalin and morphine. *Proc. Natl. Acad. Sci. U.S.A*. **1995**, *92*, 4788–4792.
(23) Haupt, K.; Dzgoev, A.; Mosbach, K. Herbicide assay using an imprinted polymer based on system analogous to competitive fluoroimmunoassay. *Anal. Chem*. **1998**, *70*, 628–631.
(24) Andersson, L. Application of molecular imprinting to the development of aqueous buffer and organic solvent based radioligand binding assay for (s)-propranolol. *Anal. Chem*. **1996**, *68*, 111–117.
(25) Vandevelde, F.; Belmont, A. S.; Pantigny, J.; Haupt, K. Hierarchically nanostructured polymer films based on molecularly imprinted surface-bound nanorilaments. *Adv. Mater*. **2007**, *19*, 3717–3720.
(26) Jakusch, M.; Janotta, M.; Mizaikoff, B.; Mosbach, K.; Haupt, K. Molecularly imprinted polymers and infrared evanescent wave spectroscopy. A chemical sensors approach. *Anal. Chem*. **1999**, *71*, 4786–4791.
(27) Schmidt, R. H.; Mosbach, K.; Haupt, K. A simple method for spin-coating molecularly imprinted polymer films of controlled thickness and porosity. *Adv. Mater*. **2004**, *16*, 719–722.
(28) Yoo, K.; Choi, H.; Dionysiou, D. D. Ionic liquid assisted preparation of nanostructured TiO2 particles. *Chem. Commun*. **2004**, *17*, 2000–2001.
(29) Liu, Y.; Wang, M.; Li, Z.; Liu, H.; He, P.; Li, J. Preparation of porous aminopropylsilsesquioxane by a nonhydrolytic sol-gel method in ionic liquid solvent. *Langmuir* **2005**, *21*, 1618–1622.
(30) Wang, H. F.; Zhu, Y. Z.; Yan, X. P.; Gao, R. Y.; Zheng, J. Y. A room temperature ionic liquid (RTIL)-mediated, non-hydrolytic sol−gel methodology to prepare molecularly imprinted, silica-based hybrid monoliths for chiral separation. *Adv. Mater*. **2006**, *18*, 3266–3270.
(31) Booker, K.; Bowyer, M. C.; Holdsworth, C. I.; McCluskey, A. Efficient preparation and improved sensitivity of molecularly imprinted polymers using room temperature ionic liquids. *Chem. Commun*. **2006**, *16*, 1730–1732.
(32) Li, J.; Liu, Z. X.; Zhou, L. Synthesis of 17$\beta$-oestradiol-6-(O-Carboxymethyl) oxime human serum albumin conjugate. *J. South. Nation. Co* **1993**, *19*, 255–263.
(33) Dean, P. D. G.; Exley, D.; Johnson, M. W. Preparation of 17$\beta$-oestradiol-6-(O-Carboxymethyl) oxime-bovine serum albumin conjugate. *Steroids* **1971**, *18*, 593–603.
(34) Wang, S.; Wang, Y.; Huang, W.; Fang, G. Z.; Duan, Z. J.; Qiao, H.; Zhang, Y. Development of a solid-phase extraction-enzyme-linked immunosorbent assay method with a new sorbent of multiwall carbon nanotube for the determination of estrone in water. *Anal. Lett*. **2007**, *40*, 2338–2350.

**Received February 12, 2009. Revised Manuscript Received April 22, 2009. This work was supported by the National Natural Science Foundation of China (project no. 30571532 and no. 20775054) and the Science and Technology Development Foundation of Tianjin Colleges and Universities (project no. 2006ZD19).**