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Electrochemical biosensor for estrogenic substance using lipid bilayers modified by Au nanoparticles

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ABSTRACT

There is a growing demand for new technologies that are capable of screening the wide variety of xenoestrogens in environment. Here, a nanostructure electrochemical biosensor was developed to directly detect and screen estrogenic substances based on estrogen receptor (ER) binding without the use of radio- or enzyme-labeled compounds. The biosensor was fabricated by immobilization of ERs in supported bilayer lipid membrane (s-BLM) modified with Au nanoparticles, and the properties of the modified electrodes were characterized by cyclic voltammetry and impedance spectroscopy. The results indicated that the biosensor was able to detect the natural estrogen 17β-estradiol with an acceptable linear correlation ranging from 5 to 150 ng/L and a detection limit of 1 ng/L. The biosensor could also detect other known xenoestrogens such as bisphenol A and 4-nonylphenol with satisfied sensitivity and quantitative results. The biosensor showed good reliability and repeatability, and the Au nanoparticles greatly enhanced the sensitivity and stability of the biosensor. Moreover, estrogenic activity of water samples determined by this biosensor was in good agreement with that determined by MCF-7 cell proliferation assay.

1. Introduction

Endocrine-disrupting chemicals (EDCs) are a defined category of environmental contaminants, which are persistent in the environment and interfere with endocrine system function (Sumpter, 1998). A major group of EDCs are called xenoestrogens that can exert or prevent estrogenic effects on the human body. They include natural and synthetic steroid-estrogens, which can interact with estrogen receptor and elicit negative effects on endocrine systems in vivo, causing a variety of problems including interference with reproductive, neurologic, and immunologic function, and even carcinogenesis (Harding et al., 2006; Nilsson et al., 2001).

For the above reasons, monitoring of these environmental endocrine disruptors is of vital importance. The methods most commonly used for the determination of xenoestrogens are gas chromatography coupled with mass spectrometry (GC/MS), liquid chromatography with electrochemical detection (LC/ED), and liquid chromatography coupled with mass spectrometry (LC/MS). Although these methods are highly sensitive and specific, however, they are rather complicated, expensive, and are hardly employed for on-site measurement (Kuch and Ballschmiter, 2001; Zhang et al., 2006). The wide variety of xenoestrogens with complex structural diversity requires new technologies that are capable of screening a wide range of chemicals in the field.

Bioassays could provide biologic potency information of either individual congeners or complex mixtures. To date, different types of bioassay for screening estrogenic pollutants based on the binding of estrogenic substance with the ERs have been reported, such as cell proliferation assays (Soto et al., 1995), cell bioluminescent in vitro assays (Pillon et al., 2005), recombinant yeast assay (Nishihara and Nishikawa, 2001; Nishikawa et al., 1999) and luciferase induction (Legler et al., 2002). Such bioassays could determine estrogen substances accurately and effectively, however, there are certain inconveniences in application with either cell-culture involved, or complex aseptic manipulations required, or time consuming.

Compared with other methods, electrochemical biosensor analysis has the following advantages, simplicity, specific recognition and requiring small amount of sample, thus offering advantages over commonly used assays. The supported bilayer lipid membranes (s-BLM) have been widely used in various experimental biomembranes models (Giess et al., 2004; Tien et al., 1997; Janshoff and Steinem, 2006; Sackmann, 1996). Bilayer lipid membranes (BLMs), Which constitutes an excellent matrix, provide a natural environment for immobilizing proteins like receptors and antibodies, and maintain the biological activities of the proteins (Ivnitski et al., 2000; Wang et al., 2000; Nikolelis and Siontorou, 1996; Lamken et al., 2004; Snejdarkova et al., 1993; Wu et al., 2001). Recently, metallic or nanoparticles layers formed in the s-BLM have been studied in membrane mimetic chemistry (Ottova et al., 1997; Tien

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and Ottova-Leitmannova, 2000; Purrucker et al., 2001; Guidelli et al., 2001). The deposition of nanoparticles onto the surface of s-BLM or inside the s-BLM can drastically alter the mechanical, electrical and optical properties of the s-BLM (Liu et al., 2009). Moreover, nanoparticles doped through the s-BLM may perform as an array of nanoelectrodes, while s-BLM retains its biocompatible microenvironment (Hicks et al., 2001). For example, Liu et al., have detected cytochrome *c* at Au nanoparticle-structured s-BLM and found that Au nanoparticles in s-BLM caused the increase in membrane capacitance and resistance (Liu and Wanzhi, 2008). It was considered, nanoparticles formed in s-BLM may have the ability to increase the change of electrochemical signals.

However, up to now, there are few biosensors for monitoring the estrogenic substances in the environment. In this paper, an electrochemical biosensor was developed using s-BLM modified with Au nanoparticles as the matrix to immobilize ERs to detect estrogenic substances. The self-assembled s-BLM was formed on a cleaned pt electrode surface followed by Au nanoparticles electrodepositing, and then ERs were adsorbed to be immobilized in the s-BLM. The specific binding of the ERs to estrogenic substance would lead to conformational changes in the s-BLM and alterations of the electrical circuit components that are detected by impedance measurements. The high surface-to-volume ratio and excellent biocompatibility of Au nanoparticles in s-BLM may produce a better microenvironment for s-BLM absorbing a larger amount of ER protein with high bioactivity. Meanwhile, the high conductivity of Au nanoparticles may improve some characteristics of s-BLM, including electric resistance, capacitance and so on.

2. Materials and methods

2.1. Reagents

17β-estradiol, 4-nonylphenol (NP), bisphenol A (BPA), recombinant human estrogen receptor-α (740 pmol per vial) and Au nanoparticles (16 nm) were purchased from Sigma (St. Louis, MO, USA). The rabbit anti-human ER polyclonal antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Goat anti-Rabbit IgG/alkaline phosphatase (ALP) secondary antibody were bought from Biosynthesis Biotechnology Co (Beijing, China). Egg phosphatidylcholine (PC, ≥99%, TLC) and cholesterol (CH) were obtained from Shanghai Chemical Reagent Company (Shanghai, China). N-octane, $K_3[Fe(CN)_6]$, $K_4[Fe(CN)_6]$ and DMSO were bought from Tianjin Kermel Chemical Reagent Corporation (Tianjin, China) Company (Wuhan, China). All platinum disk electrodes with diameter 1 mm were obtained from Tianjin Scientific Apparatus Company (Tianjin, China). All chemicals and solvents used were of analytical grade. Double distilled water was used throughout this study.

The estrogen receptor-α (ER-α) was stored at −80 °C and were freshly prepared by dissolving it in phosphate buffer solution (PBS, pH 7.4) on ice before use. 17β-estradiol, NP and BPA were dissolved in 0.01% methanol and stored at 4 °C. The s-BLM forming solution was composed of 5% PC and 2% CH in n-octane and stored at 4 °C. The working buffer was consisted of 10 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ and 0.1 M KCl.

2.2. Apparatus

Cyclic voltammetric and AC impedance measurements were performed on a CHI 660C electrochemistry workstation. Controlled potential (CPEs) were performed on an electrochemical workstation (BASi Epsilon-EC). All experiments were carried out with a conventional three-electrode system with the modified electrode as the working electrode, a platinum wire as the counter electrode and a saturated calomel reference electrode (SCE) as the reference electrode. All the potentials were referred to the SCE reference electrode.

2.3. Electrode preparations

The protocols for the fabrication of s-BLM modified Pt electrodes were according to the literature (Tien and Ottova, 1998; Wu et al., 2001) with a little modification. Briefly, the working electrode was prepared as follows, firstly, a Pt electrode (Φ = 1 mm) was polished carefully with 1.0, 0.3, and 0.05 μm alumina slurry, followed by successive sonication in nitric acid (1:1), ethanol and bi-distilled water and dried in air. Then a 5 μL aliquot of s-BLM forming solution was dropped onto the surface of the cleaned electrode and the electrode was kept in room temperature for 20 min. Following that, the lipid-coated electrode was immersed into 0.1 M KCl for 15 min, in which s-BLM was formed spontaneously (s-BLM modified electrode was described as s-BLM/Pt). Subsequently, the resulting electrode was immersed in the Au nanoparticle solution for 10 min and applied under the potential at 1500 mV in order to electrochemically deposit Au nanoparticle through s-BLM (Au nanoparticles modified s-BLM/Pt electrode was described as Au/s-BLM/Pt). For comparison with and without the s-BLM formation, Au nanoparticles were electrochemical deposited onto the bare Pt electrode surface under the same condition (Au nanoparticles modified bare pt electrode was described as Au/Pt). After that, a 5 μL aliquot of the ER solution (0.3 nmol/mL) was dropped onto the surface of Au/s-BLM/Pt electrode and the electrode was maintained at 4 °C for 12 h in a wet box, and then was gently washed with PBS (the ER modified electrode was described as ER/Au/s-BLM/Pt). The finished modified electrode (working electrode) was stored at 4 °C while not being used.

2.4. Electrochemical measurements

Electrochemical measurements were performed in a conventional electrochemical cell containing a three-electrode system in 5 mL (working buffer) 10 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (1:1) containing 0.1 M KCl at room temperature. The electrochemical characteristics of the modified electrode during the self-assembled process were characterized by using cyclic voltammetry and electrochemical impedance spectroscopy (EIS). Cyclic voltammetry measurements were swept from −0.2 to 0.6 V (vs. SCE) with a sweeping rate of 0.10 V s. EIS measurements were performed at the frequency range from 10^{-2} to 10^{6} Hz at the formal potential of 220 mV, using alternating voltage of 10 mV. Impedance data were analyzed using the ZVIEW software (Version 3.1C) (Vockenroth et al., 2007; Romer and Steinem, 2004). The change of electron-transfer resistance is calculated as the following equation: $\Delta R_{ct} = R_{ct(ER\text{-}E)} - R_{ct(ER)}$, where $R_{ct(ER)}$ is the value of the electron transfer resistance produced by ER/Au/s-BLM/Pt scanned in working buffer, and $R_{ct(ER\text{-}E)}$ is the value of the electron transfer resistance after 17β-estradiol binding to ER/Au/s-BLM/Pt.

The repeatability and stability of the biosensor was evaluated from the response to 50 ng/L 17β-estradiol at six electrodes that were fabricated according to the same procedure.

2.5. Sample pretreatment and MCF-7 cell proliferation assay

Estrogenic activity of the water samples from the Yangtze River and the Han River were detected by the biosensor and MCF-7 cell proliferation assay.

River water samples were collected at four separate locations in the Yangtze River (Y1, Y2) and the Han River (H1, H2). Each water sample (2 L) was firstly filtered under vacuum using a glass fiber filter with a 0.22 μm pore size. The filtered water samples were

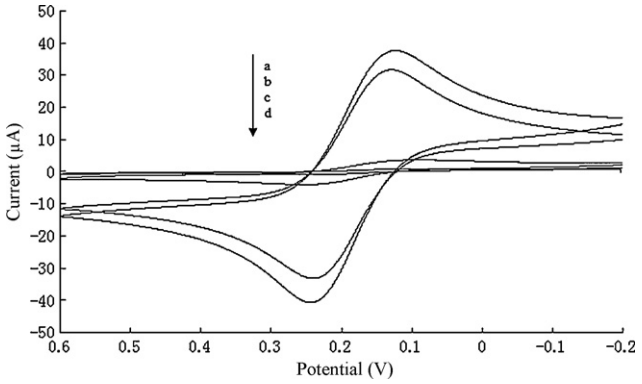

Fig. 1. Cyclic voltammograms of the different electrodes in a 10 mM $K_3[Fe(CN)_6]$:$K_4[Fe(CN)_6]$ (1:1) solution containing 0.1 M KCl: (a) Au/Pt electrode, (b) Bare Pt electrode, (c) Au/s-BLM/Pt electrode and (d) s-BLM/Pt electrode. Scan rate: 100 mV/s.

then acidified (pH 3.0) with 20% acetic acid and passed through pre-conditioned Oasis HLB cartridges (200 mg/6 cm^3, 30 μm partial size, Waters) at a rate of approximately 1 mL min^{-1}. Each sample was eluted with methanol (5 mL) and *n*-hexane (5 mL), and then evaporated to dryness under a gentle nitrogen stream at 37 °C. The residue was immediately dissolved in 1 mL 10% methanol.

MCF-7 cell proliferation assay was performed as described by Soto et al. (1995) and Silva et al. (2007). In Brief, MCF-7 cells (provided by China Center for Type Culture Collection) were seeded into 48-well plates at 5000 cells/well in Dulbecco's modified Eagle's medium (DMEM, Gibico BRL, Grand Island, New York, USA) with 5% fetal bovine serum (FBS, Gibico BRL, Grand Island, New York, USA). After 24 h, the medium was changed into experimental medium [10% charcoal dextran-treated FBS (CDFBS) in phenol red-free DMEM] containing one of the four different dilutions of water extracts. Each dilution was tested in six replications per assay. Six wells per assay without hormones comprised the negative control and 17β-estradiol, in five concentrations between 10^{-12} and 10^{-8} M, was used as positive control. After six days, the assays were terminated and the absorbance (595 nm) was determined in each well after crystal violet staining. Estrogenic activity was measured using 17β-estradiol equivalency quantity (EEQ), which was calculated by comparing samples estrogenic activity with 17β-estradiol. Briefly, EEQ was calculated by applying a linear regression equation determined in the MCF-7 cell proliferation assay.

3. Results and discussion

3.1. Cyclic voltammetric behavior of the modified electrode

Cyclic voltammograms (CVs) of the ferricyanide are valuable and convenient tools to monitor the barrier of the modified electrode, because the electron transfer kinetics of $Fe(CN)_6^{4-/3-}$ would be perturbed when the electrode surface was modified by some materials. Fig. 1 shows the CVs of different modified electrodes in 10 mM $Fe(CN)_6^{4-/3-}$ solution containing 0.1 M KCl at a scan rate 100 mV/s in the sweeping range from −0.2 to +0.6 V. As shown in Fig. 1b, a couple of well-defined redox peaks was observed at the bare Pt disk electrode. As expected, when Au nanoparticles were electrodeposited on the bare Pt surface, the anodic and cathodic peaks increased (Fig. 1a). This enhanced electrochemical performance was attributed to the presence of Au nanoparticles on the surface of the electrode which could accelerate the electron transfer (Liu and Lin, 2007; Sperling et al., 2008; Voevodin et al., 2007). Compared to bare Pt, the assembly of the s-BLM on Pt electrode led to the decrease of the anodic and cathodic peak (Fig. 1d), indicating that the s-BLM formed on the electrode surface and acted as an inert layer for blocking electron and mass transfer to hinder the diffusion of ferricyanide toward the electrode surface (Sackmann, 1996; Salamon and Tien, 1991). In the presence of Au nanoparticles, the peak current of the redox couple increased (Fig. 1c), which demonstrated that Au nanoparticles enhanced the electron transferring to the electrode surface. The s-BLM is thought to be ion-impermeable before. However, the lipid film is loosely packed on the surface of the electrode with some pinholes in it, which can provide a unique advantage for electrochemical deposition of nanoparticles (Ye et al., 2003).

Cyclic voltammetry experiments confirmed that Au nanoparticles in the s-BLM could efficiently accelerate the electron transfer and, accordingly, improve the electrochemical performance.

We selected Au nanoparticles with diameters of 16 nm to modify the s-BLM because Au nanoparticles with diameters of about 20 nm were proven to be more appropriate to modify s-BLM in previous studies. For example, Liu and Wanzhi (2008) used electrochemical deposition of Au nanoparticles through s-BLM by applying potential cycling onto the s-BLM in $HAuCl_4$ solution, and the diameters of Au nanoparticles range from 20 to 30 nm in s-BLM from scanning electron microscope (SEM) results. Similarly, Ye et al. (2003) used electrochemical deposition of Pt nanoparticles through s-BLM by applying potential cycling onto the s-BLM in K_2PtCl_6 solution, and the diameters of Pt nanoparticles range from 20 to 30 nm in s-BLM from SEM results.

3.2. The immobilization of ER in s-BLM modified by Au nanoparticles

Immobilization of protein in s-BLM has been widely studied in the last two decades. Wang et al. (2000) reconstituted membrane receptors in s-BLM and the receptor still retained its ligand binding activity after reconstitution. Ivnitski et al. (2000) presented a new ion-channel biosensor to detect bacteria using immobilization of antibody in the s-BLM. However, after the s-BLM was modified with nanoparticles, some characteristics of the s-BLM were changed, including electric resistance, capacitance and so on, which may influence the process of protein immobilizing in the s-BLM or their biological properties.

In order to further investigate whether ER-α had been successfully immobilized in s-BLM modified with Au nanoparticles, Rabbit anti-ER and ALP-labeled Goat anti-Rabbit IgG antibody were used and CVs characterization was studied. The ER/Au/s-BLM/Pt electrodes were prepared with different amount ER-α, followed by incubation with Rabbit anti-ER and ALP-labeled Goat anti-Rabbit IgG. Theses electrodes were then scanned at the potential between 0 and 600 mV (vs. SCE) with a scanning rate of 100 mV/s in Tris–HCl buffer (pH 8.0) containing 5 mM α-naphthyl phosphate. As shown in Fig. 2, well-defined oxidation peaks were observed in the region around 300 mV. The oxidation peaks came from an electron transfer reaction due to the hydrolysis of α-naphthyl under the catalytic reaction of ALP labeled on the IgG in the s-BLM.

The unspecific absorption of anti-ER and ALP labeled IgG in the s-BLM may also create oxidation with α-naphthyl in the buffer. But both the correlation between the peaks current and the receptor amount and the fact that the peak current reached a plateau when the ER-α amount approached 0.3 nmol/mL (Fig. 2) demonstrated that the ER-α had been successfully immobilized in the s-BLM and had maintained their biologic properties. So we choose 0.3 nmol/mL as the concentration of ER-α for the preparation of the biosensor.

3.3. Detection of 17β-estradiol

EIS is one of the most powerful and sensitive electroanalytical techniques frequently used in studying the electron transfer

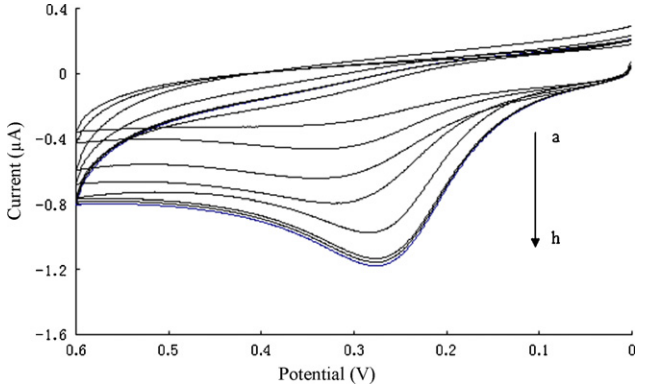

Fig. 2. Cyclic voltammograms of ER/Au/s-BLM/Pt electrodes prepared with different concentration of ER-α after incubated with Rabbit anti-ER and ALP-labeled Goat anti-Rabbit IgG in Tris–HCl buffer (pH 8.0) containing 5 mM α-naphthyl phosphate. Scan rate: 100 mV/s; ER-α concentration used in the preparation of the biosensor: (a) 0.05 nmol/L, (b) 0.1 nmol/L, (c) 0.15 nmol/L, (d) 0.2 nmol/L, (e) 0.25 nmol/L, (f) 0.3 nmol/L, (g) 0.35 nml/L and (h) 0.4 nmol/L.

kinetics. In the present study, EIS was applied to probe the feature of surface-modified electrode. The impedance was analyzed using the modified Randles equivalent circuit (insert of Fig. 3B) (Ye et al., 2003; Wu et al., 2001). It comprises several parameters: (1) electrolyte resistance, R_s; (2) the lipid bilayer capacitance, C_{dl}, which consisted of the unmodified platinum electrode (C_{Pt}) and a capacitance originated for s-BLM (C_m) on the surface of the electrode; (3) charge transfer resistance, R_{ct}; (4) Warburg element, Z_w. The complex impedance can be presented as the sum of the real, Z_{re}, and imaginary, Z_{im} components that originate mainly from the resistance and capacitance of the cell. Fitting the parameters of the

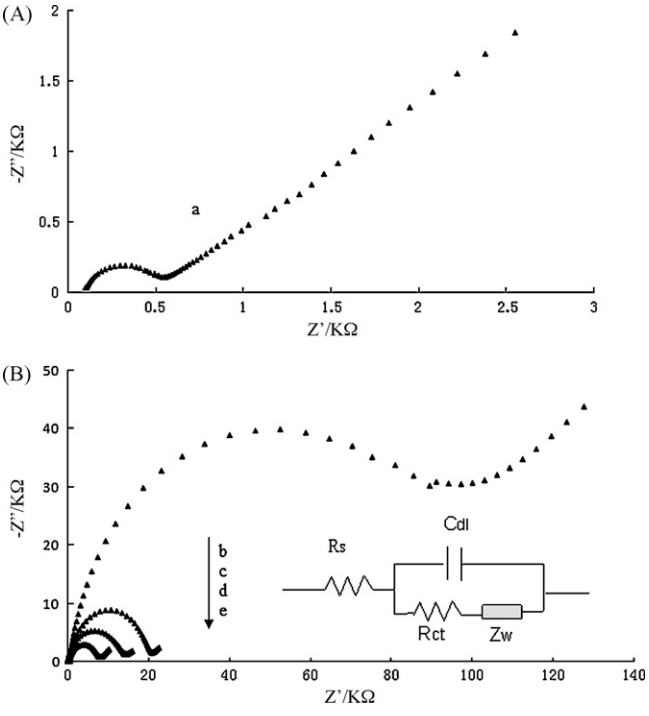

Fig. 3. Electrochemical impedance spectroscopy in 10 mM $K_3[Fe(CN)_6]$:$K_4[Fe(CN)_6]$ (1:1) solution containing 0.1 M KCl at (A.a) a bare Pt electrode, (B.b) s-BLM/Pt, (B.c) after 17β-estradiol (50 ng/L) binding to ER/Au/s-BLM/Pt, (B.d) ER/Au/s-BLM/Pt and (B.e) Au/s-BLM/Pt. A sinusoidal potential modulation of 5 mV amplitude was superimposed on the formal potential of the redox couple of $[Fe(CN)_6]^{4-}$/$[Fe(CN)_6]^{3-}$ (0.22 V vs. GCE). Applied frequency was from 10^6 to 0.1 Hz. Insert: Modified Randles equivalent circuit used to model impedance data in the presence of redox couples (ER-α concentration used in the preparation of the biosensor was 0.3 nmol/mL).

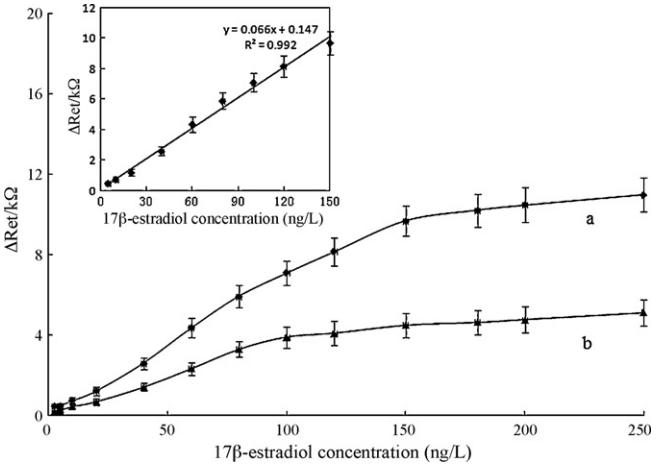

Fig. 4. Calibration curves of the electron transfer resistance change ΔR_{ct} with the different concentration of 17β-estradiol based on the ER/Au/s-BLM/Pt electrode (curve a) and the ER/s-BLM/Pt electrode (curve b). *Inset*: The linear part of the calibration curve of ER/Au/s-BLM/Pt electrode. (ER-α concentration used in the preparation of the biosensor was 0.3 nmol/mL.).

equivalent circuit to the data results in very good agreement, with fitting errors < 5%.

Fig. 3 shows the impedance spectroscopy at the formal potential on a bare Pt electrode (A.a), s-BLM modified electrode (B.b), Au/s-BLM/Pt (B.e), ER/Au/s-BLM/Pt (B.d), and after ER/Au/s-BLM/Pt electrode bind to 50 ng/L 17β-estradiol (B.c) in the presence of the redox probe $[Fe(CN)_6]^{4-/3-}$. It can be seen that the bare Pt disk electrode exhibited an almost straight line that was characteristic of a diffusional limiting step of the electrochemical process (Fig. 3A.a). When s-BLM was formed on the electrode, the resistance greatly increased, indicating that the s-BLM obstructed electron transfer of the electrochemical probe (Fig. 3B.b). Au nanoparticles electrochemical deposition through the s-BLM caused a lower interfacial electron transfer resistance (Fig. 3B.e) compared with that of s-BLM/Pt. This might due to that the Au nanoparticles serve as nanoscale conductors that facilitate the electron transfer. When ER/Au/s-BLM/Pt electrode was obtained, the resistance increased due to ERs immobilized in the s-BLM. After ER/Au/s-BLM/Pt electrode bind to 50 ng/L 17β-estradiol, the resistance increased again (Fig. 3B.c). Thus, the change of charge transfer resistance could be used as the sensor signal in this paper. The change of electron transfer resistance is calculated in accordance with the following equation: $\Delta R_{ct} = R_{ct(ER\text{-}E)} - R_{ct(ER)}$, where $R_{ct(ER\text{-}E)}$ is the value of electron transfer resistance after 17β-estradiol binding to ERs in ER/Au/s-BLM/Pt and $R_{ct(ER)}$ is the value of electron transfer resistance of ER/Au/s-BLM/Pt.

To detect 17β-estradiol, the biosensor was incubated with multiple concentrations of 17β-estradiol and then monitored by impedance spectroscopy using $[Fe(CN)_6]^{4-/3-}$ as the redox probe. Fig. 4 shows the electron transfer resistance change (ΔR_{ct}) at the ER/Au/s-BLM/Pt electrode (curve a) and the ER/s-BLM/Pt (curve b) after reaction with different concentrations of the analyte 17β-estradiol in the range of 0–250 ng/L, which indicated that the ER/Au/s-BLM/Pt electrode took on more sensitive change to ΔR_{ct} value. The ER/Au/s-BLM/Pt biosensor calibration curves are sigmoidal with 17β-estradiol concentration; however, from the insert in Fig. 4, the biosensor exhibits an acceptable linear response to 17β-estradiol concentration ranging from 5 to 150 ng/L and the linear regression equation was $\Delta R_{ct} = 0.147[17\beta\text{-estradiol}] + 0.066$ ($R^2 = 0.992$). The detection limit was 1 ng/L at the signal-to-noise ratio of 3. This higher sensitivity of ER/Au/s-BLM/Pt in detection of estrogen suggests that Au nanoparticles incorporated in the s-BLM act as an effective medium for the electron transfer.

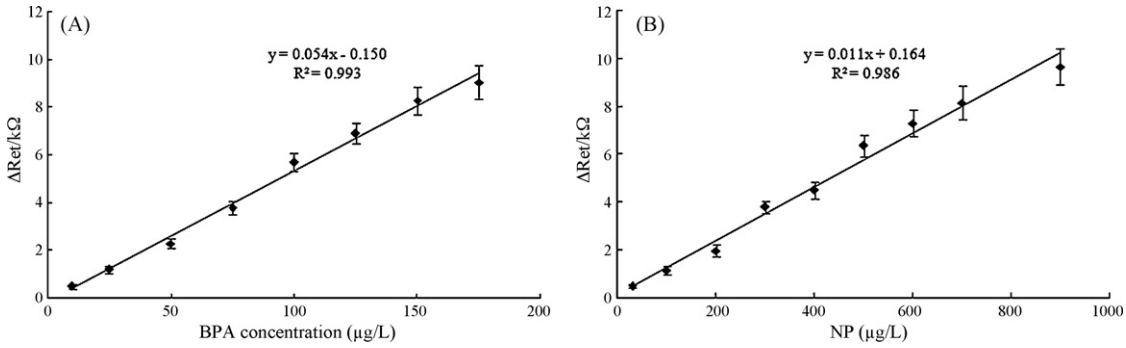

Fig. 5. Calibration curves for xenoestrogens: (A) BPA and (B) NP.

3.4. Detection of xenoestrogen

Xenoestrogen can mimic the gonadal hormone 17β-estradiol to bind to the ER-α and interfere with endocrine system function. Thus, we validate the applicability of the biosensor for the detection of xenoestrogen. BPA, largely used in polycarbonate plastics as well as polystyrene resins, and NP, a derived product of nonionic surfactants such as NP ethoxylates, are both ubiquitously present in the environment and resistant to degradation (Soares et al., 2008; Yamasaki et al., 2001). They have been received considerable attention in recent years because of their ability to induce a variety of reproductive effects in rodents. Fig. 5 shows the calibration curves for BPA (Fig. 5A) and NP (Fig. 5B). The biosensor exhibits an acceptable linear dependence in the concentration ranging from 6 to 180 μg/L for BPA and 30–890 μg/L for NP.

The reason why the sensitivity for BPA and NP is much lower than the sensitivity for 17β-estradiol is the estrogen receptor has a higher affinity for 17β-estradiol (Olsen et al., 2005; Strunck et al., 2000). Although a recent paper which also uses estrogen receptor in a carbon nanotubes field-effect transistor device (Sánchez-Acevedo et al., 2009) reports the sensitivity for BPA is much higher, the biosensor using s-BLM as matrix in this study is easier to be fabricated.

In addition, there are some points need to be explained. The biosensor described in this paper is based on the specific binding of ER with xenoestrogens. Thus the sensor cannot distinguish between different xenoestrogens in complex samples. However, this bioassay could provide biologic potency information of either individual congeners or complex mixtures. This is also the advantage of this kind of method that could be used in screening estrogenic pollutants. Positive samples could then be reanalyzed with current analytical techniques such as LC, GC or MS to identify the xenoestrogens.

3.5. Repeatability, selectivity and stability of the biosensor

The repeatability study of the biosensor was evaluated by intra-assay and inter-assay precision. We assessed intra-assay precision by using seven different ER/G/s-BLM electrodes prepared in the same procedure to test the same samples (50 ng/L 17β-estradiol) in one day. And these tests were repeated in 3 consecutive days in the same manner for inter-assay. The mean intra-assay and inter-assay coefficient variation (CV) were 4.6% and 8.9%.

The selectivity of the proposed biosensor was evaluated by measuring the impedance change of 50 ng/L 17β-estradiol with the presence of some interfering substances, such as phenol, and plumb. The results showed these compounds did not present significantly interference to the detection of 17β-estradiol. The relative standard deviation (RSD) of inhibition impedance obtained for each interfering substance was less than 9.6%.

The stability of the biosensor was tested by exposing ER/Au/s-BLM/Pt electrodes to 50 ng/L 17β-estradiol at three different periods (2nd, 4th, and 8th day) after storing in a dry box or soaking in PBS at 4 °C. It was found that an average response to 50 ng/L 17β-estradiol on day 2, 4 and day 8 were 98%, 91% and 70% of the initial response, respectively, when the electrodes were stored in a dry box at 4 °C. While continuously soaking the electrodes in PBS at 4 °C, the response decreased quickly to 60% within two days. The fast decrease of the response may be attributed to the lost of ER-α from the lipid membrane in constant solution phase. Similarly, when the Au nanoparticles were absent, the stability of ER/s-BLM/Pt electrode was also studied. The responses retained 97%, 82% and 55% of its initial sensitivity to 50 ng/L 17β-estradiol at dry environment on day 2, 4 and day 8, and 45% in PBS on day 2. The better long-term stability of ER/Au/s-BLM/Pt electrode compared to ER/s-BLM/Pt may be attributed to Au nanoparticles supplying good biocompatibility and increasing the stability of s-BLM, and preventing the leakage of bimolecular.

3.6. Comparison of ER/Au/s-BLM/Pt biosensor assay with MCF-7 cell proliferation assay

The presence of estrogenic substances in samples can be evaluated by measuring the 17β-estradiol equivalency quantity (EEQ). EEQ expressed in ng/L, is the total concentration of active estrogenic compounds in an environmental sample normalized to the natural estrogen 17β-estradiol, which can be calculated from 17β-estradiol calibration curve. To compare our method and MCF-7 cell proliferation assay, we selected water samples at four different sites in the Yangtze River (Y1 and Y2) and the Han River (H1 and H2). The results obtained from ER/Au/s-BLM/Pt biosensor were consistent with those from MCF-7 cell proliferation assay (Table 1). The

Table 1
Comparison of ER/Au/s-BLM/Pt biosensor assay with MCF-7 cell proliferation assay.

Samplings	ER/Au/s-BLM/Pt biosensor assay		MCF-7 cell proliferation assay		*P*
	EEF (ng/L)	RSD	EEF (ng/L)	RSD	
Yangtze River (Y1)	7.7	2.0	7.1	1.7	>0.05
Yangtze River (Y2)	7.1	1.8	7.8	1.9	>0.05
Han River (H1)	5.5	1.8	5.1	1.6	>0.05
Han River (H2)	5.9	1.9	5.5	1.5	>0.05

result demonstrated that the present biosensor can be used for monitoring EDCs in water samples.

4. Conclusions

In summary, a sensitive, simple and nanoscale biosensor for the detection and screening of estrogen has been developed using ERs immobilized in s-BLM modified with Au nanoparticles. Our results indicated that the biosensor was capable of detecting estrogenic substances selectively, such as 17β-estradiol, BPA and NP, even without a redox probe or enzyme labeling. Furthermore, Au nanoparticles deposition through the s-BLM has an effect on increasing sensitivity and the longer time stability of the biosensor. Comparing with other bioassays based on ERs to detect estrogen reported in the past (Legler et al., 2002; Nishihara and Nishikawa, 2001; Nishikawa et al., 1999; Pillon et al., 2005; Soto et al., 1995), the biosensor was non-cell-culture involved, more convenient in manipulations and time saving, which may provide a promising alternative approach for screening EDCs in environment in the future work.

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