Journal of Electroanalytical Chemistry 671 (2012) 106–111

Contents lists available at SciVerse ScienceDirect

Journal of Electroanalytical Chemistry

journal homepage: www.elsevier.com/locate/jelechem

Impedometric estrogen biosensor based on estrogen receptor alpha-immobilized gold electrode

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ARTICLE INFO

Article history:
Received 15 December 2011
Received in revised form 15 February 2012
Accepted 17 February 2012
Available online 3 March 2012

Keywords:
Estrogen hormone
17β-Estradiol
Estrogen receptor-α
Electrochemical impedance spectroscopy (EIS)

ABSTRACT

A label-free impedometric biosensor has been developed for the detection of estrogen hormone 17β-estradiol. The electrochemical biosensor has been fabricated by immobilizing both estrogen receptor-α and bovine serum albumin on gold electrode surfaces. The binding of 17β-estradiol to the estrogen receptors on the fabricated biosensor has increased the electron-transfer resistance which has been directly monitored by electrochemical impedance spectroscopy in the presence of 5.0 mM $K_3Fe(CN)_6$/$K_4Fe(CN)_6$ (1:1, v/v) redox couple. The association constant between the 17β-estradiol and the estrogen receptors has been found to be ca. 1.8×10^{11} M^{-1}, which indicates that the estrogen receptors are well formed on gold electrode surfaces not only with high density but also with good specificity to its corresponding 17β-estradiol. The present biosensor gives a linear response ($r^2 = 0.992$) for 17β-estradiol concentration from 1.0×10^{-13} to 1.0×10^{-9} M with a detection limit of 1.0×10^{-13} M ($S/N = 3$), which is much lower compared to those obtained with other detection methods. The present biosensor exhibits good stability, in which the biosensor has retained 88% of its initial activity after 3 weeks of storage in 50 mM phosphate buffer at pH 7.0.

1. Introduction

Estrogen hormones, including 17β-estradiol, are important bioactive materials involving not only the reproduction in females but also the development and maintenance of sexual characteristics [1]. On the other hand, estrogen hormones are known to be carcinogens due to their effects of tumor initiating and promoting [2,3]. Therefore, the development of a simple detection method for estrogen hormones with high selectivity and sensitivity is one of the important challenges in analytical sciences.

Although a number of instrumental analysis methods such as high-performance liquid chromatography (HPLC) [4], surface plasmon resonance (SPR) [5,6], and luminescent enzyme-linked receptor assay [7] have been reported, they are very complicated and require sophisticated instruments. Many researchers have tried to develop simple and sensitive biosensors for the detection of 17β-estradiol. In the estrogen biosensor, human estrogen-receptor is most widely used as the selective sensing layers in which 17β-estradiol target molecules are selectively bound to the estrogen-receptor layer [8–11]. In addition, anti-estrogen antibody [12,13] and DNA aptamer [14] have been employed as the selective sensing layers.

One of the most important points in the development of the estrogen biosensors is the development of highly sensitive transduction methods for the detection of the estrogen-receptor binding. Electrochemical transduction methods are most widely used in the development of biosensors since it is simple and cost-effective. Among the electrochemical transduction methods, electrochemical impedance spectroscopy (EIS) is a sensitive and non-destructive technique for the characterization of interfacial properties on the electrode surface [11,13,15–18]. In the present study, a highly sensitive electrochemical biosensor for the detection of estrogen hormone, 17β-estradiol, has been developed using an estrogen receptor-α immobilized on a gold electrode with electrochemical impedance spectroscopy. According to our previous preliminary work [11], the estrogen biosensor was fabricated by a stepwise manner in which, 3-mercaptopropionic acid (3-MPA) containing both thiol and carboxyl groups was first self-assembled on a gold electrode. The estrogen receptor was covalently attached to the carboxylic ends of 3-MPA self-assembled monolayers by a coupling reaction based on 3-(3-dimethylaminopropyl)-1-ethylcarbodiimide hydrochloride/N-hydroxysuccinimide. In the present work,

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doi:10.1016/j.jelechem.2012.02.022

bovine serum albumin has been covalently coupled to the remaining binding sites of the estrogen receptor-α electrode in order to eliminate the non-specific binding. The binding of target 17β-estradiol to the estrogen receptors on the fabricated biosensor has increased the electron-transfer resistance which has been directly monitored by electrochemical impedance spectroscopy in the presence of 5.0 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ (1:1, v/v) redox couple as a probe. The response characteristics of the prepared estrogen biosensor has been characterized and compared to those obtained with other biosensors in the literature.

2. Experimental

2.1. Reagents

Estrogen hormone (17β-estradiol, ⩾98%), 3-mercaptopropionic acid (3-MPA, ⩾99%), 3-(3-dimethylaminopropyl)-1-ethylcarbodiimide hydrochloride (EDC, >98%), N-hydroxysuccinimide (NHS, 98%), and bovine serum albumin (BSA, ⩾96%) were purchased from Aldrich. Estrogen receptor-α (ER-α, >85.0%) was purchased from Calbiochem (San Diego, USA). A phosphate buffered saline (PBS) solution was purchased from Gibco (NY, USA). Purification cartridge of Strata C18-T (55 μm, 1400 A) for urine sample was purchased from Phenomenex (CA, USA). All working solutions were prepared with the water purified through a Milli-Q system (Millipore, Bedford, MA) that was stored at a 4 °C before the experiments.

2.2. Instrumentation

Electrochemical impedance and cyclic voltammetry experiments were performed with EG&G 273A potentiostat (Princeton, NJ, USA) and frequency response detector (model 1025, Oak Ridge, TN, USA) with a conventional three-electrode system in a 15 mL electrochemical cell. A conventional three-electrode system was employed with a platinum-wire counter electrode, estrogen biosensor as a working electrode, and Ag/AgCl (3 M NaCl) as a reference electrode. Cyclic voltammograms and AC impedance spectra were recorded in 5.0 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ (1:1, v/v) solution prepared in 0.05 M phosphate buffer (pH 7). The impedance spectra were obtained in the frequency range from 100 MHz to 100 kHz at a bias potential of 0.2 V with an alternating voltage of 5.0 mV. The bias potential of 0.2 V is the half-wave potential estimated from the mean of the anodic and cathodic peak potential of cyclic voltammograms for 5.0 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ (1:1, v/v) solution. The impedance spectra were plotted in the form of impedance plane plots (Nyquist plots). Zsimpwin program (Princeton Applied Research, Oak Ridge, TN, USA) has been used to fit the experimental results into equivalent circuit and estimate the diameter of the semicircles. The chi square between the experimental and fitting results is 1×10^{-3} level. Atomic force microscopy images were obtained with MultiMode® atomic force microscope (Veeco Instruments Inc., USA) in tapping mode.

2.3. Preparation of estrogen biosensor

The estrogen biosensor was prepared according to the previously reported method [11]. However, an additional step was employed in order to eliminate the non-specific binding, in which bovine serum albumin was covalently coupled to the biosensor. A gold plate electrode (2 mm × 5 mm) on silicon wafer substrate was used for the preparation of estrogen biosensor. The gold plate electrode was electrochemically polished in 0.05 M phosphate buffer solution by cycling the potential from −0.6 and +1.3 V at a scan rate of 50 mV/s.

The freshly pretreated gold electrode was immersed in a 40 mM 3-MPA solution for about 4 h to produce carboxylate-terminated self-assembled monolayers (SAMs). The electrode was rinsed with distilled water to remove physically adsorbed 3-MPA molecules and then stored in distilled water. The 3-MPA modified gold electrode was immersed in a mixture of 1.0 wt.% EDC and 1.0 wt.% NHS in 0.05 M phosphate buffered saline (PBS) solution (pH 7.4) for 2 h to activate the terminal carboxylic groups. After rinsing with distilled water and drying, the active surface of the EDC-NHS/3-MPA modified gold electrode surface was treated with 15 μL of 6.7×10^{-7} M estrogen receptor-α solution prepared in PBS buffer and stored for 12 h at 4 °C. The 6.7×10^{-7} M concentration of estrogen receptor-α is good enough to fully cover the electrode surface. The unbound estrogen receptor-α was removed from the electrode surface by slowly dipping into a PBS solution (pH 7.0). Then, the estrogen receptor-α modified electrode was treated with the 3.5 wt.% BSA solution for 4 h to block the non-specific sites at room temperature, and then the electrode was thoroughly rinsed with the PBS solution to remove unbound estrogen receptor-α from the electrode surface. The schematic diagram of the biosensor fabrication and 17β-estradiol binding was illustrated in Fig. 1.

2.4. Detection of estrogen hormone

The estrogen biosensor was immersed for 90 min. in 1.0 mL of PBS solution at pH 7.0 containing various concentrations of the 17β-estradiol in the range of 1.0×10^{-9} M–1.0×10^{-13} M at room temperature, then rinsed thoroughly by 20 mM PBS solution and distilled water to remove unbound 17β-estradiol. Finally, AC impedance spectra were recorded in 5.0 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ (1:1, v/v) solution prepared in 0.05 M phosphate buffer (pH 7.0).

3. Results and discussion

3.1. Fabrication of estrogen hormone biosensor

The present estrogen biosensor was fabricated according to the modified method reported previously [11]. In order to eliminate non-specific bindings, bovine serum albumin was covalently attached to the biosensor. Since electrochemical impedance spectroscopy (EIS) has been proved to be a powerful tool for the investigation of interfacial reactions, the layer-by-layer assembling process of the present estrogen biosensor was characterized by EIS. The impedance spectra were plotted in the form of impedance plane plot (Nyquist plot) by representing the biosensor–electrolyte solution interface as a conventional four-component equivalent circuit depicted in Fig. 2A. The complex impedance can be presented as the sum of the real (Z_{re}) and imaginary (Z_{im}) components that originate from resistance and capacitance of the cell. The diameter of the semicircle lying on the Z_{re} corresponds to the electron transfer resistance (R_{et}) of the redox probe at the electrode interface, which reflects blocking behavior of the electrode surface for the redox couple and therefore could be used to characterize each modification step on the electrode surface for the fabrication of the estrogen biosensor. Based on the equivalent circuit shown in Fig. 2A, simulations were conducted in order to accurately determine R_{et}. The Nyquist plots of the gold electrode were obtained at each modification step in the presence of 5.0 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ (1:1, v/v) solution prepared in phosphate buffer (pH 7.0). A significant change in the impedance spectra could be observed in the stepwise immobilization processes on the gold electrode with 3-MPA, EDC and NHS, estrogen receptor-α and BSA in comparison to that of the bare gold electrode which showed an almost straight line indicating a typical diffusion-limited process (Fig. 2A, a). The formation of

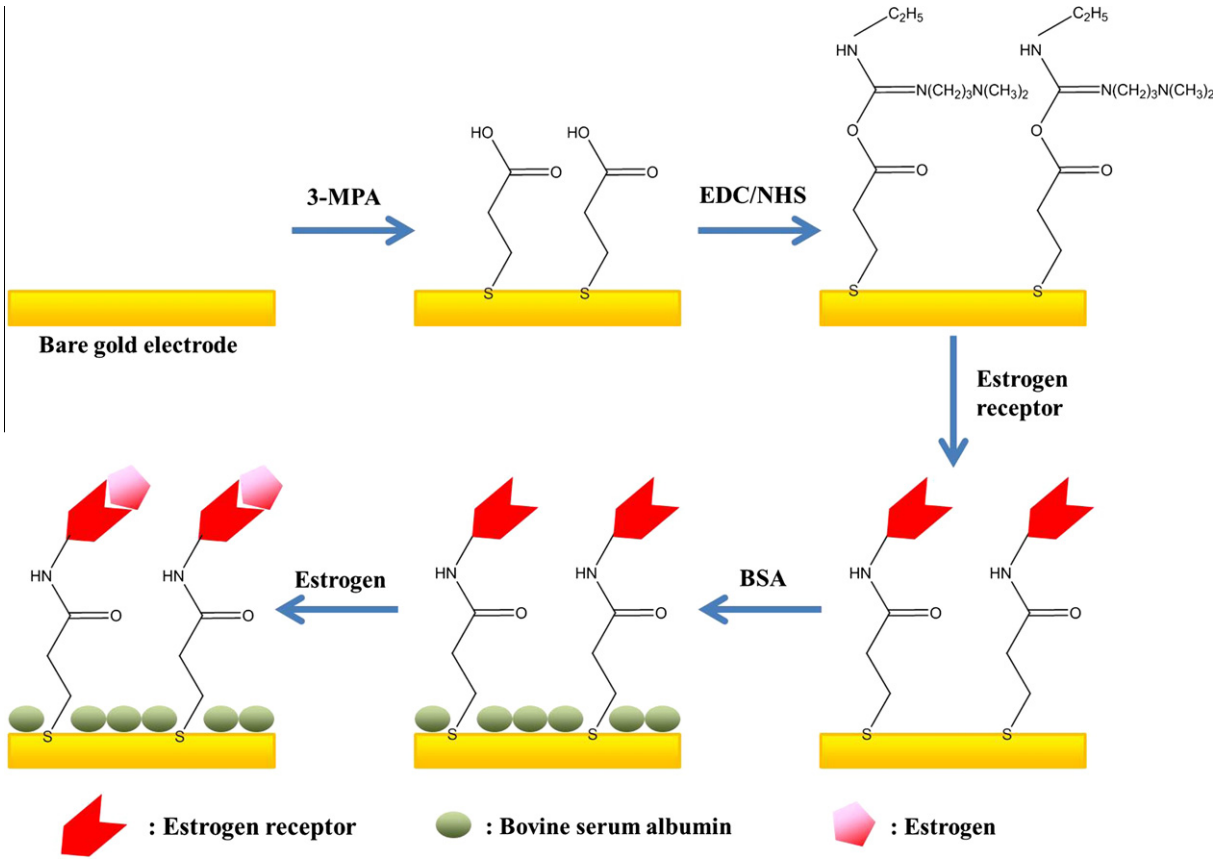

Fig. 1. Stepwise fabrication of the estrogen biosensor based on the estrogen receptor-modified electrode and the binding of estrogen hormone to the biosensor.

self-assembled monolayer of 3-MPA on a bare gold electrode resulted in an increased R_{et} from 0.26 kΩ to 4.79 kΩ (Fig. 2A, b). In addition, the 3-MPA SAM-modified gold electrode led to a dramatically decreased reduction peak from 9.01×10^{-5} A (Fig. 2B, a) to 5.67×10^{-5} A (Fig. 2B, b). These results indicate that the 3-MPA SAM has been well formed on a bare gold electrode and the carboxylate anions of the tightly organized 3-MPA SAMs on the gold electrode surface perturbed the rates of interfacial electron transfer between the electrode surface and the redox probe in the electrolyte solution. After the 3-MPA modified electrode was activated by EDC and NHS, the estrogen receptor-α was covalently attached to the modified gold electrode. The diameter of the semicircle (Fig. 2A, c) was significantly enlarged from 4.79 kΩ (Fig. 2A, b) to 12.4 kΩ A and the reduction peak was decreased from 56.7 μA ((Fig. 2A, b) to 8.40 μA (Fig. 2B, c) because the immobilized estrogen receptor-α plays as an insulating layer that inhibits the redox couple to reach the modified gold electrode. In order to eliminate the non-specific bindings, the bovine serum albumin was covalently attached to the remaining binding sites of the estrogen receptor-α-immobilized electrode. The presence of BSA on the modified electrode resulted in the increased R_{ct} from 12.4 kΩ to 22.8 kΩ (Fig. 2A, d) and the reduction peak could not be observed in the present CV experimental conditions (Fig. 2B, d) because the protein BSA also works as an insulating layer. The results obtained from the EIS and CV measurements confirm the successful fabrication of the estrogen biosensor.

Surface morphology was studied with tapping mode AFM. Fig. 3 shows the AFM images of the bare gold electrode (A), estrogen receptor-α-immobilized gold electrode before the BSA treatment (B), and estrogen receptor-α-immobilized gold electrode after BSA treatment (C). Prior to the AFM measurement, a bare electrode was thoroughly rinsed with deionized water and PBS buffer. As shown in Fig. 3A and B, a slight change was observed in AFM images after the immobilization of estrogen receptor-α on gold electrode although the gold electrode used in the AFM study was not atomically flat. The average peak to valley distance was 3.0 ± 0.6 nm and horizontal distance was 61 ± 14 nm, which was quite similar to 74 ± 34 nm for native estrogen receptor-α observed with tapping mode AFM in the previous literature [19]. The BSA treatment of the estrogen receptor-α-immobilized gold electrode resulted in a dramatically different AFM image (Fig. 3C). The roughness of estrogen receptor-α-immobilized gold electrode treated with BSA was 40% larger than that of estrogen receptor-α-immobilized gold electrode in the full range of the AFM image. This result indicates that BSA was attached to the remaining binding sites of the estrogen receptor-α-immobilized electrode.

3.2. Detection of estrogen hormone

The fabricated estrogen biosensor was applied to the detection of estrogen hormone, 17β-estradiol. For the detection of estrogen hormone, the estrogen biosensor was immersed in each 17β-estradiol solution with different concentrations 1.0×10^{-9} M–1.0×10^{-13} M in the period of 90 min. EIS spectra were then obtained at the estrogen biosensor in the presence of 5.0 mM $K_3Fe(CN)_6$/$K_4Fe(CN)_6$ (1:1, v/v) solution prepared in phosphate buffer (pH 7.0). As shown in Fig. 4A, the diameter of Nyquist plots were found to increase linearly as the concentration of the 17β-estradiol increased because the binding of the 17β-estradiol to the immobilized estrogen receptor-α of the estrogen biosensor greatly hindered the diffusion of redox couple towards the biosensor surface. In order to normalize the data, impedance change, $\Delta R_{et} = R_{et}(i) - R_{et}(0)$, was used in the data analysis, where $R_{et}(i)$ and $R_{et}(0)$ represent the electron-transfer resistance after and before the binding of 17β-estradiol to the estrogen biosensor, respectively.

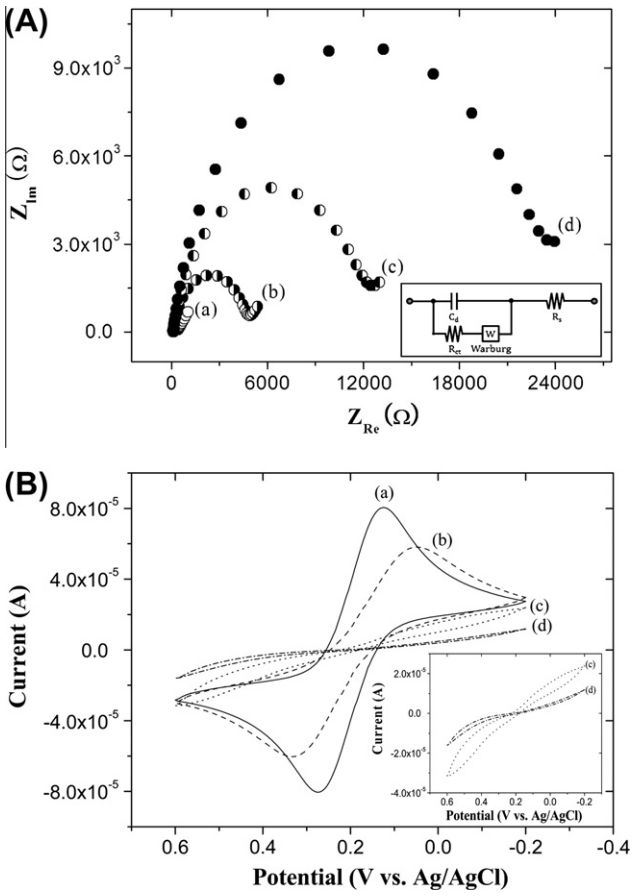

Fig. 2. The Nyquist plots (A) and cyclic voltammograms (B) of 5.0 mM $[Fe(CN)_6]^{3-/4-}$ at a bare gold electrode (a), 3-MPA-modified gold electrode (b), estrogen receptor-immobilized gold electrode (c), and estrogen receptor-immobilized gold electrode with BSA-treatment (d). Inset in (A): four-component equivalent circuit, R_{ct}: charge transfer resistance, R_s: solution resistance, W: Warburg impedance, C_d: double-layer capacitance.

As shown in Fig. 4B, the calibration plot was obtained by plotting the impedance change as a function of the 17β-estradiol concentration. A linear regression equation was obtained as $\Delta R_{et} = -1073$ (±83.7)log[17β-estradiol] + 16,321 (±1273) ($r^2 = 0.992$, $n = 3$). The detection limit of the present estrogen biosensor for 17β-estradiol was experimentally determined to be 1.0×10^{-13} M. The detection limit obtained with the present method is one or two orders of magnitude lower than those reported values in the literature as summarized in Table 1. For example, the detection limits obtained with conventional LC/MS method [20] and enzyme-linked receptor assay [7] were 7.3×10^{-11} M and 3.7×10^{-10} M, respectively. While, the detection limits obtained with aptamer-based square-wave voltammetry [14] the antibody-based EIS [13] were 1.0×10^{-11} M and 6.6×10^{-11} M, respectively.

Association constant, K_a, for the binding reaction between the 17β-estradiol and the estrogen receptor-α was determined by using Langmuir isotherm approach. The linearization of Langmuir isotherm and its relation to R_{et} leads to the equation as follows [21]:

$$\theta = 1 - \frac{R_{et}(0)}{R_{et}(i)} = \frac{K_a \cdot C}{1 + K_a \cdot C}$$

$$\frac{C}{R_{et}(i) - R_{et}(0)} = \frac{1}{R_{et}(i)} C + \frac{1}{K_a \cdot R_{et}(i)}$$

Therefore, K_a could be obtained from the calibration curve, where θ is the fractional coverage of the surface and concentration, C, is in molar concentration unit. Fig. 5 reveals that [17β-estradiol]/ΔR_{et} varied linearly with the concentration and followed a linear equation of [17β-estradiol]/$\Delta R_{et} = 1.478 \times 10^{-4}$ [17β-estradiol] + 8.419×10^{-16} ($r^2 = 0.999$, $n = 3$). From the fitting of the equation, K_a was found to be ca. 1.8×10^{11} M^{-1}. This value is larger to the reported value (5.2×10^8 M^{-1}) based on surface plasmon resonance assay [6]. The binding constant obtained in this study confirms that the estrogen receptor-α was well formed on the gold electrode surface not only with a high density but also with the specificity to its corresponding 17β-estradiol.

In order to study the binding kinetics between 17β-estradiol and estrogen receptor-α, the estrogen biosensor was exposed to the 17β-estradiol solution at room temperature for a certain period time (from 10 to 240 min) and then the relative impedance, $R_{et}(i)/R_{et}(0)$, was measured as a function of binding time. In the experiment, 17β-estradiol solutions with two different concentrations of 1.0×10^{-12} M and 1.0×10^{-13} M were used. As shown in Fig. 6,

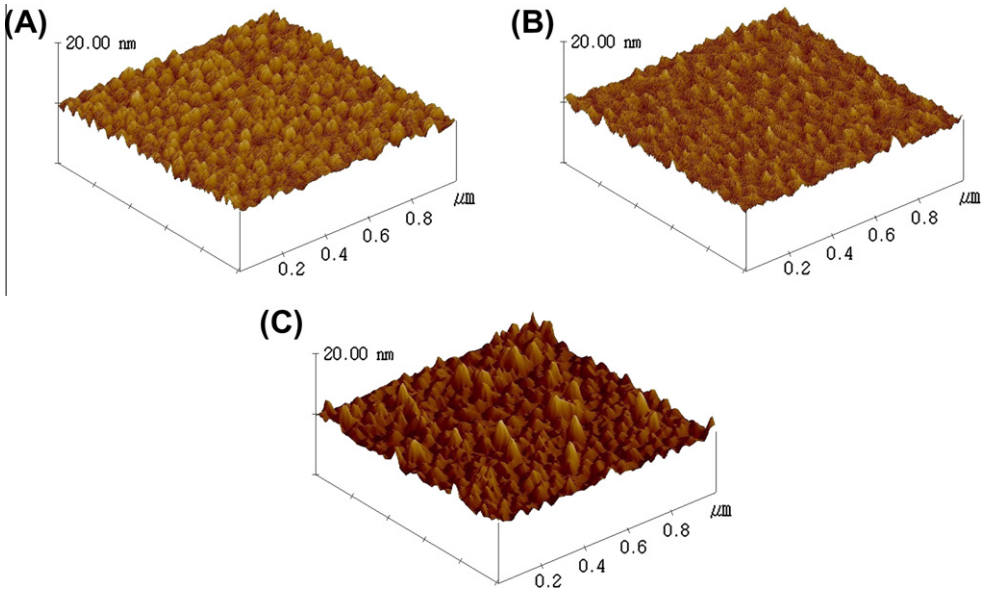

Fig. 3. AFM 3D topography images (1 μm × 1 μm × 20 nm) of a bare gold electrode (A) and estrogen receptor-immobilized gold electrode (B), and estrogen receptor-immobilized gold electrode with BSA-treatment (C).

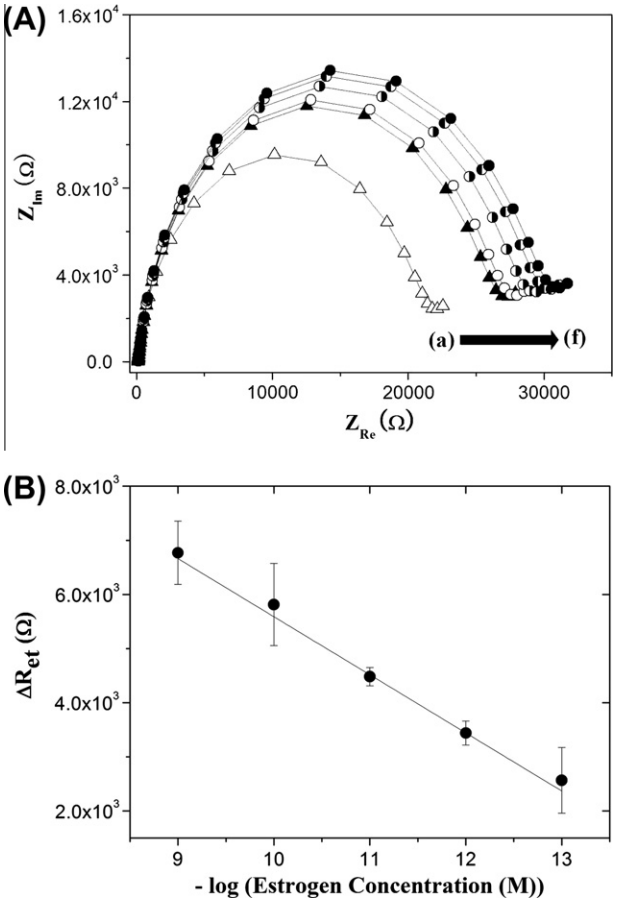

Fig. 4. (A) Nyquist plots for the faradaic impedance measurements in the presence of 5.0 mM $[Fe(CN)_6]^{3-/4-}$ at the estrogen biosensor before (a) and after the treatment of 1.0×10^{-13} M (b), 1.0×10^{-12} M (c), 1.0×10^{-11} M (d), 1.0×10^{-10} M (e), 1.0×10^{-9} M (f) estrogen hormones. (B) Plot of ΔR_{et} versus $-\log$[17β-estradiol]. $R_{et}(0)$: electron transfer resistance at the estrogen biosensor before the treatment of estrogen, $R_{et}(i)$: electron transfer resistance at the estrogen biosensor after the the treatment of a certain concentration of 17β-estradiol. $\Delta R_{et} = R_{et}(i) - R_{et}(0)$.

Table 1
Performance comparison of estrogen detection at different detection methods.

Methods	Linear range (M)	Detection limit (M)	Reference
Enzyme-linked receptor assay		7.3×10^{-11}	[7]
LC/MS		3.7×10^{-10}	[20]
Aptamer/square wave voltammetry	1.0×10^{-11}–1.0×10^{-9}	1.0×10^{-11}	[14]
Estrogen receptor/Au NPs/EIS	1.8×10^{-11}–5.5×10^{-10}	3.7×10^{-12}	[18]
Estrogen antibody/EIS	4.4×10^{-9}–6.6×10^{-11}	6.6×10^{-11}	[13]
Estrogen receptor/EIS	1.0×10^{-13}–1.0×10^{-9}	1.0×10^{-13}	This work

the relative impedances in the two estrogen solutions rapidly increased up to around 90 min and then very slowly increased. Therefore, the estrogen biosensor was immersed in the sample solution for 90 min before EIS measurement in the present work. In order to speed up the experimental time, much shorter incubation time could be used without a significant drop in the sensitivity.

3.3. Selectivity and storage stability of the estrogen biosensor

It is of interest to assess if the present estrogen biosensor exhibits good selectivity against interfering substances with similar chemical structure such as 1-aminoanthraquinone and 2-methoxynaphthalene. The response of the present estrogen biosensor to 1-aminoanthraquinone and 2-methoxynaphthalene were almost negligible (less than 3.5%). The result indicates that the present estrogen biosensor has good selectivity.

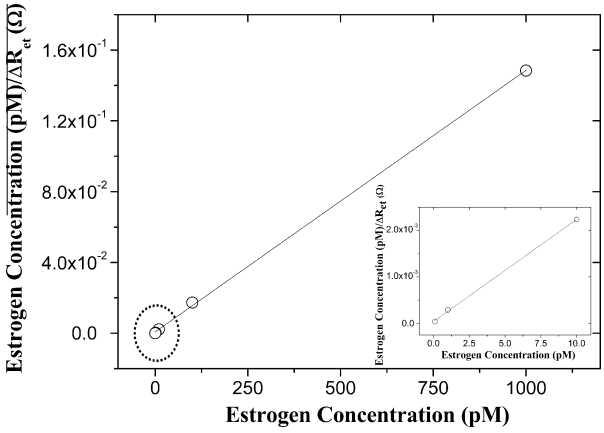

Fig. 5. Plot of [17β-estradiol]/ΔR_{et} versus [17β-estradiol] in 1.0×10^{-13}–1.0×10^{-9} M 17β-estradiol. Inset: plot of [17β-estradiol]/ΔR_{et} versus [17β-estradiol] in 1.0×10^{-13}–1.0×10^{-11} M 17β-estradiol.

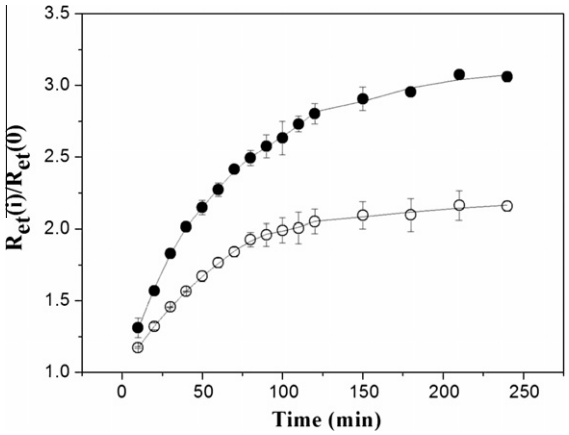

Fig. 6. Plot of $R_{et}(i)/R_{et}(0)$ versus binding time between the estrogen receptor and 1.0×10^{-12} M 17β-estradiol (●) and 1.0×10^{-13} M 17β-estradiol (○).

Operational stability and long-term stability are considered as one of the key factors in biosensor performance. First, the operational stability of the biosensor was tested by repetitive measurements over the concentration range 1.0×10^{-13}–1.0×10^{-9} M. The electron resistance values were reproducible with the relative of standard deviation (RSD) of 8.6% ($n = 3$). The long-term storage stability was studied over a certain period of time by monitoring its electron transfer resistance of the estrogen biosensor in the PBS solution with intermittent usage (every 2–3 days). The present biosensor exhibited good stability, in which the biosensor retained 88% of its initial activity after 3 weeks of storage in 50 mM phosphate buffer at pH 7.0. This stability is better than those reported previously in the literature. For example, the estrogen immunosensor based on anti-estrogen antibody retained 44% of its initial activity after 2 weeks of storage [12] and the estrogen biosensor based on estrogen receptor combined with lipid bilayer and Au nanoparticles preserved 70% of its initial activity after 8 days of storage [18].

3.4. Real sample test

To demonstrate the practical usage of the present biosensor, the recovery test of spiked 17β-estradiol in a human urine sample has been performed. The urine sample was purified with a C-18 cartridge before impedance measurement. The test result was satisfactory with the average recovery of 101 ± 6% for a spiked 1.0×10^{-10} M 17β-estradiol. It is expected that the present biosensor can be employed to the determination of 17β-estradiol in real samples such as urine and possibly serum samples.

4. Conclusions

In this paper, an electrochemical impedance biosensor for the detection of 17β-estradiol was developed by immobilizing the estrogen receptor-α on the gold electrode. The present biosensor gave a linear response ($r^2 = 0.992$) for 17β-estradiol concentration from 1.0×10^{-13} to 1.0×10^{-9} M with a remarkable detection limit of 1.0×10^{-13} M ($S/N = 3$), which is much lower compared to those obtained with other detection methods. Furthermore, the present biosensor exhibited much better stability compared to the estrogen immunosensor based on antibody. Therefore, it should complement antibody-based immunosensor or ELISA in a favorable manner and provide a versatile tool for the analysis of clinically important hormones. In addition, the present estrogen biosensor has potential applications for on-site detection of the 17β-estradiol.

Acknowledgements

This study was supported by a Grant of the Korea Healthcare technology R&D Project, Ministry for Health, Welfare & Family Affairs (A085136) and the Grant of the National Research Foundation of Korea (NRF) funded by the Korea Government (MEST) (No. 2011-0001129).

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