Development of redox-labeled electrochemical immunoassay for polycyclic aromatic hydrocarbons with controlled surface modification and catalytic voltammetric detection

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ABSTRACT

A redox-labeled direct competitive electrochemical immunoassay for polycyclic aromatic hydrocarbons (PAHs) was developed. A ruthenium tris(bipyridine)-pyrenebutyric acid conjugate was synthesized as the redox-labeled tracer. Its recognition by an anti-PAH monoclonal antibody was confirmed by surface plasmon resonance. In the immunoassay, the antibody was immobilized on (3-glycidoxypentyl)-trimethoxysilane (GPTMS)-modified indium tin oxide (ITO) electrodes. The assay was quantified by measuring the electro-catalytic current of the redox label in an oxalate-containing electrolyte which served as a sacrificial electron donor to amplify the current signal. Formation of GPTMS film on ITO and subsequent antibody immobilization were characterized by X-ray photoelectron spectroscopy (XPS) and electrochemistry. Using a ruthenium tris(bipyridine)-conjugated IgG (IgG-Ru) as the surface-bound redox probe, the highest electrochemical signal was obtained on GPTMS electrodes with 1 h modification. Under the optimized conditions for ITO modification, antibody immobilization and tracer concentration, competition curves for benzo[a]pyrene and pyrenebutyric acid were obtained with a detection limit of 2.4 and 10 ng mL⁻¹, respectively. The redox-labeled electrochemical immunoassay with signal amplification mechanism offers a potential analytical method for the simultaneous detection of multiple environmental organic pollutants on antibody biochips.

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1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a major class of environmental pollutants, which are on the priority list of many countries. Many PAHs are either carcinogens or suspected carcinogens, among which benzo[a]pyrene (BaP) is considered as one of the most carcinogenic pollutants (The International Agency for Research on Cancer, 1984). PAHs have been traditionally analyzed in the laboratories by GC–MS with acceptable accuracy, precision and specificity. In recent years, with wide spread PAHs pollution and increasing amount of test samples, there is a huge demand for rapid, low-cost and particularly on-site detection. Since antibodies against PAHs were developed in 1978 (Kado and Wei, 1978), immunoassay has become a promising tool for PAHs screening. Recently, a variety of immunochemical detection techniques for PAHs were reviewed (Fahnrich et al., 2002), including enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), fluorescence, quartz crystal microbalance (QCM) and electrochemical immunoassay (ECIA).

There are already some commercial ELISA kits on the market, such as RaPID Assay[®] and the Ensys[™] (<http://www.sdx.com/>). The detection range of PAHs RaPID Assay[®] for benzo[a]pyrene in water is between 0.1 and 5 ppb. These kits are accepted by USEPA as screening method for PAHs.

ECIA has attracted great attention for the on-site screening of environmental pollutants due to its advantages of short response time, low-cost, small size and adequate sensitivity (Liu et al., 1998, 2000; Fahnrich et al., 2003; Moore et al., 2004; Badihi-Mossberg et al., 2007; Lin et al., 2007). In particular, miniaturization of electrochemical devices can be achieved by employing well-established microelectronics fabrication techniques. The ease of miniaturization makes electrochemistry one of the most suitable methods for biochip detection. In general, there are three types of ECIA according to the labels used for signal detection, namely enzymes, nanomaterials, and small redox molecules. Enzyme-labeled ECIA is the most common assay, and it matches ELISA in terms of sensitivity (Diaz-Gonzalez et al., 2005; Li et al., 2008). However, it is not well suited for electrode array detection because the signal-generating species (either enzyme substrate or product) diffuse freely in solution and might cause cross-talk between electrodes. Nano-labels are usually dissolved into solution before signal detection. So they

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Scheme 1. Synthesis of ruthenium tris(bipyridine)-pyrenebutyric acid (PAH-Ru) conjugate.

suffer from the same problem as enzyme labels, although they possess much higher stability (Das et al., 2006, 2007; Aziz et al., 2007; Guo and Wang, 2007; Liu et al., 2007). Small molecule redox labels in immunoassays are immobilized on electrode surface via antibody/antigen binding, and detected directly (Warsinke et al., 2000; Lin and Ju, 2005). Therefore, cross-talk is not a problem in redox-labeled array detection. One obvious drawback is the lack of signal amplification of the label, and consequently low sensitivity.

Previously, we have developed a signal amplification system based on the measurement of electrocatalytic current of ruthenium(II) polypyridine derivative on indium tin oxide (ITO) electrodes in an oxalate-containing electrolyte (Zheng et al., 2004). Oxalate serves as an artificial electron donor to regenerate Ru(II) and chemically amplify its electrochemical oxidation current. Direct electrochemical oxidation of oxalate on ITO electrode is inhibited, leading to low background current. Using ruthenium(II) polypyridine derivative as a redox label, bio-assays were detected for ds-DNA and biotin/avidin binding pair, with 100-fold improvement in sensitivity over the non-amplifying detection (Li et al., 2005; Guo and Yang, 2005; Wei et al., 2006).

Another potential problem in redox-labeled ECIA is slow electron transfer kinetics between the label and electrode, which are separated by poorly conducting surface modification layer and surface-immobilized antibodies. Electrochemical kinetics is less problematic in enzyme- or nanomaterial-labeled assays, since the signal-generating species in solution can diffuse through the film and reach the electrode. Silane chemistry is a popular method for surface modification of many oxide electrodes including ITO. Previous studies have demonstrated significant detrimental effect of dense-packed silane films on ITO electrochemical reactions (Luzinov et al., 2000; Hillebrandt and Tanaka, 2001). The results point out explicitly that, to achieve efficient electron transfer, high surface coverage must be avoided and delicate control of surface

modification is needed. In a few reports about ECIA on ITO, silane-modified surface was characterized by atomic force microscopy (Yang and Li, 2005; Hao et al., 2007). The assay was quantified by impedance measurement with dissolved $\text{Fe}(\text{CN})_6^{3-}/\text{Fe}(\text{CN})_6^{4-}$. In those reports, silane surface coverage was not controlled deliberately.

In this paper, we developed a redox-labeled direct competitive immunoassay for PAHs with an aim of extending the method to biochip detection. Some key issues in the assay were studied, including (1) synthesis of a redox-labeled tracer, ruthenium tris(bipyridine)-pyrenebutyric acid conjugate; (2) controlled ITO surface modification by epoxysilane to allow for sufficient antibody immobilization and efficient electrochemistry; and (3) electrocatalytic signal amplification detection to improve assay sensitivity. Under the optimized conditions, a competition curve for benzo[a]pyrene was obtained with a detection limit of 10 nM (2.35 ng mL^{-1}), which is comparable to enzyme-labeled ECIA. The improved sensitivity has opened up the possibility of employing small molecule redox labels in ECIA on biochips.

2. Experimental

2.1. Reagents

2.1.1. Commercial reagents

N-hydroxysuccinimide (NHS), 1-[3-(dimethylamino) propyl]-3-ethylcarbodiimide (EDC), sodium oxalate, and oxalic acid were purchased from Alfa Aesar (Ward Hill, MA, USA). Benzo[a]pyrene, acenaphthene, fluoranthene, pyrene, 1-pyrenebutyric acid, (3-glycidyloxypropyl)-trimethoxysilane (GPTMS), ruthenium bis(2,2'-bipyridine)(4-methyl-4'-carboxyl-2,2'-bipyridine) NHS ester (Ru-NHS), bis(2,2'-bipyridine)-4,4'-dicarboxybipyridine-ruthenium di(*N*-succinimidyl ester) bis(hexafluorophosphate)

(Ru-2NHS), and *N, N'*-dicyclohexylcarbodiimide (DCC) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Fluorene was purchased from Shanghai Chemicals Corporation (Shanghai, China). Anti-polyaromatic aromatic hydrocarbon antibody (mouse monoclonal BAP-13) was purchased from Abcam Inc. (Cambridge, MA, USA). Bovine serum albumin (BSA) and mouse immunoglobulin (IgG) were from Xijingke Corporation (Beijing, China). Pepsin porcine (15 milliAnson units mg^{-1}) was purchased from SERVA Electrophoresis GmbH (Heidelberg, Baden-Württemberg, Germany). All other chemical reagents are of analytical reagent (A. R.) grade. Solutions were prepared in high-purity water from a Millipore milli-Q (Biocel) water purification system (Billerica, MA, USA). Unless specified, all proteins were dissolved in phosphate buffered saline (PBS, containing 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.5 mM KH_2PO_4 , pH 7.3). Polycyclic aromatic hydrocarbons (PAHs) were firstly dissolved in methanol, and then diluted with PBS to the desired concentrations with methanol content no more than 10% (v/v).

2.1.2. Ruthenium complex-PAH conjugate (PAH-Ru)

As shown in Scheme 1, the PAH-Ru conjugate was synthesized through a two-step reaction. 1-Pyrenebutyric acid was coupled to a diamine after DCC/NHS activation. The reaction mixture was purified by medium pressure liquid chromatography on a column of silica gel-60 (200–300 mesh) with a mixture of chloroform-methanol-water as the eluent. The compound was then reacted with Ru-2NHS to form the conjugate. The product was purified on a SP Sephadex C-25 column (dimensions 26 mm $\times$ 1000 mm), and then precipitated in a saturated aqueous solution of NH_4PF_6 . The purified product was characterized by UV-vis absorption spectroscopy and electrospray ionization-mass spectrometry (ESI-MS).

2.1.3. Protein labeling with ruthenium complex

Mouse IgG was labeled by mixing the protein with Ru-NHS at a molar ratio of 5 Ru-NHS for each protein in 0.1 M sodium bicarbonate (pH 9.15) for 4 h at room temperature with stirring. After the reaction, the remaining free Ru-NHS was separated from the protein by gel filtration on a D-SaltTM polyacrylamide desalting column from Pierce (Rockford, IL, USA). The labeled protein (denoted as IgG-Ru) was verified by UV-vis absorption spectroscopy, from which the labeling ratio was estimated to be 1.2 per IgG molecule.

2.1.4. PAH-BSA conjugate (PAH-BSA)

1-Pyrenebutyric acid (20 μM) was activated by mixing with NHS (20 μM) and EDC (22 μM) in 0.1 mL DMF for 3.5 h at room temperature with stirring. The mixture was then added into a BSA solution (5 mg in 1.5 mL of 0.1 M sodium bicarbonate, pH 9.15) and reacted for 4 h at room temperature with stirring. The product was run through a desalting column, and the protein fraction was collected.

2.2. Apparatus

2.2.1. X-ray photoelectron spectrometer (XPS) measurement

XPS measurements were made on a VG ESCALAB MkII spectrometer from Thermo VG Scientific (East Grinstead, West Sussex, UK) with a Mg K α X-ray source (1253.6 eV photons). All binding energies (BEs) were referenced to the neutral C_{1s} peak at 284.6 eV.

2.2.2. Surface plasma resonance (SPR) measurement

The SPR detection was performed on BI SPR-1000 System from Biosensing Instrument Inc. (Tempe, AZ, USA) with a gold chip (also from BI).

2.2.3. Electrospray ionisation-mass spectrometry (ESI-MS) measurement

ESI-MS measurement was performed on a Micromass Q-TOF Micro from Waters Corporation (Milford, MA, USA) equipped with an electrospray interface.

2.2.4. Electrochemical measurement

Cyclic voltammetry was performed on a CHI 400A electrochemistry analyzer from CH Instruments (Austin, TX, USA) with a Pt counter electrode and an Ag/AgCl (3 M KCl) reference electrode (also from CHI).

2.3. Methods

2.3.1. Recognition of PAH-Ru by antibody

Binding of the synthesized redox-labeled tracer PAH-Ru to anti-PAH antibody was investigated by SPR measurement. In such measurement, a gold chip was first coated with PAH-BSA by flowing a 50 μL solution of PAH-BSA (100 $\mu\text{g mL}^{-1}$ in PBS) over the chip surface at a flow rate of 20 $\mu\text{L min}^{-1}$. After washing with PBS and blocking with 1% BSA, a solution of anti-PAH antibody (10 $\mu\text{g mL}^{-1}$ in PBS) was injected into the flow cell at the same flow rate, and the angular shift due to antibody binding to PAH-BSA on the chip was recorded as θ_{AB} . The bound antibody was then removed by treatment with 500 $\mu\text{g mL}^{-1}$ pepsin in 200 mM glycine/HCl (pH 2.0). The regenerated PAH-BSA surface was reacted with a mixture solution of antibody (10 $\mu\text{g mL}^{-1}$) and PAH-Ru (1 $\mu\text{g mL}^{-1}$), and the angular shift was recorded as $\theta_{\text{AB+PAH-Ru}}$. In a control measurement, BSA (10 $\mu\text{g mL}^{-1}$) was used instead of the antibody, and the angular shift was recorded as θ_{BSA} .

2.3.2. ITO electrode modification

ITO conductive glass (film thickness: $900 \pm 100 \text{ \AA}$, conductivity: $19 \pm 2.1 \Omega/\square$) was obtained from Nanbo Corporation (Shenzhen, Guangdong, China), and was cut into 5 cm $\times$ 5 cm size slices. The slices were ultrasonically cleaned according to the published procedure (Guo et al., 2006), dried in an oven at 50 °C, followed by treating for 60 s with an Atmospheric Plasma Surface Treatment Systems CTD-1000z from Kulon Electronics Co., Ltd. (Nanjing, Jiangsu, China). The cleaned slices were immersed in a 1% (v/v) solution of GPTMS in dry toluene with shaking (40 rpm) at room temperature for a specified time. After the reaction, the slices were washed ultrasonically in dry toluene (5 min, twice) and anhydrous ethanol (5 min, twice) to remove physically adsorbed GPTMS from the surface. The modified slices were dried under a stream of nitrogen and cured at 115 °C for 20 h. They were stored in a sealed box till use. The modified ITO is denoted as ITO/GPTMS.

2.3.3. IgG immobilization

ITO/GPTMS slices were cut into 2.5 cm $\times$ 0.5 cm electrodes, washed with purified water, and dried under a stream of nitrogen. A 5 μL IgG (0.4 mg mL^{-1}) solution was spread evenly on the bottom of each electrode over a 0.5 cm $\times$ 0.5 cm area, and incubated in a moisturized container at 4 °C overnight. After the surface reaction, the electrodes were washed for 5 min each in PBS (with 500 mM NaCl) and PBS on a shaker, and then dried with nitrogen. The coated electrodes are denoted as ITO/GPTMS/IgG. IgG-Ru immobilization was achieved by following the same procedure, denoted as ITO/GPTMS/IgG-Ru.

2.3.4. Electrochemical immunoassay

ITO/GPTMS slices were cut into 2.5 cm $\times$ 0.2 cm electrodes. Anti-PAH antibody was immobilized on the electrodes by following the same procedure as described above for IgG, using 2 μL of 300 $\mu\text{g mL}^{-1}$ antibody for each electrode. Unreacted epoxy sites were blocked by incubating in 3% (v/v) ethanolamine at 4 °C

Fig. 1. Surface plasma resonance (SPR) angular shift of PAH-BSA adsorbed gold chip after injection of (a) $10 \mu\text{g mL}^{-1}$ anti-PAH antibody, (b) a mixture solution of $10 \mu\text{g mL}^{-1}$ anti-PAH antibody and $1 \mu\text{g mL}^{-1}$ PAH-Ru, and (c) $10 \mu\text{g mL}^{-1}$ BSA. Flow rate: $20 \mu\text{L min}^{-1}$.

overnight. To perform a competitive immunoassay, a $5 \mu\text{L}$ solution containing a PAH analyte and the tracer PAH-Ru was deposited onto the electrode and incubated in a moisturized container at 37°C for 3 h. After washing in PBS, voltammetry was measured in 30 mM oxalate/ 10 mM phosphate buffer (pH 5.8). The electrode area in contact with the electrolyte was 4 mm^2 .

3. Results and discussion

The PAH immunoassay we aimed to develop in this study is a direct competitive immunoassay using surface-immobilized anti-PAH antibody and redox-labeled tracer. To take advantage of the electro-catalytic signal amplification system we developed previously (Zheng et al., 2004; Guo and Yang, 2005; Li et al., 2005; Wei et al., 2006), i.e., ruthenium polypyridine complex signaling molecule with oxalate electron donor, a ruthenium tris(bipyridine)-pyrenebutyric acid conjugate (PAH-Ru) was synthesized as the redox-labeled tracer, as shown in Scheme 1. The product was characterized by UV-vis absorption spectroscopy and ESI-mass spectrometry. In the UV-vis absorption spectrum, a broad peak with maximum absorbance at 470 nm , characteristic of six-coordinated ruthenium polypyridine, was observed (Fig. S1 in the Supplementary Material). ESI-mass spectrometry produced a specific peak at 803.94 for $[\text{M}+2\text{NH}_4]^{2+}/2$ (Fig. S2). In the positive ion mode (ESI^+), two amino groups from residual NH_4PF_6 can be attached to the metal complex to form $[\text{M}+2\text{NH}_4]^{2+}$.

For the PAH-Ru conjugate to be used as a tracer in PAH immunoassays, it needs to be recognized by anti-PAH antibodies. The recognition reaction was investigated by SPR, in which the hapten pyrenebutyric acid-BSA was adsorbed on a gold sensor chip. When a solution of anti-PAH antibody was passed over the chip surface, an angular shift of about 160 mD was observed (Fig. 1), suggesting antibody binding to the hapten on the sensor surface. If PAH-Ru was added to the antibody solution and exposed to the same surface, SPR angular shift decreased by 40 mD . This is likely due to PAH-Ru binding to the antibody, thus reducing the amount of antibody bound to the hapten on the sensor. The change in SPR angular shift suggests PAH-Ru could be recognized by anti-PAH antibody and used as a redox tracer in competitive PAH immunoassays.

The next step in immunoassay development is antibody immobilization. In the signal amplification system we developed, ITO electrodes were used to suppress the background current from

Fig. 2. Cyclic voltammograms of ITO/GPTMS electrode as a function of GPTMS modification time: (a) 0, (b) 20 min, (c) 1 h, (d) 2 h, (e) 4 h, and (f) 24 h. Electrochemical measurement was carried out in PBS (pH 7.3) containing $50 \mu\text{M}$ $\text{Ru}(\text{bpy})_3^{2+}$. The electrode area in contact with the electrolyte was 25 mm^2 . Scan rate: 10 mV s^{-1} .

oxalate electro-oxidation. To covalently immobilize antibodies on ITO electrodes, it is necessary to introduce reactive chemical groups to the surface. 3-Glycidioxypropyl-trimethoxysilane (GPTMS) was chosen in our work for ITO modification, because the silane surface chemistry on ITO is well established; and protein coupling to the epoxy group is a simple one-step reaction (Yang and Li, 2005; Hao et al., 2007; Liao, 2007). However, it is well known that nonconductive organic silane films on electrodes have detrimental effect on electrochemical reactions (Luzinov et al., 2000; Hillebrandt and Tanaka, 2001). Therefore, GPTMS modification on ITO was investigated in details to identify conditions under which antibodies could be immobilized in sufficient amount yet without impeding significantly the electrochemical reaction of the redox label.

ITO electrodes were modified with GPTMS by the procedure described in Section 2. Film formation as a function of surface reaction time was monitored by X-ray photoelectron spectrometry (XPS) and electrochemistry. XPS signal of Si_{2p} at 102.6 eV was used as a signature for GPTMS surface coverage on ITO, since there was no such signal on bare electrodes. The intensity of Si_{2p} was found to rise as GPTMS modification time was increased, whereas the relative intensity of other elements (such as In, Sn, O, and C) decreased with time (Fig. S3). The growth of Si_{2p} intensity was significant even at the shortest reaction time of 20 min, suggesting rapid reaction kinetics in the initial phase of surface modification. The modified electrode was also characterized by cyclic voltammetry of two redox probes dissolved in solution, $\text{Fe}(\text{CN})_6^{3-}$ and $\text{Ru}(\text{bpy})_3^{2+}$. Ferricyanide is a popular redox probe because it exhibits quasi-reversible electrochemical response on many electrodes including ITO. Not surprisingly, in cyclic voltammograms a couple of well-defined redox peaks were obtained on bare ITO, with peak separation of about 70 mV (Fig. S4). Once the electrode was reacted with GPTMS, however, the peak current dropped, accompanied by an increase in peak separation. As the surface reaction time was prolonged, peak current kept decreasing, with further increase in peak separation. When the reaction time exceeded 4 h, there was no electrochemical response in the voltammogram. Apparently, formation of GPTMS film on ITO impeded electrochemical reaction of $\text{Fe}(\text{CN})_6^{3-}$. The results suggest GPTMS surface coverage was dependent on reaction time, which is consistent with XPS data. Cyclic voltammetric measurements in $\text{Ru}(\text{bpy})_3^{2+}$ produced slightly different results (Fig. 2). Voltammetric response of modified electrodes did not change from bare ITO until modification time exceeded 1 h. It seems that GPTMS film is less inhibitive toward

Fig. 3. Voltammograms of (a) ITO/GPTMS/IgG-Ru, (b) ITO/IgG-Ru, (c) ITO, and (d) ITO/GPTMS electrode. Inset: current response as a function of the modification time. Electrochemical measurement was carried out in 30 mM oxalate/10 mM phosphate buffer (pH 5.8). The electrode area in contact with the electrolyte was 25 mm². Scan rate: 10 mV s⁻¹.

electrochemical reaction of positively charged redox probes. This is a beneficial effect, since Ru(bpy)₃²⁺ is the redox label to be used in our immunoassay.

In the following experiments, protein immobilization on GPTMS-modified ITO electrodes and electrochemistry of protein-attached redox label on such electrodes were investigated. After surface modification at desired time, mouse IgG was immobilized on ITO by following the procedure described in Section 2. XPS peak of N_{1s} at 400.1 eV was used to probe surface-immobilized IgG, as there was no such peak for either bare or GPTMS-modified ITO (Qin et al., 2007). As depicted in Fig. S5, N_{1s} peak intensity increased substantially with GPTMS modification time, revealing more protein immobilized on the surface. Some signal was present for IgG incubated on bare ITO, most likely due to non-specific adsorption. Together with the data in Fig. S3, it is obvious that longer surface modification time produced higher GPTMS coverage on ITO, which consequently resulted in increased protein immobilization.

Although high antibody coverage on solid surface is generally desirable in routine immunoassays, it is not necessarily beneficial in electrochemical immunoassays. The reason has been discussed in Section 1. The detrimental effect of surface modification on electrochemical reaction of dissolved redox probes is already demonstrated in Fig. 2. However, in redox-labeled immunoassays, the signaling molecule is bound to the electrode surface, not in solution. To mimic the situation, electrochemical response of a ruthenium tris(bipyridine)-labeled mouse IgG (IgG-Ru) immobilized on GPTMS-modified ITO was investigated. Electrochemical measurement was carried out in an oxalate-containing electrolyte for electro-catalytic signal amplification. As illustrated in Fig. 3, IgG-Ru immobilized on GPTMS-modified ITO (1 h modification) displayed a current response with a plateau at the anodic end of potential scan, characteristic of an electro-catalytic reaction. A low and broad oxidation peak at 0.8 V was observed on IgG-Ru coated electrodes, which did not exist on either bare or modified ITO. This peak can be attributed to electro-oxidation of tyrosine and tryptophan residues of IgG, which has been reported for other proteins on carbon and ITO electrodes (Cai et al., 1996; Guo and Qu, 2006). The electro-catalytic current of IgG-Ru measured at 1.2 V initially increased with GPTMS modification time, then decreased when the time exceeded 1 h (Fig. 3 inset). Since the XPS data already revealed that the amount of immobilized IgG increased monotonically with modification time, the drop of current at longer hours is appar-

Fig. 4. Electrochemical response of anti-PAH antibody immobilized ITO electrodes after competitive immunoassay with 17 μg mL⁻¹ PAH-Ru and (a) 0, (b) 0.11, (c) 0.32, (d) 0.96, (e) 2.88, (f) 8.65, and (g) 26.00 μg mL⁻¹ 1-pyrenebutyric acid. Also shown are voltammograms of (h) ITO/GPTMS/Ab, and (i) ITO/GPTMS electrode. Electrochemical measurement was performed in 30 mM oxalate/10 mM phosphate buffer (pH 5.8). The electrode area in contact with the electrolyte was 4 mm². Scan rate: 10 mV s⁻¹.

ently due to inhibition of electrochemical reaction of the ruthenium complex by the organic film on ITO. It seems that 1 h modification strikes a balance between the amount of immobilized IgG and electrochemical reactivity of the label attached to the protein. Presumably, the electrochemical response observed on 1-h-modified ITO arose from electron transfer at sites where the electrode surface was exposed and the redox label could approach.

Therefore, to conduct a competitive PAH immunoassay, anti-PAH antibody was immobilized on 1-h-modified ITO. Antibody concentration (0.3 mg mL⁻¹), antibody immobilization time (12 h), and PAH-Ru concentration (12 μM) were all optimized in a series of experiments, as depicted in Figs. S6–S9. Under these conditions, the redox tracer bound to the antibody on ITO produced some electro-catalytic current (Fig. 4). The binding was very specific, since there was only residual current on mouse IgG immobilized electrode. As pyrenebutyric acid was added in, the current measured at 1.25 V (where signal-to-background ratio is the highest) dropped in an exponential fashion, reflecting the competitive binding between pyrenebutyric acid and PAH-Ru for the antibody. At the highest pyrenebutyric acid tested, the voltammetric curve was very similar to that of the mouse IgG surface. According to the competition curve, IC₅₀ and lower detection limit were calculated to be 1.28 μg mL⁻¹ and 10 ng mL⁻¹, respectively.

Benzo[a]pyrene (BaP) is considered as one of the most carcinogenic PAHs, and has thus generated considerable interest in the rapid and sensitive detection of this pollutant by immunoassays including ECIA (Liu et al., 1998, 2000). Detection of BaP was also attempted in our work. Because the solubility of BaP in aqueous solution is poor (about 10 μM), the concentration of PAH-Ru tracer used in the competitive immunoassay was lowered to 6 μM so as to obtain a competition curve with BaP at low concentrations. As shown in Fig. 5, a relationship between the normalized current and logarithmic concentration of BaP was obtained, with R² of 0.983. The RSD values were between 3% and 9% (n = 3). BaP at the highest tested concentration (10 μM) leads to a loss of signal by approximately fifty eight percent. The detection limit (S/B = 3) is estimated to be 10 nM (equal to 2.4 ng mL⁻¹), which is 10-fold lower than the result obtained in a previous work by competitive inhibition voltammetric immunoassay (Liu et al., 2000), and is as sensitive as the enzyme-labeled amperometric immunoassay for phenanthrene (Fahnrich et al., 2003). The detection limit for BaP

Fig. 5. Relationship between the electrochemical response and concentration of benzo[a]pyrene (BaP). Immunoassay and electrochemical measurement were carried out by the same procedure as in Fig. 4.

varies in other established immunoassays. Detection limit is 0.5 ppb in fluorescence immunoassay, between 0.1 and 0.7 ppb in ELISA, and 0.15 ppb in total internal reflection fluorescence immunoassay (Tschmelak et al., 2005).

To evaluate the selectivity of our immunoassay, five representative PAHs including acenaphthene, fluorene, fluoranthene, pyrene, and 1-pyrenebutyric acid were tested and compared with BaP. Cross-reactivity (CR) is often employed as a parameter for evaluating the selectivity, and is usually calculated from IC_{50} value in a competition curve. Unfortunately, IC_{50} for BaP could not be obtained due to its limited solubility. Therefore, signal inhibition with 10 μ M PAH in the competition assay was used instead of IC_{50} . And CR of a PAH is defined as signal inhibition by the PAH at 10 μ M divided by signal inhibition by BaP at the same concentration. Other PAHs including acenaphthene, fluorene, fluoranthene, pyrene and 1-pyrenebutyric acid exhibited a relative cross-reactivity of 8%, 11%, 68%, 34%, and 205% respectively (data not shown). The three-ring PAHs generally exhibited lower CR than the four-ring PAHs, and 1-pyrenebutyric acid showed the highest CR than all other PAHs. The results are in good agreement with the CR values calculated from IC_{50} in ELISA measurements (Scharnweber et al., 2001).

4. Conclusions

We have developed a direct competitive electrochemical immunoassay of PAHs, using a novel ruthenium tris(bipyridine)-pyrenebutyric acid conjugate redox tracer and electrocatalytic signal amplification system. Epoxysilane-modified ITO electrode surface was characterized by XPS as well as dissolved and surface-bound redox probes. Optimal modification condition was identified to achieve a balance between the amount of surface-immobilized antibody and the electrochemical kinetics of the redox label. Under the optimized conditions, a competitive immunoassay for benzo[a]pyrene yielded a detection limit of 10 nM (2.4 ng mL^{-1}) and acceptable cross-reactivity with other PAHs. The Maximum Contaminant Level (MCL) has been set at 0.2 ppb by USEPA. Obviously, the detection limit obtained in our work is not sufficient for

most practical applications. To improve the sensitivity, study is currently underway which employs nano-porous ITO film electrodes to enhance electron transfer kinetics of the redox label (Yang and Guo, 2009). It is hoped that the new electrochemical assay method can be used in the simultaneous detection of multiple analytes on antibody biochips.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2009.02.031.

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