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Metal ions-based immunosensor for simultaneous determination of estradiol and diethylstilbestrol

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ABSTRACT

Environmental estrogens (EEs) can cause various endocrine diseases. Herein, we designed an ultrasensitive electrochemical immunosensor for simultaneous detection of two typical EEs, estradiol and diethylstilbestrol. These two analytes were immobilized on graphene sheet (GS) modified glassy carbon electrode (GCE). Amino-group functionalized mesoporous Fe_3O_4 (Fe_3O_4–NH_2) was loaded with Pb^{2+} or Cd^{2+}, and then incubated with estradiol and diethylstilbestrol antibodies, respectively. Using an electrochemical analysis technique, two well-separated peaks were generated by the redox reaction of Pb^{2+} or Cd^{2+}, making the simultaneous detection of two analytes on the electrode possible. Subsequently, square wave anodic stripping voltammetry (SWASV) and electrochemical impedance spectroscopy (EIS) were used to investigate the electrochemical behaviors of the immunosensor. Under optimized conditions, the SWASV peak currents were proportional to the concentrations of estradiol and diethylstilbestrol in the range from 0.050 pg mL^{-1} to 100 ng mL^{-1} and 1.0 pg mL^{-1} to 100 ng mL^{-1}, respectively. The immunosensor exhibited highly sensitive response to estradiol with a detection limit of 0.015 pg mL^{-1} and diethylstilbestrol with a detection limit of 0.38 pg mL^{-1}. Furthermore, the immunosensor was satisfactorily employed to detect estradiol and diethylstilbestrol simultaneously in water samples.

1. Introduction

Environmental estrogens (EEs) have recently attracted the attention of the scientific community and the general public. The presence of low concentrations of estrogens in the environment can disrupt the endocrine system of humans, cause abnormal sexual development and decrease the average number of human spermatozoa (Chang et al., 2009). Estradiol and diethylstilbestrol are two typical EEs. Estradiol is a naturally-occurring steroid hormone. Diethylstilbestrol is an artificially synthesized non-steroidal hormone. Humans can be exposed to estradiol and diethylstilbestrol on a daily basis especially because both are usually present in water (Jiang et al., 2012). In addition, the molecular function of estradiol and diethylstilbestrol is practically equivalent according to previous studies (Schueler, 1946; Koch, 1948; Keasling and Schueler, 1950). They have the same drug mechanism and therapeutic effect, making the two compounds difficult to distinguish (Highman et al., 1980; Wiese et al., 1995; Ochi, 1999; Kipp and Ramirez, 2001). Therefore, simultaneous detection of the two compounds is particularly important.

Various techniques for the detection of either estradiol or diethylstilbestrol have been developed separately, such as high performance liquid chromatography (HPLC) (Yamada et al., 2002; Yang et al., 2012), direct electrochemistry (He et al., 2003; Bin et al., 2005; Zhang et al., 2002), molecular imprinting technology (Yuan et al., 2011) and chemiluminescence (Zhang et al., 2008; Wang et al., 2004). Recently several studies have focused on fabricating electrochemical immunosensors for ultrasensitive and fast detection of estradiol or diethylstilbestrol. These immunosensors are based on highly specific recognition elements, such as antigen–antibody complexes (X. Liu et al., 2010; S. Liu et al., 2012; X. Liu et al., 2012) and aptamers (Yildirim et al., 2012; Kim et al., 2007). In previous studies, a multiplexed electrochemical immunoassay was usually employed to detect cancer biomarkers (Song et al., 2010; Kong et al., 2013) and other biomolecules (Feng et al., 2012; Bai et al., 2012; Xiang et al., 2011). However, the simultaneous detection of two EEs was seldom reported. To the best of our knowledge, there is no report about electrochemical detection of both estradiol and diethylstilbestrol at the same time yet.

Nanomaterials have been widely employed in electrochemical immunoassays in order to improve detection sensitivity. Nanomaterials such as magnetic beads (Wei et al., 2010), colloidal gold nanoparticles (Liu and Ju, 2003), alloy particles (Cai et al., 2003; Yang et al., 2011; Xu et al., 2011), polymers (Zhang et al., 2012) and carbon nanotubes (Ye and Ju, 2005; Guan et al., 2005) have all been used as biomarker carriers. Compared with the above-mentioned nanomaterials, mesoporous Fe_3O_4 offers many advantages as an

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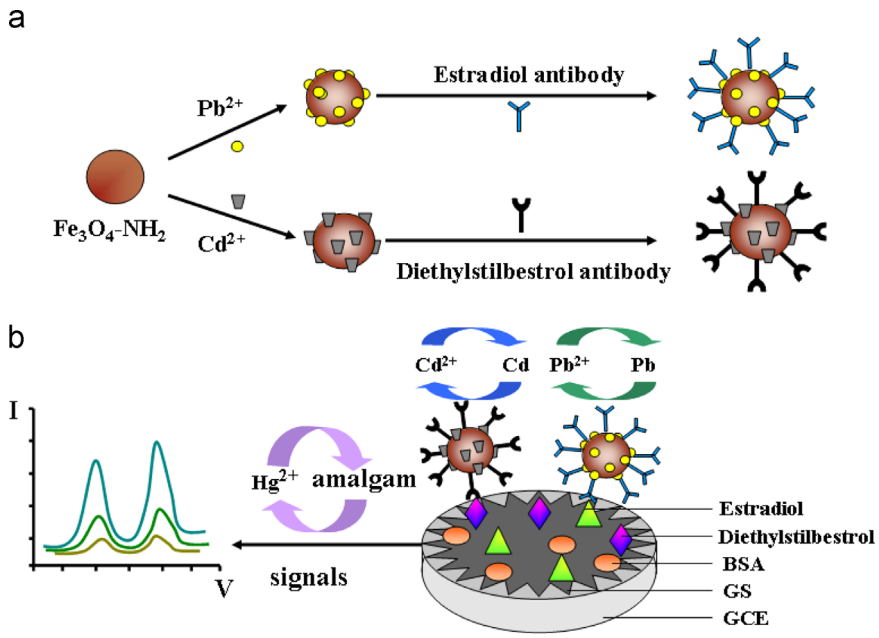

Scheme 1. Schematic representation of the preparation of labels (a) and immunosensor (b).

immobilization material for sensor construction. It possesses lower mass transfer resistance, has more specific surface area for the binding of a larger number of biomolecules, and can facilitate the separation of the immobilized biomolecules from a reaction mixture due to its magnetic properties (Li and Gao, 2008). Mesoporous Fe_3O_4 is also environmentally friendly, is easy to prepare, and possesses excellent water solubility (Cao et al., 2003; Yang et al., 2009; Gao et al., 2013). Moreover, amino-group functionalized mesoporous Fe_3O_4 (Fe_3O_4–NH_2) has remarkable adsorption performance for Pb^{2+} and Cd^{2+}. The obtained Fe_3O_4–NH_2–Pb^{2+} and Fe_3O_4–NH_2–Cd^{2+} exhibit stable and detectable electrochemical signals, enabling the immunosensor to achieve a high sensitivity.

Graphene sheets (GS) have been used in the past to fabricate immunosensors due to their unique advantages, such as high conductivity, high surface area-to-volume ratio, electronic properties, thermal conductivity and good biocompatibility (Park and Ruoff, 2009; Du et al., 2010; Zhong et al., 2010; Qin et al., 2010; Balandin et al., 2008). The large surface area of GS plays a significant role in the high sensitivity of the GS-based sensors due to a fast rate of electron transfer and a high concentration of biomolecules binding sites (Eissa et al., 2012). Moreover, compared with other carbonaceous materials, GS strongly adsorb aromatic compounds with benzene rings through π–π stacking (Cai et al., 2011). Thus, the advantages of utilizing GS in the sensor ensure the direct and stable immobilization of estradiol and diethylstilbestrol.

Metal ions as labels in immunosensors are also beneficial due to their highly sensitive electrochemical response and signals amplification capability (Wang and Tian., 1998). The introduction of different metal ions can achieve simultaneous detection of multiple analytes in a common electrode with unique peaks. Usually, a mercury film modified electrode is used because ionic metal can be reduced and dissolved in the mercury forming an amalgam (Barek et al., 2001). Significantly low detection limits can be achieved with anodic stripping voltammetry (ASV) (Doyle et al., 1982), a technology which has been developed and refined for several decades (Gardiner and Rogers, 1953; Ross et al., 1956; DeMars and Shain, 1957; Baranski, 1987; McGaw and Swain, 2006; Tarley et al., 2009; Wan et al., 2010). Furthermore, using square wave anodic stripping voltammetry (SWASV), well defined peaks can be observed reflecting the concentration of analytes with metal ions as labels.

In this work, we developed an ultrasensitive and multiplexed electrochemical immunoassay for estradiol and diethylstibestrol which were immobilized onto GS due to π–π stacking. Fe_3O_4–NH_2 loaded with Pb^{2+} and Cd^{2+} were incubated with the estradiol or diethylstibestrol antibodies (Ab) with glutaraldehyde, respectively. The immunosensor was prepared by the immunoreaction between Ab and analytes. Under electrodeposition, Hg^{2+} was introduced to reduce the metal ions and form an amalgam. The amalgam was subsequently reoxidized to ions via a stripping analysis technique to achieve simultaneous detection.

2. Experimental

2.1. Preparation of labels

The reagents and apparatus as well as the preparation of Fe_3O_4–NH_2 and GS are described in detail in the Supporting Information (S1–S3). The labels were prepared as follows: two 0.1 g samples of the as-prepared Fe_3O_4–NH_2 were added into two 100 mL solution containing 100 mg L^{-1} of the each metal ion and stirred at 170 rpm for 2 h. Thereafter, metal ion-adsorbed Fe_3O_4–NH_2 was separated from the mixture via external magnetic field. After rinsing with ultrapure water and drying under vacuum, the products (Fe_3O_4–NH_2-Pb^{2+}, Fe_3O_4–NH_2–Cd^{2+}) (1 mg) were mixed with 0.5 mL of 2.5% glutaraldehyde solution and stirred for 1 h. The products were then centrifuged and dispersed in 0.5 mL of pH 7.4 phosphate buffer solution (PBS). Finally, 5 μg of estradiol Ab were added into Fe_3O_4–NH_2–Pb^{2+} hybrids and diethylstilbestrol Ab were added into Fe_3O_4–NH_2–Cd^{2+} hybrids and shaken for 12 h. After centrifugation, the obtained bioconjugates were further washed with PBS at least three times and resuspended in 4 mL PBS as the assay solution. The preparation of labels is shown in Scheme 1a.

2.2. Modification of the immunosensor

The bare glassy carbon electrode (GCE) was first polished to a mirror-like surface using 1, 0.3, and 0.05 μm alumina powder sequentially followed by ultrasonic washing in ethanol and ultrapure water. 3 μL of GS were added onto the GCE. After drying, 3 μL