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Label-free immunosensor for the detection of kanamycin using Ag@Fe_3O_4 nanoparticles and thionine mixed graphene sheet

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ABSTRACT

A highly sensitive label-free immunosensor for the detection of kanamycin had been developed using silver hybridized mesoporous ferroferric oxide nanoparticles (Ag@Fe_3O_4 NPs) and thionine mixed graphene sheet (TH-GS). TH was used as an electron transfer mediator. The electrical signal was greatly improved in the presence of GS due to its good electron-transfer ability. With the advantages of large specific surface area and excellent electrical conductivity, Ag@Fe_3O_4 NPs could immobilize more antibodies of kanamycin and promote the electron transfer. Cyclic voltammetry and square wave voltammetry were used to characterize the recognition of kanamycin. The proposed immunosensor showed good performances such as low detection limit (15 pg mL^{-1}), wide linear range (from 0.050 to 16 ng mL^{-1}), short analysis time (3 min), high stability, and good selectivity in the detection of kanamycin. The immunosensor was evaluated for pork meat sample, receiving satisfactory results.

1. Introduction

Kanamycin is an aminoglycoside antibiotic, which is produced by the fermentation of *Streptomyces kanamyceticus* and used as sulfate or acid sulfate salt (Manyanga et al., 2010). Similar to other aminoglycosides, kanamycin exhibits comparatively narrow safety margin and may cause many side effects, such as loss of hearing, toxicity to kidneys, and allergic reactions to drugs (Oertel et al., 2004). In addition, the residual amount of kanamycin in foodstuff may lead to antibiotic resistance from the pathogenic bacterial strains, which can endanger the consumer (Zhu et al., 2012). For the sake of consumers' security, the European Union has established maximum residue limits for kanamycin in edible tissues and milk: 100 μg kg^{-1} for meat, 600 μg kg^{-1} for liver, 2500 μg kg^{-1} for kidney, and 150 μg kg^{-1} for milk (EMEA/MRL/886/03-FINAL, 2003).

A number of analytical methods, such as high performance liquid chromatography (Chen et al., 2006; Zhou et al., 2007), square-wave cathodic adsorptive stripping voltammetry (Yan, 2008), immunoassay (Elma et al., 2003), capillary electrophoresis (Kaale et al., 2003), surface plasmon resonance (Marco et al., 2010; Sabina et al., 2009), and the microbiological multi-residue system (Althaus et al., 2009), have been reported for the detection of kanamycin. Compared with these methods, electrochemical immunosensors are miniaturized analytical devices with many merits such as simple sample pretreatment, inexpensive instrument, high sensitivity, and selectivity (He et al., 2009). Hence, electrochemical immunosensors have become one of the major analytical techniques for the detection of biomolecules. Among all of the electrochemical immunosensors, label-free immunosensor especially has attracted great research interests due to their simple preparation, more cost effectiveness and well activity conservation of antibodies or antigens (Marchesini et al., 2008; Mukhopadhyay et al., 2005; Xu et al., 2005).

With the rapid development of nanotechnology, various nanomaterials have been used to develop electrochemical biosensors, such as carbon nanotubes (Zhang et al., 2010), metal oxides (Ansari et al., 2008), metal nanoparticles (Lan et al., 2010; Wang et al., 2005; Zhang et al., 2008), and semiconductors (Yang et al., 2010). When used for electroanalysis, metal nanoparticles exhibit some unique advantages, such as enhancement of mass transport, high catalytic activity, large effective surface area and easy control over electrode microenvironment (Gong et al., 2010; Guo et al., 2010; Wang et al., 2010). Ag nanoparticles have an activity for high intensity electron transfer (Hu et al., 2013; Zarchi et al., 2007) and they can facilitate the electron transfer from the redox center of protein to the electrode surface (Chen et al., 2007). Mesoporous ferroferric oxide (Fe_3O_4) nanoparticles have good performance, such as low-cost, environment friendly, easy-prepared, excellent water solubility, large specific surface area, good conductivity and high magnetic saturation (Cao et al., 2003; Gao et al., 2013; Lin and Leu, 2005; Yang et al., 2009). Fe_3O_4 nanoparticles can immobilize more antibodies and promote the electron transfer, so they are

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selected to fabricate electrochemical immunosensors. Silver hybridized Fe_3O_4 ($Ag@Fe_3O_4$) nanoparticles not only keep the advantages of both Ag and Fe_3O_4, but also reflects the excellent synergistic sensitizing effect. And the effect can further increase electron transfer efficiency on electrode surface to improve the detection sensitivity of the immunosensor.

Graphene sheet (GS) has shown several characteristics which may be very beneficial in designing electrochemical sensors, such as high electron transfer rate, large specific surface area and good biocompatibility (Akhavan et al., 2012; Xin et al., 2010). Thionine (TH) has been widely used as an electron transfer mediator for analytical applications in the development of immunosensors (Deng et al., 2008; Feng et al., 2013; Gao et al., 2003; Tang et al., 2010).

In this study, we proposed a novel label-free immunosensor for the sensitive detection of kanamycin based on $Ag@Fe_3O_4$ and TH-GS modified glassy carbon electrode (GCE). Due to its excellent electron transfer activities and abundant mesoporous structures, $Ag@Fe_3O_4$ could not only improve the electron transfer efficiency but also enhance the immobilizing amount of antibodies. Greatly amplified sensitivity was achieved by using GS owing to its large specific surface area. In this paper, the sensitive detection of a model analyte kanamycin, was demonstrated based on the peak current change of TH before and after the antigen–antibody reaction. Other antibiotics such as gentamicin, streptomycin and tobramycin can also be captured by their antibodies to fulfill the detection. The proposed immunosensor showed wide linear range, low detection limit, good reproducibility and selectivity, as well as acceptable stability.

2. Experimental

2.1. Materials and methods

2.1.1. Reagents

Thionine (TH) and glutaraldehyde (GA) were obtained from Guoyao Chemical Co. (Shanghai, China). The primary kanamycin antibody (Ab) and kanamycin were purchased from Beijing Wanger Biotechnology Co., Ltd. (Beijing, China). Bovine serum albumin (BSA, 96–99%) was purchased from Sigma (USA) and used as received. All other chemicals were of analytical reagents grade and used without further purification. Phosphate buffered saline (PBS, 0.1 mol L^{-1} containing 0.1 mol L^{-1} NaCl) was used as electrolyte for all electrochemistry measurements. Double distilled water was used throughout the experiments.

2.1.2. Apparatus

Electrochemical measurements were performed on a CHI 760D electrochemical workstation (Shanghai CH Instruments Co., China). Transmission electron microscope (TEM) images were obtained from a JEM-2100 microscope (Japan). Energy Dispersive X-Ray Spectroscopy (EDS) was recorded by a JEOL JSM-6700F microscope (Japan). X-Ray Photoelectron Spectroscopy (XPS) was recorded by a ESCALAB 250 microscope (ThermoFisher SCIENTIFIC). Raman spectroscopy was recorded by a NEXUS 670 microscope (Thermo Nicolet Co., USA). Fourier Transform Infrared Spectrometer (FTIR Spectrometer) was recorded by a VERTEX70 spectrometer (Bruker Co., Germany). Surface area measurements were performed on Micromeritics ASAP 2020 surface area and porosity analyzer (Quantachrome, United States). The samples were out-gassed overnight under nitrogen prior to adsorption measurement. Pore distributions and pore volume were calculated using the adsorption branch of the N_2 isotherms based on the BJH model. The specific surface area was calculated on the basis of the BET equation. A conventional three-electrode system was used for all electrochemical measurements: Glassy carbon electrode (GCE), 4 mm in diameter as the working electrode, a saturated calomel electrode (SCE) as the reference electrode, and a platinum wire electrode as the counter electrode.

2.2. Preparation of TH-GS

Graphene sheet (GS) used in this experiment was prepared by reducing graphite oxide (GO) according to the previously reported method (Wang et al., 2009). Typically, GO powder was produced from graphite by using a modified Hummer's method (Liu et al., 2008). Reduction of GO was carried out by addition of 0.3 mL of hydrazine into the solution of 30 mg GO in 20 mL water after sonication of 1 h and keeping stirring for 24 h at 50 °C.

In this study, we dispersed TH and GS with chitosan which contained a large amount of amino and proved to be excellent film function to obtain a layer of uniform and stable membrane for good immobilizing. 16 mg of TH was added to 4 mL of 0.5% chitosan solution to get the TH solution. 16 mg of GS was added to 4 mL of 0.5% chitosan solution to get the GS solution. The TH-GS nanocomposite was prepared by mixing GS and TH solution together and stirring at room temperature for 24 h. In this study, TH was directly adsorbed onto GS through π–π stacking (Chen et al., 2011).

2.3. Preparation of $Ag@Fe_3O_4$–Ab

Fe_3O_4 was prepared according to the previously reported method (Guo et al., 2009). Then, 30 mg of Fe_3O_4 and 30 mg of $AgNO_3$ were added into 30 mL of water and initially stirred for 24 h. The supernatant was removed by magnetic separation. 50 mmol L^{-1} of freshly prepared $NaBH_4$ solution was added until color did not change. $Ag@Fe_3O_4$ was obtained by centrifuging, and drying under high vacuum.

The synthesized $Ag@Fe_3O_4$ NPs were added into glutaraldehyde and stirred for 1 h. The supernatant was removed by magnetic separation. Then antibody (Ab) of kanamycin solution (10 μL, 100 μg mL^{-1}) was added and the mixture was stirred for 24 h. After centrifuging and washing with PBS, the $Ag@Fe_3O_4$–Ab conjugation was re-dispersed in PBS and stored at 4 °C before use.

2.4. Fabrication of the electrodes

Fig. 1 shows the schematic illustration of the stepwise procedure for the fabrication of the immunosensor. First, GCE was polished sequentially with 1, 0.3, and 0.05 μm alumina powder and then washed ultrasonically in ethanol and water respectively for a few minutes. Then, 6 μL of the TH-GS mixture was added to the electrode surface and dried. Subsequently, 6 μL of GA solution (2.5%) was added onto electrode surface and incubated for 1 h. GA as a bifunctional linker provided the reactive aldehyde groups for covalent bonding of $Ag@Fe_3O_4$–Ab with TH-GS (Migneault et al., 2004; Wei et al., 2010). After being washed, 6 μL of $Ag@Fe_3O_4$–Ab was added and incubated for 2 h. The electrode was then washed and immersed into 1% of BSA solution for another 1 h to block nonspecific binding sites. Finally, different concentrations of kanamycin solution were dropped onto the electrode to immobilize kanamycin. After being washed, the electrode was ready to be measured.

3. Results and discussion

3.1. Characterization of GS and $Ag@Fe_3O_4$ NPs

The TEM image (Fig. 2a) shows wrinkled paper-like structure of large GS. Fig. S1 shows the Raman spectrum of the GS. The main