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A non-enzymatic electrochemical immunoassay for quantitative detection of *Escherichia coli* O157:H7 using Au@Pt and graphene

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ABSTRACT

Herein, a non-enzymatic sandwich-type electrochemical immunoassay was fabricated for quantitative monitoring of *Escherichia coli* O157:H7 (*E. coli* O157:H7). Silica coated Fe_3O_4 magnetic nanoparticles (Fe_3O_4@SiO_2) were modified with mouse anti-*E. coli* O157:H7 monoclonal antibody (Ab_1) to act as capture probes to reduce detection time and increase the sensitivity of the immunoassay. The Au@Pt nanoparticles were loaded on neutral red (NR) functionalized graphene to form composite complex rGO-NR-Au@Pt. rGO-NR-Au@Pt has high specific surface area and good biocompatibility. rGO-NR-Au@Pt was used as the carriers of detection antibodies (Ab_2). Au@Pt catalyzed the reduction of hydrogen peroxide (H_2O_2) to detection of *E. coli* O157:H7 with the thionine (TH) as electron mediator to effectually amply the current signal. Under the optimized conditions, a linear relationship between the reduction peak current change (ΔI_{pc}) and the logarithm of the *E. coli* O157:H7 concentration is obtained in the range from 4.0×10^3 to 4.0×10^8 CFU mL^{-1} and the limit of detection (LOD) is 4.5×10^2 CFU mL^{-1} at a signal-to-noise ratio of 3. The immunoassay exhibits acceptable specificity, reproducibility and stability on the detection of *E. coli* O157:H7. Furthermore, the immunoassay showed good performance in pork and milk samples. The results suggest that this immunoassay will be promising in the food safety area.

Introduction

Escherichia coli O157:H7 (*E. coli* O157:H7) is a common foodborne pathogen that causes multiple food and water-borne outbreaks worldwide and is considered as serious threat to public health [1,2]. *E. coli* O157:H7 is one of the most dangerous types, causing serious illnesses such as hemorrhagic colitis and hemolytic uremic syndrome in young and immunocompromised individuals [3]. Foodborne outbreaks of *E. coli* O157:H7 are associated with consumption of contaminated foods such as fresh vegetables, juice, and ground beef [4]. Therefore, sensitive and rapid detection of *E. coli* O157:H7 in food is important to prevent the disease outbreaks and ensure health and safety. Currently, conventional culture methods for the detection and enumeration of *E. coli* O157:H7 are still the most commonly practiced techniques in the food safety area [5]. The common isolation methods using selective culturing media takes more than 48 h to complete [6]. The enzyme linked immunosorbent assay (ELISA) method requires certain enrichment procedures that take 16–24 h to achieve low LODs [7,8]. The polymerase chain reaction (PCR) considered as rapid detection methods requires skilled technical staff and expensive instrument and apparatus, restricting its application in life [9,10]. Immune-based methods have been successfully developed for the detection of *E. coli* O157:H7, such as, electrochemical biosensors [11], immunochromatographic assay [12], immunomagnetic separation method [13]. These methods exhibit simple processing, short analysis time, high sensitivity, or good potential applications in practice. Among the various immune-based analytical techniques, the electrochemical immunoassay has been widely applied for the detection of *E. coli* O157:H7, owing to their high sensitivity and specificity, and low manufacturing cost [14,15]. Li et al. designed an impedance biosensor using new kinds of screen-printed interdigitated microelectrodes and wheat germ agglutinin for signal amplification to detect *E. coli* O157:H7 with high sensitivity and time efficiency [16]. Yin et al. used silica coated silver as labels to detect *E. coli* O157:H7 with a low detection limit [17].

In order to amplify the signal and reduce the noise, enzyme is usually used for this purpose. A single enzyme molecule, such as horseradish peroxide and glucose oxidase, possesses very high catalytic efficiency [18,19]. However, the enzymes are unstable in the electrochemical immunosensor or immunoassay, and they are susceptible to external circumstances. Compared to enzyme-based immunoassay, non-enzymatic strategies are less susceptible to interferences and changes of the assay condition during the signal generation stages [20,21]. Various

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types of nanomaterials with excellent performance have been used in non-enzymatic immunoassay to amplify signal, such as quantum dots, multi-walled carbon nanotube, graphene and bimetallic nanoparticles [22,23]. Especially, bimetallic nanoparticles with intrinsic peroxidase-like activity has been widely used in nonenzymatic electrochemical immunoassay [19].

Bimetallic nanoparticles were usually used as labels in electrochemical immunosensors. This is not only because their good conductivity and biocompatibility [24], but also their excellent electrocatalytic activity [25]. Wei and his group designed a facile and sensitive non-enzymatic sandwich-type immunoassay for the detection of carbohydrate antigen 125 based on core-shell Au@Pd bimetallic nanoparticle as labels [26]. Wang and his collaborator developed an ultrasensitive sandwich-type electrochemical immunoassay based on the signal amplification strategy of mesoporous core–shell Pd@Pt nanoparticles and amino group functionalized graphene nanocomposite [27]. The use of the bimetallic nanoparticles as labels in electrochemical immunoassay due to their superior electrocatalytic activity for the reduction of hydrogen peroxide (H_2O_2) [28], but the sensitivity will be restricted when only the labels are the bimetallic nanoparticles. Reduced graphene oxide (rGO) have gained great attention in constructing electrochemical biosensors due to their large specific surface area, great electron transfer properties and good biocompatibility [29]. If the bimetallic nanoparticles are loaded on the rGO, the amount of the protein molecules loaded on the bimetallic nanoparticles will increase. This may increase the sensitivity of the immunoassay. However, during the process of graphene oxide (GO) was reduced to rGO, due to the loss of oxygen containing functional groups, rGO tends to form irreversible agglomerates via van der Waals interactions [30]. And it is also difficult for bimetallic nanoparticles and protein molecules to be immobilized on the surface of rGO directly [31]. As a consequence, different strategies have been developed to functionalize graphene. One of the most effective ways is by the noncovalent strategies [32]. A positively charged phenazine derivatives can stabilize the rGO through the strong affinity between its aromatic ring and the basal plane of graphene via π-stacking [33]. The neutral red (NR) as a positively charged dye not only can stabilize the rGO but also act as a "bridge" to connect the negative charge bimetallic nanoparticles to the rGO [34]. Besides, the bimetallic nanoparticles in the nanometer range enables sustained colloidal suspension to improve the dispersibility of the rGO in aqueous solution [27]. Therefore, the nanocomposites can distinctly improve the performance of the immunoassay.

Fe_3O_4 magnetic nanoparticles (MNPs) have received increasing attention in biosensor technology due to their high surface-to-volume ratios [35], inherent magnetic features [36] and easy to conjugate with protein molecule [37]. Therefore, the utilization of MNPs offers an excellent way for the detection of bacterial targets with improved detection sensitivity and reduced preparation time [38,39].

Herein, we demonstrate a non-enzymatic sandwich electrochemical immunoassay for quantitative detection of *E. coli* O157:H7 using Au@Pt, NR, rGO and MNPs. The MNPs were coated by silica (denoted as Fe_3O_4@SiO_2) and modified with -NH_2 (denoted as Fe_3O_4@SiO_2-NH_2) is in order to conjugate to primary antibodies (Ab_1). The MNPs modified with Ab_1 (denoted as Fe_3O_4@SiO_2-Ab_1) homogeneously dispersed in the media and captured *E. coli* O157:H7 (denoted as Fe_3O_4@SiO_2-Ab_1-*E. coli* O157:H7). The monodisperse Au@Pt, with urchinlike morphology, have good biocompatibility with biomolecules and excellent catalytic capabilities for the reduction of H_2O_2 [40]. And the negative charge Au@Pt were absorbed on the NR functionalized rGO (rGO-NR) by electrostatic interaction (denoted as rGO-NR-Au@Pt). In addition, the secondary antibodies (Ab_2) were conjugated to the rGO-NR-Au@Pt (denoted as rGO-NR-Au@Pt-Ab_2) as the non-enzyme label [27,41]. The final sandwich immunocomplexes (denoted as Fe_3O_4@SiO_2-Ab_1/*E. coli* O157:H7/rGO-NR-Au@Pt-Ab_2) were attached on the surface of the working electrodes of screen printed carbon electrode (SPCE) by an external magnet. Cyclic Voltammetry (CV) is employed to quantify *E. coli* O157:H7 via changes of the reduction peak current in the substrate solution of H_2O_2 with the thionine as electron mediator.

Experimental

Reagents and apparatus

The mouse anti-*E. coli* O157:H7 monoclonal antibody (Ab_1, Ab_2) and *E. coli* O157:H7 (strain B1409) were purchased from Prajna Biology Technique Co. ltd. (Shanghai, China). *Cronobacter sakazakii (C. sakazakii*, ATCC 29544*)*, *Escherichia coli (E. coli*, ATCC 8739*)*, *Citrobacter freundii* (*C. freundii*, ATCC 43864) were purchased from China Center of Industrial Culture Collection (Beijing, China). Chloroauric acid ($HAuCl_4$) was obtained from Hangzhou Chemical Reagent Co., Ltd. (Hangzhou, China). Thionine (TH, dye content ≥85%), chloroplatinic acid hexahydrate ($H_2PtCl_6.6H_2O$), hydrazine monohydrate, tetraethyl orthosilicate (TEOS, 98%), 3-aminopropyltri-ethoxysilane (APTES, 98%), N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC) and N-Hydroxysuccinimide (NHS) were purchased from Aladdin Industrial Inc (Shanghai, China). Ammonia (NH_4OH, 25–28%), hydrogen peroxide (H_2O_2, 30 wt %), anhydrous sodium acetate, ethylene glycol (EG) were provided by Xilong Chemical Co., Ltd (Shantou, China). Poly (4-styrenesulfonic acid-*co*-maleic acid, SS:MA = 1:1) sodium salt (PSSMA) was obtained from Macklin Reagent (Shanghai, China). Iron (III) chloride hexahydrate ($FeCl_3{\cdot}6H_2O$) was purchased from Chengdu Kelong Chemical Reagent Co., Ltd. (Chengdu, China). Phosphate-buffered solutions (PB) were prepared by mixing 0.067 mol·L^{-1} Na_2HPO_4 and 0.067 mol L^{-1} KH_2PO_4 stock solution. Phosphate-buffered saline (PBS, 0.01 M pH 7.4) contained 136.7 mmol·L^{-1} NaCl, 8.72 mmol·L^{-1} Na_2HPO_4, 2.70 mmol L^{-1} KCl and 1.41 mmol L^{-1} KH_2PO_4. Other reagents were all of analytical grade and were used as received without further purification.

All electrochemical measurements were performed on CHI760C electrochemical workstation (Shanghai, China). Screen printed carbon electrode (SPCE) was developed by Rong Bin Biotechnology Co., Ltd. (Nanjing, China). The working electrode (2 mm in diameter) and counter electrode were made of a carbon ink, the reference electrode was made of silver/silver chloride (Ag/AgCl). Scanning electron microscope (SEM) and energy dispersive X-ray spectral data (EDX) were collected on Hitachi SU-8010 field emission scanning electron microscope (Tokyo, Japan). Transmission electron microscope (TEM) images were obtained from a JEM-1200 transmission electron microscope (Tokyo, Japan). Ultraviolet–vis absorption (UV–vis) spectra were recorded with a UV 2600 UV–vis spectrophotometer (Shimadzu, Japan). Malvern Nano 2 S potential laser particle analyzer was provided by Malvern Instruments Co., LtD (Worcestershire, UK).

Preparation of bacterial antigen

E. coli O157:H7 was grown in Lysogeny broth (LB) medium at 37 °C for 18 h and used for specific experiments. The bacteria were harvested by centrifugation for 10 min (4 °C, 6000 rpm). The bacteria after centrifugation were treated by 0.4% formaldehyde solution for 24 h to ensure the personal safety. Finally, the bacteria were resuspended in 5 mL PBS and stored at 4 °C for later usage. The concentration of the bacteria was confirmed by the colony counting (CFU·mL^{-1}).

Preparation of rGO-NR

GO was synthesized according to an improved Hummer's method [42]. The rGO-NR was prepared based on Li's and coworkers' method with some revisions [33]. 2 mg GO was first dispersed in 20 mL water to yield a brown suspension with a concentration of 0.1 mg mL^{-1}. Then, 3 mL of the 0.1 mg mL^{-1} NR aqueous solution was dropped into the 20 mL GO suspension while undergoing constant ultrasound sonication to yield a burgundy, translucent dispersion. Then, 10 μL of 35%