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Sensitive electrochemical detection of *Salmonella* with chitosan–gold nanoparticles composite film

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ABSTRACT

An ultrasensitive electrochemical immunosensor for detection of *Salmonella* has been developed based on using high density gold nanoparticles (GNPs) well dispersed in chitosan hydrogel and modified glassy carbon electrode. The composite film has been oxidized in NaCl solution and used as a platform for the immobilization of capture antibody (Ab_1) for biorecognition. After incubation in *Salmonella* suspension and horseradish peroxidase (HRP) conjugated secondary antibody (Ab_2) solution, a sandwich electrochemical immunosensor has been constructed. The electrochemical signal was obtained and improved by comparing the composite film with chitosan film. The result has shown that the constructed sensor provides a wide linear range from 10 to 10^5 CFU/mL with a low detection limit of 5 CFU/mL (at the ratio of signal to noise, S/N=3:1). Furthermore, the proposed immunosensor has demonstrated good selectivity and reproducibility, which indicates its potential in the clinical diagnosis of *Salmonella* contaminations.

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1. Introduction

Sensitive detection of pathogens in food and drinking water has always been a major challenge in recent years. Due to the high specific binding of antibody against its antigen, the immunosensor based on the two types of proteins' interaction has been one of the most widely used techniques in the quantitative analysis of biomarkers [1–3]. Various immunosensors and immunoassays including radioimmunoassay, quartz crystal microbalance, surface Plasmon resonance, electrochemistry, chemiluminescence, and fluorescence have been built and reported [4–8]. Among these methods, electrochemistry has increasingly become an attractive tool due to its low cost, high sensitivity and ease of miniaturization [9–11]. The fabrication and signal amplification of the immunosensor play crucial roles in successful development of an electrochemical immunosensor. Exploring new materials and strategies for the fabrication of the electrochemical immunosensors is of great interest.

In immunoassay applications, antibodies are immobilized onto biointerface to capture specific biomarkers [3]. For improving the sensitivity and reducing the size of immunosensor, immobilizing antibodies on the substrate surface with high density is essential [12,13]. High surface area nanoparticles offer unprecedented opportunities for high protein loading, which have attracted widespread interest in their use as labels [14–16]. In recent years, various types of nanomaterials have been enlisted as labels to construct electrochemical immunosensors, including carbon-based nanomaterials [17], conducting polymers [18–20], and metal nanoparticles [21]. Gold nanoparticles, in particular, have been widely used to construct biosensors because of their excellent ability to support biomolecules. Recent reports show that GNPs provide an environment similar to the native system of the immobilized biomolecules, which effectively retains their biological function. However, the GNPs are unstable and aggregate easily in the solution or on the solid surface. Decoration of GNPs on the nanomaterials' surface or embedding GNPs in the polymer substrate is considered as an effective way to solve this problem [22,23]. Lu et al. have shown that the performance of the immunosensor can be improved by using dotted GNPs on carbon nanotube–graphene composite [24]. Chen et al. obtained a porous, conductive, stable nanocompsite by co-electropolymerized GNPs and polypyrrole (PPy) [25].

Salmonella, as one of the most common food borne pathogens worldwide, is responsible for a number of infections in warm-blooded

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animals. It is estimated that *Salmonella* causes 93.8 million human infections and 155,000 deaths annually worldwide [26–32]. The primary sources of human *Salmonella* infections are contaminated food or water. The most common clinical syndromes caused by *Salmonella* are recognized as gastroenteritis and diarrhea, which are typically self-limiting in healthy individuals, but can be fatal in immunocompromized individuals. Therefore, sensitive and rapid detection of *Salmonella* is of most importance in the field of food safety and public health. However, the conventional methods for detection of *Salmonella* involve culture pre-enrichment, plating and then identification, and it usually takes 3–4 days to obtain the results. Enzyme-linked immunosorbent assays (ELISA) provide faster detection, but are still time-consuming and can have insufficient sensitivity [33,34]. Furthermore, they are greatly restricted by the assay time at locations in the food processing or distribution network. Electrochemical detection for *Salmonella* has been performed by voltammetry using a peroxidase-labeled antibody [8,11]. Measurement is also possible by following the change in film resistance upon antigen–antibody binding [7,29]. However, the sensitive detection of *Salmonella* remains an analytical goal. This is because outbreaks of salmonellosis have been known to occur from extremely low infective doses ($<$ 10–100 cells) [10].

In the present paper, a highly sensitive electrochemical immunosensor for detection of *Salmonella* has been constructed based on chitosan/GNPs composite film. As chitosan solution is protonated and positively charged, GNPs can be easily adsorbed onto the surfaces of chitosan and form biocompatible film. However, the electron transfer between the electrode and bimolecules is inhibited due to low conductivity of chitosan film [35]. In this study, we have applied the strategy of mixing chitosan and GNPs together, which improves the performance of the composite film greatly. GNPs were well dispersed in chitosan film, and then the composite polymer was modified on the electrode. Due to the electroactive and good film-forming nature of the biopolymer, the oxidized chitosan film can be utilized to conjugate the capture antibody. After incubating the modified electrode in *Salmonella* suspension and horseradish peroxidase (HRP) conjugated secondary antibody (Ab_2) solution, a sandwich electrochemical immunosensor was constructed. The fabrication of this immunosensor is fast when compared to previous reports, but with improved sensitivity. In addition, the immunosensor exhibited a wide linear range and excellent analytical performance and it is a significant step forward for detection of *Salmonella*.

2. Experimental

2.1. Reagents and apparatus

HRP conjugated *Salmonella* species antibody, *Salmonella* antibody were purchased from BD Pharmigen (San Diego). *Samonella typhimurium* was provided by Dr. Terebiznik (University of Toronto Scarborough). The bacteria were incubated with shaking at 200 rpm for 24 h in LB broth at 37 °C. The cells were harvested and killed with 4% PFA, then washed for several times with 10 mM PBS solution (pH = 7.4). The cell suspensions were prepared in 10 mL of PBS–Tween. Optical density at 600 nm (OD_{600}) was measured and adjusted to around 0.60, corresponding to the bacteria concentration of 10^8 colony forming units (CFC)/mL. Chitosan from crab shells (85% deacetylation and 200,000 Da), $HAuCl_4 \cdot 3H_2O$, lyophilized 99% bovine serum albumin (BSA), Tween-20, Gold chloride ($HAuCl_4 \cdot 3H_2O$), $NaBH_4$, and 2-hydroxy-1,4-naphthoquinone (HNQ) were bought from Sigma-Aldrich (St. Louis, MO). All other chemicals were of analytical grade and used as received. The immunoreagents were dissolved in phosphate saline (PBS) buffer (pH 7.4, 0.01 M phosphate, 0.14 M NaCl, 2.7 mM KCl). Ultrapure water with resistivity of 18.2 MΩ (Millipore Synergy water purification system) was used in preparation of all solutions.

All electrochemical measurements were performed on a Chi-660a electrochemical workstation (CHI instrumental). Transmission electron microscope (TEM) images were obtained from using a JEM-2000EX microscope (Japan).

2.2. Preparation of GNPs and chitosan solution

GNPs were prepared by reduction of $HAuCl_4 \cdot 3H_2O$ in aqueous solution according to the established procedure [32]. In a typical experiment, a solution of 1 mL $HAuCl_4$ (1% m/v) in 50 mL milli-Q water was heated at 150 °C and stirred. When the solution was boiling, 5 mL of sodium citrate (40 mM) was added rapidly. The solution changed its color from pale yellow to red in the next 10 min of heating and stirring; it was further stirred for 15 min at 25 °C. Prepared GNPs were stored at 4 °C. A chitosan solution (1% w/w) was prepared by adding chitosan flakes to water and by slowly adding 1% HCl to achieve a pH 5.6.

2.3. Fabrication of electrochemical immunosensor

A mixture of 5 mL chitosan and 5 mL GNPs was prepared under stirring. The glass carbon (GC) (3 mM in diameter) electrode was polished carefully with 1.0, 0.3 and 0.05 μm alumina slurry, and then ultrasonicated successively in 1:1 nitric acid, acetone and deionized water, respectively. After being dried at room temperature, a suspension (10 μL) of Chitosan-GNPs blend was dropped on the cleaned GC electrode and then dried at room temperature (Fig. 1).

Chitosan is a well known biopolymer and commonly used as a substrate for immobilizing proteins. It has been demonstrated that chitosan film can be oxidized in NaCl solution and generated reactive carbonyl groups capable of reacting with proteins [36]. In the present paper, the anodic oxidation of the immobilized composite film was performed in a conjugation buffer (0.1 M NaCl and 0.1 phosphate buffer, pH 7) and by applying 0.9 V to the underlying electrode for 20 s. The films were briefly rinsed with water and protein was conjugated by incubating the activated films with *Salmonella* antibody solution (100 ng/ml in PBS) for 1 h. After incubation, the films were washed with the buffer for 3 times. In immunoassay studies, the rinsed films were then blocked by incubation with BSA (5%) for 1 h and washed with the rinsing buffer for 3 times afterward.

For detection of *Salmonella*, the Ab_1/Chi–GNPs /GC constructed as described above was incubated for 1 h with a 10 μL drop of *Salmonella* solution (1000 CFU/mL), followed by washing with 0.1% Tween-20 and PBS buffer for 3 min each to eliminate nonspecific binding. Finally, the electrode was incubated with a 10 μL drop of HRP conjugated *Salmonella species* antibody (100 ng/ml in PBS) for 1 h. After washing with 0.1% Tween-20 and PBS buffer, the electrode was ready for electrochemical measurement.

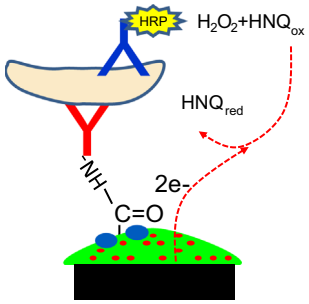

Fig. 1. Schematic illustration of the immunosensor.

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