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Hybrid Nanostructure-based Immunosensing for Electrochemical Assay of *Escherichia coli* as Indicator Bacteria Relevant to the Recycling of Urban Sludge

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ABSTRACT

Effective assay of *Escherichia coli* (*E. coli*) as indicator bacteria is critical for evaluating the recycling of urban sludge. In this work, we utilized multiwalled carbon nanotube/gold nanoparticle (CNT/AuNP) hybrid nanostructures as sensing electrode to design a novel electrochemical immunosensor for *E. coli* analysis. Herein, the hybrid nanostructures were fabricated by electrodepositing AuNPs on CNTs surface via multi-potential step technique. The CNT/AuNP hybrids were proved to facilitate the improvement of electrochemical performance for signal amplification, as well as the immobilization of *E. coli* capture antibody ($_c$Ab). The sandwich-type system was formed by specific recognition of the immobilized $_c$Ab on CNT/AuNP hybrids to *E. coli*, followed by the attachment of horseradish peroxidase-conjugated *E. coli* detection antibody ($_d$Ab-HRP) to produce the electrochemical immunosensor. On the basis of the dual signal amplification of CNT/AuNP hybrid nanostructures and enzymatic catalysis, the electrochemical immunosensor exhibits an excellent performance for *E. coli* assay ranging from 2.0×10^2 to 2.0×10^6 cfu mL^{-1}. The electrochemical method was also successfully used to evaluate *E. coli* in urban sludge. The proposed strategy provides a convenient and effective method for *E. coli* detection, and could become a promising technique for estimating the feasibility of sludge recycling.

1. Introduction

With the growing production of urban sludge on a global scale, sludge recycling is becoming increasingly important [1–3]. However, among the enormous diversity of microorganisms found in urban wastewater, some pathogens can be present and such microorganisms are concentrated in sludge during the treatment of wastewater [4,5]. Furthermore, some of these pathogens are known to survive for several months in the environment. In order to reduce the risk of spreading pathogens and protect the public health, monitoring of pathogens is essential for the routine sludge assessment prior to its recycling [6,7]. Nowadays, the main criterion for evaluating the levels of pathogens in urban sludge is the density of *Escherichia coli* (*E. coli*) as indicator bacteria, and the presence of *E. coli* suggests that pathogenic microorganisms (e.g., pathogenic bacteria, viruses and parasites) might also be present [8]. Therefore, the development of effective methods for quantitative analysis of *E. coli* plays a critical role in estimating the feasibility of sludge recycling.

Conventional methods for *E. coli* detection typically rely on the culture-based assays [9]. Although these methods can offer high accuracy, they are complicated in operation, lack of specificity and time-consuming [10–12], which greatly limit their applications for rapid, sensitive and more practical analysis. Owing to the fact that electrochemical detectors are simple, portable and inexpensive, electrochemical immunosensor has been well recognized to be a powerful tool for biological assay [13–16]. Regarding the development of a successful electrochemical immunosensor, electrical conductivity is an important requirement for obtaining high sensitivity. Recently, nanomaterials such as nanotubes [17,18], nanoparticles [19,20] and nanowires [21,22], have attracted great interest because of their unique electrocatalytic properties. For instance, graphene sheets were used for the biosensor platform to capture primary antibodies [23]. Carbon nanoparticle (CNP)-based immunosensor was developed for the electrochemical detection of a protein tumor [24]. The interdigitated capacitive transducer was modified with gold nanoparticles to detect trace amount of biomarkers in multiple cancer marker detection [25].

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A platinum nanoparticle ensemble on a polyaniline/graphene hybrid (Pt/PANI/GNs) as a novel electrode material was reported for analyzing H_2O_2 and glucose with high sensitivity [26]. When compared to a single nanomaterial, the hybrid nanostructures exhibit distinctive nature of characteristics, which make them an excellent sensing material for electrocatalytic applications. Particularly, the attachment of gold nanoparticles to the side walls of carbon nanotubes to fabricate highly efficient sensor devices are very popular for the advantages such as high surface area, favorable electronic properties, ease of biomolecule attachment, and electrocatalytic effects [27].

In this work, we reported an electrochemical immunosensor for *E. coli* detection based on multiwalled carbon nanotube/gold nanoparticle (CNT/AuNP) hybrid nanostructures as sensing platform. The hybrids were prepared by electrodepositing AuNPs on CNTs surface via multi-potential step technique, which simultaneously possessed the unique properties of CNTs and AuNPs including large surface area, excellent electrical conductivity and enhanced biocompatibility. The resulting CNT/AuNP hybrid nanostructures were proved to facilitate the improvement of electrochemical performance for signal amplification, as well as the immobilization of *E. coli* capture antibody ($_c$Ab). The analytical procedure consisted of immunoreaction of *E. coli* with the immobilized $_c$Ab, followed by attaching horseradish peroxidase-conjugated *E. coli* detection antibody ($_d$Ab-HRP) to form a sandwich-type system. By coupling with another signal amplification based on an enzymatic catalytic reaction of HRP toward the oxidation of hydroquinone (QH_2) by H_2O_2, a convenient and effective strategy was proposed for electrochemical assay of *E. coli*. The results demonstrated that the method exhibited good performance with high sensitivity, specificity and rapid response for the analysis of *E. coli* in urban wastewater, and thus provided a powerful tool for estimating the feasibility of sludge recycling.

2. Experimental

2.1. Chemicals

Bovine serum albumin (BSA) was purchased from Sigma Chemicals (St. Louis, MO, USA). *E. coli* monoclonal antibody (serving as capture antibody, $_c$Ab) was obtained from Beijing Biosea Biotechnology Co. Ltd. (Beijing, China). Horseradish peroxidase-conjugated *E. coli* polyclonal antibody (serving as detection antibody, $_d$Ab-HRP) was received from Abcam Company (Cambridge, UK). Multiwalled carbon nanotubes (CNTs, φ = 10-30 nm) were purchased from Nanotech Port Co. Ltd. (Shenzhen, China). N,N-dimethylformamide (DMF), $HAuCl_4$ and other chemical reagents were purchased from Shanghai Chemical Reagents Co. Ltd. (Shanghai, China). The 0.01 M pH 7.4 phosphate-buffered saline (PBS) contained 136.7 mM NaCl, 2.70 mM KCl, 8.72 mM Na_2HPO_4 and 1.41 mM KH_2PO_4. All the reagents used were of analytical-reagent grade and all solutions were prepared with pure water from Millipore (Milli-Q, 18.2 MΩ cm). The glassware was autoclaved prior to be used.

2.2. E. coli and plate count method

E. coli (DH5α) was supplied by School of Food and Biological Engineering, Jiangsu University (Zhenjiang, China). The pure culture of *E. coli* was grown in Luria broth (LB, containing 1% tryptone, 1% NaCl and 0.5% yeast extract) on an orbital shaker at 37 °C for 20 h before use. The culture was serially diluted to 10^{-7} using normal saline solution. To know the concentration of colony-forming units (cfu) in the bacterial solutions, *E. coli* number was determined by conventional plate counting. 0.10 mL of *E. coli* dilutions was surface plated on LB agar and incubated at 37 °C for 24 h. *E. coli* colonies on the plate were counted to determine the number of colony-forming units per milliliter (cfu mL^{-1}). The real samples were obtained from Zhengrunzhou Wastewater Treatment Plant (Zhenjiang, China).

2.3. Apparatus and instruments

All electrochemical measurements were performed on a CHI660 electrochemical working station (CH Instruments Inc., Austin, TX, USA) with a conventional three-electrode system comprised of a platinum wire as the auxiliary, a saturated calomel electrode (SCE) as the reference, and a glassy carbon working electrode (GCE, 3 mm in diameter). Electrochemical impedance spectroscopy (EIS) was carried out within frequency range of 0.10 Hz ~ 100 kHz at the amplitude of 5 mV and the applied potential of 0.24 V, and cyclic voltammetry (CV) at a scan rate of 100 mV s^{-1}. Scanning electron microscopy (SEM) analysis was performed using a HITACHI S-4800 microscope (Hitachi Co. Ltd., Tokyo, Japan).

2.4. Pretreatment of CNTs

The as-received CNTs were treated by refluxing in concentrated HNO_3 (70%) for 5 h, followed by washed with water until pH was ~7.0 and dried under vacuum at 100 °C [28,29]. Subsequently, 1.0 mg of the resulting CNTs were dispersed in 10 mL DMF and sonicated for 10 min to obtain a homogeneous dispersion.

2.5. Preparation of electrochemical immunosensor

GCE was polished into a mirror successively with 0.3 and 0.05 μm alumina slurry, and then ultrasonically cleaned by acetone, HNO_3 (1:1 v/v), NaOH (50% w/w) and water. A 10 μL of the pretreated CNTs in DMF (0.10 mg mL^{-1}) was coated on a cleaned GCE surface and dried under infrared lamp. Next, GCE/CNT was immersed into a solution of 0.50 M H_2SO_4 containing 0.10 mM $HAuCl_4$ and electrodeposited AuNPs via multi-potential step from 1.055 to–0.045 V (vs. SCE) for 15 s [30]. After being washed with water and 0.01 M pH 7.4 PBS successively, the CNT/AuNP-modified GCE was incubated with 10 μL 100 μg mL^{-1} $_c$Ab for 60 min at room temperature (RT). The unbound $_c$Ab was then removed from the electrode surface by slow dipping into PBS three times. Subsequently, the $_c$Ab-modified electrode was treated with 2.0% BSA in PBS for 30 min to eliminate nonspecific binding effect and block the remaining active groups on the surface. Finally, the GCE/CNT/AuNP/$_c$Ab/BSA was thoroughly rinsed with PBS and used for the following assay.

2.6. Enzyme-amplified electrochemical analysis

The proposed immunosensor was immersed in *E. coli* sample at a certain concentration for 60 min at RT. After being washed with PBS and dried with nitrogen, the GCE/CNT/AuNP/$_c$Ab/BSA/*E. coli* was incubated with 10 μL 100 μg mL^{-1} $_d$Ab-HRP for another 60 min at RT. Next, a last washing cycle was performed with PBS to remove the unbound $_d$Ab-HRP. The electrochemical detection was performed in degassed 0.10 M pH 7.0 PBS containing 1.0 mM H_2O_2 and 0.60 mM hydroquinone (QH_2). The differential pulse voltammetry (DPV) measurements were from 0.40 to–0.30 V (vs. SCE) with pulse amplitude of 50 mV and width of 50 ms.

3. Results and Discussion

3.1. Characteristics of the hybrid nanostructure-based electrochemical immunosensor

SEM studies were conducted to characterize GCE/CNT and GCE/CNT/AuNP, respectively. As shown in Fig. 1A, CNTs were mostly