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Single domain antibody coated gold nanoparticles as enhancer for *Clostridium difficile* toxin detection by electrochemical impedance immunosensors

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Abstract

This work presents a sandwich-type electrochemical impedance immunosensor for detecting *Clostridium difficile* toxin A (TcdA) and toxin B (TcdB). Single domain antibody conjugated gold nanoparticles were applied to amplify the detection signal. Gold nanoparticles (Au NPs) were characterized by transmission electron microscopy and UV–vis spectra. The electron transfer resistance (Ret) of the working electrode surface was used as a parameter in the measurement of the biosensor. With the increase of the concentration of toxins from 1 pg/mL to 100 pg/mL, a linear relationship was observed between the relative electron transfer resistance and toxin concentration. In addition, the detection signal was enhanced due to the amplification effect. The limit of detection for TcdA and TcdB was found to be 0.61 pg/mL and 0.60 pg/mL respectively at a signal-to-noise ratio of 3 (S/N = 3). This method is simple, fast and ultrasensitive, thus possesses a great potential for clinical applications in the future.

Keywords

Electrochemical impedance immunosensors; *Clostridium difficile* toxin detection; Gold nanoparticles; Signal amplification

1. Introduction

Clostridium difficile is a spore-forming, gram-positive and anaerobic bacterium. It is the major cause of antibiotic-associated diarrhea and almost all cases of pseudomembranous colitis [1]. During the infection, two exotoxins with similar structure and function were released by most pathogenic strains of *C. difficile*: toxin A (TcdA) and toxin B (TcdB). Both TcdA and TcdB are cytotoxic, pro-inflammatory, and enterotoxic in the human intestine [2]. They are primarily responsible for the diseases associated with the infection [3]. The incident of *C. difficile* infection (CDI) is increasing dramatically during the past few years, early diagnosis is essential for better control and management of CDI, therefore, much

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research has been focused on the rapid diagnosis and treatment of CDI in hospital settings [4–6]. The diagnosis of CDI is mainly based on clinical features and laboratory detection of *C. difficile* organisms and toxins [7]. Methods currently in use for the organism identification include stool culture, the detection of glutamate dehydrogenase (GDH), and polymerase chain reaction (PCR) [8]. The *C. difficile* toxin A&B detection assays are to detect the two toxins produced by *C. difficile* bacteria in a stool sample. There are two main assays: tissue culture assay [9,10] and enzyme immunoassay (EIA) [11,12]. A rapid and simple test with high sensitivity and specificity for detecting *C. difficile* toxins is still challenging but highly desirable.

In recent years, electrochemical biosensors have attracted considerable interest because of their intrinsic advantages such as high sensitivity, fast response, easy operation, favorable portability, and low cost [13]. Much effort has been made to design electrochemical biosensors with different technologies such as cyclic voltammetry (CV), chronoamperometry, chronopotentiometry, electrochemical impedance spectroscopy (EIS), and field-effect transistor (FET) [14]. Among these electrochemical methods, EIS is a rapid and non-destructive method with the ability to study the interfacial behavior of a wide range of materials in electrochemical system [15,16]. The electrode accessibility to the solution-based redox probe will be reduced due to the attachment of electrically insulated molecules, thus this technology is very useful to study the biorecognition event through capacitance, reactance and/or resistance changes at the electrode surface [17,18]. The electrochemical impedance immunosensors combining EIS and immunoassay have attracted extensive interest in many areas, including food industry, environmental pollution, diagnosis, biotechnology, pharmaceutical chemistry, and clinical diagnostics [19–21]. Meanwhile, researchers found that analytical signals of electrochemical impedance biosensor can be amplified by various strategies including the use of biotin–avidin/streptavidin system [16,22] and the generation of biocatalytic precipitation on the electrode surface [23].

On the other hand, it is worthy to note that with the increased understanding of nanomaterials, considerable efforts have been directed toward the design of different nanomaterial-based amplification paths aimed at achieving ultrahigh sensitivity [24–26]. For example, the application of semiconductor quantum dots (CdS) as oligonucleotide labeling tags for the detection of the target DNA by using EIS [27], which allows EIS signal to be amplified by space resistance and negative charges provided by the nanoconjugates. As one of the most widely used nanomaterials in biomedical research and clinical imaging [28], gold nanoparticles (Au NPs) have been addressed as a promising nanomaterial for the signal amplification in EIS analysis because of their good biocompatibility and ease of self-assembly through a thiol group [29,30]. It has been reported that the use of antibody modified gold nanoparticles is favorable to immobilize more antibody onto the electrode [31]. The steric hindrance, as well as the increased amount of antibody generated by the presence of the antibody-gold conjugates can be used to enhance the sensitivity of electrochemical impedance immunosensors [32,33]. So far, there is no report on the application of electrochemical impedance immunosensors for detecting TcdA and TcdB.

Herein, we designed a simple sandwich-type electrochemical impedance immunosensor with antitoxin heavy-chain-only V_H (V_{H^H}) antibodies [34] labeled gold nanoparticles as the

amplifying probe for detecting both TcdA and TcdB. Heavy chain only antibody or single domain antibody (sdAb) was used in this work against both TcdA and TcdB. A primary single domain antibody (sdAb₁) was used to bond toxin onto the electrode and a secondary single domain antibody (sdAb₂) was applied to coat Au NPs to form the enhancer. Antibody coated gold nanoparticles can bring a large amount of antibody into the immunosensor system and results in an enhancement of electrochemical impedance signal. Thus this ultrasensitive EIS assay possesses a great potential for clinical applications in the future.

2. Experimental

2.1. Reagents

Gold (III) chloride trihydrate (HAuCl₄·3H₂O) and Bovine serum albumin (BSA) were purchased from Sigma-Aldrich. Cystamine dihydrochloride (C₄H₁₂N₂S₂) was from Fluka. Sodium citrate dehydrate (Na₃C₆H₅O₇·2H₂O) was obtained from Alfa Aesar. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) were from Thermo Scientific. Recombinant TcdA and TcdB were purified from *Bacillus megaterium* as described previously [35]. Single domain V_HH antibodies (sdAbs) against TcdA and TcdB and their fusions specific to both toxins are described by us recently [34]. All other chemicals were of analytical grade and the water used in the experiment is deionized water. 0.1 M PBS solution was prepared by mixing the stock solution of KH₂PO₄ and K₂HPO₄. All working solutions of toxin and antibody were prepared by dilution in the prepared PBS.

2.2. Determination of optimal sdAb₂ concentration for coating gold nanoparticles

Gold nanoparticles were synthesized by the classic citrate reduction method [36]. The final products were incubated at 4 °C for future use. Conjugation of sdAb₂ to gold nanoparticles followed the method described by Slot & Geuze [37]. Briefly, 10 mL of Au NPs solutions were diluted with 70 mL water to give a total volume of 80 mL as a stock liquid. To prepare an antibody-conjugated Au NPs, 10–50 µL sdAb₂ (0.07 µg/µL), in a total volume of 50 µL PBS buffer (pH = 7.4), was added in 500 µL gold nanoparticles solution and incubated for 30 min at room temperature. Then 100 µL 10% NaCl solution was added, the color changed from red to purple can be observed for some of solutions. The minimum amount of sdAb₂ that did not have a color change was determined as the optimal amount for conjugation.

2.3. Preparation of sdAb₂-coated gold nanoparticles (sdAb₂-Au NPs)

The sdAb₂-Au NPs were prepared according to a documented method [38,39] with some modifications. The optimal amount of sdAb₂ was mixed into 1 mL of Au NPs solution for 30 min at room temperature. Bovine serum albumin (BSA, 100 µL of 1%) was added to the mixture to block the remaining nonspecific adsorption-reactive sites. The suspension was then rinsed with a PBS solution (pH = 7.4) containing 1% BSA by centrifugation for 3 times. The final precipitation was diluted to 1 mL in PBS solution (pH = 7.4) containing 1% BSA and kept at 4 °C for further use.

2.4. Immunoassay procedure

A sandwich electrochemical impedance immunosensor was designed for the detection of TcdA and TcdB as shown in Scheme 1. The cleaned gold electrode was first placed into a 30

mM cystamine dihydrochloride solution overnight and then rinsed with PBS solution to remove physically adsorbed dithiols. Subsequently, the cystamine self-assembled monolayers modified electrode was immersed into the sdAb₁ solution (0.045 µg/µL, EDC/NHS-activated) and allowed to react at 4 °C for 2 h. After sufficiently rinsing with PBS, the electrode was dipped in a 1% BSA solution for 30 min to block the remaining adsorption reactive sites.

After immobilized with primary antibody, the electrode was incubated in different concentration of TcdA/B solutions at 4 °C for 3 hours and thoroughly rinsed with PBS. Then, the electrode was placed in the sdAb₂-Au NP solutions to amplify the response signal of impedance spectroscopy.

2.5. Measurements

Transmission electron microscopy (TEM, JEOL 100CX-II) operating at 100 kV was applied to characterize the morphology and particle size. An Evolution 300 UV–vis spectrophotometer was used for UV–vis spectroscopic study. All electrochemical measurements were carried out using an Autolab PGSTAT12 electrochemical workstation (Metrohm, USA Inc.). A conventional cell with a three-electrode configuration was used throughout this work. The working electrode was modified gold electrode (1.6 mm dia., BASi). Platinum wire and Ag/AgCl (saturated KCl) were used as the counter electrode and the reference electrode, respectively. Cyclic voltammetry and electrochemical impedance spectroscopy were performed in the presence of 10 mM K₃[Fe(CN)₆]/K₄[Fe(CN)₆] as a redox probe in 10 mM PBS (containing 0.1 M KCl, pH = 7.4). The EIS were recorded within the frequency range of 0.1 kHz to 10 kHz at 0.17 V (vs Ag/AgCl). All the electrolytes were deaerated by bubbling nitrogen (N₂) for 20 min before the experimental procedure. All the experiments were carried out at room temperature.

3. Results and discussion

3.1. Characterization of Au NPs and sdAb₂-Au NPs

TEM image (Fig. 1a) shows a good monodispersity of as prepared Au NPs with an average spherical diameter of 13–15 nm. Fig. 1b illustrates the color change of Au NPs suspension containing sdAb₂ coated Au NPs with different ratios in the presence of NaCl. Nine aliquots of different amount (10–50 µL) of sdAb₂ (0.07 µg/µL) was diluted with PBS buffer in a total volume of 50 µL, and added separately into 500 µL Au NPs solution. The color of suspension with a low amount (lower than 25 µL) changed from red to purple after addition of NaCl to induce precipitation. Therefore, 50 µL 0.035 µg/µL sdAb₂ per 500 µL Au NPs solution was determined as the optimal ratio for the antibody coating. UV–vis spectra of Au NPs (black curve) and sdAb₂ coated Au NPs (blue curve) were found in Fig. 1c. A characteristic surface plasmon resonance peak of AuNPs was observed at 519 nm. According to Lambert–Beer law, the concentration of Au NPs solution was calculated to be 1.96 nM from the peak intensity and known extinction coefficients [40]. The adsorption peak of blue curve shifts towards the red wavelengths for several nanometers, while the adsorption intensity drastically decreased. This fact further confirmed the conjugation of sdAb₂ and Au NPs.

3.2. Electrochemical characterization of the immunosensor

The cyclic voltammogram of a fairly reversible redox couple ($\text{Fe}(\text{CN})_6^{3-/4-}$) in PBS solution ($\text{pH} = 7.4$) was studied from -0.2 V to 0.6 V at a scan rate of 50 mV s^{-1} to characterize each immobilization step on gold electrode. As can be seen in Fig. 2, $\text{Fe}(\text{CN})_6^{3-/4-}$ showed a quasi-reversible one electron redox behavior at bare gold electrode. After sdAb_1 was immobilized onto the electrode surface, the current value remarkably decreased and the peak-to-peak separation (E_p) increased at the same time. It's probably because of an effective barrier to the electronic communication from $\text{Fe}(\text{CN})_6^{3-/4-}$ to the electrode provided by the immobilized proteins. Similarly, the current further decreased and E_p increased when TcdA were absorbed on the electrode.

Fig. 3 gives the Nyquist plots of electrochemical impedance spectra of gold electrode layer by layer in 10 mM PBS solution containing 10 mM $\text{K}_3[\text{Fe}(\text{CN})_6]/\text{K}_4[\text{Fe}(\text{CN})_6]$ ($\text{pH} = 7.4$), showing the real part of impedance (Z_{Re}) versus the negative of the imaginary part ($-Z_{\text{Im}}$). Inset is the Randles model equivalent circuit for the electrochemical impedance data [18], which includes the electrolyte resistance between working and reference electrodes (R_s), the double layer capacitance of electrode/electrolyte interface (C), Warburg impedance (Z_w) causing by the diffusion of ions from the electrolyte to the interface and electron transfer resistance (R_{et}). The electrochemical impedance spectra often consist of a semicircle part at high frequencies and a linear part at lower frequencies. The linear part represents the diffusion limited process. The semicircle part corresponds to the electron transfer limited process, which shows the blocking behavior of electrode for the $\text{Fe}(\text{CN})_6^{3-/4-}$ redox couple. When proteins were attached onto the electrode surface, they would form an inert electron transfer blocking layer and hence increase electron transfer resistance. The diameter of semicircle exhibits the R_{et} of electrode surface, which is an important parameter in the measurement of electrochemical impedance immunosensor. The impedance spectrum of gold electrode (curve 1) exhibits an almost straight line which is characteristic of diffusion limited process. The immobilization of sdAb_1 onto the electrode introduces a barrier to the interfacial electron transfer, thus curve 2 exhibits a small semicircle domain at high frequencies. Then, the use of BSA to block nonspecific binding sites results in a higher electron transfer resistance and enlarges the diameter of semicircle (curve 3). After the recognition reaction of BSA/ sdAb_1 /gold electrode with TcdA solution, the semicircle diameter markedly increased (curve 4). This increase is due to the generation of toxins onto the electrode surface through antibody-antigen interaction that further blocks the electron transfer. Finally, the impedance spectrum is amplified by using sdAb_2 -Au NPs as an enhancement element to carry out a sandwich format on the electrode surface. The formation of sandwich-type immune complex generates a lot of sdAb_2 on the electrode surface and has been proved to be helpful to amplify the analytical signal (curve 5). The above results indicate that the designed sandwich immunoassay using electrochemical impedance spectroscopy technique can be employed to detect *C. difficile* toxins.

In order to investigate the sensitivity of the impedance immunosensor, the change of R_{et} after incubating the sdAb_1 modified electrode in different concentrations of toxin solutions was measured. Fig. 4a shows the Nyquist plots of BSA/ sdAb_1 /gold electrode in various concentrations of TcdA solutions without (top) with (bottom) amplification of sdAb_2 -Au

NPs. The electron transfer resistance increases regularly with increasing TcdA concentrations from 1 pg/mL to 100 pg/mL. The binding of toxins onto the electrode would reduce electrode surface area and increase electron transfer resistance. The constructed impedimetric immunosensor can detect the concentration of TcdA as low as 1 pg/mL. The effect of amplification was examined and it turns out that the impedance signal was amplified by the immobilization of sdAb₂-Au NPs. It's known that the relative resistance is often used as a more valuable parameter than absolute resistance for impedance sensing applications [41]. The impedance increment is defined as $R_{et} = R_{et}(i) - R_{et}(0)$, where $R_{et}(0)$ is the R_{et} value of BSA/sdAb₁/gold electrode (step 3 of Scheme 1), and $R_{et}(i)$ is the value of R_{et} after toxins attach to BSA/sdAb₁/gold electrode (step 4 of Scheme 1). In the case of with amplification, $R_{et}(i)$ is the value of the impedance after the binding of sdAb₂-Au NPs onto TcdA/BSA/sdAb₁/gold electrode (step 5 of Scheme 1). Herein, the relative resistance $R_{et}/R_{et}(0)$ of BSA/sdAb₁/gold electrode without and with amplification at the same TcdA concentration was compared in Fig. 4b. It can be observed that there's an increment in $R_{et}/R_{et}(0)$ for the electrode with amplified operation compared to the one without amplified operation. The calibration curves of relative resistance versus TcdA concentration with amplification of sdAb₂-Au NPs were shown in the inset of Fig. 4b. A linear relationship between the relative resistance and TcdA concentration was obtained in the range of 1 pg/mL–100 pg/mL. The linear equation is $y = 0.074 \times x + 0.933$, with a correlation coefficient r^2 of 0.99227, where y is the relative resistance and x is the TcdA concentration (unit of x : pg/mL). The limit of detection was calculated to be 0.61 pg/mL ($S/N = 3$).

The detection of TcdB shows similar results in Fig. 5a, the electron transfer resistance increases regularly with increasing TcdB concentrations from 1 pg/mL to 100 pg/mL, and the relative resistance of the electrode with amplification (top) is bigger than the one without amplification (bottom). The regression equation is $y = 0.076 \times x + 1.041$ ($r^2 = 0.99034$) for the calibration curves of $R_{et}/R_{et}(0)$ versus TcdB concentration with amplification of sdAb₂-Au NPs, and the LOD was 0.60 pg/mL ($S/N = 3$) (Fig. 5b). It's clear that the detection signal enhanced due to the amplification effect of sdAb₂-Au NPs for both TcdA and TcdB detection. Due to the sterical hindrance and the increased amount of antibody, very low detection limit (0.61 pg/mL for TcdA, 0.60 pg/mL for TcdB) was achieved.

Stability of the proposed impedance immunosensor is a key factor in practical applications. The prepared electrode was stored at 4 °C for three weeks. The impedance results show the immunosensor could retain around 90% of its initial response, indicating good stability.

3.3. Stool sample analysis

In order to investigate the performance of the designed immunosensor with stool sample, the BSA/sdAb₁/gold electrode was immersed in 1:5 diluted negative stool solution spiked with different concentration of TcdA and TcdB for 2 hours at 4 °C and then rinsed with PBS, respectively. After that, the electrode was transferred into sdAb₂-Au NP solutions to amplify the response signal. The comparison of blank single of the BSA/sdAb₁/gold electrode incubated in PBS and negative stool solution has been shown in Fig. S2. It can be found that the impedance signal of the two electrodes doesn't show significant difference, suggesting

that the negative stool sample was not responsible for the increase of R_{et} . The results of the detection of toxins diluted in negative stool solution in Fig. 6 indicated the capability of the immunosensor for the determination of both TcdA and TcdB in stool samples for clinical diagnosis.

4. Conclusion

A simple sandwich-type electrochemical impedance immunosensor with single domain antibody labeled gold nanoparticles as amplifying probe for detecting *C. difficile* toxin A and B was designed in this work. Initially, cystamine self-assembled monolayers were coated onto the gold electrode surface and utilized for the immobilization of primary antibody through amine coupling chemistry. Toxins were then bonded onto the electrode through antigen–antibody interaction. Finally, secondary antibody coated gold nanoparticles were introduced onto the electrode surface as an amplifying probe to optimize the immunosensing performance. This proposed method achieved a limit of detection for TcdA and TcdB as 0.61 pg/mL and 0.60 pg/mL ($S/N = 3$) respectively. This electrochemical impedance immunosensor exhibited convenience and high sensitivity. The pilot study with spiked clinical stool samples showed promising results, indicating the designed biosensor has a great potential in clinical applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.bioelechem.2014.10.003>.

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Fig. 1.

(a) TEM image of Au NPs; (b) photograph of AuNPs suspension with different amount of sdAb₂; (c) UV-vis spectra of Au NPs (1: black curve) and sdAb₂-Au NPs (2: blue curve).

Fig. 2. CVs of (1) bare gold electrode; (2) $sdAb_1$ /gold electrode; (3) BSA/ $sdAb_1$ /gold electrode; (4) TcdA/ $sdAb_1$ /gold electrode in 10 mM PBS containing 10 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (pH = 7.4).

Fig. 3.

Nyquist plots of (1) bare gold electrode; (2) sdAb₁/gold electrode; (3) BSA/sdAb₁/gold electrode; (4) TcdA/sdAb₁/gold electrode (50 pg/mL⁻¹); (5) sdAb₂-Au NPs/TcdA/sdAb₁/gold electrode in 10 mM PBS containing 10 mM K₃[Fe(CN)₆]/K₄[Fe(CN)₆] (pH = 7.4). Inset: the Randles model equivalent circuit for the electrochemical impedance data.

Fig. 4.

(a) Nyquist plots of BSA/sdAb₁/gold electrode (top) without and (bottom) with amplification of sdAb₂-Au NPs correspond to different concentrations of TcdA (1: 0 pg/mL; 2: 1 pg/mL; 3: 5 pg/mL; 4: 10 pg/mL; 5: 25 pg/mL; 6: 50 pg/mL; 7: 100 pg/mL) in 10 mM PBS containing 10 mM K₃[Fe(CN)₆]/K₄[Fe(CN)₆] (pH = 7.4); (b) the relative resistance

$R_{et}/R_{et}(0)$ of the BSA/sdAb₁/gold electrode without (1: black dot) and with (2: red dot) amplification. $R_{et}(0)$: the R_{et} value of BSA/sdAb₁/gold electrode. Inset: the calibration curves of relative resistance $R_{et}/R_{et}(0)$ versus TcdA concentration with amplification of sdAb₂-Au NPs. The regression equation: $y = 0.074x + 0.933$ ($r^2 = 0.99227$).

Fig. 5.

(a) Nyquist plots of BSA/sdAb₁/gold electrode (top) without and (bottom) with amplification of sdAb₂-Au NPs correspond to different concentrations of TcdB (1: 0 pg/mL; 2: 1 pg/mL; 3: 5 pg/mL; 4: 10 pg/mL; 5: 25 pg/mL; 6: 50 pg/mL; 7: 100 pg/mL) in 10 mM PBS containing 10 mM K₃[Fe(CN)₆]/K₄[Fe(CN)₆] (pH = 7.4); (b) the relative resistance

$R_{et}/R_{et}(0)$ of BSA/sdAb₁/gold electrode without (1: black square) and with (2: red square) amplification. $R_{et}(0)$: the R_{et} value of BSA/sdAb₁/gold electrode. Inset: the calibration curves of relative resistance $R_{et}/R_{et}(0)$ versus TcdB concentration with amplification of sdAb₂-Au NPs. The regression equation: $y = 0.076x + 1.041$ ($r^2 = 0.99034$).

Fig. 6.

Nyquist plots of BSA/sdAb₁/gold electrode with amplification of sdAb₂-Au NPs correspond to different concentrations of TcdA (top) and TcdB (bottom) in spiked negative stool sample (1: 1 pg/mL; 2: 5 pg/mL; 3: 10 pg/mL; 4: 100 pg/mL).

Scheme 1.

Illustration of the immobilization process of the sandwich-type electrochemical impedance immunosensor.