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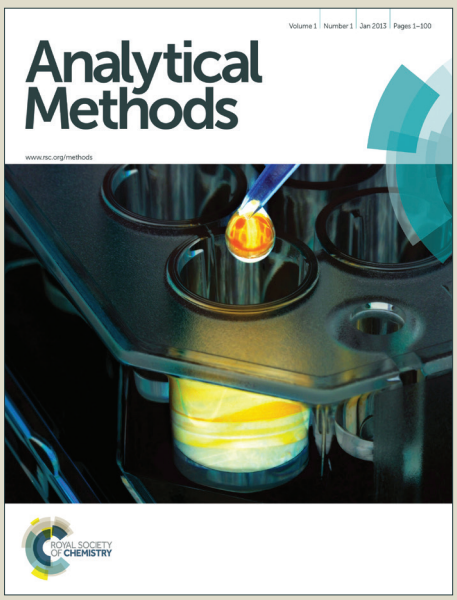

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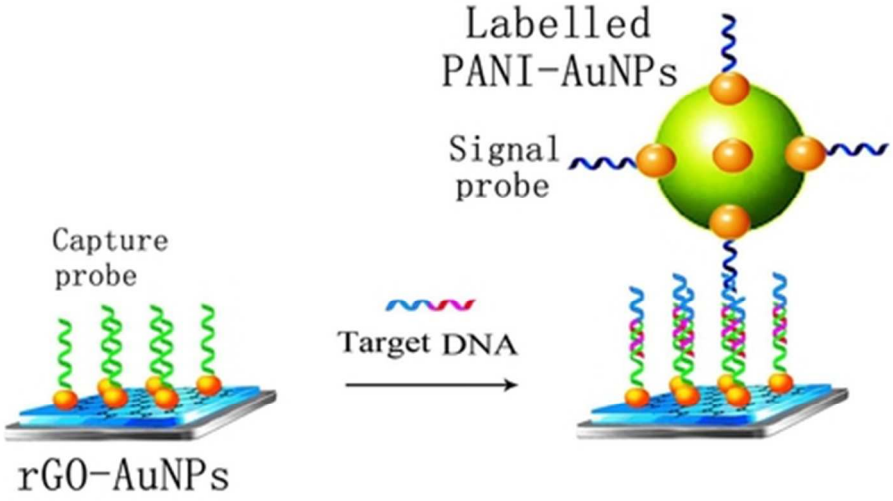

In this paper, a new electrochemical method for detection of Clostridium tetani was developed based on a reduced graphene oxide (rGO) modified electrode and polyaniline-gold nanoparticle（PANI-AuNPs）modified probe.
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ARTICLE TYPE

Electrochemical measurement of *Clostridium tetani* using a reduced graphene oxide modified electrode and polyaniline-gold nanoparticle labelled probe

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Tetanus is a neuromuscular disease in which *Clostridium tetani* exotoxin produces muscle spasms, incapacitating the host. Tetanus is especially prevalent during outbreaks of war and earthquakes. Due to the difficulty of assaying anaerobic cultures, a simple, rapid, sensitive and accurate alternative method is desirable. In this paper, a new electrochemical method was developed based on a reduced graphene oxide (rGO) modified electrode and polyaniline-gold nanoparticle（PANI-AuNPs）modified probe. The results indicate that the electroactive surface area of the rGO-AuNPs modified electrode increased approximately 95.4%, compared to the bare screen printed electrode (SPE). This electrochemical detection of *C. tetani* could be completed on a mini SPE by DPV detection with a limit of 10^{-15} mol/mL. Compared with the anaerobic culture method, this new electrochemical method has a sensitivity of 95.3%, a specificity of 97.8%, and displays high consistency (X^2 test: $p>0.05$). As a result, this new method may be an appropriate technology for point-of-care-testing (POCT) of *C. tetani* infections.

Keywords: reduced graphene oxide (rGO), gold nanoparticles (AuNPs), C. tetani, electrochemical detection, point of care testing (POCT).

1. Introduction

Tetanus is a neuromuscular disease in which *Clostridium tetani* produces muscle spasms, especially in developing countries.[1] The manifestation of the disease--spastic paralysis--is largely caused by the tetanus toxin.[2] Despite an effective vaccine, tetanus is an ongoing problem in developing countries. Tetanus is an acute toxic illness caused either by infection at a laceration, or break in the skin, or as a complication of one of the following: burns, puerperal infections, or surgical-site infections.[3] *C. tetani* has also been isolated from empyema pus.[4] Although the patient did not manifest any symptoms of localized or generalized tetanus, this was attributed to prompt management when the patient was diagnosed at the primary care facility.[5] Although tetanus is preventable by vaccination, it still claims lives around the world.[6] *C. tetani* infections can range in severity from asymptomatic to severe and life-threatening. The early detection of tetanus is essential for immediately initiating proper therapy.[7]

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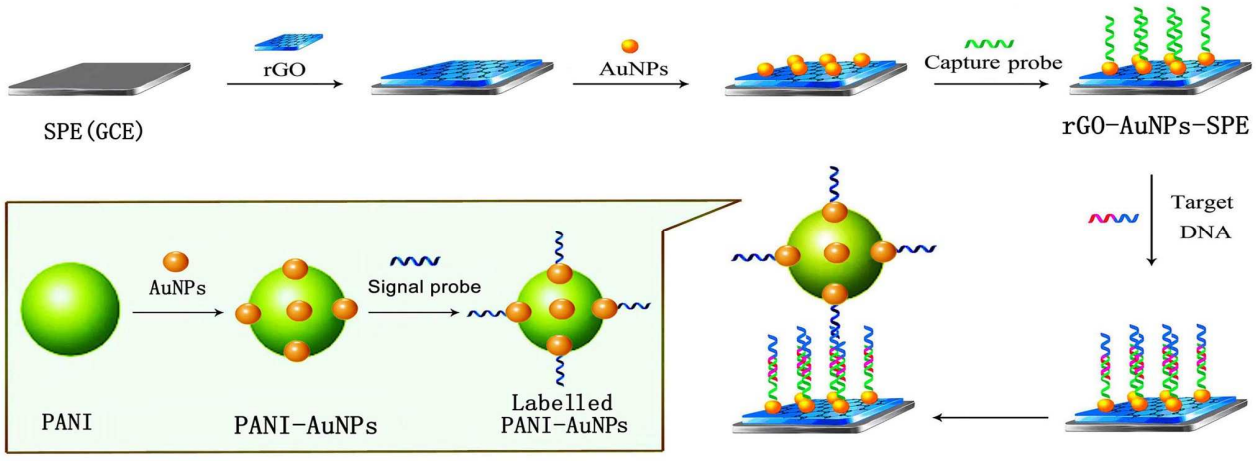
Conventionally, microscopy, cultures, or polymerase chain reaction (PCR) are employed in the diagnosis of tetanus. However, microscopy is insensitive, and the results of cultures aways take up to 2～4 days. Because *C. tetani* is an anaerobic bacterium, anaerobic culturing has been regarded as the gold standard for identification.[8] Some anaerobic bacteria, such as *C. tetani*, *C. botulinum* and *C. difficile*, are readily identifiable by anaerobic culture.[9] However, the anaerobic culture needs strict conditions, including nitrogen and carbon dioxide.[10]

Many commercially available nucleic acid amplification methods have been developed as rapid detection methods for the nucleic acids of *C. tetani*,[11] including PCR.[12] The tetanus toxin is a neurotoxin synthesised by *C. tetani* that can be used as a vaccine after detoxification with formaldehyde.[13] Because of this, the tetanus toxin has been targeted for use as a diagnostic marker.[14] A detection method for *C. tetani* was developed using loop-mediated isothermal amplification (LAMP) assay, wherein the tetanus toxin gene was used as the target gene.[15] The decryption of prokaryotic genome sequences has progressed rapidly and has provided new insights for the scientific community.[16] In another report, several homologous open reading frames (ORFs) have been identified in the genomes of the pathogen.[17] However, most of the previously mentioned molecular diagnostics have limitations, including cost and sophisticated laboratory infrastructure.

In recent years, electrochemical analysis has attracted considerable attention as a means of pathogen diagnosis because of its prompt response, sensitivity and ease of operation. Various

nanomaterials, such as gold nanoparticles[18] and single-walled carbon-nanotubes,[19] are based on an electrochemical biosensor, which facilitates the development of high sensitivity sensors. Previous research has reported that the use of graphene not only enhanced the surface area but also improved the conductivity of the electrode.[20] Conducting polymers such as polyaniline (PANI) have been proven particularly useful in the development of biosensors because of their stability, ease of electron transfer and conducting properties..[21] Additionally, hybrid polypyrrole /polyaniline polymers have been designed to exploit the synergistic and shape effects.[22] Thus, integrating PANI with gold nanoparticle sensors results in enhanced immobilisation of the DNA probe and detection sensitivity of the target DNA.

In this work, we demonstrate an electrochemical DNA sensor for the detection of *C. tetani*. We selected reduced graphene oxide (rGO) as a sensing platform and electrodeposited the AuNPs onto the decorated electrode surface to increase electronic signal transduction. We improved the PANI-AuNPs labelled probe to enhance the sensitivity of electrochemical detection. Scheme 1 shows the stepwise fabrication process for the electrochemical sensor and the corresponding signal amplification of PANI-AuNPs.



Scheme 1 The electrochemical fabrication and detection of target DNA.

2. Materials and methods

2.1 Reagents and materials

Chloroauric acid ($HAuCl_4$) and hexanethiol (HT) were purchased from Sigma (St. Louis, MO, USA). $K_3[Fe(CN)_6]$ was purchased from Beijing Chemical Reagent Co. (Beijing, China). Graphene oxide (GO) was obtained from Nanjing xianfeng Nano Co. (Nanjing, China). Poly (diallyldimethylammonium chloride) (PDDA) was obtained from Beijing Chemical Reagent Co. (China). Potassium persulfate ($K_2S_2O_8$) was purchased from Shanghai Chemical Reagent Co. (Shanghai, China). The tetanus toxin gene (GenBank: M12739) was selected to design the primers for measurement of *C. tetani* by exploring the GenBank database of the National Center for Biotechnology Information （NCBI, USA）. The specificity of the primers was positively tested by BLAST (http: //www.ncbi.nlm.nih.gov/blast). All oligonucleotides were synthesised by Sangon (Shanghai, China). The sequences of the oligonucleotides were as follows:

Capture probe: 5'-CCCAACAATCCAGAT-SH-3'
Signal probe: 5'-SH-C_6-TTTTTAACTATTACTTCTTC-3'
Target DNA: 5'-ATCTGGATTGTTGGGTTGATAATGA -AGAAG -3'

2.2 Apparatus

The size and morphology of PANI-AuNPs were characterised by Tecnai 20 transmission electron microscope (TEM) and energy-dispersive x-ray spectroscopy (EDX) (Philips, Netherlands). Cyclic voltammetry (CV) and differential pulse voltammetry (DPV) were performed with a CHI650D electrochemical workstation (Shanghai CHI, China). Electrochemical experiments were carried out in an SPE-electrode system (110454) containing a glassy carbon electrode (3 mm diameter) as the working electrode, an Ag-AgCl electrode as the reference electrode, and a carbon electrode as the auxiliary electrode.

2.3 Preparation of DNA samples

The bacteria standard strains used in this study included the following: *E. coli* (ATCC25922), *P. aenlgimosa* (ATCC 27853), *S. aureus* (ATCC25923), *P. vulgaris* (ATCC 49132), *K. pneumoniae* (ATCC 10031), *S. epidermidis* (ATCC 12228), *S. pneumoniae* (ATCC 49619), *A. baumannii* (ATCC 19606), *S. typhi* (ATCC 14028) and *S. dysenteriae* (ATCC13313). All standard strains were obtained from American Type Culture Collection (ATCC， USA), were grown on a corresponding medium, and were preserved in normal saline (NS) at -20°C while awaiting analysis. The DNA were extracted from bacteria following the instructions of the DNA extraction kit (Tiangen, China). All DNA extracts were stored at -20°C for subsequent testing. The detection was performed in a thermobath ALB64 (Finepcr, Korea).

2.4 Preparation of PANI-AuNPs and rGO-electrode

PANI-AuNPs were established by the method described by Berzina *et al*.[23] 5 mL of 0.5 M H_2SO_4 were added to 100 mL of freshly distilled aniline. 10 mL of colloidal gold nanoparticles were added into the aniline-H_2SO_4 solution. After that, 5 mL of 0.15 M $K_2S_2O_8$ were added dropwise to initiate the polymerisation reaction. After 2 hours, the polyaniline-gold nanoparticle composite was washed with water. Subsequently, 20 µL of 2.5 µM signal probe solution was dropped onto the surface of the PANI-AuNPs and incubated overnight. Following that, 20 µL of HT solution (1 mM) was added into the modified PANI-AuNPs solution and kept for 1 h to block the nonspecific binding site of the PANI-AuNPs.

rGO was prepared as follows: 0.2 mL of 30% PDDA was dropped into a 50 mL stable dispersion of exfoliated graphene oxide (GO) sheets (1 mg/mL) while stirring for 30 min. After that, 0.5 mL of 80% hydrazine hydrate was added and stirred at 90°C for 12 h. Finally, the product was centrifuged and washed with water. The SPE (No.110) electrodes (Metrohm, Switzerland) were polished with 0.05 µm Al_2O_3 and washed ultrasonically in water for 5 minutes. 6 µL of rGO solution were dropped onto the SPE surface and dried at room temperature. After that, AuNPs were electrodeposited on the rGO-modified electrode surface at a constant potential of -0.2 V for 30 s. Subsequently, 20 µL of 2.5 µM capture probe solution were dropped onto the surface of the rGO-AuNPs and incubated overnight. Following that, 20 µL of hexanethiol solution (HT, 1 mM) was dropped on the modified electrode surface and kept for 1 h to block the nonspecific binding site of the electrode. The electroactive surface area of the DNA sensor was measured in 5.0 mM Fe $(CN)_6^{4-/3-}$ at different scan rates.

2.5 Electrochemical measurements

The fabricated electrode was incubated with the required concentration of target DNA solution for 2 h at room temperature. Then, 5 µL of the prepared PANI-AuNPs labelled signal probe solution were dropped onto the electrode surface and incubated for 2 h. Following that, DPV was performed from –0.3 V to 0.4 V at a scan rate of 100 mV/s in 0.1 M PBS (pH 7.0).

2.6 Sensitivity and regression analysis

The solution of target DNA (*C. tetani*) was diluted into a series of concentrations from 1×10^{-9} to 1×10^{-15} M. Electrochemical measurement was performed by DPV from –0.3 V to 0.4 V at a scan rate of 100 mV/s in 0.1 M PBS (pH 7.0). The semi-logarithmic linear regression was analysed by SPSS 16.0 (IBM, USA).

2.7 Specificity and stability analysis

To investigate the specificity of the designed biosensor, the synthetic oligonucleotides (target DNA, 1×10^{-12} M), *C. tetani*, and 10 other types of bacteria that are associated with diarrhoea (*E. coli*, *P. aenlgimosa*, *S. aureus*, *P. vulgaris*, *K. pneumoniae*, *S. epidermidis*, *S. pneumoniae*, *A. baumannii*, *S. typhi* and *S. dysenteriae*) were detected in the same concentration (1×10^5 CFU/mL). DNA extraction and electrochemical measurement were performed in the same way. The stability of the assays was studied using 50 cycles CV measurements in 0.1 M PBS (pH 7.0) after being incubated with the target DNA (1×10^{-12} M).

2.8 Comparison with culture method

The anaerobic culture of *C. tetani* was inoculated onto high concentration blood agar culture medium (Pangtong, China). Colonies were identified by API 20A identification card (Bio-Merieux, France). The performance of the electrochemical measurement was compared with the gold standard of its difference (X^2 test), sensitivity and specificity. Differences were considered significant at $p< 0.05$.

3. Results and discussion

3.1 Modified PANI-AuNPs and rGO-electrode

The modification and monitoring of the PANI-AuNPs and rGO-electrode are presented in Fig. 1. The PANI-AuNPs is the fundamental factor of signal amplification. TEM and EDX were carried out to characterised PANI-AuNPs. In Fig. 1a, many PANI nanoparticles can be observed (40～100 nm in diameter) by TEM. However, the AuNPs on PANI are not clearly displayed. In Fig. 1b, which uses EDX, the AuNPs have been detected by 3 obvious peaks (Au). Thus, the AuNPs were modified on PANI.

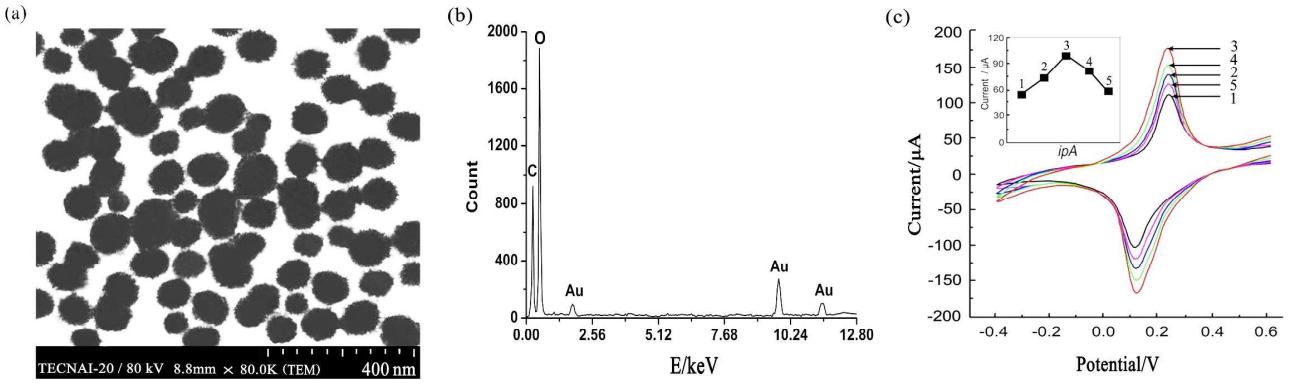


Fig. 1 Characterization of PANI-AuNPs and rGO-electrode. (a) TEM image of PANI-AuNPs; (b) EDX of PANI-AuNPs; (c) CVs of electrode in 5.0 mM Fe $(CN)_6^{4-/3-}$. (1.bare SPE; 2. rGO⁄SPE; 3. AuNPs–rGO⁄SPE; 4. capture probe⁄AuNPs–rGO⁄SPE, 5. HT⁄captuer probe⁄AuNPs–rGO⁄SPE)

In Fig. 1c, there is a well-defined redox peak from the bare SPE (curve 1). An increase of peak current was observed after the modification of the rGO (curve 2). When the AuNPs were electrodeposited onto the rGO, the peak current was to further enhanced due to the excellent electroactivity of the AuNPs (curve 3). After the capture probe was self-assembled onto the rGO-AuNPs, the peak current decreased, which suggests that the probe blocked the electron transfer (curve 4). A further decrease of peak current was observed after the electrode was treated with HT because the HT obstructed electron transfer (curve 5). The results show that the modification of rGO-electrode was successful.

3.2 Electroactive surface areas of the electrode

The electroactive surface area of an ordinary SPE and an rGO-AuNPs modified SPE were determined according to the Randles-Sevcik equation:

$$ip = 2.686\times10^{5} A \times D^{1/2} n^{3/2} \times C \times V^{1/2}$$

i_p = current maximum in amps
n = number of electrons transferred in the redox event
A = electrode area
D = diffusion coefficient
C = concentration
V= scan rate

As shown in Fig. 2, when D, C and n *are held constant*, the electroactive surface area of bare SPE was 10.8 mm^2（Fig. 2a）, while the rGO-AuNPs modified SPE was 21.1 mm^2（Fig. 2b）. Compared with the bare SPE, the electroactive surface area of rGO-AuNPs modified SPE increased approximately 95.4%.

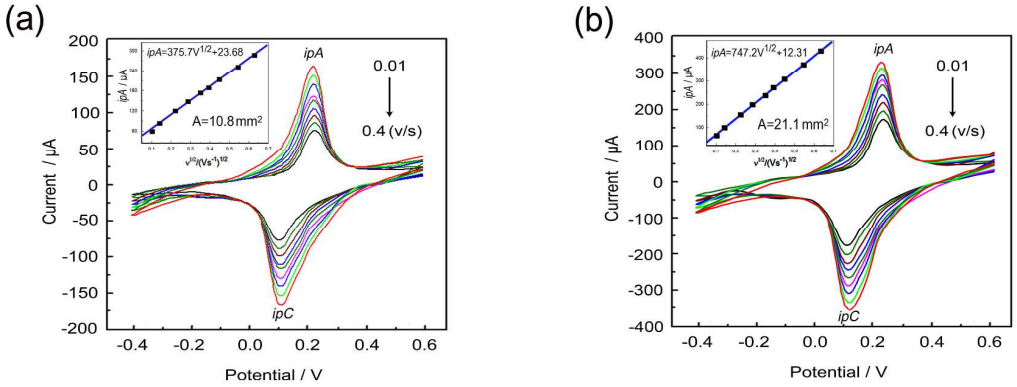


Fig. 2 The electroactive surface areas of the electrode evaluated by CVs. (a) bare SPE; (b) rGO-AuNPs modified SPE.

3.3 Sensitivity analyses of the DNA sensor

The DPV responses for synthetic target DNA at a series of concentrations were recorded in 1 mL 0.1 M PBS (pH 7.0). As shown in Fig 3a, the reduction peak current (*ipC*) increased with an increasing concentration of the target DNA solutions. The detection limit was found to be 1×10^{-15}M. A good performance was also displayed in Fig 3b. There was a good relationship between the *C. tetani* concentration and the *ipC* of DPV over a range from 1×10^{-9} to 1×10^{-15} M (y=6.7357 logx+110.01, R^2=0.9807, x: concentration of *C. tetani,* y: *ipC* of DPV).

Compared with other methods reported previously, such as anaerobic culture (limit to 10 CFU/mL) [24] and real-time PCR (20～100 CFU/mL),[25] the sensitivity of this rGO-PANI-AuNPs electrochemical detection is slightly lower (1×10^{-15}M or 10^3～10^4 CFU/mL). In addition, through the SPE and mini electrochemical instrumentation, this new method has the capacity to detect *C. tetani* in field conditions, meeting the requirements of point-of-care-testing (POCT).

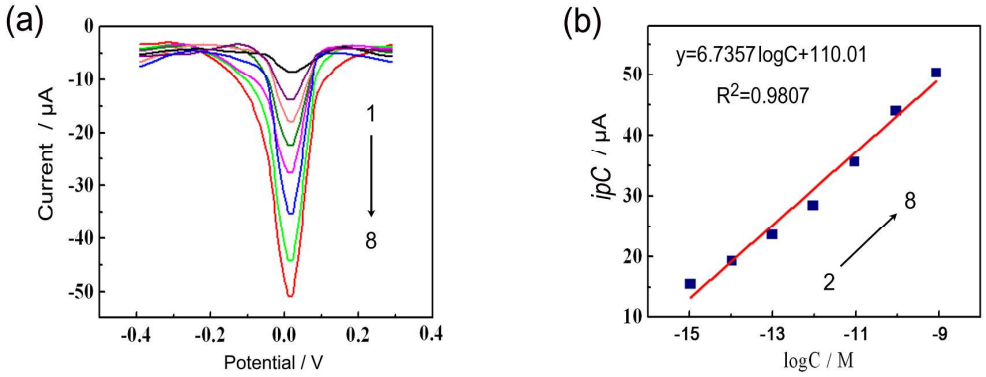


Fig. 3 Sensitivity analyses of the DNA sensor. (a) DPVs of electrochemical detection for target DNA; (b) calibration plot of *ipC* vs logC (1:0, 2～8: 1.0×10^{-15} to 1.0×10^{-9} M target DNA).

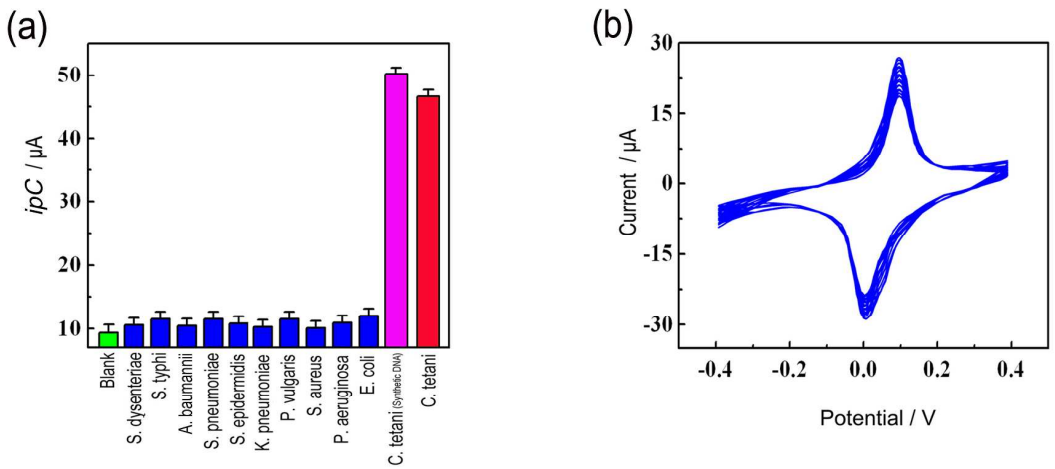


Fig. 4 Specificity and stability of the DNA sensor. (a) selectivity of the DNA biosensor by DPVs; (b) stability of the DNA biosensor by CVs scan (50 cycles with 1×10^{-12} M target DNA).

3.4 Specificity and stability of the DNA sensor

As shown in Fig 4a, there was a marked electrochemical signal (*ipC* >45μA) from the *C. tetani* (synthetic DNA and culture of bacteria). In contrast, the blank and the other 10 bacterial strains exhibited lower signals (*ipC* <13μA). The results indicate that the electrochemical measurement has a high specificity for *C. tetani*. Thus, this method minimises the effect of contamination from other bacteria at the site of a wound. In addition, as illustrated in Fig 4b, *ipA* of CVs only decreased 3～5% after 50 cycles, suggested an excellent stability.

3.5 Comparison with culture method

X^2 analysis was used to compare the performance of the electrochemical measurement to the anaerobic culture method. The results are presented in Table 1. The anaerobic culture was regarded as a gold standard. The calculation was performed according to the EP12-A approved guideline of clinical and laboratory standards institute (NCCLS,USA).

Sensitivity=100% [positive (test method) /positive (gold standard)]

Specificity=100% [negative (test method) /negative (gold standard)]

Table 1 Comparison of electrochemical measurement and anaerobic culture identify.

		Anaerobic culture	
		+	-
	+	41	1
	-	2	45
Electrochemical measurement	X^2	0.3333	
	p	0.5637	
	Sensitivity	95.3%	
	Specificity	97.8%	

The results indicate that the electrochemical measurement was consistent with the identity of the anaerobic culture (p=0.5637), which was regarded as the gold standard. Furthermore, the electrochemical measurement displayed a sensitivity of 95.3% and a specificity of 97.8% compared to the gold standard. Thus, the electrochemical measurement is a rapid, simple and reliable method for the detection of *C. tetani.*

4. Discussion

Tetanus is one of the most dramatic and globally prevalent diseases of humans and vertebrate animals. Due to the difficulty of anaerobic cultured assays, especially in the field, a simple, rapid, sensitive and accurate method is required. In this paper, a new electrochemical method is proposed, based on a rGO-AuNPs modified electrode and a probe labelled PANI-AuNPs.

The rGO-AuNPs dramatically enlarge the surface area and enhance the electronic transfer. Compared with the bare SPE, the electroactive surface area of the rGO-AuNPs modified SPE increased by approximately 95.4%. In addition, the outstanding electrical conductivity of the PANI-AuNPs could improve the sensitivity for the detection of *C. tetani* DNA down to 1×10^{-15}M. This new method also displayed a good relationship between the *C. tetani* concentration and the *ipC* of DPV over a range from 1×10^{-9} to 1×10^{-15} M. Moreover, the electrochemical measurement has a high specificity and an excellent stability. The results of the comparison analysis suggest that the measurement of electrochemical detection is very consistent with the anaerobic culture method, which is regarded as the gold standard of *C. tetani* identification. The electrochemical method displayed a high sensitivity of 95.3% and a fine specificity of 97.8% compared to the anaerobic culture method.

Thus, this electrochemical method based on the rGO-SPE and PANI-AuNPs has great potential as an easy-to-perform technology. This might lead to better management of *C. tetani* and timely implementation of infection control measures. Compared to previously reported methods, the sensitivity of this electrochemical detection is slightly lower. However, through the SPE and mini electrochemical instruments, this new electrochemical method may meet the requirements of point-of-care-testing (POCT). Therefore, this new electrochemical method may become an appropriate technology for diagnosis of *C. tetani* infection in field conditions.

Acknowledgments

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