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Label-free, electrochemical detection of methicillin-resistant *staphylococcus aureus* DNA with reduced graphene oxide-modified electrodes

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ABSTRACT

Reduced graphene oxide (rGO)-modified glassy carbon electrode is used to detect the methicillin-resistant *Staphylococcus aureus* (MRSA) DNA by using electrochemical impedance spectroscopy. Our experiments confirm that ssDNA, before and after hybridization with target DNA, are successfully anchored on the rGO surface. After the probe DNA, pre-adsorbed on rGO electrode, hybridizes with target DNA, the measured impedance increases dramatically. It provides a new method to detect DNA with high sensitivity (10^{-13} M, i.e., 100 fM) and selectivity.

Keywords:

Graphene; Reduced graphene oxide; Impedance spectroscopy; Methicillin-resistant *Staphylococcus aureus*; DNA; Label-free detection;

1. Introduction

Graphene, a new class of two-dimensional sheet materials, displays many unique properties (Huang et al., 2011). It has been used as a potential material for capacitors (Stoller et al., 2008), sensing (Ang et al., 2008; Cao et al., 2011; He et al., 2010; Sudibya et al., 2011; Wang et al., 2009b), solar cells (Yin et al., 2010a,b; Wang et al., 2008), memory devices (Liu et al., 2010a,b), electric devices (Li et al., 2010), matrices for mass spectrometry (Zhou et al., 2010; Tang et al., 2010a; Dong et al., 2010), cell imaging (Sun et al., 2008), cell cultures (Agarwal et al., 2010), and drug delivery systems (Sun et al., 2008; Liu et al., 2008).

Recently, graphene has been functionalized with different materials such as polymers (Qi et al., 2010a,b), nanoparticles (Huang et al., 2010; Zhou et al., 2009a) and biomaterials (Mohanty and Berry, 2008; Lu et al., 2009; Ang et al., 2008). In particular, the graphene–DNA hybrid materials have attracted lots of research interests due to their potential applications in the biomedicine and bioassays. Theoretically, DNA can be effectively bound onto the graphene surface (Varghese et al., 2009; Gowtham et al., 2007). Experimentally, it is reported that the single-stranded DNA (ssDNA) can be adsorbed on graphene via hydrophobic and $\pi - \pi$ stacking interactions (Tang et al., 2010b). In addition, the hydrophobic and electrostatic/hydrogen bonding interactions between DNA and graphene were used to stabilize graphene sheets in H₂O (Patil et al., 2009). All these researches inspire the application of graphene–DNA hybrid materials in the field of biotechnology and biomedicine. For example, Lu et al. (2009) has detected DNA and protein using a graphene-based platform. The Berry group designed a graphene-based biodevice for bacterium assay and DNA detection (Mohanty and Berry, 2008). However, during their DNA detection, dye molecules were used as labels (Lu et al., 2009; Mohanty and Berry, 2008).

Electrochemical impedance spectroscopy (EIS) is an effective method to detect the antigen–antibody formation, biotin–avidin complex and oligonucleotide–DNA interaction (Yang et al., 2004), as compared with other methods such as radiochemical, colorimetric and chemiluminescent methods (Li et al., 2007). It offers several advantages such as simplicity, no requirement for labeling of the analytes (Pan and Rothberg, 2005), higher sensitivity as compared with other type of DNA biosensors based on the surface plasmon resonance (SPR) or quartz crystal microbalance (QCM) techniques (Li et al., 2007). SPR is one kind of optical methods based on the measurement of changes in the refractive index due to the mass adsorption on the sensor chip surface (Kobori et al., 2004). QCM measures the change in frequency of a vibrating crystal due to the binding of molecules to the crystal (Fawcett et al., 1998). The detection limits of SPR and QCM are $\sim 10^{-9}$ (Chen et al., 2009) and 10^{-10} M (Feng et al., 2007), respectively.

In this contribution, EIS, i.e., a label-free method, is used to directly detect DNA. In our experiment, the reduced graphene oxide (rGO)-coated aminopropyltriethoxysilane (APTES)-modified glassy carbon electrode (GCE), referred to as GCE-APTES-rGO, is used to adsorb DNA. If only a bare GCE is used to detect DNA, the detection experiment is unstable since the interaction between DNA and GCE is quite weak, resulting in a little amount of DNA adsorbed on GCE (Willner and Katz, 2005). But our GCE-APTES-rGO electrode can adsorb DNA strongly because of the strong interaction between DNA and graphene, resulting in the stable impedance response (Tang et al., 2010a,b). As proof-of-concept, the methicillin-resistant *Staphylococcus aureus* (MRSA) DNA, a common pathogen that causes severe diseases in humans, such as bacterial endocarditis, pneumonia, and hospital- and community-acquired bacteremic infections (Du et al., 2002; Drummelsmith et al., 2007), is chosen as an example and detected by EIS using our GCE-APTES-rGO electrodes.

2. Experimental

2.1. Materials

ITO (10 ohm/sq, thickness: 0.7 mm) was purchased from Kintec Company (Hong Kong, China). Nature graphite, purchased from Bay Carbon (Bay City, MI, USA), was used for synthesis of grapheneoxide (GO). 3-aminopropyltriethoxysilane (APTES), H_2O_2 (30%), H_2SO_4 (98%), $\text{K}_2\text{S}_2\text{O}_8$ (99+%), P_2O_5 (97%), phosphate buffered saline, $\text{K}_4[\text{Fe}(\text{CN})_6]$ (99.9%), and $\text{K}_3[\text{Fe}(\text{CN})_6]$ (99%) were purchased from Sigma–Aldrich (Milwaukee, WI, USA) and used as received. HCl (37%, Merck) and NaCl (99.5%, Merck) were used as received. Prior to use, toluene was purified with a solvent purification system (PS-400-5, Innovative Technology Inc., USA). The oligonucleotides were purchased from 1st BASE Pte Ltd., (Singapore) and the sequences of the oligonucleotides (Tokue et al., 1991) used in this work were listed as follows.

Probe DNA (ssDNA): 5'-ATG ATT ATG GCT CAG GTA CTG CTA TCC ACC-3'

Target DNA: 5'-GGT GGA TAG CAG TAC CTG AGC CAT AAT CAT-3'

22mer noncomplementary DNA (22mer n-DNA): 5'-CAA CCT CAA ACA GAC ACC ACG G-3'

30mer noncomplementary DNA (30mer n-DNA): 5'-GCG AGA TGA TTA TGG CTC AGG TAC TGC TAT CCA CCC TCG C-3'

Stock solution of oligonucleotides was prepared in 0.01 M PBS buffer solution (pH 7.4), which was stored in refrigerator. Ultrapure Milli-Q water (Milli-Q System, Millipore, Billerica, MA, USA) was used in all experiments.

2.2. Synthesis, adsorption and reduction of GO on glassy carbon electrode (GCE)

The synthesis of GO was described in our previous reports (Zhou et al., 2009 a,b). The process for modification of GCE with GO is illustrated in Scheme 1 (Wang et al., 2009a). Briefly, after the GCE (3.0mm diameter, CHI instrument, Austin, TX, USA) was polished with alumina slurry to get a mirror-like finish, it was activated in 0.05 M H_2SO_4 by cycling the potential from 0 to 2.0 V (vs. Ag/AgCl , sat. KCl) at a scan rate of 50 mVs^{-1} . The activated GCE was washed with copious Milli-Q H_2O and then dried with N_2 gas. After the GCE was immersed in a 1% APTES anhydrous toluene solution for 15 h, the APTES molecules were assembled onto the activated GCE surface to form APTES-modified GCE electrode, i.e., GCE-APTES. After it was washed with copious anhydrous toluene and then dried with N_2 gas, the GCE-APTES electrode was immersed in a diluted GO aqueous solution for 4 h to adsorb single-layer GO sheets, and the GCE-APTES-GO electrode was obtained. Finally, the GCE-APTES-GO electrode was scanned in 0.5 M NaCl solution saturated with N_2 from 0.7 to -1.1 V at a scan rate of 50 mVs^{-1} . GO was reduced and the reduced graphene oxide (rGO) was obtained, i.e., the GCE-APTES-rGO electrode was fabricated (Wang et al., 2009a). All potentials reported in the text are with respect to the Ag/AgCl (sat. KCl) reference electrode.

2.3. Immobilization of DNA on GCE-APTES-rGO surface

$5 \mu\text{L}$ of probe DNA ($10 \mu\text{M}$) was dropped onto the surface of GCE-APTES-rGO electrode, which was then capped with a centrifuge tube to maintain the high humidity and kept at room temperature for 6 h. Then the electrode was immersed into 0.01 M PBS buffer for 10 min to remove the loosely attached probe DNA. The obtained electrode, referred to as GCE-APTES-rGO-ssDNA, was hybridized with

target DNA by dropping a 5 μ L of target DNA solution (10 M) onto its surface. The droplet of target DNA was kept on the electrode surface at room temperature for 30 min before it was immersed into 0.01 M PBS buffer in order to wash away the unreacted target DNA. The obtained electrode is referred to as GCE-APTES-rGO-dsDNA. The noncomplementary DNA (22mer and 30mer) was used in control experiments.

2.4. Assembly and reduction of GO on ITO surface

First, after 1 cm $\times$ 1 cm ITO substrates were sonicated in soap water, acetone and Milli-QH₂O, respectively, they were immersed in a mixture (28% NH₃·H₂O:30% H₂O₂:H₂O = 1:1:6, v/v/v) at 85 °C for 30 min. Then the substrates were washed with copious Milli-Q H₂O and dried with N₂ to obtain OH group-terminated ITO substrates. These substrates were immersed in a 1% APTES anhydrous toluene solution for 30 min to form the organosilane SAMs on ITO, which is referred to as ITO-APTES. After the ITO-APTES substrates were rinsed with anhydrous toluene and dried with N₂, they were annealed at 120 °C for 1 h and then cooled down to room temperature. These ITO-APTES substrates were immersed into a diluted GO aqueous solution for 30 min to adsorb GO, referred to as ITO-APTES-GO. The obtained ITO-APTES-GO was scanned in 0.5 M NaCl solution saturated with N₂ from 0.7 to -1.1 V at a scan rate of 50 mVs⁻¹ to obtain the ITO-APTES-rGO electrode.

2.5. Characterization

Raman spectra were recorded on a WITec CRM200 confocal Raman microscopy system using 488 nm laser with an air cooling charge coupled device (CCD) as detector (WITec Instrument Corp., Germany). Before measurement, the instrument was calibrated by silicon wafer.

X-ray photoelectron spectroscopy (XPS) was performed on a Kratos AXIS Ultra photoelectron spectrometer using Al K α radiation (1486.71 eV).

All electrochemical measurements were carried out in a conventional three-electrode cell using CHI 660 C Electrochemical Workstation (CHI instrument, Austin, TX, USA) with the attachment of a Faraday cage. In the experiment, GCE, platinum electrode and Ag/AgCl (*sat.* KCl) electrode were used as the working, counter and reference electrode, respectively. Impedance analyses were performed from 0.1 Hz to 100,000 Hz in the pH 7.4 phosphate buffer with 5 mV amplitude. The buffer solution was 0.1 M KCl containing 5 mM [Fe(CN)₆]^{3-/4-} (1:1) as redox mediator so as to perform faradic impedance spectroscopy. The initial potential was 0.25 V with respect to Ag/AgCl (*sat.* KCl). This potential is near the equilibrium of [Fe(CN)₆]^{3-/4-} pair, and makes the redox rates equal. Therefore, the redox species will not be depleted near the electrode surface during the measurement (Pan and Rothberg, 2005).

3. Results and discussion

Scheme 1 shows our designed strategy to detect MRSA DNA based on the electrochemically reduced GO-modified GCE (i.e., GCE-APTES-rGO electrode shown in Scheme 1). A 30mer DNA portion of MRSA specific sequence was chosen as the target DNA (Tokue et al., 1991). Its complementary DNA, i.e., probe DNA, was first adsorbed on GCE-APTES-rGO, followed by the hybridization with target DNA. The electrochemical impedance spectroscopy (EIS) was used to monitor the change of charge transfer during the DNA modification on the GCE electrode.

In order to study the adsorption of probe DNA on the rGO surface and also its hybridization with the target DNA, the ITO-APTES-rGO was first used to adsorb the probe DNA and subsequently hybrid with the target DNA (Wanget al., 2009a). These two electrodes were referred to as ITO-APTES-rGO-ssDNA and

ITO-APTES-rGO-dsDNA, respectively. Both of them were characterized by XPS and Raman spectroscopy. As shown in Fig. 1A, the peak at 400.5 eV (N 1s) is assigned to the amine group ($-\text{NH}_2$) and nonconjugated N (sp^3). After ITO-APTES-rGO was modified with ssDNA and dsDNA, the intensity of N 1s increased as compared with that of ITO-APTES-rGO (inset in Fig. 1A). In addition, one peak at 133.7 eV (P 2p) appears, which is attributed to the presence of phosphorus in DNA (Fig. 1B) (Vilar et al., 2008). For both ssDNA and dsDNA, the positions of N 1s and P 2p peaks are similar, but the intensity of the peak before and after hybridization is different. The appearance of P 2p gives evidence that the both ssDNA and dsDNA were successfully adsorbed on the ITO-APTES-rGO surface. In addition, the intensity of N 1s (curve b) and P 2p (curve d) increased after the probe DNA was hybridized with target DNA, which arises from the increased amount of DNA after the successful DNA hybridization on rGO surface.

Raman spectroscopy is an effective tool to measure the structural change of GO after reduction. In Fig. 2, the peaks at 1598 and 1348 cm^{-1} are assigned to the E_{2g} mode (G band) (Stankovich et al., 2007) and D band (Lomeda et al., 2008), respectively. Both GO and rGO have these two bands (curves a and b in Fig. 2). However, the intensity ratio of D/G increased after the electrochemical reduction of GO, confirming that rGO is obtained (Stankovich et al., 2007; Feng et al., 2007). This is consistent with our previous result (Wang et al., 2009a). Besides, the G band shifts from 1598 to 1588 cm^{-1} after the electrochemical reduction of GO, which is also observed in the chemical and thermal reduction of GO (Stankovich et al., 2007; Feng et al., 2007). Modification of rGO with ssDNA (curve c in Fig. 2) and dsDNA (curve d in Fig. 2) did not change the appearance of the D and G peaks. Although these results are obtained from the modified ITO electrodes, they can apply to the modified GCE (Wang et al., 2009a).

The Nyquist plots for the different electrodes and the corresponding equivalent circuit models are shown in Fig. 3A and B, respectively. The simulated values of the equivalent circuit elements are summarized in Table 1. In Fig. 3A, all the curves consist of a semicircle portion and a linear portion, which correspond to the electron transfer process and diffusion process, respectively. The diameter of semicircle represents the resistance of charge transfer (Yang et al., 2004). The bare GCE shows an almost straight line (the curve a in Fig. 3A), which is the characteristic of a diffusion-limited electron-transfer process. After adsorption and electrochemical reduction of GO on APTES-modified GCE, i.e., GCE-APTES-rGO electrode obtained (Wang et al., 2009a), a small interfacial electron transfer barrier was introduced to the system (for interpretation of the references to color in this text, the reader is referred to the web version of the article.). This is evidenced by the appearance of a semicircle with a small diameter (the curve b in Fig. 3A). It is well known that ssDNA is flexible and negatively charged. The presence of negative charge will reduce the conductivity because of the electrostatic repulsion between ssDNA and the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ redox mediators. Also DNA itself could be described as an insulator or a semiconductor (Wang, 2008). Thus, the conductivity of GCE-APTES-rGO undoubtedly decreases after it is modified with probe DNA through the hydrophobic and weak electrostatic/hydrogen bonding interactions between rGO and DNA (Patil et al., 2009). As expected, the resistance of charge transfer (R_{ct}) increased from ~ 23 (GCE-APTES-rGO) to 191 Ω (GCE-APTES-rGO-ssDNA) (the curve c in Fig. 3A). After the probe DNA was hybridized with target DNA, the density of negative charges at the electrode surface increased. This further reduces the charge transfer between the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ redox mediators and the electrode surface, leading to a further increase of the impedance (the curve d in Fig. 3, Table 1). Accordingly, the R_{ct} increased from 191 to 1940 Ω after DNA hybridization, proving that dsDNA still adsorbed on the rGO electrode.

In a previous report (Zhang et al., 2009), ssDNA was adsorbed on the carbon nanotube (CNT)-modified GCE. After the ssDNA was hybridized with the complementary DNA, the formed dsDNA desorbed from the CNTs because the measured impedance dropped to a value, which was similar to that obtained from the original CNT-modified GCE. However, the increased impedance in our GCE-APTES-rGO-

dsDNA electrode suggests that dsDNA remains on the rGO surface after the hybridization (Scheme 1). This may be due to the different interaction mechanisms between CNT-dsDNA and rGO-dsDNA. The ssDNA is structurally flexible and can wrap CNTs (Zhang et al., 2009). After hybridization, dsDNA is rigid and desorbs from the CNT surface. But in our experiment, the dsDNA could lie on the rGO surface to form a stable rGO-dsDNA complex (Patilet et al., 2009). Currently, although the mechanism is not fully understood, our results clearly show that the EIS can be used for label free detection of target DNA (Gebala et al., 2009).

Based on the experimental results, an equivalent circuit model of the system was simulated (Fig. 3B). The circuit consists of the solution resistance (R_s), the resistance of charge transfer (R_{ct}), constant phase element (CPE) and Warburg impedance (W). R_s and W reflect the property of the bulk solution, while R_{ct} and CPE depend on the dielectric and insulating properties of the electrode/electrolyte interface, respectively. Normally, a single element C_{dl} is used to represent the double layer capacitance. In our system, the impedance of solid electrode deviates from the purely capacitive behavior (Daniels and Pourmand, 2007). Therefore, the use of CPE, instead of C_{dl} , is required. The simulated values for all parameters are listed in Table 1, where it shows that the change of R_{ct} is most significant. Hence, the changed value of electron transfer resistance, ΔR_{ct} , before and after the GCE-APTES-rGO-ssDNA reacts with target DNA or noncomplementary DNA (n-DNA), is chosen to represent the response of our electrochemical sensor to the different concentrations of target DNA or n-DNA, respectively.

The response of impedance to different concentrations of target DNA and n-DNA were examined by using EIS (Fig. 4A). The results proved that the impedance changes with different concentrations of target DNA, which can be clearly observed from the diameter change of the semicircles. Higher concentration of target DNA produces larger impedance due to the larger amount of DNA adsorbed on rGO surface after GCE-APTES-rGO-ssDNA hybridizes with the target DNA. Even if the concentration of target DNA is as low as 10^{-13} M (100 fM), the response (ΔR_{ct}) can be easily distinguished from that obtained after GCE-APTES-rGO-ssDNA was modified with 10^{-13} M n-DNA (Fig. 4B), based on the response ratio of complementary to n-DNA, $\Delta R_{ct}^c / \Delta R_{ct}^n > 7$. The detection of 10^{-13} M target DNA by EIS is lower than that measured by fluorescence polarization (10^{-10} M) (Wang et al., 2009b). But when the concentration of target DNA is 10^{-14} M, the resistance of charge transfer of GCE-APTES-rGO-dsDNA is similar to that of GCE-APTES-rGO-ssDNA (Fig. 4B), and the target DNA cannot be detected.

Similar experiments were carried out with the n-DNA instead of the target DNA, used for hybridization with ssDNA pre-adsorbed on the rGO surface (Scheme 1). As expected, the result only shows a very small impedance change for both low and high concentration of n-DNA (Fig. 4B). It is worth noting that high concentration (10 μ M) of probe DNA was used in our experiment. After the probe DNA adsorbed on rGO surface to form GCE-APTES-rGO-ssDNA electrode, there is very few opportunity for n-DNA to adsorb on rGO since n-DNA cannot be hybridized with the probe DNA. But very little amount of n-DNA might still adsorb on rGO surface due to the nonspecific adsorption, leading to a small impedance increase in Fig. 4B. This finding shows that the EIS method can be used for selective detection of MRSA DNA.

4. Conclusion

MRSA DNA has been successfully detected by the electrochemical impedance spectroscopy (EIS) with the rGO-modified GCE. The detect limit of 100 fM is achieved. The results of XPS, Raman spectroscopy and EIS confirmed that the probe DNA was successfully anchored and then hybridized with target DNA on the surface of rGO. Compared with the bare GCE, the presence of rGO is favor to anchor both ssDNA and dsDNA, which provides the stable response of impedance. We believe that this label-free method will have potential applications in detection of other biomolecules.

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List of Schemes

Scheme 1. Schematic illustration of the surface functionalization of the glassy carbon electrode for DNA detection. The sequences of the oligonucleotides are listed in Section 2.

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- Figure 1. XPS spectra of (A) N 1s and (B) P 2p for ITO-APTES-rGO-ssDNA (curves a and c) and ITO-APTES-rGO-dsDNA (curves b and d). Inset in (A) XPS spectrum of N 1s for ITO-APTES-rGO. Fitting parameters were chosen for a consistent fit for all samples in the series (dashed lines for raw data, solid lines for total fits, and dotted lines for background).
- Figure 2. Raman spectra of (a) ITO-APTES-GO, (b) ITO-ATPES-rGO, (c) ITO-APTES-rGO-ssDNA and (d) ITO-APES-rGO-dsDNA. Laser wavelength: 488nm.
- Figure 3. (A) Nyquist plots at bare GCE (a, ▼), GCE-APTES-rGO (b, ▲), GCE-APTES-rGO-ssDNA (c, ●) and GCE-APTES-rGO-dsDNA (d, ■). Inset: magnification of the spectra of curves a–c. The concentration of target DNA used here is 10^{-6} M. Frequency range: from 0.1 to 100,000 Hz; electrolyte: $5\text{mM}[\text{Fe}(\text{CN})_6]^{3-/4-}$ (1:1) in 0.01M phosphate buffer solution (pH 7.4) containing 0.1M KCl; amplitude: 5mV. (B) The equivalent circle model for GCE-rGO-dsDNA. R_s , solution resistance; R_{ct} , resistance of charge transfer; CPE, constant phase element; W, Warburg impedance.
- Figure 4. (A) Nyquist plots of GCE-APTES-rGO-ssDNA hybridized with target DNA at different concentrations: (a) 0, (b) 10^{-14} , (c) 10^{-13} , (d) 10^{-11} , (e) 10^{-9} , (f) 10^{-8} , (g) 10^{-7} and (h) 10^{-6} M. Inset: magnification of curves a, b and c. (B) The plot of ΔR_{ct} vs. logarithm of the concentration of target DNA (■), 22mer n-DNA (●), and 30mer n-DNA (▲), respectively. Frequency range: from 0.1 to 100,000 Hz; electrolyte: $5\text{mM}[\text{Fe}(\text{CN})_6]^{3-/4-}$ (1:1) in 0.01 M phosphate buffer solution (pH 7.4) containing 0.1M KCl; amplitude: 5mV.

Scheme 1

	R_s (Ω)	CPE (F)	R_{ct} (Ω)	W (Ω)
Bare GCE	65	1.24E-05	19	0.0015
GCE-APTES-rGO	61	1.07E-05	23	0.0016
GCE-APTES-rGO-ssDNA	63	1.90E-05	191	0.0015
GCE-APTES-rGO-dsDNA	56	1.30E-05	1940	0.0015

Table 1

Figure 1

Figure 2

Figure 3

Figure 4