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Chitosan-Modified Graphene Electrodes for DNA Mutation Analysis

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Abstract

Graphene has remarkable electrochemical properties that make it an ideal material for constructing biosensors, however it has not been explored for DNA biosensing. Herein, we report on a chitosan-modified graphene platform for the electrochemical detection of changes in DNA sequences. For this purpose, graphene synthesized chemically and characterized by Raman spectroscopy and Transmission electron microscopy, was covalently modified with positively charged chitosan to facilitate the immobilization of a single-stranded DNA 'capture' oligonucleotide. The covalent attachment of chitosan to graphene was confirmed by FT-IR spectroscopy and then the capture DNA was immobilized on to the chitosan modified graphene electrode. Then, the target DNA (complementary or mismatched 'mutant' DNA) was applied to the electrode and cyclic voltammetry was performed. The results of the voltammetric experiments indicate that the chitosan modified graphene electrodes immobilized with ssDNA+complementary DNA exhibit a significantly higher magnitude of redox peak current than the chitosan modified graphene electrodes immobilized with the non-complementary mutant DNAs. Together, these results demonstrate that the chitosan-graphene platform provides a rapid, stable and sensitive detection of mismatched DNA and has the potential to be used for point-of-care diagnostic tests for specific DNA mutations associated with disease conditions.

Introduction

Graphene [1], one of the exotic new nanomaterials of the 21st century, is a two-dimensional sheet comprised solely of sp² carbon atoms arranged in a chicken-wire like framework [2–5]. Graphene possesses outstanding electronic [6–8], mechanical [9–11], thermal [12–14],

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optical [15,16] and charge transport [17,18] properties that make it an attractive choice for biosensors [19–23], fuel cells [24,25], batteries [26,27], ultracapacitors [28,29], electromechanical resonators [30,31] and field effect transistors [32,33]. Altered DNA sequences in specific genes are characteristic of many diseases from cancer to asthma and their identification is important for correct diagnosis and treatment. While DNA mutations or single-nucleotide polymorphisms can be detected by direct sequencing or PCR, these methods require costly instruments, extensive training and relatively long run times. A rapid, inexpensive, specific, and sensitive device for detecting mismatched DNAs that could be used in the field or during a clinic visit would prove invaluable to physicians, especially in resource-limited areas. In order to develop such a device, we modified graphene electrodes with biocompatible chitosan. Single-stranded DNA (ssDNA) oligonucleotides antisense to the target DNA were then electrostatically bonded to chitosan as capture molecules. Chitosan is a natural biopolymer that contains primary amine groups at the C-2 position of the glucosamine moiety and is soluble in buffers at pH < 6.5 [34,40]. In solution, chitosan acquires a positive charge due to the formation of -NH_3^+ groups and these interact electrostatically with the negatively charged phosphate backbone of the ssDNA to form a strong and stable bond that holds the molecules in position for hybridization with other ssDNAs.

In this work, we prepare a chitosan modified graphene blend and then immobilize them onto a glassy carbon electrode. The chitosan modified graphene electrode thus obtained is then modified with the capture ssDNA. A buffer solution containing the target DNA or mismatched DNA was applied to the electrode and cyclic voltammetry was performed. The chitosan-modified graphene electrodes acted as a stable and sensitive platform for the rapid and specific electrochemical detection of DNA and allowed us to rapidly and easily distinguish complementary from noncomplementary DNA.

MATERIALS AND METHODS

Hydrazine hydrate, graphite, potassium hexacyanoferrate (III), potassium hexacyanoferrate (IV), sodium phosphate (dibasic salt), sodium chloride, trisodium citrate, and bovine serum albumin were all purchased from Sigma-Aldrich (St. Louis, MO) and used without any further purification. Chitosan (33 kDa) was a gift from Transgenex (Tampa, FL). Glassy carbon electrodes, Ag/AgCl (3.0 M KCl) reference electrodes and platinum wire counter electrodes were all purchased from CH Instruments [Austin, Texas].

DNA sequences

5'-GACTCTGGTAACTAGAGATC-3' (capture DNA)

5'-GATCTCTAGTTACCAGAGTC-3' (complementary DNA)

5'-CCATATCACCTAGAACTTTA-3' (mismatch DNA)

These DNA oligonucleotides were synthesized by Integrated DNA Technologies Inc. [Coralville, IA].

Methods

Graphene synthesis

Graphene was synthesized according to the method reported earlier [19]. Initially graphene oxide (GO) was synthesized by following Hummers method [35]. GO was mixed with de-ionized water and sonicated until it becomes clear. The clear solution was treated with hydrazine hydrate and heated to 100°C in an oil-bath for 24h. This resulted in the formation of a black precipitate. This precipitate was filtered and washed several times with de-ionized

water. The precipitate was finally dried in nitrogen atmosphere for about 6 hours to get pure graphene.

Raman Spectroscopy

Raman measurements were conducted at room temperature with the help of a Reinshaw spectrometer in the back scattering configuration that operates with a 514 nm Ar⁺ laser at 50 mW. Raman spectra were collected for each sample after a 15-minute exposure time using a high throughput holographic imaging spectrograph that contains volume transmission grating, holographic notch filter and a -70°C Peltier cooled CCD with 4cm⁻¹ resolution.

Transmission Electron Microscopy (TEM)

TEM measurements were made using TECHNAI F20. The electron source is a Schottky Field emitter operating at 0.7 eV. TECHNAI F20 employed in this work has a point resolution of 0.24 nm, line resolution of 0.102 nm and information limit of 0.14 nm.

Synthesis of chitosan-modified graphene

Graphene as obtained above (5 mg/100 mL) was sonicated (Model: Branson 2510) in de-ionized water for an hour at 25°C to yield a homogeneous suspension. To this suspension, chitosan (5 mg) was added and vortexed for one hour at 25°C to yield a chitosan-modified graphene (CMG) suspension. Following this, 5 µL of CMG suspension was pipetted onto the surface of a glassy carbon electrode to serve as the electrochemical platform for the electroanalysis. The as prepared CMG platform is stable between 4°C to 50°C.

Fourier-Transferred Infra-red Spectroscopy

FTIR characterization of graphene and chitosan modified graphene nanosheets were performed using a Nicolet IR-100 spectrometer. 10 µl (of the sample was dropped onto a disposable polyethylene IR card and the solution was dried under vacuum prior taking the measurements.

Capture DNA immobilization procedure

30pg of capture DNA in 0.1 M Phosphate Buffered Saline (PBS) was pipetted onto the CMG electrode and allowed to remain at room temperature for 15 minutes. The positive charge on the CMG will exert an electrostatic interaction onto the negatively charged phosphate backbone to hold the capture DNA in place. The electrodes were rinsed twice with PBS to remove excess DNA. The amount of capture DNA (30pg) that needs to be immobilized to achieve a steady and observable signal was optimized by testing a range of DNA concentrations.

Hybridization of Target DNA with capture DNA

The hybridization was performed by pipetting an aliquot of the target DNA in 5×SSC (pH = 6.1) onto the electrode coated with the capture DNA and allowing it to remain at room temperature for 30 minutes. The electrodes were then rinsed twice with SSC to remove nonspecifically bound target DNA.

Cyclic voltammetry

The electrochemical readings were performed using a CHI-630A electrochemical analyzer (CH Instruments, Inc.). The working, reference and counter electrode(s) were CMG, Ag/AgCl (3.0M KCl) and platinum wire respectively. In all experiments, the surface areas of the CMG modified glassy carbon electrodes, Ag/AgCl and Pt-counter electrodes were identical. All the voltammetric experiments were performed in a 5 mL vial containing the redox probe

0.1 mM $K_4[Fe(CN)_6]^{3-/4-}$ in 1.0 M KCl. Before performing voltammetry, the vial containing the redox probe was purged 2 minutes with nitrogen to remove oxygen.

Results and Discussion

Initially, graphene was synthesized as detailed in the methods section. Next, the surface of graphene nanosheets was characterized by Raman spectroscopy and transmission electron microscopy. The Raman spectrum of graphene at 514 nm shows a G peak at $\sim 1590\text{ cm}^{-1}$ and a 2D peak at $\sim 2700\text{ cm}^{-1}$ (Fig. 1A). The G peak is attributed to the doubly degenerate zone center (E_{2g} mode) [36,37] and the 2D peak is due to the presence of a pair of phonons with opposite momentum in the highest optical branch near the **K** [38,39,41]. The number of layers of graphene as determined by the high resolution electron micrograph was found to be 7 and the d value (inter-layer spacing) was calculated to be 0.35 nm (Fig. 1B). Following this, graphene was subjected to FT-IR analysis. The FT-IR spectrum of graphene (Fig. 2) exhibited the IR peaks at 2920 cm^{-1} and 2849 cm^{-1} ($sp^2\text{ } \nu_{C-H}$ stretching), 1731 cm^{-1} and 1626 cm^{-1} ($\nu_{C=O}$), 1481 cm^{-1} (skeletal vibrations of graphene sheets), 1461 cm^{-1} (carboxyl ν_{C-O} stretching), 1177 cm^{-1} (alkoxy ν_{C-O} stretching at the edges of graphene) and 1051 cm^{-1} (ν_{C-O} stretching). Then, the graphene surface was covalently modified with chitosan to make the surface more positive and electrostatically trap the capture ssDNA onto the CMG platform. Chitosan modified graphene exhibited all the FT-IR bands as discussed above with the addition of a band at 1635 cm^{-1} (ν_{CONH}) indicating the covalent modification of graphene with chitosan (Fig. 2).

In order to determine whether the graphene electrode could identify a mutant DNA, electrodes were loaded with capture DNA then cyclic voltammetry was performed in 0.1 mM $K_4[Fe(CN)_6]^{3-/4-}$ / 1.0 M KCl with capture ssDNA alone, ssDNA plus complementary DNA and ssDNA plus non-complementary DNA. The introduction of ssDNA on to the CMG surface is expected to sterically hinder the diffusion of the $[Fe(CN)_6]^{3-/4-}$ ions towards the electrode surface. The steric hindrance experienced by the $[Fe(CN)_6]^{3-/4-}$ ions is mainly due to the way in which the ssDNA orients itself on the electrode surface (Fig. 3a). Furthermore, the negatively charged $[Fe(CN)_6]^{3-/4-}$ ions are expected to experience an electrostatic repulsion from the negatively charged phosphate groups on the capture ssDNA. As a result of this combined hindrance, the diffusion of the $[Fe(CN)_6]^{3-/4-}$ towards the electrode surface was restricted compared to an electrode with no DNA (data not shown). Next, we applied the complementary target DNA to the ssDNA-loaded CMG platform. The complementary DNA hybridized perfectly with the capture DNA and a double-stranded structure was formed by hydrogen bonding between the bases. Upon forming the double-stranded helix, the dsDNA is expected to orient itself perpendicular to the electrode platform as shown in scheme (Fig. 3b). This perpendicular orientation causes less steric hindrance to the negatively charged $[Fe(CN)_6]^{3-/4-}$ ions diffusing towards the electrode surface. Also, the formation of hydrogen bonds will weaken the negative charge on the phosphate backbone thereby minimizing the electrostatic repulsion between the dsDNA and the $[Fe(CN)_6]^{3-/4-}$ ions. This explanation was confirmed from the voltammogram obtained in this work using dsDNA modified CMG electrode which showed an increase in the magnitude of the voltammetric peak current (Fig. 4a & b). Though there is a significant increase in the peak current value after hybridization, the ΔE_p value ($60 \pm 3.5\text{ mV}$, $N=7$) remained the same before and after hybridization.

In the next set of experiments, we applied a non-complementary target DNA onto the ssDNA-modified CMG platform and cyclic voltammetry was performed in 0.1 mM $K_4[Fe(CN)_6]^{3-/4-}$ / 1.0 M KCl (Fig. 4c). The significant decrease in the magnitude of the peak current seen here is attributed to the major steric and repulsive effect offered to the $[Fe(CN)_6]^{3-/4-}$ ions jointly by the capture and target DNAs. The non-complementary target

DNA does not hybridize to the capture ssDNA but it is expected to bind electrostatically to the surface of the CMG electrode itself and cause a severe blockage to the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ ions diffusing towards the electrode surface (Fig. 3c). However, there was no significant change in the ΔE_p value upon comparison of the electrode with capture DNA vs capture DNA+complementary DNA. Taken together, these experiments, demonstrate that the magnitude of the voltammetric peak current varies in the following order with a negligible variation in their ΔE_p value: $i_{(\text{complementary DNA})} > i_{(\text{capture DNA alone})} > i_{(\text{noncomplementary DNA})}$

Conclusions

In this report, we have demonstrated the ability of the CMG platform to electrochemically distinguish complementary DNA from noncomplementary DNA providing a simple, sensitive, and stable platform for detecting specific mutations. Our work is continuing with the construction of a prototype CMG electrode array for multiplex DNA identification of many DNA sequence alterations in a single run. This multiplex electrode will allow development of a rapid biosensing device to characterize an individual's genetic make-up in relation to a specific disease.

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Fig. 1.
Raman spectrogram (A) and transmission electron micrograph (B) of graphene.

Fig. 2.
FT-IR spectra of graphene and chitosan-modified graphene

Fig. 3.

Illustration depicting the DNA orientation on a CMG electrode and the corresponding voltammetric response: (a) ssDNA, (b) ssDNA plus complementary DNA, and (c) ssDNA plus mismatch DNA.

Fig. 4. Cyclic voltammogram of the CMG electrode with (a) ssDNA alone, (b) ssDNA plus complementary DNA, and (c) ssDNA plus mismatch DNA.