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Biosensors | *Very Important Paper* |

VIP Rational Design of Carboxyl Groups Perpendicularly Attached to a Graphene Sheet: A Platform for Enhanced Biosensing Applications

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Abstract: Graphene oxide (GO)-based materials offer great potential for biofunctionalization with applications ranging from biosensing to drug delivery. Such biofunctionalization utilizes specific functional groups, typically a carboxyl moiety, as anchoring points for biomolecule. However, due to the fact that the exact chemical structure of GO is still largely unknown and poorly defined (it was postulated to consist of various oxygen-containing groups, such as epoxy, hydroxyl, carboxyl, carbonyl, and peroxy in varying ratios), it is challenging to fabricate highly biofunctionalized GO surfaces. The predominant anchoring sites (i.e., carboxyl groups) are mainly present as terminal groups on the edges of GO sheets and thus account for only a fraction of the oxygen-containing groups on GO. Herein, we suggest a direct solution to the long-standing problem of limited abundance of carboxyl groups on GO; GO was first reduced to graphene and consequently modified with only carboxyl groups grafted perpendicularly to its surface by a rational synthesis using free-radical addition of isobutyronitrile with subsequent hydrolysis. Such grafted graphene oxide can contain a high amount of carboxyl groups for consequent biofunctionalization, at which the extent of grafting is limited only by the number of carbon atoms in the graphene plane; in contrast, the abundance of carboxyl groups on "classical" GO is limited by the amount of terminal carbon atoms. Such a graphene platform embedded with perpendicularly grafted carboxyl groups was characterized in detail by X-ray photoelectron spectroscopy, cyclic voltammetry, and electrochemical impedance spectroscopy, and its application was exemplified with single-nucleotide polymorphism detection. It was found that the removal of oxygen functionalities after the chemical reduction enhanced the electron-transfer rate of the graphene. More importantly, the introduction of carboxyl groups promoted a more efficient immobilization of DNA probes on the electrode surface and improved the performance of graphene as a biosensor in comparison to GO. The proposed material can be used as a universal platform for biomolecule immobilization to facilitate rapid and sensitive detection of DNA or proteins for point-of-care investigations. Such reactive carboxyl groups grafted perpendicularly on GO holds promise for a highly efficient tailored biofunctionalization for applications in biosensing or drug delivery.

Introduction

Graphene and graphene oxide (GO) exhibit large surface area suitable for anchoring biorecognition elements for applications in biosensing or drug delivery.[1] Such anchoring is typically achieved by oxygen-containing groups on GO surface. Despite several decades of research on graphite oxide and almost a decade of investigations on GO, their exact chemical structures remained a mystery; many structural models have been proposed.[2,3] It is known that GO contains hydroxyl, epoxy, peroxy, carbonyl, and carboxyl groups; the last two groups listed are mostly present only at terminal carbon atoms of graphene sheets, whilst it is expected that hydroxyl, epoxy, and peroxy groups are localized on both the basal and edge planes of graphene.[4] GO can be prepared by using chlorate or permanganate oxidation routes[5–8] to provide a material with an empirical formula of approximately C_2O_1, at which the oxygen-containing groups exist in varying compositions.[9] In fact, large uncertainties in the chemical compositions of GO hamper the development for rational design of biofunctionalized graphene platforms. In addition, the most reactive oxygen-containing functionalities, carboxyl groups, which are important for biofunctionalization through carbodiimide chemistry, are only available in small amounts.[10–12] The inherent structural constraints of GO limit its availability at predominantly the edge plane.

To solve this problem, we first remove the oxygen-containing groups from GO through a reductive treatment and consequently reintroduce carboxyl groups onto the graphene surface. Carboxyl groups are inserted based on a free-radical-addition reaction that occurs not only on carbon atoms located at the edge plane but also at the much more abundant basal plane of graphene sheets. More specifically, chemically reduced graphene oxide (CRGO) was first functionalized with isobutyronitrile group, which was generated from the thermal decomposition of azobisisobutyronitrile (AIBN) to give CRGO-CN. Sub-

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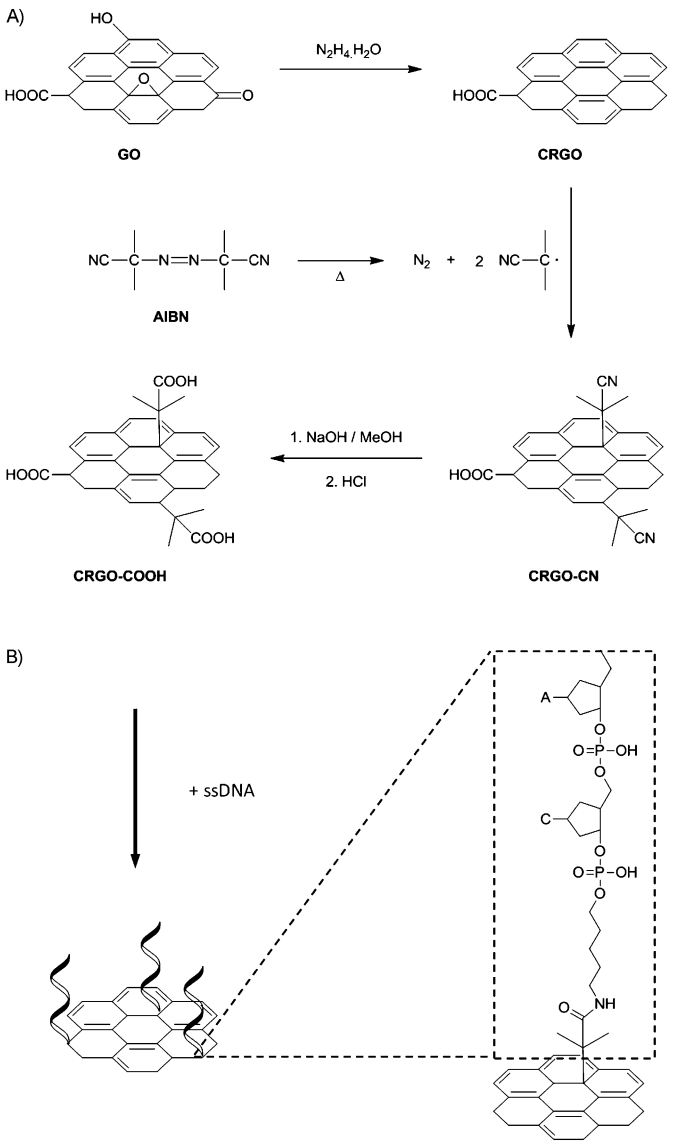
sequent heating at reflux of CRGO-CN in a mixture of methanol and sodium hydroxide aqueous solution resulted in a hydrolysis reaction to provide CRGO enriched with carboxyl groups (CRGO-COOH), as shown in Scheme 1.



Scheme 1. Schematic of functionalization method: A) synthetic route on the surface modification of GO to introduce perpendicularly grafted carboxyl groups. GO was reduced with hydrazine monohydrate to provide CRGO, which subsequently underwent free-radical addition of an isobutyronitrile moiety obtained from the thermal decomposition of azobisisobutyronitrle (AIBN) to give CRGO-CN. CRGO-CN was hydrolyzed and acidified to give CRGO-COOH; B) modification of CRGO-COOH with DNA probes.

The resulting graphene, which is rich in carboxyl groups attached perpendicularly to its basal plane, can be used as anchoring points for biomolecule binding. Additionally, such chemically uniform graphene has a low density of other oxygen functionalities that can cause detrimental effects to the subsequent biofunctionalization step. We hypothesize that this can be a stable and reproducible universal platform for the facile immobilization of biomolecule to be applied in point-of-care diagnosis for protein or DNA detection, which we will demonstrate below.

Results and Discussion

We demonstrate that rationally designed graphene with perpendicularly grafted carboxyl groups can dramatically outperform the "classical" GO, which contains carboxyl groups at mainly the edge sites (or physical defects), alongside other oxygen-containing groups, in important bioapplications, such as biosensing.[13] In particular, carboxyl moiety is exploited for anchoring DNA by carbodiimide chemistry for the application of single-stranded (ss) DNA detection. We compare the results with "classical" or untreated GO platform, which, besides carboxyl groups, contains various oxygen functionalities, such as hydroxyl, aldehyde, epoxy, carbonyl, and peroxy groups that can potentially lead to detrimental effects during the biofunctionalization step.[14]

The addition of perpendicularly grafted carboxyl groups was achieved in a three-step synthetic modification from GO (exfoliated from graphite oxide obtained by the Staudenmaier[5] oxidation method by ultrasonication treatment). The reduction of GO with hydrazine monohydrate[15] gave chemically reduced GO containing only unreduced carboxyl functionality.[16] Despite the persisting presence of carboxyl moiety in both GO and CRGO, its coverage was still minimal to benefit biofunctionalization process. Consequently, the amount of carboxyl groups on graphene sheets was enhanced by a rational design, which exploited robust chemical modification methods. Specifically, CRGO was functionalized by free-radical addition of isobutyronitrile produced by the thermal decomposition of AIBN. The nitrile group was subsequently hydrolyzed under alkaline condition to give carboxylate salt, which gave free carboxylic acid upon acidification. Akin to previous reports on free-radical functionalization of graphene surfaces, grafting occurred on both the basal and edge planes of graphene sheets.[17]

To gain more insights into the variations of functional groups on the graphene surfaces, X-ray photoelectron spectroscopy (XPS) characterizations were performed. As shown in Figure 1, high resolution C1 s core-level spectra highlighted significant changes upon chemical treatments. In general, five main types of carbon bonds can be identified at 284.6 (C=C), 285.5 (C–C), 286.4 (C–O), 287.6 (C=O), and 288.9 eV (O–C=O). A peak centered at 290.6 eV corresponded to the π–π^* shake-up satellite of the sp^2 graphitic carbon peak at 284.6 eV, indicating a highly conjugated system. Treatment of GO with hydrazine monohydrate provided CRGO with a minimal amount of oxygen functionalities, as was also indicated by an increased C/O ratio from 2.7 to 16.3 in the XPS survey scans (not shown). Following a free-radical addition with AIBN and a consequent hydrolysis reaction, the percentage distribution of C–C, C–O, C=O, and O–C=O bond types in CRGO-COOH increased by 8.1, 3.7, 1.1, and 2.0%, respectively, compared to that of CRGO. The increasing percentage distribution of these bond types strongly signified the successful grafting of isobutyric acid in CRGO-COOH. The functionalization also resulted in a lower C/O ratio

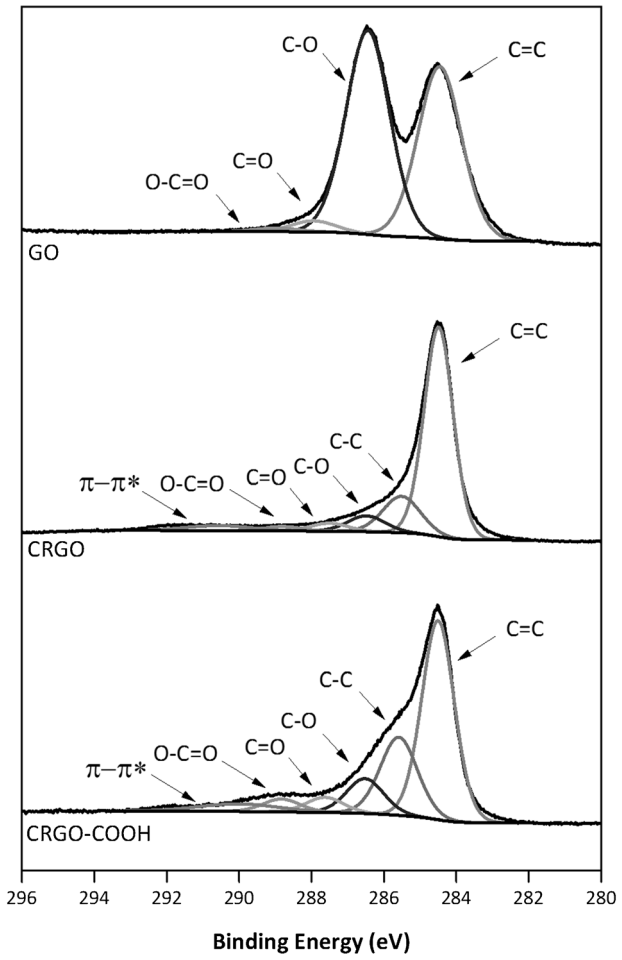


Figure 1. XPS C 1s core-level spectra of GO, CRGO, and CRGO-COOH.

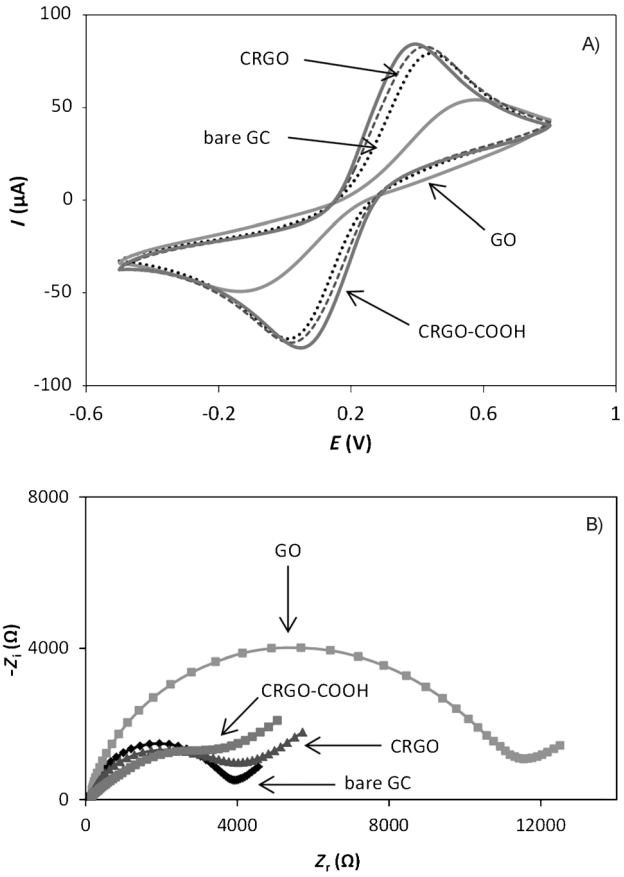


Figure 2. Electrochemical characterization of bare glassy carbon (GC), GO, chemically reduced GO (CRGO), and chemically reduced GO modified with carboxyl groups (CRGO-COOH). A) Cyclic voltammograms recorded at 100 mV s^{-1} scan rate by using Ag/AgCl reference electrode. B) Nyquist plots representing $-Z_i$ versus Z_r. All experiments were carried out in PBS (0.01 M) containing $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (10 mM).

of 6.7. More importantly, this observation was in line with a recent report and indicated the successful functionalization of graphene with isobutyric acid.[18]

The electrochemical behaviors of GO, CRGO, and CRGO-COOH were then investigated in the presence of ferrocyanide/ferricyanide redox probe. Figure 2 illustrates the cyclic voltammograms (part A) and Nyquist plots (part B) obtained for the three graphene materials. Both the electrochemical characterization methods employed the signal obtained from bare GC electrode as reference.

GO highlighted a slower charge-transfer rate as compared to CRGO and CRGO-COOH, due to the presence of various oxygen functionalities on the surface of GO. The slower charge-transfer rate of GO was shown in the cyclic voltammogram by a larger peak-to-peak separation (Figure 2, part A) and in the impedance spectrum by a larger charge-transfer resistance (R_{ct}; corresponding to the semicircle diameter in the Nyquist plot).[19,20]

Surprisingly, CRGO-COOH presented a similar peak-to-peak separation in CV studies and an R_{ct} in electrochemical impedance spectroscopy (EIS) measurements as that of CRGO, thus indicating that the presence of carboxyl groups does not pose detrimental effects to the performance of electrochemical charge transfer. Therefore, the CRGO-COOH has both the essential carboxyl functional groups needed for biofunctionalization and an improved charge-transfer rate compared to GO.

Following that, solid-state reduction measurements were conducted to further confirm the absence of oxygen functionalities other than carboxyl groups on CRGO-COOH. Figure 3 illustrates the results obtained for GO, CRGO, and CRGO-COOH. The signal obtained for bare GC is also provided as reference. It is known that the electrochemical peak provided by GO is due to the reduction of oxygen-containing groups, such as aldehyde, epoxy, and peroxy present on GO surface.[21,22] As depicted in Figure 3, a large reduction peak is shown by GO, indicating the presence of the above-mentioned oxygen functionalities on its surface. On the other hand, no significant reduction peaks were observed for both CRGO and CRGO-COOH, thus confirming the absence of electrochemically reducible oxygen functionalities on these materials. The presence of carboxyl groups on CRGO-COOH did not give rise to any reduction peak as more extreme conditions would be required for the electrochemical reduction to occur.[23]

In the following section, the superior properties of CRGO-COOH in comparison to "classical" GO upon biofunctionaliza-

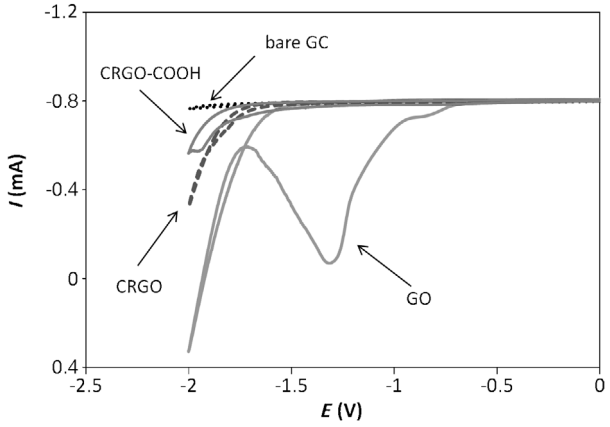


Figure 3. Cyclic voltammograms for the electrochemical reduction of bare glassy carbon (GC), graphene oxide (GO), chemically reduced GO (CRGO), and CRGO modified with carboxyl groups (CRGO-COOH). Conditions: scan rate 100 mV s^{-1}; reference electrode Ag/AgCl.

tion and application in biosensing are elucidated. DNA sequences correlated to the development of Alzheimer's disease were detected by using EIS. The relative variation of charge-transfer resistance was measured upon hybridization with complementary target (wild type), one-mismatch target (mutant) and noncomplementary target (nc). The same experiments were also carried out on GO surface for comparison purposes.

As illustrated in Figure 4, CRGO-COOH platform recorded a larger variation of charge-transfer resistance when incubated with wild-type target, meaning that hybridization has successfully taken place.[12] In fact, the formation of double-stranded (ds) DNA hybrid will give rise to a larger steric hindrance and electrostatic repulsion, which hinder the electron-transfer process of the ferrocyanide/ferricyanide redox probe at the electrode surface.[24] The recorded R_{ct} variation was not as significant when incubation was carried out with a noncomplementary sequence employed as a negative control, thus implying that hybridization did not occur and any nonspecific adsorption of the noncomplementary target on the electrode surface can be considered negligible. When the mutant sequence carrying a single base mismatch correlating to the onset development of Alzheimer's disease was used in the hybridization step, a result similar to that obtained for the hybridization with noncomplementary sequence was observed. The large disparities in R_{ct} between CRGO-COOH platform hybridized with wild-type and mutant target sequences indicated a high selectivity towards single base mismatch. The same experiments performed on GO platform highlighted an insignificant difference, thus indicating that the platform was not as selective for the detection of single nucleotide polymorphism in DNA target sequence.

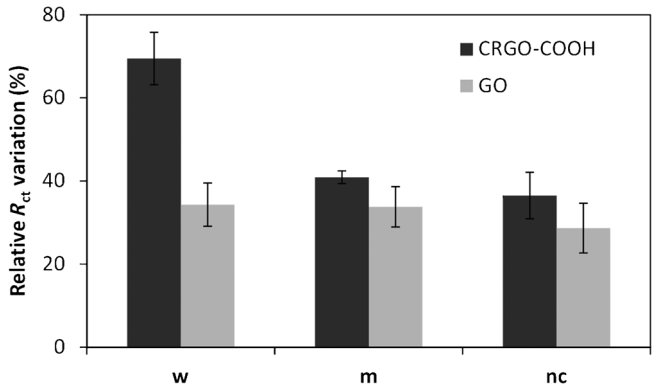


Figure 4. Histogram representing relative charge-transfer-resistance (R_{ct}) variation for hybridization experiments on graphene materials possessing carboxyl groups. CRGO-COOH: experiments performed on CRGO modified with carboxyl groups; GO: experiment performed on GO; w: wild-type DNA target; m: mutant DNA target; nc: noncomplementary DNA target.

Based on the selectivity of CRGO-COOH towards single-base nucleotide polymorphism detection, such differences in performances between GO and CRGO-COOH platforms for biosensing were assumed to be due to the more effective DNA probe immobilization on CRGO-COOH. To confirm our hypothesis, chronocoulometry experiments were performed on both CRGO-COOH and GO after being functionalized with DNA probes. The probe density was calculated to be 3.76×10^{13} and 2.41×10^{13} molecules cm^{-2} on CRGO-COOH and GO, respectively, as was derived from the extrapolation of the intercepts at time zero.[25] The larger amount of DNA probes on CRGO-COOH platform, as was resulted from chronocoulometry experiments, confirmed the improved suitability of this material for DNA probe immobilization (Figure 5).

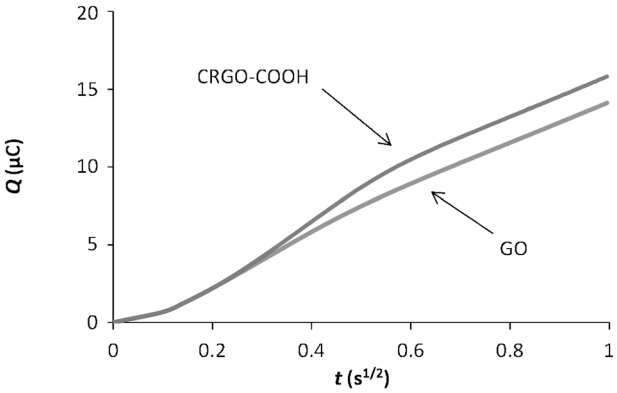


Figure 5. Chronocoulometry response curves after the immobilization of 10 μM DNA probes on CRGO modified with carboxyl groups (CRGO-COOH) and GO.

Conclusion

We have demonstrated that it is possible to considerably increase the amount of carboxyl groups on GO by grafting carboxyl groups perpendicularly to the graphene sheets by free-radical addition. Such reactive carboxyl groups can be used as anchoring points for biomolecule attachment by carbodiimide chemistry. The grafted material was carefully characterized and functionalized with ss DNA and was found to exhibit superior properties over "classical" GO.

Biosensing was performed by using impedance spectroscopy and highlighted a drastic increase of impedimetric signal on CRGO-COOH platform compared to "classical" GO, which was used as a starting material for chemical modifications. A chronocoulometry study has confirmed that this was due to a more effective immobilization of DNA probes on the CRGO-COOH surface. In summary, the new CRGO-COOH platform is ideally suitable for electrochemistry due to the inherent fast electron-transfer rate shown, as well as the enhanced immobilization and sensitive detection of DNA polymorphism. The rationally designed graphene with carboxyl groups attached perpendicularly to its surface is an advanced material highly suitable for high-density biofunctionalization and shall potentially find its way into the various applications of biofunctionalized graphene in biomedical field.

Experimental Section

Materials

Natural graphite was obtained from Asbury Carbons (Asbury, NJ, USA). Sulfuric acid (95–98%), hydrochloric acid (37%), potassium ferricyanide ($K_3[Fe(CN)_6]$), potassium ferrocyanide ($K_4[Fe(CN)_6]$), tris-(hydroxymethyl)aminomethane (Tris), hexaammineruthenium(III) chloride, sodium dodecyl sulfate (SDS), sodium hydroxide, *N*-(3-*d*imethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), *N*-hydroxysulfosuccinimide sodium salt (sulfo-NHS), and DNA sequences correlated to Alzheimer's disease were obtained from Sigma–Aldrich (Singapore). Fuming nitric acid (>90%) was obtained from J.T. Baker (Singapore). Toluene was obtained from RCI Labscan (Bangkok, Thailand). Methanol was obtained from Tedia (Singapore). Azobisisobutyronitrile was obtained from SCRC Ltd (Shanghai, China). Hydrazine monohydrate, potassium chlorate (98%), and hydroxylamine hydrochloride were purchased from Alfa Aesar (Heysham, UK). The following buffer solutions were employed: PBS (10 mM sodium phosphate, 0.1 M NaCl, pH 7.0), TSC1 (75 mM trisodium citrate, 0.75 M NaCl, pH 7.0), TSC2 (30 mM trisodium citrate, 0.30 M NaCl, pH 7.0). All solutions were prepared by using Milli-Q water (18.2 MΩ cm resistivity). Oligonucleotide sequences used in the study are the following: DNA probe 5′-NH_2-$(CH_2)_6$-ACCAGGCGGCCGCACACGTCCTCCAT-3′; complementary target 5′-ATGGAGGACGTGTGCGGCCGCCTGGT-3′ (wild type); one-mismatch target 5′-ATGGAGGACGTG**C**GCGGCCGCCTGGT-3 (mutant); noncomplementary target 5′-AAAAAAAAAAAAAAAAAAAAAAAAA-3′ (nc). Oligonucleotide stock solutions were diluted with sterilized Milli-Q water, separated into fractions, and stored at −20 °C. Single fractions were defrosted when required. Glassy carbon (GC), platinum disc, and Ag/AgCl electrodes were obtained by CHInstruments (Austin, TX). Disposable electrical printed electrodes (DEP-chips, EEPP model) were provided by Biodevice Technology (Nomi, Japan). The three-electrode system comprised a carbon-based working electrode (surface area 2.64 mm^2), a carbon-based counter electrode, and an Ag/AgCl reference electrode.

Equipment

X-ray photoelectron spectroscopy (XPS) was performed by using a Phoibos 100 spectrometer with a monochromatic Mg X-ray source (SPECS, Germany). High-resolution spectra for C1s were recorded. Electrochemical experiments were carried out with an Autolab PGSTAT302 potentiostat (Eco Chemie, Utrecht, The Netherlands; GPES software, version 4.9). CV measurements for the electrochemical reduction of graphene materials were performed in PBS buffer. CV and impedance measurements for graphene material characterization were performed in a 0.01 M PBS buffer containing $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (10 mM; 1:1 molar ratio) as a redox probe. Impedance measurements were recorded between 0.1 MHz and 0.1 Hz at 10 mV sinusoidal voltage amplitude.

Protocols

GO was produced by using the Staudenmaier[5] method, at which H_2SO_4 (17.5 mL; 95–98%) and HNO_3 (9 mL; fuming) were stirred in a round-bottom flask at 0 °C for 15 min. Graphite (1 g) was then added to the mixture under vigorous stirring to obtain a homogeneous dispersion. $KClO_3$ (11 g) was added into the mixture over 15 min to avoid any temperature spike and formation of ClO_2 gas, which is explosive at high concentrations. The mixture was stirred vigorously for 96 h at RT. Upon completion, the mixture was poured into deionized water (1 L) and filtered. GO was then redispersed and washed repeatedly with 5% HCl solution to remove sulfate ions. GO was finally washed with deionized water under centrifugation until the filtrate was of neutral pH. Slurry graphite oxide was then dried in a vacuum oven at 60 °C for 48 h before use.

GO (50 mg) was dispersed in ultrapure water to give a 1.0 mg mL^{-1} colloidal solution and ultrasonicated (150 W) for 3 h. Hydrazine monohydrate (2 mL, 32.1 mmol) was added dropwise, and the reaction mixture was left to stir at reflux for 24 h. The mixture was filtered and washed with methanol and ultrapure water to give CRGO upon drying at 50 °C in a vacuum oven for five days.

CRGO-CN was obtained by adding AIBN (2 g) to a dispersion of CRGO (10 mg) in dry toluene (10 mL). The reaction mixture was stirred vigorously at 75 °C for 4 h under inert atmosphere. The mixture was filtered and washed with toluene. Solid CRGO-CN was dried at 50 °C in a vacuum oven overnight. Subsequently, CRGO-CN (4 mg) was dispersed in methanol (4 mL), and NaOH (10 M; 4 mL) was added. The reaction mixture was stirred at reflux at 60 °C for two days. The mixture was filtered and washed with ultrapure water, acidified with HCl (1 M), and then washed with copious amount of ultrapure water, until a neutral pH was obtained. The solid was dried at 50 °C in a vacuum oven for five days to give CRGO-COOH.

Dispersion (1 mg mL^{-1}) of each graphene material either in deionized water or in DMF was prepared. Dispersion (3 μL of each) was deposited on the working electrode surface (either GC or DEP chip electrode) and left to dry under a lamp. Glassy carbon electrodes were used for material characterization, whilst DEP chips were employed for biosensing purpose.

Immobilization of amino-modified DNA probes was performed by the formation of a covalent bond with carboxyl groups on graphene surface by following the EDC/NHS coupling protocol. EDC (3 μL; 0.05 M) and sulfo-NHS (0.03 M) in PBS buffer (pH 7) were deposited on the electrode surface and incubated for 1 h to activate the carboxyl acid groups. This was followed by gentle washing the electrodes with PBS buffer; after which DNA probes (3 μL) at an optimized concentration in PBS (pH 7) was deposited onto the electrode surface and left overnight at RT in a humidified environment. After which a washing step was performed with SDS (0.05%) and hydroxylamine hydrochloride (0.04 M) to deactivate any remaining carboxyl group and remove all nonspecifically adsorbed DNA probes. DNA probe modified DEP-chips were incubated in the hybridization solution (TSC1 buffer) containing the desired concentration of DNA target (total solution volume 100 μL). The incubation was carried out at 42 °C for 30 min, under gentle stirring.

After that, two brief washing steps were performed in TSC2 buffer at same temperature.

DNA probe surface density was calculated by chronocoulometry following the method reported by Steel et al.[25] Hexaammineruthenium(III) concentration in Tris-HCl buffer used in the experiments was optimized at 100 μM (as was calculated by measuring the binding isotherm of the DNA probe modified platform). The DNA probe density on the electrode surface was calculated by the Cottrell equation considering the extrapolation of the intercept at time zero (Figure 5).

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Keywords: biosensors · charge transfer · DNA · graphene · synthetic methods

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