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Impedimetric immunoglobulin G immunosensor based on chemically modified graphenes†

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Immunosensors which display high sensitivity and selectivity are of utmost importance to the biomedical field. Graphene is a material which has immense potential for the fabrication of immunosensors. For the first time, we evaluate the immunosensing capabilities of various graphene surfaces in this work. We propose a simple and label-free electrochemical impedimetric immunosensor for immunoglobulin G (IgG) based on chemically modified graphene (CMG) surfaces such as graphite oxide, graphene oxide, thermally reduced graphene oxide and electrochemically reduced graphene oxide. Disposable electrochemical printed electrodes were first modified with CMG materials before anti-immunoglobulin G (anti-IgG), which is specific to IgG, was immobilized. The principle of detection lies in the changes in impedance spectra of the redox probe after the attachment of IgG to the immobilized anti-IgG. It was found that thermally reduced graphene oxide has the best performance when compared to the other CMG materials. In addition, the optimal concentration of anti-IgG to be deposited onto the modified electrode surface is $10 \mu\text{g ml}^{-1}$ and the linear range of detection of the immunosensor is from $0.3 \mu\text{g ml}^{-1}$ to $7 \mu\text{g ml}^{-1}$. Finally, the fabricated immunosensor also displays selectivity for IgG.

Introduction

In the last few decades, there has been rising interest in the fabrication of electrochemical immunosensors for the purpose of clinical diagnosis and environmental analysis.^{1–5} An electrochemical immunosensor is a highly sensitive and selective tool for the measurement of immunoreagents based on the specific interactions between antibodies and antigens.

Immunoglobulin G (IgG) is the most abundant type of antibody which is present in all body fluids. Upon binding specifically to anti-immunoglobulin G (anti-IgG), it activates a reaction cascade which serves as a protection against bacterial and viral infections. Based on the level of IgG present in the body, medical practitioners are able to draw conclusions about the functioning of the immune system and hence relate it to infections or autoimmune diseases.

In view of the important functions which IgG performs, tremendous efforts have been put into developing immunosensors for IgG based on several electrochemical methods such as amperometry,^{6–8} differential pulse voltammetry (DPV)⁹ and electrochemical impedance spectroscopy (EIS).^{10,11}

EIS is the study of an electrochemical system in response to an applied alternating signal over a wide range of frequency.^{12,13} EIS is highly responsive to bio-recognition events occurring at the interface of the electrode and the electrolyte. This is because in the presence of a redox probe, when the high molecular weight or electrically charged biomolecule binds to the electrode, the effect on the electron transfer kinetics between the redox probe and the electrode surface is substantial. As such, it is a very useful method for the detection of protein binding processes on the surface of the electrode.^{14,15} In addition, EIS is also less destructive to the biological interactions being studied, as compared to other electrochemical methods such as cyclic voltammetry (CV) and DPV.¹⁶ In view of all the above advantages, EIS has received much attention for immunodetection in the recent years.^{17–19} However, further studies have to be done on immunosensors in order to satisfy the desired requirements of high sensitivity, selectivity and simplicity. Therefore, the search for new materials to achieve sensitive, selective and simple detection is still ongoing.

Graphene, consisting of a single layer of carbon atoms in a closely packed honeycomb two-dimensional lattice, has garnered vast attention from researchers in recent years.²⁰ It is regarded to be a potential material for biosensing²¹ due to its unique properties such as high electron conductivity,²² fast heterogeneous electron transfer rate at the basal plane defects and edges of its sheets in the order of $\sim 0.01 \text{ cm s}^{-1}$, large specific surface area,²³ low cost and simple synthetic routes.²⁴

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On this note, we examine here for the first time the suitability of a variety of chemically modified graphene (CMG) materials (according to Ruoff *et al.*²⁵ CMG consists of graphite oxide and various forms of reduced graphene oxides) for label-free impedimetric immunosensing of IgG. The ultimate goal of this study is to fabricate an immunosensor which is capable of satisfying the desired properties. In addition, we utilize CMG modified disposable electrochemical printed electrodes which show potential for point-of-care analysis.

Experimental section

Materials

Immunoglobulin G (IgG) from rabbit serum, anti-rabbit immunoglobulin G (anti-IgG) produced in goat, albumin from bovine serum (BSA), graphite microparticles (< 20 μm), sodium borohydride, fuming nitric acid (conc. > 90%), sulfuric acid (conc. 95–98%), potassium chlorate, hydrochloric acid (conc. 37%), sodium phosphate dibasic, sodium chloride, Tween® 20, potassium hexacyanoferrate(II) trihydrate, and potassium hexacyanoferrate(III) were purchased from Sigma-Aldrich (Singapore). DEP electrodes (disposable electrochemical printed electrodes) were obtained from BioDevice Technology (Nomi, Japan). Chemically modified graphene (CMG) materials were all prepared with detailed characterization according to our previous report.²⁶

The CMG materials prepared were all characterized by transmission electron microscopy (TEM), Raman spectroscopy and X-ray photoelectron spectroscopy (XPS) and the characterization results were published in our previous study.²⁶ In summary, the D/G Raman signal ratios for graphite oxide (GPO), graphene oxide (GO), thermally reduced graphene oxide (TR-GO) and electrochemically reduced graphene oxide (ER-GO) were 1.12, 1.05, 1.35 and 1.08 respectively. In addition, XPS determined the C : O ratios of GPO, GO, TR-GO and ER-GO to be 1.9, 2.8, 23.3 and 5.1 accordingly.

Apparatus

Electrochemical experiments were performed with an Autolab potentiostat PGSTAT302 (Eco Chemie, Utrecht, The Netherlands) connected to a personal computer and controlled by NOVA software version 1.7.

Impedance measurements were recorded between 0.1 MHz and 0.1 Hz at a sinusoidal voltage perturbation of 10 mV amplitude. The experiments were carried out at room temperature using DEP electrodes and 10 mM $\text{K}_4[\text{Fe}(\text{CN})_6]/\text{K}_3[\text{Fe}(\text{CN})_6]$ (1 : 1 molar ratio) as a redox probe in PBS buffer solution (0.01 M Na_2HPO_4 + 0.135 M NaCl, pH = 7.4). Randles equivalent circuit was used to fit the obtained impedance spectra, represented as Nyquist plots in the complex plane.

Protocol

Graphite oxide (GPO), graphene oxide (GO) and thermally reduced graphene oxide (TR-GO) were immobilized onto each DEP electrode surface by physical absorption. 3 μL of chemically modified graphene (CMG) at a concentration of 1 mg mL^{-1} (ref. 27) in Milli-Q water was deposited on the electrode surface and

left to dry at room temperature overnight. Excess of CMG material that was not adsorbed on the electrode surface was then removed by gentle washing with Milli-Q water. Prior to depositing the CMG materials onto electrode surfaces, graphene oxide and TR-GO were sonicated for 30 minutes in order to ensure maximum uniform dispersion of the materials. To obtain an electrochemically reduced graphene oxide (ER-GO) modified electrode, a graphene oxide modified electrode was reduced at -1.2 V for 900 s in PBS buffer solution (pH = 7.4) at room temperature.

Anti-rabbit immunoglobulin G (anti-IgG) was next immobilized onto the modified electrode surfaces by dry physical absorption. 3 μL of anti-IgG in PBS-B buffer solution (1% BSA + PBS, pH = 7.4) at a concentration of 100 $\mu\text{g mL}^{-1}$ was deposited onto the modified electrode surface and placed under the lamp for drying for 30 minutes. The modified electrode surface which was not immobilized with anti-IgG was blocked with BSA in the same step. After which, the electrode was washed gently with PBS-T buffer solution (0.05% Tween® 20 + PBS, pH = 7.4), PBS buffer solution and finally ultrapure water to remove the excess anti-IgG and BSA which were not well adsorbed on the modified electrode surface.

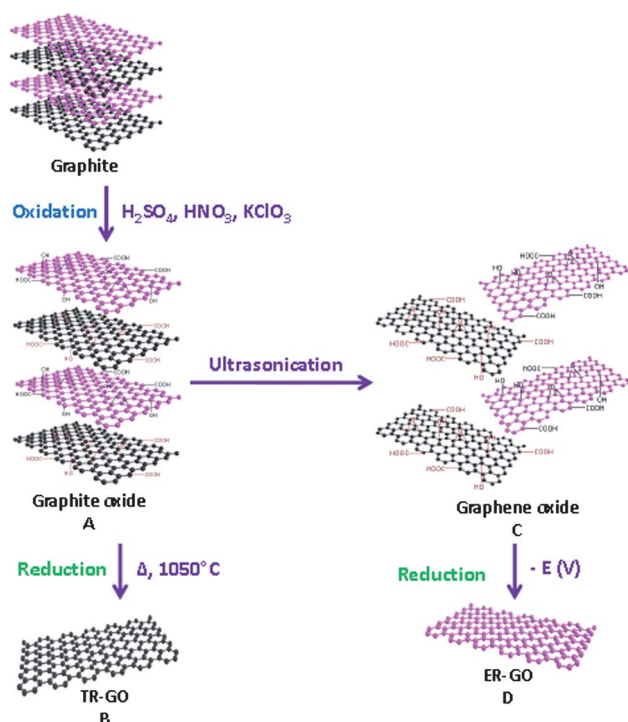
DEP electrodes modified with anti-IgG and BSA were then incubated in Eppendorf tubes containing PBS-T buffer solution with the desired concentrations of protein target (total volume: 100 μL). The incubation was performed at 37 $^{\circ}\text{C}$ for 60 minutes with gentle stirring. Finally, washings with PBS-T buffer solution, PBS buffer solution and ultrapure water were performed to remove the excess of non-specifically adsorbed IgG. For a selectivity study, negative control was performed using the above sensing protocol with BSA protein.

Results and discussion

We investigated the performance of various chemically modified graphene (CMG) platforms for the impedimetric immunosensing of immunoglobulin G (IgG) in this study. Following that, the material which exhibits the best performance compared to the others was employed for further studies.

CMG materials such as graphite oxide (GPO), graphene oxide (GO), thermally reduced graphene oxide (TR-GO) and electrochemically reduced graphene oxide (ER-GO) were utilized in this work. Scheme 1 demonstrates the methods of preparation for various CMGs.

The analytical protocol illustrated in Scheme 2 was employed in this study. In summary, the DEP electrode which was modified with CMG material shows comparatively low impedance as it is totally accessible to the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ redox probe (see Fig. S1 in the ESI†). Subsequently, anti-rabbit immunoglobulin G (anti-IgG) was deposited onto the surface and BSA was also used to block the remaining modified surface in the same step. With this, the impedance increases due to the decrease in accessibility. Lastly, the modified DEP electrode was incubated with IgG which is the specific target of the immobilized anti-IgG. Hence, it binds specifically to anti-IgG and this results in a further increase in impedance due to either the additional steric hindrance caused by the bulky IgG protein molecules or the electrostatic interaction between IgG protein molecules and $[\text{Fe}(\text{CN})_6]^{3-/4-}$ redox



Scheme 1 An illustration of the preparation of chemically modified graphene (CMG) materials using graphite as the starting material. (A) Graphite oxide (GPO) was first generated by oxidation from graphite. This was followed with: (B) thermal reduction/exfoliation of graphite oxide to generate thermally reduced graphene oxide (TR-GO); (C) ultrasonication/exfoliation to produce graphene oxide (GO); and (D) electrochemical reduction of graphene oxide (GO) to yield electrochemically reduced graphene oxide (ER-GO).

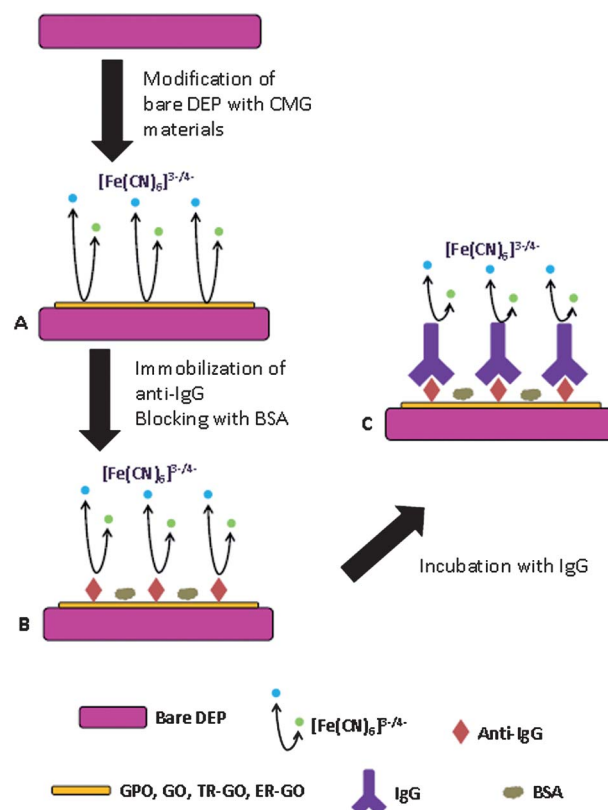
probe.^{28,29} Impedance spectra were measured after each modification stage for all four CMG materials.

For all of the four CMGs, the charge transfer resistance (R_{ct}) which relates to the diameter of the semi-circle of the impedance curve increases with immobilization of anti-IgG and BSA. R_{ct} then further increases with the incubation with IgG target.

The impedance spectra obtained were next fitted and results were represented in the form of R_{ct} ratio ($R_{\text{ct}}^{\text{IgG}} - R_{\text{ct}}^{\text{blank}} / R_{\text{ct}}^{\text{anti-IgG}} - R_{\text{ct}}^{\text{blank}}$), as depicted in Fig. 1. From Fig. 1, it can be inferred that the TR-GO surface exhibits the largest impedimetric signal variation (greatest R_{ct} ratio value) when compared to the other CMG materials. This proposes that the TR-GO platform is the most sensitive surface for impedimetric immunosensing of IgG and hence, it was the selected material to be used for further experimental studies.

Anti-IgG optimization was then performed to establish the optimum concentration of anti-IgG to be deposited onto the TR-GO modified electrode. Impedance spectra measured in the experiment were once again analyzed and illustrated in a histogram as seen from Fig. 2.

It can be observed from Fig. 2 that the impedimetric signal is the greatest when $10 \mu\text{g ml}^{-1}$ of anti-IgG was deposited onto the TR-GO modified electrode surface. As such, it can be determined that the optimum concentration of anti-IgG to be immobilized onto the modified electrode surface for maximum surface



Scheme 2 An illustration of the protocol for IgG immunodetection based on the EIS method. (A) A bare DEP electrode modified with CMG material; (B) a CMG material modified DEP electrode after the immobilization of anti-IgG and blocking with BSA; and (C) an anti-IgG and BSA modified DEP electrode after the incubation with IgG.

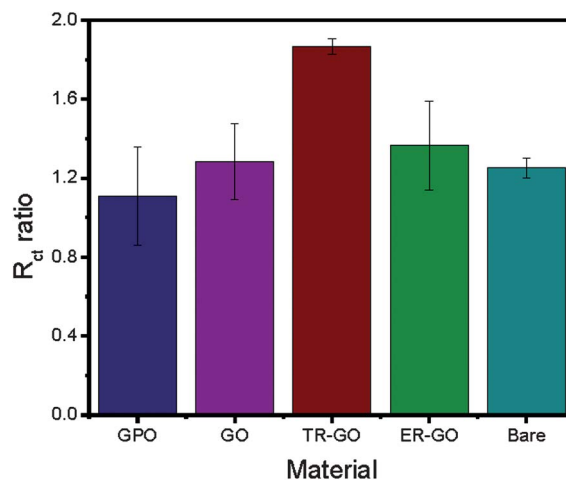


Fig. 1 Histogram representing a comparison of impedimetric signals on GPO, GO, TR-GO, ER-GO and bare DEP electrodes. Signal is represented as average R_{ct} ratio ($R_{\text{ct}}^{\text{IgG}} - R_{\text{ct}}^{\text{blank}} / R_{\text{ct}}^{\text{anti-IgG}} - R_{\text{ct}}^{\text{blank}}$). Error bars correspond to triplicate experiments. Concentration of anti-IgG used is $100 \mu\text{g ml}^{-1}$; concentration of the IgG target is $10 \mu\text{g ml}^{-1}$. All measurements were performed with $10 \text{ mM K}_4[\text{Fe}(\text{CN})_6]/\text{K}_3[\text{Fe}(\text{CN})_6]$ in PBS buffer solution (pH = 7.4) at room temperature.

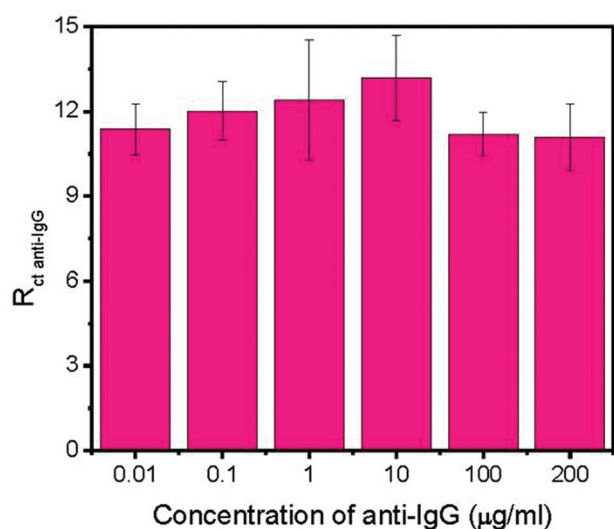


Fig. 2 Impedimetric response towards the concentration of anti-IgG deposited on the TR-GO modified DEP electrode surface. Signal is represented as $R_{ct} \text{ anti-IgG}$. Error bars correspond to triplicate experiments. All measurements were performed with 10 mM $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$ in PBS buffer solution (pH = 7.4) at room temperature.

coverage is $10 \mu\text{g ml}^{-1}$. Therefore, $10 \mu\text{g ml}^{-1}$ of anti-IgG was adopted for successive experiments.

In order to ascertain the detection range on the TR-GO platform, impedimetric response towards various IgG concentrations was examined. For this, the concentration of anti-IgG was kept unchanged at the optimal concentration of $10 \mu\text{g ml}^{-1}$ while the impedimetric signal was recorded for a series of IgG concentrations.

Fig. 3 shows that increasing concentration of IgG has led to a higher $R_{ct} \text{ IgG}/R_{ct} \text{ anti-IgG}$ value. This implies that increasing concentrations of IgG result in a higher degree of analytical signal variation. In addition, it was also observed that the linear range of detection is from $0.3 \mu\text{g ml}^{-1}$ to $7 \mu\text{g ml}^{-1}$.

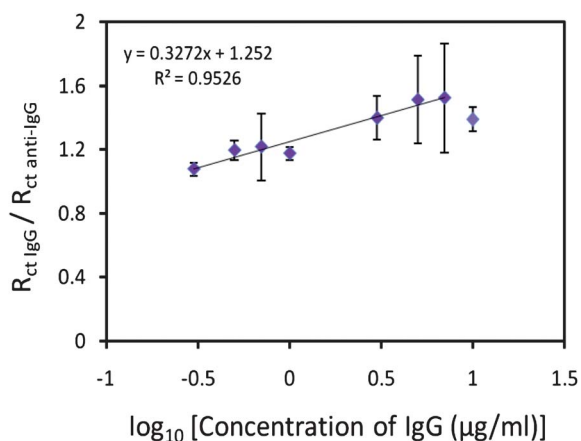


Fig. 3 Calibration plot corresponding to the changes in the charge transfer resistance (R_{ct}) after the incubation with different concentrations of IgG. Signal is represented as $R_{ct} \text{ IgG}/R_{ct} \text{ anti-IgG}$. Error bars correspond to triplicate experiments. All measurements were performed with 10 mM $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$ in PBS buffer solution (pH = 7.4) at room temperature.

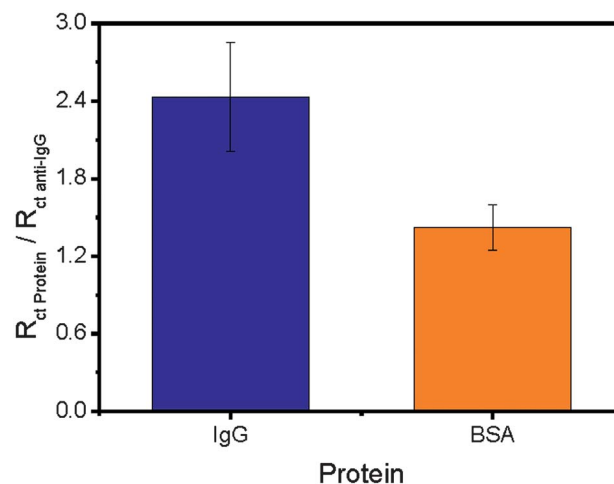


Fig. 4 Comparison of impedimetric response after incubation with a different protein target. BSA was used as negative control. Concentration of IgG and BSA used was kept constant at $5 \mu\text{g ml}^{-1}$. Signal is represented as $R_{ct} \text{ protein}/R_{ct} \text{ anti-IgG}$. Error bars correspond to triplicate experiments. All measurements were performed with 10 mM $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$ in PBS buffer solution (pH = 7.4) at room temperature.

We compared our findings with some references and found out that the range of detection obtained by adopting DPV⁹ was reported to be from 0.2 ng ml^{-1} to 320 ng ml^{-1} . This range is wider than the range obtained by our proposed method. However, it should be noted that our adopted protocol is label-free and very straightforward. Furthermore, with our acquired range of detection, the immunosensor has the potential to detect IgG in human samples as IgG is present in human serum and plasma samples in the range of 4 to 16 mg ml^{-1} .

To exemplify the selectivity of the immunosensor, TR-GO modified electrodes after the immobilization of anti-IgG were incubated with BSA protein as negative control.

As depicted in Fig. 4, it can be observed that the $R_{ct} \text{ protein}/R_{ct} \text{ anti-IgG}$ value for BSA is much smaller than that for IgG. This signifies that BSA does not interact with anti-IgG and the interference of BSA in the immunodetection of IgG is negligible. Therefore, the impedimetric immunosensor based on the TR-GO surface can be deduced to be selective towards IgG as compared to BSA.

The reproducibility of the immunosensor was evaluated by performing three replicates for every experiment conducted. An average relative standard deviation value of 11.4% was achieved. Hence, this indicates good electrode-to-electrode reproducibility by the fabrication protocol proposed above.

Conclusions

In conclusion, the performance of the various chemically modified graphene platforms, specifically graphite oxide, graphene oxide, thermally reduced graphene oxide and electrochemically reduced graphene oxide, for impedimetric immunodetection of IgG has been examined for the first time in our study. We established that thermally reduced graphene oxide is the most responsive platform for the detection of IgG based on the

protocol employed. In addition, it was also found that the optimal concentration of anti-IgG to be deposited on the modified electrode surface is $10\ \mu\text{g ml}^{-1}$. The range of detection of the immunosensor is also determined to be from $0.3\ \mu\text{g ml}^{-1}$ to $7\ \mu\text{g ml}^{-1}$. Finally, it was verified that the immunosensor is highly selective for IgG as compared to BSA. We wish to underline here the necessity to evaluate different graphene platforms in biosensing research as they exhibit different material properties and consequently different biosensing properties.

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