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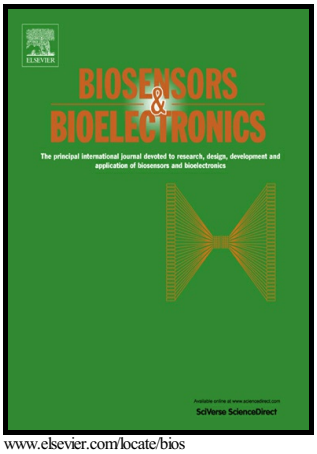

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Electroactive and biocompatible functionalization of graphene for the development of biosensing platforms

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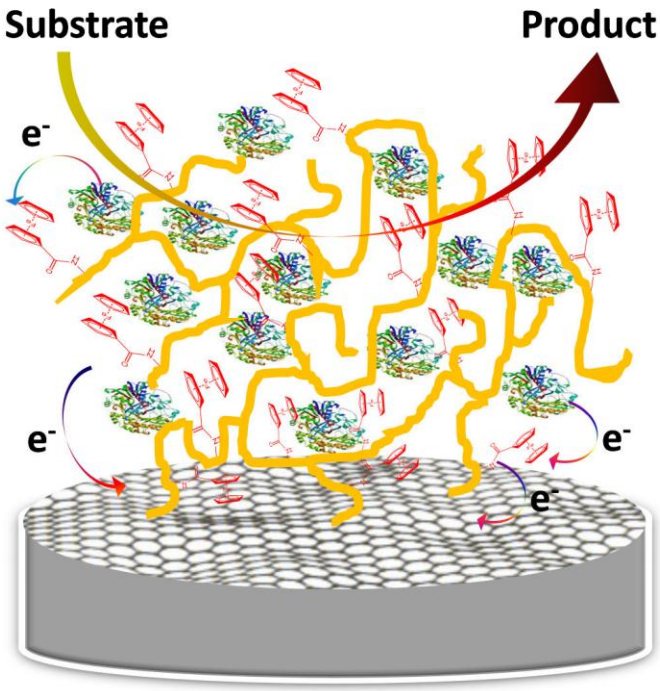
Abstract

Design and synthesis of low-cost, highly stable, electroactive and biocompatible material is one of the key steps for the advancement of electrochemical biosensing systems. To this end, we have explored a facile way for the successful synthesis of redox active and bioengineering of reduced graphene oxide (RGO) for the development of versatile biosensing platforms. A highly branched polymer (PEI) is used for reduction and simultaneous derivation of graphene oxide (GO) to form a biocompatible polymeric matrix on RGO nanosheet. Ferrocene redox moieties are then wired onto RGO nanosheets through the polymer matrix. The as-prepared functional composite is electrochemically active and enables to accommodate enzymes stably. For proof-of-concept studies, two crucial redox enzymes for biosensors (i.e. cholesterol oxidase and glucose oxidase) are targeted. The enzyme integrated and RGO supported biosensing hybrid systems show high stability, excellent selectivity, good reproducibility and fast sensing response. As measured, the detection limit of the biosensors for glucose and cholesterol is 5 µM and 0.5 µM (S/N=3), respectively. The linear response range is from 0.1 to 15.5 mM for glucose and from 2.5 to 25 µM for cholesterol. Furthermore, this biosensing platform shows good anti-interference ability and reasonable stability. The nanohybrid biosensing materials can be combined with screen-printed electrodes, which are successfully used for measuring the glucose and cholesterol level of real human serum samples.

Graphical abstract



Keywords: *Reduced graphene oxide, electroactive functionalization, electrochemical biosensor, cholesterol oxidase, glucose oxidase, human serum sample*

1. Introduction

In the 21st century, highly modernized society and our fast-pace lifestyle have increased the probability of suffering different so-called 'lifestyle diseases', such as type-2 diabetes, obesity and various cardiovascular problems (Health and Information, 2013). Therefore, regular-basis monitoring and control of these diseases are of high priority in our daily life. For these reasons, biosensors are becoming an essential part of our everyday life (Turner, 2013). Determination of clinically important human metabolites in whole blood is the most important for early stage disease diagnostics. An imbalance of these analytes in blood can cause serious illness and life threatening diseases. Among various important analytes in blood, glucose and cholesterol are considered as the most crucial (Zhang et al., 2007). The abnormality in the blood glucose level is the main cause of global epidemic diabetes and nearly 80 million people are suffering for this in today's world (Herman and Zimmet, 2012). On the other side, irregularity in blood cholesterol level is closely associated with a high risk of cardiovascular disease, which can cause coronary heart disease, stroke, and peripheral vascular disease (Goldstein and Brown, 2001). High level of cholesterol is also linked to diabetes and high blood pressure. For all these reasons, diagnosis

and management of these analytes in blood require highly sensitive, affordable and reliable biosensors.

With this high demand, the biosensor technology is going through a rapid development phase in the last few decades. According to WHO (World Health Organization) guidelines, practical biosensing devices should be simple, affordable, user-friendly, sensitive and specific to a particular analyte (Peeling et al., 2006). Recent advances in nanotechnology and materials chemistry could offer new promises for this purpose (Sekretaryova et al., 2015). Several factors are very crucial for the design of an ideal biosensing platform, including (1) electroactive and biocompatible support material, (2) stable immobilization of selected biological recognition elements on support with intact activity, and (3) efficient electronic communication between the redox active area of biomolecules and the electrode surface (Walcarius et al., 2013). A number of redox enzymes associated with nanostructured materials have been extensively studied to design various biosensors or biofuel cell elements (Willner et al., 2007).

Owing to their extraordinary physicochemical properties (Kostarelos and Novoselov, 2014; Liao et al., 2014), graphene based nanomaterials could offer a unique solution for the fabrication of biosensors. Although pristine graphene is chemically inert and electrochemically inactive, tunable functionalization and reduction of graphene oxide (GO) is very promising for development of a wide range of biosensors (Halder et al., 2016). Solution processed (or wet-chemical) synthesis is a very promising approach for low-cost production and facile functionalization of graphene based materials for the development of biosensing materials. In the last decades, several approaches have been used for the development of biosensors (Walcarius et al., 2013). Among them redox mediator based biosensors have received huge interest due to their low redox potential, high stability, solubility and efficient electron transport properties (Chaubey and Malhotra, 2002). Ferrocene is one of the mostly used electron-transfer mediators for the fabrication of electrochemical sensors due to its facile physicochemical properties (Rabti et al., 2016b). Ferrocene mediator based biosensor was introduced for the first time by the Turner's group (D'Costa et al., 1986). Among others, the Schmidtke group has used ferrocene functionalized linear polymers for glucose and H_2O_2 biosensors (Merchant et al., 2009, 2010). The Niu group used ferrocene functionalized

graphene for H_2O_2 biosensors (Fan et al., 2012). Heller and co-workers used the osmium based redox complexes for the fabrication of commercial glucose biosensor (Heller and Feldman, 2008). Dey et al used ferrocene functionalized GO architecture for the development of versatile biosensing platforms (Dey and Raj, 2013). More recently, Merkoci and co-workers have used ferrocene functionalized graphene for the development of glucose biosensor (Rabti et al., 2016a). Although several groups have already attempted to develop ferrocene-modified graphene materials for biosensing applications, most reports lack to provide a specific biocompatible matrix for environmentally-friendly accommodation of particular bio-recognition elements such as enzymes.

Herein we describe a simple way for synthesis of the redox active functionalization of polymer linked RGO as well as the development of biocompatible matrix for accommodation of different bio-recognition elements. Ferrocene redox moieties were anchored onto reduced graphene oxide (RGO) nanosheets via a branched polymer (PEI). The polymer acts as both a reducing agent and a molecular spacer for RGO. The functionalized RGO is electrochemically active and can offer a biocompatible microenvironment for immobilization of different enzymes (e.g. cholesterol oxidase and glucose oxidase). The as-constructed electroactive matrix was further used for the development of integrated biosensing platforms for cholesterol and glucose sensing. The biosensing process is dependent on bioelectrocatalytic oxidation of bio-analytes through redox functionalized graphene-enzyme nanohybrid architecture. This nano-bio-hybrid material was further combined with an integrated screen-printed electrode and used for the glucose and cholesterol level measurements of real human serum samples.

2. Materials and methods

2.1. Reagents and materials

Graphite flakes (＜20 μm), D-(+)-glucose (≥ 99%), N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC), 1-hydroxy benzotriazole (HOBt), Ferrocene carboxylic acid (≥97.0% (Fe)), poly (ethyleneimine) solution (50% (w/v) in water, M_w = 750,000), glucose oxidase (from Aspergillus Niger, 100,000-250,000 units/g solid), Cholesterol oxidase from Streptomyces sp. (lyophilized powder, ≥20 units/mg) were purchased from Sigma-Aldrich. K_2HPO_4 and KH_2PO_4

were purchased from Fluka. 10 mM phosphate buffer solutions (PBS) was used as supporting electrolyte and the pH value was adjusted to 7.0 with K_2HPO_4 and KH_2PO_4. All chemicals were used as received. All solutions were prepared with Milli-Q water (18.2 MΩ).

2.2. Synthesis of GO and Fc-RGOP

Graphene oxide (GO) was synthesized according to our previously reported method (Zhang et al., 2016) and utilized as a starting material for this work. Graphene oxide (GO) was prepared in two steps by modified Hummer's method with graphite as a starting material. In the first step, pre-oxidized graphite was prepared. During synthesis, graphite powder (5.0 g) was slowly added into concentrated H_2SO_4 solution (7.5 ml) containing P_2O_5 (2.5 g) and $K_2S_2O_8$ (2.5 g) put in a hot water bath (80°C) under strong stirring for 3 h. After cooling down to room temperature and mixing with Milli-Q water, the dark green mixture was filtered and washed several times with Milli-Q water until the waste solution pH going to be neutral. Pre-oxidized graphite powder was then dried in air at room temperature for overnight. In the second step, pre-oxidized graphite powder (1.0 g) was gently added to conc. H_2SO_4 solution (23 ml) placed in an ice bath (0°C). $KMnO_4$ (3.0 g) was then put into the mixture with slow stirring and maintaining the whole procedure below 20 °C. After removing the ice bath, the mixture was reacted at 35 °C for 2 h with stirring, and further Milli-Q water (46 ml) was added. After a few minutes, more Milli-Q water (137.5 ml) and 2.5 ml 30% H_2O_2 solution were added to the reaction mixture. The solution color rapidly changed to bright yellow. The mixture was then washed carefully with 1:10 HCl solution (v/v 250 ml). After that, the mixture was filtered to remove residual metal ions. The raw GO dispersion in Milli-Q water was centrifuged at a high rotation speed (12000 rpm min^{-1}). The supernatant was collected, which contained highly dispersed and stable GO nanosheets. To remove residual salts and acids, the GO supernatant was dialyzed using a dialysis tube (with a cut-off molecular weight of 12000-14000) for at least even days by changing water bath regularly (2-3 times per day).

The electroactive functionalization of graphene was done in a two-step procedure (Figure 1). In the first step, GO was subjected to react with the highly branched polymer, polyethyleneimine (PEI) at 95°C for 1 hour. The polymer assisted the reduction of GO to RGO, and furthermore it was attached onto the plane surface of RGO nanosheets. The as-synthesized polymer

functionalized RGO (RGOP) can provide a biocompatible environment for accommodation of different enzymes, and the free $–NH_2$ groups PEI matrix can help for further coupling reaction with ferrocene carboxylic acid. In the second step, ferrocene moieties were attached onto the polymer functionalized RGO (RGOP) through a simple coupling reaction. The coupling reaction was done in DMF using 1-Ethyl-3-(3- dimethylaminopropyl) carbodiimide (EDC) and 1-hydroxy benzotriazole (HOBt) as coupling agents with ferrocene carboxylic acid (FCA) (Albericio, 2004; Han and Kim, 2004; Hayashi et al., 2001). The mixture was incubated at room temperature for 12 hours. The carboxylic groups of FCA were activated by EDC and HOBt and participated in further coupling reaction with RGOP. In order to reveal their structure and physicochemical properties, the as-synthesized redox functionalized and polymer reduced graphene (Fc-RGOP) was systematically characterized by FT-IR, UV- vis, XPS, XRD, FEG-SEM and electrochemistry.

2.3. Material characterization

UV-vis spectra were recorded using a single-beam spectrophotometer (HP8453, Hewlett Packard). Atomic force microscope (AFM) imaging was performed in the tapping mode using a 5500AFM system (Agilent Technologies, Chandler, USA). X-ray photoelectron spectroscopy (XPS) analysis was carried out by an ESCALABMKII X-ray photoelectron spectrometer. Fourier transform infrared spectra (FTIR) were recorded by a Perkin Elmer Spectrum. Scanning electron microscope (SEM) and energy-dispersive X-ray spectroscopy (EDS) mapping images were obtained by Quanta FEG 200 ESEM (with ETD and BSE detector) from FEI Company (Oregon, USA).

All electrochemical measurements were performed at room temperature (23 ± 2 ° C) using an Autolab System (Eco Chemie, Netherlands) controlled by the NOVA 1.10 software or by CHI 760C electrochemical workstation equipped with a Faradaic cage. All the measurements (otherwise mentioned) were performed in 10 mM phosphate buffer solution (PBS) under neutral condition (pH 7) with a three-electrode system consisting of the glassy carbon electrode (GCE) (diameter = 0.5 cm) as working electrode, saturated calomel electrode (SCE) as reference electrode, a platinum coiled wire with a large surface area as counter electrode. The GCE was first polished to mirror finish with three different sizes of alumina slurry paste (0.05, 0.3 and 1 μm) and washed repeatedly with Milli-Q water followed by sonication in Milli-Q water for 20

min. The SCE reference electrode was washed with Milli-Q water prior to measurement and it was stored in saturated KCl solution when not in use. The platinum coiled as counter electrode was cleaned with hydrogen flame followed by washing with Milli-Q water repeatedly. Screen printed electrode (SPE) (Dropsens, Spain) was also used as a conducting substrate for the development of biosensing platform. The SPE contains a carbon working (0.4 cm in diameter), platinum counter and silver reference electrode. A DRP-DSC connector (Dropsens, Spain) was used to connect the SPE to the potentiostat.

2.4. Biosensor fabrication

Glassy carbon electrode (GCE) or/and screen-printed electrode (SPE) were used as the support electrodes for the fabrication of biosensing platforms. As usual, the GCE was first polished with alumina slurry and cleaned with repetitive washing in Milli-Q water and sonication. After drying, 5 mg of Fc-RGOP material was dispersed in 1 ml 0.5 % ethanolic Nafion solution by sonication. The suspension (10 µl) was drop casted on the surface of clean electrodes and dried at room temperature for 2 h. For the fabrication of glucose biosensor, 10 µl of the glucose oxidase (GOx) solution (10 mg/ml) in a phosphate buffer (PBS, 10 mM, pH 7.0) was drop casted on the Fc-RGOP electrode surface. For the fabrication of cholesterol biosensor, 10 µl of the cholesterol oxidase (ChOx) solution (10 mg/ml) was used. The electrodes were further dried at 4°C for overnight. Before electrochemical biosensing measurements, the electrodes were repeatedly washed with PBS solutions to remove the loosely adsorbed enzymes (Parlak et al., 2013).

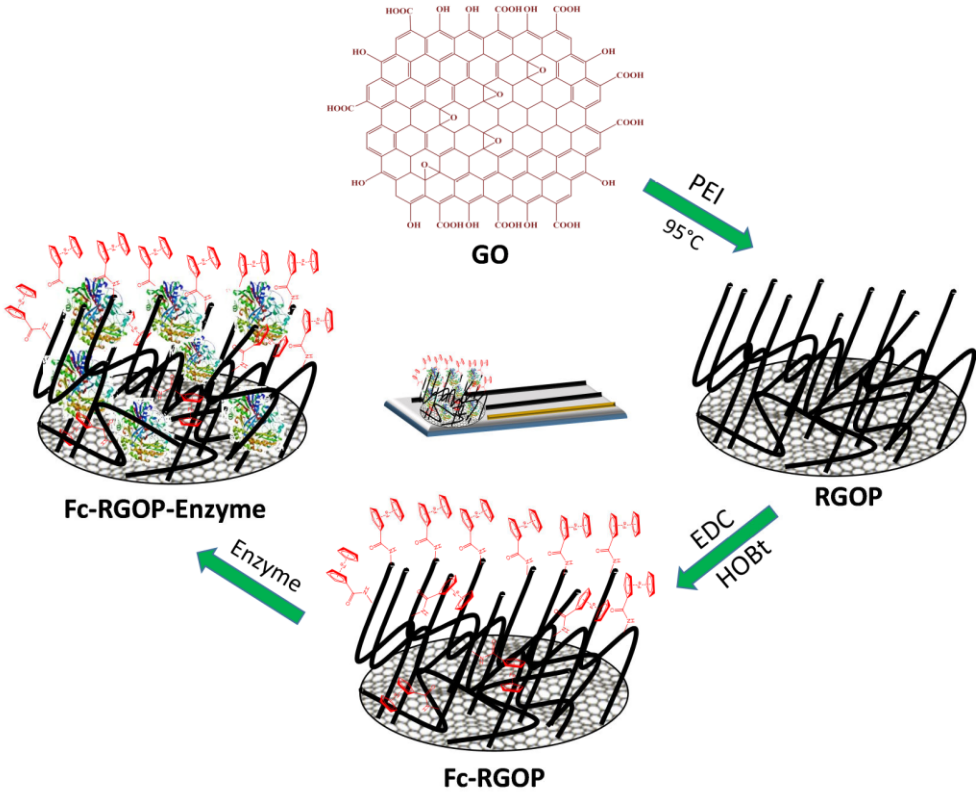


Figure 1. Schematic illustration of the procedure for the preparation of Fc-RGOP nano-bio hybrid materials including GO reduction, electroactive functionalization and enzyme loading. Not drawn to scale.

3. Results and discussion

3.1. Synthesis of Fc-RGOP nanohybrid material

Tunable derivation can enrich graphene with new physicochemical properties and functionalities. Figure 1 represents the procedure for reduction and electrochemically active functionalization of graphene as well as enzyme loading. High-quality GO was synthesized and used as a starting material for the synthesis of Fc-RGO nanohybrids. The AFM topography imaging of GO clearly shows that the average thickness of single-layer GO nanosheets is 0.8-1.2 nm and the lateral diameters are in the size range of 1-2.5 µm (Supporting Figure S1). In the first step, the highly branched polymer PEI was used to reduce the GO and it further acted as a functional linker for the redox active functionalization. With the progress of reduction reaction, the brown colour of GO dispersion changed to dark grey or black as a clear indication for the reduction of GO. The as-synthesized polymer functionalized RGO can be well dispersed in water and is stable for a long period of more than one year, while pure RGO suspension is only stable

for ca. 2-3 days. In the second step, polymer reduced graphene was further coupled with ferrocene redox unit. The main driving force for the formation of covalent bonding between the polymer reduced RGO and FCA is the rich availability of free amino groups in PEI and free carboxylic acid groups in FCA (van Staveren and Metzler-Nolte, 2004). EDC and HOBt were used as coupling reagents in order to activate carboxylic acid groups in FCA. This functionalization of graphene with low redox potential based electrochemical redox mediator (FCA) can be utilized as versatile biosensing platforms (Rabti et al., 2016b). In the final step, different enzymes were loaded onto this matrix for further biosensing tests.

3.2. Material characterization of GO and Fc-RGOP

Structural characterization of the synthesized hybrid nanomaterials was systematically performed with the key results highlighted in Figure 2. The UV-vis spectra of both GO and Fc-RGOP clearly indicate the reduction of GO upon its reaction with polymer and FCA (Figure 2a). In detail, the UV-vis spectrum of GO shows a typical absorption band at 232 nm due to π-π* transitions of C=O bond and a shoulder peak at around 302 nm due to n- π* transitions of C=O bonds. After reduction by polymer and redox functionalization, a new band emerges at 270 nm for Fc-RGP, which is close to the typical absorption band of pure RGO (260 nm) and a clear evidence for reduction of GO (Figure 2a). After reduction, the π-conjugated network in the graphitic planes was largely restored.

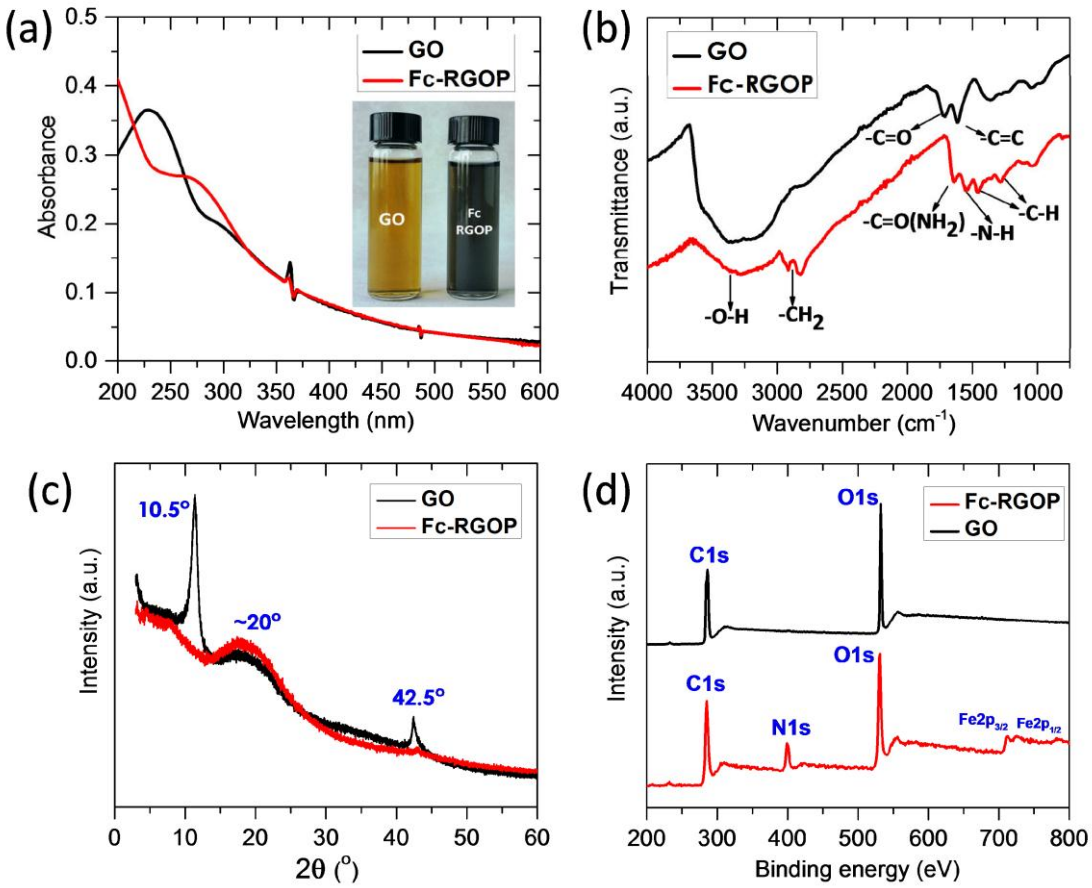


Figure 2. Structural characterization of hybrid materials (Fc-RGOP) by a variety of spectroscopic techniques: (a) UV-vis spectra (the inset is the photographs of two suspension solutions), (b) FT-IR spectra, (c) XRD profile, and (d) XPS profiles. Pure GO sample was used as a reference system, in order to reveal the changes in structures upon functionalization.

As shown in Figure 2b, the FT-IR spectrum of GO shows typical characteristic stretching frequencies for oxygen containing functional groups such as C=O (1720 cm^{-1}), O-H (3400 cm^{-1}), alkoxy C-O (1050 cm^{-1}), and epoxy C–O (1225 cm^{-1}) (Zhu et al., 2013). After reduction and functionalization with FCA, the stretching frequency (1723 cm^{-1}) corresponding to C=O is shifted to 1647 cm^{-1} due to the formation of amide bonds. Two new peaks are observed at 1115 and 1545 cm^{-1} due to the C-N stretching and N-H bending, respectively. In addition, the peaks at around 1281 and 1463 cm^{-1} corresponding to the C- H stretching and bending become profound after GO was reduced to RGO. The new peaks at around 2800 cm^{-1} from Fc-RGOP are attributed to the symmetric and asymmetric stretching of CH_2 groups from PEI polymer chains.

Therefore, the FT-IR spectra can provide rather detailed information about the chemical bonding formation of Fc-RGOP.

As expected, the XRD profile of GO samples shows a sharp peak at 2θ = 10.5° due to the reflection from (002) plane (Figure 2c). This sharp peak completely disappears after functionalization. Moreover, the appearance of a new broad peak for Fc-RGOP at around 2θ = 20° after the formation of Fc-RGOP is a strong indication of the reduction of GO (Figure 2c). The another peak for GO at 2θ = 42.5° arising from the turbostratic band of disordered carbon materials (Chen et al., 2011) significantly reduces upon functionalization.

XPS was further used to estimate the efficiency of reduction and functionalization on the surface of GO with FCA (Figure 2d). GO and Fc-RGOP show both peaks of C 1s and O 1s, but the C/O ratio in Fc-RGOP decreases notably due to the conversion of GO to RGO, which clearly indicates that the amount of oxygen containing functional groups in Fc- RGOP is significantly reduced. Furthermore, the N 1s peak at 398.6 eV is observed only in Fc-RGOP samples, due to the presence of rich amine groups containing polymer PEI on RGO surface. In addition, Fc-RGOP shows two other important characteristic peaks at 709.7 eV and 724.2 eV corresponding to Fe $2p_{3/2}$ and Fe $2p_{1/2}$ (Supporting Figure S2c). The appearance of peaks for N and Fe clearly confirms that the GO was successfully reduced and functionalized by PEI and FCA (Dey and Raj, 2013). The de-convoluted C 1s XPS spectra of Fc-RGOP show three peaks located at 284.6, 285.9 and 287.3 eV, respectively, corresponding to C=C/C-C, C-O/C-N and N-C=O functionalities (Supporting Figure S2a). The N 1s spectrum of Fc-RGOP can be de-convoluted into the two peaks with the binding energy at 399.4 eV for –NH– and 401.28 eV for free $–NH_2$, respectively (Figure S2b). These observations indicate that the PEI linker molecules were successfully grafted on the RGO nanosheets.

Scanning electron microscopy (SEM) analysis and elemental mapping were performed directly on the Fc-RGOP coated screen printed electrodes. The synthesized material was drop casted on screen-printed electrode surface and dried before SEM imaging. As shown in Figure 3a, the SEM image shows a relatively rough film with large surface area. The uniform distribution of the key elements including C, N, O and Fe is clearly evidenced by the corresponding elemental mapping images (Figure 3b-e), which further provided a clear evidence for the attachment of ferrocene

moiety on the polymer reduced graphene surface.

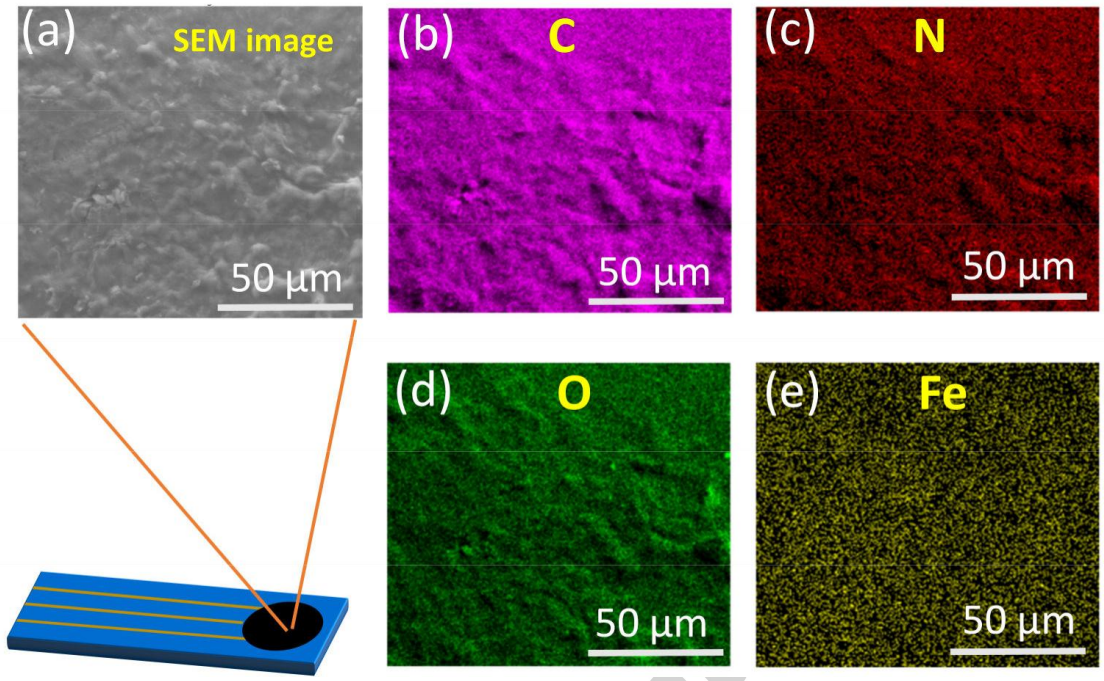


Figure 3. (a) FESEM image of Fc-RGOP drop-casted on screen-printed electrode surface and (b-e) corresponding elemental mapping images of C, N, O and Fe.

3.3. Electrochemical behavior of Fc-RGOP

The electrochemical behavior of functionalized Fc-RGOP material was evaluated by cyclic voltammetry. Figure 4a shows the cyclic voltammetric responses of Fc-RGOP in PBS (10 mM, pH 7.0) electrolyte with various scan rates applied. These voltammograms (CVs) show clearly well-defined and reversible redox peaks with the apparent formal potential of 0.35 V (vs SCE), which is invoked by the ferrocene moiety. The peak current linearly increases with increasing the scan rate, indicating that the redox active material acts as a surface-confined species (Figure 4b). Moreover, the voltammetric response of the Fc-RGOP remains unchanged after hundreds of successive cyclic voltammetric scans between the potential window of 0.0 to 0.55 V. This is consistent with the fact that Fc moiety is covalently and stably attached to the polymer matrix as evidenced by the IR analysis (Figure 2b). Overall, the results show that the covalently attached Fc moieties can reversibly communicate with the supporting electrode for fast electron transfer.

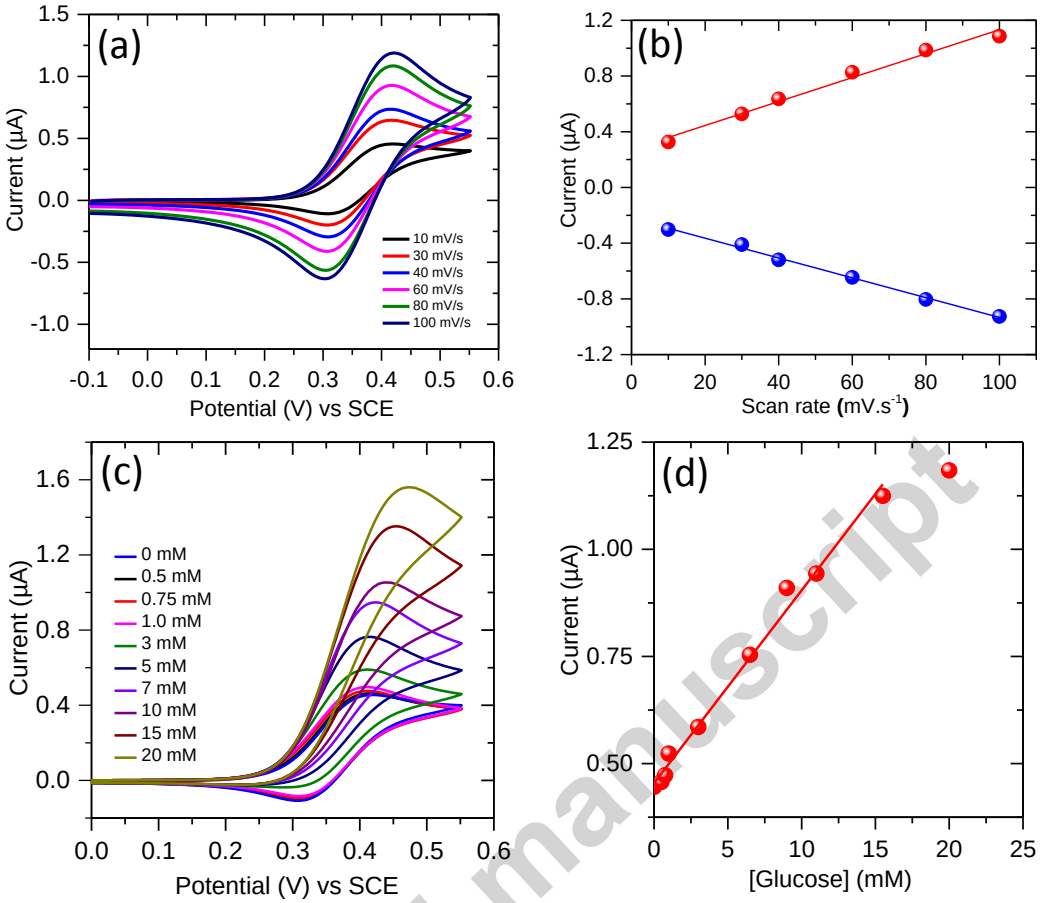


Figure 4. (a) Cyclic voltammograms of Fc-RGOP/SPE obtained with different scan rates in 10 mM PBS (pH 7.0). The scan rates from inner to outer are 10 to 100 mV/s), and (b) the linear relationship between the redox peak current and the scan rate. Electrocatalytic oxidation of glucose at Fc-RGOP-GOx/SPE: (c) Cyclic voltammograms obtained with various concentrations of glucose (0 mM - 20 mM) in pH 7.0 PBS, scan rate 10 mV s^{-1}; and (d) a corresponding relation between the glucose concentration and electrocatalytic current.

3.4. Electrochemical biosensing of glucose and cholesterol

With the systematic structural characterization described above, it is confirmed that electron transfer (ET) mediator, Fc moiety is covalently bond to the branched PEI which is covalently attached to RGO nanosheet. Such an Fc-RGOP nanostructured material offers a biocompatible microenvironment for electrostatic attraction of redox enzymes into the polymeric matrix via positively charged amine groups to form a biosensing system. In order to test electrochemical biosensing performances, we have deposited enzyme-containing hybrid materials on either glassy carbon electrodes (GCE) or screen-printed electrodes (SPE). A schematic illustration of the electrode structure, ET pathway and bioelectrocatalysis is depicted in Figure 5.

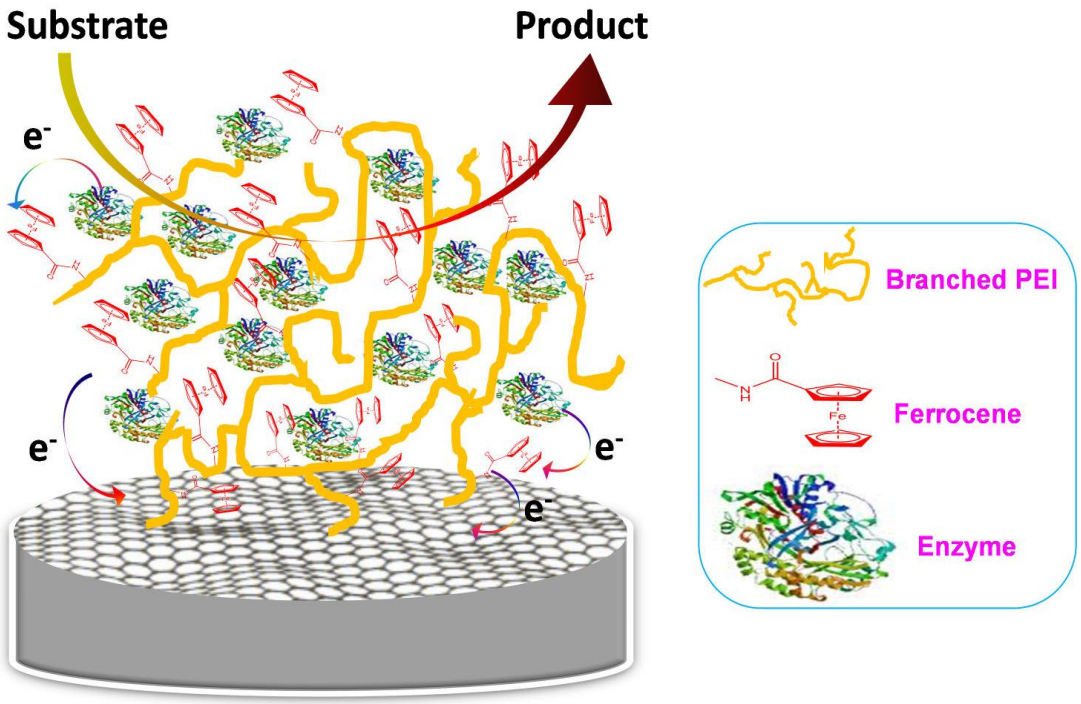


Figure 5. Schematic illustration of structure, electron transfer and bioelectrocatalysis of Fc-RGOP-Enzyme systems. Not drawn to scale.

The major goal of our research line is to develop a general biosensing platform for the detection of a wide range of clinically important analytes using the designed and synthesized hybrid materials. In this work, for proof-of-concept studies we have focused our main targets on two redox enzymes, i.e. cholesterol oxidase (ChOx) and glucose oxidase (GOx). For qualitative and quantitative analysis, we have performed two types of electrochemical measurements, i.e. cyclic voltametric and amperometric tests, to evaluate bioelectrocatalysis.

As shown in Fig. 4c, the bioelectrocatalytic oxidation of glucose at Fc-RGOP-GOx/SPE is well revealed by cyclic voltammograms (CVs). It is clearly evident that in the absence of glucose, the electrode system exhibited reversible voltametric response from Fc moieties, similar to those obtained at Fc-RGOP/GCE (i.e. without enzyme, Fig. 4a). This indicates that the accommodation of enzyme (GOx) does not block electron communication between Fc moieties and supporting electrode. Upon the addition of glucose, the anodic peak increased notably with increasing glucose concentration, accompanied by the suppression of the cathodic peak significantly (Fig. 4c). This observation shows a typical characteristic of ET-mediated bioelectrocatalysis. The

rapid disappearance of the cathodic peak in the presence of increasing analyte concentration indicated high efficient ET and bioelectrocatalytic cycles demonstrated by the Fc-RGOP system. The increase in the oxidation current is linearly associated with the glucose concentration up to 15 mM (Fig. 4d). The well- defined linear response to glucose is in the concentration range of 0.1 to 15 mM, with the dynamic range extended to appromixately 20 mM. Similar observations were obtained for Fc-RGOP-ChOx biosensing systems, except much lower concentration of cholesterol was needed.

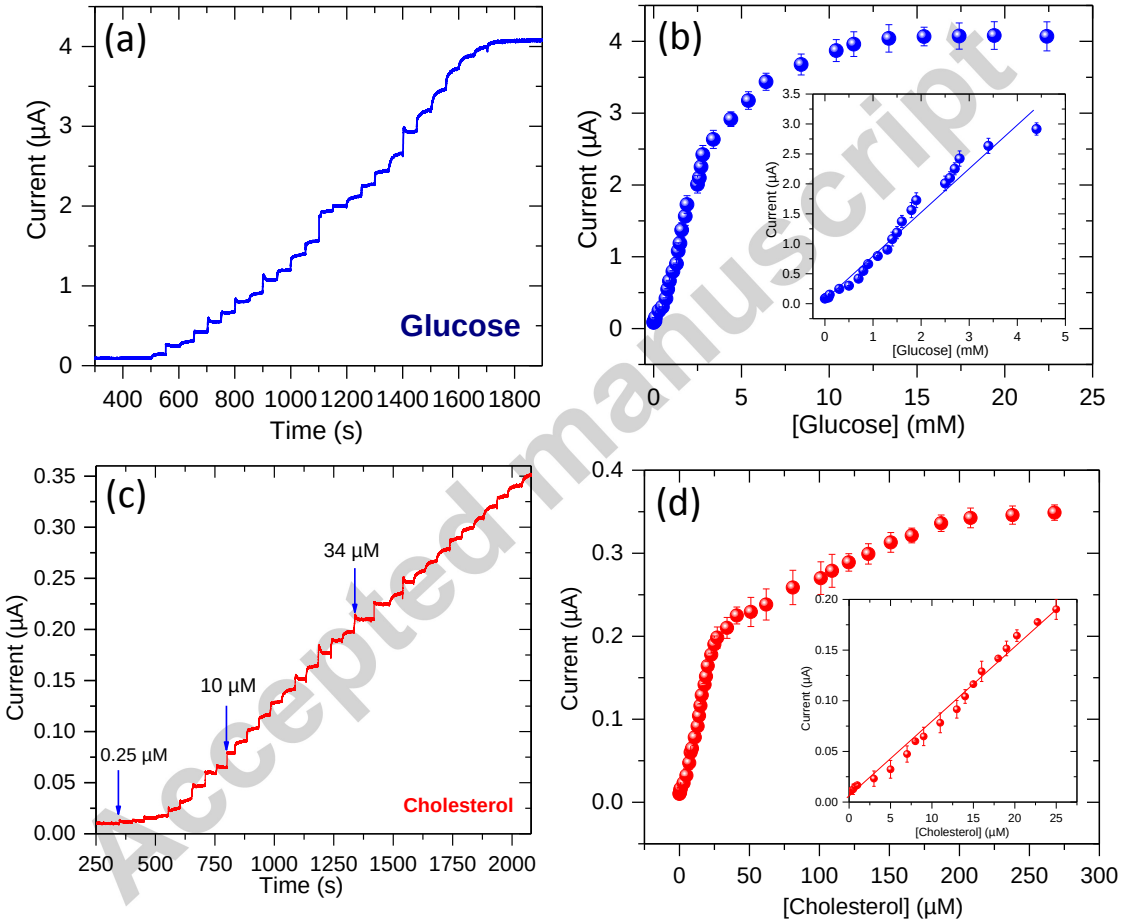


Figure 6. Amperometric determination of glucose and cholesterol. (a) Current-time curve obtained at Fc-RGOP-GOx/GCE electrode upon successive injection of glucose, (b) dependence of responsive current on glucose concentration, (c) amperometric response to cholesterol with Fc-RGOP-ChOx/GCE electrode, and (d) calibration curve for cholesterol. The insets in (b) and (d) are the linear part of current responses to the substrates. Electrolyte: 10 mM PBS (pH 7.0); the working electrode potential fixed at 0.4 V (vs SCE); substrate (either glucose or cholesterol) solution was injected at a regular time interval to the supporting electrolyte which was under stirring.

The amperometric measurements were performed in a stirring buffer solution (10 mM PBS, pH 7.0) with the working electrode potential fixed at 0.4 V (vs SCE). With the key results summarized in Figure 6, we can clearly see that the electrocatalytic currents increased with increasing the substrate concentration and achieved saturation at high concentration of substrate, as expected by Michaels-Menten enzyme kinetics (Fig. 6a and 6c). For the case of glucose, the results are largely consistent with those obtained from CVs (Fig. 4). The sensitivity of the glucose biosensor is 3.45 mA M^{-1} cm^{-2} with a limit of detection (LOD) at approximately 5 μM.

Intriguingly, Fc-RGOP-ChOx/GCE shows fast response and very high sensitivity to cholesterol, so that the detection can be readily achieved within 10 seconds and at the micromolar level (Fig. 6c). Figure 6d shows the corresponding calibration curve obtained from amperometric measurements. Cholesterol biosensors enable to respond linearly to cholesterol in the concentration range of 2.5-25 μM (the inset in Fig. 6d) with the dynamic detection range extended to 270 μM. The limit of detection goes down to 0.5 μM (S/N=3). The sensitivity of the cholesterol biosensor can reach 380 mA M^{-1} cm^{-2}. These observations suggest that the redox functionalized RGO exhibited high activity for ET communication between the redox center of enzymes and Fc moiety to enable highly efficient bioelectrocatalytic oxidation of glucose or cholesterol.

The apparent Michaels-Menten constant (K_M) for glucose and cholesterol is calculated as 4.09 and 0.32 mM, respectively. These values are lower than those previously reported in literatures (Arya et al., 2008) and suggest that the enzymes confined in the Fc-RGOP matrix well reserve their native structures with a high binding affinity to the substrates. The performances and characteristics of fabricated biosensors are compared with those previously reported for carbon- materials based glucose and cholesterol biosensors (Table S1). It is clearly evident that the present sensing platform has high performances.

3.5. Stability and reproducibility of the biosensor

To check the operational stability of this biosensor, the electrochemical performance of biosensing electrodes was tested against the storage time. Amperometric measurements were

performed using glucose biosensing electrodes stored at 4°C. The response decreases mainly occurred in the first 7 days. A 3% decrease in the oxidation current is observed after 3 days and a 11 % decrease after 7 days (Supporting Fig. S3b). The small decrease in biosensing response was observed due to the loss of bioactivity of the immobilized enzyme with the passage of time. The reproducibility of the biosensor was tested by preparing four individual biosensors under same condition on different days and the amperometric response was evaluated. The relative standard deviation (RSD) of biosensing response was 4% only, which implies the excellent reproducibility of this biosensor for potential practical applications.

3.6. Selectivity of the biosensor

The selectivity studies are focused on Fc-RGOP-GOx/GCE electrodes towards electrocatalytic oxidation of glucose. It was measured against these common electroactive species existing in human blood samples, including ascorbic acid, uric acid and cholesterol. The results are provided in the Supporting Information (Figure S3a). Amperometric measurements clearly indicate that no significant interference from these species was detected. Uric acid and ascorbic acid are tested especially, but as they are anionic in neutral pH medium and could thus be subjected to electrostatic repulsion by negatively charged polymer film such as Nafion. Thus, if Nafion is used the selectivity could further be improved for the present biosensing electrodes.

3.7. Real sample analysis

To test potential practical applications, we used Fc-RGOP-GOx/SPE as disposable sensors to analyze the glucose and cholesterol level in human serum samples. The results are compared with those obtained by the conventional clinical measurements of same samples (i.e. Trinder method) (Trinder, 1969). For determination of the glucose level in real samples, the human serum was added to the electrolyte (10 mM PBS, pH 7) on the screen printed electrode surface and the steady state current was measured at a constant potential of 0.4 V. For quantification of the cholesterol level in the serum samples, human serum sample was spiked with two different concentration of cholesterol (Eguilaz et al., 2011) (2 mM and 5 mM) and further diluted with 10 mM PBS (pH 7). The results have proven that the present biosensors can be

useful for the determination of glucose and cholesterol in serum samples without the need of sample pretreatment. Four individual measurements were performed for each sample and the RSD value was found between 3 and 6. As summarized in Table 1, the data obtained by our measurements are in a close agreement with the clinical laboratory measured results, which is encouraging and promising signal for the further development of our biosensing material into practical sensors.

Table 1. Glucose and cholesterol level measurement in human serum samples and comparison with clinical measurements. RSD was obtained from four independent measurements.

Analytes	Sample no.	Measuerd by our sensor (mM)	Measuerd by clinical method (mM)	RSD %
Glucose	1	4.17	4.3	5.2
	2	7.66	7.9	4.1
Cholesterol	1	2.12	2	3.4
	2	4.87	5	5.7

4. Conclusion

We have demonstrated the wiring of ferrocene electron transfer mediator and redox enzyme to graphene nanosheets through a highly branched polymeric matrix to synthesize biosensing hybrid nanomaterials. The PEI matrix can offer linking groups (e.g. amine groups) for covalent attachment of ferrocene units to the whole planes of graphene nanosheets, not limited to the nanosheet edges as shown by most of previous reports. The PEI matrix can also offer a biocompatible microenvironment to confine enzymes, where enzymes retain their catalytic activity and high affinity to substrates. We have tested the hybrid material for fabrication of reagent-less (i.e. all crucial components including enzyme, ET mediator and supporting graphene nanosheet in an integrated material) electrochemical biosensors. The results show that the hybrid material systems are stable and electrochemically active, indicating that the communication of electron transfer and bioelectrocatalysis between mediator, enzyme and supporting electrode is well established. Such nano-bio-hybrid material has been especially studied for potential applications in sensing glucose and cholesterol with promising outcomes.

The real-time application of biosensors for clinically important analyte detection was performed with human serum samples, and the results are validated by conventional clinical measurements. This material holds promise for the development of low-cost point of care biosensors.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version.

References

Albericio, F., 2004. Curr. Opin. Chem. Biol. 8, 211–221. doi:10.1016/j.cbpa.2004.03.002

Arya, S.K., Datta, M., Malhotra, B.D., 2008. Biosens. Bioelectron. 23, 1083–100. doi:10.1016/j.bios.2007.10.018

Chaubey, A., Malhotra, B.D., 2002. Biosens. Bioelectron. 17, 441–456. doi:10.1016/S0956-5663(01)00313-X

Chen, L., Xu, Z., Li, J., Min, C., Liu, L., Song, X., Chen, G., Meng, X., 2011. Mater. Lett. 65, 1229–1230. doi:10.1016/j.matlet.2011.01.063

D'Costa, E.J., Higgins, I.J., Turner, A.P.F., 1986. Biosensors 2, 71–87. doi:http://dx.doi.org/10.1016/0265-928X(86)80011-6

Dey, R.S., Raj, C.R., 2013. ACS Appl. Mater. Interfaces 5, 4791–4798. doi:10.1021/am400280u

Eguilaz, M., Villalonga, R., Yanez-Sedeno, P., Pingarron, J.M., 2011. Anal. Chem. 83, 7807–7814.

doi:10.1021/ac201466m

Fan, L., Zhang, Q., Wang, K., Li, F., Niu, L., 2012. J. Mater. Chem. 22, 6165. doi:10.1039/c2jm15411k

Goldstein, J.L., Brown, M.S., 2001. Science (80-.). 292, 1310–1312.

Halder, A., Zhang, M., Chi, Q., 2016. Rev. Adv. Sci. Eng. 5, 4-31.

Han, S.Y., Kim, Y.A., 2004. Tetrahedron 60, 2447–2467. doi:10.1016/j.tet.2004.01.020

Hayashi, Y., Kinoshita, Y., Hidaka, K., Kiso, A., Uchibori, H., Kimura, T., Kiso, Y., 2001. J. Org. Chem. 66, 5537–5544. doi:10.1021/jo010233o

Health, T., Information, S.C., 2013. Ment. Heal. Pract. 16, 5. doi:10.7748/mhp2013.03.16.6.5.p10726

Heller, A., Feldman, B., 2008. Chem. Rev. 108, 2482–2505. doi:10.1021/cr068069y

Herman, W.H., Zimmet, P., 2012. Diabetes Care 35, 943–944.

Kostarelos, K., Novoselov, K.S., 2014. Science (80-.). 344, 261–263. doi:10.1126/science.1246736

Liao, L., Peng, H., Liu, Z., 2014. J. Am. Chem. Soc. 136, 12194–12200. doi:10.1021/ja5048297

Merchant, S.A., Iran, T.O., Meredith, M.T., Cline, T. C., Glatzhofer, D. T., Schmidtke, D.W., 2009. Langmuir 25, 7736–7742. doi:10.1021/la9004938

Merchant, S.A., Meredith, M.T., Tran, T.O., Brunski, D.B., Johnson, M.B., Glatzhofer, D.T., Schmidtke, D.W., 2010. J. Phys. Chem. C 114, 11627–11634. doi:10.1021/jp911188r

Parlak, O., Tiwari, A., Turner, A.P.F., Tiwari, A., 2013. Biosens. Bioelectron. 49, 53–62. doi:10.1016/j.bios.2013.04.004

Peeling, R.W., Holmes, K.K., Mabey, D., Ronald, A., 2006. Sex. Transm. Infect. 82 Suppl 5, v1–v6. doi:10.1136/sti.2006.024265

Rabti, A., Mayorga–Martinez, C.C., Baptista–Pires, L., Raouafi, N., Merkoçi, A., 2016a. Anal. Chim. Acta 1–8, doi:10.1016/j.aca.2016.04.010

Rabti, A., Raouafi, N., Merkoçi, A., 2016b. Carbon 108, doi:10.1016/j.carbon.2016.07.043

Sekretaryova, A.N., Eriksson, M., Turner, A.P.F., 2015. Biotechnol. Adv. 34, 177–197. doi:10.1016/j.biotechadv.2015.12.005

Trinder, P., 1969. J. Clin. Pathol. 22, 158–61. doi:10.1136/jcp.22.2.158

Turner, A. P. F., 2013. Chem. Soc. Rev. 42, 3184. doi:10.1039/c3cs35528d

van Staveren, D.R., Metzler-Nolte, N., 2004. Chem. Rev. 104, 5931–5986. doi:10.1021/cr0101510

Walcarius, A., Minteer, S.D., Wang, J., Lin, Y., Merkoçi, A., 2013. J. Mater. Chem. B 1, 4878. doi:10.1039/c3tb20881h

Willner, I., Baron, R., Willner, B., 2007. Biosens. Bioelectron. 22, 1841–1852. doi:10.1016/j.bios.2006.09.018

Zhang, M., Halder, A., Hou, C., Ulstrup, J., Chi, Q., 2016. Bioelectrochemistry 109, 87–94. doi:10.1016/j.bioelechem.2016.02.002

Zhang, Y., Guo, K., LeBlanc, R.E., Loh, D., Schwartz, G.J., Yu, Y.-H., 2007. Diabetes 56, 1647–1654. doi:10.2337/db07-0123

Zhu, N., Han, S., Gan, S., Ulstrup, J., Chi, Q., 2013. Adv. Funct. Mater. 23, 5297–5306. doi:10.1002/adfm.201300605

Highlights

- An electroactive graphene supported composite is synthesized and characterized.
- This hybrid material is biocompatible to accommodate different redox enzymes.
- Nano-bio-hybrid materials are tested for constructing glucose and cholesterol biosensors.