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# A bio-electrochemical sensing platform for glucose based on irreversible, non-covalent pi–pi functionalization of graphene produced via a novel, green synthesis method

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ABSTRACT

In this work, pristine graphene was produced through a novel single step exfoliation of graphite in mild sonochemical alcohol–water treatment. The developed green synthesis approach successfully eradicates issues associated with conventional methods which use organic solvents, acids and oxidizers, leaving undesirable functional groups attached to the graphene surface. Results from cyclic voltammetry and amperometric analysis showed a wide linear range up to 5 mM and sensitivity improvements of more than 22 times in comparison to the control sample. Subsequently, an electrochemical glucose biosensor was fabricated by the immobilization of glucose oxidase (GOx) via bi-functional linkers. This reliable surface modification method provides irreversible non-covalent bonding between graphene and the enzymatic amide groups, while preserving the $sp^2$ graphene structure, whilst promoting better electron transfer kinetics between the FAD/$FADH_2$ redox sites of GOx at the modified electrode surface. The fabricated biosensor exhibited satisfactory long-term stability, reproducibility and high selectivity for glucose detection and showed significant improvements when compared to unmodified electrodes.



## 1. Introduction

Diabetes is a common chronic disease and brings with it debilitating health conditions affecting millions of people in the world [1,2]. This, coupled with the need for regular monitoring of blood glucose levels necessitates the development of sensitive, accurate biosensors for faster diagnosis and therapy of the disease. To date, various detection methods have been developed such as electrochemical [3,4], optical detection [5] and fluorescent [6] detection methods. However, amongst these methods, electrochemical determination of glucose concentrations remains popular due to its quick detection speed and low detection limits.

Nanoelectroanalytical chemistry is the combination of electrochemistry via nanomaterial platforms and is increasingly popular due to the rise of various nanomaterials [7]. A large variety of nanomaterials such as silver nanoparticles [8], gold nanoparticles [9], $Cu_2O$ nanocubes [2], ZnO nanoparticles [10], carbon nanotubes [11] and carbon nanofibres have been studied for constructing electrode platforms. Graphene is a one atom thick 2D planar sheet of carbon atoms arranged in an $sp^2$ hybridized configuration [12]. Owing to its stellar combination of chemical inertness, extensive surface area, and excellent electron transport [13] graphene is a viable electrode material for a glucose sensing platform. Various approaches have been studied for the implementation of graphene in the fabrication of sensing devices [7,14,15]. However, although various efforts have been done to study the application of graphene in electrochemical glucose sensors, so far the starting graphene material has been reduced from graphene oxide (GO) [10]. This is due in part to the lack of a scalable, reproducible method for producing pristine sheets of unoxidized graphene. Whilst the popular oxidation–reduction route for producing graphene is capable of mass production, the reduced GO has less than desirable qualities for sensing applications. Owing to the presence of defects and residual functional groups that cannot be fully removed even with subsequent annealing, the electrical conductivity of the reduced GO is compromised [16]. In an effort to circumvent these drawbacks, the graphene that is used in this work was produced via

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a previously published study involving mild facile exfoliation by sonicating graphite in ethanol–water mixtures [17]. This method is not only novel and green, the as-produced graphene sheets are free from any residual functional groups, and is thus suitable for various functionalization approaches for a wide variety of applications.

One of the most efficient ways of constructing a high performance enzyme electrode is to improve the detection of hydrogen peroxide, a common by-product of oxidase catalyzed reactions. Unfortunately, tranducers that function on this mechanism are susceptible to electrochemical interferences from oxidizable species such as L-ascorbic acid, uric acid, and acetaminophen [18]. As such, transducers of this form often have a coating incorporated on the electrode surface to prohibit unwanted interactions between these interfering species and the electrode. However, the integrity of these layers is often jeopardized during the process of immobilizing the enzyme on the electrode surface. This results in reduction in the efficacy of the membrane layer [18]. Therefore, efforts have been invested towards fabricating enzymatic electrodes which have a lower susceptibility to these issues.

The use of the enzyme glucose oxidase (GOx) has been extensively researched due to its easy availability and relatively low cost. More importantly, GOx is one of the most widely used enzymes for the detection of glucose as it catalyzes glucose into gluconolactone and hydrogen peroxide, as reported in [19] and is shown in Eqs. (1) and (2).

$$\mathrm{GOx(FAD)} + \beta\text{-d-glucose} \rightarrow \mathrm{GOx(FADH_2)} + \delta\text{-d-gluconolactone} \quad (1)$$

However, the $FADH_2$ flavoenzyme has to be reoxidized in order to continue the redox cycle, and this occurs as:

$$\mathrm{GOx(FADH_2)} + \mathrm{O_2} \rightarrow \mathrm{GOx(FAD)} + \mathrm{H_2O_2} \quad (2)$$

δ-D-gluconolactone is then easily hydrolysed into gluconic acid

$$\delta\text{-d-gluconolactone} + \mathrm{H_2O} \rightarrow \text{d-gluconicacid}$$

all of which can be summarized in the common general enzymatic reaction [20,21]:

$$\text{Glucose} + \mathrm{O_2} \xrightarrow{\mathrm{GOx}} \text{gluconic acid} + \mathrm{H_2O_2}$$

This flavoenzyme has been widely used both in solution or immobilized on solid substrates due to its easy availability and stability. Essentially, GOx consists of two electrochemically active flavine adenine dinucleotide (FAD) cofactors which functions as its redox centres. In general, the redox reaction that occurs at the GOx redox sites can be expressed as:

$$\mathrm{GOx(FAD)} + 2\mathrm{H^+} + 2\mathrm{e^-} \rightarrow \mathrm{GOx(FADH_2)}$$

$$\mathrm{GOx(FADH_2)} - 2\mathrm{e^-} \rightarrow \mathrm{GOx(FAD)} + 2\mathrm{H^+}$$

whereby FAD and $FADH_2$ refer to the oxidized and reduced forms of the GOx cofactors [22].

However, as these cofactors are protected in a protein shell, it is difficult to achieve direct electron transfer between the FAD redox sites and the electrode. Therefore, it is common to use a highly conductive nanomaterial such as CNTs, quantum dots [3] or graphene to facilitate direct electron transfer between the redox sites to the electrode transducer.

Whilst immobilizing the enzyme onto the surface of the nanomaterial promotes greater electron transfer kinetics, research has shown that the enzymatic activity of GOx decreases when the enzyme is directly deposited unto the nanomaterial [23,24]. Therefore, it is important to first functionalize graphene in order to anchor the enzyme. Chen et al. studied a noncovalent functionalization method using bifunctional organic molecules containing a succinylimide ester and a pyrene moiety [25]. This method has been proven to be effective in binding proteins to various carbon nanomaterials, as the pyrene moiety non-covalently bonds to the surface of graphene via a π–π hydrophobic stacking that does not disrupt the pristine electronic structure of the graphene. Despite being a non-covalent binding, the strong interaction between pyrene groups and the basal plane of graphite has long been established, and evidenced in the work of other groups in which the electrochemical stability of graphite electrodes functionalized with pyrene bifunctional groups were reported [25–27]. The work reported in this paper adapts this simple noncovalent functionalization of graphene with 1-pyrenebutyic acid *N*-hydroxysuccinimide ester, an organic molecule which acts as a mediator that anchors and promotes the electron transfer between the immobilized enzyme and the electrode. Due to the pristine nature of our novel facilely exfoliated graphene, the produced biosensor exhibits fast responses, selectivity, stability and repeatability.

## 2. Experimental

### 2.1. Materials

Graphite flakes (purity ≥98% carbon) were purchased from Bay Carbon, USA. Ethanol was purchased from Merck. Raw graphite and all solvents were used as received. Deionized water (DI) from the Millipore system was used as a co-solvent. 1-Pyrenebutyric acid *N*-hydroxysuccinimide ester, (PSE) glucose oxidase (GOx) from *Aspergillus niger*, D-(+)-glucose, uric acid (UA) ≥99%, ascorbic acid (AA) ≥99%, phosphate buffer solution (PBS) tablets were purchased from Sigma–Aldrich and diluted in DI water to produce a 0.1 M buffer solution with a pH of 7.4.

### 2.2. Enzyme electrodes modification

Screen printed electrodes (SPE) were purchased from Dropsens, Spain. The electrodes had a 4 mm diameter working electrode (WE) and counter electrode (CE), and a silver (Ag) reference electrode (RE). Graphene was prepared through a mild facile synthesis route in water–alcohol mixtures as detailed in Chia et al. [17]. The as-produced graphene was then sonicated in ethanol with different ratios of 1-Pyrenebutyric acid *N*-hydroxysuccinimide ester (PSE) for 30 min. This graphene-PSE (G-PSE) dispersion was then washed with DI water and centrifuged at 3000 rpm to wash away any unbound PSE molecules. The G-PSE composite was then redispersed in ethanol and 6 μL of the dispersion was drop-casted onto the SPE. Enzyme modification was carried out by drop-casting 5 μL of different concentrations of GOx prepared in PBS buffer onto the SPE. The G-PSE-GOx electrode was then allowed to air-dry before storing at 4 °C overnight. Prior to electrochemical measurements, the modified electrodes were pre-treated by soaking in PBS buffer for 15 min before lightly rinsed with DI water to remove any unbound moieties. Electrodes were also subjected to 30 repeated CV cycles in PBS buffer in order to condition the electrodes before measurements were taken in fresh electrolyte. For comparison purposes, G-GOx, and G-PSE-GOx electrodes with different PSE ratios were prepared in a similar manner and stored at 4 °C prior to analysis. Fig. 1 schematically illustrates the cross section of the SPE used, followed by the functionalization process via π–π stacking of PSE on the surface of exfoliated graphene sheets. The succinimidyl ester moiety then binds with amine groups in the enzyme GOx.