PRELIMINARY STUDY ON THE EFFECT OF REDUCED GRAPHENE OXIDE, GOLD NANOPARTICLES, AND NAFION® CONCENTRATION ON REDOX PEAK CURRENT FOR ELECTROCHEMICAL BIOSENSING

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Abstract

Electrochemical characterization of graphene-based nanocomposites as transducer nanomaterial for highly sensitive biosensors was performed. Parameters that were varied include graphene oxide (GO) concentration, amount of added gold nanoparticles (AuNPs), and Nafion® as binder for enhancing electrical conductivity of the transducer layer. The nanocomposite-modified glassy carbon electrode (GCE) transducer layers were fabricated via a simple two-step drop-cast and subsequent electrochemical reduction. Cyclic voltammetry (CV) was used to characterize the redox capability of the transducer layer. Electrochemical deposition of ultra-highly concentrated single-layer graphene oxide (UHC GO) suspension with a concentration of 6 mg/ml gave highest anodic peak current $I_{pa,}$ ~0.6 mA, after electrochemical reduction, compared to most peak currents reported in the literature for an electrode with an inner diameter of 3 mm; adding UHC GO:AuNPs in a 2:1 ratio followed by electrochemical reduction resulted in even higher I_{pa} (0.4 mA higher) in comparison to reduced UHC GO alone, whereas adding UHC GO:Nafion® in an 8:1 ratio shows a 1-mA increase in peak current. Therefore, AuNPs and Nafion® can be added to reduce UHC GO as nanocomposite for the development of a redox-active transducer that can result in highly sensitive biosensors.

Keywords: Cyclic voltammetry; Graphene nanocomposites; Gold nanoparticles; Nafion®; Single-layer graphene.

1. Introduction

Electrochemical sensors operate through electrochemical reactions at the electrode-analyte solution interface, where oxidation-reduction is characterized via voltammetric methods, as different analytes can be oxidized or reduced at different potentials [1]. Owing to fast, relatively low-cost, and facile methods of fabrication, electrochemical sensors have been extensively researched for various applications. In order to achieve optimal sensor performance in terms of sensitivity, accuracy, linear range, and detection limit, the electrode-solution interface requires fast charge transfer for an electrical signal to be measured [2-5]. Fast charge transfer at the electrode-solution interface can be achieved by introducing nanomaterials with high surface-to-volume ratio, like graphene, and gold nanoparticles (AuNPs), at the electrode surface; this layer is often called a transducer layer. The purpose of this paper is to understand the influence of these materials on electrochemical sensor performance by looking at the redox anodic peak current.

Huang et al. [6] commented that graphene is a two-dimensional, zero-band gap semiconductor monolayer sheet of sp^2-bonded carbon atoms, first introduced by Geim and his team in 2004, for which he won a Nobel Prize in 2010. Graphene's structure, arranged in a perfect honeycomb network lattice, displays remarkable physical, chemical, mechanical, optical, and electrochemical properties. It is the thinnest known material, with atom-sized thickness, yet is also the strongest known material, with strength 100 to 300 times that of steel. The two-dimensional planar structure contributes to the outstanding electron mobility that subsequently results in excellent electrical conductivity compared to carbon nanotubes (CNTs), which are a three-dimensional cylindrical structure of graphene. Singh et al. [7] and Justino [8] reported that graphene also exhibits high structural stability and energy-storage capability. Hence, it is one of the most attractive nanomaterials as a transducer for the development of a biosensor. Based on studies by Zhu et al. [9], its high theoretical specific surface area of approximately is 2,630 m^2 per gram and its stability at ambient temperature, graphene appears an excellent transducer candidate for the development of electrochemical biosensors.

According to Sabury et al. [10], most past research involving biosensors used graphene oxide (GO) concentrations of 0.5 to 1 mg/ml, prepared by exfoliating a GO sheet in solvent (e.g., DI water, PBS) using ultrasonication. In this study, GO prepared via exfoliation will be compared for its redox anodic peak current against a commercially available graphene, ultra-highly concentrated single-layer graphene oxide (UHC GO); the UHC GO has a concentration of 6 mg/ml and is claimed to have more than 80% single-layer graphene content in a suspension. Both exfoliated GO and UHC GO will be deposited on electrodes via drop-casting and reduced before voltammetry measurements are made to measure the anodic peak current. A higher anodic peak current at a specific potential implies that the electrodes are more capable of oxidizing the freely diffusing redox species in the measurement solution [11].

Despite its desirable physical properties, graphene does not have adequate biocatalytic properties, and therefore could not function as a catalyst in a biochemical sensor [12]. Noble-metal nanoparticles such as silver (Ag), gold (Au), platinum (Pt), and palladium (Pd), on the other hand, are known to be excellent catalysts. Therefore, conjugation of these noble-metal nanoparticles with graphene in the transducer layer could enhance the electroactive surface area of the electrode,

as well as improve the electron transfer of byproducts or ions that can be measured by the electrode. AuNPs are highly researched noble-metal nanoparticles used to enhance the measurement performance of graphene-based electrochemical biosensors. Furthermore, AuNPs have excellent biocompatibility, allowing them to serve as immobilization matrix for biomolecules such as antibodies and enzymes. Besides AuNPs, synthetic polymers such as Nafion®, an ion exchange resin that functions as a material binder and also helps improve the stability and longevity of a biosensor can also be incorporated into the transducer layer [13].

For development of enzymatic biosensors, incorporation of the aforementioned materials in a composite can help improve enzyme immobilization and possibly protect the immobilized enzyme against denaturation as well as prevent enzyme leakage from the electrode surface into the solution containing the analyte. Usage of the materials in graphene-based composites can also help reduce the π-stacking interactions between graphene sheets [14], provide larger electrochemically active surface areas for the adsorption of biomolecules [15], and effectively accelerate electron transfer from the solution containing the analyte and the electrodes[15, 13].

The redox capability of reduced graphene oxide (rGO) incorporating AuNPs and Nafion® as transducer material was characterized using cyclic voltammetry (CV) for different concentrations of GO and ratios of GO to AuNPs and Nafion®. The results of this study can be used to provide initial insight on the effect of GO types, AuNPs, and Nafion® as transducer material for electrochemical biosensors.

2. Experimental

2.1. Reagents and apparatus

Graphene oxide (GO) sheets were purchased from Abalonyx, Oslo, Norway. Ultra-highly concentrated single-layer graphene oxide (UHC GO), 6 mg/ml, was purchased from Graphene Supermarket, the United States of America (USA). Gold nanoparticles (AuNPs), 15 nm in diameter, and Nafion® perfluorinated resin solution were purchased from Sigma-Aldrich, St. Louis, MO, USA. Potassium ferricyanide ($K_3Fe(CN)_6$) was purchased from R&M Chemicals, Selangor, Malaysia. Potassium dihydrogen phosphate (KH_2PO_4) and disodium hydrogen phosphate (Na_2HPO_4) obtained from Sigma-Aldrich, St. Louis, MO, USA, were used to prepare 0.1 M PBS, pH 5. DI water was used to prepare the solutions.

All electrochemical experiments to characterize graphene and its composites were performed using a conventional three-electrode cell and pocketSTAT purchased from IVIUM Technologies, Eindhoven, the Netherlands, as a potentiostat/galvanostat/impedance analyzer. A modified glassy carbon electrode (GCE) with inner diameter of 3.0 mm was used as the working electrode (WE), platinum (Pt) was used as the counter electrode (CE), and silver/silver chloride (Ag/AgCl) in 3.0 M saturated sodium chloride (NaCl) was used as the reference electrode (RE). The WE was purchased from BASi®, West Lafayette, IN, USA, and the CE and RE were purchased from ALS Co., Ltd., Tokyo, Japan. Prior to surface modifications, the GCE was polished with a slurry of diamond and alumina (0.05 μm grain size), ultrasonicated in ethanol and then in deionized (DI) water, and dried under ambient conditions for 30 min before use. All experiments were performed at ambient temperature.

2.2. Fabrication of rGO/GCE, rGO-AuNP/GCE, and rGO-Nafion®/ GCE with Exfoliated GO and UHC GO as Precursors

Redox current from GCEs modified with GO from exfoliation of GO sheets at concentrations of 1, 3, and 5 mg/ml and UHC GO at concentration of 6 mg/ml as purchased will be compared, in order to provide insights into the advantage of using UHC GO for electrochemical sensors. For exfoliated GO, different GO concentrations of 1, 3, and 5 mg/ml were prepared by diluting a 6-mg/ml GO stock solution using the molarity equation. These concentrations were chosen randomly. The stock solution was prepared from a GO sheet that was ultrasonicated in DI water for 1 hr to disperse the graphene flakes. UHC GO of 6 mg/ml was used as purchased.

Nanocomposites with UHC GO-AuNP and UHC GO-Nafion® ratios of 2:1, 4:1, and 8:1 were prepared by adding the desired amount of AuNPs and Nafion® to the UHC GO suspension and ultrasonicating the mixture for 15 min. Deposition of GO, UHC GO, UHC GO-AuNP and UHC GO-Nafion® nanocomposites was performed by drop-casting 8-µl samples onto GCEs. After drop-casting, the modified electrodes were dried at ambient conditions for 1 hr, followed by electrochemical reduction of GO to rGO in 0.1 M PBS, pH 5, for 30 electrochemical reduction cycles using cyclic voltammetry (CV) from 0 to -1.5 V as illustrated in Fig. 1.
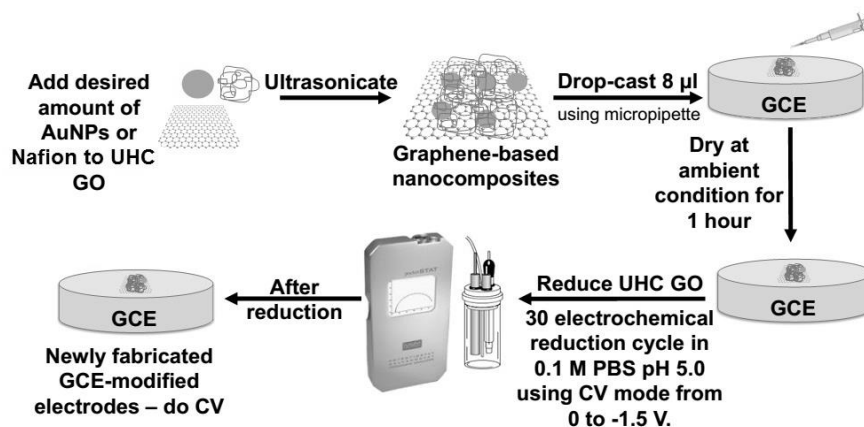
Fig. 1. Illustration of fabrication of UHC GO-based electrodes with addition of AuNPs or Nafion®, starting with adding desired amount of Nafion®/AuNPs into GO solution, followed by ultrasonication of the mixture to evenly disperse the materials in the UHC GO solution; the mixed solution was drop-cast onto a GCE. Electrochemical reduction of the drop-cast GCE in PBS pH 5.0 reduces most of the UHC GO to UHC rGO; the modified GCE was used in CV experiments.

2.3. Cyclic Voltammetry of rGO/GCE, rGO-AuNP/GCE, and rGO-Nafion®/GCE Electrodes

Cyclic voltammetry (CV) was used to electrochemically characterize the electron-transfer capabilities of the modified GCEs based on the anodic peak current, I_{pa}. Potassium ferricyanide ($K_3Fe(CN)_6$), 0.05 M, was used as the redox solution, and the potential to the electrodes was varied linearly at 100 mV/s scan rate. CV graphs are presented using the IUPAC convention. Using the IUPAC convention, a higher

peak with decline of the potential difference between the oxidation and reduction peaks indicates better voltammetry response [11].

2.4. Statistical analysis

To provide statistical support for the CV results, statistical testing was applied to look for significant differences (if any) across various applied voltage ranges within each experiment (refer to sections 3.1 - 3.3). The chosen region for the analysis is the measured current during forward scan (i.e., from -0.50 V to 1.00 V; and from 0.13 V to 0.61 V. From now onward, the former setting is addressed as region A and the latter as region B).

Prior to deciding whether to apply a parametric or nonparametric test, the homogeneity of variance was first investigated using the Fligner-Killen test. If the variances are not significantly different, this implies that it is sensible to use one-way ANOVA testing. Otherwise, its counterpart nonparametric Kruskal-Wallis statistical test was chosen instead.

A check of the data showed that the variances differ significantly (p-values for (3.1) data: 9.525×10^{-5}; (3.2) data: 5.436×10^{-3}; (3.3) data: 2.542×10^{-6}. Therefore, the Kruskal-Wallis test was chosen for subsequent analysis.

In cases where significant differences were observed resulting from Kruskall-Wallis testing, a post-hoc testing (i.e., Dunn's test, with p-values adjusted using the Bonferroni method) was carried out to perform pairwise comparisons, in order to indicate which experiment setting is significantly different from which. Significance of p-values resulting from the testing is denoted by the following notation: * for $p < 0.05$, ** for $p < 0.01$ and *** for $p < 0.001$.

3. Results and Discussion

3.1. Effect of Graphene Oxide (GO) Concentration on rGO/GCE Anodic Peak Current

GCEs modified using different concentrations of GO gave higher anodic peak current (I_{pa}) for lower GO concentrations prepared via exfoliation; a GCE/rGO fabricated with GO concentration of 1 mg/ml showed a I_{pa} of 0.453 mA while concentrations of 3 mg/ml and 5 mg/ml have $I_{pa}s$ of 0.436 and 0.305 mA, respectively (Table 1, Fig. 2). The slightly lower $I_{pa}s$ for higher concentrations is probably a result of multiple-layer graphene at those concentrations and the effects of oxygenated functional groups and crystallinity structures that can influenced the physicochemical properties of the reduced GO [16]. However, when utilizing 6 mg/ml UHC GO, which has more than 80% single-layer GO in suspension form, we observed a I_{pa} of 0.583 mA (Table 1, Fig. 2). Note that all the electrodes experienced the same number (30) of electrochemical reduction cycles. The UHC rGO could have the right balance of oxygenated functional groups and defectiveness of the graphene structures [16].

The use of a higher content of single-layer UHC GO as precursor is expected to give a higher I_{pa} value; UHC rGO/GCE shows the highest I_{pa}. However, rGO/GCE fabricated from 1-mg/ml exfoliated GO shows a redox current value that is higher than that of similar material with higher GO concentrations (3 and 5 mg/ml). These current values reflect electrochemical sensor performance with respect to

voltammetry and the effect of common materials used to enhance transduction, but in no way are conclusive about device behavior, because sensor performance is affected not only by electrode material but also by the electrolyte and analyte solution. However, we think that careful characterization of the functional groups along with the crystallinity of the GO and rGO structures are needed to understand the effect of the different types of graphene, the deposition process, and the reduction process.

Although there is a slight difference in the I_{pa}, testing for significant differences between the measured current distributions across various experimental settings has resulted in a p value $= 4.159 \times 10^{-5}$. Further investigation using Dunn's test provided strongest support for UHC rGO/GCE from a concentration of 6 mg/ml (p values for region A and B are 1.128×10^{-5} and 1.053×10^{-5} respectively). This suggests that UHC rGO with a concentration of 6 mg/ml explains the significant measured current distributions observed across the various experimental settings.
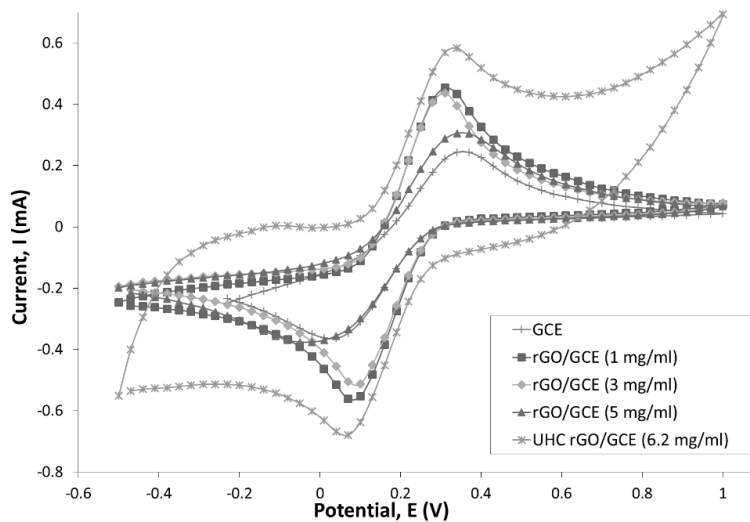
Fig. 2. Effect of different GO concentrations on the anodic peak current of rGO/GCEs as shown by the CV graphs for rGO-based GCEs prepared by reduction of exfoliated GO precursor at concentrations of 1, 3 and 5 mg/ml; in comparison to the anodic peak current of rGO-based GCEs prepared by reduction of UHC GO at concentration of 6 mg/ml.

Table 1. Effect of different GO concentrations on the measured peak current. Of rGO/GCE electrodes, UHC rGO/GCE showed a significant increase in I_{pa}, with p values for region B of 1.053×10^{-5} as compared to other GCE types; * for $p < 0.05$, ** for $p < 0.01$ and * for $p < 0.001$.**

Electrode Type	Graphene Concentration (mg/ml)	Current, I_{pa} (mA)
GCE	-	0.243
rGO/GCE	1	0.453
rGO/GCE	3	0.436
rGO/GCE	5	0.305
UHC rGO/GCE	6	0.583***

3.2. Effect of Adding Gold Nanoparticles (AuNPs) to UHC GO Suspension on the Performance of rGO-based GCEs

The effect of UHC GO-to-AuNP ratio was investigated. After reduction of the drop-cast GCE, CV was conducted and anodic peak current measured. The 2:1 ratio of UHC GO to AuNPs showed a significant increase in I_{pa} (~ 1 mA) when compare to the 8:1 ratio (0.4 mA), as shown in Fig. 3 and Table 2. The results suggest that adding more UHC GO did not necessarily contribute towards higher I_{pa}. This trend is consistent, as reported elsewhere [12]. Addition of more graphene resulted in more stacking of graphene sheets because AuNPs could no longer prevent the graphene sheets from stacking together, thus resulting in lower current [13].

Testing for significant differences between the measured current distributions across various experimental settings resulted in a p value = 3.276×10^{-5}. Interestingly, performing Dunn's test for region A yielded stronger statistical support for UHC rGO/GCE against a bare GCE (p value: 5.692×10^{-6}), compared to UHC rGO-AuNP/GCE (8:1 ratio) against a bare GCE (p value: 9.157×10^{-3}) and UHC rGO-AuNP/GCE (2:1 ratio) against a bare GCE (p value: 4.024×10^{-2}). On the other hand, interestingly, further testing on region B resulted in strongest support for UHC rGO-AuNP/GCE (2:1 ratio) type against a bare GCE (p value: 7.787×10^{-6}), followed by UHC rGO/GCE against a bare GCE (p value: 1.170×10^{-5}), UHC rGO-AuNP/GCE (4:1 ratio) against a bare GCE (p value: 4.390×10^{-4}), and UHC rGO-AuNP/GCE (8:1 ratio) against a bare GCE (p value: 2.563×10^{-2}).
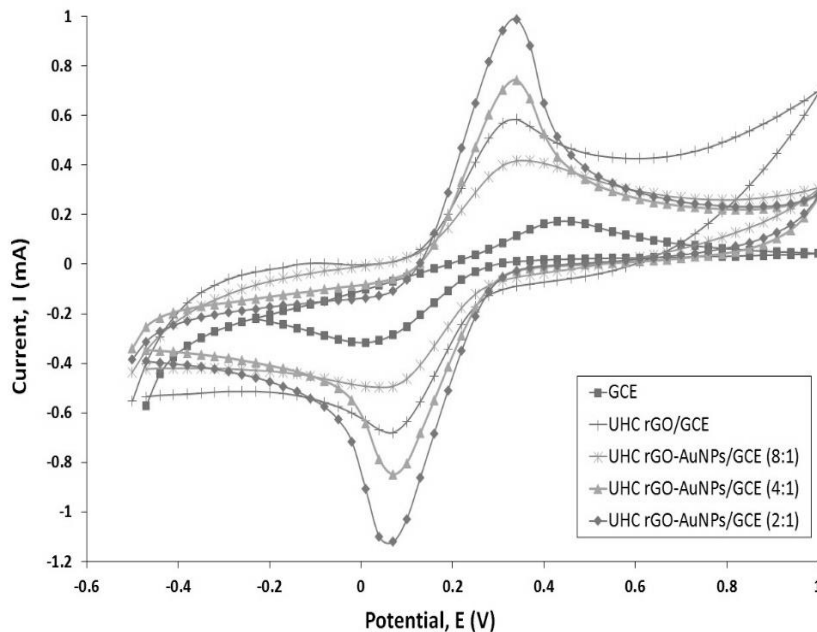
Fig. 3. Effect on redox peak current of adding gold nanoparticles (AuNPs) at different ratio of UHC rGO to AuNPs.

Table 2. Effect of UHC GO-to-AuNP ratio on rGO/GCE redox anodic peak current (I_{pa}). Strongest statistical support was observed for UHC rGO-AuNP/GCE (2:1 ratio) when testing at region B, followed by UHC rGO/GCE, UHC rGO-AuNP/GCE (4:1 ratio), and UHC rGO-AuNP/GCE (8:1 ratio) (see results in text);* for $p < 0.05$, ** for $p < 0.01$ and * for $p < 0.001$.**

Electrode Type	UHC rGO:AuNP Ratio	Current, I_{pa} (mA)
GCE	-	0.172
UHC rGO/GCE	-	0.583***
UHC rGO-AuNP/GCE	8:1	0.416*
UHC rGO-AuNP/GCE	4:1	0.742***
UHC rGO-AuNP/GCE	2:1	0.988***

### 3.3. Effect of Adding Nafion® to UHC GO Suspension on the Performance of rGO-based GCEs

The effect of different UHC GO-to-Nafion® ratios was investigated. After reduction of the drop-cast GCE, CV was conducted and anodic peak current measured. Results show an increase in I_{pa} of ~0.7 mA for electrodes drop-cast with UHC GO-to-Nafion® ratio of 8:1, as shown in Fig. 4 and Table 3. Nafion® is known to function as an ion-exchange resin that can facilitate electron transfer. However, we think that a large amount of Nafion® can hinder electron transfer to rGO layers owing to Nafion's® having both hydrophilic and hydrophobic Teflon®-like regions. The hydrophilic region consists of sulfonate groups that can swell and change in size and/or shape owing to water intake, thus facilitating ion transport, but the hydrophobic region might not mix well with the rGO, hindering electron transfer [16, 17]. Liao et al. [18]and Zhao et al. [19] explained the electrostatic repulsion force between the negatively charged Nafion® and $K_3Fe(CN)_6$ could explain this phenomenon. A high ratio of rGO to Nafion® could assist in removing the electron-hindrance effect from Nafion®. However, further investigation is needed to prove this claim.

Testing for significant differences between the measured current distributions across various experimental settings has resulted in a p-value = 5.963×10^{-4}. Investigating further using Dunn's test provided strongest support for UHC rGO-Nafion® (8:1) (*p* values for region A and B are 1.440×10^{-3} and 3.373×10^{-7} respectively). Testing at region A signalled significant support only for UHC rGO-Nafion® (8:1) but for region B, weaker statistical support was also observed for UHC rGO/GCE, UHC rGO-Nafion® (2:1 and 4:1) (*p* values are 5.676×10^{-3}, 3.995×10^{-3} and 2.306×10^{-3} respectively.

Table 3. Effect on redox anodic peak current of adding different ratios of UHC GO to Nafion®. Strongest statistical support was observed for UHC rGO-Nafion®/GCE (8:1 ratio) when testing at region B, followed by UHC rGO/GCE, UHC rGO- Nafion®/GCE (2:1 ratio) and UHC rGO- Nafion®/GCE (4:1 ratio) (see results in text); * for $p < 0.05$, ** for $p < 0.01$ and * for $p < 0.001$.**

Electrode Type	UHC rGO:Nafion® Ratio	Current, I_{pa}(mA)
GCE	-	0.172
UHC rGO/GCE	-	0.583**
UHC rGO-Nafion®/GCE	8:1	0.698***
UHC rGO-Nafion®/GCE	4:1	0.669**
UHC rGO-Nafion®/GCE	2:1	0.562**
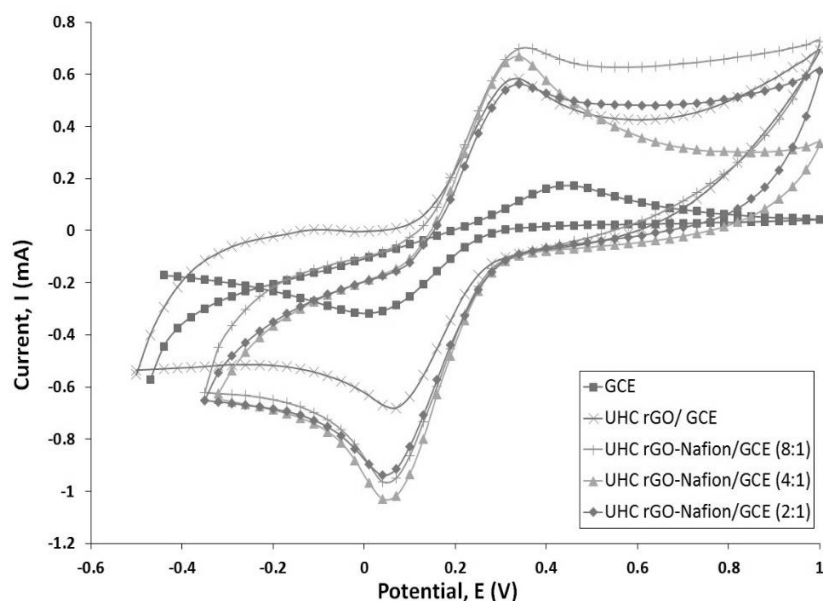
Fig. 4. Effect of different ratios of UHC rGO to Nafion® on redox peak current, showing highest anodic current value for the 8:1 ratio.

4. Conclusions

In this preliminary work, we have demonstrated that commercially available UHC GO (6 mg/ml) with more than 80% single-layer graphene content as a precursor is a promising graphene form with effective charge-transfer capability that is shown by the high anodic peak current. Adding an adequate ratio of AuNPs to the GO suspension helped increase the I_{pa}. The effect of adding Nafion® as a binder for graphene were also studied; the highest ratio of UHC rGO:Nafion® shows the highest anodic peak current in comparison to other ratios.

The role of Nafion® can be further studied in order to improve stability and longevity of the biosensor, either as part of the nanocomposite, acting as binder, or as a coating material that can protect the film deposited on the substrate from detachment. A statistical significance test was used to verify the difference in the anodic peak current values, where significant difference in current values implies that the current values are dependent on the materials used to modify the GCEs.

Future work includes observations of the modified electrodes using field emission scanning electron microscopy (FESEM) to see the morphology of the materials on electrodes and to correlate the results with the measured CV current values. Stability over time of the nanocomposite layer deposited on the substrate will also be studied.

Acknowledgement

Nomenclatures

I_{pa}	Anodic peak current
$K_3Fe(CN)_6$	Potassium ferricyanide
KH_2PO_4	Potassium dihydrogen phosphate
Na_2HPO_4	Disodium hydrogen phosphate

Abbreviations

Ag	Silver
Ag/AgCl	Silver/silver chloride
ANOVA	Analysis of variance
Au	Gold
AuNPs	Gold nanoparticles
CNTs	Carbon nanotubes
CV	Cyclic voltammetry
FESEM	Field emission scanning electron microscopy
GCE	Glassy carbon electrode
GO	Graphene oxide
Pd	Palladium
Pt	Platinum
rGO	Reduced grapheme oxide
UHC GO	Ultra-highly concentrated single-layer graphene oxide
WE	Working electrode

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