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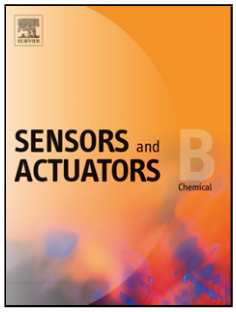

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Graphene dispersed cellulose microfibers composite for efficient immobilization of hemoglobin and selective biosensor for detection of hydrogen peroxide

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Research highlights

- Preparation of a novel and highly stable GR-CMF composite.
- Electrochemical behavior of Hb was studied at Hb immobilized GR-CMF composite.
- The high k_s of 6.17 s^{-1} was achieved for Hb immobilized GR-CMF composite.

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- A lower LOD (10 nM) was obtained for H_2O_2 using Hb immobilized GR-CMF composite

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Abstract

In the present work, we have investigated the electrochemical behavior and electrocatalysis of hemoglobin (Hb) immobilized on a glassy carbon electrode (GCE) modified with a graphene-cellulose microfiber (GR-CMF) composite. The GR-CMF composite was characterized by scanning electron microscopy, elemental analysis, and Raman and Fourier transform infrared spectroscopy. Well-defined electrochemical redox characteristics of Hb were observed for Hb immobilized on a GR-CMF composite modified GCE, with a formal potential of -0.306 V and a peak to peak separation of approximately 67 mV. Due to the high biocompatibility of the GR-CMF composite, the electrochemical behavior of the Hb heme redox couple (Fe^{II}/Fe^{III}) was enhanced for Hb immobilized on the GR-CMF composite when compared to Hb immobilized on pristine GR. The heterogeneous electron transfer constant (k_s) was calculated as 6.17 s^{-1}, and is higher than previously reported for Hb immobilized GR supports. The Hb immobilized GR-CMF composite modified electrode was used for the quantification of H_2O_2 under optimal conditions, and shows a wider linear amperometric response ranging from 0.05 to 926 μM. The limit of detection of the biosensor was 0.01 μM with the sensitivity of 0.49 $\mu A \mu M^{-1}$ cm^{-2}. The biosensor also showed high selectivity in the presence of the range of interfering compounds and exhibits good operational stability and practicality in the detection of H_2O_2.

Keywords: Graphene; cellulose microfibers; direct electrochemistry; hemoglobin; biosensor; H_2O_2

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1 1. Introduction

Recent advances in the nanomaterials indicate a wide range of promising applications for their use in biosensor systems [1] as commonly applied to the detection of toxins and pathogens in food, clinical, and environmental analysis [**2, 3**]. Given their high specificity, biosensors based on heme redox proteins are widely used for detection of small molecules such as hydrogen peroxide (H_2O_2) and nitrite (NO_2^-) in food and environmental samples [**4**]. Demonstrating greater stability than other commercially available redox heme proteins such as horseradish peroxidase, cytochrome C, and myoglobin, hemoglobin (Hb) is ideal for biosensor applications [**5, 6]**. Hb-based biosensors are particularly suited to the selective detection of H_2O_2 due to their high electrocatalytic activity and narrow target specificity. The accurate detection of H_2O_2 in food, biological and pharmaceutical samples is fundamental to a wide range of industrial applications [**7-10**]. However, effective immobilization of Hb on the electrode surface is a limiting factor in the efficiency of Hb based biosensors. Accordingly, different micro and nanomaterials or approaches have been explored as a means to anchor the redox active center of Hb to the electrode matrix.

Over recent years, carbon nanomaterials [**11-13**], metal oxides [**14**], metal nanoparticles [**15**], ionic liquids [**16**] and conducting polymers [**17**] have been utilized as immobilization matrices for Hb. In particular, the 2D carbon nanomaterial graphene (GR) exhibits electronic conductivity and thermal stability superior to other carbon nanoforms [**18, 19**], and make it an ideal support material in the fabrication of biosensors [**19, 20**]. However, the direct immobilization of Hb on pristine GR surface is problematic due to the molecules hydrophobic nature, and the Hb redox active center is located deep within the proteins tertiary structure [**21**]. Accordingly, the immobilization of redox active proteins such as Hb has necessitated modification of GR with appropriate biocompatible materials. For instance, carbohydrate polymers and supramolecular adducts are widely used as a dispersing agent for GR and the resulting composite may enrich the biocompatibility of GR for immobilization of redox active proteins [**22-26**]. As a natural, renewable, abundant, and biodegradable carbohydrate polymer, cellulose has been utilized in a wide range of industrial and medical applications [**27**]. In particular, hydrophobic, water insoluble cellulose microfibers (CMF) represent a promising biomaterial for enzyme immobilization in biosensors due to their unique chemical properties and high biocompatibility [**27**]. In addition, CMF exhibits a high surface area, high porosity, and bond with the variety of conductive materials [**27-30**]. In the present work, we have exploited the aforementioned properties of CMF and, by dispersing GR in a CMF aqueous solution, prepared a GR CMF composite for the immobilization of Hb. In doing so, the inherent nature of the hydrophilic CMFs acts to effectively prevent aggregation of GR and form a stable GR-CMF composite for immobilization of Hb.

A review of the published literature indicates the great majority of GR and cellulose composites have been prepared by chemical reduction of graphene oxide and cellulose [**28–30**], but none have demonstrated the direct preparation of GR-CMF composite. However, we have recently demonstrated the direct preparation of GR-CMF composite as an immobilization matrix for laccase [**31**]. In the present work, we evaluate the electrochemical redox characteristics of Hb immobilized on a GR-CMF composite modified electrode and discuss this in relation to comparable modified electrodes for the immobilization of Hb. An H_2O_2 biosensor was fabricated

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based on the Hb immobilized GR-CMF composite modified electrode, and the detection parameters quantified using an amperometric method.

2 2. Experimental

2.1 *2.1. Material and methods*

The cellulose microfibers (medium) powder was purchased from Sigma Aldrich. Graphene 8 nm nanoflakes were obtained from UniRegion Bio-Tech, Taiwan. H_2O_2 (30 %) was received from Wako Pure Chemical Industries. Human blood serum samples were received from valley biomedical, Taiwan product & services Inc., and was approved by the ethics committee of Chang-Gung memorial hospital in contract no. IRB101-5042A3. Commercial contact lens cleaning solution was purchased from China Chemical and Pharmaceutical, Taipei, Taiwan. The whole milk was purchased from a local department store in Taipei, Taiwan. The supporting electrolyte was pH 7 phosphate buffer and was prepared with 0.05 M Na_2HPO_4 and NaH_2PO_4 in double distilled water. Adjustments to pH were made with 0.1 M H_2SO_4 and 0.1 M NaOH.

The surface morphologies of the as-prepared materials were characterized using an Hitachi S-4300SE/N High Resolution Schottky Analytical VP scanning electron microscope (SEM). Elemental analysis (EDS) and elemental mapping of the composite were performed using Hitachi S-4300SE/N High Resolution Schottky Analytical VP SEM attached BRUKER AXS elemental analyzer. Fourier transform infrared (FTIR) spectroscopy was acquired using a JASCO FTIR-6600 spectrometer. Raman spectra for the materials were taken using a Dong Woo 500i Raman spectrometer equipped with a charge-coupled detector. Cyclic voltammograms and amperograms (amperometric *i-t* curve) were taken using CHI1205B electrochemical workstation from CH Instruments. Hb immobilized on a GR-CMF modified glassy carbon electrode (GCE) was used as a working electrode, where the apparent electrode surface of the GCE was approximately 0.079 cm^2. Saturated Ag|AgCl and Pt wire were used as a reference and auxiliary electrodes respectively. Amperometric *i-t* measurements were performed using a PRDE-3A (ALS Co., Ltd, Japan) rotating ring disc electrode (RDE), in which the geometric area of the electrode is 0.08 cm^2. The electrochemically active surface area (EASA) of the GR-CMF composite modified RDE was calculated as 0.27 cm^2, and was calculated using Randles–Sevcik equation by cyclic voltammetry response of 1 mM ferricyanide with 0.05 M KCl [**32**].

2.1.1 *2.2. Fabrication of the biosensor*

To fabricate the biosensor, first, the GR-CMF composite was prepared by dispersing GR (5 mg mL^{-1}) into the CMF solution using ultrasonication for approximately 30 min. The stable CMF solution was prepared by the addition of 10 mg mL^{-1} of CMF into the doubly distilled water and sonicated for 45 min at 10°C. Then, about 6 µL of GR-CMF

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composite solution was dropped on pre-cleaned GCE and allowed to dry in an air oven. Once dry, 6 µL of Hb solution (optimum) was dropped on the as-prepared GR-CMF composite modified electrode and dried at room temperature. Then, the resulting Hb immobilized GR-CMF (GR-CMF/Hb) composite modified electrode was used for further electrochemical studies. The schematic representation of the biosensor fabrication is shown in **Scheme 1**. The fresh Hb stock solution was prepared by dissolving 5 mg mL^{-1} of Hb at pH 7 and was stored at -4˚C when not in use. The Hb immobilized GR modified electrode was prepared by drop coating of 6 µL of Hb solution on GR modified electrode, while the GR dispersion was prepared by dispersing of 5 mg mL^{-1} of GR into the dimethylformamide (DMF) using ultrasonication for 30 min. The Hb immobilized CMF modified electrode was prepared by the same method. Optical images of GR-DMF, CMF and GR-CMF composite are shown in **Fig. 1D**. All electrochemical measurements were performed in oxygen-free atmosphere by purging high purity N_2 into pH 7 for at least 10 min, and the modified electrodes were stored under the dry condition when not in use.

3. Results and discussion

3.1. *Characterizations*

The surface morphological studies of the GR, CMF, and as-prepared GR-CMF composite were characterized by high-resolution SEM. **Fig. 1** shows SEM images of pristine GR (A), CMF, (B) and GR-CMF composite (C). The SEM images shows the closely arranged layering of relatively small individual pristine GR nanosheets, and the typical dense fiber morphology of CMF. It is clear from **Fig. 1C** that the GR nanosheets were highly exfoliated by

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CMF compared with pristine GR. In addition, the optical image of GR-CMF composite (**Fig. 1D**) confirms the formation of GR-CMF composite and CMF is a suitable dispersing agent for GR. The GR-CMF composite was found to be highly stable even after storage for six days. We also performed EDS and elemental mapping of the GR-CMF composite and the results are shown in **Fig. S1 and Fig. 2A and B**. The EDS and elemental mapping of GR-CMF composite confirm the presence of carbon and oxygen in the composite, while oxygen is absent in the EDS and elemental mapping of pristine GR (not shown). The result also supports formation of GR-the CMF composite.

FTIR spectroscopy has been a powerful tool to investigate the different functional groups present in the compounds and interactions between the compounds in the composite. **Fig. 3A** shows the FTIR spectra of GR (a), CMF (b) and GR-CMF (c). The FTIR spectrum of CMF shows a characteristic vibration band at 3300–3500 cm^{-1}, and corresponding to stretching vibrations of the OH group [**31**]. Analysis of CMF showed two additional bands at 2892 and 1652 cm^{-1}, due to stretching of CH and CH_2, and OH from absorbed water [**31**]. The FTIR spectrum of GR was found to be featureless in the finger print region and is similar to that previously reported for GR [**25**]. However, the FTIR spectrum of GR-CMF composite showed a similar characteristic band those compared with the FTIR spectrum CMF, which confirms the firm attachment of CMF to the GR surface.

Raman spectroscopy is widely used to study the allotropes of carbon, especially GR nanostructures, due to its sensitive nature to a positioning of the carbon atoms. As shown in **Fig. 3B**, the Raman spectra of GR and GR-CMF exhibits a strong G band at 1592 and 1591 cm^{-1}, and due to the in-plane vibrational modes of sp^2 hybridized carbon atoms on GR [**25**]. The less intense D band at 1888 and 1387 cm^{-1}, is ascribed to the vibrations of sp^3 carbon atoms of disordered GR and vibrations of sp^2 carbon atom domains of graphite [**33**]. Usually,

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the D band is less active and G band is highly active in few-layered GR than multi layered GR sheets. The 2D bands of were observed at 2726cm^{-1} and 2724 cm^{-1} on GR and GR-CMF composite, is due to the two phonon lattice vibrational process on GR sheets [**33**]. Peak intensity ratio (I_{2D}/I_G) of the 2D and G bands of GR and GR-CMF was found as 0.92 and 0.91. The result confirms that GR-CMF composite has few layered GR sheets with less defects than pristine GR.

2.1.2 *3.2. Electrochemical behavior of Hb at different modified electrodes*

The redox activity site of Hb is acknowledged as the cofactor heme (Fe^{II}/Fe^{III}) and is responsible for its direct electrochemical behavior on the electrode surface. Hence, we have investigated the electrochemical behavior of Hb immobilized at the surface of different modified electrodes by CV. The CV measurements were carried out in N_2 atmosphere with a scan rate of 100 mV/s and a potential range of -0.7 to 0.2 V. The electrochemical response of Hb immobilized at CMF (a), GR (b), and GN-CMF composite (c) modified electrodes is shown in **Fig. 4**. The Hb immobilized CMT modified electrode did not show the redox behavior we expected for Hb, and shows only a cathodic peak response at -0.306 V with reversible anodic peak. This result indicates that electrochemical redox of Hb is not favorable at the CMF modified electrode. A less intense electrochemical redox behavior of Hb was observed for the GR modified electrode and the anodic and cathodic peak potentials were located at -0.362 and -0.282 V. The peak-to-peak separation was 80 mV. However, the electrochemical redox behavior of Hb was greatly enhanced when immobilized on the GR-CMF modified electrode, with a redox current intensity 3 fold higher than observed on GR. The anodic and cathodic peak of redox couple was located at –0.372 and 0.242 V, with the peak-to-peak separation of 130 mV. The result indicates the direct electrochemical behavior of Hb to be greatly enhanced on GR-CMF composite when compared to Hb immobilized on pristine GR. The good biocompatibility of CMF provides a suitable matrix for orientation of Hb on the composite electrode surface. As shown in **Fig. S2a**, the GR-CMF composite did not show any obvious response in the absence of Hb, which indicates the redox electrochemical activity to be dependent on the presence of a Hb heme.

The surface concentration (Γ^-) of immobilized Hb on GR-CMF composite modified electrode was determined as 0.45×10^{-10} mol cm^{-2} and was calculated using Q/nFA. Where Q is the total charge, n is the number of electrons transferred in the redox reaction, F is the Faraday constant, and A is the electrochemically active surface area of the biosensor. The obtained Γ^- value of the biosensor is higher than that of previously reported for Hb immobilized on nanomaterial modified electrodes [**34–37**], and indicates a greater efficiency of Hb molecules adsorption on the composite electrode surface. We have also performed CV to determine the effect of scan rate (ranging from 50 to 1000 mV/s) on the electrochemical redox behavior of the Hb immobilized GR-CMF modified electrode. As shown in **Fig. S3A**, the redox peak current of Hb increases with the scan rate, and corresponding potentials showed a greater degree of positive and negative shift as the scan rate increased from 50 to 1000 mV/s. We have also made the plot for scan rate vs. anodic and cathodic peak current of Hb and the results are shown in **Fig. S3B**. It can be seen that the anodic and cathodic redox peak current of Hb has a linear dependence on the scan rate. The linear regression equations were expressed as: I_{pa} (μA) = 0.4446 + 2.9878 mV/s (R^2 = 0.9959) and I_{pc} (μA) = -0.0419 – 0.8512 mV/s (R^2 = 0.9975). The result indicates that the electrochemical redox behavior of Hb was a surface controlled electrochemical process on the GR-CMF composite electrode. Additionally, the Laviron equation was used to calculate the heterogeneous

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electron transfer rate constant (k_s) of Hb immobilized GR-CMF composite modified electrode [34], as 6.63s^{-1}. This high k_s value of the Hb immobilized GR-CMF composite indicates that the GR-CMF composite facilitates the direct electrochemical activity of the Hb heme, and facilitates fast direct electron transfer to the electrode surface. Furthermore, the calculated k_s value is higher than previously reported Hb immobilized nanomaterials modified electrodes [**6, 7, 14, 26, 35–38**], and indicates that GR-CMF composite is a more efficient Hb immobilization matrix than previously reported GR modified electrodes.

As a biomolecule, the direct electrochemical behavior of Hb is pH dependent. Hence, the effect of electrolyte pH on the electrochemical redox activity of Hb immobilized GR-CMF composite modified electrode was studied by CV (not presented here). Hb electrochemical activity was characterized in N_2 saturated different pH (pH 5, 7, 9 and 11) at a scan rate of 100 mV/s. The obtained results are plotted in **Fig. S3C**. The formal potential ($E^{0'}$) of Hb shows a linear dependence over this pH range, and the linear regression equation was $E^{0'}$ = -0.0583 + 0.1167 V/pH with the correlation coefficient of 0.9887. The obtained slope value (-58.3 mV/pH) is much closer to the reported theoretical value of the Nernstian equation for a reversible electrochemical process involving an equal number of electrons and protons [**35**]. The redox electrochemical mechanism of Hb was extensively studied on carbon nanomaterial modified electrodes, and our results are similar to the previous reports for a one proton and electron transferred redox reaction of the heme redox active center [**26, 35**].

2.1.3 *3.3. Electrocatalytic reduction of H_2O_2*

The electrocatalytic activity of Hb immobilized GR-CMF composite modified electrode was further examined by CV. **Fig. S4** shows the CV response of biosensor in the absence (a) and presence (c) of 500 µM H_2O_2 into the PBS. A stable and well-defined redox couple of Hb was observed in the absence of H_2O_2. However, the cathodic peak current of the Hb heme was dramatically increased in the presence of 500 µM H_2O_2 due to the reduction of H_2O_2 by immobilized Hb. In the absence of Hb, the GR-CMF composite modified electrode did not show the obvious reduction peak current response to H_2O_2 (curve b), confirming the enhanced reduction current of H_2O_2 is due to the presence of heme redox active center of immobilized Hb on GR-CMF composite. The result indicates that Hb immobilized GR-CMF composite modified electrode can be used for sensitive and low potential detection of H_2O_2.

Amperometric *i-t* method was used for the quantification of H_2O_2 using Hb immobilized GR-CMF modified electrode. The working potential of the biosensor was fixed at -0.3 V and is close to the reduction potential of H_2O_2 by heme redox active center of Hb (**Fig. S4)**. Under optimized conditions, the amperometric *i-t* was performed for Hb immobilized GR-CMF modified RDE according to H_2O_2 concentration by additions of H_2O_2 into a constantly stirred pH 7. The obtained amperometric results are shown in **Fig. 5A**. It can be clearly seen that the biosensor exhibited a stable and well-defined amperometric response for the addition of different concertation of H_2O_2. As shown in the upper inset, the biosensor shows a stable response for the addition of 0.05, 0.1, 0.5, 1.0, 3.0 and 5.0 µM H_2O_2. The response time of the biosensor was calculated as 4 s, and indicates a fast electrocatalytic reduction of H_2O_2. As shown in the lower inset, the amperometric response of the biosensor was linear over the H_2O_2 concentrations ranging from 0.05 to 926 µM with a correlation coefficient of 0.9965. The limit of detection (LOD) of the biosensor was calculated as 0.01 µM based on a signal-to-noise ratio equal to 3

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(S/N=3). The sensitivity of the biosensor was 0.49 $\mu A \mu M^{-1}$ cm^{-2} and was calculated using slope/EASA. We have compared the analytical performance of the as-prepared biosensor with previously reported enzymatic H_2O_2 sensors, and the results are shown in **Table 1**. The comparative results clearly show that our biosensor has a superior analytical performance (low LOD, high k_s, wider linear response range and sensitivity) towards the detection of H_2O_2 [**16, 23, 26, 35, 39–44**]. Accordingly, the as-prepared biosensor is more suitable for the sensitive and low-level detection of H_2O_2. The high conductivity and biocompatibility of the GR-CMF composite provide a matrix suited to the immobilization of Hb, and result in optimal H_2O_2 detection demonstrating a wider response range, greater sensitivity, lower LOD, and a fast response. The catalytic activity and enzyme–substrate kinetics of immobilized Hb on GR-CMF composite was evaluated using Michaelis Menten constant (K_M^{app}), and was calculated from the Lineweaver–Burk equation ($1/I_{ss} = 1/I_{max} + K_M^{app}/(I_{max} C)$). The reciprocal of the steady-state current (I_{ss}) vs. reciprocal of [H_2O_2] was plotted, and showed that I_{ss} had a linear relationship with the reciprocal of [H_2O_2] with a correlation coefficient of 0.9957. The K_M^{app} was determined from the obtained slope (11.555) and intercept (0.028), where slope and intercept is equal to K_M^{app}/I_{max} and $1/I_{max}$. The apparent K_M^{app} was calculated as 413 µM. The obtained K_M^{app} was higher than previously reported Hb immobilized graphene/carbon fiber (80.0 µM) [**16**], graphene/chitosan (344.0 µM) [**23**], graphene/zinc oxide/gold nanoparticles (170.0 µM) [**39**], multi-walled carbon nanotubes-poly-l-histidine/zinc oxide (140.0 µM) [**41**], graphene/Fe_3O_4 (3.7 µM) [**43**] and palladium nanoparticles/graphene–chitosan (16.0 µM) [**44**] modified electrodes. The high value of K_M^{app} indicates the high biological affinity of the biosensor towards H_2O_2.

2.1.4 *3.4. Selectivity of the biosensor*

Since H_2O_2 interacts with ascorbic acid, dopamine, uric acid, glucose, and L-cysteine, selectivity of the as-prepared biosensor was evaluated by amperometry in the presence of interfering species [**26**]. **Fig. 5B** shows the amperometric response of the Hb immobilized GR-CMF modified electrode in response to the addition of 1 µM H_2O_2 (a), and 500 µM additions of ascorbic acid (b), dopamine (c), uric acid (d), epinephrine (e), l-cysteine (f), norepinephrine (g) and glucose (h) to N_2 saturated pH 7 with an operational working potential of -0.3 V. The biosensor showed a sharp and stable amperometric response for the addition of 1 µM H_2O_2 (a), while the addition of each interfering species elicited no discernible current response. These results indicates the high specificity of the biosensor is due to the high specificity of immobilized Hb and negative working potential (-0.3 V). Hence, the as-prepared biosensor is suited to the selective detection of H_2O_2 in environmental or clinical samples.

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We have also investigated the operational stability of the biosensor by amperometry and the results are shown in **Fig. 5C**. The working conditions are similar to **Fig. 5A**. It can be seen that the amperometric background current response of the biosensor was 97.8% stable even after the continuously run up to 2000 s in pH 7.0, and reflecting the highly stable nature of Hb immobilized on the GR-CMF composite. The CV was further used to evaluate the repeatability and reproducibility of the biosensor for detection of 500 μM H_2O_2 and with the experimental conditions similar to **Fig. S4**. We used six independently prepared biosensors for the detection of 500 μM H_2O_2, and obtained a relative standard deviation (RSD) of approximately 4.3%. By way of comparison, a single biosensor showed an RSD of 2.7% for detection of 500 μM H_2O_2 across 10 separate solutions. The observed RSD values of the biosensor are well within acceptable limits; hence the biosensor is suited to precise detection of H_2O_2. The fabricated biosensor was also tested for long-term storage stability (up to 35 days) by CV and was stored at 4 ºC under dry conditions. The prepared biosensor was tested for every five days by CV in response to 500 μM H_2O_2 and experimental conditions similar to **Fig. S4**. As shown in **Fig. S5**, the as-prepared biosensor retained 84.2 and 81.1% of its initial sensitivity after the 20 and 35 days storage respectively, which indicating excellent stability.

3.5. Determination of H_2O_2 in food, biological and pharmaceutical samples

As a proof of concept, we have evaluated the biosensors performance in amperometric determination of H_2O_2 in real samples. The amperometric experimental conditions are similar to **Fig. 5A**. We have used whole milk, contact lens cleaning solution (3% H_2O_2) and human serum for the real sample analysis. The whole milk and human serum samples were H_2O_2 free and used as received. The contact lens cleaning solution was diluted with pH 7 and the unknown concentration of H_2O_2 was determined using the as-prepared biosensor. Known concentrations of H_2O_2 (1.0 and 2.0 μM) contained in whole milk, contact lens cleaning solution, and human

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serum were injected into the pH 7 phosphate buffer and their recoveries were calculated using a standard addition method. The obtained recoveries of H_2O_2 were summarized in **Table S1**. The average recoveries of H_2O_2 were 93.0, 98.5 and 99.0% with a RSD of 3.5, 3.1 and 2.8% in whole milk, contact lens cleaning solution, and human serum samples respectively. The good recovery and appropriate RSD of the biosensor further validates that it the application of the biosensor for real-time detection of H_2O_2 in food, biological and pharmaceutical samples.

4. Conclusions

In summary, we have developed a sensitive and selective H_2O_2 biosensor based on the electrochemical redox behavior of Hb immobilized on GR-CMF composite. Physicochemical characterizations confirms the formation of the GR-CMF composite. The combined unique properties of GR-CMF composite facilitates the direct electron transfer between Hb and electrode than GR and CMF modified electrodes. The as-prepared biosensor exhibits good electrocatalytic activity and analytical performance (low LOD (10 nM), high sensitivity (0.49 $\mu A \mu M^{-1}$ cm^{-2}), fast response (~3 s) and a broad linear response range (up to 926 μM)) towards H_2O_2. The biosensor performance for real samples demonstrates its suitability to real time monitoring of H_2O_2 in food, biological and pharmaceutical samples. The presented research points the way for the development of further biosensors based on the superior immobilization efficiency and electron transfer characteristics of the GR-CMF composite.

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Conflict of interest

We declare that we do not have any conflict of interest.

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Table 1 Comparison of the analytical performance of GR-CMF/Hb biosensor with the previously reported Hb based biosensors for the determination of H_2O_2.

Biosensor	E_{app} (V)	LOD (nM)	LRR upper limit (μM)	Ks (s)	Sensitivity ($\mu A \mu M^{-1} cm^{-2}$)	Ref.
Hb-MWCNT/ZnO/GCE	−0.34	20.0	516.0	1.26	3.66	[26]
Hb-GR-CS/GCE	−0.4	510.0	230.0	NR	NR	[23]
Hb-GR-GTN/GCE	−0.32	40.0	786.6	3.82	0.048	[35]
Hb-GR-CFE	−0.36	2000.0	210.0	1.93	1.4	[16]
Hb-GR/ZnO-AuNPs/GCE	−0.3	80.0	1130.0	1.3	NR	[39]
Hb-GR-MWCNT/CILE/GCE	NR	15.3	210.0	1.03	NR	[40]
Hb-MWCNT–His–ZnO/GCE	−0.25	10.0	18000.0	5.16	0.021	[41]
Hb-Fe_3O_4-GR/CCE	−0.3	500.0	585.0	0.91	NR	[42]
Hb-GR-Pt/GCE	−0.26	1000.0	585.0	0.92	NR	[43]
Hb-GN/CS/PdNPs/GCE	NR	660.0	1100.0	0.86	0.002	[44]
GR-CMF/Hb/GCE	−0.3	10	926	6.3	0.49	Present work

Abbreviations

LRR – linear response range; LOD – limit of detection; MWCNT – multi-walled carbon nanotubes; GCE – glassy carbon electrode; NR – not reported; GR – graphene; CS – chitosan; GTN – gelatin; CFE – carbon fiber electrode; NPs – nanoparticles; His – histidine; GN – graphene

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3 Biographies

<remove picture pageno -1>Dr. Vijayalakshmi Velusamy is a Senior Lecturer in Electronic Engineering in the Division of Electrical and Electronic Engineering at Manchester Metropolitan University, UK. She holds a PhD (2012) in DNA Biosensor Development from University of Limerick, Ireland, Master's degree (2007) in Micro and Nanotechnology from University of Ulster, UK and a Bachelor's degree (2002) in Electrical and Electronic Engineering from Bharathiar University, India. Her multidisciplinary research focuses on the development of novel integrated sensors/biosensors for a wide range of applications such as environmental monitoring, biosecurity, wearable devices, disease biomarker detection and point-of-care and smart medical devices.

<remove picture pageno -1>Dr. Selvakumar Palanisamy received his B.Sc (2006), M.Sc (2009) and M. Phil (2010) degrees in Chemistry from Madurai Kamaraj University, Tamilnadu, India. He obtained his PhD degree (2014) in Chemical Engineering from National Taipei University of Technology (NTUT), Taiwan. Currently, he is working as a post-doctoral fellow in Prof. Shen-Ming Chens' lab at NTUT. His current research interest include the synthesis of nanomaterials and applications related to the electrochemical sensors and biosensors. He has published over 75 research articles in SCI journals. He also serves as a consulting editor of Journal of reports in electrochemistry.

<remove picture pageno -1>Dr. Shen-Ming Chen received his B.S. Degree in Chemistry in 1980 from National Kaohsiung Normal University, Taiwan. He received his M.S. Degree (1983) and Ph.D. degree (1991) in Chemistry from National Taiwan University, Taiwan. He is currently a professor at the Department of Chemical Engineering and Biotechnology, National Taipei University of Technology, Taiwan. His current research interests include electroanalytical chemistry, bioelectrochemistry, fabrication of energy conservation and storage devices and nanomaterial synthesis for electrochemical applications. He has published more than 550 research articles in SCI journals.

<remove picture pageno -1>Mr. Tse-Wei Chen received his B.Sc. (2016) degrees in chemistry from Fu Jen Catholic University, Taiwan. Currently, he is a M.S. student in the department of Chemical Engineering and Biotechnology at National Taipei University of Technology. He specializes in electrochemical sensors and biosensors based on modified electrodes. His research interest also includes the synthesis of nanomaterials for different applications including graphene based composites.

<remove picture pageno -1>Dr. Sayee Kannan Ramaraj received his M.Sc. (1992) degree in Chemistry from Madurai Kamaraj University. He was the gold medalist in Master of Philosophy in School of Chemistry, Madurai Kamaraj University. He obtained his Ph.D. (2003) in School of Chemistry, Madurai Kamaraj University. He is currently an assistant professor at the Department of Chemistry, Thiagarajar College, Madurai, India. He is also a visiting professor at the National Taipei University of Technology, Taiwan. He specializes in polymeric composites, Corrosion science, electrochemical sensors and biosensors.

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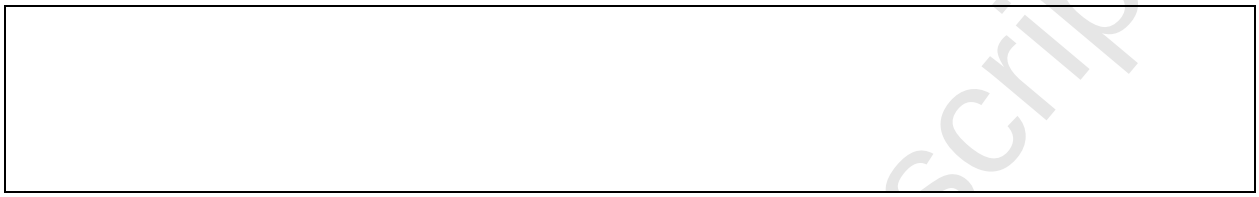
<remove picture pageno -1>Ms. Sonadevi Selvam received her B.Sc degree (2010) from Sri Meenakshi College for Women in Madurai, Tamil Nadu, India. She received her M.Sc degree (2012) from VVV College for Women, Virudhunagar, Tamil Nadu, India. She obtained her M.Phil. degree (2013) from NMSSVN College, Madurai. Currently she is working as an Assistant professor in Department of Chemistry, PSR Engineering College, Sevalpatti, Sivakasi. Her research interest mainly focuses on the synthesis of nanomaterial for electrochemical sensors, biosensors and energy devices.



<remove picture pageno -1>Dr. Bih-Show Lou received her B.S. Degree in Chemistry in 1984 at the National Kaohsiung Normal University, Taiwan. She then went to University of New Mexico, USA where she received her Ph.D. with Prof. Mark R. Ondrias in Biophysical Chemistry in 1993, then was a postdoctoral fellow at University of Arizona with Victor J. Hruby, Regents Professor. She is currently a professor at the Chemistry Division, Center for General Education, Chang Gung University, Taiwan. Her current research interests include spectroscopy for chemical and nanomaterial analysis, bioelectrochemistry for electrochemical and biosensor applications.

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4 Figure captions

Scheme 1 Schematic representation of the fabrication of Hb immobilized GR-CMF biosensor and its electrochemical reduction of H_2O_2.

Figure 1 SEM images of GR (A), CMF (B), GR-CMF composite (C). D) The corresponding optical images of GR dispersed in DMF (a), CMF (b) and GR dispersed in CMF after six days storage.

Figure 2 Elemental mapping of carbon (A) and oxygen (B) in GR-CMF composite.

Figure 3 A) FTIR spectra of CMF (a), GR (b) and GR-CMF composite (c). B) Raman spectra of pristine GR (a) and GR-CMF (b).

Figure 4 CV response of Hb immobilized on CMF (a), GR (b) and GR-CMF composite (c) modified electrodes in pH 7 at a scan rate of 100 mV/s.

Figure 5 A) Amperometri**c** *i-t* response of Hb immobilized GR-CMF modified RDE for the different concentration additions (0.05–976 μM) of H_2O_2 into the constantly stirred pH 7 with a working potential of –0.3 V. Upper inset shows the amperometric response of Hb immobilized GR-CMF modified RDE for the addition of 0.05, 0.1, 0.5, 1.0, 3.0 and 5.0 μM H_2O_2 into the constantly stirred pH 7. Lower inset is the linear plot for amperometric current response vs. [H_2O_2]. B) Amperometric *i-t* response of Hb immobilized GR-CMF modified RDE for the addition of 1 μM H_2O_2 (a), and 100 μM additions of ascorbic acid (b), dopamine (c), uric acid (d), epinephrine (e), l-cysteine (f), norepinephrine (g) and glucose (h) into the constantly stirred pH 7. Working potential = -0.3 V. C) Operational stability of the Hb immobilized GR-CMF modified RDE for the addition of 100 μM H_2O_2 in pH 7 with a working potential of –0.3 V.

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Scheme-1

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Figure-1

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