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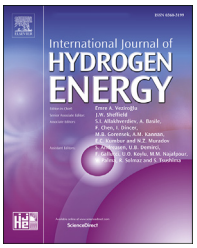

High-performance enzymatic biofuel cell based on three-dimensional graphene

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HIGHLIGHTS

- The performance of the enzymatic biofuelcell has enhanced.
- 3-dimensional graphene improve the electron transfer.
- 3-dimensional graphene decrease enzyme leaking.
- 3-dimensional graphene facilitate DET.
- Power density of 164 μW cm^{-2} achieved.

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ABSTRACT

Enzymatic biofuel cells are a subclass of biofuel cells, which employ enzymes to generate energy from renewable sources. In this study, 3-dimensional graphene (3DG)/glucose oxidase (GOx) bio-nanocomposite was fabricated in order to improve enzyme immobilisation and enzyme lifetime with an enhanced electron transfer rate. These enhancements are due to the unique physical properties of 3DG, e.g. high porosity, large surface area, and excellent electrical conductivity. A power density of 164 μW cm^{-2} at 0.4 V was achieved from this enzymatic biofuel cell (EBFC) with an acceptable performance compared to that of the other glucose biofuel cells (GBFCs). The 3DG enhances the enzyme lifetime, decreases enzyme leaking and, due to its good conductivity, facilitates the electron harvest and transfer from the enzyme active site to the electrode. This suggests that 3DG could be

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Bio-electrocatalysis
Bioanodes
3D graphene

used as effective support for enzyme immobilisation on the surface of the electrode in EBFC applications and related areas such as biosensors, bioreactors and implantable biofuel cells.

Introduction

Biofuel cells are a favourable next-generation green energy alternative [1]. Enzymatic biofuel cells, a subclass of biofuel cells, utilise enzymes to generate electricity from renewable fuels such as carbohydrates, urea and organic acids through electrochemical oxidation occurring at the anode [2,3]. They are unique compared to other conventional energy systems because they are cost-effective, generate electricity from renewable sources, enable enzyme selectivity towards the fuel and can be utilised in physiological pH and temperature [4], though they have smaller power density and energy density [5,6]. These benefits make them a suitable choice for powering up implantable medical devices such as pacemakers and micro-drug pumps, and they are even used in wastewater treatment [7–9], drug delivery [10], biosensors [11], remote sensing and communication devices in bioelectronics [12,13]. Glucose oxidase (GOx) is the most preferred enzyme in enzymatic biofuel cells (EBFCs), due to the availability and high selectivity towards glucose [14].

Biofuel cells utilise the abundance of glucose or other carbohydrates present in animal fluids. The most challenging part in cell fabrication is the efficient immobilisation of the enzyme on the electrode surface [15,16]. The limitations of GOx immobilisation on the electrode surface include the enzyme leaching, shorter enzyme lifetime and the poor electron transfer between the enzyme active site and the electrode, which accounts for the lower energy density and power density [17,18].

Based on our knowledge, several methods have been developed to increase the biological function, stability and efficacy of GOx immobilisation on the electrode surface. The entrapment or covalent immobilisation of GOx in stable matrixes such as nanostructured and mesostructured metal oxides [19,20], metal nanoparticles [21,22], conducting polymers [23–25], mesostructured silica [26], sol-gel matrixes [27,28], carbon nanotubes [29,30], graphene [31–33], etc. leads to a faster electron transfer rate and improved enzyme stability [34]. The enzymes' immobilisation platforms prepared from nanostructured composite and hybrid materials such as nanoparticles, nanofibers and nanocarbons are the focus of intense fundamental and applied research [35]. Graphene, a carbon polymorph, is a critical material which immobilises the enzyme and enhances the electrical conductivity between the enzyme active site and the electrode surface [36].

Graphene possesses extraordinary physicochemical properties such as a highly specific surface area [37,38] and an easily modified surface area [39,40], in addition to excellent mechanical, thermal and chemical stability [41] and excellent electronic properties. Thus, graphene is increasingly used in various applications such as catalysis, sensors, environmental remediation, and energy storage and conversion.

The large surface area of graphene-based nanomaterials and the excellent electrical conductivity create ideal immobilisation support for biomolecules such as enzymes [42–46]. Numerous studies on the direct electron transfer (DET) behaviour of GOx on graphene decorated glassy carbon electrode (GCE) are also reported [47–51]. In recent years, there have been several reports on graphene-based modified electrodes for biofuel cell applications [52,53]. Despite various applications of EBFCs, some drawbacks persist such as enzyme leakage and low power density due to the sluggish electron transfer between the enzyme and the electrode surface, which is the principal research focus in EBFCs [54]. In order to overcome these drawbacks, a GCE/3DG-GOx bio-nanohybrid was prepared and utilised as the bioanode in EBFCs. The results in this work were encouraging, as the 3DG biocatalyst support enhanced the electrical conductivity between the GOx enzyme and the GCE.

Experimental methodology

Chemicals

Graphite powder (<45 μm; ≥99.99%), H_2SO_4 (0.5 M), H_3PO_4 (85%), $KMnO_4$ (97%), Na_2HPO_4, NaH_2PO_4, KCl and GOx (type vii from *Aspergillus niger*) were procured from Sigma-Aldrich (Dorset, UK). The supporting electrolyte in the electrochemical experiments was 0.1M of phosphate buffer solution (pH 7), prepared by dissolving equimolar Na_2HPO_4 and NaH_2PO_4 with KCl in deionised water.

Instrumentation

All electrochemical experiments were performed using a computer-controlled 302N Autolab potentiostat/galvanostat (Ecochemie, Netherlands) with a conventional three-electrode system in a 50 ml cell. The modified glassy carbon electrode (GCE, diameter 3 mm) was the working electrode, while a thin Pt wire and Ag/AgCl (3M KCl) were the counter and reference electrodes, respectively. The Raman spectra of the 3D-graphene composite were obtained using a Renishaw inVia™ (M005-141) confocal Raman microscope employing a 532 nm excitation wavelength to confirm the reduction of graphene. The Fourier transform infrared (FTIR) spectroscopy of samples was characterised by a Bruker IFS 66/S FTIR with a 4 cm^{-1} resolution. The resolution of the FTIR was set to 4 cm^{-1}, with 16 scans in the wavelength range of 400–4000 cm^{-1}.

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Preparation of bioanode

Preparation of 3DG

The initial step for the development of 3DG is the synthesis of graphitic oxide from graphite flakes using the modified Hummer's method [55,56]. Graphene oxide (GO) was obtained through the oxidation of 1 g of graphite flakes with 120 ml of H_2SO_4, 13 ml of H_3PO_4 and 6 g of $KMnO_4$. The mixture was stirred for 3 days to complete the oxidation of graphite. During oxidation, the colour of the mixture changed from dark purple-green to dark brown. To stop the oxidation process, about 150 g of ice cube was added into the reaction, followed by 7 ml of H_2O_2 solution, with the colour of the mixture changing to bright yellow due to the severe oxidation of graphite. The graphitic oxide was washed three times with 1 M of HCl with centrifugation and repeatedly washed with deionised water until the pH reached 4–5. During the washing process with deionised water, the graphitic oxide was exfoliated, resulting in the thickening of the GO solution due to the formation of GO gel.

A mixture of 1 ml GO aqueous dispersion, and 40 ml of deionised water was stirred for 20 min to prepare a homogeneous solution. Then, 10 μl of hydrazine was added into the mixture with stirring, and the pH was adjusted to 9. The dispersion was sealed in a 50 ml stainless steel autoclave and maintained at 80 °C for 24 h. The prepared 3DG was removed with tweezers and kept in a freeze drier for 48 h in order to remove absorbed water. The dried 3DG was stored in a sealed Schott bottle for the next step.

Fabrication of 3DG modified glassy carbon electrode (GCE)

The synthesised 3DG (10 mg) was placed in a test tube containing 5 ml of ethanol, ultrasonicated for 30 min to provide a homogeneous dispersion and left to cool to room temperature for 10 min. In the following step, 5 mg of glucose oxidase (GOx) enzyme was added into the solution and mixed gently before being stored in a fridge.

The GCE (3 mm diameter) was polished with 0.05 mm of alumina slurry on a velvet pad. Before the modification, the GCE was placed in a beaker containing 1:1 v/v double distilled water and ethanol. Then the GCE was ultrasonicated for 5 min, washed with deionised water and left to dry at room temperature (25 ± 3 °C). After drying, 5 μL of the prepared 3DG-GOx dispersion was drop-casted onto the GCE surface and left to dry at room temperature (25 ± 3 °C) for 15 min.

Results and discussions

Spectroscopic studies

Raman spectroscopy is a powerful tool to characterise the presence of disorder in carbon nanostructures. Fig. 1(A) shows the comparison between the Raman spectra of GO and 3DG.

As can be seen in Fig. 1(A), the spectra of all samples show two peaks at around 1584 cm^{-1} and 1351 cm^{-1}, related to the G and D bands of RGO, respectively. The G band is assigned to the first-order scattering of the E_{2g} phonon, while the D band is attributed to the decreasing size of the sp^2 domains due to the extensive oxidation on the GO nanosheets [57]. The ratio between the D and G band intensity (I_D/I_G) represents the amount of disorder, which is expressed by the ratio of sp^2/sp^3 carbon, which increases (from 1.184 to 1.386) after the reduction of GO. Compared to GO (0.93), the 3DG (1.01) has a slightly higher I_D/I_G ratio, which is attributed to the decrease in the size of the sp^2 domains after the reduction process.

The comparison of the FTIR spectrum between GO and 3DG is presented in Fig. 1(B). The peak in the FTIR spectrum of GO at 1158 cm^{-1} is attributed to the C–O and C–OH group stretching vibration, while the peak at 1741 cm^{-1} is assigned to the carbonyl C=O stretch, and the broad wave between 2500 cm^{-1} and 3500 cm^{-1} is due to the hydroxyl O–H stretch from the OH groups and the presence of moisture in the GO [58]. The absence of the peaks attributed to the C=O, C–O and O–H groups in the FTIR spectrum of 3DG confirms the successful synthesis of 3DG and the reduction of GO.

Morphology characterisation of 3DG

The two factors which control the power density and long-term activity of biofuel cells are the enzyme stability and the diffusion towards the electrode surface. The microporous structure of 3DG can prevent the denaturation of the immobilised enzyme, enzyme leaching, and provide a sufficient concentration of both glucose and oxygen to the enzymes. The morphology of the bioanode was characterised by field emission scanning electron microscopy (FESEM) and high-resolution transmission electron microscopy (HRTEM). Fig. 2(A) shows the HRTEM image of the synthesised 3DG, while the FESEM image (Fig. 2B) reveals a highly porous structure of the 3DG, which improves the biological function, stability and efficacy of GOx immobilisation. As it is apparent in Fig. 2(C), the GOx enzyme has been trapped inside the porous structure of 3DG through the physical absorption, and we have a successful hybridisation of 3DG/GOx. This is the simplest method of immobilisation which developed base on the hydrogen bonds and Van der Waal's forces. It is based on the physical interaction of enzyme and 3-dimensional graphene. Also, due to not employing any reagent comparing to chemical methods, it is less disruptive to the enzyme protein structure. Furthermore, physical adsorption is cheap, easy to carry out, and minimum steps of activation required [59].

Electrochemical performances of bioanode

The immobilisation of GOx on the GCE/3DG was confirmed by cyclic voltammetry (CV)(five cycles repeated). Line (a), in Fig. 3(A) presents the CV of the GCE/3DG-GOx at pH 7, which shows a pair of redox peaks (oxidation and reduction reactions) at −0.43 V and −0.39 V.

In contrast, the CV of GCE/GOx (line b) does not show any electrochemical peaks. These results suggest that the fabricated 3DG-GOx bio-nanocomposite enhances the electron transfer process between the electrode surface and the enzyme.

The effect of different scan rates on the GCE/3DG-GOx was also studied by varying the scan rates from 1 mVs^{-1} to 100 mVs^{-1} in nitrogen saturated solution at pH 7. According to

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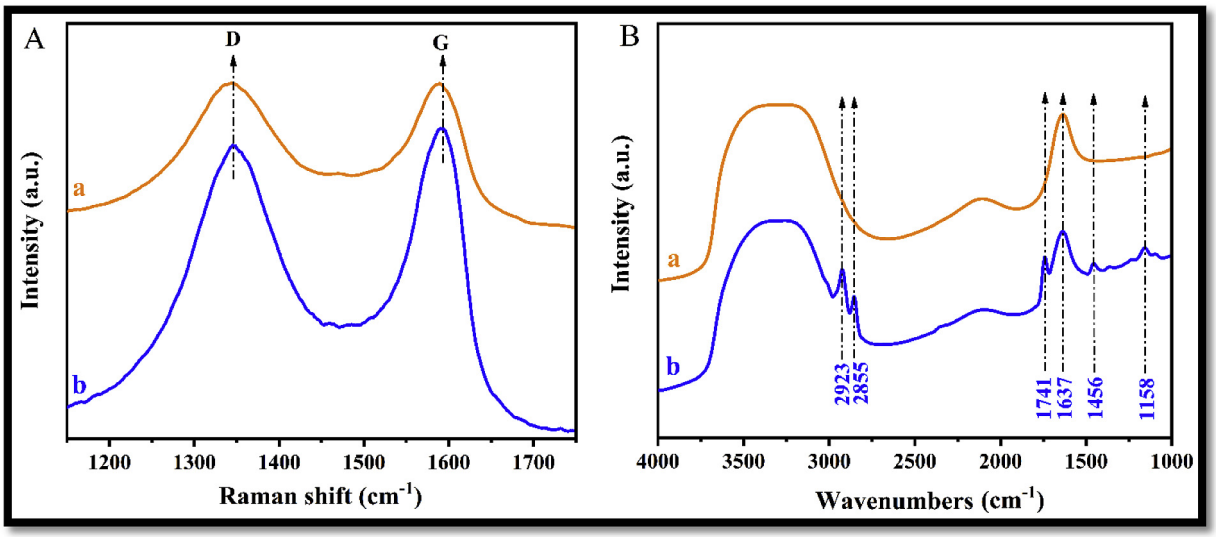

Fig. 1 – (A) Raman spectra, and (B) FTIR spectrum of a: GO and b: 3DG.

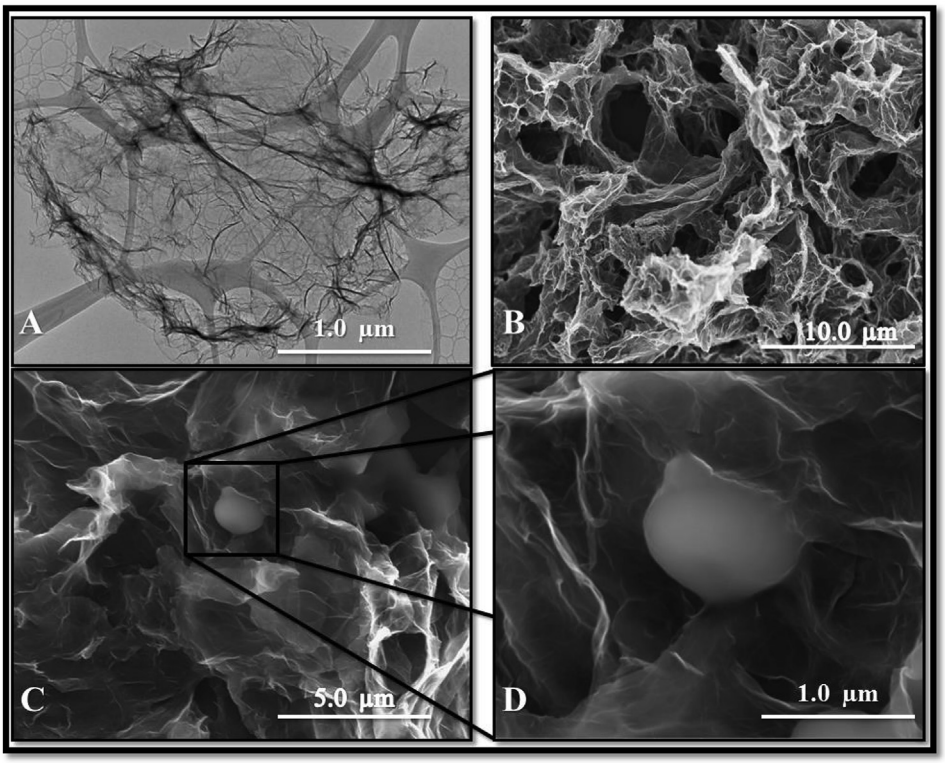

Fig. 2 – (A) HRTEM image of 3DG, (B) FESEM image of 3DG, (C) The hybrid of GOx and 3DG and (D) immobilised GOx on 3DG.

Fig. 3(B), the peak current is linearly dependent on the scan rate, while the peak potential is shifted towards the negative region with the increase of the scan rate from 10 to 200 mVs^{-1}. These results confirm that the electron transfer is a quasi-reversible process, while the diffusion is controlled through a surface diffusion process and not through solution diffusion. It means that the electron transfer rate between the working electrode and the species in the solution is fast, but still, there are some thermodynamic barriers. This suggests that the electron transfer between the GOx enzyme and the electrode surface is a quasi-reversible process, wherein the GOx retains its bioactivity even at high scan rates. Fig. 3(C) presents the chronoamperometric response of the GCE/3DG-GOx bio-nanocomposite based on the current-time plot for successive additions of glucose at −0.40 V in an O_2 saturated 0.1 M PBS (pH 7) solution. From Fig. 3(C), the current increases with the addition of glucose and reaches a steady-state within 5 s. The amperometric current increases linearly with the

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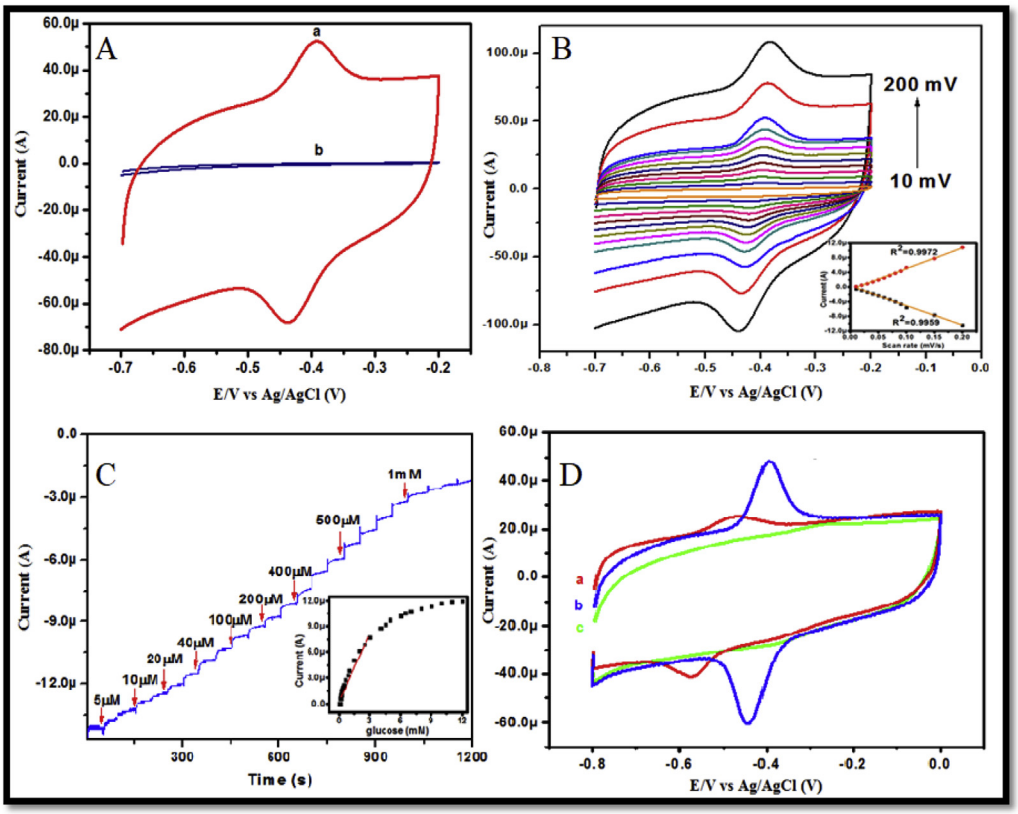

Fig. 3 – (A) CV of the GCE/3DG-GOx at pH 7; (a) bare GCE, (b) GCE/3DG-GOx, (B) GCE/3DG-GOx at different scan rates in N_2 saturated solution at pH 7. The scan rates from inner to outer are: 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 150, 200 mV s^{-1}. Inset is the linear dependence of Ipa (■), and Ipc (■) on the scan rate (10–200 mVs^{-1}), (C) Chronoamperometric response of the GCE/3DG-GOx to successive injection of glucose into air saturated 0.1 M PBS (pH 7.0) at −0.40 V, (D) The CV of GCE/3DG-GOx at 100 mV s^{-1} in buffer solutions at different pH values; (a) pH 10, (b) pH 7 and (c) pH 4.

concentration of glucose from 0.05 to 3 mM. Considering that the average blood glucose level is between 4 and 6 mM, the EBFC could be supplied with a sufficient amount of glucose for implant applications. The CV of GCE/3DG-GOx at 100 mVs^{-1} in buffer solutions with a different pH is shown in Fig. 3(D). The CV shows a negative shift of the redox peaks with increasing pH values from 4 to 10 at 100 mVs^{-1}.

Electrochemical impedance spectroscopy (EIS) of the modified electrodes was performed in order to investigate the effect of enzyme catalysts on the charge transfer process of the EBFC bioanodes. The charge transfer resistance (R_{ct}) of each bioanode was obtained from the Nyquist plot. Fig. 4(A) shows the Nyquist plots (-Z′ vs. Z'') of the enzyme immobilised 3DG modified GCE, the GCE/3DG and the GCE/GOx. From the Nyquist plots, the GCE/GOx has the highest R_{ct} value, followed by the GCE/3DG/GOx. The lowest R_{ct} value was reported for the 3DG modified GCE. These results show the significant role of 3DG in enhancing the electron transfer between the enzyme active site and surface of the GC electrode and decreasing the electrical resistance. The bioanodes show different values of R_{ct} obtained from the Nyquist plots. GOx is a protein molecule with negligible electronic conductivity. Thus, the R_{ct} increases with the loading amount of the GOx enzyme. It should be noted that the intercepts on the real axis in the Nyquist plots are similar for all bioanodes. This demonstrates that the solution resistance (Rs) of the bioanodes is unaffected by the types of biocatalysts.

Tafel polarization measurements were also performed in order to evaluate the performance of the fabricated bioanodes. The observed open circuit potential (OCP) of 0.44 V is much less than the reported OCPs for 3DG aggregated electrodes with DET. As mentioned previously, the fabricated GBFC is polarised by connecting the fabricated electrode to a range of the resistors. Fig. 4(B) represents the potential and power density of the cell as a function of current. This indicates that the 3DG/GOx modified GCE can supply a power density of approximately 160 μWcm^{-2} at 0.4 V. The higher power density observed in this work compared to previous reports indicates the critical role of 3DG in the enhancement of enzyme immobilisation and the increased electron transfer process, with both factors being crucial for improved performance of the EBFC.

In order to evaluate the stability of the 3DG/GOx modified GCE, the bioanode stored in PBS buffer (pH 7) at 4 °C and tested every day. After the first day of fabrication, it had lost 13% of its original power output. It was found out that the power output decays slowly and become 60% of the initial power density after 7 days, as illustrated in Fig. 4(C).

Table 1 present a power generation compression between the reported carbon-based EBFCs in previous studies and the

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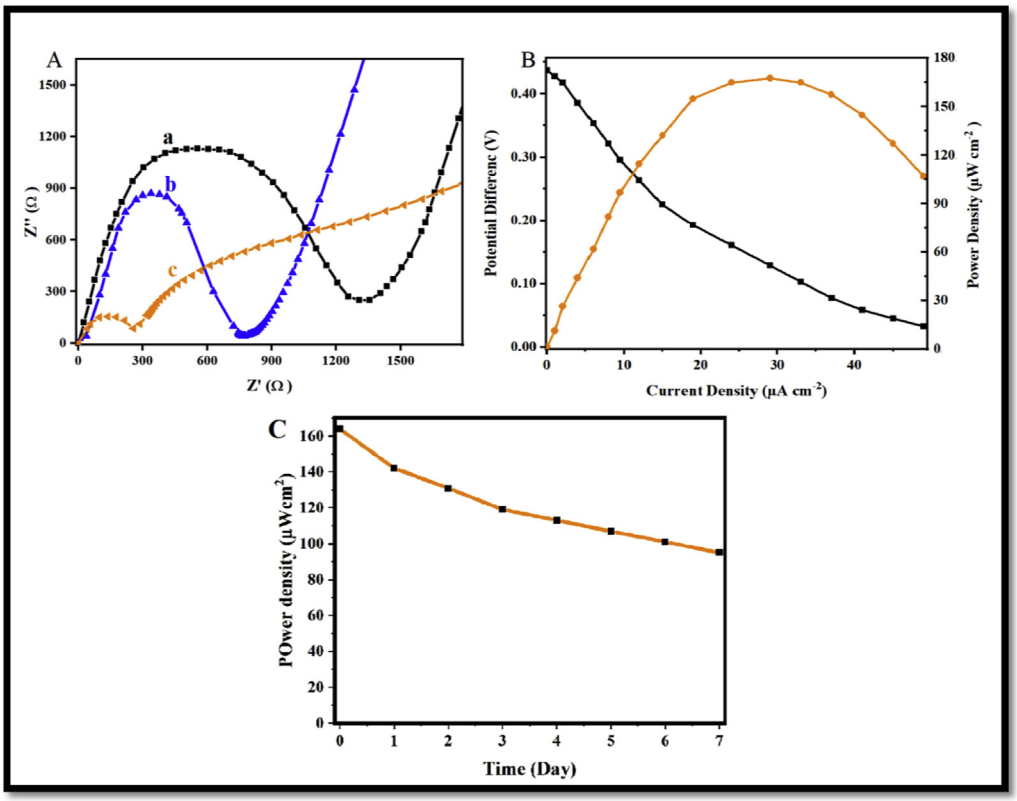

Fig. 4 – (A) Nyquist plots in 0.1 M PBS (pH 7), (a) bare GCE; (b) 3DG modified GCE; (c) 3DG/GOx modified GCE, (B) Polarization curve of the fabricated 3DG/GOx modified GCE in 0.1 M PBS (pH 7), (C) The operation stability of fabricated bioanode as a function of time.

3DG/GOx modified GCE, which have been fabricated in this study. According to this table, the power density of fabricated bioanode is among top three reported biofuels cells. The performance merit of the GCE/3DG-GOx bioanode places it right after the PABMSA modified BP and vaCNTs, but before the graphene–AuNP hybrid. This is a noticeable power density while the fabrication process of 3DG/GOx modified GCE is much easier, and no functionalization is required comparing to other competitors.

Table 1 – Carbon based EBFCs power generation comparison.

Material	Enzyme	Power density ($\mu W\ cm^{-2}$)	Ref.
Gold/MWCNT	GOx	42	[60]
NH_2-CNTs	GDH	45	[61]
PABMSA via BP & vaCNTs	GDH	122	[62]
Graphene–AuNP hybrid	GOx	196	[63]
PEDOT-coated MWCNT	GOx	640	[64]
3D Graphene	GOx	164	Present study

MWCNT: Multiwall Carbon Nanotubes.
NH_2: amino group.
CNTs: Carbon Nanotubes.
PABMSA: Poly (3-aminobenzoic acid-co-2-methoxyaniline-5-sulfonic acid).
vaCNTs: Vertically Aligned Carbon Nanotubes.
PEDOT: Poly(3,4-Ethylenedioxythiophene).

Conclusions

A 3DG modified bioanode was fabricated in this study in order to enhance the performance of the EBFCs, i.e. to increase the lifetime of the enzyme, overcome the enzyme leaching and improve the electrode transfer between the enzyme active site and the electrode surface. In this study, we synthesised a highly porous, conductive 3DG and utilised it as an electrode platform for the immobilisation of the GOx enzyme. The fabricated bioanode in this study has a maximum power density of 164 μWcm^{-2} at a cell voltage of 0.4 V.

In conclusion, the highly porous structure of 3DG entraps the GOx enzyme and decreases enzyme leaching, thus increasing the enzyme lifetime by maintaining the 3D structure. The excellent electrical conductivity of 3DG facilitates the DET between the active site of the GOx and the modified GCE and decrease the resistance toward the electron flow. The results demonstrated the high performance of the fabricated GCE/3DG-GOx as a bioanode for EBFC applications. The synthesised 3DG is a versatile electrode platform for the immobilisation of other enzymes in the construction of EBFCs and third-generation enzymatic biosensors.

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