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A Novel Glucose Biosensor at Glucose Oxidase Immobilized Graphene and Bismuth Nanocomposite Film Modified Electrode

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We have prepared graphene-bismuth nanoparticle (Bi) nanocomposite by simple solution based chemical reduction method and explored its biosensor application for the enzymatic determination of glucose. The characterization results revealed the successful formation of the composite. The graphene-Bi nanocomposite was used for the immobilization of glucose oxidase (GOx). Direct electron transfer of GOx was observed with well-defined redox peaks at the formal potential of – 0.427 V. The amount of electroactive GOx (Γ) and electron transfer rate constant (k_s) were calculated to be 5.48×10^{-10} mol cm^{-2} and 5.57 s^{-1}, respectively. An amperometric glucose biosensor has been fabricated which detects glucose in a wide linear range from 1 mM to 12 mM with high sensitivity (2.243 μAmM^{-1} cm^{-2}). Practical feasibility of the biosensor is explored in human blood serum sample which are revealed the excellent practicality of the biosensor. Moreover, the sensor exhibited appreciable stability, repeatability and reproducibility.

Keywords: Graphene, bismuth nanoparticle, glucose oxidase, glucose, biosensor, amperometry.

1. INTRODUCTION

Bismuth nanoparticle (Bi) is a semi-metal known for its wide applications in pharmaceutical and metallurgical additives [1-4]. Due to its interesting optical, electronic and electrocatalytic properties, Bi finds considerable applications in the fabrication of electrochemical sensor and biosensors [5, 6]. Its biocompatibility, low toxicity and promising electrochemical ability make it as an

alternative feasible electrode material for the mercury electrodes [7-9]. However, Bi alone is not stable on the bare electrode surface. It requires suitable support to deliver its applications. In the past decades, carbon nanotubes have been widely used as supporting mat attributed to their excellent properties [10, 11]. Recently, graphene, a two dimensional carbon nanomaterial composed of monolayered carbon atoms has grown to be hottest material due its interesting physicochemical properties such as large surface area, high conductivity, high porosity, biocompatibility, wide electrochemical window, cheaper production and less antifouling ability [12-19]. Notably, graphene has large surface area than CNTs and hence it could be an auspicious carbonaceous support to stabilize massive amounts of Bi [3]. Graphene oxide (GO), an oxygenated derivative of graphene is the ideal precursor for the preparation of graphene-metal nanoparticle composites owing to its unique advantageous such as, inexpensive and simple production from graphite, easy processing in aqueous dispersions and available sites for the functionalization [20, 21]. The electronic properties of graphene can be easily tailored by metal nanoparticles and the resulting nanocomposites exhibited exceptional stability and synergy [16, 22]. To date, several graphene based metal nanocomposites have been reported for the biosensor application. However, graphene-Bi nanocomposite has never been used for glucose biosensor applications.

There are two important limitations in immobilizing enzymes on the solid electrodes: (1) poor electrical communication between active site of enzyme and electrode, and (2) enzyme leaching [23, 24]. To overcome these issues, choosing a suitable immobilization matrix having good electrical conductivity, stability, and antifouling property is mandatory [15, 25-27]. Therefore, immobilizing enzymes at the solid electrode surface through suitable matrix and forming efficient electrical communication between active site of the enzyme and the electrode is the fundamental subject of research in biosensors and enzymatic biofuel cell applications [24]. A variety of conducting supports such as polymers, sol-gel, membranes, metal oxides, metal nanoparticles, hydrogel and carbon nanotubes based nanomaterials are reported as supports, while adsorption, covalent cross-linking and entrapment methods are developed for the immobilization [23]. Recently, graphene and GO supported electrocatalysts are proved as highly qualified supporting materials for the immobilization of enzymes, biosensor and energy device applications [28]. Designing graphene based nanocomposites as suitable matrix for the efficient immobilization enzymes is a continuous research interest in our research group [14, 15, 17, 18, 23, 29]. In this way, for the first time, we are developing a sensitive glucose biosensing platform based on the glucose oxidase (GOx) immobilized graphene-Bi nanocomposite film modified electrode.

From the medicinal perspective, development of sensitive glucose biosensors for the accurate determination of blood glucose level is of great significance to control the diabetics [30, 31]. GOx is the most widely employed model enzyme for the enzymatic determination of glucose [30]. Determination of glucose via first generation glucose biosensors requires oxygen as co-substrate. However, it has a major drawback known as ‘oxygen deficit', since oxygen may not present in all the systems and its concentration in biological fluids cannot be fixed. The ‘oxygen deficit’ issue can be addressed by replacing oxygen with redox mediators such as ferrocene monocarboxylic acid, hydroquinone etc., [14].

The main objective of the work is to develop graphene-Bi nanocomposite based biosensing platform for the enzymatic determination of glucose. We prepared graphene-Bi nanocomposite by simple solution based chemical reduction approach and used it for the immobilization of GOx. The preparation of the nanocomposite is involving simple and easily adoptable chemical reduction method which does not requiring any surfactants. The developed amperometric sensor is exhibited excellent electrocatalytic parameters such as wide linear range and wide sensitivity.

2. EXPERIMENTAL

2.1 Chemicals and Apparatus

Bismuth (III) nitrate pentahydrate, $Bi(NO_3)_2$. $5H_2O$ was purchased from Wako pure chemical industries, Ltd. Graphite (powder, <20 μm), glucose oxidase (GOx, type x-s from aspergillus Niger), glucose and all other chemicals were purchased from Sigma-Aldrich and used without further purification.

All the electrochemical measurements were carried out using CHI 611A electrochemical work station. Electrochemical studies were performed in a conventional three electrode cell using BAS GCE as a working electrode (area 0.071 cm^2), saturated Ag/AgCl (KCl) as a reference electrode and Pt wire as a counter electrode. All the electrochemical experiments were carried out at ambient temperature. Prior to each electrochemical experiment, the electrolyte solutions were deoxygenated with pre-purified nitrogen for 15 min unless otherwise specified. Amperometric measurements were performed with analytical rotator AFMSRX (PINE instruments, USA) with a rotating disc electrode (RDE) having working area of 0.21 cm^2. Scanning electron microscope (SEM) and Energy-dispersive X-ray (EDX) spectra studies were performed using Hitachi S-3000 H scanning electron microscope and HORIBA EMAX X-ACT (Sensor + 24V=16 W, resolution at 5.9 keV) respectively.

2.2 Preparation of graphene-Bi nanocomposite and immobilization of GOx

Graphite oxide was prepared by Hummer's Method as reported elsewhere [32] and suspended in DMF (1 mg mL-1) and exfoliated to graphene oxide (GO) via ultrasonication for 2 h. The homogenous dispersion of GO was separated and used for the preparation of graphene-Bi nanocomposite. $Bi(NO_3)_2$. $5H_2O$ was added to the 100 ml of GO (w/w: 2:3) and ultrasonicated for 30 min. Consequently, 1 ml of hydrazine monohydrate was added and refluxed at 160°C for 24 h under nitrogen atmosphere [3]. Upon completion of the reduction process, the prepared graphene-Bi nanocomposite has been isolated via filtration, washed with copious amount of water and ethanol. The nanocomposite was dried overnight and redispersed in DMF (0.5 mg mL-1).

GCE surface was polished with 0.05 μm alumina slurry using Buehler polishing kit, cleaned and dried. 5 μl of graphene-Bi nanocomposite was drop casted onto the GCE surface, dried at ambient conditions. Subsequently, 6 μl of GOx (10 mg mL^{-1}) was drop casted onto the graphene-Bi composite film modified GCE and dried at room temperature. Then, GCE/GOx/graphene-Bi was gently washed

with water to remove loosely adsorbed GOx. Finally, 2 µL of nafion (0.5% in ethanol) was drop casted on the GCE/graphene-Bi/GOx to avoid enzyme leaching. As a control, GCE/graphene/GOx was also prepared.

3. RESULTS AND DISCUSSION

3.1. Characterization of graphene-Bi nanocomposite

The SEM image of graphene (Figure 1A) shows the characteristic wrinkled and scrolled intrinsic sheet like morphology. EDX profile of graphene (Fig. 1C) is exhibited the signals corresponding to C and O with wt. % of 96.58 and 3.42 respectively. The SEM image of graphene-Bi nanocomposite (Fig. 1B) displays the uniform distribution of Bi at both faces of graphene sheets. The Bi nanoparticle size ranges in 40-140 nm, while thickness of the graphene sheets varies between 1 to 3 nanometers. EDX profile of graphene-Bi nanocomposite (Fig. 1D) is exhibited the signals of C, O and Bi with wt. % of 51.26, 5.27 and 43.47 respectively. The observation of 43.47% weight percentage of Bi indicating the high Bi loading into graphene sheets which indicating the efficiency of graphene nanosheets to support Bi nanoparticles.

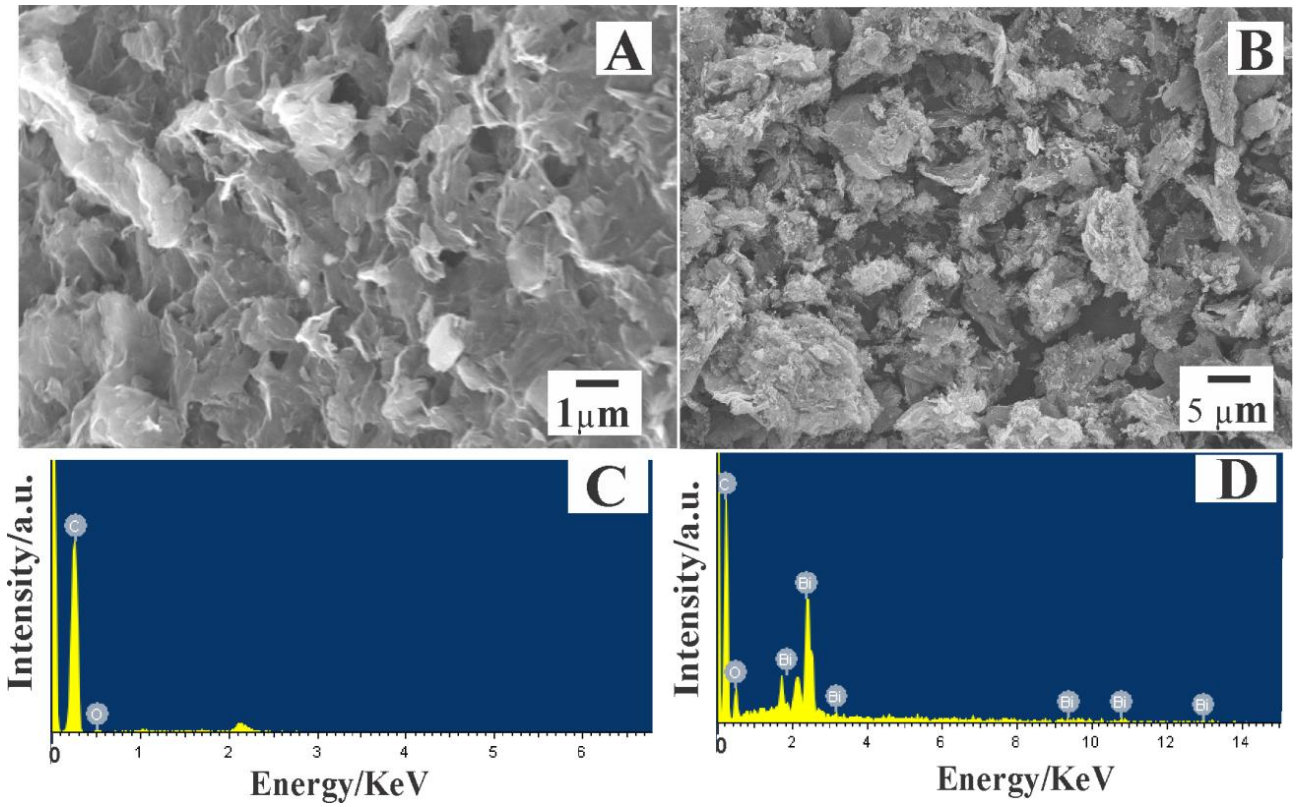

Figure 1. SEM images of graphene (A) and graphene-Bi nanocomposite (B). EDX profiles of graphene (C) and graphene-Bi nanocomposite (D).

3.2 Direct electrochemistry of GOx

Figure 2 shows the CVs obtained at GCE/graphene/GOx (a), and GCE/graphene-Bi/GOx (b) films in PBS (pH 7) at the scan rate 50 mVs^{-1}. No noteworthy redox couple was observed in the CV of graphene/GOx indicating absence of direct electron transfer between GOx and electrode surface at this electrode. However, the CV of graphene-Bi/GOx/GCE has exhibited well-defined and highly enhanced redox couple at the formal potential ($E^{o'}$) of – 0.427 V. The redox couple should be attributed to the direct electron transfer of GOx ($FAD/FADH_2$) at the graphene-Bi/GOx/GCE. The peak to peak separation value (ΔE_p) of the redox couple was calculated to be 0.30 mV. The greatly enhanced redox peaks and very low ΔE_p are indicating the attainment of direct electrochemistry of GOx which in turn revealing the successful immobilization of GOx. Graphene/GOx/GCE has not shown any obvious redox couple, however graphene-Bi/GOx/GCE has presented sharp redox pair responsible for the GOx. This result clearly reveals the significant role of Bi towards enabling the direct electrochemistry of GOx. Thus, the graphene-Bi composite is a promising support for the immobilization of GOx which could be ascribed to the large surface area, high conductivity, good porosity and good biocompatibility of the graphene-Bi composite. Perhaps, there could be a strong synergy between graphene nanosheets and Bi nanoparticles which significantly influence the direct electron transfer ability of the resulting nanocomposite.

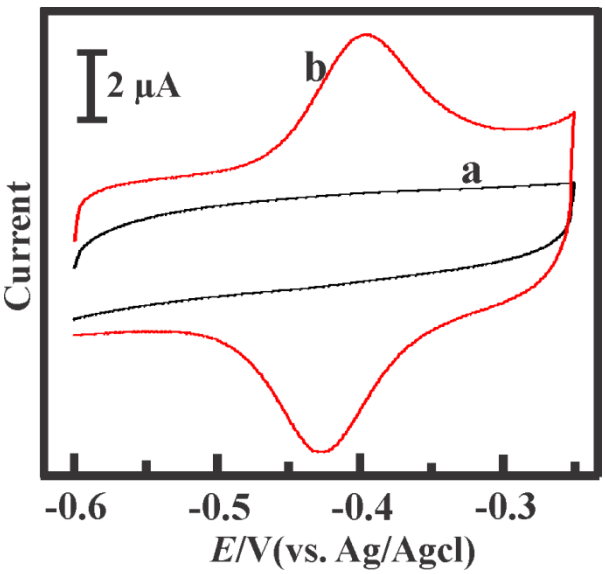

Figure 2. CVs obtained at GCE/graphene/GOx (a) and GCE/graphene-Bi/GOx (b) films in PBS (pH 7) at the scan rate of 50 mVs^{-1}.

3.3 Different scan rate studies

Figure. 3A presents the CVs of GCE/graphene-Bi/GOx in PBS (pH 7) at different scan rates from 0.1 to 1 Vs^{-1}. Both anodic (I_{pa}) and cathodic peak currents (I_{pc}) are increases linearly with scan rates (v) from 100 to 1000 mV s^{-1}. ΔE_p value also increases upon increase in scan rates. A plot of I_{pa}

and I_{pc} versus v exhibited linear relationship which indicated that the direct electron transfer of GOx occurring at GCE/graphene-Bi/GOx is a surface confined process (Figure. 3B). The corresponding linear regression equations can be expressed as eq. (1) and (2):

For anodic process, $I_{pa}/\mu A = 146.2\ v\ (\mu A/Vs^{-1}) + 14.13$; $R^2 = 0.980$ (1)

For cathodic process, $I_{pc}/\mu A = -136.4\ v\ (\mu A/Vs^{-1}) - 14.60$; $R^2 = 0.978$ (2)

The surface coverage of the electroactive GOx (Γ) at the GCE/graphene-Bi/GOx was calculated by substituting the slope values of eq. (1) and (2) in the following eq. (3) [33],

$$I_p = n^2F^2vA\Gamma/4RT \quad (3)$$

Where, v (Vs^{-1}) is the scan rate and A (cm^2) is the electrode surface area. The constants R, T and F represents their usual meanings (R=8.314 J $K^{-1}mol^{-1}$, T=298 K, F=96485 C mol^{-1}). Assuming, the number of electrons transferred (n) in direct electron transfer of GOx as 2, the value of Γ has been calculated to be 5.48×10^{-10} mol cm^{-2} which is higher than the theoretical monolayer coverage of GOx. The apparent heterogeneous electron transfer rate constant (k_s) for the direct electron transfer of GOx has been calculated using the eq. (4) [33].

$$\text{Log } K_s = \alpha \log(1-\alpha) + (1-\alpha)\log\alpha - \log(RT/nFv) - \alpha(1-\alpha)nF\Delta E_p/2.3RT \quad (4)$$

Where, α is the charge transfer coefficient (~0.5) and the other parameters stands for the similar meanings explained in the eq. (3). The k_s value for the direct electron transfer of GOx at the GCE/graphene-Bi/GOx was calculated to be 5.57 s^{-1}, which is higher than the previously reported modified electrodes [25, 34, 35].

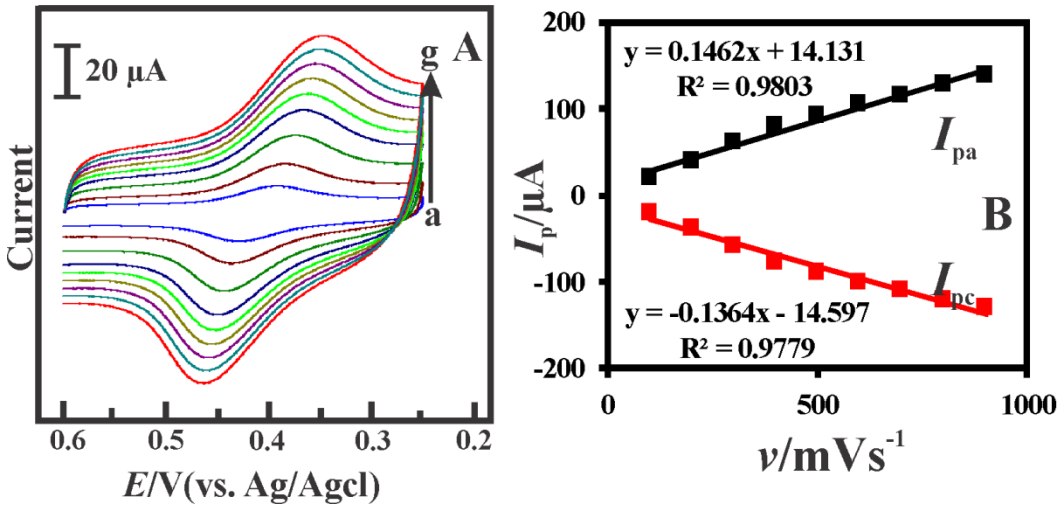

Figure 3. (A) CVs of graphene-Bi/GOx/GCE in PBS (pH 7) at different scan rates from 0.1 to 1 Vs^{-1}. (B) Plot of I_{pa} and I_{pc} versus v.

3.4 Electrocatalysis of glucose at graphene-Bi/GOx/GCE

Figure 4A presents the CVs obtained at graphene-Bi/GOx/GCE in the absense (a) and presence of 0.5 mM (b), 1 mM (c), 1.5 mM (d), 2 mM (e) and 2.5 mM (f) glucose in deoxygenated PBS (pH 7) containing ferrocene monocarboxylic acid (FMCA). The CV obtained at graphene-Bi/GOx/GCE presented a redox couple at the $E^{o'}$ of + 0.32 V responsible for the redox reaction of FMCA. Upon addition of 0.5 mM of glucose, a sharp increase in the anodic peak current was observed due to the FMCA mediated oxidation of glucose. A linear increase in the oxidation peak current was observed from 0.5 mM to 2.5 mM of glucose (Figure 4B) which revealing the excellent electrocatalytic activity of the electrode towards FMCA mediated biosensing of glucose. The FMCA mediated oxidation of glucose can be explained by the following equations (5) – (7) [14].

$$\text{Glucose} + \text{GOx(FAD)} \longrightarrow \text{Gluconolactone} + \text{GOx(FADH}_2\text{)} \quad (5)$$

$$\text{GOx(FADH}_2\text{)} + 2\text{FMCA}^+ \longrightarrow \text{GOx(FAD)} + 2\text{FMCA} + 2\text{H}^+ \quad (6)$$

$$2\text{FMCA} \rightarrow 2\text{FMCA}^+ + 2\text{e}^- \quad (7)$$

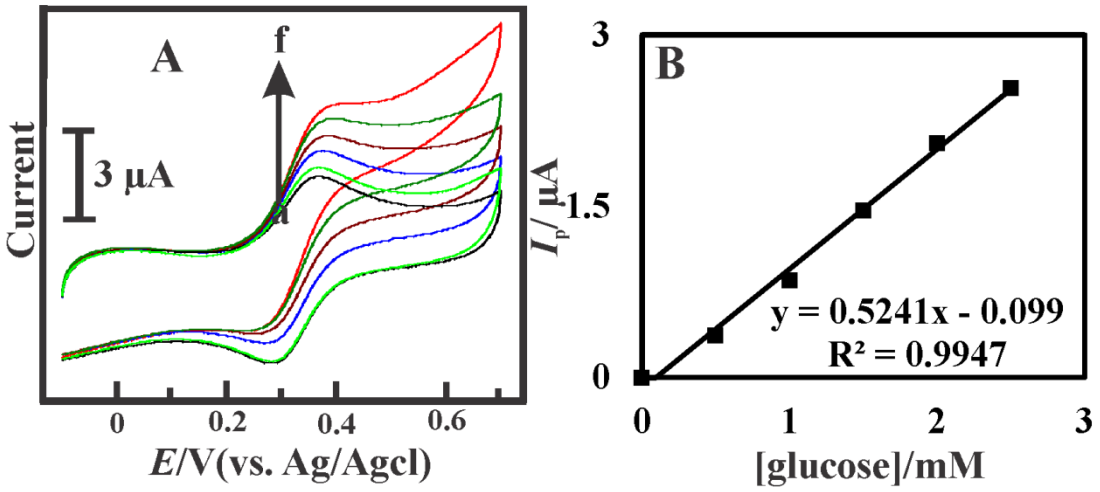

Figure 4. (A) CVs obtained at graphene-Bi/GOx/GCE in deoxygenated PBS (pH 7) containing 5 mM FMCA in the absence (a) and presence of glucose 0.5 (b), 1 (c), 1.5 (d), 2 (e) and 2.5 (f) mM concentration of glucose. (B) [glucose]/mM vs. I_p.

3.5 Amperometric glucose biosensor

Figure 5A displays the amperogram obtained at the graphene-Bi/GOx/GCE towards each sequential addition of 1 mM glucose into continuously stirred deoxygenated PBS (pH 7) containing 5 mM FMCA at regular intervals of 50 s. Applied electrode potential (E_{app}) was + 0.31 V, while the rotation speed was applied at 1500 RPM. Well defined amperometric response was observed for the each addition of glucose. The amperometric responses are linearly increased in the wide linear range from 1 mM to 12 mM. A calibration plot was made between concentration of glucose and I_p (figure 5B). The respective linear regression equation can be expressed as, I_p/µA = 0.471 [glucose]/µA mM^{-1} – 0.159, R^2=0.993. The sensitivity of the sensor has been calculated to be 2.243 µAmM^{-1} cm^{-2}. Limit of

detection (LOD) was calculated to be 0.35 mM. The important analytical parameters such as LOD, linear range and sensitivity obtained at the graphene-Bi/GOx/GCE were quite comparable with other glucose biosensors [18, 34, 36].

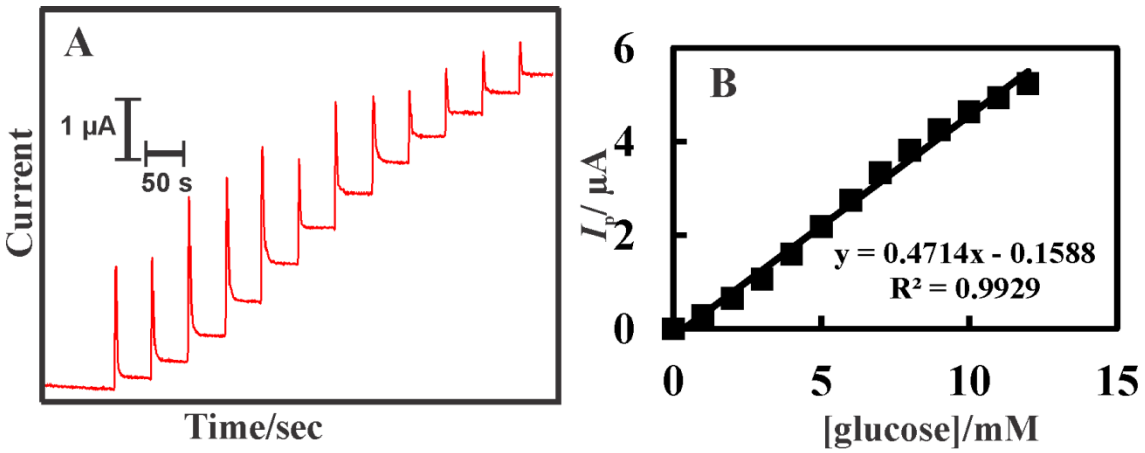

Figure 5. Amperometric i-t response obtained at graphene-Bi/GOx/GCE (E_{app} = + 0.31 V, Rotation rate: 1500 RPM) upon successive addition of 1 mM glucose into continuously stirred deoxygenated PBS (pH 7) containing 5 mM of FMCA. (B) [glucose] versus I_p.

3.6 Stability, repeatability and reproducibility studies

The graphene-Bi/GOx/GCE has retained 95.31% of its initial response current even after one month of its storage revealing its appreciable storage stability. In addition, only 5.5% of the initial peak currents are loosed even after 250 successive potential scans revealing the excellent stability of the modified electrode. The graphene-Bi/GOx/GCE biosensor exhibited appreciable repeatability with an R.S.D of 2.15% for six successive repeated measurements in a single modified electrode. Moreover, the biosensor exhibited excellent reproducibility with an R.S.D of 2.23% for six individual measurements carried out at six different modified electrodes.

3.7 Real sample analysis

The practical feasibility of the sensor has been assessed in human blood serum sample collected from a healthy man. The collected blood sample was allowed to clot. Then, the clot was removed through centrifugation (speed= 2000 × g) for 15 min. The blood serum separated as supernatant was stored at the temperature of – 20°C and used for the analysis. First, 2 ml of the serum sample was diluted to 10 ml by the addition of PBS (pH 7) containing 5 mM of FMCA. Then experiments were carried out using graphene-Bi/GOx/GCE in the diluted blood serum sample by adopting the experimental conditions of lab sample. The amount of glucose present in the serum sample was found as 5.24 ±2.8 mM by electrochemical method using graphene-Bi/GOx/GCE. The concentration of glucose was found as 4.98 mM by photometric sensor (Roche Cobas c 111 analyzer). Thus, the concentration measured by our sensor is in good agreement with the commercial sensor

which validates the accuracy of our biosensor. Therefore, the graphene-Bi/GOx/GCE could be a promising biosensor for the determination of glucose present in clinical samples.

4. CONCLUSIONS

In summary, we have prepared graphene-Bi/GOx/GCE and developed a sensitive glucose biosensor. GOx was successfully immobilized at graphene-Bi nanocomposite which was confirmed by the observation of direct electron transfer redox peaks. The high values of Γ (5.48×10^{-10} mol cm^{-2}) and k_s (5.57 s^{-1}) indicating the occurrence of fast electron transfer at the prepared modified electrode. An amperometric biosensor was fabricated for the determination of glucose. The graphene-Bi/GOx/GCE based biosensor has been fabricated which presented excellent electroanalytical parameters such as wide linear range (1 mM – 12 mM), high sensitivity (2.243 μAmM^{-1} cm^{-2}) and low LOD (0.35 mM). The practical feasibility of the biosensor has been assessed in human blood serum sample. Moreover, the biosensor possess appreciable stability, repeatability, and reproducibility. The excellent electrocatalytic ability of the graphene-Bi nanocomposite revealed its applicability for the immobilization of other enzymes or proteins for the fabrication of novel biosensors.

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