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A novel glucose sensor using lutetium phthalocyanine as redox mediator in reduced graphene oxide conducting polymer multifunctional hydrogel

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Abstract

Herein, we report a scalable synthesis of multifunctional conducting polyacrylic acid (PAA) hydrogel (MFH) integrated with reduced graphene oxide (rGO), vinyl substituted polyaniline (VS-PANI) and lutetium Phthalocyanine (LuPc₂) as three dimensional robust matrix for glucose oxidase (GOx) immobilization (PAA-rGO/VS-PANI/LuPc₂/GOx-MFH). We have integrated the multicomponents such as PAA with rGO, and VS-PANI through free radical polymerization using methylene bis-acrylamide, and ammonium persulphate as the cross linker and initiator. The LuPc₂ was then doped to form multifunctional hydrogel (PAA-rGO/VS-PANI/LuPc₂-MFH). Finally, biosensor was fabricated by immobilizing GOx into PAA-rGO/VS-PANI/LuPc₂-MFH and subsequently used for electrochemical detection of glucose. The PAA-rGO/VS-PANI/LuPc₂/GOx-MFH biosensor exhibited high sensitivity (15.31 $\mu\text{AmM}^{-1}\text{cm}^{-2}$) for the detection of glucose over a concentration range of 2–12 mM with a low detection limit of 25 μM . The PAA-rGO/VS-PANI/LuPc₂-MFH biosensor showed a fast response time (1s) to the addition of glucose with high storage stability of 3 months. The real sample analysis reveals that PAA-rGO/VS-PANI/LuPc₂/GOx-MFH could be effectively used as an electrochemical biosensor in industrial as well clinical diagnosis.

Keywords: Polyacrylic acid, reduced graphene oxide, polyaniline, lutetium Phthalocyanine, glucose biosensor, multifunctional hydrogel

1 **1. Introduction**

2

3 Despite many technological advances in biosensor research, the worldwide increase in the
4 prevalence of chronic disease (diabetes mellitus) has been the driving force for the
5 development of glucose sensors (Wang and Lee, 2015). Diabetes, occurs due to
6 malfunctioning of pancreatic β cells responsible for production of insulin that control glucose
7 level in the blood (Schuit et al., 2001). About 5 percent of the world's population (3.9 million
8 people in the UK alone) are living with diabetes (Diabetes UK, 2015). The elevated level of
9 glucose (normal range 4.4–6.6 mM) poses serious health problems in diabetic patients and
10 accounts for 4 million deaths worldwide every year (Wild, et al, 2014). Frequent self-
11 monitoring of glucose may provide data for optimizing and/or changing insulin uptake in
12 patients (Canadian Diabetes Association, 2008).

13 Although several conventional methods available for the detection of glucose,
14 electrochemical detection of glucose show unique advantages such as fast response, sensitive,
15 low cost and does not require skilled labour or large instrumentation for detection (Rahman et
16 al., 2010). Non enzymatic electrochemical sensors exhibit poor performance in selectivity
17 and suffer from interference from electrochemically active molecules (Toghill and Compton,
18 2010). Glucose oxidase (GOx) is the widely studied enzyme for amperometric glucose
19 sensing since its inception from Clark and Lyons in 1962 (Claussen et al., 2012; Wang 2001).
20 The key factor for an efficient GOx based sensor is to obtain electron transfer from the active
21 centre, flavin adenine dinucleotide (FAD) enwrapped within thick protein layer, maintain
22 biological activity, high enzyme loading and avert enzyme leeching. Additionally, an ideal
23 biosensor has to be stable for long-term application, accurate in sensor-to-sensor
24 measurements with significant operational lifetimes. To achieve these factors, large number
25 of substrates as recognition elements such as carbon nanotubes (Zhu et al., 2012), graphene
26 (Unnikrishnan et al., 2013), inorganic nanostructures (Wu et al., 2015), conductive polymers
27 (Chen et al., 2006; Garjonyte and Malinauskas, 1999), cellulose acetate (Ren et al., 2009),
28 polyacrylonitrile (Zheng et al, 2002) have been proposed for GOx immobilization. However
29 fewer reports are available on the utilization of a robust three dimensional multifunctional
30 conducting hydrogel as an enzyme immobilization matrix and subsequent biosensing of
31 glucose.

32 Hydrogels are physically or chemically three-dimensional cross-linked, insoluble
33 hydrophilic polymeric network with the capability to swell in the presence of aqueous

1 solution or biological fluids (Barakat and Sahiner, 2008; Sezgin and Balkaya,
2 2015). Hydrogel networks formed from polyacrylic acid (PAA) have the ability to absorb
3 more than hundred times their weight in water and they are classified as super absorbents
4 (Dhodapkar et al., 2009). PAA hydrogel plays an important role as pH stimuli-responsiveness
5 and bioadhesive characteristics because of the presence of $-\text{COOH}$ groups in their chain
6 (Arunbabu et al., 2013). However, pristine polymeric hydrogels are mechanically weak and
7 poses insufficient electrical conductivity that limits their application as electrochemical
8 sensors (Damia Mawad, 2016). The incorporation of nanomaterials into robust hydrogels
9 generates tailored functionalities feasible to construct sensor and other drug delivery vehicles
10 (Puoci and Curcio, 2013; Gaharwar et al., 2014). Especially, integration of graphene with
11 polymeric hydrogel offer enhanced actuation performance due to its excellent physico-
12 mechanical properties and facile conjugation reactions (Worsley et al., 2012; Bhattacharjee et
13 al., 2013). The presence of hydroxyl, epoxy and carboxyl end functional groups on the
14 surface of GO provide multiple conjugation avenue and endow electrochemical properties to
15 hydrogel matrices (Bai et al., 2010; Liu et al., 2011).

16 PANI based conducting hydrogels that combine the properties of both conducting polymers
17 and hydrogels, find potential application in the fields of electrochemical energy storage,
18 metal corrosion resistance, biological and chemical sensor (Guo et al., 2015; Wang et al.,
19 2014; Nath et al., 2014; Ding et al., 2015). The inbuilt properties of hydrogels such as good
20 biocompatibility and permeability to water-soluble biochemicals in combination with
21 excellent electronic properties of PANI would be an ideal matrix for enzyme immobilization
22 and preserves their bioactivity for electrochemical detection. Moreover, the 3D conducting
23 polymer network offers sufficient charge collection capacity to hydrogel. Overall the
24 synthetic method can be easily manipulated to construct intricately patterned electrodes at
25 low cost (Heller, 2006; Åsberg and Inganäs, 2003). Pan et al (2012) have reported on the
26 synthesis of hierarchical nanostructured PANI hydrogel, in which phytic acid is used as the
27 gelator and dopant to directly form a conducting polymer network. Here we propose vinyl
28 substituted polyaniline (VS-PANI) forming hydrogel in the presence of PAA.

29 Metallophthalocyanines (MPc) show good electrocatalysis in the oxidation of several
30 industrially and biologically important molecules (Barrera et al., 2006; Pereira-Rodrigues et
31 al., 2002). d-block MPc (Fe, Co) complexes with macrocyclic extended π -systems endow
32 them to undergo fast redox processes with minimal reorganizational energies have been
33 intensively studied as redox mediators for glucose enzyme electrodes (Hart and Wring,

1 1997; Wang et al., 2005). GOx catalyses the oxidation of glucose, in which the natural
2 electron-acceptor (oxygen) is converted to the H₂O₂. Modification of the electrode surface
3 with highly biocompatible MPc will reduce the voltage requirement for H₂O₂ oxidation
4 (Ozoemena et al., 2005). Among numerous MPc families, double-decker lanthanide
5 derivatives, lutetium phthalocyanine (LuPc₂) attract interest due to their high intrinsic
6 conductivity and electrochemical properties (Basova et al., 2008). The electrochromic
7 behaviour of lanthanide phthalocyanine films has been used for the detection of nicotinamide
8 adenine dinucleotide (Pal et al., 2011; Galanin and Shaposhnikov, 2012a). Synthesis and
9 spectral properties of lanthanide double-decker complexes with tetrabenzoporphyrin and
10 phthalocyanine have also been studied (Galanin and Shaposhnikov, 2012b).

11 Herein, we report a scalable synthesis of multifunctional conducting PAA hydrogel (MFH)
12 integrated with rGO, VS-PANI and LuPc₂ as three dimensional robust matrix for GOx
13 immobilization (PAA-rGO/VS-PANI/LuPc₂/GOx-MFH). We have integrated the
14 multicomponents such as PAA with rGO, VS-PANI through free radical polymerization
15 using methylene bis-acrylamide as the cross linker and initiator. The LuPc₂ was then doped to
16 form multifunctional hydrogel (PAA-rGO/VS-PANI/LuPc₂-MFH). Finally, biosensor was
17 fabricated by immobilizing GOx into PAA-rGO/VS-PANI/LuPc₂-MFH and subsequently
18 used for electrochemical detection of glucose.

19 **2. Experimental**

20 *2.1. Chemicals*

21 rGO (surface area: 450 m²/g), poly(ethylene glycol) diamine (NH₂-PEG-NH₂), *N*-(3-
22 dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC hydrochloride), acrylic
23 acid, 3-vinylaniline (97%), *N,N'*-methylenebis(acrylamide) (MBA), ammonium persulfate
24 (APS), D-(+)-glucose, glucose oxidase from aspergillus niger, Type X-S, lyophilized powder,
25 100,000-250,000 units/g solid (without added oxygen), glutaraldehyde solution (Grade II,
26 25% in H₂O), Potassium ferrocyanide, Potassium ferricyanide, potassium chloride (KCl),
27 sodium chloride (NaCl), phosphate buffer solution (PBS, pH 7.0) were all purchased from
28 Sigma Aldrich (UK) and used as received. Synthesis and characterisation of lutetium bis-
29 phthalocyanine (LuPc₂) has appeared in an earlier publication (Gurek et al., 2001). *N,N*-
30 Dimethylformamide (DMF) is of analytical grade obtained from Sigma Aldrich.

31 *2.2. Apparatus*

1 The morphologies of the prepared MFH were examined by FEI-Nova scanning electron
2 microscopy (SEM) with a low magnification (160,000 \times) and high voltage (10 kV). UV–
3 Visible spectrophotometer (Varian 50-scan UV–Visible) in the range 190–1100 nm was used
4 to measure the absorption spectra of MFH. FT-IR spectra were recorded on a Perkin Elmer
5 Spectrum 100 spectrophotometer. The electrochemical measurements of the electrodes were
6 conducted using a portable bipotentiostats μ Stat 200 that was purchased from DropSens
7 (Spain) and controlled by PC with DropView 200 software. The sensor platforms were
8 disposable screen-printed carbon electrodes DRP-C110 also from DropSens with 4 mm
9 diameter working electrode. The working and auxiliary electrodes are carbon, while silver
10 forms the reference electrode and the trager (carrier) is ceramic. The working electrodes were
11 modified with PAA-rGO/VS-PANI/LuPc₂/GOx-MFH or PAA-VS-PANI/LuPc₂/GOx-MFH
12 or PAA-LuPc₂/GOx-MFH. The electroactivity of MFH modified electrode was evaluated by
13 recording cyclic voltammogram in potassium ferro/ferricyanide solution containing 0.1 M
14 NaCl in the potential range from -0.5V to +0.5V. The biocatalytic activity of PAA-rGO/VS-
15 PANI/LuPc₂/GOx-MFH biosensor was evaluated by cyclic voltammetry for a solution of
16 4 mM glucose in 0.1 M PBS. The amperometric responses of PAA-rGO/VS-
17 PANI/LuPc₂/GOx-MFH biosensor towards glucose detection were recorded under stirred
18 conditions in 0.1 M PBS (pH 7.0) by applying a constant potential of +0.3 V at the working
19 electrode.

20 *2.3. Preparation of PAA-rGO/VS-PANI/LuPc₂/GOx-MFH biosensor*

21 The preparation of MFH involves three steps: (i) preparation of amine functionalized
22 rGO, (ii) formation of PAA-rGO/VS-PANI-MFH, (iii) incorporation of LuPc₂ and
23 subsequent immobilization of GOx to form PAA-rGO/VS-PANI/LuPc₂/GOx-MFH
24 biosensor.

25 *2.3.1. Preparation of amine functionalized rGO*

26 Amine functionalized rGO preparation was carried out according to earlier reports (Lei et al.,
27 2016). Briefly, about 10 mg of rGO was dispersed in 10 mL of water containing NH₂-PEG-
28 NH₂ (0.1 g) and EDC.HCl (0.1 g) under vigorous stirring overnight. rGO-PEG-NH₂ thus
29 obtained were allowed to settle down and filtered through polycarbonate membrane (pore
30 size=200 nm). The samples were dried in vacuum oven at 60 $^{\circ}$ C for further use.

31 *2.3.2. Formation of PAA-rGO/VS-PANI-MFH*

1 5 mg of rGO-PEG-NH₂ was dispersed in 10 mL of HCl (0.1M) containing 10 mg of
2 EDC.HCl under sonication. Subsequently, AA (2.4 mL), MBA (2 M) 3-vinylaniline (0.1 M)
3 and APS (0.2 M) were added into the mixture with continued stirring. The above solution
4 mixture was purged with N₂ gas to remove the dissolved oxygen content and poured into a
5 pre-cleaned vial. The vial was placed in the vacuum oven maintained at 70 °C for about an
6 hour. A solid hydrogel was formed at around 45 min. The solid hydrogel was cooled to room
7 temperature. The colour of the hydrogel turned to green with black spots to form PAA-
8 rGO/VS-PANI-MFH. For comparative purposes, PAA/VS-PANI-MFH and PAA-rGO-MFH
9 were prepared separately. This was achieved by excluding rGO-PEG-NH₂ and 3-vinylaniline
10 respectively in the hydrogel preparation step. It took around 90 min for gelation in the
11 absence of rGO-PEG-NH₂ (PAA/VS-PANI-MFH) and 60 min in the absence of 3-
12 vinylaniline (PAA-rGO-MFH). The hydrogels thus obtained were systematically analyzed by
13 SEM, UV-visible, FTIR spectroscopy to determine the morphology and chemical structure of
14 the hydrogels.

15 2.3.3. Fabrication of PAA-rGO/VS-PANI/LuPc₂/GO_x-MFH biosensor

16 Initially PAA-rGO/VS-PANI-MFH (2 μl) was formed as detailed before on the surface of
17 screen printed electrode (Area = 0.1256 cm²). Then LuPc₂ (2 μl) (5 mg dissolved in 1 mL
18 chloroform) was dropped and allowed to dry in oven at 60°C for 2 h. The fabrication of PAA-
19 rGO/VS-PANI/LuPc₂/GO_x-MFH biosensor was achieved by immobilizing GO_x (1 μl) (10
20 mg in 1 mL PBS (pH 7.0)) along with glutaraldehyde (1 μl) and dried at room temperature
21 for further analysis. Similarly, the other two PAA/VS-PANI/Pc₂/GO_x-MFH biosensor and
22 PAA-rGO/LuPc₂/GO_x-MFH biosensor were fabricated.

23 3. Results and Discussion

24 3.1. Synthesis of PAA-rGO/VS-PANI-MFH

25 The steps involved in the preparation of the PAA-rGO/VS-PANI/LuPc₂/GO_x-MFH are
26 illustrated as scheme in Fig. 1A. Initially rGO-PEG-NH₂ was obtained through covalent
27 grafting of PEG diamine chains onto carboxyl sites in the surface of rGO sheets via
28 amidation in aqueous media. PAA-rGO/VS-PANI-MFH was formed through free radical
29 polymerization of AA along with rGO-PEG-NH₂ and 3-vinyl aniline in the presence of MBA
30 and APS (Layek and Nandi, 2013) (Fig. 1A). The inclusions of rGO-PEG-NH₂ and VS-
31 PANI in the hydrogel were aimed to improve the binding sites and favourable conditions for

1 loading LuPc₂ and GOx immobilization. PAA containing –COOH groups in MFH protect
2 VS-PANI in its protonated form. Fig. 1B shows a photograph of the prepared PAA-rGO/VS-
3 PANI-multifunctional hydrogel (MFH). This hydrogel exhibits green colour which is
4 originated from the protonated and doped VS-PANI in MFH with dispersed black spots.

5 3.2. Morphology

6 Fig.1B(a and b) shows SEM images of PAA-rGO/VS-PANI-MFH and PAA/VS-
7 PANI-MFH respectively. PAA-rGO/VS-PANI-MFH clearly showed randomly distributed
8 micro porous 3D inter-linked network, due to the integration of the components involved
9 (Fig. 1B(a)). The average pore diameter of PAA-rGO/VS-PANI-MFH was estimated to be
10 approximately 7µm. Such 3D interconnected hierarchical porous structure offers greater
11 effective surface areas for LuPc₂ and GOx immobilization. However, the PAA/VS-PANI-
12 MFH showed a layered structure characterised with smooth surface and much smaller pore
13 size as shown in Fig. 1B(b). This suggests that VS-PANI twists with the PAA component and
14 fills up the existent voids in PAA/VS-PANI-MFH. Further, the surface area of the MFH was
15 also studied through Brunauer, Emmett and Teller (BET) measurements. From the BET
16 results, the surface area of PAA-rGO/VS-PANI-MFH, PAA-rGO-MFH are calculated to be
17 $1.3653 \pm 0.1416 \text{ m}^2/\text{g}$ and $0.9794 \pm 0.1095 \text{ m}^2/\text{g}$ respectively. We presume that the harsh
18 pretreatment process (degassing condition – 3 h at 120 deg C) before N₂ physisorption has
19 induced a collapse of the pores structure of MFH (Iza et al., 2000). The surface area value is
20 slightly lower than previously reported polyacrylamide–polyacrylic acid copolymer hydrogel
21 ($4.9 \text{ m}^2/\text{g}$) (Li et al., 2002).

22 3.3. UV visible analysis

23 UV-visible spectrum of pristine LuPc₂ and hydrogels decorated with LuPc₂ (PAA-rGO/VS-
24 PANI/LuPc₂, PAA/VS-PANI/LuPc₂ and PAA-rGO/LuPc₂), were recorded by using Cary 50
25 UV-Visible Spectrophotometer (190-1100 nm), as shown in Fig. 2. Thin films of LuPc₂ and
26 the hydrogels were formed on the surface of pre-cleaned glass substrate. Main absorption
27 peaks derived from the measured absorption spectra are presented in Table 1 (Supporting
28 Information (SI-1)). Bis-phthalocyanine complex of LuPc₂ thin film exhibited characteristic
29 UV-visible B and Q absorption bands. A strong N-band at 317 nm, Soret B band at 390 nm
30 region and intense visible Q band of the macrocycles at 706 nm can be seen (Fig. 2inset)
31 (Basova et al., 2008). Also two weak p-radical blue valence (BV)/ red valence (RV) related
32 bands attributed to 2eg–a_{1u} and 1eg–a_{1u} transitions were observed at 510–580 (BV) and

1 930–970 nm (RV), as reported earlier (Nekelson et al., 2007; Zheng et al., 2015; Pushkarev et
2 al., 2012). In the hydrogels that includes VS-PANI, PAA-rGO/Vs-PANI/LuPc₂ and
3 PAA/Vs-PANI/LuPc₂ showed a shoulder peak around 430 nm corresponding to the
4 benzenoid to quinoid transitions of backbone PANI structure (Fig 2a,b). Additionally the N
5 band of LuPc₂ was coupled with π - π^* absorption peak of VS-PANI. These results
6 demonstrate the presence of interconnected networks of VS-PANI in the hydrogel formed
7 (Ipek et al., 2014). The Q band was shifted bathochromically in the case of PAA-rGO/Vs-
8 PANI/LuPc₂ to 712 nm (Fig 2a), PAA-rGO/LuPc₂ to 718 nm (Fig 2c) and hypsochromically
9 in the case of PAA/Vs-PANI/LuPc₂ to 702 nm (Fig 2b). This reveals the perturbation in the
10 electronic states of LuPc₂ in MFH due to the electron transfer from rGO to phthalocyanine
11 ring which leads to the formation of π - π stacking interaction between them and thus lower
12 the band gap. This ensures that the studied MFHs were well decorated with double-decker
13 lutetium (III) phthalocyanine moieties (Mani et al., 2015).

14 3.4. FTIR analysis

15 The FTIR spectra of PAA-rGO/Vs-PANI-MFH, PAA/Vs-PANI-MFH and PAA-rGO-MFH
16 are shown in the SI-2. PAA-rGO/Vs-PANI-MFH exhibited the main characteristic
17 absorption peaks of PAA around 2900-3600 cm⁻¹ (-OH stretching), 1700 cm⁻¹ (C=O
18 stretching) and VS-PANI around 1450 cm⁻¹ (benzenoid rings), 1620 cm⁻¹ (quinoid rings)
19 (Kennedy and Ratner, 1995; Saravanan et al., 2006; Nabid et al., 2007) (SI-2a). The absence
20 of 1180 cm⁻¹ peak in PAA/Vs-PANI/LuPc₂ (SI-2b) and the suppression of 1450 cm⁻¹
21 absorption peak in PAA-rGO/LuPc₂ (SI-2c) can be noticed which illustrates the difference in
22 structure among the three prepared MFH.

23 3.5. Electrochemical performance of modified electrodes

24 Cyclic voltammograms (CVs) were recorded for the PAA-rGO/Vs-PANI/LuPc₂/GOx-MFH,
25 PAA/Vs-PANI/LuPc₂/GOx-MFH, and PAA-rGO/LuPc₂/GOx-MFH biosensor using 5 mM
26 potassium ferro/ferricyanide solution containing 0.1M NaCl as the bench mark redox system
27 in the potential range from +0.5 to -0.5 V at a scan rate of 0.025 Vs⁻¹, as shown in Fig. 3.
28 CVs of MFH based biosensor showed well-defined reversible peaks corresponding to the
29 Fe(II)/Fe(III) redox transition process. The efficacy of the electrode was compared in terms
30 of anodic to cathodic peak potential separation ($\Delta E_p = E_{pa} - E_{pc}$) and using oxidation or
31 reduction peak currents (I_{pa} or I_{pc}) of the Fe(CN)₆^{3-/4-}. The I_{pa} of PAA-rGO/Vs-
32 PANI/LuPc₂/GOx-MFH Fig. 3(a) was ~2.32 and ~6.38 times higher than those exhibited by

1 PAA/VS-PANI/LuPc₂/GOx-MFH Fig. 3(b) and PAA-rGO/LuPc₂/GOx-MFH biosensor Fig.
2 3(c). However the I_{pa} of PAA-rGO/VS-PANI/LuPc₂/GOx-MFH (14.55 μA) was typically
3 lower than the pristine PAA-rGO/VS-PANI/LuPc₂-MFH (21.6 μA) Fig. 3(d) prepared in the
4 absence of GOx. This ensures the effective immobilization of GOx onto PAA-rGO/VS-
5 PANI/LuPc₂-MFH matrix (Hua et al., 2012). Furthermore the ΔE_p value increased from 105
6 mV for the PAA-rGO/VS-PANI/LuPc₂/GOx-MFH to 240 mV for the PAA-rGO/LuPc₂/GOx-
7 MFH. The results demonstrates that the presence of VS-PANI as interconnecting network in
8 MFH augment electronic conductivity and promote faster electron transport kinetics at PAA-
9 rGO/VS-PANI/LuPc₂/GOx-MFH (Dou et al., 2016; Gopalan et al., 2013). CVs of PAA-
10 rGO/VS-PANI/LuPc₂/GOx-MFH were also recorded for different scan rates (10–100 mV/s)
11 (SI-3). A plot of I_{pa} and I_{pc} were linearly proportional to square root v , which indicates a
12 diffusion controlled process of Fe(CN)₆^{3-/4-} redox reaction at PAA-rGO/VS-
13 PANI/LuPc₂/GOx-MFH (Mashazi et al., 2006). From the plot of I_{pa} versus $v^{1/2}$ a slope of
14 $1.194 \times 10^{-4} \text{ A V}^{-1/2} \text{ s}^{1/2}$ was obtained. For the diffusion controlled reversible process the
15 electrochemical active surface area (A) was determined using Randles–Sevcik equation
16 (Siswana et al., 2006). From the D value for K₃[Fe(CN)₆] = $6.7 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ (Ye et al.,
17 2005), and n = 1, the area (‘A’) of PAA-rGO/VS-PANI/LuPc₂/GOx-MFH was estimated to
18 be 0.244 cm². The value of ‘A’ of PAA-rGO/VS-PANI/LuPc₂/GOx-MFH was found to be
19 higher than that of the bare GCE (0.078 cm²), GOx/PANI microtube/GCE (0.156 cm²)
20 (Zhang et al., 2015) and rGO-C60/GOx (0.12 cm²) (Thirumalraj et al., 2015) respectively. It
21 is understood that the high value of ‘A’ results from the porous structure of PAA-rGO/VS-
22 PANI-MFH as seen in Fig. 1B(a). Furthermore, swelling ratio of PAA-rGO/VS-PANI-MFH
23 is found to be approximately 3 and 1.4 times higher than PAA/VS-PANI -MFH and PAA-
24 rGO-MFH respectively at a fixed time. The higher swelling ratio value results from more
25 pore volume at PAA-rGO/VS-PANI-MFH, where the larger the pore volume leads to lower
26 diffusional resistance.

27 3.6. Electrochemical impedance measurements

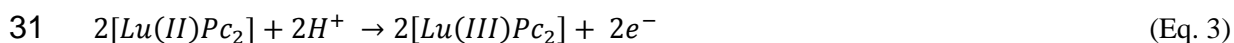
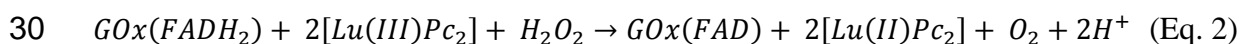
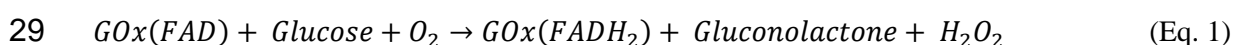
28 The electrochemical surface characteristics of the electrodes modified with PAA-rGO/VS-
29 PANI/LuPc₂/GOx-MFH (a) and PAA/VS-PANI/LuPc₂/GOx-MFH (b) were monitored
30 through electrochemical impedance measurements in PBS containing 0.1M NaCl [SI-4A].
31 The immobilization of GOx enzyme onto the porous sites of the hydrogel alters the interfacial
32 capacitance and electron-transfer resistance of the conductive electrodes (Ding et al., 2005;

1 Katz and Willner 2003; Pei et al., 2001). The nyquist plot of PAA-rGO/VS-
 2 PANI/LuPc₂/GOx-MFH biosensor (a) exhibited a much smaller semicircular diameter
 3 ascribed to electron-transfer resistance than those observed on PAA/VS-PANI/LuPc₂/GOx-
 4 MFH (b). The smaller semicircular diameter indicated faster electron transfer kinetics at high
 5 frequency region and the linear part at lower frequency region ensures mixed kinetics and
 6 diffusion controlled process. The lower charge transfer resistance (R_{ct}) for PAA-rGO/VS-
 7 PANI/LuPc₂/GOx-MFH biosensor arises from the inter-connected PAA-rGO/VS-PANI
 8 network. The LuPc₂ decorated inside PAA-rGO/VS-PANI MFH provides easy electron
 9 conduction pathways. The impedance spectra obtained were fitted by a simple Randle circuit
 10 made up of electrolyte resistance (R_s), electron transfer resistance (R_{et}), double layer
 11 capacitance (C_{dl}) and Warburg coefficient (W). Data fitting was carried out using **THALES**
 12 software with a **complex non-linear least-squares** method. The equivalent circuit model is
 13 presented in [SI-4B].

14

15 3.7. Electrochemical detection of glucose

16 Fig. 4A shows the CV of PAA-rGO/VS-PANI/LuPc₂/GOx-MFH biosensor Fig. 4A(red
 17 curve) in bare PBS (pH 7.0) and in the presence of glucose (4mM) Fig. 4A(black curve). At
 18 PAA-rGO/VS-PANI/LuPc₂/GOx-MFH a reversible redox peak which corresponds to
 19 Lu(II)Pc₂/ Lu(III)Pc₂ was observed at -0.05 V/0.1 V under our experimental conditions, and
 20 this result is similar to results reported by Kadish et al. (2001). In the latter reference only one
 21 reduction and oxidation peak of Lu(Pc)₂ have been reported in a cast film in aqueous media
 22 (0.5 M KCl). For further confirmation, CV was also recorded for PAA-rGO/VS-PANI/LuPc₂-
 23 MFH in the absence of GOx, where a sharp redox couple at -0.12/ 0.1 V was obtained under
 24 identical conditions (Fig. 4A inset). Fig. 4A (black curve) shows the electrocatalytic
 25 oxidation of glucose (4 mM) at PAA-rGO/VS-PANI/LuPc₂/GOx-MFH. With the addition of
 26 glucose a large increase in anodic current, starting above 0.2 V was observed. This suggests
 27 that Lu(III)Pc₂ mediates electron transfer between the immobilized GOx and the electrode
 28 through oxidation of the reduced form of the enzyme. The plausible mechanism is as follows



1 Glucose is transferred from bulk solution to the PAA-rGO/VS-PANI/LuPc₂/GOx-MFH
2 biosensor through diffusion. The enzyme GOx(FAD) in the presence of the natural co-
3 substrate O₂, converts glucose to gluconic acid and O₂ to H₂O₂. The reduced GOx(FADH₂)
4 will be reoxidized by Lu(III)Pc₂ which exists in the MFH. The reduced mediator, Lu(II)Pc₂
5 which remains intact inside the MFH, is completely oxidized back to Lu(III)Pc₂ when the
6 anodic potential is applied, resulting in high currents. A similar mechanism was reported for
7 the electrochemical determination of glucose by GOx and the mediator by the Mn(III)-M(IV)
8 tetra(o-aminophenyl)rphyrin couple (Rishpon et al., 1991), cobalt(II) phthalocyanine-
9 cobalt(II) tetra(5-phenoxy-10,15,20-triphenylporphyrin), (CoPc-(CoTPP)₄) pentamer
10 (Ozoemena and Nyokong, 2006) and Au-ME-CoTCAPc SAM (Mashazi et al., 2006).
11 However electrocatalytic oxidation of glucose at PAA/VS-PANI/LuPc₂/GOx-MFH and PAA-
12 rGO/LuPc₂/GOx-MFH showed only a marginal increase in the anodic current value (SI-5).
13 This confirms the importance of individual component (PAA, rGO, and VS-PANI) as
14 interconnected network in the hydrogel. To ascertain the importance of LuPc₂ and GOx in
15 MFH for glucose sensing, a similar MFH biosensor was fabricated without loading LuPc₂ and
16 GOx immobilization. MFH biosensor in the absence of GOx does not show glucose response
17 in the applied potential region. However, for MFH biosensor in the absence of LuPc₂, the
18 oxidation glucose response current was feeble (figures not shown). This further ensures the
19 significance of combined presence of LuPc₂ and GOx in MFH for good electrochemical
20 glucose determination.

21 *3.8. Amperometric response of glucose at PAA-rGO/VS-PANI/LuPc₂/GOx-MFH biosensor*

22 To demonstrate the functioning of PAA-rGO/VS-PANI/LuPc₂/GOx-MFH as a potential
23 glucose biosensor, amperometric measurements were recorded for varied glucose
24 concentration as shown in Fig 4B. Optimization of parameters for recording amperometric
25 measurements were presented in SI-6. PAA-rGO/VS-PANI/LuPc₂/GOx-MFH biosensor was
26 kept at +0.3 V in 0.1M PBS (pH 7.0). The bioelectrocatalytic current response for the
27 successive additions of 1 mM glucose was monitored amperometrically under stirred
28 condition. The PAA-rGO/VS-PANI/LuPc₂/GOx-MFH biosensor showed a rapid response to
29 the changes in glucose concentration and the steady-state response current reached within
30 1s. The response time is much lower than PANI incorporated silica particles (Manesh et al.,
31 2010), MWNT-g-SPAN-NW GOx based biosensors (Lee et al., 2010), GOx/PtNP/PAni
32 hydrogel/Pt (Zhai et al., 2013), and GOx/GNp/CS/GOx/GNp/PAA/Pt (Wu et al., 2007). The
33 reduced amperometric response time observed here is attributed to the faster diffusion of

1 glucose into the porous three-dimensional PAA-rGO/VS-PANI MFH. The rapid response to
2 glucose was also achieved due to the existence of LuPc₂ that transfers electrons at a fast pace
3 from GOx to MFH (Koide and Yokoyama, 1999; Dervisevic et al., 2014). The
4 amperometric *i-t* response current of the biosensor is linear in the range of 2–12 mM
5 concentration of glucose ($R^2 = 0.9903$) as shown in Fig. 4B(Inset, line (a)) with the
6 sensitivity of $15.31 \mu\text{A mM}^{-1} \text{cm}^{-2}$ calculated from the slope of the calibration plot. The
7 sensitivity is higher than those of $5.2 \mu\text{A cm}^{-2} \text{mM}^{-1}$ for GOD immobilized in an osmium-
8 based three-dimensional redox hydrogel (Ohara et al., 1994), $1.7 \mu\text{A cm}^{-2} \text{mM}^{-1}$ for GOD
9 formed upon cross-linking with the copolymer hydrogels (Lumley-Woodyear et al., 1995).
10 To illustrate the importance of LuPc₂ and GOx in the fabrication of glucose biosensor, the
11 amperometric *i-t* response for glucose was also observed for electrodes PAA-rGO/VS-
12 PANI/LuPc₂-MFH and pristine PAA-rGO/VS-PANI-MFH (both fabricated without GOx).
13 Typically, the electrodes exhibited a linear range from 2-5 mM (PAA-rGO/VS-PANI/LuPc₂-
14 MFH) as shown in Fig. 4B(Inset, line (b)) and 1-3 mM (pristine PAA-rGO/VS-PANI-MFH)
15 as shown in Fig. 4B(Inset, line (c)) of glucose respectively. The linear range of glucose
16 detection for pristine PAA-rGO/VS-PANI-MFH and PAA-rGO/VS-PANI/LuPc₂-MFH are
17 relatively lower than PAA-rGO/VS-PANI/LuPc₂/GOx-MFH biosensor. Furthermore, the
18 sensitivity of PAA-rGO/VS-PANI/LuPc₂-MFH ($0.912 \mu\text{A mM}^{-1} \text{cm}^{-2}$) and pristine PAA-
19 rGO/VS-PANI-MFH ($0.455 \mu\text{A mM}^{-1} \text{cm}^{-2}$) are ~16.8 and ~33.6 times lower than PAA-
20 rGO/VS-PANI/LuPc₂/GOx-MFH biosensor. The smaller linear range along with lowered
21 sensitivity value in the case of PAA-rGO/VS-PANI/LuPc₂-MFH and pristine PAA-rGO/VS-
22 PANI-MFH might be due the blockage of pores in the hydrogel structures by the oxidised
23 products of glucose which in-turn hampered the diffusion of glucose molecules to the
24 electrode surface. However, the obtained longer linear response of PAA-rGO/VS-
25 PANI/LuPc₂/GOx-MFH biosensor (2-12 mM) to glucose is suitable for glucose detection in
26 human blood samples (normal glucose range in human blood is 4.4–6.6 mM). The wide
27 linear range with high sensitivity of PAA-rGO/VS-PANI/LuPc₂/GOx-MFH biosensor for the
28 amperometric detection of glucose may be due to the following facts. The porous three
29 dimensional PAA-rGO/VS-PANI MFH matrices offer low diffusional resistance to glucose
30 and hence reduces the mass transport barrier. rGO and VS-PANI as interconnected network
31 within hydrogel provide electronic conductivity to achieve fast electron transport. PAA based
32 hydrogel craft favorable environment for loading LuPc₂ and GOx enzyme immobilization
33 (Zhao et al., 2013). The results obtained from the amperometric current response of glucose
34 at PAA-rGO/VS-PANI/LuPc₂-MFH and pristine PAA-rGO/VS-PANI-MFH electrodes

1 illustrate that GOx plays the key component in the detection of glucose and LuPc₂ effectively
2 transfers electrons from GOx to the electronically conducting hydrogel matrix.

3 The apparent Michaelis–Menten constant (K_M) was calculated as 17.05 mM using the slope
4 and intercept values from Lineweaver–Burk plot for PAA-rGO/VS-PANI/LuPc₂/GOx-MFH
5 biosensor (Iwuoha and Smyth, 1997). The K_M value obtained for this biosensor is higher than
6 other GOx-mediator based glucose sensors (Ghica and Brett, 2005). The high value
7 of K_M informs that the GOx in the PAA-rGO/VS-PANI/LuPc₂/GOx-MFH biosensor
8 possesses higher affinity for glucose. The limit of detection (LoD) for glucose at PAA-
9 rGO/VS-PANI/LuPc₂/GOx-MFH biosensor was calculated as 25 μ M (signal to noise ratio =
10 3). The detection limit is estimated as three times of the standard deviation of the
11 background. The LoD value was lower than Ru mediator attached PVPAA copolymer/GOx
12 biosensor (Deng, et al, 2014). In enzymless detection of glucose a LoD in the nano mole
13 range has been found for Cu/3D graphene hybrid-based electrode (Hussain et al., 2015).

14 The analytical performance of the proposed sensor are compared with similar
15 enzyme/mediator based glucose biosensors and presented in [Table 1](#). From the [Table 1](#) values
16 it is understood that the proposed PAA-rGO/VS-PANI/LuPc₂/GOx-MFH biosensor exhibits
17 wide linear range response to glucose, high sensitivity and low LoD.

18 *3.9. Repeatability, Reproducibility and stability of PAA-rGO/VS-PANI/LuPc₂/GOx-MFH* 19 *biosensor*

20 Different aspects concerning the utility of PAA-rGO/VS-PANI/LuPc₂/GOx-MFH as sensible
21 glucose biosensor were evaluated. The repeatability of PAA-rGO/VS-PANI/LuPc₂/GOx-
22 MFH as sensible glucose biosensor was tested through amperometric measurements for two
23 different concentrations of glucose, 4 mM and 6 mM ([SI-7](#)). Seven repetitive measurements
24 of a single PAA-rGO/VS-PANI/LuPc₂/GOx-MFH biosensor (washed with 0.1M PBS
25 between individual measurements) yielded a relative standard deviation (RSD) value of 6.1%
26 (4 mM) and 6.5% (6 mM) of glucose. To demonstrate the stability of fabricated PAA-
27 rGO/VS-PANI/LuPc₂/GOx-MFH biosensor, amperometric response to 4 mM glucose
28 solution was periodically registered for a period of 3 months keeping the electrodes stored at
29 4 °C refrigerated condition. It has been found that the PAA-rGO/VS-PANI/LuPc₂/GOx-MFH
30 biosensor retained > 98% of current response which indicates that the functioning of GOx
31 well protected within the new MFH without leaching under ideal conditions. The leaching
32 effect of LuPc₂ from the fabricated PAA-rGO/VS-PANI/LuPc₂-MFH was investigated by

1 recording UV-visible spectrum before and after immersion into 0.1M PBS for a time period
2 of 30 minutes (time taken to record amperometry). From the characteristic B and Q band
3 absorption peaks of LuPc₂ it is confirmed that there is no leaching of LuPc₂ from PAA-
4 rGO/VS-PANI/LuPc₂-MFH electrode. This may be due to the π - π interaction between the
5 rGO, VS-PANI with double ducker LuPc₂ (Fukuda et al., 2012). The reproducibility of PAA-
6 rGO/VS-PANI/LuPc₂/GO_x-MFH biosensor was estimated with ten similarly fabricated
7 sensor assemblies. The results revealed that the biosensor has a satisfactory reproducibility
8 (RSD = 2.2%, $n = 10$). Under the applied potential of +0.3 V, the addition of interfering
9 substances such as ascorbic acid and uric acid hardly affects the amperometric response
10 current of glucose (4 mM) (SI-8). With three repetitive measurements of glucose (4 mM)
11 along-with interfering substances in a single PAA-rGO/VS-PANI/LuPc₂/GO_x-MFH
12 biosensor, the coefficient of variation (COV) was found to be 10.03%. Also the COV was
13 calculated for the reproducibility test results of glucose (4 mM) in the presence of
14 interference and was found to be 11.36% ($n=5$). This shows that PAA-rGO/VS-PANI/LuPc₂-
15 MFH biosensor exhibit selective detection of glucose even in the presence of
16 electrochemically active substances.

17 *3.10. Glucose determination in real samples at PAA-rGO/VS-PANI/LuPc₂/GO_x-MFH* 18 *biosensor*

19 To illustrate the feasibility of PAA-rGO/VS-PANI/LuPc₂/GO_x-MFH as potential biosensor
20 in industrial as well in clinical diagnosis, real sample analysis was carried out. In our work,
21 commercial glucose samples and human serum obtained from Biomolecular Sciences
22 Research Centre at Sheffield Hallam University were suitably diluted using 0.1 M PBS (pH
23 7.0) to obtain required concentration sample solutions. A continuous amperometry was
24 recorded for varied additions of real samples under identical experimental conditions as
25 similar to Fig. 4B. PAA-rGO/VS-PANI/LuPc₂/GO_x-MFH biosensor exhibited response to all
26 the real samples added. The data obtained from analysis are summarized in SI-9. The results
27 obtained are satisfactory with an error percentage of +3.0. The satisfactory results
28 demonstrate good practical applicability of the proposed biosensor.

29 **4. Conclusions**

30 In this work, we demonstrate the synthesis of multifunctional conducting polyacrylic acid
31 (PAA) hydrogel (MFH) integrated with reduced grapheme oxide (rGO) and vinyl substituted
32 polyaniline (VS-PANI) (PAA-rGO/VS-PANI-MFH). A redox mediator based glucose

1 biosensor was constructed by loading lutetium phthalocyanine (LuPc_2) and glucose oxidase
2 (GOx) into PAA-rGO/VS-PANI-MFH matrix (PAA-rGO/VS-PANI/ LuPc_2 /GOx-MFH). The
3 structural stability and hierarchical porosity of PAA-rGO/VS-PANI-MFH resulted in large
4 surface area for GOx immobilization. The high electrical conductivity is achieved through
5 interconnected VS-PANI network for rapid mass/charge transport. LuPc_2 functions as an
6 excellent redox mediator and reduces the amperometric glucose detection potential to +0.3
7 V. The present work demonstrates that the multi-components (rGO, VS-PANI, and LuPc_2)
8 have synergistic influences on the performance of biosensor. The PAA-rGO/VS-
9 PANI/ LuPc_2 /GOx-MFH biosensor exhibits wide linear range and high sensitivity to glucose
10 with a low detection limit. PAA-rGO/VS-PANI/ LuPc_2 /GOx-MFH could be effectively used
11 as an electrochemical biosensor in industrial as well clinical diagnosis. Further, the MFH
12 provides scope for loading of other redox enzymes to fabricate high performance biosensors.
13

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19

20 **6. Supporting informations are available for this paper**

21

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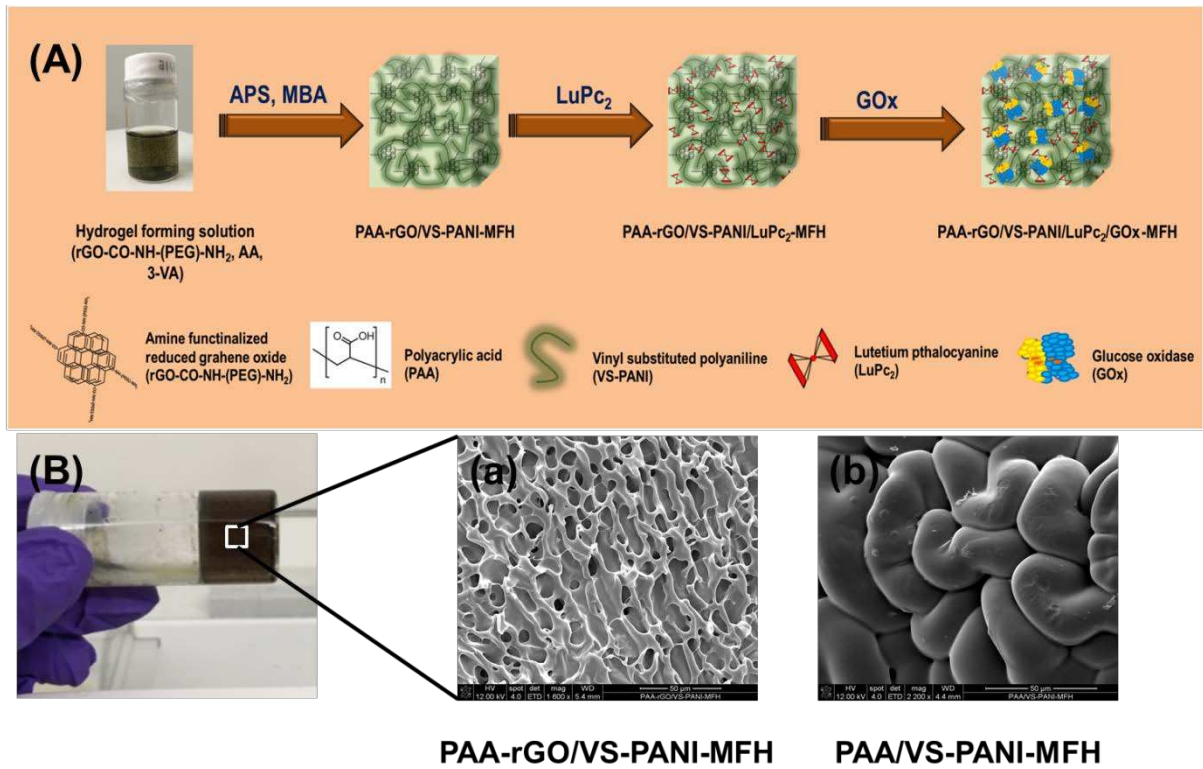
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Fig. 1. (A) Schematic representation of the formation of PAA-rGO/VS-PANI/LuPc₂/GOx-MFH; (B) SEM images of (a) PAA-rGO/VS-PANI-MFH, (b) PAA/VS-PANI-MFH

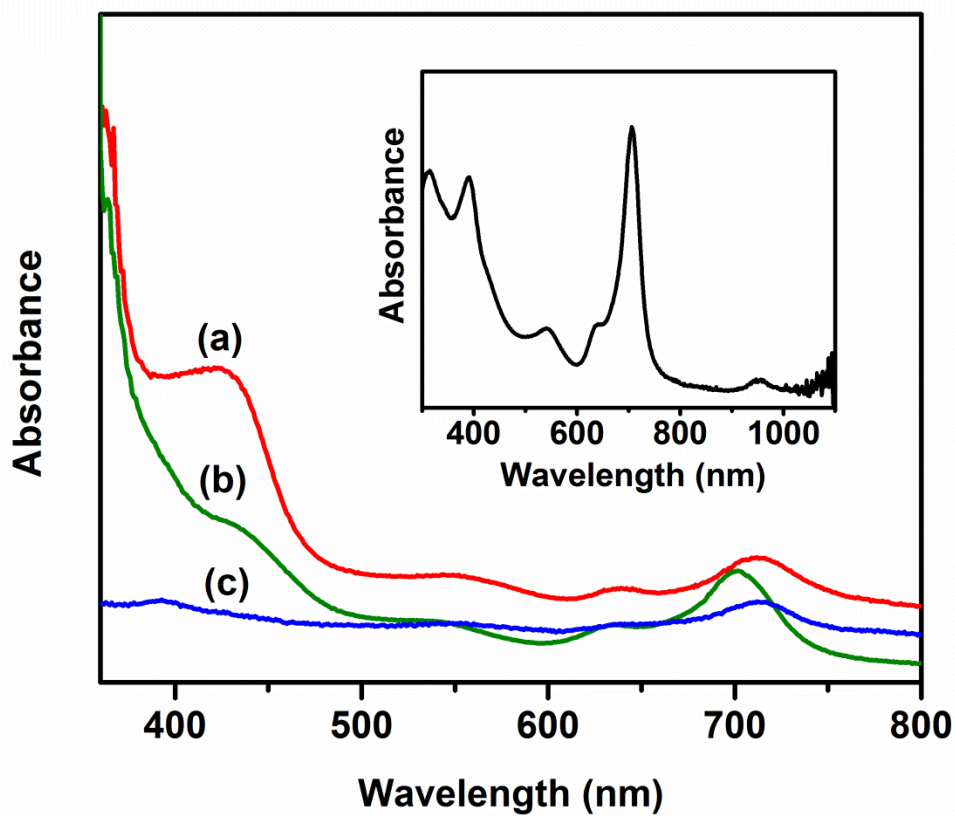


Fig. 2. UV-visible spectrum of (a) PAA-rGO/VS-PANI/LuPc₂-MFH, (b) PAA/VS-PANI/LuPc₂-MFH, (c) PAA-rGO/LuPc₂ -MFH. Inset UV-visible spectrum of LuPc₂ film

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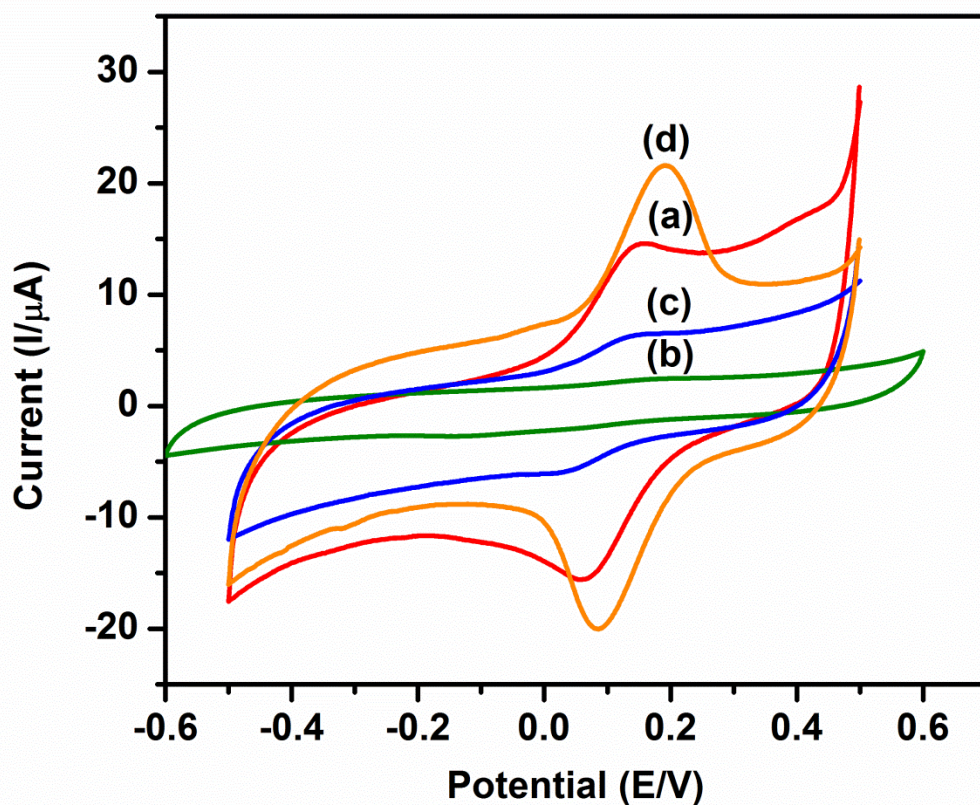


Fig. 3. Cyclic voltammogram of (a) PAA-rGO/VS-PANI/LuPc₂/GOx-MFH, (b) PAA/VS-PANI/LuPc₂/GOx-MFH, (c) PAA-rGO/LuPc₂/GOx-MFH, (d) PAA-rGO/LuPc₂-MFH recorded in 5 mM Potassium ferro/ferri cyanide solution containing 0.1M NaCl; scan rate = 25 mV/s

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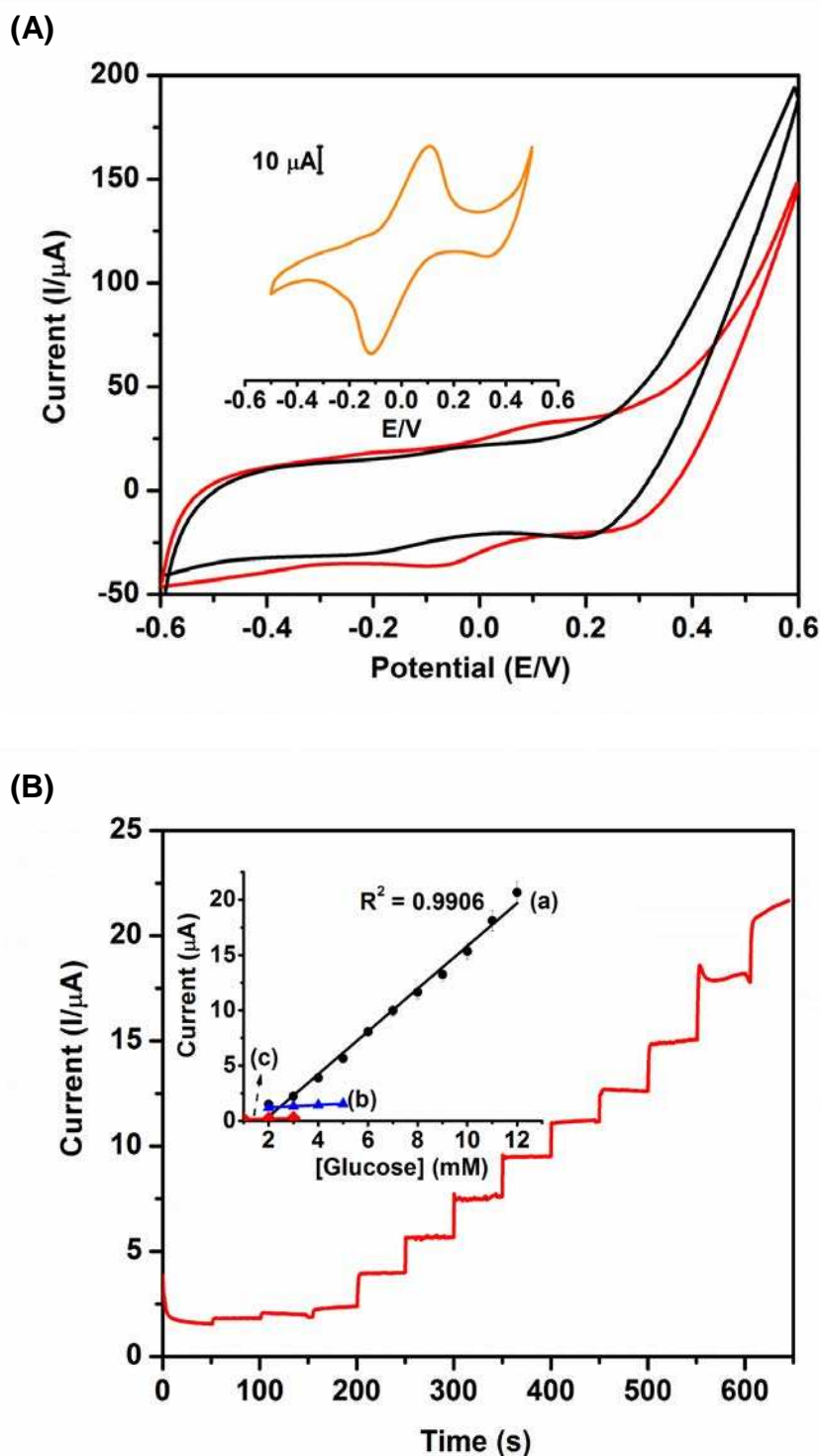


Fig. 4. (A) Cyclic voltammogram of PAA-rGO/VS-PANI/LuPc₂/GOx-MFH (red line) 0 mM glucose and (black line) 4 mM glucose in 0.1M PBS (pH 7.0). Inset Cyclic voltammogram of PAA-rGO/VS-PANI/LuPc₂-MFH in 0.1M PBS (pH 7.0); (B) Amperometry of PAA-rGO/VS-PANI/LuPc₂/GOx-MFH for successive addition of glucose in 0.1M PBS (pH 7.0). Inset: calibration plot peak current versus [glucose].

1 **Table 1. Comparison of analytical performance of some glucose biosensors**

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| Electrode | Response time (s) | linear range (mM) | Sensitivity ($\mu\text{A mM}^{-1} \text{cm}^{-2}$) | LOD (μM) | Ref. |
|--|-------------------|-----------------------|--|-----------------------|------------------------------|
| Au-MPS-OsPVP-GOx | 5 | 0.1–10 | - | 50 | (Hou et al., 1998) |
| SPCE/CoPC/GOD | 60 | 0.2–5 | 1.12 | - | (Crouch et al., 2005) |
| GCE-CoPc-(CoTPP) ₄ -GOx | ~5 | upto 11 | 0.024 | 10 | (Ozoemena and Nyokong, 2006) |
| {GOx/Au-(SH)PANI-g-MWNT} _n /ITO | ~8 | 1–9 | 3.97 | 0.06 | (Komathi et al., 2009) |
| GOx-nanoPANI/Pt | 3 | 0.01-5.5 | 97.18 \pm 4.62 | 0.3 \pm 0.1 | (Wang et al., 2009) |
| GOx-PANI nanowire | 10 | 0–8 | 2.5 | 0.05 | (Horng et al., 2009) |
| GOD/ERGO/PLL/GC | - | 0.25 - 5 | - | - | (Hua et al., 2012) |
| Nafion-GOx-ordered mesoporous carbon | 9 \pm 1 | 5-15 | 0.053 | 156.52 | (Zhou et al., 2008) |
| Os(2,2'bpy) ₂ -RP/GOx | <5 | 0-10 | 16.5 | - | (Deng et al., 2013) |
| poly(GMA-co-VFc)-GOx | 1 | 1-17 | 0.27 | 33 | Dervisevic et al., 2014 |
| Au/CA/(GOx/TFGn) _n | 6 | 1-13 mM | 19.9 | - | (Ren et al., 2016) |
| Hedgehog-like NiO nanostructures | - | 0.1–5.0 μM | 1052.8 | 1.2 | (Soomro et al., 2015) |
| PAA-rGO/Vs-PANI/LuPc ₂ /GOx-MFH | ~1 | 2-12 | 15.31 | 25 | Our present work |

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