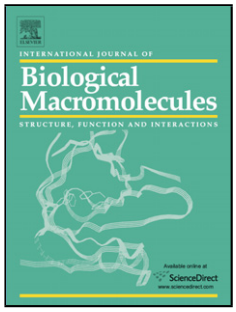

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PII: S0141-8130(16)31516-1
DOI: http://dx.doi.org/doi:10.1016/j.ijbiomac.2016.12.020
Reference: BIOMAC 6834

To appear in: *International Journal of Biological Macromolecules*

Received date: 5-9-2016
Revised date: 8-12-2016
Accepted date: 8-12-2016

Please cite this article as: Ece Buber, Abdulcelil Yuzer, Saniye Soylemez, Melis Kesik, Mine Ince, Levent Toppare, Construction and amperometric biosensing performance of a novel platform containing carbon nanotubes-zinc phthalocyanine and a conducting polymer, International Journal of Biological Macromolecules http://dx.doi.org/10.1016/j.ijbiomac.2016.12.020

Construction and amperometric biosensing performance of a novel platform containing carbon nanotubes-zinc phthalocyanine and a conducting polymer

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Abstract

A novel glucose oxidase (GOx) based amperometric biosensor utilizing a conducting polymer (CP), multi walled carbon nanotubes (MWCNTs) and a novel water soluble zinc phthalocyanine (ZnPc) was constructed. For this purpose, a novel ZnPc was synthesized to examine the role of being a part of support material for enzyme deposition. High water solubility was achieved with the introduction of tetra quaternized imidazolyl moieties at the peripheral positions of phthalocyanine. In order to fabricate the proposed biosensor, a graphite electrode was firstly modified with poly[9,9-di-(2-ethylhexyl)- fluorenyl-2,7-diyl] end capped with N,N-Bis(4- methylphenyl)-4-aniline (PFLA) and MWCNTs. Then, GOx was co-immobilized with ZnPc onto the modified surface. To the best our knowledge, a sensor design which combines conjugated polymer/MWCNTs/ZnPc was attempted for the first time and this approach resulted in improved biosensor characteristics. The constructed biosensor showed a linear response for glucose between 0.025 - 1.0 mM with a detection limit of 0.018 mM. K_M^{app} and sensitivity values were calculated as 0.53 mM and 82.18 $\mu Amm^{-1}cm^{-2}$, respectively. Moreover, scanning electron microscopy (SEM) and cyclic voltammetry (CV) techniques were used to investigate the surface modifications. Finally, fabricated biosensor was tested on beverages for glucose detection successfully.

Keywords: Amperometric biosensor, conducting polymer, zinc phthalocyanine, carbon nanotube, glucose biosensor

1. Introduction

Accurate detection and quantification of glucose concentration are vital in clinical and biological samples and food processing [1]. Glucose is an important carbohydrate circulating in the physiological system since abnormal blood glucose concentration level in human body results in diabetes mellitus which is a serious health problem throughout the world [2]. Monitoring blood glucose level is crucial for the diagnosis and treatment of diabetes mellitus. Hence, there is a growing demand to develop sensitive, selective, reliable and low cost glucose biosensors in the field of health care, medicine and food and agriculture product

processing. Glucose oxidase (GOx) is the most widely used model enzyme for glucose detection [3]. The most important step during biosensor construction is choosing a suitable immobilization matrix. For this purpose, different types of the materials can be used for the biosensing platform. One of them is the phthalocyanines (Pcs). Pcs are planar 18 π-electron aromatic compounds with a considerable large π-delocalized surface that accounts for their unique optical properties. Pcs derivatives have recently received much attention for their application in the field of functional materials, especially in photovoltaic solar cells, light emitting diodes and field effect transistors because of their unique physicochemical properties [4,5]. The important photochemical advantage of Pcs is based on their extended photo response in the red/near infrared region and good thermal and chemical stability. Pcs are also promising electroactive molecules for biosensor applications due to their excellent electronic properties, rich redox chemistry and high physico-electrochemical stability. Owing to their catalytic property as well as low raw material cost and biocompatibility with various biological molecules, Pcs are widely employed as electrode modifiers for the enzyme immobilization during biosensor construction [6]. In this regard, several metallated Pc (MPcs) derivatives have been reported for the purpose of using as an electrocatalyst in biosensor applications [7]. However, since pristine MPcs are adsorbed physically to the electrode surface, they usually leach out from the electrode surface resulting in poor stability and low reproducibility [6,8,9]. Furthermore, their low electrical conductivity and aggregation tendency limited their electrochemical performance [10]. Thus, suitable support materials are required to construct stable electrodes by presenting their excellent electrochemical properties. The incorporation of Pc with carbon nanostructures such as graphene and carbon nanotubes by means of covalent or non-covalent interactions appears as a possible way in order to increase their catalytic activity and construct stable bioanalytical sensors [11]. In particular, multi walled carbon nanotubes (MWCNTs) appeared attractive matrix for the fabrication of efficient electronic devices due to high electrical and thermal conductivity, mechanical strength, and good chemical stability. They are also promising nanomaterials in the fabrication of electrochemical biosensors since they enhance electrochemical conductivity and facilitate electron transfer of MPcs [12,13]. Due to their π-extended aromatic surface, Pcs are able to connected to sidewalls of carbon nanotubes by means of π- π interaction giving rise not only to the successful formation of hybrid system but also the preservation of the chemical and electronic structure of carbon nanotubes [14]. Moreover, the use of MWCNTs in biosensor fabrication has subjected keen interest due to their unique three-dimensional

structures, high mechanical and chemical stability, large surface-to-volume ratios and good biocompatibility [15].

Recently, several research groups have examined MPcs based support materials for development of glucose biosensors. Shi et al. developed a glucose biosensor constructed using copper phthalocyanine functionalized graphene [16]. Luong and co-workers studied that GOx was immobilized into the TiO_2 gel matrix layered on the surface of iron phthalocyanine (FePc) vertically-aligned carbon nanotube (CNT) modified electrode [17]. In an another study, a nanocomposite of cobalt phthalocyanine nanorods and graphene was developed for the construction of glucose sensing device [18]. Yu et al developed an amperometric glucose biosensor based on electropolymerized tetraaminophthalocyanatocobalt(II) and phenol films coated glassy carbon electrode [19].

On the other hand, many Pcs are practically insoluble in common organic solvents due to the strong interactions between ring systems which limit their potential applications. Since, Pc based devices cannot be easily prepared by solution processes. Nevertheless, the chemical flexibility of Pcs from the structural point of view allows the introduction of suitable substituents either in peripheral or in the axial position of the macrocycle which leads to modulate their electronic properties, increases the solubility of these compounds in organic solvents.

In this context, a novel zinc phthalocyanine (ZnPc) have been synthesized in order to investigate the role of being a part of active layer in biosensor construction. Introduction of tetra quaternized imidazolyl moieties at the peripheral positions of phthalocyanine leads to high solubility in water. Immobilization platform was generated via incorporation of ZnPc with MWCNT into the conducting polymeric matrix. In bio-sensing systems, another important active material is the conducting polymers (CPs). Enzyme electrodes, constructed with conducting polymers, possess superior features since CPs exhibit excellent conductivity and high mechanical strength and processability. In addition, CPs provide high surface area, adjustable morphology by arranging thickness of the polymer film and offer extensive stability of the enzymes incorporated [20, 21]. Herein, poly[9,9-di-(2-ethylhexyl)- fluorenyl-2,7-diyl] end capped with N,N-Bis(4-methylphenyl)-4-aniline (PFLA) was used as the conducting polymer utilizing a matrix for the MWCNT and ZnPc. Since the biomolecule have both hydrophobic and hydrophilic parts in its structure, interaction between alkyl chains on the polymer backbone and the biomolecule resulted in the enhanced stability; thus durable biosensor architecture. Also, owing to π-π interactions between the enzyme and polymer , the

strong and stable interactions was obtained. By this way, any dialysis membrane is not required in order to entrap the enzyme molecules.

By taking advantages of all properties, for the first time, we developed a novel glucose biosensor via combining CP, MWCNT and ZnPc. Our results showed that the proposed biosensor has a good biosensor characteristics compared to already published articles. The combination of this three materials may possess complementary properties due to synergistic effects and thus increase the total performance of the sensing device.

2. Materials and Methods

2.1. Materials

Poly[9,9-di-(2-ethylhexyl)- fluorenyl-2,7-diyl] end capped with N,N-bis(4- methylphenyl)-4-aniline (PFLA) was obtained from American Dye Source, Inc (Quebec, Canada; www.adsdyes.com). Glucose oxidase (GOx, β-D-glucose: oxygen 1-oxidoreductase, EC 1.1.3.4, 17300 units/g solid) from *A. niger* and D-glucose were purchased from Sigma (St. Louis, USA; www.sigmaaldrich.com). Glutaraldehyde (GA), multi walled carbon nanotubes (MWCNTs) and chloroform were purchased from Sigma–Aldrich Co., LCC. (St. Louis, USA) and N-N dimethylformamide (DMF) was obtained from Carlo Erba Reagents SAS (Reuil, France: www.carloerbareagents.com). For enzyme immobilization, a 50 mM, pH 7.0 phosphate buffer solution (PBS) consisting of 0.025 M Na_2HPO_4 (Fisher Scientific Company) and 0.025 M NaH_2PO_4 (Fisher Scientific Company) was used. As the substrate, glucose solution (0.1 M) was prepared by dissolving 0.18 g of glucose in 10 ml pH 7.0 PBS buffer solution. All chemicals for Zn (II)Pc synthesis were purchased from Aldrich and used without further purification. All chemicals were of analytical reagent grade.

2.2. Apparatus

All the amperometric studies and cyclic voltammetry measurements were performed using PalmSens potentiostat (PalmSens, Houten, The Netherlands). Three electrode system containing graphite electrode (Ringsdorff Werke GmbH, Bonn, Germany, type RW001, 3.05 mm diameter and 13% porosity) as the working electrode, platinum electrode (Metrohm, Switzerland) as the counter electrode and Ag wire as the reference electrode was used for all electrochemical measurements. In amperometric analyses, the data were given as the average

of three measurements and standard derivations were recorded as ±SD. All the measurements were conducted under ambient conditions (25°C). For surface investigation of the fabricated biosensor, scanning electron microscope (SEM) (JEOL JSM-6400 model) was used.

For the synthesis and characterization of Zn(II)Pc, TLC was carried out on aluminum sheets percolated with silica gel 60 F254 (E. Merck). The IR spectra were performed with Perkin-Elmer, FT-IR/MIR-FIR (ATR, Attenuated total reflectance) spectrophotometer. ^{1}H-NMR (400 MHz) spectra were recorded with a Bruker AC-400 instrument. UV/Vis spectra were recorded with an Analytic JENA S 600 UV-Vis spectrophotometer.

2.3. Synthesis of Zn(II)Pc complex

A novel zinc phthalocyanine consisting of a tetra quaternized imidazolyl moieties was prepared. The Zn(II)Pc (**1**) was synthesized following the route depicted in Scheme 1.

The synthesis of imidazole substituted phthalonitrile (**3**) was carried out following a modification of a literature procedure [22]. The reaction of 4- nitrophthalonitrile with 2-methylimidazole in the presence of K_2CO_3 in DMF gave compound **3** in 60% yield. Zn(II)Pc (**2**) was synthesized by cyclotetramerization reaction of phthalonitrile in the presence of $Zn(OAc)_2$ in N,N, dimethylaminothanol (DMAE) in 38 % yield. Subsequently quaternization of Zn(II)Pc (2) with an excess methyl iodide in DMF, afforded water soluble tetra cationic Zn(II)Pc (1) with 88% yield.

All compounds were fully characterized by ^{1}H-NMR, UV-Vis, and FT-IR spectroscopy. The ^{1}H NMR spectrum (Figure 1) of final compound Zn(II)Pc (**1**) in d-DMSO that is polar coordinating solvent revealed well resolved sharp signals, with the Pc macrocycle protons and the aromatic protons of the imidazole fragment assigned between 9.7 and 8.1 ppm. In addition, the methyl groups (N-CH_3) at 4.1 ppm are also appear in the spectra.

The UV-Vis spectrum of Zn(II)Pc (**1**) is depicted in Figure 2, and shows the Q-band centered at 631 nm. Zn(II)Pc (**1**) aggregates in aqueous solutions, as indicated by the broadening of its Q band.

Synthesis of 4-(2-Methyl-1H-imidazol-1-yl)phthalonitrile (3):

To a solution of 4-nitrophthalonitrile (500 mg, 2.89 mmol) and 2-methylimidazole (237.26 mg, 2.83 mmol) in dry DMF (5 ml) was added K_2CO_3 (1.2 g, 8.67mmol). The reaction mixture was stirred for 48 h at room temperature. The solution was poured into water and the solid was then filtered and dried. Recrystallization from methanol led to pure compound (360 mg, 1.73 mmol) in 60% yield as a white solid.

^{1}H NMR (DMSO-d_6, 400MHz): δ (ppm) = 8.38 (d, J = 4 Hz, 1H), 8.32 (d, J = 8 Hz, 1H), 8.05 (dd, J = 4, 8 Hz, 1H), 7.48 (d, J = 4 Hz, 1H), 6.98 (d, J = 4 Hz, 1H), 2.39 (s, 3H).

IR (ATR): ῡ = 3118, 3097, 2242, 1599, 1502, 1420, 1403, 1273, 1174, 1138, 985, 919, 848, 781.

Zinc (II) 2(3),9(10),16(17),23(24)-tetra-(2-Methyl-1H-imidazol-1-yl)-phthalocyaninato (2-)-N^{29}, N^{30}, N^{31}, N^{32} (2):

A solution of 4-(2-methyl-1H-imidazol-1-yl)phthalonitrile (300 mg, 1.44mmol) and $Zn(OAc)_2$ (88.11, 0.45 mmol) in DMAE (6 mL) was heated at reflux overnight under argon atmosphere. After cooling to room temperature, the solvent was evaporated *in vacuum* and the crude mixture was washed with common organic solvents. In this way, Zn(II)Pc (**2**) was obtained as a blue solid in 38% yield (123 mg, 0.137 mmol).

^{1}H-NMR (CD_3OD/ with drop of TFA, 400 MHz): δ (ppm) = 9.5-9.1 (m, 6H), 9.09-8.8 (m, 2H), 8.5-8.1 (m, 8H), 7.9-7.8 (m, 4H), 2.95 (d, J = 4 Hz, 6H), 2.88 (d, J =4 Hz, 6H).

UV-vis (DMSO): λmax, nm (log ε): 676 (5.32), 610 (4.53), 350 (4.72).

IR (ATR): ῡ = 3533, 2549, 1650, 1512, 1492, 1409, 1298, 1132, 1086, 1035, 899, 760.

MS (MALDI-TOF) m/z: calcd for $C_{48}H_{32}N_{16}Zn$: 898.265; found 899.028 $[M+H]^+$.

Synthesis of quaternised phthalocyanine (1):

To a solution of Zn(II)Pc 2 (200 mg, 0.22 mmol) in DMF (15 mL) an excess of methyl iodide (22.12 g, 155.86 mmol) was added and the solution was stirred for 12 h at 50 ℃ under argon. Afterwards, the solvent was removed under vacuum and the crude product was washed with common organic solvents. Zn(II)Pc (**1**) was obtained (285 mg, 0.18 mmol) in 88% yield as a blue solid.

^{1}H-NMR (DMSO-d_6, 400 MHz): δ (ppm) = 9.7-9.2 (m, 8H), 8.8-8.4 (m, 8H), 8.16 (d, J =8 Hz, 4H). 4.11 (d, J =8 Hz, 12H), 2.95 (s, 12H).

UV/Vis (H_2O): λmax (log ε) = 663 (4.66), 631 (4.84), 337 (4.75).

IR (ATR): ῡ = 3402, 2910, 1613, 1523, 1487, 1413, 1319, 1247, 1150, 1093, 1057, 944.

MS (MALDI-TOF) m/z: calcd. for $C_{52}H_{44}I_4N_{16}Zn$: 1466.021; found 1340.552 $[M–I]^+$, 1211.715 $[M–2I]^+$, 1084.428 $[M–3I]^+$.

2.4. Biosensor preparation

Spectroscopic grade graphite rods were polished on emery paper and washed with distilled water before surface modification. A 10 μL aliquot of CP solution prepared by dissolving 2.0 mg PFLA in 2.0 mL chloroform was deposited on the surface of a cleaned graphite electrode. Then, 0.5 mg MWCNT was dispersed in 10 mL DMF and ultrasonicated for 15 min to obtain a black suspension. After the CP modified electrode was dried at room temperature, 10 μL aliquot of MWCNT solution was casted on the surface and the modified electrode was left to dry at room temperature for 3 h. For the co-immobilization of GOx and ZnPc (**1**), the certain amount of GOx was dissolved in 3.0 μL of PBS (50 mM, pH 7.0). Then, 3.0 μL of ZnPc solution (1.0 mg of ZnPc in 1.0 mL distilled water) was added into the GOx solution. Then, 6.0 μL of this solution was immobilized on the modified electrode surface. After a while, 3.0 μL of GA solution (1% in 50 mM PBS pH 7.0) was spread over the surface in order to prevent leaching out of the enzyme molecules from the electrode surface. High crosslinking activity of GA results in enhanced immobilization. It improves the compact structure of enzyme molecules by providing the proper enzyme conformation [23]. The electrode was left to dry for 2 h at room temperature. Finally, the fabricated biosensor was rinsed with distilled water to remove the unbound molecules and impurities. Scheme 1 illustrates schematic representation of the procedure for the construction of the proposed amperometric glucose biosensor.

2.5. Amperometric measurements

All amperometric measurements were carried out at room temperature in a reaction cell filled with 10 mL PBS, pH 7.0 under mild stirring by applying -0.7 V constant potential. The buffer

solution was refreshed after each measurement. As a result of the enzymatic reaction between GOx and the substrate, the decrease in the oxygen level associated with substrate concentration was monitored at -0.7 V potential [24]. During the amperometric measurements, when the baseline current reaches to the equilibrium, certain amount of glucose substrate was added into the reaction medium. After the current is changed, equilibrium is established again. The difference between these two constant current values is equal to the biosensor response.

In biosensor construction, several parameters affecting the biosensor performance should be optimized to obtain a stable and reproducible biosensor. For this purpose the amounts of CP, MWCNTs, ZnPc, and enzyme together with pH were investigated. In order to perform the optimization studies, different electrodes were prepared by changing only the amounts of the parameter to be optimized. It means that all other parameters were kept constant except for the one to be optimized. All optimization studies were performed this way

3. Results and discussion

3.1. Optimization studies

To obtain a stable and reproducible biosensor, all parameters affecting the biomolecule immobilization and biosensor performance should be optimized. For this purpose several parameters such as the amounts of CP, MWCNTs, ZnPc, enzyme and also pH were investigated.

Firstly, the effect of CP was investigated since improper CP amounts may result in low and unstable biosensor responses. To determine the optimum value, 1.0, 1.5, 2.0, 2.5 and 3.0 mg of PFLA were dissolved in 2.0 mL of chloroform and 10 μL aliquots of these solutions were casted on graphite electrodes and their biosensor responses were compared by keeping all the other parameters constant. As shown in Figure 3A, the highest response was obtained with 2.0 mg PFLA.

Then, the effect of MWCNTs was achieved to evaluate biosensor performance. Higher amounts of MWCNTs resulted in lower biosensor responses since it may cause diffusion problem for the substrate and limited orientation for the enzyme molecules. On the other hand, lower MWCNTs amounts may affect the biosensor responses since enzyme molecules may not be fixed properly onto electrode surface [25]. To find the optimum MWCNTs amount, 0.25, 0.40, 0.50, 0.60, 1.00, 1.50 and 1.75 mg of MWCNTs were dissolved in 10.0 mL of DMF and 10 μL aliquots of these solutions were deposited on the CP coated electrode

surfaces. According to the recorded signals, biosensor prepared using 0.50 mg MWCNT gave the highest response (Figure 3B).

Moreover, ZnPc amount on biosensor response was also determined. Metal phthalocyanine (MPc) complexes are strongly adsorbed on carbon-based electrode materials. Also, their excellent catalytic properties and high physicochemical stability may increase the biosensor responses. Therefore, the biosensor responses increase with increasing MPc amount. However, further increase in MPc results in lower response and sensitivity of the biosensor as a result of increased diffusion constraints [26]. In order to find the best biosensor response among different MPc amounts, ZnPc solutions were prepared by dissolving 0.25, 0.50, 1.00, 1.50 and 1.75 mg of ZnPc in 1.0 mL of distilled water and 3.0 μL aliquots of these solutions were co-immobilized with GOx solution. As seen in Figure 3C, 1.00 mg ZnPc resulted in the highest response.

Furthermore, enzyme amount should also be optimized to obtain the highest biosensor performance. The immobilization matrix has an enzyme loading capacity. Therefore, if there is an excess enzyme loading on the electrode surface, the excess enzyme molecules may leach out from the surface. Whereas, if the enzyme amount is too low, the desired responses corresponding the sensitivity cannot be recorded [23]. To optimize the enzyme amount, five different electrodes having 0.5 mg (8.7 U), 1.0 mg (17.3 U), 1.3 mg (22.5 U), 1.5 mg (26.0 U) and 2.0 mg (34.6 U) GOx were prepared by keeping all the parameters constant. The highest signal was obtained with 1.3 mg GOx as shown in the Figure 4A.

Finally, the optimum pH value was investigated for an effective biosensor construction since enzyme molecules are affected by pH changes. The enzyme conformation changes at different pH values for any system. For each enzyme, there is a pH range within which they can function properly. The pH value at which the net charge of the enzyme equals to zero is called as isoelectronic point. At pH values different than the isoelectronic point of the enzymes, they may form or break intra and intermolecular bonds resulting in the change in the shape of the enzyme. As a result, the activity of the enzyme is affected [27]. Herein, the pH effect was investigated using 50 mM buffer solutions in a pH range of 4.0-9.0 (sodium acetate buffer at pH 4.0-5.5, sodium phosphate buffer at pH 6.0-7.5, tris buffer at pH 8.0-9.0, 25 °C). The optimum enzyme activity is obtained with pH 7.0 as shown in the Figure 4B.

In order to achieve the best combination for outstanding biosensor performance, the effect of PFLA, MWCNT, ZnPc and their different combinations were investigated. Different biosensors were prepared using their optimized parameters to compare their biosensor responses. As seen in Figure 5, MWCNTs, which are well known suitable charge transfer reagents, enhance the charge transfer ability and increase the surface area of the electrodes via improving biosensor performance. In addition, ZnPc complex also improves the biosensor responses due to its high chemical stability, non-toxicity, redox activity and semi-conducting properties. Moreover, since conjugated polymers have the ability to mimic the natural environment for biomolecules, they are charming materials for the biomolecule immobilization. However, when these three components were considered separately, designed surfaces may not be sufficient enough to bind biomolecule onto the electrode surface. Also, 3D structure of the enzyme molecules may not be protected and that may cause a decrease in the amperometric signal and shelf life of the biosensor. In addition, due to the insufficient enzyme immobilization, stability and sensitivity values of the biosensor also may be affected badly. Hence, the combination of these three electrode modifiers results in enhanced biosensor performance relative to individual use of MWCNT, ZnPc or PFLA species.

3.2. Surface design characterizations

Cyclic voltammetry (CV) studies were carried out to characterize the effective surface area for each modification. Experiments were performed in a solution containing 5.0 mM $Fe(CN)_6^{3-/4-}$, 0.1 M KCl and 50.0 mM PBS pH 7.0 at the potential between 0 and 1.0 V with a scan rate of 100.0 mV s^{-1}. Figure 6 shows the voltammograms of four different electrodes; pristine PFLA, PFLA/MWCNT, PFLA/MWCNT/ZnPc and PFLA/MWCNT/ZnPc/GOx under optimum conditions. Different electrode constructions can lead to different interfacial structures. Also, determination of average electroactive surface area can be determined using Randles–Sevcik equation:

$$Ip = 2.69 \times 10^5 A D^{1/2} n^{3/2} v^{1/2} C$$

where n is the number of electrons involved in the redox reaction, A is the electrode area (cm^2), D is the diffusion coefficient of the molecule in solution (cm^2 s^{-1}), C is the

concentration of the probe molecule in the bulk solution (mol cm^{-3}),and v is the scan rate (V s^{-1}).

This equation suggests that peak current and effective surface area is directly proportional. That is, increase in the peak current means an increase in the effective surface area. The electroactive surface area for pristine PFLA, PFLA/MWCNT, PFLA/MWCNT/ZnPc and PFLA/MWCNT/ZnPc/GOx modified electrodes were 0.038 cm^2, 0.052 cm^2, 0.061 cm^2 and 0.046 cm^2, respectively. The electroactive surface area of pristine PFLA is comparably lower than the other electrodes since PFLA formed additional layer onto the graphite electrode surface passivating the electrode surface. Incorporation of MWCNT and ZnPc lead to increase in effective surface coverage. Enhancement of peak currents demonstrated that these materials promote the electron transfer rate and facilitate the redox reaction of the probe. Also, strong π-π interaction between the ZnPc and MWCNT/PFLA was a strong evidence of the highest electroactive surface area of PFLA/MWCNT/ZnPc. Furthermore, enzyme immobilization caused the decrease in oxidation current and the surface area. This result is attributed to the insulating character of the biological molecules.

Scanning Electron Microscopy (SEM) technique was used for surface morphology characterization of different surfaces. Figure 7 shows SEM images of pristine PFLA, PFLA/MWCNTs/ZnPc and PFLA/MWCNTs/ZnPc/GOx modified electrode surfaces, respectively. As seen in Figure 7A, each surface changes prove the surface modification properly. PFLA deposition resulted in fully covered homogenous film. When PFLA coated electrode was modified with MWCNT and ZnPc complex, the molecules of the ZnPc complex well distributed with the help of uniform MWCNT distribution. The layered structures were rougher than the morphology of PFLA itself, indicating the successful PFLA/MWCNT/ZnPc combination. After GOx immobilization onto the modified electrode, a significant morphology change was observed. Homogeneous coating of the enzyme showed that the proposed electrode modification serves as a great host–guest platform for biomolecule deposition.

3.3. Analytical characterization of the biosensor

After all optimum conditions for the proposed biosensor were determined, a calibration curve for glucose was plotted (Figure 8). A linear response range was obtained between 0.025-1.0 mM glucose in 50 mM PBS pH 7.0 as given with the equation; $y = 5.312x + 0.311$ with $R^2 = 0.997$. At the higher glucose concentration than 1.0 mM, substrate saturation was observed. Moreover, the limit of detection (LOD) and sensitivity values were calculated by setting the intercept of the linear range of the calibration curve to zero using S/N (signal-to-noise ratio) = 3 criterions and they were found as 0.018 mM and 82.18 $\mu A m M^{-1} cm^{-2}$, respectively.

In order to prove the repeatability of the fabricated biosensor, the biosensor responses corresponding to 0.5 mM glucose solution were recorded for ten times. The standard deviation (SD) and the relative standard deviation (RSD) values for these measurements were calculated as 0.06 and 3.09% respectively. The lifetime of the biosensor was also measured by taking amperometric measurements for 30 days with regular time intervals. Only 5% of activity loss was observed at the end of this time.

Furthermore, the kinetic parameters of the proposed biosensor were determined using Michaelis-Menten enzyme kinetics model. Since the studies were conducted as amperometric method which measures the current change with respect to time, I_{max} and K_M^{app} values were calculated from Lineweaver-Burk plot (1/I vs 1/[S]). From the equation of this plot, K_M^{app} was calculated as 0.53 mM and I_{max} was found as 6.12 µA. When we compared our results with the literature, K_M^{app} value was found to be superior. Chen et al. [28] used a pyrolytic graphite electrode (PGE) and modified it with Nafion and nanoscaled cobalt phthalocyanine (NanoCoPc). The K_M^{app} of this biosensor was 12.4 mM. Nyokong at al. [29] prepared CoPc–CoTPP complexes and Nafion based glucose biosensor and K_M^{app} value was calculated as 14.91 mM. In another study, Luong et al. [17] designed a glucose biosensor using a nanoporous TiO_2 film/multi walled carbon nanotubes (MWCNT)/iron phthalocyanine/Nafion film. In addition, the constructed biosensor shows better characteristics such as low LOD and high sensitivity than some other studies in the literature as can be seen in Table 1. These enhanced characteristics can be attributed to the combination of the conjugated polymer/MWCNT/ZnPc which provides an improved support material for immobilization.

The main purpose of GOx biosensors is to analyze glucose amount in blood samples. Since there are various biological molecules in the blood, biosensor should give a response only to

the target analyte in order to perform successful analysis with different samples. For this reason, 0.5 mM ascorbic acid and urea solutions were prepared and the effect of these interfering substances was investigated. During amperometric measurements, these species were injected to the reaction cell instead of glucose. As seen in Figure 9, the proposed biosensor did not give any significant response.

3.4. Sample application

The applicability of the proposed biosensor for real sample detections was investigated by analyzing different commercial beverages. The samples were injected into the reaction medium without any pretreatment and the glucose contents of these samples were determined using the calibration curve. As demonstrated in Table 2, results are very close to the product label values showing that the fabricated biosensor is feasible for practical sample testing with reliable accuracy and precision.

4. Conclusions

In this study, a novel glucose biosensor based on a conducting polymer, multi walled carbon nanotubes and zinc phthalocyanine was fabricated. Herein, for the first time the combination of conjugated polymer (PFLA)/MWCNT/ZnPc was constructed to detect glucose in the beverages. PFLA/MWCNTs/ZnPc/GOx biosensor showed superior kinetic parameters, high sensitivity, low detection limit in a good linear range and presented long term stability. In addition, electrochemical properties were characterized by CV method and the surface morphology was investigated by SEM technique which resulted in excellent homogeneity. Moreover, after optimization and characterization studies, the biosensor was tested on beverages to determine their glucose content. Satisfactory results were obtained indicating that the proposed sensing system is an important tool for real time analyses for glucose determination.

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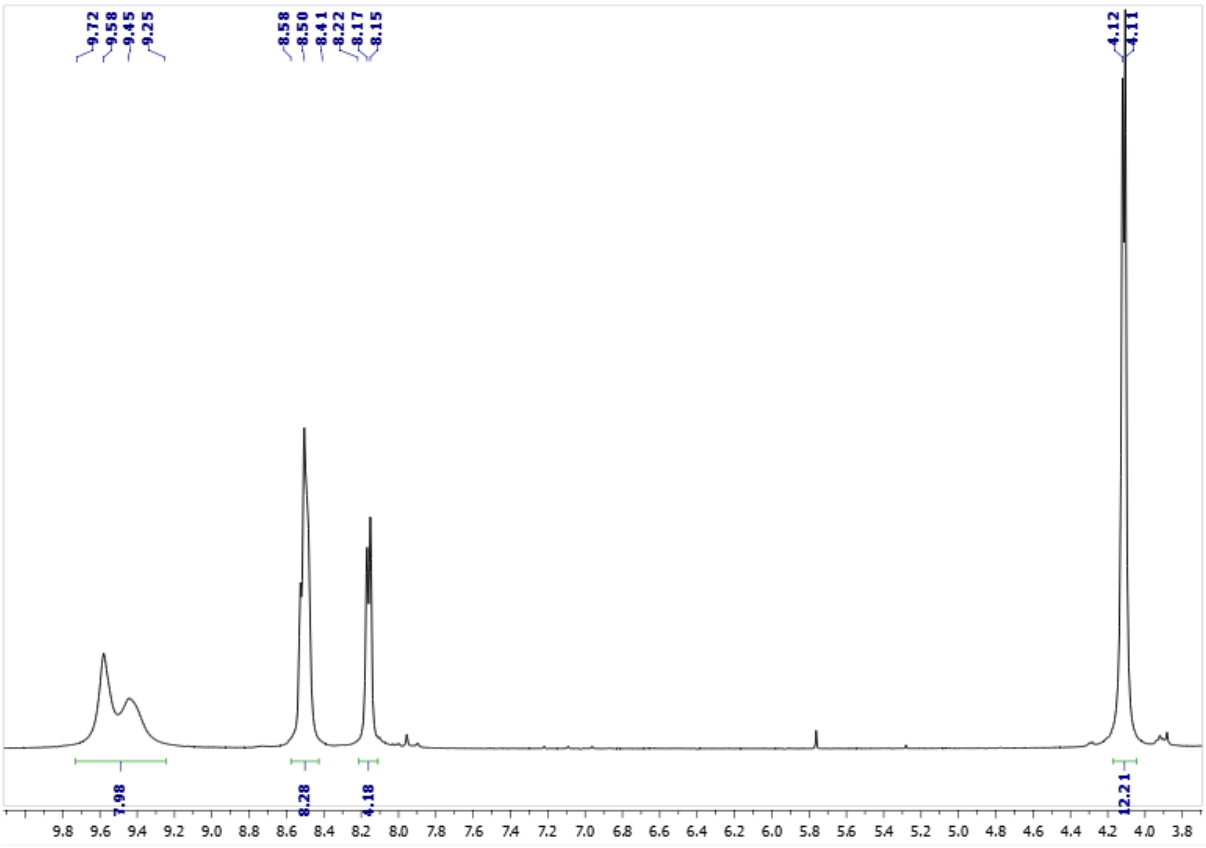


Figure 1. 1H NMR (DMSO-d_6) spectrum of dyad Zn(II)Pc **1**.

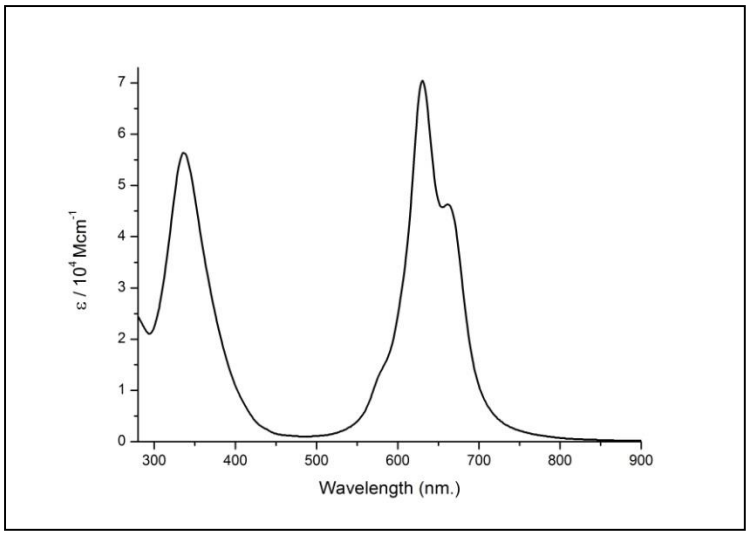


Figure 2. UV-Vis spectrum of Zn(II)Pc (**1**) in water~$1x10^{-5}$M.

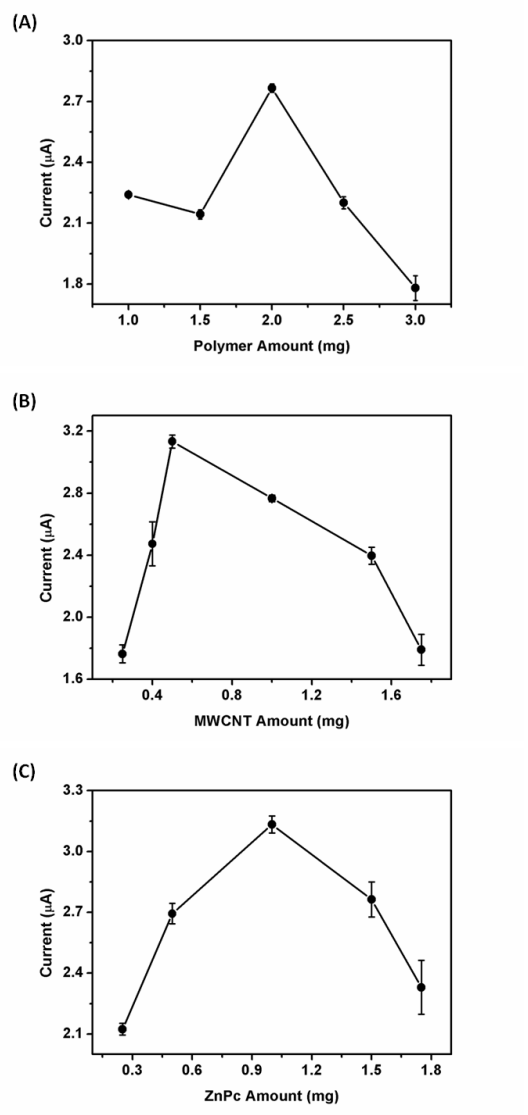


Figure 3. The effect of (A) polymer amount, (B) MWCNT amount, (C) ZnPc amount on biosensor response (in 50.0 mM phosphate buffer, pH 7.0, 25 °C). Error bars show the standard deviation (SD) of three measurements.

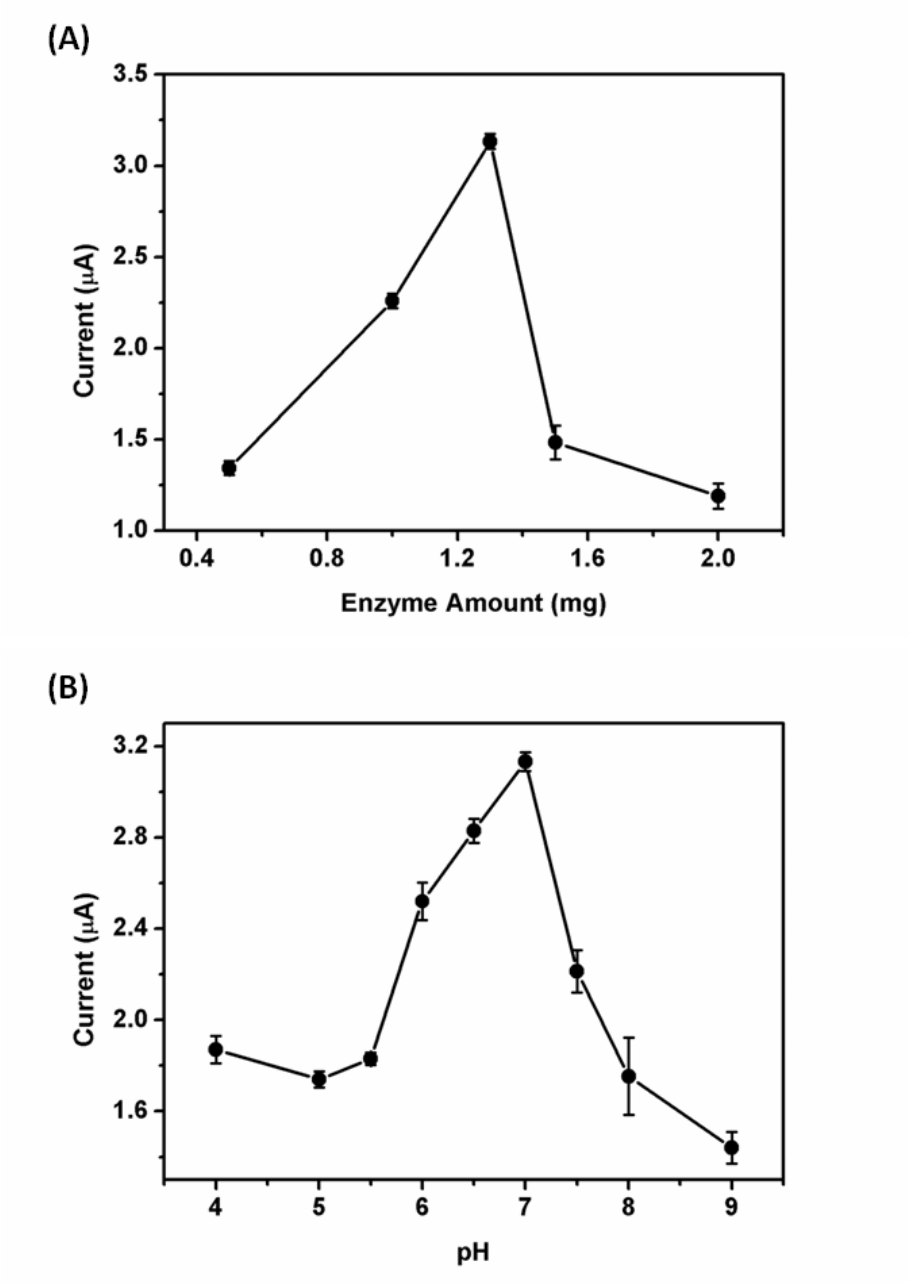


Figure 4. (A) The effect of loaded enzyme amount on biosensor response (in 50.0 mM phosphate buffer, pH 7.0, 25 °C). (B) The effect pH on biosensor response (in 50 mM sodium acetate buffer at pH 4.0; 5.0; 5.5, 50 mM phosphate buffer at pH 6.0; 6.5 7.0; 7.5 and 50 mM Tris buffer at pH 8.0 and 9.0, 25 °C). Error bars show the standard deviation (SD) of three measurements.

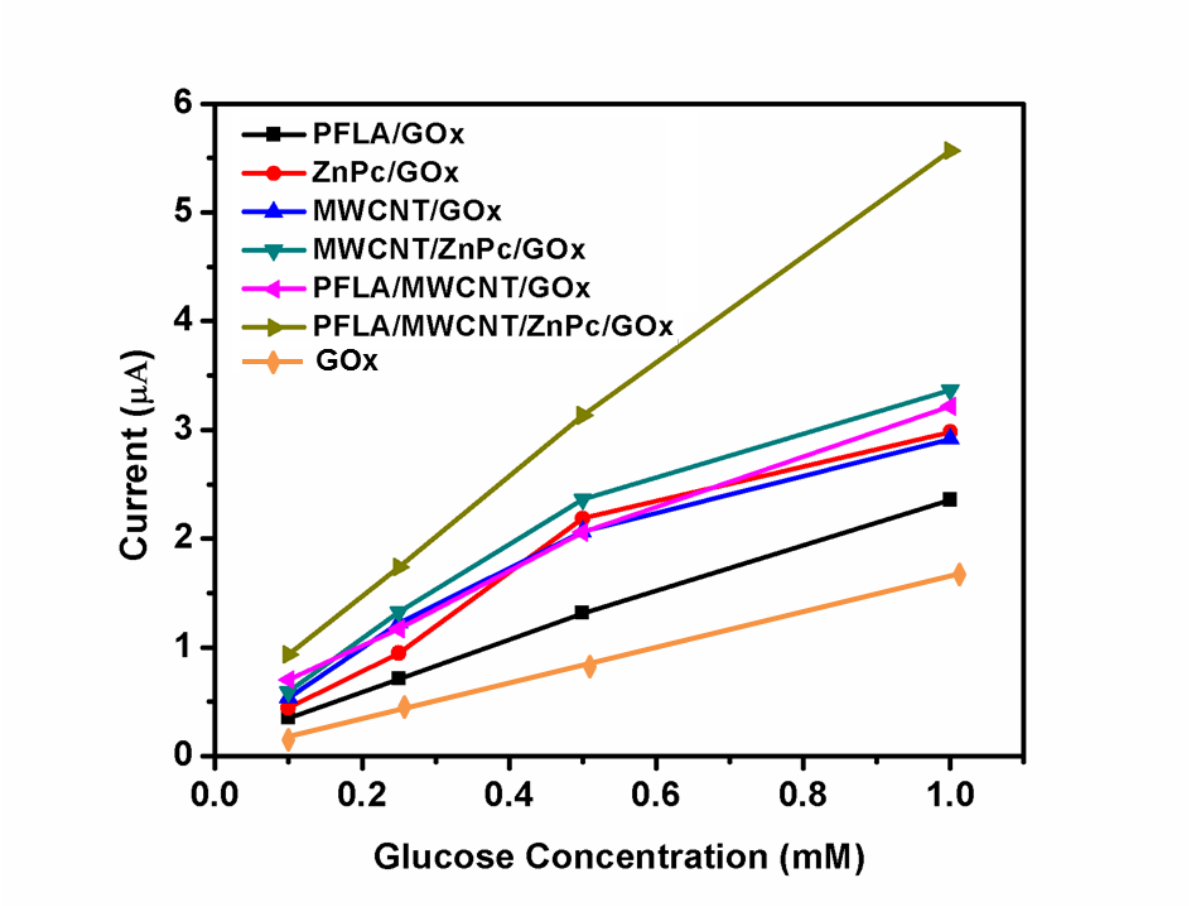


Figure 5. The effect of surface modification on performance of glucose biosensors (in 50.0 mM phosphate buffer, pH 7.0, 25 °C). Error bars show the standard deviation (SD) of three measurements.

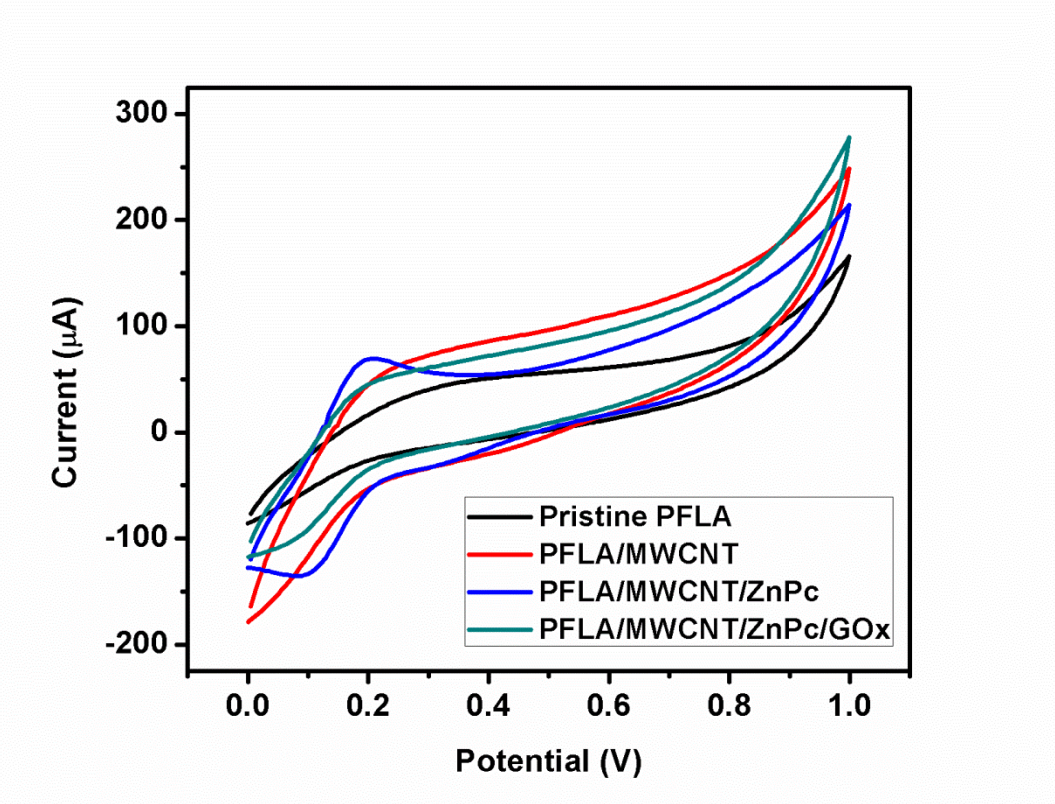


Figure 6. Cyclic voltammograms resulting from pristine PFLA, PFLA/MWCNT, PFLA/MWCNT/ZnPc and PFLA/MWCNT/ZnPc/GOx in 5.0 mM $Fe(CN)_6^{3-/4-}$ containing 0.1 M KCl.

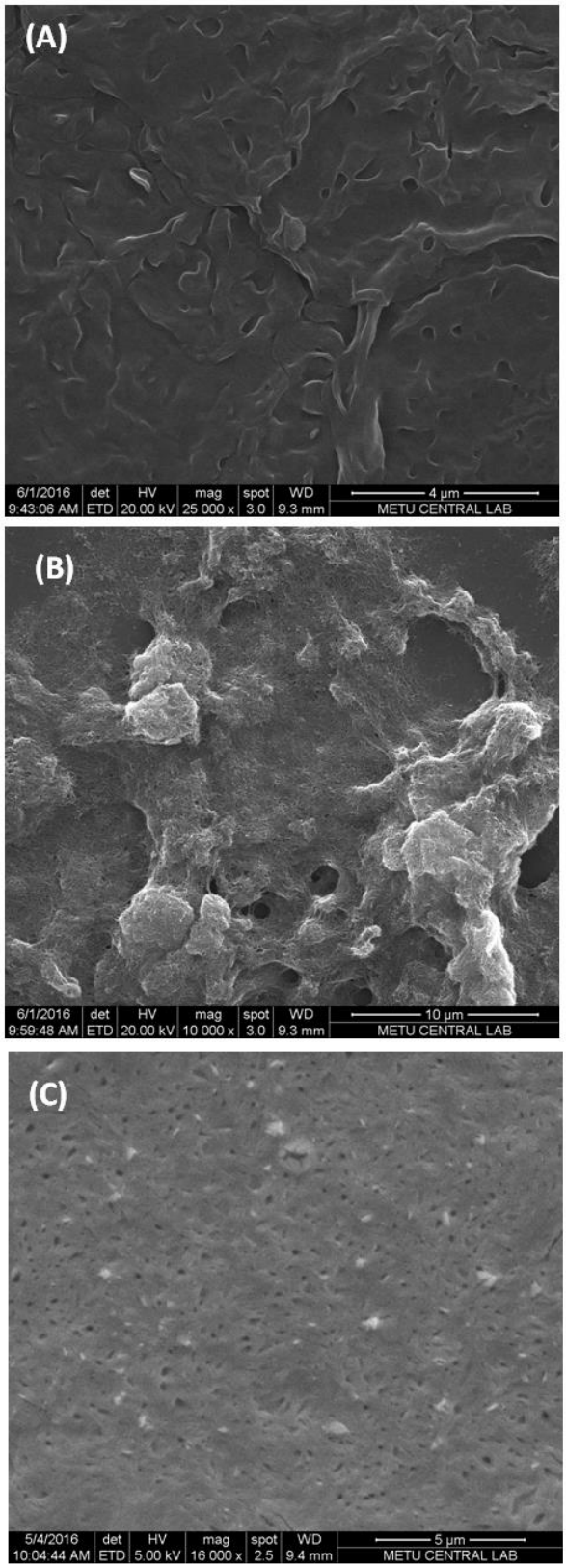


Figure 7. SEM images of (A) pristine PFLA; (C) PFLA/MWCNT/ZnPc; (D) PFLA/MWCNT/ZnPc/GOx under optimized conditions.

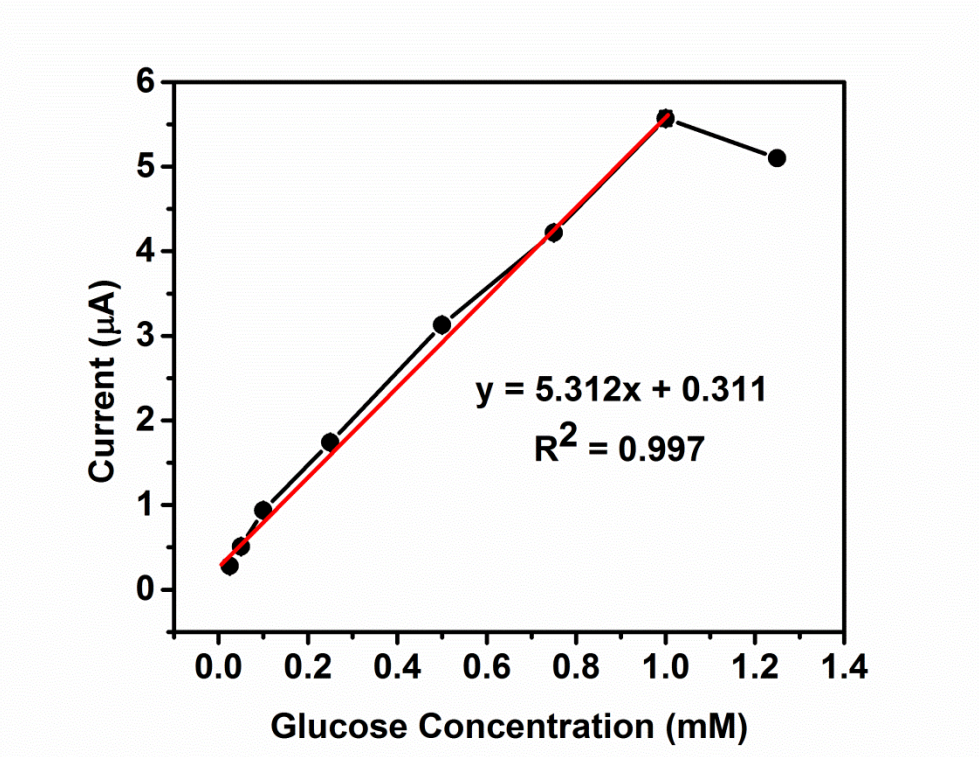


Figure 8. Calibration curve for glucose (in 50 mM phosphate buffer, pH 7.0, 25 °C). Error bars show standard deviation of three measurements (A typical amperometric response to 0.5 mM glucose in 50 mM phosphate buffer, pH 7.0 given as inset).

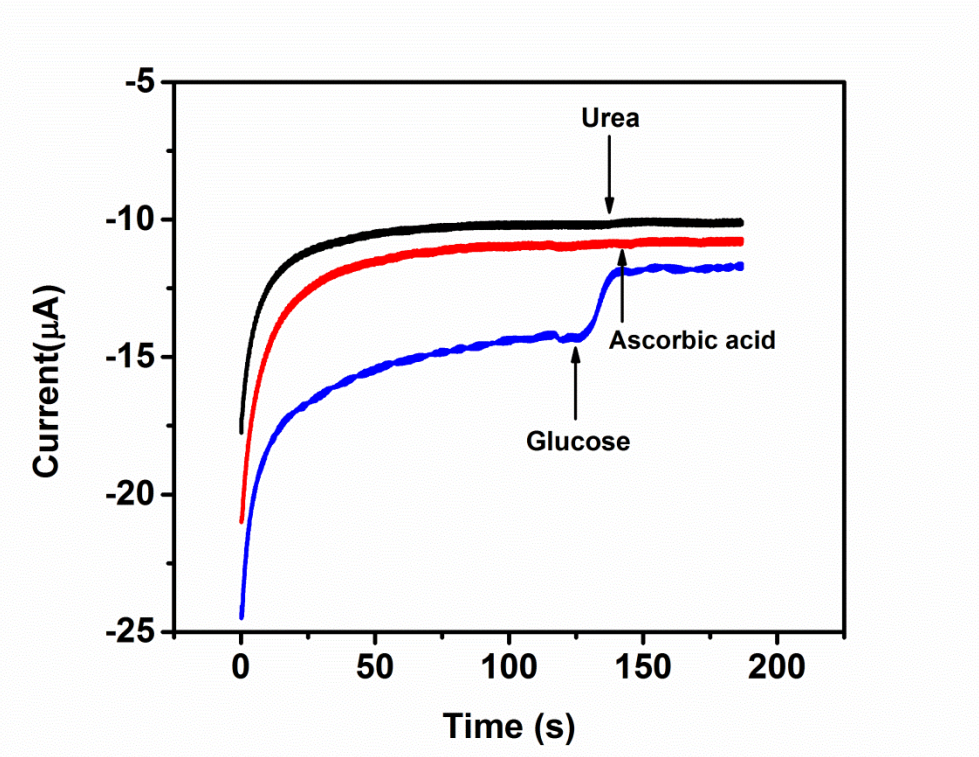
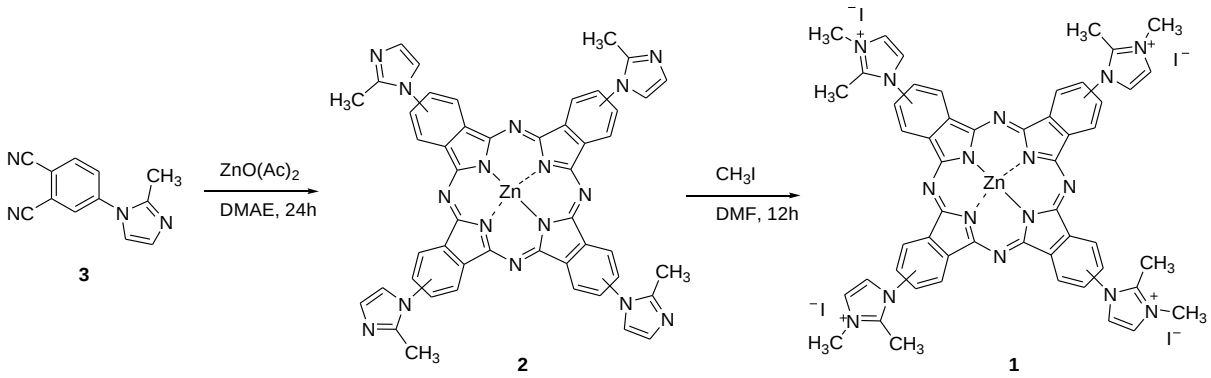
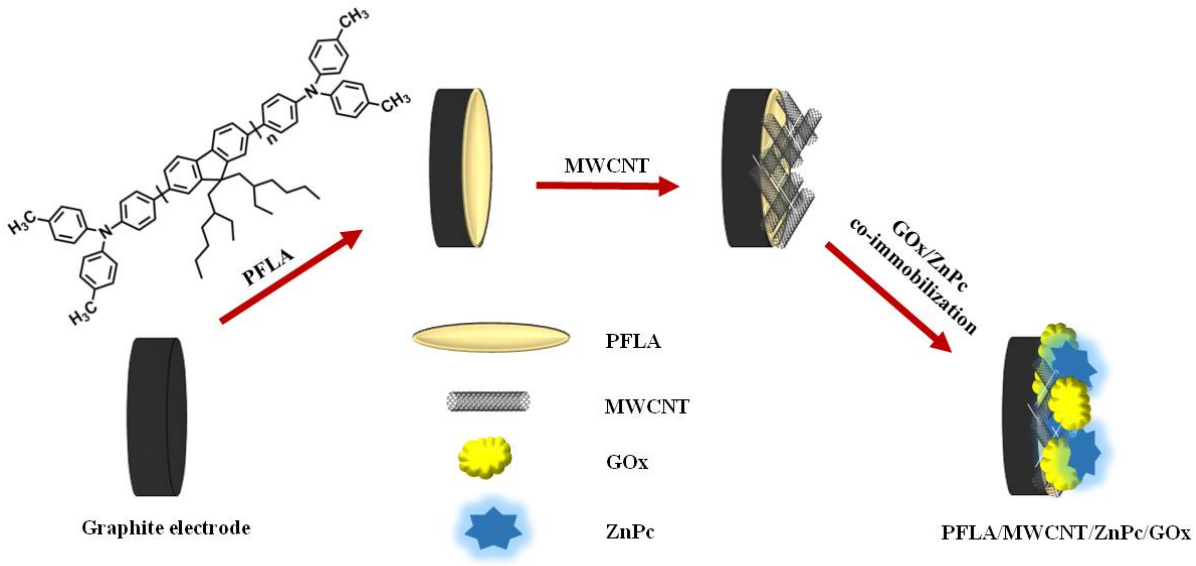


Figure 9. Biosensor responses to glucose and interfering substances (in 50 mM phosphate buffer, pH 7.0, 25°C).



Scheme 1. Synthetic route to Zn(II)Pc (**1**).



Scheme 2. Schematic representation of PFLA/MWCNT/ZnPc/GOx biosensor

Table 1. Comparison of glucose biosensors in the literature

Matrices on electrodes	**LOD (mM)**	**Sensitivity ($\mu AmM^{-1}cm^{-2}$)**	**K_m^{app} (mM)**	**Ref**
Poly/MWCNT/ZnPc/GOx	**0.018**	**82.18**	**0.53**	**This work**
GOD/Nafion/$(LbL)_{3.5}$/ABS	0.05	17.50	NR	[30]
PDDA/PSS/{PDDA-MWCNTs/GOx}$_5$	0.058	5.60	NR	[31]
Nf/GOx/TiO_2/FePc-CNTs	0.03	8.25	NR	[17]
Poly(adamantanepyrrole)/SWCNT/β-cyclodextrin/GOx	NR	31.02	5.00	[32]
Au/MPTS-solgel/Au NPs/cysteamine/GOx	0.023	8.3	NR	[33]

Table 2. Results of glucose analyses in beverages

Sample	**Glucose Content (mM)**		
	Product Label	**Poly/MWCNT/ZnPc/Gox**	**%Error**
S® Milk	0.250	0.247	-1.20
L® Ice tea	0.370	0.350	-5.41
C® Coke	0.620	0.598	-3.55