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Synthesis and characterization of a novel polyphosphazene and its application to biosensor in combination with a conducting polymer

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ABSTRACT

An amperometric glucose biosensor was prepared successfully based on a conducting polymer, poly(4-(2,5-di(thiophen-2-yl)-1*H*-pyrrol-1-yl)benzenamine) (poly(SNS-NH_2)) and a flexible hydrophilic polyphosphazene polymer, poly[(methoxyethoxy)ethoxy-*co*-3-formylphenoxy]phosphazene (PPA). Poly(SNS-NH_2) was electrochemically polymerized on a graphite electrode to achieve a conducting immobilization matrix to improve the enzyme immobilization on the transducer surface. Moreover, to strengthen the immobilization, a polyphosphazene derivate bearing functional aldehyde group was designed, synthesized and used in the immobilization of glucose oxidase. Not only the amino groups in the structure of poly(SNS-NH_2), but also the aldehyde groups in PPA were contributed to the covalent immobilization as well as the entrapping the biomolecules in PPA network during the immobilization process. This afforded an effective and long-life analysis of glucose. Amperometric measurements were conducted at −0.7 V vs. Ag/AgCl in 50 mM sodium acetate buffer at pH 4.5. K_M^{app} (0.677 mM), I_{max} (20.91 μA), LOD (1.3 μM) values were determined. Moreover, biosensor showed an extremely high sensitivity as 237.1 μA mM^{-1} cm^{-2} owing to the newly synthesized and combined highly flexible hydrophilic polymeric immobilization matrix. Finally, the proposed biosensor was successfully applied for determination of glucose content in several beverages, successfully.

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1. Introduction

Over the past decade, major advances have occurred in the development of enzymatic biosensors and they started to be used as alternatives or complementary devices in detection and quantification of certain substances. Biosensors are easy to construct and use, cheap, rapid, selective, sensitive and generally do not require sample pre-treatment. Nevertheless, the crux in the preparation of a new enzyme-based biosensor is the selection and fabrication of immobilization matrix for the enzyme molecules in order to have a long-life, sensitive and stabile biosensor. Nowadays, material science is the heart of all scientific applications. The choice of true material for an application is the most crucial step of the point of operation. In an enzyme-based amperometric biosensor, a convenient immobilization matrix must not only fix the enzyme molecules onto the transducer surface in a proper conformation for an efficient catalytic activity, but also keep the enzyme functional during the measurements as long as possible without denaturation or loss of activity. Immobilization matrix firstly has to serve as an appropriate platform for the enzyme, secondly transfer the electrons efficiently in electrochemical measurements. Hence, to provide and enhance the electron transfer between active site of enzymes and electrode, numerous materials such as metal nanoparticles [1,2], carbon nanotube derivatives [3,4] or inorganic or polymeric nanocomposite materials [5,6] were used in biosensor fabrications. Here, we developed a novel immobilization platform based on the polymers with organic and inorganic backbones for an efficient and durable enzymatic biosensor.

Polymers with inorganic backbone have attracted a growing interest during the last few decades due to their superior properties. Polyphosphazenes, bearing two side chains attached via macromolecular substitution reactions to each phosphorous atom

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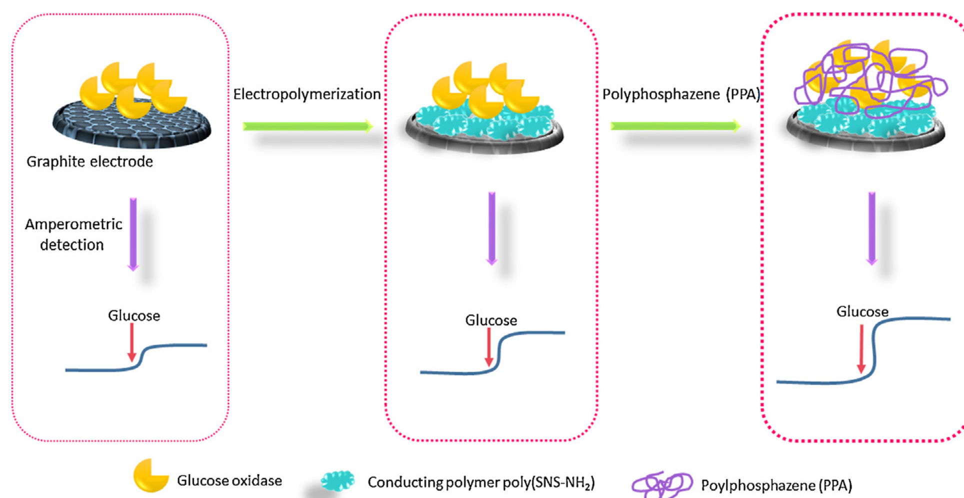

Scheme 1. Preparation of the glucose biosensor.

of the repeating unit, have a special characteristic among them. A wide variety of the polyphosphazenes with different physical and chemical properties can be synthesized in this way. Moreover, the possibility to design polyphosphazenes having different organic groups statistically distributed along the main chain allows to fine tune these properties further [7,8].

The flexibility in the synthesis of polyphosphazenes let the research expand in this field tremendously and the distinct polymer architectures find diverse potential applications. Biomedical applications are one of them and numerous reviews can be found in this field [9–15]. However, polyphosphazenes have been scarcely investigated as a platform to chemically link a biocatalyst. The first example of enzyme immobilization was given for glucose-6-phosphate dehydrogenase and trypsin on the surface of alumina coated with a thin film of $[NP(OPh)_2]_n$ [16]. Later, high invertase activity for the system immobilized on the surface of $[NP(OCH_2CF_3)_2]_n$ spherical particles activated by $NaOCH_2CH_2NH_2$ was reported [17]. In another study, urease was encapsulated in a hydrogel composed of crosslinked $[NP(OCH_2OCH_2OCH_3)_2]_n$ [18]. Cuetos et al. studied first the immobilization of alcohol dehydrogenase and lipase on $[NP(O_2C_{12}H_{7.5}(NH_2)_{0.5})]_n$ for the stereoselective bioreductions of ketones in aqueous solutions and the letter was for kinetic resolutions in organic solvents respectively [19] and recently reported the first selective co-immobilization of a Baeyer–Villiger monooxygenase (phenylacetone monooxygenase) and a NADPH recycling enzyme (glucose-6-phosphate dehydrogenase) on the same polyphosphazene carrier $[NP(O_2C_{12}H_{8-x}(NH_2)_x)]_n$ (x ranging from 0.5 to 2) [20].

Conducting polymers with easily tailored and functional architectures are durable and stabile. Such conductive structures are always considered as one of the most convenient materials for biosensors [21–25]. Using conducting polymers with functional pendant groups, enzyme molecules can be covalently immobilize onto them in a controlled manner to have a long-lasting and stabile biosensor.

In this study, a conducting polymer, poly(4-(2,5-di(thiophen-2-yl)-1*H*-pyrrol-1-yl)benzenamine) (poly(SNS-NH_2)) and a novel flexible hydrophilic polyphosphazene, poly[(methoxyethoxy)ethoxy-*co*-3-formylphenoxy]phosphazene (PPA), were synthesized and utilized for immobilization of glucose oxidase (GOx) on the graphite electrode. GOx was covalently immobilized onto the polymers containing transducer surface. The electrochemical properties of the conducting polymer was used to enhance the electron transfer during amperometric measurements; besides, the stability, durability and sensitivity of the biosensor were boosted with the use of polyphosphazene polymer bearing aldehyde functional groups. Moreover, due to the hydrophilicity of PPA, the polymer interacted in a better way with the enzyme molecules and provided an easy and efficient immobilization. During immobilization, biomolecules were entrapped in polyphosphazene network and also covalently bound to the polymers with the help of both amine and aldehyde groups in the structures of the polymers in a restrained and efficient manner, which brought the superior stability and activity of the biosensor. Scheme 1 displays the steps of amperometric glucose biosensor preparation representatively. The biosensor fabrication was optimized and characterized. The proposed poly(SNS-NH2)/PPA/GOx biosensor was utilized for the estimation of glucose contents in various beverages in a great agreement with a reference method.

2. Materials and methods

2.1. Materials

Glucose oxidase (GOx, β-D-glucose: oxygen 1-oxidoreductase, EC 1.1.3.4, 17,300 units/g) from *Aspergillus niger*, D-glucose, acetonitrile (ACN) and glutaraldehyde were purchased from Sigma (St. Louis, USA; www.sigmaaldrich.com). Tetrabutylammonium hexafluorophosphate ($TBAPF_6$) was supplied by Aldrich. All chemicals for the synthesis of the monomer were purchased from Aldrich except tetrahydrofuran (THF) which was obtained from Acros (Geel, Belgium, www.acros.comAll other chemicals were analytical grade.

All reactions for the synthesis of polyphosphazene were carried out under an atmosphere of dry nitrogen gas and using standard vacuum-line Schlenk techniques. Tetrahydrofuran (THF, 99.0%, Merck), toluene (99.8%, Sigma–Aldrich) and pentane (99.0%, Merck) were distilled over sodium benzophenone ketyl prior to use. Sodium hydride (NaH, 60% dispersion in mineral oil Sigma–Aldrich) were washed with excess petroleum ether (boiling range 40–60 °C, Merck), dried and stored in a Glove-box under dry argon gas. Phosphorous pentachloride (PCl_5, 99.0%, Merck) was sublimed and kept in Glove-Box. 3-Hydroxybenzaldehyde (97%, Sigma–Aldrich) was stored in a Glove-Box, diethylene glycol methyl ether (98.0%, Merck) was stored over activated molecular sieves (type 4A) and used without further purification.

2.2. Apparatus

For the amperometric studies and cyclic voltammetry measurements, Palm Instrument potentiostat (PalmSens, Houten, The Netherlands) was used. All electrochemical measurements were performed in a conventional three-electrode cell containing a graphite electrode (Ringsdorff Werke GmbH, Bonn, Germany, type RW001, 3.05 mm diameter and 13% porosity) as the working electrode, platinum electrode as the counter electrode, a Ag/AgCl (3 M KCl saturated with AgCl) electrode (Metrohm, Switzerland) as the reference electrode. In amperometric analyses, the data were given as the average of three measurements and standard derivations were recorded as ±SD. All measurements were performed at ambient conditions (25 °C). JEOL JSM-6400 model scanning electron microscope (SEM) was used for surface imaging studies.

NMR spectra were measured using a Bruker Spectrospin Avance DPX-400 spectrometer and Bruker Biospin UltrashieldTM 300 MHz) spectrometers. The spectra were recorded using $CDCl_3$ as the solvent and tetramethylsilane (TMS) the internal reference. The molecular weights and molecular weight distributions (PDI) of the polymers were determined by a gel permeation chromatograph, Viscotek TDA Max (Malvern Instruments). The column was calibrated with polystyrene (polyCAL TDS PS-99K and PS-235K) standards. The flow rate was 1 mL/min of tetrahydrofurane. Differential Scanning Calorimetry (DSC) was used to obtain the glass transition temperatures (T_g) and other primary transitions of the polymers. The DSC thermograms were recorded using Perkin Elmer diamond differential scanning calorimetry (DSC) at a heating rate of 10 K/min between −150 °C and +150 °C under an atmosphere of dry nitrogen. The second heating run was used for the determination of glass transition temperature (T_g). FT-IR spectra of the polymers and polymer electrolytes were measured on a Varian 1000 FTIR spectrometer.

3. Experimental

3.1. Synthesis of the monomer, SNS-NH_2

4-(2,5-Di(thiophen-2-yl)-1*H*-pyrrol-1-yl)benzenamine (SNS-NH2) was synthesized and characterized according to a previously described method [26]. Starting with 1,4-di(2-thienyl)-1,4-butanedione and combining it with benzene-1,4-diamine in the presence of propionic acid, the monomer was synthesized and purified with column chromatography. The product was obtained as pale yellow solid representing the desired monomer.

3.2. Synthesis of trichloro (trimethylsilyl)phosphoranimine (monomer) (**1**)

The synthesis of the monomer trichloro (trimethylsilyl)phosphoranimine ($Cl_3 = NSiMe_3$) was carried out according to the procedure described by Wang et al. [27] with minor modifications [28].

3.3. Synthesis of poly(dichlorophosphazene) (**2**)

The polymer was synthesized from trichloro (trimethylsilyl)phosphoranimine via living cationic polymerization using the route developed by Allcock et al. [29]. The molar ratio of monomer to PCl_5 was kept around 350:1. The concentration of PCl_5 in the polymerization solution was 0.0027 mol/L. This polymer was used as the precursor polymer for the synthesis of target polymers via macromolecular substitution reaction (Fig. 1).

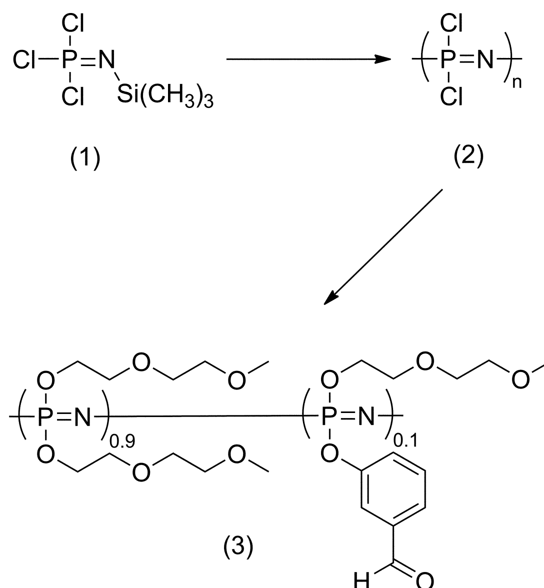

Fig. 1. The representative synthesis of poly(dichlorophosphazene) (2) and poly[(methoxyethoxy)ethoxy-*co*-3-formylphenoxy]phosphazene (3) starting from the monomer (1).

3.4. Synthesis of poly[(methoxyethoxy)ethoxy-co-3-formylphenoxy] phosphazene (**3**)

3-Hydroxybenzaldehyde (0.437 g, 3.59 mmol) was dissolved in 10 mL THF and slowly added to the 5 mL THF suspension of NaH (0.086 g, 3.59 mmol) in 250 mL round bottom three necked flask equipped with a reflux condenser in ice bath. Then the mixture was stirred for half an hour at room temperature in order to be sure that the reaction was complete. To this solution poly(dichlorophosphazene) (4.15 g, 35.85 mmol) in 40 mL THF was then added and the reaction mixture was stirred at reflux for 6 h and cooled to room temperature. A sodium salt solution of diethylene glycol methyl ether was prepared by slowly adding diethyleneglycol methyl ether (10.3 g, 88.5 mmol) to 65 mL THF suspension of NaH (1.96 g, 81.7 mmol) in ice bath and then refluxing until a clear solution was obtained in 30 min. This solution was then added slowly to the reaction mixture and refluxed for 18 h. The reaction mixture was concentrated and dialyzed against water for two days, then 0.1 M NaOH solution for 1 day and then water for another two days. The solution was filtered and the solvent was removed under vacuum. The polymer obtained was dissolved in THF and precipitated in petroleum ether and dried under high vacuum at least for two days until a constant weight is reached. 7.89 g (78%) of polymer was isolated (Fig 1). ^{1}H NMR ($CDCl_3$): δ = 3.99 (br, 2H), 3.67 (br, 2H), 3.56 (br, 2H), 3.44 (br, 2H), 3.28 (3H) for (methoxyethoxy)ethoxy moiety and around 7.5 aromatic signals (4H) and 9.91 aldehyde group (1H) for 3-formylphenoxy moiety. ^{13}C NMR ($CDCl_3$): δ = 72.0, 70.3, 68.0, 65.2, 58.9 for (methoxyethoxy)ethoxy moiety and not seen for 3-formylphenoxy moiety. ^{31}P NMR ($CDCl_3$): δ = 7.71 (sharp big s) and −2.82 (broad small s). FT-IR: P=N (1231 cm^{-1}), P—O—C (1200 and 1046 cm^{-1}), C—O—C (1107 cm^{-1}). One peak was observed in the GPC measurement with the average molecular weight M_n = 628.800 D and a polydispersity index (PDI) of 1.06 (refer to Supporting Information for the NMR and FTIR spectra).

3.5. Preparation of the poly(SNS-NH_2)/PPA/GOx biosensor

The conducting polymer, poly(SNS-NH_2), was electrochemically synthesized on to the previously polished and cleaned graphite

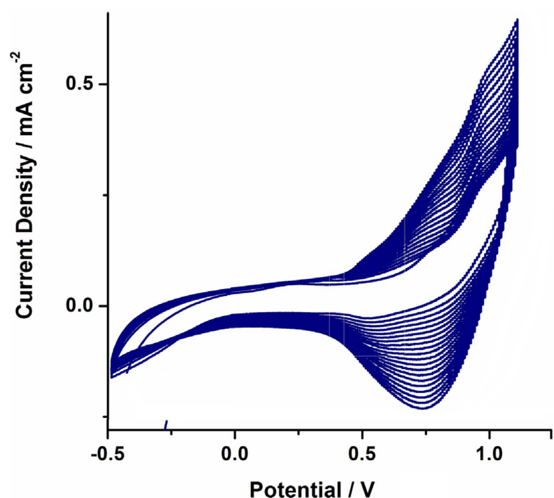

Fig. 2. Cyclic voltammogram for polymerization of SNS-NH_2 in 0.1 M $NaClO_4$/$LiClO_4$/ACN electrolyte-solvent system at a scan rate of 100 mV s^{-1} on graphite (up to 20 cycles).

electrodes. The polymer was electropolymerized on the electrode in 0.1 M $NaClO_4$/$LiClO_4$/ACN electrolyte/solvent system with 40 cycles via scanning the potential between −0.5 V and +1.2 V by cyclic voltammetry. After polymerization, the polymer coated electrode surface was rinsed with deionized water in order to get rid of the organic impurities. First 20 cycles of the polymerization are shown in Fig. 2.

Prior to immobilization of GOx, 91.5 mg/mL PPA solution was prepared by dissolving PPA in deionized water and sonicated for 15 min to obtain a clear solution. Suitable amount of this solution was diluted with 50 mM phosphate buffer solution (pH 7) in 1:1 ratio to yield 50% PPA solution in buffer. This solution was prepared before each immobilization, freshly. For the immobilization of enzyme, 5.0 μL aliquot of this PPA solution, suitable amount of GOx solution (2.60 mg (45.0 U) in 5.0 μL, 50 mM sodium phosphate buffer (pH 7.0)) were mixed in a vial and immobilized on the poly(SNS-NH_2) coated graphite electrode surface with the help of 1.0% glutaraldehyde in 5.0 μL 50 mM sodium phosphate buffer (pH 7.0). This is followed by drying at room temperature to evaporate the solvent for 2 h. The enzyme electrodes were then rinsed with distilled water to remove unbound enzyme and crosslinker. The electrodes were kept in a refrigerator at 4 °C overnight. The solutions were prepared freshly prior to preparation of the biosensors.

3.6. Electrochemical measurements

Amperometric measurements using the prepared biosensor were performed in a reaction cell containing 10 mL 50 mM sodium acetate buffer solution (pH 4.5) under gentle stirring. Measurements were carried out under constant potential; −0.7 V versus Ag/AgCl. After the current reached a steady state, the substrate solution, glucose, was injected to the reaction cell in proper amounts and the current change (μA) generated from the catalytic activity of the enzyme was followed. In that reaction, β-D-glucose is oxidized to gluconolactone which is then hydrolyzed to gluconic acid. Simultaneously, oxygen is consumed to generate hydrogen peroxide [30]. At the defined potential, oxygen consumption was followed and correlated with the consumed glucose in the amperometric measurements showing the activity of the biosensor. The buffer solution used in the reaction cell was refreshed and biosensor was rinsed with distilled water after each measurement. Substrate, glucose, stock solutions were prepared in buffer solution and stored in dark and refrigerator.

3.7. Sample application

The biosensor was tested on various beverages measuring the glucose contents in them. The beverages were used as they are without a need for pre-treatment. During the measurements, instead of glucose, beverages were injected into the reaction cell and the biosensor responses were recorded. Using the calibration curve for glucose, the glucose content was calculated. The same samples were also tested with a commercial enzyme assay kit for glucose measurements (HUMAN 10260, Glucose Liquicolor, Wiesbaden, Germany). This enzyme assay kit is based on GOx-PAP method including glucose oxidase, peroxidase, phenol and 4-aminophenazone. Colorimetric test depends on enzymatic oxidation of glucose by the help of glucose oxidase. Hydrogen peroxide, the product, reacts with phenol and 4-aminophenazone in the presence of peroxidase which gives a red-violet color via quinoneimine dye indicator [31,32].

4. Results and discussion

4.1. Characterization of polyphosphazene (PPA) (**3**)

The target polymer was investigated using NMR, FT-IR, DSC and GPC. The macromolecular substitution of the polymer was performed mainly using oligoether side chains to maintain the water solubility with small amount of aldehyde moiety for further interactions with the enzyme. ^{1}H NMR spectrum of the polymer showed mainly the characteristic peaks of the oligoethers, the signals of 3-formylphenoxy groups were as expected very week. The ratio of the methyl protons at 3.28 ppm to the aldehyde proton at 9.91 ppm must be statistically 30 and this ratio was confirmed by integration of the corresponding signals in ^{1}H NMR spectra (as shown in supporting Information, Fig. S1). Although the proton NMR gave hints for the structure of the synthesized polyphosphazene, ^{31}P NMR of the polymer gave more significant results in terms of substitution pattern and the completeness of the macromolecular replacement (as shown in Supporting Information, Fig. S3). First of all, the disappearance of the single sharp peak of PDCP at around −18 ppm was accompanied by the expected two signals of the synthesized polyphosphazenes at around −8 ppm and −3 ppm. This showed the complete replacement of the chlorine atoms of the poly(dichlorophosphazene). The big signal at around −8 ppm was assigned for the repeating units fully substituted by oligoether [7] and the small signal at around −3 ppm was attributed to the mixed substituted repeating units. The ratio of the repeating units were calculated as 0.91–0.09 by taking into these two signals into account and the result was quite close to the target polymer composition of 0.90–0.10.

Number and weight average molecular weights of the obtained polyphosphazenes were estimated by GPC relative to polystyrene. The living cationic polymerization with the monomer to polymer ratio of 350:1 implied a narrow molecular weight distribution and the polymer chains of the polyphosphazenes ought to contain around 700 repeating units (as two initiator species were used per chain). This assumption mean the molecular weight should be around 2×10^5 D, however much higher molecular weights (around 6×10^5 D) were estimated by the measurements. Although, the overestimation of the molecular weight of the polyphosphazenes by GPC is known, such a high molecular weight jump could be resulted either from the loss of the initiator during the course of the polymerization (as the monomer to initiator ratio increases, the molecular weight increases but the narrow distribution is maintained) or the partial crosslinking of the polyphosphazene chains during the macromolecular displacement reactions due to impurities mainly water (both the molecular weight and the

polydispersity increases). The former explanation could be taken into account as the polydispersity index of the molecular weight distributions of the polymers was estimated to be very close to unity. No crosslinking was observed and the polymer was soluble in common solvents like THF, dioxane, chloroform and water.

The structure was also confirmed with the FT-IR spectrum. The P=N stretching at 1231 cm^{-1} was attributed to the presence of high polymeric phosphazene structure. The two expected P—O—C peaks were assigned to 1200 cm^{-1} and 1046 cm^{-1}. The presence of the oligoether side chain was identified by the strong C—O—C stretching band at 1107 cm^{-1}. Since the concentration of 3-formylphenoxy groups on the polymer chain is rather low, the expected aldehyde peak at around 1700 cm^{-1} was shown as a weak shoulder of the broad oligoether signal at 1640 cm^{-1}.

Thermal properties of the polymer were investigated using DSC. As expected, there were no crystallinity observed on the thermogram. The T_g value of the polymer was −81.5 °C and in accordance with the reported T_g value of poly[bis(methoxyethoxy)ethoxy phosphazene] in the literature [7].

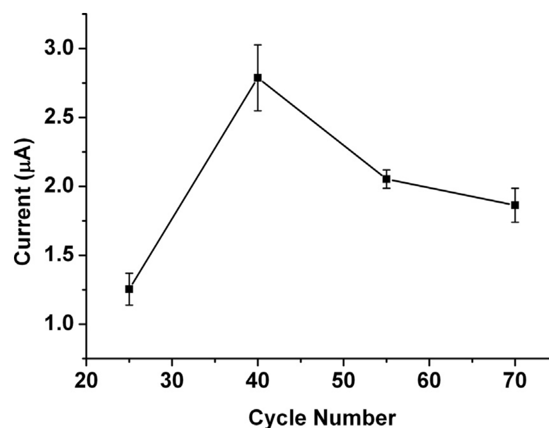

Fig. 3. The effect of poly(SNS-NH_2) film thickness on biosensor response (in pH 4.5 50 mM sodium acetate buffer, 25 °C, −0.7 V, [glucose]: 0.7 mM). Error bars show the standard deviation (SD) of three measurements.

4.2. Optimization studies in biosensor preparation

A biosensor must be designed for repetitive and long-run uses. The biosensor should be stable and reproducible for real sample applications. In order to have such a biosensor, all the conditions that affect the biosensor performance should be optimized and kept unchanged during the measurements. As a result, all the parameters affecting the biosensor activity were optimized. In each case, one of the parameter such as enzyme amount was changed in multiple biosensors and in all of them other parameters such as conducting polymer layer thickness, PPA and glutaraldehyde amount, pH were kept constant to see the only effect of one parameter.

Especially in enzyme-based biosensors, immobilization matrix has a major impact on biosensor quality and performance. A well-designed matrix can prolong the biosensor shelf-life by maintaining the 3D structure of enzymes where they are embedded in. For this purpose, conducting polymers are perfect candidates to be able to keep enzyme alive at the same time fixed on a transducer surface [33,34].

In this study, covalent binding and entrapment immobilization techniques were used together. Excessive covalent binding of biomolecules to a support material may cause an irreversible change in the natural conformation of the biomolecules due to the improper fixation. Comparably, entrapment immobilization technique is hard to apply when it comes to choose a material as the encapsulation network. The membrane-like material should keep the biomolecules on the electrode surface as well as allowing the substrate to reach the active site of the biomolecule. Both techniques have pros and cons; however, using them in combination in a controlled fashion can bring the better result compared to the single techniques.

To obtain a proper orientation and binding of enzyme on the amino functionalized conducting polymer, poly(SNS-NH_2), polymer layer thickness was optimized. It was adjusted during the electropolymerization. The cycle (scan) number in the polymerization determines the deposited charge on the polymer film so do the thickness of the conductive polymer layer. To seek for the optimum thickness, poly(SNS-NH_2) was deposited onto the graphite electrode with 25, 40, 55, 70 scan numbers while the other parameters were kept same (Fig. 3). The biosensor responses to same amount of glucose were recorded and compared. The highest performance of the biosensor was observed with the electrode having 40 cycle-polymer. This shows that in higher polymer thicknesses, due to the excessive presence of amino groups on the polymer layer, covalent binding is more than enough which brings the loss of activity of enzyme resulting in low signal. In contrast, when the polymer layer is thinner than expected, immobilization is poorer; hence the enzyme molecules are immobilized loosely causing again low activity. As a result, 40 cycles were chosen as the optimum scan number in electropolymerization which corresponds to 64.0 nm (equivalent of 2.80 mC charge) in thickness and used in further studies.

To enhance the immobilization, a polyphosphazene derivative polymer, poly[(methoxyethoxy)ethoxy-*co*-3-formylphenoxy] phosphazene (PPA) was used together with the conducting polymer. PPA is thought and designed for enzyme immobilization as a perfect candidate since it has free aldehyde pendant groups open to linkage and it is water soluble. It is easily dissolved in water and combine with the enzyme while casting on the desired support surface. Since it is comfortable to have an enzyme–polyphosphazene mixture in one phase medium, it is easy to apply on a surface. After evaporation of water on the surface, the polymer can encapsulate enzyme molecules as a network and with the pores in the structure, it allows an easy diffusion of the analyte and product molecules to and from the biomolecule environment while keeping the enzyme molecules immobile within the polymer matrix the substrate to diffuse the biomolecule environment whereas keeping the enzyme molecules on the surface. Moreover, the synthesized polyphosphazene has free aldehyde groups. They take place in imine bond formation with the free amino groups of the enzyme molecules when they interact each other for a certain time [35,36]. Hence, the entrapment was supported with the covalent binding. To get the best combination yielding highest biosensor response, PPA amount in the enzyme solution during the immobilization was optimized. After poly(SNS-NH_2) was coated on the electrodes, several electrodes with different PPA ratios in enzyme mixture were prepared. 9.51 mg/mL PPA solution in water was diluted with 50 mM phosphate buffer solution (pH 7.0) (PBS) in 0.5:1, 1:1, 1.5:1, 2:1 ratios for PPA in water: PDS to yield 33, 50, 60, 67% PPA solution in buffer. 5 μL of those solutions were used to mix with 45.0 U GOx containing buffer solution for immobilization. The biosensor responses for different glucose concentrations were recorded and depicted in Fig. 4. It is clearly seen that polyphosphazene has a great contribution to the biosensor sensitivity in terms of higher signals showing the improvement in the immobilization. However, when the PPA ratios were compared, not much difference was seen between first three. With the one containing 50% PPA, it is possible to detect lower concentrations of glucose with higher signals; likewise, while the others reach saturation around 0.5 mM glucose level, it gives superior signals compared to them. Due to the excessive entrapment and possible covalent linkage, 67% PPA containing biosensor gives

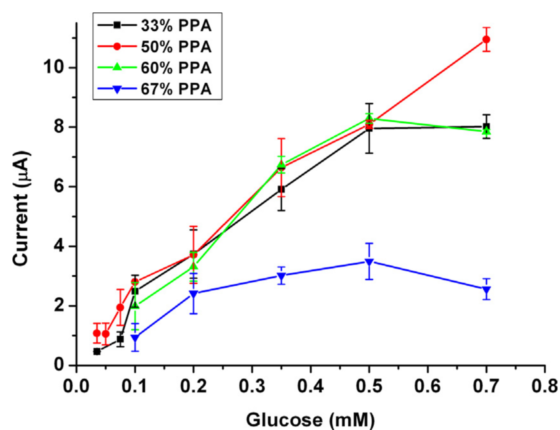

Fig. 4. The effect of polyphosphazene on biosensor response (in pH 4.5 50 mM sodium acetate buffer, 25 °C, −0.7 V). Error bars show the standard deviation (SD) of three measurements.

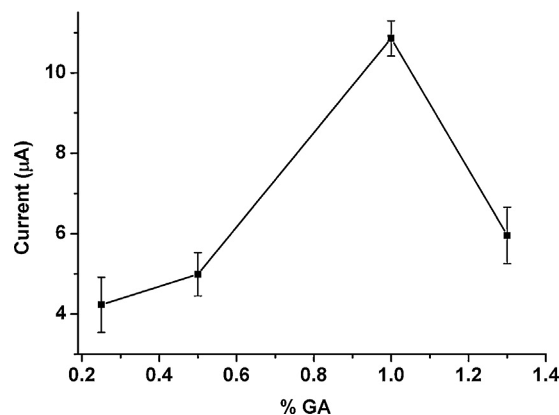

Fig. 6. The effect of crosslinker, GA, on biosensor response (in pH 4.5 50 mM sodium acetate buffer, 25 °C, −0.7 V, [glucose]: 0.7 mM). Error bars show the standard deviation (SD) of three measurements.

the lower signals as a result of diffusion problem and possible denaturation of the enzyme molecules. Therefore, 50% PPA amount was chosen as the optimum polyphosphazene amount.

To have higher biosensor signal, to increase enzyme amount may be a good strategy; however, enzyme amount should be also optimized to get the highest biosensor responses. Since the immobilization matrix has an enzyme loading capacity, more than that cannot be fixed on the electrode surface and during the measurements then enzyme loss can be observed. In contrast, in lower enzyme amounts, due to the absence of higher responses, the biosensor response to lower substrate concentrations may be too small to detect which causes the lower sensitivity. Considering all these cases, four different electrodes were prepared with changing enzyme amounts from 37.2 U to 50.9 U (2.15–2.94 mg) GOx. Biosensor responses are shown in Fig. 5. The highest responses were obtained using 45.0 U GOx in the preparation of the biosensor as a result of a better interaction and linking with the polymeric matrix. Thus, 45.0 U enzyme was used for further experimental steps.

Glutaraldehyde (GA) was used as the crosslinking agent in the biosensor preparation. In addition to the imine bond formation between PPA matrix and enzymes, GA with its high activity was used to enhance the immobilization via linking on the amino functionalized conducting polymer. Moreover, with its long chain crosslinking property, it supports the proper enzyme conformation and enhances the compact structure of the enzyme molecules on the polymer. 0.25%, 0.5%, 1.0% and 1.3% GA values were tested (Fig. 6). 1% GA was found as the optimum value.

Furthermore, pH of the working environment also affects the performance of a biosensor. Insuitable pH values of the soluions in which the biosensor kept may cause denaturation of the enzyme moleucles. pH dependence of the responses was investigated over a pH range between 4.0 and 6.5 with 50 mM sodium acetate buffer at 4.0–5.5 and 50 mM phosphate buffer at 6.0; 6.5, 25 °C. The best result was achieved at pH 4.5 sodium acetate buffer solution as given in Fig. 7.

Moreover, in order to see the effectiveness of the immobilization matrix, importance and contribution of the all components, three biosensors were prepared and results are shown in Fig. 8. When the enzyme was casted on the bare electrode surface (GOx electrode), it was truly hard to record significant responses. Singularly, when poly(SNS-NH_2) was used as alone, lower responses were obtained. Conversely, when PPA is contributed into the immobilization matrix, the signals were enhanced greatly. Linear range and sensitivity were improved which shows the effectiveness of the combined immobilization platform.

4.3. Characterization

Scanning electron microscopy (SEM) technique was used to analyze the morphological properties of the biosensor after each modification. Fig. 9 displays morphologies after each modification on the surfaces. Fig. 9(A) exhibits the coarse morphology of

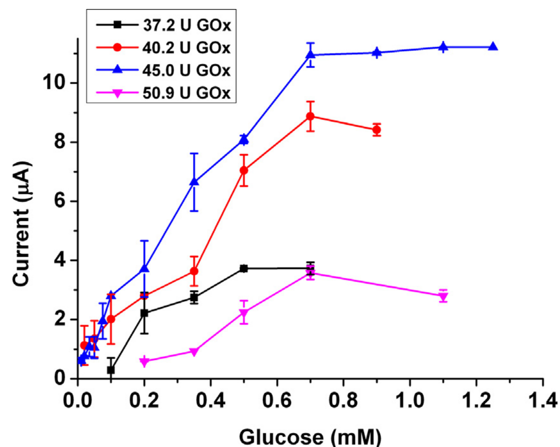

Fig. 5. The effect of enzyme amount on biosensor response (in pH 4.5 50 mM sodium acetate buffer, 25 °C, −0.7 V). Error bars show the standard deviation (SD) of three measurements.

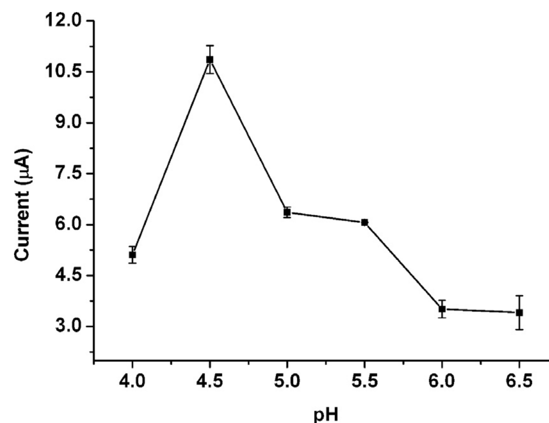

Fig. 7. Effect of pH (in sodium acetate buffer, 50 mM, at pH 4.0, 4.5, 5.0, 5.5 and in sodium phosphate buffer, 50 mM, at pH 6.0, 6.5, 25 °C, −0.7 V, [Glucose]: 0.7 mM). Error bars show standard deviation of three measurements.

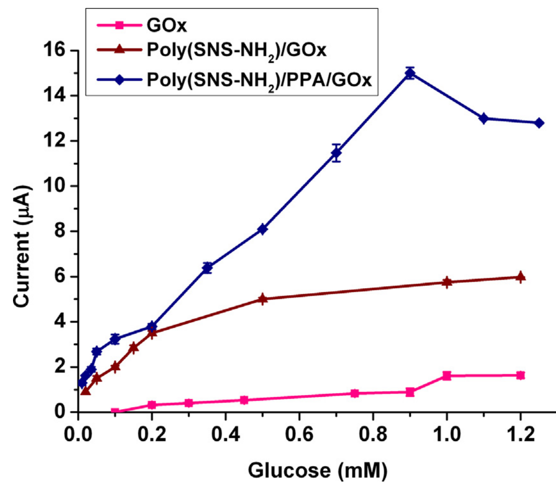

Fig. 8. Effect of immobilization matrix on biosensor response under optimized conditions (in pH 4.5 50 mM sodium acetate buffer, 25 °C, −0.7 V). Error bars show the standard deviation (SD) of three measurements.

polyphosphazene (PPA). In Fig. 9(B), the combination of PPA and GOx is seen. Due to the solubility of PPA in water, GOx and PPA are integrated into each other very well and a rich interfacial interaction is observed. In Fig. 9(C), GOx immobilized poly(SNS-NH_2) surface is seen. Compared to (B), a finer morphology was obtained. The conducting polymer coats the electrode surface homogeneously and uniformly which behaves as an excellent candidate for enzyme immobilization. Therefore, in the combined final structure, Fig. 9(D), all three components were integrated and the image for poly(SNS-NH_2)/PPA/GOx represents a 3D and freely occurred biolayer was achieved. The porous and indented structure shows that on this immobilization platform, enzyme molecules can freely be oriented and preserve their significant 3D structure after immobilization. This brings the effectiveness of the platform as an immobilization matrix. After immobilization, enzyme molecules should retain their activity so do 3D structure in order to be able to catalyze the enzymatic reactions. Moreover, as seen, PPA does not excessively cover the biolayer; instead, it supports the activity of enzymes while holding them in an appropriate conformation.

Analytical characterization of the glucose biosensor was done with the preparation of a calibration curve for glucose. Biosensor responses for varying glucose concentrations were recorded and a calibration curve with a linear range was obtained (Fig. 10). The biosensor shows a perfect linearity in a wide range, 0.01–0.9 mM concentration range for glucose. A wide linear range is always desired for a biosensor to be able to work in a wide range of samples. When it is compared with the literature, the linear range of poly(SNS-NH_2)/PPA/GOx biosensor is good compare to 0.01–05 mM for Ru-Py-HRP-GOx/Nafion microsensor [37], 0.05–1.1 mM for Nafion/GOx/Ag-Pdop@CNT/GCE biosensor [38] and 0.01–0.8 mM for GOx-CS/AgNWs/GCE biosensor [39]. A typical biosensor response for glucose was given as an inset proving the fast response to glucose. Due to the superiority of the immobilization matrix, the substrate can easily reach to the active sites of the enzyme molecules on the electrode surface and reaction is catalyzed by the enzymes. Hence, the fast response can be comfortably seen in 19 seconds with the help of electroactive immobilization matrix. The limit of detection (LOD) shows the capacity of

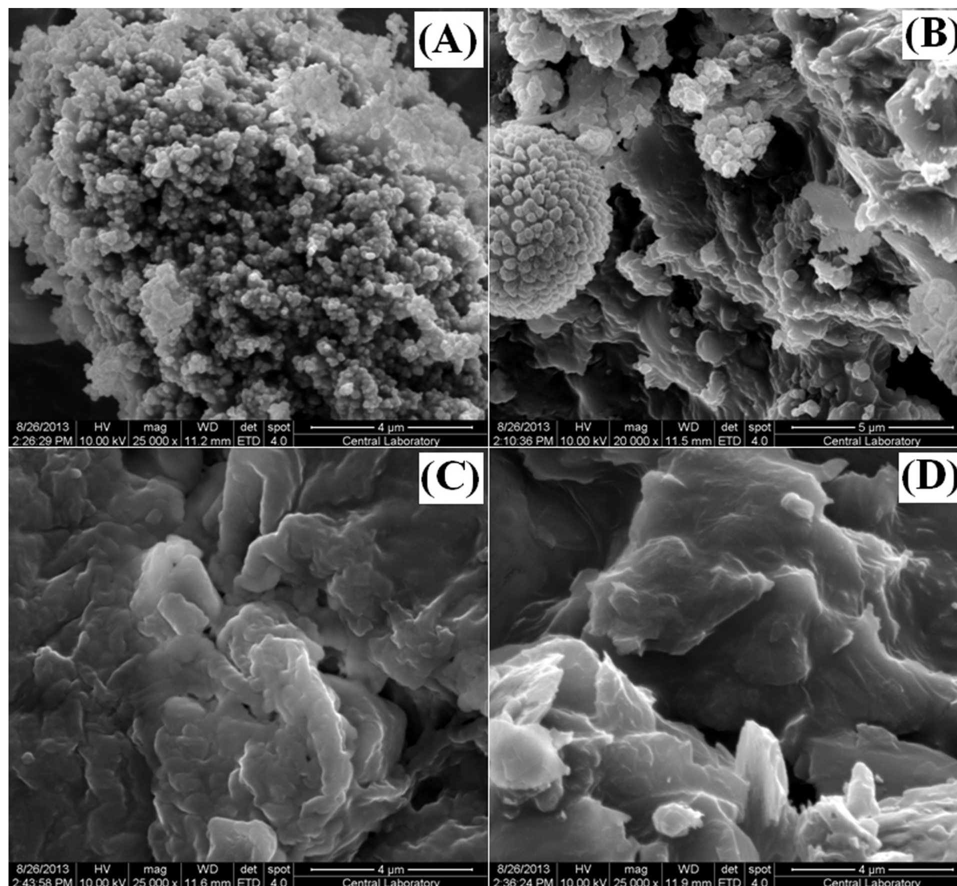

Fig. 9. Surface characteristics of (A) polyphosphazene (PPA), (B) GOx immobilized PPA, (C) GOx immobilized poly(SNS-NH_2) and (D) GOx immobilized poly(SNS-NH_2)/PPA surface via SEM images.

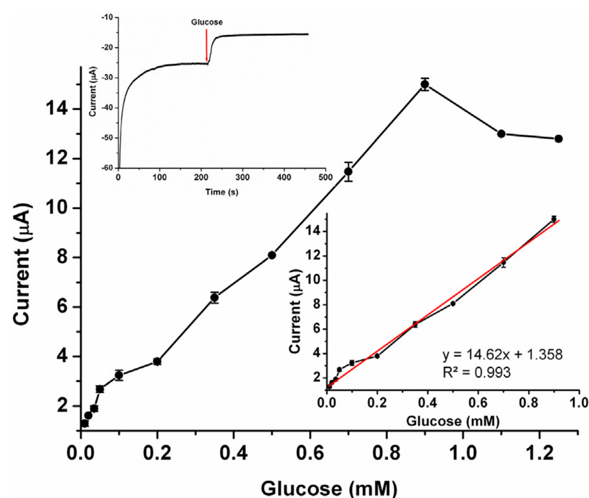

Fig. 10. Calibration curve for glucose (in pH 4.5 50 mM sodium acetate buffer, 25 °C, −0.7 V). Error bars show the standard deviation (SD) of three measurements. (A typical amperometric signal to 0.70 mM ethanol in pH 4.5 50 mM sodium acetate buffer given as inset.)

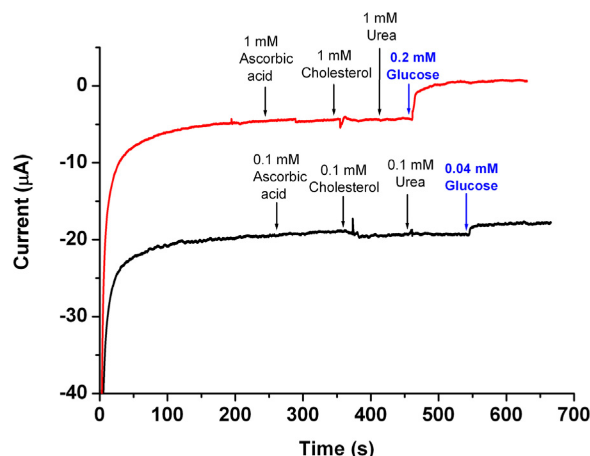

Fig. 12. Biosensor responses to glucose and possible interfering species (in pH 4.5 50 mM sodium acetate buffer, 25 °C, −0.7 V).

the biosensor to sense the small quantities of the substrate. The detection limit was estimated to be 1.3 µM according to S/N = 3 which is lower compared to the other studies. For example, a glucose oxidase biosensor prepared with ordered mesoporous carbon (OMC) supported platinum nanoparticles (Pt/OMC) gives an LOD as 50 µM [40]. In another example, based on glucose oxidase immobilized by *o*-phenylenediamine (*o*PD) on platinum nanoparticles (PtNPs) electrodeposited polyvinylferrocenium perchlorate matrix ($PVF^+ClO_4^-$), the LOD was found as 18 µM. [41]. Moreover, Sensitivity was estimated as 237.1 mA/M cm^2 which extremely high when it is compared to the other glucose biosensor such as graphene-nano Au–GOx–GCE biosensor having 56.93 mA/M cm^2 [42], GOx–In_2O_3–chitosan/GCE having 7.3 mA/M cm^2 [43] and GOx/TCS-TiO_2/chitosan/GCE biosensor with 23.2 mA/M cm^2 [44] sensitivities (Fig. 11).

The Michaelis–Menten constant (K_M) is considered as the indicator of enzyme affinity to its substrate which is used to understand the reaction kinetics. This can be used to understand the behavior of the enzyme or bioactivity and bioavailability of the enzyme after immobilization showing the effect of immobilization on the enzyme molecules. The apparent Michaelis–Menten constant (K_M^{app}) was calculated for poly(SNS-NH_2)/PPA/GOx biosensor as 0.676 mM and I_{max} as 20.91 µA according to the Lineweaver–Burk equation [45]. The lower K_M value is a sign of higher affinity of immobilized GOx molecules toward glucose. We obtained a highly lower K_M^{app} than those of 9.85 mM, 5.46 mM, 11.1 mM, 10.0 mM and free GOx [38,41,46–48].

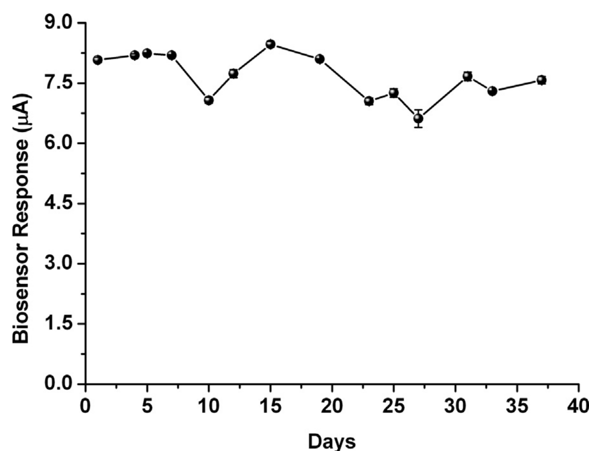

Fig. 11. Biosensor responses for 37 days (in pH 4.5 50 mM sodium acetate buffer, glucose: [0.5 mM], 25 °C, −0.7 V). Error bars show the standard deviation (SD) of three measurements.

The outstanding performance of the biosensor in terms of sensitivity, response time LOD can be attributed to the immobilization matrix and biosensor preparation. The conducting polymer not only expands the electrode surface with higher surface area and provides an improved surface for immobilization but also enhances the catalytic reactions on the electrode surface with its excellent electroactivity; while polyphosphazene generates a network proper for entrapment of the enzyme molecules taking the advantage of water solubility and conjugation via aldehyde groups.

4.4. Stability and interference study

The operational stability of poly(SNS-NH_2)/PPA/GOx biosensor was estimated by measuring 0.7 mM glucose for 10 times during a period of 8 h and relative standard deviation (RSD) was 4.86% indication a good detection reproducibility. Shelf-life and storage stability of the biosensor was detected by recording the biosensor responses for 0.5 mM glucose every day for 37 days. Between measurements, the biosensor was kept at 4 °C. The biosensor showed a superior activity over this period with 95% retained its initial activity. The possible reasons for this obvious stability may be the perfect microenvironment provided by the combination of organic and inorganic polymers. Moreover, the membrane-like structure of polyphosphazene and sufficient covalent binding contributed the maintenance of the structure and activity of the GOx molecules on the electrode surface. This is noticeable that the biosensor can be used for long-time studies with its favorable immobilization platform.

To have a reliable and applicable biosensor, it should work interference-freely. To see the effects of some possible interferents, oxidizable interfering species such as ascorbic acid, cholesterol and urea were tested in a concentration range of 0.01 mM to 0.1 M. During amperometric measurements, instead of glucose, these species were injected to the reaction cell. No interference effect was observed in all applications (Fig. 12). However, upon addition of various amounts of glucose, a clear signal to glucose was obtained showing the proper working and selectivity of the biosensor in an interferent-rich environment.

Table 1
Glucose detection in beverages (All measurements were conducted three times; standard deviations were calculated and given as ± SD.).

Sample	Glucose (M)	
	Spectrophotometric	Poly(SNS-NH_2)/PPA/GOx
C® Soda	0.076	0.077 ± 0.0071
E® Lemonade	0.183	0.170 ± 0.0030
L® Ice tea	0.035	0.052 ± 0.0110
C® Orange juice	0.107	0.100 ± 0.0108

4.5. Sample application

The feasibility of the biosensor for real sample detections as a potential detector was investigated by analyzing several commercial beverages. Glucose contents of the samples were calculated from the calibration curve by measuring them in amperometric measurements instead of glucose without any pretreatment. As shown in Table 1, the determined glucose levels are close to the values obtained by enzymatic catalytic spectrophotometry. The results are in a good agreement with our data showing that the novel biosensor can be utilized for practical sample testing with a reliable accuracy and precision.

5. Conclusion

In this study, we synthesized a novel flexible hydrophylic polyphosphazene derivative, poly[(methoxyethoxy)ethoxy-*co*-3-formylphenoxy]phosphazene, PPA, and developed a novel glucose biosensor based on a conducting polymer, poly(4-(2,5-di(thiophen-2-yl)-1*H*-pyrrol-1-yl)benzenamine) (poly(SNS-NH_2)), in combination with PPA as a new immobilization platform for enzymes. The graphite electrode was coated with conducting polymer. Then, enzyme was immobilized together with water soluble PPA. Due to the presence of amino groups in the conducting polymer and aldehyde groups on PPA, GOx easily immobilized covalently and effectively. The conditions for immobilization were optimized. The biosensor was characterized in terms of surface morphology, linear range, LOD, sensitivity and affinity toward glucose. It is observed that the biosensor shows an extreme sensitivity and affinity toward glucose which brings the higher signals and lower detections limits for glucose. This situation can be attributed to the effectiveness and performance of the newly designed immobilization matrix. Moreover, the performance of the glucose biosensor was tested on glucose containing beverages. With its interference-free working capacity, the GOx biosensor showed satisfied performances in real sample measurements with high accuracy.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.snb.2014.05.040.

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