RSC Advances

PAPER

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Cite this: *RSC Adv.*, 2015, **5**, 35940

A novel architecture based on a conducting polymer and calixarene derivative: its synthesis and biosensor construction

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Received 5th March 2015
Accepted 13th April 2015

DOI: 10.1039/c5ra03933a

www.rsc.org/advances

In this study, a novel amperometric glucose biosensor based on a selenium comprising conducting polymer and calixarene was developed. Firstly, poly(2-(2-octyldodecyl)-4,7-di(selenoph-2-yl)-2*H*-benzo[*d*][1,2,3]-triazole), poly((SBTz)) was electrodeposited onto a graphite electrode by an electropolymerization technique. Then, a newly synthesized calixarene and gold nanoparticle (AuNP) mixture was used for the improvement of biosensor characteristics. GOx, as a model enzyme was immobilized on the modified electrode surface. The constructed surface serves as a sufficient immobilization platform for the detection of glucose. Calixarenes and their derivatives may be a favouring agent for enzyme immobilization due to their specific configurations. Moreover, through the covalent binding between the carboxylic groups of the calixarenes and amino groups of the biomolecule, effective enzyme immobilization can be achieved while protecting the well-ordered structure of the enzyme molecule. Amperometric detection was carried out following oxygen consumption at −0.7 V *vs.* the Ag reference electrode in phosphate buffer (50 mM, pH 6.5). The proposed biosensor showed a linear amperometric response for glucose within a concentration range of 0.005 to 0.5 mM (LOD: 0.004 mM). K_m^{app} and sensitivity were calculated as 0.025 mM and 102 μA mM^{-1} cm^{-2}, respectively. Scanning Electron Microscopy (SEM) was used to investigate the surface morphologies of successive modifications. Finally, the constructed biosensor was tested successfully to detect glucose in beverage samples.

1. Introduction

Biosensors are valuable devices for food technology, biotechnology, pharmaceutical studies and clinical diagnosis[1,2] due to their simplicity, low cost, quick response and portability. Because of the demand of the technology for sensitive and reliable detection of glucose,[3] cholesterol[4] and pesticides,[5] it is significant to design new biosensor systems. One important factor in manufacturing a new generation of biosensors is to immobilize biomolecules on a surface without losing their activity. In order to fabricate excellent enzyme biosensors and improve their functionality, various supports have been used to keep recognition molecules on an electrode surface.[6–8] Conducting polymers (CPs) have been widely suggested in the architecture of biosensors as immobilization matrices due to the number of advantages such as their easy preparation and direct deposition on the electrode surface.[9,10] The charge transport properties of π-conjugated materials play important role in the design and fabrication of biosensors. Taking into consideration of the advantages of their homogenous and feasible film character, biocompatibility and easy production; it is thought that they can be effectively used in biosensors. Moreover, they may minimize the access of interfering effects of compounds to biosensor surface.[11]

Glucose oxidase (GOx) is an oxido-reductase enzyme which catalyses the oxidation of glucose to hydrogen peroxide and D-glucono-δ-lactone. GOx shows high resistance against such micro environmental conditions like denaturing agents and acidic environment. GOx based amperometric electrodes have played a leading role for testing sugar of drinks, especially monitoring glucose.[3,12,13] For this type of analyses, GOx should be immobilized onto a substrate. Variety of substrate surfaces which are chemically and physically diverse can be applied according to desired purposes. They can be used for either direct electrical connection or for increasing surface area.[14]

In recent years, nanomaterials have attracted great interest in the construction of biosensing devices due to their outstanding properties. In particular, gold nanoparticles

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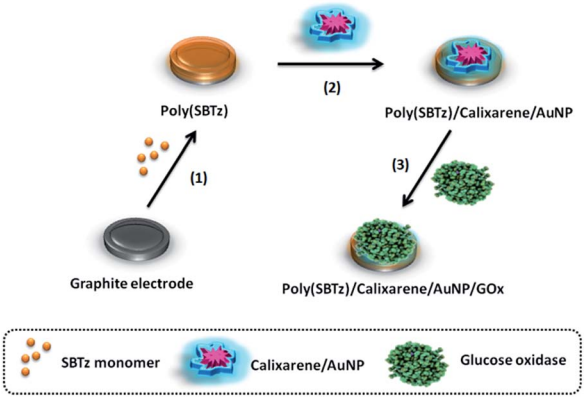
(AuNPs) were found to be charming due to their high surface to volume ratios. They allow the achievement of enhanced analytical performance with respect to other designs. AuNPs provide robust immobilization platform of proteins while retaining their bioactivity.[15] Furthermore, they have such important advantages as noncytotoxicity, excellent biocompatibility, ease of synthesis and surface functionalization, strong light absorption and scattering effect and photostability. However, they have a tendency to easily aggregate in the presence of biomolecules. Therefore, some compounds especially thiol containing ones are used to modify the nanoparticle surface. By this way, their stability, dispersibility and biocompatibility can be improved. This is mainly attributed to the fact that thiol groups can bind to the AuNPs *via* an Au–S bond.[16]

Among the immobilization materials, the use of supramolecular compounds; calixarenes for the construction of biosensing devices constitutes one of the most exciting approaches. Calixarenes, which have a cavity shaped architecture, composed of several cyclic phenolic oligomers. Two adjacent phenol rings are connected by an *ortho*–ortho CH_2 bridge or an analogous homo CH_2 bridge.[17,18] The word "calixarene" stems from an analogy of the space filling model and the ancient Greek vase the calix crater.[19] They enable structural modifications to accomplish desired properties. Chemical modification can be readily achieved *via* several one-step procedures. Furthermore, the structure of calixarene provides easy deposition of the guest molecules on the surface due to their cavity properties.[20] Therefore, they might be one of the remarkable host macrocyclic molecules and promising materials for immobilizing an enzyme.

Various types of biosensors modified with a conducting polymer and calixarene have been designed and tested for the analysis of important biological components. The main aim of the studies is to obtain superior biosensors for the detection of several analytes. In previous studies, especially those making use of functionalized conducting polymers having a variety of functional groups such as amino, carboxylic acid or moieties such as selenium, have received great attention for their strong bonding capability and better interaction with biomolecules.[21] Emre *et al.*[22] utilized a novel platform where GOx was successfully immobilized on the poly(4,7-di(2,3)-dihydrothienol[3,4-*b*]-[1,4]dioxin-5-yl-2,1,3-benzoselenadiazole) (PESeE) coated surface. They also reported that Se shows better ability of adsorption towards biomolecules and biocompatibility for biosensor fabrication. In another study, Soylemez *et al.*[23] reported that a novel immobilization matrix; 2-(4-fluorophenyl)-4,7-di(thiophene-2-yl)-1*H*-benzo[*d*]imidazole (BIPF) was electrochemically deposited on a graphite electrode and used as a matrix for the immobilization of cholesterol oxidase. Additionally, Le Goff *et al.*,[24] described the advantages of using electrogenerated polymer matrices for designing electrochemical sensors. Hence, use of conducting polymer for immobilization of biomolecule improved the electron transfer during the enzymatic reactions and that brings reliable, accurate and much effective detection of different analytes while maintaining their high selectivity and sensitivity. In addition to this, Kesik *et al.*[25] designed a glucose biosensor based on a conducting polymer and functionalized AuNPs. AuNPs were modified with mercaptopropionic acid (MPA) yielding Au–S bonds. The presence of both conducting polymer and functionalized AuNPs together bring the wiring effect on electron transfer between active site of the enzymes and polymer coated transducer which accelerates and facilitates the electron transfer and diminishes the diffusion problems. Calixarenes can be used as an alternative to many of materials for the immobilization of the biocomponent. Demirkol *et al.*[26] fabricated an amperometric GOx biosensor using thiol-containing calixarenes (Calix-SH) as an immobilization matrix to obtain better biosensor performance with good linearity and low limit of detection. Additionally, *p-tert*-butylthiacalix[4]arene tetra-amine (TC4TA) were used as the enzyme immobilization matrix to explore their unique molecule recognition function property.[27] Finally, they concluded that calixarenes may be a charming material for enzyme immobilization owing to GOx was strongly adsorbed on the calixarene modified surface. These results refer to a reality that calixarenes are cup-shaped molecules which can form complexes with a wide range of guest species.[28] Herein, considering the benefits of CPs, calixarene and AuNPs, we have integrated them in a biosensor fabrication to accomplish a good biosensor performance.

In this study, we constructed an amperometric biosensor using SBTz and newly synthesized calixarene derivative with AuNPs to detect of glucose. Elaboration of polymer film was investigated by direct electropolymerization of functionalized monomer, (SBTz), and then the conducting polymer coated surface was modified with a mixture of calixarene and AuNPs to form a better surface for enzyme immobilization. The model enzyme, GOx, was immobilized onto the modified surface using cross linking agent. Carboxylic acids in the top rim of the calixarene were used for selectively capturing the biomolecule through covalent interactions. The formation of covalent bonding between the biomolecule and calixarene form the strongest immobilization procedure. The primary aim of the technique is to prevent leaching of the enzyme from the electrode surface without losing its activity. By this motivation, CPs were used to improve biosensor performance as well as enhance the charge transfer properties, hence the sensitivity of the biosensor can be improved. Moreover, calixarenes have been used for the enzyme immobilization in the fabrication of excellent enzyme biosensors. Calixarenes are a class of cavity shaped with a hydrophilic outer surface and hydrophobic inner cavity that provides better interaction with the enzyme molecule.[20,28] The hydrophobic and hydrophilic features of cavities enable calixarenes to interact with a wide range of guest species. These properties could enhance the enzyme loading and also produce an ideal site for selective bindings with a guest molecule, enzyme *via* covalent bonding. In addition to excellent properties, gold nanoparticles (AuNPs) were used for the specific binding of the biomolecules on the surface because of their large specific area, excellent biocompatibility.[29] The thiol groups serve as binding sites for covalent attachment of calixarene to AuNPs *via* Au–S bond. Considering the importance of the CPs, calixarenes and AuNPs, we investigate the role of these combinations on the characteristics of the biosensor. Overall,



Scheme 1 (1) Electropolymerization of the SBTz monomer. (2) Casting calixarene–gold nanoparticle (calixarene/AuNPs) suspension onto the polymer coated graphite electrode. (3) Immobilization of glucose oxidase (GOx) onto the modified electrode.

poly(SBTz)/calixarene/AuNPs/GOx biosensor is illustrated in Scheme 1. Results of the characteristics and performance of the biosensor were studied in detail. SEM was used for investigate the surface morphology. After optimization of enzyme loading, cycle number, pH, and calixarene amount for biosensor response and analytical properties were determined. Finally, the poly(SBTz)/calixarene/AuNPs/GOx biosensor was tested successfully to detect the glucose content in the real samples.

2. Materials and methods

2.1. Materials

Glucose oxidase (from *Aspergillus niger*, 17 300 units per g solid) and glucose (from Sigma-Aldrich) were used without any purification. Dichloromethane (DCM), acetonitrile (ACN) were purchased from Merck (Darmstadt, Germany). *N*-Hydroxysuccinimide (NHS), *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (EDC) was purchased from Fluka Buchs, Switzerland and Sigma, respectively. A phosphate buffer solution (PBS) (pH 6.5) consisting of 0.05 M Na_2HPO_4 (Fisher Scientific Company) and 0.05 M NaH_2PO_4 (Fischer Scientific Company) was used for amperometric studies. Gold nanoparticles, 10 nm diameter, stabilized suspension in 0.1 mM PBS, was obtained from Sigma-Aldrich. The chemicals used in the synthesis of the monomer were purchased from Sigma-Aldrich.

2.2. Apparatus

Electropolymerization was carried out by a Voltalab 50 potentiostat in three electrode cell hosting a graphite electrode (RingsdorffWerke GmbH, Bonn, Germany, type RW001, 3.05 mm diameter and 13% porosity) as the working electrode. Ag wire as the reference electrode and a platinum wire as the counter electrode were used. All amperometric measurements were performed using Palmsens (Palm Instruments, Houten, The Netherlands). Amperometric analysis measurements were calculated as the average of three measurements and ±SD. Scanning electron microscope (SEM) (JEOL JSM-6400 model) was used for the characterization of the surface characteristics of the biosensor. Structures were proven by nuclear magnetic resonance (NMR) spectra recorded on a Bruker Spectrospin Avance DPX-400 Spectrometer with trimethylsilane (TMS) as the internal reference.

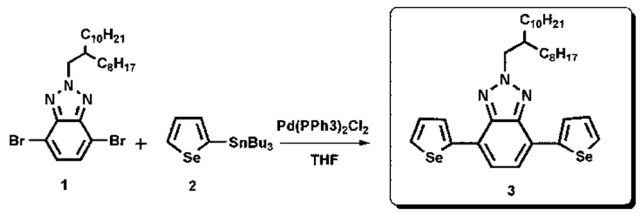
3. Experimental

3.1. Synthesis of 2-(2-octyldodecyl)-4,7-di(selenoph-2-yl)-2*H*-benzo[*d*][1,2,3]triazole (SBTz) (3)

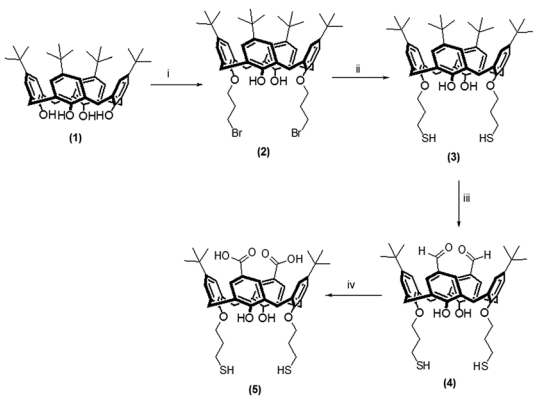
2-(2-Octyldodecyl)-4,7-di(selenoph-2-yl)-2*H*-benzo[*d*][1,2,3]triazole was synthesized according to previously described method.[30] 4,7-Dibromo-2-(2-octyldodecyl)-2*H*-benzo[*d*][1,2,3]-triazole (**1**) and tributyl-(selenophen-2-yl)stannane (**2**) were subjected to Stille coupling reaction to obtain SBTz as a yellow solid (Scheme 2).

3.2. Synthesis of the calixarene

The compounds **1**, **2** and **3** were synthesized by procedures published in the literature.[26,31,32] 5,17-Bis-*tert*-butyl-11,23-dicarboxaldehyde-25,27-dihydroxy-26,28-bis(3-thiol-1-oxypropane)-calix[4]arene (**4**) and 5,17-bis-*tert*-butyl-11,23-dicarboxylic acid-25,27-dihydroxy-26,28-bis(3-thiol-1-oxypropane)-calix[4]arene (**5**) are herein reported for the first time (Scheme 3).



Scheme 2 Synthetic route to SBTz.



Scheme 3 The synthetic route for synthesis of 5,17-bis-*tert*-butyl-11,23-dicarboxylic acid-25,27-dihydroxy-26,28-bis(3-thiol-1-oxypropane)-calix[4]arene (**5**). Reaction conditions: (i) K_2CO_3, CH_3CN, 1,3-dibromopropane; (ii) thiourea, KOH, CH_3CN; (iii) TFA, hexamethylenetetramine; (iv) sulfamic acid, sodium chlorite, $CHCl_3$/acetone.

3.3. Synthesis of 5,17-bis-*tert*-butyl-11,23-dicarboxaldehyde-25,27-dihydroxy-26,28-bis(3-thiol-1-oxypropane)-calix[4]arene (4)

A mixture of **3** (0.3 g, 0.376 mmol) and hexamethylene tetraamine (2.12 g, 15.090 mmol) in TFA (20 mL) was refluxed for 27 h, and added into ice-cold water (60 mL), and extracted with $CHCl_3$ (3 × 40 mL). The combined organic phases were neutralized with water and dried over $MgSO_4$. The filtrate was evaporated; the resultant crude was washed with hexane, and finally dried in oven. The structure of target calixarene was characterized using NMR technique. Yield: 0.15 g (55.6%), m.p.; 262–264 °C.

^{1}H NMR (400 MHz $CDCl_3$): δ 9.71 (s, 2H, –CHO), 7.59 (s, 4H, ArH), 7.14 (s, 4H, ArH), 4.22 (d, 4H, J = 12.8 Hz, Ar–CH_2–Ar), 4.06 (t, 4H, J = 4.8 Hz, O–CH_2–), 3.53 (d, 4H, J = 12.8 Hz, Ar–CH_2–Ar), 3.44–3.46 (m, 4H, –CH_2–S), 3.44–3.45 (m, 4H, –CH_2–), 2.42–2.48 (m, 2H, –SH), δ 1.19 (s, 18H, but). ^{13}C NMR (400 MHz $CDCl_3$): δ 190.79 (C═O), 159.40 (ArO–C), 148.95 (ArO–C), 148.81 (ArC), 132.61 (ArC), 130.97 (ArC), 129.15 (ArC), 128.68 (ArC), 126.53 (ArC), 75.16 (O–CH_2), 34.43 (C), 33.82 (–CH_2), 32.10 (Ar–CH_2–Ar), 31.29 (CH_3), 28.22 (S–CH_2). Anal. calcd for $C_{44}H_{52}O_6S_2$: C, 71.32; H, 7.07; S, 8.65. Found (%); C, 71.29; H, 7.17; S, 8.56.

3.4. 5,17-Bis-*tert*-butyl-11,23-dicarboxylic acid-25,27-dihydroxy-26,28-bis(3-thiol-1-oxypropane)-calix[4]arene (5)

To a solution of **4** (0.034 g, 0.0459 mmol) in acetone (20 mL) were added sulfamic acid (0.01 g, 0.103 mmol) and sodium chlorite (0.005 g, 0.055 mmol). The reaction mixture was stirred for 3 h and concentrated under reduced pressure. The residue was taken up in EtOAc to wash with 1 N HCl (3 × 50 mL) and brine (50 mL), dried over $MgSO_4$ and concentrated by rotary. The crude was recrystallized from MeOH to give final product. Yield: 0.015 g (43%), m.p.; 160–162 °C.

^{1}H NMR (400 MHz DMSO): δ 7.82 (s, 4H, ArH), 7.16 (s, 4H, ArH), 4.08–4.22 (m, 8H, Ar–CH_2–Ar and O–CH_2–), 3.48–3.68 (m, 8H, Ar–CH_2–Ar and CH_2–S), 2.27–2.47 (m, 4H, –CH_2–), 1.99 (brs, 2H, –SH), 1.11 (s, 18H, but). ^{13}C NMR (400 MHz DMSO): δ 167.66 (C═O), 157.56 (ArO–C), 149.95 (ArO–C), 147.86 (ArC), 132.77 (ArC), 130.81 (ArC), 128.45 (ArC), 126.51 (ArC), 121.98 (ArC), 76.20 (O–CH_2), 34.54 (C), 34.11 (–CH_2), 31.53 (Ar–CH_2–Ar), 31.38 (CH_3), 26.52 (S–CH_2). Anal. calcd for $C_{44}H_{52}O_8S_2$: C, 68.37; H, 6.78; S, 8.30. Found (%); C, 68.31; H, 6.67; S, 8.48.

3.5. Preparation and amperometric measurements of the biosensor

First of all, the desired monomer, SBTz, was electrochemically polymerized on the previously cleaned bare graphite electrode, and then washed with distilled water to remove impurities. Electropolymerization of the SBTz was achieved *via* CV by sweeping the potential between 0.0 and 1.5 V in 0.1 M $TBAPF_6$/DCM : ACN at a scan rate of 100 mV s^{-1}.[30] Then, 0.5 mg calixarene was dissolved in 0.1 mL of dimethylformamide (DMF) in an Eppendorf and stirred at room temperature. Calixarene–gold nanoparticle (calixarene/AuNPs) suspension was obtained by mixing calixarene (5 mg mL^{-1}) and AuNPs with 1 : 9 volume ratios. The solution was dropped on a polymer coated electrode surface. The constructed biosensor was allowed at room temperature for one day to achieve covalent attachment between thiol group of calixarene and AuNPs. After that, 1.25 mg GOx was dissolved in 3 μL of 50 mM, pH 7.0 PBS and immobilized on the modified surface using *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (EDC)/*N*-hydroxysuccinimide (NHS) crosslinking agents. EDC and NHS were used to activate the free carboxylic groups of the calixarene backbone.[4] Then, the transducer was allowed to dry for 3 h at room temperature. Before use, electrode was rinsed with distilled water to remove un-bound enzyme molecules and reagents. Hence, poly(SBTz)/calixarene/AuNPs/GOx biosensor was obtained successfully.

Amperometric measurements of the biosensors were carried out at room temperature in the reaction cell containing 10 mL of PBS (50 mM, pH 6.5) at −0.7 V with respect to Ag wire electrode. Under the constant potential, the current change due to enzymatic reaction was measured. After the background current reached a steady state, a certain amount of glucose was injected in the reaction medium and the current change was recorded as the biosensor response. All the experiments were carried out at ambient conditions.

4. Results and discussion

4.1. Optimization studies of the proposed biosensor

The design of enzyme based biosensor is a current research priority to development of novel immobilization matrix without losing the biomolecule activity. For this purpose, we aimed to achieve a long life and robust biosensor for the detection of glucose. Firstly, we designed and synthesized a novel calixarene for a proper attachment of both biomolecule and AuNPs. Moreover, SBTz monomer was used for the improvement of biosensor characteristics. In this study, covalent immobilization technique was used. During the covalent immobilization procedure, the amino groups of the enzyme molecules and carboxylic groups of the calixarene were used. Using EDC/NHS as the cross linking agent; enzyme molecules were stabilized on the electrode surface.

All the parameters affecting the biosensor performance were optimized for a best biosensor performance. After fabrication of poly(SBTz)/calixarene/AuNPs/GOx biosensor, optimization and characterization studies were performed. Firstly, cycle number was optimized to obtain a proper orientation of enzyme onto the electrode surface. To study the impact of the optimum thickness, poly(SBTz) was deposited on the graphite electrode with 15, 30, 45 and 60 scan numbers and their biosensor responses were compared by keeping the other parameters constant. The effect of cycle number on biosensor responses is shown in Fig. 1B. 30 cycle was detected as the optimum value. As shown in the figure, the amperometric response was decreased when cycle number was increased. Increasing of the polymer thickness caused lower charge transfer due to the diffusion problem. In a very thick film, enzyme molecule cannot be oriented on the electrode surface properly.[33] Moreover, decreasing the thickness caused lower amperometric response due to decreased

biomolecule stability on the surface. For 30 cycle polymer film, the charge and film thickness were calculated as 96.12 mC and 149.52 nm, respectively. The effect of enzyme amount was also investigated. To optimized enzyme amount, five different electrodes were prepared with several enzyme amounts ranging from 17.3 U to 34.6 U (1–2 mg). Highest response was obtained for the biosensor with 21.62 U GOx (Fig. 1C). For lower and higher values of optimum value, enzyme molecule is not in a better interaction with the modified surface. Moreover, pH was optimized since working conditions also affects the biosensor performance. To determine the optimum pH of the proposed biosensor, different buffers were prepared in the range of 4.5–8.0. The effect of pH on the biosensor response is shown in Fig. 1A. Optimum value shows the best biosensor response. Also, the optimum value of the study is a good agreement with published studies.[34,35] Furthermore, to enhance the immobilization, calixarene was used together with the conducting polymer since calixarene chemistry is a well known modifying technique to stabilize biomolecules on a surface. In the absence of calixarene a reproducible response could not be obtained. When a biosensor only with poly(SBTz) was constructed, during the immobilization, due to the incompatibility of the enzyme with the hydrophobic polymer pendant alkyl chains, it was hard to fix the enzyme molecules on the polymer coating surface. It was noted that enzyme molecules leached from the electrode surface. In addition, in the absence of AuNPs and calixarene, the biomolecule could not be properly oriented on the electrode surface. Hence the constructed biosensor; poly(SBTz/AuNPs/calixarene/GOx) shows superior properties and gives the best immobilization matrix in sensing the targets. The effect of calixarene on the biosensor performance was investigated by measuring the current responses.

Optimum amount was found as 0.5 mg of calixarene (Fig. 1D). This shows that in higher amounts of calixarene, due to the excessive presence of carboxylic groups, covalent binding is more than enough which brings a certain loss in enzyme activity. On the other hand, a lower amount of calixarene may not create a sufficient environment for enzyme immobilization which results in low signal.

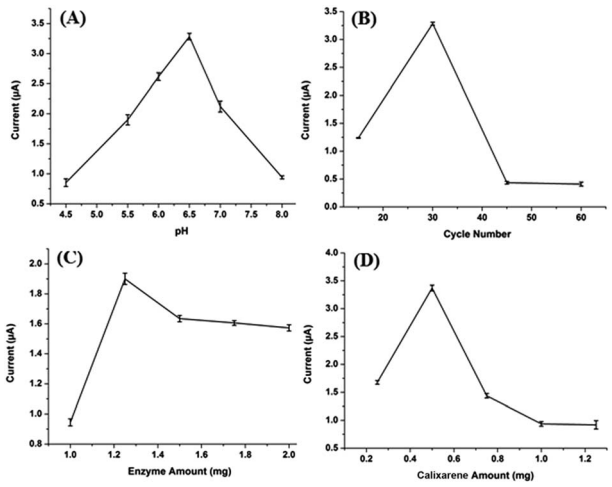


Fig. 1 Effect of pH (in sodium acetate buffer, 50 mM, at pH 4.5, 5.5 and in sodium phosphate buffer, 50 mM, at pH 6.0, 6.5, 7.0 and 8.0, 25 °C, −0.7 V, [glucose]: 0.5 mM) (A), cycle number (B), enzyme and calixarene amount (C and D) (in sodium phosphate buffer, 50 mM, pH 6.5, 25 °C, −0.7 V, [glucose]: 0.5 mM). Error bars show standard deviation of three measurements.

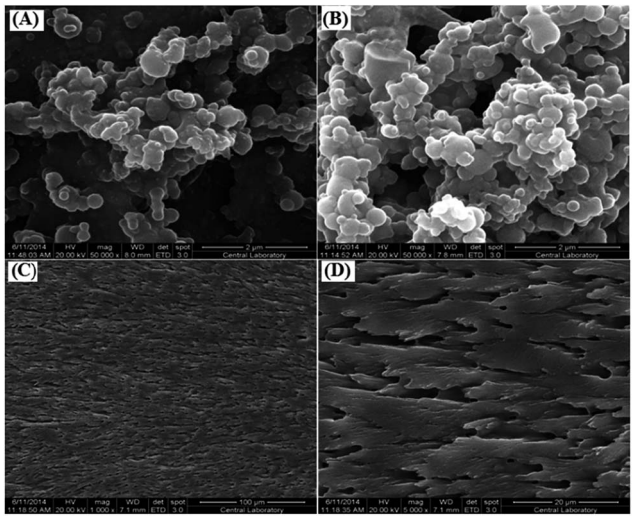


Fig. 2 SEM images of the surface characteristics of (A) pristine conducting polymer, poly(SBTz), (B) poly(SBTz)/calixarene/AuNPs modified electrode, (C) and (D) GOx immobilized poly(SBTz)/calixarene/AuNPs surface with different magnifications.

4.2. Characterization of the modified electrodes

Scanning electron microscopy (SEM) technique is used to monitor the surface properties of the biosensors. Before and after immobilization of biomolecules on the electrode surface, the surface morphology was examined by SEM. Fig. 2 shows SEM images of pristine poly(SBTz), poly(SBTz)/calixarene/AuNPs and poly(SBTz)/calixarene/AuNPs/GOx modified

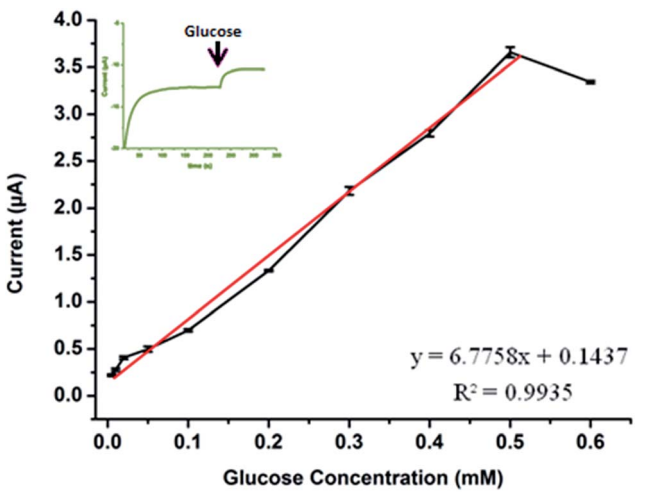


Fig. 3 Calibration curve for glucose (in pH 6.5, 50 mM PBS, 25 °C, −0.7 V). Error bars show the standard deviation of three measurements (a typical amperometric signal was given as inset).

Table 1 Different studies from the literature based on conducting polymer containing glucose biosensors[a]

Biosensors	K_m^{app} (mM)	Linear range (mM)	Sensitivity ($\mu A\ mM^{-1}\ cm^{-2}$)	LOD (mM)	Ref.
Poly(BEDOA-6)/AuNPs/MPA/graphite	0.81	0.025–1.25	14.97	0.025	25
PEDOT/GOD/platinum	NR	0.1–10	12.42	0.130	38
Ru–Py/Nafion/ring–disc	NR	0.01–0.50	15.40	NR	39
P(NMP)/PVS/ITO	12.5	1.0–10	1.40	NR	40
GCE/PSG/Pan/PAA/Nafion/GC	5.04	0.001–2.0	1.05	0.005	41
Au/dithiol/AuNPs/cysteamine/gold	4.30	0.02–5.7	8.80	0.008	42
Teflon/CNT/AuNPs	14.90	0.05–1.0	2600	0.017	43
PVS/PAMAM–AuNPs@CoHCF/ITO	2.03	Up to 1.5	0.033	0.017	44
Poly(SBTz)/calixarene/AuNP/graphite	0.025	0.005–0.50	102	0.004	This study

[a] NR: not reported.

graphite electrodes. In the Fig. 2A, the image was taken after a 30 cycle electropolymerization on graphite electrode. The morphology of the conducting polymer, poly(SBTz) (Fig. 2A), has a homogeneous cauliflower-like structure. In Fig. 2B, after the modification of electrode with calixarene and AuNPs, surface morphology was remarkably changed. This change may be due to the cage structure of calixarene which provides a support for the polymer. Due to the strong interaction and convenient organization of conducting polymer and calixarene molecule, homogenous surface can be achieved. Therefore, constructed surface presents a good platform where enzyme molecules are freely adapted. After biomolecules deposition, apparent change in morphology is a direct result of well immobilization of the enzyme. The homogenous surface was observed due to the interaction between the enzyme and calixarene.

This way provides a high probability for substrate to reach the biolayer. Therefore, the morphology of the proposed biosensors shows significantly different from each other. This refers to the formation of modification and successful immobilization of enzymes.

4.3. Analytical characterization and interference study

Fig. 3 represents the calibration curve for glucose. The linear response interval was found between 0.005 and 0.50 mM. Moreover, the limit of detection (LOD) for glucose was calculated as 0.004 mM in criteria of $S/N = 3$. The value of K_m^{app} was calculated as 0.025 mM using Lineweaver–Burk plot and the sensitivity were calculated as 102 $\mu A\ mM^{-1}\ cm^{-2}$, respectively. The operation stability of poly(SBTz)/calixarene/AuNPs/GOx biosensor was determined by measuring 0.5 mM glucose for 9 times during a period of two hours. The standard deviation (SD) and relative standard deviation (RSD) were calculated as 0.021 and 1.78% respectively. The results indicate good detection reproducibility. Moreover, during the 9 measurements, only 6% activity loss was found in the biosensor response. The constructed biosensor shows extremely wide linear range compared to those reported in previous studies.[25,36] It is reported that Au NPs assembly around GOx brings an environment congruent with the native system where the redox proteins can be oriented freely.[37] The results also verified this situation with long-term stability and amplified signals. Very low K_m^{app} value was observed from those reported in previous studies (Table 1).

The interference studies of poly(SBTz)/calixarene/AuNP/GOx biosensors have been performed on the presence of some compounds such as ascorbic acid and urea. Glucose biosensors can be used to analyze glucose amount in blood samples which is the main purpose of GOx sensors. However, the proposed sensors should not give any response to other biological molecules except glucose. In human blood plasma, the reference range of uric acid and ascorbic acid are between 214–494 μM and 17–66 μM, respectively. According to those limits in the blood, 0.5 mM of urea and ascorbic acid solutions were prepared. For this study, instead of the substrate, ascorbic acid and urea solutions (0.50 mM) were injected into the reaction cell containing phosphate buffer solution (pH 6.5, 50 mM). No

Table 2 Results of determination of glucose content using poly(SBTz)/calixarene/AuNPs/GOx biosensor in beverages

	Glucose content		
Sample	Product label (mM)	Poly(SBTz)/calixarene/AuNPs/GOx biosensor (mM)	Relative error (%)
L® ice tea (peach)	0.217	0.224	−3.23
L® ice tea (lemon)	0.140	0.144	−2.86
U® lemonade	0.389	0.381	2.06
U® pomegranate juice	0.274	0.267	2.55
U® mandarin juice	0.299	0.296	1.00

interference effect was observed in practice. Just after glucose was injected into the reaction mixture, current change was clearly determined whereas ascorbic acid and urea did not change the current. Consequently, the biosensor can be easily used in various applications.

4.4. Detection of glucose content in beverages

The proposed biosensor was used to analyze the glucose in several beverages. The target sample was injected to the reaction cell without pretreatment and the concentration of glucose was calculated using the calibration curve. The comparison of the results obtained from both product label and poly(SBTz)/calixarene/AuNPs/GOx biosensor was shown in Table 2. The results revealed that poly(SBTz)/calixarene/AuNPs/GOx biosensor can be applied for the glucose detection in beverages, successfully. Moreover, the target system can be applied for real time analysis and for the routine analysis; the proposed biosensor is feasible due to advantages such as the simple measurement procedure, short response time, adequate sensitivity and the selectivity.

5. Conclusions

Present work describes a novel poly(SBTz)/calixarene/AuNPs/GOx biosensor for detection of glucose. Firstly, conducting polymer was designed and then a newly synthesized calixarene and AuNPs were used for the improvement of biosensor performance. GOx was immobilized on the modified electrode using EDC/NHS cross linking agents. Covalent bonding technique was used for the construction of the biosensor. Amperometric response was measured as a function of glucose concentration, at a potential of −0.7 V *vs.* Ag wire in a buffer solution (pH 6.5). The surface morphology was investigated by SEM. The proposed biosensor gives the desired kinetic parameters, like low K_m^{app}, large linear range and high sensitivity. After optimization and characterization studies, the poly(SBTz)/calixarene/AuNPs/GOx biosensor was used to determine the glucose in the beverages and the satisfactory results were obtained. The results show that the proposed measurement system is an important tool for real time analyses for the glucose determination.

Acknowledgements

One of us, HB Yildiz, acknowledges the support from Turkish Scientific and Research council through project 113T022.

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