Journal of the Korean Applied Science and Technology
Vol. 36, No. 1. March, 2019. 269~278
ISSN 1225-9098 (Print)
ISSN 2288-1069 (Online)
http://dx.doi.org/10.12925/jkocs.2019.36.1.269

Fabrication of enzymatic biosensor based on the poly(3-thiophenecarboxylic acid-*co*-thiophene) polymer as electron-transfer materials

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(Received January 28, 2019; Revised March 27, 2019; Accepted March 29, 2019)

Abstract : We fabricated glucose oxidase (GOx)-modified biosensor for detection of glucose by physical immobilization of GOx after electrochemical polymerization of the conductive mixture monomers of the 3-thiophenecarboxylic acid (TCA) and thiophene (Th) onto ITO electrode in this study. We confirmed the successfully fabrication of GOx-modified biosensor via FT-IR spectroscopy, SEM, contact angle, and cyclic voltammetry. The fabricated biosensor has the detection limit of 0.1 μM, the linearity of 0.001-27 mM, and sensitivity of 38.75 $mAM^{-1}cm^{-2}$, respectively. The fabricated biosensor exhibits high interference effects to dopamine, ascorbic acid, and L-cysteine, respectively. From these results, the fabricated GOx-modified biosensor with long linearity and high sensitivity could be used as glucose sensor in human blood sample.

Keywords : Immobilization of glucose oxidase, Electrochemical polymerization, 3-thiophenecarboxylic acid;thiophene, Electron transfer, Human blood sample

1. Introduction

Blood glucose level is usually used as a clinical indicator of diabetes, which is a global health problem with vicious social and economic impact [1]. Moreover, the determination of glucose concentration is also very important fact in food processing and fermentation. An electrochemical biosensor is a potential tool and more suitable for point of care devices than normal conventional methods including spectrophotometry [2], HPLC [3], fluorescence [4] and chemiluminescence [5]. Glucose oxidase (GOx) modified electrodes have played a major role in highly selective blood sugar testing by electrochemical method. GOx is one of the most extensively researched enzymes for the fabrication of enzyme-based glucose biosensors. Because it contains two flavin adenine dinucleotide cofactors as a redox center [6], direct electron transfers between redox enzymes and the surface of electrodes can be used to investigate enzyme-catalyzed reactions in biological systems and electrochemical basis for the study of the structure, kinetics and thermodynamics of

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redox transformations of enzymemolecules and metabolic processes involving redox transformations [7-9].

On the other hand, the conducting polymers as electron transfer and immobilization site of enzyme were electrochemically synthesized through the anodic oxidation of appropriate electroactive functional monomers or by cathodic reduction respectively. Electrochemical synthesis of conducting polymers was a very important method as a result of its simplicity, cost-effectiveness and the performance of the process in a single section glass cell. Most often, the potential of monomer oxidation directing to polymerization was higher than that of charging of oligomeric intermediates polymer. A simplified means of electro polymerization of an electro active monomer, such as pyrrole or thiophene engage alternate chemical and electrode reaction steps [10].

Poly(thiophene) was a conducting polymer which is used as electrical super capacitor [11], non-linear optics [12], PLEDs [13], eletrochromics [14], photoresists [15], antistatic coatings [16], sensors [17], batteries [18], electromagnet shielding materials [19], solar cells [20], memory devices [21], transistors and imaging materials [22]. However, the poly (thiophene) without functional group could not be applied as electron transfer in biosensor field because enzymes could not be immobilizing onto the conductive poly (thiophene).

In this study, we firstly introduced carboxylic acid (-COOH) onto the ITO electrode surface by electrochemical copolymerization of thiophene and 3-thiophenecarboxylic acid, and the GOx subsequently was immobilized onto the -COOH-modified ITO electrode via physical immobilization. The fabricated GOx-modified biosensor was confirmed the successfully fabrication via FT-IR spectroscopy, SEM, AFM, contact angle, and cyclic voltammetry. The fabricated GOx-modified biosensor was determined the sensing range, detection limit, sensitivity to glucose using chronoamperometry method. Furthermore, we also examined the interference effect determination of the fabricated GOx-modified biosensor to dopamine, ascorbic acid, and L-cysteine, respectively.

2. Experiments

2.1. Reagents

Thiophene (Th), Tetrabutylammoniumtetra-fluoroborate, $[NCH_3CH_2CH_2CH_2]^+[BF_4]^-$, glucose oxidase (GOx), dopamine, ascorbic acid, L-cysteine, sodium phosphate monobasic dihydrate, sodium phosphate dibasic dihydrate, and sodium hydroxide (NaOH) was purchased from Sigma-Aldrich (St. Louis, MO, USA). 3-Thiopheneacetic acid (TCA) was also obtained from Thermo Fisher Scientific Inc. (Incheon, Korea). ITO electrode (<10 Ω cm) was purchased from DHSLKOREA.com (Seoul, Korea). All other chemicals were of analytical grade. Water was purified using a Millipore purification system (Millipore Corporation, Bedford, MA, USA).

2.2. Instrumentation

Cyclic Voltametry (CV, VersaSTAT 3 PotentiostatGalvanostat, AMETEK PAR, U.S.A.) was performed with a conventional three-electrode system such as working electrode, a platinum wire as counter electrode, and an Ag/AgCl (saturated KCl) reference electrode. Surface properties were characterized by scanning electron microscopy (FE-SEM (S-4800), Hitachi, Tokyo, Japan), contact angle (PHOENIX-300, Surface Electro Optics, Suwon, South Korea), and FT-IR (FTS-175C, BioRadLabroatories, Inc., USA).

2.3. Fabrication of GOx-modified biosensor after electrochemical copolymerization

Fig. 1 shows the fabrication of GOx-modified biosensor after electrochemical polymerization

of the conductive thiophene derivatives. In detail, electrochemical polymerization was performed using CV system with ITO electrode as working electrode, Pt counter electrode, and Ag/Ag+ reference electrode. The feed solution was prepared by adding of thiophene (0.65mmol) and 3-thiopheneacetic acid (0.65 mmole) in acetonitrile electrolyte (65mL) with $NBu_4^+BF_4^-$ (0.65mol). After then the electrochemical polymerization was performed at scan rate 75mV/S for 20 cycles in feed solution. The detail experimental condition was shown in Table 1. In order to immobilize GOx, we selected poly(TCA-co-Th)-modified electrode because this electrode have carboxylic acid group as binding site with enzyme (see, Fig. 1-c). GOx (3.00mg/mL) was dissolved in PBS solution (pH=7.4), and then the GOx solution (20 ㎖) was dropped onto the surface of poly(TCA-*co*-Th)-modified electrode. After then the GOx-modified samples were dried at 4 ℃ for 24 hrs. The GOx-modified

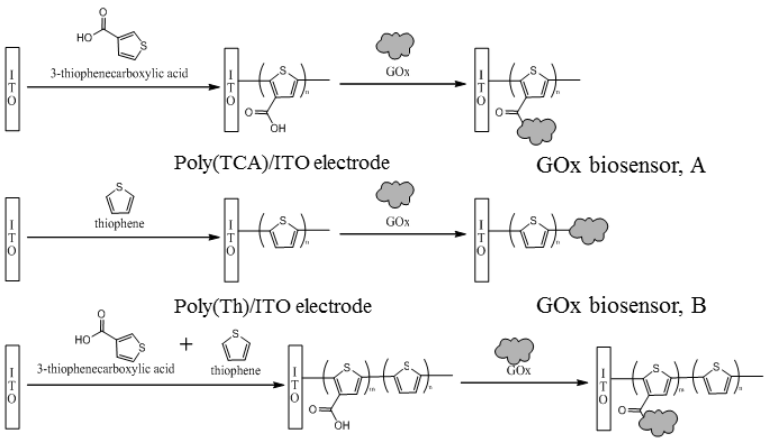

Fig. 1. Preparation of GOx-midified biosensor based on poly(3-thiophenecarboxylic acid, TCA), poly(thiophene, TCA), and poly(TCA-*co*-Th).

Table 1. Electrochemical polymerization condition of the TCA, Th, and the mixture of TCA/Th [a)]

No.	TCA	Th	Physical Properties
1	20 mM	-	Contact angle: 47° / FT-IR peaks: C=O, C=C
2	-	20 mM	Contact angle: 40.1° / FT-IR peaks: C=C
3	15 mM	5 mM	Contact angle: 50.1°
4	10 mM	10 mM	Contact angle: 38.2° / thickness: 119 nm from SEM analysis / FT-IR peak: C=O, C=C
5	5 mM	15 mM	Contact angle: 54.5° / FT-IR peaks: C=O, C=C

a) Electrolyte: acetonitrile with tetrabutylammonium tetrafluoroborate, $[N(Bu)_4]^+[BF_4]^-$.

biosensor could be reacted with glucose as following reaction mechanism:

3. Results and Discussion

Fig. 2 exhibits the electrochemical polymerization of 20mM 3-TCA (No. 1), 20mM Th (No. 2), and the mixture of 20mM 3-TCA/Th (various mole ratio, see No. 3, 4, 5 in Table 1) in acetonitrile with 10mM $[NCH_3CH_2CH_2CH_2]^+[BF_4]^-$, as electrolyte at scan rate 75mV/s. In oxidation peak (E_{OX}) and reduction peak (E_{RE}) of 3-TCA (No. 1) was not observed during 20 cycles. The current values were also observed no change during 20 cycles. These results means that the electrochemical polymerization of 3-TCA was not occurred because the carboxylic acid group may be used as radical scavenger which is formed oxidation during electrochemical polymerization (see, the radical formation in Fig. 3). However, in Fig. 2-b, the E_{ox} of Th is dramatically increased, while the E_{RE} was also remarkably decreased. The current value was reached the maximum value to 20 cycles. From these results means the electrochemical polymerization was successfully obtained in Th. In order to introduce carboxylic acid as reaction site, the mixture solution of 3-TCA/Th (No. 3, 4, 5) was performed in acetonitrile with 10mM $[NCH_3CH_2CH_2CH_2]^+[BF_4]^-$ as electrolyte at scan rate 75mV/s. The maximum current values of the mixture solution with 3-TCA/Th (No. 4) were obtained on 20 cycles. This is indicated that the electrochemical polymerization of the mixture solution with 3-TCA/Th was successfully occurred in acetonitrile electrolyte. In other words, we could the carboxylic acid as reaction site was successfully introduced onto the conducting polymer during electrochemical polymerization. Fig. 3 exhibits the electrochemical polymerization mechanism of thiophene derivatives in acetonitrile with 10mM $[NCH_3CH_2CH_2CH_2]^+[BF_4]^-$ as electrolyte at scan rate 75mV/s.

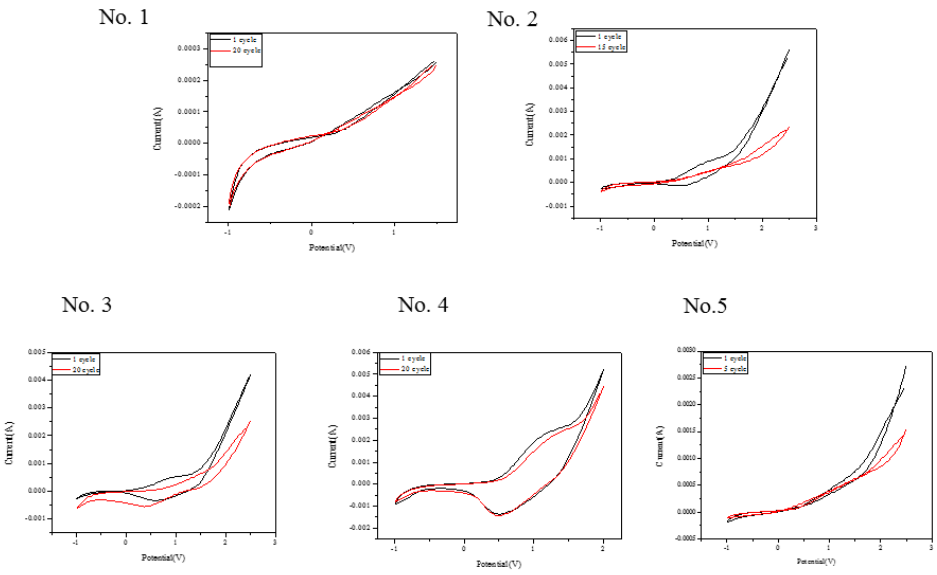

Fig. 2. Electrochemical polymerization of the TCA, Th and the mixture of TCA/Th in acetonitrile with 10mM$[N(Bu)_4]^+[BF_4]^-$ at scan rate 0.075V/S(see, Table 1).

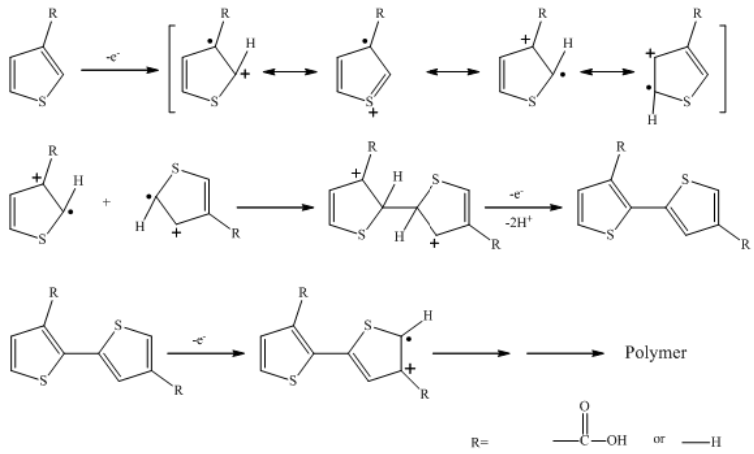

Fig. 3. Electrochemical polymerization mechanism of thiophene derivatives in acetonitrile with 10mM $[NCH_3CH_2CH_2CH_2]^+[BF_4]^-$ as electrolyte at scan rate 75mV/s.

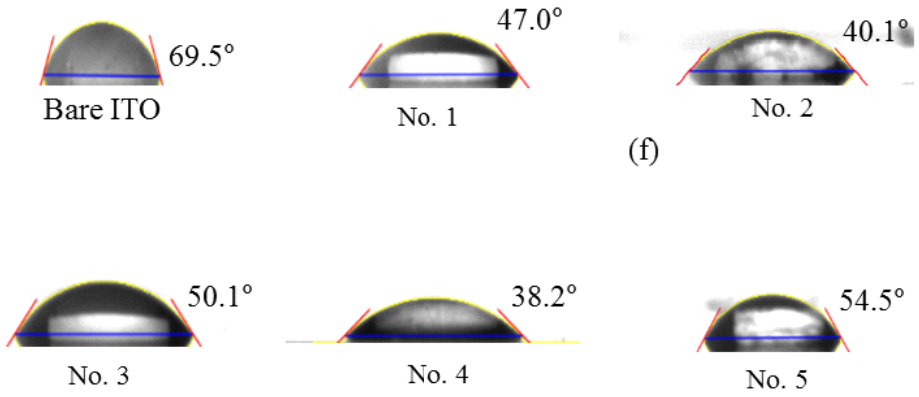

Fig. 4. Contact angles of bare ITO, No. 1, 2, 3, 4 and 5 (see, Table 1).

The contact angles (Fig. 4) of the bare ITO electrode, No. 1, 2, 3, 4, and 5 electrodewere determined at 69.7° , 47.0° , 41.0° ,50.0° , 38.2° and 54.0° respectively. The lower contact angle of the polymer-modified electrode compared to bare ITO electrode because of hydrophilic properties due to the coated conductive polymer. The lowestcontact angles of TCA-modified ITO electrode (No. 1) in spite of no electrochemical polymerization of TCA (see, Fig. 2), was also observed compared to that of the bare ITO electrode surface. This may be considered that TCA was slightly coated as monomer form onto the surface of ITO electrode. The maximum lower contact angle was appeared the poly(TCA-*co*-Th)-modified ITO electrode (No. 4 in Table 1).

Fig. 5 shows the FT-IR spectra of the No. 1, No, 2 and No. 4 electrode (see. Table 1), respectively. The characteristic peaks of carbonyl (>C=O) was observed about 1730

cm^{-1} due to carboxylic acid as shown in Fig. 5. The ring stretch was observed about 1500 cm^{-1} in No. 1,2, and 4 electrodes. From these results, the poly(TCA-*co*-Th)-modified electrode (No. 4) was successfully prepared via electrochemical copolymerization of the mixture solution of 3-TCA/Th (see, Table 1).

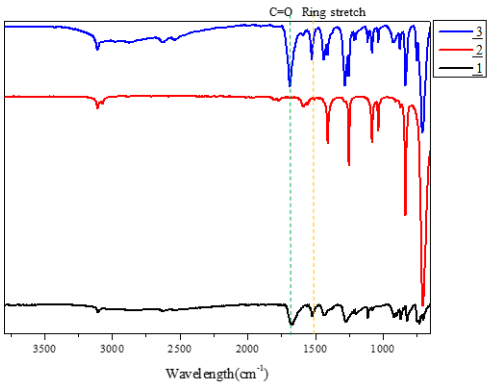

Fig. 5. FT-IR spectra of No. 1, No. 2 and No. 4 (see, Table 1).

Fig. 6 exhibits cross-section SEM images of the ITO electrode and after electrochemical copolymerization of the mixture of 3-TCA/Th (No. 4). As you can see the amorphous polymer film (thickness 119nm) onto the surface of electrode was observed. This means that the poly(TCA-*co*-Th)-modified ITO electrode was successfully prepared via electrochemical polymerization of the mixture of TCA and Th.

In order to conform the successfully preparation of the conductive polymer-modified ITO electrode, we also characterized the prepared electrode via measurement of Fe(II)/Fe(III) redox peaks in 0.1M 0.1MKCl electrolyte. Fig. 7 exhibits the cyclic voltammograms of 1.0 mM $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$ (1:1 ratio) using the prepared electrodes in 0.1M KCl electrolyte. The redox peaks of $Fe^{2+/3+}$ was appeared at 0.45V and 0.35V, respectively. The current value after electrochemical copolymerization of the mixture of 3-TCA/Th (No. 3,4,5) was slightly increased. From this results, we confirmed the successfully preparation of the poly (TCA-co-Th)-modified ITO electrode using electrochemical copolymerization.

We selected poly(TCA-co-Th)-modified ITO electrode in order to immobilize GOx. Fig. 8 exhibits cyclic voltammograms of glucose in 0.1 M PBS (pH=6.8) using GOx-modified biosensor at a scan rate of 100 mV/s. The oxidation peaks of H_2O_2 which is produced by reaction of GOx and glucose as described above, was observed at -0.175V by the fabricated GOx-modified biosensor. The current value was increased with increasing glucose concentration. From these results, the fabricated GOx-modified biosensor could be applied as sensor to detect glucose concentration.

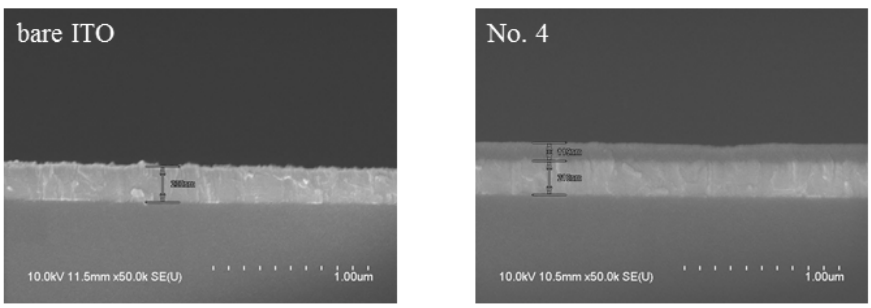

Fig. 6. Cross-section SEM images of poly(TCA-*co*-Th)/ITO electrode (see, Table 1).

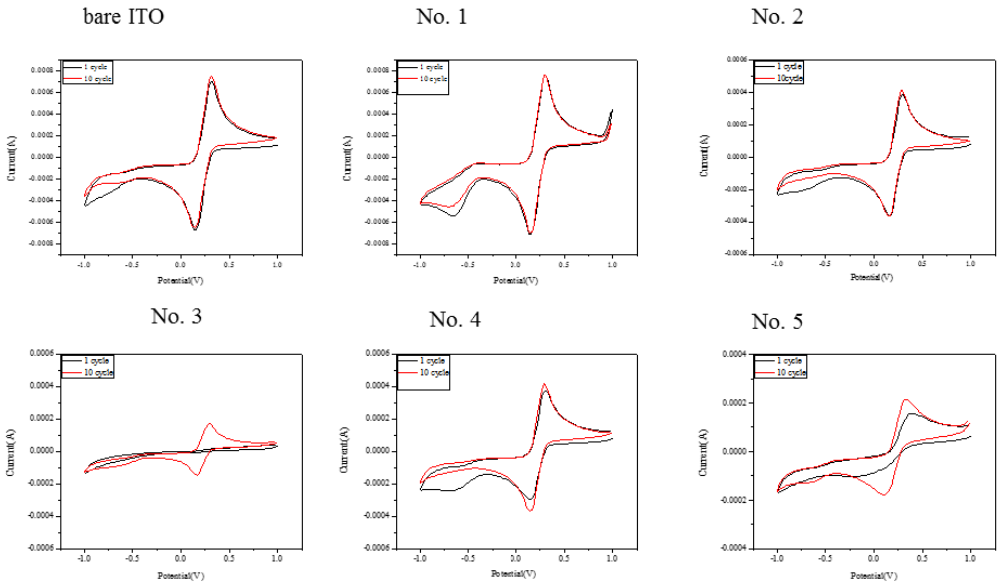

Fig. 7. Cyclic voltammograms of 1mM $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$ (1/1, mol-%) using poly(TCA), poly(Th), and poly(TCA-co-Th)-modified ITO electrode in 0.1M KCl.

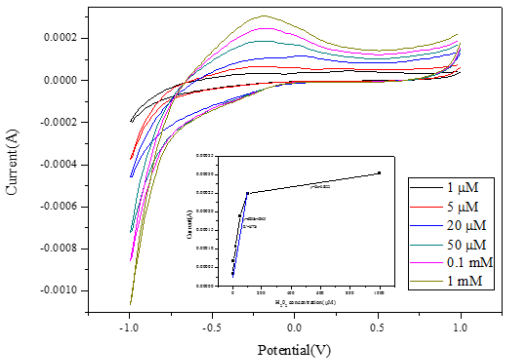

Fig. 8. Cyclic voltammograms of glucose in 0.1 M PBS (pH 6.8) using GOx-modified biosensor (No. 4) at a scan rate of 100 mV/s.

Fig. 9 exhibits the current-time curve of the fabricated GOx-modified biosensor upon successive addition of 1 mM glucose into PBS 0.1M solution (pH 7.4). at -0.175V. The current reached a steady state within 5s after adding glucose, which demonstrates a good electrocatalytic activity of the fabricated biosensor. A wide linear relationship in the range from 0.001mM to 27 mM, and a low detection limit of 0.1μM were also obtained. The sensitivity of the fabricated biosensor was calculated to be 38.75 $mAM^{-1}cm^{-2}$. These results suggest that the GOx-modified biosensor has a good performance for glucose sensing.

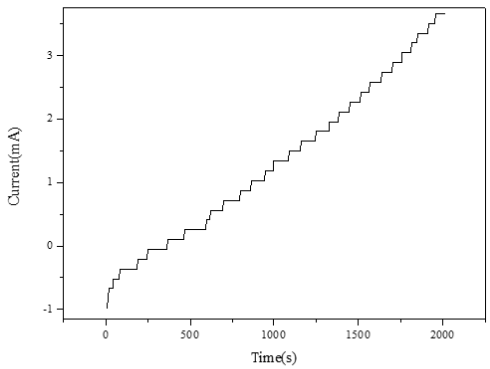

Fig. 9. Chronoamperometry electrochemical response according to glucose concentration using GOx biosensor (No. 5) in 0.1 M PBS solution at a -0.175 V potential.

In order to know the selectivity of the fabricated biosensor, we selected three chemicals such as dopamine, ascorbix acid and L-cysteine. After then weinvestigatedthe glucose concentration in the presence of the mentioned above chemical, respectively, using GOx-modified biosensor. As shown in Table 2, the relative response value was obtained. No significant increase of current was obtained (less than 10%), which indicates a high selectivity of the GOx-modified biosensor.

Table 2. Interference effect of various compounds on the assay of glucose using GOx-modified biosensor (No. 4)

Interferons	Relative response (%)
Dopamine	108.13
Ascorbic acid	105.41
L-Cysteine	106.29

$$\text{Relative response (\%)} = \frac{\text{Intensity in the mixture of glucose and interferent}}{\text{intensity in the glucose}} \times 100\%$$

Table 3 listed the reported GOx-modified biosensor and the prepared GOx-modified biosensor prepared in this study. The GOx-modified biosensor in this study exhibits a widest linear range (0.001 mM-27 mM) and a lowest detection limit (0.1 μM) among these biosensors. A remarkably high sensitivity of the GOx-modified biosensor was also observed.

4. Conclusions

In this study, we prepared a GOx-modified biosensor by immobilization of GOx after electrochemical copolymerization of TCA/Th mixture monomers on the surface of ITO electrode. After then, the GOx-modified biosensor was characterized via FT-IR spectroscopy, SEM, contact angle, and cyclic voltammetry in order to know the successfully fabrication. From these results, we concluded as follows:

(1) The fabricated biosensor has the detection limit of 0.1 μM, the linearity of 0.001-27 mM, and sensitivity of 38.75 $mAM^{-1}cm^{-2}$, respectively.

Table 3. Comparison of the reported glucose biosensors

Electrode	Linearity (mM)	Sensitivity (mA M^{-1} cm^{-2})	Detection limit (μM)	References
GOx/Pt/FGS/chitosan/GCE	Sub 0.001-5	117.89	0.6	[23]
GOx/BSA/PtNP-SWCNT	0.04-0.87	4.54	40	[24]
MSN-PtNP-GOx	0.001-26	4.35	0.8	[25]
MS/Pt-DENs/GOx	0.02-10	15.7	4	[26]
GOx/Pt/OMC/Au	0.05-3.7	12	50	[27]
ITO/poly(Th-*co*-TCA)/GOx	**0.001-27**	**38.75**	**0.1**	**This study**

(2) The fabricated biosensor exhibits high interference effects to dopamine, ascorbic acid, and L-cysteine, respectively.

(3) The fabricated GOx-modified biosensor could be used as glucose sensor in human blood sample.

Acknowledgments

This work was supported by the Hannam University Research Fund (2018).

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