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Enzyme biosensor based on an N-doped activated carbon fiber electrode prepared by a thermal solid-state reaction

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ABSTRACT

The sensitivity of a biosensor electrode was increased by introducing hydrophilic N-groups onto the surface of a polyacrylonitrile (PAN) based activated carbon fiber. The electrospun carbon fiber was activated using KOH to improve the adsorption of glucose oxidase (GOx) enzymes through pore production and the introduction of oxygen functional groups. The activated carbon fibers (ACFs) were then reacted with urea to increase their hydrophilicity by doping their nitrogen groups. The sensor sensitivity and the Michaelis-Menten constant, K_m, were altered by varying the percentages of functional groups on the electrode surfaces. Whereas the value of K_m was affected by the kind of functional groups, the sensitivity of the biosensor electrode was chiefly affected by the amount of functional groups introduced urea modification because the enzyme was better immobilized onto urea-modified activated carbon fibers. Quantitatively, the sensitivity was two- to three-fold higher for the biosensors based on urea-treated ACFs than those based on untreated ones. This increased sensitivity is attributed to the nitrogen and oxygen functional groups on the urea-modified ACFs.

1. Introduction

Glucose biosensor design is an active area of research that aims to enhance sensor performance, which is critical in clinical applications such as diabetes testing and in the food industry for quality control and the detection of hazardous contaminants [1]. Due to the large demand for glucose biosensors in various fields, many glucose biosensors are fabricated, and improvements to glucose detection methods using biosensors are actively pursued. The detection method is used to analyze glucose concentrations during one-time blood sugar testing or during continuous real-time glucose monitoring [2]. Generally, the human body regulates blood glucose levels within the range of 4–8 mM (70–120 mg dL^{-1}). Therefore, glucose biosensors are designed to detect blood glucose levels of 2–30 mM (30–500 mg dL^{-1}) [3,4].

Biosensors rely on electrochemical transduction, employing glucose oxidase (GOx) or glucose dehydrogenase (GDH) enzymes and the measuring materials appropriate for the analyzed matrices. Electrochemical biosensors are based on the entrapment of these enzymes in polymer matrices or membranes deposited on a metal or carbon working electrode, which is utilized as the transducer, and a mediator, which is utilized as the medium that transfers the electrons from the reduced enzyme to the electrode. The mediator has the several functions: One is the direct electrical communication between the enzyme and the electrode surface. The other function is to reduce any re-oxidation at the electrode. The mediator also regenerates its oxidized form [5–8].

Carbon nano-materials are used as substrates in the adsorption of biomaterials because they allow for the loading of high quantities of enzymes and for the sensing of electrical signals in a microenvironment. In addition, these materials can be incorporated for rapid electron transfer and enhanced sensitivities. Carbon nanomaterials have several forms including graphite, activated carbon fiber, and carbon nanotubes, all of which have been studied for use as biosensor electrodes [7–12].

However, carbon nano-materials have a weakness that prevents their wide use as biosensors; the surface of the carbon nanomaterials is composed of hydrophobic elements, but the biosensor must operate in a hydrophilic environment. The poor interfacial affinity between the surface of hydrophobic carbon nanomaterials and the hydrophilic biomaterial results in low sensitivity due to decreased electron transfer. Thus, the hydrophilic properties of the carbon surface must be enhanced by doping it with hydrophilic groups [13–15]. The doping elements include various heteroatoms (N, B, and P) that network with the carbon materials. Doping with these heteroatoms has been found to enhance the electrical conductivity

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and electrode biomaterial interaction of carbon nano-materials by increasing their charge-transfer ability and surface hydrophilicity, respectively [16,17].

In this study, biosensor electrodes were prepared by using N-doped activated carbon fibers (ACFs) based on polyacrylonitrile (PAN). The prepared carbon fiber was activated using KOH to increase the quantity of immobilized glucose oxidase enzyme. To increase the sensitivity of the biosensor, nitrogen groups were doped onto the surface of the activated carbon fiber by urea treatment. The amperometric and the current-voltage curve methods were utilized to examine the effects of the conductivity, and interfacial affinity at the contact area between the N-doped activated carbon electrode and glucose oxidase (GOx) on the glucose-sensing ability.

2. Experimental

2.1. Materials

The polymer and the solvent for the electrospinning solution were PAN (polyacrylonitrile, Aldrich, San Louis, MO, USA) and DMF (N,N-dimethyl formamide, 99.0%, Samchun, Korea), respectively. Potassium hydroxide (KOH, 95.0%, Samchun, Korea) was used to produce pores and introduce oxygen groups onto the carbon surface. Urea (H_2NCONH_2, 99.0–100.5%, ACS reagent) was used as a reacting agent to produce amine groups onto the carbon surface. Glucose (D-(+)-glucose, ACS reagent, San Louis, MO, USA) was prepared in a stock solution that was further used for glucose sensing analysis. Glucose oxidase (GOx, Type X-S, *Aspergillus niger*, 211 U mg^{-1}, San Louis, MO, USA) was used as an enzyme. Potassium hexacyanoferrate ($K_4[Fe(CN)_6]$, 98.5–102.0%, ACS reagent, San Louis, MO, USA) was used as the electron-transfer mediator between the GOx and the electrode. A-screen-printed electrode (SPE, Pine Research Ins., Canada,) was used as the sensing electrode to load the prepared glucose-sensing material for the electrochemical measurements.

2.2. Methods

2.2.1. Preparation of the PAN based fibers by electrospinning

The 10-wt% PAN solution was prepared by DMF and then placed into a 30-cm^3 syringe with a capillary tip (18 G; inner diameter: 1.27 mm). The PAN solution was electrospun using an apparatus equipped with a power supply (NT-PS-25 K, NTSEE Co, Korea). The experimental conditions, such as the concentration of the solution, the voltage, the TCD (tip-to-collector distance), and the pump rate of the syringe, were those previously established by our research group and others for manufacturing PAN-based carbon fibers by electro-spinning [18,19]; thus, the following conditions were applied: an applied voltage of 15 kV, a TCD of 10 cm, a syringe pump rate of 1.5 mL h^{-1}, and a collector speed of 200 rpm [20].

2.2.2. Preparation of activated carbon fiber

The electrospun fiber prepared was stabilized at 260 °C for 4 h in an air atmosphere. To carbonize these stabilized electrospun fibers, they were thermally treated at 1050 °C for 1 h in a nitrogen atmosphere at a heating rate of 5 °C min^{-1}. After stabilization and carbonization, the PAN-based carbon fiber was then activated with 6 M KOH solution as a chemical activation agent to produce mesopores [18,21]. The carbon fiber was immersed in a KOH solution (20 mL/g carbon fiber) for 4 h, with shaking. The immersed sample was placed in an alumina boat and activated in a 750 °C furnace for 3 h in a nitrogen atmosphere. The heating rate was 5 °C min^{-1} and the feed rate of the nitrogen gas was 50 mL min^{-1}. After chemical activation, the resulting sample was washed several times with distilled water until the filtered wash water had a neutral pH. The washed samples were then dried at 110 °C for 1 h [22].

2.2.3. N-doping of the surface of activated carbon fiber

N-doped ACF was prepared by a thermal solid-state reaction method. In a typical experiment, 1 g of as-prepared samples were first finely milled with a different g of urea (1, 3, 5, and 8 g) and then transferred onto an alumina boat. The boat with mixture was placed in the center of a furnace under flowing N_2. After flushing the tube with N_2 for about 30 min, the furnace was heated up to 200 °C at a rate of 5 °C min^{-1} and kept at the temperature for 2 h. After that, the furnace was cooled down to room temperature. The N-doped ACFs was washed with deionized water to remove any residual species adsorbed on the sample surface, and then dried at 110 °C for 1 h [23]. The samples for the sensing electrode were prepared with these ACF: urea weight ratios of 1:1, 1:3, 1:5, and 1:8, which are hereafter denoted as 1U-ACF, 3U-ACF, 5U-ACF, and 8U-ACF, respectively. The weight ratios of prepared electrode were decided by basic experimental results obtained in our laboratory and referencing reported paper [9].

2.2.4. Preparation of the glucose sensor electrode

The final product, which consisted of N-doped ACF (10 mg), was mixed with a GOx solution (4 mg mL^{-1} in 1 mL double-distilled water) by vortexing for 10 s and was stored for 24 h to immobilize the enzyme on the carbon surface. This ACF/GOx mixture (6 μL) was dropped by micropipette onto a working electrode with an area of 4 mm × 5 mm in the prepared SPE. The mixture-loaded SPE was then dried at room temperature for 1 h. This SPE (enzyme electrode) was rinsed with double distilled water to remove unbound enzyme molecules and it was stored at 4 °C for overnight. Hence, this modified electrode was successfully achieved.

2.2.5. Surface analysis of the samples and glucose-sensing measurement

X-ray photoelectron spectra (XPS) were obtained using a MultiLab 2000 spectrometer (Thermo Electron Corporation, UK) and were used to identify the elements present on all sample surfaces.

Cyclic voltammetry (CV) and amperometric studies were performed in a reaction cell containing 30 mL PBS buffer solution (0.1 M, pH 7.0). The glucose-sensing measurements using the N-doped ACFs were processed by cyclic voltammetry (CV) and amperometric methods using Ivium CompactStat (The Netherlands) potentiostat in a reaction cell equipped with three electrodes consisting of N-doped ACFs as the working electrode, platinum electrode as the counter electrode and Ag/AgCl electrode as the reference electrode [24]. The sensitivities of the electrodes prepared with various quantities of N-doped ACFs were measured at glucose concentrations ranging from 0 to 30 mM at pH 7.0. Measurement of amperometric analyses were calculated as an average of five measurements and standard derivations were given as ±SD. All experiments were carried out at ambient conditions.

3. Results and discussion

3.1. The surface element of the N-doped carbon fiber

An XPS elemental analysis was performed to investigate the number of O- and N-containing functional groups on the urea-treated ACF surface. The survey data and deconvoluted C1s and N1s peaks are presented in Figs. 1, 2 and 3, respectively. These results, including the atomic ratio of each element on the surface, are listed in Tables 1 and 2. Fig. 1 provides the XPS survey graphs of both the raw (RACF) and N-doped ACFs (U-ACF), which exhibit distinct carbon and oxygen peaks at approximately 284.5 and 531.0 eV.

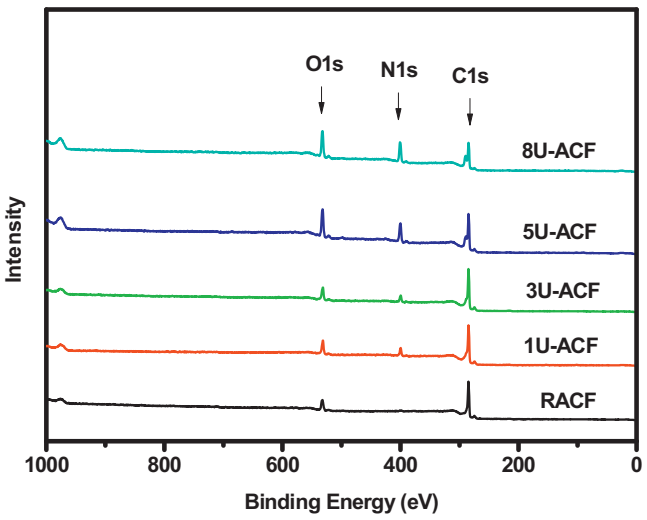

Fig. 1. Elemental-survey data for the RACF and U-ACFs.

Table 1
The elemental surface analysis of the samples used in this study.

Samples	Elemental content (at.%)			O/C (%)	N/C (%)
	C1s	O1s	N1s		
RACF	88.7	11.21		12.64	
1U-ACF	85.98	8.02	6.00	9.33	6.98
3U-ACF	82.50	9.94	7.56	12.05	9.16
5U-ACF	67.46	15.9	16.64	23.57	24.67
8U-ACF	61.15	17.76	21.09	29.04	34.49

A nitrogen peak was also found at approximately 399.7 eV on the U-RACF. In general, the elemental release of nitrogen and oxygen from the ACF occurs at the basal plane during pre-carbonization at temperatures of 550–700 °C. Dehydrogenation takes place at temperatures below 600 °C, and denitrogenation occurs at temperatures above 600 °C evolving gases such as HCN, NH_3 and N_2 [25]. Therefore, the majority of the nitrogen at the basal plane should be removed above 1050 °C. Thus, the N1s peak of the PAN-based RACF is unconfirmed in this study.

The reaction temperature of the ACF with urea was fixed at 200 °C because most nitrogen is doped on the ACF surface at that temperature. Mou et al. demonstrated that the nitrogen ratio

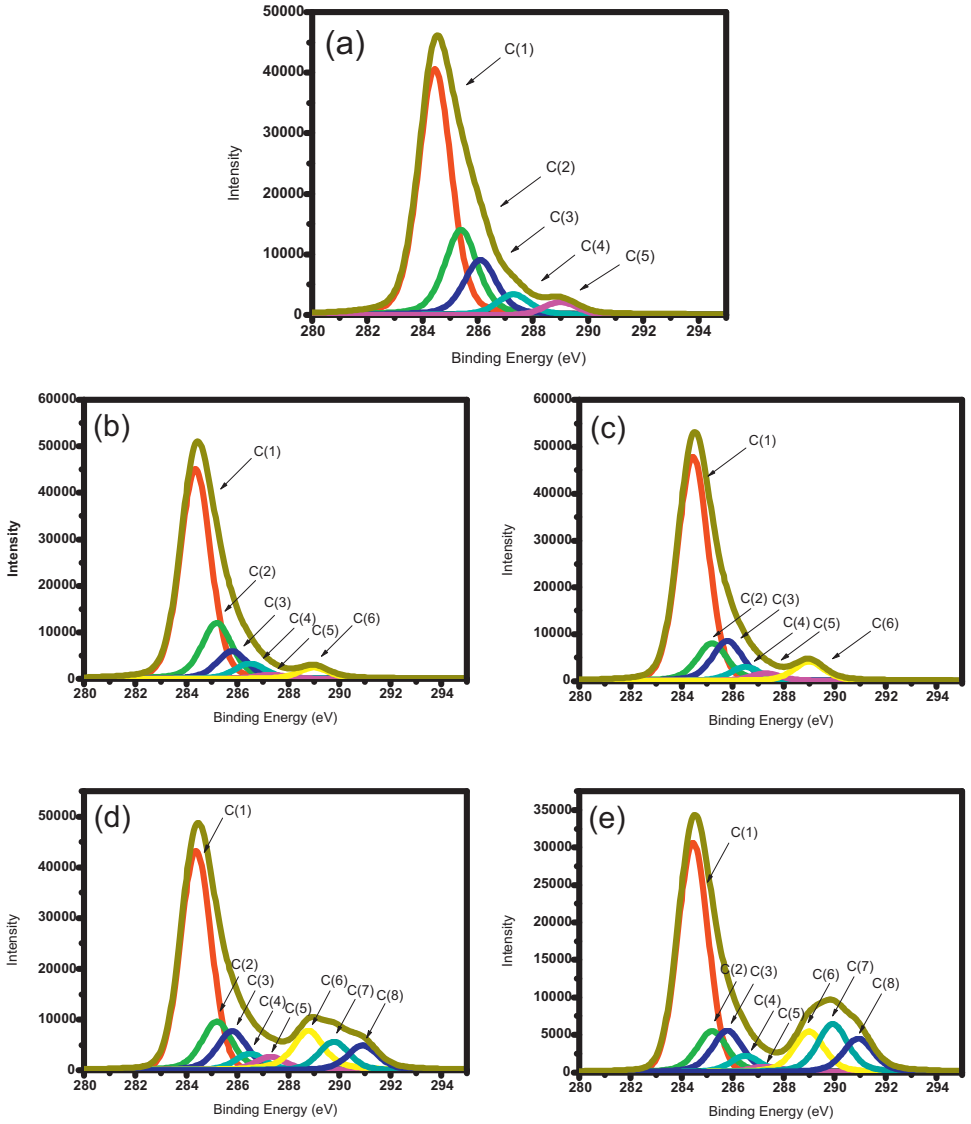

Fig. 2. The C1s X-ray spectra of the RACF (a), 1U-ACF (b), 3U-ACF (c), 5U-ACF (d), and 8U-ACF (e).

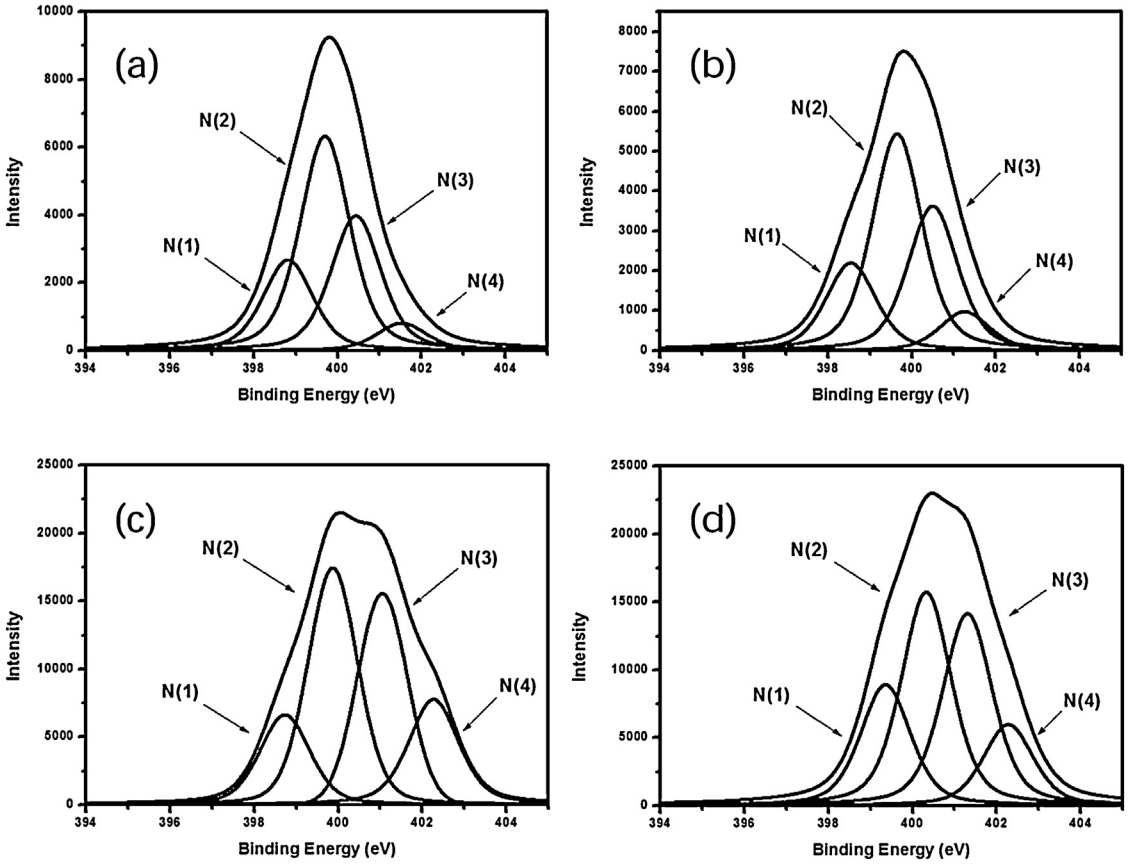

Fig. 3. The N1s X-ray spectra of the 1U-ACF (a), 3U-ACF (b), 5U-ACF (c), and 8U-ACF (d).

was highest at 200 °C, and that the nitrogen content decreased as additional nitrogen escaped into the more volatile materials above 200 °C, the temperature at which graphite oxide reacts with urea according to the following equations [9,26,27]:

$$NH_2CONH_2 + COOH,\ C{-}OH,\ C{=}O \rightarrow CNCOCN + 2H_2O + CO \quad (1)$$

$$NH_2CONH_2 \rightarrow NH_3 + HNCO \rightarrow NH4^{+}NCO^{-}\ (\text{over } 200\,^{\circ}C) \quad (2)$$

In the case of RACF, the ratio of included oxygen was approximately 11.21%, which was generated during the activation with KOH. The oxygen ratios for two other samples (1U-ACF and 3U-ACF), which were produced using low urea concentrations, were slightly lower. However, the oxygen ratios for 5U-ACF and 8U-ACF, produced with high urea concentrations, were also higher. The reaction mechanism indicated that the urea reacted with the oxygen on the carbon surface by replacing it and generating water as a by-product and volatile CO [27]. Above 200 °C, the urea decomposed to form NH_3, and HNCO, which were further transformed into material that were more volatile. The nitrogen and oxygen ratios were the highest at 200 °C [9].

The ratio of elemental nitrogen in the sample increased from 6.66 to 21.09%, and that of oxygen increased from 8.02 to 17.76% by reacting the ACFs with increasing weight ratios of urea as indicated in Table 1. Samples 1U-ACF and 3U-ACF indicate that the nitrogen groups formed by replacing the oxygen on the ACF surface when the carbon surface reacted with urea. Therefore, the oxygen content decreased significantly as it was replaced with nitrogen. However, the number of oxygen functional groups on 5U-ACF and 8U-ACF was increased over those of 1U-ACF and 3U-ACF due to an excess of urea with a –C=O functional group. The nitrogen contents of these samples increased simultaneously under these conditions.

The C1s and N1s deconvolution results for the prepared samples are provided in Table 2. In the case of the C1s deconvolution [28–31], the 3U-ACF had a C–N bond content of 12.63%, the highest among the urea-treated samples. In the case of the N1s deconvolution [28–31], the N–O bond content increased with increasing urea weight ratios of urea. The nitrogen and oxygen contents were highest in the 8U-ACF. However, the C–N bond content, which affects the conductivity, was highest in the 3U-ACF. These differences in chemical composition at the surface were expected to affect the electrochemical properties of the corresponding ACF-based biosensor.

3.2. Cyclic voltammetry during glucose sensing

Cyclic voltammetry (CV) measurements were performed using glucose, glucose oxidase, and a mediator. The results are provided in Fig. 4. The electrodes were prepared using the various U-ACF samples, and the electrodes were analyzed at voltages ranging from −0.4 to 0.6 V. In this analysis, the anodic peak appeared at approximately 0.22 V, a value representative of oxidation, and the cathodic peak appeared at approximately 0.18 V, a value representative of reduction. The data displayed in Fig. 4 were produced in a 0.1 M of phosphate buffer solution (PBS) (pH 7.0), 10 mM of glucose and 5 mM of potassium hexacyanoferrate as a mediator at a scan rate 50 mV/s [32,33].

The reaction mechanism is as follows [12]: First, glucose is oxydized by glucose oxidase (see, Eq. (3)). The reduced glucose oxidase is oxidized by a mediator resulting in an effective electron transfer from the reduced glucose oxidase to the carbon material on the electrode (see, Eq. (4)). The reduced mediator is oxidized by transferring the acquired electron to the carbon material on the electrode

Table 2
C1s and N1s peak positions and peak assignments for the samples used in this study.

Component	Assignment	Binding energy (eV)	Relative quantity (%)				
			RACF	1U-ACF	3U-ACF	5U-ACF	8U-ACF
C(1)	C–C(sp^2)	284.5	57.06	62.36	62.55	46.74	45.60
C(2)	C–C(sp^3)	285.2	21.26	18.36	11.89	12.13	10.53
C(3)	C–N	285.8		9.09	12.63	9.73	10.04
C(4)	C–O	286.1	13.72	5.08	4.43	4.16	3.36
C(5)	C=O	287.3	5.18	1.36	2.35	3.37	1.42
C(6)	O–C=O	289	2.79	3.74	6.16	10.97	10.03
C(7)	series	289.79				6.71	11.29
C(8)	π–π^*	290.93				6.19	7.72
N(1)	C–N=C	398.8		19.40	17.95	14.71	19.90
N(2)	C–N	399.7		45.87	44.45	37.03	35.15
N(3)	C–N^+–C	400.4		28.85	29.62	30.20	31.62
N(4)	N–O	401.5		5.89	7.98	18.05	13.33

(see, Eq. (5)). The mediator repeats the oxidation and reduction processes by transferring electrons from the reduced glucose oxidase to the carbon material on the electrode. Additionally, the oxidation and reduction cycles are repeated on the electrode as evidenced by CV current signal typical of oxidation and reduction.

$$\text{Glucose} + \text{GO}_{(ox)} \rightarrow \text{glucose acid} + \text{GO}_{(red)} \quad (3)$$

$$\text{GO}_{(red)} + 2\text{M}_{(ox)} \rightarrow \text{GO}_{(ox)} + 2\text{M}_{(red)} + 2\text{H}^+ \quad (4)$$

$$2\text{M}_{(red)} \rightarrow 2\text{M}_{(ox)} + 2\text{e}^- \quad (5)$$

The current peaks were also analyzed according to the functional groups at the surfaces of the RACF and U-ACFs. The RACF sample, used as the reference material without urea treatment, had various oxygen groups such as C–O, C=O, and O–C=O on its surface, which could increase its hydrophilicity and conductivity. The oxygen functional groups have dipole moments that promote the transfer of electrons to increase the conductivity of the RACF. In addition, the hydrophilic functional groups immobilize additional enzymes on the surface of the RACF. The current peak of the RACF appeared at 191.69 μA. The other current peaks appeared at 163.408, 223.044, 99.729, and 127.642 μA for the 1U-ACF, 3U-ACF, 5U-ACF, and 8U-ACF, respectively. The high current peaks correlate with an increase in the electron transfer at the surface of the prepared samples and are related to the network of functional groups. In XPS, the current peaks increased with increasing C–N bond content because the conductivity of a C–N bond is higher than that of an N–O bond [9]. Electrons could be transferred to the electrode more effectively with a higher C–N bond content and a lower N–O bond content. Fig. 4 indicates that the current peak for 3U-ACF is the highest, which can be attributed to the increased C–N bond content and reduced N–O bond content. This is due to redox reaction by the higher affinity between the enzyme and substrate. However, other functional groups, such as the nitrogen- and oxygen-containing groups, at the surfaces of the others samples 5U-ACF and 8U-ACF also affected complexly to the current. In these samples, the current was reduced due to increasing electron transfer resistance (R_{ct}) [11] (see Fig. 5).

3.3. Amperometric measurements at various glucose concentrations

Fig. 5 depicts a typical current-glucose concentration plot. This result was analyzed using the amperometric method at a fixed voltage of 0.6 V in a 0.1 M phosphate buffer solution (PBS) (pH 7.0) without mediator. The current peak for each sample was analyzed at glucose concentrations ranging from 0 to 30 mM. The current peak achieved a steady-state (leveling-off) at glucose concentration above 10 mM due to low conductivity and immobilization of the enzyme. The error bars represent the standard deviation obtained by the measurements.

The calibration curves display the steady-state current as a function of the glucose concentration. The calibration curve of each electrode indicates non-linear at high glucose concentration. The slopes of the linear limits show the sensitivities 1.86 μA mM^{-1} cm^{-2} for the RACF enzyme electrode. The sensitivi-

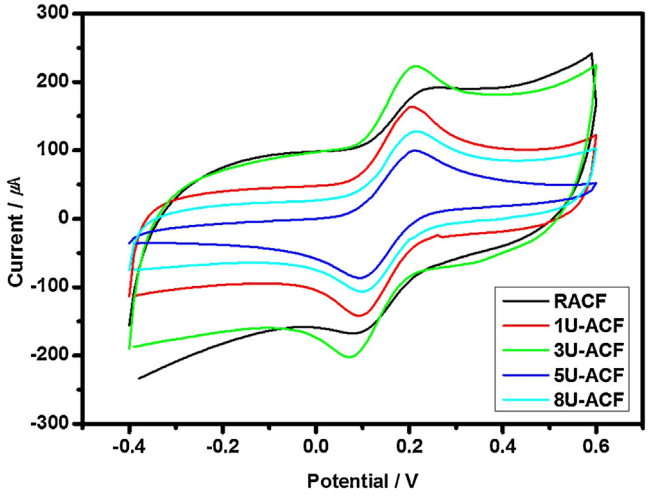

Fig. 4. Cyclic voltammetry of the electrode at various weight ratios.

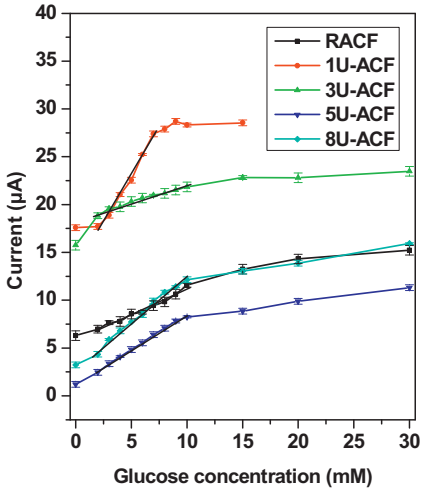

Fig. 5. Calibration curve for glucose in the amperometric responses (in 0.1 M phosphate buffer, pH7.0). Error bars show standard deviation (SD) of five measurements.

Table 3
Analytical parameters of the glucose sensor resulting from the calibration experiments.

Sample	Linear range (mM)	Sensitivity ($\mu A\,mM^{-1}\,cm^{-2}$)	I_{max} (μA)	K_m (mM)	LOD (mM)	R^2
RACF	2–10	1.86	11.66	1.51	2.4	0.986
1U-ACF	2–7	9.85	31.35	1.69	1.2	0.988
3U-ACF	2–10	1.78	22.17	0.39	2.3	0.987
5U-ACF	2–10	3.68	18.18	13.07	0.8	0.997
8U-ACF	2–10	4.89	19.61	7.07	0.7	0.989

ties of four other enzyme electrodes are 1.78–9.85 $\mu A\,mM^{-1}\,cm^{-2}$ for the other electrodes and are improved more than three times than that of the RACF enzyme electrode. This could be attributed to the role of nitrogen as a large anion in enhancing electrical conductivity of the ACF. It is expected that increasing the conductivity of the ACF facilitates the electron transfer between the ACF and the enzyme, thus the sensitivity of the glucose biosensor would grow higher. Although our study is focusing on the sensitivity of biosensor, we achieved a wide sensing linear range of 2–10 mM comparing to other reports GOx/Nitrogen/CNT with increasing concentration of glucose up to 1.02 mM [34].

We performed an enzymatic kinetic study to determine the enzyme activity on the electrodes using the Michaellis–Menten equation (6) [35]:

$$E + S \underset{\Leftarrow}{K_1} C \xrightarrow{K_2} E + P \tag{6}$$

where E, S, P and C represent the enzyme, substrate, product, and enzyme-substrate complex, respectively. Also, K_i is the rate coefficient associated with the elementary step *i*. The concentration of the complex C remains practically constant, that behaved as if it were stationary. This simplication allows the derivation of the classic Michaelis–Mentne equation (7) [36]:

$$I_S = \frac{I_{max} \times S}{K_m + S} \tag{7}$$

where I_S is the steady-state current measured at each glucose concentration, I_{max} is the maximum current measured under saturated substrate solution and indicates the intrinsic characteristics of the enzyme, K_m is the apparent Michaelis–Mentenrate constant and reflects the substrate concentration at which the reaction rate, and *S* is the substrate concentration. Which is an indicator of the affinity between the enzyme and the substrate, can be derived from the mocified Lineweaver–Burk equation (8) [37]:

$$\frac{1}{I_S} = \left(\frac{K_m}{I_{max}}\right) \times \left(\frac{1}{S}\right) + \left(\frac{1}{I_{max}}\right) \tag{8}$$

The modified Lineweaver–Burk equation show the slope (K_m/I_{max}) of the regression lines and normalizing it with respect to the geometric area of the electrode (cm^2), the sensitivity value for the electrode was calculated.

Kinetic parameters (K_m, I_{max}), from the Lineweaver–Burk plot and sensitivity were calculated. With the optimum conditions, calibration curve for glucose was plotted according to the responses of biosensor for different concentration of glucose (Fig. 5). Table 3 summarizes the analytical parameters resulting from the calibration experiments of Fig. 5. As shown in Fig. 5, the functionalized ACF's electrode has current and different values of sensitivities itself even though concentration of glucose is zero. The amount of immobilized enzyme on each electrode was not equal to the other samples due to the amount and structure of functional group [38].

In Table 3, an increase in sensitivity and a decrease in the limit of detection (LOD) were observed upon increasing the weight ratio of urea, reaching a highest sensitivity and minimum limit of detection in the linear range of 2–10 mM at 8U-ACF.

The sensitivity is indicated by the slope of the current-glucose concentration plot, which depicts the change in current with the glucose concentration. The sensitivity of the as-prepared glucose sensor system was calculated from the linear region of the calibration curve and eventually reached 4.89 $\mu A\,mM^{-1}\,cm^{-2}$ in the linear range of 2–10 mM. These data indicate that the 8U-ACF sensor leads to a highly linear response up to 10 mM glucose with a sensitivity of 4.89 $\mu A\,mM^{-1}\,cm^{-2}$. This fabricated electrode could be used as the indicator in clinical applications such as diabetes testing because the human body regulates blood glucose levels within the range of 4–8 mM (70–120 mg dL^{-1}) [3,4] and this electrode could be analyzed in these ranges by covering glucose concentration of 2–10 mM.

The K_m values were calculated using a Lineweaver–Burk plot [37]. K_m is the indication for affinity of enzyme molecule to substrate. The K_m for different electrodes showed different values due to the functional groups which increase immobility of the enzyme. The values of K_m were decreased when GOx reacted with more urea on the surface of the ACFs, and sensitivity and linear range of electrode were increased simultaneously. Eadie–Hofstee plots presented a linear response from glucose concentrations higher than 10.0 mM. The kinetics parameters obtained from this plot were $K_m^{app} = 1.77$ mM and $I_{max} = 19.61$ μA for the 8U-ACF. This K_m^{app} value is smaller than the K_m for GOx in solution [39,40].

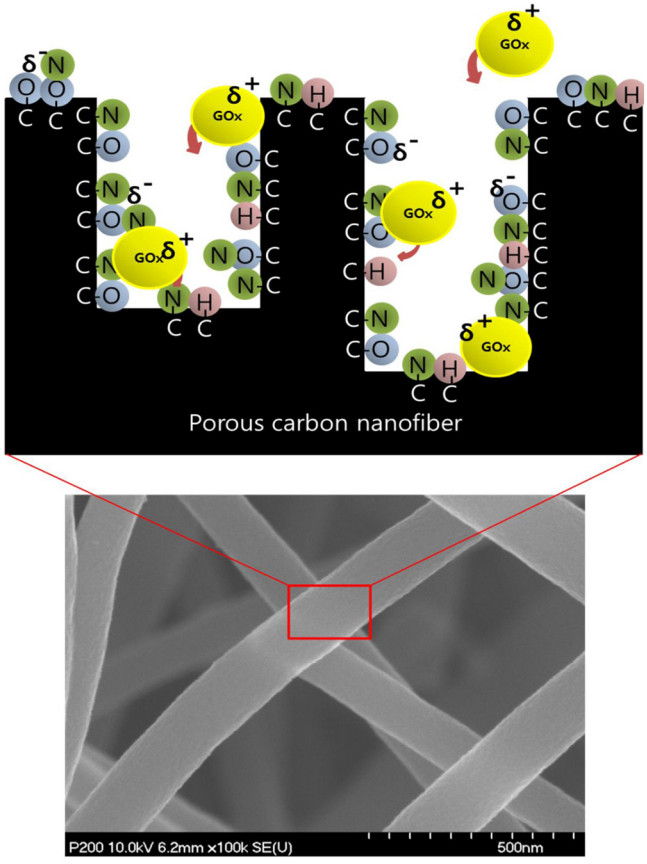

Fig. 6. Conceptual model of the conductivity and interactions between the hydrophilic groups and the enzyme.