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Effects of the gold nanoparticles including different thiol functional groups on the performances of glucose-oxidase-based glucose sensing devices

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Abstract−Thiol-based self-assembled anchor linked to glucose oxidase (GOx) and gold nanoparticle (GNP) cluster is suggested to enhance the performance of glucose biosensor. By the adoption of thiol-based anchors, the activity of biocatalyst consisting of GOx, GNP, polyethyleneimine (PEI) and carbon nanotube (CNT) is improved because they play a crucial role in preventing the leaching out of GOx. They also promote electron collection and transfer, and this is due to a strong hydrophobic interaction between the active site of GOx and the aromatic ring of anchor, while the effect is optimized with the use of thiophenol anchor due to its simple configuration. Based on that, it is quantified that by the adoption of thiophenol as anchor, the current density of flavin adenine dinucleotide (FAD) redox reaction increases about 42%, electron transfer rate constant (k_s) is $9.1\pm0.1\ s^{-1}$ and the value is 26% higher than that of catalyst that does not use the anchor structure.

Keywords: Glucose Oxidase, Biosensor, Gold Nanoparticles, Polyethylenimine, Thiol-based Self-assembled Anchor

INTRODUCTION

As attention and demand for the development of glucose biosensors increase, it is needed to develop more sensitive electrode, including enzyme based biocatalyst because glucose biosensors can utilize glucose oxidase (GOx) as a biocatalyst to oxidize the glucose within the blood that is used as a substrate for producing electrons and protons [1-5].

During the time period of development, glucose biosensors using the enzyme-based biocatalyst have faced several problems such as the low loading amount of GOx molecules, poor catalytic activity, and the sluggish charge transfer rate of biocatalysts [6]. To address the issues, the proper selection of supporting materials is crucial. With the supporting materials, the electron transfer mobility between electrode and flavin adenine dinucleotide (FAD) that is the active site of GOx is promoted, while the amount of GOx immobilized in the biocatalyst structure increases.

In this regard, gold nanoparticles (GNPs or AuNPs) have received strong attention and been considered as one of the support materials. This can offer advantages, such as large active surface area, superior electrical conductive properties, and less toxicity to biological materials like blood or human skin. In addition, this is well attached to the thiols group with a strong bond energy of 418 kJ·mol^{-1} [7]. With the thiols attached on the surface of AuNPs, the amount of immobilized GOx can increase by the formation of strong conjugation bond [8]. Furthermore, specialized spacers can be introduced to the biocatalysts including AuNPs, thiol group and GOx for enhancing catalytic activity further.

Carbon nanotube (CNT) has been also widely recognized as the supporter of GOx based biocatalytic structure for glucose biosensor because this could facilitate direct electron transfer (DET) from the FAD that is placed deep inside the GOx to the surface of the electrode, lower overpotential and increase surface area. Yet, there are still some drawbacks in the utilization of CNT. First, its severe toxicity to biological materials, especially the human body, and second, its hydrophobic nature that makes a facile immobilization of GOx on the CNT surface difficult [9,10]. To alleviate the difficulties, wrapping the CNT with a specific supporter material is suggested, and as the candidate, entrapping polymer (EP) is considered. With the use of EP, the surface polarity of CNTs wrapped by the EP is positively charged and the AuNPs-GOx composite is negatively charged [11-13]. This opposite polarity between them enables the physical entrapment by electrostatic interaction although the interaction is relatively weak. In turn, for strengthening the bonding energy, a new anchor can be adopted. In this study, as the purpose, three types of thiols, such as thiophenol, biphenylthiol, and aminothiophenol, which are immobilized by the self-assembled method, are proposed. To investigate the performance of each thiol group, three biocatalyst structures like CNT/PEI/[AuNPs-GOx]/Thiophenol, CNT/PEI/[AuNPs-GOx]/Biphenylthiol, and CNT/PEI/[AuNPs-GOx]/Aminothiophenol were fabricated. In addition, CNT/PEI/[AuNPs-GOx] formed without the adoption of thiol was also used as a control. Cyclic voltammogram (CV) was used to evaluate the catalytic activity and performance of the biocatalysts, such as the Michaelis-Menten constant and biosensor sensitivity, while to measure and predict the

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amount of GOx immobilized in the biocatalyst structures, UV-Vis spectrophotometry was used.

EXPERIMENTAL

1. Materials

Multiwall carbon nanotubes (MWCNT, MR99, Average diameter 20 nm, purity is higher than 99%) were obtained from Carbon NanoTech (Gyeongbuk, Korea). Glucose oxidase (GOx, from *Aspergillus niger* type X-S, 150.000 $U \cdot g^{-1}$ solid) and Polyethylenimine (PEI, 50% (w/v) solution in water, MW 750,000), gold nanoparticles (AuNPs, 20 nm diameter, stabilized suspension in 0.1 mM PBS), 4-aminotiophenol (97% purity), biphenyl-4-thiol (97% purity), thiophenol (97% purity), and D-glucose were purchased from Sigma Aldrich (Milwaukee, WI, USA).

2. Preparation of CNT/PEI/GOx

CNT/PEI/[AuNPs-GOx] catalyst was prepared by depositing AuNPs-GOx composite on the PEI-coated MWCNT. For fabricating the AuNPs-GOx composite, 5 $m \cdot mL^{-1}$ of GOx was immersed into AuNPs solution and mixed for 2 h. At the same time, the PEI coated CNT was manufactured by dissolving 50 mg of CNT into 10 mL of 2.5 $mg \cdot mL^{-1}$ PEI solution with the sonication of 10 min and was then stirred for 1 h. The PEI coated CNT (CNT/PEI) solution was centrifuged and washed using deionized (DI) water to remove excess PEI. After two kinds of composite materials (AuNPs-GOx and CNT/PEI) were prepared, AuNPs-GOx solution was mixed with the CNT/PEI for 2 h and was then filtered to form the CNT/PEI/[AuNPs-GOx] powder.

To produce biocatalysts anchored by the different kinds of thiols, CNT/PEI/[AuNPs-GOx] was immersed into 2 $mg \cdot mL^{-1}$ of thiophenol, biphenylthiol, or aminotiophenol (in ethanol), sonicated for 10 min, was then stirred and centrifuged. As a result, CNT/PEI/[AuNPs-GOx]/Thiophenol, CNT/PEI/[AuNPs-GOx]/Biphenylthiol, and CNT/PEI/[AuNPs-GOx]/Aminothiophenol powders were formed. All catalysts were stored at 4 °C when not in use to prevent protein denaturation. Meanwhile, enzyme activity measurement was determined using colorimeter method that was already reported.

3. Electrochemical Characterization

The electrochemical measurements were carried out using a computer connected to a potentiostat (BioLogic SP-240, USA). In three-electrode cell measurements system, a Pt wire and Ag/AgCl (soaked in 3.0 M NaCl) were used as the counter and reference electrodes, respectively, while biocatalyst ink coated glassy carbon electrode (GCE) acted as working electrode with the active area is 0.1936 cm^2. To fabricate the working electrode, 10 μL of catalytic ink was dropped on the glass GCE and dried under room temperature. After drying, 5 wt% Nafion solution was dropped on the surface of catalytic ink-loaded GCE [14]. Phosphate buffer saline (PBS) was used as an electrolyte to promote redox reaction of the active sites within GOx, whereas high purity N_2 and air gases were provided to the electrolyte to create N_2-state and air-state conditions.

RESULTS AND DISCUSSION

1. Bonding Formation Mechanism and Enzyme Activity Measurement of Biocatalysts

Predicting bonding formation in each biocatalytic structure is important because it is one of the main factors that affect the performance of biocatalyst, especially, regarding the electron transfer by biocatalyst. The bonding structure of biocatalysts is represented in Fig. 1. Here, the basic composite structure is CNT/PEI and AuNP-GOx. As explained previously, the PEI as entrapping polymer is coated on the surface of CNT. When the PEI is well coated on the CNT, two notable things can be observed. First, the charge of CNT surface is changed from negative to positive because the PEI placed at the outmost surface has a positive charge at neutral pH; and second, the PEI changes the surface property of CNT/PEI from hydrophobic to hydrophilic due to the abundant amine groups contained in PEI. Besides the CNT/PEI, in AuNP-GOx composite, the possible bonding formation is an AuNP-thiol bond. The AuNP-thiol

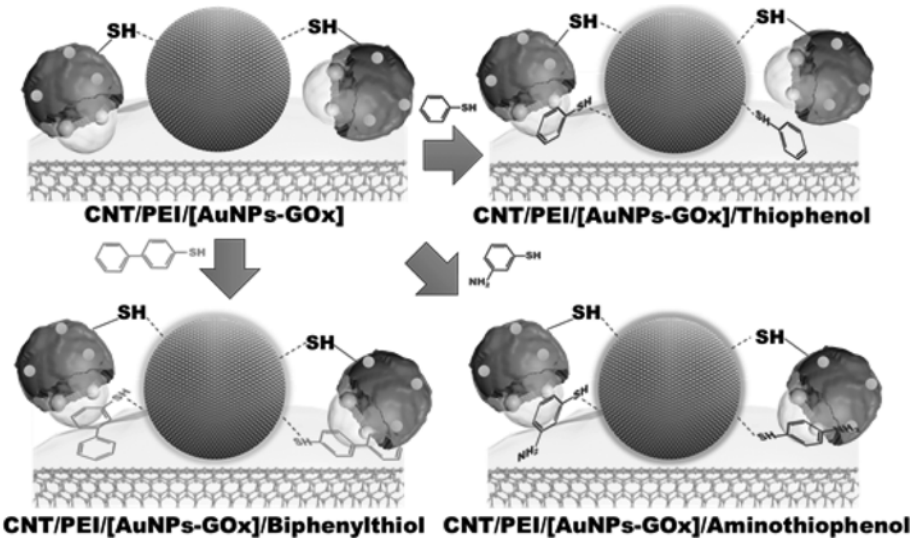

Fig. 1. Schematic illustration of CNT/PEI/[AuNPs-GOx], CNT/PEI/[AuNPs-GOx]/Thiophenol, CNT/PEI/[AuNPs-GOx]/Biphenylthiol, and CNT/PEI/[AuNPs-GOx]/Aminothiophenol catalysts.

bond whose bonding energy is high can provide superior properties, such as excellent electron transfer between GOx and electrode and increase in the amount of immobilized GOx [15,16]. For optimizing the performance of biocatalysts, the AuNP-GOx composite is coated on the surface of CNT/PEI. Since AuNP and GOx have both negative charges, they can be easily attached to the surface of CNT/PEI that is positively charged by the electrostatic interaction. Moreover, the amine groups of PEI can play a role as the entrapper for AuNP-GOx composite.

After the bonding between AuNP-GOx and CNT/PEI, the attachment of thiols is processed using the self-assembly process. Of the three thiols suggested for this study, thiophenol is initially attached to CNT/PEI/[AuNPs-GOx] surface (CNT/PEI/[AuNPs-GOx]/Thiophenol). When the thiophenol is applied, its thiol group is bonded with the AuNPs to form the Au-thiol bond, while the benzene ring of thiophenol that has a hydrophobic nature is bonded with the hydrophobic active site of GOx by hydrophobic interaction. These bonds play a role in strengthening the bonding between GOx and AuNPs. Besides that, some of the benzene rings of thiophenol are bonded with the active site of GOx. Namely, while the GOx molecules are mostly bonded with the benzene ring of thiophenol, free benzene rings remaining without bonding with GOx are bonded with the amine group of PEI to form p-hydrogen bond. This bond also increases the bonding between GOx, AuNPs, and CNT/PEI. In brief, thiophenol is acted as a linker among them.

Second, when biphenylthiol is applied to CNT/PEI/[AuNPs-GOx] surface (CNT/PEI/[AuNPs-GOx]/Biphenylthiol), three different bonds are probably formed. Initially, the thiol group of biphenylthiol is bonded with the surface of AuNPs to form Au-thiol bond, and some benzene rings of biphenylthiol are bonded with the active site of GOx to form hydrophobic interaction, while other benzene rings are bonded with the amine group of PEI to form p-hydrogen bond.

Third, even when aminothiphenol is applied to CNT/PEI/[AuNPs-GOx] surface (CNT/PEI/[AuNPs-GOx]/Aminothiophenol), similar to other catalysts, the thiol group of aminothiphenol is bonded with the surface of AuNPs and its benzene ring is bonded with active site of GOx, while its amine group is bonded with both the amine group of PEI and the lysine residue of GOx to form p-hydrogen bond.

Next, it is important to measure the amount of GOx immobilized by the above-mentioned bonding mechanism. For doing that, colorimetric based measurement is used. During the measurements, the supernatant of biocatalysts is extracted and examined using a UV-Vis spectrophotometer (Fig. 2) [12,13]. According to Fig. 2, the absorbance of supernatant of CNT/PEI/[AuNPs-GOx], CNT/PEI/[AuNPs-GOx]/Thiophenol, CNT/PEI/[AuNPs-GOx]/Biphenylthiol, and CNT/PEI/[AuNPs-GOx]/Aminothiophenol was 0.289, 0.275, 0.220, and 0.226. The higher absorbance of supernatant means the amount of GOx immobilized in the biocatalytic structure is low. Based on these absorbance curves and initial amount of GOx measured during fabrication process, the relative amount of GOx immobilized in the CNT/PEI/[AuNPs-GOx], CNT/PEI/[AuNPs-GOx]/Thiophenol, CNT/PEI/[AuNPs-GOx]/Biphenylthiol, and CNT/PEI/[AuNPs-GOx]/Aminothiophenol was 76.1, 77.3, 81.8, and 81.4%. This result indicates that thiols acting as the cross-linker in the biocatalysts play a role in preventing leaching out of the GOx molecules.

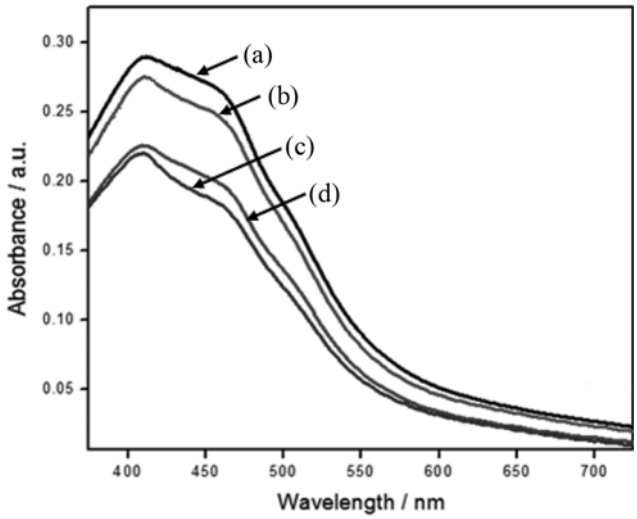

Fig. 2. Absorbance peaks of (a) CNT/PEI/[AuNPs-GOx], (b) CNT/PEI/[AuNPs-GOx]/Thiophenol, (c) CNT/PEI/[AuNPs-GOx]/Biphenylthiol, and (d) CNT/PEI/[AuNPs-GOx]/Aminothiophenol catalysts measured using UV-Vis spectrophotometer at 420 nm.

2. Electrochemical Characterizations

The catalytic activity of CNT/PEI/[AuNPs-GOx], CNT/PEI/[AuNPs-GOx]/Thiophenol, CNT/PEI/[AuNPs-GOx]/Biphenylthiol, and CNT/PEI/[AuNPs-GOx]/Aminothiophenol is electrochemically characterized by measuring CV curves (Fig. 3). There are three notable things to be explained from the catalytic activity analysis. First, in each catalyst, the redox reaction peak appeared in

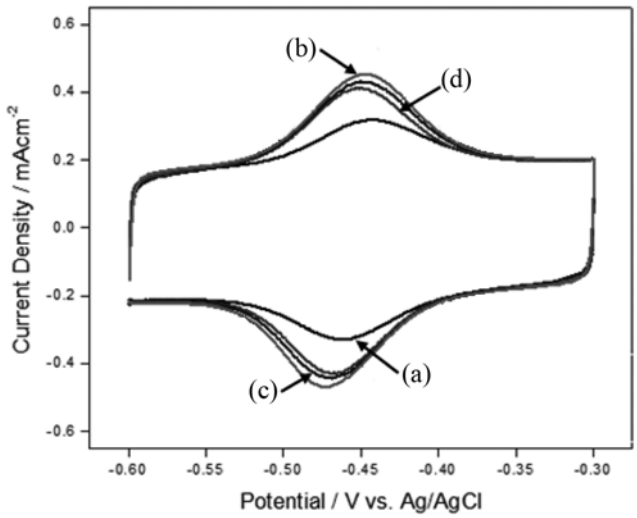

Fig. 3. CV curves of (a) CNT/PEI/[AuNPs-GOx], (b) CNT/PEI/[AuNPs-GOx]/Thiophenol, (c) CNT/PEI/[AuNPs-GOx]/Biphenylthiol, and (d) CNT/PEI/[AuNPs-GOx]/Aminothiophenol catalysts. For the tests, 1 M PBS (pH 7.4) was used as the electrolyte under N_2 state and potential scan rate was 100 mV s^{-1}.

−0.46 V vs. Ag/AgCl that corresponds to $FAD/FADH_2$ redox reaction occurring FAD inside GOx ($FAD+2H^++2e^-\leftrightarrow FADH_2$) [17,18]. Second, the peak intensity of biocatalysts containing thiol groups was about 33-42% higher than that of biocatalyst fabricated without thiol group (CNT/PEI/[AuNPs-GOx]), proving that the redox reactivity of $FAD/FADH_2$ was enhanced by the adoption of thiol group-based materials. Third, catalysts containing thiol groups have more or less similar redox reaction peak intensity, although that of CNT/PEI/(AuNPs-GOx)/Thiophenol was a little higher than others. It demonstrates that the three catalysts have a similar capability in

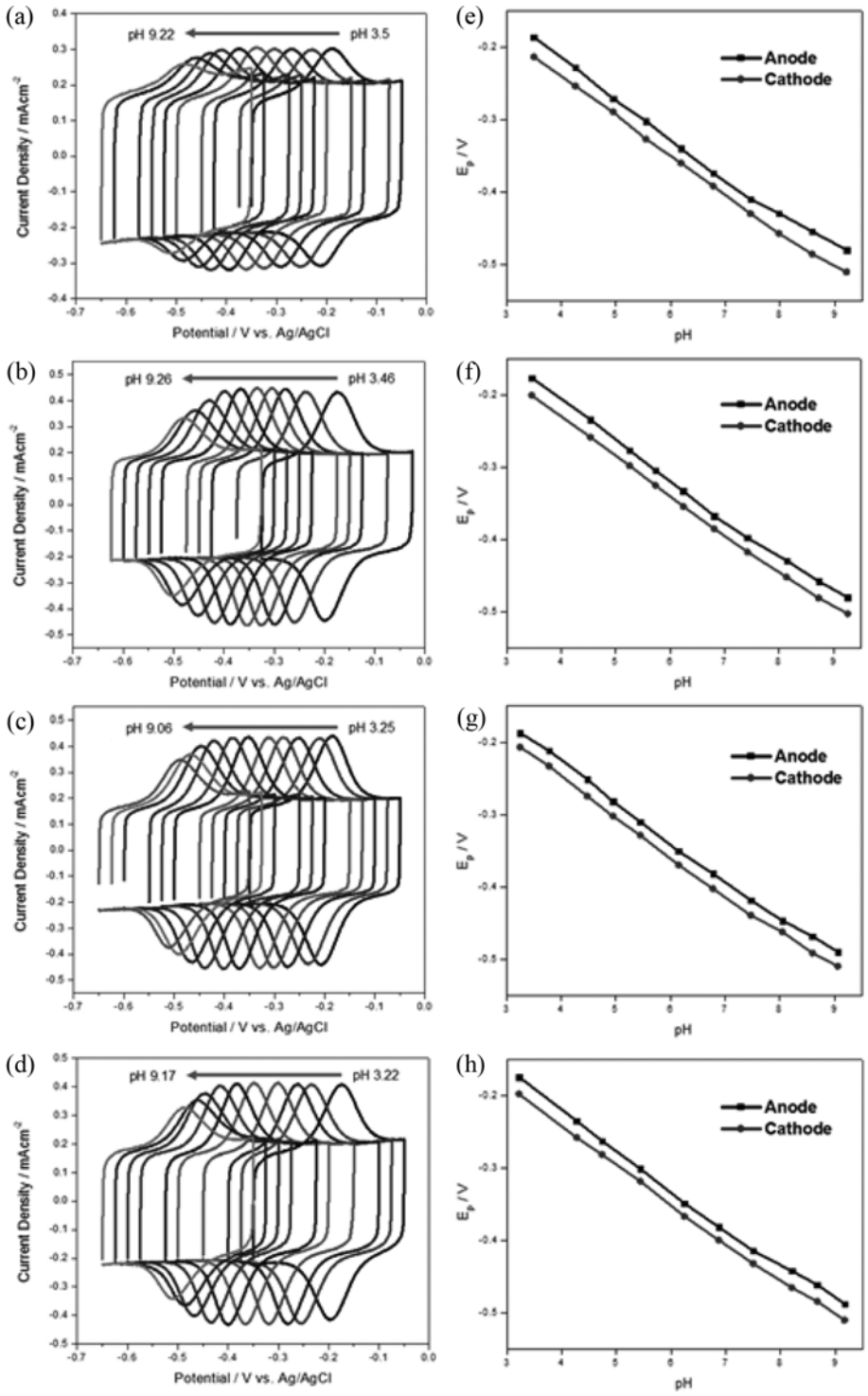

Fig. 4. CV curves of (a) CNT/PEI/[AuNPs-GOx], (b) CNT/PEI/[AuNPs-GOx]/Thiophenol, (c) CNT/PEI/[AuNPs-GOx]/Biphenylthiol, and (d) CNT/PEI/[AuNPs-GOx]/Aminothiophenol catalysts in different pHs from acid to base under N_2 state. (e)-(h) show a correlation between pH and peaks potential of each catalyst.

terms of electron transfer and reaction rate. Especially, although the amount of GOx immobilized in CNT/PEI/[AuNPs-GOx]/Thiophenol was lower than that in CNT/PEI/[AuNPs-GOx]/Biphenylthiol and CNT/PEI/[AuNPs-GOx]/Aminothiophenol, it showed a higher peak current density. It implies that the performance of biocatalyst is not only dependent on the amount of immobilized enzyme, but also the configuration of the electron transfer pathway.

Besides that, a correlation between redox reaction peak potential of FAD/$FADH_2$ and pH of the electrolyte was measured to estimate whether the catalysts can provide desirable two protons/electrons related reaction. To do that, their CV curves were measured in a pH range of 3 to 9 (Fig. 4). According to the Fig. 4, the peak

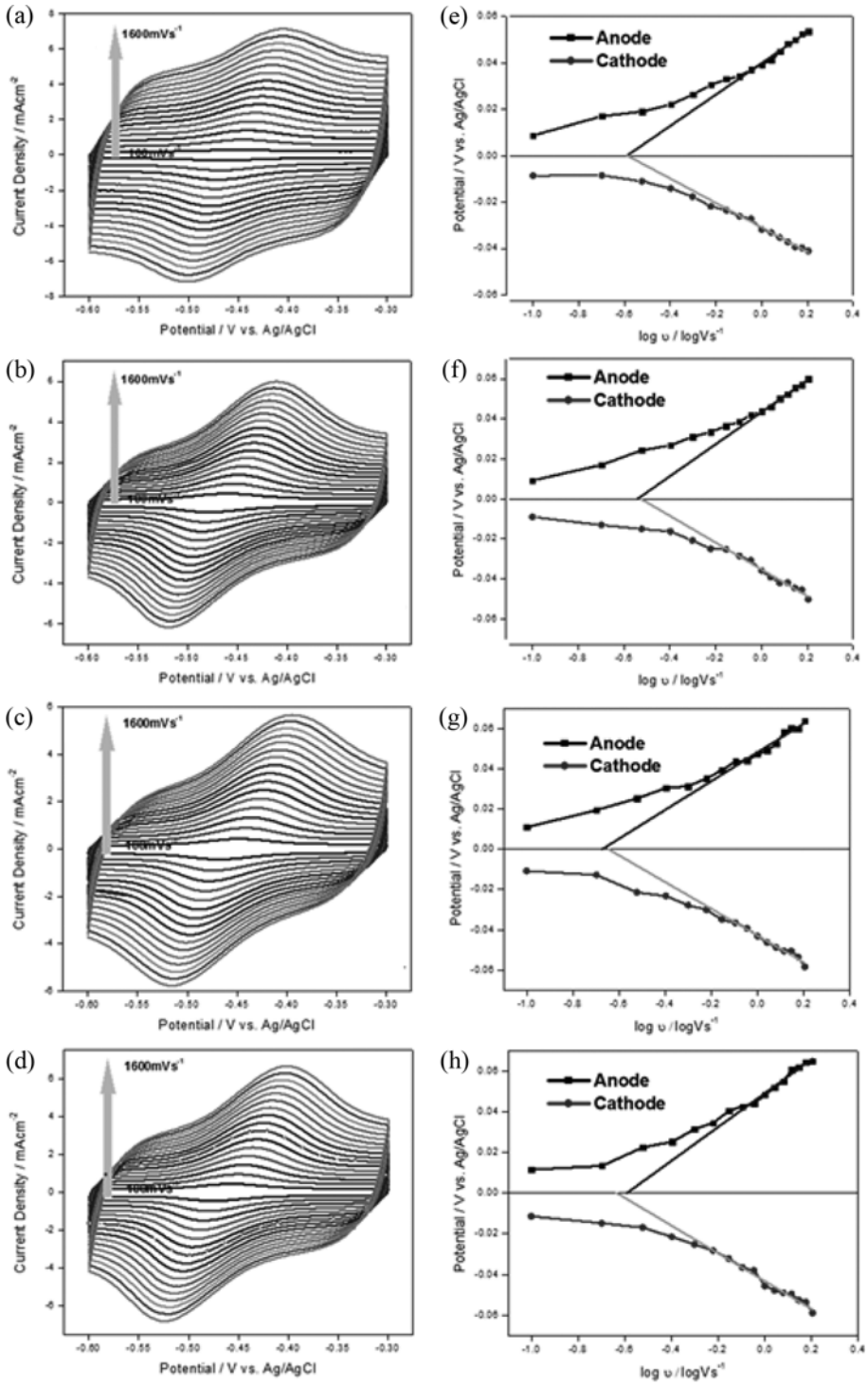

Fig. 5. CV curves of (a) CNT/PEI/[AuNPs-GOx], (b) CNT/PEI/[AuNPs-GOx]/Thiophenol, (c) CNT/PEI/[AuNPs-GOx]/Biphenylthiol, and (d) CNT/PEI/[AuNPs-GOx]/Aminothiophenol catalysts in different scan rates from 100 to 1,600 mVs^{-1} under N_2 state. (e)-(h) represent Laviron's graph of each catalyst.

potential was linearly decreased as pH increased. The average slope of CNT/PEI/[AuNPs-GOx], CNT/PEI/[AuNPs-GOx]/Thiophenol, CNT/PEI/[AuNPs-GOx]/Biphenylthiol, and CNT/PEI/[AuNPs-GOx]/Aminothiophenol was −52.1, −53, −52.8, −52.6 $mV \cdot pH^{-1}$ and the values are close to theoretical one of −58.6 $mV \cdot pH^{-1}$ [19]. It is a clear evidence that the catalysts follow a favorable two protons/electrons FAD/$FADH_2$ redox reaction.

Another approach for elucidating the electron transfer kinetics of catalysts is to measure electron transfer rate constant (k_s) and rate-determining step (RDS). The k_s was determined by measuring their redox reaction peak potential observed at various scan rates from 0.1 to 1.6 $V \cdot s^{-1}$ (Figs. 5(a)-(d)) and calculated using Laviron's equation (Figs. 5(e)-(h)) [20,21]. According to the measurements, the k_s value of CNT/PEI/[AuNPs-GOx], CNT/PEI/[AuNPs-GOx]/Thiophenol, CNT/PEI/[AuNPs-GOx]/Biphenylthiol, and CNT/PEI/[AuNPs-GOx]/Aminothiophenol was 7.2±0.2, 9.1±0.1, 7.3±0.2, and 7.4±0.3 s^{-1}, demonstrating that k_s of CNT/PEI/(AuNPs-GOx)/Thiophenol is highest and it can be reasonably speculated that because thiophenol can make a shorter length for fast electron transfer and a direct connection between CNT/PEI and hydrophobic pocket near FAD within GOx [22]. In other words, by using the thiophenol, electron transfer can be enhanced due to the electron collection effect caused by hydrophobic interaction stemmed from the phenyl group of aromatic rings [22], and this effect was more clearly incorporated with the thiophenol than the others through the shorter pathway of thiophenol attributed to the non-functionalized aromatic ring structure. In the same prospect, it can be interpreted that regarding the electrons transfer, the presence of biphenylthiol and aminothiophenol is relatively less useful because their branched structure has a limitation to promote the electron transfer occurring by the hydrophobic bonds within biocatalytic structure. In turn, the limitation is further ascribed to the functional groups which can interrupt a direct connection and induce other types of interactions between GOxs and linkers, while the lower k_s values than the biocatalyst containing thiophenol are a clear evidence for that.

Meanwhile, the RDS of catalysts was determined by measuring the redox reaction peak current density at various scan rates (Figs. 6(a)-(d)). According to the measurements, the peak current density of biocatalysts linearly increased with an increase in scan rate, substantiating that the catalysts are controlled by the surface reaction [22,23].

To evaluate the catalytic activity of biocatalysts, the effects of anaerobic (N_2-state) and aerobic (air-state) conditions on the catalytic activity of biocatalysts were examined (Fig. 7). As shown in Fig. 7, even though a high concentration (20 mM) of glucose was injected on the electrolyte, there was no peak change under anaerobic condition. It indicates that FAD/$FADH_2$ redox reaction cannot occur prop-

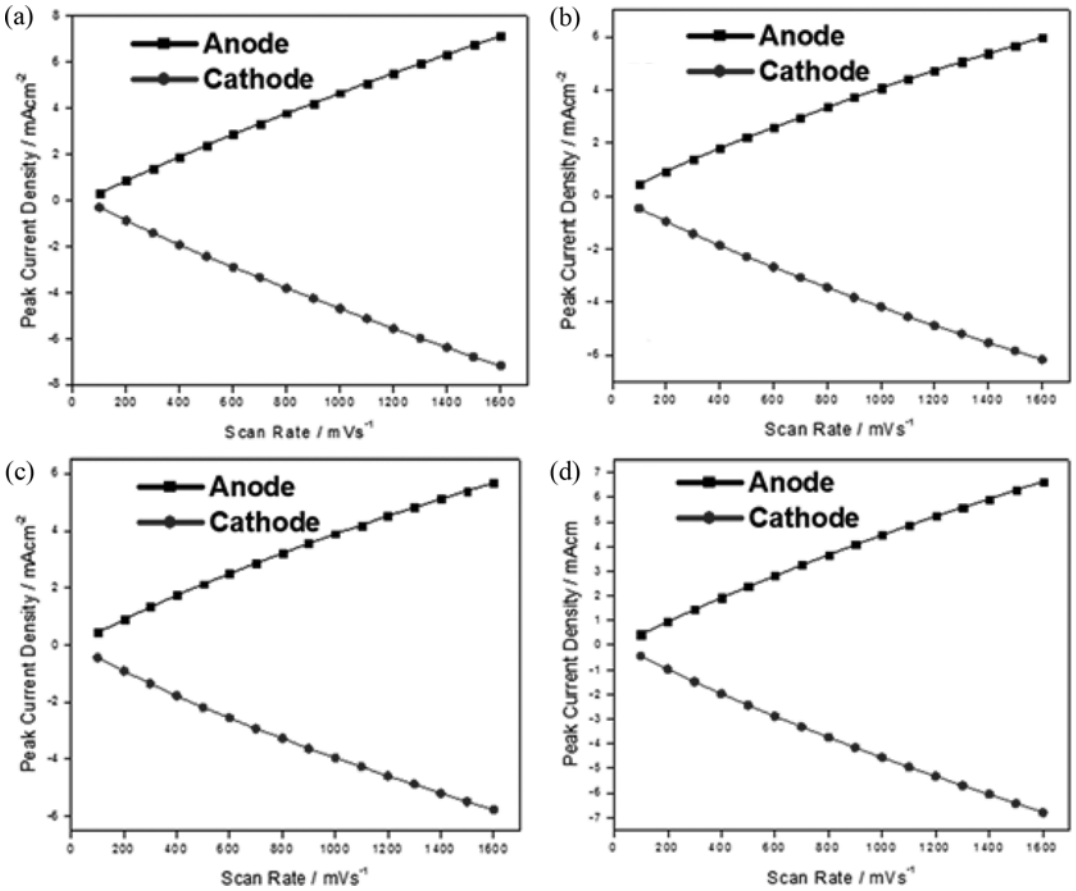

Fig. 6. Correlations between redox peaks current density and scan rate for (a) CNT/PEI/[AuNPs-GOx], (b) CNT/PEI/[AuNPs-GOx]/Thiophenol, (c) CNT/PEI/[AuNPs-GOx]/Biphenylthiol, and (d) CNT/PEI/[AuNPs-GOx]/Aminothiophenol catalysts.

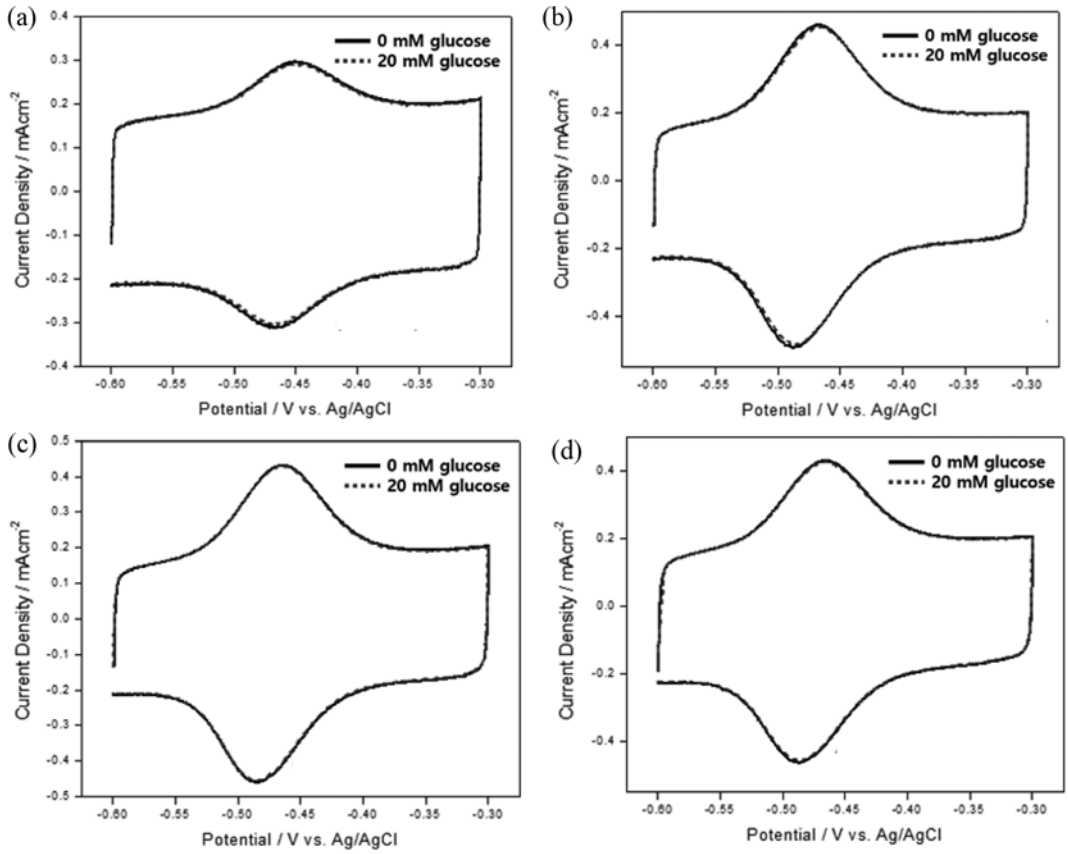

Fig. 7. CV curves of (a) CNT/PEI/[AuNPs-GOx], (b) CNT/PEI/[AuNPs-GOx]/Thiophenol, (c) CNT/PEI/[AuNPs-GOx]/Biphenylthiol, and (d) CNT/PEI/[AuNPs-GOx]/Aminothiophenol catalysts measured at N_2 state at the condition of 0 and 20 mM glucose. For the tests, 1 M PBS (pH 7.4) was used as the electrolyte and potential scan rate was 100 mV s^{-1}.

erly and the corresponding catalysts do not play as an anodic biocatalyst for glucose oxidation reaction (GOR). In contrast, Fig. 8 presents how the FAD/$FADH_2$ redox reaction is affected by the sequential injection of glucose under the aerobic condition. Here, the injected glucose concentration is 0.1-60.0 mM. According to the measurements, there are two main observations. First, the peak current density was up-shifted. What this means is that in this condition, the protons and electrons produced by GOR are used for the FAD reduction reaction ($FAD+2e^-+H^+ \rightarrow FADH_2$), and thus the reduction reaction peak of FAD was up-shifted [24]. Second, the two electrons and protons produced by $FADH_2$ oxidation reaction are consumed for the partial or total ORR by GOx.

Based on Fig. 8(a)-(d), the Michaelis-Menten constant that represents the sensitivity of corresponding catalysts was calculated by measuring the different reduction peak current density [29-31]. From the calculation using Michaelis-Menten's equation, the K_m of CNT/PEI/[AuNPs-GOx], CNT/PEI/[AuNPs-GOx]/Thiophenol, CNT/PEI/[AuNPs-GOx]/Biphenylthiol, and CNT/PEI/[AuNPs-GOx]/Aminothiophenol was 0.84, 0.77, 0.83, and 0.82 mM. The lowest K_m value means the biocatalyst plays a proper role in transferring electrons. In this case, the electron transfer pathway is more important in terms of electron transfer than the type of bonding formed in biocatalyst structure. Although there are biphenylthiol and aminothiophenol in CNT/PEI/[AuNPs-GOx]/Biphenylthiol and CNT/PEI/[AuNPs-GOx]/Aminothiophenol, which also act as a crosslinker, they cannot play the role because the distance from catalysts using them is longer than the thiophenol in CNT/PEI/[AuNPs-GOx]/Thiophenol. Interestingly, CNT/PEI/[AuNPs-GOx]/Thiophenol has lower K_m value than CNT/PEI/[AuNPs-GOx] that is a control structure. In this case, this can be explained that the addition of thiol bond between thiophenol and AuNPs is more crucial than the distance determining electron transfer pathway.

CONCLUSIONS

Thiophenol self-assembled anchor was introduced to link GOx and AuNP cluster to enhance the performance of glucose biosensor. By the use of thiophenol anchor (CNT/PEI/[AuNPs-GOx]/Thiophenol), its current density and electron transfer rate constant (k_s) was 42 and 26% higher than that of biocatalyst that was fabricated without anchor (CNT/PEI/[AuNPs-GOx]). By the adoption of three different thiol-based anchors (thiophenol, biphenylthiol or aminothiophenol), the amount of immobilized GOx increased and the activity and sensitivity of biocatalysts were significantly improved due to the strong hydrophobic interaction (i) preventing the leaching out of GOx and (ii) enhancing the electron collection and transfer. Among these three anchors, thiophenol mostly affected the enhancement of the activity and sensitivity of biocatalyst, and it was

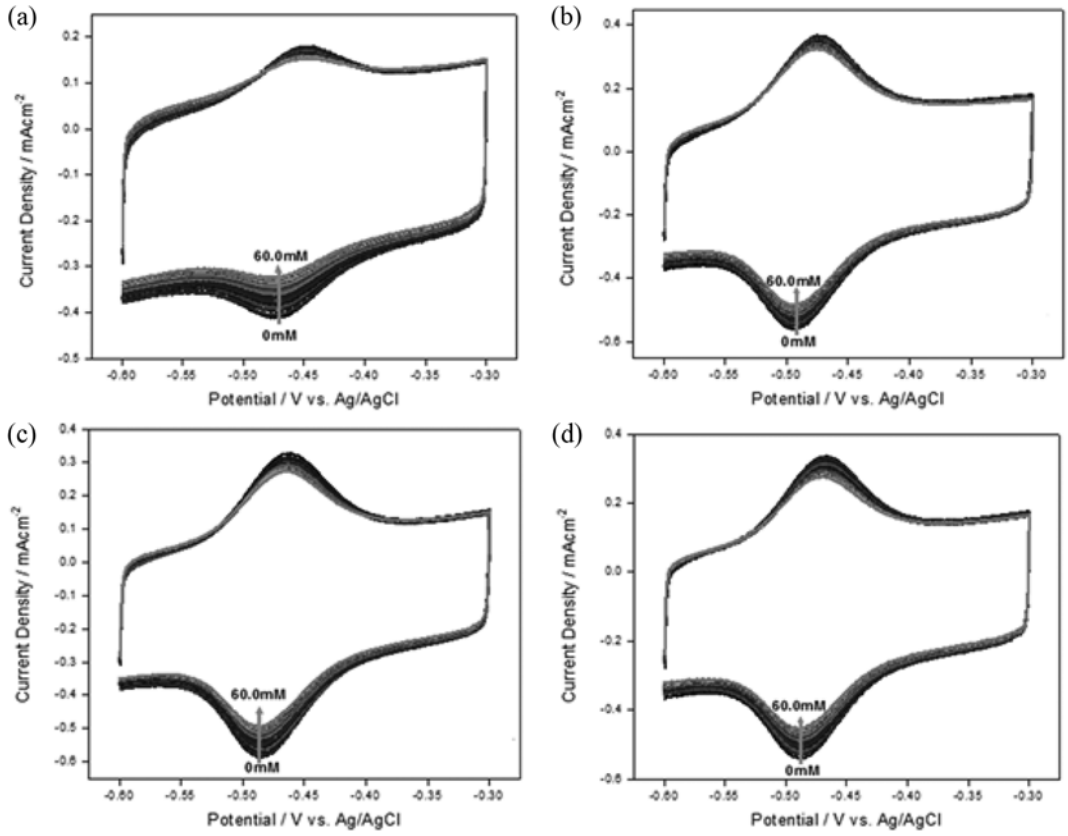

Fig. 8. CV curves of (a) CNT/PEI/[AuNPs-GOx], (b) CNT/PEI/[AuNPs-GOx]/Thiophenol, (c) CNT/PEI/[AuNPs-GOx]/Biphenylthiol, and (d) CNT/PEI/[AuNPs-GOx]/Aminothiophenol measured at air state at the condition of 0 and 20 mM glucose. For the tests, 1 M PBS (pH 7.4) was used as the electrolyte and potential scan rate was 100 mV s^{-1}.

attributed to the simple configuration connecting directly between the hydrophobic pocket of GOx and the phenyl ring of anchor. In terms of the glucose biosensor, the calculated Michaelis-Menten constant of CNT/PEI/[AuNPs-GOx]/Thiophenol was 0.77 mM at the glucose concentration range of 0-60 mM, confirming that this was a proper catalytic structure as the glucose sensor.

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