Bioelectrochemistry 101 (2014) 114–119

Contents lists available at ScienceDirect

Bioelectrochemistry

journal homepage: www.elsevier.com/locate/bioelechem

Enhanced electrochemical sensitivity of enzyme precipitate coating (EPC)-based glucose oxidase biosensors with increased free CNT loadings

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ARTICLE INFO

Article history:
Received 9 April 2014
Received in revised form 13 August 2014
Accepted 19 August 2014
Available online 29 August 2014

Keywords:
Enzymatic glucose sensors
Glucose oxidase
Carbon nanotubes
Electron generation and transfer
Enzyme precipitate coating

ABSTRACT

Enzymatic electrodes were fabricated by using three different immobilizations of glucose oxidase (GOx): covalent enzyme attachment (CA), enzyme coating (EC), and enzyme precipitate coating (EPC), here referred to as CA-E, EC-E, and EPC-E, respectively. When additional carbon nanotubes (CNTs) were introduced from 0 to 75 wt% for the EPC-E design, its initial biosensor sensitivity was improved from 2.40×10^{-3} to 16.26×10^{-3} $A \cdot M^{-1} \cdot cm^{-2}$, while its electron charge transfer rate constant was increased from 0.33 to 1.47 s^{-1}. When a fixed ratio of CNTs was added for three different electrode systems, EPC-E showed the best glucose sensitivity and long-term thermal stability. For example, when 75 wt% of additional CNTs was added, the initial sensitivity of EPC-E was 16.26×10^{-3} $A \cdot M^{-1} \cdot cm^{-2}$, while those of EC-E and CA-E were only 6.42×10^{-3} and 1.18×10^{-3} $A \cdot M^{-1} \cdot cm^{-2}$, respectively. Furthermore, EPC-E retained 63% of its initial sensitivity after thermal treatment at 40 °C over 41 days, while EC-E and CA-E showed only 12% and 1% of initial sensitivities, respectively. Consequently, the EPC approach with additional CNTs achieved both high sensitivity and long-term stability, which are required for continuous and accurate glucose monitoring.

1. Introduction

Electrochemical applications of enzymes have attracted growing attention due to their potential viability in continuous glucose monitoring [1,2] and enzymatic biofuel cells [3–7]. Two main problems arise when using enzymes in electrochemical applications; enzyme activity must be maintained for a reasonable length of time, and the initial performance of enzymatic electrodes (e.g., sensitivity and power output) must be improved. These challenges impose a bottleneck in biofuel cell advancements which may be circumvented by improving enzyme stability and their electron transfer rate. The practical applications of enzymes for continuous glucose monitoring and enzymatic biofuel cells cannot be fully realized until these two major problems are resolved [4]. Efforts have been made to improve the electrochemical performance of enzyme electrodes by developing new enzyme immobilization techniques. Recent success in using conductive nanomaterials for enzyme immobilization has demonstrated great potential for developing high-performance, long-lived enzyme electrodes with high electrochemical activity [8–13].

One of the most successful strategies for enzyme immobilization is the enzyme precipitate coating (EPC) method on carbon nanotubes (CNTs) [9]. EPC of glucose oxidase (GOx) was immobilized on CNTs via a three-step process consisting of covalent attachment, enzyme precipitation, and cross-linking. Chemical cross-linking of highly concentrated enzyme molecules form an enzyme coating over the CNT surface when the enzymes are immobilized on CNTs via the EPC method. This multipoint covalent linkage can effectively prevent enzyme denaturation and leaching, allowing for high enzyme loading and stabilization. High enzyme loading enables a large amount of electrons to be generated per unit time, while the good electrical conductivity of CNTs in the EPC design help to transfer electrons from the enzymes to the electrode. Consequently, the EPC-GOx on CNTs shows a much higher enzyme stability and activity per unit weight of CNT than conventional enzyme immobilization methods based on the covalent attachment (CA) approach [9]. Despite their improved stability and activity, Laviron's experiments showed that the electron transfer rate constant of the EPC-based enzyme electrode (EPC-E) design was lower than that of the electrode with covalently attached enzymes (CA-E) [9]. Because enzymes are not electrically conductive, high enzyme loadings in the form of EPC would lower the electron transfer

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http://dx.doi.org/10.1016/j.bioelechem.2014.08.017
1567-5394/

rate in EPC-E. In the present work, the enzymatic electrodes with EPC were prepared by entrapping EPC together with additional CNTs as an electron transfer promoter. This can potentially improve the electrode's electron transfer rate constant by introducing additional conductive pathways within the EPC-Nafion® matrix. For comparative studies, additional CNTs were also introduced to the enzymatic electrodes based on CA and enzyme coating (EC) methods. EC was prepared as one of the control samples in a two-step process of covalent attachment (CA) and enzyme cross-linking without the enzyme precipitation step that was present in the EPC preparation. The electrochemical performance of these enzymatic electrodes and their stability were investigated with potential applications of EPC-E in continuous and accurate glucose monitoring in mind.

2. Materials and methods

2.1. Synthesis of CA, EC, and EPC

Immobilized GOx samples in the forms of CA-GOx/CNT (CA), EC-GOx/CNT (EC), and EPC-GOx/CNT (EPC) were prepared by following the procedure outlined by [9]. CNTs (multi-walled, 30 ± 15 nm in outer diameter and 1–5 μm in length, purity >95%) were purchased from Nanolab (Newton, MA, USA). CNTs (100 mg) were treated in an acid solution containing H_2SO_4 (7.5 mL of 18 M of H_2SO_4) and HNO_3 (2.5 mL of 16 M of HNO_3) at room temperature under shaking conditions (200 rpm) overnight. Acid-treated CNTs were washed with distilled water, dried at 80 °C in a vacuum oven, and stored at room temperature. The dried CNTs (20 mg) were suspended in a MES buffer solution (pH 6.5) containing *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and *N*-hydroxysulfosuccinimide (NHS). After rigorous stirring at room temperature for one hour, the suspension was thoroughly washed with 100 mM MES buffer (pH 6.5). CA-GOx/CNT was prepared by mixing 2 mL of the suspension of functionalized CNTs (1 mg mL^{-1}) with 1 mL of GOx solution (10 mg mL^{-1}), followed by incubation at room temperature under shaking conditions (100 rpm) for one hour and at 4 °C for overnight.

EPC-GO_x/CNT, also called EPC, was prepared by adding ammonium sulfate solution (26 wt% concentration). This mixture was gently shaken for 30 min to precipitate GOx. Following this enzyme precipitation step, the glutaraldehyde (GA) solution was added into the mixture to a final concentration of 0.5 wt% GA for enzyme cross-linking. Unreacted aldehyde groups were capped by incubating the samples in 100 mM Tris-HCl buffer (pH 7.4), and the samples were thoroughly washed with 100 mM sodium phosphate buffer (pH 7.0). For the preparation of EC-GOx/CNT (also called EC) as a control sample, the precipitation step in the preparation of EPC was omitted. The activities of CA, EC, and EPC were measured by a conventional GOx assay [14].

2.2. Enzyme electrodes of CA, EC, and EPC with and without additional CNTs

Enzyme electrodes were fabricated on glassy carbon electrodes (GCE, 3 mm diameter; CH Instruments, Austin, TX, USA). The appropriate amount of CA, EC, and EPC samples were dispersed in 0.5 wt% Nafion® solution to fix the final CNT concentration at 4 mg mL^{-1}. Twenty microliters of each of these mixtures were deposited on polished GCE surfaces and dried under ambient conditions for one hour. Additional CNTs were introduced to act as electron transfer promoters in the enzyme electrode designs by preparing the buffer solution containing both additional CNTs and GOx-immobilized CNTs at various ratios. These buffers were dispersed in 0.5 wt% Nafion® solution with the total concentration of CNT fixed at 4 mg mL^{-1}. For EPC + CNT electrodes (i.e., EPC electrode with additional CNT as the electron transfer promoter), the amount of additional CNTs was varied in the range of 0 to 90 wt% while the final amount of CNTs in each enzyme electrode was fixed at 0.08 mg by reducing the EPC amounts accordingly. Thus, the total enzyme amount was decreased as the amount of additional CNT was increased. After Nafion® entrapment, all the electrodes were washed and stored in the 100 mM sodium phosphate buffer (pH 7.0) at 4 °C until use.

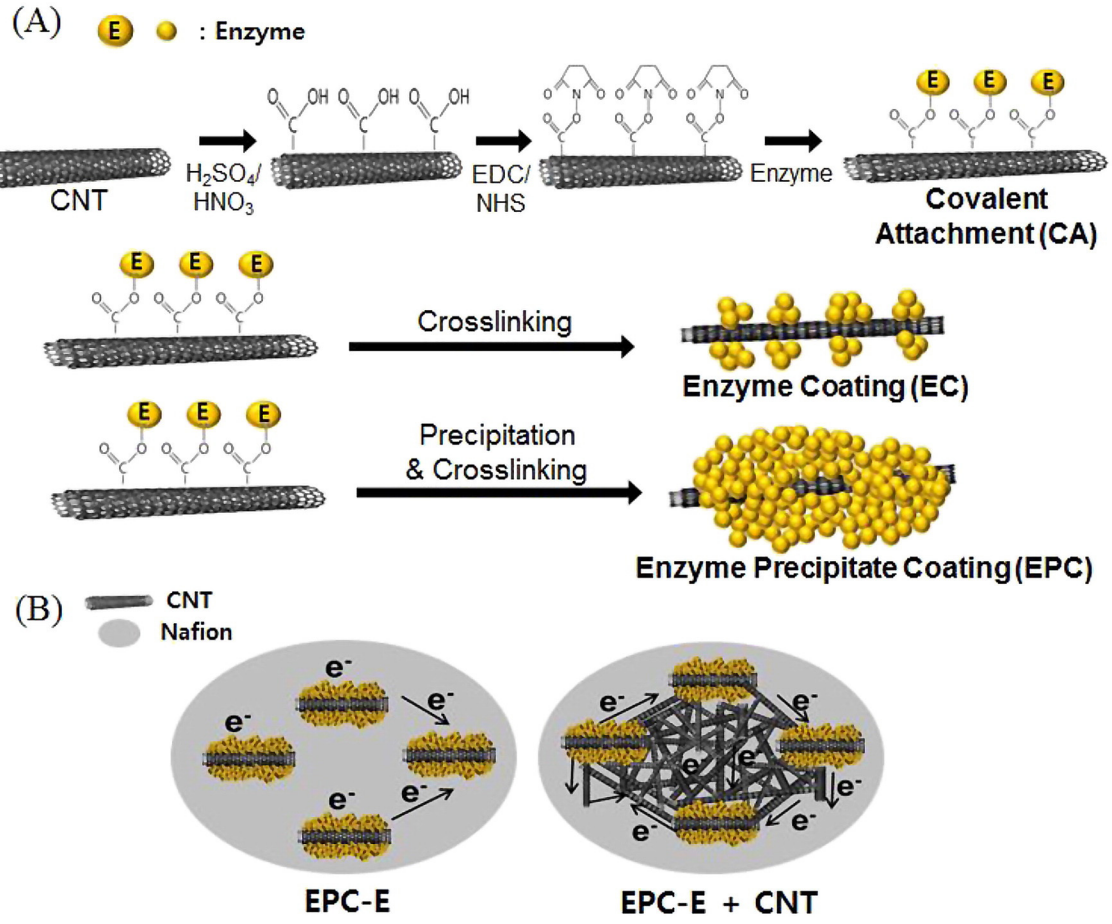
2.3. Electrochemical measurements in glucose sensing

All electrochemical measurements were conducted in 100 mM sodium phosphate buffer (pH 7.0) at room temperature. The enzyme electrode, Ag/AgCl, and a platinum wire were used as the working, reference, and counter electrodes, respectively. A commercial standard Ag/AgCl electrode was purchased from CHI (Austin, TX, USA). The diameter and length of the platinum counter electrode were 0.5 mm and 32 mm, respectively. For the amperometry study, 600 mV vs. Ag/AgCl was applied to the enzyme electrodes. Their steady current responses were measured under magnetic stirring conditions as 2 μL of 5 M glucose stock solution was added. Measurements were performed after allowing for an appropriate current response stabilization period of 60 seconds. Cyclic voltammetry (CV) was performed in the electrolyte solution containing 5 mM glucose in 10 mL of 100 mM sodium phosphate buffer (pH 7.0) using a scan rate of 100 mV s^{-1}. For the long-term stability test, the electrochemical performance of each enzyme electrode was checked by performing an amperometric measurement after incubation of each electrode in the 100 mM sodium phosphate buffer (pH 7.0) at 40 °C.

3. Results and discussion

3.1. Electrochemical performances of GOx-immobilized electrodes without and with additional CNTs

Scheme 1A outlines the synthesis of CA, EC, and EPC. Even though EPC showed an impressive stabilization of enzyme activity, the enzymatic electrode with EPC (EPC-E) showed poor electrical conductivity when compared to the enzymatic electrode with CA (CA-E) [9]. Enzymatic electrodes with free CNTs (CA/EC/EPC + CNT electrodes) were prepared by entrapping GOx-immobilized CNTs together with additional CNTs in a Nafion® matrix (Scheme 1B). Enzyme electrodes were also prepared by entrapping GOx-immobilized CNTs with no additional CNTs and used as control samples. The sensitivity of each electrode toward glucose oxidation was obtained in the linear range of responsive currents to glucose additions (Figs. S1 and S2). According to Fig. S2, at the applied potential of 600 mV vs. Ag/AgCl, the initial amperometric responses from the sensing tests display a linear response as a function of glucose concentration. This indicates the glucose diffusion control characteristic of the biosensors. However, the amperometric response of both EC-E and EPC-E deviates from linear behavior once the glucose concentration increases to a point where the system is no longer limited by diffusion [15,16]. The glucose concentration at which the sensor response deviates from linear behavior defines its maximum sensing range. Depending on the design of the electrode, this sensing range will vary, and is determined by a combination of the rate of both electron generation and transfer. The sensitivities of CA-E, EC-E (the enzymatic electrode with EC), and EPC-E with no additional CNTs were 0.13×10^{-3}, 0.82×10^{-3}, and 2.40×10^{-3} $A{\cdot}M^{-1}{\cdot}cm^{-2}$, respectively (Fig. 1). Because the cross-linked enzymes of EPC do not effectively conduct electrons, the EPC-E shows one order of magnitude lower charge transfer rate when compared to that of the CA-E [9]. It is important to point out that the total amount of CNT used in each electrode was fixed for the present study. Under these conditions, the EPC-E would possess a higher total amount of enzyme than that of the CA-E due to its higher enzyme loading per unit weight of CNT. Since the electron generation rate increases as the enzyme amount increases, the EPC-E generates a higher number of electrons per unit time than that of the CA-E. Thus, despite its lower charge transfer rate, the EPC-E still shows higher sensitivity than the CA-E because it possesses a higher electron generation rate.



Scheme 1. (A) Illustrations for three different enzyme immobilization methods used in this study: CA, EC, and EPC [9]. (B) Schematic illustrations for the preparation of enzyme electrodes without and with additional CNTs. The EPC electrode (EPC-E) was prepared by using EPC immobilization only while the EPC + CNT electrode (EPC-E + CNT) was fabricated by co-entrapping EPCs together with free CNTs as the electron transfer promoters in a Nafion® matrix.

To evaluate the effect of CNT addition as the electron transfer promoter on glucose sensing, free CNTs with no immobilized enzymes were added into the CA-E, EC-E, and EPC-E as described in Section 2.2. The weight percentage of free CNTs to the GOx-immobilized CNTs was fixed to be 75 wt% for all of electrodes. As shown in Fig. 1, all enzyme-immobilized electrodes showed significant improvements in their sensitivities upon the addition of free CNTs. These improvements reveal that free CNTs can provide an efficient pathway for quick electron transport from the active site of GOx to the main GCE surface (Scheme 1B).

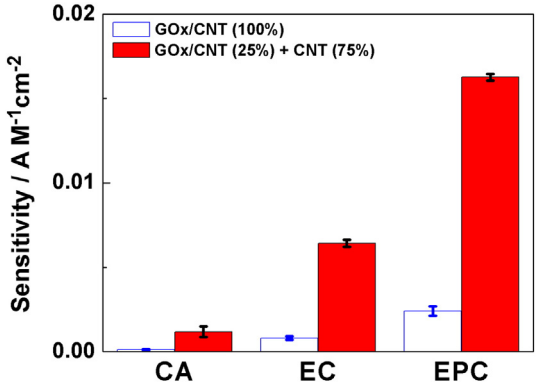


Fig. 1. Sensitivities of enzyme electrodes without and with additional CNTs. The sensitivity of each enzyme electrode was calculated from the linear slope of amperometric responses to the increases of glucose concentration (see Fig. S2). (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

To further investigate the effect of CNT addition, EPC-E were prepared by adding different amounts of free CNTs at five different weight ratios: EPC (100%) + CNT (0%), EPC (75%) + CNT (25%), EPC (50%) + CNT (50%), EPC (25%) + CNT (75%), and EPC (10%) + CNT (90%). Fig. 2 shows the sensitivities of these five different EPC-based electrodes, which were obtained in the linear range of amperometric responses to glucose additions (Fig. S3). As the weight percentage of additional CNTs increased from 0 to 75 wt%, their sensitivities were improved from 2.40×10^{-3} to 16.26×10^{-3} $A \cdot M^{-1} \cdot cm^{-2}$ as shown in Fig. 2A. Further increasing the weight percentage of additional CNT from 75 to 90 wt% only improved sensitivity marginally. Since the total amount of CNTs (that were used as either the support to immobilize enzymes or the electron transfer promoter) was fixed, the total enzyme loading of an EPC-based electrode would decrease as the amount of additional free CNTs used to promote the electron transfer was increased. A specific sensitivity was defined to partially account for this difference in the total enzyme loading of the various EPC-based electrodes with different amounts of free CNT and to isolate the effect of the additional free CNTs. To calculate this specific sensitivity, the sensitivity values from Fig. 2A were normalized using the weight of CNTs used for the enzyme immobilizations. Fig. 2B illustrates the specific sensitivity of EPC-based electrodes as a function of the quantity of free CNTs used. A much more dramatic relationship between sensitivity and weight percent of additional CNTs is revealed by employing specific sensitivities (compare Fig. 2A and B). These results suggest that the additional CNTs are acting as an "electron transport highway" to increase the efficiency of electron

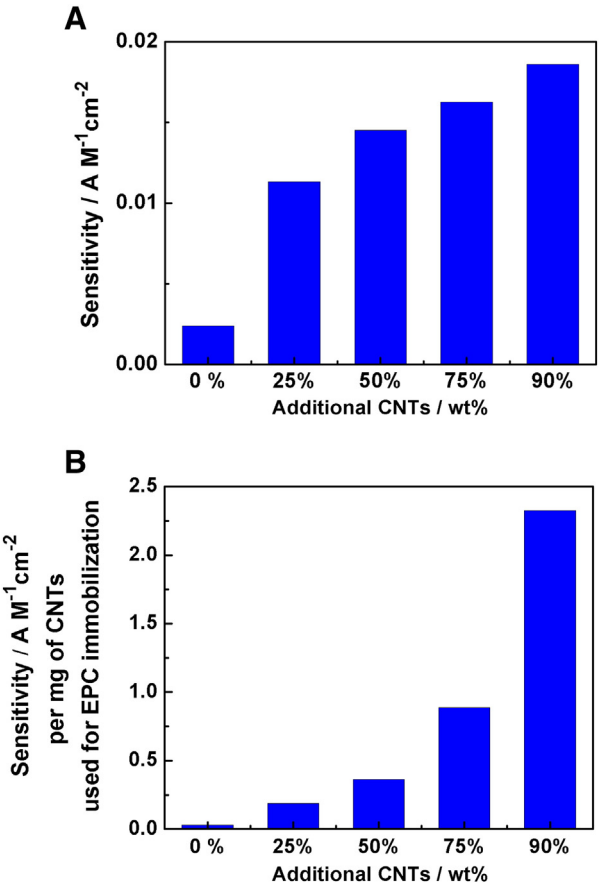


Fig. 2. (A) Effect of additional CNTs on the sensitivity of EPC-E. The ratio of additional CNTs to GOx-immobilized CNTs was varied as shown on the *x*-axis, while the total amount of CNTs in each enzyme electrode was fixed at 0.08 mg. In other words, the amount of GOx-immobilized CNTs decreased as the amount of free CNTs was increased in the enzyme electrode. The sensitivity was calculated from the linear slope of initial current responses to glucose concentration increases (see Fig. S3). (B) Effect of additional CNTs on specific sensitivity. The specific sensitivity is defined by the sensitivity per unit weight (1 mg) of CNTs that were used for the enzyme immobilization in the form of EPC. (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

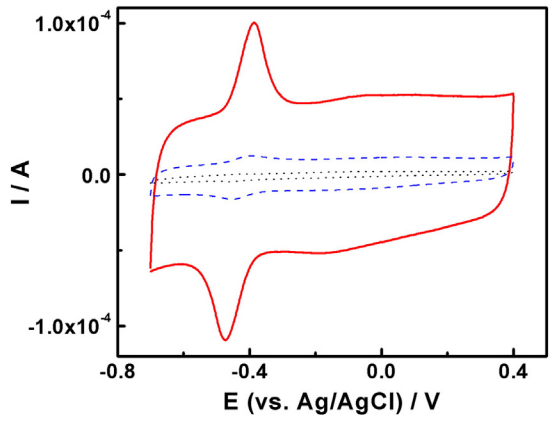


Fig. 3. Cyclic voltammetry of EPC (25%)+CNT (75%) (red solide curve), EPC (100%)+CNT (0%)(blue dashed curve), and control electrode (black dotted curve) in 100 mM sodium phosphate buffer(pH 7.0) at room temperature. The control electrode is essentially the same as the EPC (25%) + CNT (75%) electrode except that it uses no CNT supports for the enzyme immobilization. The scan rate was 100 mV s^{-1}. (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

movement from the enzyme active sites to the main electrode surface. Thus, despite a decreased electron generation rate from the lower amount of enzyme in the enzyme electrode, the overall sensitivity of the EPC-GOx/CNT electrode increases as the amount of additional CNTs increases. In other words, the positive effect of the increased electron transfer rate is much greater than the negative effect of the reduced electron generation rate for the EPC-GOx/CNT electrode as the weight percentage of additional CNT increases.

Cyclic voltammetry (CV) is another way of measuring the electrochemical activity of enzyme-immobilized electrodes. Fig. 3 shows the CVs of the EPC and EPC (25%) + CNT (75%) electrodes. More importantly, the redox peaks shown in the CV correspond to the redox peaks of the GOx active site. Its active site reduces to form $FADH_2$ when glucose is oxidized. $FADH_2$ must be re-oxidized electrochemically back to FAD so that it can be reused for additional glucose oxidation reactions. This re-oxidation reaction of $FADH_2$ can occur by transferring electrons either to molecular oxygen present in the solution (Eq. (1)) or to the electrode if the pathway for the electrons to flow between the redox center of GOx and the electrode surface exists (Eq. (2)) [17,18]:

$$FADH_2 + O_2 \rightarrow FAD + H_2O_2 \quad (1)$$

$$FADH_2 \leftrightarrow FAD + 2H^+ + 2e^- \quad (2)$$

The formal redox potential of FAD/$FADH_2$ (i.e., the active site of GOx) at pH 7.0 is about −0.44 V vs. Ag/AgCl [19]. The fact that the redox peaks of the CVs shown in Fig. 3 are close to the formal potential of FAD/$FADH_2$ suggests that some of active sites of GOx for the enzymatic electrodes are making ideal contact with the electrode to promote efficient electron transfer via Eq. (2). At this point, it is unknown what percentage of GOx in the samples used for this study are making ideal contact with free CNT and what percentage of GOx is not. However, based on the CV test shown in Fig. 3, it is likely that a significant amount of GOx is in good contact with free CNT to promote efficient electron transfer. For this study, the high positive potential of 600 mV vs. Ag/AgCl is chosen because it accelerates electron transfer from the enzyme to the electrode via the CNTs if the separation between the active site of the enzyme and the CNTs is below a critical value [20,21]. For those enzymes that are beyond this critical distance, oxygen takes up the electrons and is reduced to hydrogen peroxide; however, at the applied potential of 600 mV vs. Ag/AgCl, hydrogen peroxide is oxidized to oxygen on the electrode surface and hence the electrons coming from glucose oxidation by the enzyme are recovered by the electrode. Thus, by applying 600 mV vs. Ag/AgCl to the biosensor (i.e., working electrode), not only the electrons from GOx enzymes that make good contact with free CNTs but also those from GOx enzymes that do not make good contact with free CNTs could be collected at the electrode surface.

According to Fig. 3, the peak current of the EPC (25%) + CNT (75%) electrode improved by more than 60 times compared to that of an EPC-E without the additional free CNTs (i.e., EPC (100%) + CNT (0%) electrode). By conducting CV tests at various scan rates and applying the Laviron equation, the electron transfer rate constant was also estimated. According to this Laviron study, the electron transfer rate constant for the EPC (100%) + CNT (0%) electrode was 0.33 s^{-1}, while that of the EPC (25%) + CNT (75%) electrode was improved up to 1.47 s^{-1}. The control sample was prepared by precipitating and cross-linking GOx aggregate without CNT support and preparing the electrode with the additional CNT. This control electrode is very similar to the EPC (25%) + CNT (75%) electrode, except that it has no CNTs within the chemically cross-linked enzyme aggregates. In other words, for this control electrode, CNTs are only present outside of the enzyme aggregates. Under the same experimental conditions, the control sample did not show any significant electrochemical responses as shown in Fig. 3. This result indicates that simply increasing the free CNT amount without the enzyme aggregate in the form of EPC will not allow for

high glucose sensitivity. It is the unique combination of both EPC and additional free CNT that leads to the high glucose sensitivity. This highly sensitive EPC-E also displayed no interference at the applied potential of 600 mV vs. Ag/AgCl by various chemicals such as uric acid, dopamine, and ascorbic acid (Fig. S4). This is another important advantage for applications in glucose sensing.

3.2. Long-term electrochemical stability of EPC-E without and with additional CNTs

Achieving long-term stability and excellent initial electrochemical performance are important milestones for successfully applying enzyme electrodes in glucose sensing. To evaluate the long-term stability of the enzyme-immobilized electrodes presented in this study, their relative sensitivities over long operation times were measured. The relative sensitivity is defined as a ratio of residual sensitivity at the time of measurement to the initial sensitivity of each enzyme electrode. With the potential application of implantable biosensors for continuous glucose monitoring in mind, all the enzyme electrodes were incubated in an aqueous solution at 40 °C for 66 days. One thing to note is that the EPC (25%) + CNT (75%) electrode was used for this long-term stability test, instead of the EPC (10%) + CNT (90%) electrode, although the latter showed better biosensor initial performance. One of the main reasons for using the electrode with a lower initial performance is that there was a high tendency for EPC samples to detach from the GCE surface if the amount of additional CNTs exceeds 75%. The long-term stability data of enzyme electrodes without and with additional CNTs are shown in Fig. 4A and B, respectively. According to Fig. 4A, the CA-E showed a continuous drop in sensitivity, while the EC-E displayed marginally stable sensitivity. From these results, the half-lives of each were calculated to be 4 and 11 days, respectively. In contrast, the sensitivity of the EPC-E was clearly stabilized, showing no decrease in its sensitivity after incubation at 40 °C for 66 days. The thermal stability of CA-E, EC-E, and EPC-E with additional CNTs followed a similar trend to those samples without additional CNTs (Fig. 4B). However, their overall stability decreases with the addition of free CNTs. The half-lives of the CA + CNT and the EC + CNT electrodes were 2 and 6 days, respectively. The EPC + CNT electrode maintained 90% of its initial sensitivity after 9 days and 40% after 66 days in the same condition.

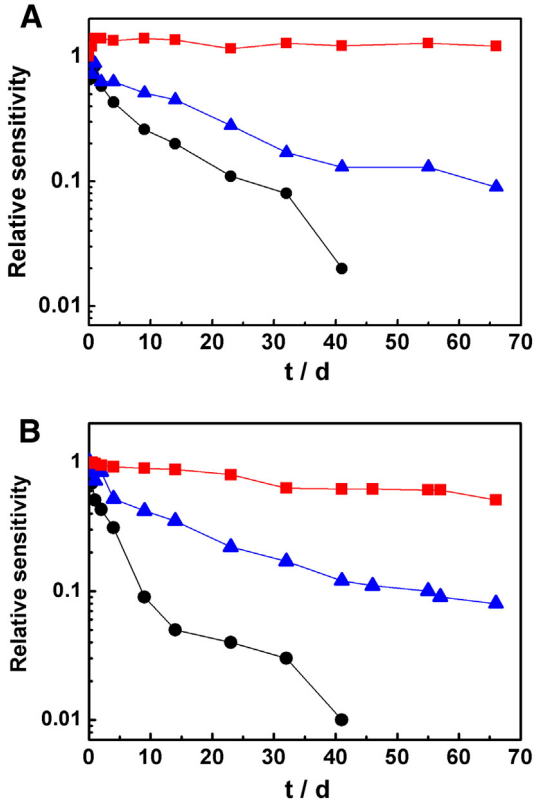


Fig. 4. Glucose sensitivity of CA-E (black circles and curve), EC-E (blue triangles and curve), and EPC-E (red squares and curve) with no additional CNTs (A) and with 75 wt% additional CNTs (B) as a function of time. Enzyme electrodes were incubated in an aqueous buffer solution (100 mM sodium phosphate; pH 7.0) at 40 °C. The sensitivity was calculated from the linear slope of initial amperometric responses to glucose concentration increases. Relative sensitivity is defined as the ratio of sensitivity at each measurement point to the initial sensitivity of each electrode. (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

Fig. 4 reveals that EPC-based electrodes were more stable than either the CA or the EC-based electrodes. The sensitivities of the CA-based electrodes were not measurable after 40 days, while the sensitivities of the EC-based electrodes were reduced by more than 80%–90% after 66 days. On the other hand, the sensitivities of the EPC-based electrodes were either well-maintained in case of no CNT addition or reduced by less than 40% after 66 days when free CNTs were added. The sensitivity reduction in CA and EC-based electrodes was attributed to both enzyme denaturation and leaching of poorly linked or adsorbed GOx molecules. The long-term stability of the EPC-based electrodes demonstrates the significant role that the enzyme precipitation step and multipoint cross-linking of GOx molecules plays in stabilizing the enzyme-immobilized electrodes. Cross-linking increases the number of chemical linkages between GOx molecules and prevents denaturation and leaching of enzymes [9]. The addition of free CNTs marginally decreased the long-term stability of the EPC-E, which can be partially explained by poor physical contact between the Nafion®-entrapped enzyme electrode and the GCE surface in the presence of additional free CNTs as observed in the detachment of the EPC (10%) + CNT (90%) electrode after long-term incubation. One of the main characteristics for the diffusion-controlled biosensor is that its sensitivity is stable over long operation times [15]. Thus, the long-term stability of the EPC-based electrodes shown in Fig. 4 suggests that their sensing operations would be controlled by the glucose diffusion process before they deviate from linear behavior.

4. Conclusions

Enzyme precipitate coatings on CNTs (EPC), with high enzyme loading and stability, were co-entrapped with free CNTs in a Nafion® matrix to prepare highly sensitive enzyme electrodes with long lifetimes. The addition of free CNTs greatly improved the initial electrochemical performance of EPC electrodes because free CNTs in the enzyme electrodes play the role of "electron transport highway" to efficiently transfer electrons from the enzyme active sites to the main electrode. The presence of multipoint cross-linking of enzymes also improved the long-term stability of the EPC electrodes' electrochemical performance, even after incubating the sample at 40 °C for 66 days. EPC electrodes with high sensitivity and long-term stability can be employed for important electrochemical applications such as continuous glucose monitoring and enzymatic biofuel cells. It is also anticipated that the procedure presented here for the addition of CNTs can be employed for the improvement of electron transfer rates of various stabilized enzyme systems to achieve practical uses of enzymes in myriad electrochemical applications.

Acknowledgements

This work was supported by the grant from the Agency for Defense Development (ADD-14-70-04-01).

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bioelechem.2014.08.017.

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