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Simple Preparation of Diaphorase/Polysiloxane Viologen Polymer-Modified Electrode for Sensing NAD and NADH

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ABSTRACT :

Nicotinamide adenine dinucleotide, NAD^+, and its reduced form, NADH, play important roles as coenzymes in many enzymatic reactions. Electrochemical methods for NAD^+ or NADH detection or generation are drawn attention because it can provide the simple and low cost platform with fairly good sensitivity. In this study, the polysiloxane viologen polymer/diaphorase/hydrophilic polyurethane (PSV/DI/HPU) modified electrodes were simply prepared and demonstrated for bio-electrocatalytic NAD^+ sensors. The electrodes were co-immobilized with diaphorase and polysiloxane viologen polymer as an electron mediator followed by the overcoating with HPU membrane. The mixture of the enzyme and the electron mediator was well stabilized within HPU membrane and exhibited good reversibility and stability. The sensitivity was 0.2 $nA \cdot \mu M^{-1}$ and the detection limit was 28 μM with a response time of 50 s ($t_{90\%}$). The capability for NADH sensor was also observed on the PSV/DI/HPU electrode.

Key words: NAD^+ reduction, NADH generation, Electrochemical sensor, Diaphorase, Polysiloxane viologen polymer, Co-immobilization

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1. Introduction

Nicotinamide adenine dinucleotide NADH is known to be a coenzyme or cofactor in many enzyme-catalyzed reactions found in living organisms. It is indispensable for the synthesis of ATP through cellular energy metabolism including glycolysis, TCA cycle, and oxidative phosphorylation, being a substrate of several hundreds of cellular dehydrogenases in redox reactions.[1] NADH is both electron and hydride donor. It also participates in nonredox systems including DNA repair and gene expression.[2] A lot of researches have been focused on the generation of NADH from NAD^+ and NAD^+ or NADH determination.[3-7] Particularly, the electrochemical methods have attracted much attention due to the possibility to make simple and low cost sensors with reasonable sensitivity.[8-10] Nevertheless, the direct electrochemical reaction gives several problems including fairly large overpotentials for both reduction and oxidation, deactivation of electrodes, and formation of undesirable forms such as NAD_2 dimer and inactive 1,6-NADH.[11-13] To overcome the drawbacks, indirect electrochemical regeneration of NADH was developed by using various metal complexes or NAD(H)-accepting oxidoreductase enzymes.[4-6,8,15-25] The enzyme-catalyzed generation systems are of great interest and most frequently studied with formate dehydrogenase,[6,15] glucose-6-phosphate dehydrogenase,[16] lipoamide dehydrogenase,[17] and diaphorase.[18-25] Among them, diaphorase (DI) has been most commonly used because of its thermal stability, insensitivity to dioxygen, and the high rates for enzymatic reaction.[26] For enzymatic-catalyzed reactions, the suitable electron mediators are required to transfer electrons between the enzyme and

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electrode, and methyl viologen has been widely used as an electron mediator.[21-25] In most of the system, mediator and DI are dissolved in solution. Only a few were focused on the co-immobilization of enzymes and electron mediators.[27-29] The present study introduces a very simple co-immobilization method by applying a mixture of polysiloxane viologen polymer (PSV) and diaphorase, followed by the encapsulation with hydrophilic polyurethane polymer. The modified electrode shows good electrocatalytic activities for both NAD^+ reduction and NADH oxidation.

2. Experimental

2.1. Materials

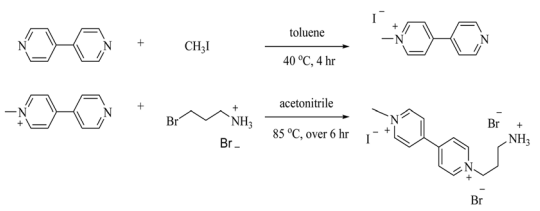
Diaphorase (DI, NADH: dye oxidoreductase) was purchased from Roche in Germany and all chemicals were purchased from Sigma-Aldrich.

2.2 Synthesis of 1-methyl-1′-aminopropyl-4,4′-bipyridyinium

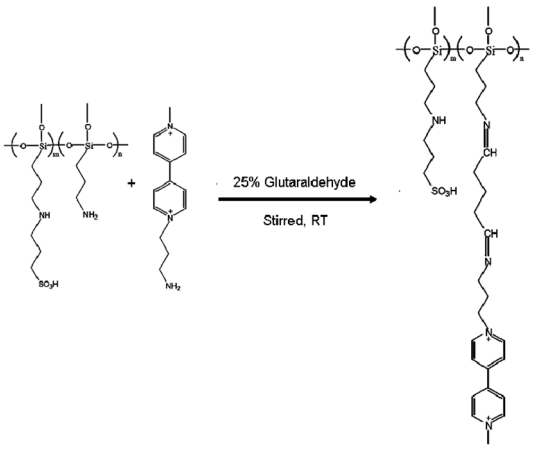
Iodomethane was dissolved in toluene and two equivalents of 4,4-bipyridinein toluene were added to the solution. The mixture was refluxed at 40ºC for over 4 hours and led to precipitation of 1-methyl-[4,4′]bipyridinyl-1-ium iodide derivatives as yellow crystals. And then, 1 equivalent of above compound was dissolved in acetonitrile and mixed with the solution of equimolar 3-bromopropylamine hydrobromide in acetonitrile. The mixture was refluxed at 85ºC for over 6 hours and collected the asymmetric viologen derivative on evaporation and purified by dissolving in methanol and re-precipitating by addition of cold ethyl acetate. The final product, 1-methyl-1′-aminopropyl-4,4′-bipyridyinium salt was obtained as yellow crystals. The synthetic pathway is shown in scheme 1.

2.3. Synthesis of polysiloxane viologen polymer

Polysiloxane viologen (PSV) was synthesized as



Scheme1. Scheme of synthesis of asymmetric viologen derivative.



Scheme 2. Synthetic procedure of polysiloxane viologen (PSV).

shown in scheme 2. First, 18 mg of 3-aminopropyltrimethoxysilane and 36 mg of 1-methyl-1′-aminopropyl-4,4′-bipyridyinium were dissolved in 1.5 mL of distilled water. Then, the pH of the mixture was adjusted to pH 8. After that, 150 μL of 25 wt% glutaraldehyde (GA) was added into the mixture to crosslink 3-aminopropyltrimethoxysilane and1-methyl-1′-aminopropyl-4,4′-bipyridyinium. The solution was stirred continuously at room temperature and it turned brown slowly. After more than 24 hours stirring, the brown polysiloxane viologen solution was purified by ultrafiltration.

2.4. Preparation of the modifiedelectrode

A 3 mm disk glassy carbon electrode was cleaned by polishing with 0.3 μm alumina powder and sonicating in distilled water, and dried with nitrogen gas. 2.5 μL of 2 mg/mL diaphorase solution prepared in 0.1 M phosphate buffer (pH 7) was mixed with 0.5 μL of 5 mg/mL polysiloxane viologen. Then, 3 μL of this mixture was dropped on a glassy carbon electrode surface and dried in the air. After drying thoroughly, the electrode surface was overcoated by 5 μL of 15 mg/mL hydrophilic polyurethane (HPU) and dried.

2.5. Electrochemical measurements

Cyclic voltammetry and chronoamperommetry measurements were carried out using CHI 1000 potentiostat (CH instruments, USA) in a three electrode single-compartment cell containing 5.0 mL of 0.1 M phosphate buffer (pH 7) at room temperature. The modified glassy carbon, coiled platinum wire, and Ag/AgCl (saturated

KCl) electrode were used as working, counter, and reference electrode, respectively. When measuring electrocatalytic reaction under Ar, the concentrations of NAD^+ or NADH in solution were increased by adding incremental amount of concentrated NAD^+ or NADH solutions in 0.1 M phosphate buffer. In amperometric detection, the aliquots of a stock solution of NAD^+ were added to the solution under constant stirring after the background current reached to a steady state value. A potential of −0.7 V vs Ag/AgCl was applied for NAD^+ reduction at room temperature.

3. Results and Discussion

3.1. Electrochemical characterization of polysiloxane viologen (PSV)

2 μL of 10 mg/mL polysiloxane viologen solution was dropcoated on glassy carbon electrode. Cyclic voltammograms of PSV modified glassy carbon electrode were recorded in 0.1 M PB (pH 7) and shown in Fig. 1. A reversible redox reaction with $E^{o'} = -0.55$ V vs. Ag/AgCl with a peak separation of less than 40 mV was obtained. It indicates that polysiloxane viologen has good reversibility and stability enough to co-immobilize with diaphorase as an electron-mediator.

3.2. Electrochemical properties of PSV/DI/HPU electrode

The simple electrocatalytic reaction mechanism occurring at the PSV/DI/HPU electrode is shown in scheme 3. Cyclic voltammograms of PSV/DI/HPU electrode were observed at the different scan rates ranging from 2 to 200 mVs^{-1}. Fig. 2(A) shows that the peak potentials were almost independent on the scan rate. The plot of peak currents versus the square root of scan rate shows a linear relationship (Fig. 2(B)). It indicates that the electro-

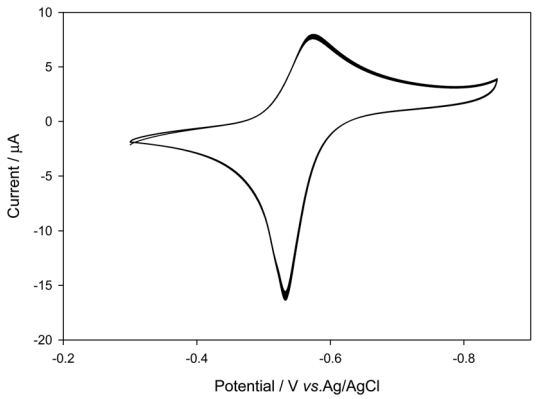
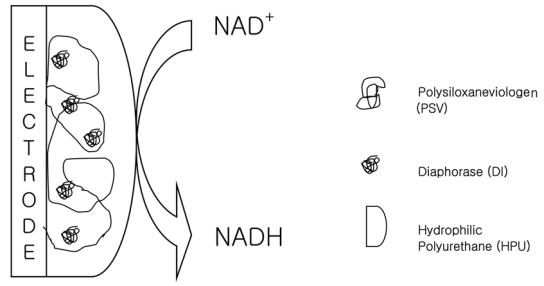


Fig. 1. Cyclic voltammograms of a glassy carbon electrode modified with polysiloxane viologen in 0.1 M phosphate buffer (pH 7) under Ar at a scan rate of 50 mVs^{-1}.



Scheme 3. Scheme of Electrocatalytic Reduction of NAD^+ at the PSV/DI/HPU electrode.

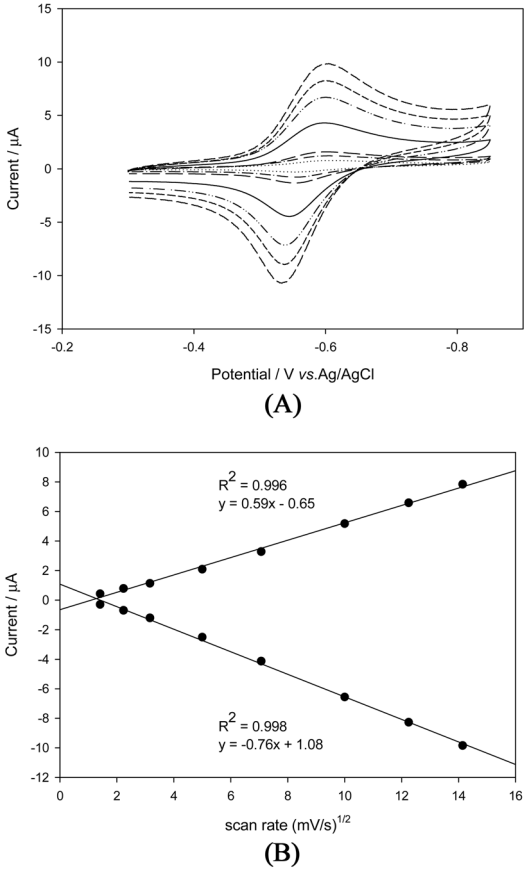


Fig. 2. (A) Cyclic voltammograms of the PSV/DI/HPU electrode in PB (pH 7) under Ar at different scan rates; from inner to outer are 2, 5, 10, 50, 100, 200 mVs^{-1}, respectively. (B) Plots for the peak currents of polysiloxane viologen vs. square root of scan rates.

chemical reaction follows the solution behavior of diffusion controlled process even though polysiloxane viologen is immobilized. Polysiloxane in the film diffuses between electrode and diaphorase within HPU as similar to the electron-mediator in solution behaves. Swelling of the polymer is responsible for the phenomena.

3.3. Electrocatalytic reduction of NAD^+on the PSV/DI/HPU electrode

The capability for electroenzymatic reduction of NAD^+ was examined by using the PSV/DI/HPU electrode. A reversible behavior is observed in the absence of NAD^+ under Ar. Once $NAD^+$is added, electrocatalytic reduction of $NAD^+$is observed (Fig. 3). The cathodic currents increased as increasing the concentration of NAD^+ to 0.5, 1, and 3 mM. The concentration was increased by adding appropriate aliquot of 1 M NAD^+. Itindicates that the PSV/DI/HPU electrode can convert NAD^+ to NADH electrocatalytically. Interestingly, the corresponding anodic current was also increased by increasing NAD^+ concentration. The main cause can be explained by the entrapment effect of HPU. The products could not easily penetrate through the HPU membrane quickly. Thus, the generated NAD Hupon the reduction on the electrode was re-oxidized to NAD^+ by diaphorase within HPU membrane. Consequently, incremental peaks for both reduction and oxidation were exhibited by increasing concentration of NAD^+ due to reversible activities of diaphorase and entrapping effect of HPU.

To verify the performance, the amperometric response of the modified electrode was examined at –0.7 V vs. Ag/AgCl in phosphate buffer with constant stirring. Fig. 4(A) showed that the reduction currents increased upon 0.2 mM incremental addition of NAD^+ with response time of 50 s ($t_{90\%}$). The plot of cathodic currents versus concentration of $NAD^+$is illustrated in Fig. 4(B). A good linear dependency was observed up to 2 mM NAD^+. By extrapolating a calibration curve with linearity, the sensitivity of the PSV/DI/HPU electrode is determined to 0.22 $nA{\cdot}\mu M^{-1}$ and the detection limit is about 28 μM (S/N = 3).

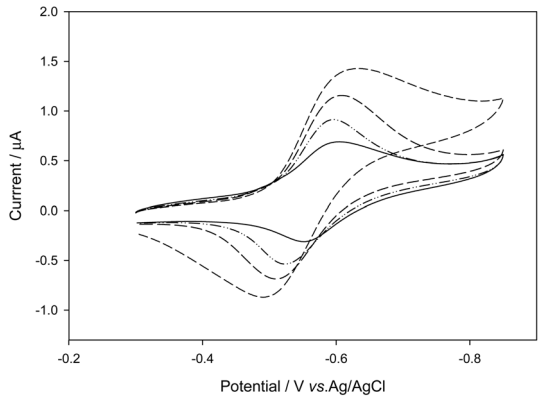


Fig. 3. Cyclic voltammograms of the PSV/DI/HPU electrode as a function of NAD+ concentration in 0.1 M phosphate buffer(pH 7) under Ar at the scan rate of 2 mVs^{-1}. [NAD^+] = 0, 0.5, 1, 3 mM.

3.4. Electrocatalytic oxidation of NADH on the PSV/DI/HPU electrode

Since diaphorase has a capabilty of NADH oxidation, the NADH sensor capability was measured. Cyclic voltammetric measurements were performed to see a

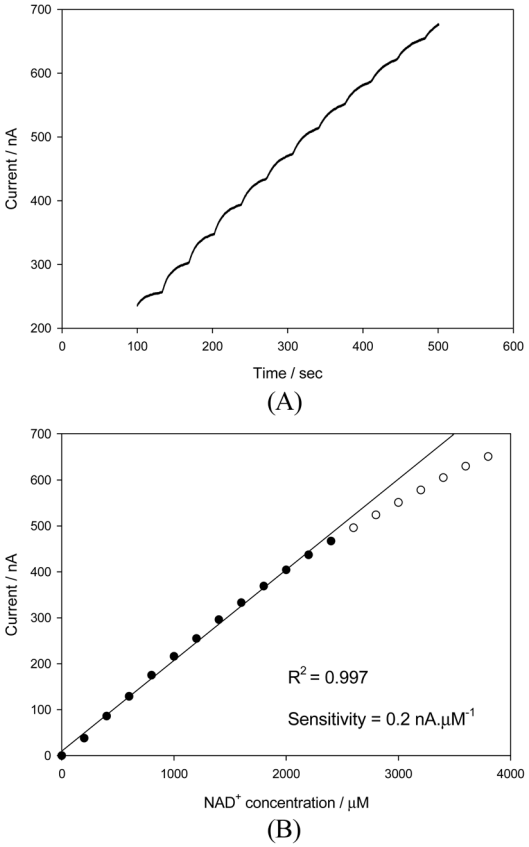


Fig. 4. (A) Current-time curve for detection of amperometric response on the PSV/DI/HPU electrode to incremental additions of 0.2 mM NAD^+ into stirred phosphate buffer (pH 7, 0.1 M) under Ar at −0.7 V (vs. Ag/AgCl). (B) Plot for dependency of the currents on the concentrations of NAD^+.

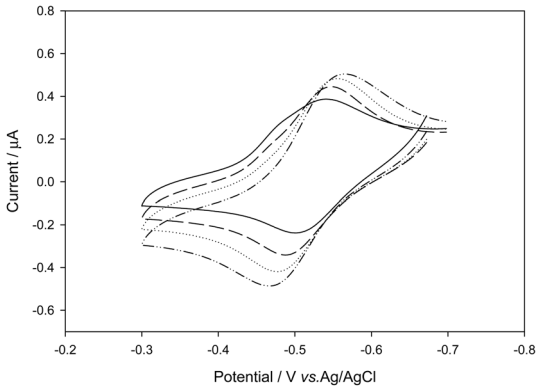


Fig. 5. Cyclic voltammograms of the PSV/DI/HPU electrode as a function of the concentration of NADH in phosphate buffer (pH 7, 0.1 M). [NADH] = 0, 0.2, 0.5, 1 mM.

catalytic activity towards the NADH oxidation. As you can see in Fig. 5, the anodic currents were increased in the presence of 0.2, 0.5, and 1 mM NADH. It demonstrates that the PSV/DI/HPU electrode has a capability to be utilized as both NAD^+ sensor and NADH sensor.

4. Conclusions

In the presentstudy, a simple method was introduced for preparing bio-electrocatalytic NAD^+conversion. The PSV/DI/HPU modified electrode was composed of diaphorase and polysiloxane viologen polymer and well entrapped within HPU membrane. It showed good reversibility and stability. Also, both NAD^+ reduction and NADH oxidation were demonstrated to occur on the PSV/DI/HPU electrode as a dual biosensor. In amperometric detection, the sensitivity of 0.2 $nA \cdot \mu M^{-1}$ and detection limit of 28 μM were determined with a response of 50 s ($t_{90\%}$). Still, there are many drawbacks and further studies should be required to increase the sensitivity.

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