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Rhodium Complex and Enzyme Couple Mediated Electrochemical Detection of Adenosine

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Abstract Adenosine is one of the nucleoside which plays an important role in signal transduction and neuromodulation. This work proposes a simple electrochemical assay, comprising two enzymes and rhodium complex based electron transfer mediator, for the detection of adenosine. Sequential reaction of adenosine deaminase and L-glutamic dehydrogenase and the supporting cycle between β-NADH and mediator enable quantitative analysis of adenosine. Role of electron transfer mediator is the conveyance of proton from electrode to β-NAD^+ for regeneration of β-NADH. The electrochemical characteristics of electron transfer mediator were also studied. Real-time adenosine detection was carried out using this multiple enzyme based chronoamperometric assay. The analysis results show a low limit of detection (140 μM) and good correspondence between current signal and the adenosine concentration (R^2=0.997).

Keywords Adenosine · Enzymatic reaction · Electrochemical · Electron transfer mediator · Nafion

Introduction

Adenosine has several functions in the central nervous system, such as neurotransmission modulation and in cerebral blood flow (1). In ischemic/hypoxic conditions, adenosine is

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released to protect heart cells from damage. Four adenosine receptors regulate a variety of adenosine functions with a range of affinities from low nanomolars to tens of micromolars (2). Measurement of adenosine in vivo, therefore, is needed to understand the amount of adenosine required to activate the receptors and hyper/hypo-adenosine condition.

In the past, analysis of numerous neurotransmitters has been performed (3). Many analytical techniques for neurotransmitters like adenosine and adenosine triphosphate were developed. Common analytical methods of these researches are chromatography and electrophoretic separations with spectrometry, which are bulky and need expensive equipment. Recently, Fan Li et al. reported a colorimetric-based method to detect adenosine using gold nanoparticles (AuNPs) and aptamer (4). Different aptamers, which are bound on AuNPs, formed a tertiary structure with adenosine. Although the colorimetric determination is easy to judge by the bare eyes, the limit of detection of adenosine is too high (>1 mM). Surface-modified microelectrodes in biosensors for the detection of nucleosides has gained much popularity, recently (5, 6). The advent of electrochemical methods has led to the analysis of biomolecules in picomolar levels with high sensitivity and smaller setup size as compared with optical analyzers.

Fangfang Yan and coworkers developed an electrochemical, adenosine detection method using aptamer and methylene blue (MB) as an electrochemical indicator (7). The change in the current signal due to association and disassociation of MB was used as a reference point for the adenosine detection. An additional washing step in the above report prevents it from real-time detection of adenosine. Additionally, the direct redox reaction of adenosine is not sufficient to perform electrochemical analysis due to its relatively high oxidation potential, which is +1.3 V (6). High voltage can also lead to oxidation of other biomolecules resulting in interference in the signal. For this reason, we performed an indirect study, which involves a one-pot homogenous enzymatic oxidation of reduced β-nicotinamide adenine dinucleotide (β-NADH) and its measurement, with adenosine as one of the reactants. Enzymatically enhanced detection of biomolecules holds a great significance in terms of sensitivity and specificity and has been reported by several groups (8, 9).

The assay is composed of two enzymes and its substrates including adenosine. A chain enzymatic reaction ensures that measurement results are from adenosine, in other words, has good selectivity. At first, the adenosine was converted to inosine in the presence of adenosine deaminase (ADA) with a release of an ammonia molecule. Later, α-ketoglutarate in the presence of ammonia and β-NADH was converted into glutamate, catalyzed by L-glutamic dehydrogenase (GLDH). During the reaction, β-NADH is continuously used up by GLDH which stops the adenosine analysis. Therefore, a synthetic rhodium-based electron transfer mediator was used to regenerate β-NADH. The regeneration of β-NADH using the mediator represents precise working of enzymes. During this regeneration, mediator produces a current signal which can be used for indirect detection of adenosine.

The main reactants which are ADA, GLDH, and mediator were immobilized on electrode surface while other reagents were present in the buffer solution. The optimal enzyme immobilization condition should have strong binding force to keep enzyme on the surface and not make them lose their characteristic. Considering all the factors, entrapment is the best way to immobilize enzyme on electrode surface. Entrapment by polymers helps in immobilizing enzymes on electrode surface without any destruction. Therefore, Nafion was used to immobilize the ADA, GLDH, and mediator on the glassy carbon electrode surface in this work.

In summary, this work proposes a novel enzymatic reaction based electrochemical detection of adenosine. The rhodium complex mediator helped in the continuity of the reaction and in indirect detection of adenosine.

Experimental

Materials and Reagents

Adenosine deaminase (EC 3.5.4.4), glutamic dehydrogenase (EC 1.4.1.3), adenosine, α-ketoglutarate, β-nicotinamide adenine dinucleotide (β-NAD), reduced β-nicotinamide adenine dinucleotide (β-NADH), adenosine, dimethyl sulfoxide (DMSO), bovine serum albumin (BSA), ethylenediaminetetraacetic acid (EDTA), and phosphate-buffered saline (PBS; pH 7.5) were purchased from Sigma-Aldrich for the enzymatic assay. Glassy carbon electrode (GCE), platinum wire electrode, and Ag/AgCl electrodes were purchased from CH instruments. Rhodium (III) chloride hydrate and hexamethlydewarbenzene were purchased from Sigma-Aldrich for the synthesis of rhodium-based electron mediator. Nafion 5 % solution in 90 % low-chain aliphatic alcohols was also purchased from Sigma-Aldrich for enzyme immobilization. Ultrapure de-ionized water was used throughout the experiment.

Mediator Synthesis

The electron transfer mediator rhodium complex [Cp*Rh(bpy)Cl]Cl (mediator) was synthesized by following the procedure published by Hollmann et al. (10). The synthesis of rhodium complex is composed of two steps. The first step is synthesis of [Cp*$RhCl_2$] and second step is the addition of bipyridine into [Cp*$RhCl_2$] to synthesize the [Cp*Rh(bpy)Cl]Cl. The synthesis protocol is as follows. Excess of pentamethylcyclopentadiene (1.0 mL) was added to $RhCl_3 \cdot 3H_2O$ (1.02 g, 3.89 mmol) in 25 mL of methanol. The mixture was stirred under reflux for 21 h. After the mixture was cooled to room temperature, the product was isolated by filtration and washed with ether (11). Next, 0.25 g of [Cp*$RhCl_2$] was mixed with bipyridine (1.2 equivalent) in 10 mL methanol, and then the mixed solution was stirred at 30 °C for 1 h. After the solution changed into a dark yellow color, 90 mL diethyl ether was added resulting in the yellow precipitate. The yellow solid was isolated by washing with diethyl ether and filtration. The solid was rewashed with diethyl ether and hexane and recrystallized from acetonitrile/diethyl ether to give [Cp*Rh(bpy)Cl]Cl as yellow crystals.

Enzyme Immobilization on Electrode

Adenosine deaminase, L-glutamic dehydrogenase, and electron transfer mediator were freshly mixed in a tube. Five percent Nafion solution was then added to the mixed enzyme solution to make Nafion-enzyme mixture. Final concentration of ADA, GLDH, mediator, and Nafion are 2.2 U/mL, 2 U/mL, 1 mM, and 0.25 %, respectively. Two microliters of this Nafion-enzyme mixture was then dropped on the GCE surface by micropipette and dried at 4 °C for 4 h. The dried Nafion formed a thin milky white layer on the electrode surface.

Optical Absorbance Multi-enzyme Activity Assay

The UV absorption of β-NADH (Δ 340 nm of wavelength) was observed to analyze the chain reaction activity of the two enzymes. The reaction mixture containing

adenosine (0.14 mM), α-ketoglutarate (39.6 mM), ADA (0.049 U/mL), GLDH (2 U/mL), BSA (0.012 mg/mL), NADH (0.18 mM), EDTA (0.75 mM), and PBS (0.037 mM) was used for the study under UV/Vis spectrophotometer (Perkin Elmer Inc.). The temperature of the cuvette was set to 25 °C for the reaction.

Electrochemical Multi-enzyme Activity Assay

All the electrochemical analyses in this study were carried out by CHI 830B potentiostat. Cyclic voltammetry (CV) and chronocoulometry (CC) were performed to get diffusion coefficient (D_o) of electron transfer mediator. Two millimolars of mediator in 10 mM PBS was used to study the D_o. CV scan in a range of −1.3 to 1 V with various scan rates, 20, 50, 100, 200, 500, and 1000 mV/s, was performed. The parameters for CC are as follows: final voltage= −0.881 and −0.94 V, step=2, and pulse width=0.5 s. Chronoamperometry (CA) was performed using enzyme-immobilized GCE and manually spiking adenosine solution (70 mM in DMSO) at an applied voltage of −0.59 V. The total volume of the buffer for CA in electrochemical cell was 5 mL. Various volumes of adenosine solution were injected in electrochemical cell during CA to check the linearity of current versus adenosine concentration. The concentrations of each reactant in the cell are as follows: 40 mM of α-ketoglutarate, 0.75 mM of EDTA, 0.2 mM NADH, and 73.6 mM PBS.

Results and Discussion

Adenosine Detection Mechanism and Role of Mediator

In this work, electrochemical and optical analyses were carried out to prove the feasibility of adenosine detection based on multiple enzymatic reaction. The enzyme mixture was composed of adenosine deaminase (ADA) and L-glutamate dehydrogenase (GLDH). The two enzymatic reactions are as follows:

Adenosine deaminase:

$$\text{Adenosine} + H_2O + (\text{ADA}) \rightarrow \text{Inosine} + NH_3 \quad (1)$$

Glutamic dehydrogenase:

$$\alpha\text{-Ketoglutarate} + \beta\text{-NADH} + NH_4^+ + (\text{GLDH}) \rightarrow_{\text{L}} \text{-Glutamate} + \beta\text{-NAD}^+ + H_2O \quad (2)$$

These two sequential enzyme reactions were linked through ammonia (NH_3) generation and uptake (Fig. 1). The ammonia generated by ADA during deamination of adenosine was used by GLDH as a substrate. Therefore, the change in the level of reduced β-nicotinamide adenine dinucleotide (β-NADH) was a signal of successful multiple enzymatic reaction. However, it is difficult to monitor the change in β-NADH level electrochemically in real time. Generation of β-NADH in the reaction is limited, which gives discontinuous signals. To analyze the adenosine in high quantities and for longer period, electron transfer mediator was introduced. The mediator conveys electron from electrode to β-nicotinamide adenine dinucleotide (β-NAD^+) and make it reduced as β-NADH. During the reduction of mediator on the electrode, the reduction current generated was monitored by electrochemical analyzer to quantify the adenosine.

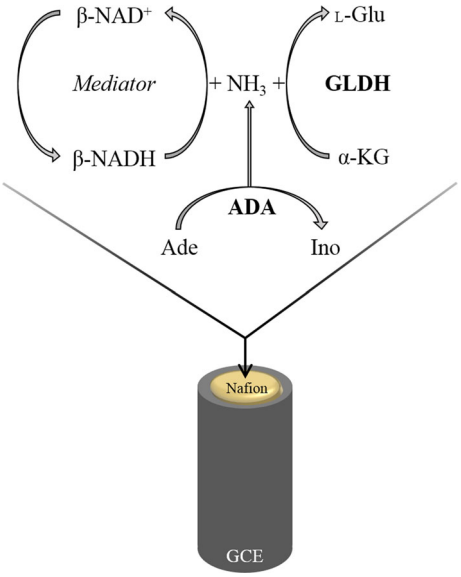

Fig. 1 Schematic of multiple enzymatic system for adenosine detection using electron transfer mediator. *ADA* adenosine deaminase, *GLDH* glutamate dehydrogenase, *Ade* adenosine, *Ino* inosine, *α-KG* α-ketogluratate, *L-Glu* L-Glutamate, *Mediator* electron transfer mediator (rhodium complex), *GCE* glassy carbon electrode

Characteristic of Synthetic Mediator: Electron Transfer

The synthetic rhodium complex mediator was electrochemically analyzed to check its electron transfer characteristics. The redox cycle of mediator consists of two reduction steps (electrochemical, E step, and chemical, C step) and one oxidation step (O step; Fig. 2). In O step, dissociated proton from mediator is transferred to β-NAD^+ which was generated during the working of GLDH, and then the reduced β-NADH facilitated continuous GLDH enzymatic reaction again. The oxidized mediator gets reduced by taking up an electron from the electrode (E step) followed by proton from the buffer (C step). This spontaneous proton uptake reaction is the rate-determining step of β-NADH regeneration. Rhodium complex has great reducing power because of its high reactivity. The ability as reducing agent of mediator was proven by

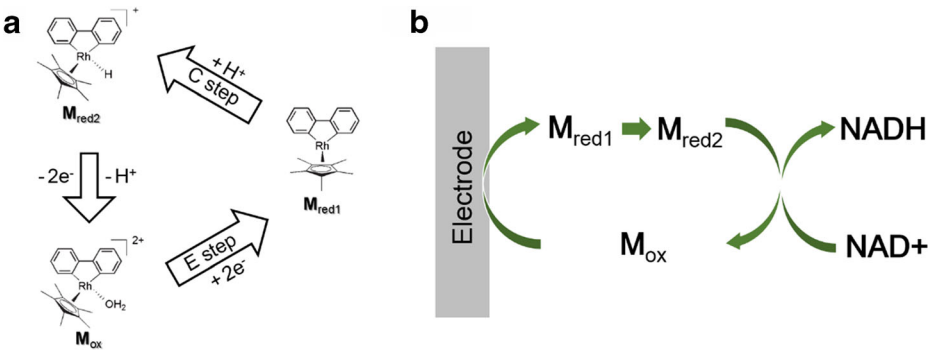

Fig. 2 **a** Redox cycle of mediator; where E step=electrochemical step, C step=chemical step. **b** Redox cycle of NADH and mediator

several other groups. It is widely used for β-NADH regeneration in various research areas (10, 12, 13). Song et al. used nanoparticulate platinum to promote C step of mediator for enhancement of β-NADH regeneration (14).

Cyclic voltammetry and chronocoulometry of mediator was performed to obtain diffusion coefficients, D_o. The reduction potential ($E_{1/2}$) mid-way voltage between anodic and cathodic peak was obtained from cyclic voltammograms. CV was performed with different scan rates ranging from 20 to 1000 mV/s (Fig. 3a and Table 1). The average $E_{1/2}$ value was used for the detection during chronocoulometry analysis. To get diffusion coefficient D_o from Eq. (3), the charge (Q) was measured by chronocoulometry. The average of slopes in Fig. 3b is equal to that in Eq. (4), because when we consider Eq. (3) as a quadratic equation, the x-axis can be root of time and Eq. (4) can be the slope of the graph. F is the Faraday constant, A is area of the electrode, C_o is the concentration of species.

From this equation, the diffusion coefficient was calculated to be 9.24×10^{-6} cm^2/s.

$$Q = \frac{2FADo^{1/2}Co^{*}t^{1/2}}{\pi^{1/2}} \tag{3}$$

$$\text{Slope} = \frac{2FADo^{1/2}Co^{*}}{\pi^{1/2}} \tag{4}$$

Working of Multiple Enzyme in Bulk System

At first, the feasibility of multiple enzymatic reaction was examined in a bulk system using optical measurement system. Two enzymes, ADA and GLDH, were mixed with other reactants except mediator, and then the UV absorbance change of β-NADH at 340 nm was observed. The enzyme assay was followed as per the protocol from Sigma-Aldrich. The rate (slope) of change of absorbance is related to the concentration of adenosine substrate. The first ADA reaction controls the activity of GLDH. In Fig. 4, a decrease in the absorbance was seen due to the reduction in adenosine and β-NADH content. In other words, the result proves the feasibility of adenosine detection using sequential multiple enzyme system.

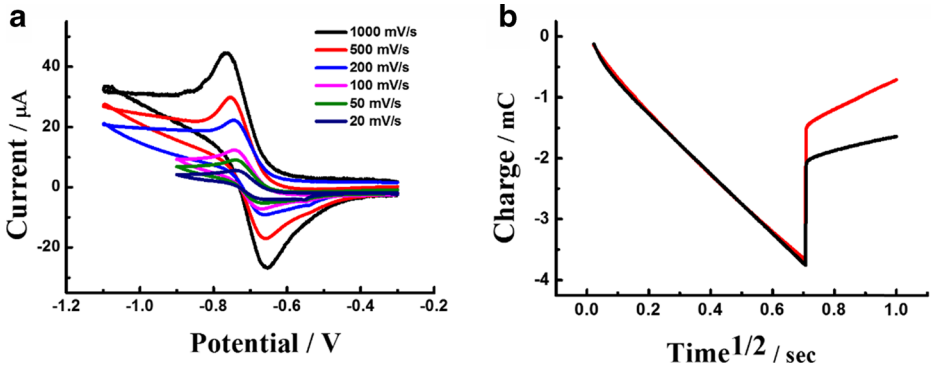

Fig. 3 Electrochemical characteristics of mediator by cyclic voltammetry and chronoamperometry. **a** Cyclic voltammogram of 10 mM mediator in PBS with different scan rates (from 10 to 1000 mV/s) versus Ag/AgCl. **b** Chronocoulometric plot for 2 mM mediator in 10 mM PBS versus Ag/AgCl. Final voltage is −0.881 V (*black*) and −0.94 V (*red*), step=2, pulse width=0.5 s

Table 1 $E_{1/2}$ of rhodium complex at different scan rates

Scan rate (mV/s)	20	50	100	200	500	1000	Avg.
$E_{1/2}$	−0.691	−0.703	−0.706	−0.706	−0.707	−0.711	−0.704

Adenosine Detection by Multiple Enzyme Reaction System on Electrode

The protocol of larger-scale multiple enzyme reaction was adopted in electrochemical electrode as it is. To immobilize enzyme on electrode surface, the entrapment method using polymer was selected. Nafion polymer was used to entrap enzymes because of its high bio-affinity and easy application. Nafion was mixed with the enzymatic solutions and dropped on the glassy carbon electrode. The mixture was dried at 4 °C for 1 h, which resulted in a firm thin film layer on the electrode surface. The Nafion film was physically and chemically stable and did not lose its integrity during the electrochemical measurement.

Enzyme-functionalized electrode was employed as a working electrode in the electrochemical analysis cell. The working electrode was dipped with Pt counter electrode and Ag/AgCl reference electrode in the electrolyte solution containing all the reactants for the multiple enzyme assay, except the adenosine.

Reduction current of mediator (E step) was measured in the cell with spiking it with adenosine at a short interval of time (25~50 s) with an applied bias of 0.59 V (Fig. 5a). A different volume of adenosine solution was injected for each measurement. During measurement, the concentration of adenosine solution in cell continuously increased after each injection. High adenosine concentration resulted in higher signal and produced a serial step-like graph. Linearity of current versus concentration of adenosine was also observed in Fig. 5b. The limit of detection (LOD) of adenosine in this assay was found to be 140 μM (n=3, R^2= 0.997). Based on these results, we propose an enzyme-based electrochemical assay for the detection of adenosine. Further analyses will be performed in the future to enhance the sensitivity and signal output.

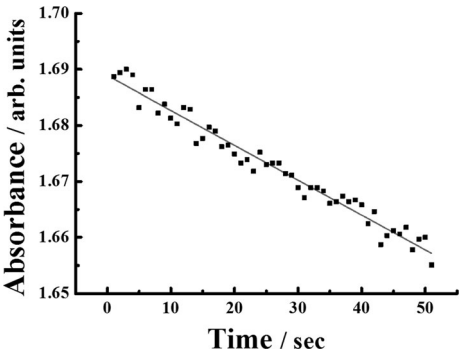

Fig. 4 UV absorbance of β-NADH during multiple enzymatic reaction with 0.13 mM of adenosine and without mediator

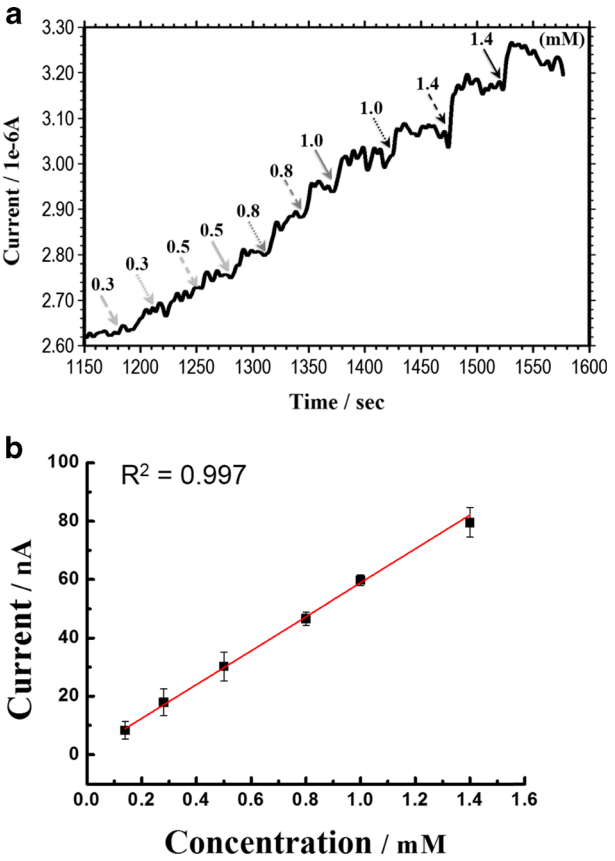

Fig. 5 Adenosine detection. (**a**) Chronoamperogram of mediator after regular injection of adenosine at enzyme-modified electrode, V= −0.59; (**b**) standard curve of obtained from (**a**)

Conclusion

Adenosine, an important neurotransmitter, was detected using a multiple enzyme assay with rhodium complex as an electron transfer mediator to enhance the electrochemical signal. Vital components of the assay, enzymes and electron transfer mediator, were immobilized on electrode surface by Nafion. The enzymatic analysis helped in highly sensitive detection of adenosine with low detection level (140 μM) and reproducibility (R^2=0.997). The proposed enzymatic method can be used to detect some adenosine—a biomarker in several medical conditions and biomolecular reactions. Further, the assay can also be miniaturized for the analysis in a microfluidic biochip with integrated electrochemical detection system for a more rapid and portable detection.

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