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Tris(2,2′-bipyridyl)ruthenium(II) electrogenerated chemiluminescence ethanol biosensor based on ionic liquid doped titania-Nafion composite film

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ABSTRACT

A highly sensitive tris(2,2′-bipyridyl)ruthenium(II) [$Ru(bpy)_3^{2+}$] electrogenerated chemiluminescence (ECL) biosensor for the detection of ethanol based on ADH and NAD^+ cofactor with ionic liquid (IL) doped sol-gel titania-Nafion composite film has been developed. Regardless of the type of the ILs, the incorporation of IL in the composite resulted in the enhancement in both the electron transfer and diffusion rates in the composite modified electrode, thus leading to dramatic increase in ECL intensity. Among several ILs tested, 1-butyl-3-methylimidazolium with chloride counter anion was most effective for the construction of the $Ru(bpy)_3^{2+}$ ECL biosensor. Two-layer system with the inner layer of $Ru(bpy)_3^{2+}$ immobilized in IL-titania-Nafion composite film and the outer layer of ADH immobilized in titania-Nafion composite film has been used to maintain the activity of the enzyme and the overall stability of the ECL biosensor. The present biosensor exhibited linear response towards ethanol with a linear dynamic range of 1.2×10^{-5} M–2.5×10^{-1} M and a detection limit of 5.0×10^{-6} M (S/N = 3). The outstanding dynamic range showed the applicability of the present biosensor for ethanol detection not only in human blood but also in a variety of different fields such as alcoholic beverages or other commercially available products.

1. Introduction

Ethanol has often been used in a wide range of fields including alcoholic beverage, disinfectant, cleaning agents, cosmetics, fuels, antifreeze and synthetic ingredients. Since these fields are intimately related to human life, personal health-related issues associated with ethanol is attracting attention. According to the Material Safety Data Sheet (MSDS), ethanol intake can cause gastrointestinal irritation, nausea, vomiting and diarrhea. Further ingestion may result in death due to unconsciousness, coma or dyspnea. Also, exposure to ethanol by skin contact and inhalation gives a strong stimulus. In addition to the damage caused by short-term exposure to ethanol, the harmful use of alcohol causes approximately 2.5 million deaths each year [1]. For these reasons, accurate determination of ethanol in both alcoholic beverages and in human body fluids is imperative for industrial and clinical purposes.

Numerous research has been performed on the development of ethanol detection methods with high accuracy and sensitivity using gas chromatography [2], liquid chromatography [3], spectroscopy [4], electrochemical analysis [5], or ECL analysis [6–9]. Among them, the electrochemical and ECL detection methods are advantageous for making portable and easy-to-use sensors for the general public [10], many of which involve the use of alcohol dehydrogenase (ADH) with nicotinamide adenine dinucleotide (NAD^+) as a cofactor. However, detection of ethanol using an electrochemical method often requires high overpotential because NADH must be directly oxidized. Such high overpotential can induce unwanted reactions of other electrochemically active species, and direct oxidation of NADH causes gradual passivation of the sensor [5].

ECL is widely known for its excellent selectivity, sensitivity, and reproducibility, since it provides spatial and time control [11]. Due to such merits of ECL process, numerous studies have been conducted in a variety of fields including its use in analytical applications [12, 13], or studies that focus on developing ECL systems to maximize the efficiency of the performance in analytical applications [14, 15]. As one of the ruthenium complexes, $Ru(bpy)_3^{2+}$ is the most representative ECL reagent due to their high chemical stability, long lifetime of excited state, good reactivity of excited state, and high luminescence efficiency [16]. One of the most import properties of $Ru(bpy)_3^{2+}$ is that its luminophore is regenerable during the ECL process. The use of ECL analysis in determination of ethanol allows for detection of ethanol without direct oxidation of NADH since it measures light from the electrochemical

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reaction between $Ru(bpy)_3^{2+}$ and NADH.

ECL biosensors for ethanol based on $Ru(bpy)_3^{2+}$ ECL with the use of ADH have been studied extensively in recent years [6–9]. One of the common materials used in such studies is carbon nanomaterials. Numerous studies on ethanol biosensors have incorporated carbon nanomaterials because of their excellent electric conductivity and stability, and their ability to be used as a platform for immobilization of organic and inorganic molecules due to their inherent properties of high surface area and hydrophobic properties [17–19]. However, due to their poor dispersibility, handling of these materials is time consuming, or even requires further modifications.

In the present study, a $Ru(bpy)_3^{2+}$ ECL biosensor for ethanol based on ADH and NAD^+ cofactor with ionic liquid (IL) doped titania-Nafion composite-modified electrode has been developed. It has been reported that the incorporation of titania sol-gel into Nafion film greatly increases the diffusion rate of $Ru(bpy)_3^{2+}$ into the composite film compared to pure Nafion films due to the increased pore size of the composite films containing $Ru(bpy)_3^{2+}$, leading to the enhanced ECL signals [20]. In addition, we have also reported the feasibility of controlling the selectivity of solid-state $Ru(bpy)_3^{2+}$ ECL by selecting ILs with appropriate hydrophobicity in the sol-gel titania-Nafion composite film [21]. Therefore, the incorporation of appropriate IL in titania-Nafion composite can improve the performance of the $Ru(bpy)_3^{2+}$ ECL sensor for the detection of NADH without the use of nanoparticles or carbon-based nanomaterials, and hence the IL-doped titania-Nafion composite-based biosensor is an ideal candidate as a sensitive dehydrogenase based ECL biosensing platform without compromising the simplicity of the biosensor. Two-layer system with the inner layer of Ru $(bpy)_3^{2+}$ immobilized in IL-titania-Nafion composite film and the outer layer of ADH immobilized in titania-Nafion composite film has been used to maintain the activity of the enzyme and the overall stability of the ECL biosensor.

2. Experimental

2.1. Materials and reagents

Titanium(IV) isopropoxide ($Ti(OR)_4$, $R = CH(CH_3)_2$, 99.999%) (Merck KGaA, Germany), 2-propanol (≥99.9%) (Fluka, Germany) and hydrochloric acid (35–37%) (Samchun, Korea) were used to make a titania sol-gel. Nafion® 117 solution (perfluorinated ion-exchange resin, ~5% in a mixture of lower aliphatic alcohols and water), tris(2,2′-bipyridyl)dichlororuthenium(II) hexahydrate ($Ru(bpy)_3Cl_2{\cdot}6H_2O$), alcohol dehydrogenase (from *Saccharomyces cerevisiae*, ≥300 units/mg protein) and ionic liquids were purchased from Merck KGaA (Darmstadt, Germany). β-Nicotinamide adenine dinucleotide sodium salt (β-NAD^+), β-Nicotinamide adenine dinucleotide reduced disodium salt hydrate (β-NADH), ethanol (pure), L-ascorbic acid, and acetic acid were used for optimization of the biosensor for the detection of ethanol. All reagents were purchased from Merck KGaA (Darmstadt, Germany). Water used in all experiments was deionized using a Milli-Q water purification system (Millipore, Bedford, MA, USA). Solutions for electrochemical experiment were prepared with phosphate buffer solution at pH 7.0.

2.2. Instrumentation

Cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) experiments were performed with an EG&G Princeton Applied Research 263A potentiostat (Pennsylvania, USA) and a frequency response detector (Model 1025, Oak Ridge, TN, USA). All experiments were carried out with a conventional three-electrode system in a 10 mL electrochemical cell made of quartz. Glassy carbon electrode (GCE) was used as a working electrode (0.071 cm^2), on which composite films were cast directly. A platinum wire was used as the counter electrode and all electric potentials stated in the experiment were relative to Ag/AgCl (3 M NaCl) reference electrode. Light emitted during ECL experiments was measured with a Hamamatsu Photonics H9319-12 photon counting head (Shizuoka, Japan). The counted photon signals were collected with EVAL40 program. The electrochemical cell was placed just before the window of the photomultiplier tube. The entire ECL experiments were performed in a light-tight box. All experiments were conducted under ambient conditions.

2.3. Preparation of the $Ru(bpy)_3^{2+}$ ECL sensor

The ECL sensor was fabricated by modifying the glassy carbon electrode (GCE) with an IL-titania-Nafion composite and immobilizing $Ru(bpy)_3^{2+}$ on the surface of composite-modified GCE. The composite solution was prepared as follows. First, the titania sol-gel was made by mixing deionized water with titanium(IV) isopropoxide dissolved in 2-propanol containing hydrochloric acid as a catalyst. The concentration and proportion of each substance were based on the results of a previously reported study [20]. Then, a Nafion solution diluted to the desired concentration with an 80 v/v% ethanol was mixed with the same volume of prepared titania sol-gel *via* ultrasonication. During this process, the temperature of water in the soni-bath was kept constant at a low temperature because Nafion is structurally unstable in high temperature [22]. After ultrasonication for about 40 min, a certain amount of IL was added to the solution and then the mixture was stirred for more than half a day to form an IL-titania-Nafion composite solution. Finally, 0.1 M $Ru(bpy)_3^{2+}$ solution was added to the IL-titania-Nafion composite solution (7:93, v/v). A 3 μL aliquot of the Ru $(bpy)_3^{2+}$/IL-titania-Nafion composite solution was cast on the GCE. The composite-modified electrode surface was dried for 20 min in dark room under ambient conditions to form a thin film. The modified electrode was then immersed in 50 mM phosphate buffer solution (PBS) at pH 7.0 for 20 min to swell the film and remove excess materials that are not sufficiently bound to the electrode surface. The modified electrode was then electrochemically stabilized by running consecutive cyclic potential scans in 50 mM PBS at pH 7.0, from +0.8 V to +1.35 V *vs.* Ag/AgCl (3 M NaCl) at a scan rate 100 mV/s until constant cyclic voltammogram was obtained. Once the oxidation current of Ru $(bpy)_3^{2+}$ immobilized on the electrode was saturated, an ECL sensor based on $Ru(bpy)_3^{2+}$/IL-titania-Nafion composite was ready for use.

2.4. Preparation of the ECL biosensor for ethanol detection

The ECL biosensor was fabricated based on the double-layer format involving a layer for the immobilization of alcohol dehydrogenase (ADH) and another for the immobilization of $Ru(bpy)_3^{2+}$ on the electrode. The ADH layer was made by mixing an ADH solution prepared in 50 mM PBS at pH 7.0 with the same volume of titania-Nafion or IL-titania-Nafion composite solution. To prepare the biosensor, a 3 μL aliquot of ADH solution was cast on top of the $Ru(bpy)_3^{2+}$ immobilized electrode, and it was dried for 20 min in dark room under ambient conditions. Finally, the biosensor was immersed for 10 min in 50 mM PBS at pH 7.0 to swell the composite film and wash away the unbound ADH from the electrode surface. The biosensor was stored in 50 mM PBS buffer at pH 7.0 at 4 °C when it was not in use. All ECL measurements were performed in the potential range from +0.0 V to +1.35 V *vs.* Ag/AgCl (3 M NaCl) at a scan rate 100 mV/s.

3. Results and discussion

3.1. Electrochemical and ECL behavior of composite modified ECL sensor

The analytical performance of the current ECL sensor for NADH detection greatly affects the overall performance of the ECL ethanol biosensor since NADH is the product of the enzymatic oxidation of ethanol into acetaldehyde with NAD^+ as a cofactor. As shown in Fig. 1, the overall mechanism of ethanol detection based on $Ru(bpy)_3^{2+}$ ECL