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Short communication

Electrogenerated chemiluminescence ethanol biosensor based on alcohol dehydrogenase functionalized $Ru(bpy)_3^{2+}$ doped silica nanoparticles

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ABSTRACT

An ethanol biosensor, based on the electrogenerated chemiluminescence of $Ru(bpy)_3^{2+}$-doped silica nanoparticles (RuSiNPs), was investigated in this study. The biosensor was a modified glassy carbon electrode, where alcohol dehydrogenase was crosslinked to RuSiNPs, and then immobilized on the electrode surface using chitosan. The results indicated that the biosensor exhibited excellent performance during ethanol determination with a wide linear range (10^{-7} to 10^{-2} M), low detection limit (5.0×10^{-8} M) and good stability.

1. Introduction

Nowadays, alcohol abuse has become a worldwide social problem and a public sanitation issue. In order to prevent the damage caused by overdrinking and to promote pharmaceutical and food safety, fast, sensitive and selective analytical methods are required for ethanol determination (Martinis et al., 2004; Niculescu et al., 2002; Santos et al., 2003). At present, many methods are available for ethanol determination, such as gas chromatography (Kitagawa et al., 1991; Zilly et al., 2003), high-performance liquid chromatography (Liden et al., 1998), infrared spectroscopy (Gallignani et al., 1993), as well as fluorescence, colorimetric methods, and redox titrations. However, some of these approaches suffer from drawbacks including difficulties during operation, a lack of sensitivity and a requirement for expensive instruments. The enzymatic method is an effective approach to overcome these problems. Enzymes, biological catalysts produced by cells, are able to catalyze various biochemical reactions efficiently in vivo. Their excellent characteristics, including high efficiency and specificity for enzymatic reactions reveal that enzymes could be taken as powerful agents for chemical analysis, and could be applied together with many analytical methods, such as the widely available spectroscopic and electrochemical methods (Azevedo et al., 2005). With this in mind, biosensors based on an enzymatic reaction offer promise for application in biomolecular detection.

Electrochemiluminescence (ECL) has been applied in many analytical areas due to its versatility, simplified optical setup, reproducibility and high sensitivity (Knight, 1999; Richter, 2004; Yin et al., 2004; Gorman et al., 2006). As early as 1992, tri(2,2′-bipyridyl) ruthenium(II) ($Ru(bpy)_3^{2+}$) ECL was first employed to quantify nicotinamide-adenine dinucleotide phosphate (NADH) (Downey and Nieman, 1992). Jameison et al. (1996) propose ECL based assay as an alternative to quantify clinical analytes such as ethanol that can be linked to the NADH-forming enzyme. The immobilization of $Ru(bpy)_3^{2+}$ on an electrode surface reduces the reagent consumption and gives brighter luminescence, and Martin and Nieman (1997) describe the immobilization of an enzyme and $Ru(bpy)_3^{2+}$ as adjacent layers on an electrode using Nafion film. In the past few years, a sol–gel technique was developed for sensor probe immobilization. In view of the compatibilities of sol–gel materials, alcohol dehydrogenase (ADH) and $Ru(bpy)_3^{2+}$ were immobilized in a sol–gel hybrid film for ethanol determination (Xu et al., 2005). Subsequently, Zhang et al. (2007) made a novel ethanol biosen-

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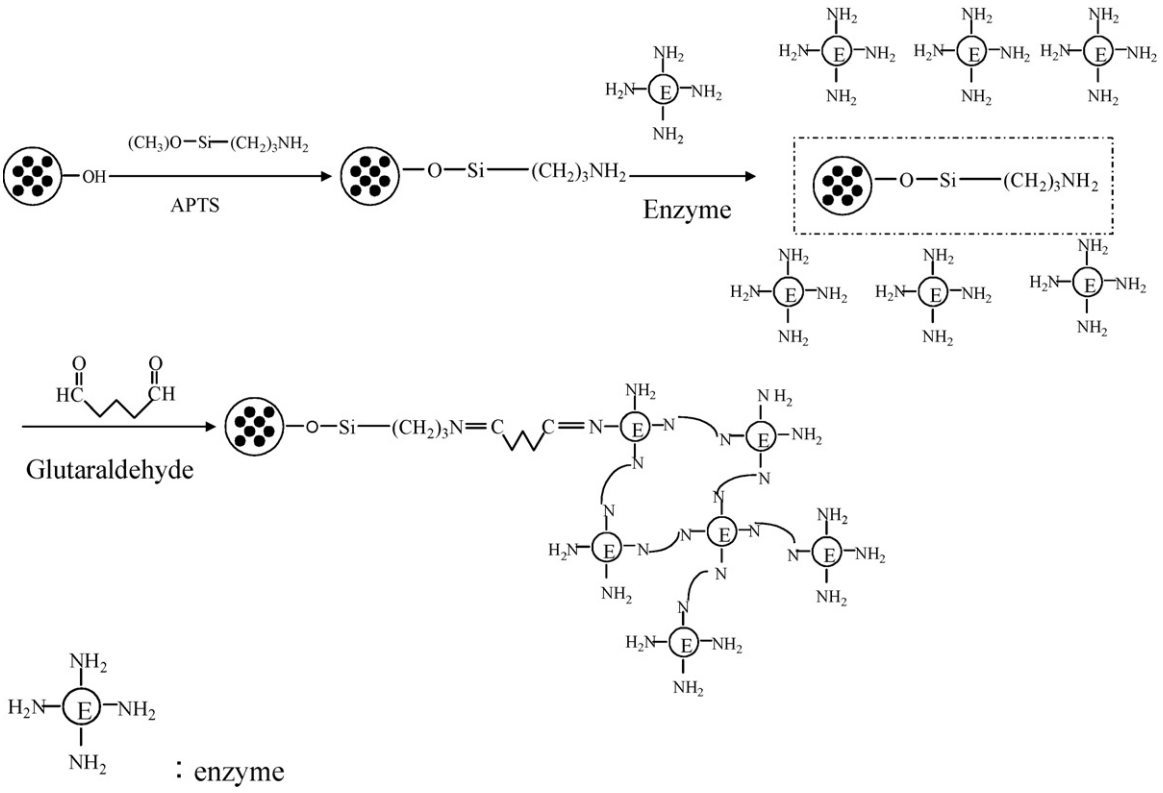

Scheme 1. Schematic diagram of crosslinking enzyme clusters on RuSiNPs.

sor involving the self-assembling of ADH and $Ru(bpy)_3^{2+}$–gold nanoparticles aggregated on an indium tin oxide electrode surface. However, these methods have some disadvantages. Generally, when ADH and $Ru(bpy)_3^{2+}$ are immobilized in the same layer, ADH activity decreases obviously because the high concentration of $Ru(bpy)_3^{2+}$ increases the hydrophobicity of the layer microenvironment (Martin and Nieman, 1997). In addition, the leakage of $Ru(bpy)_3^{2+}$ during continuous determination causes the modified electrode to be unstable.

$Ru(bpy)_3^{2+}$-doped silica nanoparticles (RuSiNPs) incorporate the $Ru(bpy)_3^{2+}$ into silica material in order to prevent them leaking from the electrode surface, and provide a suitable surface for enzymes to contact directly (Santra et al., 2001a,b; Chang et al., 2006; Zhang and Dong, 2006; Wang et al., 2007; Wei et al., 2008a,b; Miao, 2008; Zanarini et al., 2009). In this study, taking glutaraldehyde (GA) solution and aminopropyltrimethoxysilane (APTS) as linking agents, we produced a novel ethanol biosensor by crosslinking ADH to RuSiNPs. Chitosan (CHIT) was used to immobilize the composites onto the surface of a glassy carbon electrode (GCE). To the best of our knowledge, there is no report of such a combination between ECL of RuSiNPs and enzyme reactions. Compared with reported ethanol biosensors, this novel biosensor exhibited high sensitivity, wide linear range and good stability (Scheme 1).

2. Experimental

2.1. Reagents and instrumentation

ADH (from baker's yeast, 451 U/mg solid), β-nicotinamide adenine dinucleotide (β-NAD^+) (sodium salt, from yeast), and Triton X-100 (TX-100) were purchased from the Sigma Chem. Co. (USA). Tri(2,2′-bipyridyl) dichlororuthenium(II) hexahydrate ($Ru(bpy)_3Cl_2{\cdot}6H_2O$); triethoxysilane, $HSi(OCH_2CH_3)_3$, (TEOS); and dimethyldimethoxysilane, $(CH_3)_2Si(OCH_3)_2$, (DiMe–DiMOS) were obtained from Fluka AG (Buchs, Switzerland) and used as received. CHIT (from crab shells, practical grade), and APTS were supplied by Aldrich (Milwaukee, WI, USA). A 1% CHIT solution was prepared by dissolving CHIT in 1% acetic acid solution with magnetic stirring for ~2 h. GA (25% aq) was obtained from Alfa Aesar (Karslruhe, Germany). Cyclohexane, 1-hexanol, acetone, ethanol, toluene and ammonia (25 wt%) were purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). All other chemicals were of analytical grade, and the water used throughout all the experiments was purified using a Millipore purification system (Millipore, MA, USA).

Cyclic voltammetric experiments were performed on a CHI 830 Electrochemical Analyzer (Chenhua Inc., China). ECL experiments were carried out on the CHI 830 and an IFFM-D FIA Luminescence Analyzer (Ruimai Co., China) at room temperature. The three-electrode system was composed of a modified GCE (BAS, Tokyo, Japan) coated with ADH–RuSiNPs/CHIT composite film as the working electrode, a platinum wire as the auxiliary electrode and Ag/AgCl (saturated KCl) as the reference electrode. The size of RuSiNPs and amino-functionalized RuSiNPs were examined under a Tecnai F30 (FEI Co., USA) transmission electron microscope (TEM) operated at an accelerating voltage of 300 kV. Fourier transform infrared (FTIR) spectra were obtained using a FT-IR7400SX spectrometer (Nicolet, USA).

2.2. Preparation of modified electrodes

Before each modification, a GCE was polished with 1 and 0.3 μm aluminum slurry and sonicated in pure water thoroughly. Then, the GCE was allowed to dry in nitrogen.

RuSiNPs were prepared using the microemulsion method (Santra et al., 2001a,b). First, the microemulsion was produced by mixing 1.77 mL of TX-100, 7.5 mL of cyclohexane, 1.8 mL of 1-hexanol, 400 μL 20 mM $Ru(bpy)_3^{2+}$ and 100 μL of water. In the

presence of 100 μL of TEOS, a polymerization reaction was initiated by adding 60 μL of $NH_3 \cdot H_2O$ solution (25%). The reaction was stirred for 24 h, and after the reaction was completed, 2 mL of acetone was added to the mixture to precipitate RuSiNPs. The products were washed with acetone, ethanol, and water in sequence and dried before use. These RuSiNPs were suspended in 3 mL toluene by ultrasonication, and then, 10 μL of APTS was added. The mixture was heated to 85 °C and kept at this temperature for 24 h with magnetic stirring. The final products were rigorously washed with a phosphate buffer solution (PBS; 150 mM, pH 7.5). To crosslink ADH on the amino-functionalized RuSiNPs, 1 mg of ADH was mixed well with 0.5 mg of the amino-functionalized RuSiNPs in 2 mL PBS. Then, GA solution was added to the mixture to obtain a final solution of 0.1% (w/v). The mixture was incubated at 4 °C with a magnetic stirrer (200 rpm) for 17 h (Lee et al., 2008).

ADH-functionalized RuSiNPs were also prepared using the covalent attachment method. The amino-functionalized RuSiNPs were incubated in 1% GA solution for 30 min. After excess GA was removed by thorough washing in buffer solution, the sample was incubated in enzyme solution for 17 h. After immobilization, the products were washed five times with PBS.

These functionalized RuSiNPs were then suspended in PBS using ultrasonication and mixed with 1% CHIT solution. A 2-μL aliquot of ADH–RuSiNPs/CHIT composite was coated on the GCE surface and allowed to dry at 4 °C.

2.3. Measurement of enzyme activity

The activities of free and immobilized enzymes were measured using the NAD^+ and ethanol reaction in the presence of ADH. The formation of NADH was evaluated by measuring the increase in absorbance at 340 nm using a spectrophotometer (Rella et al., 1987; Schoevaart et al., 2004). For the activity measurement of the immobilized enzyme systems, an aliquot of sample was mixed with ethanol and NAD^+ and shaken at 200 rpm. The change of absorbance at a wavelength of 340 nm was measured time-dependently after centrifugation at 20,000 rpm (Kim et al., 2006).

3. Results and discussion

3.1. Characterization of RuSiNPs

Spherical RuSiNPs were prepared using a microemulsion method (Santra et al., 2001a,b). TEM images of RuSiNPs and amino-functionalized RuSiNPs are shown in Fig. S1. The diameter of these nanoparticles is around 40 nm. No changes in shape and size could be found after their amino-functionalization.

3.2. Amino-functionalization of RuSiNPs

For application of RuSiNPs as a host material for enzymes, grafting of a functional group onto their surfaces is important (Lee et al., 2008). APTS is generally used to functionalize RuSiNPs by forming an amino group on their surfaces. The existence of an amino group can be confirmed using one of the two methods: First the reaction of fluorescamine, a reagent used for the detection of the amino group is almost instantaneous with amines at room temperature in aqueous media. The products are highly fluorescent, whereas the reagent and its degradation products are nonfluorescent (Sidney et al., 1972; Santra et al., 2001a,b; Qhobosheane et al., 2001). As shown in Fig. S2, the product has a maximum absorption at 387 nm and maximum fluorescence intensity at 498 nm. The other method is to apply the FTIR technique, since its spectrum (Fig. S3) can be used to confirm the existence of amino groups on the surface of RuSiNPs and the silica network from the characteristic Si–O–Si asymmetric stretching vibration.

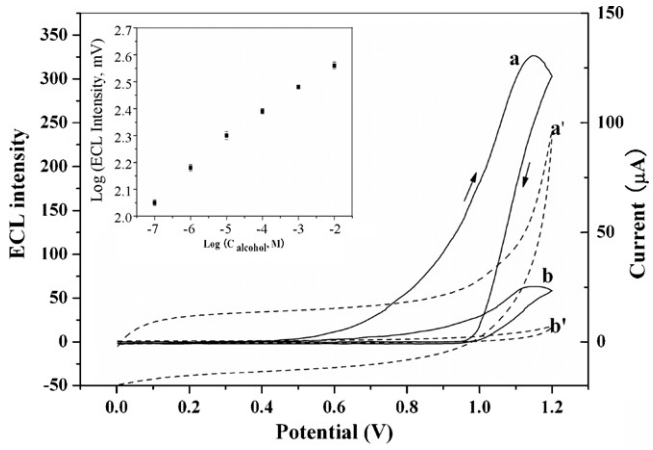

Fig. 1. ECL–potential curves for the modified electrode without (b) and with (a) 1 mM ethanol in PBS (150 mM, pH 7.5) containing 1 mM NAD^+, and the corresponding cyclic voltammogram in the absence (b′) and the presence (a′) of ethanol. (Inserted figure) Calibration curve of ethanol with the prepared ECL biosensor in PBS (150 mM, pH 7.5).

3.3. Immobilization of ADH on amino-functionalized RuSiNPs

GA is generally applied to bind ADH to functional materials (Betancor et al., 2007). Here, we compared the immobilization of ADH on the surfaces of RuSiNPs using crosslinking and covalent attachment methods (Kim et al., 2006; Torabi et al., 2007; Dai et al., 2008). The main difference between the two approaches is the sequence of GA and ADH addition (Lee et al., 2008). Based on the experimental results, the crosslinking immobilization strategy provided an increase in enzyme specific activity compared to that of the covalent attachment method. Shear stress and collisions caused by the severe shaking during the immobilization and washing processes can damage the enzyme molecules (Lee et al., 2008). However, the crosslinking approach is expected to form multilayer enzyme molecules on the surfaces of nanoparticles via intermolecular crosslinking (Lee et al., 2005), which can prevent enzyme inactivation during immobilization.

3.4. ECL response of the biosensor to ethanol

The ECL intensity–potential curves and the corresponding cyclic voltammogram of the modified electrode in the absence and the presence of ethanol at the scan rate of 0.06 V/s are presented in Fig. 1. The onset of luminescence occurred near 0.9 V, at which point $Ru(bpy)_3^{2+}$ was oxidized to $Ru(bpy)_3^{3+}$. The ECL intensity rose until it reached a maximum near 1.16 V, which was consistent with the oxidation potential of the $Ru(bpy)_3^{2+}$. The detection was based on coupling the enzymatic reaction between ethanol and NAD^+ with $Ru(bpy)_3^{2+}$ ECL reaction. In the solution of ethanol, the ECL intensity increased markedly, indicating the occurrence of the ECL reaction between NADH and $Ru(bpy)_3^{3+}$ (Xu et al., 2005). The ECL intensity increased with the addition of ethanol, which was applied for the ECL biosensing determination of ethanol.

3.5. Optimization of the experimental conditions and calibration curve

As mentioned above, enhancement of the ECL intensity resulted from the enzyme catalyzed reaction between ethanol and NAD^+. Since the buffer solution pH could play a key role in enzyme activity, we studied the effect of pH on the response of the biosensor (Fig. S4). It is reported that the pH range for optimal ADH activity is from 8.4 to 9.5 (Xu et al., 2005; Zhang et al., 2007), and that the ECL intensity in the detection of NADH is nearly constant when pH increases

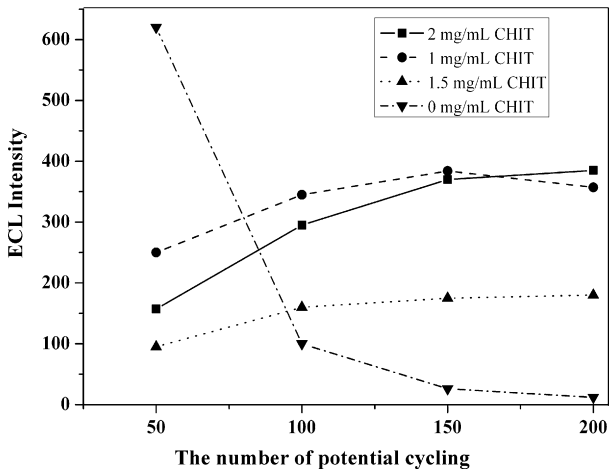

Fig. 2. Effect of CHIT concentration on ECL intensity in PBS (150 mM, pH 7.5) containing 1.0 mM ethanol and 1.0 mM NAD^+.

above 5.5 (Downey and Nieman, 1992; Martin and Nieman, 1993). In our sensing system, the observed ECL response was the integrated result of both enzyme catalysis and ECL reactions. Although the observed maximum ECL response of the biosensor could be obtained in the pH range 8.0–8.5, NAD^+ became unstable in this pH range. Based on the experimental results, a pH 7.5 buffer solution was selected in all experiments.

The effect of the applied potential scan rate on ECL intensity was studied. The ECL intensity decreased with the increase in scan rate in the range 20–200 mV s^{-1} in the presence of 1.0 mM ethanol. Two factors, the formation and diffusion of the intermediate at different scan rates, affected ECL intensity. When the scan rate increased, shorter reaction time led immediately to lower concentration and smaller transmission of the reaction, resulting in a lower ECL intensity. Although the ECL intensity increased at a lower scan rate, the poor repeatability and the long reaction time limited the ECL application. Considering the above factors, 60 mV s^{-1} was chosen as the suitable applied potential scan rate.

CHIT is a polysaccharide biopolymer, which displays excellent film-forming ability, high water permeability, and good adhesion to the electrode surface (Zhang et al., 2004, 2006). Generally, acetic acid, used as the solvent for CHIT, decreases ADH activity and, in addition, the addition of CHIT increases steric hindrance and limits substrate diffusion to the modified electrode. Although the use of CHIT for the immobilization of the functional nanoparticles resulted in a slight decrease in the ECL response, a suitable amount of CHIT reduced the negative effect and obviously increased the modified electrode stability and usage life (Fig. 2). In the present work, 1 mg mL^{-1} CHIT was used in all experiments.

3.6. Sensitivity and stability of the biosensor

The present ECL biosensor was used for ethanol determination and it was found that the sensor response to ethanol increased with increasing ethanol concentration. Based on the experimental results (Fig. 1(insert)), the calibration curve for ethanol is linear in the ethanol concentration range 10^{-7} to 10^{-2} M ($R^2 = 0.9953$) with a detection limit of 5.0×10^{-8} M (S/N = 3). Each point was shown in Fig. 1(insert) is a mean of three ECL signals obtained by consecutively cyclic potential scanning at the same concentration of ethanol. Compared with the value reported in the literature (Xu et al., 2005; Zhang et al., 2007), the present ECL biosensor gave a higher sensitivity and wider linear concentration range. The improved sensitivity could be attributed to the large amount of $Ru(bpy)_3^{2+}$ doped in the film, which greatly increased the ECL response.

Experimental results (Fig. S6) indicated that the ECL response remained nearly constant under continuously cyclic potential scanning for 15 cycles in PBS (pH 7.5) containing 1 mM ethanol and NAD^+, suggesting its excellent operation stability. The long term storage stability of the present sensor (Fig. S7) was studied over 2 weeks by monitoring its ECL response to 1.0 mM ethanol with intermittent usage (every 2–3 days) and by storing at 4 °C when not in use. After 2 weeks, the biosensor retained 80% of its original ECL response. The good stability of the sensor could be attributed to the following interactions: (1) the strong electrostatic interaction between positively charged $Ru(bpy)_3^{2+}$ and negatively charged silica nanoparticles; (2) multiple-point attachment of the enzyme molecules, which effectively prevented the denaturation of the enzyme molecules; and (3) the H-binding interaction between ADH and CHIT.

4. Conclusions

Combination of the ECL of RuSiNPs and the ADH enzymatic reaction was used for the detection of ethanol. The ECL reagent, $Ru(bpy)_3^{2+}$, was concentrated in SiNPs, and presented a high ECL intensity after it was immobilized onto a GCE. Moreover, using the crosslinking immobilization method for enzymes maintained both the activity and stability of the ADH–RuSiNPs composite. With the application of CHIT, the resulting biosensor displayed remarkable reproducibility and stability. The ECL intensity presented good linearity with different concentrations of ethanol in the wide range 10^{-7} to 10^{-2} M, which ensured that the ECL biosensor could be used for ethanol determination in a variety of substrates and revealed great potential for its use in other biological assays.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2009.06.030.

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