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Electrochemiluminescence glucose biosensor based on glucose dehydrogenase functionalized $Ru(bpy)_3^{2+}$ doped silica nanoparticles

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A novel electrochemiluminescence (ECL) sensing approach was developed for glucose detection based on crosslinking $Ru(bpy)_3Cl_2$-doped silica nanoparticles (RuSiNPs) with glucose dehydrogenase on a glassy carbon electrode (GCE). Glutaraldehyde and aminopropyltrimethoxysilane were used as linking agents, and chitosan was used to immobilize the composites onto the GCE surface. The ECL sensor presented good characteristics in terms of stability and reproducibility. Under optimized conditions, the linear response of ECL intensity to glucose concentration was valid in the range of 0.2 to 20 mmol/L (R^2 = 0.9962). The application results indicated that the proposed approach is with great potential in the determination of glucose.

electrochemiluminescence, sensing, silica nanoparticles, tris(2,2'-bipridine) ruthenium, glucose, glucose dehydrogenase

1 Introduction

Diabetes mellitus is a chronic but treatable disease affecting about 200 million people around the world [1]. Determination of glucose in human blood is the sole standard of diagnosing diabetes. A fast, exact and reliable detection approach is still important and a pursuant quaesitum in the early diagnosis, classification, observation and prognosis of diabetes. Recently, the interest in practical electrochemiluminescence (ECL) glucose sensors has been centered on the efforts to find a breakthrough in the sensitivity and applications. In the glucose biosensors based on luminol ECL [2–6], hydrogen peroxide produced by enzymatic reaction of glucose oxidase (GOD) is used to enhance luminol ECL in the detection of glucose. The luminescence efficiency of luminol ECL is higher than those of other ECL reagents; however, the poor measurement stability caused by the alkaline media in luminol ECL is still a serious problem. In addition, the alkaline media restrain the activity of enzymes, resulting in lower sensitivity in the glucose determination. Ju group [7] fabricated the first biosensor based on the intrinsic ECL of quantum dots (QDs) coupled with an enzymatic reaction with GOD as a model. Using GOD as a model enzyme, the biosensor for oxidase substrate is constructed by incorporating GOD in thioglycolic acid-capped CdSe QD film. The sensing approach shows sensitive ECL responses to glucose in a wide linear range, satisfying reproducibility and acceptable stability. This consideration can be applied in more bioanalytical systems for oxidase substrates.

$Ru(bpy)_3Cl_2$-based ECLs have been applied in many analytical areas due to their versatility, simplified optical setup, good reproducibility and acceptable sensitivity [8–11]. Recently, as a new immobilization approach, $Ru(bpy)_3Cl_2$-doped silica nanoparticles (RuSiNPs), incorporating $Ru(bpy)_3Cl_2$ into silica material, have got great attention in ECL studies since this approach prevents $Ru(bpy)_3Cl_2$ leaking from the electrode surface effectively, and provides a suitable surface for enzymes to contact directly [12–14]. In addition, the concentrated $Ru(bpy)_3Cl_2$ in silica nanoparti-

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cles provides a higher effective concentration of $Ru(bpy)_3Cl_2$ on the electrode surface, resulting in higher or even visible luminescence [15].

In this study, we produced a novel ECL biosensor for glucose determination by crosslinking glucose dehydrogenase (GDH) to RuSiNPs using glutaraldehyde (GA) and aminopropyltrimethoxysilane (APTS) as linking agents. Chitosan (CHIT) was employed to immobilize the composites onto the surface of a glassy carbon electrode (GCE). The effect factors for the ECL responses were also discussed. In addition, the proposed approach was applied to the determination of glucose in glucose-NaCl injection and beer samples.

2 Materials and methods

2.1 Chemicals and instruments

Glucose dehydrogenase (GDH, thermoplasma acidophilum, 5.5 mg Protein/mL, 279 U/mg Protein), β-nicotinamide adenine dinucleotide (β-NAD^+) (sodium salt, from yeast), and Triton X-100 (TX-100) were purchased from Sigma Chem. Co. (USA). Tri(2,2′-bipyridyl) dichlororuthenium(Ⅱ) hexahydrate ($Ru(bpy)_3Cl_2·6H_2O$), triethoxysilane (TEOS), and dimethyldimethoxysilane (DiMe-DiMOS) were obtained from Fluka AG (Buchs, Switzerland) and used as received. Chitosan (CHIT, from crab shells, practical grade) and aminopropyltrimethoxysilane (APTS) were supplied by Aldrich (Milwaukee, WI, USA). 1% CHIT solution was prepared by dissolving CHIT in 1% acetic acid solution with magnetic stirring for ~2 h. Glutaraldehyde (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).

Glucose injections were acquired from a local hospital and diluted using phosphate buffer solution (0.5 mol/L Na_2HPO_4 and KH_2PO_4, pH 7.0, PBS). Beer samples were bought from a local supermarket and directly detected after being filtrated and degassed.

Cyclic voltammetric (CV) 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. A 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 a Ag/AgCl (saturated KCl) electrode 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.

2.2 Preparation of the modified electrode

Before modification, a GCE was polished with 1 and 0.3 μm aluminum slurry and sonicated in pure water thoroughly, and then the GCE was allowed to dry in nitrogen. RuSiNPs were prepared as the reported microemulsion methods [14, 17]. In the preparation of amino-functionalized RuSiNPs, APTS was used to functionalize RuSiNPs by forming an amino group on their surfaces. The existence of an amino group can be confirmed by fluorescence or FTIR technique as shown in reports [14, 16]. To crosslink GDH on the amino-functionalized RuSiNPs, 1 mg of GDH 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 ^oC with a magnetic stirrer (200 r/min) for 17 h [17]. These functionalized RuSiNPs were then suspended in PBS using ultrasonication and mixed with 1% CHIT solution. All the procedure could be described as shown in Scheme 1. After the preparation, a 2-μL aliquot of ADH-RuSiNPs/CHIT composite was coated on the GCE surface and allowed to dry at 4 °C for further usage.

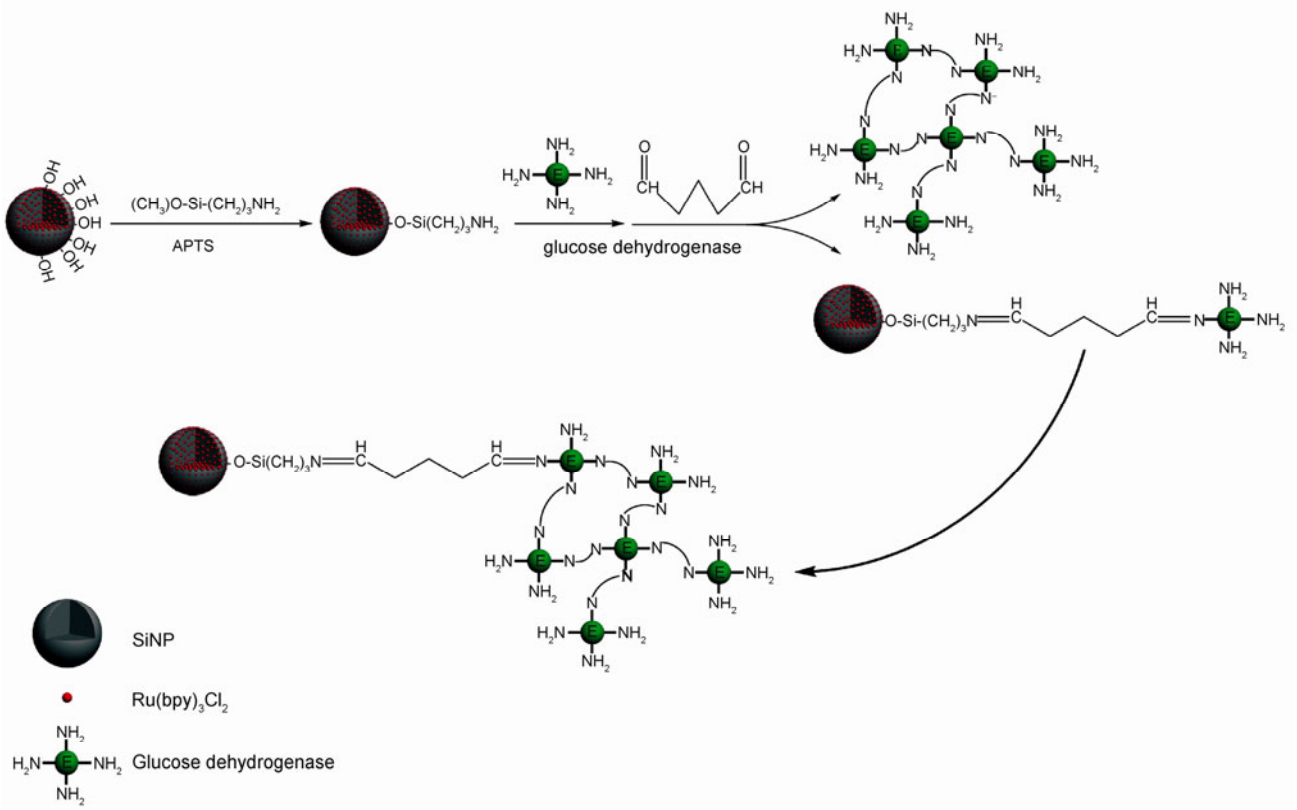
3 Results and discussion

3.1 Characterization of RuSiNPs

Spherical RuSiNPs could be obtained using the microemulsion method [14, 17]. Direct morphology observation of the RuSiNPs and amino-functionalized RuSiNPs by TEM tests are shown in Figure 1. From the figures, we can find that formed RuSiNPs were well separated and their average size was around 40 nm. From Figure 1(b), no change in shape or size could be found after their amino-functionalization.

3.2 ECL response of the modified electrode to glucose

The catalytic ECL sensing mechanism between $Ru(bpy)_3^{2+}$ and enzyme was reported by Marquette *et al*. [18]. Similarly, NADH could be generated after the reduction reaction between NAD^+ and glucose. In the meantime, at a suitable applied potential, $Ru(bpy)_3^{2+}$ could be oxidized to $Ru(bpy)_3^{3+}$, and NADH lost a proton to be its radical, $NAD^•$, which would react with $Ru(bpy)_3^{3+}$ and result in ECL emission. In the experiments, using the GDH-RuSiNPs modified electrode at the scan rate of 0.06 V/s, the ECL intensity-potential responses and the corresponding CV curves in the absence or the presence of glucose are presented in Figure 2. The onset of luminescence occurred near +0.9 V, where $Ru(bpy)_3^{2+}$ began to be oxidized to $Ru(bpy)_3^{3+}$. The ECL intensity increased until the applied potential reached a maximum value near +1.06 V, which was consistent with the oxidation potential of $Ru(bpy)_3^{2+}$. In the solution of



Scheme 1 Schematic diagram of crosslinking GDH clusters on RuSiNPs.

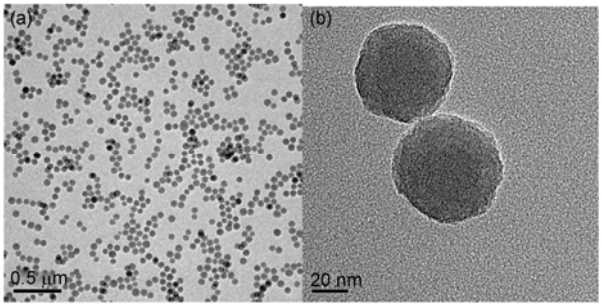


Figure 1 TEM images of prepared RuSiNPs (a) and amino-functionalized RuSiNPs (b).

glucose, since the high specific enzymatic reaction between glucose and NAD^+ coupled $Ru(bpy)_3^{2+}$ ECL reaction, the ECL intensity increased obviously, indicating the occurrence of the ECL reaction between NADH and $Ru(bpy)_3^{3+}$. The ECL intensity increased with the addition of glucose. This result indicated that this approach was available for the glucose ECL sensing.

3.3 Optimization of the experimental conditions

As mentioned above, the enzyme catalyzed the reaction between glucose and NAD^+ enhanced the ECL intensity. Since the pH of the buffer solution would take an important role in the enzyme activity, the effect of pH on the response of the ECL responses was studied. As shown in Figure 3, the maximum ECL intensity could be obtained at pH 8.5. It is reported that the pH for optimal GDH activity is at 8.5, and the ECL intensity in the detection of NADH keeps nearly constant when pH is above 5.5 [19, 20]. The observed ECL response was the integrated result of both enzyme catalysis and ECL reactions. Although the maximum ECL response could be obtained in pH 8.5, NAD^+ became unstable at this pH value. Therefore, pH 7.5 buffer solution was selected in the all experiments.

The effect of the potential scan rate on ECL intensity was studied. Experimental results revealed that the ECL intensity decreased greatly with the increase of scan rate in the

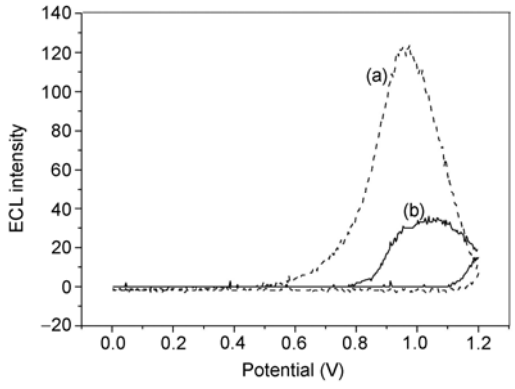


Figure 2 ECL-potential curves for the modified electrode without (b) and with (a) 10 mM glucose in PBS (150 mM, pH 7.5) containing 2.2 mM NAD^+. Conditions: scan rate: 0.06 V/s; scan potential range: 0–1.2 V.

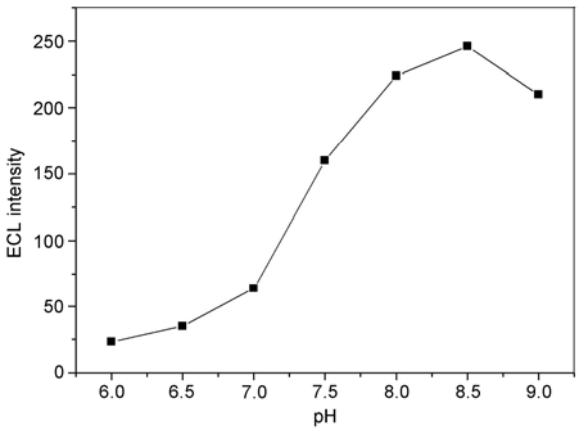


Figure 3 Effect of pH on ECL intensity.

range of 0.02 to 0.10 V/s. ECL intensity was related to the concentrations of the oxidation products of $Ru(bpy)_3^{2+}$ and NADH, $Ru(bpy)_3^{3+}$ and $NAD^{\bullet}$. The higher potential scan rate caused lower concentration and smaller transmission of the reactants to the electrode, and resulted 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 sensing applications. Considering both efficiency and sensitivity, 0.06 V/s 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 an electrode surface [21, 22]. The addition of CHIT increases steric hindrance and limits substrate diffusion to the modified electrode. In the research, GDH modified RuSiNPs were linked with CHIT by hydrogen bond interaction, then the composites were fixed on the electrode surface. In our experiments, the CHIT content in the composite for the modified electrode greatly affected the sensitivity and stability of ECL responses. Without CHIT immobilization, RuSiNP easily desquamated from the GCE surface and lost their ECL capability. Using the modification composite without CHIT, ECL intensity obviously decreased from 4700 mV to 130 mV after 150 cycle measurements, but, in the same experiment, ECL intensity kept constant if the volume ratio of CHIT and GDH-RuSiNPs reached 1:1. Increasing the volume ratio of CHIT to GDH-RuSiNPs (such as 2:1), very stable but lower ECL intensity could be obtained. 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 stability and usage life for the GDH-RuSiNPs modified electrode. Considering the ECL stability and sensitivity, the volume ratio of CHIT and GDH-RuSiNPs was chosen as 1:1 in all experiments.

3.4 Evaluation of the modified electrode for glucose ECL sensing

The long-term storage and operational stability of the electrode is essential for the continuous monitoring of glucose. The stability of the present electrode was examined using the same GDH-RuSiNPs modified electrode for 20 repetitive measurements in successive addition of 2.0 mmol/L glucose. The relative standard deviation was 4.2% (Figure 4), confirming that the as-synthesized electrode for glucose ECL sensing was stable. In the preparation of the modified electrode, the electrode-to-electrode reproducibility was found to be 6.2% using six electrodes prepared in the same procedure. The long-term storage was evaluated by measuring its sensitivity to glucose within two weeks. The GDH-RuSiNPs modified electrode was stored in air and the sensitivity was tested every 2 days. The result demonstrated that the sensitivity was 95% of its initial sensitivity after two months storage. These results indicated that GDH-RuSiNPs modified electrode displayed good long-term storage and excellent stability, promising a desirable glucose sensor for most routine analyses. The good stability of the modified electrode could be attributed to the following interactions: (1) in RuSiNPs, 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) H-binding interaction between GDH and CHIT.

To evaluate the sensitivity of the GDH-RuSiNPs modified electrode, ECL responses were measured under different concentration of glucose at the above optimal condition. It was found that ECL intensity increased with the increase of glucose concentration. Under the optimal experimental conditions, ECL intensity linearly increased with the glucose concentration in the range of 0.2 to 20 mmol/L (R^2 = 0.9962).

3.5 Sample analysis

The ECL sensing approach using the GDH-RuSiNPs modified electrode was applied to the analysis of glucose samples. Commercial glucose injection and beer samples were detected using both spectrometric method (Sigma-Aldrich

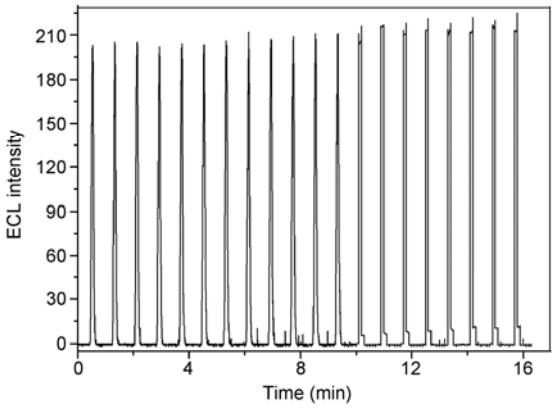


Figure 4 ECL intensity from the biosensor under continuous potential scanning for 20 cycles.

Table 1 ECL determination results and recoveries of glucose samples using a GDH-RuSiNPs modified GCE (n = 6)

Sample	Found (mmol/L)		Content (%)		RSD (%)	Added (mmol/L)	Found (mmol/L)	Recovery (%)
	ECL	GAGO20[a)]	ECL	GAGO20[a)]				
Glucose injection 1#[b)]	1.28±0.3	1.35±0.3	4.58	4.83	3.8	1.0	2.48	105.6±3.7
Glucose injection 2#[b)]	1.22±0.2	1.27±0.2	4.37	90.3	3.1	1.0	2.24	98.6±3.3
Glucose injection 3#[b)]	1.38±0.1	1.34±0.2	4.94	88.2	3.2	1.0	2.30	107.0±3.5
Beer 1#[c)]	0.26±0.03	0.30±0.03	0.0047	0.0054	4.2	0.5	0.78	102.8±3.8
Beer 2#[c)]	0.22±0.02	0.20±0.02	0.0039	0.0036	4.6	0.5	0.81	112.3±4.5

a) Samples were determined using Sigma-Aldrich Glucose Assay Kit (GAGO20); b) 5% glucose NaCl injection samples were diluted 200 folds; c) beer sample was detected without dilution.

Glucose Assay Kit, GOGA20) and the present method. As shown in Table 1, compared with the GOGA20 method, the results obtained from the ECL sensing approach were acceptably accurate. These results indicated that the GDH-RuSiNPs modified electrode displays good analytical performance, promising a desirable glucose sensor for most routine analyses.

4 Conclusions

This study fabricated an ECL glucose biosensor by cross-linking GDH to functionalized RuSiNPs and immobilizing GDH-RuSiNPs on a GCE using CHIT. ECL intensity was detected with a GDH-RuSiNPs modified electrode as the working electrode. The effect of pH, scan rate of cycle voltammetry and volume rate between CHIT and GDH-RuSiNPs on the biosensor was also explored. Combination of the ECL of RuSiNPs and the GDH enzymatic reaction was used for the detection of glucose. In the optimal experimental conditions, ECL intensity is linear with glucose concentration in the range from 0.2 to 20 mM. With the application of CHIT, the resulting biosensor displayed remarkable reproducibility and stability. The application results revealed that the proposed approach may have great potential in the determination of glucose.

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