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# Electrochemical and electrochemiluminescence study of $Ru(bpy)_3^{2+}$-doped silica nanoparticles with covalently grafted biomacromolecules

Hui Wei [a,b], Lingling Zhou [a,b], Jing Li [a,b], Jifeng Liu [a,b], Erkang Wang [a,b,*]

[a] *State Key Laboratory of Electroanalytical Chemistry, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Changchun, Jilin 130022, People's Republic of China*

[b] *Graduate School of the Chinese Academy of Sciences, Beijing 100039, People's Republic of China*

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**Abstract**

Spherical $Ru(bpy)_3^{2+}$-doped silica (RuSi) nanoparticles were prepared via a water-in-oil microemulsion approach. The electrochemical and electrochemiluminescent properties of the RuSi nanoparticles immobilized on an indium tin oxide (ITO) electrode were investigated. Further, electrochemiluminescence (ECL) of the RuSi nanoparticles with covalently coated biomacromolecules was studied. By covalent cross-linking with glutaraldehyde, $\gamma$-(aminopropyl) triethoxysilane (APTES)-pretreated RuSi nanoparticles were coupled with different concentrations of bovine serum albumin (BSA), hemoglobin, and myoglobin, respectively. ECL from these biomacromolecule-coated RuSi nanoparticles decreased with the increase of the loading of biomacromolecules. Moreover, the ECL of coreactants with different sizes was studied. The ECL decrease could be assigned to the steric hindrance and limited diffusion of coreactant molecules into the RuSi nanoparticles after biomacromolecule conjugation. Since tens of thousands of $Ru(bpy)_3^{2+}$ molecules are contained in the silica particles and the RuSi nanoparticle surface modification could improve their biocompatibility, the biomacromolecule-coated RuSi nanoparticles could be readily used as efficient and stable ECL tag materials in the future.
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*Keywords:* Electrochemiluminescence; Silica nanoparticles; Biomacromolecules; ECL tag

## 1. Introduction

Small molecules, such as transition metal complexes and organic dyes, can be encapsulated into sol–gel to form doped polymer materials, and this technique provides an attractive alternative to conventional organic polymers [1–6]. Among all the doped sol–gel systems, the tris(2,2′-bipyridyl)ruthenium(II) [$Ru(bpy)_3^{2+}$]-doped sol–gel system has been extensively studied due to its application in optical sensors [7], photosensitizers in solar energy conversion schemes [8], quantification of surface binding of molecules to metallic nanoparticles [9], fluorescence sensors [10–12], and electrochemiluminescence (ECL) [13–21].

ECL is luminescence from excited molecules generated by electrochemical redox reactions [22–25]. Among many organic and inorganic ECL systems, ECL based on $Ru(bpy)_3^{2+}$ has proven to be the most valuable since its discovery [26] due to its strong luminescence and its inherent sensitivity, selectivity, and wide linear range in utility in different analytical areas [22–25, 27–40].

Silica-based materials, especially silica nanomaterials, show great promise for application in many research areas, such as bionanomaterials and bioassays because of their biocompatibility, chemical stability, and easy surface modification [41–43]. Dye-doped silica nanomaterials as functional materials have been intensively investigated and used in biosensors [10–12]. Spherical $Ru(bpy)_3^{2+}$-doped silica (RuSi) nanoparticles have been prepared and used for multiplexed signaling in bioanalysis [11]. Recently stable and sensitive ECL biosensors based on RuSi nanoparticles have been prepared via a water-in-oil microemulsion method [39,40]. Fang's group has realized ECL detection of DNA using RuSi nanoparticles [44]. Very recently,

* Corresponding author at: State Key Laboratory of Electroanalytical Chemistry, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Changchun, Jilin 130022, People's Republic of China. Fax: +86 431 85689711.

*E-mail address:* ekwang@ciac.jl.cn (E. Wang).

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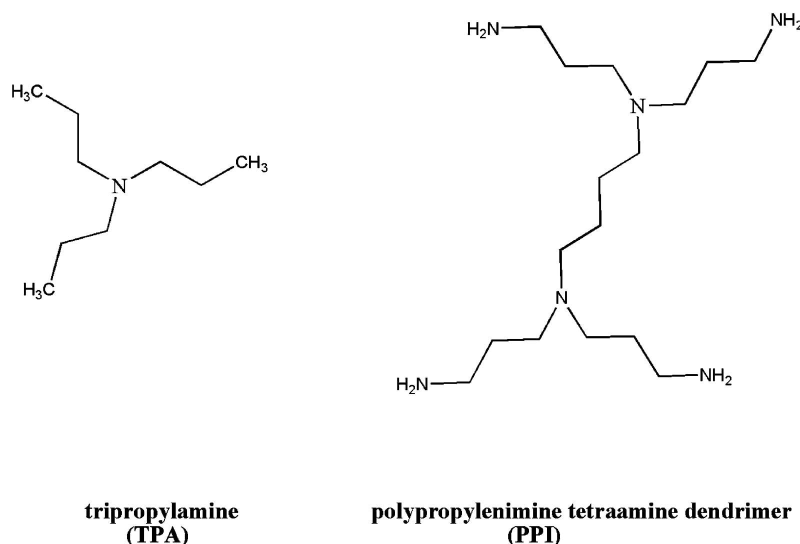


Fig. 1. Molecular structures of ECL coreactants used in this study.

we have studied the RuSi nanoparticles with layer-by-layer biomolecular surface modification [45].

In this work, we further extended the work to study the interactions between the RuSi nanoparticles and some biomacromolecules through covalent attachment. ECL from these biomacromolecule-coated RuSi nanoparticles decreased with the increase of the loading of biomacromolecules, which is similar to our previous results [45]. Moreover, the ECL of coreactants with different sizes was studied.

## 2. Materials and methods

### 2.1. Materials

Tris(2,2′-bipyridyl)ruthenium(II) chloride hexahydrate [Ru-(bpy)$_3$Cl$_2$·6H$_2$O], tetraethyloxysilane (TEOS), $\gamma$-(aminopropyl) triethoxysilane (APTES), polypropylenimine tetraamine dendrimer (PPI) (Fig. 1), and polystyrene ($M_w = 280{,}000$) were obtained from Aldrich (WI, USA). Myoglobin was obtained from Sigma (Milwaukee, WI). Bovine serum albumin (BSA) was from Randox (Antrim, UK). Hemoglobin was from Xinjiang Institute of Chemistry (Urumqi, China). Glutaraldehyde (50% aqueous solution) was from Tianjin Tiantai Fine Chemicals Company (Tianjin, China). Tripropylamine (TPA) was obtained from Acros (Morris Plains, NJ) (Fig. 1). Other reagents and chemicals were at least analytical reagent grade. All aqueous solutions were prepared with water purified by a Milli-Q system (Millipore, Bedford, MA) and stored at 4 °C in a refrigerator.

### 2.2. Preparation of RuSi nanoparticle-modified electrode

The RuSi nanoparticles used for preparation of modified electrode were prepared according to the previous method [45]. The amounts 5.31 mL of Triton X-100, 22.5 mL of cyclohexane, 5.4 mL of $n$-hexanol, and 1020 µL of water were mixed together to form the water-in-oil microemulsion. Concentrated Ru(bpy)$_3^{2+}$ solution was then added into the microemulsion system to a final concentration of 1.2 mM. After addition of 300 µL of TEOS and 180 µL of NH$_4$OH, the hydrolysis reaction was allowed to continue for 24 h. Acetone was then added to destroy the emulsion and to isolate the deep orange-colored nanoparticles, followed by centrifuging and washing with ethanol and water and by ultrasonication, removing any surfactant molecules. Finally, the orange-colored RuSi nanoparticles were obtained and stored in a refrigerator (4 °C) until use. The size of this prepared RuSi nanoparticles was about 39 nm according to transmission electron microscopy.

The immobilization of RuSi nanoparticles on an ITO electrode was similar to the procedure adopted by Villemure's method [46]. The amount of 50 µL of a suspended solution of polystyrene in tetrahydrofuran (1 mg/mL) containing 1 mg of RuSi nanoparticles was spread on a $0.5 \times 2.5$ cm$^2$ piece of ITO-coated glass substrate, and the solvent was allowed to evaporate. The final film was left to be $0.5 \times 1.5$ cm$^2$.

### 2.3. Covalent binding of proteins to RuSi nanoparticles

For covalent binding of protein to silica particles, BSA, hemoglobin, and myoglobin were dissolved in 0.15 mol/L, pH 7.4, phosphate-buffered saline (PBS) with the concentration range from $10^{-9}$ to $10^{-3}$ mol/L. About 2 mg RuSi nanoparticles was added into 1.5-mL tube and treated with 0.5 mL of 10% APTES alcoholic solution, centrifuged, rinsed with PBS solution, reacted with glutaraldehyde (25% aqueous solution), centrifuged, and washed with PBS thoroughly. Different concentrations of BSA, hemoglobin, and myoglobin PBS were immediately added into the tubes for coupling, followed by centrifuging and washing of the RuSi nanoparticles with PBS, and finally dispersed into PBS.

### 2.4. Instrumentation

The electrochemical and ECL measurements were performed with an MPI-A ECL detector (Xi'an Remax Electron-

ics, Xi'an China in association with Changchun Institute of Applied Chemistry, Changchun, China), using a three-electrode system consisting of an ITO electrode as working electrode, a KCl-saturated Ag/AgCl electrode as reference electrode, and a platinum wire electrode as auxiliary electrode, respectively. The voltage of photomultiplier tube was set at 800 V in the process of detection. A cyclic potential (0–1.35 V) was applied to the ITO electrode, ECL intensity was recorded during potential scanning. The ECL peak intensities were used for quantitative analysis in our study.

TEM measurements were made on a JEM1011 transmission electron microscope operated at an accelerating voltage of 120 kV. The samples for TEM characterization were prepared by placing a drop of colloidal solution on a carbon-coated copper grid and drying at room temperature.

## 3. Results and discussion

### 3.1. Electrochemical and ECL properties of RuSi nanoparticle-modified ITO electrode

To check the electrochemical and ECL properties of a RuSi nanoparticle-modified ITO electrode, the cyclic voltammograms (CV) were recorded in a detection reservoir with and without 10 mmol/L TPA (in 150 mmol/L phosphate buffer solution, pH 7.4). As shown in Fig. 2, the RuSi nanoparticle-modified ITO electrode exhibited irreversible but obvious CV response in the absence of TPA. The presence of TPA caused the anodic peak current to increase clearly while the cathodic peak current decreased, which was consistent with a previously reported electrocatalytic reaction mechanism. The corresponding ECL intensity-potential curves were also recorded (Fig. 2). The onset of luminescence occurred at ca 1.05 V, at which $Ru(bpy)_3^{2+}$ was oxidized to $Ru(bpy)_3^{3+}$. The ECL signal arose quite deeply when 10 mmol/L TPA was added into the reaction buffer due to the electrocatalytic oxidation of TPA with $Ru(bpy)_3^{2+}$.

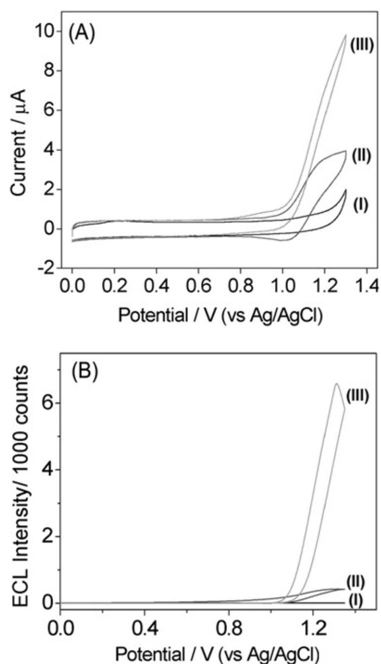


Fig. 2. (A) Cyclic voltammograms and (B) corresponding ECL intensity-potential curves of unmodified ITO electrode in phosphate buffer (I); RuSi nanoparticle-modified ITO electrode in phosphate buffer (II); and RuSi nanoparticle-modified ITO electrode immersed in 10 mM TPA (III). Electrolyte, 150 mM phosphate buffer (pH 7.4); scan rate, 50 mV/s.

### 3.2. ECL of RuSi nanoparticles covalently bound with proteins

Here we immobilized various concentrations ($10^{-9}$–$10^{-3}$ mol/L) of proteins (i.e., BSA, hemoglobin, and myoglobin) by conventional covalent binding on the RuSi nanoparticles after treatment with APTES and glutaraldehyde. (*Note*: the actual amount of protein conjugated onto the nanoparticles was not determined and the concentrations of proteins here were the amount of protein in the corresponding aqueous solution.) The outside silica particle surface has the same property as that of silica glass microbeads. Thus, the RuSi nanoparticles can be easily modified employing existing silica surface chemistry to attach desired functional groups.

When the RuSi nanoparticles were covalently bound with proteins, the ECL emission intensity decreased with the increase of concentration of proteins exposed to the particles (Fig. 3). The reason for the decreased ECL intensity may be due to the steric hindrance and limited diffusion of the coreactant TPA mobilized into the RuSi nanoparticles caused by the protein layers covalently bound. In fact, TPA can enter the porous system to have translational and rotational mobility despite the existing specific surface interactions between the amine and the walls of the silica [40,45] and obtain access to $Ru(bpy)_3^{2+}$ to react. So ECL could be produced via the reaction of TPA with $Ru(bpy)_3^{2+}$ at the external surface of the RuSi nanoparticles or could involve the permeation of TPA into the porous sol–gel system. When the RuSi nanoparticles were bound with extra biopolymer layers, the diffusion behavior of TPA became more difficult because of the hindrance effect of biopolymer layers, which inhibited the diffusion of TPA. When more proteins were bound, this hindrance effect was more obvious, and the chance for $Ru(bpy)_3^{2+}$ to react with TPA became less; thus the ECL intensity decreased (Fig. 3).

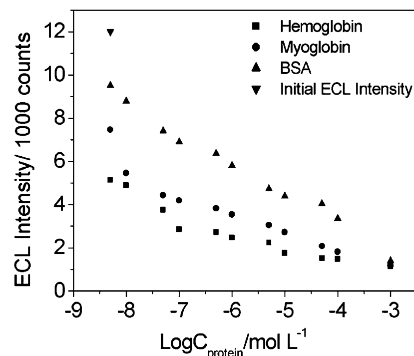


Fig. 3. ECL intensity of RuSi nanoparticles (in solution) processed with protein solutions. (▲) BSA, (■) hemoglobin, (●) myoglobin, and (▼) initial ECL intensity without molecular coating.

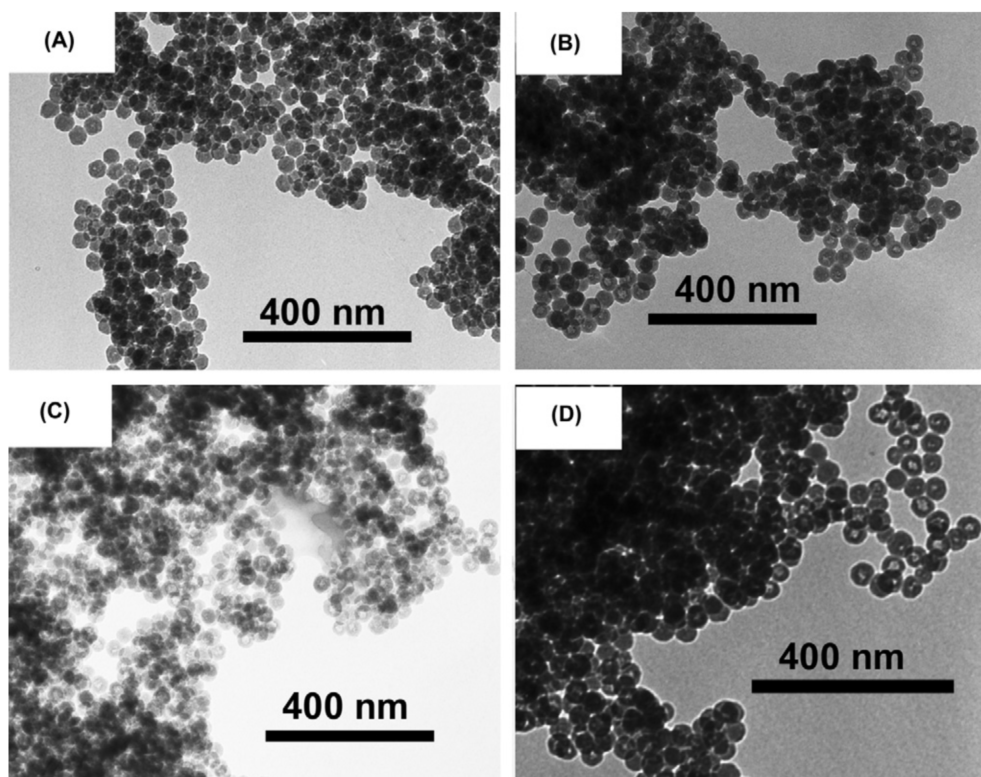


Fig. 4. TEM images of the RuSi nanoparticles (A) before and after covalent binding with (B) BSA, (C) hemoglobin, and (D) myoglobin.

### 3.3. *Steric effect of the coated biomacromolecules on the RuSi nanoparticle ECL*

To inspect the difference of BSA, hemoglobin, and myoglobin, the changes of particle size, morphology, amount of $Ru(bpy)_3^{2+}$, and permeability of the RuSi nanoparticles on coating with proteins were investigated. As shown in Fig. 4, the sizes of RuSi nanoparticles before and after BSA, hemoglobin, and myoglobin conjugation were $39.4 \pm 4.7$, $42.5 \pm 3.5$, $39.6 \pm 3.2$, and $41.6 \pm 3.9$ nm, respectively. For all the three protein coatings, the size of RuSi nanoparticles after protein conjugation was slightly larger than that of the uncoated RuSi nanoparticles. This result confirmed the successful surface modification of the RuSi nanoparticles with the protein coatings. Further examination of the TEM images in Fig. 4 indicated that there was no significant change of the morphology of the RuSi nanoparticles on coating with BSA and myoglobin. However, the morphology of the RuSi nanoparticles coated with hemoglobin was different from the uncoated RuSi nanoparticles (Fig. 4C). This difference might be assigned to the nature of the protein coatings. Despite the difference of the morphology of the RuSi nanoparticles after surface modification, all three protein coatings exhibited obvious ECL inhibition. The amount of $Ru(bpy)_3^{2+}$ was the same before and after coating with proteins since $Ru(bpy)_3^{2+}$ has been capsulated into the RuSi nanoparticles. Thus the permeability change should play an important role. As shown in Fig. 5, in the presence of protein coatings, the ECL signals were inhibited and the larger ECL coreactant PPI exhibited a larger ECL inhibition than the smaller TPA (for the chemical structures of PPI and TPA, see Fig. 1). This result suggested that the coated proteins could inhibit the diffusion of the ECL coreactants and thus inhibit the ECL response due to the steric effect.

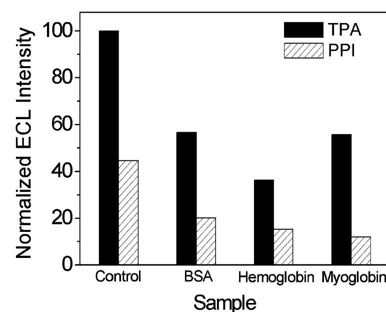


Fig. 5. ECL responses of RuSi nanoparticles before (sample, control) and after covalent binding with different proteins (samples, BSA, hemoglobin, and myoglobin) in the presence of TPA and PPI as coreactants.

## 4. Conclusion

In summary, spherical $Ru(bpy)_3^{2+}$-doped silica nanoparticles were synthesized through a water-in-oil microemulsion approach. The as-prepared nanoparticles were further functionalized with covalently attached biomacromolecules. ECL from these biomacromolecule-coated RuSi nanoparticles decreased with the increase of the loading of biomacromolecules. The biomacromolecule coatings should have greater obstacles for ECL coreactant diffused into the RuSi nanoparticles, thus causing a significant ECL decrease. Our work is significant for the following reasons. First, these RuSi nanoparticles have a large surface

area and a high surface free energy. Second, a single biological recognition event can be amplified using tens of thousands of $Ru(bpy)_3^{2+}$ dye molecules entrapped in the nanoparticles; therefore, it is a more sensitive method than conventional ECL detection schemes. Third, the surfaces of the RuSi nanoparticles are further modified with biopolymers via covalent coatings, and thus the biocompatibility of the particles could be improved. Therefore, they hold great promise in ECL analysis and detection and as efficient and stable ECL tag materials in the future.

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