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Electrochemiluminescence immunosensor for ultrasensitive detection of biomarker using $Ru(bpy)_3^{2+}$-encapsulated silica nanosphere labels

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ABSTRACT

Here, we describe a new approach for electrochemiluminescence (ECL) assay with $Ru(bpy)_3^{2+}$-encapsulated silica nanoparticle (SiO_2@Ru) as labels. A water-in-oil (W/O) microemulsion method was employed for one-pot synthesis of SiO_2@Ru nanoparticles. The as-synthesized SiO_2@Ru nanoparticles have a narrow size distribution, which allows reproducible loading of $Ru(bpy)_3^{2+}$ inside the silica shell and of α-fetoprotein antibody (anti-AFP), a model antibody, on the silica surface with glutaraldehyde as linkage. The silica shell effectively prevents leakage of $Ru(bpy)_3^{2+}$ into the aqueous solution due to strong electrostatic interaction between the positively charged $Ru(bpy)_3^{2+}$ and the negatively charged surface of silica. The porous structure of silica shell allowed the ion to move easily through the pore to exchange energy/electrons with the entrapped $Ru(bpy)_3^{2+}$. The as-synthesized SiO_2@Ru can be used as a label for ultrasensitive detection of biomarkers through a sandwiched immunoassay process. The calibration range of AFP concentration was 0.05–30 ng mL^{-1} with linear relation from 0.05 to 20 ng mL^{-1} and a detection limit of 0.035 ng mL^{-1} at 3σ. The resulting immunosensors possess high sensitivity and good analytical performance.

1. Introduction

Electrochemiluminescence (ECL) analysis has received considerable attention due to its versatility, low-cost, low background signals, applicability to a wide range of analytes, and high sensitivity [1,2]. A series of ECL analysis methods have been developed for clinical diagnostics, environmental assays, food and water testing, as well as biowarfare agent detection [3]. Two main ECL reactions are used in ECL analysis: one uses ruthenium complexes as the coreactants [4–6] and the other is based on the luminol-H_2O_2 system [7]. The $Ru(bpy)_3^{2+}$-tripropylamine (TPA) or $C_2O_4^{2-}$ anodic ECL system is the favorite at the moment for commercially available testing due to its advantages in chemical stability, reversible electrochemical behavior, and high luminescence efficiency over a wide range of buffer pH levels [8–10]. However, $Ru(bpy)_3^{2+}$ dissolved in solution is used in most cases which emits from the diffusion layer near the electrode surface. Hence, it requires continuous delivery of the chemiluminescence reagent into the electrochemical cell, which limits the use of $Ru(bpy)_3^{2+}$ ECL from broader applications. Great efforts have been made toward immobilization of $Ru(bpy)_3^{2+}$ on a solid electrode surface to reduce consumption of expensive reagents and simplify experimental design [11]. To date, two primary approaches have been developed to realize immobilization of $Ru(bpy)_3^{2+}$ on a solid electrode surface. The first method immobilizes $Ru(bpy)_3^{2+}$ directly on a solid electrode surface by embedding $Ru(bpy)_3^{2+}$ in polymer layers on electrode surfaces [12–14], in sol–gels [15,16], the formation of a monolayer on an electrode surface using the Langmuir–Blodgett technique [17], or by a self-assembly technique [18,19]. A cation-exchange polymer, such as Nafion, can effectively prevent immobilized $Ru(bpy)_3^{2+}$ from leaching into the solution due to its high ion-exchange selectivity [20,21]. Some nanostructured materials such as SiO_2 [21], TiO_2 [22], ZrO_2 [23,24], and carbon nanotubes [25–27] have been also incorporated into these Nafion composite films to improve diffusion of the analytes into the film and accelerate charge transportation. Guo and Dong have fabricated the $\{SiO_2/Ru(bpy)_3^{2+}\}_n$ multilayer films through a layer-by-layer assembly process [25]. This process is based on the electrostatic interaction between positively charged $Ru(bpy)_3^{2+}$ and negatively charged silica nanoparticles. This layer-by-layer assembled sensor possesses a large surface area for maximum loading of $Ru(bpy)_3^{2+}$, and has been successfully applied in TPA determination.

The second method involves the use of $Ru(bpy)_3^{2+}$ as electroactive ECL labels immobilized onto an electrode through a series of recognition reactions. For example, a sandwiched assay

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was developed to differentiate DNA or protein using $Ru(bpy)_3^{2+}$ conjugated proteins as labels [28,29]. DNA, or anti-C-reactive protein (anti-CRP), was first immobilized on a gold electrode surface for capturing target DNA or CRP. The ECL intensity of the $Ru(bpy)_3^{2+}$ labels, attached on gold through hybridization or specific recognition interaction between antigen and antibody, was used to quantify the concentrations of DNA or CRP. Another promising method described recently was based on encapsulation of hydrophobic $Ru(bpy)_3^{2+}$ compound in polymer microspheres [30] or liposome [5,31]. Signal amplification was achieved by adding multiple $Ru(bpy)_3^{2+}$ to a single antibody. In a typical liposome-based system, $Ru(bpy)_3^{2+}$ was encapsulated into liposome nanospheres followed by introducing anti-hemagglutinin onto the liposome surface to obtain immunoliposome labels. Hemagglutinin antigen was immobilized onto Au electrodes during competitive capturing of immunoliposome labels from the solution containing the fixing concentration of anti-hemagglutinin and the immunoliposome. The amount of attached immunoliposome labels proportionally related to the concentration of hemagglutinin and can be detected by the destruction of immunoliposomes with ethanol, followed by adsorption of $Ru(bpy)_3^{2+}$ onto the electrode surface. Similarly, $Ru(bpy)_3^{2+}$-doped silica nanoparticles were prepared by a microemulsion method and used for ECL amplification [32,33]. Amplification of the ECL signal by about 3 orders with respect to single tag detection was achieved in DNA or protein detection.

Studies on immunosensors for rapid, selective, sensitive, and low-cost sensing of antigen–antibody interactions have attracted considerable attention recently. Our previous studies have provided several strategies to construct sensitive and reusable immunosensors to detect α-fetoprotein (AFP) including a competitive assay based on antigen's reversible binding to 3-aminophenylboronic acid (APBA)-conjugated thiol-mixed monolayer on gold using cyclic voltammetric (CV) measurement [34] and a non-competitive flow injection chemiluminescence immunosensing system using a boronate immunoaffinity column packed with boronic acid-modified sepharose gel [35] or a phenylboronic acid immunoaffinity reactor by immobilizing APBA on glass microbeads [36] as the glycated antigen collector. Recently, we have also reported a sandwich-type immunosensor using enzyme-functionalized silica nanoparticles as sensitive labels in flow injection chemiluminescence measurements [37] and CdTe quantum dot-functionalized silica nanosphere labels in electrochemical stripping techniques [38] with the detection limit of 10 pg mL^{-1} and 5 pg mL^{-1}, respectively.

Our present work is motivated by the promising applications of $Ru(bpy)_3^{2+}$-doped silica nanoparticle labels for ultrasensitive detection of biomarkers. To develop a highly sensitive immunosensor, $Ru(bpy)_3^{2+}$-encapsulated silica nanoparticles (SiO_2@Ru) were synthesized by an improved one-pot water-in-oil (W/O) microemulsion method. The as-synthesized SiO_2@Ru nanoparticles possess good monodispersity and uniformity, which ensure same loading of $Ru(bpy)_3^{2+}$ and secondary antibodies (Ab2) on each SiO_2@Ru nanoparticle. Sensitive detection was accomplished in ECL measurement after attaching SiO_2@Ru onto Au electrodes following sandwiched immunoreactions.

2. Experimental

2.1. Reagent and materials

AFP and anti-AFP were purchased from Nanjing Sunshine Biotechnology Co. Ltd (Nanjing, China). The photometric concentration (determinated at 280 nm) of AFP antibody is 12 μg mL^{-1}. Tris(2,2′-bipyridyl) dichlororuthenium(II)hexahydrate ($Ru(bpy)_3Cl_2 \cdot 6H_2O$), 11-mercapto-1-undecanol (MU), 11-mercapto-1-undecanoic acid (MUA), *o*-(5-norbornene-2,3-dicarboximido)-N,N,N′,N′-tetramethyluronium tetrafluoroborate (TNTU), bovine serum albumin (BSA), tetraethoxysilane (TEOS), 3-aminopropyl triethoxysilane (APTS), and glutaraldehyde (25% in water) were purchased from Sigma–Aldrich Chemical Co. TNTU activation solution was prepared by dissolving 2.9 mg of TNTU in 100 μL N,N-dimethylformamide containing 1 μL N-methylmorpholine and was incubated at room temperature for 15 min. All other reagents were of analytical grade and were used without further purification. 0.1 M of pH 7.0 phosphate buffer solution (PBS), containing 3 mM $C_2O_4^{2-}$, was used as the electrolyte in our measurements. Distilled water was used throughout the study.

2.2. Apparatus

The electrochemical and ECL measurements were carried out on a MPI-E multifunctional electrochemical and electrochemiluminescent analytical system (Xi'an Remex Analytical Instrument Ltd. Co., China) at room temperature. All the experiments were performed in a 5 mL homemade quartz cell containing an Au wire as the working electrode, a platinum counter electrode, and an Ag/AgCl (saturated KCl solution) reference electrode.

Photoluminescence (PL) spectra were obtained on a RF-540 spectrophotometer (Shimadzu, Japan). Fluorescence microscopy images were carried out with an Olympus IX71 Inverted Optical Microscope (Olympus, Japan). X-ray photoelectron spectroscopy (XPS) measurements were performed using a PHI 5300 X-ray photoelectron spectroscopy (PERKIN-ELMER Corporation, USA). The morphology and size of the SiO_2@Ru nanoparticles were analyzed with a transmission electron microscope (TEM, S-3400N, HITACHI, Japan). The SiO_2@Ru nanoparticles on the anti-AFP-modified Au substrates, having gone through a sandwiched immunoassay, were characterized using a scanning electron microscope (SEM, JEM-2100, JEOL, Japan) at an acceleration voltage of 30 kV. A thin gold film was applied using argon plasma sputtering for 45 s to the specimens for SEM measurements.

2.3. One-pot synthesis of Ru@SiO_2 nanoparticles

One-pot synthesis of SiO_2@Ru nanoparticles with W/O microemulsion method was according to Zhang's description [39] with slight modification as shown in Fig. 1. 1.77 mL of Tween-80 were mixed with 7.5 mL of cyclohexane, 1.8 mL of n-hexanol and 340 μL of 5 mg mL^{-1} $Ru(bpy)_3^{2+}$ solution in water. Then 100 μL of TEOS was added to the mixture. The polymerization reaction was initiated by adding 60 μL of $NH_3 \cdot H_2O$. After 20 h, SiO_2@Ru nanoparticles with diameter of 140 ± 5 nm, characterized by using TEM (inset in Fig. 1A), were deposited with acetone. After that, it was sonicated for 10 min before centrifugation. The colloidal solution was centrifuged at 8000 rpm for 10 min and an orange precipitate was observed (see Supporting Information Fig. S1). The precipitation was washed three times with distilled water and dispersed with ethanol to a final volume of 2 mL.

2.4. Immobilization of anti-AFP onto the SiO_2@Ru nanoparticles

The process for the immobilization of anti-AFP onto the SiO_2@Ru nanoparticles is shown in Fig. 2. 2 mL of the above SiO_2@Ru nanoparticles suspension was diluted to 6 mL with ethanol followed by the addition of 400 μL APTS. After being vigorously stirred for 30 min, the mixture was centrifuged, washed with water and ethanol several times, to remove excess APTS, and the amino-terminated SiO_2@Ru nanoparticles were thus obtained. Then, the amino-functionalized SiO_2@Ru nanoparticles were reacted with