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Sandwich-type electrochemiluminescence immunosensor based on Ru-silica@Au composite nanoparticles labeled anti-AFP

Shirong Yuan, Ruo Yuan*, Yaqin Chai, Li Mao, Xia Yang, Yali Yuan, Huan Niu

Key Laboratory on Luminescence and Real-Time Analysis, Ministry of Education, The Key Laboratory of Eco-environments in Three Gorges Reservoir Region, College of Chemistry and Chemical Engineering, Southwest University, Chongqing 400715, PR China

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ABSTRACT

A simple and sensitive sandwich-type electrochemiluminescence immunosensor for α-1-fetoprotein (AFP) on a gold nanoparticles (nano-Au) modified glassy carbon electrode (GCE) was developed by using Ru-silica ($Ru(bpy)_3^{2+}$-doped silica) doped Au (Ru-silica@Au) composite as labels. The primary antibody, anti-AFP was first immobilized on the gold nanoparticles modified electrode due to the covalent conjugation, then the antigen and the Ru-silica@Au composite nanoparticles labeled secondary antibody was conjugated successively to form a sandwich-type immunocomplex through the specific interaction. The surfaces of Ru-silica nanoparticles were modified via the assemble of Au nanoparticles. The prepared Ru-silica@Au composite nanoparticles own the large surface area, good biocompatibility and highly effective electrochemiluminescence properties. The morphologies of the Ru-silica@Au composite nanoparticles were investigated by using transmission electronic microscope (TEM). The Ru-silica@Au composite nanoparticles labeled anti-AFP/AFP/bovine serum albumin (BSA)/anti-AFP/nano-Au modified GCE electrode was evaluated by means of cyclic voltammetry (CV) and electrogenerated chemiluminescence (ECL). The immunosensor performed high sensitivity and wide liner for detection AFP in the range of 0.05–50 ng/mL and the limit detection was 0.03 ng/mL (defined as $S/N = 3$).

1. Introduction

Electrochemiluminescence (ECL) involves the generation of species at the surface of an electrode that then undergo electron-transfer reactions to form excited states and produce light [1–3]. ECL has recently become an important and powerful analytical tool and been applied in many fields, such as environmental pollutant determination, pharmaceutical analysis, and immunoassay because of its high sensitivity and selectivity, simple instrumentation and low cost [2,4–7]. Among the ECL systems, Tris(bipyridine)-ruthenium(II) ($[Ru(bpy)_3]^{2+}$) has been widely used in electrochemiluminescence analysis due to its superior properties including high sensitivity and stability under moderate conditions in aqueous solution [8,9]. In comparison to the solution-phase $Ru(bpy)_3^{2+}$ ECL system, solid-state $Ru(bpy)_3^{2+}$-ECL can reduce the consumption of expensive ECL reagent, enhance the ECL signal, simplify experimental design and create a regenerable sensor. Up to now, great efforts have been made toward immobilization of $Ru(bpy)_3^{2+}$ on electrode to develop cost-effective, and regenerable chemical sensors and biosensors [10,11], such as the use of the Langmuir–Blodgett technique [12], self-assembled films [13,14], carbon paste [15], polymer films, and sol–gel composites [16,17]. Furthermore, electrochemiluminescence immunoassay (ECLIA) has attracted much attention owing to wide dynamic range, low background, and simple formats of ECL [18].

Recently, nanoparticles have been used in ECL detection because of their special physical and chemical properties. Especially, silica nanoparticles are considered as a good matrix because of their biocompatibility, chemical stability and their surfaces are easily functionalized and modified. It is noted that dyes or quantum dots have been successfully immobilized in silica nanoparticles. In these methods, the properties of dyes and quantum dots are not affected and this strategy is efficient in preventing the immobilized dye molecules from leaking out. More recently, Qian and Yang successfully prepared Ru-silica nanoparticles by using the Stöber method [19]. Stable and sensitive ECL biosensors based on Ru-silica nanoparticles have been intensively investigated [20–22]. The metal nanoparticles and biomolecules can be linked onto the Ru-silica nanoparticles because of the relatively easy modified silica surfaces [19]. In addition, gold nanoparticles have attracted much interest due to their good biocompatibility and stability, showing great applications in construction of modified electrodes for protein immobilization because it facilitated the transfer of electrons between the electrode and the biomolecules [23]. Actually, the

* Corresponding author. Tel.: +86 23 68252277; fax: +86 23 68252277.
E-mail address: yuanruo@swu.edu.cn (R. Yuan).

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Ru-silica@Au composite nanomaterials have a synergistic effect of each component [24]. Compared with the individual components, the composites could exhibit better physical and chemical properties [25,26]. These composites could combine the advantages both superior conductivity and favorable biocompatibility together and the large surface area could be used to adsorb more biomolecules as well. Therefore, the Ru-silica@Au composite nanoparticles were hopeful to label secondary antibody.

To develop a highly sensitive immunosensor, in this study, Ru-silica nanoparticles were successfully prepared by using the convenient and simple Stöber method. Then, BSA, an inert protein with many reactive amino groups [27], was chosen to functionalize Ru-silica nanoparticles. BSA was easy to be absorbed on Ru-silica surface through the hydrogen bonding between –NH group of BSA and –OH group of Ru-silica [28]. Furthermore, the BSA covered Ru-silica could load gold nanoparticles by the reactive amino groups of BSA, the resulting Ru-silica@Au composite nanoparticles could possess better biocompatibility, more electronic conductivity and high surface area for labeling more secondary antibody. So a sensitive sandwich-type electrochemiluminescence immunosensor based on Ru-silica@Au composite nanoparticles labeled anti-AFP/AFP/BSA/nano-Au film was constructed successfully. The experimental results indicated that it exhibited good performance for detection of AFP with a wide linear range and a low detection limit.

2. Experimental

2.1. Reagents and materials

AFP antibody (Anti-AFP, 0.312 μg/mL) and antigen (AFP) were purchased from Biocell Company (Zhengzhou, China). Tris(2,2′-bipyridyl)ruthenium(II)chloride hexahydrate, tripropylamine (TPA), gold chloride ($HAuCl_4$) and BSA (96–99%) were bought from Sigma–Aldrich (St. Louis, MO, USA). The prepared Au colloids (16 nm) were kept at 4 °C. Gold nanoparticles were produced by reducing gold chloride tetrahydrate with citric acid at 100 °C for half an hour [29].

All chemicals and solvents used were of analytical grade and were used as received. Double distilled water was used throughout this study. Phosphate buffered solutions (PBS, pH 7.4) were prepared using 0.1 M Na_2HPO_4, 0.1 M KH_2PO_4 and 0.1 M KCl. The AFP was stored at 4 °C, and its standard solution was prepared daily with double distilled water as in use.

2.2. Apparatus

ECL experiments were carried out with a model MPI-A electrochemiluminescence analyzer (Xi'an Remax Electronic Science & Technology Co. Ltd., Xi'an, China) with the voltage of the photomultiplier tube (PMT) set at 800 V. Cyclic voltammetric measurements (CVs) were performed with a CHI 610B electrochemistry workstation (Shanghai CH Instruments, China). The morphologies of Ru-silica@Au nanoparticles were characterized with a transmission electron microscope (TEM) (TECNAI 10, PHILIPS, Holland). All experiments were carried out with a conventional there-electrode system with the modified glassy carbon electrode (GCE, =4 mm) as the working electrode, a platinum wire as the counter electrode and an Ag/AgCl (sat. KCl) reference electrode.

2.3. Preparation of Ru-silica@Au nanoparticles

According to Ref. [19], the Ru-silica nanoparticles were obtained and dispersed in distilled water to a final volume of 2 mL. Then, the prepared Ru-silica nanoparticles suspension was surface-chemically functionalized by adding 4 mL of BSA. Subsequently, the modified Ru-silica nanoparticles was mixed with 4 mL of Au colloid and stirred for 4 h, after that, the mixture was centrifuged and washed with water several times. Finally, the Ru-silica@Au nanoparticles were obtained and dispersed in 3 mL distilled water. The morphologies of Ru-silica@Au nanoparticles were characterized by TEM. In Fig. 1, the Ru-silica substrate was covered with a great deal of small spherical gold nanoparticles, which demonstrated that the Ru-silica@Au composite nanoparticles were prepared successfully.

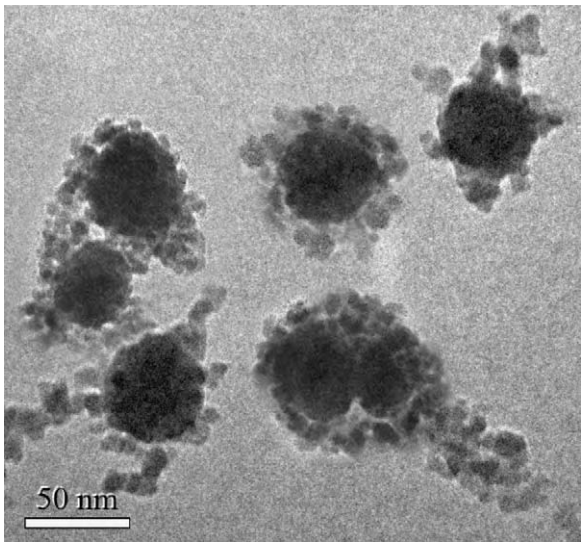

Fig. 1. TEM image of Ru-silica@Au nanoparticles.

2.4. Preparation of Ru-silica@Au composite nanoparticles labeled anti-AFP

Excess antibodies solution was added to the obtained Ru-silica@Au nanoparticles suspension and was stirred at 4 °C for 24 h. Then the mixture was centrifuged to remove residual antibodies and dispersed in distilled water. After that, 4 mL of BSA was added to avoid the non-specific adsorption. Subsequently, the mixture was centrifuged and washed with water several times to obtain the Ru-silica@Au composite nanoparticles labeled anti-AFP. Finally, the Ru-silica@Au composite nanoparticles labeled anti-AFP were dispersed with 0.1 M of pH 7.4 PBS to a final volume of 2 mL. It was stored at 4 °C for later usage.

2.5. Fabrication of the sandwich-type electrochemiluminescence immunosensor

The steps of preparing the modified electrode were shown schematically in Fig. 2. A GCE electrode (Φ = 4 mm) was polished repeatedly with 1.0, 0.3 μm alumina powder to remove adsorbed organic matter, followed by successive sonication in distilled water and ethanol and dried in air. The cleaned electrode was immersed in 1% of 5 mL $HAuCl_4$ solution, and treated by applying −0.2 V for 30 s for electrodeposition of nano-Au film firstly and then soaked in anti-AFP solution at 4 °C for more than 12 h. Following that, it was incubated in BSA solution for about 1 h at 4 °C in order to block possible remaining active sites of the nano-Au monolayer. Subsequently, the electrode was incubated in antigen solution for 20 min at the room temperature. At last, 15 μL of secondary antibody (Ru-silica@Au composite nanoparticles labeled anti-AFP) solution was dropped on the modified electrode incubated for 20 min at the room temperature, then rinsed with distilled water to remove unbound Ru-silica@Au composite nanoparticles labeled anti-AFP.

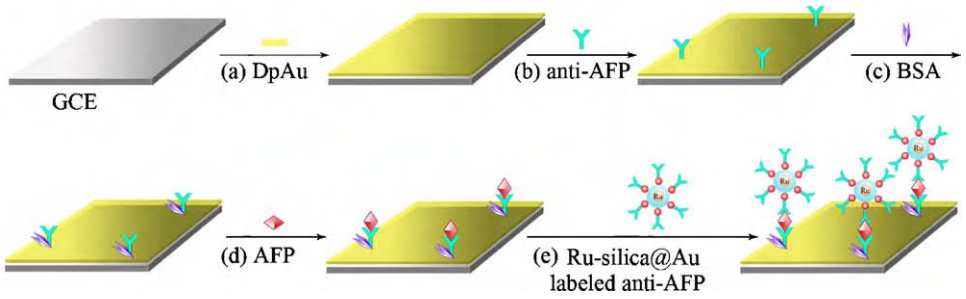

Fig. 2. Schematic illustration of fabrication of the immunosensor.

2.6. Experimental measurements

Electrochemical measurements were done at 25 °C (room temperature) and the potential swept from −0.2 to 0.6 V with scan rate of 100 mV/s. The electrochemical measurements were carried out in a background solution of 2.5 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ PBS (0.1 M KCl, pH 7.4). ECL measurements were performed in 3 mL of PBS (pH 7.4) containing 6.67×10^{-6} M TPA with a photomultiplier tube voltage of 800 V.

3. Results and discussion

3.1. Electrochemical characterization of the sandwich-type ECL immunosensor

CV is a simple and easy means of showing the changes of electrode behavior after each assemble step. Fig. 3 shows CVs of differently modified electrodes in 2.5 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ in PBS of pH 7.4. Well-defined CVs, characteristic of diffusion-limited redox processes, are observed at the bare GCE electrode (Fig. 3a). The peak currents increased after nano-Au electrodepositing on the bare electrode (Fig. 3b), the reason is that nanometer-sized gold plays an important role similarly to a conducting wire or electron-conducting tunnel. However, the peak currents decreased (Fig. 3c) after anti-AFP were immobilized on the electrode surface because the antibody would block the electron transfer. The currents were further decreased after immersing BSA to block the non-specific adsorption (Fig. 3d), for that BSA would act as the inert electron and hind the electron transfer. When AFP antigen molecules were coupled covalently onto the antibody molecules, an obvious decreased current response was obtained (Fig. 3e) due to the immunocomplex blocking layer. Finally, when the secondary antibody (Ru-silica@Au composite nanoparticles labeled anti-AFP) reacted to the antigen on the electrode, a significant decreased current response was obtained (Fig. 3f).

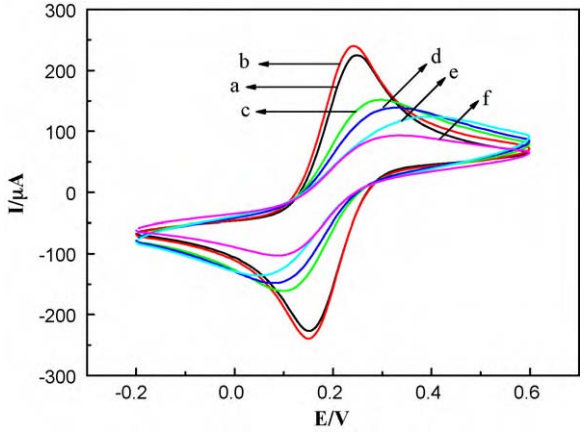

Fig. 3. Cyclic voltammograms of the electrode measured in 2.5 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ (pH 7.4) solution after different steps of modification: (a) bare GCE electrode; (b) nano-Au/GCE; (c) anti-AFP/nano-Au/GCE; (d) BSA/anti-AFP/nano-Au/GCE; (e) AFP/BSA/anti-AFP/nano-Au/GCE; (f) Ru-silica@Au labeled anti-AFP/AFP/BSA/anti-AFP/nano-Au/GCE.

3.2. Optimization of experimental conditions

Electrodeposition is a facile approach for the deposition of gold nanoparticles onto the electrode surfaces. It is known that the deposition time is very important. Therefore, the effect of deposition time was studied (Fig. 4) in this study. The response current increased with the increase of deposition time and reached the maximum when deposition time was 30 s and after that the variation was mild. So the optimal electrodeposition time was 30 s.

The formation of immunocomplex on the electrode surface depended on the time of incubation. As shown in Fig. 5, the oxidation current increased with the increasing incubation time and reached a maximum value at 20 min and after that the variation slowed, indicating a saturated binding of the immobilized anti-AFP and AFP. Thus, the incubation time of 20 min was chosen in this study.

3.3. Performance of the sandwich-type ECL immunosensor for AFP detection

Under the optimal conditions, Fig. 6 shows the ECL profiles of the immunosensor before (a) and after (b–h) incubating in different concentrations of AFP. It is found that the ECL intensity enhanced linearly with the concentration of AFP. The standard calibration curve for AFP detection was shown in insert of Fig. 6. The linear range for detection of AFP was 0.05–50 ng/mL and the limit of

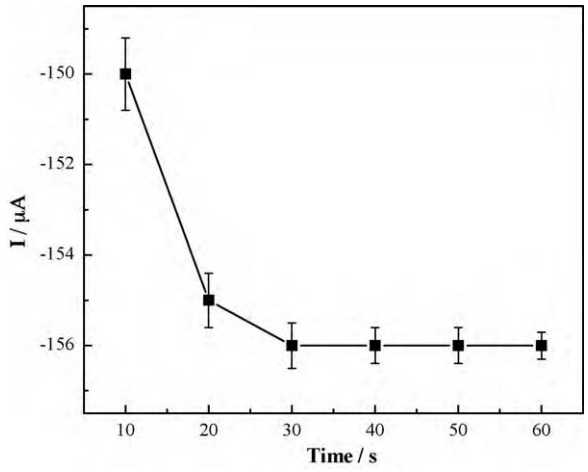

Fig. 4. Effect of electrodeposition time on the response signals.

Table 1
Result obtained compared with those of other immunoassay biosensing systems.

Detection methods	Linear range (ng/mL)	Detection limit (ng/mL)	References
Electrochemiluminescence	0.05–50	0.03	Present work
Differential pulse voltammetry	0.5–80	0.25	[30]
Flow injection chemiluminescence	5.0–100	2.7	[31]
Cyclic voltammetry	6.0–500	6	[32]

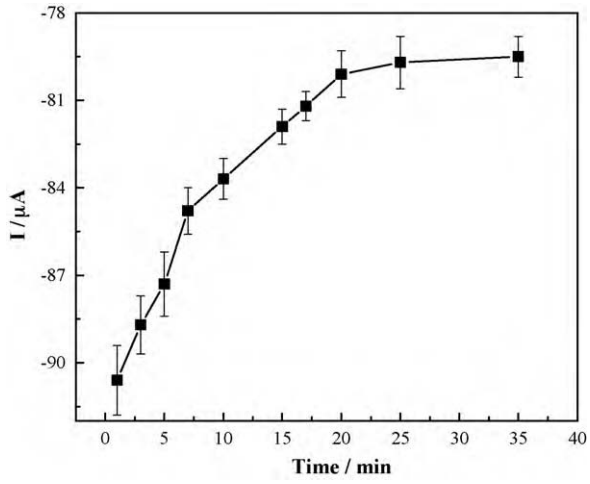

Fig. 5. Effect of incubation time on the response signals.

detection was 0.03 ng/mL ($S/N = 3$). The results demonstrated that the proposed method could be used for the determination of AFP. Moreover, Table 1 shows the linear range and detection limit of immunosensors with previous reports [30–32]. Compared to other methods, the immunosensor has a relative large linear range and low detection limit.

3.4. Application

In order to evaluate the feasibility of the immunosensor for clinical applications, recovery experiments were performed by standard addition methods in human serum. The experimental results are shown in Table 2 and the recovery was in the range of 95.6–104.6%, which confirmed that the developed immunosensor could detect AFP in human serum.

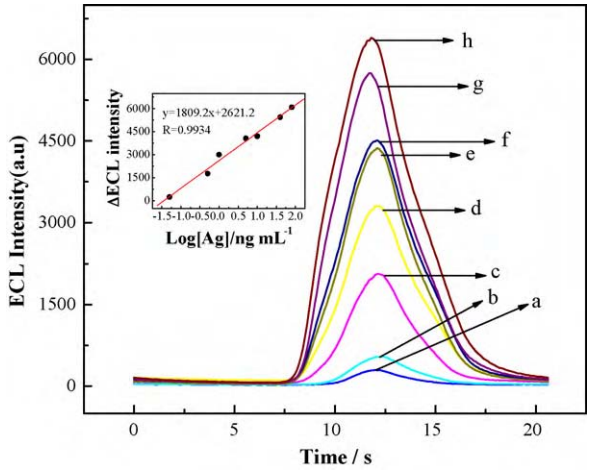

Fig. 6. ECL profiles of the immunosensor in the absence (a–h) of different concentrations of Ag in pH 7.4 PBS containing 0.1 M KCL and 6.67×10^{-6} moL L^{-1} TPA. Ag concentration (ng mL^{-1}): (a) 0, (b) 0.05, (c) 0.5, (d) 1, (e) 5, (f) 10, (g) 40, (h) 80 (the voltage of the photomultiplier tube was set at 800 V). Insert is the calibration curve of the immunosensor.

Table 2
The recovery of the proposed immunosensor in human serum.

Sample number	Added (ng/mL)	Found (ng/mL)[a]	Recovery (%)
1	0.50	0.52 ± 0.02	104.0
2	1.00	0.97 ± 0.03	97.0
3	5.00	5.23 ± 0.2	104.6
4	10.0	9.6 ± 0.4	96.0
5	25.0	23.9 ± 0.9	95.6

[a] Mean value ± SD of three measurements.

4. Conclusions

In conclusion, the Ru-silica@Au composite nanoparticles could be used as a promising label for detection of AFP in a sandwiched electrochemiluminescence immunoassay due to good biocompatibility, large surface area and the high ECL intensity. A simple and sensitive sandwiched electrochemiluminescence immunosensor was fabricated successfully. The resulting immunosensor demonstrated high sensitivity and good analytical performance. The simple proposed method may lead to an attractive approach for the other analyte determination.

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