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ORIGINAL PAPER

Electrochemiluminescence DNA sensor based on $Ru(bpy)_3^{2+}$-doped silica nanoparticle labeling and proximity-dependent surface hybridization assay

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Abstract A new electrochemiluminescence (ECL) method based on the proximity-dependent surface hybridization assay and $Ru(bpy)_3^{2+}$-doped silica nanoparticles (Ru-DSNPs) as labels were proposed for detecting DNA. The hybridization process involves two steps: firstly, the 3′ thiolated capture probe was self-assembled on the gold electrode. Secondly, the proximity-dependent surface hybridization assay was carried out. This proximity-dependent surface hybridization assay depended on the simultaneous recognition of a target DNA by a capture probe and Ru-DSNP-labeled probe and the formation of a duplex complex, which results in the luminophor approach to the electrode surface. Thus, sensitive ECL signals were obtained. Under optimum conditions, the intensity of the ECL of Ru-DSNPs was linearly related to the concentration of the target sequence in the range of 2.0×10^{-15} to 2.0×10^{-11} mol/L. The detection limit was 1.0×10^{-15} mol/L (S/N=3).

Keywords Electrochemiluminescence · DNA · Sensor · $Ru(bpy)_3^{2+}$-doped silica nanoparticles

Introduction

The detection of DNA sequences is very important for the diagnosis and treatment of genetic diseases, detection of infectious agents, and drug discovery [1–3]. Various approaches for the detection of DNA have been developed, including optical [4–6], electrochemical [7, 8], quartz-crystal microbalance [9], and electrochemiluminescence (ECL) [10–12]. Among these techniques, ECL methods have attracted significant attention for their high sensitivity, low background signals, low cost, and applicability to a wide range of analytes [13–15]. Recently, a variety of strategies including the use of new luminophores and co-reactants, solid state ECL detection, and multiple ECL labeling have been proposed to enhance ECL sensitivity [16–18]. Xu's group reported a highly sensitive $Ru(bpy)_3^{2+}$ ECL system using 2-(dibutylamino)ethanol as co-reactant for immunoassays and DNA probe assays [19]. Zhu et al. used PCR-free ECL based on bio bar code assay for the quantitative detection of genetically modified organisms from raw materials [20]. Our group also developed novel ECL DNA assays using quantum dots as DNA labels [21]. In fact, multiple ECL labeling is also a very efficient way for signal amplification. Among numerous ECL reagents, $Ru(bpy)_3^{2+}$ is most studied and exploited due to its strong luminescence, solubility, and ability to regenerate during the ECL process [22]. Many protocols for labeling multiple $Ru(bpy)_3^{2+}$ species to a single biomolecule have been reported [23–25]. Bard's group provided sensitive ECL bioassays by labeling polystyrene microspheres [26, 27] preloaded with a large number of $Ru(bpy)_3^{2+}$ to target molecules and then releasing the $Ru(bpy)_3^{2+}$ into an acetonitrile solution and measuring the ECL of $Ru(bpy)_3^{2+}$. Thus, a large amplification factor of $Ru(bpy)_3^{2+}$ for each biomolecule is obtained. Our group used 350-nm diameter magnetic nanobeads (MNBs) as the carrier of $Ru(bpy)_3^{2+}$ species to enhance the ECL signal and developed a new ECL immunoassay [28].

The rapidly evolving fields of nanoscience and nanotechnology have opened up a new and promising era. In the past few years, several kinds of nanostructured materials such as gold nanomaterials [29], quantum dots [30], carbon

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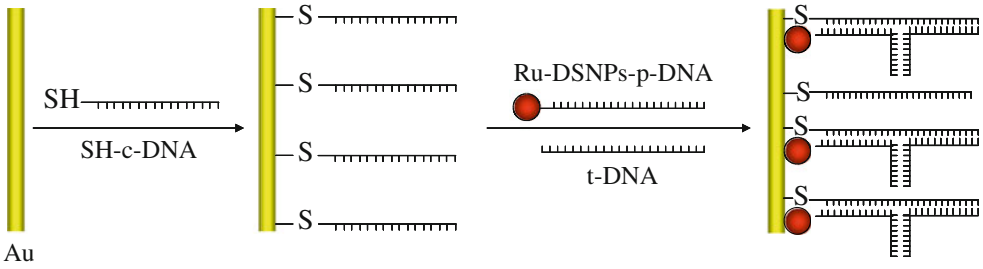
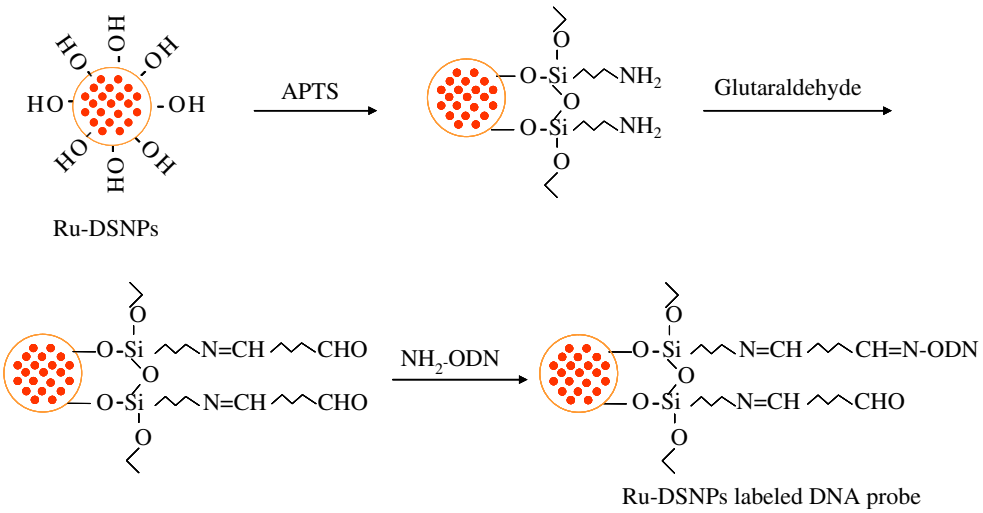


Scheme 1 Schematic representation of the process of DNA determination

nanotubes [31], and silica nanomaterials [32] have been used in the field of ECL. Dye-doped silica nanomaterials have been intensively investigated and used in biosensors [33]. Tan and co-workers [34] prepared $Ru(bpy)_3^{2+}$-doped silica nanoparticles (Ru-DSNPs), in which $Ru(bpy)_3^{2+}$ could still retain its ECL property, and the exterior nano-silica prevented the luminophor from leaching out into the aqueous solution due to the electrostatic interaction. Hence, the Ru-DSNPs are considered as excellent luminophor labels in the ECL sensor.

Usually, the DNA hybridization reaction mode with a sandwich structure was adopted to fabricate a DNA sensor for detecting DNA sequences in ECL assays. However, when there are more base pairs of DNA sequences, the detection probe is far away from the electrode, meaning the luminophor-tagged tail sequence of the detection probe is far away from the electrode, which is unfavorable for ECL. Fredriksson and co-workers developed a method for the detection of proteins, termed proximity ligation assay [35]. The proximity ligation assay depends on the simultaneous recognition of a target molecule by a pair of affinity probes. This brings the tail sequences of the affinity probe pair in close proximity to hybridize together with a connector oligonucleotide, giving rise to an augmented detection signal. Zhang et al. [36, 37] have also reported an electrochemical apta sensor for the detection of proteins and electrochemical DNA biosensor for the detection of nucleic acids based on a proximity-dependent surface hybridization assay. This DNA biosensor was proven to be simple, single-step, and sensitive, with a low detection limit and a wide linear dynamic range.

The aim of the present work was to develop a sensitive and simple ECL DNA sensor based on the principle of the proximity-dependent surface hybridization mode. Because of the specific hybridization among the capture probes, Ru-DSNP-labeled probe, and target DNA, as well as the predesigned melting temperature, the surface proximity assay was realized. This ECL DNA sensor was constructed as follows: a 3′ short thiolated capture probe was first self-assembled on the gold electrode for the first step. Subsequently, the target DNA and Ru-DSNP-labeled probe were introduced into the system. The target DNA was complementary to the 3′ thiolated capture probe at one half of the segment and complementary to the 5′ Ru-DSNP-labeled probe at the other half of the segment, resulting in the formation of a duplex complex (shown in Scheme 1). As a result, the Ru-DSNPs were proximate to the electrode surface. Thus, a higher ECL signal from the luminophor, Ru-DSNPs, was obtained. The DNA sensor showed a wide linear range, from 2.0×10^{-15} to 2.0×10^{-11} mol/L, low detection limit of 1.0×10^{-15} mol/L, and good stability for the determination of the special DNA sequence.



Scheme 2 Schematic representation of the oligonucleotide conjugation process onto the Ru $(bpy)_3^{2+}$-doped silica nanoparticle surface

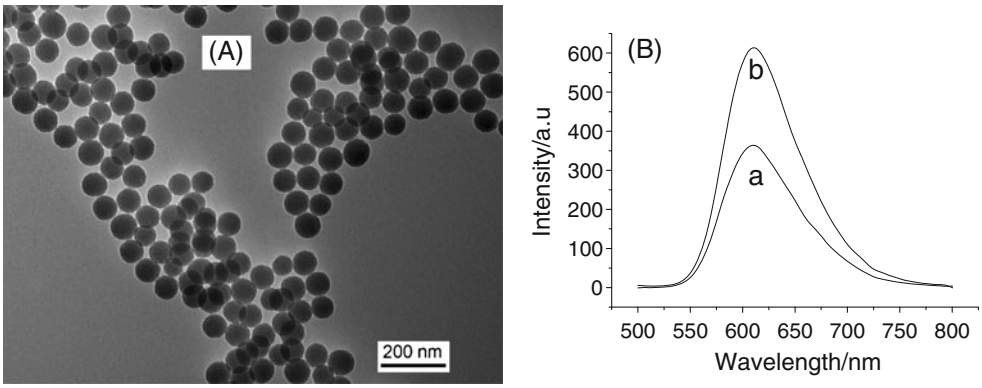


Fig. 1 **a** TEM image of Ru-DSNPs (the *bar scale* is 200 nm) **b** PL spectra. *a* free $Ru(bpy)_3^{2+}$ and *b* Ru-DSNPs (excited at 460 nm) in 10 mmol/L PBS, respectively

Experimental

Apparatus

The voltammetric and ECL measurements were performed using a CHI802 electrochemical analyzer (CH Instruments, Austin, TX, USA). A PMT (model H9305-04, Hamamatsu Photonics K. K., Japan) with a spectral width of 185–830 nm was used to measure ECL emission. During measurement, a potential was applied to the working electrode via a CHI 802 electrochemical analyzer (CH Instruments, Austin, TX, USA), and ECL emission was detected by a H9305-4 photomultiplier tube (Hamamatsu, Japan). The transmission electron microscopy (TEM) image was obtained with a JEM-1011 microscope (JEOL, Japan) operating with a 100-kV accelerating voltage.

Reagents

Tris (2,2-bipyridyl) ruthenium (II) chloride ($Ru(bpy)_3^{2+}$) and Triton X-100 were purchased from Sigma–Aldrich (St. Louis, MO, USA). 3-Aminopropyltriethoxysilane (APTS) was obtained from Acros (Belgium). Tetraethylorthosilicate (TEOS) was purchased from Damao Chemical Reagents Co. Ltd. (Tianjin, China). TPrA (≥98%) was obtained from Fluka (Buchs, Germany). Other chemicals were obtained from standard reagent suppliers. The DNA oligonucleotides used in this paper were synthesized by Sangon Biotech Co. Ltd. (Shanghai, China). Sequences were the following: capture probe 5′-GAG GAG TTG AGT CAG T-$(CH_2)_6$-SH-3′, target 5′-TGA GCA ATG TGC TCC CCC AAC TCC TCT CCA G-3′, detect probe 5′-NH_2-$(CH_2)_6$-ACT GAC TGG GGA GCA CAT T-3′. The phosphate buffer (PBS) consisted of 7.6×10^{-3} mol/L NaH_2PO_4 and 2.4×10^{-3} mol/L Na_2HPO_4 (pH=7.4). All reagents were of analytical grade, and doubly distilled water was used throughout.

Preparation of Ru-DSNP-labeled DNA probe

The Ru-DSNPs were prepared according to previously published protocols [38]. Briefly, 1.77 mL of Triton X-100, 7.5 mL of cyclohexane, 1.8 mL of n-hexanol, and 340 μL of $Ru(bpy)_3^{2+}$ (40 mmol/L) were added to a 20-mL glass vial with constant magnetic stirring. In the presence of 100 μL of TEOS, a polymerization reaction was initiated by adding 60 μL of NH_4OH (28–30 wt.%). After 24 h, the Ru-DSNPs were isolated with acetone and washed with ethanol and water several times to remove any surfactant molecules.

The oligonucleotide conjugation process onto the Ru-DSNPs' surface was shown in Scheme 2. Firstly, the Ru-DSNPs were functionalized according to the literature [39]; 0.01 g of Ru-DSNPs and some APTS were added into anhydrous toluene to make 5 mL of mixture solution. The

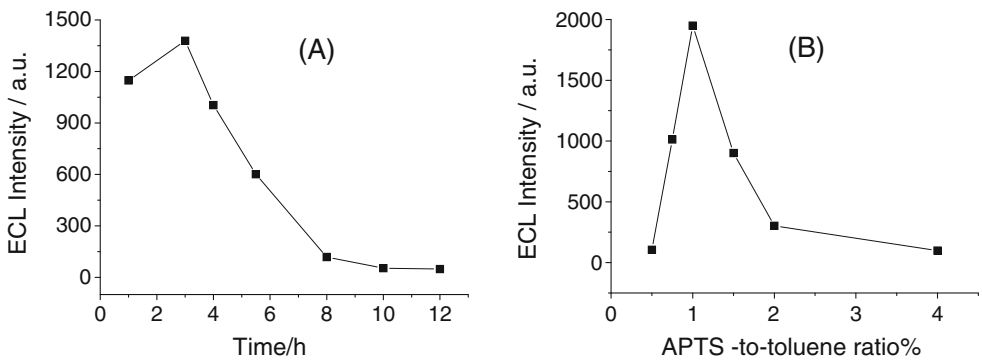


Fig. 2 **a** Ru-DSNPs' silylation time **b** Effect of APTS-to-toluene ratio on ECL intensity

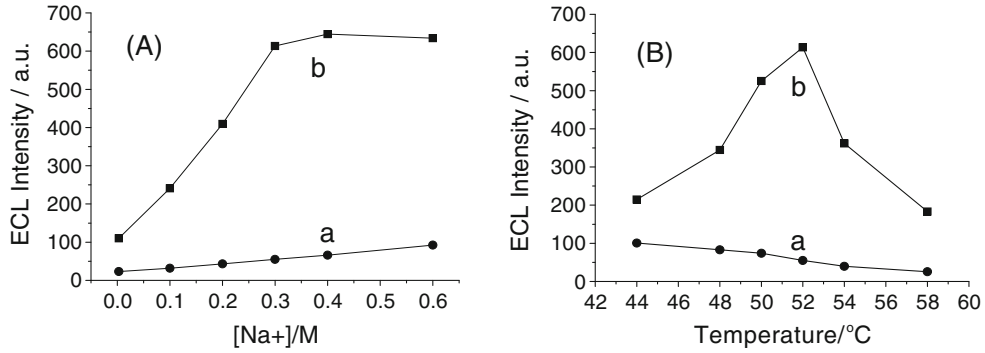


Fig. 3 Dependence of the ECL intensity on the concentrations of NaCl (**a**) and hybridization temperature (**b**). *a* blank and *b* 2 p mol/L target DNA detection. Reaction conditions: *a* 10 mmol/L PBS (pH 7.4) incubation at 52 °C for 2.5 h. *b* 10 mmol/L PBS containing 0.3 mol/L NaCl (pH 7.4)

mixture was refluxed under dry nitrogen. The resulting APTS-modified silica particles were separated by centrifugation, washed with toluene, and dried with nitrogen. The above amine-functionalized Ru-DSNPs were dispersed into the PBS buffer containing 5% glutaraldehyde for 2 h at 37 °C with shaking, followed by washing with PBS buffer to remove excess glutaraldehyde, and re-dissolved in PBS. Then, 10 μL of 5′-amine-capped oligonucleotide (1×10^{-4} mol/L) was added into the above solution, and the reaction continued for 2 h with stirring in a water bath of 37 °C, resulting in the formation of the Ru-DSNP-labeled DNA probe. Finally, the Ru-DSNP-labeled probe was treated with 1 mL glycine (30 mmol/L) for 30 min, followed by dispersing in 10 mmol/L PBS (pH 7.4) and storing at 4 °C for later use.

Preparation of the ECL sensor

A gold disk electrode with 4-mm diameter was polished carefully with 0.5 and 0.05 μm γ-Al_2O_3 powder on a microcloth and washed ultrasonically with water. Before modification, the bare electrode was cleaned in Piranha solution (98% H_2SO_4/30% H_2O_2, 70:30, *v*/*v*) for 30 min. Then, the electrode was rinsed thoroughly with doubly distilled water and allowed to dry in nitrogen. After that, 10 μL thiolated oligonucleotides (2×10^{-6} mol/L) and 30 μL PBS (10 mmol/L) were dropped to the electrode and incubated for 12 h at room temperature. The resulting thiolated oligonucleotide functionalized electrode was incubated in 10 mmol/L PBS containing the 0.1 μmol/L Ru-DSNP-tagged probe and 500 μL of target solutions of different concentrations at 52 °C for 2.5 h.

ECL detection for target DNA

ECL determination was performed at room temperature in a 5-mL homemade quartz cell. A three-electrode system was used with the modified Au electrode (4 mm in diameter) as the working electrode, a saturated calomel electrode as the reference electrode, and a platinum wire as the counter electrode. ECL measurement was performed at a potential, scanning from 0.0 to 1.6 V in 0.15 mol/L PBS containing 0.10 mol/L TPA and 0.10 mol/L KNO_3 (pH 7.4).

Results and discussion

Characterization of Ru-DSNPs

The Ru-DSNPs prepared by a W/O microemulsion method possess high monodispersion and uniformity in size. The TEM micrographs showed that the size of Ru-DSNPs were about 75 nm (Fig. 1a). Fluorescence emission spectra showed that Ru-DSNPs have a characteristic emission at 610 nm when excited at 460 nm (shown in Fig. 1b), similar to that of free $Ru(bpy)_3^{2+}$. The results indicate that $Ru(bpy)_3^{2+}$ was successfully entrapped inside the SiO_2 nanospheres, and the entrapped $Ru(bpy)_3^{2+}$ upheld its optical properties. The ECL intensity of Ru-DSNPs was compared with that of simple Ru $(bpy)_3^{2+}$ molecules. In order to immobilize $Ru(bpy)_3^{2+}$ onto the electrode, the $Ru(bpy)_3^{2+}$ was first functionalized to become the ruthenium bis (2,2′-bipyridine)-(2,2′-bipyridine-

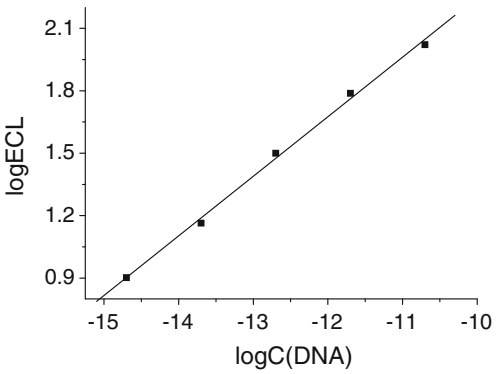


Fig. 4 Calibration curve of ECL intensity versus DNA concentration. PB (0.15 mol/L) (pH 7.4) containing 0.1 mol/L KNO_3 and 0.1 mol/L TPrA; scan rate was 50 mV/s

4,4′-dicarboxylic acid)-*N*-hydroxysuccinimide ester ($Ru(bpy)_3^{2+}$–NHS). The ECL intensity was obtained via the following steps: firstly, cysteine was self-assembled on the activated Au electrode. Secondly, Ru-DSNPs or $Ru(bpy)_3^{2+}$–NHS were immobilized onto the electrode surface through the reaction of the amino groups. Thirdly, the ECL detection of Ru-DSNPs or $Ru(bpy)_3^{2+}$–NHS was performed at room temperature (figure not shown). The results indicate that the ECL intensity of Ru-DSNPs was about three orders of magnitude higher than that of the $Ru(bpy)_3^{2+}$ molecules, which was in conformity with the literature [40] reported.

Optimization of silylation conditions

Different silylation times resulted in the changes of shape and quantity of APTS on the Ru-DSNPs, which could influence their ECL behaviors. As shown in Fig. 2a, the ECL signals of the Ru-DSNPs became stronger by increasing the silylation time within 3.5 h. However, when the silylation time exceeded 3.5 h, the ECL intensity decreased. Figure 2b showed the influence of the ratio of APTS-to-toluene to the ECL intensity. The ECL intensity increased from 0% to 1% (*v*/*v*) and then went down with more silylation reagents. Therefore, 3.5 h and 1% were respectively chosen as the optimum silylation time and ratio of APTS-to-toluene for the functionalization of Ru-DSNPs.

Selection of Na^+ concentration and hybridization temperature

In order to obtain a higher sensitivity of the ECL sensor, the experimental conditions, such Na^+ concentration and experimental temperature, were optimized. The ECL intensity increased with the increasing of the NaCl concentration and reached plateau regions at the 0.3-mol/L concentration of NaCl, as shown in Fig. 3a. Therefore, 0.3 mol/L was chosen as the optimum salt ion concentration. The melting temperatures of the oligonucleotide probes were calculated by using the Zuker program [41]. The melting temperatures for the Ru-DSNP-labeled probe and target DNA are designed to be higher than the reaction temperature, while melting temperatures for the capture probe and target DNA as well as the Ru-DSNP-labeled and capture probes are both lower than the reaction temperature. The ECL intensity ratios of target and blank changed with the hybridization temperature, which was like a parabola, and its peak was at 52 °C as shown in Fig. 3b. So, 52 °C was chosen as the optimal hybridization temperature.

ECL quantification of DNA

ECL of Ru-DSNPs connected with DNA can be utilized for DNA determination. Under optimal conditions, the logarithm of ECL intensity increased linearly with the logarithm of target DNA concentration in the range from 2.0×10^{-15} to 2.0×10^{-11} mol/L (R=0.9984) (shown in Fig. 4.), and the detection limit was 1.0×10^{-15} mol/L (S/N=3). The relative standard derivation for 1.0×10^{-12} mol/L target DNA was 3.9% (n=6).

Conclusions

A novel ECL DNA sensor with Ru-DSNPs as labels was constructed based on the proximity-dependent surface hybridization mode. The main advantages of the present sensor contributed to two aspects. First, the ECL intensity of Ru-DSNPs increased about three orders of magnitude compared to the simple $Ru(bpy)_3^{2+}$ molecules. Second, the proximity-dependent surface hybridization mode made the luminophor approach to the electrode surface as aloes as possible, which led to the following effects: electron transfer became easy, and ECL signal was enhanced.

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