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[Ru(bpy)$_2$(dcbpy)NHS] Labeling/Aptamer-Based Biosensor for the Detection of Lysozyme by Increasing Sensitivity with Gold Nanoparticle Amplification

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Abstract: A novel [Ru(bpy)$_2$(dcbpy)NHS] labeling/aptamer-based biosensor combined with gold nanoparticle amplification for the determination of lysozyme with an electrochemiluminescence (ECL) method is presented. In this work, an aptamer, an ECL probe, gold nanoparticle amplification, and competition assay are the main protocols employed in ECL detection. With all the protocols used, an original biosensor coupled with an aptamer and [Ru(bpy)$_2$(dcbpy)NHS] has been prepared. Its high selectivity and sensitivity are the main advantages over other traditional $[Ru(bpy)_3]^{2+}$ biosensors. The electrochemical impedance spectroscopy (EIS) and atomic force microscopy (AFM) characterization illustrate that this biosensor is fabricated successfully. Finally, the biosensor was applied to a displacement assay in different concentrations of lysozyme solution, and an ultrasensitive ECL signal was obtained. The ECL intensity decreased proportionally to the lysozyme concentration over the range 1.0×10^{-13}–1.0×10^{-8} mol L^{-1} with a detection limit of 1.0×10^{-13} mol L^{-1}. This strategy for the aptasensor opens a rapid, selective, and sensitive route for the detection of lysozyme and potentially other proteins.

Keywords: aptamers · biosensors · gold · luminescence · ruthenium

Introduction

Protein detection plays an important role in many areas for fundamental research.[1] In recent years, a number of protein-binding methods with high degrees of specificity have been developed, including antigen–antibody systems, nucleic acid hybridization techniques, and protein–ligand systems.[2] Among them, antigen–antibody systems are the most versatile techniques. In 1990, a new ligand which showed high affinity and selectivity, referred to as an aptamer, was reported independently by the laboratories of Joyce, Szostak, and Gold.[3] Aptamers are artifical synthetic oligonucleotides with small size and are easily and reproducibly synthesized compounds which show high affinity and sensitivity to recognize a certain target.[4] Recent works have indicated that target–aptamer systems offer more considerable advantages compared with antigen–antibody systems and present an alternate candidate to antigen–antibody systems.[5] Taking into account the advantages of aptamers, a biosensor fabricated with an aptamer holds great promise for applications in proteomic microarrays and therapeutic agents owing to their small size, easy modification, high binding affinity, and resistance against denaturation.

Recently, a variety of aptamer-based analytical methods have been developed for protein recognition and detection, including fluorescence,[6] surface plasma resonance (SPR),[7] AFM,[8] quartz crystal microbalance,[9] electrochemistry,[10] electrochemiluminescence (ECL),[11] and so on. Among all these methods, different ECL detection routes have been recently developed and hold obvious potential for the multifaceted advantages of ECL. Since Noffsinger and Danielson[12] first reported the chemiluminescence of tris(2,2′-bipyridine)ruthenium(III) and then Leland and Powell[13] re-

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ported the coreactant tri-*n*-propylamine (TPrA), which exhibited the highest ECL efficiency, ECL has received much attention owing to its inherent sensitivity, selectivity, low cost, wide linear range, and utilization in different analytical areas such as clinical tests and biomolecule detection.[14] In view of these advantages, a wide range of electrochemiluminescence probes based on tris(2,2′-bipyridine)ruthenium(III) have been proposed and tested in different sensing systems in recent years.[15]

Aptamer-based displacement immunoassays are ultrasensitive since the labeled protein has an obviously lower affinity to the aptamer compared with the unmodified one.[16] Wang et al.[17] developed quantum-dot/aptamer-based mutianalyte electrochemical aptamer biosensors with subpicomolar detection limits 3–4 orders of magnitude lower than most advanced aptamer biosensors reported.[18]

Herein, by adopting lysozyme as a model analyte, we combine displacement assays with the ECL detection method to present a ruthenium tris(2,2′-bipyridine-4,4′-dicarboxylic acid) *N*-hydroxysuccinimide ester ([$Ru(bpy)_2$(dcbpy)NHS]) labeling/aptamer-based biosensor for protein detection. First, the gold electrode was immersed in *p*-aminothiophenol (*p*-ATP) solution to assemble a *p*-ATP monolayer, and then the gold electrode was dipped into the colloidal gold to improve the adsorption capacity. A thiolated-oligonucleotide solution was dropped onto the gold electrode surface to generate the aptamer monolayer, which was subsequently treated with 6-mercapto-1-hexanol to block the electrode. [$Ru(bpy)_2$(dcbpy)NHS]-labeled lysozyme was bound to the aptamer monolayer for the next step. Last, the displacement assay was carried out in different concentrations of lysozyme solution, and then the ultrasensitive ECL signal was obtained. The ECL intensity decreased proportionally to the lysozyme concentration over the range 1.0×10^{-13}–1.0×10^{-8} mol L^{-1} with a detection limit of 1.0×10^{-13} mol L^{-1}. Furthermore, this biosensor was applied successfully to determine the content of lysozyme in egg, which guaranteed the feasibility of this method to analyze biological samples. Through this protocol, ECL probes, synthetic, and labeling techniques are successfully brought into aptamer-based bioassays, which makes the protein detection more sensitive and provides a new model for the applications of ECL probes at the same time. Moreover, gold nanoparticle amplification further increases the sensitivity of the detection and makes the method more practical. Finally, with lysozyme as a model analyte, this protocol could be utilized as a versatile and powerful tool for the detection of further proteins and molecules in the clinical, pharmaceutical, and bioassay fields.

Abstract in Chinese:

摘要： 本文报道了一个新颖的以三联吡啶钌活化酯标记以及适配子为基础的同时结合金纳米放大用电化学发光检测溶菌酶的传感器。本文用适配子、电化学发光探针结合金纳米放大以及竞争反应进行电化学发光检测。使用这些方法，我们制作了一种新颖的结合适配子和三联吡啶钌活化酯的生物传感器，比其它以三联吡啶钌为基础的生物传感器，具有高选择性高灵敏度的优点。所有阻抗以及原子力谱图都表明了该生物传感器组装成功。最后，我们把这个生物传感器用于不同浓度的溶菌酶溶液进行置换反应，得到了很灵敏的电化学发光信号。电化学发光信号在 1.0×10^{-13} mol L^{-1}–1.0×10^{-8} mol L^{-1}范围内随着溶菌酶浓度的增大相应减小，检出限达 1.0×10^{-13} mol L^{-1}。这种适配子传感器为一种快速、灵敏、高选择性的用于检测溶菌酶和其他蛋白质的方法。

Results and Discussion

Effect of Electrolyte Buffer on ECL Intensity

Tris(2,2′-bipyridine)ruthenium(III) coupled with the coreactant tri-*n*-propylamine (TPrA) has been demonstrated to exhibit the highest ECL efficiency. Many early works have investigated the ECL mechanism of the $[Ru(bpy)_3]^{2+}$/TPrA system. It is clear that the ECL intensity depends significantly on the concentration of both $[Ru(bpy)_3]^{2+}$ and TPrA; the solution pH value and the electrode material are also important.[19] It has been reported that a strong ECL was produced when tris(2,2′-bipyridine)ruthenium(III) was dissolved in MeCN containing TPrA as the ECL coreactant.[20] In addition, a similar result is also obtained for electrochemiluminescence labels. In our experiment, the ECL intensity is enhance with increasing pH value. However, when the pH value is over 10.70, a white deposition appears. Thus, PB buffer (pH 10.55, 100 mmol L^{-1}) containing TPrA (100 mmol L^{-1}) and MeCN (50 mmol L^{-1}) was selected as the electrolyte for ECL study.

AFM Characterization of the Fabricated Biosensor

The surface structure and morphology of the fabrication progress of the biosensor is characterized by AFM as shown in Figure 1. Figure 1a exhibits an image of the bare gold substrate. Figure 1b shows a clustered surface morphology, covered by irregularly shaped gold nanoparticles (GNPs) with an average size of 13 nm. This morphology is greatly different from that of the bare Au surface shown in Figure 1a. Figure 1c shows the image of an aptamer layer immobilized on the GNP-amplified gold substrate, which shows a slight increase in fabrication density compared to Figure 1b in this step. However, when [$Ru(bpy)_2$(dcbpy)NHS]-labeled lysozyme is immobilized on the aptamer, one can see the difference from Figure 1c in terms of both particle size and surface morphology, which demonstrates that the biosensor is successfully fabricated.

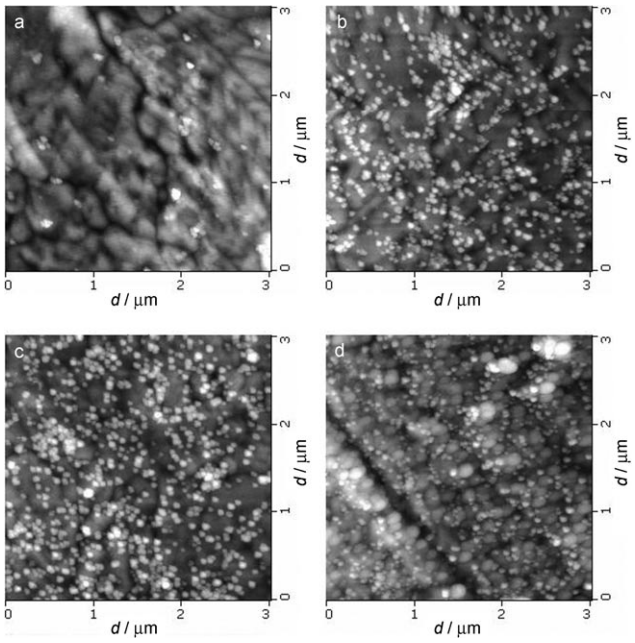


Figure 1. AFM images of a) the Au surface, b) gold nanoparticles on Au, c) the aptamer surface, and d) the [Ru(bpy)$_2$(dcbpy)NHS]-labeled lysozyme surface.

The Selection of Monolayer for GNP Amplification

Gold nanoparticle amplification is a well-known method for signal enhancement in biosensors.[21] The difficulty is that gold nanoparticle sols are unstable especially when electrolytes are added, owing to inevitable rapid precipitation and flocculation. To make sure of the stability of the self-assembled monolayer (SAM) for GNP amplification, three SAMs including 1,6-hexanedithiol, cysteamine, and *p*-ATP were investigated. Among the three SAMs cysteamine is the best conductor because of its shorter chain, and GNPs are easy to precipitate when it is added. 1,6-Hexanedithiol shows a better stability than cysteamine, whereas its conductivity is not satisfactory. In considering both stability and conductivity, the monolayer of *p*-ATP was utilized for anchoring GNPs. Then, this process was characterized using electrochemical impedance spectroscopy (EIS). As shown in Figure 2, curve a represents the electrochemical impedance of the bare Au electrode, which shows a very small semicircular domain. When *p*-ATP is assembled on the Au electrode surface, we can see a slight change in the impedance in curve b. In the next step, the Au electrodes were dipped into colloidal gold to adsorb on the surface of *p*-ATP. We observed a strange phenomenon, as shown in curve c, which is very similar to curve a.[22] The reason may be attributed to the fact that GNPs are good conductors that can improve the adsorption capacity of the electrode. Therefore, the electron transfer from $[Fe(CN)_6]^{3-/4-}$ to the electrode would be enhanced and thus induces a decreased semicircle domain compared to curve b. Meanwhile, this result also illustrates that the GNPs could increase the effective electrode surface area and thus displays the potential to immobilize more aptamer molecules to amplify the signal. This is consistent with the ECL measurement as well. Compared to the sensing surface without GNPs, six times the enhancement of the final ECL signal is obtained in our experiment (data not shown here), fully proving the amplification function of the GNPs.

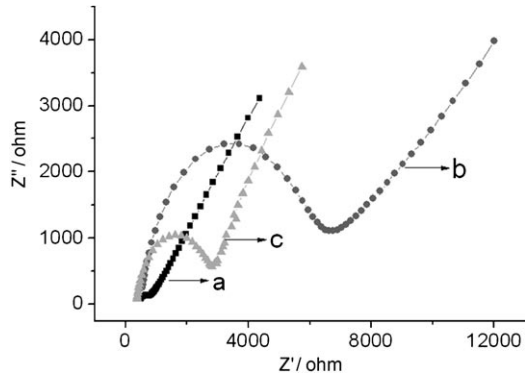


Figure 2. Impedance plot of the bare Au electrode (a), *p*-ATP modified Au electrode (b), and gold nanoparticles on the Au electrode (c) in the presence of 5 mmol L^{-1} $[Fe(CN)_6]^{3-/4-}$ with 0.1 mol L^{-1} KCl as the supporting electrolyte. The electrode potential was 0.24 V versus Ag/AgCl. The frequency range was 0.01 Hz to 10 kHz.

ECL Measurement of Lysozyme

Typical ECL intensity–potential curves of [Ru(bpy)$_2$(dcbpy)NHS]-labeled lysozyme immobilized on a gold electrode and [Ru(bpy)$_2$(dcbpy)] solution on a gold electrode are shown in Figure 3. As exhibited in curve a, the ECL intensity of [Ru(bpy)$_2$(dcbpy)] reaches a maximum at a potential of about 1.30 V versus Ag/AgCl. However, two ECL waves occur at the gold electrode in curve b, with the first ECL wave at a potential of 0.8 V, which is less positive than that for the oxidation of [Ru(bpy)$_2$(dcbpy)] (whereas the

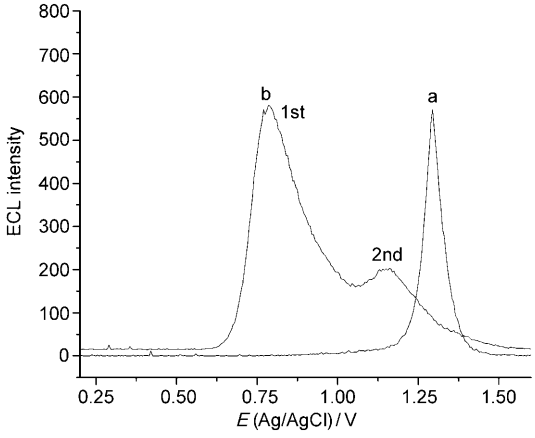


Figure 3. ECL profiles of [Ru(bpy)$_2$(dcbpy)] solution on the gold electrode (a) and [Ru(bpy)$_2$(dcbpy)NHS]-labeled lysozyme immobilized on the gold electrode (b); 100 mmol L^{-1} PB buffer containing 100 mmol L^{-1} TPrA and 50 mmol L^{-1} MeCN (pH 10.55) scanned from 0.20 to 1.60 V with a scan rate of 50 mV s^{-1}.

second wave occurs at a potential of 1.14 V for the oxidation of [Ru(bpy)$_2$(dcbpy)NHS]-labeled lysozyme). Some earlier works also reported a similar behavior when a much lower concentration of $[Ru(bpy)_3]^{2+}$ was used (just 1.0 nM in the presence of 0.10 M TPrA). The first ECL wave probably originates from TPrA and the second ECL wave may be [Ru(bpy)$_2$(dcbpy)NHS]-labeled lysozyme.[23] There may be two reasons for this behavior. 1) The concentration of TPrA is much higher than that of [Ru(bpy)$_2$(dcbpy)] at the surface of the electrode; thus, there is more opportunity for TPrA to be oxidated. 2) When [Ru(bpy)$_2$(dcbpy)NHS] is labeled on lysozyme, it can not transfer freely like TPrA to reach the electrode surface; hence, TPrA has a quicker oxidation rate. We thus draw the conclusion that the two ECL peaks are attributable to the oxidation of TPrA and [Ru(bpy)$_2$(dcbpy)NHS]-labeled lysozyme, respectively. It can be seen that lysozyme is well labeled, and the best potential for ECL detection is 0.80 V.

It is important to consider that the oxidation of the thiol layer and aptamers may induce loss of sensitivity toward the analytes. Thus, only the first-cycle ECL signal of the CV was recorded for the standard calibration curve. Almost no background ECL signal is observed before the [Ru(bpy)$_2$(dcbpy)NHS]-labeled lysozyme incubates with the biosensor. However, a great increase of ECL is observed when the [Ru(bpy)$_2$(dcbpy)NHS]-labeled lysozyme is captured on the electrodes. Figure 4 shows the ECL intensity at different concentrations of lysozyme after incubation with the biosensor. The ECL signal decreases with an increase in lysozyme concentration because the labeled lysozyme has a lower affinity to the aptamer than the unmodified one, which agrees well with the principle of displacement immunoassays described above. The detection limit of this method is 1.0×10^{-13} mol L^{-1}, which is more sensitive than other ECL methods reported.[24]

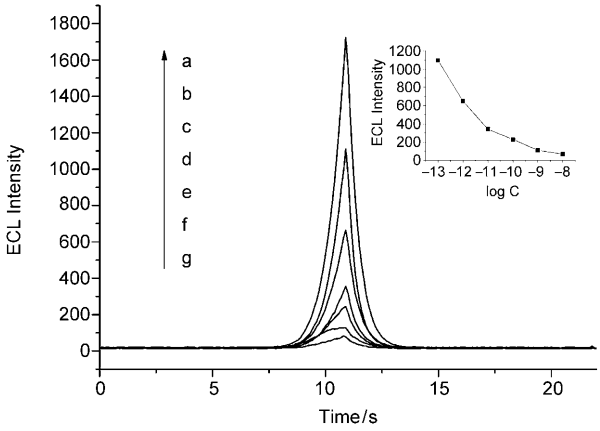


Figure 4. The ECL profiles of different concentrations of lysozyme after incubation with the fabricated electrodes. Concentration of lysozyme: a) 0, b) 1.0×10^{-13} mol L^{-1}, c) 1.0×10^{-12} mol L^{-1}, d) 1.0×10^{-11} mol L^{-1}, e) 1.0×10^{-10} mol L^{-1}, f) 1.0×10^{-9} mol L^{-1}, g) 1.0×10^{-8} mol L^{-1}; 100 mmol L^{-1} PB buffer, containing 100 mmol L^{-1} TPrA and 50 mmol L^{-1} MeCN (pH 10.55) scanned from 0.25 to 0.80 V with a scan rate of 50 mV s^{-1}. Inset: Maximum ECL intensity as a function of the logarithm of the lysozyme concentration in mol L^{-1}.

The specificity analysis of the ECL biosensor was examined using two common proteins, BSA and CytC, as shown in Figure 5. It was found that, when BSA, CytC, and lysozyme with the same concentration (1.0×10^{-13} mol L^{-1}) were incubated and detected individually with the biosensor, only in the presence of lysozyme was an obvious decrease in ECL displayed. Therefore, it can be seen that no significant interference was obtained for these proteins, and a high selectivity for this biosensor was observed.

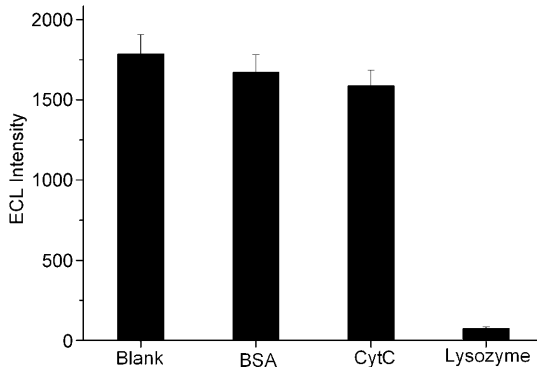


Figure 5. Specificity analysis of the ECL biosensor. The concentration of BSA, CytC, and lysozyme were 1.0×10^{-8} mol L^{-1}. The results are the average of three experiments.

Applications

To investigate the feasibility of this biosensor for the analysis of biological samples, the content of lysozyme in egg was examined. Lysozyme is a familiar small enzyme with well-known structure and properties, making up 3.5% content in egg. The lysozyme in an egg white sample was diluted to 10^{-10} mol L^{-1} and then analyzed by this method. The average concentration of detection sample according to the standard curve is 6.2×10^{-11} mol L^{-1} with a relative error of 10.6% ($n=5$). In view of the activity of lysozyme in egg, the result is still satisfactory.

Conclusions

A sensitive and selective electrochemical aptasensor for lysozyme has been developed by self-assembly, gold nanoparticle amplification, and displacement immunoassay protocols. This method shows remarkably high sensitivity as a result of all these protocols used. A sixfold enhancement of the ECL intensity was obtained in our experiment when *p*-ATP was used for the GNP amplification. The biosensor has been utilized to measure lysozyme with a detection limit of 1.0×10^{-13} mol L^{-1}. Finally, this protocol could be applied to

a real sample, and it could potentially be applied to the detection of other proteins.

Experimental Section

Reagents

For synthesis, commercially available chemicals were used without further purification unless specified. $RuCl_3 \cdot xH_2O$, $HAuCl_4$, sodium hexafluorophosphate, lysozyme, 4-aminothiophenol (*p*-ATP), 1,6-hexanedithiol, cysteamine, and 6-mercapto-1-hexanol were purchased from Sigma (St.Louis, MO); tri-*n*-propylamine (TPrA), *N*,*N*′-dicyclohexylcarbodiimide (DCC), and *N*-hydroxysuccinimide (NHS) were purchased from Pierce (Rockford, IL); 2,2′-bipyridine (bpy) was purchased from First Reagent Factory in Shanghai, China. Oligonucleotides were obtained from Shenggong Bioengineering Ltd. Company (Shanghai). The sequence of aptamer was 5′(SH)-$(CH_2)_6$-TTTTTTTTTTATCTACGAATTCATCAGGGCTAAAGAGTGCAGAGTTACTTAG3′. Other reagents were all of analytical reagent grade. Ultrapure water from a Milli-Q plus system (Millipore Co.) was exclusively used in all aqueous and rinsing procedures. The 0.1 mol L^{-1} PB buffer (pH 10.55) containing 0.1 mol L^{-1} TPrA was used as the electrolyte for the ECL intensity–potential measurement.

Apparatus

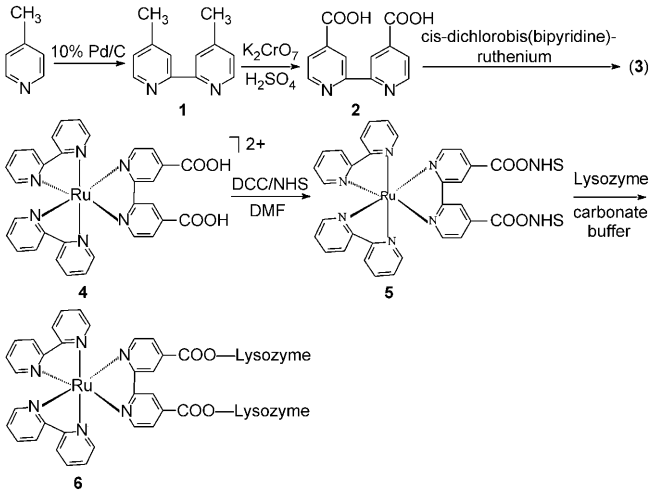
The electrochemical measurements were performed with a three-electrode system. The working gold electrodes were fabricated as a lysozyme biosensor before use. A KCl-saturated Ag/AgCl electrode and a platinum wire electrode were used as the reference and the auxiliary electrode, respectively. The scan rate was fixed at 50 mV s^{-1}. Because the ECL performance depended on the gold electrode pretreatment history, the gold electrodes were polished with 0.30 and 0.05 μm α-Al_2O_3 power, then ultrasonically cleaned in ethanol and water for 3 min each, and washed with water thoroughly. The gold electrodes were subsequently scanned in H_2SO_4 solution (0.10 mol L^{-1}) over the range −0.20 to +1.6 V for about 200 cycles to achieve a constant cyclic voltammogram and then washed with water again. The ECL emission was detected with a Model MPA Chemiluminescence Analyzer Systems (Xi′an Remax Science & Technology Co. Ltd., Xi′An, China). The voltage of the photomultiplier tube used in the Model MPA Chemiluminescence Analyzer was set at 850 V in the process of detection. The ECL peak intensities were used for quantitative analysis in our study. For [Ru(bpy)$_2$(dcbpy)NHS]-labeled lysozyme the ECL peak intensities at +0.80 V were used for quantitative analysis. Only the ECL generated from the first circle of cyclic voltammetry was recorded because of the oxidation of thiolayer and lyozyme-binding aptamer would lead to an ECL intensity loss for further use. The ^{1}H NMR spectra were recorded on a Varian Gemini 400 (MHz) spectrometer. Transmission electron microscopy (TEM) samples were examined by a HITACHI H-8100 electron microscope operated at an accelerating voltage of 200 kV. All the AFM images were taken with an SPI3800N microscope instrument (Seiko Instruments, Inc.) in tapping mode in air at ambient temperature.

Preparation of Gold Nanoparticles

Gold nanoparticles with a diameter of approximately 13 nm was prepared according to a well-known method.[25] In brief, 10% $HAuCl_4$ solution (170 μL) was heated to reflux and then sodium citrate (2 mL, 0.1 mol L^{-1}) was introduced to the boiling solution with stirring. The solution was kept boiling for another 15 min and cooled to room temperature. The diameter of gold the nanoparticles was about 13 nm according to TEM (picture not shown).

Preparation of [Ru(bpy)$_2$(dcbpy)NHS]-Labeled Lysozyme

This compound was prepared similarly to some published procedures with several modifications. All the ruthenium complexes were characterized by ^{1}H NMR spectra, and the results agreed with those in the literature.[26] The synthetic route to the ruthenium complexes is shown below.



4,4′-Dimethyl-2,2′-bipyridine (**1**): This compound was prepared essentially as described elsewhere .[27] Freshly distilled 4-picoline (50 mL) with 10% Pd/C (2 g) was heated at reflux for 3 days. Benzene (20 mL) was added to the mixture over 30 min. The solution was filtered while still hot and washed with benzene. The filtrate was concentrated in vacuum overnight. The white crystals were recrystallized from ethyl acetate, yielding 5.8 g.

4,4′-Dicarboxy-2,2′-bipyridine (**2**): The procedure described by Zhou et al.[28] was used to obtain a much higher yield (95%) than the traditional method.[29] The solution containing **1** (1 g) and sulfuric acid (95–98%, 25 mL) was stirred, then potassium dichromate (4.8 g) was added to the solution gradually. The process was highly exothermic, so the reaction temperature should be controlled between 70 and 80 °C with water cooling. The reaction mixture was stirred for an additional 30 min until the temperature fell to room temperature. The dark green solution was poured into 180 mL of ice water and allowed to stand for 4 h in the ice water bath. Removal of the dark green precipitate by filtration resulted in a light yellow solid, which was heated at reflux with nitric acid (50%, 30 mL) for 4 h. After acidification, the solution was poured into ice water (80 mL) and filtered again. White crystals (1.18 g) were obtained after washing and drying.

cis-Dichlorobis(bipyridine)ruthenium (**3**): $RuCl_3$ (1.5 g) and 2,2′-bipyridine (1.8 g) were heated at reflux in DMF (60 mL) for 3 h. Most of the DMF was evaporated and cooled to room temperature. Acetone (50 mL) was added to the remaining solution and kept at 0 °C overnight. Crystals formed after filtration and were washed with water. The crude product was heated at reflux in a mixture of water–ethanol (40 mL, 1:1) for 1 h, filtered, and treated with lithium chloride (30 g) carefully. The ethanol in the solution was distilled, and after cooling in an ice bath, dark brown crystals were obtained, which were washed with water and dried in vacuo for 3 h. Yield: 2.1 g.

4: A mixture of methanol (15 mL) and water (10 mL), **2** (300 mg), $NaHCO_3$ (300 mg), and **3** (500 mg) were heated for 4 h under reflux. After heating, the solution was cooled to room temperature and saturated ammonium hexafluorophosphate (5 mL) was added to precipitate the product, which was refrigerated overnight. The dark purple precipitate was collected by filtration and washed with water. Yield of hexafluorophosphate salt of **4**: 250 mg.

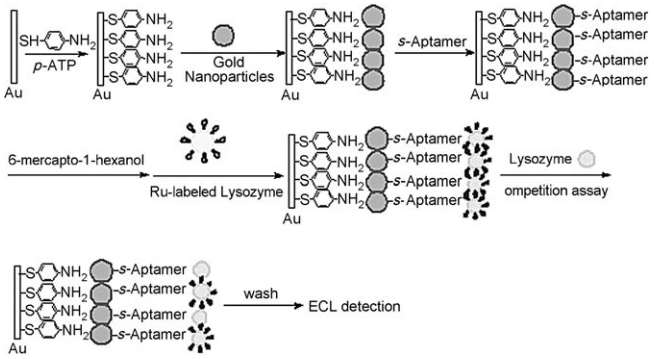
5: In this experiment *N*,*N*′-dicyclohexylcarbodiimide (DCC) and *N*,*N*′-diisopropylcarbodiimide (DIC) were considered as potential coupling reagents. Because the by-products from DCC can be easily separated from the product, DCC was used as the coupling reagent. *N*-hydroxysuccinimide (NHS) is a popular active ester to simplify the workup progress. Compound **4** (40 mg, 40 μmol) was dissolved in anhydrous acetonitrile (2 mL) and DCC (17.3 mg, 84 μmol) and NHS (8.8 mg, 88 μmol) were added and stirred in an ice bath for 48 h in the dark. The filtrate of the crude product was added to anhydrous 2-propanol (15 mL) to precipitate

at 0 °C for 4 h. The orange-colored NHS ester was collected by filtration and washed with dry ether (5 mL) three times.

6: Compound **5** (2 mg) was dissolved in anhydrous DMF (100 μL), and lysozyme (2 mg) was dissolved in carbonate buffer (900 μL, 50 mmol L^{-1}, pH 9.0). Then the [Ru(bpy)$_2$(dcbpy)NHS] solution was added dropwise to the lysozyme solution with stirring at room temperature. Six hours later, the formed [Ru(bpy)$_2$(dcbpy)NHS]–lysozyme conjugate was stored at 4 °C.

Fabrication of the Biosensors

The fabricated biosensors were prepared in four steps. First, the pretreated gold electrodes were immersed in *p*-ATP ethanol solution (100 μmol L^{-1}) at room temperature for 24 h in the dark to prepare thiol self-assembled monolayers (SAM) on the surface of the gold electrodes. Before the next step the newly formed SAM was rinsed with ethanol and water and dried with a N_2 stream. Second, the gold electrodes were dipped into the colloidal gold for 10 h. The gold nanoparticle modified electrodes were rinsed with water and dried with a N_2 stream. Third, the lysozyme-binding aptamer (10 μL, 2.0 μmol L^{-1}) in TE buffer (50 mmol L^{-1} Tris-HCl, 1 mmol L^{-1} EDTA) was dropped onto each gold electrode for 20 h and then rinsed with water and dried with a N_2 stream. The surface of the gold substrates and the GNPs were blocked with 200 μL of 6-mercapto-1-hexanol (20 mmol L^{-1}) for 30 min, followed by rinsing with water. Finally, [Ru(bpy)$_2$(dcbpy)NHS]-labeled lysozyme (10 μL) in phosphate buffer (50 mmol L^{-1}, pH 7.5) was dropped on the Au/SAM/S-aptamer surface, and 30 min later the desired Au/SAM/S-aptamer/[Ru(bpy)$_2$(dcbpy)NHS]-labeled lysozyme structure was obtained. The schematic diagrams of the procedures for the fabrication of biosensor are shown below.



Displacement Assay and ECL Detection

The fabricated biosensors above were incubated with different concentrations of lysozyme solution at 37 °C for 1 h, followed by washing with water, and put in PB solution (100 mmol L^{-1}, pH 10.55) containing TPA (0.1 mol L^{-1}) and then scanned from 0.25 to 0.80 V.

Acknowledgements

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