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Label-Free and Signal-On Electrochemiluminescence Aptasensor for ATP Based on Target-Induced Linkage of Split Aptamer Fragments by Using $[Ru(phen)_3]^{2+}$ Intercalated into Double-Strand DNA as a Probe

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Aptamers are specific nucleic acids (DNA or RNA) selected from random sequence libraries using SELEX (systemic evolution of ligands by exponential enrichment)[1,2] with high affinity and specificity in the binding to small molecules, proteins and other macromolecules.[3,4] Owing to their simple synthesis, good stability, easy storage, and simple modification for further immobilization procedure,[5–10] aptamers have attracted increasing interest as the ideal recognition elements for biosensor applications.[11–14]

Sandwich-type assays have been widely used in biosensors for their specificity and low detection limits.[15–18] However, the approach largely excluded aptamer-based sensors due to the requirement that the target exposes two distinct epitopes.[19] Hence, rather few aptamer-based sandwich-type assays have been reported for proteins, much less small molecules.[20–23] Recently, the construction of aptasensors for small molecules based on linkage of split-aptamer fragments, in the presence of the analyte-substrate, creating a "sandwich assay", was introduced as a general platform for aptasensors.[19,24–26] However, most aptasensors need chemical labeling procedures, which are usually complex, time-consuming, and labor-intense. Therefore it is desirable to establish a label-free aptamer-based sandwich-type assay with high specificity and low detection limit for small molecules.

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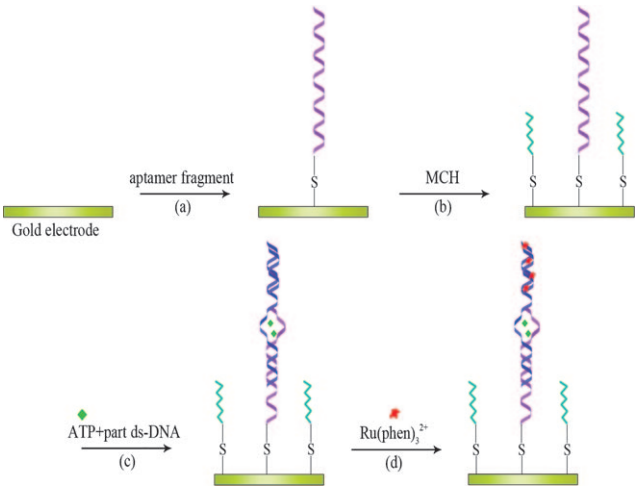
It has been reported that the double-strand DNA (ds-DNA) has the capacity to be intercalated with some small molecules into its grooves with high affinity;[27–31] some aptamer-based sensors have been developed based on the intercalation of small molecule probes into the DNA structures.[32–36] Nevertheless, most of these sensors are based on the competing reaction between the analytes and complementary strands, which might be more difficult than only target–aptamer interaction. The factor could lead to relatively slow response to the target compared with some non-competition assays. Recently, electrochemiluminescence (ECL) aptasensors, which integrate the advantages of electrochemical detection[37] and chemiluminescent techniques, have received particular attention due to their high sensitivity and selectivity, wide linear ranges, as well as low production cost.[32] As a popular ECL reagent with high ECL emission efficiency for bioassays, $Ru(phen)_3^{2+}$ (phen = 1,10-phenanthroline) can intercalate into the grooves of ds-DNA.[30,31] In comparison to the commonly used DNA-binding fluorescing intercalator ethidium bromide, $Ru(phen)_3^{2+}$ is more expensive, but has the advantages of lower toxicity, better stability, and easy use. Moreover, $Ru(phen)_3^{2+}$ can be used not only in fluorescence study but also ECL studies, which do not require expensive light source as in fluorescence study.[38–40]

Herein, a label-free sandwich-type ECL sensing system based on target-induced conjunction of split aptamer fragments has been developed by the use of $Ru(phen)_3^{2+}$ intercalated into ds-DNA as the ECL probe. ATP was selected as the model target to demonstrate the principle of the present ECL aptamer-based assay. The design concept of the sensing system and the ATP detection are displayed in Scheme 1.

To assemble the aptasensor, the 27-mer anti-ATP DNA aptamer was divided into two different fragments which do not interact with each other in the absence of ATP. One of them, modified with thiol at 5′ terminus **(1)**, was immobi-



Scheme 1. Schematic for the preparation of the ECL ATP aptasensor. a) Assembling the aptamer fragment; b) 6-mercaptohexanol (MCH) block to the electrode; c) binding with ATP and part ds-DNA; d) intercalating $Ru(phen)_3^{2+}$ into the ds-DNA structure.

lized on the gold electrode through interaction of thiol-Au; the other one, lengthened with other 23-mer DNA sequence **(2)**, was hybridized with its complementary 20-mer single strand DNA **(3)** to form a part DNA duplex (ds-DNA). In the absence of ATP, the thiolated 5′ fragments interacted weakly with part ds-DNA. The part ds-DNA intercalated with $Ru(phen)_3^{2+}$ could be easily removed via washing, resulting in a weak ECL signal. In the presence of ATP, a linkage of the thiol modified aptamer fragment and part ds-DNA was induced to form strong aptamer-target complex on the electrode surface. As the concentration of ATP increases, more aptamer–target complexes are formed on the electrode surface, and can immobilize more $Ru(phen)_3^{2+}$ by intercalation, which leads to the increase in ECL signals with increasing ATP concentrations. The change of the ECL intensity was thus able be used to quantify the ATP content.

Cyclic voltammetry (CV) was used to characterize the modified procedure of gold electrode. The CVs of different modified electrodes in 0.2 M PBS (pH 7.5) in the presence of a 5 mM $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$ mixture as a redox probe are shown in Figure 1. Curve a displays the CV of a bare

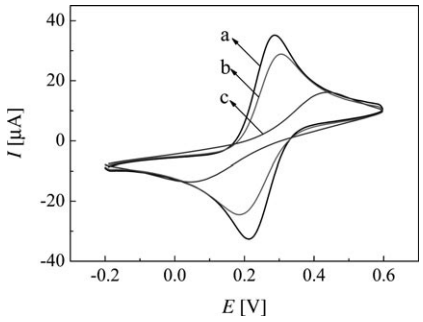


Figure 1. CVs of the different electrodes in 0.2 M PBS (pH 7.5) containing 5 mM $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$: a) a bare gold electrode; b) thiolated 5′ fragment modified gold electrode; c) electrode b) after incubated in the part ds-DNA **4** mixed with ATP solution. Scan rate: 50 mV s^{-1}.

gold electrode and shows a pair of well-defined current peaks at 0.21 and 0.28 V (vs. Ag/AgCl). After self-assembled thiolated 5′ fragments layer on gold electrode, the current response obviously decreased (Figure 1b), which indicates that the thiolated 5′ fragments are immobilized on gold electrode surface and decreased the electron-transfer efficiency. After the thiolated 5′ fragments reacts with the part ds-DNA and ATP, the peak current decreased greatly owing to the formation of aptamer–target complex blocking layer (Figure 1c).

The CV and ECL intensities of the sensor are shown in Figure 2. Curves a and b are the CV and the corresponding ECL response of the $Ru(phen)_3^{2+}$/part ds-DNA/ATP/aptamer fragment modified electrode in 0.2 M PBS (pH 7.5) containing 20 mM oxalate. It does not exhibit any ECL response in the potential range from 0 to 0.95 V. As the potential increased, the ECL intensity increased and achieved its maximum at 1.15 V (Figure 2b). At the same time, an obvious oxidation peak was obtained at this potential (Figure 2a), because of the electrochemical oxidation of oxalate and $Ru(phen)_3^{2+}$.

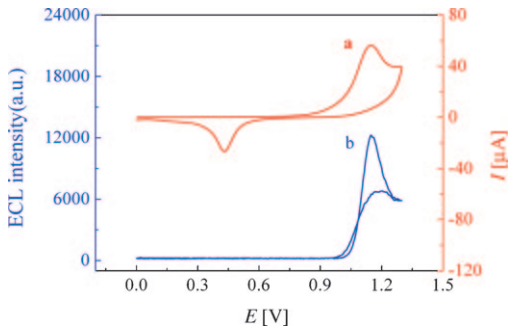


Figure 2. CV (curve a) and ECL intensity response (curve b) of gold electrode modified with $Ru(phen)_3^{2+}$/part ds-DNA/ATP/aptamer fragment in 0.2 M PBS (pH 7.5) containing 20 mM oxalate. Scan rate: 50 mV s^{-1}.

The detection of ATP is based on the change of the ECL intensity before and after the aptamer–target recognition reaction. In the absence of ATP, the thiolated 5′ fragments interacted weakly with part ds-DNA. The part ds-DNA intercalated with $Ru(phen)_3^{2+}$ could be easily removed by washing, resulting in weak ECL signal. The value of the ECL intensity was recorded as I_{0ECL}. In the presence of ATP, thiolated 5′ fragments would combine with ATP and part ds-DNA to form compound on the electrode surface. Then, $Ru(phen)_3^{2+}$ was intercalated into ds-DNA and resulted in the increase of ECL signals, which was recorded as I_{ECL}. The change of the ECL intensity given by $\Delta I_{ECL} = I_{ECL} - I_{0ECL}$ is dependent on the concentration of ATP.

The aptasensor was incubated with different concentrations of ATP and then ECL responses were recorded in PBS (pH 7.5) containing 20 mM oxalate. The changes of ECL intensity (ΔI_{ECL}) were proportional to the ATP concentration ranging from 6.4×10^{-7} to 1.0×10^{-3} M with a regression equation of the form $\Delta I_{ECL}(a.u.) =$

$7.7714C_{ATP}(\mu M)+314.04$ and a linear correlation of $r=0.9986$. A low detection limit of 0.64 μM ATP was calculated according with the $3s_b/m$ criterion, where m is the slope of the calibration graph, and s_b is the standard deviation ($n=3$) of the signals of the blank. As shown in Table 1, the result is comparable with those of other reported methods.[41–43]

Table 1. Comparison of the present method with other reported methods.

Detection method	Concentration range [M]	Detection limit [M]	Ref.
electrochemical method	1.0×10^{-6}–4.0×10^{-3}	1.0×10^{-6}	[19]
fluorometric method	4.0×10^{-5}–5.0×10^{-6}	1.0×10^{-7}	[41]
chemiluminescence	3.9×10^{-7}–9.96×10^{-5}	1.38×10^{-7}	[42]
colorimetry	4.4×10^{-6}–1.327×10^{-4}	6.0×10^{-7}	[43]
electrochemiluminescence	6.4×10^{-7}–1.0×10^{-3}	6.4×10^{-7}	present work

In order to investigate the selectivity of the aptasensor for ATP, the changes in the ECL response induced by the possible interference, such as CTP, GTP, and UTP were measured. The change in ECL response induced by the non-specific binding between the interference above was much lower than that of the response induced by the specific binding of ATP (Figure 3a–d). We had also studied the changes in the ECL response induced by the ATP analogues, including ADP, AMP and adenosine. Similar to other studies, our study shows that there was no significant difference between the changes in the ECL response induced by the ATP analogues and that induced by ATP since the aptamer bind ATP, ADP, AMP, and adenosine with similar affinity.[44,45] The selectivity can be improved if more selective ATP aptamers can be found in the future.

Control experiments with point mutant of the aptamer were performed. Oligonucleotide **4** (see Experimental Section for its sequences) was used to replace **2** for the detection of ATP. Oligonucleotide **4** is a point mutant of the aptamer fragment **2** with a G to A change (indicated in italics) at the aptamer fragment domain of **2**. When **2** was replaced with oligonucleotide **4**, ECL responded negligibly after reaction with 0.1 mM ATP (Figure 3e). This indicates that a G to A change at this position eliminated binding to ATP.

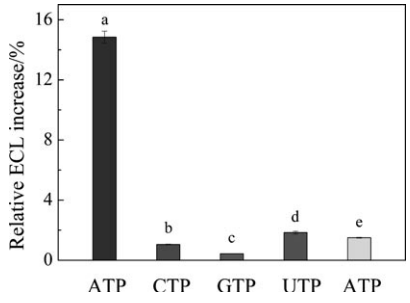


Figure 3. ECL signal increase of the aptasensor brought by 0.1 mM a) ATP, b) CTP, c) GTP, and d) UTP in 0.2 M PBS (pH 7.5) containing 20 mM oxalate; e) 0.1 mM ATP detected by **4** in 0.2 M PBS (pH 7.5) containing 20 mM oxalate. The error bars represent the relative standard deviation of three measurements.

The long-time stability of the aptasensor was also investigated during a 15-day period. When the aptasensor was stored in the refrigerator at 4 °C, the ECL response of the aptasensor retains 94.3 % of its initial response after the first seven days storage and 92.13 % after 15-day storage. This indicates that the developed aptasensor has good stability.

In summary, a label-free sandwich-type ECL aptasensor for highly sensitive detection of ATP had been developed based on target-induced conjunction of split aptamer fragments by the use of $Ru(phen)_3^{2+}$ intercalated into ds-DNA as the ECL probe. The sensor works in a wide linear range and good sensitivity for ATP. The proposed technique offers a new approach to generate a wide variety of signaling aptamer-based assays for small molecules or protein detection.

Experimental Section

Materials: All DNA oligonucleotides used in this study were obtained from Sangon Inc. (Shanghai, China) with the following sequences:

1: 14-mer thiolated 5′ fragment: 5′HS-$(CH_2)_6$-**ACC TGG GGG AGT AT**-3′

2: 36-mer lengthened 3′ fragment: 3′-**TGG AAG GAG GCG T**CA A*GT TTT TCT AGT CTA TTA TTC*-5′

3: 20-mer part complementary ss-DNA of **2**: 5′-*CA AAA AGA TCA GAT AAT AAG*-3′

4: 3′-**TGG AAG GAG ACG T**CA A*GT TTT TCT AGT CTA TTA TTC*-5′

Apparatus: Electrochemical measurements were carried out in a conventional three-electrode cell with a CHI 840B electrochemistry work station (Shanghai CH Instruments Co., China), using an Ag/AgCl reference electrode (saturated KCl), a platinum wire counter electrode, and the modified gold electrode ($\Phi=3$ mm) as working electrode. ECL intensities were monitored through the bottom of three-electrode cell with a BPCL Ultra-Weak luminescence analyzer (Institute of Biophysics, Chinese Academy of Science). Unless otherwise noted, the PMT was biased at −1200 V.

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Keywords: aptamers · biosensors · electrochemistry · luminescence · ruthenium

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