Supporting Information for

Functional Nucleic Acid-based Electrochemiluminescent Biosensor for Interaction Study and Detection of Ag⁺ Ions and Cysteine

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Experimental Section

Apparatus. The electrochemical measurements for ECL emission were carried out on a Model LK98BII Microcomputer-based Electrochemical Analyzer (Tianjin Lanlike High-Tech Company, Tianjin, China). A traditional three-electrode system was used with Ag/AgCl/KCl (satured) as reference electrode, a 3-mm diameter Au disk modified electrode as working electrode, and Pt wire as counter electrode. The ECL emission was detected by using a model MPI-A Chemiluminescence Analyzer (Xi'An Remax Electronic Science & Technology Co. Ltd, Xi'An, China) at room temperature. The voltage of the PMT was set at -900 V in the detection process. The fluorescence is measured using an F-4500 spectrofluorometer (Hitachi, Japan) with a 1 cm quartz cell at room temperature.

Reagents. HPLC-purified DNA oligonucleotides were provided by Takara Biotechnology (Dalian, China) with the following sequences:

1: 5'-SH-(CH₂)₆-CCCCC-3' (SS1)

2: 5'-CCCCC *GTT TGT AAT AGA ACA A* **GAG** *T TGT TCT ATT ACA AAC* -3' (denoted as Functional Oligonucleotides, **FO**). C₅ section is used to recognize of Ag^+ ion and form C- Ag^+ -C complex. The italic part can form intermolecular duplex and is used for $Ru(phen)_3^{2+}$ intercalation. Black GAG is a loop for the formation of duplex.)

3: 5'- GGGGG-3' (SS2)

All oligonucleotides were diluted to 5 μ M in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) as single strand concentration. Tris(1,10-phenanthroline)ruthenium chloride hydrate (Ru(phen)₃Cl₂·H₂O) and tripropylamine (TPA) were obtained from Sigma-Aldrich, Shanghai, China. 2-Mercaptoethanol (MCE) used to block the active sits was obtained from Yangguang Yunneng Biotechnology Company, Tianjin, China. Saline solutions were prepared with Mn(Ac)₂, Mg(NO₃)₂, Pb(NO₃)₂, Zn(Ac)₂, Cd(NO₃)₂, Fe(NO₃)₃, CaCl₂, Co(Ac)₂, Cu(NO₃)₂, AgNO₃, and Hg(NO₃)₂. Various amino acids were used to investigate the potential interference and to validate the selectivity of this biosensor for the Cys.

Sensing Ag⁺ ions. The procedure for determination of Ag^+ ions using the ECL biosensor was presented in Scheme 1. The new gold electrode (3-mm in diameter) was polished with 0.3- and 0.05- μ m

aluminum slurry and ultrasonicated with distilled water for 3 minutes. The electrodes were cleaned further in 0.1 M H₂SO₄ by potential scanning between -0.2-1.6 V until a remarkable voltammetric peak was obtained. The gold electrode was modified with SS-DNA (C₅, **SS1**) using 6 μ L of 5 μ M thiolated oligonucleotide solution for 0.5 h at 36 °C. Then, 2-mercaptoethanol (MCE) was used to block the active sites for 0.5 h at 36 °C. At the same time, 10 μ M FO was mixed with 1 mM Ru(phen)₃²⁺ at 4 °C in refrigerator over night for the probe intercalation (Scheme 1A). 10 μ M G₅ (**SS2**) was added into FO intercalated with Ru(phen)₃²⁺ for the formation of G-C double helix structure for 1 h at 36 °C. 5 μ L asprepared FO and 10 μ L sample containing Ag⁺ ions were dropped onto the **SS1**-modified electrode for 0.5 h at 36 °C. During this period, the C-Ag⁺-C construction formed and the probe, Ru(phen)₃²⁺, was introduced into the electrode surface (Scheme 1C). After the modified electrode was cleaned thoroughly, the potential was scanned linearly from 0 to 1.25 V vs Ag/AgCl with 20 mM TPA as coreactant for ECL determination of Ag⁺ ions. The selectivity of the biosensor was tested in the presence of the competing metal ions such as Mn²⁺, Mg²⁺, Pb²⁺, Zn²⁺, Cd²⁺, Fe³⁺, Ca²⁺, Co²⁺, Ag⁺, and Cu²⁺.

Sensing Cys. For sensing Cys, the mixture of 10 μ L of 100 nM Ag⁺ ions and 5 μ L of as-prepared FO prepared freshly was incubated onto the C₅-modified electrode. Further, 10 μ L of the sample containing Cys was dropped onto the FO-modified electrode (Scheme 1D). The interaction between Cys and Ag⁺ ion dissociated the C-Ag⁺-C complex and the ECL probe, Ru(phen)₃²⁺ was released. The change (ΔI_{ECL}) in the emission before and after reacting to the sample was used to quantify the content of Cys.

The recovery experiment of Cys and the result comparison between the present method and ESI-MS were used to validate the performance of the biosensor. Human serum samples were obtained from volunteers and provided by a local hospital. 10 μ L of the serum sample was diluted to 1000 μ L for Cys determination. Similar to the standard solution, 10 μ L of the serum sample with and without spiked Cys was dropped onto the FO-modified electrode for intramolecular displacement between Cys and cytosine bases in C-Ag⁺-C complex.

The construction of ECL aptasensor for detection of Ag⁺ ions and Cys

The formation of C-Ag⁺-C complex and the intercalation of $Ru(phen)_3^{2+}$ into the formed duplex play an important role in the present biosensor for Ag⁺ and Cys. Ag⁺ has a strong interaction with nucleic acid containing multi-cytosine, and thus, the complex formed between Ag⁺ and cytosine can be used as a platform for probing Ag^{+} . ¹⁻⁹ Ru(phen)₃²⁺ has the capacity to be intercalated into the preformed duplex of DNA and is used to develop various ECL DNA-based biosensors. ¹⁰ Other, Cys is much strong Ag⁺ ion-binder and can interact with Ag⁺ to dissociate the C-Ag⁺-C complex. ¹¹⁻¹² A functional oligonucleotide (FO) is therefore designed to integrate a cytosine-rich section for Ag⁺ ion recognition and an intermolecular duplex for intercalation of $Ru(phen)_3^{2+}$. The duplex was designed as only 4 cytosine bases in the total 35 bases, so Ag⁺ ion has weak interaction to the duplex section in FO. To eliminate the hybrid between Ag⁺ ions and the duplex section, the strand for duplex in FO was designed as cytosine-poor sequence as shown in Experimental section. With this design, the ECL sensor for Ag⁺ ion and Cys was constructed as illustrated in Scheme 1. The incubation of FO and $Ru(phen)_3^{2+}$ achieved the intercalation of $Ru(phen)_3^{2+}$ into the intermolecular duplex (Scheme 1A). The interaction between $Ru(phen)_3^{2+}$ and the duplex was investigated with fluorescent titration experiment as shown in Fig. S1. To avoid the self-assembly between cytosine-rich sections in different FOs and Ag⁺ ions, a G-C duplex preformed with the Ag⁺ ion recognition section and a G₅ oligonucleotide (Scheme 1B). To introduce FO onto a gold electrode surface, a thiolated C₅ oligonucleotide was immobilized onto the electrode surface through gold-thiol bond. In the presence of Ag⁺ ions, C-Ag⁺-C complex formed between FO and the immobilized C₅ sequence (Scheme 1C). In this mode, the FO containing ECL probe $Ru(phen)_3^{2+}$ was introduced onto the electrode surface. An ECL emission was observed when a potential was applied with tripropylamine (TPA) as co-reactant. The emission was employed to monitor Ag^+ ion concentration. The strong interaction between Cys and Ag^+ ion can dissociate the C- Ag^+ -C complex. Then, the FO was released from the electrode surface as shown in Scheme 1D. Correspondingly, the decreased emission was used to quantify the content of Cys.

Investigation of intercalation of Ru(phen)₃²⁺ into the duplex with fluorescent titration

Functional oligonucleotide functions as the intercalation of $Ru(phen)_3^{2+}$ into the duplex and the formation of C-Ag⁺-C complex. To validate the formation of intermolecular duplex, the intercalation of $Ru(phen)_3^{2+}$ and that whether the two parts interfere to each other, a fluorescent titration experiment was carried out (Fig. S1). $Ru(phen)_3^{2+}$ has natural fluorescent emission but DNA strand not. Moreover, the intercalation can enhance the fluorescent emission. As shown in Fig. S1, the fluorescent emission was enhanced as FO concentration increased from 0 to 0.4 µM and then the intensity levelled off. There are two kinds of interaction between $Ru(phen)_3^{2+}$ and adjacent DNA base pairs (bps), one is the strong intercalation and the other is the electrostatic interaction. ^{10, 13-14} The strong intercalation between $Ru(phen)_3^{2+}$ and DNA bps resulted in the enhanced luminescence. ^{10, 13} Thus, once $Ru(phen)_3^{2+}$ was totally intercalated into the duplex, no further increase in fluorescence emission was observed even the concentration of FO increased. Binding constant and site size can be calculated from the fluorescence data.^{10, 13} Using the data in Fig. S1, the calculated ratio (i.e. site size) between base pair number of duplex in FO and $Ru(phen)_3^{2+}$ molecules was 3.97:1, similar to the previous result of 4:1. ^{10, 13} The consistent result indicated the successful formation of intermolecular duplex and the intercalation of $Ru(phen)_3^{2+}$. It also validated that the Ag⁺ ion recognition section did not interfere to the formation of duplex and intercalation of $Ru(phen)_3^{2+}$. There are 16 bps in the duplex section of FO and every 4 bps can be intercalated with one Ru(phen)₃²⁺ molecule, so four Ru(phen)₃²⁺ molecules were integrated into one FO strand. Comparing with the chemical labelling signal molecule at the end of DNA strand,¹⁵⁻¹⁷ the intercalation strategy provides a simple but efficient method to integrate the probe into the DNA strand because no chemical labelling is needed and one strand can be integrated with four signal molecules.^{10, 13-14} The duplex was designed as only 4 cytosine bases in the total 35 bases, so Ag⁺ ion has weak interaction to the duplex section in FO.

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Fig. S1. (A) Fluorescent titration of 1.0 μ M Ru(phen)₃²⁺ using different concentration of FO (where an intermolecular duplex section formed for the intercalation of Ru(phen)₃²⁺). (B) The profile of the peak emission intensity and the concentration of FO.

The electrochemical behavior of the biosensor

The electrochemistry during the preparation procedure of the biosensor was investigated to validate the possibility of the biosensor. Fig. S2 shows the electrochemistry in 0.1 M phosphate buffer solution (PBS) containing 20 mM TPA. A low TPA oxidation current appeared at the bare gold electrode (Fig. s2a). Once the electrode was modified with C_5 , an improved peak current was observed. The integration of FO on the electrode surface resulted in an enhancement of the anionic currents, while the oxidation peak split into two parts. The first one was related to the oxidation of TPA and the second peak, as shown by the arrow, was from the oxidation of Ru(phen)₃²⁺. The previous works ^{10, 18-19} validated that dsDNA enhanced the TPA oxidation through the preconcentration and the improved deprotonation to TPA ^{10, 18} or the high conductivity of double strand of DNA. ²⁰ The catalytic action of Ru(phen)₃²⁺ also increase the oxidation of TPA (Fig. S2c). The second peak, from the oxidation of Ru(phen)₃²⁺, validated that the intercalated Ru(phen)₃²⁺ was successfully introduced onto the electrode surface.



Fig. S2. Cyclic voltammograms of (a) bare gold electrode, (b) C_5 -modified gold electrode, and (c) electrode modified with FO in 0.1 M PBS solution containing 20 mM TPA. Scan rate: 0.2 V s⁻¹.

The electrochemical behaviors of TPA and the intercalated $Ru(phen)_3^{2+}$ were further investigated with the cyclic voltammograms at different scan rates (Fig. S3). At C₅-modified electrode, the anion currents for TPA oxidation increased as the scan rates increased and the peak currents were directly proportional to the square root of the scan rates (Fig. S3A and B). The results indicated TPA oxidation was controlled by its diffusion from the bulk solution to the electrode surface. After FO integrated with $Ru(phen)_3^{2+}$ was modified on the C₅-modified electrode, an increase in the oxidation current was observed as a comparison with the that at C₅-modified electrode at the same scan rate. An improved TPA oxidation was observed at dsDNA-modified electrode^{10, 18} and a low potential ECL biosensor was successfully developed using the ECL emission from the improved TPA oxidation. ¹⁸ At high scan rate (>0.15 V s⁻¹), a shoulder peak appeared at about 1.10 V, that is the oxidation potential of ruthenium complex (Ru(phen)₃²⁺ and Ru(bpy)₃²⁺). Moreover, the oxidation currents from the shoulder peaks increased linearly with the increase in scan rate (Fig. S3D), indicating a surface-controlled electrochemistry for Ru(phen)₃²⁺ oxidation. The results was same as the previous reports ²¹⁻²² and proved that Ru(phen)₃²⁺ was immobilized onto the electrode surface.



Fig. S3. Cyclic voltammograms of the gold electrode modified with C_5 (A) and the FO- Ru(phen)₃²⁺ complex via Ag⁺ ions (C) at different scan rates. (B) The relationship between the anodic peak currents at Profile A and the square root of scan rates. (D) The relationship between the peak current as indicated

by arrow in Profile C and scan rates. Electrolyte 0.1 M phosphate buffer solution (pH 7.5) containing 20 mM TPA.

Selectivity of the present biosensor for Ag⁺ ions

The selectivity of the biosensor for Ag⁺ ions was tested with the emission from various metal ions in the absence or presence of Ag⁺ ions. As shown in Fig. S4, those non-target metal ions resulted in emission higher than the blank signal even no evidence that the metal ions can be interacted with C-C base pair. The complex and/or electrostatic interaction between the interfering ions and DNA bases may achieve the introduction FO containing $Ru(phen)_3^{2+}$ probe onto the electrode surface. However none of interfering metal ions produced the ECL emission higher than one tenth of that observed from 1 nM Ag⁺ ions even at their 100 nM level. Therefore, the selectivity of the biosensor was at least 1000-fold higher for Ag⁺ ions than the interfering ions. Similar emission was observed for 1 nM Ag⁺ ions with and without interfering mental ions at their 10 nM level. Compared with G-quadruplex-based DNAzyme enhanced by Pb^{2+} and inhibited by Hg^{2+} , ⁶ the present biosensor shows a high anti-interference capacity because Ag⁺ ion recognition section and probe intercalation section has no interference to each other. Because the modified electrode was cleaned thoroughly and the matrix was removed, so the ECL sensor does not encounter the matrix interference. Moreover, the present sensor gives a good reproducibility with a relative standard deviation (RSD, n=6) of 1.9 % for Ag⁺ ions and 5.0 % for Cys as shown in Fig. S5. These results demonstrated the high sensitivity, broad responding range, good anti-interference capacity and reproducibility of the biosensor for quantitative analysis of Ag^+ ions.

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Fig. S4. Selectivity of the biosensor for Ag^+ ion. All competing ions were at their 100 nM level. As a comparison, the responses of 1.0 nM Ag^+ ions, and the mixtures of 1 nM Ag^+ and 10 nM for each other ions were presented.



Fig. S5. The reproducibility of the present biosensor for (A) 1 nM Ag^+ ions and (B) 5 nM Cys with six repetitive determinations.

Selectivity of the biosensor for Cys

To evaluate the selectivity of the sensor for detecting Cys, the ECL response of the biosensor to some amino acids and GSSG (at 10 nM level) with and without 1.0 nM Cys was recorded. As shown in Fig. 6, Cys exhibited a significant decrease on the ECL emission, but those amino acids and GSSG did not induce obvious ECL emission change. The signals from those amino acids and GSSG were less than one tenth of that from 1.0 nM Cys even their concentration was ten times higher than Cys. In addition, the ECL signal change by Cys was less than 5 % in the presence of amino acid and GSSG except for phenylalanine and glycine (9.1 and -11.4 %, respectively). These results illustrated that none of the tested amino acids have the interaction strong enough to dissociate the C-Ag⁺-C complex and the biosensor has high selectivity for Cys over those amino acids and GSSG. As shown in Fig. 3B, GSH may interfere to the determination of Cys because it contains free thiol group. However, once GSH is oxidized and transferred into GSSG, the interference is eliminated.

The comparison of analytical performance of various biosensors for Ag⁺ ions and Cys

Table S1 presented a comparison of the detection limits for Ag^+ ions and Cys based on C- Ag^+ -C strategy. We can find the detection limits of this biosensor were a few orders of magnitude lower than those obtained by fluorescent and Colorimetric methods. Besides the high sensitivity of ECL and the increased probe molecule number with the intercalation strategy for probe introduction, the low background also contributed to the low detection limit. Gong and Li⁸ designed a Y-type C-rich DNA strand and immobilized the strand onto the electrode surface. A label-free electrochemical biosensor for Ag^+ ions and Cys was constructed using electrochemical impedance spectroscopy (EIS) with the detection limits of 10 and 100 fM for the two species. Both the present work and Gong and Li's work ⁸ validated the merits of electrochemical and related detections, such as simple setup and high sensitivity.

Method	Notes	DLs/nM		Ref
wiethou		For Ag ⁺	For Cys	
Colorimetry	Ag ⁺ -induced disruption of G- quadruplex-hemin DNAzyme	64	50	1
Colorimetry	G-quadruplex-hemin DNAzyme- amplified colorimetric detection	6.3	-	2
FL ^a	Quench of FI of dye-labeled ss-DNA by single-walled carbon nanotubes	1	9.5	3
Colorimetry	Ag ⁺ -induced formation of G-quadruplex DNAzyme for colorimetric detection	2.5	40	5
Colorimetry	Ag ⁺ ion induced formation of G- quadruplex DNAzyme for colorimetric detection	20	25	6
FL ^a	Ag ⁺ -induced disruption of triphenylmethane dye/ G-quadruplex complex	36	59	7
EC ^a	Electrochemical impedance spectroscopy with Y-type, C-rich DNA probe	10 fM	100 fM	8
Colorimetry	Ag ⁺ -induced aggregation of DNA-coated Au nanoparticles	12	-	23
FL ^a	Ag ⁺ -induced formation of duplex for intercalation of SYBR Green I probe	32	-	24
ECL ^a	A functional oligonucleotide for Ag identification and ECL probe intercalation	0.005	0.050	This work

Table S1. Method detection limits (DLs) for Ag⁺ ions and Cys with C-Ag⁺-C strategy using different detection techniques.

a ECL: electrochemiluminescence; EC: electrochemistry; FL: fluorescence.

Validation of the performance of the biosensor based on C-Ag⁺-C complex

The determination of Cys in serum and the result comparison between the present biosensor and ESI-MS were used to validate the practicability of the biosensor for real sample. After 10 μ L of the sample with and without spike was incubated on the electrode modified with FO integrated Ru(phen)₃²⁺ probe. The electrode was washed thoroughly with 0.1 M PBS to reduce the non-specific binding. The analytical results were presented in Table 1. No Cys was found in the diluted serum sample and thus the serum spiked Cys with different concentration level was tested. We can find the recovery was range from 107 to 110 %. The sample spiked with 2.15 μ M Cys was analyzed by the present biosensor and electrospray ionization-tandem mass spectrometry (ESI-MS). The mass flow charts from standard and spiked sample were showed in Fig. S6. The results by the two methods was consistent to each other. It implied that no significant interference was encountered in the serum sample and the present biosensor has a promising feature for the analysis of the biological samples. Because the modified electrode was washed thoroughly before ECL determination, the interference from the sample matrix was significantly eliminated and thus the background interference was avoided. The other merit of this biosensor is little sample consumption (only 10 μ L) and makes the present method for diagnostic potential with scarce sample. Supplementary Material (ESI) for Chemical Communications This journal is © The Royal Society of Chemistry 2011



Fig. S6. Mass spectra of (A) diluted serum spiked 2.15 μ M cysteine and (B) the 2.00 μ M cysteine standard solution with electrospray ionization-tandem mass spectrometry.

The comparison of "turn-on" and "turn-off" detection strategies

Generally, there are two modes for the signal output of a biosensor; one is "turn-on" mode, i.e. the signal increase with the increased target concentration and the other is "turn-off" mode, i.e. the signal decrease along with the increased target concentration. The present biosensor integrated the two modes together and provided a direct comparison between the two modes. As shown in Table S2, the work provided direct evidence that "turn-on" mode shows high sensitivity, broad linear ranges, and good reproducibility over the "turn-off" strategy.^{4, 25-26}

Table S2. Analytica	l performance of the	present biosensor	for Ag ⁺	ions and Cys.
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	Turn-on mode for Ag^+ ions	Turn-on mode for Cys
Detection limits	5 pM	50 pM
Linear ranges	10 pM to 100 nM	100 pM to 50 nM
Reproducibility (RSD, %, n=6)	1.9	5.0

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