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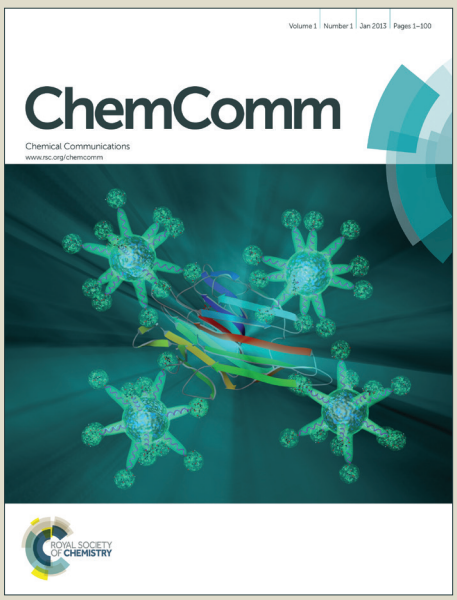

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Highly sensitive "signal-on" electrochemiluminescent biosensor for the detection of DNA based on dual quenching and strand displacement reaction

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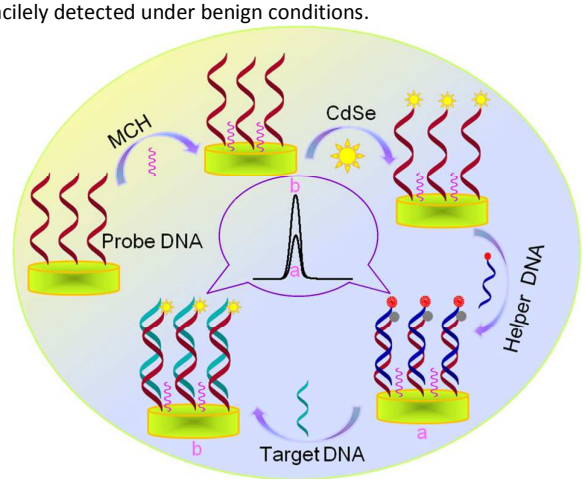
A "signal-on" electrochemiluminescent (ECL) DNA biosensing platform was proposed based on the dual quenching and strand displacement reaction. This novel "signal-on" detection strategy revealed its sensitivity in achieving a detection limit of 2.4 aM and its selectivity in distinguishing single nucleotide polymorphism of target DNA.

Combining the outstanding advantages of the electrochemical and luminescent techniques, electrochemiluminescent (ECL) biosensors have been widely used in sensing diverse moleculars such as protein,[1] DNA,[2] RNA[3] and metal ions.[4] Based on different manners of the signal corresponding to the target, ECL biosensors could be classified into two types: "signal-off" and "signal-on". Currently, most of the ECL biosensors belong to "signal-off" manners, which suffer the ingrained drawback of strong background signal.[5] Therefore, to pursue better analytical performances, more efforts should be devoted to establish "signal-on" ECL biosensors.

With the development of nanotechnology, various nanomaterials have been employed in ECL biosensors. Among them, quantum dots (QDs) are the promising one, due to its intrinsic low background, size or surface trap-control luminescence, and good stability against photobleaching.[6-8] For example, owing to the property of the QDs-based resonance energy transfer, an analytical system based on CdS:Eu nanoclusters has been designed, which can detect target DNA sensitively and selectively.[9] Recently, it is demonstrated that Ferrocene (Fc) can serve as a quencher of the QDs-based photoluminescence (PL) emission *via* energy transfer and photoexcited electron transfer.[10, 11] That is to say, by approaching Fc to QDs, both electron and energy can effectively transfer from excited QDs to the Fc, leading a dual quenching of ECL signal.[12] Ju and coworkers designed a "signal-off" platform and obtained a low detection limit and a wide calibration range for the detection of carcinoembryonic antigen (CEA) through this dual quenching mechanism.[12] But the "signal-off" analytical system suffered a strong background signal. In DNA biosensors, this drawback can be overcame by employing strand displacement reaction.[13,14] Strand displacement reaction is the process through which two strands with partial or full complementarity hybridize to each other, displacing one or more pre-hybridized strands in the process.[15] Only if eligible to this principle, each nucleic acid strand can trigger a strand displacement reaction with superior specificity,[16,17] which is of great value in fabricating versatile analytical approaches. Besides, strand displacement reaction can take place at room temperature without the participation of any enzymes. Therefore, by adopting strand displacement reaction in a biosensor, target strand may be facilely detected under benign conditions.



Scheme 1 ECL DNA sensing platform based on dual quenching and strand displacement reaction.

In the present work, a novel "signal-on" ECL DNA biosensor has been proposed based on a dual quenching of QDs and strand displacement reaction on a solid surface (scheme 1). Well-defined ECL signal of QDs can be effectively quenched by Fc *via* electron and energy transfer, and recovered by target DNA (taking a typical sequence of oral cancer gene as a model)[18, 19] *via* strand displacement reaction, thus target DNA can be determined in a

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"signal-on" manner. Benefited from the sensitivity of electrochemiluminescence, selectivity of strand displacement reaction and the advantages of "signal-on" detection manner, this biosensor can quantify the label-free target DNA in a wide range with an ultra-low detection limit, and differentiate one-base mutation in the target strand. Besides, it is demonstrated that target DNA in serum sample can be measured with this ECL DNA biosensor directly and accurately, indicating great potentials in clinical nucleic acid analysis.

The TEM image of the CdSe QDs is shown in Fig. 1A. The formation of the CdSe QDs is observed and the average size is about 2.5 nm. They are well dispersed in water. The good dispersion of CdSe QDs in aqueous solution was beneficial for electron transfer between CdSe QDs and the electrode, leading to the construction of a highly sensitive ECL DNA biosensor. UV-vis spectra of CdSe are displayed in Fig. 1B, the CdSe QDs have an absorption peak in UV-vis spectrum at 467 nm. The concentration of the as-prepared CdSe QDs solution could be estimated according to the absorption peak in the UV-vis and several empirical equations reported previously.[20] The concentration of the as-prepared QDs in aqueous solution is about 5.69 μM. The synthesized CdSe QDs have a photoluminescence peak at 660 nm (Fig. 1C), indicating the consequence of quantum confinement.[21]

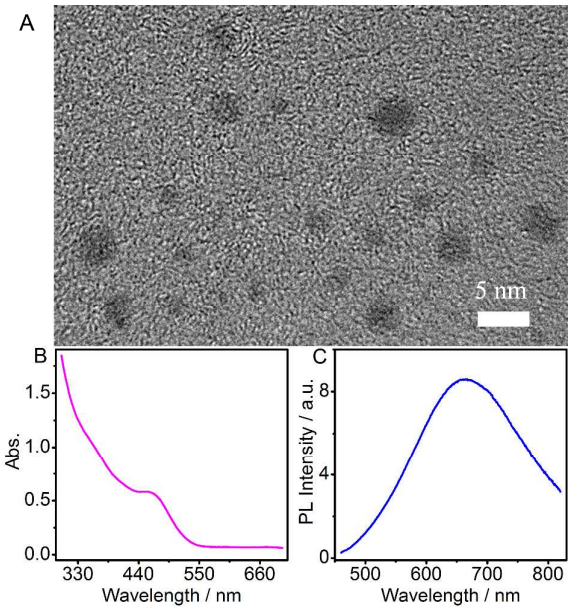

Fig. 1 (A) TEM image of CdSe QDs. (B) UV-vis absorption spectrum of the CdSe QDs. (C) PL spectrum of the CdSe QDs.

EIS was used to investigate the stepwise assembly of the ECL DNA biosensor. As shown in Fig. 2A, bare GE exhibits a small semicircle (curve a), which indicates a low electron-transfer resistance (R_{et}) towards the redox couple. Because of the self-assembly of the probe DNA on the GE, R_{et} increases significantly (curve b), which is attributed to an electrostatic repulsion force to $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ from the negatively charged phosphate backbone of the probe DNA. After the probe DNA modified electrode is immersed into MCH solution, R_{et} further increases due to its poor conductivity (curve c). Subsequently, with the EDC/NHS activation, MPA-CdSe QDs are covalently modified on GE surface, leading to an enlarged semicircle (curve d). This experimental result proves that the CdSe QDs can be successfully bonded to the probe DNA. The R_{et} further increases after hybridizing with helper DNA (curve e), implying an efficient DNA hybridization on the modified GE surface. Finally, after introduction of target DNA, a largest R_{et} is obtained, because target DNA triggers the strand displacement reaction, leading to a long DNA strand immobilized on the GE surface (curve f).

Meanwhile, the PL responses were also recorded to monitor the fabrication of the DNA biosensor (Fig. 2B). After the probe DNA is modified with CdSe QDs, a typical and intensive fluorescence can be obtained (curve a). By introducing helper DNA, the fluorescence intensity decreases significantly (curve b), which could be explained as follows. Ferrocene (Fc) is a quencher of the QDs-based PL emission through both energy transfer and photoexcited electron transfer.[12] Because of the hybridization of probe DNA and helper DNA, Fc and CdSe, both of which are modified on the terminal of DNA, approach close, causing the decrease of ECL signal *via* dual quenching. However, after further introduction of the target DNA that can trigger the strand displacement reaction, helper DNA is released from the GE surface, resulting in the recovery of ECL signal (curve c).

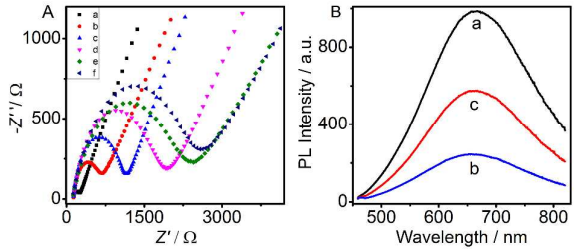

Fig. 2 (A) EIS spectra of (a) bare GE and GE modified with (b) probe DNA, (c) probe DNA/MCH, (d) probe DNA/MCH/CdSe, (e) probe DNA/MCH/CdSe/helper DNA, and (f) probe DNA/MCH/CdSe/helper DNA/target DNA. (B) PL spectra of GE modified with (a) probe DNA/MCH/CdSe, (b) probe DNA/MCH/CdSe/helper DNA and (c) probe DNA/MCH/CdSe/helper DNA/target DNA.

Fig.S1 shows the ECL-potential curve and cyclic voltammogram (CV) of the CdSe QDs on the GE surface. CdSe QDs display a strong cathodic ECL emission in Tris-HCl buffer (Fig.S1A). The main ECL peak at -1.4 V is resulted from the reaction of the QDs with $S_2O_8^{2-}$. In Fig. S1B, it can be found that two reduction peaks appear at about -0.7 V and -1.4 V, respectively. The peak at -0.7 V should be attributed to the reduction of $S_2O_8^{2-}$ on the electrode surface, while the peak at -1.4 V is ascribed to electrochemical reduction of CdSe QDs, which produce negatively charged radicals of $CdSe^{-\bullet}$. According to the previous reports,[22, 23] The $S_2O_8^{2-}$ is reduced to a strong oxidant ($SO_4^{-\bullet}$), and the product $SO_4^{-\bullet}$ that reacts with the $CdSe^{-\bullet}$, could further produce an excited state species ($CdSe^{*}$) that emits light. In addition, the oxygen can be reduced to OOH^- and used to catalyze ECL emission of the QDs.[24]

Effects of some factors on the ECL response were investigated to identify the optimal conditions for biosensor fabrication. Fig.S2A shows the relationship between the concentration of helper DNA

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and the ECL intensity. The ECL intensity decreases with the concentration of helper DNA increasing from 0.1 to 1.5 μM, while tends to level off at higher concentration. These results evidently indicate that the quenching efficiency reaches a maximum when the helper concentration is 1.5 μM. Therefore, 1.5 μM was selected as the optimal concentration of helper DNA for the following assays Fig.S2B and Fig.S2C display the effects of incubation time of helper DNA and target DNA on the ECL intensity. With the increase of the incubation time (from 0 to 60 min), more and more helper DNA can hybridize with probe DNA, resulting in the continuous decrease of the ECL intensity (Fig.S2B). However, more incubation time would not further enhance the hybridization and the quenching effect. The dependence of ECL intensity recovery on the incubation time of target DNA was also investigated (Fig.S2C). The intensity of ECL signal increases dramatically during the first 75 min of target DNA incubation, and then tends to level off. There experimental results demonstrate that target DNA can successfully trigger the strand displacement reaction and the helper DNA is released within 75 min. As a result, 60min and 75 min were chosen as the optimal incubation time of helper DNA and target DNA, respectively.

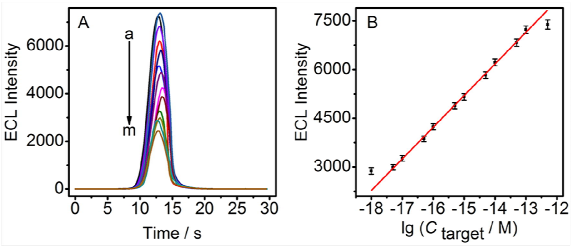

Fig. 3 (A) ECL responses of the proposed DNA biosensor to different concentrations of target DNA (from top to bottom: 500 fM, 100 fM, 50 fM, 10 fM, 5 fM, 1 fM, 500 aM, 100 aM, 50 aM, 10 aM, 5 aM and 1 aM) in 50 mM Tris-HCl buffer containing 0.1 M $K_2S_2O_8$ and 0.1 M KCl. (B) Linear calibration curve between the ECL intensity and the logarithm of the target DNA concentration.

On the basis of the "signal-on" strategy, the ECL responses to different concentrations of target DNA were investigated. Fig. 3A depicts the ECL intensity measured at different concentrations of target DNA. The ECL intensity exhibits a linear increase with increasing target DNA concentrations from 5 aM to 100 fM (Fig. 3B). If the target DNA concentration is more than 100 fM or less than 5 aM, the ECL intensity would deviate the linear calibration curve. The calibration plot is constructed by plotting ECL intensity (I) against $\lg^C$ (C was the concentration of target DNA). The regression equation is I = 19820+975$\lg^C$ with the correlation coefficient of 0.998. The detection limit of for target DNA is 2.4 aM (S/N = 3). For comparison, the analytical performance of several typical DNA biosensors is presented in (Table 1)[25-30], which proves the superiority of this ECL biosensor in linear detection range and detection limit.

Table 1. Comparison of the performance of several typical DNA biosensors.

Method	Detection limit (M)	Linear range (M)	Refs.
Fluorescence	2×10^{-11}	5×10^{-11}-1×10^{-10}	25
Electrochemiluminescence	1.7×10^{-15}	5×10^{-15}-1×10^{-12}	26
Colorimetry	5×10^{-11}	5×10^{-11}-6×10^{-9}	27
Electrochemistry	1×10^{-13}	1×10^{-13}-1×10^{-10}	28
Chemiluminescence	3.4×10^{-12}	1×10^{-10}-3×10^{-9}	29
Photoelectrochemistry	3.5×10^{-14}	1×10^{-13}-5×10^{-9}	30
Electrochemiluminescence	2.4×10^{-18}	5×10^{-18}-1×10^{-13}	This work

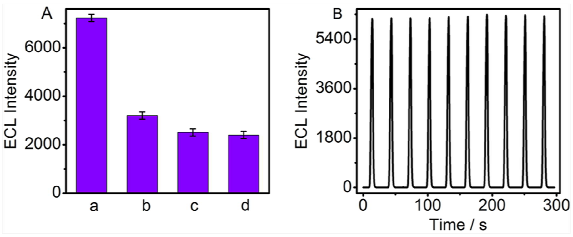

Fig. 4 (A) ECL responses of the proposed DNA biosensor for the detection of (a) target DNA, (b) single-base mismatched sequence, (c) two-base mismatched sequence and (d) three-base mismatched sequence in 50 mM Tris-HCl buffer containing 0.1 M $K_2S_2O_8$ and 0.1 M KCl. The concentration of different DNA sequences was 100 fM. (B) ECL response of the DNA biosensor with 10 fM target DNA under continuous cyclic potential scan between 0 and -1.5 V for 10 cycles at 100 mV s^{-1}.

Table 2. Serum sample tests of target DNA

Sample	Target DNA added (fM)	Target DNA found (fM)	Recovery (%)	RSD (%, n=3)
Sample 1	50.00	48.66	97.32	1.52
Sample 2	5.00	4.89	97.80	1.64
Sample 3	0.01	0.0098	98.00	1.02

The selectivity of the present ECL DNA biosensor was challenged with four similar DNA strands that included target DNA, one-base mismatched DNA, two-base mismatched DNA and three-base mismatched DNA. At the same concentration (100 fM) of DNA strands, the ECL response from target DNA is manifestly larger than those from other three DNA strands (Fig. 4A). This difference essentially suggests that the ECL biosensor has a better ability of mismatched discrimination than previously reported DNA

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biosensor,[31] and can be implemented for single nucleotide polymorphism (SNP) detection. Fig. 4B shows the ECL signal-time curve under consecutive potential scans for 10 cycles. There is no apparent change in ECL emission, and the relative standard deviation is 0.9 %, indicating good stability and reliability of the proposed strategy.

Recovery experiments were performed to verify the feasibility of the ECL DNA biosensor in practical applications. Different concentrations of target DNA were first spiked in fetal bovine serum, and then detected with the proposed method. According to the experimental results shown in Table 2, no serious interferences has been found, suggesting that this biosensor is suitable for the detection in real samples.

In summary, dual quenching *via* energy and electron transfer has been integrated with stand displacement reaction for the first time on a solid surface. Based on this principle, a novel "signal-on" detection manner was proposed and employed in an ECL DNA biosensor. Benefited from the high efficiency of signal quenching and recovery, this "signal-on" biosensor exhibited excellent analytical performance with a wide linear range (5 aM to 100 fM) and a low detection limit (2.4 aM, S/N = 3). Besides, this biosensor revealed selectivity in determining single nucleotide polymorphism and feasibility in real sample detection. By changing the helper DNA sequence, this versatile biosensing platform could be applied for the determination of different DNA species. Considering that the target DNA was label-free and no enzyme was necessary in the whole procedure, this biosensor may provide a powerful tool for disease diagnostics and clinical analysis.

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