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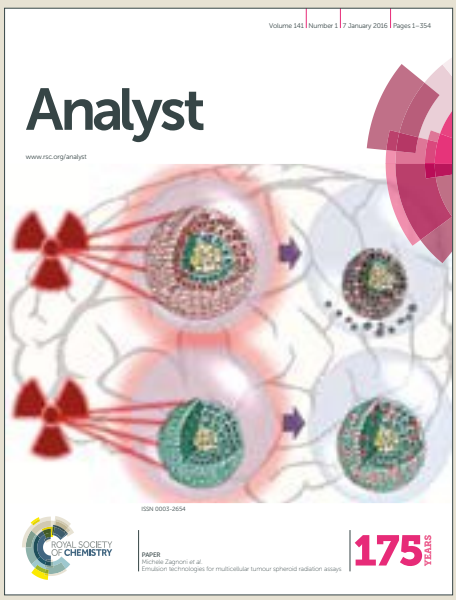

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PAPER

Sensitive ratiometric electrochemical biosensor based on DNA four-way junction formation and enzyme-assisted recycling amplification

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A simple ratiometric electrochemical biosensor is developed for sensitive detection of target DNA based on DNA four-way junction (DNA-4WJ) formation and enzyme-assisted recycling amplification. This biosensor can be easily fabricated by one-step assembly of ratiometric probes and simply performed by one-step incubation procedure. In the presence of target DNA, two unmodified DNA oligonucleotides may cooperatively hybridize with the hairpin probe in the triple-helix molecular beacon (THMB) to form a DNA-4WJ, which may cause the conformational transduction and induce the change of the distance between two redox labeling probes and the electrode surface. The subsequent recognition and cleavage of DNA-4WJ quadripartite complexes by RNase HII may result in significant signal amplification. Due to the introduction of DNA-4WJ formation, enzyme-assisted recycling amplification and ratiometric measurement, this biosensor exhibits high sensitivity with a detection limit of as low as 0.063 pM and long dynamic range from 0.1 pM to 100 nM. Moreover, this biosensor demonstrates good performance with excellent selectivity, high reliability and good reproducibility, holding great potential for further applications in biomedical research and clinical diagnostics.

Introduction

Ratiometric measurement has found wide applications in the development of novel biosensors for sensitive detection of various analytes due to its accuracy and reliability. The introduction of ratiometric measurement can remove the signal fluctuation caused by complex experimental conditions, offering the capability of self-reference. In comparison with the use of only single-signal readout, the ratio calculation of dual-signal readouts may lead to improved sensitivity. In addition, ratiometric measurement may significantly enhance the robustness and reproducibility of the biosensors. The reported ratiometric biosensors include fluorescent,[1-4] electrochemical,[5-11] electrochemiluminescent[12,13] and photoelectrochemical[14] ones, and they have demonstrated excellent performance in practical applications. Among these biosensors, the ratiometric electrochemical biosensor has attracted increasing attention because of their rapid response, simplicity and cost-effectiveness. The use of two electrochemical redox labels may offer a built-in correction toward the signal transduction for accurate detection of metal ions,[15-17] small molecules,[18] DNA[19] and proteins.[8,9]

As a common feature of nucleic acid structure, DNA four-way junctions (DNA-4WJ) are formed by four DNA strands and synthesized as immobile structural components of 2D DNA lattices.[20] The DNA-4WJ is structurally polymorphic and is a key intermediate in DNA recombination.[21-24] The 4WJ motif may precisely place different molecules in close proximity with a dynamic control over their spacing, offering the capability of discriminating even a single nucleotide difference at ambient temperatures without the need for precise temperature control.[25-27] Moreover, DNA-4WJ structure with conformational versatility may provide a well-defined structural target for specific detection of drugs and small-molecules.[28-31] Recently, Labib et al.[29] developed a DNA-4WJ-based electrochemical sensor for miRNA assay. Zhu et al.[24] demonstrated the use of DNA-4WJ-driven toehold-mediated strand displacement for the building of DNA logic circuit.

In this research, we developed a ratiometric electrochemical biosensor for sensitive detection of DNA based on DNA-4WJ formation and enzyme-assisted recycling amplification. We designed

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a triple-helix molecular beacon (THMB) which consisted of a methylene blue (MB)-labeled hairpin DNA (HP) as a central, two unmodified stands α and β-specific DNA sequence flanked by two arm segments and a ferrocene (Fc)-labeled universal trigger (UT) oligonucleotide,[28] and we obtained a ratiometric probe through the integration of Fc-labeled UT and MB-labeled HP in THMB. The presence of target DNA may induce the formation of DNA-4WJ structure and the breakdown of THMB structure, leading to the release of MB from the electrode surface and the close of Fc to the electrode surface for the ratiometric measurement. Moreover, the quadripartite complexes of DNA-4WJ may be cleaved by RNase HII to initiate recycling signal amplification. This biosensor may be easily fabricated by one-step assembly of ratiometric probes and simply performed by one-step incubation procedure, and it exhibits high sensitivity with a detection limit of as low as 0.063 pM and long dynamic range from 0.1 pM to 100 nM.

Experimental

Materials and reagents

All oligonucleotides (Table 1) were synthesized and purified with HPLC by Takara Biotech. Co. Ltd. (Dalian, China). Mercaptohexanol (MCH), tris-(2-carboxyethyl) phosphine hydrochloride (TCEP), 3-(N-Morpholino)propanesulfonic acid (MOPS), tris-(hydroxymethyl)aminomethane (Tris) and bovine serum albumin (BSA) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). RNase HII containing 20% glycerol, 20 mM Tris-HCl, 100 mM potassium chloride, 1 mM DTT, 0.2 mM EDTA, pH 8.0, was obtained from New England Biolabs (Ipswich, MA, USA). The buffers used in this research were as follows: DNA immobilization buffer (M1 buffer, 10 mM MOPS, pH 6.0), triple-helix buffer (M2 buffer, 10 mM MOPS + 20 mM NaCl + 2.5 mM $MgCl_2$, pH 6.0), reaction buffer (T buffer, 20 mM Tris-HCl + 0.1 M NaCl, pH 8.8), and MCH incubation buffer (1 mM MCH dissolved in M1 buffer). The serum samples were used directly without any treatment. Other reagents were all of analytical grade and purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). All solutions were prepared with ultrapure water obtained from a Milli-Q water purification system (Millipore Corp., Bedford, MA, USA) with a resistivity of 18.2 MΩ·cm.

Table 1. Sequences of the oligonucleotides

Name	Sequence (from 5' to 3')
HP [a]	**TCT TTC T**GT TAa CAT ACA ATa GAT C**TC TTT CT**-MB
UT [b]	HS-*CCT CC***A GAA AGA** AGA GAG AGA *GGA GG*-Fc
Strand α	GAT CTA TTG GCG GCG AGA CGA T
Strand β	AAC CAG TGG TAC TTA TGT TAA C
Target DNA	TTC ATC GTC TCG CCG CAG TAC CAC TGG TTT GTG CAA GAA T
SM 1 [c]	TTC ATC GCC TCG CCG CAG TAC CAC TGG TTT GTG CAA GAA T
SM 2 [d]	TTC ATC GTC TCG CCG CAG TAC CGC TGG TTT GTG CAA GAA T
CM [e]	CCA CGA TCA CAC AAT AGC AGT AGA CAC AAG TAC AGC GGC C

[a] Hairpin probe; [b] Universal trigger; [c] Single-base mismatched DNA 1; [d] Single-base mismatched DNA 2; [e] Complete mismatched DNA

The binding regions in target DNA, HP, strand *α* and strand *β* were shown in underlined, respectively. The binding regions in HP and UT were shown in bold. The binding regions in UT hairpin were shown in italics. The cleavage sites in HP for RNase HII were shown in lower case.

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Fabrication of ratiometric electrochemical biosensor

Prior to modification, the Au electrode was polished to a mirror-like surface with 1.0 μm, 0.3 μm and 0.05 μm α-Al_2O_3 slurry, respectively, followed by successive sonication with pure water and ethanol for 3 min to remove the residual alumina powder. The electrode was then immersed into fresh piranha solution (3:1 (v/v)) mixture of concentrated H_2SO_4 and 30% H_2O_2) for 10 min, followed by a thorough rinse with ultrapure water and drying with nitrogen. The resultant electrode was electrochemically cleaned in 0.1 M H_2SO_4 by potential scanning between −0.2 V and +1.6 V at a scan rate of 100 mV s^{-1} until a stable reproducible cyclic voltammogram was obtained. After washing with pure water and drying in a nitrogen stream, the electrode was incubated with 6 μL of 1 μM UT probe which contained 50 μM TCEP (TCEP was used to reduce the disulfide bonded oligonucleotides) at room temperature for 12 h to make UT immobilize onto the gold electrode surface via gold-sulfur chemistry. The electrode was then thoroughly rinsed with M1 buffer and dried under a stream of nitrogen gas. Subsequently, 6 μL of 1 mM MCH was dropped on the electrode surface for 30 min to block the unmodified sites. After rinsing thoroughly with M1 buffer and dried in nitrogen, the electrode was incubated with 1 μM HP probes in M2 buffer for 2 h. At last, the electrode was thoroughly rinsed and dried with nitrogen, and the sensing layer of THMB was obtained and stored at 4 °C.

Measurement procedure

All electrochemical measurements were performed at room temperature on a CHI 660e electrochemical workstation (CH Instruments Inc., U.S.A.) with a three-electrode system which is composed of a platinum wire, a saturated calomel and a gold electrode (d = 2 mm) as counter, reference and working electrodes, respectively. To evaluate the assay performance of the ratiometric electrochemical biosensor, 6 μL of different-concentration target DNA, strand α (100 nM), strand β (100 nM) and RNase HII with optimal concentration in T buffer was pipetted to the electrode surface and incubated at 37 °C for 15 min. The electrode was rinsed with Tris-HCl buffer (pH 7.4) and dried under N_2 atmosphere. For the measurement of target DNA, ACV responses of the ratiometric probe before and after the addition of target were monitored in 10 mM PBS (pH 7.4).

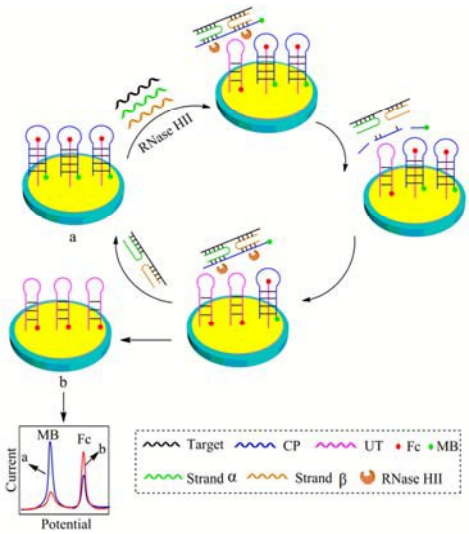
Alternating current voltammetry (ACV) was performed using a potential window of -0.5 V to +0.6 V with a pulse amplitude of 50 mV and a pulse width of 200 ms in 5 mL of T buffer (note: T buffer was degassed with nitrogen for 30 min prior to the measurement). Electrochemical impedance spectroscopic (EIS) measurements were carried out in 0.1 M KCl containing 5 mM $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$ in the frequency range from 0.1 Hz to 100 kHz with the amplitude of 5 mV at a potential of 0.22 V (vs SCE).

Results and discussion

Principle of ratiometric electrochemical biosensor

The strategy for the detection of target DNA is illustrated in Scheme 1. The ratiometric electrochemical biosensor is constructed by the assembly of a THMB on the surface of gold electrode, with two

electro-active molecules of MB and Fc as both the electrochemical indicator and the built-in control. The THMB consists of a MB-labeled HP as a central, two unmodified stands α and β-specific DNA sequence flanked by two arm segments and a Fc-labeled UT oligonucleotide.[32] Each unmodified strands α and β has a fragment complementary to HP and a fragment complementary to target DNA. The integration of a Fc-labeled UT and a MB-labeled HP in THMB enables the preparation of a ratiometric probe. The stem-loop structure of HP brings MB close to the electrode surface with the generation of a strong electrochemical signal, while the open configuration of UT [33] makes Fc away from the electrode surface with the generation of a weak electrochemical signal. In the absence of target DNA, the THMB structure is thermodynamically stable. On the contrary, the presence of target DNA may induce the formation of DNA-4WJ structure and the breakdown of THMB structure, resulting in the release of MB from the electrode surface and the close of Fc to the electrode surface and consequently the decrease of MB electrochemical signal and the increase of Fc electrochemical signal. The measurement of ratiometric signal (I_{Fc}/I_{MB}) generated from two electro-active molecules (Fc and MB) may be used to quantify the concentration of target DNA. Moreover, the quadripartite complexes of DNA-4WJ may be cleaved by RNase HII,[34] dissociating HP from the quadripartite complex. The released target-strand α-strand β tripartite complex may bind another HP in THMB to form a new DNA-4WJ quadripartite complex which may be subsequently cleaved by RNase HII, inducing recycling signal amplification. Notably, this ratiometric biosensor has significant advantages of simplicity, good selectivity and high sensitivity, and the formation of DNA-4WJ structure ensures the good selectivity, and the subsequent cleavage of DNA-4WJ quadripartite complex by RNase HII ensures the high sensitivity.



Scheme 1 Schematic illustration of the ratiometric electrochemical biosensor coupling with enzyme-assisted recycling signal amplification for DNA assay.

Characterization of the ratiometric electrochemical biosensor

We investigated the electrochemical feature of the modified Au electrodes and characterized the assembly of sensing interface in 5 mM $[Fe(CN)_6]^{3-/4-}$ containing 0.1 M KCl, with the peak currents and the separation of peak potential (ΔE_p) in cyclic voltammetric (CV) being used to reflect the electron transfer of the redox probe at the sensing interface. As shown in Fig. 1A, a well-defined redox peak of $[Fe(CN)_6]^{3-/4-}$ with a ΔE_p of 101 mV is observed at the bare Au electrode (Fig. 1A, curve a), indicating the fast electron transfer of the redox probe. The self-assembly of UT onto the bare Au electrode causes the decrease of redox peak current and the increase of ΔE_p to 126 mV (Fig. 1A, curve b) due to the blocking of interfacial electron transfer by UT. The subsequent self-assembly of MCH onto the UT/Au electrode may block the remaining active sites, resulting in the decrease of redox peak current and the increase of ΔE_p (Fig. 1A, curve c). The further self-assembly of HP onto the MCH/UT/Au electrode may prevent the diffusion of ferricyanide toward the electrode surface, leading to the decrease of redox peak current and the increase of peak-to-peak separation (Fig. 1A, curve d). Interestingly, the presence of target DNA may induce the formation of DNA-4WJ and the increase of active sites, resulting in the increase of peak current and the decrease of ΔE_p (Fig. 1A, curve e).

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We further employed EIS for interfacial investigation. The electron-transfer resistance (R_{et}) is controlled by the electron transfer kinetics of $[Fe(CN)_6]^{3-/4-}$ on the modified Au electrodes and may be characterized by the semicircle diameter of Nyquist plot. As shown in Fig. 1B, the bare Au electrode displays a small semicircle diameter and a long tail, indicating the diffusion of the redox probe $[Fe(CN)_6]^{3-/4-}$ (Fig. 1B, curve a). In comparison with the bare Au electrode, the UT-modified Au electrode exhibits a higher R_{et} (Fig. 1B, curve b) because the self-assembly of the negatively charged UT may repel the $[Fe(CN)_6]^{3-/4-}$ to the electrode surface. The subsequent surface blocking by mercaptohexanol may induce the increase of R_{et} (Fig. 1B, curve c). The hybridization of HP with UT on the MCH/UT/Au electrode causes the increase of R_{et} (Fig. 1B, curve d) due to both the steric hindrance of THMB structure and the electrostatic repulsion effect between the negative charged phosphate backbone in THMB and negatively charged $[Fe(CN)_6]^{3-/4-}$. On the contrary, the presence of target DNA causes significant decrease of R_{et} (Fig. 1B, curve e) due to target DNA-induced formation of DNA-4WJ and the subsequent dissociation of THMB from the electrode surface. These results clearly demonstrate the success fabrication of electrochemical sensing interface for DNA assay.

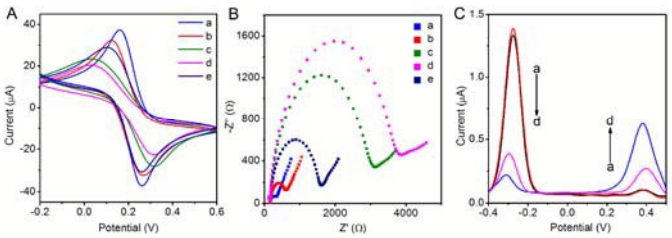

Fig. 1 (A) Cyclic voltammograms and (B) electrochemical impedance spectra (Nyquist plots) of different modified electrodes in 0.1 M KCl solution containing 5 mM $[Fe(CN)_6]^{3-/4-}$: (a) bare Au electrode, (b) UT/Au electrode, (c) MCH/UT/Au electrode, (d) HP/MCH/UT/Au electrode and (e) after reaction with mixture solution containing 1 nM target DNA, 100 nM strand α, 100 nM strand β and 0.1 U.μL^{-1} RNase HII. (C) ACV responses of the THMB-modified Au electrode (curve a), the THMB-modified Au electrode incubated with100 nM strand α and 100 nM strand β in the absence (curve b) and in the presence of target DNA without (curve c) and with RNase HII (curve d). The concentration of target DNA is 1 nM, and the RNase HII concentration is 0.1 U.μL^{-1}.

Feasibility of the ratiometric electrochemical biosensor

To verify the feasibility of the ratiometric electrochemical biosensor for DNA assay, we carried out the ACV measurement on the THMB modified Au electrode. As shown in Fig. 1C, a high oxidation peak of MB at -0.28 V and a low oxidation peak of Fc at +0.39 V are observed

at THMB modified electrode (Fig. 1C, curve a), indicating the successful construction of the THMB-modified Au electrode with two well-distinguished potentials corresponding to two electroactive molecules (Fc and MB), respectively. The incubation of THMB-modified electrode with the solution containing 100 nM strands α, β and RNase HII induces negligible response in the absence of target DNA (Fig. 1C, curve b), suggesting the thermodynamically stability of THMB structure without the formation of DNA-4WJ structure. On the contrary, the incubation of THMB-modified electrode with the unmodified strand α and β and target DNA leads to the decrease of MB electrochemical signal and the increase of Fc electrochemical signal (Fig. 1C, curve c) due to the formation of DNA-4WJ structure induced by target DNA. Moreover, the addition of RNase HII induces further decrease of MB electrochemical signal accompanied by further increase of Fc electrochemical signal (Fig. 1C, curve d), indicating the recycling signal amplification induced by RNase HII. These results demonstrate the feasibility of the ratiometric electrochemical biosensor for DNA assay.

Optimization of the ratiometric electrochemical biosensor

To ensure the good assay performance, we optimized the concentration of RNase HII and the reaction time. As shown in Fig. 2A, the ratiometric ACV peak current of I_{Fc}/I_{MB} improves with the increasing amount of RNase HII and reaches the maximum at 0.1 U.μL^{-1}. Further inhibition effect caused by high-concentration RNase HII on the reaction process may probably result from non-conducting of RNase HII-induced decrease of the electron transfer between the redox molecules and electrode surface. Thus, 0.1 U.μL^{-1} RNase HII was used in subsequent experiments. As shown in Fig. 2B, the ratiometric ACV peak current of I_{Fc}/I_{MB} increases with the reaction time and reaches the maximum at 15 min. Notably, the decrease of I_{Fc}/I_{MB} with longer reaction time beyond 15 min may be ascribed to the non-specific absorption of RNase HII on the surface of electrode. With increasing reaction time, more and more RNase HII may absorb non-specifically on the surface of electrode. Because RNase HII is non-conductive, it may hinder the electron transfer from Fc molecule to the electrode, resulting in the decrease of I_{Fc} and consequently the decrease of I_{Fc}/I_{MB} with longer reaction time. Thus, the reaction time of 15 min was used in subsequent experiments.

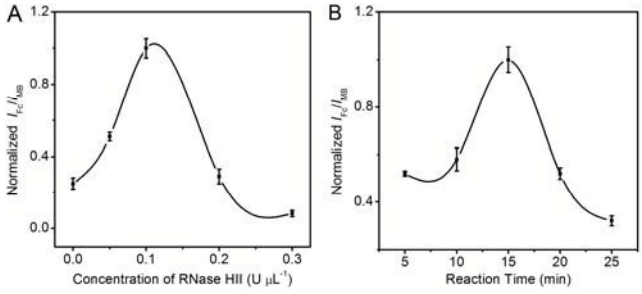

Fig. 2 Optimization of (A) RNase HII concentration and (B) reaction time in the presence of 1 nM target DNA, 100 nM strand α, 100 nM strand β and 0.1 U.μL^{-1} RNase HII. Error bars represent standard deviations of three independent experiments.

Assay performance of the ratiometric electrochemical biosensor

We further evaluated the assay performance of the ratiometric electrochemical biosensor under the optimally experimental conditions by monitoring the ACV peak current ratio (I_{Fc}/I_{MB}) in response to different concentrations of target DNA. As shown in Fig. 3A, the ACV peak current ratio of I_{Fc}/I_{MB} improves with the increasing concentration of target DNA. Notably, a good linear relationship between the ACV peak current ratio of I_{Fc}/I_{MB} and the logarithmic of target DNA concentration is obtained in the range from 0.1 pM to 100 nM with a linear regression equation $\log_{10}(I_{Fc}/I_{MB}) = 0.3364 \log_{10}C + 0.4938$ ($R^2 = 0.9938$) (Fig. 3D). The limit of detection (LOD) is calculated to be 0.063 pM by evaluating the average value of the control plus 3 times standard deviation. The LOD of this ratiometric electrochemical biosensor was at least 3 orders of magnitude lower than those of reported electrochemical biosensors,[6, 35] at least 4 orders of magnitude lower than those of fluorescent biosensors [1,2,4] and at least 2 orders of magnitude lower than those of electrochemiluminescent[36,37] and photoelectrochemical [38,39] biosensors (Table 2). In addition, the LOD obtained by using individual electrochemical indicator is 2.5 nM for MB and 3.8 nM for Fc (Fig. 3B), much poorer than that obtained by dual-signal ratiometric readout with a LOD of 0.063 pM. These results demonstrate the high sensitivity of the ratiometric electrochemical biosensor for DNA assay.

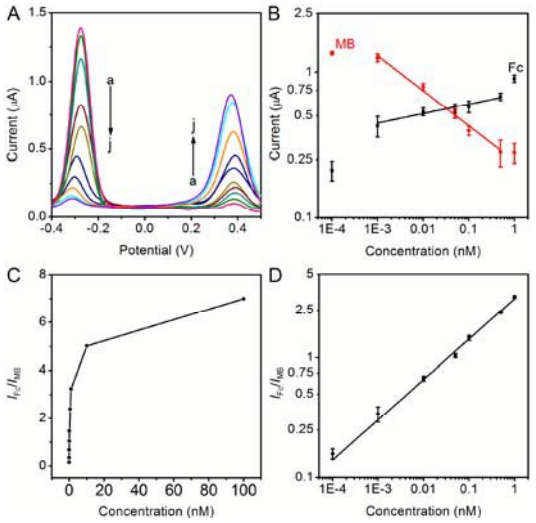

Fig. 3 (A) Variance of ACV peak current in response to different concentrations of target DNA: (a) 0, (b) 0.1 pM, (c) 1 pM, (d) 10 pM, (e) 50 pM, (f) 0.1 nM, (g) 0.5 nM, (h) 1 nM, (i) 10 nM and (j) 100 nM; (B) Logarithmic dependence of MB and Fc electrochemical signals upon the concentration of target DNA; (C) Variance of ACV peak current ratio of I_{Fc}/I_{MB} with the concentration of target DNA; (D) Logarithmic dependence of the ACV peak current ratio of I_{Fc}/I_{MB} upon the concentration of target DNA. The error bars represent the standard deviation of three independent experiments.

Selectivity, stability and reproducibility of the ratiometric electrochemical biosensor

To investigate the selectivity of the ratiometric electrochemical biosensor for DNA assay, we monitored the ACV peak current ratio of I_{Fc}/I_{MB} in response to target DNA, single-base mismatched DNA and complete mismatched DNA. As shown in Fig. 4, a negligible electrochemical signal is observed in the presence of complete mismatched DNA, similar to the control with only the buffer. A low electrochemical signal is obtained in the presence of single-base mismatched DNA. On the contrary, the target DNA may generate a distinct electrochemical signal, because only the target DNA may trigger the DNA hybridization and induce the formation of DNA-4WJ and consequently the release of MB from the electrode surface and the close of Fc to the electrode surface for the ratiometric measurement. These results clearly demonstrate the good selectivity of the ratiometric electrochemical biosensor for DNA assay with the

capability of discriminating even single-base mismatched.

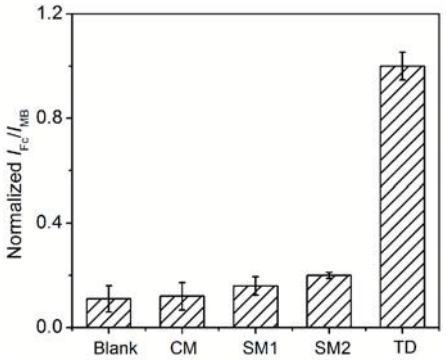

Fig. 4 Variance of ACV peak current ratio of I_{Fc}/I_{MB} in response to target DNA (TD), single-base mismatched DNA 1 (SM1), single-base mismatched DNA 2 (SM2), and the complete mismatched DNA (CM). The concentration of target DNA is 10 pM. The SM1 concentration is 1 nM, and the SM2 concentration is 1 nM, and the CM concentration is 1 nM. Error bars represent standard deviations of three independent experiments.

We also investigated the stability and reproducibility of the ratiometric electrochemical biosensor. The repeatability of the electrochemical biosensor was monitored by repetitively determining 1 nM target DNA. The relative standard deviation (R.S.D.) of peak currents was calculated to be 2.1% (n = 6), and no significant change in peak current was observed even after two-week storage at 4 °C, suggesting the excellent stability of the ratiometric electrochemical biosensor. The reproducibility was investigated by preparing six electrodes from the same batch for the detection of same-concentration target DNA. The obtained R.S.D. was 5.9%, indicating the good reproducibility of the ratiometric electrochemical biosensor. The feasibility of the ratiometric electrochemical biosensor for real sample analysis was investigated by detecting target DNA spiked in the BSA samples. We spiked 5 pM, 30 pM and 900 pM target DNA in 10-fold diluted BSA sample, and calculated the recovery to be 106.8%, 97.3% and 94.1%, respectively, indicating the good accuracy of the ratiometric electrochemical biosensor for real sample analysis.

Table 2. Comparison of the ratiometric biosensors with the reported methods for DNA assay.

Analytical methods	Detectable range	LOD	Refs
Electrochemical	1 pM − 100 nM	0.063 pM	This work
Electrochemical	50 pM − 1 μM	25 pM	6
Electrochemical	0 − 4 nM	85 pM	35
Fluorescent	0.5 nM − 140 nM	0.29 nM	1
Fluorescent	10 nM − 1000 nM	7.3 nM	4
Fluorescent	1.0 mM − 100 mM	0.47 mM	2
ECL[a]	0 − 1 nM	1 pM	36
ECL	2 nM − 0.2 pM	0.1 pM	37
PEC[b]	20 nM − 400 nM	4.5 nM	38
PEC	1 pM − 1000 pM	0.8 pM	39

[a] Electrochemiluminescence; [b] Photoelectrochemistry

Conclusions

In summary, we have developed a simple ratiometric electrochemical biosensor for sensitive detection of target DNA based on DNA four-way junction formation and enzyme-assisted recycling amplification. This biosensor may be easily fabricated by one-step assembly of ratiometric probes and simply performed by one-step incubation procedure. We design a THMB which consists of a MB-labeled hairpin DNA as a central, two unmodified stands α and β-specific DNA sequence flanked by two arm segments and a Fc-labeled universal trigger oligonucleotide, and we obtained a ratiometric probe through the integration of a Fc-labeled universal trigger and a MB-labeled hairpin DNA in THMB. The presence of target DNA may induce the formation of DNA-4WJ structure and the breakdown of THMB structure, leading to the changes in both MB and Fc electrochemical signals for the ratiometric measurement. Moreover, the electrochemical signal may be further amplified by cyclically cleavage of DNA-4WJ quadripartite complexes by RNase HII. Due to the introduction of DNA-4WJ formation, enzyme-assisted recycling amplification and ratiometric measurement, this biosensor exhibits excellent selectivity with the capability of discriminating even sing-base mismatched and high sensitivity with a detection limit of as low as 0.063 pM and long dynamic range from 0.1 pM to 100 nM. The sensitivity of this biosensor is superior to those of most of currently used approaches for DNA assay.[1,2,,4,6,35-39] Importantly, this biosensor demonstrates excellent performance with high stability and good reproducibility, holding great potential for further applications in biomedical research and clinical diagnostics.

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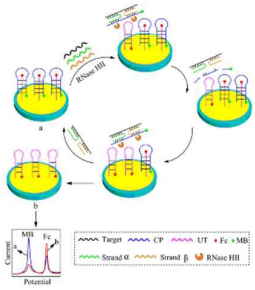

We develop a ratiometric electrochemical biosensor for DNA assay based on DNA four-way junction formation and enzyme-assisted recycling amplification.