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Label-free electrochemical DNA sensing with a one-target-multitriggered hybridization chain reaction strategy

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A one-target-multitriggered hybridization chain reaction (MHCR) strategy was designed for ultrasensitive electrochemical detection of DNA by combining the isothermal strand-displacement polymerase reaction (ISDPR) with the DNA self-assembly on a DNA sensor surface. The sensor was constructed by immobilizing a hairpin-like capture probe (CP) on a gold electrode *via* an Au–S bond. The ISDPR was triggered by the hybridization of the target DNA to open the CP and primer to anneal the complementary part in the bottom of the exposed stem and the extension of the primer in the presence of dNTPs and polymerase. Each target copy could produce a few opened CPs. Afterwards, the other part of the exposed stem acted as an initiator to trigger the hybridization chain reaction (HCR) when incubated with two hairpin monomers. Using $[Ru(NH_3)_6]^{3+}$ as an electrochemically active indicator to interact with the MHCR product, the amperometric response demonstrated a perfect multiple amplification effect. The constructed sensor showed a high sensitivity for detection of the target DNA in a linear range from 0.1 fM to 10 pM, a detection limit down to 0.02 fM (3σ) and good selectivity for base discrimination. This method did not need any modification or labelling process. The proposed strategy provides a powerful tool for cascade signal amplification and has a wide potential application in bioanalysis.

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Introduction

The trace detection of sequence specific DNA is of vital importance in clinical diagnosis, mutational analysis, and gene therapy. Therefore, the signal amplification strategies to achieve sensitive detection of DNA have become a rapidly emerging research field. Typically, template (target) recycling-, superstructure- and nanomaterial-based signal amplification methods have been designed. The nanomaterial based amplification strategies greatly improve the detection sensitivity due to the function of accelerating electron transfer or loading a large number of signal reporters.[1] Meanwhile, the DNA-based amplification strategies by assembly of the DNA superstructure and target recycling have recently been evolved as the more widely used techniques for sensitive detection of DNA.[2–14]

DNA superstructure can be formed by supersandwich self-assembly or hybridization chain reaction (HCR). The supersandwich self-assembled suprastructure was proposed for electrochemical detection of DNA with a methylene blue labeled signal probe that contains a "sticky end".[2] The "sticky end" can hybridize another target to create a supersandwich structure containing multiple labels. In order to avoid the need for numerous target DNA sequences for forming the superstructure, an auxiliary probe that can hybridize two different regions of the signal probe has been introduced for electrochemical detection of DNA.[3] HCR for DNA superstructure assembly is performed using an initiator strand to open two species of DNA hairpins coexisting in solution.[4] To further improve the amplification efficiency, the DNA hairpins have been modified with biotin that can further conjugate enzyme-labeled streptavidin for amplifying the signal,[5] pyrene molecules to form numerous pyrene excimers,[6] fluorophore/quencher pair resulting in quenching of the luminescence of fluorophore[7] and ferrocene each of which can produce an electrochemical signal within the applied potentials.[8] A label-free strategy using $Ru(phen)_3^{2+}$ as the signal indicator has also been developed for HCR-based electrochemiluminescent detection of DNA.[9] These traditional HCR-based assays usually use the target DNA strand as the initiator, and each copy of the target can trigger only one chain of hybridization event, limiting the signal readout. Here a novel strategy for enhancing the amplification efficiency was designed by combining an isothermal strand-displacement polymerase reaction (ISDPR) with label-free HCR-based electrochemical assay (Scheme 1). The ISDPR process could provide multiple DNA strands to initiate the HCR by target recycling-based amplification, generated by numerous long dsDNAs, thus greatly improving the detection sensitivity of target DNA.

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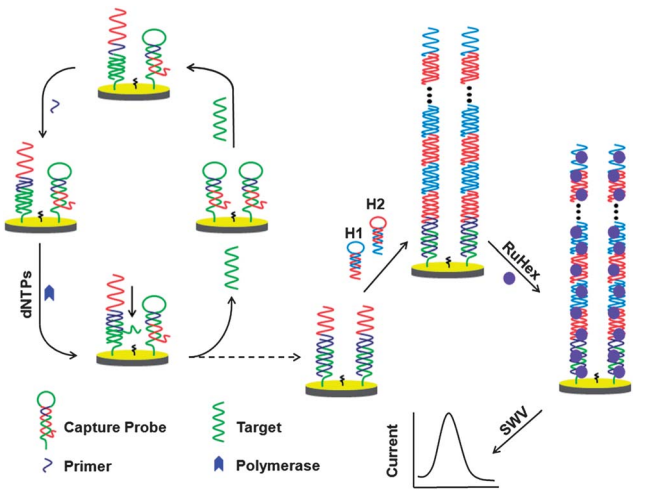

Scheme 1 Schematic illustration of the label-free electrochemical DNA sensing with a one-target-multitriggered hybridization chain reaction strategy.

The target recycling-based amplification can be achieved using various nucleases *e.g.* endonuclease,[10] exonuclease[11] and polymerase.[12] Among these techniques, the polymerase-based ISDPR has been combined with other amplification strategies such as nicking endonuclease scission to further push down the detection limitation.[13] An electrochemical detection method for DNA has been developed by combining the ISDPR with HCR, which is performed using the primer to trigger the HCR of two biotin labeled DNA hairpin monomers for introducing the streptavidin–alkaline phosphate as a signal tag to an electrode surface.[14] Here the signal tag was introduced onto the electrode by the strong electrostatic interaction between the anionic DNA phosphate backbones and $[Ru(NH_3)_6]^{3+}$ (RuHex). The HCR was then triggered by a single-stranded segment of the opened hairpin-liked triple-functionalized capture probe (CP). After the specific recognition of the target DNA to the immobilized CP to open CP, the primer could hybridize the bottom of the exposed stem and propagate ISDPR in the presence of dNTPs and polymerase, and the other part of the exposed stem acted as an initiator to trigger the HCR. This process can be defined as a one-target-multitriggered hybridization chain reaction (MHCR), which generates numerous dsDNAs. Thus, a large amount of the redox indicator, $[Ru(NH_3)_6]^{3+}$, could be electrostatically bound to the MHCR product to obtain a significantly higher signal than traditional HCR-based assay. The proposed strategy led to the femtomolar detection of the target DNA, and provided an amplifying tool for biosensing.

Experimental

Materials and reagents

Tris-(2-carboxyethyl) phosphine hydrochloride (TCEP), hexaammineruthenium(III) chloride ($Ru(NH_3)_6^{3+}$, RuHex) and 6-mercapto-1-hexanol (MCH) were purchased from Sigma-Aldrich, Inc. (USA). Klenow fragment, exo-, 10× reaction buffer (500 mM Tris–HCl, 50 mM $MgCl_2$, 10 mM DTT) and dNTP Mix were obtained from Fermentas Inc. (Canada). 6× DNA loading buffer was bought from Solarbio Co. Ltd (Beijing, China) and UltraPower™ dye was ordered from BioTeke Co. Ltd (Beijing, China). Tris(hydroxymethyl) aminomethane (tris)–HCl (10 mM, pH 7.4) containing 1 mM ethylenediaminetetraacetic acid (EDTA) and 50 mM NaCl was used as the DNA immobilization buffer. 1× SPSC buffer (1 M NaCl, 50 mM Na_2HPO_4, pH 7.5) was used for all hybridization reactions. 0.05% Tween-20 was spiked into Tris–HCl buffer (10 mM Tris–HCl, 50 mM NaCl) as wash buffer to minimize unspecific adsorption. MCH incubation buffer was 1 mM MCH dissolved in 10 mM Tris–HCl (pH 7.4). 10 mM Tris–HCl (pH 7.0) solution was made as the detection electrolyte in the square-wave voltammetric (SWV) experiments and 10 mM Tris–HCl (pH 7.0) solution containing 50 μM RuHex was used in the chronocoulometric (CC) experiments. All other reagents were of the analytical grade. All aqueous solutions were prepared using ultra-pure water (≥18 MΩ, Milli-Q, Millipore).

The oligonucleotides were purchased from Sangon Biological Engineering Technology Co. Ltd (Shanghai, China) and purified using high-performance liquid chromatography. Their sequences were listed as follows.

Three-base mismatched oligonucleotide (tmDNA): 5′-ACT TT*C* GCC TGC *G*AA AGT AGT CTA TT*G* GAA-3′.

Single-base mismatched oligonucleotide (smDNA): 5′-ACT TTG GCC TGC *G*AA AGT AGT CTA TTC GAA-3′.

Target: 5′-ACT TTG GCC TGC CAA AGT AGT CTA TTC GAA-3′.

Capture probe (CP): 5′-HS-C_6TTC GAA TAG ACT ACT TTG GCA GGC CAA AGT GCC TGC CAA AGT AGT CTA GGA TTC GGC GTG-3′; primer: 5′-GCA GGC-3′.

Hairpin 1 (H1): 5′-AGT CTA GGA TTC GGC GTG GGT TAA CAC GCC GAA TCC TAG ACT ACT TTG-3′.

Hairpin 2 (H2): 5′-TTA ACC CAC GCC GAA TCC TAG ACT CAA AGT AGT CTA GGA TTC GGC GTG-3′.

Instrumentation

SWV and CC experiments were carried out on a CHI 630D electrochemical workstation (Chenhua Instrument Company, China). A three-electrode cell consisted of a bare gold working electrode (2 mm in diameter), a platinum counter electrode, and a saturated calomel electrode (SCE) as the reference. All electrochemical measurements were carried out at room temperature. The gel electrophoresis was performed on the DYCP-31BN Electrophoresis Analyser (Liuyi Instrument Company, China) and imaged on the Bio-rad ChemDoc XRS (Bio-Rad, USA) to study the products after each step.

Preparation of sensor

A gold electrode was polished thoroughly with 0.05 μm alumina paste, followed by ultrasonic cleaning with absolute ethanol and Milli-Q water for 5 min respectively to remove the residual alumina powder. The electrode was then electrochemically pretreated in 1 M H_2SO_4 by a cyclic sweep between 0 and +1.6 V at 100 mV s^{-1} to remove the remaining impurities, rinsed thoroughly with water and dried by blowing pure nitrogen.

Prior to use, all the hairpin oligonucleotides were heated to 95 °C for 2 min and then allowed to cool to room temperature

for at least 2 h. The thiolated CP was activated by treatment with 1 mM TCEP for 1 h. 5 μL of the CP was pipetted onto the pretreated electrode and then incubated overnight at room temperature in 100% humidity. After rinsing, the CP modified gold electrode was immersed into 1 mM MCH solution for 2 h to remove nonspecific adsorption sites and optimize the orientation of the CP. After washing, 5 μL target solution at different concentrations was dropped on the electrode and incubated for 1 h to hybridize the immobilized CP. The modified electrode was then soaked in 10 μL of 1× duplication buffer containing 1 μM primer, 5 U polymerase Klenow fragment, exo-, and 50 μM dNTP mix (each at a concentration of 50 μM). The duplication process was allowed to proceed for 3 h at 37 °C. After washing thoroughly, 10 μL of 1× SPSC solution containing H1 and H2 was dropped on the electrode surface and incubated for 3 h at 37 °C. The whole procedure is shown in Scheme 1.

SWV measurements

The quantification of DNA was performed by SWV detection of RuHex. After MHCR, the sensor was placed in 10 mM Tris–HCl buffer containing 50 μM RuHex for 1 min to reach the equilibrium adsorption of RuHex, since the equilibrium time is only seconds.[15] The RuHex attached sensor was then rinsed with Tris–HCl buffer and scanned from 0 to −0.6 V with an amplitude of 25 mV and a step potential of 4 mV at a frequency of 15 Hz in 10 mM Tris–HCl buffer to obtain the SWV response. The Tris–HCl buffer was thoroughly purged with pure nitrogen for 15 min before the experiment.

Gel electrophoresis performance

12% native polyacrylamide gel (PAGE) was prepared using 1× TBE buffer. The loading samples were prepared by mixing 14 μL DNA sample, 3 μL 6× loading buffer and 3 μL UltraPower™ dye, which were kept for 3 min so that the dye could integrate with DNA completely. The gel was run at 90 V for 90 min in 1× TBE buffer and then illuminated with UV light to obtain the image.

Results and discussion

Sensing principle

As shown in Scheme 1, the CP, which possesses a stem-loop hairpin-like structure with a sticky end at the 3′ end, is immobilized on the gold electrode *via* Au–S chemistry. In the absence of the target DNA, the short primer cannot anneal with the CP, thus the CP remains the stable hairpin-like conformation even co-existing with dNTP/polymerase and hairpin monomers, and the HCR event cannot be triggered for signal amplification. After the immobilized CP hybridizes with the target DNA to open the hairpin conformation, the primer anneals with the complementary part in the bottom of the exposed stem (colored in purple in Scheme 1) to switch on a polymerization reaction in the presence of dNTPs and polymerase. With the process of primer extension, the target is displaced and hybridizes with another CP to start the polymerization reaction again. Each polymerization reaction generates a partial double-strand DNA (dsDNA) with a 24-base single-stranded fragment as the initiator to trigger the HCR event when incubated with hairpin 1 (H1) and hairpin 2 (H2). Compared with the traditional HCR based amplification strategy, this approach can achieve multiple amplification since one copy of the target can yield numerous long-range nicked dsDNA for adsorbing RuHex *via* strong electrostatic interaction. The RuHex is used as the quantitative signal indicator for electrochemical readout of the target DNA.

Feasibility of sensor

The feasibility of the proposed MHCR based sensing strategy in detecting sequence-specific DNA was explored by SWV. The typical SWV curves of the adsorbed RuHex at different experimental stages were shown in Fig. 1. Before the target DNA was introduced, only a small reduction peak was observed due to the small amount of RuHex electrostatically adsorbed to the immobilized CP (curve a). After 10 pM target DNA was added, the reduction current increased to 108%, indicating the hybridization of the trace amount of the target with the CP (curve b). Subsequently, the CP modified electrode was incubated with 5 U Klenow, 50 μM dNTPs and 1 μM primer for 3 h to trigger the polymerization reaction for generating numerous partial dsDNA with a single-stranded fragment. Thus much more RuHex was introduced onto the electrode surface to produce a relatively remarkable electrochemical signal (curve c). After integrating with the HCR, the peak current further increased by 3.2 times (curve d), indicating an efficient signal amplification capability.

Amplification capacity

In order to further study the amplification efficiency of the proposed MHCR strategy, the CC experiment was employed to measure the amount of adsorbed RuHex that reflected the quantity of immobilized DNA. The integrated current, or charge (Q), as a function of time (t) is given by the integrated Cottrell expression:[16]

$$Q = \frac{2nFAD_0^{1/2}C_0^{*}}{\pi^{1/2}}t^{1/2} + Q_{dl} + nFA\Gamma_0$$

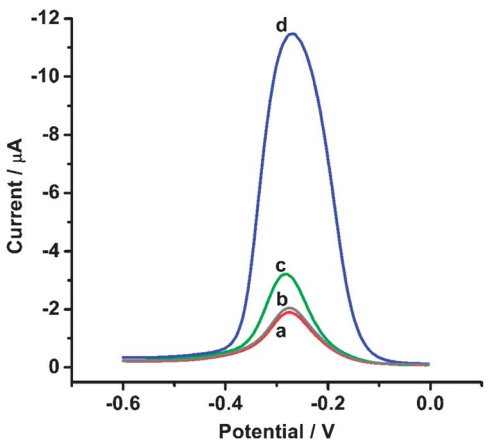

Fig. 1 SWV curves of RuHex adsorbed at the sensor surface in the presence of a capture probe (a), (a) +10 pM target (b), (b) +5 U Klenow, 50 μM dNTPs and 1 μM primer (c), and (c) with HCR amplification (d).

where the first term expresses the charge owing to the reaction of RuHex that diffuses into the electrode surface, Q_{dl} is the capacitive charge, and $nFA\Gamma_0$ represents the charge produced by the adsorbed RuHex. The amount of RuHex adsorbed to the phosphate backbone of the immobilized DNA (Γ_0), which can be determined from the difference in intercepts (Δ_{int}) at $t = 0$ in the presence and absence of RuHex, is quantitative to the quantity of the immobilized DNA (Γ_{DNA}) by dividing the number of bases in the single immobilized DNA strand.

From Fig. 2A, the recycling index of the ISDPR could be calculated to be about 13, and the number of hybridization cycles in the mere HCR event was obtained to be approx. 5, which identified that five copies of hairpins were linked in one nicked dsDNA chain. After combining the ISDPR and HCR to perform MHCR, the hybridization cycles were calculated to be approx. 57. This value was slightly smaller than the integrated value of 65 for individual ISDPR and HCR cycles, indicating a perfect multiple amplification effect.

The gel electrophoresis was performed to observe the result of each step (Fig. 1B). The CP and target showed individual lanes (lane 1 and 2). After mixing with the CP and target, a new band of the hybridization product was observed, and the band of target disappeared (lane 3), suggesting that the CP was opened. After adding the dNTP/polymerase/primer to incubate for 3 h, the band of CP also disappeared due to the protraction of the primer and re-hybridization of the displaced target (lane 4). The products of HCR (lane 7) and MHCR (lane 8) showed the bands of high-molecular weight structures, indicating the successful growth of the double helix. Considering that the amount of initiator increased greatly after ISDPR, the average molecular weight of MHCR products was lower than that in traditional HCR. These results verified the processes of ISDPR, HCR and MHCR.

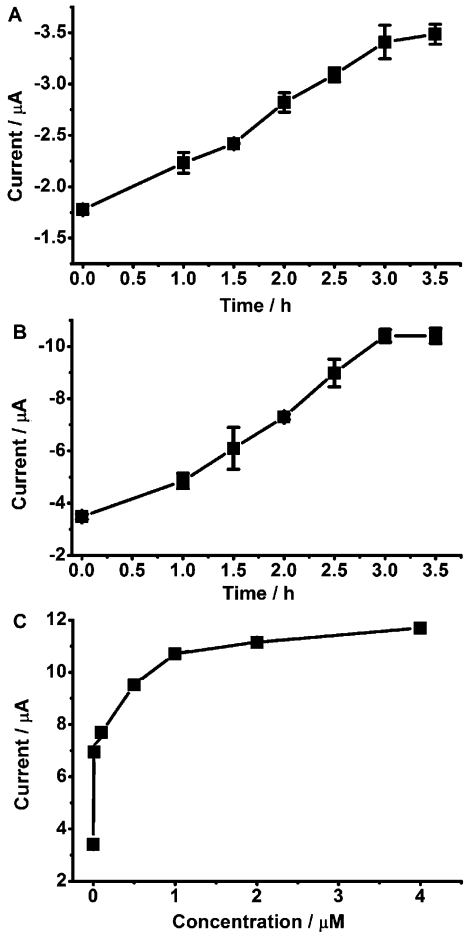

Fig. 3 Effects of (A) duplication time of ISDPR, (B) reaction time of HCR, and (C) concentrations of H1 and H2 on the peak current of RuHex ($n = 3$).

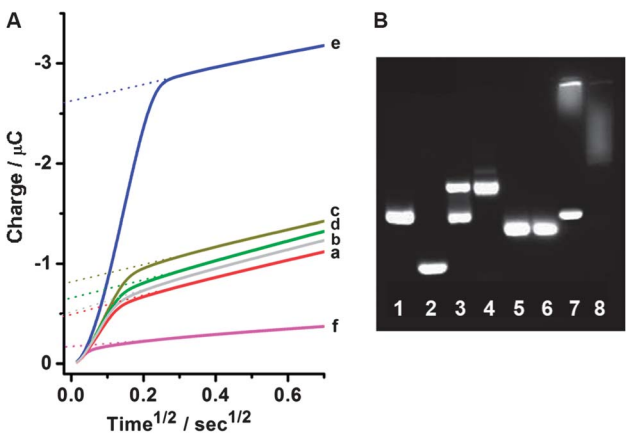

Fig. 2 (A) Chronocoulometric responses at the capture probe modified electrode (a) and the electrode incubated with 10 pM target (b) followed by ISDPR (c), HCR (d), and MHCR (e) in the presence of 50 μM RuHex. (f) is response of (e) in the absence of RuHex. (B) PAGE analysis of the capture probe (lane 1), target (lane 2), and hybridization product of the capture probe with target for 1 h (lane 3) followed by ISDPR (lane 4), HCR (lane 7) and MHCR (lane 8). Lanes 5 and 6 are the PAGE images of H1 and H2, respectively.

Optimization of detection conditions

The ISDPR efficiency was related to the reaction temperature, duplication time and the amount of polymerase. According to the previous work,[17] 5 U Klenow was applied and 37 °C was chosen as the suitable polymerization temperature. The effect of the duration time for the duplication process on the peak current is shown in Fig. 3A. The current increased as the reaction proceeded, and trended to a maximum value after reacting for 3 h, thus 3 h was selected as the optimal polymerization reaction time.

The hybridization time and the concentration of H1/H2 during the HCR course were also optimized. At the target DNA concentration of 10 pM in the presence of 1 μM H1 and H2, the electrochemical signal increased with the increasing hybridization time and reached a plateau at 3 h (Fig. 3B). This phenomenon resulted from the distant-sensitive electrochemical reaction. The electrochemical response also increased with the increasing concentration of two hairpins (Fig. 3C). Similarly, the peak current trended to a plateau at high hairpin concentrations. Thus, 3 h and 2.0 μM were used as the optimal hybridization time and hairpin concentration, respectively.

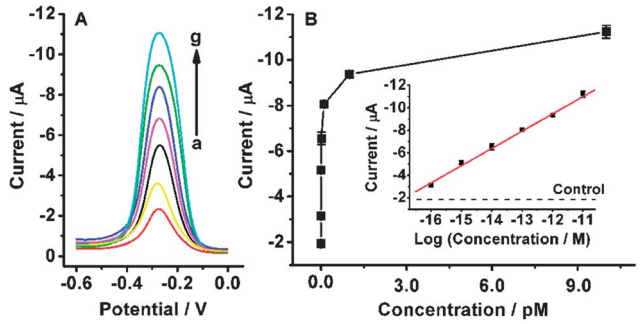

Fig. 4 (A) SWV responses at target DNA concentrations of 0 (a), 0.1 (b), 1 (c), 10 (d) fM, and 0.1 (e), 1 (f), 10 (g) pM from (a) to (g). (B) Plot of peak current *vs.* target DNA concentration. Inset: linear relationship between the peak current and the logarithm of target DNA concentration with the negative control. The illustrated error bars represent the standard deviations of three repetitive measurements.

Electrochemical detection of the target DNA

Under the optimal conditions, the analytical performance of the sensor was investigated by varying the target DNA concentration. Fig. 4A shows the typical SWV curves of the sensor at different target DNA concentrations. The peak current increased as the target DNA concentration changed from 0 to 10 pM (Fig. 4B). The plot of the peak current *vs.* the logarithm of target concentration showed a linear relationship in the detection range from 1×10^{-16} M to 1×10^{-11} M with a sensitivity of 1.5 μA per decade (inset in Fig. 4B). The correlation equation was $I = -1.54 \lg c - 27.90$ ($R = 0.9980$). The detection limit was calculated to be 0.02 fM at three times the standard deviation for the negative control measurement, which is much lower than those of the nicking endonuclease assisted colorimetric method (0.5 fM)[10b] and ISDPR-based fluorescent assay (6.4 fM).[12a] This capability for low level DNA detection owes to the multiple amplification effect by combining the ISDPR with HCR.

Selectivity of target detection

The specificity of the DNA sensing method was investigated by exposing the sensor to three kinds of DNA sequences, including the perfectly complementary target, single-base mismatched oligonucleotide (smDNA) and three-base mismatched oligonucleotide (tmDNA) at the same concentration (1 pM). As shown in Fig. 5, the response of the smDNA was only 39% that of the perfectly complementary target, indicating that the sensor exhibited good performance to discriminate the perfectly complementary target and smDNA. Meanwhile, the peak current of tmDNA showed only 20% that of the perfectly complementary target, which is almost as low as the background. The high specificity of the proposed electrochemical DNA sensing strategy was due to the long stem–short loop conformation of the immobilized CP as well as the two hairpins designed to perform the HCR event.

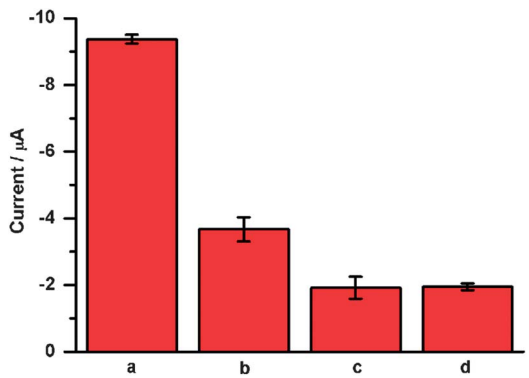

Fig. 5 SWV responses at 1 pM of the perfectly complementary target (a), single-base mismatched oligonucleotide (b), three-base mismatched oligonucleotide (c) and blank (d).

Conclusions

A one-target-multitriggered hybridization chain reaction strategy was designed for ultrasensitive electrochemical detection of DNA by using a triple-functionalized capture probe to combine the ISDPR with HCR. The hairpin-like capture probe could hybridize the target DNA to open the hairpin conformation, which led to the hybridization of the bottom of the exposed stem with a primer to trigger ISDPR. Thus, each copy of the target produced a few opened CPs. The remaining single-stranded fragments of each exposed stem then acted as an initiator to trigger the HCR event when incubated with two hairpin monomers. The HCR process produced numerous long-range nicked dsDNA for adsorbing RuHex as the signal reporter. The multiple amplification of MHCR resulted in high sensitivity of the strategy. The one-target-multitriggered HCR strategy provides a possibility for DNA to act as an amplifying transducer to construct an ultrasensitive electrochemical DNA biosensor, and has great potential for applications in bioanalysis and biomedicine.

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