Supporting Information

A New Hybrid Signal Amplification Strategy for Ultrasensitive Electrochemical Detection of DNA Based on Enzyme-assisted Target Recycling and DNA Supersandwich Assemblies

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Experimental Section

Chemicals and Materials: Hexaaminеruthenium(III) chloride ($[Ru(NH_3)_6]Cl_3$, RuHex), Tris-HCl, Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) and MCH were purchased from Sigma-Aldrich (St. Louis, MO). The nicking endonuclease (N.BstNB I) and 10×NEBuffer 3 (50 mM Tris-HCl, 10 mM $MgCl_2$, 100 mM NaCl, 1 mM dithiothreitol, pH 7.9) were obtained from New England BioLabs (Ipswich, MA, USA). Streptavidin-coated magnetic dynabeads (STV-MB, MyOneTM Streptavidin C1, 1.0 μm in diameter) were from Invitrogen Corp. (Oslo, Norway). Ethylenediaminetetraacetic acid (EDTA), sodium phosphate monobasic, sodium phosphate dibasic, sodium chloride, potassium chloride, potassium phosphate dibasic and potassium phosphate monobasic were obtained from Kelong Chemical Inc. (Chengdu, China).

The following buffer solutions were prepared in our laboratory. 2×binding & washing (B&W) buffer consisting 10 mM Tris-HCl, 1 mM EDTA and 2 M NaCl (pH 7.5) was used for coupling the biotin-CP_1 with STV-MB. Immobilization buffer was made of 10

mM Tris-HCl, 1 mM EDTA, 10 mM TCEP, 0.1 M NaCl (pH 7.4). The hybridization buffer (HB) was 50 mM sodium phosphate buffer containing 0.5 M NaCl (pH 7.4). The detection buffer was made of 10 mM Tris-HCl containing 10 μM RuHex (pH 7.4).

All synthetic oligonucleotides were ordered from Shanghai Sangon Biological Engineering Technology and Services Co., Ltd. (Shanghai, China), and the sequences were listed below.

Hairpin capture probe (biotin-CP_1):

5'-biotin-CCCTACCCGAGTCTTCC↓AGTGTGATGAGGGTAGGG-3';

(The underlined letters are the recognition sequence of N.BstNB I, and the arrow indicates the nicking position.)

p53 gene target: 5'-TCATCACACTGGAAGACTC-3';

Single-base mismatch sequence (sDNA): 5'-TCATCACACTGGAAGAATC-3';

Non-complementary sequences (nDNA):5'-GACGTCTGACTTCCTGCGA-3'; Thiolated capture probe (SH-CP_2): 5'-SH-CCCTACCCT-3';

Helper probe 1 (DNA1): 5'-CATCACACTCAAAGTAGT-3';

Helper probe 2 (DNA2): 5'-AGTGTGATGACTACTTTG-3';

All reagents were analytical grade and solutions were prepared using ultrapure water (specific resistance of 18 MΩ-cm).

Preparation of STV-MB/biotin-CP_1 bioconjugates: Firstly, the STV-MB was washed three times and resuspended in 2×B&W buffer to a final concentration of 5 μg μL^{-1} (twice original volume). Then, an equal volume of the biotin-CP_1 (20 μM in H_2O) was added to dilute the NaCl concentration in the 2×B&W buffer from 2 M to 1M and incubated with the STV-MB for 30 min at room temperature with gentle mixing.

Subsequently, the STV-MB/biotin-CP_1 conjugates were separated with a magnet and the supernatant was removed. Finally, the conjugates were washed twice with 1×B&W buffer, resuspended and stored in 4 ºC for further use.

Collection of the amplified intermediate DNA products *via* N.BstNB I-assisted target recycling: In brief, a solution containing 4 μL 10×NEBuffer 3, STV-MB/biotin-CP_1 bioconjugates (to a final concentration of 2.5 μg μL^{-1}) and different concentrations of the target DNA sequence were added to a centrifuge tube and diluted to 37 μL. After 30 min of hybridization with gentle mixing, 3 μL of N.BstNB I enzyme (10 units μL^{-1}) was added and incubated at 55 ºC for 60 min.[1] Then, the mixture was heated to 90 ºC and kept for 10 min to deactivate the N.BstNB I enzyme. After cooling down to room temperature, the STV-MB/biotin-CP_1 conjugates were separated by a magnet and the supernatant was collected as the DNA intermediate product containing solution for next-step incubation.

Fabrication of sensors: First of all, gold electrodes (3 mm in diameter, CH Instruments Inc., Shanghai, China) were immersed in a fresh warm piranha solution (volume(concentrated H_2SO_4): volum(30% H_2O_2)=3:1) for 30 min. After rinsing thoroughly with water, the electrodes were polished with 0.3 and 0.05 μm aluminum slurry and sonicated sequentially in distilled water, ethanol and distilled water for 5 min each. Then, the electrodes were electrochemically cleaned in 0.5 M H_2SO_4 with potential scanning from 0.2 to 1.6 V until remarkable voltammetric peaks were obtained, followed by sonication again and drying with nitrogen. Next, an aliquot of 10 μL SH-CP_2 (1 μM) was cast onto the pretreated electrode and incubated overnight at room temperature in humidity. The electrode surface was rinsed with water and blocked with 1 mM MCH for 2 h. After washing, the modified electrode surface was soaked in 10 μL of N.BstNB I

enzyme-cleaved DNA intermediate products corresponding to various target concentrations for 1 h. Subsequently, the electrodes were rinsed with PBS, dried with N_2, and incubated with a mixture of 5 μL DNA1 (1 μM) and 5 μL DNA2 (1 μM) in HB for 2 h. Finally, the current intensity of the resulting functionalized electrode was recorded in detection buffer.

EC characterizations and measurements: Cyclic voltammetry (CV) and differential pulse voltammetry (DPV) were performed on a CHI 852C electrochemical workstation (CH Instruments, Shanghai, China). A conventional three-electrode configuration was used, with the modified gold working electrode (3 mm in diameter,), an Ag/AgCl (3 M KCl) reference electrode, and a platinum wire counter electrode. The newly prepared detection buffer (10 mM Tris-HCl, 10 μM RuHex, pH 7.4) was purged thoroughly with nitrogen for 10 min and the modified electrode was immersed in the detection buffer for 5 min prior to the DPV tests. DPV measurements were performed from 0.1 to -0.5 V with a pulse amplitude of 0.05 V and a pulse width of 0.05 s.

Surface coverage of the SH-CP_2 on the gold electrode:

The surface coverage of the SH-CP_2 on the gold electrode was estimated to be 8.7×10^{12} molecules per cm^2 by the Cottrell's experiment according to equations (1) and (2).[2,3]

$$\Gamma_{\mathrm{Ru}} = \frac{Q_{ds} - Q_{dl}}{nFA} \qquad (1)$$

$$\Gamma_{DNA} = \Gamma_{\mathrm{Ru}}(z/m)(N_A) \qquad (2)$$

In these equations, Γ_{Ru} is the amount of the redox marker confined near the electrode surface, Q_{ds} and Q_{dl} are the surface charges, n is the number of electrons in the reaction, F is the Faraday constant, A is the area of the working electrode, Γ_{DNA} is the surface

density of DNA, z is the charge of the redox marker, m is the numbers of the bases in DNA, and N_A is Avogadro's number.

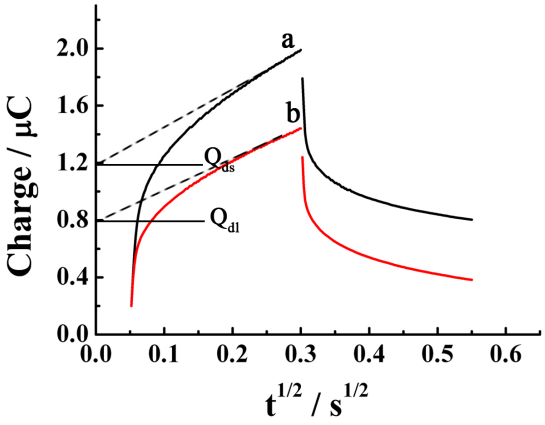
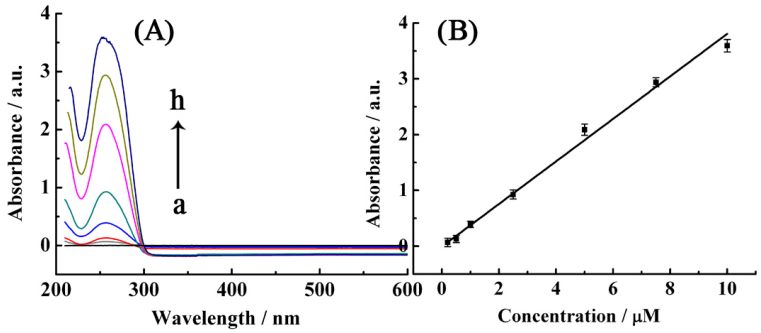


Fig. S1 Chronocoulometric curves for (a) capture probes/MCH and (b) MCH modified electrodes in the presence of 50 μM RuHex. The chronocoulometric intercept at t = 0 represents the charges of the redox marker confined near the electrode surface.

Surface coverage of the biotin-CP_1 on the magnetic beads:

The surface coverage of the biotin-CP_1 on the magnetic beads was estimated to be 2.2 nmol DNA per mg beads by comparing the intensity of the UV-Vis absorbance at 260 nm before and after the incubation of biotin-CP_1 with magnetic beads.



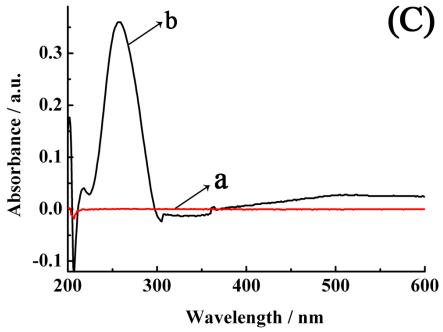


Fig. S2 (A) UV-Vis spectrums of different concentrations of biotin-CP_1 in B&W buffer: (a) 0 μM, (b) 0.2 μM, (c) 0.5 μM, (d) 1 μM, (e) 2.5 μM, (f) 5 μM, (g) 7.5 μM, (h) 10 μM. (B) The resulting calibration plot for biotin-CP_1concentration vs. intensity of UV-Vis absorption. (C) UV-Vis spectrums: (a) B&W buffer, (b) 5-fold dilution of the supernatant after incubation and separation of STV-MB/biotin-CP_1 conjugates.

EC behavior of RuHex on different electrodes:

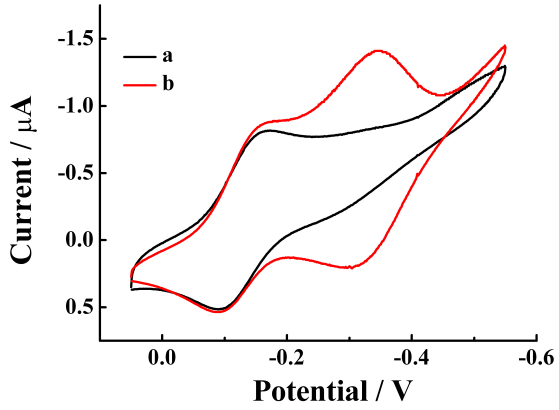


Fig. S3 Cyclic voltammograms of gold electrodes modified with (a) MCH and (b) SH-CP_2/MCH in 10 mM Tris-HCl (pH 7.4) containing 50 μM RuHex. Scan rate: 50 mV s^{-1}.

According to previous studies,[4] RuHex, one of the charged transition metal complexes, has unique electroactive behavior on the surface of DNA/MCH modified electrode. Considering cyclic voltammetry (CV) is a useful EC technique that provides important information of electron-transfer reactions at electrode surfaces, we employed this technique to characterize the redox reactions of RuHex at gold electrode surfaces with

SH-CP_2/mercaptohexanol (MCH) monolayers. According to the cyclic voltammograms shown in Fig. S3, only a pair of peaks corresponding to the reduction and oxidation of RuHex at a MCH-assembled gold electrode surface is observed in a 50 μM RuHex-containing solution (curve a), which correlates well with previous report.[4] However, two pairs of peaks are observed on the SH-CP_2/MCH-assembled gold electrode (curve b). One pair of peaks at about -0.12 V, similar to that of a MCH-assembled gold electrode surface, is ascribed to the direct diffusion of RuHex in solution to the electrode surface in the absence of SH-CP_2, consistent with the diffusion-controlled electron-transfer process, while the other pair of peaks at about -0.30 V is resulted from the cationic RuHex bound to the anionic phosphate backbones of SH-CP_2 *via* electrostatic interaction. Therefore, this peak pair could be affected by the amount of DNA strands localized at the electrode surface and used as the basis for EC DNA quantification.[5] From this point of view, the diffusion-controlled peak pair (-0.12 V) might interfere the analytical signal response. However, the distinct differences in redox potential of the two peak pairs provide opportunities to distinguish the interference current from the analytical current, thus ensuring the accuracy as well as reliability of our proposed strategy.

Optimization of RuHex concentration:

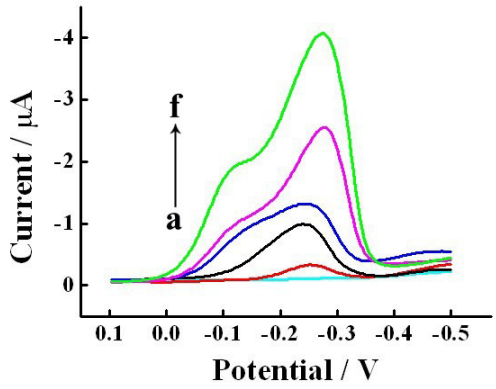


Fig. S4 DPV curves for SH-CP_2/MCH modified electrodes with the presence of different concentrations of RuHex (a) 0, (b) 5, (c) 10, (d) 15, (e)25, and (f) 50 μM in 10 mM Tris-HCl (pH 7.4) with a pulse amplitude of 0.05 V and a pulse width of 0.05 s.

In order to suppress the influence of the interference current caused by direct diffusion of RuHex to the electrode surface and to screen the suitable concentration of RuHex for high sensitivity, the concentration of RuHex in the detection buffer was optimized. Considering the fact that differential pulse voltammetry (DPV) offers excellent resolution for current responses especially when analyzing low concentrations of redox species compared to other conventional sweep techniques such as CV. Therefore, in our work, we employed DPV to characterize the electron transfer of the surface-confined RuHex on the sensing surfaces and to investigate the optimal concentration of RuHex by immersing SH-CP_2/MCH/Au electrodes in detection buffers with the presence of different concentrations of RuHex. As shown in Fig. S4, only the surface-confined cathodic peaks are observed when the RuHex concentration transforms from 5 to 10 μM (curves b and c) because the diffusion-controlled current is not able to be produced at such low concentrations of RuHex. However, by increasing concentration of RuHex from 15 to 50 μM, the diffusion-controlled current located at -0.1 V becomes obvious and both peaks are augmentative. It has been reported that the current response of the diffusion-controlled RuHex could be larger than that of the surface-defined RuHex when the RuHex concentration is higher than 75 μM.[6] Obviously, an excessively high concentration of RuHex is unsuitable for our DNA analysis. Based on the optimizations, 10 μM of RuHex is selected as the optimal experimental condition to minimize the interference current.

Table S1: Detection limit (DL) comparison between our method and other reported ones

Ref	Detection method	DL	Signal amplification strategy
7	Differential Pulse Voltammetry	0.9 pM	DNA-wrapped carbon nanotubes as electrochemical labels
8	Colorimetric Detection	0.5 fM	Nicking endonuclease-assisted nanoparticle amplification
9	Fluorescence	0.25 pM	Hybridization chain reaction amplification and pyrene-excimer
10	Square Wave Voltammetry	100 fM	supersandwich assembly with methylene blue labels
11	Differential Pulse Voltammetry	0.81 nM	PCR amplification and guanine oxidation signal
12	Chemiluminescence	71 aM	Rolling circle amplification and DNAzyme amplification
this work	Differential Pulse Voltammetry	0.36 fM	Coupling of enzyme-assisted target recycling with DNA supersandwich assemblies

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