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Scaling Up Electrochemical Signal with Catalytic Hairpin Assembly Coupling Nanocatalyst Label for DNA Detection

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1 Experimental

1.1 Reagent

All synthetic oligonucleotides were purchased from SBS Genetech Co. Ltd. (Beijing, China). Their sequences were presented in the followings. The red portions in H1 and probe DNA indicated complementary sequence. The green portions in H1 and target DNA indicated complementary sequence. The upperlined sequence in H1 and H2 formed stems of hairpins. The underlined portions in H1 and H2 indicated complementary sequence.

Capture DNA (H_1): SH-$(CH_2)_6$-AAACTTGGGCAGCGCCTCACAACCCATCGTGTACGTTGTGAGGCGCTGC AAGCGTACTTGTACAG
Target DNA: GGTTGTGAGGCGCTGCCCAAGCGA
Linker DNA (H_2): GCGCCTCACAACGTACACGATGGGTTGTGAGGCGCTGCCATCGTGTACG
Probe DNA: SH-$(CH_2)_6$-GTTTACACTGTACAAGTAC

The DNA used for the generality of the developed method as followings.

Capture DNA(H_1): SH-$(CH_2)_6$-AAAGTTATTCAAAGATGAGTAGGAAAGGAGTTAC CCTACTCATCTTTGAGACATGTTCATGCGAA
Target DNA: TCCTACTCATCTTTGAATAACTAC
LinkerDNA(H_2): AAGATGAGTAGGGTAACTCCTTTCCTACTCATCTTTGAAGGAGTTACC
Probe DNA: SH-$(CH_2)_6$-GTTTACATTCGCATGAACA

All the other chemicals and solvents were of analytical grade and used without further purification. Hydrogen tetrachloroaurate trihydrate ($HAuCl_4 \cdot 3H_2O$), chloroplatinic acid ($H_2PtCl_6 \cdot 6H_2O$) were obtained from Fluka. p-nitrophenol (PNP), $NaBH_4$ was provided from Dingguo Biotech. Co. Ltd. (Beijing, China). Ferrocenecarboxylic acid (FCA), Tris(2-carboxyethyl) phosphine hydrochloride (TCEP) were obtained from Shanghai Chemical Reagent Co. (Shanghai, China). All solutions were prepared with doubly distilled water.

1.2 Apparatus

A TGL-16G centrifuge (Shanghai Anting Science Instrument Co., China) was used for centrifugation. CV and DPV experiments were performed with a CHI 760B electrochemical workstation (CH Instruments). A three-electrode cell was employed. A coiled Pt wire and a saturated calomel electrode (SCE) were used as the counter and the reference electrodes, respectively. CV was employed to sense the assembly of AuNPs, the immobilization of DNA, the hybridization of DNA and capture of signal probe.

1.3 Fabrication of gold nanoparticles and core/shell Au@Pt nanoparticles

Prior to synthesis of gold nanoparticles (AuNPs), all glassware were cleaned with aqua regia (HCl:HNO_3 volume ratio = 3:1) [Caution! aqua regia is corrosive in nature and should be handled with care]. Approximately 20 nm AuNPs were prepared according to the method reported previously with a slight modification.[1] Briefly, $HAuCl_4$ and trisodium citrate solutions were filtered through a 0.22 μm microporous membrane filter prior to use, and then 0.08 g trisodium citrate dissolved in 1.0 mL water was added rapidly to 100.0 mL of boiling 3×10^{-4} mol/L $HAuCl_4$ solution and stirred for 10 min at the boiling point. The solution color turned to a wine red, indicating the formation of AuNPs.

Au@Pt nanoparticles (Au@PtNPs) with nanostructured Pt shells were synthesized by seed-induced growth methods and chemically reducing H_2PtCl_6

species.[2] Briefly, 10 mL as-synthesized AuNPs, 1 mL of $HAuCl_4$ (0.03 mol/L), and 0.1 g PVP was mixed with 50 mL water in a 250 mL beaker. The Pt shell thicknesses on Au cores can be easily tuned by controlling the Au@Pt molar ratios in the starting precursor or solutions. H_2PtCl_6 could be reduced well by ascorbic acid when the mole ratio of ascorbic acid and H_2PtCl_6 was 4:1. The mixed solution was heated to boiling and sustained for 10 min. Then 7 mL of H_2PtCl_6 (1%) and a certain amount of ascorbic acid was slowly added. The reaction continued for a period of time with stirring and heating until the solution became dark brown. Then the resulting solution was placed at ambient temperature and stopped stirring. The sizes of the nanoparticles were verified by scanning electron micrograph (SEM) using a JEOLJSM-6700F microscope (Hitachi, Japan) operated at 200 kV. The final AuNPs prepared by this method have an average diameter of approximately 13 nm. The prepared AuNPs and 62 nm Au@PtNPs were stored in brown glass bottles at 4 ◦C.

1.4 Bioconjugation of probe DNA with core/shell Au@Pt nanospheres

Firstly, 10 μL of 1.0×10^{-5} mol/L probe DNA, 10 μL of 50 mM Tris-HCl (pH 8.2) and 10 μL of 10 mM TCEP were added into a 2 mL tube for 1 h to activate the -SH of probe DNA. Then, 1 mL Au@PtNPs was added and incubated at 37 ℃ for 16 h with shaking. The probe DNA would binding with the Au@PtNPs through Pt-S binding.[3] The production was washed with 1.0 mL of 0.1 mol/L phosphate buffer solution three times, and resuspended in 1.0 mL phosphate buffer solution and stored at 4 ℃ for further use.

1.5 Preparation of gold nanoparticle modified electrode

Briefly, a gold electrode (3 mm in diameter, Jiangsu Jiangfen Electroanalytical Instrument Co., Ltd.) was polished carefully with alumina slurries (1, 0.3, 0.05 μm) and washed ultrasonically with deionized and doubly distilled water. Then it was electrochemically cleaned in 0.5 mol/L H_2SO_4 solution by cyclic potential scanning between 0.3 and 1.5 V until a standard CV was obtained. Subsequently, the gold electrode was rinsed with deionized and doubly distilled water and absolute ethanol in turn and dried with nitrogen gas. As-pretreated bare gold electrode was immersed in a 2 mM 1,3-propanedithiol-ethanol solution, and was incubated at room temperature for 10 h. The thiol self-assembled monolayer on the surface of the gold electrode was rinsed with ethanol and ultrapure water. After that, 10 μL gold colloid solution was dropped onto the gold electrode and incubated for 10 h at 4 °C. After rinsing with ultrapurewater, the AuNPs modified gold electrode was allowed to dry at room temperature and was ready for further experiments.

1.6 Fabrication of catalytic hairpin assembly biosensor

The procedure of the fabrication of catalytic hairpin assembly biosensor and the principle of nanocatalyst label-based EC detection of target are illustrated in Scheme 1. The AuNPs modified gold electrode was immersed into 0.5 mL of 1.0×10^{-7} mol/L H1 in 10 mM phosphate buffer (pH 6.8) and incubated for 1 h. Then the H1 modified gold electrode was immersed in the phosphate buffer containing 1 mM MCH for 1 h

to block the uncovered gold surface. Then, the modified electrode was immersed into 10 mM phosphate buffer containing target DNA at 37 °C. Half an hour later, 10 μL of 1.0 × 10^{-7} mol/L H2 was droped, and incubated certain time. Then the resulting electrode was washed with phosphate buffer three times to remove nonspecifically adsorbed sequences. Finally, the gold electrode was immersed into 0.5 mL of the above Au@PtNPs probe solution. After incubated certain time, the resulting gold electrode was washed with phosphate buffer three times and taken for EC detection.

1.7 Electrochemical measurements

Electrochemical measurements including cyclic voltammetry and electrochemical impedance spectroscopy (EIS) was performed with CHI 660D electrochemical analyzer (Shanghai CH Instrument, China). A conventional three-electrode system was used with a gold electrode as working electrode, a platinum wire as auxiliary electrode and a saturated calomel electrode (SCE) as reference electrode. The concentration of target DNA was quantified by a increase of EC peak height ΔI ($\Delta I = I_0 - I$), where I_0 is the EC peak height before the prepared biosensor incubated with the target DNA solution, H2 and nanocatalyst label and I is the EC peak height after incubation. The electrochemical response of the biosensor was performed in pH 7.4 phosphate buffer solution containing 5 mM *p*-nitrophenol, 5 mM $NaBH_4$, and 5 mM ferrocenecarboxylic acid (FCA) by using cyclic voltammetry from -200 to 600 mV (*vs*. SCE) at 50 mV s^{-1}, unless otherwise stated. For establishing the analytical method, the DPV experiments were employed and I-E curve was recorded in the

potential window between -0.1 to 0.6 V. The optimum parameters for DPV were: amplitude 0.05 V, pulse width 0.05 s, Incr E 0.001 V, pulse period 0.2 s. All electrochemical measurements were done in an unstirred 8 electrochemical cell at room temperature (RT, 25 ± 1.0 °C).

2 Resultion and discussion

2.1 Conditions for catalytic hairpin assembly.

The amount of target DNA hybridized with H1 on the surface of electrode and the amount of H2 displaced the target DNA increase with the increasing times of incubation and become saturated in a certain time. The time required for saturation increases with decreasing the concentration of target DNA. To examine the saturation time, electrochemical signals were obtained in different times of incubation. The target DNA was dropped onto an H1 modified electrode. Then the electrode was kept at 37 ℃. After 30 min, H2 with the concentration of 1.0×10^{-6} mol/L was dropped onto the surface. After incubation for a certain time at 37 ℃, the nanocatalyst label was added onto the surface. Then the electrode was taken for EC detection. From Fig. S1 (B), it is apparent that EC intensity obviously increases with increasing the incubation time from 0.5 h to 3 h and then reaches a plateau in 4 h. This suggested that 4 h was enough for the hybridization and displacement, and thus it was chosen as the extension time in the following experiments.

The incubation time of the nanocatalyst label on electrode was also investigated. The incubation time and the amount of nanocatalyst label immobilized on the electrode were closely related, so the EC intensity also was influenced by the

incubation time of the nanocatalyst label. From Fig. S1 (D), it is apparent that EC intensity obviously increases with the increasing hybridization time from 5 min to 30 min and then reaches a plateau in 50 min. This suggested that 50 min was enough for the nanocatalyst label to hybridize with H2. Thus, 50 min was the hybridization time chosen in the following experiments.

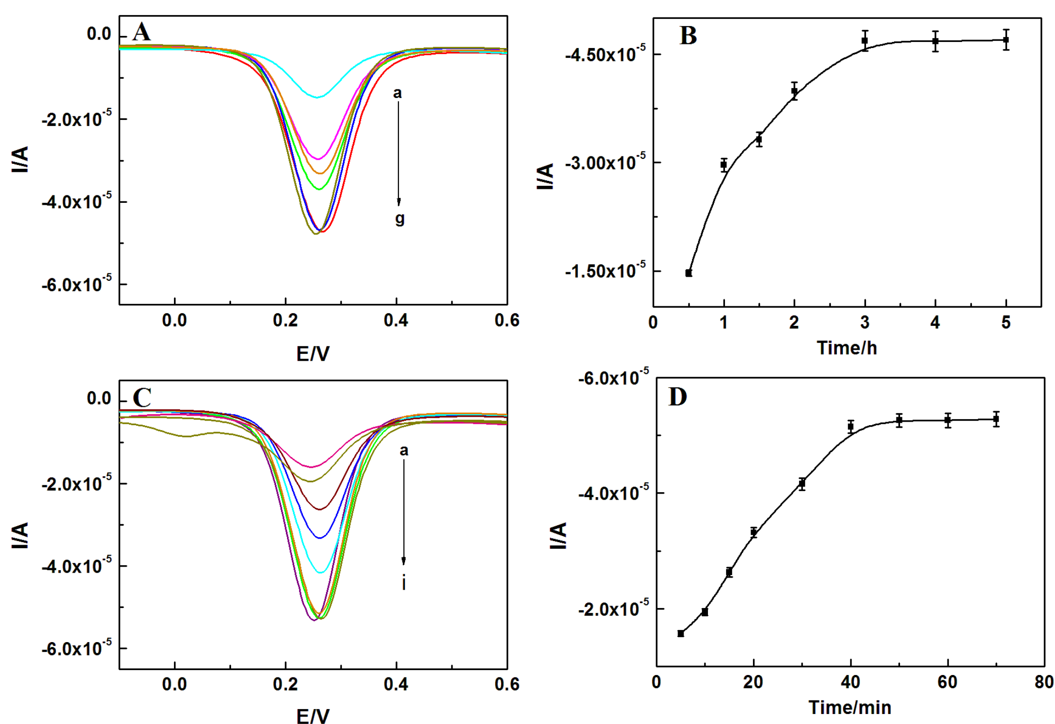

Fig. S1. The effect of time of catalytic hairpin assembly reaction time on EC intensity (A) and (B). a, 0.5 h; b, 1 h; c, 2 h; d, 3 h; e, 4 h; f, 5 h. And the effect of the EC nanoparticle probe hybridization time on EC intensity (C) and (D). a, 5 min; b, 10 min; c, 15 min; d, 20 min; e, 30 min; f, 40 min; g, 50 min; h, 60 min; i, 70 min.

2.2 Comparison

Analytical performance characteristics of the method were determined with DPV with varying concentrations of target DNA. Fig. S2 shows the calibration curves for the detection of target DNA. Under the optimized test condition, the peak current

increased with the increasing of the concentration of target DNA. The linear range and detection limit of this bioassay to target DNA were measured. The results showed that the peak current linear increased with the concentration of target DNA in the range from 1.0×10^{-18} to 1.0×10^{-7} mol/L (Fig. S2 (B)) and the detection limit was 3.0×10^{-19} mol/L. The regression equation $I_p = 5.010 \log C + 95.38$ with a regression coefficient of 0.9990 (C, mol/L; I_p, μA).

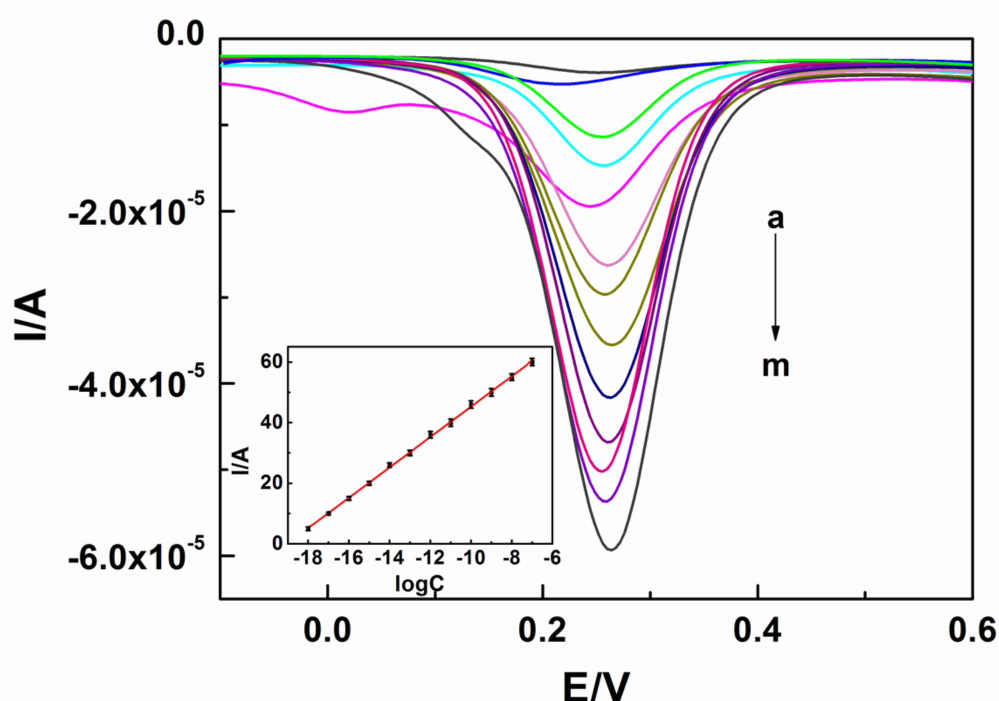

Fig. S2. Differential pulse voltammograms of various concentrations target DNA. From a to m are 0.0, 1.0×10^{-18}, 1.0×10^{-17}, 1.0×10^{-16}, 1.0×10^{-15}, 1.0×10^{-14}, 1.0×10^{-13} 1.0×10^{-12}, 1.0×10^{-11}, 1.0×10^{-10}, 1.0×10^{-9}, 1.0×10^{-8}, 1.0×10^{-7} mol/L target DNA we have used. The calibration curve of peak height versus the concentration of target DNA from 1.0×10^{-18} to 1.0×10^{-7} mol/L (insert).

In order to confirm the contribution of the catalytic hairpin assembly and nanocatalyst label amplification to the high sensitivity, a control experiment was carried out in catalytic hairpin assembly with FAC labeled AuNPs (CHA with AuNPs label method) or sandwich hybridization with nanocatalyst label (sandwich with nanocatalyst label method). The experimental results suggested that CHA with AuNPs label method gave the linearity range from 1.0×10^{-14} mol/L to 1.0×10^{-10} mol/L with LOD of 3.0×10^{-15} mol/L. And sandwich with nanocatalyst label method gave the linearity range from 2.0×10^{-15} mol/L to 1.0×10^{-11} mol/L with LOD of 7.0×10^{-16} mol/L. The detection limit of the catalytic hairpin assembly and nanocatalyst label amplification was 10 000 or 2 300 times higher than that obtained in the CHA with AuNPs label method or sandwich with nanocatalyst label method. Thus the high sensitivity of this method was mainly attributed to the amplification of CHA and redox cycling process, which increased the repeat use of target DNA and the number of electrons on the surface of AuNPs modified electrode.

2.3 Testing the generality of the developed method

To test the generality of the developed method, other one target DNA sequence was detected, the relative DNA sequences were provide in the section of “Reagent”. Under the above optimal condition, the peak current also increased with the increasing of the concentration of target DNA. The linear range and detection limit of this bioassay to target DNA were measured. The results showed that the peak current linear increased with the concentration of target DNA in the range from 5.0×10^{-18} to 2.0×10^{-7} mol/L and the detection limit was 1.0×10^{-19} mol/L. The regression equation I_p

= 5.017 log C + 93.42 with a regression coefficient of 0.9989 (C, mol/L; I_p, μA).

2.4 Selectivity of the gene-sensor

The specificity of the electrochemical bioassay was monitored by challenging the system with mismatch base DNA, namely, full-matched bases, one mismatch bases, two mismatch bases, three mismatch bases and artificial complex samples by mixing target DNA and random sequences DNA respectively. The experiments were done under the same condition and the concentration of these four strands was 1.3×10^{-8} mol/L. And target DNA is 4.0×10^{-9} mol/L and random sequences DNA is 4.0×10^{-8} mol/L in the artificial complex samples. The gene-sensor exhibits the different response signals. As is shown in Fig. 3 (B), the response signals gradually decreased with the increasing of the mismatch bases. The response signal for artificial complex samples was almost the same as that for target DNA. The results indicated that the selectivity of the developed gene-sensor is sufficient for DNA detection.

Table S1. Comparison between the catalytic hairpin assembly coupling nanocatalyst label method and other reported techniques for the determination of DNA

Detection method	Assay format	Linearity range	Detection limit	Reference
ACV[a]	Pt nanoelectrodes	-[b]	20 nM	4
EIS	ECPs	nM range	-	5
Am	NATR and DNAzyme	80 aM - 8.0 fM	20 aM	6
Flu	NE and MB	1 pM - 10 nM	200 fM	7

Flu	IEA with CdTe quantum dots	10 aM - 10 pM	8.5 amol	8
MRI	MNP assembly induced by PCR	10 aM - 10 pM	4.26 aM	9
SWV	Ratiometric method	-	25.1 pM	10
Flu	FRET from PFP to P	0.05 - 20nM	40 pM	11
Flu	CtCSa	100 - 10 nM	-	12
Flu	λ exo hydrolysis	-	30 pM	13
Flu	T7 hydrolysis	10 -10 nM	4 pM	14
Flu	Nicking enzyme, RCA and CHA	4.0 -10 aM	1.2 aM	15
DPV	T7 hydrolysis	0.5 - 100 fM	0.17 aM	16

[a] ACV, alternating current voltammetry; EIS, Electrochemical impedance spectra; ECPs, Electrochemically active conducting polymers; Am, Amperometric detection; NATR, nuclease-assisted target recycling; Flu, fluorescent; NE, nicking endonuclease; MB, molecular beacon IEA, isothermal exponential amplification; MRI, magnetic resonance imaging, SWV: square wave voltammetry; FRET from PFP to P; Pfluorescence resonance energy transferfrom poly [(9,9-bis(6′-N,N,N-trimethylammonium)hexyl)-fluorenylene phenylene dibromide] (PFP) to P (fluorescein (FAM)-labeled single-stranded DNA); CtCSa: cross-triggered cascading self-amplification; DPV: Differential pulse voltammetry.

-[b] Not reported in their articles.

2.5 Application of the developed method for the spiked samples

To test application in real samples, serum was isolated from healthy blood donors according to standard procedures and was pooled; 200 μL were artificially spiked with known concentrations of target DNA in serial dilutions. The result of detection was shown in **Table S2.** It suggested that the developed method can be used in the real samples.

Table S2 Recovery results for the added standard target from serum samples obtained by the developed method

Sample no	Initial target DNA content	Added standard target DNA content	Detected content (mean±SD) [a]	Recovery ratio (%)
1	0	0.50 nM	0.49 ± 0.56	98.0
		1.00 nM	104 ± 0.98	104.0
		5.20	5.29 ± 1.21	105.8
2	0	2.50 fM	2.47 ± 0.21	98.8
		10.00 fM	9.89 ± 0.34	98.9
		50.0 fM	48.95 ± 0.37	97.9

[a] The average of five determinations, SD standard deviation

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