Experimental Section

Reagents. The nicking enzyme (N.BstNB I) and NEBuffer 3 (50 mM Tris-HCl, 10 mM MgCl₂, 100 mM NaCl, 1 mM dithiothreitol, pH 7.9) were obtained from New England BioLabs. All oligonucleotides were synthesized by TaKaRa biotechnology Co., Ltd. (Dalian, China), and their base sequences were as follows: Target DNA: T1-5′-AGC AGG ACG GAC TCA T-3′; Hairpin capture probe (HP): 5′-HS-T GCA CGCTAGAT GAGTCCGTC↓CTGCTGCGTGC-3′-Ferrocene (The italic bold letters at the two ends represent the sequence of the stem arms; the underlined bold letters are the recognition sequence of N.BstNB I, and the arrow indicates the nicking position.); single-base mismatched probe: T2-5′-AGC AGG AGG GAC TCA T-3′ (The underlined bold indicates the mismatched position.); non-complementary probe: T3-5′-GAT GAA GAA AGA GAG A-3′. Tris-(hydroxymethyl) aminomethane was purchased from Cxbio Biotechnology Co. Ltd. (Denmark). Ethylenediaminetetraacetic acid (EDTA), Hexaammineruthenium (III) chloride (RuHex, 98%), mercaptohexanol (MCH) and tris (2-carboxyethyl) phosphine hydrochloride (TCEP) were purchased from Sigma-Aldrich (USA).

Solutions. Hybridization buffer was the mixture of 100 mM NaCl and 10 mM TE (pH 7.4). DNA immobilization buffer was the mixture of 10 mM TE, 10 mM TCEP, 100 mM NaCl and 10 mM MgCl₂ (pH 8.0). MgCl₂ was added into the electrolyte to induce the formation of the hairpin structure of probe DNA, as reported previously. Washing buffer solution was 100 mM NaCl and 10 mM PB solution (pH 7.4). To avoid the instability of ferrocenium (the oxidized form of the ferrocene), 1.0 M NaClO₄ solution was used as the supporting electrolyte when electrochemical behavior of the working electrode was investigated. All solutions were prepared with MilliQ water (18.2 MΩ).

Electrochemical measurements. All electrochemical measurements were performed by using CHI 660D Electrochemical Workstation (CH Instrument, USA). The electrochemical system consisted of a working electrode (a gold disk electrode modified with hairpin capture probe), a platinum wire as the
auxiliary electrode, and a reference electrode (Ag/AgCl). Cyclic voltammetric (CVs) were carried out at a scan rate of 100 mV/s. Differential pulse voltammograms (DPVs) were registered in the potential interval 0.0 to +0.6 V vs Ag/AgCl under the following conditions: modulation amplitude 0.05 V, pulse width 0.06 s, and sample width 0.02 s.

**DNA self-assembly, hybridization and the nicking reaction at gold electrode.** A gold disk electrode (2 mm diameter, GE) was firstly polished to obtain mirror surface with 0.05 μm alumina powder, followed by sonication in ethanol and water for 5 min respectively. Then, the GE was electrochemically cleaned to remove any remaining impurities.® After drying with nitrogen, the electrode was immediately used for DNA immobilization. Firstly, 5 µL of 2 µM HP solution was first spread on the pre-cleaned gold electrode surface for 16 hours in the 100 % humidity. Next, this electrode was immersed in 1 mM MCH for 2 hours to remove the nonspecific DNA adsorption and optimize the orientation of the capture probes to make hybridization easier. The DNA surface density could be measured with chronocoulometry (CC) as previously described.7,8 At low surface densities, it was difficult to obtain well-defined DPV curves of Fc. At the same time, at high surface densities, the reaction rate slowed down because the binding of target or nicking endonuclease to the capture probe was hindered due to steric/conformational effects. Our results showed that the ideal surface density for the sensor is between 1.8×10¹² and 3.5×10¹² molecules/cm². In above range, there is a largest current change of the sensor, thus allowing easy quantification for various concentrations of target. In this case, the 2.6×10¹² molecules/cm² was chosen as the optimal surface density. Then, 5 µL of the hybridization solution containing complementary T1, single-base mismatch T2 or noncomplementary T3 was placed on the DNA SAM (self-assembly monolayer) of HP modified GE for 1 h at room temperature, respectively. After hybridization, the GE was extensively rinsed with washing buffer solution and dried under a stream of nitrogen gas. Afterwards, the sensor was then incubated in a solution of N.BstNB I reaction buffer (10 units/Ul) in NEBuffer 3 at 55 °C (such temperature could facilitate faster hybridization and subsequent dissociation of DNA in a nicking reaction.9). After a specified period of time, the electrodes were rinsed with wash buffer and stored at 4 °C until analysis by electrochemical measurement.
CVs of RuHex in the different stage of the electrochemical DNA biosensor. Previous studies have demonstrated that RuHex can bind to DNA by electrostatic interaction since DNA backbone has negative charge. In order to further test whether indeed the NEACA DNA biosensor worked as our expected, the CV behavior of RuHex was investigated at different stages of the biosensor preparation. The CVs of RuHex at different GE surfaces was shown in Fig. S1. As shown in Fig. S1, a pair of peaks corresponding to the reduction and oxidation of RuHex can be observed at a relatively dilute concentration (5 μM) at HP/MCH/GE (curve b), indicating that the electron-transfer reaction of RuHex at HP/MCH surface was a surface-confined redox process since the peak separation was close to zero and peak currents were linearly proportional to scan rates. This pair of peaks was ascribed to RuHex electrostatically binding to DNA surface, and reflected the amount of DNA on the electrode surface. When HP/MCH/GE was incubated in the solutions containing target DNA (T1), the hairpin capture probe hybridized with T1 to form a double helix structure. As a result, the amount of the RuHex electrostatically binding to the GE surface increased and finally resulted in a much higher current signals (curve a). From Fig. S1, it could be clearly observed that there was a lowest signal on HP-T1/MCH modified GE after incubating with nicking enzyme (curve c). It could be attributed to the fact that the nicking enzyme bound to and nicks the DNA at the cleave site. Then the target DNA and the ferrocene tag fragment of the cleaved probe dissociated from GE surface. As a result, the binding amount of RuHex on GE surface decreased due to the decreasing of negative charges on GE surface.

Reaction rate and target concentration. As anticipated, nicking reaction was extremely rapid. The current intensity plateaued after 30 minutes in the presence of 10 fM complementary DNA and 10 U of enzyme, indicating that the nicking endonuclease cleaved approximately 100% of the HP in 30 minutes (Fig. S2, curve a). By decreasing the target concentration 10-fold to 1 fM, the reaction rate slowed down and the reaction went to completion by 80 min (Fig. S2, curve b). With the target concentration decreasing to 0.5 fM, the reaction rate slowed down further and the reaction didn’t plateau until 120 min (Fig. S2, curve c). When the target concentration was reduced even further to 0.05 fM (Fig. S2, curve d), we didn’t see apparent changes in current signal compared with 0 fM (negative control, Fig. S2, curve e).
until after 120 min. Therefore, it was concluded that the nicking reaction rate slowed down with the target concentration decreasing, which was consistent with the result obtained in the previous report \(^9\).

**References**

**Fig. S1** CVs of RuHex on HP-T1/MCH/GE (a), HP/MCH/GE (b) and HP-T1/MCH/GE after being incubated in the nicking enzyme buffer solution (c). The concentration of RuHex was 5 μM, data was obtained in 10 mM Tris-HCl buffer solution (pH 7.0), scan rates =500 mV/s.
**Fig. S2** Time courses were recorded at target concentrations of 10 fM (a), 1 fM (b), 0.5 fM (c), 0.05 fM (d), and 0 fM (e), respectively.
Fig. S3 A cyclic voltammogram for a gold electrode modified with the ferrocene-tagged, hairpin probe in the absence of target DNA (scan rate 0.1 V/s). The electrolyte is 1 M NaClO₄. Inset shows the background-subtracted DPVs for the sensor in the presence of complementary DNA at 0 pM (d), 1 pM (c), 10 pM (b) after 60 min and the DPV for the sensor in the presence of complementary DNA at 1 pM after 30 min nicking reaction (a).