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Exonuclease III-based and gold nanoparticle-assisted DNA detection with dual signal amplification

Qi Fan [a], Jing Zhao [a], Hao Li [b], Li Zhu [a], Genxi Li [a,b,*]

[a] Laboratory of Biosensing Technology, School of Life Sciences, Shanghai University, Shanghai 200444, PR China
[b] Department of Biochemistry and State Key Laboratory of Pharmaceutical Biotechnology, Nanjing University, Nanjing 210093, PR China

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ABSTRACT

Herein we report a sensitive electrochemical biosensor for DNA detection by making use of exonuclease III and probe DNA functionalized gold nanoparticles. While probe DNA P1 modified on a gold electrode surface can self-hybridize into a stem-loop structure with an exonuclease III-resistant 3′ overhang end, in the presence of target DNA, P1 may also hybridize with the target DNA to form a duplex region. Therefore, exonuclease III may selectively digest P1 from its 3′-hydroxyl termini until the duplex is fully consumed. Since a single target DNA can trigger exonuclease III digestion of numerous P1 strands, the first signal amplification is achieved. On the other hand, since the digested P1, exposing its complementary sequence to probe DNA P2, can further hybridize with P2 that has been previously modified on the surface of gold nanoparticles, many nanoparticles loaded with numerous DNA strands are immobilized onto the electrode surface. Consequently, large amount of electroactive molecules $[Ru(NH_3)_6]^{3+}$ can bind with the DNA strands to produce an intense electrochemical response as the second signal amplification. Based on the studies with cyclic voltammetry (CV) and chronocoulometry (CC) techniques, the proposed biosensor can sensitively detect specific target DNA at a picomolar level with high specificity.

1. Introduction

Ultrasensitive detection of specific DNA sequences plays a critical role in clinical diagnostics of genetic diseases, accurate identification of pathogens and early detection of cancers (Staudt, 2001; Debouck and Goodfellow, 1999). Target amplification and signal amplification are two commonly used approaches to increase the detection sensitivity (Saiki et al., 1985). As a classical target amplification method, polymerase chain reaction (PCR) can amplify a few copies of target DNA across several orders of magnitude through thermal cyclic reaction (Heid et al., 1996). But PCR is prone to yielding false-positive results from the artificial amplification that may have effect on the detection specificity (Halford, 1999; Schweitzer and Kingsmore, 2001; Song et al., 2009). In contrast, signal amplification that directly enhances the detection signals from a single analyte-probe recognition event can efficiently improve the detection sensitivity without sacrificing the specificity. In recent years, the use of nicking endonuclease that cleaves only one strand of double-stranded DNA at specific recognition nucleotide sequences has largely facilitated the development of signal amplification (Tan et al., 2007; Xu et al., 2009). Yet, only 13 nicking endonucleases are commercially available, which extremely restricts the application of nicking endonuclease-based technology (Zhao et al., 2011).Different to the sequence-specific nicking endonuclease, exonuclease does not require a specific recognition sequence, so it may be more beneficial to the development of universal signal amplification strategies for DNA detection. Exonuclease III (ExoIII) is a kind of exonuclease catalyzing the stepwise removal of mononucleotides from 3′-hydroxyl termini of double-stranded DNA, which is not active on 3′-overhang ends of double-stranded DNA or single-stranded DNA (Cui et al., 2010). In the reported ExoIII-assisted signal amplification approaches, the fluorescent signaling probes are selectively digested by ExoIII with the target cycling, generating multiple signaling events and achieving signal amplification (Zhang et al., 2011; Zuo et al., 2010; Cui et al., 2010). These ExoIII-based fluorescent methods can sensitively detect target DNA at a picomolar level.

Fluorescent technique often suffers from several drawbacks, such as high cost, easy contamination and high complexity (Xu et al., 2009), so electrochemical technique is suggested to be an alternative due to its advantages in cost, portability and convenience (Liu et al., 2009; Xiao et al., 2008; Cao et al., 2010). Hsieh et al. have reported an electrochemical DNA detection method by using λ exonuclease-assisted signal amplification, and demonstrated 16-fold signal amplification at low target concentrations. However, the detection limit in this assay is only 10 nM, which cannot be as good as the result in the fluorescent methods (Hsieh et al., 2010).

* Corresponding author. Tel.: +86 25 83593596; fax: +86 25 83592510.
E-mail address: genxili@nju.edu.cn (G. Li).

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In this work, we have designed an improved electrochemical DNA detection strategy by coupling probe DNA functionalized gold nanoparticles (Au NPs)-assisted electrochemical signal amplification with ExoIII-assisted signal amplification. DNA functionalized Au NPs have been often used as colorimetric probes for DNA detection. Combined with ExoIII, a sensitive colorimetric method for DNA detection has been proposed (Cui et al., 2011). Since Au NPs have large surface to volume ratio, huge amount of DNA strands can be loaded onto their surfaces for the hybridization with the complementary sequences immobilized at an electrode surface, thus intense electrochemical response can be achieved to realize a very high sensitive electrochemical detection (Wang et al., 2009; Miao et al., 2011a; Zhang et al., 2006; Miao et al., 2009a; Steel et al., 1998). Therefore, due to dual signal amplification, our assay can have not only the advantages of electrochemical technique but also the same high detection sensitivity as the fluorescent method.

2. Experimental

2.1. Materials and chemicals

Chloroauric acid ($HAuCl_4$) was obtained from Shanghai Sinopharm Chemical Reagent Co., Ltd. Mercaptohexanol (MCH), ethylenediamine-tetraacetic acid (EDTA), hexaammineruthenium(III) chloride ($[Ru(NH_3)_6]Cl_3$), and tris(2-carboxyethyl) phosphine hydrochloride (TCEP) were from Sigma. ExoIII (specific activity, 100,000 units/mL) was purchased from New England Biolabs. Other chemicals were of analytical grade and were used without further purification. For all experiments, Milli-Q water (>18.0 MΩ) was used, purified by a Milli-Q Plus 185 ultrapure water system (Millipore purification pack).

DNA oligonucleotides were synthesized by Sangon (Shanghai, China) and used without further purification. The DNA sequences are as follows:

P1: 5′-SH-AAAAAAATTCTGCAGCAAGCGGCATCAATGAGGAAGC TGCAGAATGGGAT-3′;

P2: 5′-SH-TTTTTTTTTTTTCCGCTTGCTGCAG-3′;

Target DNA: 5′-ATCCCATTCTGCAGCTTCCTCATTGATGGTCTC-3′;

Control DNA: 5-'TTGAGCTGCAGAATGGGATCGGTAGCCAAATAG CTC-3′.

2.2. Preparation of P1 modified gold electrode

The substrate electrode was a 5 mm disk gold electrode obtained from Shanghai Chenhua Instrumental Co. It was wrapped by inert material polytetrafluoroethylene. Before use, the gold electrode was firstly polished on fine sand papers and alumina (particle size of about 0.05 μm)/water slurry on silk. Then it was thoroughly ultrasonicated in ethanol and doubly distilled water for about 5 min, respectively. After that, the electrode was electrochemically cleaned to remove any remaining impurities in 0.5 M H_2SO_4. After drying with nitrogen, the gold electrode was immersed in a solution containing 1 μM P1, 10 mM Tris–HCl, 10 mM TCEP, 5 mM $MgCl_2$ and 0.1 M NaCl (pH 7.4) for 16 h, followed by a 1 h treatment with an aqueous solution of 1 mM MCH. After thoroughly rinsed with pure water, P1 modified electrode was immersed into the buffer (10 mM Tris–HCl, 5 mM $MgCl_2$ and 0.1 M NaCl, pH 7.4) for at least 1 h to equilibrate the probe structures prior to the further treatment.

2.3. Preparation of P2 functionalized gold nanoparticles

The 13 nm Au NPs were firstly prepared using a standard citrate method (Jin et al., 2003). They were then functionalized with thiol-modified P2 according to the previous report (Liu and Lu, 2006). After activating by the treatment with TCEP at room temperature, the deprotected P2 (9 μL, 1 mM) was added into 3 mL citrate-stabilized nanoparticles solution (17.5 nM). The resulting solution was stored in a drawer at room temperature for at least 16 h. After that, 1 M NaCl solution was gradually added to enhance the NaCl concentration of the solution to 100 mM, and the solution was incubated for another day at room temperature. P2 functionalized Au NPs (P2-Au NPs) was purified three times by centrifugation (16,600 g for 15 min) in Tris–HCl (10 mM, 0.1 M NaCl, pH 7.5), and the solution was stored in the same buffer at 4 °C.

2.4. Dual signal amplification reactions on P1 modified electrode

P1 modified gold electrode was firstly immersed into ExoIII digestion buffer (10 mM Bis Tris Propane–HCl, 10 mM $MgCl_2$, 0.25 U/μL Exo III, pH 7.0) with different concentrations of target DNA for 10 min at 37 °C. Then, the electrode was thoroughly rinsed with pure water, and subsequently immersed into 100 μL P2-Au NPs solution for 30 min at 30 °C to ensure the hybridization with P2 modified on the surface of Au NPs. After that, the electrode was thoroughly rinsed with double-distilled water and prepared for electrochemical measurements.

2.5. Electrochemical measurements

Cyclic voltammetry (CV) and chronocoulometry (CC) were carried out on a CHI440A electrochemical analyzer in 10 mM Tris–HCl solution (pH 7.4) containing 50 μM $[Ru(NH_3)_6]^{3+}$. Electrochemical impedance spectroscopy (EIS) was carried out on a CHI660 C electrochemical analyzer in 0.1 M PBS (pH 7.2) containing 0.1 M KCl and 5 mM $[Fe(CN)_6]^{3-/4-}$. A three-electrode system consisting of the modified gold electrode, saturated calomel reference electrode (SCE) and platinum counter electrode was used for all the electrochemical measurements. All test solutions were thoroughly deoxygenated by bubbling high-purity nitrogen through the solution for at least 10 min. A stream of nitrogen was then blown gently across the surface of the solution in order to maintain the solution anaerobic throughout all the experiments.

3. Results and discussion

In this work, we have chosen a short DNA sequence related to human immunodeficiency virus (HIV) as the target DNA, since the nucleic acid-based tests are most accurate for HIV detection (Weiss, 1993; Baur et al., 2010). Fig. 1 may illustrate the mechanism of the proposed method. Firstly, probe DNA P1 immobilized on the surface of the gold electrode can self-hybridize into a stem-loop structure with an ExoIII-resistant 3′ protruding termini (Fig. 1a). Nonetheless, in the presence of target DNA, P1 will hybridize with the target DNA to form a 28 bp double-stranded DNA, which is then recognized and digested by ExoIII (Fig. 1b). For the digestion, 5′-mononucleotides of P1 are selectively removed from its 3′-hydroxyl termini until the duplex region is fully consumed. Consequently, target DNA spontaneously dissociates from the digested P1 to hybridize with another intact one, thereby initiating a new round of strand digestion. With the cycling, a single target DNA is able to trigger the digestion of multiple P1, which is defined as the first signal amplification in our strategy (Fig. 1c). After the digestion, P1 is transformed from the stem-loop conformation to a flexible linear structure, and the complementary sequence to P2 is exposed (Fig. 1d). Therefore, the digested P1 can hybridize with P2 that has been previously modified on the surface of Au NPs, thus DNA functionalized Au NPs are loaded onto the surface of the gold electrode. Subsequently, a large number of electrochemically active molecules $[Ru(NH_3)_6]^{3+}$ electrostatically bind to DNA molecules modified on the surface of Au

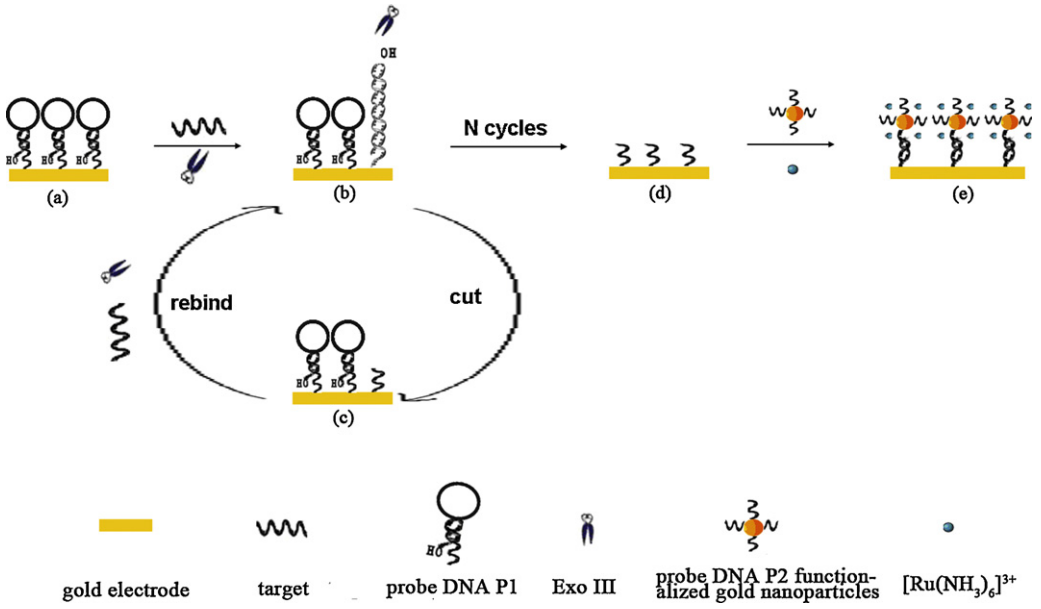

Fig. 1. Schematic illustration of the strategy for the target DNA detection based on ExoIII and Au NPs-assisted dual signal amplification.

NPs, resulting in an intense electrochemical response as the second signal amplification (Fig. 1e).

3.1. Characterization of the modified gold electrodes with EIS

The stepwise reactions on the surface of the gold electrode have been characterized with EIS. As is shown in Fig. 2, no impedance can be observed to the bare gold electrode. After P1 has been immobilized on the electrode surface, the interfacial electron resistance is increased evidently, ascribing to the electrostatic repulsion between the negatively charged P1 and the electrochemical species $Fe(CN)_6^{3-/4-}$. Nevertheless, in the presence of the target DNA, the target DNA will trigger the digestion of P1 by ExoIII, so the digested P1 may carry less negative charges, weakening its electrostatic repulsion with $Fe(CN)_6^{3-/4-}$, thus a reduced resistance is observed. Furthermore, after the digested P1 has hybridized with P2 modified on the surface of Au NPs, the immobilized P2-Au NPs with a large amount of negatively charges will more strongly repel $Fe(CN)_6^{3-/4-}$, so more evidently increased interfacial electron resistance can be observed.

3.2. Electrochemical detection of target DNA by cyclic voltammetry

The electrochemistry of the modified electrode has been characterized by CV with $[Ru(NH_3)_6]^{3+}$. As shown in Fig. 3, two pairs of peaks can be observed at the P1 modified electrode. While peak I is assigned to $[Ru(NH_3)_6]^{3+}$ that electrostatically binds to DNA, peak II reflects $[Ru(NH_3)_6]^{3+}$ diffusion to MCH (Lao et al., 2005; Miao et al., 2009b). Moreover, it can be observed that a dramatic increase of the peak current of peak I can be observed upon addition of target DNA to the test solution, which is reasonable because P2-Au NPs

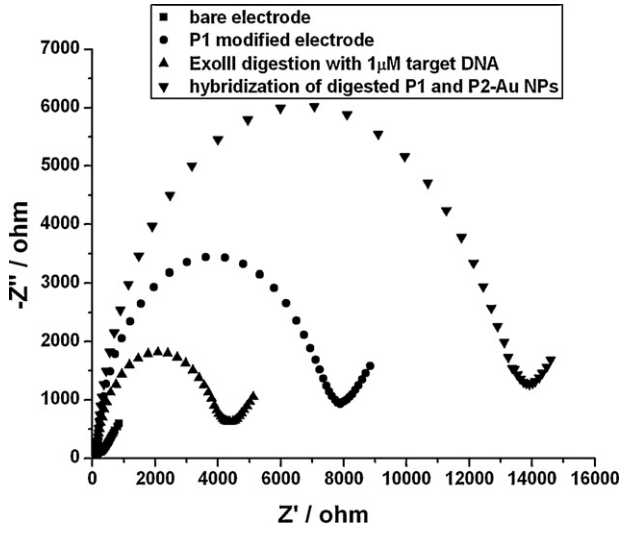

Fig. 2. Electrochemical impedance spectra (Nyquist plots) obtained at the bare gold electrode, P1 modified electrode, P1 modified electrode after the treatment with ExoIII digestion in the presence of 1 μM target DNA, and the electrode after further treatment with P2-Au NPs. The test solution: 100 mM PBS buffer solution containing 5 mM $[Fe(CN)_6]^{3-/4-}$ and 0.1 M KCl with pH 7.0. Bias potential: 0.224 V. Frequency range: 1 Hz–100 kHz. Amplitude: 5 mV.

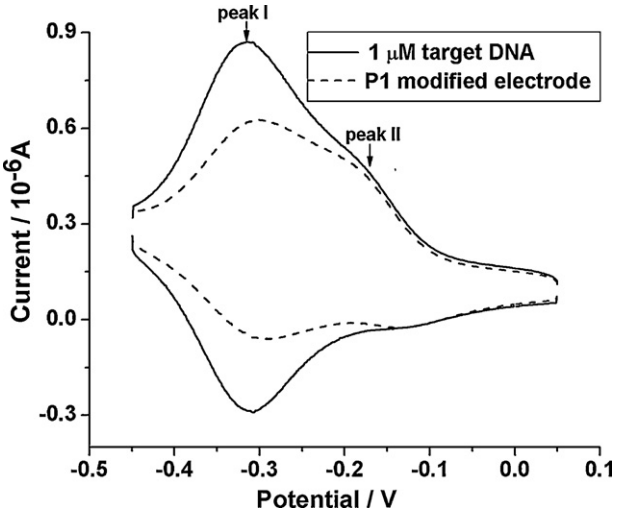

Fig. 3. Cyclic voltammograms for a 10 mM Tris–HCl buffer (pH 7.4) containing 50 μM $[Ru(NH_3)_6]^{3+}$ obtained at the P1 modified electrode and the electrode after the Exo III digestion in the presence of 1 μM target DNA. Initial potential: −0.45 V. Final potential: 0.05 V. Scan rate: 50 mV/s.

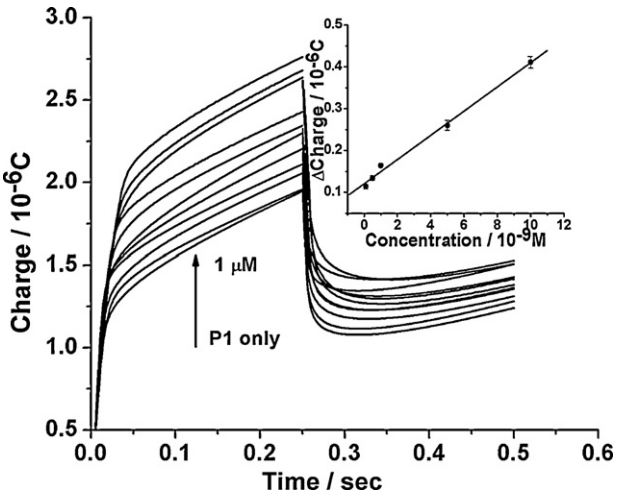

Fig. 4. Chronocoulometric curves obtained at the modified electrode in the presence of different concentrations of target DNA (from bottom to up: 0 M, 100 pM, 500 pM, 1 nM, 5 nM, 10 nM, 50 nM, 100 nM, 500 nM and 1 μM). Inset shows a linear relationship between the increase of surface charge density (ΔCharge) and target DNA concentration from 100 pM to 10 nM. Error bars represent the standard deviations of three independent measurements. Pulse period: 250 ms. Pulse width: 700 mV. Buffer: 10 mM Tris–HCl buffer (pH 7.4) containing 50 μM $[Ru(NH_3)_6]^{3+}$.

will be immobilized onto the surface of the gold electrode through the hybridization of P2 with ExoIII digested P1, thus larger amount of $[Ru(NH_3)_6]^{3+}$ molecules will be loaded on the electrode surface.

3.3. Quantitative detection of target DNA by chronocoulometry

In order to more sensitively detect target DNA, we have further employed a much more accurate electrochemical technique CC for this study (Katz et al., 2005; Miao et al., 2011b). Fig. 4 shows the relationship between the surface charge density of the gold electrode and the concentration of target DNA. Similar to CV results, with the addition of target DNA, the surface charge density of the gold electrode is increased. Furthermore, ΔCharge is found to be linear with target DNA concentration (x) from 100 pM to 10 nM. The regression equation is ΔCharge (10^{-6} C) = 0.12062 + 0.02891x (10^{-9} M), R = 0.997 (Fig. 4, inset). The limit of detection (LOD) for the target DNA detection is estimated to be 33 pM (3 times signal-to-noise ratio), much lower than that in the previous exonuclease-based electrochemical detection (Hsieh et al., 2010) and comparable to that by fluorescent technique (Cui et al., 2010) and colorimetric method (Cui et al., 2011). A series of three repetitive measurements with different concentrations of target DNA may all have a relative standard deviation within 10%, so the reproducibility of the detection can be also satisfactory.

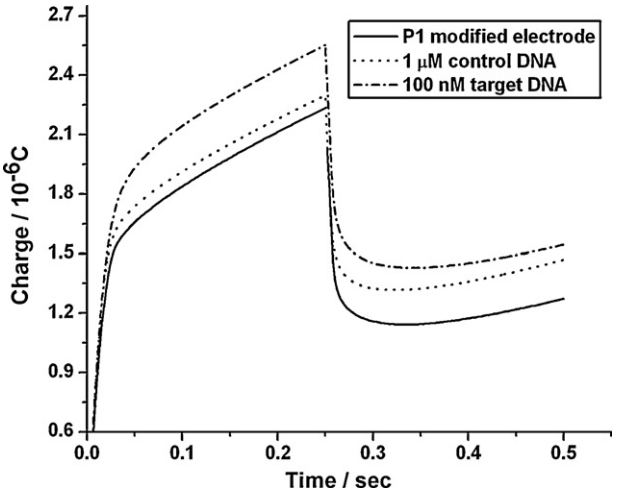

Fig. 5. Chronocoulometric curves obtained at the modified electrode in the presence of 1 μM control DNA and 100 nM target DNA. Others same as in Fig. 4.

3.4. Studies of the detection specificity

We have also conducted a control experiment to demonstrate the selectivity of our assay. Fig. 5 shows that nearly no change of the surface charge density can be observed even with 1 μM control DNA, while the surface charge density has increased significantly with only 100 nM target DNA. So, the control DNA that is not complementary to P1 is not able to initiate the signal amplification reactions, confirming the high specificity of our detection.

4. Conclusions

In conclusion, based on ExoIII and Au NPs-assisted dual signal amplification, we have proposed a very sensitive and simple, convenient, easily-operated electrochemical approach for DNA detection with improved sensitivity and high specificity. The recyclable use of target DNA promotes the efficiency of the digestion of P1 on the electrode surface, and the employment of DNA functionalized Au NPs can further enlarge the obtained electrochemical responses. Moreover, the enzymatic digestion reaction in our strategy is carried out at a constant temperature, avoiding the complex temperature cycling protocol like PCR. In addition, since ExoIII do not require specific recognition sequences, the DNA detection method proposed in this work may offer much better flexibility for choosing a target sequence and may have a wider application range in clinical diagnostics, mutation detection, and biodefense applications in the future.

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