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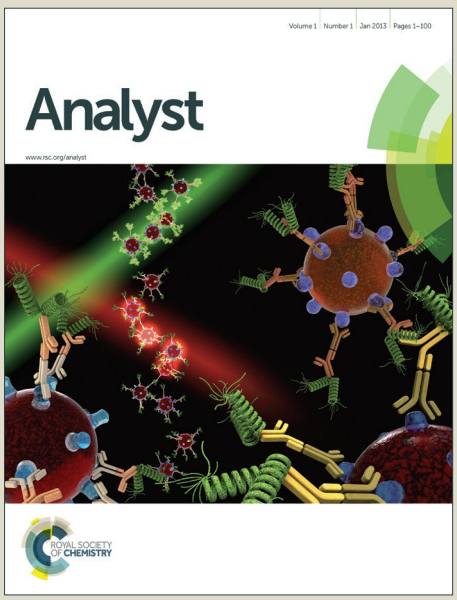

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Journal Name

ARTICLE

Electrochemical detection of PCR amplicons of Escherichia coli genome based on DNA nanostructural probes and polyHRP enzyme

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Abstract: Fast, portable and sensitive analysis of E. coli is becoming an important challenge in many critical fields (e.g., food safty, environmental monitoring and clinical diagnosis). Thus, electrochemical biosensing of PCR amplicons from bacterial genome is attracting reasonable research attention. In this work, we utilized a 3D DNA tetrahedral probe to establish a "sandwich-type" electrochemical DNA biosensor for sensitive and specific analysis of a 250 bp unpurified PCR amplicon from uidA gene of E.coli genome. Asymmetric PCR was used to produce single-strand PCR product. Streptavidin-polyHRP80 was employed to improve the signal gain during electrochemical detection. We optimized important experiment conditions for DNA sensing, including the streptavidin-polyHRP, signal probe and the ion strength. Finally, we achieved a remarkable sensitivity of 10 fM synthetic DNA target, and successfully performed the analysis of PCR amplicons from as low as 0.2 pg/μL of E.coli genome. Compared with traditional single stranded DNA (ssDNA) proe based detection, our present work demonstrated 3-order of magnitude improvement in sensitivity. In addition, our electrochemical DNA biosensor was 4 orders of magnitude more sensitive than normal electrophoretic analysis of PCR products. Our work made an important progress in DNA nanostructured probe-based biosensors toward application in real applications.

Introduction

The detection of pathogenic microorganisms[1-3] is becoming a more and more critical health issue in food safety[4], environmental monitoring[5] and clinical diagnosis[6, 7]. In recent years, many disease outbreaks happened worldwide, due to foodborne or waterborne bacterial pathogens such as *Escherichia coli* (*E.coli*)[8, 9] O157:H7, *Staphylococcus,* etc., causing death and injury of so many people. The World Health Organization (WHO) estimated that 1.5 million children die from diarrheal diseases every year, 88% of which were caused by unsafe water contaminated with *E. coli*[10]. Thus *E. coli* is becoming so representative that it is taken as an indicator for microorganism contamination in many fields, although some of its serotypes are nonpathogenic.

For *E. coli* screening, routine methods based on bacterial culturing need about 18 hours, while genetic analysis including PCR and other molecular biological methods show obvious advantage in analysis speed and specificity for the detection of pathogenic microorganism genome DNA, most of which can be finished in about 1~2 hours. Based on the fast development of quantitative real-time PCR, highly sensitive analysis of genetic DNA was realized using fluorescence probes. However, the fluorescence detection instrument is usually not competent for miniaturization and field analysis. Aiming at more portable and low economy/labor cost methods, several scientists researched the electrochemical detection of the PCR amplicons[11-14], because electrochemical biosensor[15] is recognized to possess great potential in multi-channel analysis[16, 17] based on minimized chips[18-20]. For example, Rebecca Y. Lai et al. [13] established an electrochemical sensor for the detection of unpurified PCR amplicons of gyrB gene of *Salmonella typhimurium*. Thibaut et al.[21] reported a real-time monitoring of the PCR product of bacterial genome by analyzing the electrochemical redox catalysis of $Os(bpy)_3^{3+}$. Eiichi Tamiya's group[22] combined a continuous-flow polydimethylsiloxane (PDMS) microfluidic PCR chip with disposable electrical printed chips for sensing of influenza virus.

Unfortunately, the development of electrochemical analysis method for PCR amplicons still remains a challenge. On one hand, several reported works were based on "signal off" strategies[13], which hindered the improvement of sensitivity, on the other hand, DNA recognition and hybridization was limited by the accessibility of long target DNA to probes on the heterogeneous electrode surface due to the reduced mass transport and the surface crowding effects,

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especially when facing the very complex matrix of PCR amplicons. Our previous work[23-26] proved that a three-dimensional (3D) DNA tetrahedral nanostructure[27-30] can improve the ability for biomolecular sensing[31, 32], through the improved accessibility of the probes and the better density control of the DNA probes on the surface[33].

In this work, we utilized the 3D DNA probes to establish a "sandwich-type"[34] electrochemical DNA biosensor for electrochemical analysis of a 250 bp unpurified PCR amplicons of uidA gene of *E.coli* genome. Asymmetric PCR[35] was used to produce single-strand PCR product. In order to improve the signal gain during electrochemical detection, we employed the streptavidin-polyHRP80 which consists of up to 400 horse radish peroxidase (HRP) molecules per conjugate. By optimizing the critical conditions for probe assembling and target hybridization, we achieved an increased signal-to-noise (S/N), and successfully detected PCR amplicons from as low as 0.2 pg/μL genome template.

Experimental section

Materials

All oligonucleotides and PCR primers were synthesized and purified by Invitrogen Inc., and the DNA sequences are shown in Table 1. Horseradish peroxidase-conjugated streptavidin (streptavidin-HRP, SA-HRP) was from eBioscience. Polymerized streptavidin-HRP conjugates including poly-HRP20 (SA-HRP20) and poly-HRP80 (SA-HRP80) and their diluent buffer were obtained from Fitzgerald Industries International (Acton, MA). Bacteria genomic DNA isolation kit was obtained from Tiangen Biotech (Beijing) Co., LTD. E. coli Dh5α, dNTP mixture, Ex Taq DNA polymerase and 10×Ex Taq Buffer were from Takara. Tris (2-carboxyethyl) phosphine hydrochloride (TCEP) was purchased from Sigma. The TMB substrate (TMB= 3, 3', 5, 5' tetramethylbenzidine) was purchased from Neogen in the format of a ready-to-use reagent (K-blue low activity substrate, H_2O_2 included). All other chemicals were of analytical grade and all chemicals were used without further purification. All solutions were prepared with Milli-Q water (18 MΩ·cm resistivity).

Construction of the DNA tetrahedral nanostructure-based biosensing surface

Gold electrodes (2 mm in diameter, CH Instruments Inc.) were firstly polished on microcloth with micropolish deagglomerated alumina suspensions (0.05 μm in diameter). Residual alumina powder was removed by sonicating electrodes sequentially in ethanol and Milli-Q water for 3 min. Then the electrodes were electrochemically cleaned in a freshly prepared 0.5 M H_2SO_4 solution. Finally, the electrodes were washed thoroughly with Milli-Q water and dried lightly with N_2 before immobilization.

Tetrahedral nanostructure-based capture probes (TSP) for DNA detection were formed as follows: four strands (tetra-A, B, C, D in table 1) were dissolved in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) yielding a final concentration of 50 μM. 1 μL of each strand was mixed with 5 μL of TCEP (30 mM) and 41 μL of TM buffer (20 mM Tris, 50 mM $MgCl_2$, pH 8.0), and the resulted mixture was firstly heated to 95 °C for 2 min, then cooled to 4 °C over 30 s using a peltier thermal cycler (PTC-200, MJ. Research Inc., SA). After that, 3μL TSP probes (1 μM) were pipetted onto the cleaned electrode surface and incubated overnight at room temperature. For bacteria genomic DNA detection, we just conveniently changed the recognition part of the capture probe (*E.coli* -A), while all the other conditions for the construction of the DNA tetrahedral nanostructure were the same.

Table 1. DNA sequences information

Name	Sequence (5'-3')
Tetra-A	GTATCCAGTGGCTCATTTTTTTTTTACATT CCTAAGTCTGA AACATTACAGCTTGCTACACGAGAAGAG CCGCCATAGTA
Tetra-B	SH-C_6- TATCACCAGGCAGTTGACAGTGTAGCAA GCTGTA ATAGATGCGAGGGTCCAATAC
Tetra-C	SH-C_6- TCAACTGCCTGGTGATAAAACGACACTA CGTGGG AATCTACTATGGCGGCTCTTC
Tetra-D	SH-C_6- TTCAGACTTAGGAATGTGCTTCCCACGTA GTGTC GTTTGTATTGGACCCTCGCAT
signal probe	BIOTIN-GCATGCTAGTAATGCTCTTG
Target	TGAGCC**A**CTGGATACTTTTTCAAGAGCAT TACTAGCATGC
Mis-T	TGAGCCA**T**TGGATACTTTTTCAAGAGCAT TACTAGCATGC
Mis-A	TGAGCCA**A**TGGATACTTTTTCAAGAGCAT TACTAGCATGC
Mis-G	TGAGCCA**G**TGGATACTTTTTCAAGAGCA TTACTAGCATGC
Mis-2	TGAGCC**GG**TGGATACTTTTTCAAGAGCA TTACTAGCATGC
E.coli -A	ACATTCCTAAGTCTGAAACATTACAGCTT GCTACACGAGA AGAGCCGCCATAGTATTTTTTTCCCACCA ACGCTG
E.coli signal probe	ATCAATTCCACAGTTTTCGC-BIOTIN
E.coli target	GCGAAAACTGTGGAATTGATCAGCGTTG GTGGGAA
E.coli PCR primer1	GCGAAAACTGTGGAATTGAT
E.coli PCR primer2	TGATGCTCCATCACTTCCTG
250 bp PCR product	GCGAA AACTG TGGAA TTGAT CAGCG TTGGT GGGAA AGCGC GTTAC AAGAA AGCCG GGCAA TTGCT GTGCC AGGCA GTTTT AACGA TCAGT TCGCC GATGC AGATA TTCGT AATTA TGCGG GCAAC GTCTG GTATC AGCGC GAAGT CTTTA TACCG AAAGG TTGGG CAGGC CAGCG TATCG TGCTG CGTTT CGATG CGGTC ACTCA TTACG GCAAA GTGTG GGTCA ATAAT CAGGA AGTGA TGGAG CATCA

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Bacteria culture and PCR amplification

E.coli Dh5α was cultured overnight, and its genomic DNA was extracted using a bacteria genomic DNA isolation kit (Tiangen, Beijing). The asymmetric PCR amplification of *E.coli* genomic DNA was performed on a thermal cycler (S1000, Bio-Rad). A pair of asymmetric primers (primer1/primer2=10:1) was employed to generate ssDNA strands as many as possible. The amplification protocol is as follows: 2 min at 95 °C followed by 50 cycles of 95 °C for 30 s, 61 °C for 30 s, and 72 °C for 30 s. The reaction system was further incubated at 72 °C for 10 min to extend any incomplete products. The PCR products were diluted with hybridization buffer for certain concentrations.

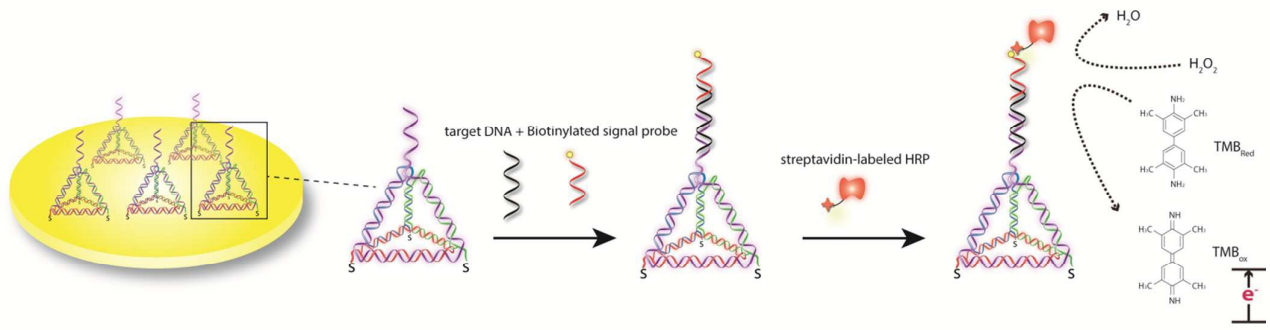

Fig. 1 Scheme for DNA tetrahedral nanostructure-based "sandwich-type" biosensing strategy for electrochemical DNA analysis.

Electrochemical Measurements

The synthetic DNA and the PCR amplicons were respectively analyzed with a "sandwich-type" biosensing strategy. Firstly, the target DNA was mixed with the biotinylated reporter probe (P1, 500 nM) in 10 mM phosphate buffer (PB) containing 1 M NaCl and 20 mM $MgCl_2$ (pH 7.4), then heated to 80 °C for 5 min, and then cooled down to room temperature for more than 20 min. Secondly TSP probe modified gold electrode was immersed into 100 μL of the above mixture in a microtube at 37 °C for 2 hr, to perform the hybridization between the TSP probe and the target DNA. Thirdly, the gold electrode was rinsed with 0.01 M PBS (10 mM PB containing 0.01 M NaCl) buffer and dried lightly with N_2, and then incubated with 3 μL of HRP (SA-HRP, SA-HRP20 or SA-HRP80) (10 ng/μL) for 15 min at room temperature. Lastly, the sensors were extensively rinsed with 0.01 M PBS and subjected to electrochemical measurements.

Cyclic voltammetry (CV) and amperometric detection were performed with a CHI 630 electrochemical workstation (CH Instruments Inc., Austin) and a three-electrode configuration was employed all through the experiment, which involved a gold working electrode, a platinum wire auxiliary electrode and an Ag/AgCl (3 M KCl) reference electrode. Cyclic voltammetry (CV) was carried out at a scan rate of 100 mV/s. Amperometric detection was performed at 100 mV (versus Ag/AgCl reference) and the electrocatalytic reduction current was measured at 100 s after the HRP redox reaction reached steady state.

Electrochemical impedance spectroscopy (EIS) measurements were performed using a CHI 660b electrochemical workstation (CH Instruments Inc., Austin) and a three-electrode system with an Ag/AgCl reference electrode, a Pt counter electrode, and a gold working electrode. The EIS analysis solution was 10 mM PBS (pH 7.4) containing 5 mM $Fe(CN)_6^{3-/4-}$ and 0.1 M KCl.

Results and Discussions

Detection strategy of TSP-based electrochemical DNA sensor

In this study, we constructed a "sandwich-type" strategy on a novel DNA nanostructure-based surface. A synthetic target DNA was firstly analyzed to construct and optimize our biosensor, and then a 250 bp PCR amplicon from the uidA gene of *E.coli* genome was finally analyzed. As shown in Fig. 1, firstly the DNA tetrahedron nanostructure-based capture probes were firmly immobilized on gold surface by three thiol groups at three vertices, and the pendant capture probe on the top vertex was designed to hybridize with the target DNA. Our previous studies demonstrated that the rigid DNA tetrahedron nanostructures could hold the capture probe standing upright and thus reduce the surface steric hindrance effect. Then, with the presence of the target DNA, the DNA tetrahedron nanostructure-based capture probe and biotinylated signal probe formed a stable sandwich structure together, and then the biotin tag of signal probe could specifically capture a streptavidin-labeled HRP, catalyzing the reduction of hydrogen peroxide to generate a quantitative electrochemical current signal in TMB co-substrate. Importantly, a streptavidin-labeled polyHRP conjugate (SA-HRP80) was applied in place of normal SA-HRP, to further increase the electrochemical catalysis signal.

Electrochemical behavior of TSP-based DNA sensor

Cyclic voltammetry (CV) and chronoamperometry were applied to study the electrochemical behavior of TSP-based genomic DNA sensor. We firstly employed the sensor to detect a synthetic target DNA. As expected, clear electron communication between TMB and the underlying gold electrode was obtained on the DNA tetrahedron nanostructure. Either with or without the presence of the target, we all observed two pairs of well-defined redox peaks in the CV results on the TSP modified Au electrode, which were assigned to the two-electron reduction and oxidation reactions of TMB. After hybridization with the target DNA, the typical HRP-based electrocatalytic process occurs at about 250 mV (Fig. 2A). By employing the amperometry method, we observed a more visualized

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HRP-catalysed electrochemical process due to the current (I) versus time (t) curve, and the catalytic current increased with the increase of target DNA concentration (Fig. 2B). Electrochemical impedance spectroscopy (EIS) was performed to characterize the establishing process of the TSP-based sensor (Fig. S1) by using Fe $(CN)_6$ $^{3-/4-}$ redox couples.

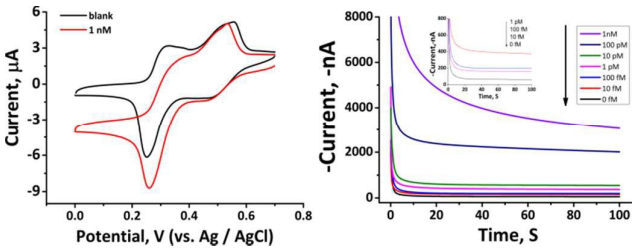

Fig. 2 (A) CV results of our biosensor in the absence (black line) and presence (red line) of 1 nM synthetic target DNA. Scan rate: 100 mV/s. (B) Amperometric curves (I-t curves) of a series of synthetic target DNA concentrations. From top to bottom: 1 nM, 100 pM, 10 pM, 1 pM, 100 fM, 10 fM, 0 fM. Insert shows the I-t curves of low concentration range target DNA between 10 fM to 1 pM. The potential for I-t analysis was 100 mV.

Comparison of different HRP

Normal SA-HRP conjugate contains only one HRP molecule, while SA-HRP20 contains up to 100 HRP molecules per conjugate and SA-HRP80 contains up to 400 HRP molecules per conjugate with much higher catalytic activity, however, the applifiation of poly-HRP for electrochemical biosensing is still a main challenge. Firstly, larger molecules may bring higher molecule crowding, which will affect the avidin-biotin combination and HRP acitivity, secondly, unspecific adsorption of polyHRP may cause even higher background signal. Thus, the probe assembling definitely need to be improved for better surface density regulation and to resist the possible HRP unspecific adsorption. In this work, by using the 3D DNA probe, we developed a polyHRP-based biosensor and successfully realized absolutely higher S/N.

We compared different streptavidin-HRP conjugates (SA-HRP, SA-HRP20 and SA-HRP80) in analyzing unpurified PCR amplicons of genomic DNA against the complex biological matrix.SA-HRP80 showed much higher current signal than SA-HRR and SA-HRP20, as it brought more HRP molecules to each signal probe. What is more important, the background current of poly-HRP80 almost remained as low as that of single SA-HRP, which clearly demonstrated the strong resistance of non-specific HRP enzyme adsorption of our TSP covered electrode surface. Thus the S/N of SA-HRP80 was obviously higher (Fig. 3B). As Fig. 3 showed, the S/N of 1 nM target DNA was as high as 37.7 by using poly-HRP80, which was almost 4 times higher than of SA-HRP20.

Optimization of the ion strength in the hybridization buffer

Ion strength in the buffer concentration was believed to influence the hybridization, and divalent magnesium is found to be more efficient than monovalent sodium[36]. On one side, proper concentration of ion strength could improve the reaction kinetics between DNA strands through cation shielding, on the other side, Mg^{2+} is also reported to specifically interact with the oligonucleotide bases[37], which may result in non-complementary target hybridization or formation of secondary structure. It is widely recognized that the Mg^{2+} concentration needed for DNA hybridization is much higher in self-assembled monolayer (SAM) than in solution, to compensate for the negative charge between the high density DNA probes on the surface. Our 3D DNA tetrahedral nanostructure raised up the capture probe and provided a sub-aqueous phase environment, which decreased the Mg^{2+} needed for the cation shielding. In our work, the concentration of Mg^{2+} in hybridization buffer was optimized in the range between 1 mM to 1 M, in 10 mM phosphate buffer (PB) containing 1 M NaCl, while all the other conditions were fixed. The results are summarized in Fig. 4: When 20 mM Mg^{2+} was added into the system, the DNA hybridization was obviously improved, generating a 5 times enhance of the current signal, however, when we continuously increased the concentration of Mg^{2+}. Thus, in our work, 20 mM Mg^{2+} was chosen as an optimized condition for the hybridization.

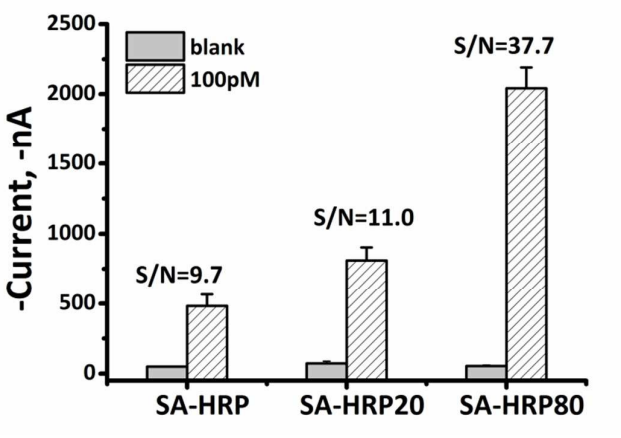

Fig. 3 Analysis results of 100 pM target DNA using different streptavidin-labelled HRP. The Error bars represent standard deviations for measurements taken from at least three independent experiments.

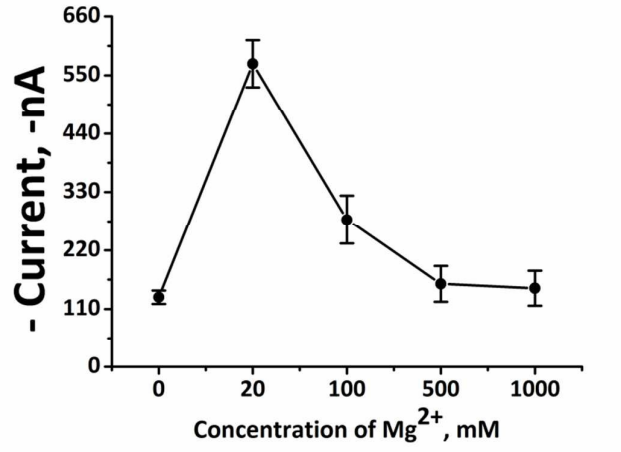

Fig. 4 Analysis results of TSP-based DNA sensor using different concentration of Mg^{2+} in the hybridization buffer. The

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concentration of target DNA was 10 pM. Error bars represent standard deviations for measurements taken from at least three independent experiments.

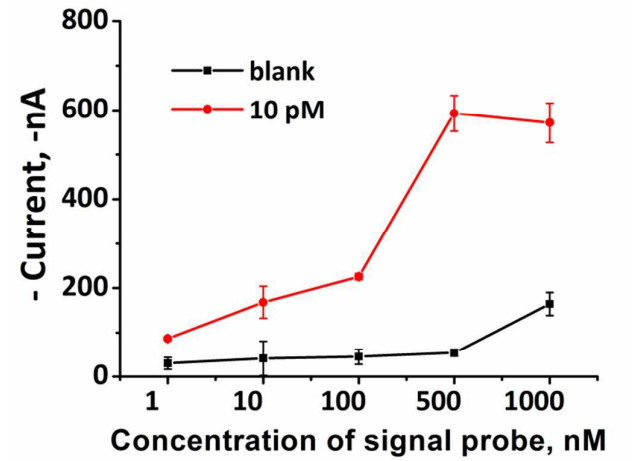

Fig. 5 Investigation of the signal probe concentration for TSP-based electrochemical DNA biosensor. The concentration of target was 10 pM. Error bars represent standard deviations for measurements taken from at least three independent experiments.

Investigation of signal probe concentration

The electrochemical signal of "sandwich-type" DNA sensor was greatly influenced by the concentration of signal probe. Apparently, an insufficient supply of the signalling probe would limit the signal, while excessive addition of the signal probe would increase the risk of unspecific adsorption. In our experiment, we take 10 pM synthetic DNA as the target and studied the effect of signal probe in the range of 1 nM to 1000 nM. The result was shown in Fig. 5: The current signal of DNA hybridization increased with the increase of signal probe, and reached a plateau at 500 nM. Interestingly, the background signal kept stably low, indicating that only negligible unspecific adsorption occurred on our TSP covered electrode surface. However, when the signal probe concentration exceeded 500 nM, a relatively higher background current was observed, which may due to the large amount of signal probe adsorped onto the electrode surface by nonspecific hybridization resulting in an increased background. Thus, a signal probe concentration of 500 nM was proved to be a critical upper limit for the background control on TSP-based DNA biosensors, which was, to the best of our knowledge, high enough for most of the "sandwich-type" biosensor research[38, 39] As a result, 500 nM signal probe concentration was chosen for the highest S/N for DNA hybridization analysis.

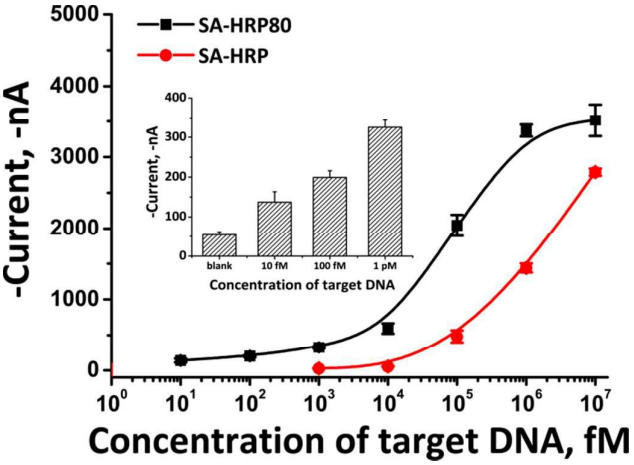

Fig. 6 Logarithmic plot of amperometric current versus target DNA concentration for TSP-based DNA sensor (black line: SA-HRP80, red line: SA-HRP). The inserted histogram is the analysis result of low concentration DNA and the blank. Error bars represent standard

Sensitivity of TSP-based DNA sensor

Based on the detailed optimization and investigation above, we finally settled the optimized conditions, and achieved ultra-high sensitivity of DNA analysis. As shown in Fig. 6, with the application of SA-HRP80, the signal for 10 fM target DNA (-137 nA) could be easily distinguished from the background (-54.16 nA) (Insert in Fig. 6), and a broad dynamic range (7 orders of magnitude) was realized. As a comparison, when we used the SA-HRP to replace the SA-HRP80, the detection limit was only 1 pM (Red line in Fig. 6), which was ascribed to the much lower catalysis activity of SA-HRP than SA-HRP80. Significantly, the TSP probe modified electrodes kept in the 4 °C refrigerator could remain their analysis capability for at least one week with a stable S/N value (Fig.S1).

Selectivity of TSP-based DNA sensor

The selectivity of TSP-based DNA sensor was challenged through single nucleotide polymorphisms (SNPs) analysis. DNA sequences with 1~2 mismatched bases were analyzed by our biosensor, and their sequence information were listed in Table 1. As shown in Fig.7, the fully matched target generated obviously higher signal than other mismatched DNA targets, which improved the high selectivity of the DNA nanostructured probes at the Au surface.

Analysis of PCR amplicons of E.coli genomic DNA

Finally, our TSP-based DNA biosensor was applied to analyze the PCR amplicons of bacterial genomic DNA to test the real applicability of TSP-based DNA sensor. We selected a 250 bp sequence of uidA gene in *E. coli* genome as the target. The capture probe on the top vertex of the TSP (*E.coli* A) and the biotinylated signal probe (*E.coli* signal probe) were redesigned to hybridize with a 35 bp sequence (*E.coli* target) on the 250 bp PCR amplicons. By using an asymmetric PCR amplification, ssDNA targets of 250 bp was obtained which was directly analyzed by the TSP-based genomic DNA sensor without any denaturing step.

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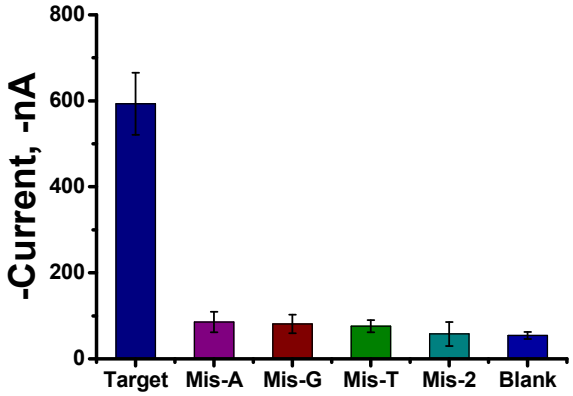

Fig. 7 The single-base mismatch discrimination ability of the TSP-based DNA sensor. All target concentrations are 10 pM.

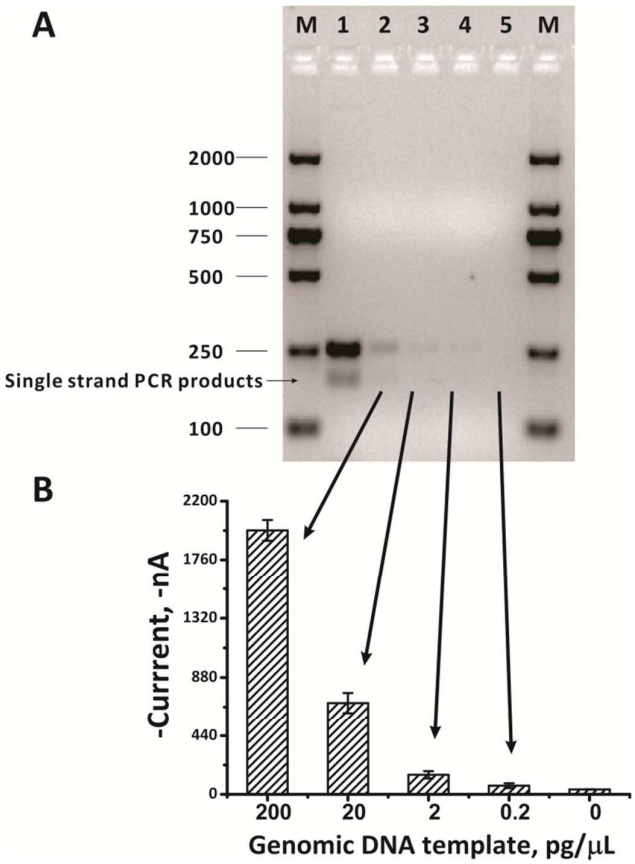

Fig. 8 A) Electrophoretic analysis of the asymmetric PCR amplicons from uidA gene of *E.coli* with starting template concentrations of: 1: 2 ng/μL, 2: 200 pg/μL, 3: 20 pg/μL, 4: 2 pg/μL, 5: 0.2 pg/μL. M is the marker. The final volume of PCR solution is 100 μL. B) Concentration profile for the detection of *E. coli* genomic DNA by using the TSP-based genomic DNA sensor. The DNA template was the genomic DNA isolated from *E. coli* and serially diluted to the required amounts. The long arrows indicated the correspondence of the samples for electrophoretic analysis and electrochemical analysis.

However, challenges still remained for our biosensor toward sensitive detection of PCR amplicons: firstly, the asymmetric PCR is usually much less efficient in amplification than normal PCR, because the depletion of one primer will lead to a linear, nonexponential amplification, and moreover, the produced amount of ssDNA in the whole PCR amplicons is usually limited. Secondly, the PCR amplified DNA is 250 bp which is much longer than the model system as described above. Interestingly, our sensor showed excellent performance by coupling asymmetric PCR. As Fig. 8B showed, this sensor could selectively detect PCR amplicons starting from as few as 0.2 pg/μL *E. coli* genomic DNA in a 100 μL unpurified PCR solution. This electrochemical DNA biosensor was at least 4 orders of magnitude more sensitive than normal electrophoretic analysis of PCR products (Fig. 8A), which clearly demonstrated the promising application of our sensor for the detection of bacterial genome.

Conclusions

We here report the construction of a novel TPS-based electrochemical DNA biosensor, towards sensitive detection of PCR amplicons from *E.coli* genome. By optimizing the most critical conditions, TSP probes on the electrode surface showed significant performance in reducing unspecific HRP adsorption and facilitating DNA hybridization. On the other side, streptavidin-polyHRP was utilized to induce higher HRP activity and thus generate an amplified current signal. Finally, we achieved a remarkable sensitivity of 10 fM synthetic DNA target, and successfully performed the analysis of PCR amplicons from as low as 0.2 pg/μL of *E.coli* genome. Our work in bacterial genome analysis is a representative progress of TSP-probe based electrochemical biosensor, which shows a great potential in development of fast and portable PCR-electrochemical chips instead of conventional fluorescent DNA detection methods.

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