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ORIGINAL PAPER

Voltammetric determination of DNA based on regulation of DNA strand displacement using an allosteric DNA toehold

Shengqiang Li[1] · Xu Liu[1] · Shuchao Pang[1] · Ruojun Lu[1] · Yonghua Liu[1] · MeiHong Fan[1] · Zhijie Jia[1] · Hongxue Bai[1]

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Abstract

An electrochemical biosensor for determination of DNA is described that is based on the reaction of regulated DNA (reg-DNA) first with substrated DNA (subs-DNA) to form a reaction intermediate. The intermediate binds target DNA (T) by hybridization and initiates a branch migration leading to the production of complex of substrated DNA and target DNA (TC). Once TC is produced, it reacts with assisted DNA (ass-DNA) through a toehold exchange mechanism, yielding the product complex of substrated DNA and assisted DNA (CS). The target is then released back into the solution and and catalyzes the next cycle of toehold-exchange with the reaction intermediate of substrated DNA and regulated DNA (CPR). Unlike in a conventional DNA toehold that is hardwired with the branch migration domain, the allosteric DNA toehold is designed into a reg-DNA which is independent of the branch migration domain. Under the optimal experimental conditions and at a working potential as low as 0.18 V, response to DNA is linear in the 1 fM to 1000 pM concentration range, and the detection limit is 0.83 fM. The assay is highly specific and can discriminate target DNA even from a single-base mismatch. It was applied to the analysis of DNA spiked plasma samples.

Keywords Cycle amplification · Serum analysis · Gold electrode · Electrochemical sensor · Streptavidin-alkaline phosphatase

Introduction

The special and quantitative detection of sequence-specific DNA in body fluids or tissues plays essential roles in pathogen detection, gene therapy, and clinical diagnostics [1–4]. Chemiluminescent [5], fluorescent [6, 7], colorimetric [8], surface plasmon resonance [9] photoelectrochemical [10] and electrochemical methods [11–15] have been used for the detection of DNA. Among these techniques, electrochemical biosensor has received particular attention owing to its small-sized commercial detector, easy operation, rapid response, low cost, high sensitivity and selectivity [16–18]. However, the sensitivity of traditional electrochemical assay is not satisfied for direct detection. Thus, it is very urgent to develop ultrasensitive methods for detection of low abundant sequence-specific DNA.

A variety of amplification strategies have been established for sensitive detection of DNA, because they can overcome the inherent limitation of target-to-signal ratio of 1: 1 in the traditional assay and acquire higher sensitivity [19, 20]. Such as polymerase chain reaction (PCR) [21], loop-mediated isothermal amplification [22], exonucleaseIII aided signal amplification [23], catalytic hairpin assembly [24], rolling circle amplification [25–27] and strand displacement amplification [28]. Among them, strand displacement amplification has received particular interest due to its excellent property of signal amplification [29]. Unlike the enzyme amplification method which has been reported by cheng et al. [4], the electrochemical biosensor for detection of DNA base on strand displacement amplification is more convenience and operational. Although strand displacement amplification has a lot of advantages, the rates of strand-exchange reactions between stable DNA duplexes and identical invading strands are very slow. Thus, extensive effort is still an urgent demand to improve the sensitivity of strand displacement amplification.

Since the concept of toehold-mediated DNA strand displacement was introduced by Yurke et al. in their work in 2000, how to control activation of a toehold has attracted more

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✉ Shengqiang Li
yixuelishengqiang@126.com

[1] First Teaching Hospital of Tianjin University of Traditional Chinese Medicine, Tianjin 300000, People's Republic of China

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and more attention [30]. The traditional method is typically achieved by sequestering a toehold into an inter- or intramolecular DNA duplex, which can then be activated by external stimuli and toehold-exchange reactions [31]. For example, Chen described an associative DNA toehold that attached a DNA toehold to a branch migration domain whenever needed through hybridization, expanding the rule set to control DNA circuits [32]. However, as the branch migration domain and the toehold are "hardwired" into the same invading strand, it is not possible to adjust activation of a toehold only altering toehold concentration or branch migration concentration. So to simplify the design of complex strand displacement systems with fewer molecular components and to achieve the flexible control over the activation of a toehold, it is worthwhile to add new toehold regulation mechanisms to the current strand displacement toolbox. As far as we know, there is no report to detect DNA on an electrochemical platform based on regulation of DNA strand displacement, although this regulation mechanism was introduced some years ago [33].

Aiming at improving the analytical performance of toehold-mediated DNA strand displacement strategy for special DNA detection, we introduce an allosteric DNA toehold to regulate DNA strand displacement, which can effectively separate toehold from branch migration domains and significantly improve signal amplification. An ultrasensitive electrochemical DNA biosensor was established based on regulated DNA strand displacement using an allosteric DNA toehold for the first time. The inherent properties of regulated DNA strand displacement would ensure ultrasensitivity and high specificity of the designed biosensing strategy. The biosensor exhibited excellent analytical performance towards DNA detection, which might provide a promising sensing platform for bioanalysis and clinical molecular diagnostics.

Materials and methods

Materials and reagents

DNA oligonucleotides were synthesized by SangonInc., (Shanghai, China, http://www.sangon.com). Their sequences are listed in Table 1. bovine serum albumin (BSA),6-Mercapto-1-hexanol (MCH), streptavidin-alkaline phosphatase (ST-AP) and a-naphthyl phosphate (a-NP) were purchased from Sigma-Aldrich (Saint Louis, Missouri, USA, http://www.sigmaaldrich.com). DNA hybridization buffer was phosphate buffered saline (PBS,137 mM NaCl, 2.5 mM Mg^{2+}, 10 mM Na_2HPO_4, 2.0 mM KH_2PO_4, pH 7.4). DNA was stored in Tris-HCl (10 mM, pH 8.0) containing 1 mM ethylenediaminetetraacetic acid. The washing buffer was PBS (0.1 M, pH 7.4) containing 0.05% (*w/v*) Tween-20. Diethanolamine (DEA) buffer (pH 9.6) contained 0.1 M DEA, 1 M $MgCl_2$ and 100 mM KCl. All other reagents were of analytical grade, and purified using high-performance liquid Chromatography and Millipore-Q water (≥18 MΩ.cm, Milli-Q, Millipore, http://www.mollipore.com) was used in all experiments.

Apparatus

All electrochemical measurements were performed on a CHI660D electrochemical workstation (Chenhua Instru-ments Co.Ltd., Shanghai China, http://www.chinstruments.com) with a conventional three-electrode system composed of platinum wire as auxiliary, Ag/AgCl electrodeas reference, and a 3-mm diameter gold electrode (GE) as working electrode.

Fluorometry was performed on a Cary Eclipse fluorescence spectrophotometer (Agilent, California https://www.agilent.com).

DNA hybridization

Substrated DNA, regulated DNA, assisted DNA and different target DNA concentration were mixed in DNA hybridization buffer for 45 min at room temperature. Then the amplification products were stored in at 4 °C for further use.

Preparation of electrochemical biosensor

The gold electrode was polished to a mirror sequentially with 0.3 μm and 0.05 μm alumina powder, followed by ultrasonic cleaning with water, ethanol and water. Then, the electrode was soaked in the piranha solution (H_2SO_4:H_2O_2 = 3:1) for 10 min, followed by rinsing thoroughly with ultrapure water to eliminate other substances. 10 μL of 0.1 μM thiolated capture probe was dropped on the pretreated gold electrode surface and incubated overnight at 4 °C. After washed with the buffer, the electrode was immersed into 100 μL of 1 mM MCH solution for 1 h to block nonspecific binding sites at room temperature. The electrode was further rinsed with the washing buffer and treated in salmon sperm DNA and 1% BSA for 30 min to block then on-specific binding sites on its surface to obtain the electrochemical DNA biosensor.

Target DNA detection protocol

A-toehold-mediated DNA strand displacement amplification system consisted of 10 μL of substrated DNA (contain C and P) (10 nM), 25 μL of allosteric regulated DNA (25 nM), 15 μL of assisted DNA (20 nM) and 10 μL target DNA (various concentrations). After 45 min amplification reaction, 10 μL of the products was dropped on the surface of electrode to hybridize with capture probe. The electrode was then rinsed with diethanolamine buffer (DEA, 0.1 M diethanolamine,1 mM $MgCl_2$, 100 mM KCl, pH 9.6). Subsequently, 10 μL of 1.25 μg mL^{-1} ST-AP was dipped onto the electrochemical biosensor at 37 °C for 45 min, The electrochemical sensor was

Table 1 Sequences of oligonucleotides

Oligonucleotide	Sequence (5′ - 3′)
Capture probe	SH-$(CH_2)_6$-TTTTTT GA ACA GC
substrated DNA C (C)	Biotin-T GAC ACA TTC GGT GGC TGT TC
substrated DNA P (P)	GTC TCT CGA ACA GCC ACC GAA TGT GTC A
substrated DNA C (C)	FAM-T GAC ACA TTC GGT GGC TGT TC
substrated DNA P (P)	GTC TCT CGA ACA GCC ACC GAA TGT GTC A-BHQ1
regulated DNA (R_{16})	T GC TGT TCG AGA GGAC
regulated DNA (R_{15})	GGC TGT TCG AGA GAC
regulated DNA (R_{14})	GC TGT TCG AGA GAC
regulated DNA (R_{13})	C TGT TCG AGA GAC
regulated DNA (R_{12})	TGT TCG AGA GAC
assisted DNA (S)	C ACC GAA TGT GTC A
Target DNA (T)	GA ACA GCC ACC GAA T
Single-base-mismatched	CA ACA GCC ACC GAA T
Single-base-mismatched	GA ACA GCG ACC GAA T
Single-base-mismatched	GA ACA GCC ACC GAA A
Two-base-mismatched	CAACA GCC GCC GAA T
Non-complementary	CT TGT CGG TGG CTT A

washed thoroughly with DEA buffer containing 0.05% Tween-20. Finally, the electrochemical sensor surface was regenerated with 50 mM sodium hydroxide for 5 min and washed with hybridization buffer. The DPV measurement was performed in DEA buffer containing 1 mg mL^{-1} of a-NP substrate with modulation time of 0.05 s, interval time of 0.017 s and potential scan from 0.0 to +0.6 V.

Fluorometric measurements

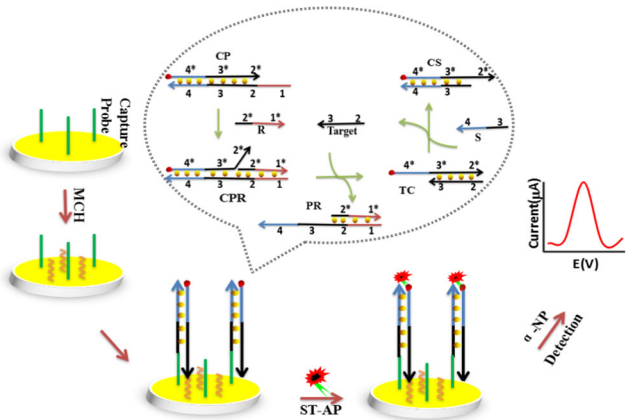
All fluorescence measurements were performed on a Cary Eclipse Fluorescence Spectrophotometer, using a quartz fluorescence cell with an optical path length of 1.0 cm. The maximum fluorescence emission intensity was obtained at 518 nm, The emission spectra were obtained from 490 to 600 nm with an excitation wavelength of 480 nm at room temperature in steps of 1 nm. Both the excitation and emission slit widths were set at 5 nm. Prior to each experiment, all the cuvettes were washed with 70% ethanol and distilled water.

In this assay, 5 μL of target DNA,10 μL of substrated DNA (contain C and P), 25 μL of allosteric regulated DNA, 15 μL of assisted DNA were added into the reaction buffer (10 mM Tris-HCl, 50 mM NaCl, 10 mM $MgCl_2$, 10 mM KCl, pH 7.6) to give a total volume of 100 μL and the final concentrations of target DNA, subs-DNA, reg-DNA, ass-DNA were1 pM, 10, 25, 20 nM respectively. The above solutions were incubated at 37 °C for 45 min before fluorescence measurements.

Results and discussion

Design of electrochemical biosensor

The biosensing process of target DNA detection is illustrated in Scheme 1. The length of regulated DNA is very important for the overall circuit. Upon increasing of the base pair number of regulated DNA, the rates of strand-exchange reactions between stable DNA duplexes and regulated DNA are increased. As the rates of reaction increases, the signal amplification is



Scheme 1 Schematic representation of DNA detection based on regulation of DNA strand displacement using an allosteric DNA toehold. (i) reg-DNA first with subs-DNA to form a reaction intermediate (CPR); (ii) target DNA and CPR hybridize to generate TC; (iii) TC reacts with assisted DNA yielding CS

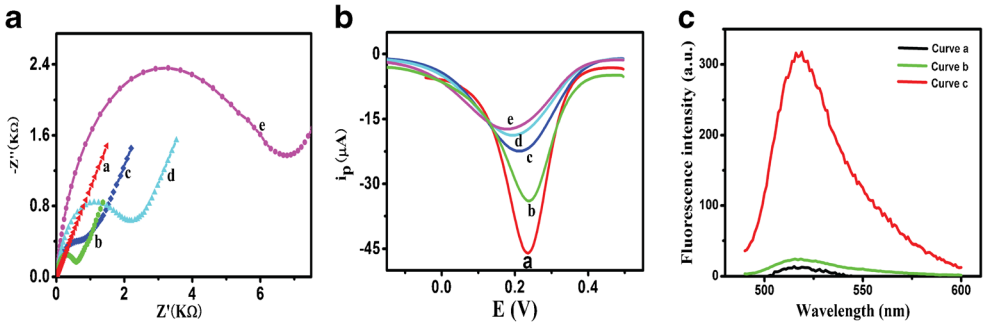


Fig. 1 EIS (**a**) and DPV (b) in 0.4 M KCl containing 0.5 mM $Fe(CN)_6^{3-/4-}$ at bare electrode (**a**), capture DNA modified electrode (**b**), capture DNA modified electrode after sealed with MCH and BSA (**c**), after complex CS (d) and ST-AP (e), respectively. fig. 1c the signal amplification performance of regulation of DNA strand displacement was evaluated. Typical fluorescence spectra of (a) blank, (b) subs-DNA + reg-DNA + ass-DNA (c) subs-DNA + reg-DNA + ass-DNA + target DNA. The final concentrations of target DNA, subs-DNA, reg-DNA, ass-DNA were 1 pM, 10, 25, 20 nM respectively

strengthened. However, the regulated DNA dissociation rate favors the shortest toehold as possible. In order to keep the balance of the signal amplification and the reaction rate, 7 nt is chosen as the toehold, and 15 nt is chosen as the length of the regulated DNA. the domain 1* of reg-DNA first with the domain 1 of subs-DNA (contain C and P chain) to form a reaction intermediate (CPR). In the presence of target DNA, the domain 2 of which hybridizes and initiates a branch migration with the domain 2* of CPR and produces complex of subs-DNA (C chain) and target DNA (TC). Once TC forms, the domain 4* of which then reacts with the domain 4 of ass-DNA through a toehold-exchange mechanism, yielding the product complex of subs-DNA (C chain) and assisted DNA (CS). Target DNA is released back into the solution and can then be used to catalyze the next cycle of toehold-exchange with CPR. The products then hybridize with the capture probe on the surface of biosensor. Finally streptavidin-alkaline phosphatase (ST-AP) is introduced to the sensing system via biotin-streptavidin binding. After the addition of the enzyme substrate a-naphthyl phosphate, alkaline phosphatase (ALP) catalyzes a-naphthyl phosphate to an electroactive product a-naphthyl. Due to the fast electron transfers between the electroactive species and the DNA modified electrode, current response is thus amplified. The catalytic current response is dependent upon the amount of ALP, which in turn reflects the amount of target DNA attached to the electrode surface.

Characterization of electrode surface modification

In order to follow the stepwise biosensor assembly, electrochemical impedance spectroscopy (EIS) were used to characterize the different stage of modified electrode in the presence of 5 mM $[Fe(CN)6]^{3-/4-}$ with 0.1 M KCl and the semicircle diameter was equal to electron-transfer resistance (Ret) (Fig. 1a). As seen in the EIS spectra (Fig. 1a). Bare electrode exhibits an almost straight line (curve a), reflecting the outstanding electrochemical conductivity. When the capture probe is self-assembled onto the bare electrode via Au-S bond, the Ret increases to 612 Ω (curve b), which is mostly due to repulsing forces between the negatively charged redox probe. When MCH and BSA are immobilized on the electrode surface, the Ret obviously increases to 926 Ω (curve c), The reason may be the MCH and BSA resist the electron transfer kinetics of the redox probe at the electrode interface, resulting in the increasing impedance of the electrode. Afterwards, the capture DNA hybridized with the complex CS, the Ret further increases to 2136 Ω (curve d), which indicated that in the presence of target DNA, a large amount of complexes are

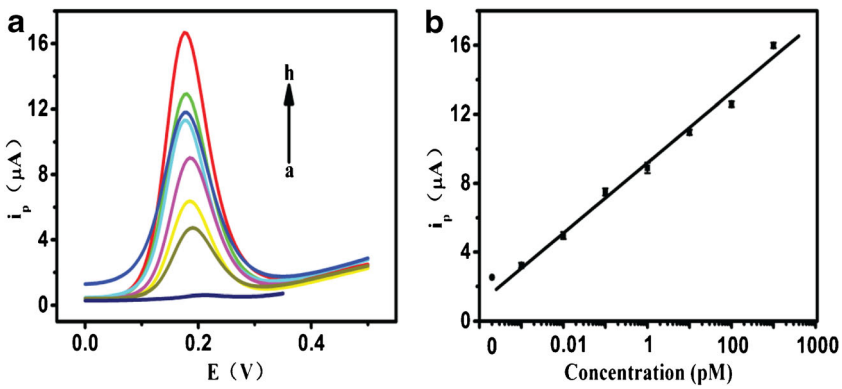


Fig. 2 (**a**) DPV responses to 0, 0.001, 0.01, 0.1, 1, 10, 100 and 1000 pM of target DNA (from a to h) using A-toehold amplification. (**b**) The calibration plot of the designed strategy for DNA detection. The error bars represent the standard deviations calculated from three different spots

Table 2 Comparison between the proposed assay and other reported methods for DNA detection

Signal amplification method	Analytical technique	Detection limit mol (mol L^{-1})	References
RCA	ECL	10^{-15}	[34]
SRP	Raman	5×10^{-11}	[35]
CSDPR/HCR	DPV	8×10^{-15}	[36]
MB-mediated CSD/RCA	DPV	9×10^{-16}	[4]
Exonuclease/CHA	DPV	9.2×10^{-15}	[37]
allosteric DNA toehold	DPV	8×10^{-16}	this work

formed and the negatively charged DNA molecules further block electron transfer. Finally, upon the streptavidin binding onto the electrochemical sensor surface, the Ret significantly increases to 6816 Ω (curve e). The above results are in a good accordance with differential pulse voltammetry (DPV) curves as shown in Fig. 1b. In addition, the signal, which is gain by the regulation of strand displacement amplification, is at least 8.4 times that of the traditional sandwich assay (Fig. S1).

To further characterize the signal amplification performance of regulation of DNA strand displacement, Fluorescence spectrophotometer measurements were performed. As shown in Fig. 1c, in the presence of target DNA, a dramatically high fluorescence signal value is obtained, which is much higher than that of background signal (curve c). The control experiments, in presence of all sensing components except target DNA, showed only small fluorescence signal value (curve b), which is almost equal to the background signal (curve a). These results shown here clearly demonstrate the remarkable signal amplification performance of the designed regulation of strand displacement using an allosteric DNA toehold.

Optimization of assay

The following parameters were optimized: (a) bare number; (b) concentration of Reg-DNA; (c) incubation time; (d) concentration of Ass-DNA. Respective data and Figures are given in the Electronic Supporting Material (Fig. S2). The following experimental conditions were found to give best results: (a) Optimal bare number: 15 nt; (b) Optimal concentration of Reg-DNA: 70 nM; (c) Optimal incubation time: 45 min; (d) Optimal concentration of Ass-DNA: 20 nM.

Analytical performance

Under the optimal experimental conditions, the DPV peak currents at 0.18 V increased with the increasing target DNA concentrations. As shown in Fig. 2, The calibration plot shows a good linear relationship between the peak currents and the logarithm of DNA from 1 fM to 1000 pM with a correlation coefficient of 0.995. The electrochemical sensitivity is 2.04 μA.pM^{-1}. The error bars represent the standard deviations in three different measurements for each concentration. According to the sum of blank response and 3 times standard deviation (3σ) ($n = 3$), the detection limit (LOD) is calculated to be 0.83 fM. Compared with the reported methods the established DNA biosensor has preferable performance, which can be seen in Table 2. Moreover, the binding molecules can be removed completely from the sensor surface without irreversible loss of its activity by sodium hydroxide. Up to 3 cycles of binding/regeneration processes on the same sensor, the signal decreased <12%. These results indicated that a well-immobilized sensor can be regenerated more than 3 times. More importantly, the whole assay time of the is only 90 min. Thus, this biosensor can be applied to quantification of DNA with a wide linear range and low detection concentration.

Specificity, reproducibility and repeatability

To evaluate the specificity of the established method, four different kinds of control DNA sequences, including the

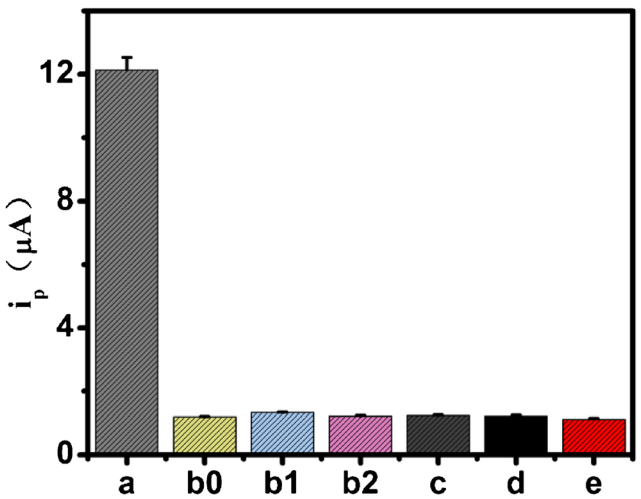


Fig. 3 DPV peak currents responding to 1 pM of target oligonucleotides (**a**), 100 pM of single-base-mismatched oligonucleotides (b0, b1, b2 at the first, middle and terminal region respectively), 100 pM of two-base-mismatched oligonucleotides (**c**), 100 pM of non-complementary oligonucleotides (**d**) and blank (**e**)

perfectly complementary sequence, three single-base mismatched sequences (sDNA), double-base mismatched sequence (ssDNA) and the non-complementary sequence (nDNA) were analyzed. As shown in Fig. 3, the current responses to 100 pM of sDNA (b0, b1, b2), ssDNA (c) and 100 pM of nDNA (d) are similar to that of the blank (e), respectively. In the presence of target DNA only 1 pM concentration results in a significant increase in the current response (a), which is about 12 times as much as that of sDNA,ssDNA and nDNA. More importantly, this method can discriminate not only one base mismatched DNA in the first region, but also in the middle or terminal region. These results guarantee the specificity of the biosensor.

The reproducibility of the biosensor was also evaluated by measuring the target DNA at 100 fM and 10 pM with five replicates and the coefficients of variation for both concentrations were about 2.4 and 5.6%, respectively. These results demonstrate that the acceptable reproducibilities are achieved.

The intraday and interday precisions of the sensor were also investigated by the variation coefficients (CVs), by making 6 successive measurements of three concentrations of DNA using an identical sensor. The CVs of the intra-assay are determined as 3.8%, 3.2%, and 2.3% at 0.1, 1, and 10 pM of DNA, respectively. Similarly, a set of 6 sensors was prepared to detect 0.1, 1, and 10 pM of DNA, and the inter-assay CVs of the measurement are 4.6%, 4.3%, and 4.8%, respectively. The results present that the sensor exhibits acceptable precision.

Real sample analysis

To demonstrate the practical applicability of the established sensor in real sample analysis, we performed the DNA assay using human serum. Human serums were got from the First Teaching Hospital of Tianjin University of Traditional Chinese Medicine. Various amounts of synthetic DNA were spiked into human serum, respectively. Then, the method was used to detect target DNA in these serum samples. The results were listed in Table 3. However this regulation of DNA strand displacement amplification system needs several kinds of DNAs with well defined base sequences, which requires skills. On the other hand, there are some problems with proposed method being used in blood sample directly. Although the biosensor has a certain defect, the recoveries are acceptable when they are determined under the same experimental conditions. Therefore, The strategy might become a potential approach for DNA assay in real biological samples.

Table 3 The recoveries determined using the method via spiking synthetic DNA into human serum samples ($n = 3$)

Sample	Spiking value	Assayed value	Recovery
No.	$C_{DNA\ (fM)}$	$C_{DNA\ (fM)}$[a] ± SD[b]	(%)
1	5	5 ± 0.05	100.0
2	50	49.2 ± 0.02	98.4
3	500	513.5 ± 0.03	102.7

[a] The data represent the average value of three independent determinations

[b] SD, standard deviation

Conclusion

In summary, an allosteric DNA toehold (A-toehold) strategy for ultrasensitive and specific detection of DNA was demonstrated. With the introduction of regulated DNA, the method can simplify DNA sequence design to achieve control of DNA strand displacement. The designed strategy also shows a broad dynamic range, ultrasensitivity, excellent specificity and good reproducibility. However, high-throughput detection cannot be achieved by this method. Although the biosensor has some defects, this electrochemical biosensor can be conveniently applied for the detection of many other nucleic acid molecules by substituting the target-specific sequences. In additional, the electrochemical sensor can be used in combination with other toehold activation mechanisms to build complex DNA devices or circuits for detection of target DNA in biomedical research.

Compliance with ethical standards The author (s) declare that they have no competing interests.

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