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# A novel ultrasensitive electrochemical DNA sensor based on double tetrahedral nanostructures

Dongdong Zeng [a,1], Huan Zhang [a,1], Dan Zhu [b,1], Jiang Li [b], Lili San [a], Zehua Wang [a], Chenguang Wang [a], Yunsheng Wang [a], Lihua Wang [b], Xiaolei Zuo [b,*], Xianqiang Mi [a,*]

[a] Laboratory of System Biology, Shanghai Advanced Research Institute, Chinese Academy of Sciences, Shanghai 201210, China
[b] Division of Physical Biology and Bioimaging Center, Shanghai Synchrotron Radiation Facility, Shanghai Institute of Applied Physics, Chinese Academy of Sciences, Shanghai 201800, China

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ABSTRACT

Electrochemical DNA (E-DNA) sensor is an important tool for detecting DNA biomarker. In this work, we have demonstrated a novel strategy of E-DNA sensor based on DNA tetrahedral nanostructures for the sensitive detection of target DNA. In our design, thiol and biotin modified DNA tetrahedral nanostructures were used as capture and report probes respectively. The biotin-tagged three dimensional DNA tetrahedral nanostructures were employed for efficient signal amplification by capturing multiple catalytic enzymes. Such improved E-DNA sensor can sensitively detect DNA target as low as 1 fM with excellent differentiation ability for even single mismatch. And a mean recovery rate of 90.57% in DNA solution extracted from human serum was obtained. We have also compared this new method of attaching catalytic enzymes with the other two typical methods: One is through biotinylated single-stranded DNA (SSDNA) and the other is through gold nanoparticles (GNPs). Results indicated that the RTSPs-based enzyme amplification system showed much better performance than the other two systems.



## 1. Introduction

As DNAs are important biomarkers for disease diagnosis, ultrasensitive DNA sensors are essential for detection of low abundance of DNA biomarkers in clinical samples (Cosnier and Mailley, 2008; Wan et al., 2013). Among different types of DNA biosensors, electrochemical DNA sensor (E-DNA sensor) is the most promising one with the ability to produce a fast response, easy to use, low cost platform for DNA sensing. The E-DNA sensor family members have demonstrated great potential in detection of DNA. (Fan et al., 2003; Hvastkovs and Buttry, 2010). The "sandwich" type strategies are used widely to develop various E-DNA sensors. A typical "sandwich" type E-DNA sensor consists of a recognition layer that is a single-stranded DNA capture probe immobilized on electrodes and a single-stranded DNA report probe that converts target recognition event into electrochemical signals. The capture probe and report probe are usually complementary to partial of the target DNA, which makes the whole system like a "sandwich".

To achieve high sensitivity of "sandwich" type E-DNA sensor, two key components need to be improved: "the recognition layer" and "the signal transduce layer". For "the recognition layer", the most commonly used capture probe for E-DNA sensor is thiolated single-stranded DNA. However, control of the density and orientation of recognition probes at the electrode surface is a challenge. Recently, a DNA tetrahedral nanostructure recognition probe has attracted great interests. The three dimensional DNA tetrahedral nanostructure was first developed in 2004 (Goodman et al., 2004) and then first used in E-DNA sensor in 2010 (Pei et al., 2010). According to previous reports, the DNA tetrahedron could be immobilized on gold electrode surface by modifying three of its vertices with thiol groups, while the fourth vertex was designed with an extended DNA anchor probe as capture which was complementary to partial of the target (Ge et al., 2014; Lin et al., 2014; Pei et al., 2010; Wen et al., 2013). Taking advantage of its rigid structure, the DNA tetrahedral nanostructure can ensure that the capture probes were with well controlled density and orientation, which as a result enhanced the E-DNA sensor's sensitivity by minimizing the surface crowding effect, increasing the target accessibility, providing solution-like environment, etc (Mitchell et al., 2009; Pei et al., 2010). For "the signal transduce layer", a large number of signaling molecules such as redox enzymes, nanoparticles have been used to amplify the signal (Mao et al., 2008; Pinijsuwan et al., 2008; Zhang et al., 2007). The Horseradish

* Corresponding authors.
*E-mail addresses:* zuoxiaolei@sinap.ac.cn (X. Zuo), mixq@sari.ac.cn (X. Mi).
[1] These authors contribute equally to this work.

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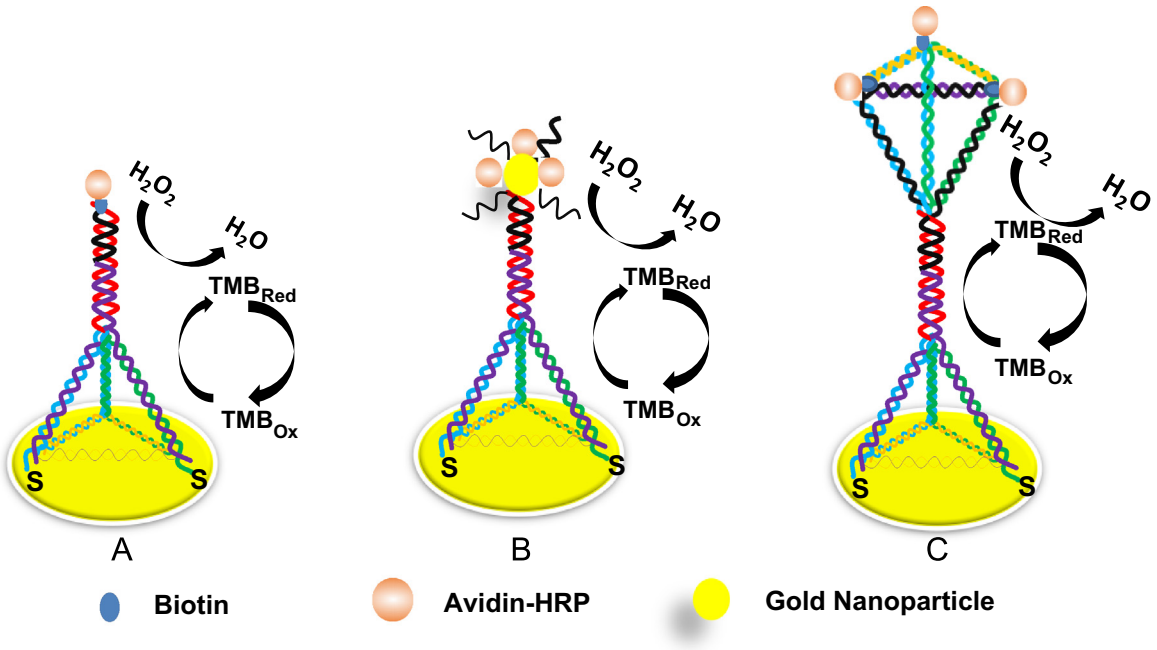


**Scheme 1.** Schematic illustration of (A) single-stranded DNA report probes (SSRPs), (B) gold nanoparticles report probes (GNPRs) and (C) report tetrahedral structured probes (RTSPs)-based electrochemical DNA sensor (E-DNA sensor). The thiolated three-dimensional DNA tetrahedral nanostructures attached on gold electrode surface were used for recognition of target DNA. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

peroxidase-conjugated avidin (avidin–HRP) enzyme is one of the most popular redox enzymes used for E-DNA sensor due to its advantages of stable, efficient, commercially available, etc. Additionally, avidin–HRP can be easily attached to biotinylated DNA strands covalently.

However, it is not easy to control the immobilization of avidin–HRP because of the uncontrolled and unordered surface, often resulting in low efficiency of enzyme reactions. Herein we employ biotin-tagged DNA tetrahedral nanostructures as report probes named report tetrahedral structured probes (RTSPs) for attaching avidin–HRP, which can amplify the enzymatic electrochemical signal and providing a novel approach to ultrahigh sensitive detection of target DNA (Scheme 1). Here, the biotin-tagged RTSPs were actually used as labels and bridges between the capture probes and Horseradish peroxidase (HRP). This strategy was to overcome the uncontrolled and unordered drawbacks of using single-stranded DNA as report probes and realize multiple signal amplification. To further evaluate the RTSPs-based E-DNA sensor, we also tried to use biotinylated single-stranded DNA report probes (SSRPs) and gold nanoparticles report probes (GNPRs) as HRP enzyme binding sites for the sandwich type E-DNA sensor (Scheme 1).

## 2. Experimental section

### 2.1. Materials

DNA Oligonucleotides were synthesized by Sangon Biological Engineering Technology & Services Co. Inc. (Shanghai, China) with their sequences listed in Table S1 (see details in supporting information). The TMB substrate (TMB=3,3,5,5-tetramethylbenzidine) was purchased from Neogen in the format of a read-to-use reagent (K-blue low activity substrate, $H_2O_2$ included). Horseradish peroxidase-conjugated avidin (avidin–HRP) was purchased from eBioscience (San Diego, CA, USA). Tris-(hydroxymethyl) aminomethane (Tris base), tris (2-carboxyethyl) phosphine hydrochloride (TCEP) and 5 nm diameter gold nanoparticles were purchased from Sigma-Aldrich (St. Louis, MO, USA). The other chemicals were of analytical grade and purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). All chemicals were used without further purification.

The buffer solutions involved in this study were as follows: The DNA tetrahedral nanostructure forming buffer was TM buffer (20 mM tris buffer and 50 mM $MgCl_2$, pH 8.0). The washing buffer for gold electrodes(W-buffer) was phosphate buffer saline (10 mM $Na_2HPO_4$, 2 mM $KH_2PO_4$, 37 mM NaCl and 2.7 mM KCl, pH7.4). The hybridization buffer was H-buffer (10 mM $Na_2HPO_4$, 10 mM $KH_2PO_4$, 1 M NaCl, 20 mM $MgCl_2$). The TCEP solution was 30 mM in water. Avidin-HRP was diluted 1000 times by 0.5% casein solution. Buffer for electrochemical measurement was TMB substrate. All solutions were prepared with Milli-Q water (18 MΩ cm resistivity) from a Millipore system.

### 2.2. Formation of "sandwich" type E-DNA sensor based on double DNA tetrahedral nanostructures

The design principle of oligonucleotides' sequences for assembling capture tetrahedral structured probes (CTSPs) was the same as previous report (Wen et al. 2013). Four strands were used to form CTSPs: three of them (tetra-b, tetra-c and tetra-d) were designed with thiol modification at their 5′-terminal. The last strand (tetra-a) had an extended probe (12 bases) complementary to part of target DNA (22 bases). The sequences of oligonucleotides for assembling report tetrahedral structured probes (RTSPs) were similar with those for CTSPs, but with two differences: (1) biotin modification of three oligonucleotides (tetra-b-biotin, tetra-c-biotin, tetra-D-biotin) at the 5′-terminal instead of thiol modification; (2) the extended probe was complementary to the other part of target DNA (10 bases).

The CTSPs were prepared as the previous reported process (Wen et al., 2013) (see details in Supporting information). The synthesis process of RTSPs was similar. Strand tetra-a-reporter, tetra-b-biotin, tetra-c-biotin, tetra-d-biotin without TCEP were used instead of tetra-a, tetra-b, tetra-c, tetra-d respectively. The gold electrodes (2 mm in diameter) were cleaned as the previously

reported protocol (Zhang et al., 2007). Then 3 μl of CTSPs solution was injected on the cleaned gold electrode and incubated for 16 h at room temperature for immobilization. Target DNA solutions with different concentrations were first mixed with 100 nM RTSPs in H-buffer, then the mixture was incubated at 4 °C for 20 min. Next, the electrodes with CTSPs were rinsed with W-buffer and dried with $N_2$, and incubated with the above target solution (100 μL) for 3 h at 4 °C for hybridization. Later, the electrodes were again rinsed with W-buffer and dried by $N_2$, 3 μL diluted avidin–HRP was added on each gold electrode surface for 15 min at 4 °C. At last, the electrode was rinsed thoroughly again with W-buffer and subjected to electrochemical measurements. The reaction condition of mismatched DNAs was the same as target DNA. Spiked DNA experiments were performed to determine the recovery rate. Total plasmid solution was extracted from human serum samples following the process of QIAamp DNA Mini Kit instruction (Qiagen). Then 1 pM target DNA was spiked in the plasmid solution. The reaction condition was the same for the control experiments of SSRPs-based E-DNA sensor and the processes were similar while SSRPs were used instead of RTSPs.

## 3. Results and discussions

### 3.1. Design strategy of the E-DNA sensor based on double DNA tetrahedral nanostructures

In our newly design, DNA tetrahedral nanostructures were employed not only as capturing probes but also as reporter probes, as Scheme 1 shows. The RTSPs have two important designs: (1) biotin modification at three of oligonucleotides (tetra-b-biotin, tetra-c-biotin, and tetra-d-biotin) to produce three avidin–HRP binding sites at three vertices of RTSPs; (2) the extended probe (black) was complementary to part of target DNA (red). With such designs, the RTSPs can hybridize the target DNA and form "sandwich" type together with CTSPs, and bind the avidin–HRP to produce enzyme catalytic signal together with TMB substrate. Comparing to the conventional E-DNA sensor designed with single-stranded DNA (SSDNA) as report probes, three fold enzyme catalytic signal for each target DNA were produced because each DNA tetrahedral nanostructure could bind three avidin–HRP enzymes at its vertices. Moreover, by designing more biotin–avidin binding sites on the RTSPs (e.g. on the edge of DNA tetrahedral nanostructure), one hybridization-event can bring multiple HRP molecules, leading to even larger signal amplification.

### 3.2. Detection performance of target DNA

Cyclic voltammetry and amperometry were employed to characterize the avidin–HRP enzyme based electrocatalytic process of E-DNA sensor for the detection of target DNA. As shown in

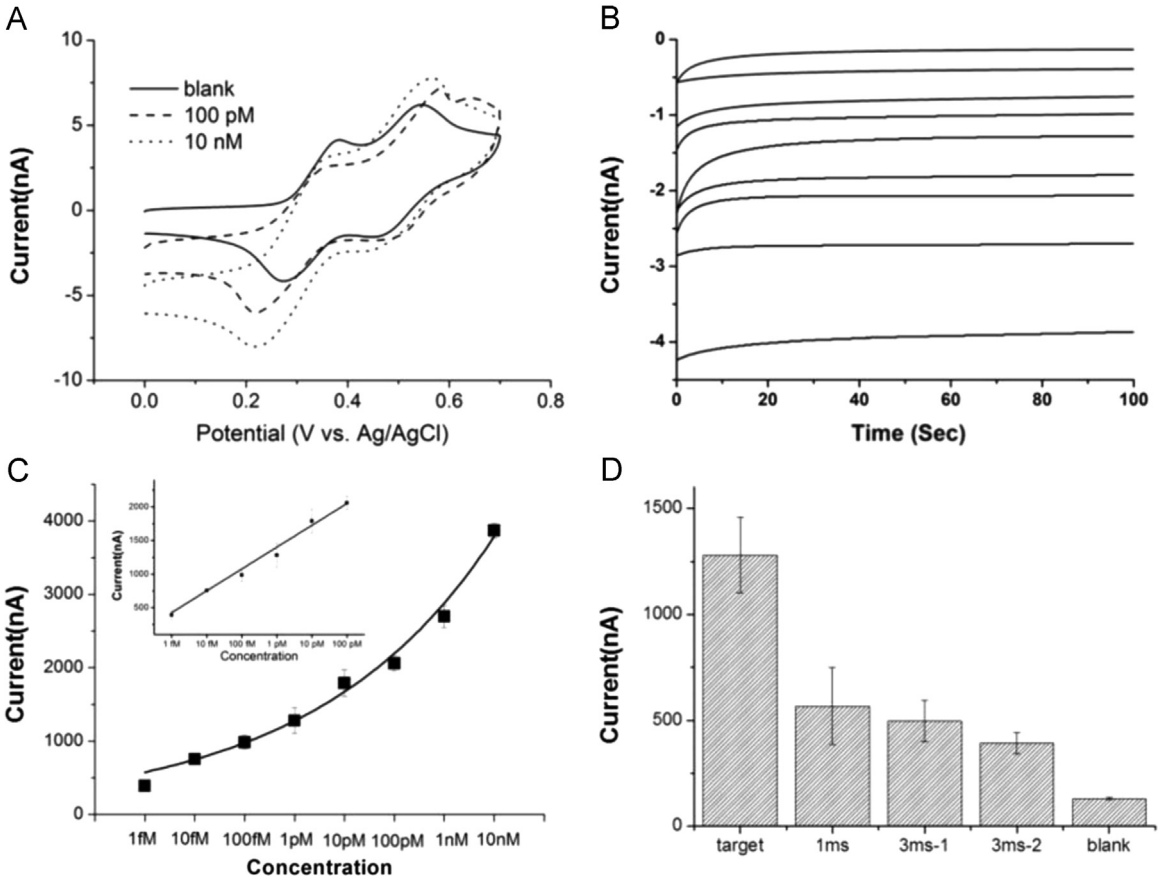


**Fig. 1.** (A) Cyclic voltammogram (CVs) for DNA tetrahedral structured probe-based E-DNA sensor in the absence (solid) and presence of 100 pM (dash), 10 nM (dots) of target DNAs. Scan rate: 100 mV/s. The representative redox peaks of TMB were observed and the increasing electrocatalytic current indicated the successful target detection. (B) Amperometric response of 0, 1 fM, 10 fM, 100 fM, 1 pM, 10 pM, 100 pM, 1 nM (from top to bottom) target DNA on DNA tetrahedral structured probe-based E-DNA sensor. The potential was held at 100 mV (vs. Ag/AgCl) and the reduction current was recorded at 100 s. (C) Logarithmic plot of amperometric current vs. target DNA concentration with RTSPs-based E-DNA sensor. The amperometric signal increased monotonically with the logarithm concentration of target DNA, resulting in a dose-response curve. A calibration linear curve for the target concentration range from 1 fM to 100 pM was obtained. The linear equation was $y = 103.3 + 324.5\log(X)$. (Error bars represent standard deviations of at least three independent experiments.) (D) Selectivity of our RTSPs-based E-DNA sensor for the detection of a series of DNA targets at 1 pM: perfectly matched target DNA (target), 1-base mismatched DNA (1ms), 3-bases mismatched DNA (3ms-1 and 3ms-2). Data were collected from at least three independent sets of experiments.

Fig. 1A, Electrocatalytic current has been recorded by cyclic voltammogram (CVs) for DNA tetrahedral structured probe-based electrochemical sensor. In the absence of target DNA, two pairs of well-defined redox peaks that were assigned to two-electron reduction and oxidation reactions of TMB, the electroactive co-substrate of HRP. Of note, since HRP cannot exchange electrons directly with the electrode, TMB served as electron shuttle that diffuses in and out the redox site of HRP. When in the presence of target DNA, we found that the reduction peak located at ~200 mV apparently increased, leading to a pair of asymmetric redox peaks that was characteristic of the occurrence of electrocatalysis. This implied that both target DNA and RTSPs were captured on the gold electrode surface and formed the "sandwich" structure.

Amperometry was employed for quantitative measurement. Upon the onset of the potential at 100 mV, we instantly observed a decay curve for current vs. time, which reached a plateau (steady-state current) within ~100 s, as demonstrated in Fig. 1B. The amperometric current (1280 ± 177 nA) for 1 pM target was observed. In contrast, when in the absence of target DNA, the background current was as low as ~130 ± 11 nA. This high signal-to-background ratio reflected the amplification of RTSPs design. Impressively, we can detect as low as 1 fM target DNA. Additionally, amperometric current for detection of target DNAs with concentrations across the range of 1 fM to 10 nM were discriminated. We found that the amperometric signal increased monotonically with the logarithm concentration of target DNA concentration across the range from 1 fM to 10 nM, spanning a response region of at least 8 orders of magnitude (Fig. 1C). A regression equation of $y = 103.3 + 324.5 \log(X)$ ($R^2 = 0.990$) for range from 1 fM to 100 pM was obtained (Fig. 1C, inset), where y is the amperometric signal value in nA and x is the concentration of target in fM. This large dynamic range of the RTSPs-based E-DNA sensor provided an unprecedented opportunity to screen target DNA that exists quite diversely in cells (range from ~10 to 50,000 copies/cell).

Then, we challenged the sensor with a series of targets to evaluate the selectivity (Fig. 1D). The signal intensity for the 1 pM noncognate DNA was statistically significant from that of target DNA, suggesting that this sensor was highly selective towards even a single-base mismatched DNA. The accuracy of the sensor for detecting DNA was evaluated by recovery study. Target DNA (1 pM) were added in DNA solution extracted from human serum and percentage recoveries were analyzed to be 75.92–114.3% with an average value of 90.57%.

### 3.3. Comparison of RTSPs-based E-DNA sensor with GNPRs-based and SSRPs-based E-DNA sensor

To further substantiate the advantages of RTSPs, we compared it with SSRPs system and GNPRs system.

Biotinylated single stranded DNA has been popularly employed to develop E-DNA sensors. For SSRPs system, similar sandwich assays were established by using the same CTSPs and biotinylated SSRPs that flanked the target DNA. Again, the avidin–HRP was used to generate electrocatalytic signals. This type of sensors showed poorer performance than RTSPs-based E-DNA sensor. As Fig. 2A shows, the background current for SSRPs-based E-DNA sensor was ~68 ± 15 nA, while the signal for 1 pM of target DNA was ~218 ± 38 nA, corresponding to a signal-to-background ratio of ~3, which was smaller than that of RTSPs-based E-DNA sensor (signal-to-background ratio was ~9). Also, when target DNA concentration was lower than 1 pM, the signal was indistinguishable from the background ( < 3SD). Hence, the sensitivity of SSRPs-based sensors was lower than that of RTSPs-based E-DNA sensors.

It is well-known that GNPs possess a very high surface-to-volume ratio (Georganopoulou et al., 2005). This offers an opportunity to attach both DNA and avidin–HRP at the surface of a single gold nanoparticle. An elegant example is an ultrasensitive DNA detector using multi-component GNPs-based nanoprobes (Li et al., 2008). These nanoprobes are GNPs assembled with thiolated oligonucleotide (detect probe), horseradish peroxidase (HRP) and bovine serum albumin (BSA). Such GNPs-based nanoprobes could detect target DNA as low as 25 pM. For our GNPRs-based E-DNA sensor, AuNPs with diameter of 5 nm were carefully chosen considering their similar sizes to DNA tetrahedral nanostructures. The surface area of 5 nm GNPs is about 78.5 $nm^2$. Therefore, one GNP can theoretically bind ~3 avidin–HRP enzyme. To make sure GNPs can be immobilized on gold electrode surface, we first attached thiolated DNA with the same sequence as the appended probe from "report tetrahedral structured probes" (RTSPs) onto GNPs by using protocol as previous reported (Li et al. 2009). In this way, GNPs conjugated with DNA (named GNPRs) could hybridize to target DNA and formed a sandwich structure together with CTSPs. And as high concentration as 80 nM of GNPs and theoretically 3 μM DNA probe were used. Then, the same amount of avdin–HRP (3 μL) was added. Here, the avidin–HRP was immobilized through protein–GNPs adsorption. When in the presence of target DNA, the GNPs and HRP were captured by hybridization. Hence, electro signal could be observed. However, we found that the GNPRs-based signal amplification was not significant, even lower than SSRPs-based E-DNA sensor. The background current was almost

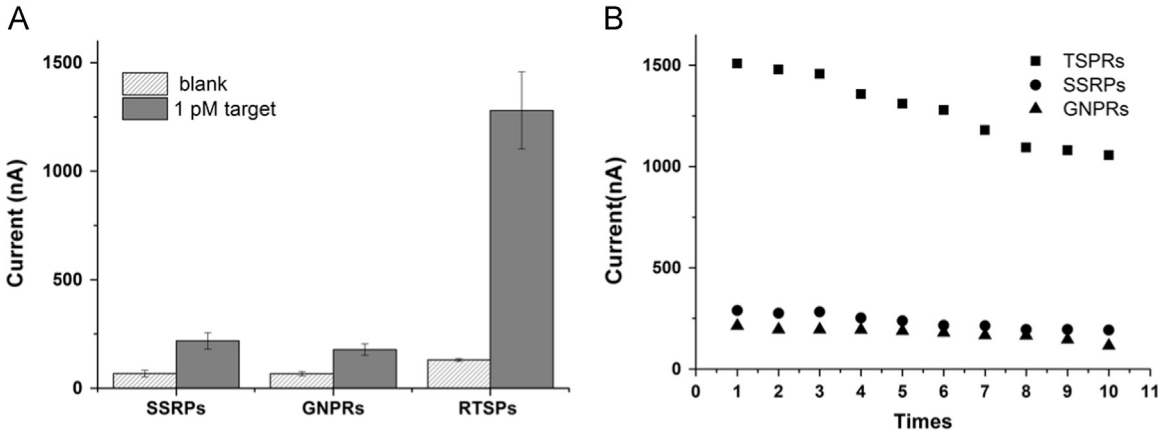


**Fig. 2.** (A) Signal of SSRPs, GNPRs, RTSPs-based E-DNA sensor in the absence (light grey) and the presence of 1 pM (dark grey) of target DNA. Error bars represent standard deviations for measurement taken from at least three independent experiments. (B) Comparison of reproducibility among SSRPs, GNPRs and RTSPs-based E-DNA sensors by testing 10 times for 1 pM target DNA.

the same as SSRPs-based E-DNA sensor ($68 \pm 15$ nA), and the signal for 1 pM of target DNA was only $\sim 178 \pm 26$ nA (Fig. 2A), reflecting a low signal-to-background ratio. The low signal-to-background ratio may be due to the steric hindrance effect of avidin–HRP enzyme to GNPs surfaces. In addition, the reproducibility of three sensors was evaluated by testing them for 10 times for 1 pM of target DNA (Fig. 2B). It was also an evidence of that RTSPs-based E-DNA sensor was superior to the other two sensors.

## 4. Conclusion

In summary, we developed a novel E-DNA sensor with ultrahigh sensitivity based on double DNA tetrahedral nanostructures. This E-DNA sensor employed DNA tetrahedral nanostructures as both capture probes and report probes. The newly design of RTSPs can incorporate multiple biotin labels and bring large HRP enzyme amplification. By using this new strategy, we demonstrated that this E-DNA sensor was able to detect as low as 1 fM DNA target and obtained a large dynamic range. Additionally, single base mismatch can be discriminated. Therefore, we expect this highly sensitive and selective E-DNA sensor will become a promising DNA biomarker detecting method. Given the unprecedented addressability and programmability of DNA nanostructures, our work may broaden their application in biodetection. More research work for in-field and point-of-care quantitative test of disease-related DNA, miRNA and protein using this RTSPs system should be done in the future.

## Author contribution

Dongdong Zeng design and carried the experiments; Huan Zhang and Dan Zhu analyzed the data and wrote the main manuscript.

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## Appendix A. Supplementary information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2015.04.065.

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