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PCR-free colorimetric DNA hybridization detection using a 3D DNA nanostructured reporter probe

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ABSTRACT: A "sandwich-like" biosensor was developed based on the magnetic bead platform for sensitive detection of breast cancer 1 **(**BRCA1) DNA. In the present study, a tetrahedron-structured reporter probe (TSRP) was designed, in which 3 vertices of the tetrahedron were labelled with digoxin (Dig) and the other one was labelled with a detection probe. TSRP here provided accurate enzyme loading and well-organized spatial arrangement for optimized signal amplification. The detection limit of this biosensor was as low as 10 fM, which is at least 4 orders of magnitude lower than the single DNA probe (100 pM), and the signal gain was 2 times higher than the analysis using three one-dimensional (1D) reporter probes. We could distinguish DNA sequences with only 1 base mismatch, and the performance of our TSRP biosensor was proved to be equally good in both PCR products and real fetal calf serum (FCS) sample as in buffer. We believe this work provided a novel avenue for the development of signal amplification strategies.

KEYWORDS: DNA biomarker, signal amplification, magnetic microparticles, DNA tetrahedron, BRCA1

1. INTRODUCTION

The specific detection of DNA molecules[1-2] with high sensitivity has attracted more and more research interest, as a pressing technical demand in many areas including environmental monitoring, anti-bioterrorism[3], and particularly diagnostics[4-6]. Currently, a large amount of DNA biomarkers[7] have been demonstrated to be highly

related to the development of cancers, and the collection and analysis of low abundance circulating nucleic acids such as cell-free tumor DNA (ctDNA)[8], have been known to be critical strategies for diagnosis. Compared to tissue biopsy which is notoriously painful, costly, and time-consuming, DNA biomarker analysis is minimally invasive and more informative. More importantly, DNA is more chemically and biochemically stable than RNA[9] and cells[10-11].

Magnetic beads[12] have been widely utilized for low abundance DNA enrichment towards highly sensitive detection, based on its unique superparamagnetic property, operational convenience and high separation efficiency[13-14], that provides us an excellent platform for the development of DNA biosensor[15]. Generally, a sandwich-like structure is constructed on surface of the magnetic microparticles (MMPs), connecting a signal tag with the MMPs, which has been demonstrated to be a powerful and utility tool for detection of DNA[15-18], protein[19,20-22] and small molecules[23]. However, the sensitivity is usually limited due to sample loss during the magnetic separation and the limited amount of the signal tags on MMP surface.

Aiming at high analysis sensitivity, several signal amplification strategies were developed by designing optimized reporter probes and introducing more signal tags. Fan and coworkers developed a novel nanoprobe by integrating DNA detection probe, horseradish peroxidase (HRP) and bovine serum albumin (BSA) on gold nanoparticle (AuNP) surface, that was then utilized in the sandwich complex instead of single detection probe[16], however, the sensitivity was still limited due to the unspecific

loading of biomolecules on the nanoparticle surface. Later they reported a second generation of signal amplification strategy for MMP biosensor. They developed a novel reporter probe with a two-dimensional (2D) DNA belt with biotin tags that is produced by using rolling circle amplification (RCA) product and stamp strands[22]. However, the space crowing effect of the 2D reporter probes hindered the development of MMP-based biosensors[24].

DNA nanostructures[25-38], especially, three-dimensional (3D) DNA tetrahedron nanostructure[39-45] have attracted considerable research interest for optimized interfacial engineering in biosensor development in various fields due to its unique structure controllability and precision[25, 46-47]. In this work, we developed a new generation of 3D reporter probe for a MMP-based sandwich-structure DNA biosensor. A tetrahedron-structured reporter probe (TSRP)[48-49] was designed with 3 vertices labelled with digoxin (Dig) and the other one labelled with a detection probe complementary to the target sequence. TSRP provided not only accurate loading of the Dig tags to improve the repeatability of the analysis, but also well-organized spatial arrangement for improved enzyme binding and catalysis activity, based on its stable and solid 3D scaffold. In the detection of target sequence which is a segment from the breast cancer-associated BRCA1 gene[50-51], excellent detection performance was achieved both in buffer and fetal calf serum (FCS) sample.

2. EXPERIMENTAL SECTION

2.1 Reagents and Materials

The DNA sequences shown in Table 1 were synthesized and purified by Invitrogen Biotechnology Co., Ltd (Shanghai, China). The magnetic microparticle (MagnaBind^{TM} streptavidin beads, 1-4 μm diameter, 5 mg/mL) was purchased from Thermo Fisher Scientific Inc. (Rockford, USA). BSA was obtained from Sigma-Aldrich Inc. (St Louis, USA). The anti-Dig antibody (HRP) (1000 mg/mL) was purchased from Abcam Trading Co., Ltd (Shanghai, China), and its diluent buffer was obtained from Fitzgerald Industries International (Acton, USA). The custom TMB substrate was obtained from Neogen Co. (USA). All other reagents were of analytical grade. The chemicals mentioned were used without further purification, and Milli-Q water (18 MΩ·cm resistivity) was used throughout all experiments.

Table 1. Sequences of oligonucleotides in this work.

Name	Sequence（5'-3'）
target	GAGCATACATAGGGTTTCTCTTGGTTTCTTTGATTATAATTCATAC
Capture Probe	GAAACCCTATGTATGCTCTTTTTTTTTT-(Biotin)
A-tail	ATTACAGCTTGCTACACGAGAAGAGCCGCCATAGTATTTTTTTGTATGAATTATAATCAAA
B-DIG	Dig-TATCACCAGGCAGTTGACAGTGTAGCAAGCTGTAATAGATGCGAGGGTCCAATAC
C-DIG	Dig-TTCAGACTTAGGAATGTGCTTCCCACGTAGTGTCGTTTGTATTGGACCCTCGCAT
D-DIG	Dig-TCAACTGCCTGGTGATAAAACGACACTACGTGGGAATCTACTATGGCGGCTCTTC
SNP DNA:	GAGCATACATAGGGTTTCTCTTGGTTTCTTTGATTATXATTCATAC ("X" is T, C, or G)

NTC	ACACGCTTGGTAGACTTTTTTTTTTAGCATCGATAACGTT
Probe 1	Dig-GTATGAATTATAATCAAA
Probe 2	Dig-GTATCCAGTGGCTCAGTATGAATTATAATCAAA
Probe 3	TGAGCCACTGGATACTTTTCAAGACGATTACTAG-Dig
Probe 4	Dig-GCATGCTAGTAATCGTCTTG
primer1	AGAAAACAAGGACTCTAAATAAC
primer2	CTGTAAAATACAAGGGAAAAC

The single/double underlined, blue or red segments the target and probes are complementary.

2.2 Solutions

The buffers used in the experiments are as follows: washing buffer (10 mM phosphate sodium buffer solution, pH 7.4, 100 mM NaCl), hybridization buffer (10 mM phosphate sodium buffer solution, pH 7.4, 1 M NaCl), blocking buffer (10 mM phosphate sodium buffer solution, pH 7.4, 1 M NaCl, 1% BSA), stock buffer (10 mM phosphate sodium buffer solution, pH 7.4, 1 M NaCl, 1% BSA, 1% casein) and TM buffer (20 mM Tris, 50 mM $MgCl_2$, pH 8.0). All solutions were prepared with Milli-Q water.

2.3 Preparation of capture probe functionalized magnetic microparticles (MMPs)

The MMPs modified with streptavidin were first washed twice with washing buffer before use. Then, the biotinylated capture probe DNA was added to the collected MMPs. After 30 min of incubation at 25 °C with gentle shaking on a thermomixer

comfort (Eppendorf, Germany), the mixed solution was washed twice with washing buffer. Then, the beads were resuspended in blocking buffer, and stored in stock solution at 4 ℃ for further use.

2.4 Preparation of DNA tetrahedral structure reporter probe (TSRP)

A DNA tetrahedral structure reporter probe (TSRP) was prepared mainly according to a previously reported paper[52] with 3 short DNA strands labeled with Dig at the 5' terminus (B-Dig, C-Dig, D-Dig) and a long DNA strand (A-tail). The A-tail consists of 3 parts: (1) an 18-base fragment complementary to the target, (2) a T7 spacer, (3) three 17-base fragments with a 2-base interval between each other that are complementary to the B-Dig, C-Dig and D-Dig respectively. Four DNA strands (A-tail, B-Dig, C-Dig, D-Dig) were dissolved in TM buffer with equimolar ratio with a final concentration of 50 μM. Then, the solution was heated to 95 ℃ for 5 min, and then quickly cooled down to 4 ℃ for 30 s using a S1000 thermal cycler (BIO-RAD, USA). The final TSRP solution was stored in 4 ℃ for the next step.

2.5. TSRP based Magnetic DNA sensors for target DNA detection

Our biosensing reaction was performed on a 96-well plate. To eliminate the unspecific adsorption, the 96-well plate was firstly treated with a blocking buffer containing 1% BSA overnight. In a typical experiment, 10 μL capture probe coated MMPs were washed with washing buffer, and then added to a BSA-blocked 96-well plate, and then magnetically collected by the BioMag 96-Well Plate Separator (Bangs Laboratories, Inc.). Then 25 μL of the hybridization buffer containing target DNA of

various concentrations and 10 μL of 1 μM TSRP were added together and incubated with the MMPs for 2 h at 37 °C with gentle shaking. Subsequently, the complexes were magnetically collected again and rinsed with washing buffer. This washing procedure was repeated for 5 times to remove unbound reporter probes. Then, 10 μL per well of anti-Dig labeled horseradish peroxidase (anti-Dig-HRP) solution (1 mg/mL) were dispensed and incubated at room temperature for 10 min with gentle shaking. After 5 times washing in buffer, 100 μL of TMB substrate solution per well was added to react with the resulted complexes for 10 min at 25 °C with gentle shaking. Then, 50 μL 0.5 M H_2SO_4 per well was added into the solution as a stop solution for 10 min.

After a magnetic separation step, the supernatant was then removed to another clean 96-well plate and measured with the microplate reader (iMark Microplate Reader, BIO-RAD) to achieve the absorbance (OD450). Meanwhile, the system background (OD450) signal of water on 96-well plate was read on the same microplate reader, the average background signal of the 96 wells was calculated to be 0.06 (Data was shown in Table S1). Thus, in the following analysis, a signal normalization was performed by deducting the system background.

2.6 Cell culture and PCR amplification

The genomic DNA of Michigan Cancer Foundation-7 (MCF-7) cell line was extracted using a human genomic DNA isolation kit (Tiangen, Beijing) and provided by Shanghai Bingxin Biological Technology Co., Ltd. The concentration of DNA

template was evaluated to be 224 ng/μL by using a NanoDrop 2000 (Thermal Scientific, USA). The asymmetric PCR amplification of MCF-7 genomic DNA was performed on a thermal cycler (S1000, Bio-Rad, USA). A pair of asymmetric primers (primer1 and primer2) were employed to produce a 234 nt ssDNA strand. The 20 μL PCR reaction system consisted of 10 μL 2×premix Ex Tap, 1 μL each primer, 1 μL DNA sample and 7 μL water. The PCR program consisted of an initial denaturation step at 95 ℃ for 10 min and 55 cycles of 95 ℃ for 30 s, 50 ℃ for 30 s, and 72 ℃ for 30 s. Then an incubation step at 72 ℃ for 10 min was performed to finish any potential incomplete extension. Finally, gel electrophoresis was utilized to confirm the PCR product: 3% agarose gel electrophoresis was carried out in 1×TAE at a constant voltage of 130V for 25 min. The solution consisted of 2.5 μL diluted PCR products, 2.5 μL H_2O and 1 μL 6×loading buffer.

3. Results and discussion

3.1 Design and preparation of TSRP-based biosensor

In the present study, a "sandwich-like" detection strategy was employed (Figure 1), and the analysis system involved a capture probe on MMP and a 3D reporter probe, that flanked the DNA target sequence. The 3D TSRP was designed based on a DNA tetrahedral structure, with 3 vertices labelled with Dig tags and the other vertex appended with a DNA probe which is complementary to part of the target DNA. By using the TSRP instead of single strand probe, 3 Anti-Dig-HRP molecules were captured for one DNA target, and more importantly, by modulating the distance

between the Dig tags on the 3D DNA scaffold, we decreased the space crowing effect between the enzymes, and thus improved the enzyme binding and catalysis efficiency. The capture probe was assembled on the surface of a streptavidin-MMPs (SA-MMPs) through the strong streptavidin-biotin interaction, which could hybridize to the target DNA. In each detection process, when the target strands were added into the analysis system, the MMPs covered by capture probes and the TSRP would be linked together through specific hybridization, and then, the anti-Dig-HRP bound to the Dig tags at 3 vertices of the DNA tetrahedron. This complex could be magnetically separated for subsequent optical detection. In contrast, in the absence of target DNA, the reporter probe was not captured onto the surface of the MMP, and would be cleaned out during the magnetic separation step.

The magnetically collected and rinsed MMPs were then added to the substrate solution containing TMB and H_2O_2, that showed blue color under HRP catalysis. As anticipated, the color change of the TMB substrate was highly related to the presence of target DNA: when the concentration of the target was increased, the color intensity increased accordingly. The light absorption at 450 nm (OD450) was recorded as the result for the sensitive quantification of target DNA.

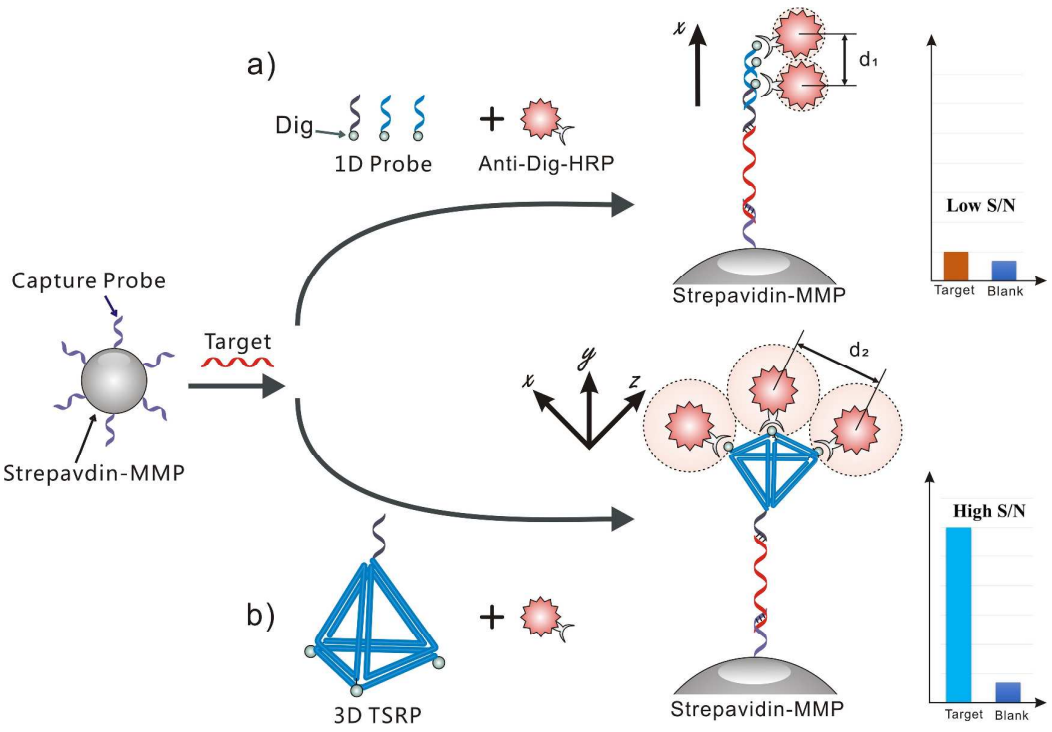

Figure 1. Schematic illustration of the MMP-assisted DNA sensor based on signal amplification using a) 1D probe and b) 3D TSRP.

3.2 Enhanced detection performance of TSRP based on 3D nanostructure

The 3D DNA tetrahedron played key role for the performance of our biosensor. To better evaluate the effect of DNA tetrahedron scaffold, we designed four 1D linear Dig-labelled reporter probes (Probe 1, Probe 2, Probe 3, Probe 4 in Table 1), that were utilized in different groups: Probe group I (PI) was a single probe (Probe 2), probe group II (PII) consisted of 2 reporter probes (Probe 2/Probe 3), and probe group III (PIII) consisted of 3 reporter probes (Probe 2/Probe 3/Probe 4). As shown in Figure 2,

PII and PIII were designed to form linear double-strand structures through hybridization between the reporter probes, and thus provide more than one Dig tags.

The detection performance of 3D TSRP and linear reporter probes were compared in the presence of 100 pM target DNA (Fig 6). When the amount of the reporter probes was increased from 1 (PI) to 3 (PIII), the signal gain increased gradually from 0.39 to 0.64, because more anti-Dig-HRP were captured. As anticipated, the signal of TSRP was obviously higher than 1D reporter probe (PI) or groups of them (PII and PIII). It is worth to note that we achieved 2 times higher current signal with TSRP than PIII, with the same amount of Dig tags, while the background was extremely stable for different probes, which strongly demonstrated the signal amplification effect of our TSRP by not only increasing the amounts of Dig labels but more importantly improving the interfacial engineering on surface of the MMP.

The improved performance was attributed to the unique 3D structure of the TSRP. In our design, each side of our DNA tetrahedron had 17 base pairs with a length of 5.78 nm, that was comparable to the molecular dimension of the anti-Dig-HRP (~6 nm). The 3D DNA structure provided critical steric support for HRP enzyme, with optimized molecular distance and improved binding efficiency.

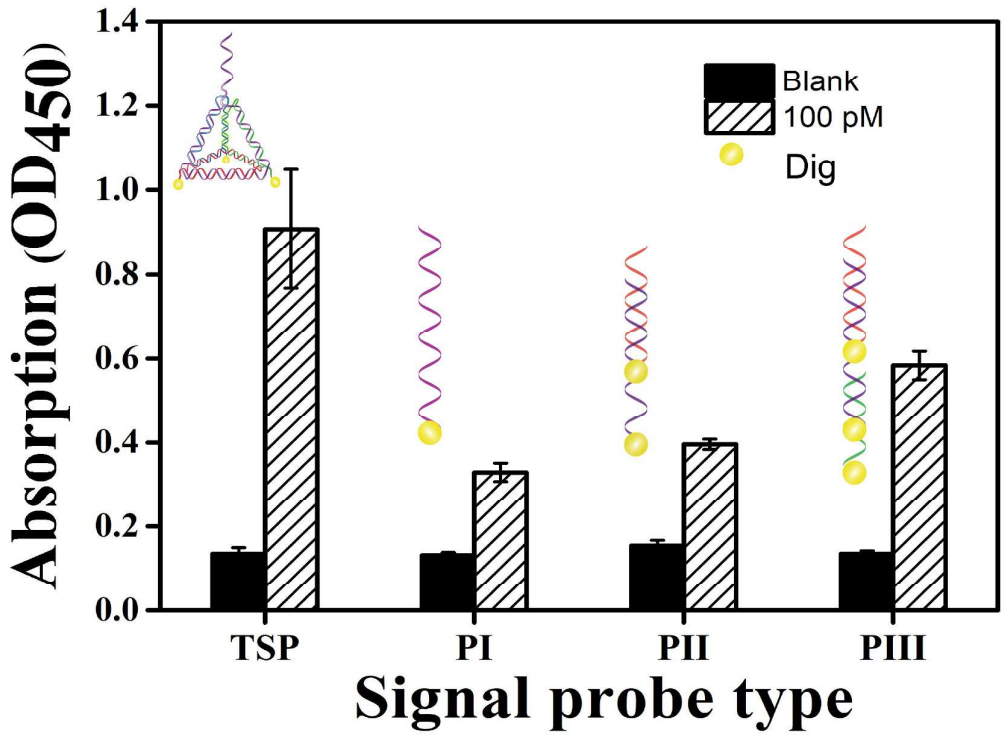

Figure 2. Comparison of the detection performance of TSRP and 3 different groups of 1D linear reporter probes in the presence of 100 pM target DNA. PI, PII and PIII consisted of 1, 2 and 3 Dig-labelled reporter probes, respectively. The concentration of TSRP and 3 different groups of 1D linear reporter probes is 40 pM.

3.3 High sensitivity of DNA detection

A synthetic DNA which is a fragment of MCF-7 gene was chosen as a target. After optimizing the key experimental conditions (data was shown in Figure S1), we investigated the sensitivity of our biosensor in the presence of different concentrations of target DNA. As shown in Figure 3, a dramatic increase of OD450 was observed with the target concentrations increased from 0 to 10 nM. The OD450 values were then plotted against the target DNA concentration, resulting in a sigmoid working

curve in a wide concentration range between 10 fM to 10 nM. The highest signal growing rate happened between target concentration approximately 10 pM and 5 nM, and the working curve reached a platinum at the target concentration of 10 nM. The inserted histogram showed the signal change in the presence of low-concentration targets, which clearly demonstrated the detection limit of our sensor ranging from 10 fM to 10 pM.

To better evaluate the detection performance of the TSRP biosensor, a 1D reporter probe (SSP, Probe 1 in table 1) was applied to perform the analysis under the same conditions, and the results were also shown in Figure 3. At very high concentration levels (≥10 nM), TSRP and SSP achieved comparable detection signal, presumably because when the target concentration exceeded 10 nM, the molecular density on MMP surface is too high to bind adequate TSRP for every target strand, thus limited the signal gain for TSRP analysis. However, when target concentrations were lower than 10 nM, detection performance of TSRP surpassed SSP at every concentration point with the highest signal difference of 50% at 10 pM (Fig 3). As the inserted histogram showed, the detection capability SSP against low concentration DNA targets is relatively poor with only one Dig tag on a flexible single DNA strand. All of these results together indicated the LOD of TSRP (10 fM) is at least 4 orders of magnitude lower than SSP (100 pM).

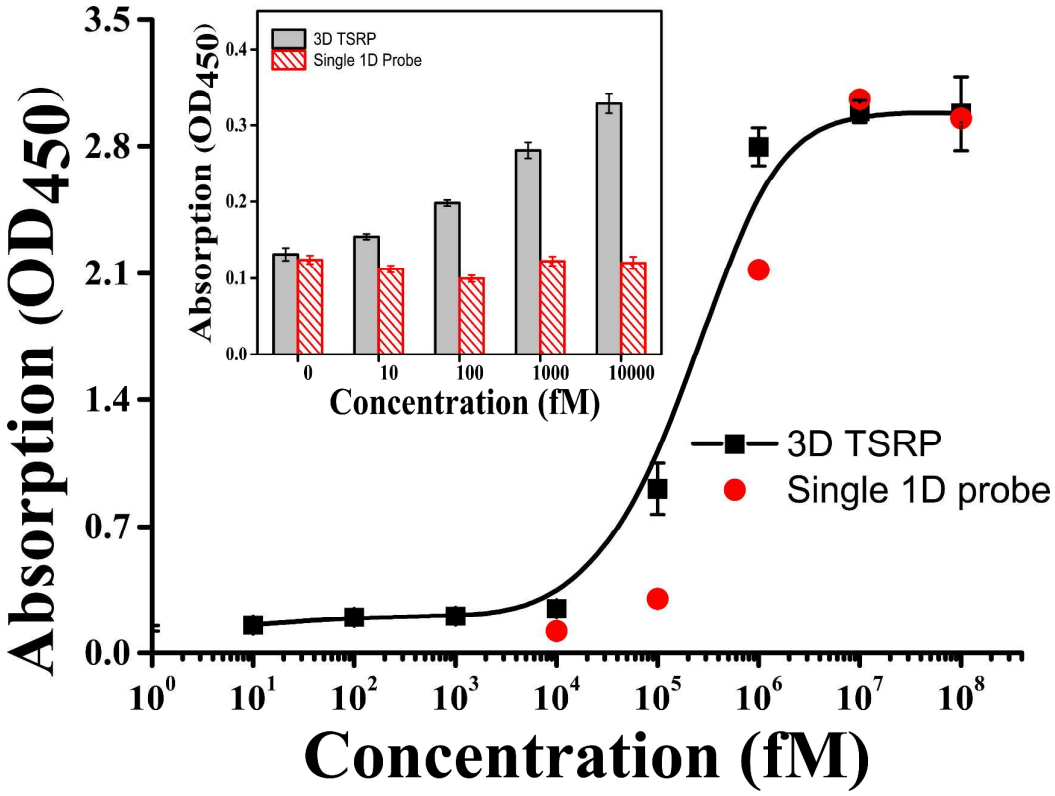

Figure 3. A dose-response curve for colorimetric detection of target DNA using TSRP (black) and a single stranded DNA probe (red) labelled with Dig. The inserted histogram showed the detection results of low concentration targets, ranging from 0 to 10 pM.

3.4 High specificity of DNA detection

We then investigated the specificity of our TSRP biosensor facing single nucleotide polymorphism (SNP). 3 SNP DNA targets (table 1) and a non-target control (NTC in table 1) were analyzed together with the fully complementary target DNA at the concentration of 10 pM. As shown in Figure 4, NTC showed only negligible signal, that was very close to the blank signal. Our TSRP biosensor discriminated 3 SNP

DNA even with only 1 base mismatch, by producing much higher signal for the fully complementary target than SNP DNA. This assay clearly demonstrated the excellent specificity of our biosensor.

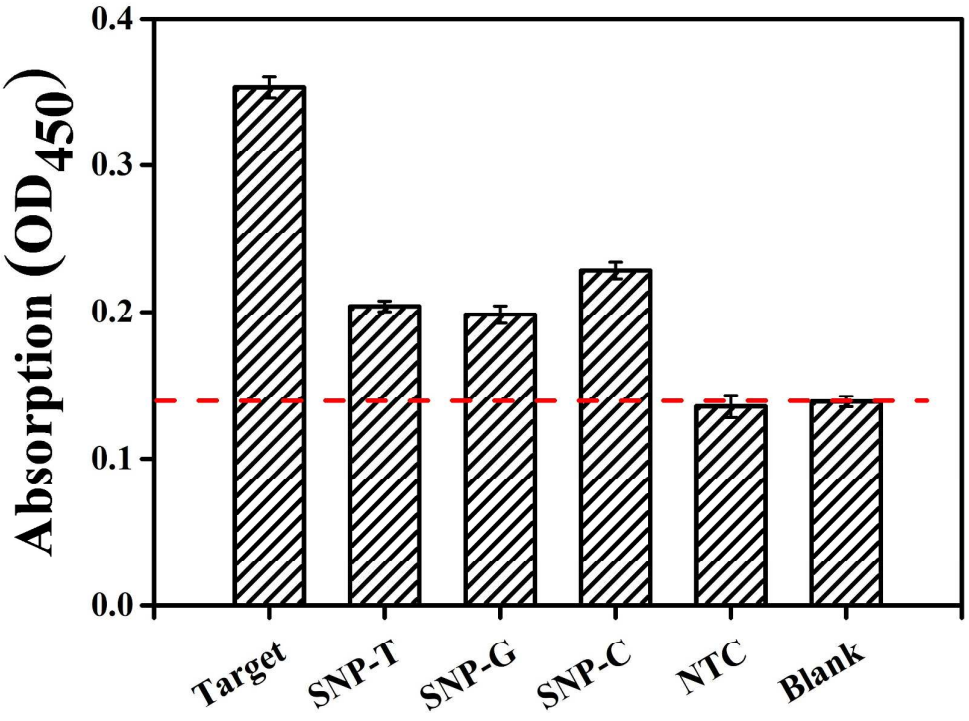

Figure 4. Detection specificity of TSRP biosensor against SNP. The concentration of all targets was 10 pM. The red dash line indicated the value of the blank signal.

3.5 Detection of PCR products

To challenge the practicability of our biosensor, we analyzed a product of asymmetric PCR, that is known to be capable of producing much more single-strand DNA (ssDNA) [53-54] target than normal PCR. A 234 nt ssDNA fragment on MCF-7 gene was taken as the target. As shown in Figure 5, our sensor could detect a 12500 times

diluted PCR product, even though this target was much longer than the synthetic DNA strand. The sensitivity was at least 4 orders of magnitude better than normal agarose gel electrophoresis method of PCR products analysis (Fig S2), demonstrating our sensor was a promising fast analysis method for DNA biomarkers.

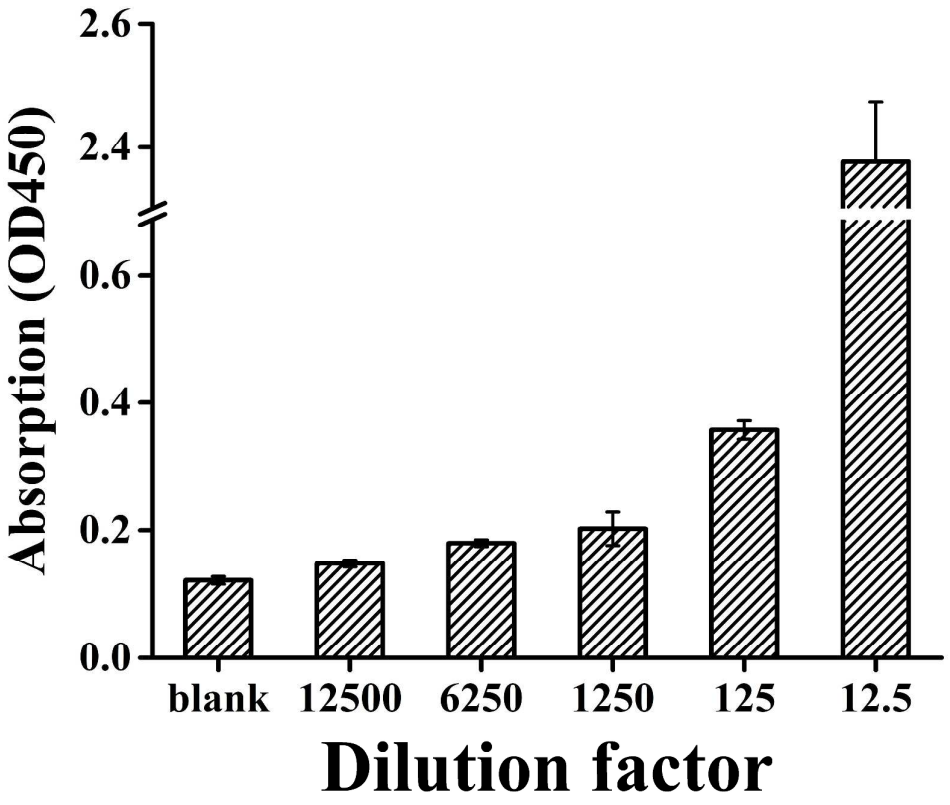

Figure 5. Detection of PCR products with TSPR biosensor. The concentration of DNA template (genomic DNA of MCF-7) was 224 ng/μL. After PCR amplification, the PCR products were diluted 12500, 6250, 1250, 125 and 12.5 times, respectively, and then analyzed with TSRP biosensor. A buffer solution was analyzed as the Blank control.

3.6 Real sample analysis

Furthermore, we investigated the performance of our biosensor in fetal calf serum (FCS) sample, and the performance of our biosensor was demonstrated to be equally good as in buffer. As shown in Figure 6, we indicated the signal of LOD in FCS solution using a blue dash line, and clearly 100 fM concentration was recognized from the blank signal. Interestingly, when detecting targets in low concentrations, a slight signal increase was observed in FCS, presumably due to the unspecific adsorption of the complex FCS matrix on TSRP. Though the signal increase in FCS samples happened accordingly for every concentration including the blank, thus the analysis capability was not significantly disturbed. This assay further demonstrated the applicability of our TSRP biosensor in real sample.

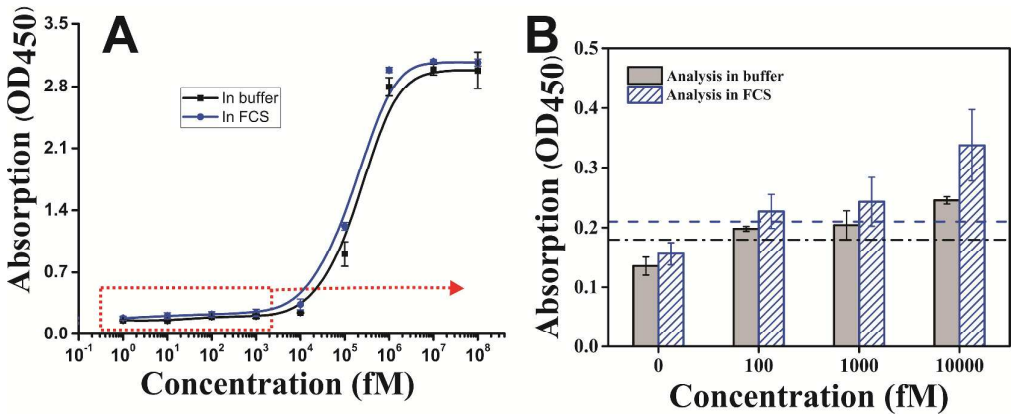

Figure 6. Comparison of the detection performance of TSRP biosensor in buffer and FCS. A) A dose-response curve for colorimetric detection of target DNA in FCS (blue line) and the buffer (black line). B) Detection performance of TSRP biosensor in the presence of targets of low concentration range (0-10 pM) in FCS (blue columns) and in buffer (black columns). The black dash line indicated the LOD in buffer (3SD higher than the blank signal in buffer) and the blue dash line indicated the LOD in

FCS (3SD higher than the blank signal in FCS).

4. Conclusions

In summary, we herein developed a novel signal amplification strategy combining the DNA tetrahedron with magnetic microbeads, for the highly sensitive detection of BRCA1 DNA biomarker. Benefit from the advantages of MMP platform, our biosensor was demonstrated to be fast and convenient. By using DNA tetrahedron as a scaffold, we optimized the enzyme molecule combination and its spatial arrangement on MMP surface. Thus, the signal-to-noise ratio was significantly increased. The detection limit was as low as 10 fM, which is at least 4 orders of magnitude lower than single DNA probe (100 pM) (Fig 3), and more convincingly, 2 orders of magnitude lower than a nanoprobe (10 pM) based on unspecific adsorption of HRP on the AuNPs[16]. Our work for the first time demonstrated the fact that the optimization of the interfacial engineering for the reporter probe plays key role for the performance of the biosensor. By using this novel TSRP strategy, we can distinguish DNA sequences with only 1 base mismatch, and the practicability of our TSRP biosensor was proved to be equally good in both PCR product and real FCS sample as in buffer. Given the high sensitivity, practicability and operation convenience, we believe our sensor is a promising method for point-of-care testing without the need of PCR amplification, with quite competitive performance among previously reported works (Table S2). Our work provided a novel avenue for the development of signal amplification strategies, especially that need larger molecular space and smarter interfacial regulation.

ASSOCIATED CONTENT

Supporting Information:

Background analysis of the microplate readout system, optimization of main experimental conditions, and gel electrophoresis results of the 234 bp double-strand and single-strand DNA amplified from the MCF-7 genomic DNA. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

Xue Yang and Yanli Wen contributed equally to this work.

Notes

The authors declare no competing financial interest.

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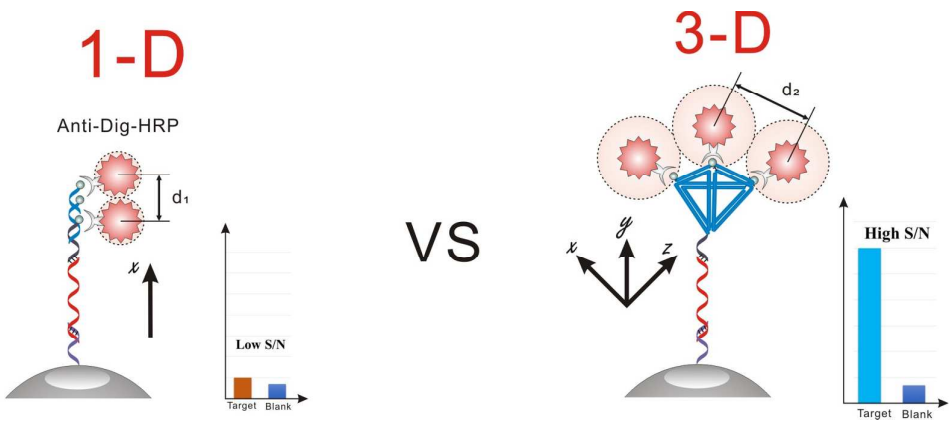
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