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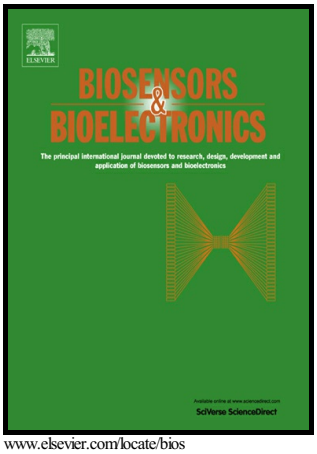

www.elsevier.com/locate/bios

PII: S0956-5663(16)31064-8
DOI: http://dx.doi.org/10.1016/j.bios.2016.10.044
Reference: BIOS9266

To appear in: *Biosensors and Bioelectronic*

Received date: 23 August 2016
Revised date: 4 October 2016
Accepted date: 19 October 2016

Cite this article as: Mengqi Zou, Daxiu Li, Ruo Yuan and Yun Xiang, Metal-ion dependent DNAzyme recycling amplification for sensitive and homogeneou immuno-proximity binding assay of α-fetoprotein biomarker, *Biosensors an Bioelectronic,* http://dx.doi.org/10.1016/j.bios.2016.10.044

Metal-ion dependent DNAzyme recycling amplification for sensitive and homogeneous immuno-proximity binding assay of α-fetoprotein biomarker

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Abstract: Based on target-induced immuno-proximity binding and metal ion-dependent DNAzyme recycling signal amplification, we describe the development of a homogeneous and sensitive fluorescent method for the detection of protein cancer biomarkers by using α-fetoprotein (AFP) as the model target. Two DNA strands with short complementary regions are conjugated to the AFP antibodies to prepare the recognition probes. The hybridization of the two DNAs in the absence of the AFP target molecules is inhibited due to the low melting temperature (T_m) of the complementary regions. The binding of the antibody-linked DNAs to the AFP target molecules can increase the local effective concentrations of the two DNAs and facilitate their hybridization with significantly increased T_m to form the catalytic cores of the metal ion-dependent DNAzymes. The fluorescently quenched hairpin substrate sequences further bind the catalytic cores to form the DNAzymes, in which the substrate sequences are cyclically cleaved by the corresponding metal ions to generate remarkably enhanced fluorescent signals for sensitive detection of AFP in the dynamic range from 2 to 500 pM with the detection limit of 1.8 pM. The sensing approach also possesses high selectivity toward the target AFP over other interfering proteins, and can be employed to detect AFP in human serum samples. The significant signal amplification in our approach avoids the involvement of any enzyme or nanomaterial labels and sensitive protein detection can be realized in homogeneous solution, which makes the developed method holds great potential for convenient and sensitive detection of various protein biomarkers for early disease diagnosis.

Keywords: DNAzyme; Proximity binding; Biosensor; Signal amplification; Biomarker.

1. Introduction

Protein, the basic material of life, plays an important role in various life activities. Numerous studies have proven that changes in the concentrations of protein biomarkers in blood or tissues are often closely associated with the occurrence and development of different diseases (Zong et al., 2012; Rusling, 2013). However, in the disease nascent stage, the levels of the specific biomarkers are extremely low. Thus, the development of sensitive and accurate methods for the detection of protein biomarkers is crucial for early diagnosis, prevention and treatment of diseases to increase patient survival rate. Taking α-fetoprotein (AFP), a major serum protein produced by adult hepatic cell, as an example, the content of AFP in health adults is generally very low while increased concentration of AFP has a close correlation with the occurrence of several cancerous diseases, such as gastric, nasopharyngeal and testicular cancers (Wang and Xie, 1998; Taketa, 1990). Thus, the identification and detection of AFP in human serums is of great significance for clinical research.

The enzyme-linked immunosorbent assays (ELISAs) have been the most conventional and widely used methods for protein detection up to date. Although ELISA can achieve sensitive detection of proteins with specificity and reliability, it encounters the drawbacks of time-consuming, insufficient sensitivity, the need of sophisticated equipment, high cost and complexity (Zhou et al., 2012; Ahirwar et al., 2015; Liu et al., 2013). Especially, the application of ELISA for detecting trace amounts of protein biomarkers is significantly limited. To address these issues, recent research attentions have been directed toward the development of new signal amplification approaches for highly sensitive detection of protein biomarkers (Chinen et al., 2015; Kadimisetty et al.,

2015), and polymerase chain reaction (PCR) (Ma et al., 2013), rolling circle amplification (RCA) (Gao et al., 2015; Zhang et al., 2015), biobarcodes (Xia et al., 2014) and enzymes (Deng et al., 2014) or functional nanomaterial-assisted (Wei et al., 2016) amplifications have been successfully coupled with electrochemical (Li et al., 2015; Zhang et al., 2007) or optical (Gokulrangan et al., 2005; Tang et al., 2011) signal transduction means to achieve this purpose. Despite the fact that these newly developed signal amplification techniques can improve the sensitivity for detecting low levels of proteins, they still suffer several limitations. For example, PCR requires multiple sample preparation and precise temperature control, and RCA is considerably difficult to operate. While the enzyme-assist methods have the drawbacks of high cost and the risk of fake positive signal outputs, and the nanomaterial-based method usually accomplish in heterogeneous systems and need tedious washing, separation and labeling steps. Considering these facts, the development of simple and homogeneous signal amplification strategies will significantly facilitate sensitive detection protein biomarkers.

The proximity ligation assay (PLA) first reported by Landegren and co-workers (Fredriksson et al., 2002) involves the binding of two DNA-linked affinity probes to one target protein molecule that promotes the ligation of the two DNA to generate an amplifiable DNA sequence for highly sensitive detection of proteins. The PLA approach expands the capabilities of traditional immunoassays for indirect detection of proteins with high specificity and sensitivity. Based on similar principles, Heyduk (Heyduk et al., 2008) and Le (Zhang et al., 2011) further extends the PLA method to binding-induced DNA annealing and DNA assembly for protein detection, respectively. In addition, the endonuclease-assisted recycling amplification has been incorporated into the

binding-induced assembly assays to enhance the signal output for achieving high sensitivity (Zhang et al., 2015). Indeed, the PLA-based signal transduction methods offer convenient and sensitive detection of a variety of protein molecules in homogeneous solutions by avoiding the washing steps. However, the involvement of enzymes (ligase and polymerase in RCA or endonuclease in DNA assembly recycling amplifications) in amplified PLA assay of proteins potentially increases the cost and complexity of these detection methods.

Herein, to explore enzyme-free amplification-based PLA, we report the development of a metal-ion dependent DNAzyme recycling signal enchantment strategy for sensitive and homogeneous immuno-proximity binding assay of protein biomarkers. DNAzyme is a kind of functional catalytic nucleic acid sequence selected by systematic evolution of ligands by exponential enrichment (SELEX) in vitro (Ren et al., 2015). In comparison with the protein enzymes or ribozymes, DNAzymes have the inherent advantages of high catalytic activity, stability and excellent biocompatibility, which enable them promising amplification labels for detecting different target molecules. Particularly, metal ions are indispensable cofactors to activate several DNAzymes, and the specific nucleic acid substrates of the activated metal ion-dependent DNAzymes can be cyclically cleaved by the corresponding metal ions to amplify the analytical signals for highly sensitive detection of metal ions (Zhao et al., 2011) and nucleic acids (Xu et al., 2016). We show here the proximity binding of two DNA-conjugated AFP antibodies to the AFP targets can facilitate the formation of DNAzyme catalytic core structures, which bind the fluorescently quenched hairpin substrate DNA sequences to form the Mg^{2+}-dependent DNAzymes. The Mg^{2+} ions thus cyclically cleave the substrate DNA sequences to

generate significantly amplified fluorescent signals for sensitive detection of the AFP biomarker in a homogeneous and enzyme-free way.

2. Experimental section

2.1. Materials and reagents

The monoclonal AFP antibodies and AFP antigen were purchased from Abcam Co., Ltd. (Shanghai, China). The Mg^{2+}-dependent DNAzyme was designed by following previous publications with slight modifications (Wang et al., 2011; Breaker et al., 1995). The fluorescently quenched hairpin DNAzyme substrate sequence (HS), the oligonucleotides used for antibody conjugation (DNA1, DNA2), [Tris(hydroxymethy-l)aminomethane] hydrochloride (Tris-HCl), dithiothreitol (DTT) and ethylenediaminetetraacetic acid (EDTA) were all provided by Shanghai Sangon Biological Engineering Technology and Services Co., Ltd. (Shanghai, China). The oligonucleotides sequences were listed in Table 1. Thrombin, mouse immunoglobin G (IgG) and carcinoembryonic antigen (CEA) were purchased from Sigma (St. Louis, MO, USA). Sulfosuccinimidy l-4-[N-maleimidomethyl]-cyclohexane-1-carboxylate (sulfo-SMCC) was provided by Thermo Scientific (Rockford, IL, USA). The serum samples from healthy volunteers were provided by the 9th People's Hospital of Chongqing (Chongqing, China) and stored at −20 °C before usage. Other chemical reagents were obtained from Sinopharm Chemical Reagents, Co., Ltd. (Shanghai, China). All reagents involved in the tests were of analytical grade and directly used without further purification. All solutions were prepared by ultrapure water with specific resistance of 18.25 MΩ•cm.

Table 1 The sequences of the oligonucleotides used in the experiments.

Name	Sequence (from 5'to 3')
DNA1	5'-SH-$(CH_2)_6$-T_{38}-**CCTCAC**CACCCATGTTACTCTCT-3'
DNA2	5'-CTGATATCAGCGAT**GTGAGG**-T_{38}-$(CH_2)_6$-HS-3'
DNA2′ (4 bp)	5'-CTGATATCAGCGAT**GTGA**AA-T_{38}-$(CH_2)_6$-HS-3'
DNA2′ (6 bp)	5'-CTGATATCAGCGAT**GTGAGG**-T_{38}-$(CH_2)_6$-HS-3'
DNA2′ (8 bp)	5'-CTGATATCAGCGAT**GTGAGGAA**-T_{36}-$(CH_2)_6$-HS-3'
DNA2′ (10 bp)	5'-CTGATATCAGCGAT**GTGAGGAAAA**-T_{34}-$(CH_2)_6$-HS-3'
HS	5'-FAM-CACCACAGCATTAGAGAGTAT/rA/GGATATCAGC TTGACGTGGTG-Dabcyl-3'

*The binding regions between DNA1 and DNA2 or DNA2′ were shown in bold.

2.2. Preparation of DNA-conjugated AFP antibodies

The conjugation of the DNAs to the AFP antibodies was performed by following previous publication with slight modifications (Söderberg et al., 2006). Briefly, the anti-AFP antibody (2.0 mg mL^{-1}) was reacted with 30-fold excess of sulfo-SMCC for 2 h at room temperature in PBS (55 mM phosphate buffer, 150 mM NaCl, 20 mM EDTA, pH 7.2). Meanwhile, 15 µL of 100 µM thiolated DNA was incubated with 20 µL of 100 mM DTT in PBS for 1 h at 37 °C to reduce the disulfide bonds, followed by further purification by using the Illustra MicroSpin G-50 Columns (GE Healthcare). Next, the sulfo-SMCC activated antibodies were incubated with the DTT-treated DNAs at 4 °C

overnight. This was followed by dialyzing (Slide-ALyzer, Thermo Scientific) the mixture against PBS buffer overnight to obtain the DNA-anti-AFP conjugates.

2.3. AFP assay procedures

Prior to experiments, HS was heated to 90 °C for 5 min and subsequently cooled down to room temperature for 2 h to obtain stable hairpin loop–stem structure. Next, various concentrations of AFP were incubated with the mixture of DNA1-anti-AFP1 (100 nM), DNA2-anti-AFP2 (100 nM) and HS (200 nM) in 50 μL of Tris-HCl buffer (20 mM Tris-HCl, 100 mM NaCl, 10 mM KCl, 10 mM $MgCl_2$, pH 7.4) for 60 min in the dark at room temperature to allow the formation of Mg^{2+}-DNAzyme and the cyclic cleavage of HS. Then, the mixture was diluted with Tris-HCl buffer to a total volume of 200 μL, and fluorescence measurements were performed to obtain the data.

2.4. Fluorescence measurements

The fluorescence intensities of the solutions were measured on a RF-5301-PC fluorescence spectrophotometer (Shimadzu, Tokyo, Japan) by using a 150 W Xenon lamp (Ushio Inc., Japan) as the excitation source. The fluorescence emission spectra were collected in the range from 450 nm to 650 nm with the excitation wavelength of 490 nm. The excitation and emission slit widths were both set at 5 nm.

3. Results and discussion

3.1. Amplified fluorescent protein detection principle

Our proximity binding homogeneous immunoassay approach for amplified fluorescent detection of AFP is depicted in Scheme 1. In the design, DNA1 and DNA2 are conjugated to two antibodies that recognize the AFP target at different epitopes. The DNAs conjugated to the antibodies contain three functional regions: the $polyT_{38}$ sequence

(the black regions) next to the antibody to reduce the steric hindrance for partial hybridization between DNA1-anti-AFP1 and DNA2-anti-AFP2 upon the binding of the antibodies to AFP, the catalytic core (the red region) and arm (the green region) sequences of the DNAzymes. The fluorescently quenched hairpin DNA substrate (HS) containing the ribonucleobase (rA) recognition site in the loop region is used for signal generation. DNA1 and DNA2 are elaborately designed to contain 6 complementary base pairs after the polyT_{38} region, exhibiting a melting temperature (T_m) of 15.3 °C between DNA1 (100 nM) and DNA2 (100 nM, verified by the OligoAnalyzer 3.1 online software by Integrated DNA Technologies). Thus, under the experimental conditions (room temperature at 25 °C) and without the presence of the AFP target, DNA1-anti-AFP1 and DNA2-anti-AFP2 are unable to hybridize with each other to form the catalytic core necessary for the DNAzymes to function. In other words, the formation of the Mg^{2+}-dependent DNAzymes and the cleavage of the HS are inhibited, and minimal fluorescence emission is expected. However, in the presence of AFP, simultaneous binding of the antibody affinity ligands to the target protein brings DNA1-anti-AFP1 and DNA2-anti-AFP2 into close proximity, which greatly increases their local effective concentrations and accelerates the hybridization between their complementary sequences. In this case, the antigen-bound complex can be regarded as a stem-loop hairpin structure with 6 complementary base pairs as the stem and the polyT in the middle as the loop region (Tang et al., 2015), and the T_m value of the complex is estimated to be 42.6 °C, which is remarkably higher than the DNA1-DNA2 partial duplex (15.3 °C) alone. The sharp difference in T_m after the association with AFP enables the successful formation of the catalytic core component of the Mg^{2+}-dependent DNAzyme due to the binding

between the complementary regions of DNA1-anti-AFP1 and DNA2-anti-AFP2. Subsequently, the arm sequences bind HS to form Mg^{2+}-dependent DNAzymes, in which HS can be cleaved by the Mg^{2+} ion cofactor, leading to the separation of the fluorophore/quencher pair because of the low T_m (19.3 °C) of the cleaved partial dsDNA of HS) and the recovery of the fluorescent signal. In addition, the liberated catalytic core component upon the cleavage of HS again binds another intact HS to imitate cyclic cleavage of HS, resulting in significantly amplified fluorescent signals. In this way, quantitative detection of the AFP target protein can be successfully converted into the fluorescent signal readout.

Scheme 1

3.2. Feasibility demonstration of the method

The fluorescent emissions of different solutions were recorded to verify the feasibility of our strategy for sensitive AFP detection. Take the fact into consideration that at high concentrations of DNA1-anti-AFP1 and DNA2-anti-AFP2, they could potentially hybridize with each other spontaneously even without the presence of the target AFP, which could lead to the increase in background noise and interfere the final assay results. In addition, due to the enzymatic multiple turnover strategy, once the DNAzymes are formed, they can be repeatedly used in cyclic cleavage of many HS. Thus, there should be excessive HS in solution. Based on these facts, the concentrations of DNA1-anti-AFP1, DNA2-anti-AFP2 and HS were fixed at 100 nM, 100 nM, 200 nM, respectively. The fluorescent emission signals were obtained by incubating the mixtures at room temperature for 60 min.

As shown in Fig. 1, the solution of HS shows a small fluorescent emission peak (curve a) at 520 nm, revealing the sufficient quenching of FAM by Dabcyl. The mixture of DNA1-anti-AFP1, DNA2-anti-AFP2 and HS causes a neglect change in fluorescence intensity (curve b) compared to the HS solution, indicating the co-existence of the three components. As expected, the addition of the target AFP (300 pM) to the above mixture leads to a significant increase in fluorescence intensity (curve c). Such apparent fluorescence intensity difference between the absence and presence of AFP demonstrates that only in the presence of the target AFP can lead to the formation of Mg^{2+}-dependent DNAzymes and cyclic cleavage of HS to generate significantly amplified fluorescent signals. Such comparison also shows the promising potential of the strategy for sensitive detection of AFP.

Figure 1

3.3 Experimental optimizations

To minimize the background signal and to achieve the best performance of the sensing system, the complementary base pairs between DNA1-anti-AFP1 and DNA2-anti-AFP2 and incubation time were subsequently optimized. It should be noted that the optimization process is achieved by keeping other parameters unchanged except for the tested ones. The length of the stem sequences between DNA1-anti-AFP1 and DNA2-anti-AFP2 was first optimized. According to Fig. 2A, In the presence of 300 pM AFP (with 60 min reaction time), we can see that the increase in the complementary base pairs from 4 to 10 leads to the increase of the fluorescence intensity (red bars in Fig. 2A). However, the background noise also increases (black bars in Fig. 2A) accordingly due to part of the non-target induced assembly process. That is to say, with longer

complementary bases, DNA1 of DNA1-anti-AFP1 could spontaneously hybridize with DNA2 of DNA2-anti-AFP2 even without the help of the target AFP, which produces a large background noise (F_0) and a lower sensor sensitivity. The maximum ratio of fluorescence and background signal (F/F_0) is observed at 6 base pairs, which is chosen for subsequent assays. The incubation time is also a key parameter to affect the analytical performance including both the amount of the formed Mg^{2+}-DNAzymes and the cleavage of HS. We investigated the reaction time from 20 to 70 min with a time interval of 10 min. As depicted in Fig. 2B, in the presence of AFP (300 pM), the fluorescence intensity increases quickly with the tested time scale from 20 to 50 min, and reaches a plateau thereafter. Therefore, 50 min is chosen as the optimal reaction time for the assay protocol.

Figure 2

3.4 Analytical performance of the sensing method

Under optimized experimental conditions, the analytical performance of the method for quantitative detection of AFP was evaluated with various concentrations of AFP. From Fig. 3A, we can clearly see that the fluorescent emission intensity gradually increases with increasing concentration of AFP from 0 pM to 500 pM (from curves a to i), indicating that AFP plays a major role in the formation of the Mg^{2+}-DNAzyme structures. When plotting the fluorescence intensity vs. the concentration of AFP in the range from 2 pM to 500 pM, a calibration curve is obtained with a regression equation of $y = 1.0138\ x + 106.69$ (y and x represent the fluorescence signal and the concentration of AFP, respectively) and a correlation coefficient (R^2) of 0.9984 (Fig. 3B). The detection limit of AFP is calculated to be 1.8 pM based on the 3σ rule. Such detection limit shows significant improvement over conventional ELISA-based methods (Xing et al., 2012) and

is comparable with other previously reported methods coupled with complex signal amplification approaches (Zhang et al., 2016; Li et al., 2014) for AFP detection. Besides, our method also has a good reproducibility with six parallel measurements of AFP at the standard deviation of 6.4%.

Selectivity is also an essential factor to access the analytical performance of the sensing method. Other interfering proteins, including mouse IgG, carcinoembryonic antigen (CEA), thrombin, were examined under the optimized conditions to evaluate the selectivity of the method. As shown in Fig. 3C, in the addition of the non-target proteins (each at the concentration of 3 nM, 10-fold excess over the concentration of target AFP) to the sensing solution, the fluorescence intensities are almost similar to the blank test (in the absence of target protein). In contrast, with much lower concentration of target AFP (0.3 nM), substantial increase in fluorescence intensity be observed. Such an obvious comparison demonstrates that only the target AFP can induce the assembly process to form the active DNAzyme structures and to amplify the fluorescence signal, which verifies the specificity of the developed method.

Figure 3

3.5 Detection of AFP in real samples

The feasibility and the potential applicability of the sensing method for detecting AFP in real complex samples was also examined. Based on the developed sensing method, recovery tests for AFP in 10-fold Tris-HCl buffer diluted human serum samples from healthy volunteers were performed, and the final results are shown in Table 2. It is clear that the method exhibits good recovery rates for AFP in the range of 96.4-103.8% with

the relative standard deviations below 3.1%, demonstrating that the proposed method has great potential for monitoring AFP in human serum samples

Table 2. Recovery tests for AFP in 10% diluted serum samples (n=6).

Samples	Added (pM)	Found[a] (pM)	Rate of recovery (%)	RSD (%)
0	0	53.2	/	3.0
1	10.0	62.8	96.4-100.2	3.1
2	50.0	104.3	100.5-103.8	2.5
3	100.0	153.6	101.2-103.5	2.3

[a] The average value of six measurements.

4. Conclusions

In conclusion, we have demonstrated that homogeneous and enzyme-free amplification detection of low levels of AFP cancer biomarkers can be achieved by coupling immuno-proximity binding and Mg^{2+}-dependent DNAzyme recycling signal enhancement. The binding of the DNA-conjugated antibodies to the AFP target molecules leads to the formation of Mg^{2+}-dependent DNAzymes, in which the fluorescently quenched hairpin substrate sequences are cyclically cleaved to generate significantly amplified signals for realizing sensitive detection of AFP down to 1.8 pM. Due to the specific recognition between the AFP antigens and antibodies，the method possesses high selectivity and can be applied to detect AFP in diluted human serum samples. Besides, our method avoids the use of enzymes or nanomaterials for signal amplification, eliminating the cumbersome separation steps or complicated synthesis process for the nanomaterials in other commonly reported approaches. With these

advantages, the method demonstrated herein can be extended as a general sensitive and homogeneous sensing platform for detecting various protein cancer biomarkers upon choosing the appropriate affinity pairs.

Acknowledgment

This work was supported by National Natural Science Foundation of China (Nos. 21275004 and 21675128), the New Century Excellent Talent Program of MOE (NCET-12-0932) and Fundamental Research Funds for the Central Universities (XDJK2014A012 and XDJK2015A002).

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Figure captions

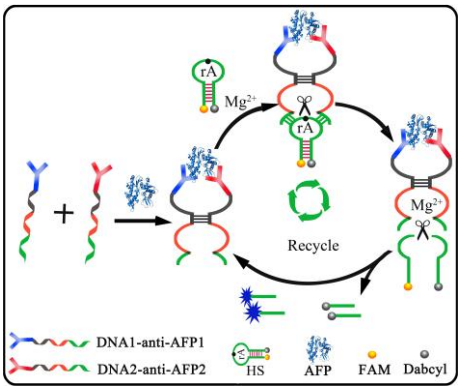
Scheme 1. Schematic principle for homogeneous and sensitive fluorescent detection of AFP based on Mg^{2+}-dependent DNAzyme recycling amplification and immuno-proximity binding.

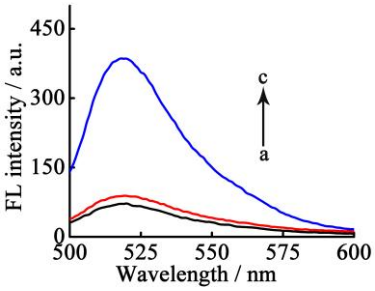
Fig. 1. Fluorescent emission spectra of different solutions incubated at room temperature for 60 min: (a) HS; (b) DNA1-anti-AFP1, DNA2-anti-AFP2 and HS; (c) AFP,

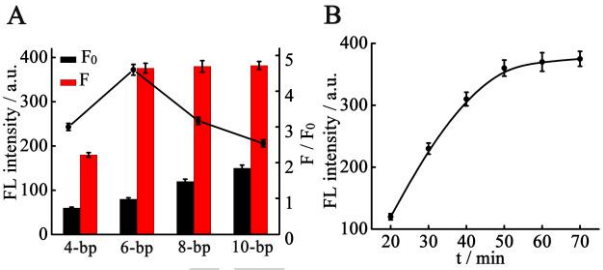
DNA1-anti-AFP1, DNA2-anti-AFP2 and HS. The concentrations of HS, DNA1-anti-AFP1, DNA2-anti-AFP2 and AFP were 200 nM, 100 nM, 100 nM, and 300 pM, respectively.

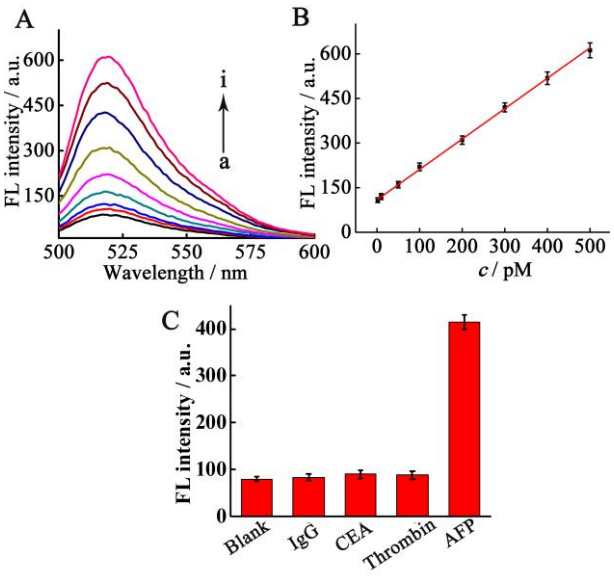
Fig. 2. (A) Effect of the length of the complementary sequence between DNA1-anti-AFP1 and DNA2-anti-AFP2 (with 60 min incubation time) on the fluorescence intensity. The histogram F (red) and F_0 (black) corresponded to the fluorescence responses of the solution in the presence or absence of the target AFP (300 pM), respectively. The black curve represented the signal-to-background ratio (F/F_0) under the corresponding conditions. (B) Effect of incubation time from 20 to 70 minute with a time interval of 10 minute (with 6 base pairs of the stem sequence) on the fluorescence intensity. Error bars represented the standard deviations of three measurements.

Fig. 3. (A) The fluorescence emission spectra of the proposed method for detecting various concentrations of AFP. From a to i: (a) 0 pM, (b) 2 pM, (c) 10 pM, (d) 50 pM, (e) 100 pM, (f) 200 pM, (g) 300 pM, (h) 400 pM, (i) 500 pM. (B) The corresponding calibration plot of the concentration of AFP vs. the fluorescence intensity in the range from 2 pM to 500 pM. Error bars represented the standard deviations of three measurements. (C) Specificity evaluation of the proposed method for the detection of the target AFP over other interfering proteins (IgG, CEA and thrombin). The concentration of AFP was 0.3 nM and all other proteins were 3 nM.









Highlights of our manuscript include:

● The binding of DNA-linked antibodies to antigen leads to the formation of DNAzymes.

● Cyclic cleavage of DNAzymes generates significantly amplified detection signals.

● Enzyme-free amplified detection of proteins in human serums can be realized.