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Sensitive Detection of cytokine in Complex Biological Samples by using MB track mediated DNA walker and nicking enzyme assisted signal amplification method combined biosensor

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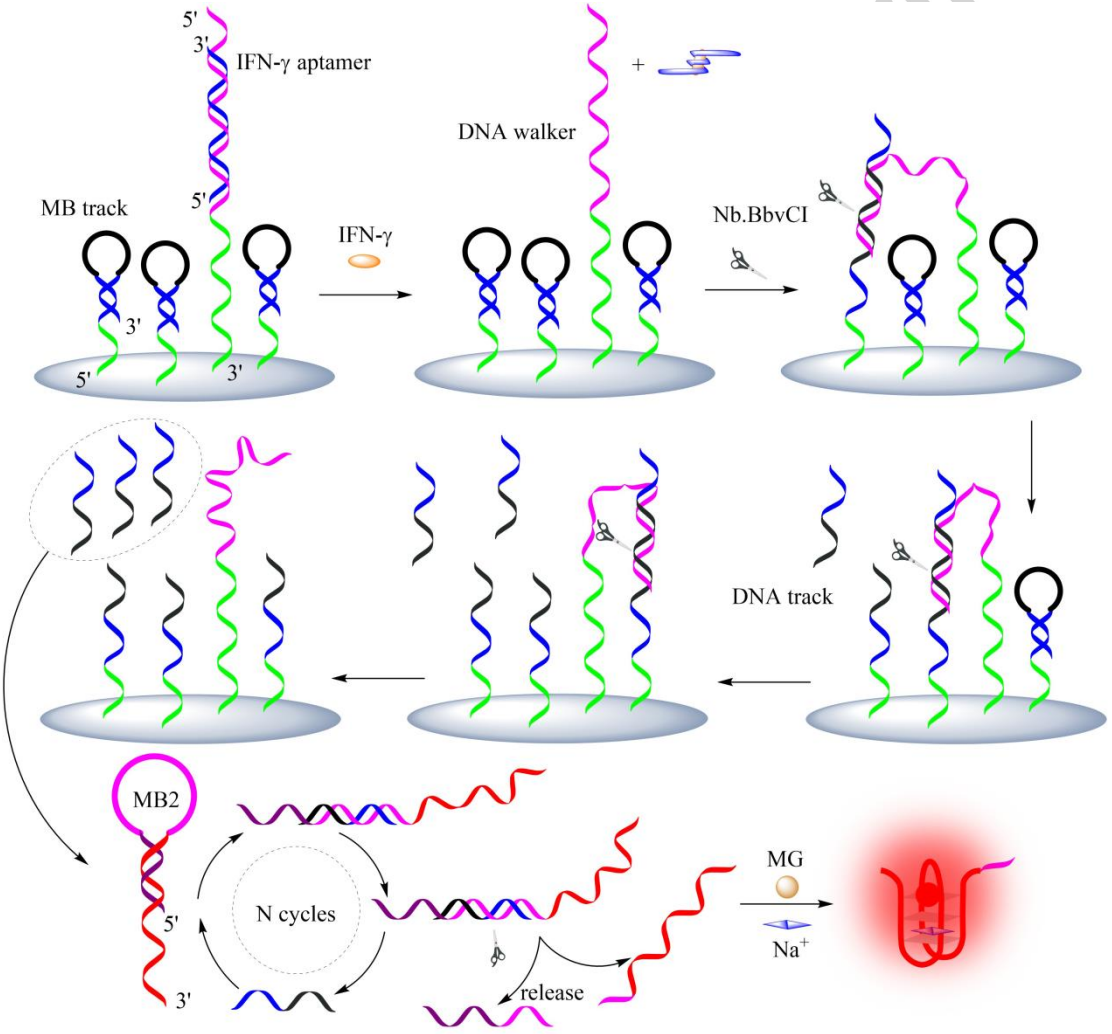
Abstract

Highly sensitive detection of proteins is essential to biomedical research as well as clinical diagnosis. However, usual detecting methods are complicated operating, time-costing and extensive label preparing. Here, we combined molecular beacon (MB) track mediated DNA walker and nicking enzyme assisted signal amplification method to develop a simple and ultrasensitive malachite green fluorescence biosensor for specific detection cytokine, interferon-γ (IFN-γ). The association of the IFN-γ with the corresponding aptamers of the dsDNA strands leads to free of DNA walker which trigged the generation of DNA track at the help of Nicking endonuclease (Nb.BbvCI).

The released MB track opens MB2 to form MB track/MB2 duplex and the duplex cleaved the MB track with the help of Nb.BbvCI. The DNA track is subsequently released to hybridize with another MB2. And the cleavage also generates the G-rich oligomer, this released G-rich oligomer folds into a G-quadruplex structure and thus allows the formation of a fluorescence transducer in the presence of Malachite green (MG). The formed fluorescence transducer can give a high fluorescence intensity. So, one IFN-γ can initiate the cleavage of numerous MB track and MB2, resulting in the highly sensitive detection of IFN-γ with the detection limit of 7.65 fM. This new methodology can be expected to provide a highly sensitive platform for the amplified analysis of various target molecules.

Graphical abstract



Keywords: cytokine; molecular machine; amplification method; malachite green; cell

lysates

1. Introduction

Molecular beacon (MB), a hairpin-shaped single-stranded oligonucleotide, comprised of antisense oligonucleotide labeled with a “reporter” fluorophore at one end and a quencher at the other end, are being increasingly adopted as promising tools for the detection and analysis of a given nucleic acid sequence in solution.[1-5] Because of their advantages of nontoxic, high sensitivity, and especially excellent selectivity to differentiate single-base mismatched targets, MBs have been widely used in different areas including oligonucleotide assay, and real-time gene monitoring. [1, 2, 5, 6] Interestingly, although molecular beacons were originally developed to assay oligonucleotide, more molecular beacons employed strategies were used for the detection of protein. [3, 4, 7, 8] Such molecular beacons-assisted approaches, with its inherent stability, specificity, and simplicity, has recently emerged as a potential amplification technique for rapid and cost-effective detection of protein.

On the other hand, inspired by nature, various artificial molecular machines have been created, including DNA walkers, molecular gears, and molecular spiders. [9-14] The success in constructing such machines proves DNA to be versatile and robust building blocks for artificial devices due to the well-defined Watson–Crick base pairing rule. The autonomous movements of molecular machines consume energy. One of the most common used energy inputs for DNA walking machines is through the cleavage of DNA substrates by using endonucleases or deoxyribozymes. During DNA walking, multiple substrates will be released as a result of the cleavage. [15-19] Recently, Li et al. reported an interesting three-Dimensional DNA nanomachine which based DNA walking to build a biosensor. By using this biosensor, they specifically detect nucleic acid target by incorporating a toehold exchange mechanism with high sensitivity.[19]

Although much effort has been emphasized on utilizing such DNA walking devices to achieve DNA computing or DNA biosensing, there are fewer reports of using DNA walking machines for building biosensors for protein assay. To this end, we aim to design a DNA nanomachine that is amenable for tasks including rapid and sensitive protein assay.

Recently, various amplification methods have been established for sensitive detection of target DNA, RNA or protein, such as nicking enzyme assisted signal amplification, strand displacement amplification, hybridization chain reaction (HCR), rolling circle amplification (RCA) and catalytic hairpin assembly (CHA). [20-37] Among them, nicking enzyme assisted signal amplification, as an ideal isothermal amplification technique, has been used in a variety of assays owing to its simplicity and high efficiency. [38] However, the sensitivity of the nicking enzyme assisted isothermal amplification technique based biosensors is not perfect. To achieve ultrasensitive detection, multiple-stages amplification method has been developed derived from nicking enzyme assisted signal amplification, which is an isothermal amplification through an oligonucleotide strands generation. [39-42] The performance of multiple-stages amplification method is simple and cost-effective compared with PCR, which has been widely applied for sensitive detection of viral RNA, gene mutation and proteins. MB mediated amplification strategy, as another isothermal DNA amplification method, can further improve the specificity and sensitivity of the biosensor. As an efficient and specific hybridization assay, it has become an essential strategy for oligonucleotide or protein detection and quantification in the process of designing DNA or protein sensors.

Label-free sensors combining fluorescence enhancement of dyes by intercalation with oligonucleotides have been widely used. Malachite green (MG) is a positively charged triphenylmethane (TPM) dyes with almost no fluorescence when in solution. [1, 43, 44] It has been reported that the fluorescence of MG increased more than

hundreds of fold when MG was bound to the aptamer or G-quadruplex. [45-49] This strong enhancement of fluorescent signal was utilized to design label-free fluorescent sensors.

In this study, we combined MB track mediated DNA walker and nicking enzyme assisted signal amplification method to develop a simple and ultrasensitive malachite green fluorescence biosensor for specific detection of IFN-γ, in which target recycling and isothermal signal amplification are successfully achieved. Compare with other fluorescent, colorimetric and electrochemical methods for the detection of IFN-γ, our strategy exhibits superior specificity towards target IFN-γ and has a lower detection limit. Due to these advantages of this method, it may provide a simple and powerful platform for IFN-γ screening in bioanalysis and clinical diagnosis.

2. Experimental Section

2.1 Reagents.

IFN-γ was obtained from Takara (Dalian, China). The strand sequences were purchased from Genscript Biotech. Co., Ltd. (Nanjing, China) with the sequences as shown in Table 1. Nicking endonuclease (Nb.BbvCI) and 10 ×NEBuffer buffer were purchased from New England Biolabs (Beijing, China). Malachite Green (MG) was obtained from J&K Scientific Ltd. (Shanghai, China). Fluorescence was measured by RF-5301PC Spectrofluorophotometer (Shimadzu, Japan). The samples were excited at 590 nm. All solutions were prepared with Milli-Q (Branstead) purified double distilled water having specific resistance of > 18 MΩ cm.

2.2 Preparation of DNA walker/IFN-γ aptamer duplex, MB track and MB2

Firstly, the mixture of IFN-γ aptamer and DNA walker in 14 μL 10 mM phosphate buffer solution (PBS, 0.1 M NaCl, pH 7.4), the solution of MB track and the solution of MB2 were heated to 90 °C for 10 min and allowed to cool to room temperature in each tube for about 4 h to form the partially complementary dsDNA strands and the

stem-loop structure DNA, respectively.

2.3 Preparation of DNA walker/IFN-γ aptamer duplex and MB track modified plate

In a typical experiment, the DNA walker/IFN-γ aptamer duplex and MB track oligonucleotides were mixed at a ratio of 1 to 20 as previous report. [19] So, to immobilize DNA walker/IFN-γ aptamer duplex and MB track on the 96 well microtiter plate, 200 μL DNA walker/IFN-γ aptamer duplex (1 μM) and MB track (0.05 μM) in phosphate buffer (0.1 M NaCl, pH 7.4) was added in each well (with an active ester surface) by incubation at room temperature for 12 h. After rinsing each well twice with 300 μL of washing buffer ((2.7 mM KCl, 1.47 mM KH_2PO_4, 137 mM NaCl, 8.1 mM Na_2HPO_4, and 0.05 w/v % Tween-20), the plate was coated with 300 μL blocking buffer (2.7 mM KCl, 1.47 mM KH_2PO_4, 137 mM NaCl, 8.1 mM Na_2HPO_4 and 0.1 w/w % bovine serum albumin (BSA)).

2.4 Polyacrylamide hydrogel electrophoresis.

Nondenaturing polyacrylamide gel electrophoresis (PAGE) (15%) was carried out in 1× TBE buffer at a 120 V constant voltage for 120 min at room temperature. The mixture of DNA walker and IFN-γ aptamer (1 μM each) was heated to 90 °C for 10 min and allowed to cool to room temperature for about 5 h to form the dsDNA. Then the above solution was incubated with IFN-γ aptamer (1 μM) at 37 °C for 30 min. The gel was taken photograph under UV light after staining with GelRed for 10 min.

2.5 Amplification reactions

Next, the IFN-γ (200 μL) with different original concentrations was added to each well and incubated at 37 °C for 30 min in 270 μL of PBS buffer (200 mM NaCl, 100 mM sodium phosphate buffer solution (pH 7.4)). Subsequently, the solution was incubated with 30 μL of 10× NEBuffer solution and 10 U Nb.BbvCI at 37 °C for 90 min. Finally, the resulting mixtures were heated at 80 °C for 20 min to deactivate the

Nb.BbvCI. And then cool to room temperature for about 4 h. Samples were then incubated with 1 μM MG at room temperature for 1 h. After these steps, fluorescence intensities were measured by using Spectrofluorophotometer.

3. Results and discussion

3.1 The working principle

The working principle of the MB track mediated DNA walker and nicking enzyme assisted signal amplification method is illustrated in Scheme 1. In this strategy, two molecular beacons (MBs), which named MB track and MB2, were ingeniously designed. MB track mainly consists a middle loop region, contains the specific nicking cleavage sequence 5'-GC!TGAGG-3' (! is the cleavage site) of Nb.BbvCI, which was designed to hybridize DNA walker. The DNA walker is designed to have a 23 nt sequence to open the MB track and 26 nt sequence to hybridization with IFN-γ aptamer (the sequence in bold). MB2 consists of three fragments (we employed the sequences as our pervious report[43]): a G-quadruplex-forming sequence at the 3' end; an 8 nt sequence "interfering tail" at the 5' end that is complementary to part of the G-rich sequence which prohibits the formation of the G-quadruplex; and a middle loop region that was designed to probe DNA track. In the absence of IFN-γ, the G-quadruplex shows low fluorescence intensity since MB track and DNA walker will not be recognized and cleaved by Nb.BbvCI, which will not trigger the cycle reaction. It should be noted that a 9 base sequence (table 1) was well-designed which can hybridize with G-quadruplex-forming sequence effectively to inhibit the G-quadruplex formation in the present of K^+ and MG. [43] Upon addition of MG, the fluorescence intensity was very weak due to the weak binding ability of the locked G-quadruplex to MG. When the target IFN-γ is introduced into the sensing system, it associates with IFN-γ to form IFN-γ aptamer complexes and releases the free DNA

walker from the DNA walker/IFN-γ aptamer duplexes. The hybridization of the DNA walker with the loop part of DNA track opens the DNA track to form the double stranded DNA including the specific cleavage site, and hence cleaved the MB track with the help of Nb.BbvCI, resulting in the release of the DNA track. Then, the free DNA walker hybridized with a new MB track and therefore triggered the next circulation of cleavage. By this mean, multiple DNA track was released. The released MB track opens MB2 to form MB track/MB2 duplex and the duplex cleaved the MB track with the help of Nb.BbvCI. The DNA track was subsequently released to hybridize with another MB2. And the cleavage also generates the G-rich oligomer, this released G-rich oligomer folds into a G-quadruplex structure and thus allows the formation of a fluorescence transducer in the presence of MG. The formed fluorescence transducer can give a high fluorescence intensity. So, one IFN-γ can initiate the cleavage of numerous MB track and MB2, resulting in the highly sensitive detection of IFN-γ.

3.2 Feasibility Study

To further confirm the availability of the DNA walker and isothermal amplification biosensor, the resulting products were first detected by using a fluorescence technique via the mechanism that MG can be embedded into G-quadruplex and thus yields a strong fluorescence signal intensity. [43, 45, 47, 49] According to Fig. 1A, a low fluorescence signal was recorded when the DNA walker/IFN-γ aptamer duplex and MB track plate separately lacked the MB2, Nb.BbvCI, or IFN-γ (curves a, b, and c) even treated with MG, and a high fluorescence intensity was observed in the presence of these compounds simultaneously (curve d) after adding MG. These results indicate that the dual amplification strategy occurs and large amounts of DNA track in the first DNA walking cycle and large amounts of G-quadruplex in the second cycle are produced. These results indicate that (1) the DNA walker/IFN-γ aptamer duplex can be activated by the target IFN-γ, (2) the DNA walker can open the MB track and the

DNA walkers are well established and (3) the MB2 based Nb.BbvCI based isothermal amplification was all well established.

Although the DNA walker opens the MB track with a high ability, it may have low activity against long stem duplexes of MB track. Therefore, we shortened the stem sequences to less bases. The fluorescence signal to background ratio (Fsig/Fback) was highly sensitive to the length of stem part of MB track (Fig. 1B). 100 pM IFN-γ were employed and a series of MB track by changing the length of stem from 6 to 10 base pairs (Table 1). As shown in Fig. 1A, the best fluorescence signal to background ratio was obtained when the length stem is 8 base pairs. This interesting phenomenon may be caused by the low melting temperatures (Tm) stability of the hairpin structure (6 base pairs (MB track a) and 7 base pairs (MB track b)) and the high stability of the long stem (9 base pairs (MB track c) and 10 base pairs (MB track d)). Therefore, we chose the stem length of 8 base pairs for the biosensing in this study.

To understand the time-dependent signal change of the method, kinetic studies have been performed by obtained the fluorescence intensity at designated time points after addition of 10 U Nb.BbvCI for amplification step evaluating. As can be observed in Fig. 1C, most of the fluorescence change occurred in the first 60 min and the signals reached stable in about 90 min. Therefore, 90 min was enough for the two-stage amplification reaction in this experiment and we chose the 90 min reaction time for the assay.

The DNA walker/IFN-γ aptamer duplex and IFN-γ reaction was then verified by polyacrylamide gel electrophoresis (PAGE) (Fig. 1D). As shown in the picture, after incubation with IFN-γ, DNA walker band was observed (Lane b). In contrast, no distinguishable DNA walker band was observed without the addition of IFN-γ (Lane a). This study proved that the walker/IFN-γ aptamer duplex effectively bound the IFN-γ and released the DNA walker.

3.3 Sensitivity of the Sensing System

To test whether the DNA walker based sensor can be used to detect IFN-γ effectively and quantitatively, experiments were carried out and the results were depicted in Fig. 2. As shown in Fig. 2A, the fluorescence signal intensity increased when the concentration of target IFN-γ was increased from 0 to 100 pM, indicating that the release of the G-quadruplex strand is highly dependent on the concentration of target IFN-γ. Fig. 2B shows the corresponding calibration plot of concentration of IFN-γ versus the G-quadruplex/MG complex fluorescence intensity. The fitting equation of the range of linearity curve 0 fM to 20 pM shown in Fig. 2B insert is Y = 175.27 + 0.073X (R^2 = 0.994), where Y is the MG fluorescence intensity and X is the concentration of target IFN-γ. The detection limit was calculated to be 76.53 fM according to the responses of the blank tests add 3 times the standard deviation (3σ) according to the equation. This detection limit for cytokines is even lower than some other existing fluorescence signal amplification methods. [50, 51]

3.4 The specificity test

The selectivity of this assay was further investigated by adding IFN-γ, thrombin, alpha fetoprotein (AFP), and carcinoembryonic antigen (CEA) with the same concentration (100 pM) into the reaction system. As shown in Fig. 3, low fluorescence intensity was detected in the presence of thrombin, AFP, and CEA, which was slightly higher than that in the control experiment, whereas a significant increase of fluorescence intensity was observed when IFN-γ was present. The results demonstrated that the well-designed biosensing approach showed a high selectivity toward the target IFN-γ. Thus, the as-proposed homogeneous amplification IFN-γ

assay exhibited high sequence specificity to discriminate target IFN-γ from other proteins.

3.5 Measurement of IFN-γ in human serum

We also challenged our sensor for detecting target IFN-γ in a complicated sample matrix by spiking targets of varying concentrations into human serum samples of different dilution levels (10-fold-diluted, 5-fold-diluted, 2-fold-diluted, and undiluted). As shown in Fig. 4, the sensors are functional in different time-diluted human serum samples. The results which were compared with the standard curve in buffer solution at one time are given in Fig. 5. If there is little or even no matrix interference in nuclear extract, the curve from the dilution experiment should be parallel with the standard cure of IFN-γ in buffer. Also, Fig. 5 indicated that the interference from the diluted human serum analysis was negligible. Table S1 shows the experimental results obtained in IFN-γ-spiked serum samples. IFN-γ concentration recoveries of 95.29–118.08% were achieved (Accuracy in table S1). These results showed that the interference of serum could be overcome. Therefore, the as proposed biosensor exhibits excellent selectivity for IFN-γ detection and has great potential to be applied in clinical tests.

The measurements of the biosensor variability were utilized to determine the precision of the method. Four different concentrations (50, 100, 500 and 1000 fM) of IFN-γ were prepared in human serum samples of different dilution levels (10-fold-diluted, 5-fold-diluted, 2-fold-diluted, and undiluted). The relative standard deviation (RSD) was used as a measure of precision. The precisions are shown in Table 1 where the RSDs ranged from 1.81% to 2.99%. These results showed that this biosensor has a high precision.

4. Conclusions

In summary, we have developed a DNA walker biosensor for sensitive detection of IFN-γ coupling the Nb.BbvCI-aided isothermal amplification strategy with the fluorescence property of MG bond G-quadruplex. In addition to good sensitivity for IFN-γ assay, this biosensor system exhibits excellent selectivity to distinguish proteins. Furthermore, this method exhibits additional advantages of feature of easy operation. Therefore, the as-proposed DNA walker fluorescence IFN-γ assay may become an alternative method for cytokines assay and has great potential to be applied in cytokines-related clinical diagnostics and biochemical research.

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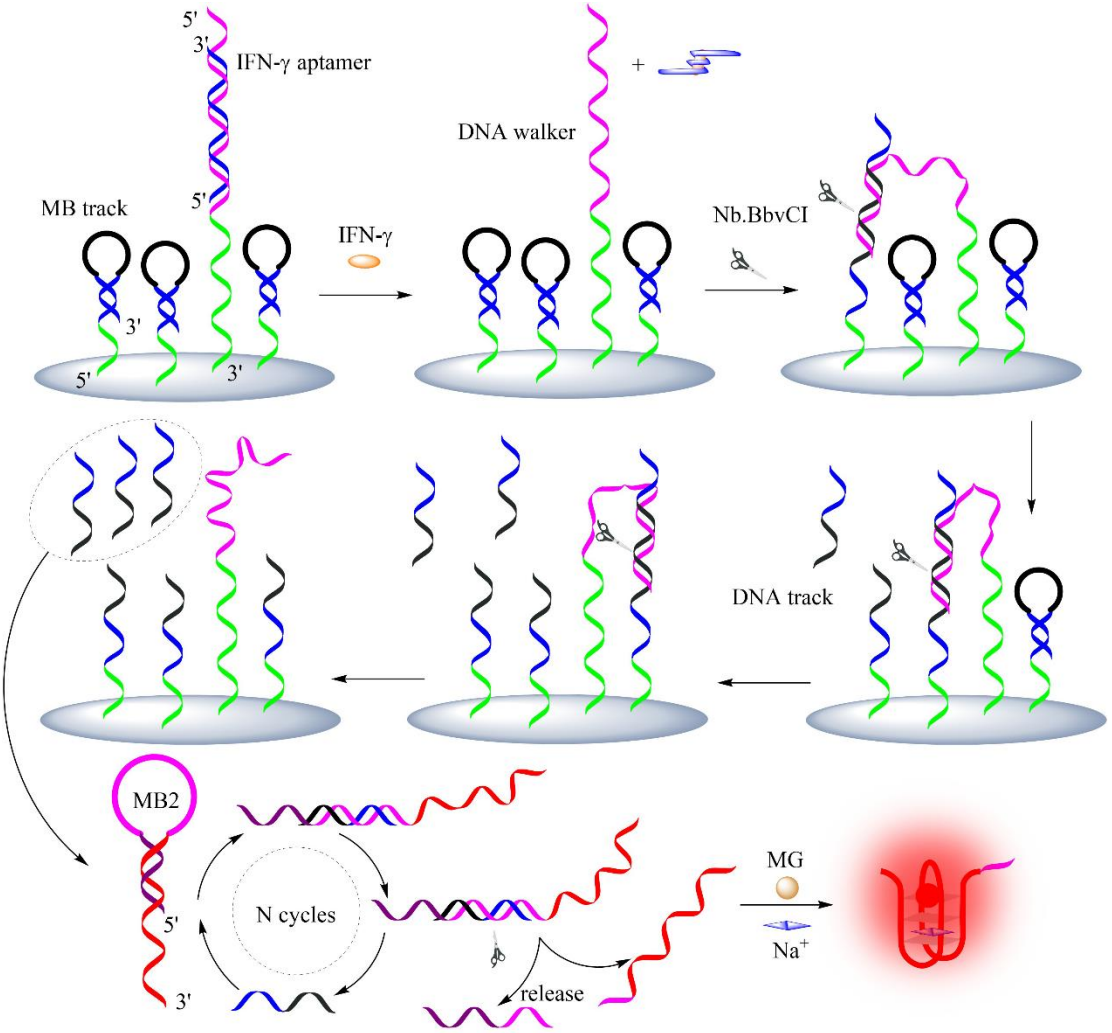

Scheme 1. Schematic diagram showing the principle of DNA walker and nicking enzyme assisted signal amplification method based biosensor for simple and ultrasensitive detection of IFN-γ.

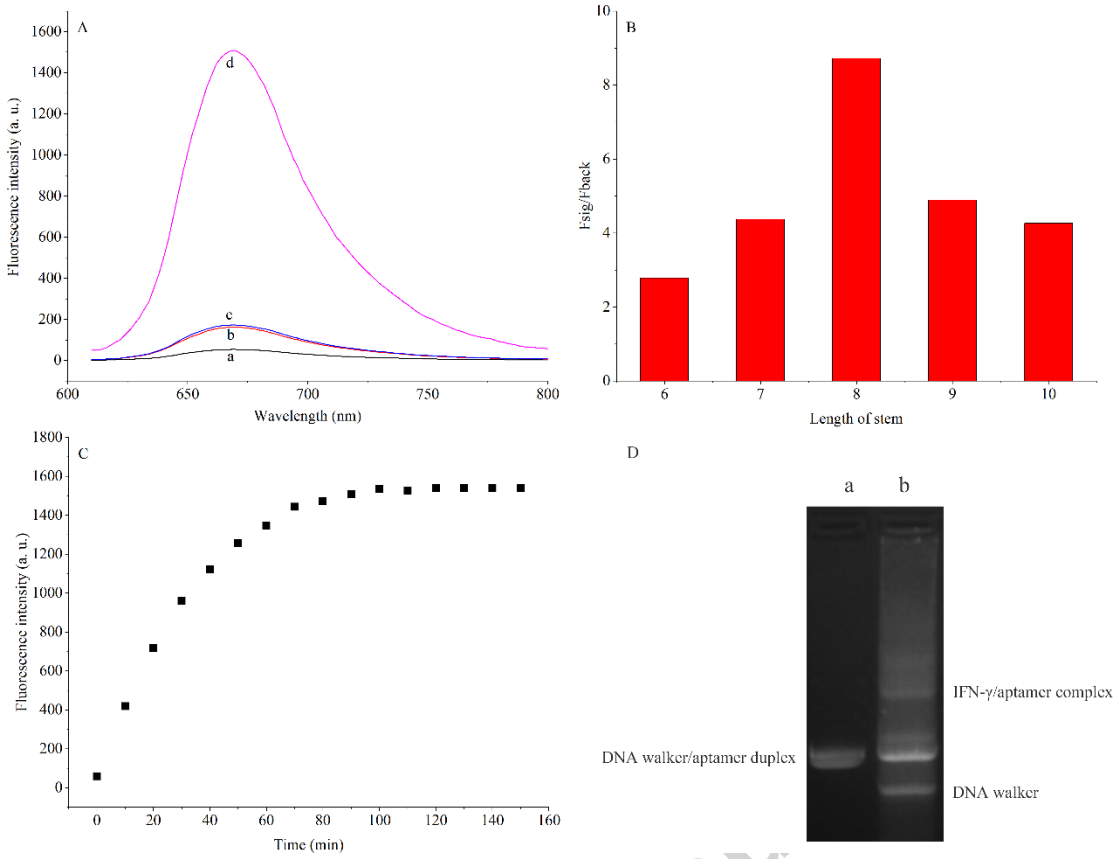

Fig. 1. Optimization of the detection conditions: (A) The fluorescence emission under different conditions: DNA walker/ IFN-γ aptamer duplex and MB track plate incubated with 100 pM IFN-γ and 10 U Nb.BbvCI (curve a), DNA walker/IFN-γ aptamer duplex and MB track plate incubated with 100 pM IFN-γ and MB2 (curve b), DNA walker/IFN-γ aptamer duplex and MB track plate incubated with MB2 and 10 U Nb.BbvCI (curve c) and DNA walker/IFN-γ aptamer duplex and MB track plate incubated with MB2, 10 U Nb.BbvCI and 100 pM IFN-γ (curve d). All the samples were incubated with 1 μM MG at room temperature for 1 h. (B) The performance of the assay was evaluated by the signal-to-background (S/B) ratio of different length of the stem of MB track. (C) Fluorescence intensity versus the time in the presence of 100 pM IFN-γ after addition of 10 U Nb.BbvCI. (D) Nondenaturing polyacrylamide gel electrophoresis (PAGE). Lane a: DNA walker/IFN-γ aptamer duplex (1 μM), and Lane b: DNA walker/IFN-γ aptamer duplex treated with 1 μM IFN-γ.

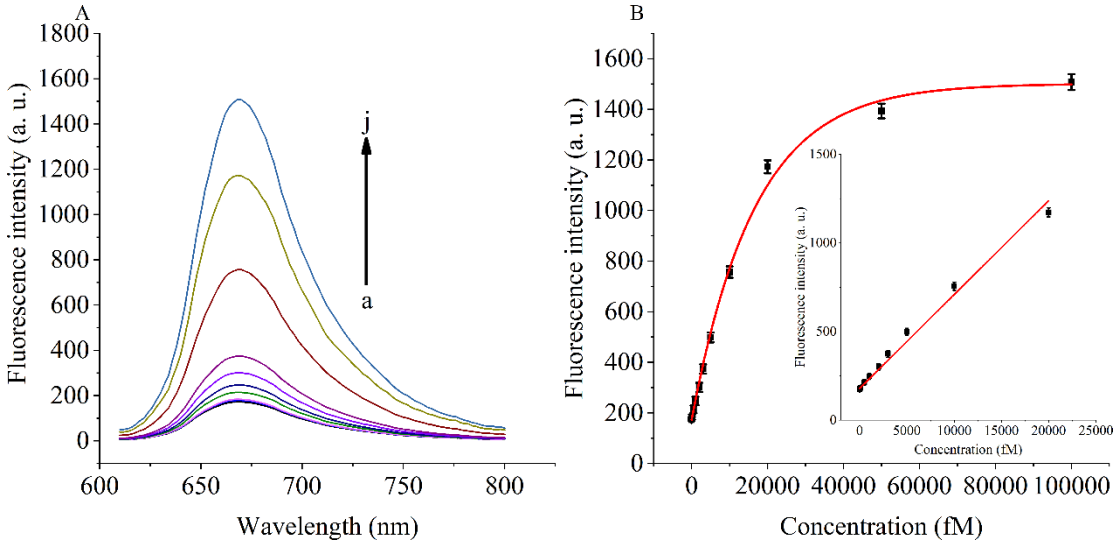

Fig. 2. (A) Fluorescence emission spectra at different IFN-γ concentration (0, 50 fM, 100 fM, 500 fM, 1 pM, 2 pM, 3 pM, 10 pM, 20 pM and 100 pM from a to j). (B) Relationship between the fluorescence intensity and the concentration of IFN-γ. The inset shows the linear relationship over the concentration ranging from 0 to 10 pM. All the data were obtained from three independent experiments and error bars denote standard deviations.

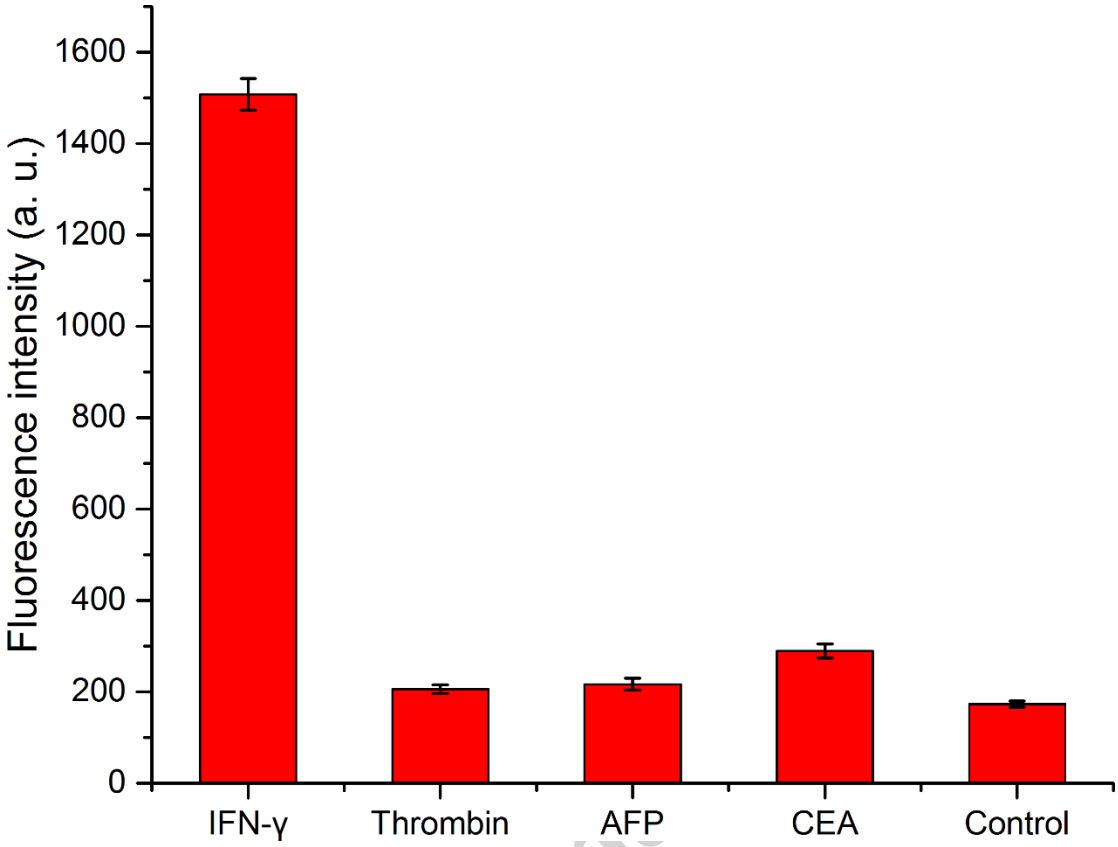

Fig. 3. Test of IFN-γ and other proteins, showing the specificity of the assay. The concentration of the proteins was all 100 pM.

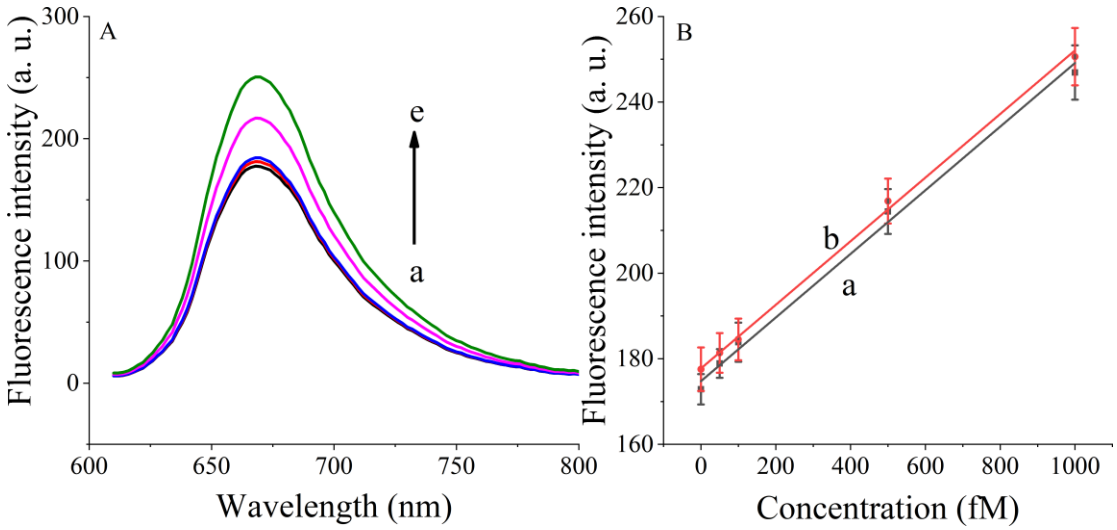

Fig. 4. (A) Fluorescence emission spectra at different IFN-γ concentration added in 10-fold-diluted human serum samples (0, 50 fM, 100 fM, 500 fM, and 1000 fM from a to e). (B) Relationship between the fluorescence intensity and the concentration of IFN-γ added in 10-fold-diluted human serum samples (b) and in buffer solution (a). All the data were obtained from three independent experiments and error bars denote standard deviations.

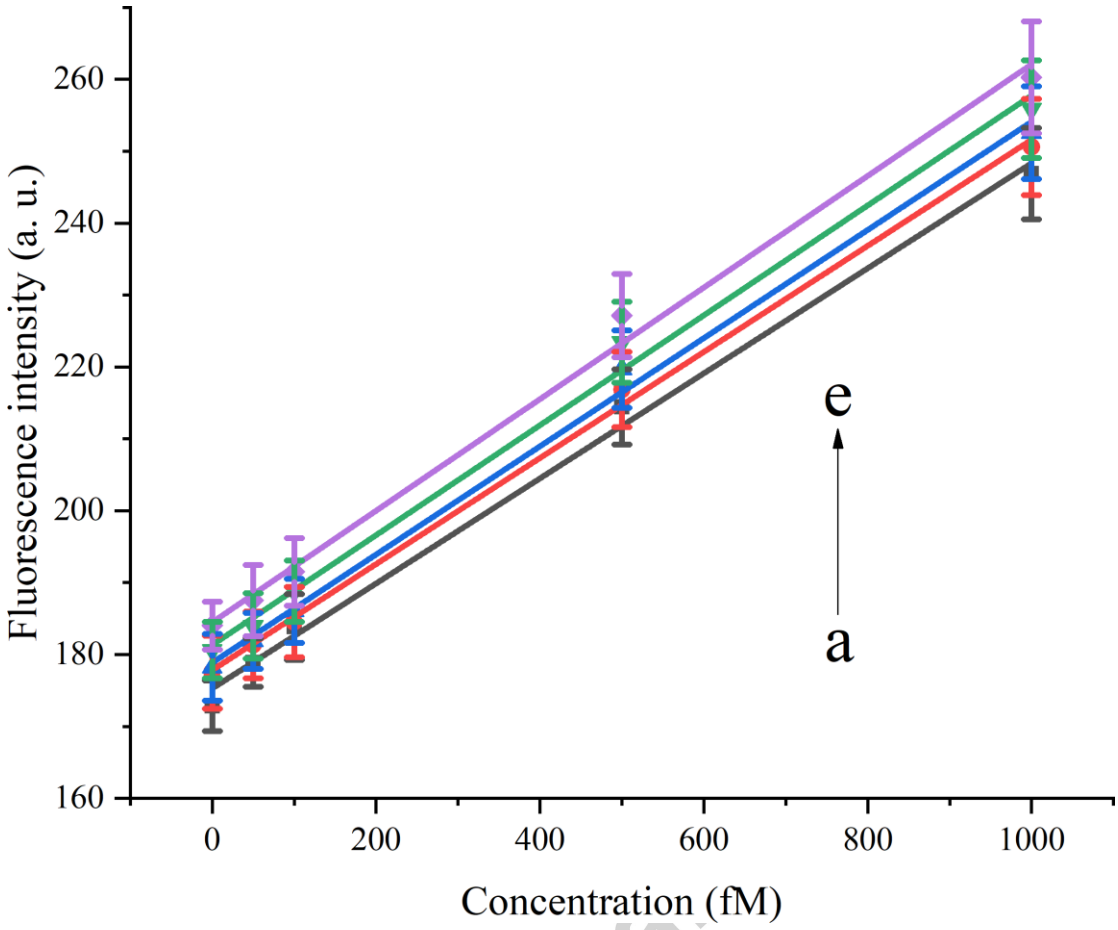

Fig. 5. the dose-response behavior of the sensor to the protein IFN-γ is robust and sensitive in buffer (a),10-fold-diluted human serum samples (b), 5-fold-diluted human serum samples (c), 2-fold-diluted human serum samples (d), and undiluted human serum samples (e).

Table 1. Sequence information for the IFN-γ aptamer, MB track (MB track a, MB track b, MB track c, and MB trackd) and MB2 used in this study. In MB track, MB track a, MB track b, MB track c, MB track d, and MB2,! is the cleavage site of Nb.BbvCI. The double-line marked bases DNA walker and (MB track, MB track a, MB track b, MB track c, and MB track d) are the hybridization part. Single-line marked sequences are the inhibiting part of G-quadruplex.

note	sequence (5' to 3')
IFN-γ aptamer	GGGGTTGGTTGTGTTGGGTGTTGTGT
DNA walker	AGGCCTCAGC**ACACAACACCCAACACAACCAACCCC** T_{40}-NH_2
MB track	NH_2-T_7-GCATGCTGGGTGTTGTGTGC!*TGAGGCCTCAGCATGCGA*
MB track a	NH_2-T_7-ATGCTGGGTGTTGTGTGC!*TGAGGCCTCAGCATGA*
MB track b	NH_2-T_7-CATGCTGGGTGTTGTGTGC!*TGAGGCCTCAGCATGGA*
MB track c	NH_2-T_7-GGCATGCTGGGTGTTGTGTGC!*TGAGGCCTCAGCATGCCGA*
MB track d	NH_2-T_7-GGGCATGCTGGGTGTTGTGTGC!*TGAGGCCTCAGCATGCCCGA*
MB2	CCACCACC*TCGCATGC*!*TGAGGCCTCAGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTG*

Highlights

- A DNA walker and nicking enzyme assisted biosensor was reported here.
- Strong enhancement in fluorescent signal of malachite green was utilized.
- This biosensor was employed for the detection of IFN-γ with a detection limit of 7.65 fM.