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An amplified fluorescent aptasensor based on single-stranded DNA binding protein, copper and silica nanoparticles for sensitive detection of interferon-gamma

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Abstract

It is great significance to identify interferon-gamma (IFN-γ), as a biological marker for diagnosis of latent tuberculosis, in serum samples. In this paper, a novel fluorescent aptasensor was fabricated and applied for sensitive and specific detection of IFN-γ. This biosensor was based on hairpin structure of oligonucleotide, single-stranded DNA-binding protein (SSB), copper nanoparticles (CuNPs) and silica nanoparticles coated with streptavidin (SNPs-Streptavidin). The presences of double-stranded DNA (dsDNA) region and poly thymine (T) in the hairpin structure of the oligonucleotide, SSB and SNPs-streptavidin caused IFN-γ determination with high selectivity and sensitivity. Upon addition of IFN-γ, the hairpin structure of the oligonucleotide was disassembled and therefore, poly T strand interacted with SSB and a weak fluorescence signal was obtained. Without introduction of IFN-γ, the hairpin structure of oligonucleotide was preserved and fluorescent CuNPs were formed on the stem region of hairpin structure of oligonucleotide, resulting in strong fluorescence intensity. Under optimal conditions, concentration as low as 1 pg/mL IFN-γ could be detected, with a linear range between 10 pg/mL and 4 ng/mL. The presented method was further performed for detection of IFN-γ in the spiked human serum samples and the recoveries were 92.52%-98.32%, showing the great potential of the proposed analytical method in biomedical analysis.

Keywords: Interferon-gamma; Single-stranded DNA-binding-protein; Copper nanoparticles; Silica nanoparticles; Fluorescent aptasensing platform

1. Introduction

Tuberculosis (TB) is an infectious bacterial disease which is a major cause of mortality worldwide. Most of the people infected with TB have a latent infection with no symptom. So, this is of great importance to detect people with latent TB infection and prepare protective treatment for them [1, 2]. Interferon-gamma (IFN-γ) is an important cytokine which acts as a biological marker for diagnosis of tuberculosis and its release increases at the early stages of the TB infection [3].

Analytical techniques based on enzyme-linked immunosorbent assay are the clinical standards for detection of IFN-γ. While these immunoassays have good sensitivity, they suffer from some limitations, including need for highly trained staff, performing time-consuming protocols and the use of costly devices [2, 4, 5].

Aptamers are artificially-synthesized single-stranded DNA (ssDNA) or RNA structures with high selectivity and affinity for different targets, ranging from whole cells and proteins to organic small molecules and metal ions [6, 7]. The in vitro selection procedure of aptamer is named Systematic Evolution of ligands by Exponential enrichment (SELEX) [8]. Compared to antibodies, aptamers have several advantages for application in different types of sensors, including resistance to denaturation, easy regeneration capabilities, high

temperature stability, easy synthesis and low cost [8-10]. Therefore, aptamers have been used in a variety of analytical techniques [11, 12].

Silica nanoparticles (SNPs) have been widely used in therapeutic delivery systems and sensing platforms, owing to their features, including biocompatibility, low cost, good solidity and dispersibility in various sensing environments. Moreover, SNPs can serve as enhancers of fluorescence intensities of fluorophores [13-15].

Among different sensing methods, aptamer-based fluorescent sensors have received much attention, because of their simplicity, rapidness and low cost detection [16-18].

In the present study, we designed a fluorescent aptasensor for IFN-γ detection, based on single-strands DNA-binding protein (SSB), silica nanoparticles coated with streptavidin (SNPs-Streptavidin), fluorescent copper nanoparticles (CuNPs) and hairpin structure of oligonucleotide containing aptamer sequence and poly thymine (T) sequence (Fig. S1). The high specificity of the proposed aptasensor was generated by the hairpin structure of oligonucleotide. Also, SSB caused the formation of fluorescent CuNPs only in the absence of IFN-γ, leading to a very significant difference in the fluorescence signal of the presented aptasensor in the absence and presence of IFN-γ and increasing of the sensitivity of the aptasensor. Furthermore, the SNPs-Streptavidin could amplify

the fluorescence signal of CuNPs and improved the sensitivity of aptasensor more.

2. Materials and Methods

2.1. Reagents and chemicals

The oligonucleotide, 5'-Biotin-CCAAAAAAAAAAAAAAAAAAAAAGGGGTTGGTTGTGTTGGGTGTTGTGTATTTTTTTTTTTTTTTTTTTTT-3' (the underlined sequence is IFN-γ aptamer [5, 19]), was provided from Bioneer (South Korea). Interferon-gamma (IFN-γ), bovine serum albumin(BSA), L-Cysteine (L-Cys), myoglobin (MB), $CuSo_4$, ascorbic acid, plasma from human, 3-(N-Morpholino)propanesulfonic acid (MOPS) were obtained from sigma-Aldrich (USA). Single-stranded DNA-binding protein (SSB) was ordered from Affymetrix (Canada). Silica nanoparticles coated with streptavidin (SNPs-Streptavidin) were purchased from Micromod (100 nm, Germany). Salmon sperm DNA was ordered from Thermo Fisher Scientific (USA).

2.2. Preparation of the Oligonucleotide-modified SNP-Streptavidin complex

SNPs-Streptavidin (120 µg/mL) was incubated with 10 µL oligonucleotide (2 µM) in 80 µL SSB buffer (10 mM Tris-HCl, 20 mM $MgCl_2$, 1 mM EDTA, 200 mM NaCl, pH 7.4) for 1 h at room temperature. 2.5% agarose gel electrophoresis was applied to assess the formation of the Oligonucleotide-modified SNP-Streptavidin complex (Fig. S2).

2.3. Optimization of SSB concentration

First, 200 µL reaction systems containing 100 µL Oligonucleotide-modified SNP-Streptavidin complex and 100 µL IFN-γ (4 ng/mL) were pre-incubated for 30 min at room temperature. Then, the samples were centrifuged at 11000 g for 10 min and the supernatants were removed. Next, 80 µL SSB buffer and different concentrations of SSB (0-1200 nM) were introduced to the pellets and incubated for 15 min at room temperature. Then, the samples were centrifuged again at 11000 g for 10 min and the supernatants were discarded. After that, 80 µL MOPS buffer (10 mM MOPS, 150 mM NaCl, 2 mM $MgCl_2$, pH 7.6), 10 µL ascorbic acid (20 mM) and 10 µL $CuSO_4$ (1 mM) were added to the pellets and the mixtures were kept in dark for 10 min at room temperature. Finally, the fluorescence intensities of the mixtures, λ_{Ex}= 340 nm and λ_{Em}= 615 nm, were recorded by a Synergy H4 microplate reader (BioTeK, USA).

2.4. Optimization of the incubation time with SSB

Oligonucleotide-modified SNP-Streptavidin complexes, treated with 4 ng/mL IFN-γ, were incubated with 80 µL SSB buffer and 800 nM SSB at room temperature from 0 to 25 min. After centrifugation, the pellets were treated with 80 µL MOPS buffer, 10 µL ascorbic acid (20 mM) and 10 µL $CuSO_4$ (1 mM) for 10 min at room temperature and the fluorescence signals were measured.

2.5. Assay procedure

100 µL Oligonucleotide-modified SNP-Streptavidin complexes were incubated with 100 µL various concentrations of IFN-γ (0-6 ng/mL) for 30 min at room

temperature, followed by centrifugation at 11000 g for 10 min. Thereafter, the supernatants were eliminated and the solutions containing 80 µL SSB buffer and 20 µL SSB (800 nM final concentration) were added to the pellets. After incubation for 15 min at room temperature, samples were centrifuged and supernatants were removed. Then, pellets were incubated with 80 µL MOPS buffer, 10 µL $CuSO_4$ (1 mM) and 10 µL ascorbic acid (20 mM) for 10 min in dark at room temperature. Finally, the fluorescence intensities were measured.

2.6. Selectivity of the aptasensor

The presented aptasensing platform was employed for analyzing other substances, including BSA, L-Cys and MB (the concentration of each substance was 4 ng/mL) as mentioned before.

2.7. Monitoring of IFN-γ in serum samples

The feasibility of the developed aptasensing platform was monitored by assaying IFN-γ in the spiked serum samples, treated with 0.03 mg/mL salmon sperm DNA.

3. Results and Discussion

3.1. Principle of the proposed fluorescent method

In this assay, we fabricated a novel fluorescent aptasensor for IFN-γ detection, based on CuNPs nanoparticles as the fluorescent reporters, high specificity of SSB for ssDNA, SNPs-Streptavidin-assisted fluorescent amplification and conformational structure of oligonucleotide as a template for the formation and binding of CuNPs to the surface of SNPs-Streptavidin in the absence of IFN-γ.

In the experiments, high concentrations of NaCl and Mg^{2+} were applied to make sure all four subunits of the SSB tetramer could bind to the ssDNA (poly T) in the presence of target and remove nonspecific interaction of SSB [20, 21]. It has been shown in the presence of ascorbic acid and copper ions, double-stranded DNA (dsDNA) and poly T strand can act as sites for the formation of fluorescent CuNPs [22, 23].

Scheme 1 depicts the principle mechanism of the proposed assay. Without introduction of IFN-γ, the oligonucleotide preserved its hairpin structure on the surface of SNPs-Streptavidin. So, there was no ssDNA for SSB binding. Upon the addition of copper ions and ascorbic acid, CuNPs were formed via binding to the stem region of oligonucleotide structure which contained both dsDNA and poly T. DNA-templated CuNPs acted as fluorescent reporters and strong fluorescence intensity was recorded. Because of the formation of fluorescent CuNPs on the surface of SNPs-Streptavidin, the surrounding environment of

CuNPs changed and became more hydrophobe and the fluorescence intensity was more amplified.

Upon the introduction of IFN-γ to the Oligonucleotide-modified SNP-Streptavidin complex, the hairpin structure of oligonucleotide was dismantled via the formation of Aptamer/IFN-γ complex. So, the SSB could interact with the 3'-end of oligonucleotide as ssDNA, containing poly T. When copper ions and ascorbic acid were added to the mixture, there was no dsDNA and free poly T strand in the sample which acted as a template for the formation of fluorescent CuNPs. Therefore, a very weak fluorescence signal was obtained.

3.2. Optimizations for IFN-γ detection

Before quantification of IFN-γ by the established assay, the detection conditions, including concentration and incubation time of SSB were optimized. In this study, the responses of the Oligonucleotide-modified SNP-Streptavidin complex, treated with 4 ng/mL IFN-γ, in the presence of different concentrations of SSB were monitored using fluorescence signals as a function of concentration. Fig. 1 (a) indicated that by increasing the concentration of SSB, the relative fluorescence intensity of the aptasensor rapidly enhanced and kept unchangeable at 800 nM SSB, implying that poly T strands completely bound to SSBs at this concentration.

The effect of SSB incubation time on the sensing system was then investigated. As shown in Fig. 1 (b), the relative fluorescence signals of the presented sensing

method increased and reached to plateau in 15 min incubation time of SSB, confirming that poly T strands interacted with SSBs completely within 15 min. So, the incubation time of 15 min after the addition of SSB was applied in the next experiments.

3.3. Proof of concept for the developed sensing strategy

Fluorescence emission spectra were used to confirm the function of the presented sensing assay. Without introduction of IFN-γ, the hairpin structure of oligonucleotide was preserved on the surface of SNPs-Streptavidin and SSB could not bind to poly T strand, which was hybridized with its complementary stand on the stem region of the hairpin structure of oligonucleotide. So, in the presence of ascorbic acid, the copper ions used the stem of the hairpin structure of oligonucleotide as a template for the formation of fluorescent CuNPs. As expected a strong fluorescence intensity was measured (Fig. 2, red curve). When IFN-γ and SSB were added to the Oligonucleotide-modified SNP-Streptavidin complex, a very weak fluorescence intensity was recorded (Fig. 2, green curve), indicating the formation of Aptamer/IFN-γ complex, destruction of the hairpin structure of oligonucleotide and access of SSB to poly T strand as a ssDNA. Upon addition of ascorbic acid and copper ions, very less fluorescent CuNPs were formed as there was no dsDNA region (stem region) in the environment and also, the poly T strand was covered by the SSB.

3.4. Analytical performance of aptasensor

To confirm the ability of the developed aptasensor to detect IFN-γ, a series of various concentrations of IFN-γ were measured under the optimal conditions Fig. 3 (a) showed the relative fluorescence intensity exhibited a good linear relationship with the logarithm of IFN-γ concentrations ranging from 10 pg/mL to 4 ng/mL. The limit of detection (LOD) was determined to be 1 pg/mL (3σ/slope, where σ was the standard deviation in blank sample, n=4)

Compared to recent assays, as summarized in Table 1, the presented analytical method exhibited a satisfying LOD. Also, the designed method did not need any complicated sample pre-treatment.

3.5. Selectivity of the proposed analytical method for IFN-γ

Selectivity is a critical issue in the development of aptasensors for utilization in biomedical systems. Different materials were employed as interfering reagents to investigate the selectivity of the assay. The selectivity test was carried out by measuring and comparing the relative fluorescence signal of the aptasensor to such interfering substances as L-Cys, BSA and MB. As shown in Fig. 3 (b), the relative fluorescence signal of IFN-γ was much higher than other interfering materials. These results showed that the biosensor was capable to detect IFN-γ with high specificity.

3.6. Monitoring of IFN-γ in real samples

To analyze analytical reliability and application potential of the presented aptasensing platform, known concentrations of IFN-γ in the spiked serum

samples were detected by the developed aptasensor. As indicated in Table 2, the relative standard deviation (RSD) of analytical results were less than 7.1% and the recoveries for the spiked serum samples ranged from 92.52% to 98.32%, confirming that the matrix effects were not severe.

4. Conclusion

In this study, a sensitive fluorescent aptasensing platform for the detection of IFN-γ was reported. The presence of both dsDNA region and poly T in the hairpin structure of oligonucleotide made a very suitable template for the formation of fluorescent CuNPs. Also, the presence of SSB in the sample could guarantee no fluorescent CuNPs could be formed on the surface of SNPs-Streptavidin in the absence of IFN-γ, leading to the improvement of the sensitivity of the developed aptasensor. The proposed aptasensor exhibited a satisfying linear range (10 pg/mL-4ng/mL) and very low LOD (1 pg/mL) in the buffer. The developed method was also successfully utilized to determine the concentrations of IFN-γ in the spiked serum samples. It seems the proposed aptasensor has potential to be modified and used for detecting other molecules in biological samples.

Conflict of interest

There is no conflict of interest about this article.

Acknowledgment

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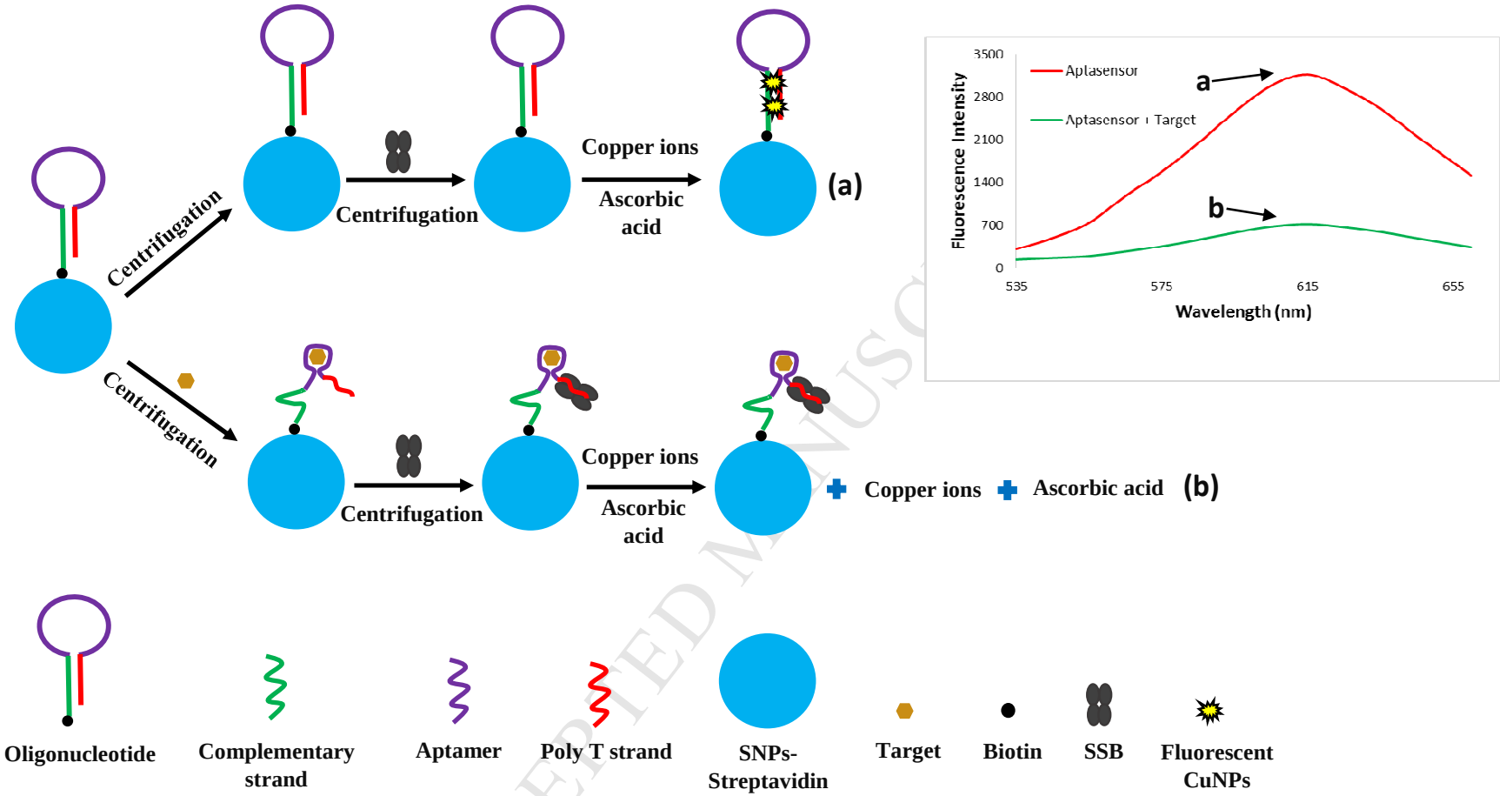


Scheme 1. Schematic illustration of IFN-γ detection using the presented fluorescent aptasensor. In the absence of IFN-γ, the hairpin structure of oligonucleotide is preserved on the surface of SNPs-Streptavidin and SSB could not bind to poly T strand. So, fluorescent CuNPs were formed on the surface of SNPs-Streptavidin, leading to a strong fluorescence signal (a). In the presence of IFN-γ, the hairpin structure of oligonucleotide is destructed and SSB could bind to the released poly T strand. Thus, the fluorescent CuNPs were not formed on the surface of SNPs-Streptavidin, resulting in a weak fluorescence intensity (b).

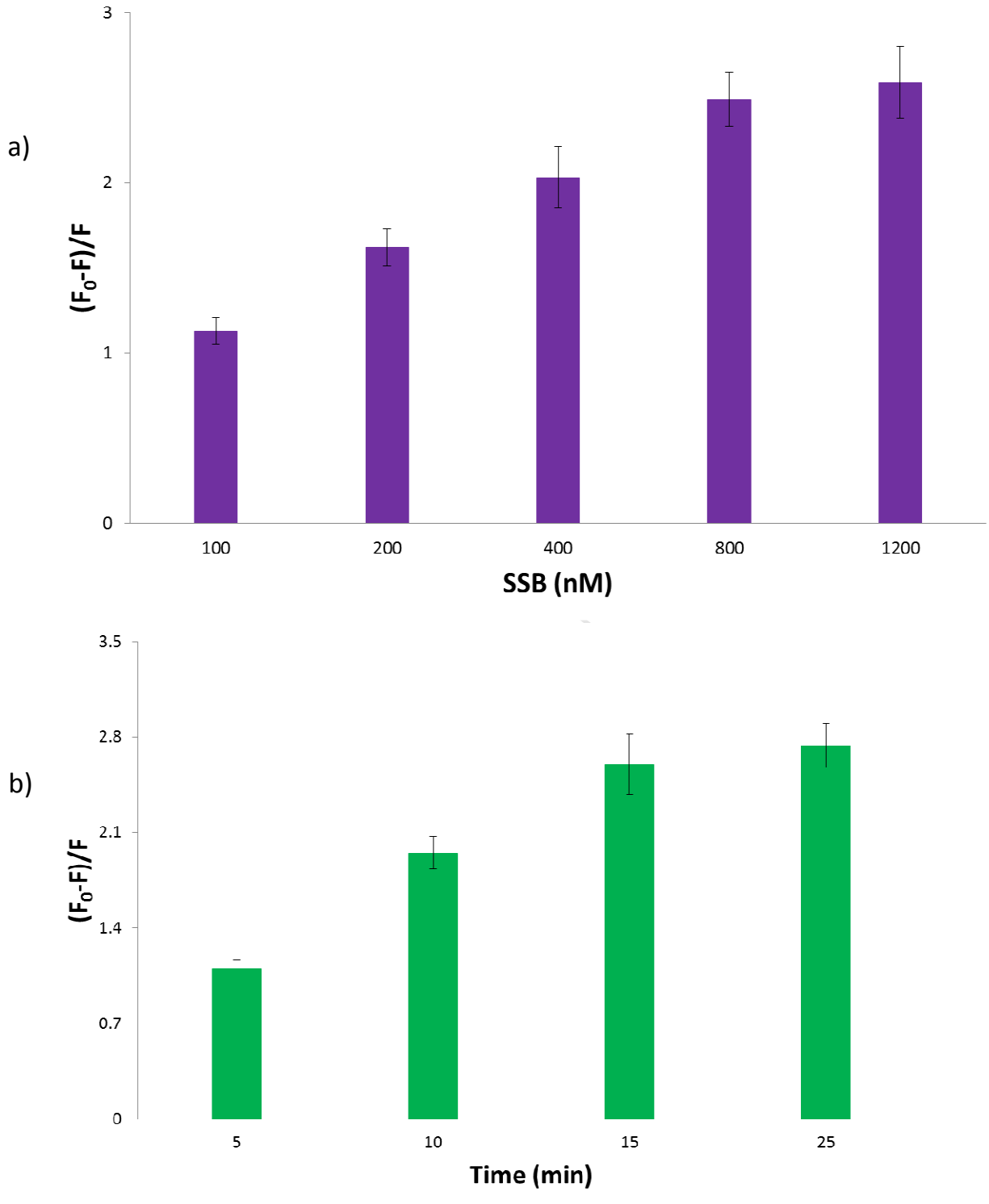


Fig. 1. Optimization of the factors involved in the performance of the proposed method. (a) Relative fluorescence signals of Oligonucleotide-modified SNP-Streptavidin complex in the presence of different concentrations of SSB. (b) Relative fluorescence signals of Oligonucleotide-modified SNP-Streptavidin complex as a function of SSB incubation time. F_0 and F are the fluorescence intensities at 615 nm before and after addition of SSB, respectively.

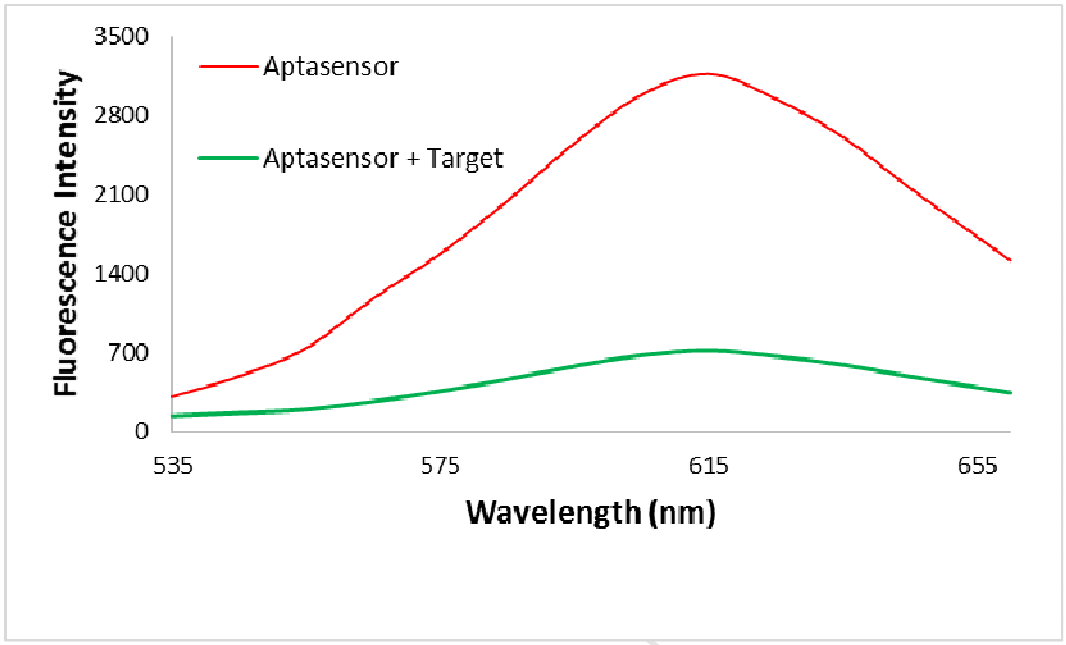
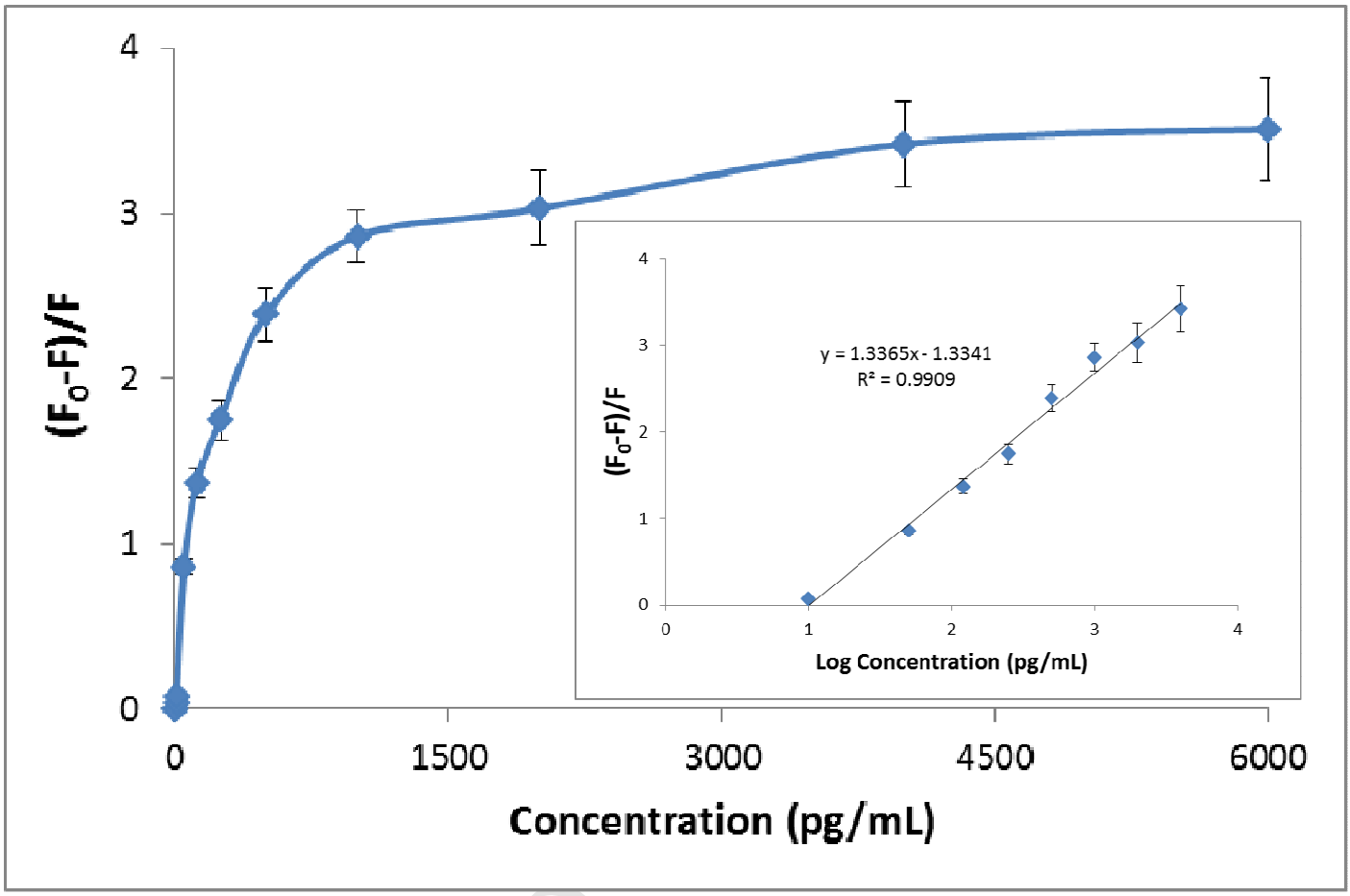


Fig. 2. Validation of the performance of the developed sensing platform. Fluorescence spectra of the proposed analytical method in the absence (red curve) and presence of IFN-γ (green curve).

a)


4
3
2
1
0
(F0-F)/F
0
1500
3000
4500
6000
Concentration (pg/mL)
y = 1.3365x - 1.3341
R² = 0.9909
(F0-F)/F
Log Concentration (pg/mL)

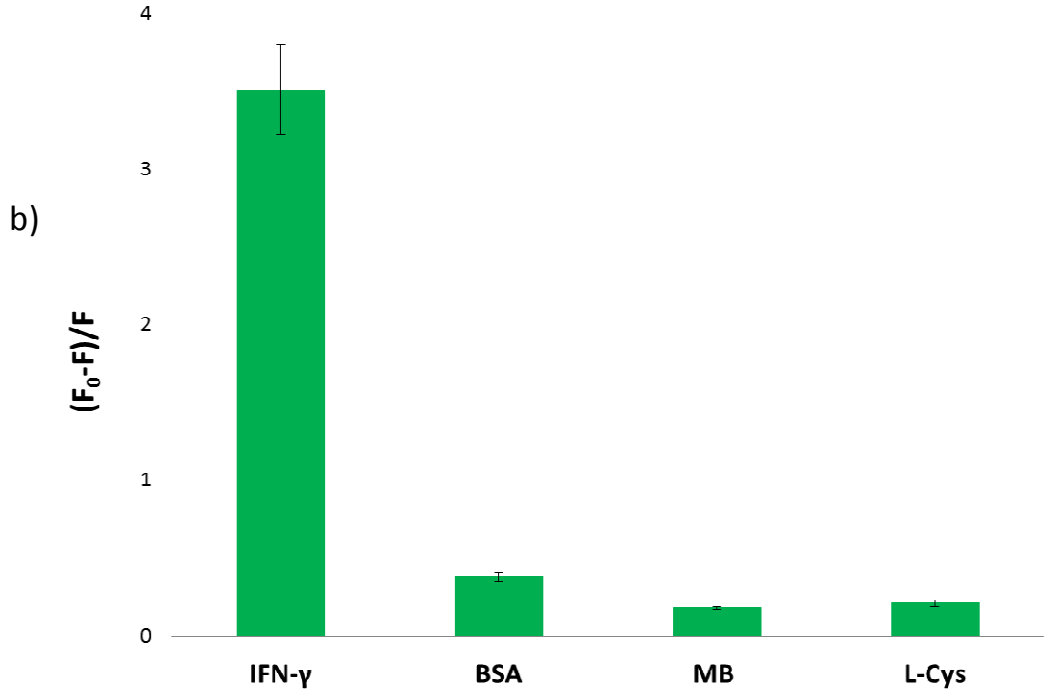


Fig. 3. (a) Relative fluorescence responses of the developed method after interaction with different concentrations of IFN-γ. The insert figure is calibration plot in the presence of different amounts of IFN-γ. F_0 and F are the fluorescence intensities at 615 nm before and after addition of IFN-γ, respectively. (b) Relative fluorescence signals of the designed sensing method in the presence of different interfering materials (the concentration of each substance was 4 ng/mL). F_0 and F are the fluorescence intensities at 615 nm before and after addition of each substance, respectively.

Table 1. Reported detection limits of IFN-γ in different manuscripts.

Method	LOD	Linear range
Amperometric IFN-γ immunosensors [3]	28.22pg/mL	Not reported
An electrochemical aptasensor detection of IFN-γ using graphene and a dual signal amplification strategy based on the exonuclease-mediated surface-initiated enzymatic polymerization [4]	3 pg/mL	10 pg/mL-50 ng/mL
Aptamer based field effect transistors (FET)-like electrochemical biosensor [5]	1400 pg/mL	Not reported
LSPR biosensing using DNA-functionalized gold nanorods [24]	160 pg/mL	160 pg/mL-16 ng/mL
A cyclometalated iridium(III) complex used as a conductor for the electrochemical sensing of IFN-γ [25]	0.275 pg/mL	0.843-50.637 pg/mL

Table 2. Recovery of IFN-γ from serum samples (n=4). Data are mean ± standard deviation (SD).

Serum samples	**Found (pg/mL)**	**Added IFN-γ (pg/mL)**	**Total Found (pg/mL)**	**Recovery (%)**	**RSD (%, n=4)**
1	11	60	65.69	92.52	4.9
2	11	200	204.33	96.84	5.3
3	11	600	587.35	96.13	2.5
4	11	2000	1977.21	98.32	7.1

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Table 1. Reported detection limits of IFN-γ in different manuscripts.

Table 2. Recovery of IFN-γ from serum samples (n=4). Data are mean ± standard deviation (SD).

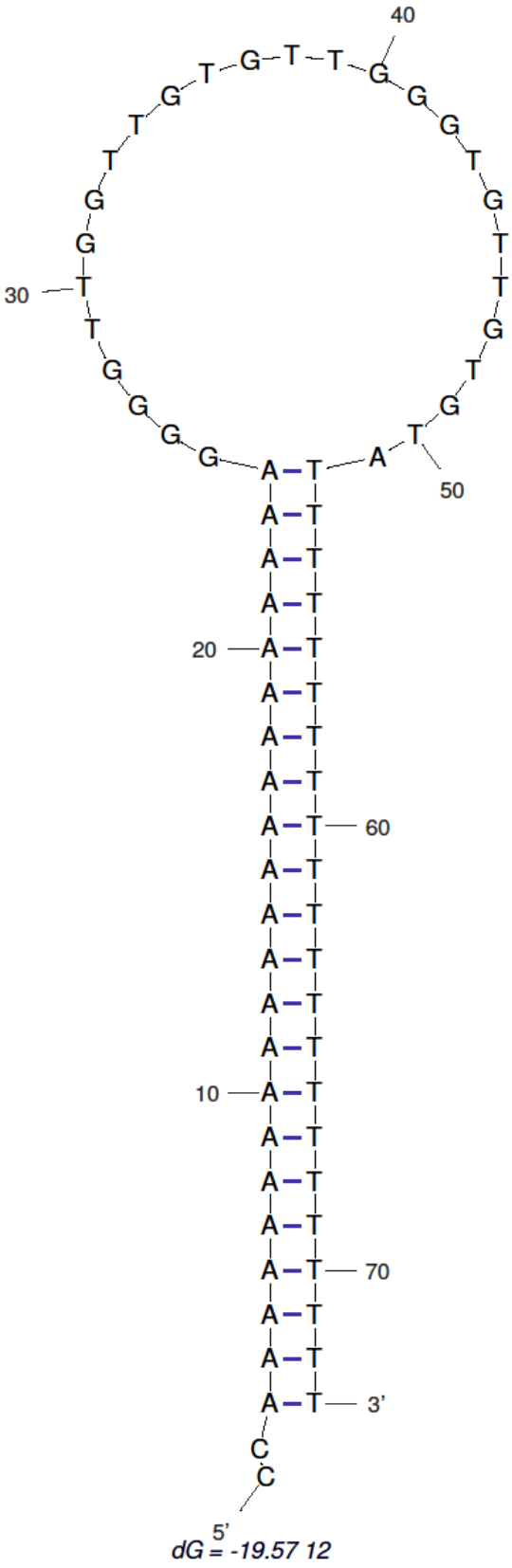


Fig. S1. Hairpin structure of oligonucleotide predicted by Mfold web server.

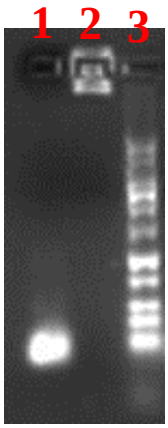
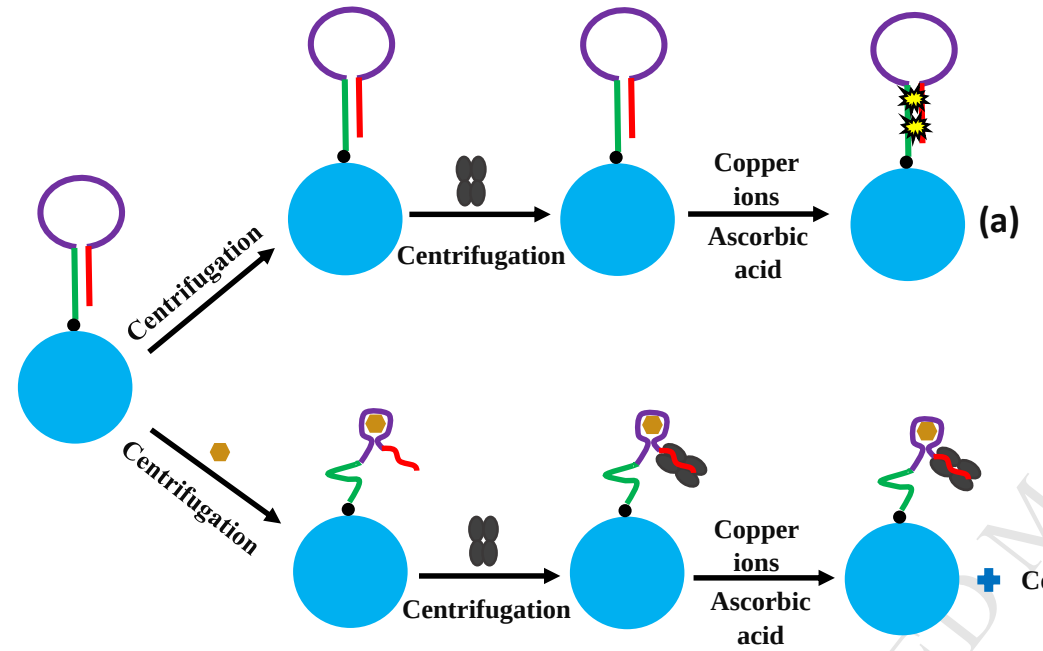
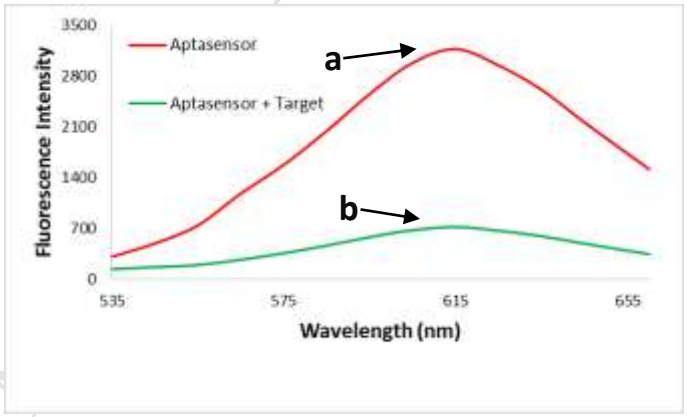
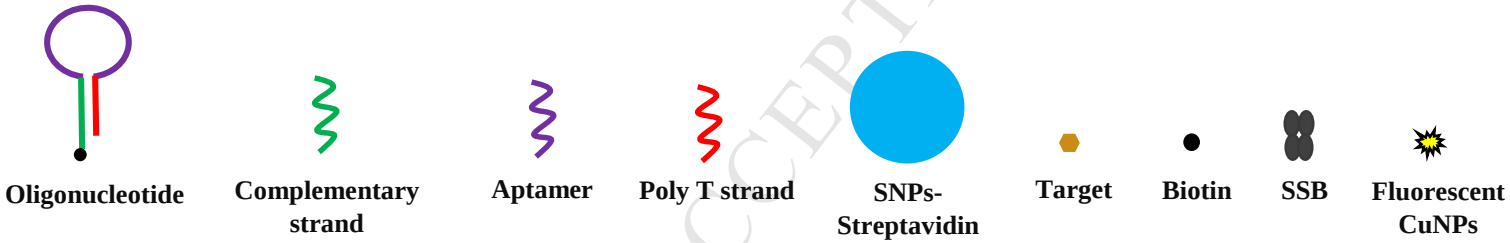


Fig. S2. Confirmation of the formation of Oligonucleotide-modified SNP-Streptavidin complex. Agarose gel electrophoresis of Oligonucleotide-modified SNP-Streptavidin complex. Lane 1: Oligonucleotide, Lane 2: Oligonucleotide-modified SNP-Streptavidin complex; Lane 3: 50 bp ladder. As shown in the in figure, the band of Oligonucleotide-modified SNP-Streptavidin complex was retarded relative to the oligonucleotide band.







* Interferon-gamma (IFN-γ) is a biological marker for diagnosis of latent tuberculosis.

* A fluorescent aptasensor was designed for IFN-γ detection.

* Under optimal conditions, concentration as low as 1 pg/mL IFN-γ could be detected.

* The sensor was performed for detection of IFN-γ in the spiked human serum samples.