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Fluorescent aptasensor based on D-AMA/F-CSC for the sensitive and specific recognition of myoglobin

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ABSTRACT

A novel fluorescent biosensor based on dabcyl [(E)-4-((4-(dimethylamino) phenyl) diazenyl)benzoic acid] -modified anti-Mb aptamer (D-AMA) and 6-FAM(6-carboxyfluorescein) -modified complementary short chain (F-CSC)for the specific and sensitive detection of Mb levels is presented in this study. In PBS buffer solution, D-AMA bound to F-CSC, and then dabcyl quenched the fluorescence of 6-FAM. After adding Mb into the system, D-AMA bound to Mb and separated from F-CSC. The fluorescence of 6-FAM was restored after it separated from dabcyl. The assay exhibited high specificity and sensitivity toward Mb, with a low limit of detection of 0.07 ng/mL (S/N = 3) and linear relationships of 0.1−5 ng/mL. It was further applied to detect Mb levels in spiked human blood sera samples.

1. Introduction

Acute myocardial infarction (AMI) is one of the cardiovascular diseases that cause human morbidity and mortality worldwide [1]. Therefore, the accurate and rapid diagnosis of AMI is crucial and has an important clinical value in patient survival and successful prognosis. Myoglobin (Mb) is one of the important markers of early AMI, and it is the earliest biomarker released by cells after myocardial injury [2]. The normal concentration range of Mb in human serum is 6−100 ng/mL. When

AMI occurs, the Mb level increases to 600 ng/mL in a short time [3]. Hence, the rapid and accurate detection of Mb is of great importance for the early diagnosis of AMI.

At present, the main methods for detecting Mb include mass spectrometry [4], liquid chromatography [5], electrochemical method [6,7], fluorescence method [8], surface plasmon resonance method [9], colorimetric method [10], molecular imprinting [11,12], immunoassay [13,14], and chemiluminescence method [15]. Mass spectrometry has high sensitivity and specificity, however, it requires expensive instrument, and its database is not perfect. Thus, it has not been widely used. High-performance liquid chromatography has the advantages of good separation effect and high sensitivity. However, due to the low concentration of Mb in serum, samples need to be enriched and concentrated before detection. The operation is tedious and time-consuming and cannot meet the requirements of rapid determination of Mb. In addition, most of the above methods for detecting Mb are based on the mutual recognition of antibody and antigen. Though immunoassay has high selectivity and sensitivity toward Mb, the preparation of antibodies requires immune animal or cell experiments, which is cumbersome, time-consuming, and costly. Moreover, antibodies are susceptible to the influence of external conditions, especially temperature, and require strict storage conditions, greatly limiting the flexible application of these methods.

Aptamers are small single-stranded oligonucleotide sequences screened by SELEX (index enrichment ligand phylogenetic technology) [16]. They are a kind of small RNA or single-stranded DNA with specific three-dimensional conformation. Their length is generally 20−80 bases, and their relative molecular weight is 6−30kD. Given the structural diversity and spatial conformation of aptamers, some stable three-dimensional structures (e.g., hairpins, knots, convex rings, G-tetrads) can be formed by pairing with some complementary bases and electrostatic and hydrogen bonding interactions with target molecules. Moreover, aptamers can specifically recognize target molecules by binding them with high

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affinity. Therefore, small molecules, proteins, bacteria, viruses, and cells can be specifically recognized by aptamers [17]. Compared with antibodies, aptamers have high specificity, high affinity, no (or low) immunogenicity, low cost, easy synthesis and modification, easy fixation, wide target range, reusability, and long-term preservation.

The fluorescence method has low detection limit and high sensitivity. It is rapid, and requires simple instruments. Thus, a reliable, fast, highly selective, and sensitive analytical method for detecting Mb may be developed by combining fluorescence method with aptamer technique. Here, we developed a low-cost, simple, and accurate fluorescent biosensor based on dabcyl[(E)-4-((4-(dimethylamino)phenyl)diazenyl) benzoic acid]-modified anti-Mb aptamer (D-AMA) and 6-FAM (6-carboxyfluorescein)-modified complementary short chain (F-CSC) for the specific detection of Mb.

2. Experiment section

2.1. Materials and apparatus

Bovine sera albumin (BSA), human sera albumin (HSA), immunoglobulin A (IgA), and immunoglobulin G (IgG) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cardiac troponin I (cTnI) was provided by Shanghai Linc-Bio Science Co., Ltd. (Shanghai, China). Myoglobin (Mb) was obtained from Shanghai Anyan Trade Co., Ltd. (Shanghai, China). Alpha-fetoprotein was purchased from Fitzgerald Company (USA). Human sera samples were provided by Xin'an International Hospital (Jiaxing, China). All other chemicals, including NaCl, KCl and $MgCl_2$ were provided by Aladdin (Shanghai, China). All chemicals are analytical grade and can be used without further purification. Amicon ultra centrifugal filters (30 kD) were supplied by Merck Millipore Corporation (China). Dabcyl-modified anti-Mb aptamer (D-AMA) (5′-CCCTCCTTTCCTTCGACGTA GATCTGCTGCGTTGTTCCGA-Dabcyl-3′), 6-FAM-modified complementary short chains (F-CSC) including DNA1 (5′-6-FAM-TCGGAACAAC-3′), DNA2 (5′-6-FAM-CGGAACAACG-3′), DNA3 (5′-6-FAM-GGAACAACGC-3′) and DNA4 (5′-6-FAM-GAACAACGCA-3′) were provided by Sangon Biotech (Shanghai, China) Co., Ltd. 0.01 M PBS buffer (pH = 7.4) consists of 5 mM NaCl, 2 mM KCl and 1 mM $MgCl_2$. Milli-Q purification systems were used to prepare ultrapure water and the water was used throughout the experiment.

The fluorescence spectra of the samples were recorded by F-7000 fluorophotometer (Hitachi, Japan). The fluorescence intensity was tested at 517 nm with excitation at 495 nm at 25 °C.

2.2. Exploration of DNA chain base sequences of F-CSC

On the basis of the base sequence of D-AMA, we designed four 6-FAM-modified complementary short chains (F-CSC) with different initial hybridization sites (DNA1—DNA4). Their hybridization with D-AMA chains is described below (Fig. 1).

The hybridization process was performed as follows: 50 μL of 500 nM D-AMA, 50 μL of 500 nM DNA1 (DNA2, DNA3, or DNA4), and 900 μL of 0.01 M PBS buffer solution (pH = 7.4) were added to a 5 mL plastic tube. After 20 min of hybridization at 25 °C, the fluorescence intensity of the above systems was determined. The best complementary short chain was DNA3, whose fluorescence intensity decreased greatly (Fig. 2). The final concentration of D-AMA and F-CSC in the buffer was 25 nM.

2.3. Optimization of hybridization time of D-AMA and DNA3

We explored the hybridization time of D-AMA and DNA3. The procedure was performed as follows: 50 μL of 500 nM DNA3, 50 μL of 500 nM D-AMA, and 900 μL of 0.01 M PBS buffer solution (pH = 7.4) were added to a 5 mL plastic tube, and the fluorescence intensity of the system was measured after hybridization at 25 °C for a period of time.

2.4. Optimization of incubation time of D-AMA and Mb

We also explored the incubation time of the specific binding of D-AMA to Mb. The steps are as follows: 50 μL of 500 nM DNA3, 50 μL of 500 nM D-AMA, and 880 μL of 0.01 M PBS buffer solution (pH = 7.4) were added to a 5 mL plastic tube; after hybridization for a period of time at 25 °C, 20 μL of Mb solution with a certain concentration was added and incubated for a period of time, and their fluorescence intensity was measured.

2.5. General steps for Mb detection

First, 1760 μL of 0.01 M PBS buffer solution (pH = 7.4), 100 μL of 500 nM F-CSC, and 100 μL of 500 nM D-AMA were added to a 5 mL plastic tube. The mixture was hybridized at 25 °C for a certain time. Then, the mixture was evenly divided into two equal parts, with one being labeled as the blank sample. Approximately 20 μL of 0.01 M PBS buffer solution (pH = 7.4) was added to the blank sample. The other part was mixed with 20 μL of a certain concentration of Mb standard solution (i.e., 5, 25, 50, 100, 150 or 250 ng/mL). Then, both samples were incubated at 25 °C for a period of time. Finally, their fluorescence intensity was measured with an F-7000 fluorescence spectrophotometer. The difference between the two samples was the fluorescence recovery intensity.

2.6. Specificity detection of the fluorescent aptasensor

Several common proteins (e.g., HAS, BSA, IgA, IgG, cTnI, and AFP)

Fig. 1. Hybridization of D-AMA and F-CSC.

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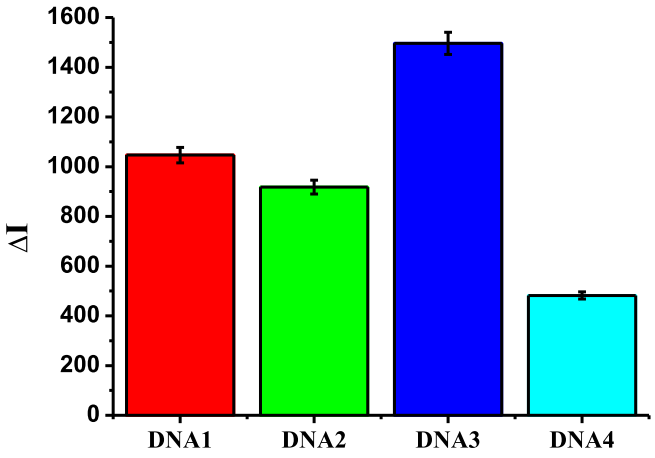


Fig. 2. Comparison of quenching effects of D-AMA on F-CSC.

were used to test the selectivity and specificity of the fluorescent aptasensor. The protein concentration was 5 ng/mL. The detection steps were the same as mentioned in Section 2.5. The fluorescence data were compared with Mb data.

2.7. Detection of human serum samples

To evaluate the practicability of the assay, spiked healthy human serum samples were tested. First, the spiked serum samples were prepared by adding Mb of known concentration into the serum samples of healthy people. Then, the resulting serum samples were diluted 10 times with 0.01 M PBS buffer (pH = 7.4) and further filtered with an ultrafiltration centrifuge tube (30 kD) to remove some macromolecule proteins [1], then the pretreated spiked serum samples were obtained. The preparation and detection processes of the spiked human serum samples were introduced as follows.

Firstly, 50 μL of human serum sample was mixed with 50 μL of a certain concentration of Mb standard solution. After fully mixing, the spiked human serum sample was obtained. Then the mixture was diluted 10 times with 0.01 M PBS buffer solution (pH = 7.4), and it was further filtered with an ultrafiltration centrifuge tube (30 kD) to remove some macromolecule proteins, the filtrate was named pretreated spiked serum sample.

Secondly, 1760 μL of 0.01 M PBS buffer solution (pH = 7.4), 100 μL of 500 nM F-CSC solution and 100 μL of 500 nM D-AMA solution were successively added into 5 ml plastic tubes (The final concentration of F-CSC and D-AMA were 25 nM), the mixed solution was then incubated at 25°Cfor 20 min.

Next, the mixed solution (1960 μL) was divided into two equal parts, the volume of each part was 980 μL, 20 μL of 0.01 M PBS buffer solution (pH = 7.4) was added into one part and mixed evenly (the final volume was 1000 μL), the part was marked as the blank sample. 20 μL of the pretreated spiked serum sample was added into the other part and mixed evenly (the final volume was also 1000 μL), and the part was marked as the test sample. Then the two samples were incubated at 25°Cfor 50 min.

Finally, their fluorescence intensity was measured with an F-7000 fluorescence spectrophotometer. The difference between the two samples was the fluorescence recovery intensity.

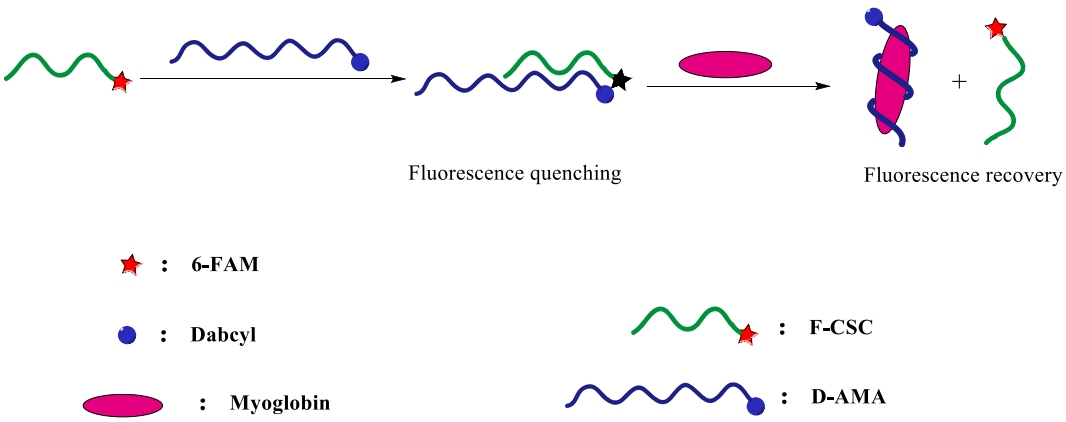
3. Result and discussion

3.1. Detection principle of the fluorescent aptasensor

In the PBS buffer solution, D-AMA and F-CSC were hybridized. The fluorescent group 6-FAM and the quenching group dabcyl contacted each other. The fluorescence of 6-FAM was quenched by dabcyl. When a certain concentration of Mb was added to the system, D-AMA was separated from F-CSC and bound to Mb because of its high affinity to Mb. The fluorescence of 6-FAM of F-CSC was restored after it separated from dabcyl of D-AMA (Scheme 1). The intensity of fluorescence recovery is proportional to the Mb concentration, which can be used to determine the Mb level.

3.2. Effect of DNA base sequences of different F—CSCs on fluorescence quenching

As shown in Fig. 2, when D-AMA combined with different F—CSCs (e.g., DNA1, DNA2, DNA3, or DNA4), the fluorescence intensity of the system decreased at varying degrees. The experimental results showed that D-AMA had the best fluorescence quenching effect on DNA3. In theory, the fluorescent group of DNA1 is the closest to the quenching group of D-AMA, thus, the quenching effect should be the best. However, the experimental results were not satisfactory. This result may be due to the space barrier of the two labeled groups themselves. Although the two groups are close to each other, they are not in good contact, leading to less decline of the fluorescence intensity. The experimental results indicated that the fluorescence intensity of DNA3 decreased most, possibly due to the moderate distance between their fluorescence and quenching groups. After comprehensive consideration, we chose DNA3 as the best complementary short chain.



Scheme 1. Schematic of Mb detection.

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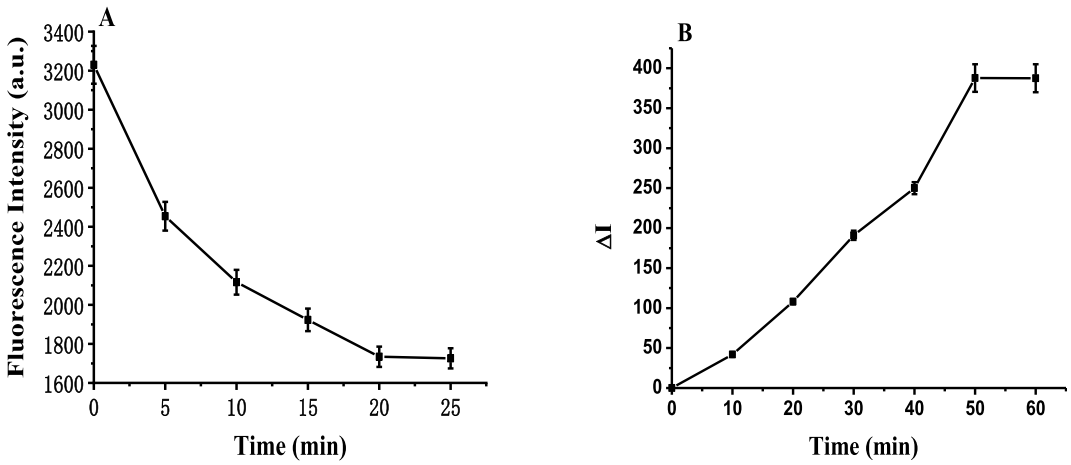

Fig. 3. (A) Effect of hybridization time of D-AMA and F-CSC on fluorescence intensity. (B) Effect of binding time of D-AMA and Mb on fluorescence recovery intensity.

3.3. Optimization of detection conditions of Mb

To detect Mb accurately, the fluorescence quenching time and fluorescence recovery time were studied. Considering the influence of time, we divided the test time into two parts. One involves the hybridization of D-AMA and DNA3 to determine the optimal fluorescence quenching time. The other part involves the specific binding of D-AMA and Mb after fluorescence quenching to determine the optimal fluorescence recovery time. Fig. 3A shows that the fluorescence intensity hardly decreases when the quenching time exceeds 20 min. Therefore, 20 min was set as the optimum fluorescence quenching time. As shown in Fig. 3B, the fluorescence intensity increases to the highest level and tends to be stable after 50 min. Thus, 50 min was set as the best time for the fluorescence recovery.

3.4. Quantitative detection of Mb

Under the above optimum conditions, different concentrations of Mb were determined by the fluorescent aptasensor. As shown in Fig. 4, the fluorescence intensity of DNA3 was restored to varying degrees when different concentrations of Mb were added into the system. The dynamic range of Mb level is 0.1–100 ng/mL. However, no linear relationship was observed between Mb concentration and the recovery value of fluorescence intensity within 5.0–100 ng/mL. Fig. 5 shows a linear relationship between the fluorescence recovery intensity and the Mb level in the concentration range of 0.1–5.0 ng/mL. The linear equation is $I = 79.4C + 22.59$, where I is the fluorescence recovery intensity and C is the Mb concentration. The correlation coefficient (R) is 0.995. The limit of detection (LOD) was 0.07 ng/mL (S/N = 3). Table 1 shows that the method has higher sensitivity for Mb than most of the previously reported assays.

3.5. Selectivity of fluorescent aptasensor

To test the selectivity and specificity of the proposed fluorescence sensor, six common proteins, namely, BSA, AFP, IgA, IgG, HSA, and cTnI, were tested under the same detection condition. Fig. 6 shows that Mb had the highest fluorescence recovery intensity compared with the other six proteins. This result indicates that the method has high specificity and selectivity for Mb.

3.6. Detection of Mb in human serum samples

Considering the complexity of serum samples, high

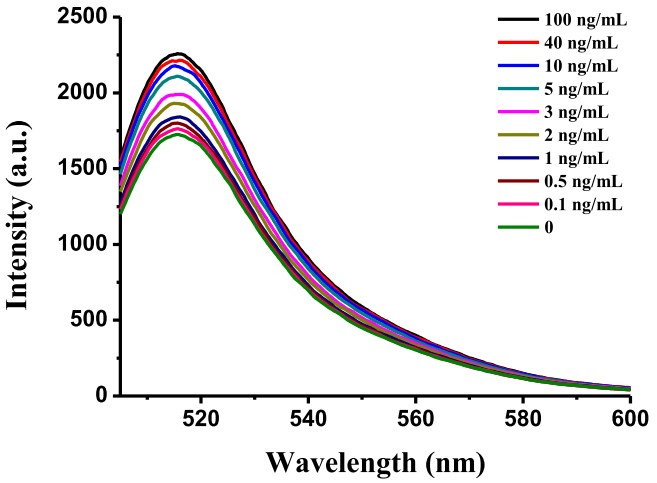

Fig. 4. Fluorescence spectra of the D-AMA/DNA3 buffer in the presence of Mb with different concentrations ($\lambda_{ex} = 495$ nm, $\lambda_{em} = 517$ nm).

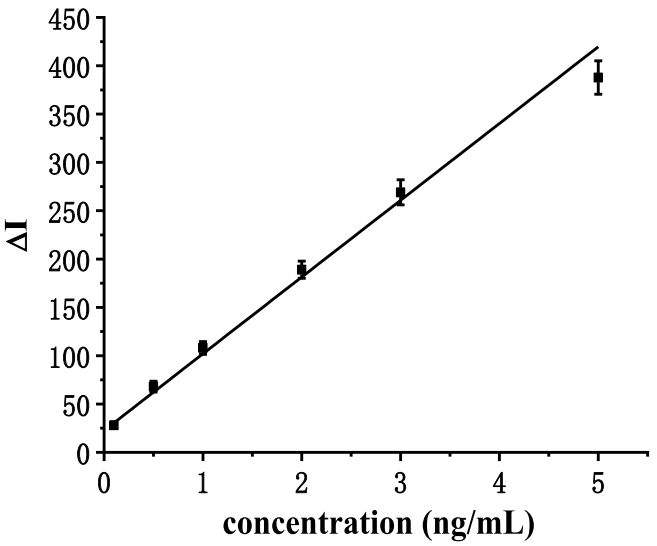

Fig. 5. Fluorescence recovery intensity of D-AMA/DNA3 buffer in the presence of Mb with different concentrations (0.1–5 ng/mL). $\lambda_{ex} = 495$ nm, $\lambda_{em} = 517$ nm.

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Table 1
Comparison of the proposed method with other assays in the detection of Mb.

No.	Method	LOD (ng/mL)	References
1	Impedimetric immunosensor	0.83	[13]
2	Point-of-care assay	8.9 (0.5 nM)	[18]
3	Lateral flow assay	0.21	[19]
4	Label-free aptasensor	34.6	[6]
5	Fluorescent biosensor	0.02	[8]
6	Nanostructured aptamer-based sensing platform	0.04 (2.2 pM)	[3]
7	Surface-enhanced Raman spectroscopy	10	[20]
8	Graphene–CNT nanohybrid aptasensor	0.34	[21]
9	Electrochemical immunosensor	0.0004	[7]
10	Giant magnetoimpedance based immunoassay	0.5	[14]
11	Colorimetric biosensor	44.5 (2.5 nM)	[22]
12	Molecularly imprinted poly(o-phenylenediamine)	8.9 (0.5 nM)	[23]
13	Biomembrane-like films on carbon electrodes	5340 (0.3 μM)	[24]
14	Aptamer and ubiquitous personal glucose meter	0.89 (50 pM)	[1]
15	Electrochemiluminescence aptasensor	0.21 (12 pM)	[25]
16	Electrochemical aptasensor	0.48 (27 pM)	[26]
17	Fluorescent aptasensor based on silica nanoparticles	0.93 (52 pM)	[27]
18	Fluorescent aptasensor	0.07	This work

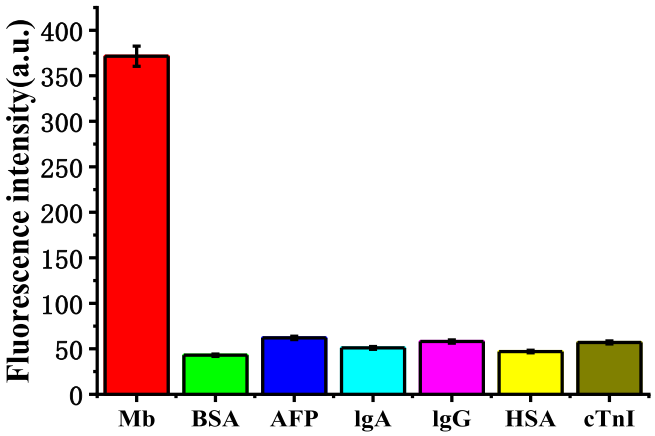

Fig. 6. Recovery fluorescence intensity of D-AMA/DNA3 buffer in the presence of different proteins.

concentrations of coexisting proteins such as HSA (the normal concentration of HSA in human serum is 35–50 mg/mL) have significant interference effect on the detection of Mb. Moreover, other macromolecular proteins in the serum may produce strong fluorescence background. Therefore, we diluted the spiked serum sample 10 times with 0.01 M PBS buffer (pH = 7.4) and then filtered with an ultrafiltration centrifuge tube (30 kD) to remove some macromolecule proteins. Then, the pretreated spiked serum sample was tested. Table 2 shows that the recoveries of the spiked serum samples ranged from 99.1% to 107.2% (n = 5). The experimental results revealed that the proposed method has potential application value in the early diagnosis of AMI.

4. Conclusions

A simple fluorescent aptasensor based on the interaction between D-AMA and F-CSC was developed in this study. The developed aptasensor is inexpensive and easy to prepare and has high specificity and sensitivity for Mb. Given that the aptasensor displays good test performance, it has potential application value in the immediate detection of AMI.

Table 2
Detection results of Mb in spiked human serum samples.

No.	Found (ng mL^{-1})	Added Mb (ng mL^{-1})	Total found (ng mL^{-1})	Recovery (%)
1	33.4	50	82.6 ± 11.40	99.1
2	33.4	200	244.7 ± 10.62	104.8
3	33.4	450	518.2 ± 19.35	107.2

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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