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RESEARCH ARTICLE

Label- and modification-free-based in situ selection of bovine serum albumin specific aptamer

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Systematic evolution of ligands by exponential enrichment is a traditional approach to select aptamer, which has a great potential in biosensing field. However, chemical modifications of DNA library or targets before selection might block the real recognition and binding sites between aptamers and their targets. In this study, a label- and modification-free-based in situ selection strategy was developed to overcome this limitation. The strategy is an attempt to screen bovine serum albumin aptamers according to the principle of electrophoretic mobility shift assay, and allowed single-stranded DNA sequence to be fully exposed to interact with bovine serum albumin which was mixed with the agarose gel beforehand. After eight rounds of selection, specific aptamer with low dissociation constant (K_d) value of 69.44 ± 7.60 nM was selected and used for subsequent establishment of fluorescence biosensor. After optimization, the optimal aptasensor exhibited a high sensitivity toward bovine serum albumin with a limit of detection of 0.24 ng/mL (linear range from 1 to 120 ng/mL). These results indicated that the label- and modification-free-based in situ selection strategy proposed in this work could effectively select specific aptamer to develop aptasensor for sensitive detection of bovine serum albumin or other targets in actual complicated samples.

KEYWORDS

actual sample analysis, aptamers, aptasensors, bovine serum albumin, in situ selection

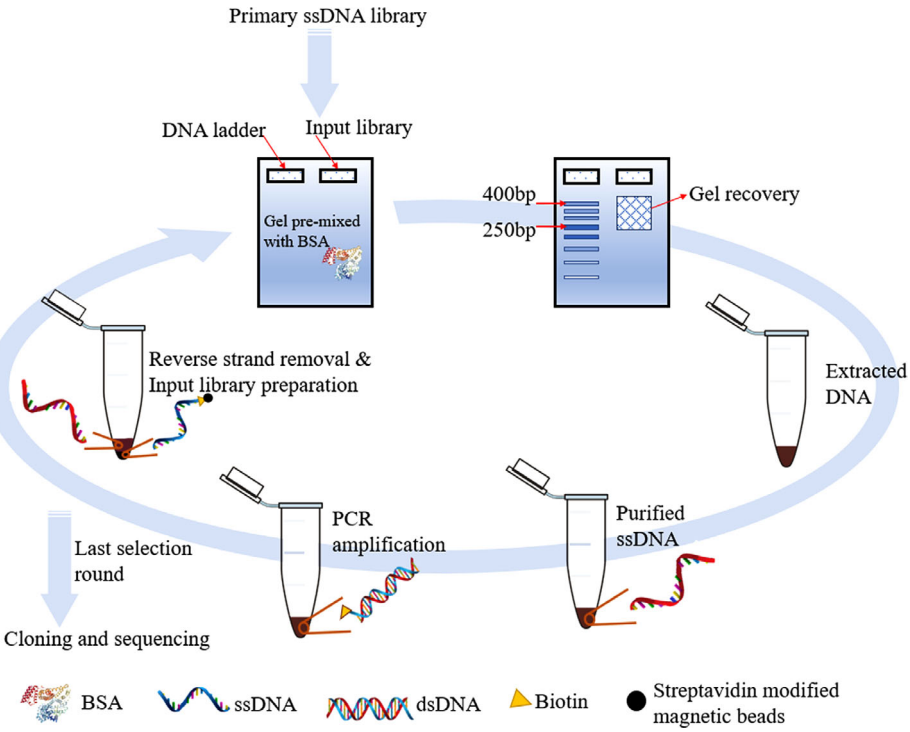
1 | INTRODUCTION

Aptamers, which are DNA or RNA ligands that can specifically recognize and bind with their targets, have some obvious characteristics superior to antibodies, such as wide range of targets, convenient preparation, chemical stability, and flexible modification [1]. Due to these advantages, aptamers have been widely applied in developing sensitive biosensors for detecting drugs, toxins, pesticides, proteins, viruses, bacteria, etc. [2]. Besides applications in food safety control [3,4] and environmental monitoring [5], specific molecular recognitions between aptamers and targets have also been investigated deeply in clinical diagnostic [6] and therapeutic fields [7].

Traditionally, aptamers were mainly selected by the classical method known as systematic evolution of ligands by exponential enrichment (SELEX). SELEX strategy, which originated in 1990, has been proven to be an effective method for isolating the specific aptamer through iterative process [8]. However, there are still some intrinsic restrictions on the traditional selection strategy. The SELEX procedure is tedious, laborious, and time-consuming. Advancements have been made to enhance the SELEX specificity and efficiency during the past decades. Since the selection of desired aptamer

Article Related Abbreviations: K_d, low dissociation constant; MNP, magnetic nanoparticle; SA, streptavidin; SELEX, systematic evolution of ligands by exponential enrichment; ssDNA, single-stranded DNA.

Chuchu Wang and Xiaoyan Du contributed equally to this paper.



SCHEME 1 Schematic illustration of label- and modification-free-based in situ selection process of specific aptamer against BSA

candidates from the enriched single-stranded DNA (ssDNA) library is the most critical step, some creative strategies, such as Mag-SELEX, cell-SELEX, CE-SELEX, etc., have been adapted to enhance the selection efficiency [9].

Magnetic beads coated with target or ssDNA are widely used to assist the separation process [10]. On the basis of Mag-SELEX, FluMag-SELEX [11] and Capture-SELEX [12] are designed to respectively immobilize target and DNA library on magnetic beads. With considerable competence in recognizing biomarkers and molecular signatures of cell surfaces, cell-targeted SELEX has also attracted attentions and some novel strategies, such as fluorescence-activated cell sorting-assisted SELEX, cell-internalization SELEX, 3D cell-SELEX, microfluidic based cell-SELEX, ligand-guided selection, and cross-over SELEX, with great significance to futuristic oligonucleotide based diagnosis, and therapy have been developed during the past few years [13,14]. CE-SELEX, which partitions aptamer sequences that bound to target from the non-specific unbound sequences through a gel free CE, allows reducing the number of aptamer selection rounds and eliminates stationary support and linker biases [15]. And a modified version, micro free flow electrophoresis brought a dramatic increase in aptamer partitioning efficiency [16]. Moreover, monoclonal surface display SELEX [17], domain targeted SELEX [18], and double library SELEX strategy [19] also provide new clues to researchers.

Despite these improvements, the strategies mentioned above cannot cover up the flaws as follows. First, the iterative manual manipulation requires multiple successive cycles of selection and amplification, thereby retarding the selection process. In general, though several improved strategies have been applied, around 8–15 cycles of affinity selection and amplification are still needed [20]. Second, chemical modifications such as biotin modification of DNA library before selection may have influence on the binding process with the target. To some extent, covalent modification and other modifications might cause changes in spatial structures of compounds and disturb their recognizing and binding process with their targets. For example, covalent modification by phenolic compounds could result in changes of conformational properties of target proteins [21] and the secondary even tertiary structure shifts of sunflower protein [22]. Similarly, some real specific binding sites between aptamer candidates and their targets might also be changed and covered by these modifications. Moreover, chemical modification needs extra performance and makes the selection process more labor-intensive and time-consuming. Therefore, it is necessary to explore a novel aptamer selection method which can not only save time and effort, but also, what's more, avoid influence of chemical modifications on binding sites and real recognition.

In this work, a novel label- and modification-free in situ selection strategy was developed through agarose gel electrophoresis to select specific ssDNA aptamers of BSA, a typical pattern protein, from the random oligonucleotide library (Scheme 1). After eight rounds of selection, BSA specific aptamer with low dissociation constant (K_d) value of 69.44 ± 7.60 nM was selected and used for subsequent

establishment of fluorescence biosensor. After optimization, the optimal aptasensor exhibited a high sensitivity toward BSA with a LOD of 0.24 ng/mL (linear range from 1 to 120 ng/mL).

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents

The carboxyl (-COOH) modified magnetic beads and streptavidin modified magnetic beads were respectively purchased from Beaver Biomedical Engineering (Suzhou, China) and PuriMag Biotech (Xiamen, China). Smooth muscle actin antibody was purchased from Proteintech Group (Wuhan, China). Anti-His tag antibody was purchased from Sino Biological (Beijing, China). HSA was purchased from Shanghai Acmec Biochemical (Shanghai, China).

All the chemicals and reagents used in this study were of analytical grade.

2.2 | SsDNA library and primers

The random ssDNA library consisting of a region of 40 nucleotides (N40) flanked by two constant regions (5′-AGCAGCACAGAGGTCAGATG-N40-CCTATGCGTGCT-ACCGTGAA-3′) and the primer pair were synthesized by Sangon Biotech (Shanghai, China). The primer pair used to amplify the aptamers included the forward primer (5′-AGCAGCACAGAGGTCAGATG-3′) and the biotinylated reverse primer (5′-Biotin-TTCACGGTAGCACGCATAGG-3′).

2.3 | Preparation of in situ selection gel

Electrophoresis was performed in a mixture gel consisting of 2% agarose solution and 500 ng/mL BSA solution with a volume ratio of 3:2. During this process, structure of BSA should not be damaged to guarantee the subsequent selection. Therefore, the agarose solution was heated to near-boiling point alone and then stood to cool down. Before it became solidified, agarose solution was mixed with the BSA solution prior to pouring into the cast.

2.4 | In situ-immersion-based selection of aptamer candidates against BSA

The random ssDNA library was incubated at 95°C for 5 min and subsequently cooled at 4°C for 15 min to make the ssDNA denatured and stretched. Once the gel had set, the primary ssDNA library without any modification mixed with the loading buffer was loaded into the wells.

The separation of aptamer candidates from those unbound ssDNA sequences was realized according to the principle of electrophoretic mobility shift assay [23]. Instead of mixing BSA with ssDNA before electrophoresis, the recognition and affinity interaction between BSA and ssDNA came out through the electrophoretic mobility shift, which not only simplified the selection process but also resulted in a better separation. While the molecular weights of BSA and ssDNA (80 bp) are about 66.5 and 24.4 kDa, respectively, the combination of the two molecules could form a complex with a molecular weight of 90.9 kDa (i.e. corresponds to 298 bp and lies in the range of 250–400 bp). Therefore, the gel slices between 250 and 400 bp containing desired DNA fragments were cut and purified using agarose gel DNA extraction kit. The selected oligonucleotides candidates were amplified through PCR using biotinylated reverse primers to get an enriched secondary library. After amplification, the PCR product was denatured and then the biotinylated complementary strands could bind to streptavidin modified magnetic beads before elution of the desired single strands with 0.2 M NaOH solution. The neutralized supernatant would be served as secondary library and used for the next selection cycle. Selection rounds were performed until the selected library was well enriched and converged to a collection of sequences with specific affinity for target BSA.

2.5 | Cloning, sequencing, and structural analysis of selected aptamer candidates

The selection process would stop until the recovery rate of the selected ssDNA pools did not increase any more. The recovered ssDNA library was amplified by PCR. The PCR products were cloned and sequenced by Sangon Biotech (Shanghai, China). The internet tool *mfold* (http://mfold.rna.albany.edu) was used to predict the secondary structures of the selected aptamer candidates.

2.6 | Measurement of dissociation constant value

The aptamer candidate K_d values were determined based on the method described previously [24–26]. A more detailed description of the method is given in Supporting Information.

2.7 | Establishment and optimization of a fluorescence aptasensor

The aptamer candidate with the lowest K_d value was chosen to establish the aptasensor for specifically detecting BSA. The aptasensor for detecting BSA was constructed according to the principle and process described in our previous work [27].

2.8 | Measurement of the specificity and sensitivity of the aptasensor

The specificity of the aptasensor was investigated by competitive binding assay using other proteins (i.e. smooth

muscle actin antibody, anti-His tag antibody, and HSA). The competitors prepared in 100 ng/mL with binding buffer were added into the detection system and incubated at 37°C for 30 min. After magnetic separation, the fluorescence intensity of the supernatant was measured in at least triplicates for error analysis.

The optimized aptasensor was applied to the quantification of BSA. Different concentrations of BSA were dissolved in binding buffer and detected by the aptasensor system. Binding buffer without BSA was considered as the negative control.

2.9 | Determination of BSA in spiked pear juice samples

To evaluate the feasibility and applicability of the aptasensor for detecting BSA in actual complicated samples, pear juice sample (Uni-President China Holdings, Shanghai, China) purchased from local store were spiked with BSA to different final concentrations (i.e. 40, 60, 80, and 100 ng/mL). BSA content spiked in juice samples were quantified by the established aptasensor. The reliability of this aptasensor in actual juice samples was evaluated by recovery rate of BSA. The recovery rate (y (%)) was calculated according to the following equation:

$$y(\%) = x_1/x \times 100\% \quad (1)$$

In this equation, y (%) refers to the recovery rate; x_1 refers to the measured BSA amount; x refers to the additive BSA amount.

3 | RESULTS AND DISCUSSION

3.1 | Selection of aptamers specific for BSA

After eight rounds of in situ-immersion-based selection, the recovery rate of the selected ssDNA pools did not increase any more (Figure 1). The recovered ssDNA library from the eighth round of selection was amplified by PCR for subsequent cloning and sequencing. Of course, the number of selection rounds might not obviously be different from previous reports about aptamer selection [28]. However, the emphasis of this strategy was on the elimination of any chemical modifications which might influence the binding site and real recognition between BSA and its specific aptamer. As not any pre-modifications were performed on the input ssDNA library, the entire sequence aptamer candidates could be sufficiently exposed to BSA, thereby contributing to the real recognition.

3.2 | Sequences of the selected aptamer candidates and their secondary structures

After cloning and sequencing, eight potential BSA aptamer candidates were identified and named as No. 1, No. 2, No. 3, No. 4, No. 5, No. 6, No. 7, and No. 8, respectively. Sequences of the selected candidate aptamers are shown in Table 1. To our knowledge, this is the first study to report the aptamer sequences that specifically bind to BSA up to now.

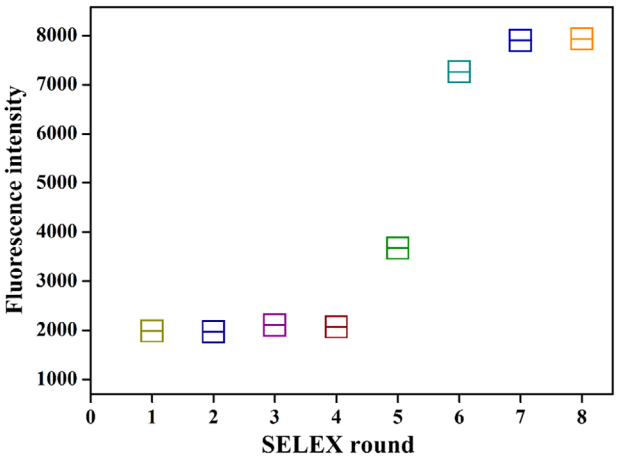


FIGURE 1 The recovery rate of each round in the label- and modification-free-based in situ selection process

After sequence analysis, the secondary structures of these eight potential aptamers were predicted by *mfold* software (http://mfold.rna.albany.edu) according to the principle of minimum free energy (Figure 2A; Supporting Information Figure S1). These aptamers were mainly made up of ring structures and hairpin structures. Different secondary structures of these eight candidates demonstrated that they might belong to different families. The "G four-couplet" structure can contribute to the recognition and binding between aptamers and their targets [29]. Among these eight potential candidates, No. 3, No. 4, and No. 8 candidates had the G four-couplet; moreover, No. 1, No. 4, and No. 6 candidates had different lengths of G continuous segment that also contributed to the total G content. However, it is not that the higher G content the aptamer possesses, the greater performance would be detected. To some extent, exceeded G content might discount the aptamer's performance [30]. In addition, the secondary structure prediction results indicated that conservative sequences (i.e. 'CAGG' and 'CGAG'), which might consist of the functional sequences of aptamer [31], located at the loop area (Figure 2A; Supporting Information Figure S1). Conservative sequence (CAGG) was present in No. 2, No. 3, and No. 5 candidates, and segment (CGAG) existed in No. 1, No. 4, and No. 8 candidates. Based on these results, No. 3 and No. 4 aptamer candidates might have higher affinity than the other candidates.

3.3 | Dissociation constant values of the aptamer candidates

K_d value is an important criterion for evaluation of affinity of aptamers [32]. The lower K_d value the aptamer possesses,

TABLE 1 Random sequence of the selected aptamer candidates

Aptamers	The random sequences (5′-3′)	G content (%)
No. 1	CGAG TGCATATTTGTCAAGCACGCGACAACGGGTGAGGGCCC	33.33
No. 2	GCGGAAAGTCCACGTGAC CAGG CAGTGTGATTCTCCCCAG	30.00
No. 3	CGGGGTGAGTCTACGCG CAGG ATCCGTGACGTTGACGCGG	42.50
No. 4	GTAT CGAG CGCAGGGGCGCCTTTGTTAATGATTACGGGCG	37.50
No. 5	TG CAGG AAGAAGTCGATCATGCCAGCGTCGGTAGGTGCTG	37.50
No. 6	CCAAATGTACACGGGGGCTTGGGGGGGAAAGTACGGCCAG	42.50
No. 7	CCTACGCGTGCTACCGTGAAAGCTAGCAGCACAGAGGTCAGATG	29.55
No. 8	AGGGGCAGAACGACCACCGTCTATGGACTGGAGTTG CGAG	37.50

The underlined sequences were continuous G sequences. Gray-shaded parts were segments of conserved sequences (i.e. CAGG and CGAG).

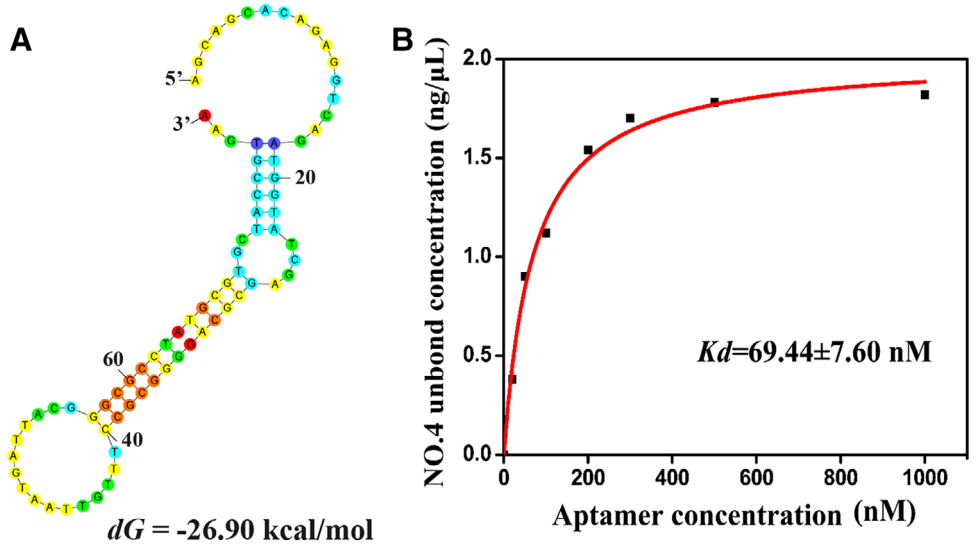


FIGURE 2 (A) Predicated secondary structure, and (B) K_d value of No. 4 aptamer

the higher affinity would be displayed. The affinity of each aptamer candidate was determined by K_d value, which was measured by the non-linear regression analysis of the binding data of the aptamer candidates (Figure 2B; Supporting Information Figure S2).

Among the eight candidates, No. 4 aptamer had the lowest K_d value (i.e. 69.44 ± 7.60 nM), indicating it possessed the highest affinity with BSA. Moreover, No. 3 aptamer had the second lowest K_d value (i.e. 109.52±40.48 nM) (Supporting Information Figure S2C). These results further confirmed the secondary structure-based prediction result. Therefore, No. 4 aptamer was chosen for further establishment of fluorescence aptasensor for sensitive detection of BSA.

3.4 | Optimization of the aptasensor

The competition ability of BSA with probe for aptamer was dependent on the ability of probe to hybridize with the aptamer. Therefore, four FAM-labeled ssDNA probes with different lengths (i.e. 13, 16, 20, and 24 bases) were investigated to evaluate the influence of probe length on the aptasensor performance. The double-stranded DNA (dsDNA) was attached to the streptavidin magnetic nanoparticles (SA-MNPs) through the biotin modification on the aptamer strand. In the presence of BSA, the competitive binding affinity of probe and BSA toward the aptamer would result in the release of FAM-labeled probe. The greater difference of fluorescence intensity in the supernatant between the presence and the absence of BSA, the better performance of the probe was. The probe with a length of 13 bases reached the highest fluorescence intensity difference (Figure 3A) and was chosen as the optimum probe for subsequent works.

Considering the aptamer concentration and time needed for synthesis of SA-MNP-aptamer-probe complex, 3 μM was chosen as the optimal aptamer concentration in the subsequent experiments (Figure 3B), while 80 min was chosen as the optimal time needed for synthesis of SA-MNP-aptamer-probe complex in the subsequent experiments (Figure 3C).

Finally, the reaction time of target with the aptasensor system would also determine the feasibility of the aptasensor. The fluorescence intensity in the detection system increased over time as the FAM-labeled probes were released into the

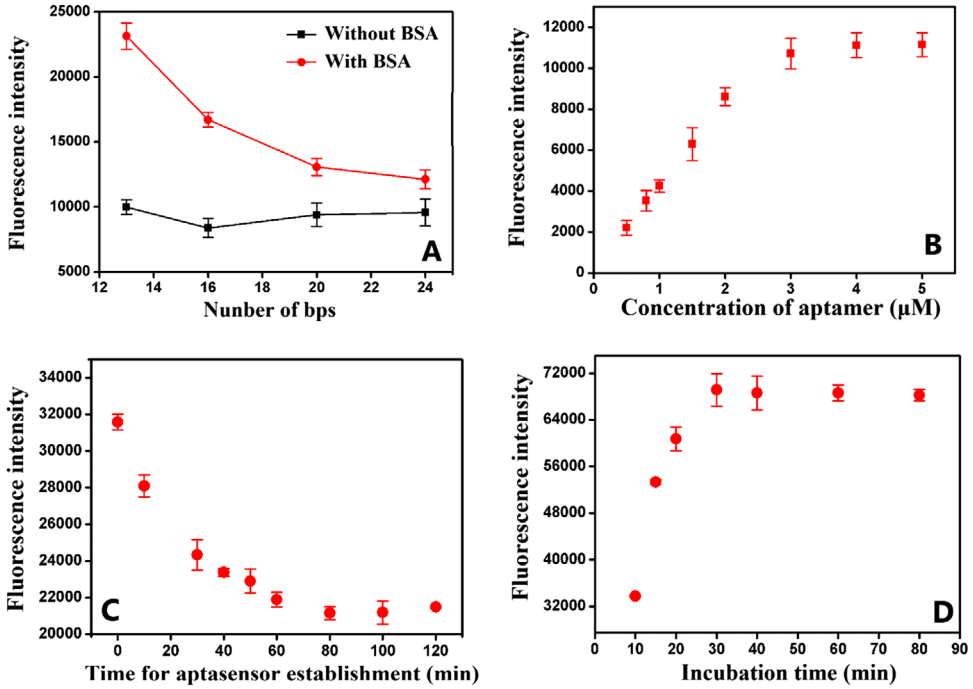


FIGURE 3 Influence of the relevant exprerimental factors on the aptasensor. (A) Optimization of probe length; (B) optimization of aptamer concentration; (C) optimization of time needed for synthesis of SA-MNP-aptamer-probe complex; and (D) optimization of detective incubation time

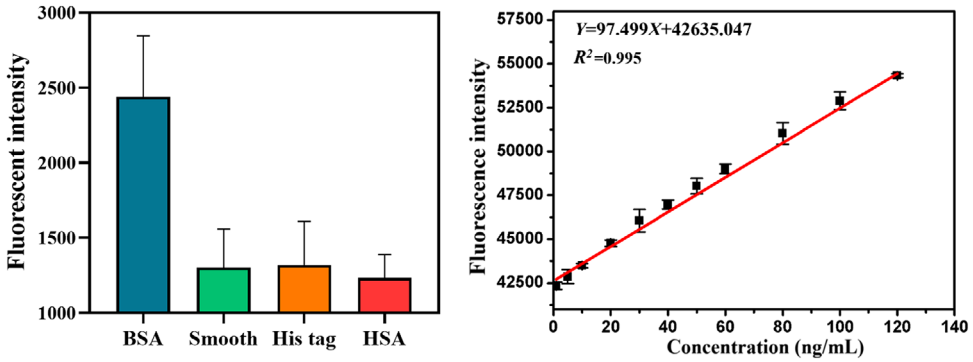


FIGURE 4 (A) The specificity of the developed aptasensor. The competitors are smooth muscle actin antibody, anti-His tag antibody and HSA, respectively; (B) the sensitivity of the developed aptasensor for detection of BSA in spiked buffer solution

supernatant due to the stronger competitive affinity between target and aptamer. Then the fluorescence intensity reached a plateau after 30 min (Figure 3D). Therefore 30 min was chosen as the optimal incubation time for the established aptasensor.

3.5 | The specificity and sensitivity of the optimized aptasensor

For investigating the specificity of the established aptasensor, different proteins with same concentration were added to the system. The fluorescence intensity of BSA group was significantly stronger than that of other proteins (Figure 4A), indicating that aptasensor has high specificity to BSA.

The fluorescence intensity of the aptasensor increased with the increased amount of added BSA, demonstrating that the aptasensor responds well to BSA. However, further increases in the added BSA content above 120 ng/mL did not yield higher fluorescence intensity. Therefore, there was a linear relationship between the BSA concentration (X) and the fluorescence intensity (Y) in the linear range of 1–120 ng/mL. The equation of this linear relationship was fitted as $Y = 97.499X + 42635.047$ with a regression coefficient of 0.995 (Figure 4B). Moreover, the LOD of 0.24 ng/mL was obtained by the equation (i.e. LOD = 3SD/K). Besides ELISA, studies of detecting trace BSA through RIA required enzyme-labeled or radioisotope beacons, which might have a pretty good detectability but did have harmful effect on environment and human

TABLE 2 Determination of the concentration of BSA in the pear juice samples

Samples	Spiked BSA (ng/mL)	BSA found* (ng/mL)	Recovery rate (%)
1	40	38.30 ± 0.02583	95.75%
2	60	59.01 ± 0.07851	98.35%
3	80	77.55 ± 0.02943	96.94%
4	100	98.72 ± 0.09236	98.72%

*BSA found is presented as mean ± SD.

health [33]. The detection system in this study was more acceptable to detect BSA with simplicity and practicality.

3.6 | Detection of BSA in spiked juice sample

Analysis of the spiked juice samples with BSA of different concentrations was performed to evaluate the feasibility of the aptasensor system for detection of complicated samples. The recovery rates of BSA from spiked juice samples ranged from 95.75 to 98.72% (Table 2), demonstrating that this aptasensor could be feasible for BSA detection in actual complicated samples.

4 | CONCLUDING REMARKS

An innovative label- and modification-free in situ selection strategy was performed to select specific aptamer against BSA. BSA was mixed with the agarose gel in advance to decrease or eliminate the influence of any chemical modifications on selection procedure. None of the chemical pre-modifications on ssDNA library sequences or BSA could contribute to sufficient exposure of aptamer candidates or BSA to each other, thereby realizing selection of real specific aptamer against BSA. After eight rounds of selection, BSA specific aptamer with low dissociation constant (K_d) value of 69.44 ± 7.60 nM was selected and used for subsequent establishment of fluorescence biosensor. After optimization, the optimal aptasensor exhibited a high sensitivity toward BSA with a LOD of 0.24 ng/mL (linear range from 1 to 120 ng/mL). Moreover, the selection strategy would provide a useful platform for selection of specific aptamers against more other targets.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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