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A universal amplified strategy for aptasensors: Enhancing sensitivity through allostery-triggered enzymatic recycling amplification

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ABSTRACT

A universal amplified sensing strategy based on endonuclease was developed for designing fluorescence aptasensors. By employing hairpin-structured design for both recognition and reporter probes to decrease background signal, and a nicking endonuclease to perform target-triggered enzymatic recycling amplification, the proposed biosensor showed high sensitivity to target protein. To demonstrate the feasibility of the design, immunoglobulin E (IgE) was studied as a model target. Upon the addition of target protein, the specific formation of IgE/aptamer complex induced the releasing of the 37-mer fragment which partially hybridized with the molecular beacon (MB) probe. In the presence of endonuclease Nt.BbvCI, the MB was cleaved into two parts. Then, the released 37-mer fragment hybridized with another MB, and triggered the second cycle of cleavage, leading to an accumulation of fluorescence signals. Under the optimal conditions, a detection limit of 5 pM was obtained. The proposed sensing system was used for detection of IgE in complex biological samples with satisfactory results.

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1. Introduction

Aptamers are a class of synthetic DNA/RNA oligonucleotides which recognize and bind a wide range of target molecules including various proteins with strong affinity and excellent specificity (Ellington and Szostak, 1990; Tuerk and Gold, 1990). Compared to antibodies, aptamers have similarly high affinity and selectivity for proteins (Wilson and Szostak, 1999; Shamah et al., 2008). Moreover, aptamers have many advantages over antibodies, including simple synthesis, easy storage, good stability, design flexibility, and easy modification for wide applications (Fang and Tan, 2010; Deng et al., 2010; Tombelli et al., 2005). Therefore, aptamers can be useful alternatives to antibodies as recognition elements in developing sensing systems for proteins (Cho et al., 2009; Liu et al., 2009). Immunoglobulin E (IgE) plays an important role in the most powerful immune reactions (Sutton and Gould, 1993), such as allergic asthma, atopic dermatitis and other immune deficiency-related diseases. Therefore, IgE is usually chosen as a model protein to fabricate aptasensors (Cole et al., 2007; Zhang et al., 2008; Ohno et al., 2010; Cheow and Han, 2011). Several groups have converted anti-IgE aptamer into highly selective electrochemical (Xu et al., 2005), fluorescent (Jiang et al., 2004; Gokulrangan et al., 2005) and quartz crystal microbalance (QCM) (Liss et al., 2002) biosensors. While most of these aptasensors show satisfying sensitivity for detection of protein in buffered aqueous solutions, an even higher sensitivity is desired if these biosensors are used in complex biological or environmental samples to counteract background interference.

An efficient way to improve the sensitivity of a biosensor is through enzymatic signal amplification. Nicking endonuclease is an enzyme with high specific activity which recognizes a short specific sequence of a hybridized double-strand DNA and cleaves only one specific strand at a fixed position (Heiter et al., 2005; Morgan et al., 2000), which makes it favorable for constructing highly sensitive biosensors by using a template-triggered enzymatic recycling cleavage. As a consequence, in the past three years, quite a few nicking endonuclease-based biosensors have been reported for isothermal amplified detection of target DNA (Li et al., 2008; Kong et al., 2011; Chen et al., 2010; Zou et al., 2011) or its cofactor (Wang et al., 2010) by using DNAzyme as the molecular recognition module (Lin et al., 2011; Niu et al., 2010). Recently, aptamer–protein–aptamer conjugate-based nicking endonuclease amplified assay method has also been developed for highly sensitive detection of thrombin (Xue et al., 2010, 2012). However, this strategy employed two different aptamers (Apt15 and Apt29) to bind with two different sites of thrombin, which will limit its applications for other targets with just one aptamer. Although a large number of aptamers have been selected in the past twenty years, just a few aptamers could bind

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with two different sites of the same target. In most cases, a target molecule could bind with only one aptamer. Therefore, a new and universal design strategy with endonuclease-based signal amplification is desirable for aptasensors (Xue et al., 2010). In this work, by employing hairpin-structured design for both recognition and reporter probes to afford low background signal, and a nicking endonuclease to perform target-triggered enzymatic recycling signal amplification, we propose a universal amplified strategy for constructing ultra-sensitive fluorescence aptasensors for proteins. To demonstrate the general applicability of our strategy, IgE was chosen as a model protein.

2. Experimental

2.1. Chemicals

Oligonucleotides designed in this study were synthesized by invitrogen Bio Inc. (ShangHai, China), and their sequences are listed as follows:

RP1: 5′-GCGCGGGGCACG TACA GCTGAGGACACT TAAGACTA GGGGCACGT TTATCCGTCCCTCCTAGTGG CGTGCCCCGCGC-3′;
RP2: 5′-GCGCGGGGCACG TACA GCTGAGGACACTG AAAGACTA GGGGCACGT TTATCCGTCCCTCCTAGTGG CGTGCCCCGCGC-3′;
RP3: 5′-GCGCGGGGCACGTACA GCTGAGGACACTGT AAGACTA GGGGCACGT TTATCCGTCCCTCCTAGTGG CGTGCCCCGCGC-3′;
MB: 5′-(FAM)-CCACGAGTCAGTGT**CCTCAGCG**TGG-(DABCYL)- 3′.

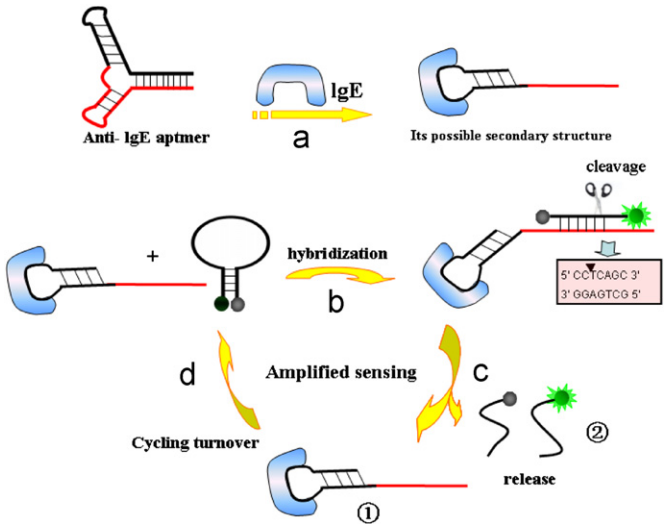
The underlined portion in the aptamer probe is the specific binding sequence for IgE, while the italicized portions can hybridize with the MB sequence. Immunoglobulin E (IgE) was purified from human plasma was obtained from Meridian Life Science, Inc. (Saco, ME USA). Human IgG and human serum albumin (HSA) were purchased from Dingguo Biotech (Beijing, China). Nicking Endonuclease Nt.BbvCI and NEB buffer 4 were purchased from New England Biolabs (Ipswich, MA). Other reagents were purchased from Sigma (St. Louis, USA). All chemicals were analytical grade unless otherwise specified. All solutions were prepared with Milli-Q water (resistance $>18\,M\Omega\,cm$) from a Millipore system. All the oligonucleotides involved in our experiment were dissolved in 10 mM Tris–HCl (pH 7.9), 10 mM $MgCl_2$ containing 50 mM NaCl prior to use.

2.2. Assay process

10 μL IgE of a specific concentration was mixed with 10 μL RP 2 (1 μM) for 30 min at 37 °C. Subsequently, 30 μL MB (167 nM), 50 μL 1 × NEB buffer 4 (20 mM Tris-Ac, 10 mM $Mg(Ac)_2$, 50 mM KAc, 1 mM DTT, pH 7.9) and endonuclease Nt.BbVCI (2 U) were successively added. The cleavage reaction was allowed to occur for 30 min at 37 °C.

2.3. Apparatus and fluorescence measurements

Fluorescence spectra were measured using a Hitachi F-7000 fluorescence spectrometer (Hitachi, Japan) with both excitation and emission slit set at 5.0 nm. A quartz fluorescence cell with an optical path length of 1.0 cm was used. Excitation wavelength was excited at 494 nm, and the emission spectra from 505 to 600 nm were collected. All measurements were carried out at room temperature unless stated otherwise.



Scheme 1. Schematic illustration of the sensing strategy based on allostery-triggered enzymatic recycling amplification: (a) the binding of target with aptamer triggering the allostery of the recognition probe, black line represents IgE aptamer, red line is the additional 37-mer fragment which can hybrid with the MB; (b) hybridization between the liberated 37-mer fragment and MB to form the recognition site for Nt.BbvCI; (c) Nt.BbVCI digestion of the MB; (d) recycling amplification.

3. Results and discussion

3.1. Sensing mechanism

Scheme 1 displays the amplified sensing principle for our aptasensor. The aptasensor employs a hairpin-structured probe contains the anti-IgE aptamer sequence (the red portion) and a 37-mer fragment (the black portion) as the recognition probe. In our design, a molecular beacon (MB) with a nicking endonuclease site was chosen as the signal probe which can partially hybridize with the 37-mer fragment. In the absence of target protein, both the recognition probe and MB folded into hairpin structures due to the favorable formation of the stem hybrids. The 37-mer fragment was masked in the hairpin structure and thus could not hybridize with the MB. As a consequence, since the recognition site for Nt.BbvCI was not formed and the MB can not be cleaved, no remarkable fluorescent signal was trigged. However, in the presence of target protein, the specific formation of IgE/aptamer complex induced the intramolecular folding of the recognition probe, and thus releasing the 37-mer fragment. Then, the released 37-mer fragment partially hybridized to hybridize with the MB and open its hairpin structure, as well as form the double-stranded recognition sequence for Nt.BbvCI. Once the MB was cleaved into two parts and dissociated from the sensing system, the fluorescence signal was dramatically increased because of the complete separation of the fluorophore from the quencher. The released recognition probe-IgE complex with 37-mer fragment was then hybridized with another MB, and triggered the second cycle of cleavage, leading to an accumulation of fluorescence signals. Eventually, each recognition probe-IgE complex can undergo many cycles to trigger the cleavage of many MBs, providing amplified fluorescence signal for the target.

3.2. Feasibility of the proposed signaling principle and signal amplification capability of the aptasensor

To verify the recognition probe allostery induced by target IgE could trigger the opening of the hairpin structure of the MB, fluorescence assay in the absence of endonuclease was first

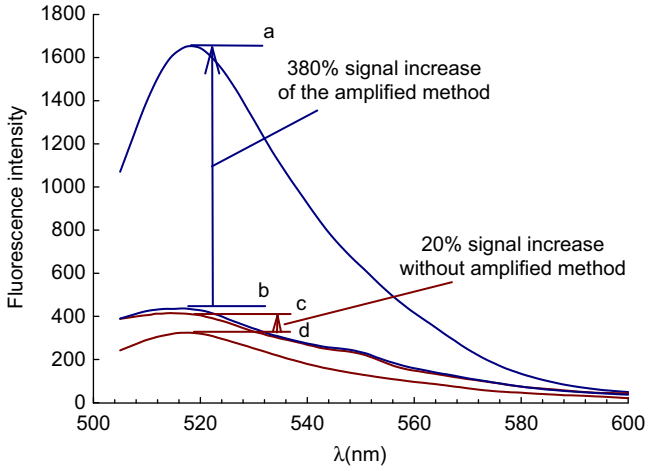


Fig. 1. The fluorescence spectra of the sensing system under different conditions: (a) RP2+MB+Nt.BbVCI+IgE, (b) RP2+MB+Nt.BbVCI, (c) RP2+MB+IgE, and (d) IgE+MB. The IgE concentration is 1 nM.

carried out (see the Supporting Information, Fig. S1). The addition of IgE could significantly induce the fluorescence enhancement of the sensing system, while the absence of IgE could only induce a slight fluorescence enhancement. The results indicated that the specific binding of IgE by the aptamer sequence could trigger the conformational transition of the recognition probe, thus releasing the 37-mer fragment to hybridize with MB probe and open its hairpin structure. Therefore, the fluorescent intensity was increased.

To assess the amplification function of the proposed sensing system, the allostery-triggered fluorescence enhancements in the presence and absence of Nt.BbVCI were then recorded. The RP2 was first incubated with target protein IgE for 30 min at 37 °C. After the simultaneous addition of MB and Nt.BbVCI with 1 × NEB buffer 4, the mixture was incubated for another 30 min at 37 °C, then the fluorescent spectra were recorded. As shown in Fig. 1, under these conditions, an increased background fluorescence was observed (curve b), which could be due to the partially hybridization of RP2 with MB. Fortunately, the IgE-induced signal enhancement was much larger than the background (curve a), assuring a high sensitivity for detection of target protein. As could be observed in Fig. 1, in the presence of endonuclease Nt.BbVCI, there was a (380 ± 21)% fluorescent signal increase upon addition of 1 nM IgE. In contrast, under the same condition, in the absence of endonuclease, only a (20 ± 5)% signal increase was observed. All these results indicated that the endonuclease-based amplification strategy could substantially afford an amplified fluorescence signal. Furthmore, the amplification based on the nicking endonuclease was obvious, which lad to a dramatic increase in the final fluorescence intensity.

3.3. Optimization of the recognition probe sequence

In order to achieve optimum sensing performance for the sensing system, the sequence of the recognition probe was investigated. The sequences RP1, RP2, and RP3 contain 12, 13 and 14 complementary bases with the MB probe were chosen as the recognition probes, and the fluorescent responses in the presence and absence of IgE (2 nM) were recorded. As shown in Fig. 2A, the fluorescence responses increased with the increasing of the number of the complementary bases between recognition probe and MB probe, which indicated that the hybridization stability was increased. However, too many complementary base pairs could lead to strong hybridization between the recognition

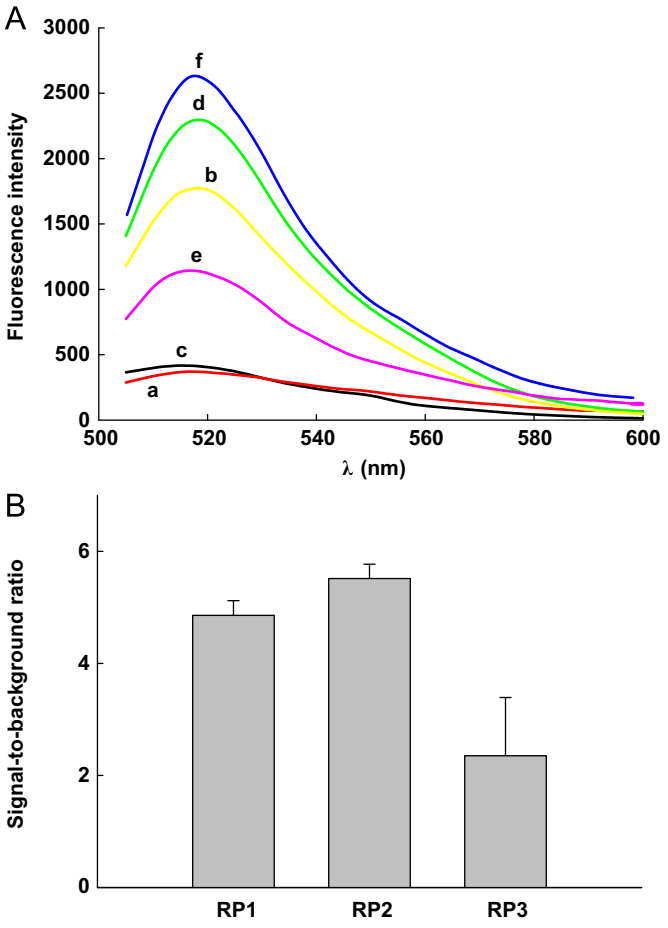


Fig. 2. (a) Fluorescence responses of RP 1, RP 2, and RP 3, respectively. Lines (a, c, e) represent fluorescence signal in the absence of target (2 nM) and lines (b, d, f) represent fluorescence signal in the presence of target. (b) The corresponding signal-to-background ratio.

probe and MB, resulting in the increase of the background signal. As shown in Fig. 2B, by using the recognition probe RP2, a largest signal-to-background ratio (SBR) was observed. Therefore, RP2 was chosen as the optimized recognition probe for further investigation.

3.4. Optimization of the endonuclease and MB concentration

The performance of the developed sensing system is still strongly influenced by the assay conditions such as the concentration of endonuclease and MB. In order to obtain an optimal assay conditions and achieve a high signal-to-noise ratio, the effects of endonuclease and MB were investigated (see the Supporting Information, Fig. S2). Endonuclease Nt.BbvCI provides a unique opportunity to monitor the reaction of its recognition site on a specified strand of the DNA. Its activity on ssDNA is limited when the recognition sequence does not form a complementary dsDNA. Although the recognition probe and MB could not hybridize to each other in the absence of target protein, the nonspecific hybridization still enhance the background fluorescence. The fluorescence responses were recorded with different concentrations of endonuclease. Our experimental results showed that the fluorescence intensity increased when the concentrations ranging from 0.005 to 0.04 U/μL, but the background fluorescence increased either. A final concentration of endonuclease at 0.02 U/μL could provide a maximum S/N ratio for the sensing system.

Therefore, 0.02 U/μL was chosen as the optimized endonuclease concentration.

Due to the self-quenching of the unique stem-loop structure, the MB has a weak background. The high MB concentration could produce high background fluorescence and interfere with the *S*/*N* ratio. Therefore, the effect of MB concentration was further investigated. Experiment results showed low concentration of MB could produce a very low background signal. Considering the sensitivity of the instrument and the *S*/*N* ratio, 50 nM MB as the optimized assay concentration was chosen.

3.5. Analytical performance of the Sensor

To investigate the sensitivity of the proposed sensing system for the detection of target protein, the fluorescence enhancement upon the addition of different concentrations of IgE were recorded. As shown in Fig. 3a, the fluorescence intensity increased gradually with the increasing of the IgE concentration, indicating that the signal-on sensing mechanism possesses preferable sensing properties. Fig. 3b shows the calibration curve of the amplified sensing system with a dynamic range from 10 pM to 20 nM for IgE with a detection limit of 5 pM. In contrast, control experiments for the biosensor with IgE at various concentrations in the absence of endonuclease were also carried out with a detection limit of only 1 nM (see the Supporting Information, Fig. S3). Its sensitivity is two orders of magnitude poorer than that of the endonuclease-amplified method. These results indicate that the introduction of endonuclease could remarkably improve the sensitivity of the biosensor. As a result, the proposed aptameric amplified sensing system shows obviously improved sensitivity than previously reported aptameric biosensors for IgE. A similar design for protein detection based on target-triggered aptamer hairpin switch and nicking enzyme assisted fluorescence signal amplification was reported recently by Xing group (Xue et al., 2012). In contrast to linear signal probe used in their design, MB shows a low fluorescent background with a high signal-to-noise (*S*/*N*) ratio. In addition, the hairpin structure of MB can effectively lower the non-specific hybridization with the aptamer probe.

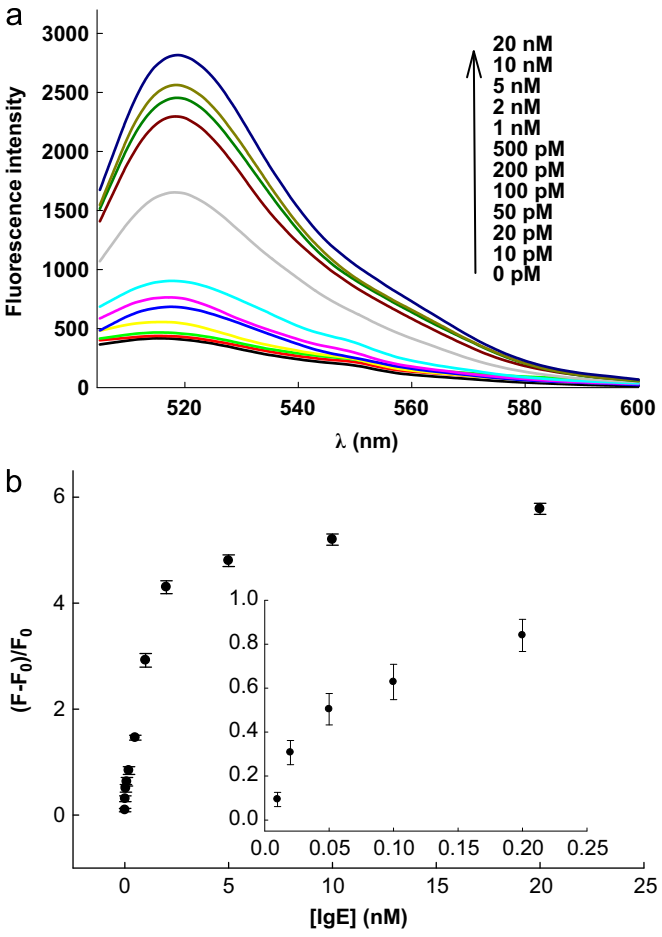


Fig. 3. (a) Fluorescence spectra of assay systems at various concentrations of IgE. (b) The relative fluorescence intensity enhancement with various concentrations of IgE. Inset shows the responses of the sensing system to target IgE at low concentration. The error bars indicated the standard deviations of three independent experiments. F_0 and F are the fluorescence intensity of the sensor in the absence and presence of target, respectively.

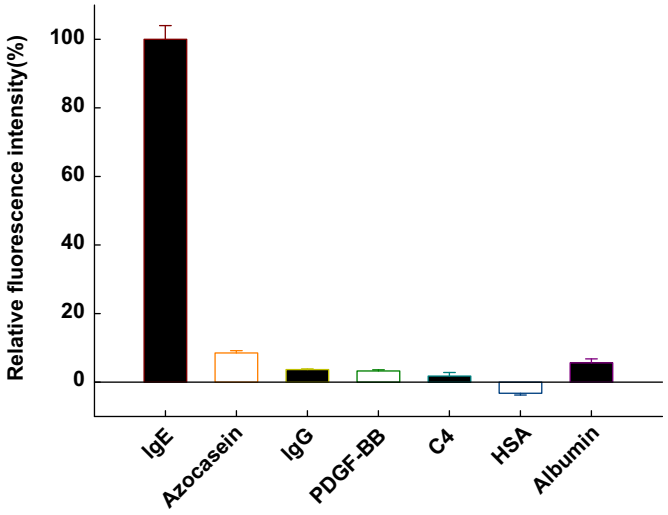


Fig. 4. Selectivity of the aptameric biosensor for IgE over other potential interferences (the proteins concentrations: 5 nM). Relative fluorescence intensity = $\Delta F/F_0 \times 100\%$, where signal change ΔF was calculated as $F - F_0$. The target fluorescence response is defined as 100%.

The amplified sensing system also shows a high specificity for IgE. The fluorescence changes induced by several potential interferents were also studied under the same conditions as those involved for IgE assay. As shown in Fig. 4, when protein concentrations are all 5 nM only IgE caused a dramatic fluorescence enhancement, indicating that other proteins can't drive the allostery of the recognition probe to trigger the enzymatic cleavage of the MB reporter probe. These results demonstrate the high specificity of our proposed sensing system for IgE.

3.6. Analysis of complex biological samples

To test the practicality of this aptasensor in complex biological samples, we further conducted the IgE detection in human serum, one of the most challenging media containing a variety of proteins and other potential interferents. Varying amounts of IgE were added to the diluted (20-fold) serum samples, and then the fluorescence responses of the aptasensor induced by these samples were recorded. It is found in Fig. 5 that the fluorescence enhancement obtained in serum was similar to that measured in buffered aqueous solution, although the background fluorescence showed a slight increase. These results revealed that the proposed aptameric biosensor was applicable for practical IgE detection in real biological samples with other potential interferents co-existing.

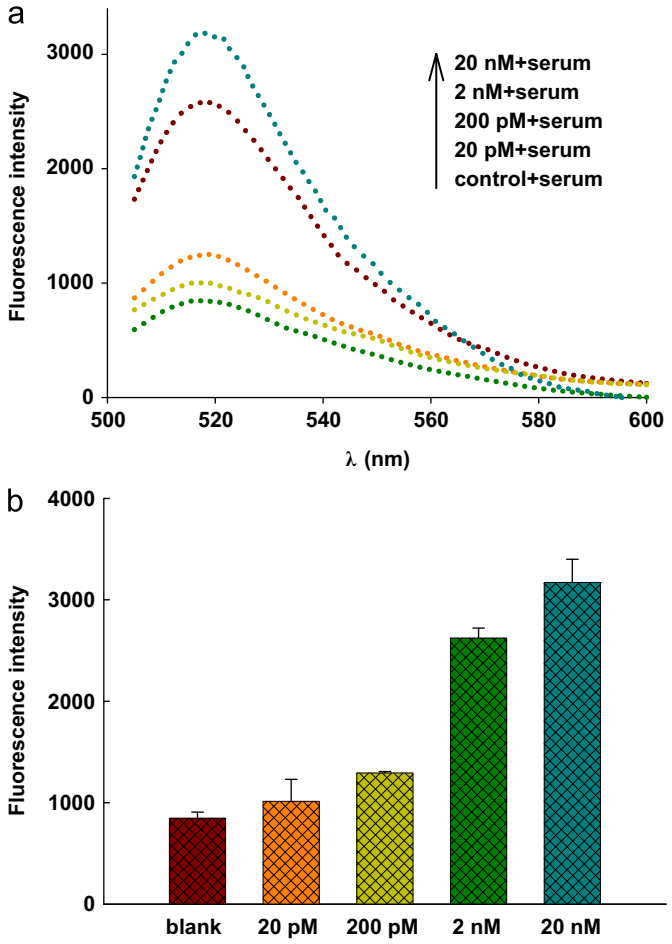


Fig. 5. Fluorescence response of the aptasensor with IgE in diluted (20-fold) serum samples in the concentration range from 0.02, 0.2, 2 to 20 nM.

4. Conclusion

In conclusion, we have developed a universal, nicking endonuclease-based, amplified sensing strategy for the design of fluorescence aptasensors. By combining the lowing background signal strategy through hairpin-structured design for both recognition and reporter probes with the enzymatic recycling amplification, the proposed amplified aptasensor shows high sensitivity to IgE with a detection limit of 5 pM, which is much lower than that of previously reported aptasensors for IgE. This novel sensing system is simple in design and can be easily carried out by simple mixing and incubation. Moreover, the proposed sensing system was applied in human serum for the detection of target protein with results similar to that in buffer solution, demonstrating the potential of practical applications in biological samples. Since numerous aptamers have been selected for a wide range of targets including small molecules, proteins and even cells, our strategy provides a new general platform for constructing highly sensitive biosensors for various targets, and holds a promising potential in biomedical applications.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2012.05.008.

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