

Label-free homogeneous detection of immunoglobulin E by an aptameric enzyme subunit

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Abstract We have developed an aptameric enzyme subunit (AES) for immunoglobulin E (IgE) sensing. AES is an artificial enzyme subunit constructed from two different aptamers and does not require any modification. Using the AES, the target molecule can be detected by measuring enzymatic activity in homogeneous solution. We connected IgE-binding aptamer and its complementary strand to split thrombin-inhibiting aptamer. The hybrid of these two oligonucleotides inhibited thrombin activity and it decreased in the presence of IgE. We were able to detect IgE by using this AES in homogeneous solution with a detection limit of 50 pmol.

Keywords Aptameric enzyme subunit · Enzyme-inhibiting aptamer · Protein detection · Immunoglobulin E

Introduction

Aptamers are nucleic acid ligands which are selected from random sequence pools (Ellington and Szostak 1990; Tuerk and Gold 1990). Numerous aptamer-based sensors have been developed (for review, see

Mairal et al. 2007) because aptamers can recognize their target molecules with high affinity and specificity. On the other hand, aptamers have some advantages over antibodies. One of them is the structure change of aptamers can be designed by the straightforward hybridization rules. Recently, homogeneous sensing systems which used structure change of aptamers have been reported (for review, see Nutiu and Li 2005). These sensor systems mainly use fluorescence resonance energy transfer (FRET) to detect their target molecules therefore these sensor systems are required to modify fluorescence to aptamers. Moreover, FRET-based techniques are not so sensitive assays because they cannot amplify detection signals. The allosteric ribozyme (Soukup and Breaker 1999) and deoxyribozyme (Levy and Ellington 2002) can amplify detection signals however their signal amplification are not effective because of their low catalytic activity.

We have reported aptameric enzyme subunit (AES) that can detect target molecules by measuring enzymatic activity in homogeneous solution (Yoshida et al. 2006a, b; Ikebukuro et al. 2007). AES is constructed by connecting an enzyme-inhibiting aptamer to a target molecule-binding aptamer. AES can allosterically control the corresponding enzymatic activity by binding to target molecules, that is, AES enables the homogeneous detection by measuring enzymatic activity. Enzymes can amplify the detection signals effectively therefore highly sensitive assay can be expected compared with FRET-based techniques,

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allosteric ribozyme or deoxyribozyme. Moreover, AES can be made by simply oligonucleotides which do not contain any modification. AES would archive highly sensitive, easy and rapid assay.

We have already developed AESs for small-molecule and DNA sensing, however, protein sensing is one of most important task for clinical diagnosis such as cancer diagnosis. In this paper, we report an AES which can detect the target protein. We used a thrombin-inhibiting aptamer (Ikebukuro et al. 2005) as an enzyme-inhibiting aptamer and the IgE-binding aptamer (Wiegand et al. 1996) as a target molecule-binding aptamer and connected them to construct an AES for IgE sensing (Fig. 1).

Materials and methods

Materials

Human thrombin and bovine serum albumin (BSA) were purchased from Sigma and human fibrinogen was purchased from Calbiochem and IgE was purchased from Athens Research & Technology. All oligonucleotides were obtained from Invitrogen (Table 1). The other reagents were of analytical grade.

Measurement of the inhibitory effect of AES on thrombin

The oligonucleotides were mixed in the buffer (8.1 mM Na_2HPO_4 , 1.1 mM KH_2PO_4 , 138 mM NaCl, 2.7 mM

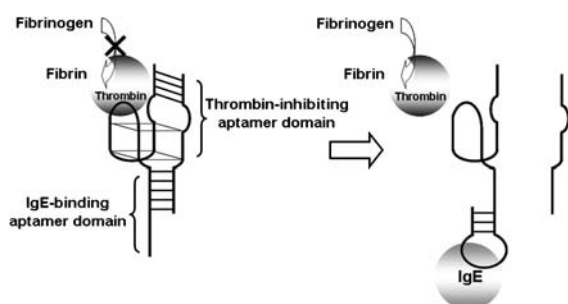


Fig. 1 Schematic of IgE sensing by AES. The IgE-binding aptamer and its part of complementary strand were attached to split thrombin-inhibiting aptamer. The structure change of IgE-binding aptamer domain leads to the disruption of the thrombin-inhibiting aptamer domain

KCl, 1 mM MgCl_2 , pH 7.4) and annealed by heating the solution to 95°C for 3 min and slowly cooling down to 25°C over 30 min. The thrombin was added to oligonucleotides and then IgE or BSA was added. The final volume of the reaction was 0.2 ml. After 5 min incubation at 37°C, the fibrinogen was added to the oligonucleotides–thrombin–IgE mixture. The clotting time was measured using an automated fibrometer (Amelung KC-4A Micro Coagulation Analyzer) at 37°C. The concentration of oligonucleotides and proteins are provided in each legend to figures.

Results

Design and characterization of AES

The thrombin-inhibiting aptamer might fold into G-quartet structure which is important to inhibit thrombin activity. Therefore if the G-quartet structure is destroyed by binding to target molecules, the inhibitory activity of the aptamer can be decreased. To construct AES which has above property, we split the thrombin-inhibiting aptamer into two oligonucleotides at 3' T-T loop site on G-quartet structure and the one of the split thrombin-inhibiting aptamer was attached with the IgE-binding aptamer (5' AES) and the other strand was attached with 8-mer complementary strand of the IgE-binding aptamer (3' AES). We expected that these oligonucleotides would hybridize with each other to fold the G-quartet structure which inhibits thrombin activity and they would dissociate by binding to IgE because the folding of IgE-binding aptamer would prevent the hybridization between the IgE-binding aptamer and its complementary strand. This structure change which is induced by IgE would result in a decrease thrombin inhibition.

The thrombin enzymatic activities were determined by measuring the clotting time of fibrinogen solution, because the thrombin-inhibiting aptamer inhibits clot formation by binding to fibrinogen binding site of thrombin. The mixture of 5' AES and 3' AES did not inhibit thrombin enzymatic activity (data not shown). This result indicated that the 8-mer complementary strand of the IgE-binding aptamer was not enough to hybridize with the IgE-binding aptamer site. Therefore, we inserted the 6- or 4-bp linker DNA between the thrombin-inhibiting

Table 1 Sequences of the oligonucleotides used in this study

Oligonucleotide	Sequence (5' to 3')
Thrombin aptamer	cactgtaggttggtgtggtgggcccagtg
5' AES	cactgtaggttggtgtggtTTCACGTTTATCCGTC CCTCCTAGTGGCGTGA
3' AES	AAACGTGATtggggccagtg
5' AES-4	cactgtaggttggtgtggtTATTGTACGTTTATCCGTC CCTCCTAGTGGCGTGA
3' AES-4	AAACGTGACAATTtggggccagtg
5' AES-6	cactgtaggttggtgtggtTGAATTGTACGTTTATCCGTC CCTCCTAGTGGCGTGA
3' AES-6	AAACGTGACAATTCTtggggccagtg

The thrombin-inhibiting aptamer is shown as lower case, the IgE-binding aptamer is underlined, and the complementary strand of IgE-binding aptamer is shown in italics

aptamer domain and the IgE-binding aptamer domain to promote hybridization each other (5' AES-6 is 5' AES which bears 6-mer linker DNA; the other oligonucleotides were named in the same way). The hybrid of the 5' AES-4 and 3' AES-4 and the hybrid of the 5' AES-6 and 3' AES-6 were designated as AES-4 and AES-6, respectively. The presumed structure of the AES-4 is shown in Fig. 2.

The 5' AES-6, 3' AES-6, 5' AES-4 and 3' AES-4 did not inhibit thrombin enzymatic activity alone (data not shown), however the AES-6 and AES-4 inhibited thrombin enzymatic activity (Fig. 3, bar 5 and 8). These results indicate that linker DNAs promote hybridization and these hybrids would fold a G-quart structure to inhibit thrombin activity. The inhibitory activity of the thrombin-inhibiting aptamer and the AES-6 did not change by addition of IgE compared with it in the presence of BSA (bar 3, 4, 6, and 7), however the inhibitory activity of the AES-4 decreased by addition of IgE (bar 9 and 10, the clotting time was changed from 24 to 20 s). These results indicate that the thrombin-inhibiting aptamer is not affected by IgE and the 6-bp linker DNA is too long to induce structure change of the IgE-binding aptamer domain. However the structure change of the AES-4 might be induced by binding to IgE and result in a decrease thrombin activity.

Calibration of IgE using AES-4

In the above experiment, 1.7 mg fibrinogen/ml was used as a substrate of thrombin. However the difference of clotting time between in the presence and absence of IgE was only 4 s when the AES-4 was used. To get a clearer difference, a lower concentration of

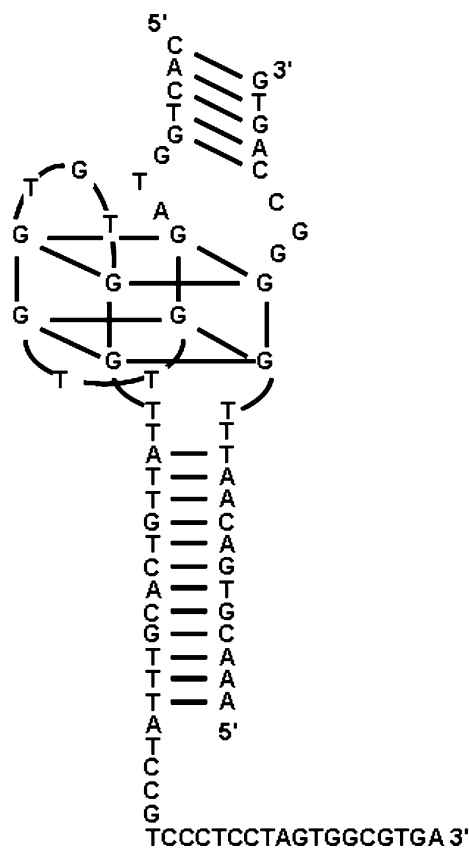


Fig. 2 Presumed secondary structure of the AES which has 4-bp linker DNA between two aptamers domain (AES-4)

fibrinogen (0.34 mg/ml) was used. Under this condition, the clotting time was approximately 26 s in the presence of thrombin only (data not shown). Although inhibition of thrombin activity was hardly influenced by the addition of BSA, it did decrease with IgE in a concentration-dependent manner and the lower IgE detection limit was 250 nM (Fig. 4).

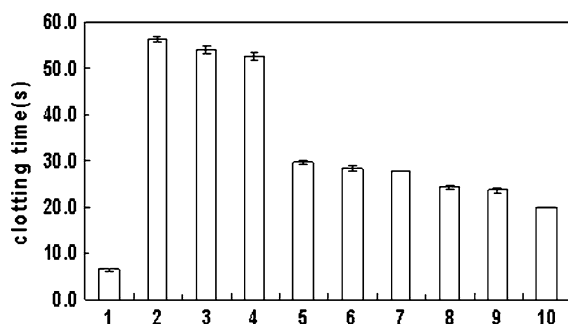


Fig. 3 Inhibition of clotting by addition of oligonucleotides with IgE or BSA. Bar 1, thrombin only; bar 2, thrombin-inhibiting aptamer; bar 3, thrombin-inhibiting aptamer with BSA; bar 4, thrombin-inhibiting aptamer with IgE; bar 5, AES-6; bar 6, AES-6 with BSA; bar 7, AES-6 with IgE; bar 8, AES-4; bar 9, AES-4 with BSA; bar 10, AES-4 with IgE. These experiments were performed using 250 nM oligonucleotide, 250 nM IgE, 250 nM BSA, 54 nM thrombin and 1.7 mg/ml fibrinogen at 37°C. Clotting times are the average of three experiments and error bars are indicated

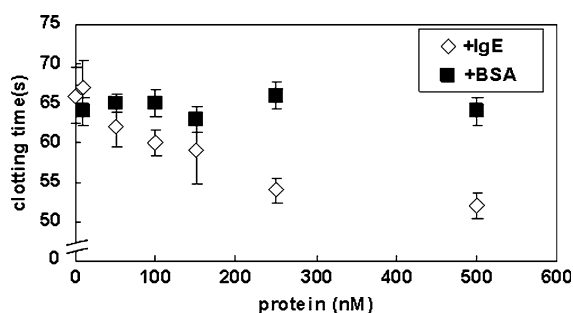


Fig. 4 Calibration graph of IgE using the AES-4. These experiments were performed using 250 nM AES-4, 54 nM thrombin and 0.34 mg fibrinogen/ml with appropriate proteins in a final volume of 0.2 ml at 37°C. Clotting times are the average of three experiments and error bars are indicated

Discussion

The sensitivity of the AES-4 was not higher than ELISA because we used the thrombin-inhibiting aptamer as an enzyme-inhibiting aptamer. To determine thrombin enzymatic activity, we measured the clotting time which cannot amplify the detection signal effectively. The thrombin enzymatic activity can also be measured by using the peptide modified with fluorescence because thrombin is one of serine protease. However the thrombin-inhibiting aptamer cannot inhibit the binding of peptide to active site of thrombin because it binds to fibrinogen binding site.

We previously suggested the split AES for universal design (Yoshida et al. 2006) and it was also successfully applied to IgE detection. Many aptamers form intramolecular hybridization to fold their three-dimensional structures therefore aptamers can be split into two oligonucleotides at the stem-loop forming site. There are other reports that describe the split aptamers into two oligonucleotides that hybridize when their target molecules are present (Stojanovic et al. 2000). In this study, we used the thrombin-inhibiting aptamer; if a more suitable enzyme-inhibiting aptamer for AES is used, the sensitivity of the AES would be improved.

Conclusion

We constructed an AES for IgE sensing by connecting IgE-binding aptamer and its part of complementary strand to split thrombin-inhibiting aptamer. Using this AES, we were able to detect IgE by measuring thrombin enzymatic activity in homogeneous solution. This means split AES design is applicable to many kinds of aptamers. We have already developed AESs for small-molecule and DNA sensing. Therefore DNAs, RNAs, low-mass molecules and proteins can be detected by AESs in a common assay.

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