Highly sensitive protein detection based on aptamer probe and isothermal nicking enzyme assisted fluorescence signal amplification†

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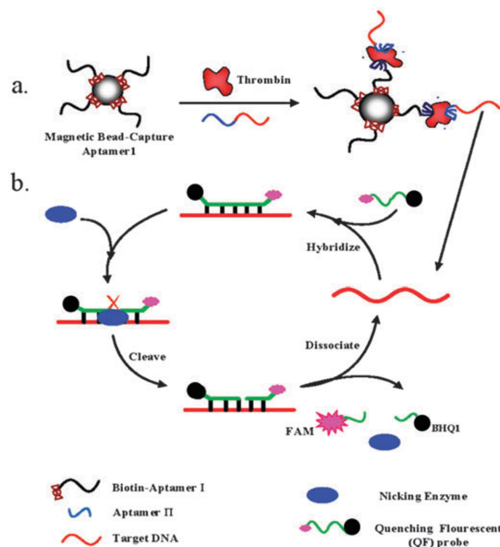
An isothermal and sensitive fluorescence assay for protein detection using aptamer–protein–aptamer conjugates based on nicking enzyme amplification has been developed, which was combined with magnetic microparticles separation, making this assay suitable for protein detection in biological samples.

The detection of proteins plays a key role in biomedical research as well as in clinical diagnostics. Current assays for proteins are mostly based on the use of antibodies, which are not well adapted to rapid, sensitive protein detection. Aptamers are DNA or RNA oligonucleotides that have been designed through *in vitro* selection experiments termed SELEX[1,2] (Systematic Evolution of Ligands by Exponential Enrichment). They are selected from random sequence libraries, which has wide applicability and productivity. Aptamers also deliver optimized nucleic acids for high-affinity binding to given ligands such as proteins, lipids or small molecules.[3] They offer several advantages over antibodies as they can be completely engineered *in vitro*, synthesized chemically, and labeled by chemical synthesis.[4,5]

In recent years, several methods for protein detection using aptamers have been proposed. Aptamer-based protein detections have been illustrated in conjugation with electrochemistry,[6] colorimetry,[7] fluorescence,[8] surface plasmon resonance[9] and chemiluminescent techniques.[10] However, most of these strategies are time-consuming, not sensitive enough, or need a multistep process. And, as we know, effective disease diagnosis is highly dependent on the development of a sensitive method, so the development of a highly sensitive signal amplification strategy is a highly significant task. As to amplification assays, immuno-polymerase chain reaction[11] (immuno-PCR) and immuno-rolling circle amplification[12] (immuno-RCA) can achieve a very low detection limit. However, both of them require the conjugate of antibody and DNA, thus are subject to the same shortcomings faced by other assays applying antibodies, including labor intensive synthesis, being time-consuming and potential batch-to-batch variation. To circumvent the aforementioned limitations, other alternative strategies, such as aptamer based PCR[13] and aptamer based RCA[14] assays, have been developed. Both of the assays are highly sensitive and can overcome some disadvantages brought by antibodies. For aptamer-PCR, the highly precise temperature cycling needed for PCR hampers its widespread use for routine analysis. Aptamer-based RCA can be carried out isothermally. But, the design of an effective aptamer probe that can undergo a complex conformational change to effectively initiate RCA is fairly difficult.[14] Besides, several assays using nanoparticles and enzyme as tools for signal amplification have been developed.[15] Nanoparticles-based materials (gold nanoparticles, Cd nanoparticles *etc.*) show excellent prospects for protein detection because of their unique physical and chemical properties. However, the labelling of these nanoparticles is really time-consuming and the chemical modification processes are complicated.

In this report, we developed a sensitive and isothermal nicking enzyme assisted fluorescence signal amplification assay (NEFSA) for protein detection. In order to demonstrate the principle (Scheme 1), we chose human α-thrombin as the model analyte of interest. Thrombin is an important protein that plays a central role in thrombosis. The thrombin-binding aptamer[16] (Apt15, 5′-GGTTGGTGTGGTTGG-3′) is the first one chosen *in vitro*, specific for a protein. After the thrombin-binding aptamer G-quarter had been established and the binding site had been identified, this aptamer has been coupled to different transduction principles and has shown wide applicability. These studies clearly indicate the suitability and the advantage of aptamer-based systems. Moreover, a different thrombin-binding aptamer, Apt29[17] (single-stranded DNA with a K_d of 0.5 nM), binding to the heparin-binding site of thrombin, has also been selected. Two different aptamers can be used for the same target, which allows the design of



Scheme 1 Schematic representation of the nicking enzyme assisted fluorescence signal amplification (NEFSA) assay for protein.

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different assay systems, such as sandwich assays, making it possible for a real diagnostic application.[17] To assay human α-thrombin, aptamer I (Apt29) which was labeled by biotin, was immobilised onto streptavidin-coated magnetic beads. This binding interaction between streptavidin and biotin is quick, reliable, and strong. We chose magnetic beads, instead of another surface immobilization strategy, because we used the beads not only as the immobilization matrix but also as the separation tool. Moreover, the use of the magnetic beads can reduce the incubation time, facilitating the kinetics of the binding process.[18] The second aptamer, aptamer II (Apt 15), was designed containing an extended DNA sequence (the ex-DNA). The binding of the two aptamers to the same thrombin molecule resulted in a sandwich complex (aptamer I–thrombin–aptamer II). Subsequently, these complexes were easily separated and excess probes were washed off using buffer with the assistance of a magnetic separator. It dramatically minimized the matrix effect due to improved washing and separation. The ex-DNA was designed to be complementary to the BHQ-quenching fluorescence (BQF) probe.[19] The BQF probe is a short oligo-DNA, which carried the recognition sequences and cleavage site for the nicking endonuclease Nb.Bbvc I,[20] and was labeled with the fluorescent dye 6-carboxyfluorescein (6-FAM) and its quencher Black Hole Quencher I (BHQ I) at the 5′- and 3′-ends, respectively.[21] Nicking endonuclease is an enzyme that binds to its asymmetrical recognition sequence in double-stranded DNA and nicks only one specific strand of the duplex.[22] Therefore, a fluorescence signal appears only when the BQF probe was cleaved by Nb.BbvC I. After nicking, the hybrid BQF probe became less stable, and the cleaved strand dissociated from the target, thus resulting in the complete disconnection of the fluorophore from the quencher. The released target strand could then hybridize to another BQF probe and initiated the second cycle of cleavage. Finally each target strand could go through many cycles, resulting in cleavage of many probes. In traditional fluorescence methods, such as the molecular beacon based strategy, one target can only cause one quenched fluorescent probe to fluoresce. Therefore, the current method will have a higher detection limit than the traditional ones. And, for other proteins, it is easy to desire an advisable DNA structure that can effectively initiate NEFSA by changing the corresponding aptamer.

To provide convincing proofs of the detection mechanism of the BQF probe and the nicking enzyme reaction, spectra and/or relative fluorescence intensities of the various phases of the BQF probe were tested. Fig. S3 shows that the fluorescence intensities of the BQF probe alone or hybridized to its complementary sequence in aptamer II are rather low. When the BQF probe hybridized to aptamer II and in the presence of nicking enzyme, significant fluorescence responses were achieved. To confirm the ability of the described strategy to sensitively detect target protein, a series of different concentrations of thrombin were measured. All the fluorescence intensities were measured after 80 min of enzymatic reaction. As shown in Fig. 1, the fluorescence intensities were increased with the increase of thrombin. The insert shows fluorescence spectra of the sensing system after addition of different concentrations of thrombin. This phenomenon that the fluorescence

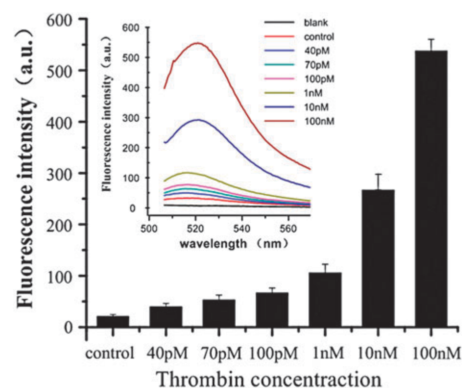

Fig. 1 Fluorescence intensities and emission spectra obtained in the presence of control or different concentrations of thrombin. The error bar was calculated from three independent experiments. All the fluorescence intensities were measured after 80 min of enzymatic reaction. Signal from the blank sample is the instrument background.

intensity increased gradually with the concentrations of thrombin was related to the accumulation of the fluorescence and it implied that more and more BQF probes were cleaved by nicking enzyme in solution. Additionally, the fluorescence of the control sample (the thrombin was absent, but all the other compositions and reaction steps were the same as in the detection of thrombin samples. See the detail in supplementary information†) is relatively low because the ex-DNA can be hybridized only in the presence of target protein. We estimate the limit of detection is 40 pM based on a signal to noise ratio of 3. In contrast with other aptasensors, the detection limit is lower,[23,24] which represents that this nicking enzyme amplification method for protein detection is sensitive.

Sensitivity and specificity are the two key factors for a successful assay system for protein. To verify the specificity of the procedure, a series of comparative studies between the thrombin and the other proteins were performed, using the same procedures (see the detail in supplementary information†) as those used for human thrombin. The concentrations of the proteins were 1 nM thrombin, BSA and trypsase. As shown in Fig. 2, only thrombin induced a marked response, which

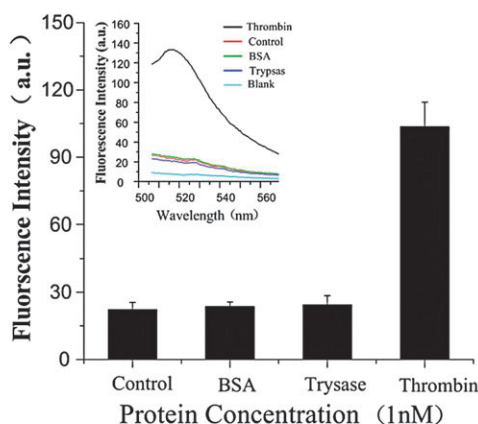

Fig. 2 Test of human thrombin and other proteins, showing the specificity of the assay. The protein concentration of the control sample is 0 nM. All the fluorescence intensities were measured after 80 min of enzymatic reaction. Signal from the blank sample is the instrument background.

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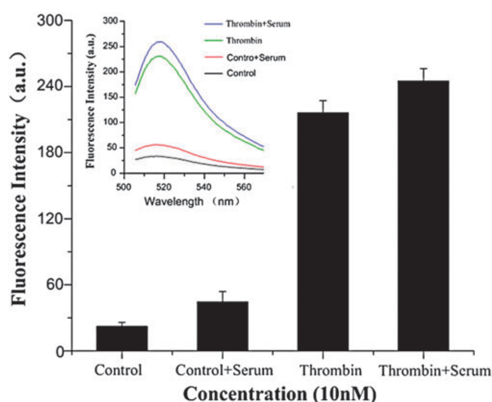

Fig. 3 Results obtained from the testing of serum samples spiked with thrombin and thrombin in buffer (thrombin concentration is 10 nM). All the fluorescence intensities were measured after 80 min of enzymatic reaction.

indicated that the aptasensor is responsive to its target protein with extraordinary specificity. Compared with previous studies,[24,25] the target protein binding to two different aptamers rather than one aptamer as chosen in our method can improve the specificity.

To demonstrate the feasibility of the approach in complex biological matrixes, the amount of thrombin in serum was detected by this aptasensor. Serum is what remains from whole blood after coagulation, whose chemical composition is similar to plasma, but does not contain coagulation proteins such as thrombin or other factors. Serum, diluted 10 times (see the detail in supplementary information†), was tested alone or spiked with thrombin (at a concentration of 10 nM), and the results were compared with the same concentrations prepared in buffer.

As shown in Fig. 3, comparable responses were found for thrombin in both buffer and serum. We noted that the signal obtained in serum was slightly higher than the signal measured in buffer. Probably this increase is due to the nonspecific binding of the aptamer to the beads caused by the interaction with some matrix components. And, the serum contained some DNA nucleases not present in the standard solution which caused the increase of fluorescence intensity. Overall, the current assay shows potential for application in protein detection in a biological system.

To conclude, we have successfully developed a new isothermal reaction to detect protein by taking advantage of two affinity aptamers for increased specificity and nicking enzyme for signal amplification. Firstly, the proposed NEFSA assay differs from most other detection methods based on aptamers since the amplification does not require thermal cycling and the read-out does not require further preparation steps. Therefore the method has also the potential to be integrated in simplified devices for distributed diagnostic applications. Secondly, compared to other amplified strategies such as PCR and RCA, the design of an effective detection probe for NEFSA is fairly easy. By changing the corresponding aptamer, NEFSA assay can be easily used for other proteins. Thirdly, magnetic beads serving as the solid support make it easy to collect the target from the complex sample matrix by a magnetic field, which makes this assay suitable for protein detection in biological samples.

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