Nucleic Acids Research Advance Access published July 8, 2011

Nucleic Acids Research, 2011, 1–7
doi:10.1093/nar/gkr559

Fluorescence protection assay: a novel homogeneous assay platform toward development of aptamer sensors for protein detection

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Received May 16, 2011; Revised June 15, 2011; Accepted June 19, 2011

ABSTRACT

Development of novel aptamer sensor strategies for rapid and selective assays of protein biomarkers plays crucial roles in proteomics and clinical diagnostics. Herein, we have developed a novel aptamer sensor strategy for homogeneous detection of protein targets based on fluorescence protection assay. This strategy is based on our reasoning that interaction of aptamer with its protein target may dramatically increase steric hindrance, which protects the fluorophore, fluorescein isothiocyannate (FITC), labeled at the binding pocket from accessing and quenching by the FITC antibody. The aptamer sensor strategy is demonstrated using a model protein target of immunoglobulin E (IgE), a known biomarker associated with atopic allergic diseases. The results reveal that the aptamer sensor shows substantial (>6-fold) fluorescence enhancement in response to the protein target, thereby verifying the mechanism of fluorescence protection. Moreover, the aptamer sensor displays improved specificity to other co-existing proteins and a desirable dynamic range within the IgE concentration from 0.1 to 50 nM with a readily achieved detection limit of 0.1 nM. Because of great robustness, easy operation and scalability for parallel assays, the developed homogeneous fluorescence protection assay strategy might create a new methodology for developing aptamer sensors in sensitive, selective detection of proteins.

INTRODUCTION

Rapid and selective assays of protein biomarkers play crucial roles in proteomics and clinical diagnostics. Such assays are typically started using certain affinity ligands such as antibodies and aptamers that specifically interact with the protein targets. Then, the molecular recognition events are detected via a cascade of signal transduction to render target-specific responses. Classic protein detection strategies include enzyme-linked immunosorbent assays, protein microarrays (1), magnetic-separation assay (2), lateral-flow assay (3,4) and biosensors (5,6). These techniques commonly rely on immobilization of the biomolecular ligands to the protein targets. Although technically endowed with the capacity of multiplexed assay of multiple targets, these surface-based methods may interfere with interactions between the target proteins and the biomolecular ligands, thus requiring cautious washing and blocking to combat non-specific adsorption. Homogeneous assay such as fluorescence polarization (7), fluorescence resonant energy transfer (8,9) and protein-fragment complementation (10), which can be implemented without any surface operations, then offers a rapid, selective and robust technology for the detection of protein biomarkers.

Aptamers are short single-stranded oligonucleotides selected for their high affinity to certain targets (11,12). Compared with conventional biomolecular ligands as antibodies, aptamer-based ligands may exhibit prominent advantages such as site-specific labeling, structure-controlled design and sequence-dependent amplification, which makes them an ideal molecular recognition tool for biomedical detection and biosensor developments (13,14). In the context, the development of aptamer sensors with unique response strategies for homogeneous assays has been a subject of intensive interest, besides the proliferated uses of aptamers in place of antibodies in established immunoassay techniques (15). Aptamers often undergo conformational changes on interacting with their cognate targets, which renders a generic homogeneous assay strategy for the construction of aptamer sensors (16,17). Adaptive binding of aptamers to the targets is possible to displace a complementary sequence

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from the aptamer-target binding region. This structure switching strategy creates another useful homogeneous assay platform for aptamer sensors (18,19). On the other hand, aptamer sensors may be devised based on re-assembly of two split aptamer subunits as a result of aptamer–target interactions (20,21). Alternatively, aptamer sensors can be constructed for homogeneous assays based on proximity-dependent hybridization of two aptamer probes simultaneously interacting with target proteins (22,23). Apart from these response strategies, aptamer recognition can be readily combined with nucleic acid-based enzymatic treatments for downstream signal transduction or amplification in aptamer sensors (24–27). For example, aptamer recognition could result in the formation of a folded hairpin structure, from which a signal-reporting DNA sequence was amplified via nickase-based strand displacement amplification (24), target-mediated strand displacement amplification (25) or rolling circle amplification (26,27).

Herein, we report a novel aptamer sensor strategy for homogeneous fluorescence detection of protein targets. This strategy is based on the inhibition of antibody-induced quenching of fluorescein isothiocyannate (FITC) label by binding of aptamer to its protein target. Because interferences from non-specifically interacting proteins could be precluded by competitive interaction of antibody–FITC, this strategy possesses improved specificity as compared with other aptamer sensor strategies. Moreover, it is possible to adjust the resistance to non-specific interactions via selection of antibodies with varying binding affinities. Furthermore, the developed technique is implemented in homogeneous format with no need of washing and separation steps. This makes the assays more robust, easily automated, scalable for parallel assays of patient samples, as desired in clinical applications. As a proof-of-principle, the strategy was demonstrated using a model protein target of immunoglobulin E (IgE), a known biomarker associated with atopic allergic diseases (28).

MATERIALS AND METHODS

Chemicals and materials

IgE, monoclonal antibody to FITC were purchased from Sigma Aldrich Chemical Co. Human serum albumin (HSA), immunoglobulis G (IgG), lysozyme, thrombin, α-fetoprotein (AFP), hemoglobin and carcinoembryonic antigen (CEA), bovine serum were obtained from Dingguo Biotech. Co. (Beijing, China). All other chemicals were of analytical grade and obtained from Sinopharm Chemical Reagent Co. All solutions were prepared using ultrapure water, which was obtained through a Millipore Milli-Q water purification system (Billerica, MA, USA) and had an electric resistance >18.3 MΩ. DNA aptamer against IgE was designed to have the following sequence: 5′-GCG CGG GGC ACG TTT ATC CGT CCC TCC TAG TGG CGT GCC CCG CGC-3′, and the FITC labeling sites are given in Figure 2A. A control DNA was designed to have the following sequence: 5′-ATG TAA GAG GTT TCA TAT(-FITC) TGC TAA TAG CAG CTA CAA TCC AGC TAC-3′. All oligonucleotides were synthesized from Takara Biotechnology Co. Ltd. (Dalian, China). Thermodynamic parameters and secondary structures of all oligonucleotides were calculated using bioinformatics software (http://mfold.rna.albany.edu/).

Fluorescence protection assay of IgE

A 50-μl aliquot of reagent solution containing a FITC-labeled aptamer probe (20 nM) in a binding buffer (8 mM Na_2HPO_4, 1 mM KH_2PO_4, 1 mM $MgCl_2$, 3 mM KCl and 150 mM NaCl, pH 7.4) was added in a given sample (50 μl, final IgE concentrations ranging from 0 to 50 nM with other protein concentration otherwise specified). The mixture was incubated at room temperature for 10 min to allow complete interaction between aptamer and IgE. Then, 5 μl antibody to FITC (18.5 μM) was added in the mixture and incubated for another 10 min to perform the immunoreaction. The resulting solution was immediately subjected to fluorescence measurements. The fluorescence spectra were recorded at room temperature in a quartz cuvette on an F-7000 spectrofluorometer (Hitachi, Japan). The excitation wavelength was 490 nm and the emission wavelengths were in the range from 510 to 610 nm with both excitation and emission slits of 5 nm.

Fluorescence anisotropy assay of aptamer–IgE interaction

The fluorescence anisotropy response of the aptamer probe was measured directly using 10 nM FITC-labeled aptamer solution. The aptamer–IgE complex was prepared by adding 50 μl reagent solution in 50 μl IgE solution (final concentration of IgE and aptamer were 20 and 10 nM, respectively) followed by incubation at room temperature for 10 min. The resulting mixture was directly taken to measure the fluorescence anisotropy response of aptamer–IgE complex. Then, 5 μl antibody to FITC (18.5 μM) was added in the mixture and incubated for another 10 min. Again, the fluorescence anisotropy response of the mixture was measured. The complex between antibody and FITC-labeled aptamer was prepared by adding 50 μl reagent solution, 50 μl ultrapure water and 5 μl FITC antibody (18.5 μM) followed by incubation at room temperature for 10 min. The resulting mixture was used to measure the fluorescence anisotropy response of complex between antibody and FITC-labeled aptamer. The fluorescence anisotropy measurements were performed at room temperature in a quartz cuvette on a FL3-P-TCSPC fluorescence spectrophotometer (Jobin Yvon, France). The excitation wavelength was 490 nm and the emission wavelengths were in the range from 510 to 580 nm with both excitation and emission slits of 5 nm.

Capillary electrophoresis assay of aptamer–IgE interaction

All samples as prepared in fluorescence anisotropy assay were subjected to capillary electrophoresis assay. The capillary electrophoresis experiment was performed using a capillary electrophoresis system equipped with UV

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absorption detection (P/AGE MDQ, Beckman, Germany) under an applied potential of 20 kV using a quartz capillary with 75 μm diameter (total length, 50 cm; effective length, 30 cm) in 50 mM borate running buffer (pH 9.2). Samples were injected into the capillary using the pressure injection mode at 0.5 psi for 5 s and detected at 254 nm for 30 min. The temperature of the separation, 25°C, was controlled by immersion of the capillary in a cooling liquid circulating in the cartridge. Between successive runs, the capillary was rinsed with ultrapure water and running buffer for 5 min, respectively.

RESULTS AND DISCUSSION

Analytical principle of fluorescence protection assay-based aptamer sensor

This developed aptamer sensor strategy relied on protection of fluorophores against antibody-induced quenching upon aptamer–protein binding, as illustrated in Figure 1. A fluophore is labeled in proximity to the target-binding site of the aptamer. In the absence of IgE target, the fluophore antibody is able to bind to the fluophore moiety, efficiently quenching the fluorescence of the fluophore. Upon binding of aptamer to its protein target, the big size of proteins (with reference to aptamer) can dramatically increase steric hindrance at the fluophore site. This prevents the big size antibody from interacting the fluophore, resulting in the protection of fluorescence from antibody-based quenching. It is known that there are some fluophores with fluorescence efficiently quenched by their antibodies (29,30). Preferentially, FITC can be chosen as the fluophore because of its high fluorescence response and high quenching efficiency obtained when bound by fluorescein antibody (31).

It is noteworthy that the developed strategy simply utilizes the fact that after binding of protein target to aptamer, steric hindrance at the binding pocket will be increased substantially such that the sites at the binding pocket cannot be accessed to other macromolecules as antibody. This mechanism is conceptually straightforward and, to our knowledge, is innovative with comparion to existing four unique mechanisms for aptamer sensors (16–23). Specifically, it is also substantially different from other IgE aptamser sensors reported previously (32,33), which are essentially based conformational changes of the aptamer probes induced by aptamer–IgE binding. For example, it was supposed that the free-state aptamer exhibits a sticky-end pairing dimeric duplex conformation in the absence of IgE, while, on binding to IgE, the aptamer changed its conformation into a stem–loop secondary structure. This inhibited the cross-linking of gold nanoparticles by the thiolated dimeric duplex, thus rendering a light-up colorimetric sensor for IgE (32).

Probe design and optimization of label sites on aptamer sequence

The aptamer against IgE comprised a 45-nt hairpin-structured DNA sequence with a 4-bp extension in the stem region as shown in Figure 2A, which was

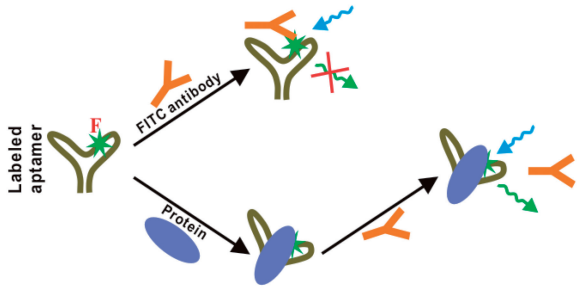

Figure 1. Fluorescence protection assay strategy for aptamer sensor. Fluorophore label (green star) on free aptamer binds to its antibody giving quenched fluorescence. Interaction of protein with aptamer prevents antibody from binding to fluorophore, strong fluorescence recovered.

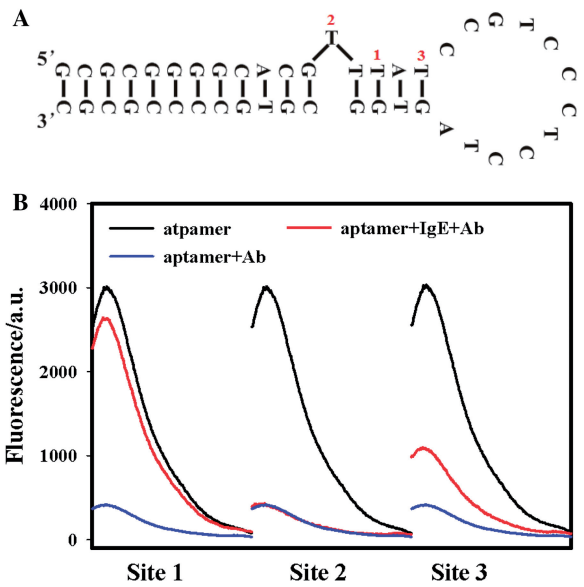

Figure 2. (**A**) Secondary structure of the IgE DNA aptamer used in the experiments. Three sites at T nucleotide for FITC labeling are symbolized in red numbers. (**B**) Typical fluorescence spectra measured for IgE aptamer (10 nM) with FITC label at three different sites in response to 0.88 μM FITC antibody (Ab) and 40 nM IgE plus 0.88 μM FITC Ab.

reported to improve its affinity to IgE target (37). Like other aptamer sensor strategies requiring sequence optimization (14–22), the FITC fluorophore should be optimized to be labeled in adjacency to the aptamer–protein binding site in order to obtain fluorescence protection against antibody-induced quenching. Because DNA synthesis had been able to incorporate various FITC-modified nucleotides at any site of a DNA sequence, we designed aptamer probes with FITC label at different sites to screen for the location with desired fluorescence protection characteristics.

Figure 2B depicts fluorescence spectral responses of aptamer probes with FITC labeled at three distinct sites. In the absence of IgE and FITC antibody, strong fluorescence peaks were observed for these aptamer probes. After incubated with antibody to FITC, these aptamer probes

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displayed much smaller fluorescence peaks (~14% in peak intensity compared to that for free aptamer probes), indicators of efficient fluorescence quenching by FITC antibody for free aptamer probes. Interestingly, for aptamer probe with FITC label at Site 1, incubation of aptamer probe with IgE followed by FITC antibody was observed to give a strong fluorescenc peak with ~90% intensity retained. This signal was substantially enhanced (>6-fold) with reference to the antibody-quenched fluorescence. Though a fluorescence decrease still appeared because of the equilibrium between aptamer–IgE complexes and free aptamers with a dissociation constant ~3.6 nM (37), this finding implied that binding antibody to FITC was substantially inhibited by aptamer–IgE interaction, an immediate evidence for fluorescence protection. In contrast, for aptamer probes with FITC at Site 2 after incubation with IgE followed by FITC antibody, we observed a very weak fluorescence peak, its intensity approximating to those for free aptamer probes directly quenched by FITC antibody. This suggested that aptamer–IgE interaction almost had no effect on the binding of antibody to FITC at Site 2. Site 3 was an intermediate case between Sites 1 and 2 for which the fluorescence peak was between those of Sites 1 and 2 after the aptamer probe incubated with IgE followed by FITC antibody. This might be attributed to a decrease of binding affinity to IgE for the aptamer with FITC at Site 3. Combining these observations, we obtained that aptamer probe with FITC at Site 1 exhibited a desirable response characteristic for our fluorescence protection assay strategy. Hence, this aptamer probe was used for subsequent studies.

Typical characteristics of fluorescence protection assay-based aptamer sensor

A close inspection of interactions between the aptamer probe and IgE together with FITC antibody was performed using capillary electrophoresis analysis as shown in Figure 3. It was noticed that after incubated aptamer probe with IgE or FITC antibody, a new peak appeared at an increased migration time with concomitant decreases in electrophoretic peaks for free aptamer or proteins. This indicated that aptamer probe was bound to IgE or FITC antibody, both increasing the frictional drag of aptamer and exhibiting a prolonged migration time (34,35). Intriguingly, after addition of FITC antibody in the mixture of aptamer and IgE, only the free aptamer peak disappeared accompanying with the appearance of a peak for FITC antibody-bound aptamer, the aptamer–IgE complex peak remaining unaltered with no new peak emerging accordingly. This finding disclosed that aptamer–IgE interaction could inhibit the binding of antibody confirming our hypothesis of fluorescence protection.

A further investigation of interactions between the aptamer probe and IgE together with FITC antibody was performed using fluorescence anisotropy analysis as shown in Figure 4. It is observed that after incubated with IgE or FITC Ab, the aptamer probe showed appreciable rises in fluorescence anisotropy responses, indicating the formation of aptamer–IgE or antibody FITC-labeled aptamer complex with substantially increased molecular weights. Intriguingly, no further appreciable ascent of fluorescence anisotropy responses was obtained after the IgE-bound aptamer probe incubated with FITC antibody, revealing that there was no substantial change in the molecular weight for the major fluorescence component, aptamer–IgE complex. Thus, this finding actually disclosed that aptamer–IgE interaction could inhibit the binding of antibody to FITC label.

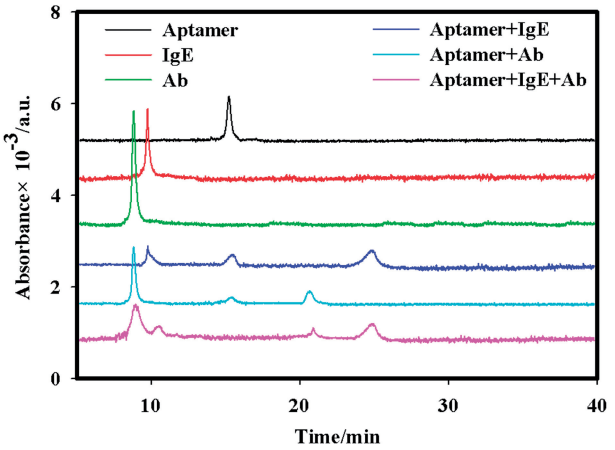

Figure 3. Capillary electrophoretograms obtained with 180 nM IgE, 150 nM aptamer, 0.88 μM FITC Ab and their mixtures.

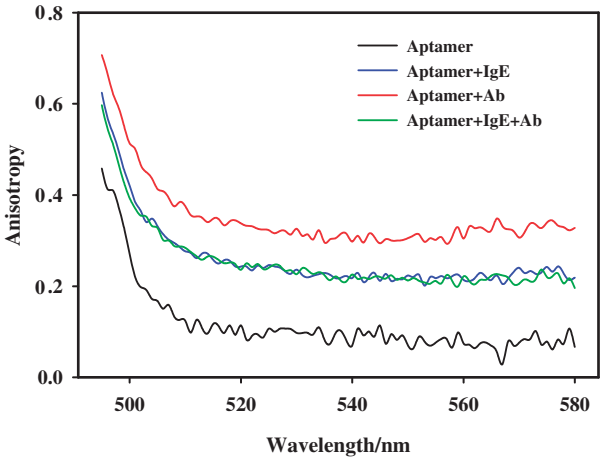

Figure 4. Fluorescence anisotropy profiles obtained in 510 nm through 580 nm for 10 nM FITC-labeled aptamer, mixture of 10 nM FITC-labeled aptamer and 0.88 μM FITC antibody, mixture of 10 nM FITC-labeled aptamer and 20 nM IgE, and mixture of 10 nM FITC-labeled aptamer and 20 nM IgE with later addition of 0.88 μM FITC antibody.

Quantitative analysis of IgE using fluorescence protection assay-based aptamer sensor

Figure 5 depicts typical fluorescence spectral responses of the aptamer sensor to IgE of varying concentrations. One observed dynamically increased fluorescence peaks with increasing IgE concentration ranging from 0.1 to 50 nM. A quasilinear response was obtained in the concentration range from 1.0 to 40 nM with a readily achieved detection

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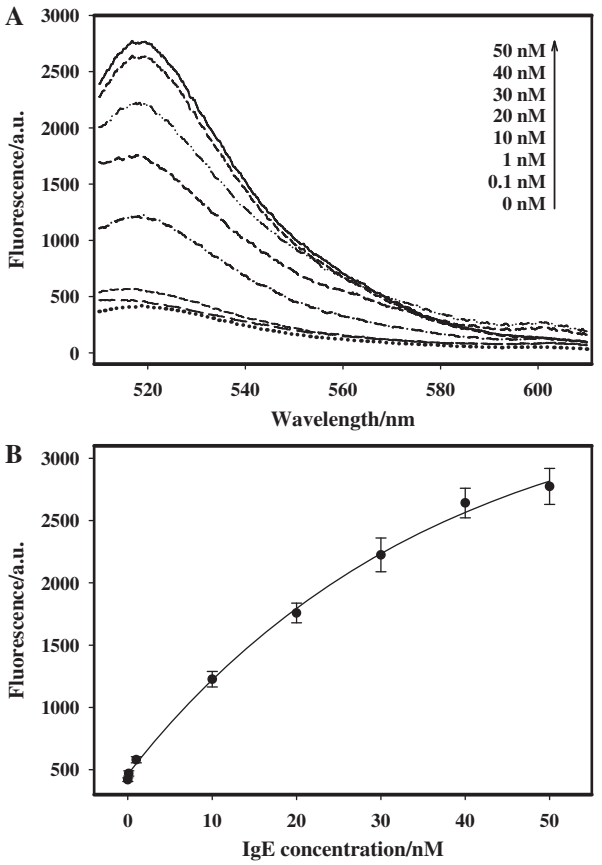

Figure 5. (**A**) Typical fluorescence spectral responses for FITC-labeled aptamer sensor (10 nM) to IgE of varying concentrations. FITC antibody concentration was 0.88 μM. (**B**) Plot of fluorescence peak responses at 520 nm versus IgE concentrations. Error bars are standard deviation of four repetitive experiments.

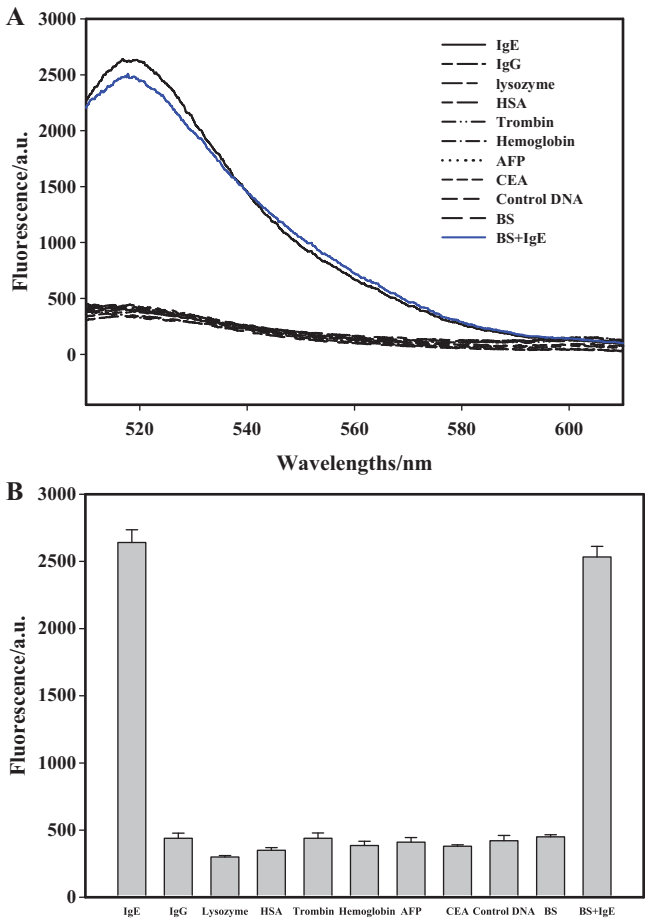

Figure 6. (**A**) Typical fluorescence spectral responses for FITC-labeled aptamer sensor (10 nM) to 40 nM IgE, 1 μM IgG, 1 μM nM lysozyme, 1 μM HSA, 1 μM thrombin, 1 μM AFP, 1 μM hemoglobin, 1μM CEA, 10-fold diluted bovine serum (BS) or 40 nM IgE in 10-fold diluted BS. FITC antibody (Ab) concentration was 0.88 μM. Fluorescence spectrum obtained in the control using FITC-labeled non-aptamer DNA (10 nM) in assay of 40 nM IgE is also inlcuded. (**B**) Bar plot of corresponding fluorescence responses at 520 nm to varying proteins and control DNA. Error bars are standard deviation of four repetitive experiments.

limit of 0.1 nM. Such detection sensitivity was adequate for routine clinical applications (36), and was comparable to many exisitng homogenous assay techniques for IgE detection (33,38). Because of its homogeneous assay format, the strategy also exhibited excellent reproducibility, fluorescence peak readouts showing relative standard deviations of 3.7, 5.1 and 4.5% in four repetitive assays of 1, 10 and 40 nM IgE, respectively.

A major advantage of fluorescence protection assay was its high resistance to non-specific interactions of proteins with aptamer probe. As shown in Figure 6, it was observed that the aptamer sensor yielded very weak fluorescence signals in response to other proteins such as HSA, IgG, lysozyme, thrombin, AFP, CEA, which contrasted clearly with the strong fluorescence response to IgE. In a control experiment using a DNA probe with sequence different from the aptamer, we also obtained very small fluorescence peak, implying that fluorescence protection was specifically originated from the interaction of aptamer with IgE protein. Notably, fluorescence responses to bovine serum with and without IgE exhibited only slight discrepancies from those for IgE in the buffer and for the blank, respectively. This revealed excellent selectivity of fluorescence protection assay, which might support its potential in clinical applications. As a matter of fact, the fluorescence protection assay involved two competitive interactions, FITC with its antibody and aptamer with target protein. If non-specific interactions showed faster dissociation kinetics than that for FITC-antibody complex, binding of antibody to FITC could become dominant in the competition, thereby allowing eliminating the interferences from non-specifically interacting proteins in fluorescence protection assay.

CONCLUSION

We developed a novel fluorescence protection assay strategy toward aptamer sensors for homogeous detection of IgE. This strategy relied on inhibition of antibody-induced quenching of FITC label by aptamer–IgE binding. Because interferences from non-specifically interacting proteins could be precluded by competitive

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interaction of antibody FITC, this strategy could provide improved specificity as compared with other aptamer sensor strategies (16–22). Moreover, it was possible to adjust the resistance to non-specific interactions via selection of antibodies with varying binding affinities. The method could be applied directly to detecting other proteins by use of corresponding aptamers through optimization of the FITC labeling site. Also, the strategy could be implemented for multiplex detection of multiple proteins by using different fluorophore labels with non-overlapping emission peaks, provided that their quenching antibodies were available (29,30). Because of its homogeneous assay format, this technique could show great robustness, easy operation and scalability for parallel assays of hundreds of samples. In view of these advantages, the fluorescence protection assay strategy might create a new methodology for developing sensitive, selective aptamer sensors in homogeneous detection of proteins.

FUNDING

National Natural Science Foundation of China (grants 21025521, 21035001 and 20875027); National Key Basic Research Program (grant 2011CB911000); European Commission FP7-HEALTH-2010 Programme-GlycoHIT (grant 260600); Program for Changjiang Scholars and Innovative Research Team in University; Natural Science Foundation of Hunan Province (grant 10JJ7002). Funding for open access charge: National Key Basic Research Program (grant 2011CB911000).

Conflict of interest statement. None declared.

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