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Molecular pincers – new antibody-based homogenous protein sensors

Ewa Heyduk[1], **Benjamin Dummit**[2], **Yie-Hwa Chang**[2], and **Tomasz Heyduk**[1,*]

1*Edward A. Doisy Department of Biochemistry and Molecular Biology, St. Louis University Medical School, 1100 S. Grand Blvd., St. Louis, MO 63104, USA*

2*Mediomics, LLC, 5445 Highland Park Ave, St. Louis, MO 63110, USA*

Abstract

We describe here a new homogenous antibody-based protein sensor design (molecular pincers) that allows rapid and sensitive detection of a specific protein in solution. In the presence of the target protein these sensors produce fluorescence signal derived from target-dependent annealing of short complementary fluorochrome-labeled oligonucleotides attached to a pair of target-specific antibodies via nanometer-scale flexible linkers. The sensors allow near-instantaneous detection of the target with sensitivity and specificity approaching ELISA but requiring no sample manipulation other then the addition of the sample to the sensor mix. We used cardiac troponin I and C-reactive protein as the targets to validate these desirable properties of the sensors. Due to the availability of antibodies to thousands of interesting targets and the straightforward design blueprint of the sensors we expect their wide-ranging applications in research and medical diagnosis, especially when simplicity, high throughput, and short detection time are essential.

Introduction

Detection and determination of a level of a specific protein is the most commonly performed assay in biomedical research and clinical diagnosis. A number of powerful techniques utilizing antibodies have been developed for detecting proteins. ELISA (Enzyme-linked Immunosorbent Assay) represents the gold standard of specificity and sensitivity of protein detection [1-5]. The outstanding utility of antibody-based assays was a motivation for the development of thousands of antibodies. Despite the unquestionable value of currently available immunoassays, there is a significant demand for further development of antibody-based protein detection methodologies with a specific emphasis on increasing the speed of the detection, improving sensitivity, increasing multiplexing capabilities, and reducing the cost of the assays. Reducing the complexity of assays such that they could be adaptable to point-of-care use is also a worthy goal. The current most widely used specific protein detection methodology, ELISA, is a heterogeneous assay requiring a number of manipulations (coating of the plates with antibodies, incubation with a sample, extensive washings, adding reagents for enzyme amplification reaction, and reading of the signal) that require up to several hours to perform. The goal of the work presented here was to develop homogenous antibody-based protein sensors that would retain most of the positive aspects of ELISA but would eliminate the complexity and greatly reduce the time necessary for protein detection.

Design of these sensors (Fig. 1A) utilizes target protein-induced increase of local concentration of complementary oligonucleotides with which the pair of the antibodies recognizing non-

*Corresponding author: Tomasz Heyduk, E-mail: heyduk@slu.edu, Fax: (314) 977 9205, Phone (314) 977 9238.

overlapping epitopes of the protein are labeled. This increase in the local concentration results in the annealing of the complementary oligonucleotides producing efficient FRET between the fluorescence probes incorporated into the oligonucleotides. This FRET signal can be used for sensitive, near-instantaneous detection of the target protein. Oligonucleotide-labeled antibodies have been previously employed in conjunction with PCR or Rolling Circle Amplification to achieve great enhancements in sensitivity of immunoassays [6-10]. The emphasis of our sensor design was on homogenous and rapid detection.

Since we expected the speed and reduced complexity to be the major attributes of our new protein sensors, we have chosen cardiac troponin I as an example protein target for sensor development. Cardiac troponin is a perfect example of a target protein requiring a rapid, specific and inexpensive assay compatible with bed-side applications. Troponin is the contractile regulating protein complex of striated muscle [11]. Cardiac troponin I test has been extremely useful in the differential diagnosis of patients admitted to the Emergency Room with chest pains [12-14]. The levels of troponin I are very low in normal serum. After acute myocardial infarction (AMI), the levels increase in 4-6 hours and remain elevated for 6-12 days [15, 16]. Thus, detection of troponin I in the serum allows highly specific and sensitive diagnosis of AMI. Some data also suggest a correlation between troponin levels and a long-term outcome after a chest discomfort episode [16]. Troponin has become a preferred biomarker of myocardial damage [17, 18].

Our data confirmed the desirable properties of the sensors. Cardiac troponin could be detected in ~ 15 min with ~ 1ng/ml (~40 pM) sensitivity. The sensor was compatible with detection of the protein in plasma. Generality of sensor design was confirmed by preparing sensor detecting C-reactive protein (CRP) and by preparing mixed design sensors (in which an antibody and ds DNA were used as sensing molecules) to detect Nf-κB and p53. In principle, the sensors could be developed for any protein for which a pair of antibodies recognizing non-overlapping epitopes of a protein is available. Straightforward and general design blueprint for preparing the sensors should assure their wide applications in biomedical research and clinical diagnosis.

Experimental Section

Reagents

The oligonucleotides were obtained from IDT (Coralville, IA) or from W.M. Keck facility at Yale University (New Haven, CT). The following oligonucleotides were prepared (names are given in parentheses, X corresponds to Spacer18 phosporamidate): 5′amino-TA GGA GAG AGA GAG AGG A (A1), 5′amino-TAG GTG CTC GAC GCT GAC (A2), 5′ fluorescein-GCT CAT TGT CAG CGT CGA GCA CCT A (A3), 5′ Cy5-ATG AGC TTC CTC TCT CTC TCT CCA T (A4), 5′-amino-XXX XXX CGC ATC T 3′ C3 S-S CPG (A5), CAA TAA ATG TGA TCT AGA TCA CAT TTT AGG XXX XXX AGA TGC G 3′SS (A6), CCT AAA ATG TGA TCT AGA TCA CAT TTA TTG (A7), 5′ fluorescein-CGC ATCT XXX XXX GTC AGC GTC GAG CAC (A8), CCT CGG AGG ACA GTA CTC CGC C TA GGA GAG AGA GAG AGG A (A9), GGC GGA GTA CTG TCC TCC GAG G (A10), Cy5-AGA TGC G XXX XXX TCC TCT CTC TCT CTC C (A11), TAC AGA ACA TGT CTA AGC ATG CTG GGG (A12), CCC CAG CAT GCT TAG ACA TGT TCT GTA (A13), and 5′amino-GTG CTC GAC GCT GAC (A14).

Fluorescently labeled oligonucleotides were purified using reversed-phase HPLC[19]. 5′-amino-labeled oligonucleotides that were used for antibody modification were ethanol-precipitated and were used without further purification. Monoclonal cardiac troponin antibodies (clones M18 and M4) were from RDI division of Fitzgerald Industries (Concord, MA), goat anti-GST antibody from Rockland, Inc. (Gilbertsville, PA) and anti-p53 antibody was from Santa Cruz

Biotechnology (Santa Cruz, CA). Purified cardiac troponin I (Standard Reference Material (SRM) 2921) was purchased from NIST (Gaithersburg, MD), porcine muscle troponin was from Sigma (St. Louis, MO), C-reactive protein (CRP) and corresponding antibodies were from Biospacific (Emeryville, CA), GST-tagged Nf-κB was from Upstate (Lake Placid, NY), and p53 was from ProteinOne (Bethesda, MD). NHS-PEO_8-maleimide, SMCC and Traut's reagent were from Pierce (Rockland, IL) whereas NHS-$dPEG_{24}$-maleimide was from QuantaBiodesign (Columbus, OH).

Antibody modification and purification

Antibodies modified with short oligonucleotides through long flexible linkers are a crucial component of the sensors illustrated in Fig. 1A. A typical antibody modification procedure is described below. While several variants of this protocol were used to prepare labeled antibodies, the procedure described below most reproducibly resulted in antibodies labeled extensively with the oligonucleotide (typically ~ 2-4 oligonucleotides/antibody). The antibodies labeled with oligonucleotides in this range resulted in the best signal-to-background ratio.

The first step of the procedure involves preparation of a thiol-reactive oligonucleotide that is subsequently used to react with thiolated antibody. When the labeling strategy illustrated in Fig. 1B(c) was used, 200 μl of 5′-amine containing 18nt oligonucleotides (A1 or A2) at ~250 μM in 20mM NaH_2PO_4 (pH-7.4), 150mM NaCl and 2.5mM EDTA buffer (conjugation buffer) were mixed with 5 μl of ~250mM of a bifunctional crosslinker containing long flexible linker (in most experiments, NHS-PEO_8-maleimide) dissolved in DMF. The reaction mixtures were incubated for 1-1.5hr at room temperature. Oligonucleotide was purified from the excess of the crosslinker by ethanol precipitation in the presence of 1mg/ml of glycogen. Precipitated oligonucleotides were dried in Speed-Vac and were stored at -20° C until they were used for antibody modification. Typically, ~50-75% of the oligonucleotides were successfully conjugated with the linker as estimated by native 10% polyacrylamide gel electrophoresis. When antibody labeling strategies shown in Fig. 1B (a) or 1B (b) were used, the protocols for preparing thiol-reactive oligonucleotide were very similar but a different crosslinker (SMCC) was used since the long flexible linker in these cases was introduced into the oligonucleotide during the oligonucleotide synthesis.

50-75 μl antibody solutions containing 0.3-0.4 mg of the protein were run on a spin column (Zeba™, Pierce, Rockford, IL)) equilibrated with the conjugation buffer. Antibodies were thiolated for 1.5hrs at room temperature with 40 molar excess of Traut's Reagent added as ~14mM stock solution in DMF. The excess of Traut's Reagent was removed on Zeba™ spin column equilibrated in the conjugation buffer. The thiolated antibody was then reacted with a 15-20 molar excess of linker-conjugated oligonucleotide (calculated assuming that 50-75% of the oligonucleotides were conjugated with the crosslinker). Reaction mixtures were incubated for 4 hrs at room temperature followed by an overnight incubation at 4° C.

Modified antibodies were purified from the excess of the oligonucleotides by size exclusion FPLC chromatography using 10/30GL Superdex™ 200 column (Pharmacia) equilibrated with 10 fold-diluted 20 mM Tris (pH 8.0), 100 mM NaCl, 10 μM EDTA buffer. Fractions containing modified antibodies were pooled and concentrated 10-fold in the Speed-Vac. The protein concentration was estimated using Bradford Assay and DNA content was determined by fluorescence assay using Sybr-Green. The typical overall yield of this antibody modification procedure was ~ 40%. Modified antibodies were stable for several weeks in 4°C and for longer storage they were frozen at -80 °C in 50% glycerol. Protein A chromatography could be also used to purify labeled antibodies although the results were less reproducible and the yields were lower compared to size-exclusion chromatography.

Fluorescence measurements

Troponin sensor measurements were performed in 20mM Tris (pH 8.0), 100mM NaCl, 10 μM EDTA and 0.2mg/mL BSA. Measurements with CRP were conducted in the same buffer + 5 mM $CaCl_2$. Measurements with p53 were conducted in the same buffer but with 0.1 mg/ml BSA and the measurements with GST-NF-κB were in the same buffer + 5% glycerol. Fluorescence spectra were recorded on Aminco-Bowman AB2 spectrofluorometer. Fluorescence intensity measurements were performed in 20 μl volumes in 384-well low-volume black microplates (Corning, Lowell, MA) using Analyst AD (LJL Biosystems, Sunnyvale, CA) or SpecroflourPlus (Tecan, San Jose, CA) fluorescence plate readers. Luminescence Energy Transfer (LRET) was measured on Analyst AD using 1000 lamp flashes, 50 μsec delay and 1000 μsec integration time. Results were expressed as fold change of FRET signal calculated by dividing the ratio of acceptor and donor emission intensities in the absence and in the presence of the target protein, respectively.

While various assay conditions have been used throughout this work, a typical assay procedure involved preparing a desired volume of the assay mix by premixing the oligonucleotide-labeled antibodies (40 nM of fluorescein-labeled antibody and 50 nM of Cy5-labeled antibody) followed by 30 min room temperature incubation. This diluted assay mix can be stored in a refrigerator for at least 24 hours without any effect on assay performance. Ten μl of the assay mix were then added to the samples diluted to 10 μl with the assay buffer followed by gentle mixing. Reaction mixtures were incubated at room temperature for 30 min followed by a readout of the fluorescence in 384-well microplate.

Results and Discussion

Sensor design

The overall design of molecular pincer sensors is illustrated in Fig. 1A. This concept is derived from molecular beacons for detecting DNA binding proteins and from aptamer-based molecular beacons for detecting proteins which were previously developed in our laboratory[20, 21]. A pair of antibodies recognizing non-overlapping epitopes of the protein is labeled with a pair of short complementary signaling oligonucleotides using a long flexible PEG-based crosslinker, respectively. Oligonucleotides are modified with a pair of fluorophores which could function as a donor and an acceptor in Fluorescence Resonance Energy Transfer (FRET)[22]. The length and the sequence of the oligonucleotides was chosen to obtain the dissociation constant for the duplex of ~5 μM. Thus, when the free antibodies labeled with these oligonucleotides are mixed at nanomolar concentrations (that are well below the K_d for duplex formation), an insignificant amount of the duplex will be formed and no FRET signal should be observed. In contrast, when both antibodies bind to the target protein, the local concentration of the oligonucleotides attached to the antibodies will be greatly increased (to tens of micromolar, that is well above K_d for the duplex formation; Ling and Heyduk, manuscript in preparation) resulting in almost complete association of the oligonucleotides to form a duplex. This in turn will bring the fluorophores to close proximity resulting in efficient FRET that can be used as a signal for target protein detection. The use of very long flexible linkers in our sensor design is an essential design feature since they will allow enough of room and flexibility for effective formation of complexes of labeled antibodies with even a very large target protein or a multiprotein complex.

Signaling oligonucleotides have two important roles in this assay. The first role is to provide means for generating FRET signal reporting the presence of the target protein. It is important to emphasize that the use of the signaling oligonucleotides (as opposed to direct labeling of antibodies with fluorescence probes) allows reliable generation of FRET signal regardless of the specific configuration of the complex and the size of the complex (within the range of the

reach of flexible linkers). This is because the FRET signal is generated due to target protein dependent annealing of signaling oligonucleotides which brings the fluorescence probes into predictably close distance. This distance does not depend on the architecture of the complex but is determined by a simple and predictable geometry of duplex DNA. This eliminates one of the difficulties in designing assays based on FRET where the relatively short distance between the probes (~ 50 Å or less) necessary for efficient FRET can be difficult to achieve and difficult to incorporate into assay design. Furthermore, FRET probes that exhibit large spectral separation could be used in the sensors (for example, fluorescein and Cy5) which can significantly reduce the background signal in the absence of FRET. Such probes require close physical proximity for FRET to occur. Close proximity of the probes in our sensors is guaranteed by the assay design.

The second role of signaling oligonucleotides is less obvious but equally important. Favorable free energy of association between the signaling oligonucleotides together with their high local concentration resulting from their attachment through flexible linkers increases the stability of the complex illustrated in Fig. 1A. We have developed a simple model to study the rules of free energy additivity in multivalent ligands connected by flexible linkers (such as the one in Fig. 1). This analysis indicated that the stability of the complex shown in Fig. 1 could be 10-10,000 times better (depending on the affinity of individual antibodies, length of signaling oligonucleotides, and the length of flexible linkers) compared to the same complex without signaling oligonucleotides component (Ling and Heyduk, manuscript in preparation). Increased stability of the complex illustrated in Fig. 1A will have a beneficial effect on sensitivity and specificity of the assay. Specificity of molecular pincer sensor for the target protein is assured by the necessary coincidence of three molecular events (the recognition of the target protein by each of the two antibodies and the association of the complementary signaling oligonucleotides). In the absence of any one of these, the sensor will generate no signal.

Modification of antibodies with signaling oligonucleotides

We have tested three procedures illustrated in Fig. 1B to label antibodies with the signaling oligonucleotides. For the procedure illustrated in Fig. 1B(a) signaling oligonucleotide is synthesized with a 5′ amino group and with a long flexible non-DNA linker composed of multiple Spacer18 linkers (for example, oligonucleotide A5 (Methods)). The oligonucleotide is then reacted with a short NHS-maleimide bifunctional crosslinker (SMCC) producing 5′ maleimide oligonucleotide that can be then conjugated with thiolated (by reaction with Traut's reagent) antibody. For the procedure illustrated in Fig. 1B(b) antibody is first conjugated using procedure outlined above with an unlabeled 18 nt oligonucleotide of a unique sequence. This is followed by annealing of a construct containing at its 3′ end an 18 nt oligonucleotide complementary to the oligo used for labeling the antibody and at 5′ end a signaling oligonucleotide connected with a long flexible linker. For the procedure illustrated in Fig. 1B (c) an 18 nt unlabeled oligonucleotide with the 5′ amino group is first reacted with a long chain bifunctional NHS-maleimide crosslinker producing 5′ maleimide oligonucleotide which can be conjugated with thiolated antibody. This is then followed by annealing of a simple signaling oligonucleotide (which does not contain a flexible linker) that has an 18 nt region complementary to the oligo used for labeling the antibody and a short single-stranded extension. In our studies we used antibody constructs containing various lengths of the flexible linker. When the NHS-PEO_8-maleimide was used to attach signaling oligonucleotides (a crosslinker containing an 8 ethylene oxide spacer) the flexible linker was ~ 5 nm long. When NHS-$dPEG_{24}$-maleimide was used (a crosslinker containing 82 atom spacer), the flexible linker was ~10 nm long. When A5 oligonucleotide was used to label the antibody, the flexible linker was ~14 nm long. We did not observe large differences in sensor performance between these various linker lengths. We considered the three variants of labeling procedure (Fig. 1B)

since we expected that they have different cost and expected performance characteristics. We thought that the procedure in Fig. 1B(a) should exhibit the best performance since it represent the “cleanest” construct lacking any unnecessary components. However, this is also the most costly procedure since the expensive component (fluorophore and flexible-linker derivatized oligonucleotide) has to be used in large excess over the antibody for effective conjugation. In contrast, we expected the procedure in Fig. 1B(c) to be the most straightforward and the least expensive since the antibody is labeled with a simple oligonucleotide lacking fluorophore and the flexible linker incorporated during oligo synthesis. Flexible linker in this case is derived from the bifunctional crosslinker that is much less expensive compared to the linker incorporated during oligonucleotide synthesis. However, we expected that the procedure in Fig. 1B(c) would likely result in a diminished performance of the molecular pincer assay due to the additional bulk of DNA duplex present in the construct and due to limited length of commercially available bifunctional crosslinkers. We further expected that the procedure illustrated in Fig. 1B(b) should provide a balanced compromise between the cost and performance. Throughout the work described in this paper, we have used all three strategies and the surprising result was that the procedure illustrated in Fig. 1B(c) resulted in best performance (the largest signal change in the presence of troponin) of the sensor. Thus, fortuitously the least complicated and the least expensive procedure is also the best for the performance of the assay. All experiments described below for troponin were obtained using labeling procedure illustrated in Fig. 1B(c).

Sensor for cardiac troponin I

We modified a pair of cardiac troponin I monoclonal antibodies (M18 and MF4 clones from Fitzgerald Industries International, Inc., Concord, MA) with fluorescein and Cy5 labeled signaling oligonucleotides (using the strategy illustrated in Fig. 1B(c)). When the labeled antibodies were mixed in the absence of cardiac troponin, no significant FRET signal was observed when fluorescence spectra of donor-labeled antibody in the absence and the presence of acceptor-labeled antibody were compared (Fig. 1C). Upon addition of troponin, a large FRET signal was observed illustrated by several fold increase of emission at 670 nm with the excitation at 490 nm and quenching of donor emission at 520 nm. The data shown in Fig. 1C validate sensor design illustrate in Fig. 1A.

Fig. 2A illustrates experiments designed to probe relationship between concentrations of assay components (labeled antibodies) and sensitivity and dynamic range of the assay. Response of the assay at five different concentrations of labeled antibodies with four concentrations of cTnI was measured. Sensitivity of the assay increased with the decrease of antibody concentration. For example, 0.2 nM cTnI could not be measured using 100 or 50 nM antibodies, whereas a measurable signal was observed with lower antibody concentration (Fig. 2A). This is because at low antibody concentrations even a small amount of troponin could produce the amounts of target-antibody complex constituting a significant fraction of the total antibody concentration (and thus could produce measurable signal). At high antibody concentrations the same amount of the complex becomes an insignificant and immeasurable fraction of total antibodies. Reducing concentrations of the antibodies will produce an increase of sensitivity as long as these concentrations would not become comparable or lower than the apparent dissociation equilibrium constant of target-antibody complex. At high end of troponin concentrations, when the concentration of troponin becomes higher than antibody concentration the assay exhibits saturation and with further increase of troponin, a decrease of the signal could be observed. This is because the very high troponin concentrations will dissociate a ternary antibody-troponin complex in favor of two binary antibody-troponin complexes. This high-end limit of sensor response can be easily overcome by simply using higher antibody concentrations. Thus, very simple manipulations of sensor components concentrations can be used to tailor the assay performance (sensitivity and dynamic range) to a particular range of target protein

concentrations that needs to be measured. For example, Fig. 2B illustrates the excellent response to sub-nanomolar concentrations of troponin obtained with the sensor employing low concentrations of antibodies (i.e. optimized for detection of low concentrations of the target).

Data shown in Fig. 2A also illustrate excellent reproducibility of the sensor. The average CV of the sensor for all conditions where the signal was above the background in Fig. 2A was 4.5% (range: 0.7 to 21.4%). Largest CV's were for the samples at low target concentrations (approaching assay detectability limit). Sensitivity of the sensor at 3 nM antibody concentration can be estimated to be ~ 40 pM (~ 1 ng/ml) (assuming 3 × standard deviation over the zero troponin sample as the lowest detectable signal change in the presence of troponin).

All data described above were obtained using a pair of signaling oligonucleotides labeled with fluorescein and Cy5, respectively. These two probes were selected because of very low background signal in the absence of FRET (direct excitation of Cy5 at the 480 nm used for excitation of fluorescein is minimal and residual fluorescein emission at 670 nm is also very low). However, various other labeling and signaling schemes for the sensor could be considered and could be utilized for optimizing the assay or for increasing assay sensitivity. To demonstrate such possibilities we have prepared a variant of a sensor for troponin in which the signaling oligonucleotides were labeled with Eu^{3+} chelate and Cy5, respectively. Luminescence energy transfer (LRET) using lanthanide chelates has been shown to offer significant advantages for homogenous assays based on energy transfer[23, 24]. Long luminescence life-times of these probes allow elimination of the background derived from light scattering and direct excitation of the acceptor which can significantly improve signal-to-background ratio[24]. This could be very beneficial when turbid or autofluorescent samples need to be analyzed. Fig, 3A shows a comparison of signals obtained with molecular pincers labeled with fluorescein/Cy5 and Eu^{3+} /Cy5 donor-acceptor pairs. Signals at the same troponin concentrations were much higher with LRET compared to FRET. Using these data we estimated that the use of LRET vs. FRET for signaling would produce at least ~ 2 fold increase of sensitivity of the sensor. These data illustrate the flexibility of the sensor design in incorporating various signaling schemes.

In the next step, we investigated the kinetics of sensor response. Different concentrations of troponin were added to the sensor and the FRET signal as a function of time was observed (Fig. 3B). FRET signal reached maximum after ~ 10 min of incubation and remained stable until the recording was stopped (60 min). These data demonstrated that the response of the sensor is rapid and no lengthy incubation times will be required for detecting the target protein.

Specificity of the sensor for cardiac troponin was tested by the experiment illustrated in Figs. 4A. No FRET signal was observed when nonspecific related control protein (porcine muscle troponin) was added to the sensor whereas a robust response with cardiac troponin was observed. Also, unlabeled cardiac troponin antibodies acted as competitors (data not shown) indicating that the signal generated by molecular pincer in the presence of troponin was due to specific interactions between labeled antibodies and the protein.

An important application of our sensors will be to use them for rapid detection of a target protein in complex biological samples such as, for example, serum or plasma. We have thus tested if the sensor could detect cardiac troponin when measured in plasma. We have compared the signal generated by various concentrations of troponin in a buffer and when spiked into pooled normal human plasma. No significant difference in assay outcome with and without plasma was observed (Fig. 4B) demonstrating compatibility of the sensors with protein determination in plasma.

To demonstrate the generality of sensor design we selected CRP (C-reactive protein), also a marker of cardiovascular disease[25], as another target for sensor development. CRP is a pentameric protein synthesized in the liver and normally present at trace amounts in plasma[26]. CRP has been long recognized as a general marker for inflammation[27]. More recently, a strong association of CRP plasma levels with cardiovascular disease has been established. Clinical studies have shown a correlation between increased mortality among patients with angina and elevated levels of CRP[28]. These observations suggested that measurement of CRP in patients with ischemic heart disease could provide a useful method for detecting high risk of plaque rapture.

Strategy illustrated in Fig. 1B(c) was used to label two monoclonal anti-CRP antibodies recognizing non-overlapping epitopes of the protein with the same signaling oligonucleotides as previously used in troponin sensor. Addition of purified CRP to the sensor produced large highly reproducible protein dependent FRET signal (Fig. 5). The average CV of the sensor was 10% (range: 2.4 to 26.8%). Largest CV's were for the samples at low target concentrations (approaching assay detectability limit). Estimated sensitivity of CRP detection was ~ 2ng/ml (~ 17 pM). Specificity of the sensor was verified by the lack measurable FRET signal when negative control (cardiac troponin) was added to the sensor (not shown). The data shown in Fig. 5 demonstrate the generality of the sensor design since by using the same blueprint as used in the case of troponin, a sensor for another target could be readily prepared exhibiting very similar FRET signal changes and sensitivities as in the case of troponin.

Variants of the sensor design

Various interesting combinatorial variations of sensor design could be envisioned where instead of a pair of antibodies a pair of heterologous molecules could be utilized. For example, an antibody and a small molecule ligand conjugated with signaling oligonucleotides or an antibody and an aptamer conjugated with signaling oligonucleotides could used to prepare a sensor. Fig. 6 illustrates one possible example demonstrating feasibility of such sensors. A sensor for detecting a DNA binding protein is composed of DNA fragment (containing a binding site for the protein) with a signaling oligonucleotide added at the end and an antibody labeled with a complementary signaling oligonucleotide.

We used two examples of DNA binding proteins to demonstrate feasibility of this sensor variant. The first example (Fig. 6A) was an eukaryotic transcription factor NF-κB[29] tagged with GST at the N-terminus. This illustrates a sensor design which could be generally used for analyzing DNA binding proteins fused with commonly used tags. Only a single signaling oligonucleotide-labeled anti-tag antibody would be needed for all tagged proteins. The second example (Fig. 6B) is p53, a multifunctional DNA binding protein mutated in many human cancers[30]. This example illustrates the use of a specific antibody targeting an epitope that does not overlap with DNA binding region of the protein. In all both cases functional sensors exhibiting target protein concentration dependent FRET signal were obtained illustrating versatility of sensor design.

Conclusions

We have described here a new homogenous antibody based protein sensors that we believe have several important advantages. Since the assay is homogenous and it requires only the addition of the sample to the sensor mix, it will be possible to employ it in high-throughput applications. The assay is extremely simple to perform, as no complicated sample manipulation is necessary. The assay is fast, requires only simple fluorescence readout and is amenable to miniaturization limited only by technical capabilities of the instrumentation used to detect the fluorescence. Very small amounts of labeled antibodies are used per each measurement since our sensors utilize low (nanomolar) antibody concentrations and the assays can be done in very

small volumes. These desirable properties of the assay should allow its wide-ranging application in research and medical diagnosis.

The design of the sensors is not specific to any given protein. In principle, the sensors could be developed for any protein for which a pair of antibodies compatible with ELISA is available. Modification of antibodies with signaling oligonucleotides is relatively straightforward. All of the above mentioned characteristics of the sensors indicate that they could be rapidly developed for many targets of research or diagnostic importance. This generality of the sensors has been confirmed in our laboratory by the development of the sensor for detecting CRP (Fig. 5) and by the preliminary data demonstrating successful development of sensors for C-peptide and insulin using design shown in Fig. 1A (Heyduk and Heyduk, unpublished).

Sensitivity of the sensors will be an important characteristic that will determine the range of the targets for which the sensors could be applied. Sensitivity observed with troponin I (~ 1 ng/ml) is comparable to the sensitivity of a typical commercial troponin ELISA assay (for example, ~1ng/ml for ELISA kit from Biocheck, Inc., Foster City, CA; cat# BC-1105)). Sensitivity of CRP sensor is also in a range of ELISA assays for CRP (for example, ~100 ng/ml for ELISA kit from Biocheck, Inc., Foster City, CA; cat# BC-1105). In general, ELSA assays can achieve sensitivities in pg/ml range. Such sensitivity will be difficult to achieve by our sensors so far since in contrast to ELISA they do not employ a signal amplification step. We are currently working on implementing signal amplification strategies that will not compromise the major advantages resulting from simplicity derived from the homogenous nature of molecular pincers. Nevertheless, even without signal amplification, the current sensitivity obtained with troponin will be sufficient for applying the sensors for rapid measurement of troponin I or CRP in blood to aid prompt diagnosis. Current sensitivity of the sensors will be also sufficient for practical application of the sensors for detecting many other proteins of research or diagnostic importance. For example, required sensitivities for detecting other markers of cardiovascular disease, such as myoglobin (~ 5 ng/ml) or CK-MB (~2.5 ng/ml), are all within the range currently achievable by our sensors. The simplicity of assay employing our sensors, the speed and the potential for miniaturization will be the main assets of the sensors.

Successful development of mixed sensors composed of an antibody and ds DNA illustrates a more general potential of building the sensors with various alternative specific protein binders. In addition to the antibodies and natural nucleic acid binders, DNA or RNA aptamers, peptide ligands, and small molecule ligands can be potentially used in the sensors. The only requirement would be the availability of chemistry to attach the binder to the signaling oligonucleotide without a diverse effect on the binding affinity of the binder.

In addition to protein detection and quantification, other interesting applications of the sensors could be envisioned. Antibodies specific to the different proteins that form a heterologous complex could be used to develop sensors detecting the presence of such complex. One obvious application of such sensors would be a development of rapid high throughout assays for screening for drugs disrupting formation of such complexes. Long, nanometer scale flexible linkers used in the sensors should allow their use for detecting large macromolecular complexes where the binding sites for the antibodies could be separated by large distances. By using antibodies targeting various components of such complexes the composition of such complexes could be interrogated as well. Antibodies specific to a particular post-translational modification of the protein (e.g. phosphoserine) together with antibody specific to a protein of interest could be used to develop sensors for rapid detection of a specific protein isoform. We are currently investigating the applicability of molecular pincers for studying such complexes and molecular events *in vitro* and for imaging them *in vivo*.

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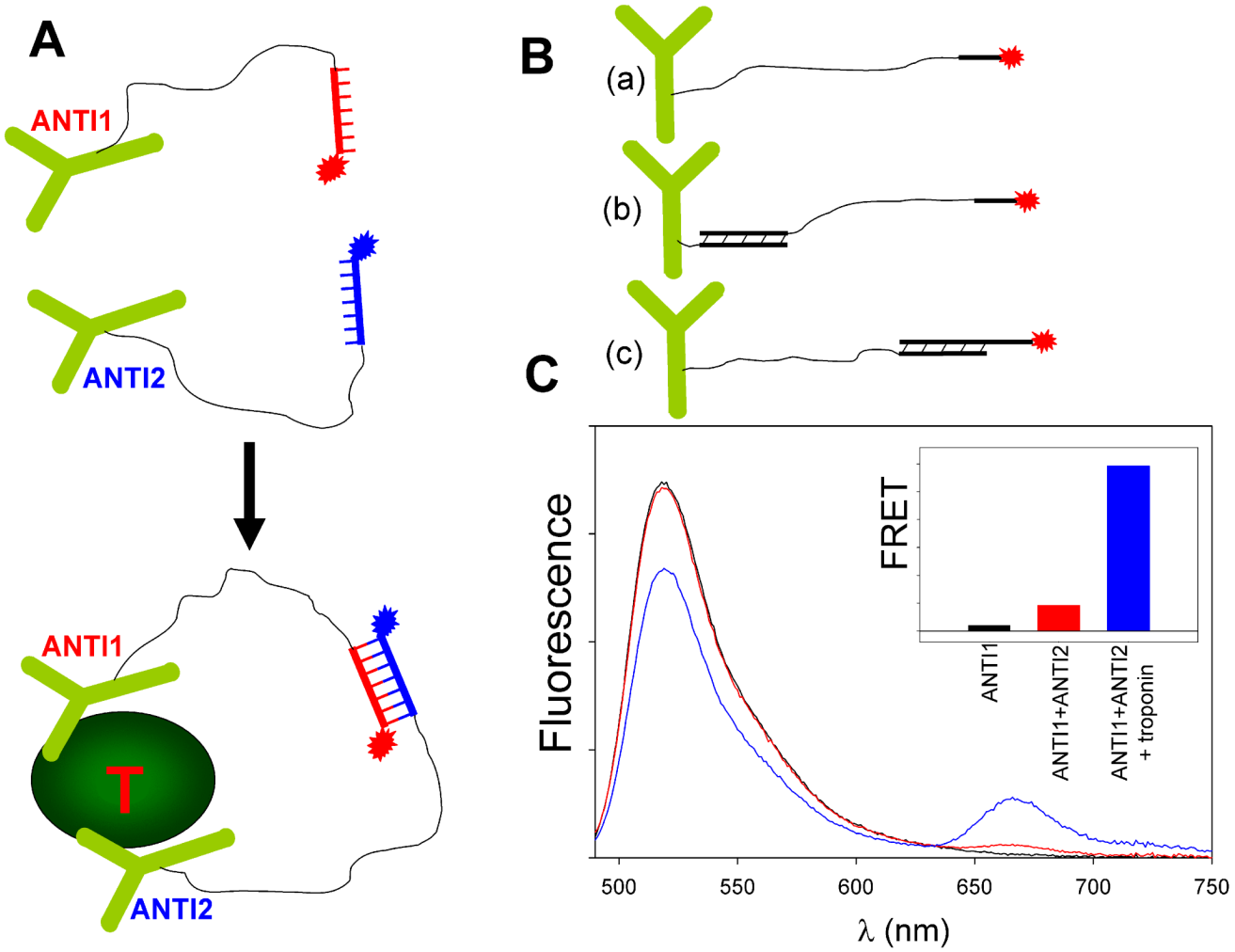

Fig. 1.

(A) Design of the sensor. ANTI1 and ANTI2 depict antibodies labeled with signaling oligonucleotides. T corresponds to the target protein. (B) Strategies for modifying the antibodies with signaling oligonucleotides. The three strategies (a,b and c) are explained in the text. (C) Proof-of-principle for molecular pincers. Black line: emission spectrum of 20 nM ANTI1 labeled with fluorescein (using the strategy illustrated in panel B(c) employing A1 and A3 oligonucleotides). Red line: Emission spectrum of a mixture of 20 nM ANTI1 (labeled with A1/A3) and 25 nM ANTI2 labeled with Cy5 (using the strategy illustrated in panel B(c) employing A2 and A4 oligonucleotides). Blue line: 20 nM ANTI1 (labeled with A1/A3) and 25 nM ANTI2 (labeled with A2/A4) in the presence of 20 nM human cardiac troponin I. Excitation was at 490 nm. Inset: FRET signals for each sample.(emission at 670 nm with the excitation at 490 nm).

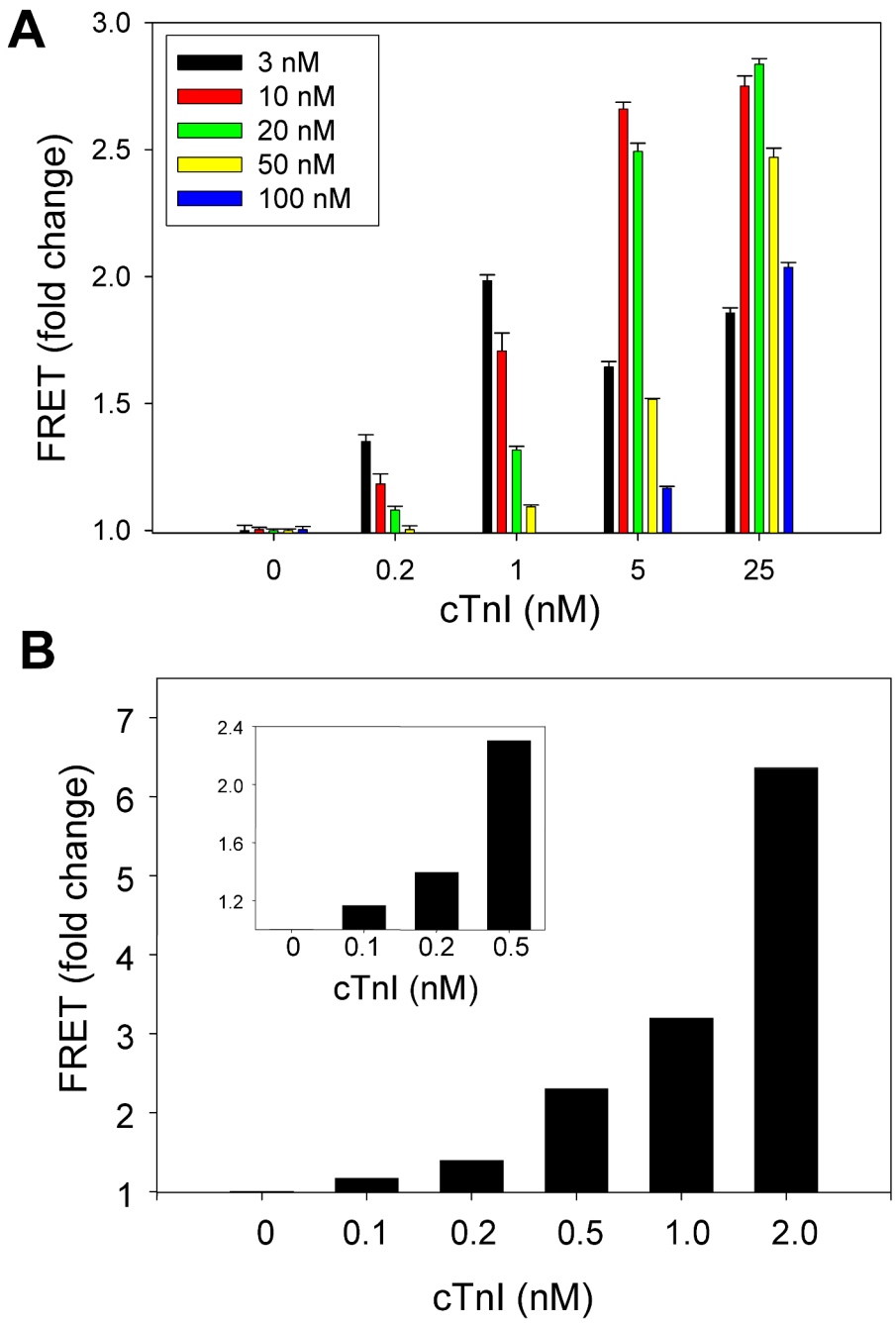

Fig. 2.
(A) FRET signal of the sensor for troponin at different concentrations of target. (B) FRET signal for the sensor optimized for detecting low concentrations of troponin.

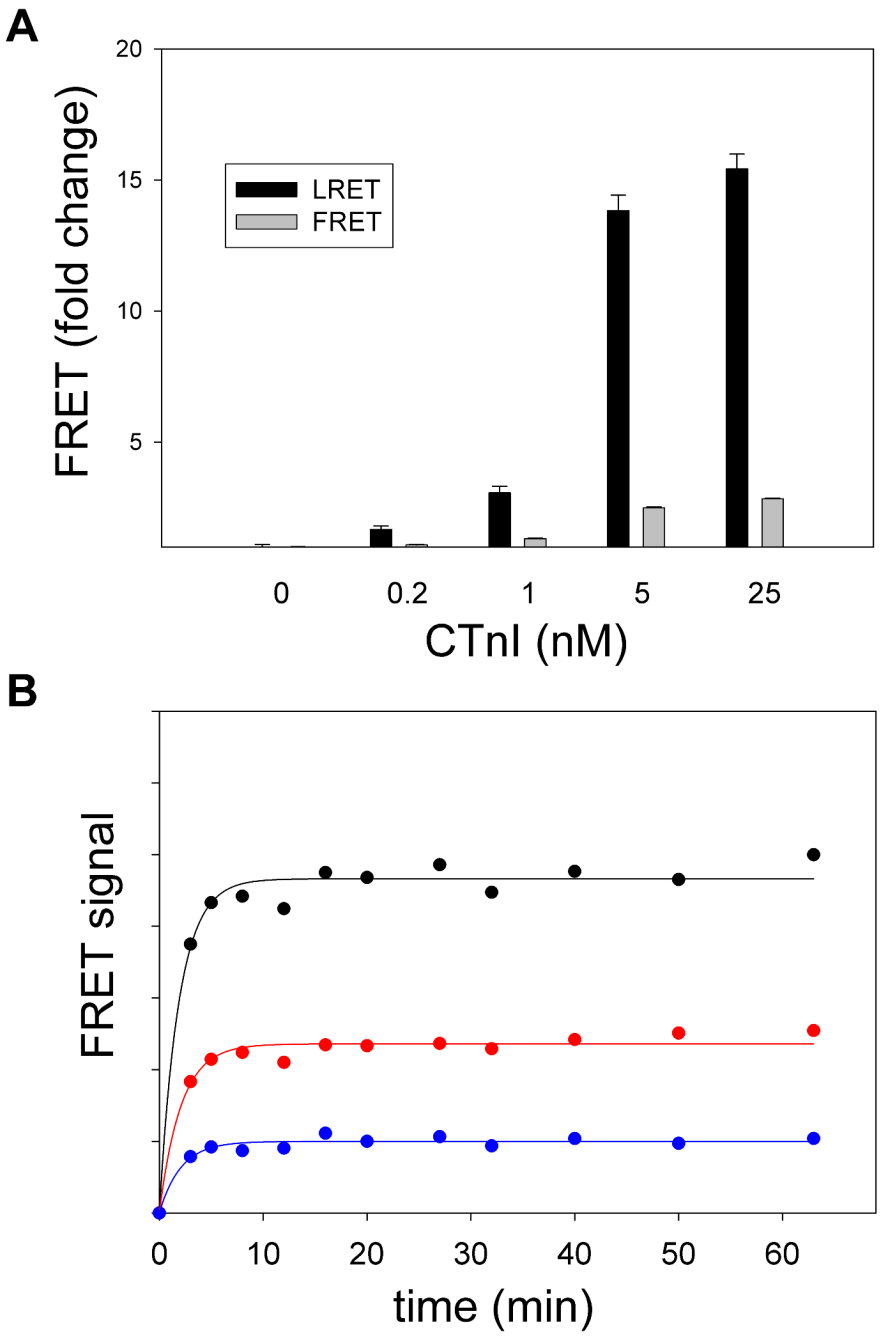

Fig. 3.

(A) Comparison of troponin detection using FRET between fluorescein and CY5 and LRET between Eu^{3+} chelate and Cy5. (B) Time course of FRET signal after addition of troponin to the mixture 20 nM ANTI1 (labeled with A1/A3) and 25 nM ANTI2 (labeled with A2/A4) antibodies. Concentrations of troponin were: 20 nM (black circles), 10 nM (red circles), and 5 nM (blue circles).

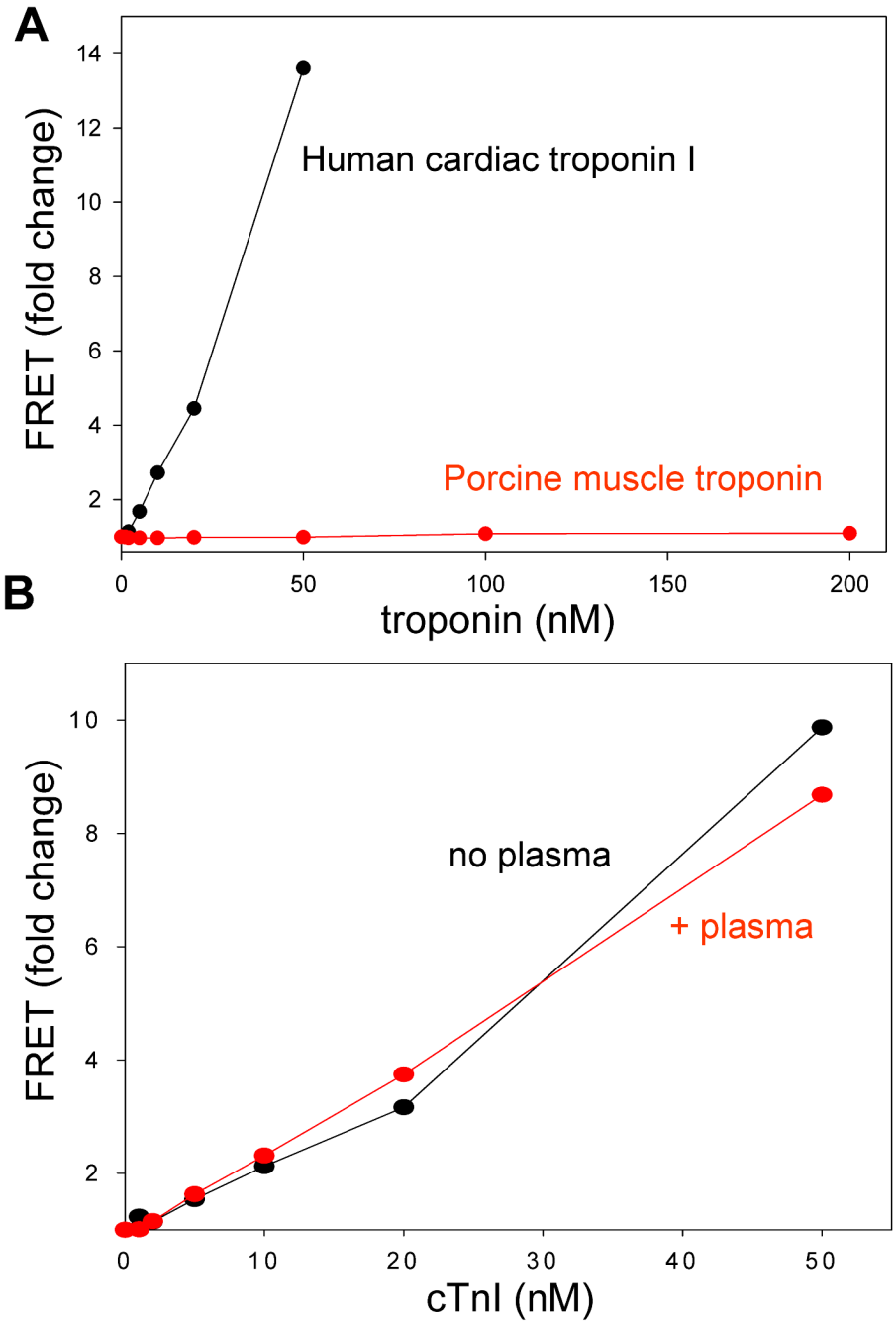

Fig. 4.

(A) Specificity of the sensor for cardiac troponin. 20 nM ANTI1 (labeled with A1/A3) and 25 nM ANTI2 (labeled with A2/A4) antibodies were used in this experiment. (B) Compatibility of the sensor for troponin with detection of the protein in plasma. 2 μl of spiked plasma was used for 20 μl assay.

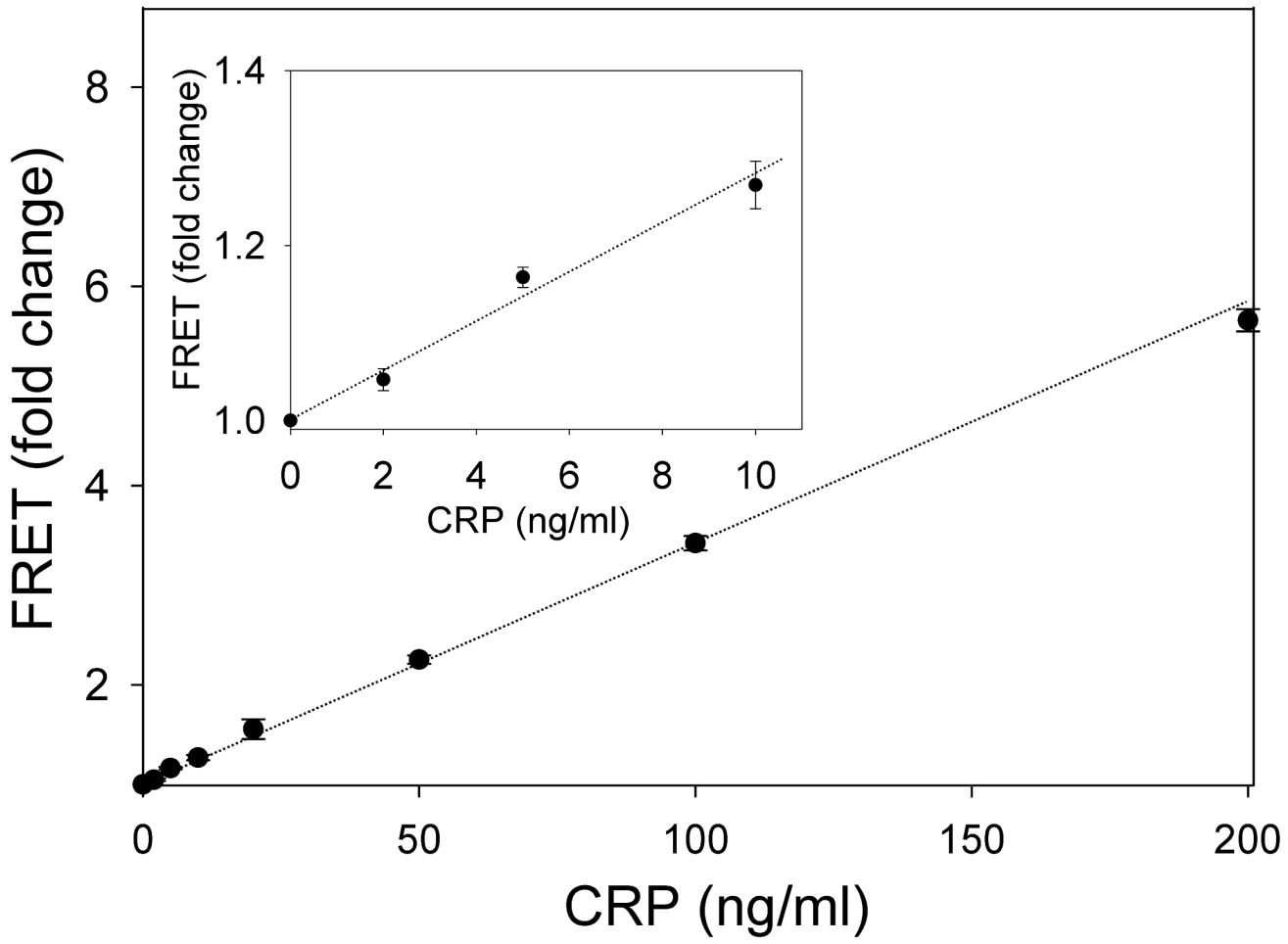

Fig. 5.
Molecular pincer sensor for detecting C-reactive protein (CRP). 10 nM ANTI1 (labeled with A1/A3) and 12.5 nM ANTI2 (labeled with A2/A4) were used.

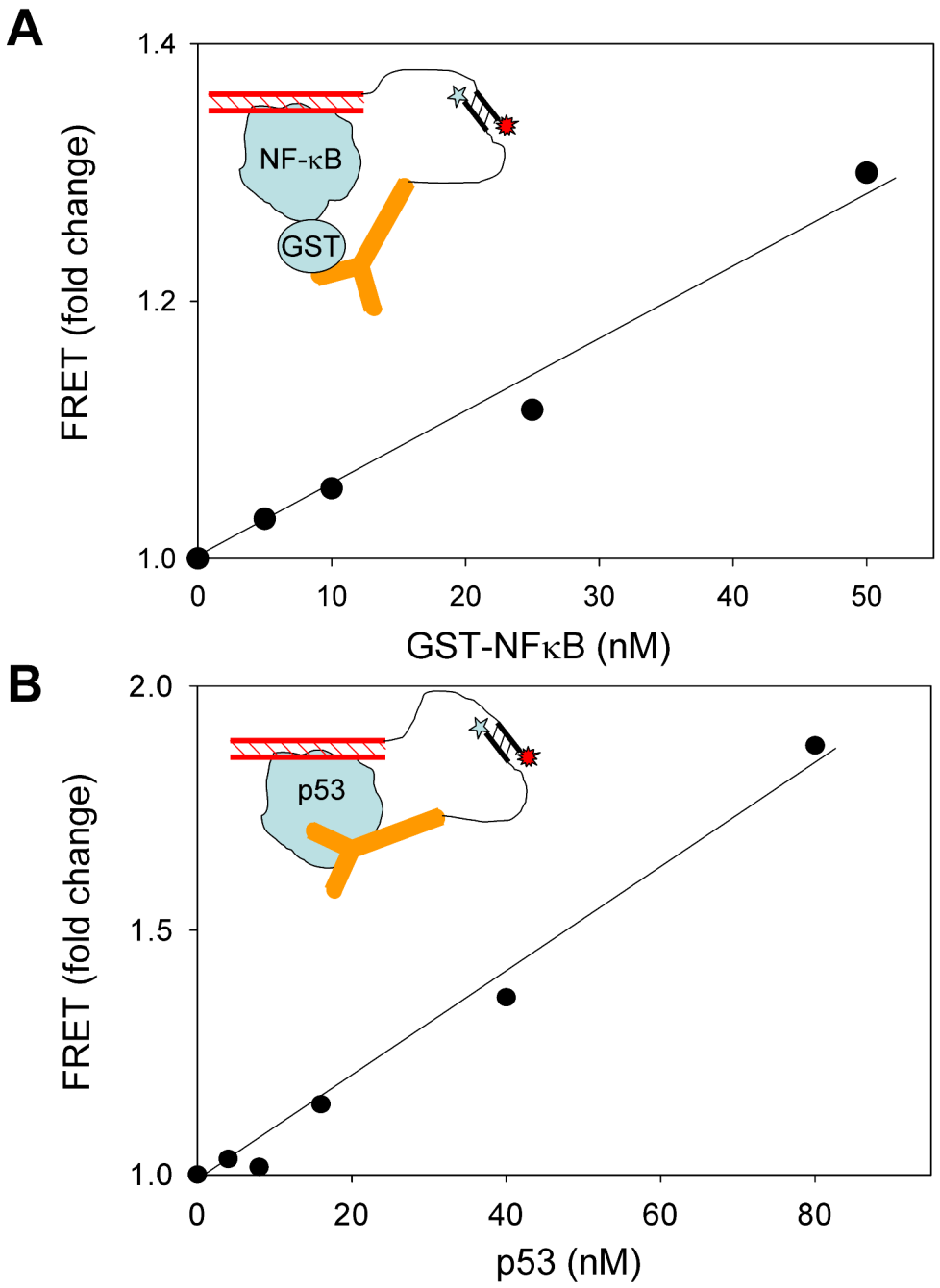

Fig. 6.

Mixed sensors utilizing an antibody and a ds DNA containing a binding site for a DNA binding protein. (A) Sensor for GST-tagged NF-κB. Anti-GST antibody (10 nM) labeled with a signaling oligonucleotide (using the strategy illustrated in Fig. 1B(b) employing A2 and A8 oligonucleotides) and 40 bp DNA fragment (A9A/A10A11 duplex) containing NF-κB binding site at 25 nM were used. (B) Sensor for p53 protein. Anti-p53 antibody (10 nM) labeled with a signaling oligonucleotide (using the strategy illustrated in Fig. 1B(b) employing A14 and A8 oligonucleotides) and 43 bp DNA fragment (A12/A13A11 duplex) containing p53 binding site at 10 nM were used.