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Antigen peptide-based immunosensors for rapid detection of antibodies and antigens

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Abstract

The homogenous immunosensor design described here utilizes bivalent nature of the antibody. Antigen peptide is conjugated using flexible linkers with short complementary oligonucleotides (signaling oligonucleotides) containing each a fluorochrome that can form a Fluorescence Resonance Energy Transfer (FRET) donor-acceptor pair. The complementary signaling oligonucleotides are short enough to prevent their annealing on their own. Binding of the peptide-signaling oligonucleotide constructs to bivalent antibody results in a large increase in local concentration of signaling oligonucleotides causing their annealing and appearance of FRET signal. We used simple model system (anti-biotin antibody) to obtain proof-of-principle validation of the sensor design. We then constructed two sensors based on two peptides corresponding to the antigens of two antibodies raised against human cardiac troponin I. We demonstrated that these sensors could be used for sensitive detection of the antibody and for competition-based detection of the intact troponin I. Furthermore, we showed that these sensors could be used for detection of kinase activity targeting the antigen peptide. These simple and robust immunosensors may find applications in antibody detection (for example, in diagnosis of autoimmune or infectious disease), in protein detection (especially when speed of detection is essential), and in assays for detecting enzymatic activities involved in posttranslational modifications of proteins.

Introduction

Antibodies have found wide-ranging applications for highly specific and sensitive detection of target molecules[1, 2]. In addition to classical immunochemical techniques (such as, for example, ELISA[3, 4]) various antibody-based sensor technologies are being developed[5-7] to further increase the utility of antibody-based detection methodologies. We have recently developed antibody-based homogenous sensors (molecular pincers) that allow rapid and sensitive detection of proteins in solution[8]. These sensors utilize a pair of antibodies recognizing non-overlapping epitopes of the target protein. The antibodies are conjugated with short complementary oligonucleotides (using long flexible linkers) that are modified with fluorescence probes. These oligonucleotides are designed to be short enough that in the absence of the target they do not hybridize. In the presence of the target protein, labeled antibodies bind to their respective protein epitopes and as a consequence, the local concentration of the oligonucleotides attached to the antibodies is greatly increased resulting in efficient hybridization of the oligonucleotides. This in turn brings the fluorescence probes incorporated into the oligonucleotides into the close proximity resulting in efficient FRET (Fluorescence Resonance Energy Transfer[9]) between the probes signaling target protein detection.

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Successful implementation of molecular pincer design provided a motivation for further exploration of signaling possibilities afforded by a hybridization of the short complementary oligonucleotides induced by a change in their local concentrations. The bivalent character of antibodies together with local concentration-driven annealing of complementary oligonucleotides could be used to design novel antigen-peptide based sensors illustrated in Fig. 1. These sensors could be used for rapid homogenous detection of antibodies recognizing peptide antigens, for detection of protein targets with antibodies detecting solvent-accessible antigens utilizing competition-based assay format and for designing assays for enzymatic activities involved in posttranslational modifications of proteins. The goal of this work was to provide experimental validation of the sensor design and to verify its applicability for the above-mentioned applications.

Experimental Section

Materials

The oligonucleotides were obtained from Keck Oligonucleotide Synthesis Facility at Yale University. The following constructs were used in this work (X = spacer18):

A1: 5′-C6-amino-XXXXXX-AGATGCG-S-S-CPG-3′;

A2(FL): 5′-C6-amino-XXXXXX-CGCATCT-Fluorescein-3′;

A4: 5′-C6-amino-GCAGCCGATTCGACTTGC-3′;

A5(FL): 5′-GCTCATGCAAG(dT-fluorescein)-CGAATCGGCTGC-3′;

A6: 5′-GCTCATGCAAGTCGAAT(dT-C6-amino)-CGGCTGC-3′;

A7: 5′-A(dT-C6-amino)GAGCGGCAAGTCGAATCGGCTGC-3′.

3′-Fluorescein was incorporated into oligonucleotide A2(FL) during oligonucleotide synthesis. A1(Cy5) (A1 labeled at 3′ end with Cy5) was prepared by postsynthetic modification of DTT cleaved A1 with Cy5 maleimide (Invitrogen). A6(Eu3+) (A6 modified with europium chelate) was prepared using a two-step labeling procedure described previously[10]. A7(Cy5) (A7modified with Cy5) was prepared by post-synthetic modification with Cy5-NHS (Invitrogen). A1(Cy5) and A2(FL) were labeled at 5′ end with biotin (A1(Cy5;biot), A2(FL) (biot)) by post-synthetic modification with biotin-NHS (Pierce, Rockland, IL). All modified oligonucleotides were purified by reversed-phase HPLC[11]. Concentrations of the oligonucleotides were calculated from UV absorbance at 260 nm after correction for the contribution of the fluorophore absorbance at 260 nm. Biotin and biotin polyclonal antibody (goat) were from Sigma (St. Louis, MO). Fab and $F(ab)_2$ fragments of anti-biotin antibody were prepared using Mouse IgG1 Fab and $F(ab')_2$ Preparation Kit (Piece, Rockford, Il) according to manufacturer's instructions. Troponin peptides P1 (residues 1-15; MADGSSDAAREPRPAC) and P2 (residues 13-29; RPAPAPIRRRSSNYRAYC) were obtained from Keck Peptide Synthesis Facility at Yale University. Polyclonal antibody G-131-C (goat) specific to human cardiac troponin I first 15 residues was from BiosPacific Inc. (Emeryville, CA). Monoclonal antibody RD1-TRK4T21-M18 specific to human cardiac troponin I residues 13-29 was from Fitzgerald Industries International, Inc. (Concord, MA). Human cardiac troponin complex was from National Institute of Standards and Technology (Gaithersburg, MD). Human skeletal muscle troponin I protein was from HyTest (Turku, Finland). Troponin from porcine muscle and bovine protein kinase A catalytic subunit was from Sigma (St. Louis, MO). SMCC (Succinimidyl 4-[N-maleimidomethyl] cyclohexane-1-carboxylate) and TCEP (tris (2-carboxyethyl) phosphine) were from Pierce (Rockford, IL). MAL-$dPEG_{24}$-NHS crosslinker was from Quanta Biodesign, Ltd (Powell, Ohio).

Preparation of peptide-oligonucleotide conjugates

Two strategies for conjugating fluorochromes-labeled signaling oligonucleotides with the peptides were employed (Fig. 1 D&E). For direct conjugation of the peptide with the signaling oligonucleotide (Fig. 1D), A1 (or A2(FL)) oligonucleotides were incubated with a 10-fold excess of SMCC in 0.1M $NaHCO_3$ (pH 8.3) at room temperature for 3 hr, followed by the addition of 50-fold excess of the peptide. Incubation was continued for another 6 hrs. Oligonucleotide-peptide conjugates were purified by native polyacrylamide gel electrophoresis. For the indirect conjugation of the signaling oligonucleotide with the peptide (Fig. 1E), the peptides were first conjugated to a short unlabeled oligonucleotide (A4) followed by annealing of fluorochrome-labeled signaling oligonucleotides (A5(FL), A6 or A7). A4 oligonucleotide was incubated with a 10-fold excess of MAL-$dPEG_{24}$-NHS crosslinker in 0.1M $NaHCO_3$ pH 8.3 at room temperature for 3 hr followed by incubation with 50 fold excess of the peptide in the same buffer for 6 hr. Peptide-oligonucleotide conjugate was purified by native polyacrylamide gel electrophoresis. Signaling oligonucleotides were annealed by incubating A4-peptide conjugate with equimolar concentrations of A5(FL) (or A6, or A7). This procedure simplifies the labeling since only one covalent conjugate of the peptide needs to be made. However, signaling oligonucleotides produced this way are bulkier since they contain dsDNA segments (Fig 1D vs. Fig. 1E).

Fluorescence Measurements

All fluorescence measurements were performed in 20 μL of binding buffer (20 mM Tris-HCl pH 8.0, 100 mM NaCl and 10 μM EDTA) in 384-well low-volume black microplates (Corning cat #3676) at 25 °C. The donor (fluorescein; excitation at 485 nm, emission at 535 nm) and sensitized acceptor emission (Cy5; excitation at 485 nm, emission at 665 nm) signals were read with Analyst AD plate reader (LJL Biosystems, Sunnyvale, CA) or Spectra FluorPlus microplate reader (Tecan, Research Triangle Park, NC). Luminescence Resonance Energy Transfer (LRET)[12] was measured on Analyst AD plate reader. Donor (europium) emission was measured with the excitation at 330 nm and emission at 620 nm. Sensitized acceptor (Cy5) emission was measured at 670 nm with excitation at 330 nm. Gated emission was measured with 50 μsec delay and 1000 μsec integration time. All reaction mixtures were incubated for 40 min before fluorescence measurements were made.

Results of fluorescence measurements in the case of titration with antibodies were expressed as a fold change of FRET signal:

$$\text{FRET (fold change)}=(F_{SA}/F_D)/(F^0_{SA}/F^0_D) \quad (1)$$

where F_{SA}, F_D, $F_{SA}{}^0$ and $F_D{}^0$ are sensitized acceptor and donor emission intensities in the presence and absence of the antibody, respectively. Buffer background was subtracted from the measured fluorescence intensities before FRET values were calculated. Using the ratio of sensitized acceptor and donor emission intensities for calculating FRET according to eq. 1 reduces the variability of FRET measurements due to dilution and instrumentation errors. Results of competition experiments were expressed at each competitor concentration as the percentage of the signal observed in the absence of the competitor.

Determination of limits of detection and sensitivities of the assays

To determine limit of detection (LOD)[13], the lowest concentration of the antibody (or troponin I) that could be detected with 99% confidence, FRET signals for 10 evenly spaced analyte concentrations were measured in triplicate (Figs. 3B & 4B). Ten repeats of the blank (no analyte) sample were also measured. The data were fitted to appropriate calibration curves

(straight line in the case of antibody and four parameter logistic curve in the case of troponin). LOD was calculated from calibration curves as the analyte concentration corresponding to FRET signal equal to blank+3×(standard deviation of the blank)[13]. Sensitivity of the assay[13] (FRET signal change per unit of analyte concentration) was obtained from the slope of calibration curve (in the case of antibody) or from slope of a line through first four data points in the case of troponin where the calibration curve was nonlinear.

Phosphorylation of troponin peptide by PKA

Bovine protein kinase A catalytic subunit was reconstituted in H_2O containing 20 mM DTT at a concentration of 2 U/μl. P2 peptide conjugate with A4 (P2-A4) was annealed with A6(Eu^{3+}) and A7(Cy5) to yield P2-A4/A6(Eu^{3+}) and P2-A4/A7(Cy5), respectively. A 1μM mixture of P2-A4/A6(Eu^{3+}) and P2-A4/A7(Cy5) in 5μl buffer (100 mM Tris, pH 8.0, 100 mM NaCl, 0.5 mM EDTA, 2 mM $MgCl_2$, with or without 300 μM ATP) was incubated with 0.01 U, 0.02 U, 0.05 U, 0.1 U, 0.2 U and 0.5 U of PKA at 30°C for 10 min. The reaction was stopped by heating the sample to 70°C for 20 min. Samples from each reaction mixture were withdrawn and were mixed with monoclonal anti-troponin I antibody recognizing residues 13-29 of human cardiac troponin. The final concentration of antibody and peptide-oligonucleotide conjugates was 40 nM in 20 μL of the buffer (20 mM Tris-HCl pH 8.0, 100 mM NaCl and 10 μM EDTA). Time-resolved emission of europium chelate donor and Cy5 acceptor were measured in 384-well plates at 25 °C after 40min incubation on Analyst AD plate reader.

Results and Discussion

Proof-of-principle for the sensor design

Fig. 1 illustrates the design of the sensors. Design of the sensors is based on two simple thermodynamic considerations. The first is a large change of local concentration of the signaling oligonucleotides attached via flexible linkers to the peptide recognized by the antibody when two molecules of peptide-oligonucleotide conjugate bind to one bivalent antibody. We used 8.6 nm long linker to prepare peptide-oligonucleotide conjugates. Our recent analysis of the properties of ligands containing long flexible linkers [14] indicated that linkers up to at least 50 nm length should be compatible with the design illustrated in Fig. 1 and should produce local concentration of oligonucleotides in antibody-peptide-oligonucleotide complexes in tens of micromolar range. Thus, complementary signaling oligonucleotides can be easily designed such that at nanomolar concentrations in the absence of the antibody essentially no annealing will take place. We used a pair of complementary oligonucleotides with predicted hybridization free energy of -7.4 kcal/mole (melting temperature of 2.6°C for 10 nM oligonucleotide concentration at 100 mM salt)[15]. In contrast, when the peptide-oligonucleotide constructs bind to the antibody, the large increase of the local concentration of signaling oligonucleotides attached to the peptides will result in almost 100% annealing. This annealing in turn will bring the two fluorophores attached to oligonucleotides to close proximity producing FRET signal that could be used to detect peptide-antibody complex formation. The second is a preferential formation of peptide-oligonucleotide complexes with the antibody in which each molecule of the antibody binds a pair of peptide-oligonucleotide constructs containing complementary signaling oligonucleotides (Fig. 1C). Although the two peptide binding sites of the bivalent antibody are equivalent, when a mixture of peptide-oligonucleotide conjugates containing complementary oligonucleotides is incubated with the antibody, the most stable complex will be the one which allows for annealing of the complementary oligonucleotides due to the additional favorable free energy provided by the annealing (ΔG_2 in Fig. 1C). The complexes containing either two donor-labeled or two acceptor-labeled oligonucleotides (and thus not able to produce FRET signal) will be disfavored and will not be formed in significant amounts. This built-in selection of complexes

that produce FRET signal assures maximal possible FRET signal. The combination of the two phenomena discussed above allows efficient functioning of the sensors.

Placing FRET fluorescence probes on the signaling oligonucleotides that undergo annealing when peptide-oligonucleotide conjugates bind to the antibody allows reliable generation of FRET signal. The probes in this case are brought to predictably close distance (defined by well established geometry of the DNA duplex formed by annealed signaling oligonucleotides) that could be designed to match the distance required for most effective FRET between a given set of fluorescence probes.

Additional benefit of the enhanced stability of antibody-peptide-oligonucleotide complex derived from additional free energy of oligonucleotide hybridization (ΔG_2) will be that functional sensors utilizing peptide-antibody pairs of modest binding affinity should be possible to prepare.

We envision that the sensors could be used either in a direct assay format (Fig. 1A) for detecting antibodies or in a indirect competitive assay format (Fig. 1B) for detecting proteins containing the exposed peptide epitope corresponding to the peptide in the peptide-oligonucleotide conjugate.

We first utilized a simple model system consisting of anti-biotin antibody and Cy5 and fluorescein labeled biotinylated A1(Cy5) and A2(FL) oligonucleotides (A1(Cy5;biot), A2 (FL;biot) to validate the sensor design illustrated in Fig. 1. This model system, due to its simplicity, can be viewed as “the best case scenario” reference to which any peptide-based sensor can be compared to evaluate its performance. Addition of anti-biotin antibody to a mixture of A1(Cy5;biot) and A2(FL;biot) resulted in a large antibody-concentration dependent FRET signal (Fig. 2A). The signal decreased at high antibody concentration after reaching the maximum value at intermediate antibody concentration. This was expected since at high antibody concentrations the complexes containing only one oligonucleotide bound to the antibody should start to form and these complexes could not produce FRET signal. No FRET signal was detected when a mixture of A1(Cy5;biot) and A2(FL;biot) was titrated with a monovalent Fab anti-biotin antibody fragment although it could bind efficiently the biotinylated oligonucleotides (as determined by native gel electrophoresis mobility shift experiments (not shown)). This confirmed that as illustrated in Fig. 1A the signal could be only generated with a bivalent antibody. The data shown in Fig. 2A provided a proof-of-principle for the sensor design illustrated in Fig. 1A.

FRET signal produced by a mixture of anti-biotin antibody, A1(Cy5;biot) and A2(FL;biot) decreased upon addition of unlabeled biotinylated oligonucleotide and was unchanged upon addition of the oligonucleotide lacking biotin (Fig. 2B). These data provided proof-of-principle for using the sensor in a competitive assay format. We also used $F(ab)_2$ antibody fragment in these competition experiments (Fig. 2B, empty circles). $F(ab)_2$ fragment exhibited reduced biotin binding affinity (as determined by native gel electrophoresis mobility shift experiments (not shown)) and it was interesting to note that the complex of A1(Cy5;biot) and A2(FL;biot) with $F(ab)_2$ fragment was more readily competed with the biotinylated oligonucleotide compared to the intact antibody. This suggests that the performance of the competitive assay could be improved by using $F(ab)_2$ fragment in place of the intact antibody.

Detection of the antibody using direct format of the assay

Having confirmed the feasibility of sensor design using anti-biotin antibody model, we then selected cardiac troponin I as a target for testing the sensor design involving antigen peptides. We selected troponin I since we previously used this protein as a target for the development of novel rapid assay for troponin (molecular pincers) that utilized a pair of anti-troponin

antibodies [8]. Testing the epitope peptide sensor design depicted in Fig. 1 with troponin peptides provided the opportunity of comparison between the two assays. Troponin is the contractile regulatory protein complex of striated muscle [16]. Levels of cardiac troponin I in the serum raise dramatically after acute myocardial infarction (AMI) [17, 18]. Determination of the level of cardiac troponin in the serum allows specific and sensitive diagnosis of AMI.

One of the practical applications our epitope peptide-based sensors that we envision will be the detection of the antibodies (for example, in infectious or autoimmune disease detection and diagnosis) using direct assay format (Fig.1A). We thus tested the performance of troponin peptide based sensors for detecting anti-troponin antibody. The N-terminal cardiac troponin peptide (P1) was conjugated with A1(Cy5) and A2(FL) oligonucleotides to yield P1-A1(Cy5) and P1-A2(FL), respectively. Titration of the mixture of P1-A1(Cy5) and P1-A2(FL) with the goat anti-troponin I polyclonal antibody that was raised against the above peptide produced a large antibody-concentration dependent FRET signal (Fig. 3A). FRET signal observed was only slightly smaller compared to a simple biotin model system (Fig. 2A) demonstrating robust performance of the peptide-based sensor. No FRET signal was observed when the mixture of P1-A1(Cy5) and P1-A2(FL) was titrated with mouse IgG or with the anti-troponin antibody targeting different region of the protein (Fig. 3A). These data demonstrated the functionality and specificity of the sensor utilizing peptide antigen.

To determine limits of detection (LOD)[13] for the antibody detection we measured response of the sensor to a series of low concentrations of the antibody (Fig. 3B). These measurements were done at two different sensor concentrations since we expected that LOD and assay sensitivity should be dependent on sensor concentration. The LOD (determined as described in Experimental Section) was 44 pM and 430 pM at 3nM and 10 nM sensor concentration, respectively. Assay sensitivity[13] was ~ three time higher at 3 nM sensor concentration compared to 10 nM sensor concentration. Thus, the assay characteristics can be conveniently and simply manipulated with assay components concentrations. However, the increase in LOD of the assay by lowering assay component concentrations will have its limits. First, if the concentrations of assay components and the antibody fall below the equilibrium dissociation constant of antibody-peptide-oligonucleotide complex, the performance of the assay will be compromised. Second, at very low sensor concentrations fluorescence signal intensities will become too low for accurate measurement. This will depend on the sensitivity of instrumentation used to read the signals. With the fluorescence plate reader used in this work, sensitized acceptor emission intensities for 3 nM sensor were 5-13 times over the buffer background (donor emission intensities were 90-100 times over the buffer background) allowing accurate fluorescence measurements (as demonstrated by small standard deviations of FRET signals (Fig. 3B)). Lowering sensor concentrations further below 3 nM is not likely to result in lowering of LOD since the increase in assay sensitivity will be offset by an increase in error of FRET measurements.

Detection of the protein using indirect competitive format of the assay

Another application our epitope peptide-based sensors that we envision is the detection of the protein target containing the corresponding peptide epitope utilizing a competitive assay format (Fig. 1B). We have thus tested if cardiac troponin I could be detected by the decrease of FRET signal when the protein is added to a mixture of P1-A1(Cy5), P1-A2(FL) and goat anti-troponin I polyclonal antibody. Titration of the of sensor mixture with the purified troponin produced protein concentration dependent decrease of FRET signal (Fig. 4A). Similar decrease of FRET was observed when the mixture of P1-A1(Cy5), P1-A2(FL) and goat anti-troponin I polyclonal antibody was titrated with the free P1 peptide (Fig. 4A). This competitive assay was specific for human cardiac troponin I. No decrease of FRET signal was observed when much higher (up to 20-fold) concentrations of unrelated competitor (BSA) or human skeletal troponin I or

porcine muscle troponin were added (Fig. 4A, inset). The data shown in Fig. 4A demonstrated the functionality and specificity of the sensors utilizing antigen peptides for detecting the proteins containing corresponding exposed antigen peptides.

To determine limits of detection (LOD)[13] for the troponin detection we measured response of the sensor to a series of low concentrations of the protein (Fig. 4B). As in the case of antibody detection (Fig. 3B). these measurements were done at two different sensor/antibody concentrations. The LOD for troponin (determined as described in Experimental Section) was 11 ng/ml (470 pM) and 23 ng/ml (990 pM) at 3nM and 10 nM sensor concentrations, respectively. Assay sensitivity[13] was ~ three time higher at 3 nM sensor concentration compared to 10 nM sensor concentration. Thus, similarly to the case of antibody detection, the assay characteristics can be conveniently and simply manipulated with assay components concentrations.

Use of sensors to detect protein kinase activity

Proteins undergo a myriad of post-translational modifications (for example, phosphorylation by protein kinases) that are involved in regulation of their cellular activities and in disease development. There is a significant interest in developing convenient assays for detecting and quantifying these activities for research, drug screening and diagnostic purposes. It appeared to us that the sensor design depicted in Fig. 1A could be utilized for detecting any activity that produces post-translational modification in the epitope peptide recognized by the antibody. Such modification is likely to greatly decrease or eliminate binding of the peptide by the antibody resulting in dissociation of peptide-oligonucleotide-antibody complex and disappearance of the FRET signal. Alternatively, an antibody specific to the peptide bearing a given post-translation modification could be used. In this case, modification of the peptide would induce binding of the peptide to the antibody and subsequent appearance of the FRET signal. These changes of FRET signal induced by modification of the peptide in the peptide-oligonucleotide conjugate could be thus used to detect the corresponding post-translation modification activity.

We utilized cAMP-dependent protein kinase (PKA) [19] activity to test the feasibility of using the sensor for detecting protein kinase activity. Cardiac troponin peptide corresponding to residues 13-29 of the protein (P2; RPAPAPIRRRSSNYRAYC) contains two well-established PKA phosphorylation sites (Ser 23 and Ser 24) [20]. To prepare the conjugates of P2 peptide with signaling oligonucleotides we utilized an alternative simplified labeling strategy illustrated in Fig. 1D. P2 peptide was first conjugated with the A4 oligonucleotide using MAL-dPEG$_{24}$-NHS crosslinker. Fluorochrome–labeled signaling oligonucleotides were then annealed to the P2-A4 conjugate. When a monoclonal antibody specific to human cardiac troponin I residues 13-29 was added to a mixture of P2-A4/A5(FL) and P2-A4/A7(Cy5), ~ 2-fold increase of FRET signal as observed (not shown). This FRET signal was significantly lower compared to 5-6 fold increase of FRET signal observed with peptide-oligonucleotide conjugate prepared by direct labeling procedure (Fig. 3). This data demonstrated that the price for simplified methodology for preparing peptide-signaling oligonucleotide maybe a decrease in signal-to-background. These data also provided support for the generality of sensor design illustrated in Fig. 1 since using a different peptide and a different antibody a functional sensor was obtained. To improve signal-to-background ratio of this sensor we prepared its version that used signaling oligonucleotides labeled with europium chelate and Cy5, respectively, by annealing A6($Eu3^+$) and A7(Cy5) oligonucleotides to P2-A4 conjugate (producing P2-A4/A6 ($Eu3^+$) and P2-A4/A7(Cy5)). We have previously shown[8] that use of luminescence resonance energy transfer (LRET)[12] vs FRET can produce much larger analyte-dependent signals. Indeed, when a monoclonal antibody specific to human cardiac troponin I residues 13-29 was

added to a mixture of P2-A4/A6($Eu3^+$) and P2-A4/A7(Cy5), ~30-50 -fold increase of FRET signal as observed (not shown).

Phosphorylation of P2 peptide with PKA resulted in a drastic decrease of P2 peptide binding to the antibody as demonstrated by native gel electrophoresis mobility shift assay (Fig. 5A). Addition of the antibody to the unphosphorylated P2 resulted in formation of a complex with greatly reduced electrophoretic mobility. When the same concentration of the antibody was added to the P2 that was previously incubated with PKA and ATP, no complex with reduced mobility could be detected. We hypothesized thus that we should be able to use the dissociation of the complex between the antibody and P2-oligonucleotide conjugates caused by P2 phosphorylation by PKA and the resulting decrease of FRET to detect PKA activity. Consistent with this expectation, incubation of a mixture of P2-A4/A6($Eu3^+$) and P2-A4/A7(Cy5) with increasing amounts of PKA in the presence of ATP produced PKA-dependent decrease of LRET (Fig. 5B). The decrease of LRET reached plateau at high amounts of PKA added where LRET signal returned almost to the background observed in the absence of the antibody. No decrease of LRET was observed when PKA was added in the absence of ATP (Fig. 5B) confirming that PKA phosphorylation of the peptide was responsible for the observed decrease of LRET. The data shown in Fig. 5 demonstrated the functionality of the sensors utilizing antigen peptides for the detection of enzymatic activities involved in post-translational modifications of the proteins.

The effect of serum on the assay

Since we expected that one of the uses of the epitope peptide-based sensors could be for measuring analytes in human blood samples, we tested the effect of human serum on the sensors. We measured the response of the sensor to the same concentration of the antibody in assay mixtures containing varying amounts of serum (Fig. 6). At each amount of added serum a robust FRET signal in the presence of the antibody was observed. Only at the highest amount of serum added (5× dilution; 4 μl serum in 20 μl assay mix) FRET signal observed differed significantly from the signal observed in the absence of any serum added (Fig. 6). The larger FRET signal change observed at the highest amount of serum added (and the larger errors of FRET signals) are artifacts of large background serum autofluorescence subtraction. Serum exhibits very significant autofluorescence at 485 nm excitation. To improve the function of the sensors in serum-containing samples, different sets of florescence probes could be used to avoid exciting at 485 nm. Nevertheless, the data shown in Fig. 6 demonstrate that complex samples such as serum do not interfere with functioning of the assay.

Conclusions

The data presented here for the anti-biotin antibody model and for the two troponin peptides and the corresponding antibodies validated the sensor design illustrated in Fig. 1. The sensors can be used for quantitative determination (if appropriate standards are available) or semi-quantitative determination of antibodies or target proteins (in the absence of appropriate standards). The obtained data provided experimental support for the three applications of the sensors that we envision.

The first application is for the detection of the antibody. As long as the peptide antigen for the antibody is known, a sensitive, rapid, homogenous assay detecting this antibody according to the design shown in Fig. 1A should be possible to develop. Such sensors detecting specific antibodies could be used in research and also for diagnosis of infectious and autoimmune diseases. Observed limits of detection for the antibody seem to be well within the range of what would be required for practical use of the sensors for diagnostic applications. For example, from the data published by Ferrer-Miralles et al.[21] the concentration of anti-HIV antibodies in serum of infected patients is at least in few hundred nanomolar range. Thus, a hundred-fold or

more diluted serum could be used for anti-HIV antibody detection using our sensors. Similar concentration ranges of antibodies as estimated for anti-HIV antibodies can be expected for other infectious diseases. Similarly, from the data published by Feng et al.[22] serum concentrations of autoantibodies in rheumatoid diseases are in hundreds to few thousand-nanomolar range (again well within the range of our sensors).

The second application we envision is the use of a competitive variant of the assay (Fig.1B) for detecting the protein containing the corresponding peptide epitope. Many antibodies are raised using synthetic peptides derived from the target proteins. If the epitope corresponding to this synthetic peptide is exposed to the solvent in the native protein, it should be possible to develop a competitive assay for the protein. The advantages of this competitive assay will be the ease of its preparation and its rapid homogenous format. Furthermore, in comparison to molecular pincer assay described previously[8] that utilizes a pair of oligonucleotide-labeled antibodies, only a single unmodified antibody is required for the competitive assay depicted in Fig. 1B. The competitive assay could detect troponin at ~ 10 ng/ml (400 pM) concentrations. This is about 10 times higher compared with our direct molecular pincer assay (~ 1ng/ml;~ 40 pM)[8]. While the LOD and sensitivity of peptide-based sensors could be likely improved by optimizing fluorophores for FRET signaling, due to the competitive nature of the assay, it is unlikely that the LOD's as good as those observed with molecular pincer assay will be obtained. This could limit the applicability of the competitive assay for protein targets requiring very high sensitivity of detection. For example, normal levels of cardiac troponin I in serum are < 1 ng/ml and can reach hundreds of ng/ml in a patient with acute myocardial infarction[23]. Typical commercial ELISA for troponin has detection limits of 1 ng/ml or better. Epitope-peptide based sensors could not be thus used to detect troponin I at its lower concentration range in serum. There will be protein targets which concentrations in serum would be within the range measurable by the sensors. For example, physiological levels of C-reactive protein, a general inflammation marker, are from 68 to 8,200 ng/ml[23] which will be within the range measurable by the sensors. Practical applicability of the sensor for protein target detection will be thus defined (and limited) by the LOD and sensitivity requirements.

The third application we envision is for detection of enzymatic activities involved in post-translational modifications of the proteins. One obvious example of such application is for detecting protein kinase activities as demonstrated by the data presented for PKA phophorylation of the troponin peptide (Fig. 5). There is a great interest in general kinase assays and specific kinase assay for defined targets. Our sensors would be particularly useful for the latter application. Applicability of the sensors is not limited to detecting protein kinase activity. In principle, the sensor could be used to detect any activity resulting in peptide modification that either eliminates its interaction with the antibody raised against unmodified peptide or for which there is an antibody available specific for the modified version of the peptide. Since the list of post-translation modifications of biomedical significance is enormous and appropriate antibodies for many of these are available, we believe that the detection of enzymatic activities involved in post-translational modifications of the proteins would be a very useful application of the sensors. The use of our sensors for antibody detection and for detection of enzymatic activities involved in post-translational modifications of the proteins seem to be their most promising potential practical applications.

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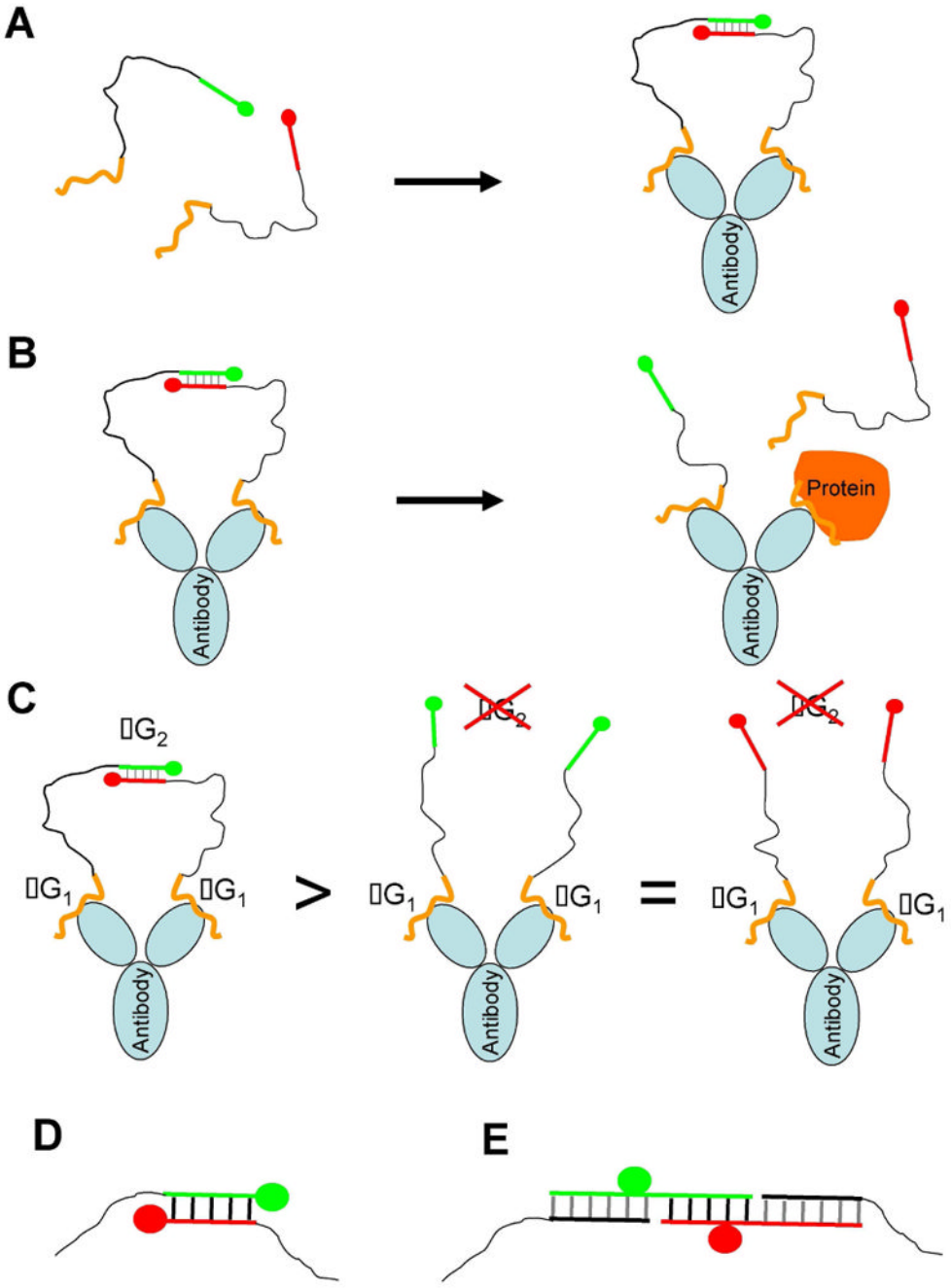

Fig. 1.

Design of epitope peptide-based immunosensor. (A) Direct sensor format for detecting antibodies. (B) Competitive sensor format for detecting proteins containing the epitope peptide. As shown in the figure, a single competitor protein bound to the antibody will be sufficient to induce FRET signal change. At higher competitor concentrations complexes containing 2 protein/antibody could be also formed but these complexes will not produce further FRET signal change (this could contribute to the nonlinearity of the assay at high competitor concentrations). (C) Enhanced stability of the complex between the antibody and the peptide-oligonucleotide conjugates containing donor and acceptor fluorochromes-labeled complementary signaling oligonucleotides. (D) Direct attachment of fluorochrome-labeled

signaling oligonucleotides to the peptide via long flexible linker. (E) Indirect attachment of signaling oligonucleotides to the peptide. The peptide is first attached to an 18 nt oligonucleotide (black) via long flexible linkers. Fluorochrome-labeled signaling oligonucleotides are annealed to this oligonucleotide using a segment of the signaling oligonucleotide containing a sequence that is complementary to the peptide-conjugated oligonucleotide.

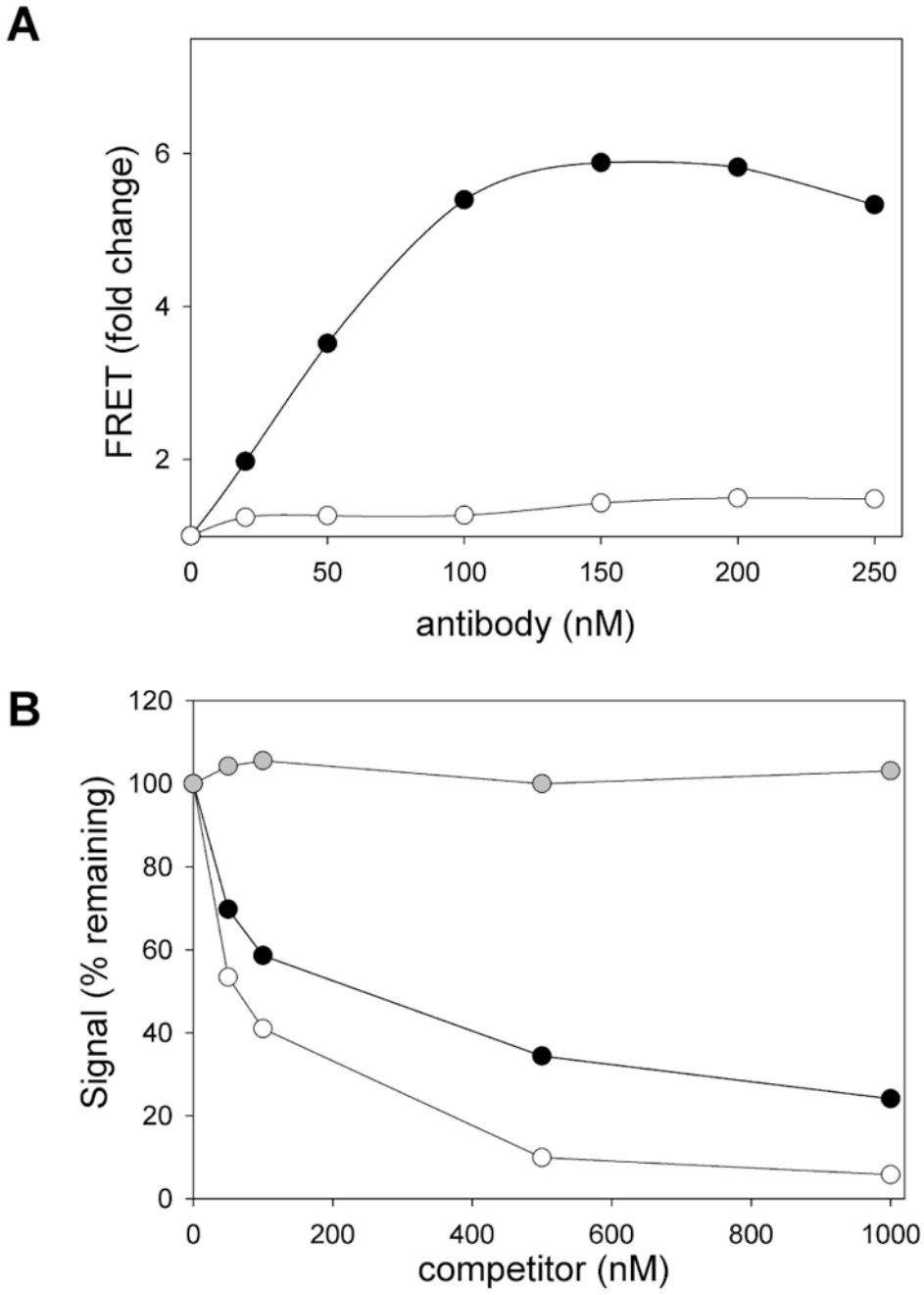

Fig. 2.

Proof-of-principle experiments with anti-biotin antibody and biotinylated signaling oligonucleotides. (A) Titration of the 50 nM mixture of A1(Cy5;biot) and A2(FL;biot) with anti-biotin antibody (black circles) or Fab fragment of anti-biotin antibody (empty circles). (B) Titration of the mixture of anti-biotin antibody, A1(Cy5;biot) and A2(FL;biot) (each at 50 nM) with unlabeled biotinylated oligonucleotide (black circles) or unlabeled no biotin oligonucleotide (grey circles), Open circles correspond to the experiment performed with F $(ab)_2$ fragment of anti-biotin antibody.

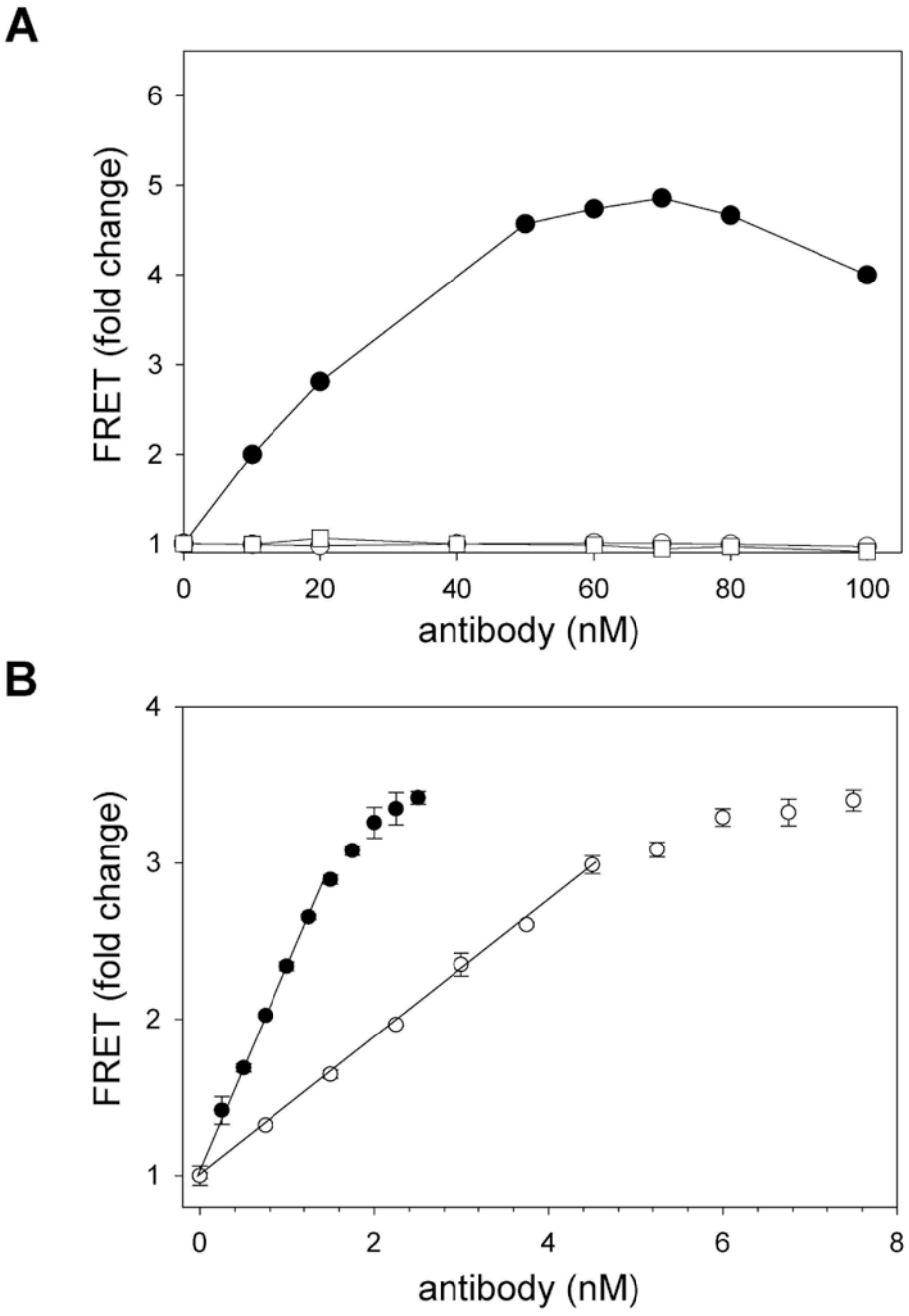

Fig. 3.

Detection of anti-troponin antibody using epitope peptide-based immunosensor. (A) Titration of the 50 nM mixture of P1-A1(Cy5) and P1-A2(FL) with goat anti-troponin polyclonal antibody raised against this peptide. To demonstrate specificity, control titrations with mouse IgG (open circles) and monoclonal antibody specific to human cardiac troponin I residues 13-29 (open squares) are shown as well. (B) Limit of detection determination. Response of the sensor to indicated concentrations of the antibody was measured for two concentrations of sensor components: 3 nM P1-A2(FL), 3.6 nM P1-A1(Cy5) (filled circles), and 10 nM P1-A2 (FL), 12 nM P1-A1(Cy5) (open circles). Error bars correspond to standard deviation of the mean of 3 measurements except for the blank (no antibody sample) for which the error bars

correspond to standard deviation of the mean of 10 measurements. Solid lines mark the linear portions of response curves.

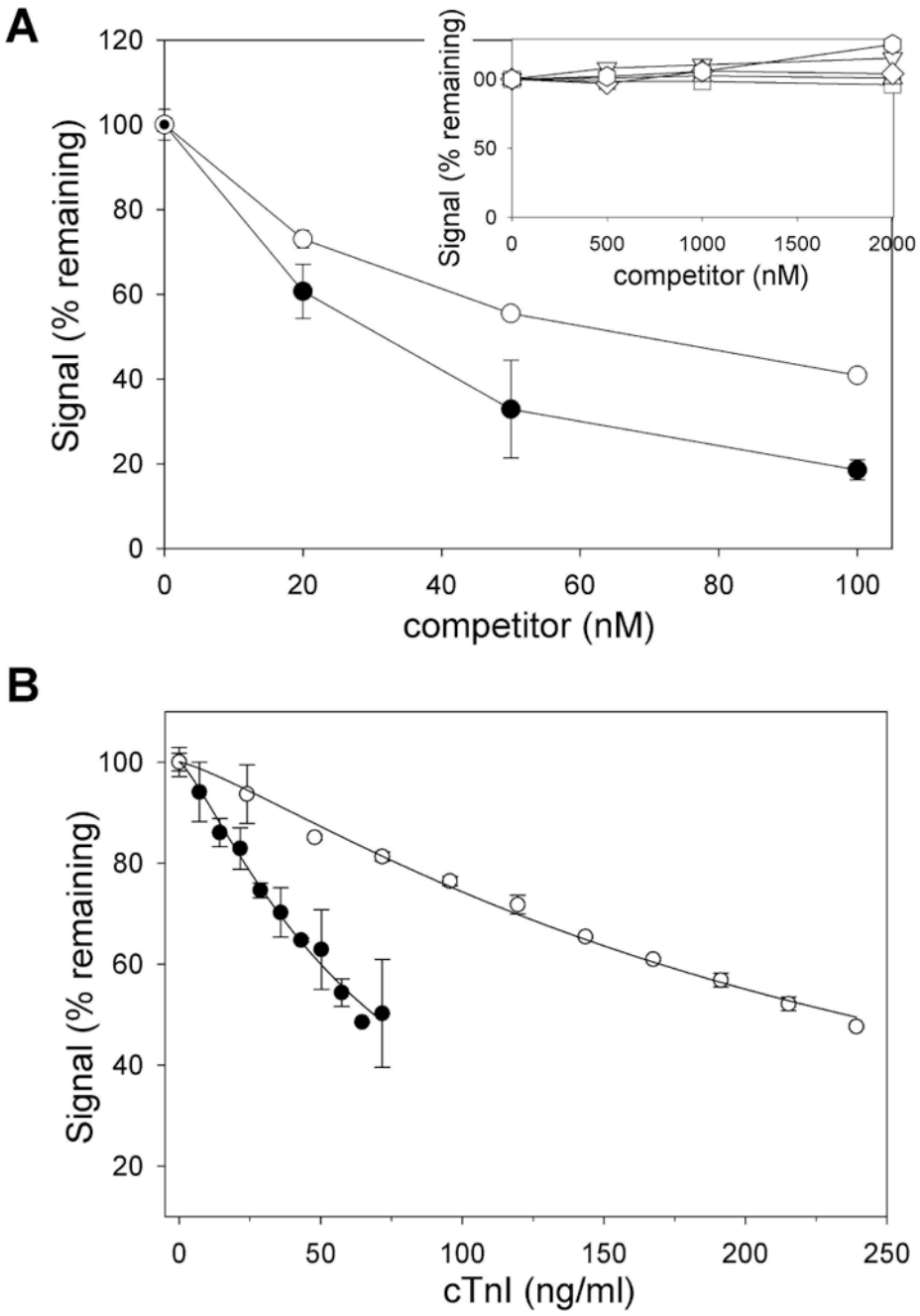

Fig. 4.

Detection of cardiac troponin using the competitive format of epitope peptide-based immunosensor. (A) Titration of a mixture of P1-A1(Cy5), P1-A2(FL) and goat polyclonal anti-troponin antibody (20 nM each) with purified cardiac troponin (black circles) or 1-15 N-terminal cardiac troponin peptide (open circles). Inset: Specificity controls. A mixture of P1-A1(Cy5), P1-A2(FL) and goat polyclonal anti-troponin antibody (20 nM each) was titrated with unconjugated oligonucleotide (black circles), BSA (open circles), nonspecific peptide (inverted triangles), human skeletal troponin I (squares) and porcine muscle troponin (triangles). (B) Limit of detection determination. Response of the sensor to indicated concentrations of the cardiac troponin I was measured for two concentrations of sensor

components: 3 nM P1-A2(FL), 3.6 nM P1-A1(Cy5), 1.5 nM antibody (filled circles), and 10 nM P1-A2(FL), 12 nM P1-A1(Cy5), 4.5 nM antibody (open circles). Error bars correspond to standard deviation of the mean of 3 measurements except for the blank (no troponin sample) for which the error bars correspond to standard deviation of the mean of 10 measurements.

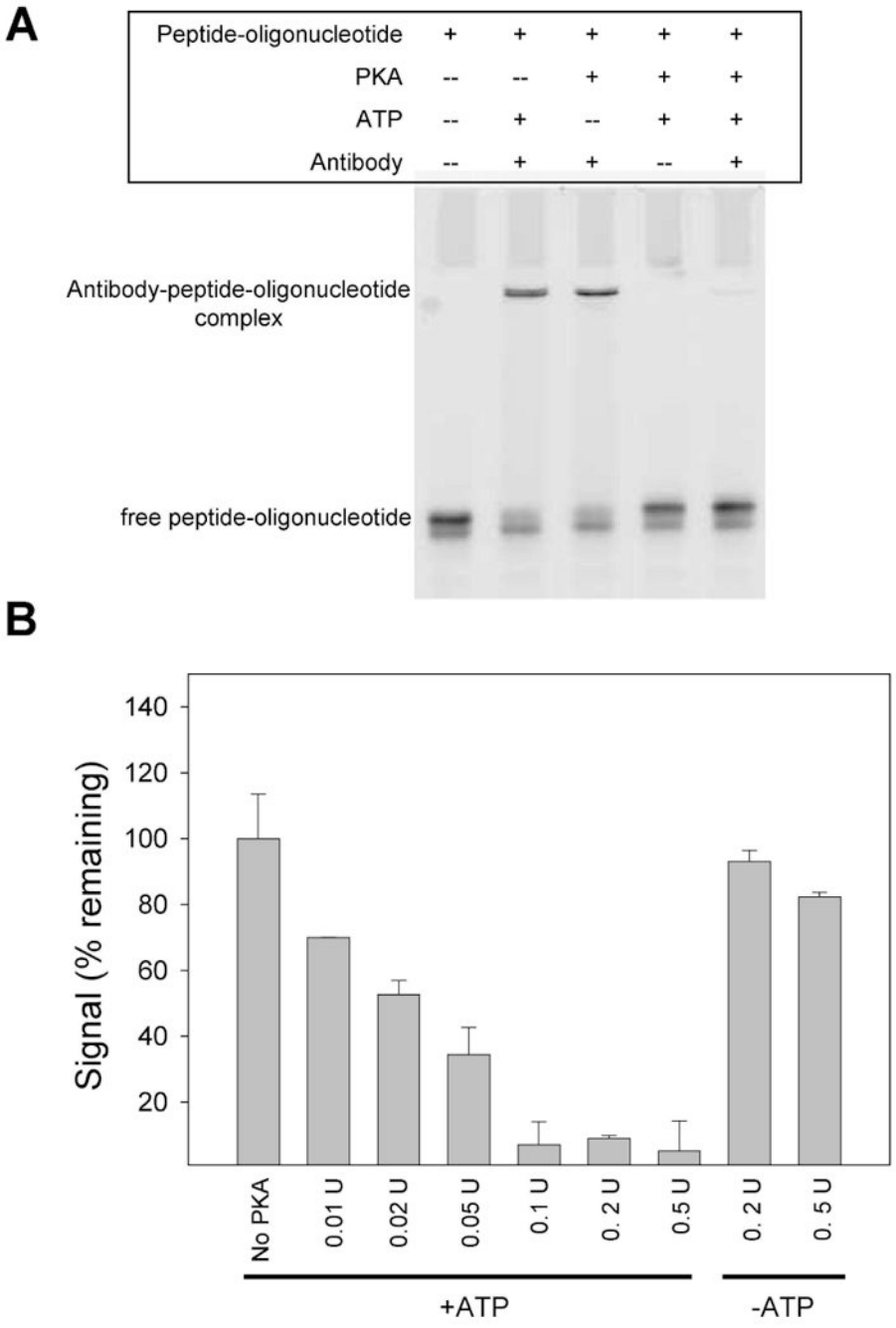

Fig. 5.

Application of epitope peptide-based immunosensor for detecting kinase activity. (A) Phosphorylation of Ser 23 and/or Ser 24 of cardiac troponin peptide (residues 13-29) by protein kinase A abolishes recognition of this peptide by the antibody. Binding between fluorescein-labeled peptide-signaling oligonucleotide conjugate (A5(FL)) and the antibody was analyzed by 10% native PAGE. (B) LRET signal of the 40 nM immunosensor mix exposed to indicated amounts of PKA in the presence or absence of ATP. LRET signals were normalized to the signal obtained in the absence of PKA (100%).

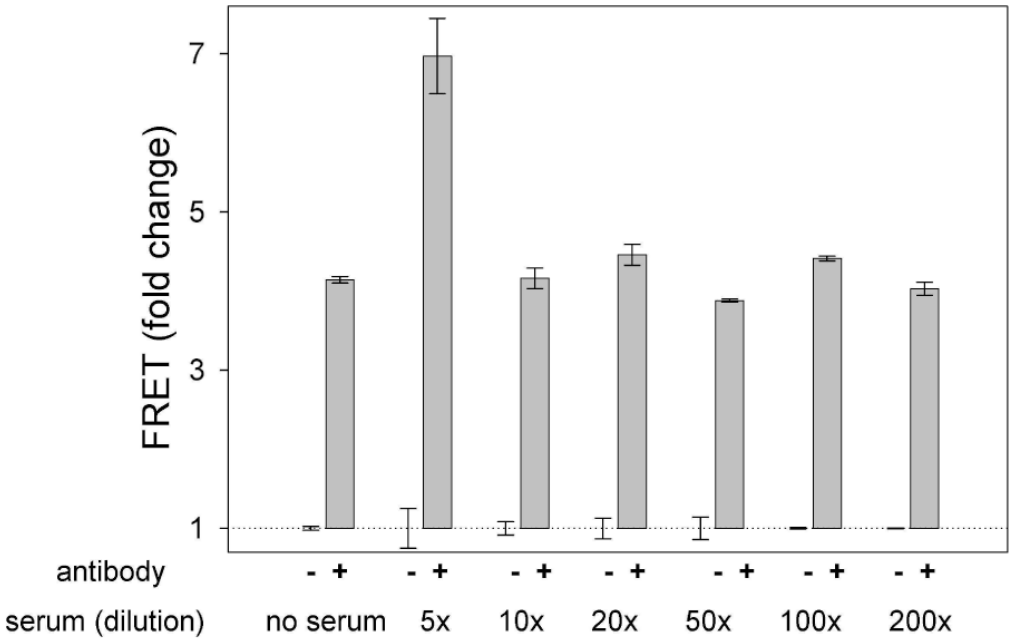

Fig. 6.

Effect of serum on epitope peptide-based immunosensor. Response of the sensor (10 nM P1-A2(FL), 12 nM P1-A1(Cy5)) to 4 nM antibody measured in the absence and presence of various amounts of human serum. The amount of serum added is expressed as its final dilution in 20 μl of the assay mix. Error bars correspond to standard deviation of the mean of 3 measurements.