Antigen Peptide-Based Immunosensors for Rapid Detection of Antibodies and Antigens

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The homogeneous immunosensor design described here utilizes the bivalent nature of the antibody. Antigen peptide is conjugated using flexible linkers with short complementary oligonucleotides (signaling oligonucleotides), each of which containing 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 (antibiotin 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 post-translational modifications of proteins.

Antibodies have found wide-ranging applications for highly specific and sensitive detection of target molecules. In addition to classical immunochemical techniques (such as, for example, ELISA), various antibody-based sensor technologies are being developed to further increase the utility of antibody-based detection methodologies. We have recently developed antibody-based homogeneous sensors (molecular pincers) that allow rapid and sensitive detection of proteins in solution. These sensors utilize a pair of antibodies recognizing nonoverlapping 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 that have been incorporated into the oligonucleotides into close proximity, resulting in efficient fluorescence resonance energy transfer (FRET) between the probes signaling target protein detection.

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, as illustrated in Figure 1. These sensors could be used for rapid homogeneous detection of antibodies recognizing peptide antigens, for detection of protein targets with antibodies detecting solvent-accessible antigens utilizing a competition-based assay format and for designing assays for enzymatic activities involved in post-translational modifications of proteins. The objective of this work was to provide experimental validation of the sensor design and to verify its applicability for the aforementioned applications.

EXPERIMENTAL SECTION

Materials. The oligonucleotides were obtained from the Keck Oligonucleotide Synthesis Facility at Yale University. The following constructs were used in this work (X = spacer 18):

A1: 5′-C6-amino-XXXXX-AGATGCG-S-S-CPG-3′;
A2(FL): 5′-C6-amino-XXXXX-CGATCT-fluorescein-3′;
A3: 5′-C6-amino-GCAGCCATCACTGC-3′;
A5(FL): 5′-GCTCATGCAAG(dT-fluorescein)-CGAATCGGCTGC-3′;
A6: 5′-GCTCATGCAAG(dT-fluorescein)-CGAATCGGCTGC-3′;
A7: 5′-Ad(T-C6-amino)-GAGCGGAAGTCACTAGGCTGC-3′.

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 antibiotin antibody were prepared using Mouse IgG1 Fab and Fab (ab)2 Preparation Kit (Pierce, Rockford, IL), according to manufacturer’s instructions. Troponin peptides P1 (residues 1–15; MADGSSDAAREPRPA) and P2 (residues 13–29; RPAPAPIRRSSNYRAYC) were obtained from the 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 obtained from Sigma (St. Louis, MO). Succinimidyl 4-[N-maleimidomethyl] cyclohexane-1-carboxylate (SMCC) and tris-(2-carboxyethyl) phosphine (TCEP) were obtained from Pierce (Rockford, IL). MAL-dPEG24-NHS cross-linker was obtained from Quanta Biodesign, Ltd. (Powell, OH).

Preparation of Peptide—Oligonucleotide Conjugates. Two strategies for conjugating fluorochromes-labeled signaling oligonucleotides with the peptides were applied (see Figures 1D and 1E). For direct conjugation of the peptide with the signaling oligonucleotide (Figure 1D), A1 (or A2(FL)) oligonucleotides were incubated with a 10-fold excess of SMCC in 0.1 M NaHCO3 (pH 8.3) at room temperature for 3 h, followed by the addition of 50-fold excess of the peptide. Incubation was continued for another 6 h. Oligonucleotide—peptide conjugates were purified via native polyacrylamide gel electrophoresis. For the indirect conjugation of the signaling oligonucleotide with the peptide (Figure 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-dPEG24-NHS cross-linker in 0.1 M NaHCO3 pH 8.3 at room temperature for 3 h, followed by incubation with a 50-fold excess of the peptide in the same buffer for 6 h. Peptide—oligonucleotide conjugate was purified by native polyacrylamide gel electrophoresis (PAGE). Signaling oligonucleotides were annealed by incubating the A4—peptide conjugate with equimolar concentrations of A5(FL) (or A6 or A7). This procedure simplifies the labeling because only one covalent conjugate of the peptide must be made. However, signaling oligonucleotides produced this way are bulkier, because they contain dsDNA segments (compare Figures 1D and 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 Catalog No. 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 an Analyst AD plate reader (LJL Biosystems, Sunnyvale, CA) or Spectra FluorPlus microplate reader (Tecan, Research Triangle Park, NC). Lumii-
nescence resonance energy transfer (LRET)\textsuperscript{12} was measured on
Analyst AD plate reader. Donor (europium) emission was mea-
sured 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 a delay
of 50 μs and an integration time of 1000 μs. 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:

\[
\text{FRET (fold change)} = \frac{(F_{SA}'/F_D')}{(F_{SA}/F_D)}
\]

where \(F_{SA}\) and \(F_D\) are the sensitized acceptor intensities in the
presence of the antibody, respectively, and \(F_{SA}'\) and \(F_D'\) are
the sensitized donor emission intensities in the 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 to calculate FRET, according to eq 1, reduces the variability of FRET measurements that is due to
dilution and instrumentation errors. Results of competing experi-
ments 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 the limit of detection (LOD),\textsuperscript{13} which
is 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 (see
Figures 3B and 4B, presented later in this paper). 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). The LOD was calculated from calibration curves as the
analyte concentration corresponding to a FRET signal that was
equal to blank + 3σ (standard deviation of the blank).\textsuperscript{13} The
sensitivity of the assay\textsuperscript{13} (FRET signal change per unit of analyte
concentration) was obtained from the slope of the calibration curve
(in the case of antibody) or from the slope of a line through the
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\textsubscript{2}O that
contained 20 mM DTT at a concentration of 2 U/μL. The P2
peptide conjugate with A4 (P2–A4) was annealed with
A6(Eu\textsuperscript{3+}) and A7(Cy5) to yield P2–A4/A6(Eu\textsuperscript{3+}) and P2–A4/
A7(Cy5), respectively. A 1 μM mixture of P2-A4/A6(Eu\textsuperscript{3+}) and
P2–A4/A7(Cy5) in 5 μL buffer (100 mM Tris, pH 8.0, 100 mM
NaCl, 0.5 mM EDTA, 2 mM MgCl\textsubscript{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 mixed with monoclonal
antitroponin 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 the europium chelate donor
and the Cy5 acceptor were measured in 384-well plates at 25
°C after 40 min of incubation on an Analyst AD plate reader.

RESULTS AND DISCUSSION

Proof-of-Principle for the Sensor Design. Figure 1 il-
ustrates the design of the sensors. The design of the sensors is
based on two simple thermodynamic considerations. The first is
a large change of local concentration of the signaling oligonucle-
tides 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 an 8.6-nm-long
linker to prepare peptide–oligonucleotide conjugates. Our recent
analysis of the properties of ligands containing long flexible
linkers\textsuperscript{14} indicated that linkers up to at least 50 nm in length
should be compatible with the design illustrated in Figure 1 and
should produce a local concentration of oligonucleotides in
antibody–peptide–oligonucleotide complexes in the range of
tens of micromolars. Thus, complementary signaling oligonucle-
tides can be easily designed such that, at nanomolar concentra-
tions in the absence of the antibody, essentially no annealing will
happen. We used a pair of complementary oligonucleotides with a
predicted hybridization free energy of −7.4 kcal/mol (melting
temperature of 2.6 °C for a 10 nM oligonucleotide concentration
at 100 mM salt).\textsuperscript{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 pep-
tides will result in almost 100% annealing. This annealing, in turn,
will bring the two fluorophores attached to oligonucleotides to
close proximity, producing a 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 that contain complementary
signaling oligonucleotides (see Figure 1C). Although the two
peptide binding sites of the bivalent antibody are equivalent, when
a mixture of peptide–oligonucleotide conjugates that contain
complementary oligonucleotides is incubated with the antibody,
the most stable complex will be the one that allows for annealing
of the complementary oligonucleotides, because of the additional
favorable free energy provided by the annealing (ΔG° in Figure
1C). The complexes that contain either two donor-labeled or two
acceptor-labeled oligonucleotides (and, thus, are not able to
produce a FRET signal) will be disfavored and will not be formed
in significant amounts. This built-in selection of complexes that
produce a FRET signal ensures the maximal possible FRET signal.

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hybrid/).
The combination of the two phenomena discussed previously 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 a 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 the most-effective FRET between a given set of fluorescence probes.

An additional benefit of the enhanced stability of antibody–peptide–oligonucleotide complexes derived from additional free energy of oligonucleotide hybridization (ΔG°) will be that functional sensors that utilize 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 (see Figure 1A), to detect antibodies, or in an indirect competitive assay format (see Figure 1B), to detect proteins that contain the exposed peptide epitope corresponding to the peptide in the peptide–oligonucleotide conjugate.

We first utilized a simple model system that consisted of an antibiotin 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 Figure 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. The addition of antibiotin antibodies to a mixture of A1(Cy5;biot) and A2(FL;biot) resulted in a large antibody-concentration-dependent FRET signal (see Figure 2A). The signal decreased at high antibody concentrations after reaching the maximum value at intermediate antibody concentration. This was expected, because, at high antibody concentrations, the complexes that contain only one oligonucleotide bound to the antibody should start to form and these complexes could not produce a FRET signal. No FRET signal was detected when a mixture of A1(Cy5;biot) and A2(FL;biot) was titrated with a monovalent Fab antibiotin antibody fragment, although it could bind the biotinylated oligonucleotides efficiently (as determined by native gel electrophoresis mobility shift experiments (not shown)). This confirmed that, as illustrated in Figure 1A, the signal could be only generated with a bivalent antibody. The data shown in Figure 2A provided a proof-of-principle for the sensor design illustrated in Figure 1A.

FRET signal produced by a mixture of antibiotin antibody, A1(Cy5;biot) and A2(FL;biot) decreased upon addition of unlabeled biotinylated oligonucleotide and was unchanged upon addition of the oligonucleotide that lacked biotin (Figure 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 (Figure 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 using a F(ab)2 fragment in place of the intact antibody.

Figure 2. Proof-of-principle experiments with antibiotin antibody and biotinylated signaling oligonucleotides. (A) Titration of the 50 nM mixture of A1(Cy5;biot) and A2(FL;biot) with antibiotin antibody (black circles) or Fab fragment of antibiotin antibody (empty circles). (B) Titration of the mixture of antibiotin antibody, A1(Cy5;biot) and A2(FL;biot) (each at 50 nM) with unlabeled biotinylated oligonucleotide (black circles) or unlabeled no-biotin oligonucleotide (gray circles); empty circles correspond to the experiment performed with the F(ab)2 fragment of the antibiotin antibody.

Detection of the Antibody Using Direct Format of the Assay. Having confirmed the feasibility of the sensor design using the antibiotin antibody model, we then selected cardiac troponin I as a target to test the sensor design that involved antigen peptides. We selected troponin I because we previously used this protein as a target for the development of a novel rapid assay for troponin (molecular pincers) that utilized a pair of anti-troponin antibodies.8 Testing the epitope peptide sensor design depicted in Figure 1 with troponin peptides provided the opportunity to make a comparison between the two assays. Troponin is the contractile regulatory protein complex of striated muscle.15 Levels of cardiac troponin I in the serum increase 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 of 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 (see Figure 1A). Therefore, we tested the performance of troponin peptide-based sensors for detecting the 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 aforementioned peptide produced a large antibody-concentration-dependent FRET signal (see Figure 3A). The FRET signal observed was only slightly smaller, compared to a simple biotin model system (see Figure 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 antitroponin antibody targeting different regions of the protein (see Figure 3A). These data demonstrate the functionality and specificity of the sensor that utilizes the peptide antigen.

To determine the limits of detection (LODs) for the antibody detection, we measured the response of the sensor to a series of low concentrations of the antibody (see Figure 3B). These measurements were made at two different sensor concentrations, because we expected that LOD and assay sensitivity should be dependent on sensor concentration. The LOD (determined as described in the Experimental Section) was 44 pM and 430 pM at sensor concentrations of 3 nM and 10 nM, respectively. Assay sensitivity was ∼3 times higher at a sensor concentration of 3 nM, compared to a sensor concentration of 10 nM. Thus, the assay characteristics can be conveniently and simply manipulated with the concentrations of the assay components. However, the increase in LOD of the assay that occurs by reducing the assay component concentrations will have its limits. First, if the concentrations of the assay components and the antibody fall below the equilibrium dissociation constant of the 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 be dependent on the sensitivity of instrumentation used to read the signals. With the fluorescence plate reader used in this work, sensitized acceptor emission intensities for the 3 nM sensor were 5–13 times greater than the buffer background (donor emission intensities were 90–100 times over the buffer background), which allowed accurate fluorescence measurements (as demonstrated by small standard deviations in the FRET signals (see Figure 3B)). Reducing the sensor concentrations further below 3 nM is not likely to result in reducing of LOD, because the increase in assay sensitivity will be offset by an increase in the error in the 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 that contains the corresponding peptide epitope, utilizing a competitive assay format (see Figure 1B). Therefore, we have tested if cardiac troponin I could be detected as the decrease in the 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 sensor mixture with the purified troponin produced a protein-concentration-dependent decrease in the FRET signal (see Figure 4A). A similar decrease in the 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 (see Figure 4A). This competitive assay was specific for human cardiac troponin I. No decrease in the 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 (see inset in Figure 4A). The data shown in Figure 4A demonstrate the functionality and specificity of the sensors that utilize antigen peptides to detect the proteins that contain the corresponding exposed antigen peptides.

To determine the limits of detection (LODs) for the troponin detection, we measured the response of the sensor to a series of low concentrations of the protein (see Figure 4B). As in the case
of antibody detection (Figure 3B), these measurements were made at two different sensor/antibody concentrations. The LOD for troponin (determined as described in the Experimental Section) was 11 ng/mL (470 pM) and 23 ng/mL (990 pM) at sensor concentrations of 3 nM and 10 nM, respectively. Assay sensitivity was 3 times higher at a sensor concentration of 3 nM than at 10 nM, respectively, by annealing A6(Eu³⁺) and A7(Cy5) oligonucleotides to the P2–A4 conjugate (producing P2–A4/A6(Eu³⁺) and P2–A4/A7(Cy5)). We have previously shown⁸ that the use of luminescence resonance energy transfer (LRET)¹² versus 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/A5(FL) and P2–A4/A7(Cy5), an ~2-fold increase in the FRET signal is observed (not shown). This FRET signal was significantly lower, compared to the 5–6-fold increase in the FRET signal that was observed with the peptide–oligonucleotide conjugate prepared using a direct labeling procedure (see Figure 3). This data demonstrate that the price for a simplified methodology for preparing peptide-signaling oligonucleotides may be a decrease in the signal-to-background ratio. These data also provide support for the generality of sensor design that is illustrated in Figure 1, because a functional sensor was obtained using a different peptide and a different antibody. To improve the signal-to-background ratio of this sensor, we prepared its version that used signaling oligonucleotides that were labeled with europium chelate and Cy5, respectively, by annealing A6(Eu³⁺) and A7(Cy5) oligonucleotides to the P2–A4 conjugate (producing P2–A4/A6(Eu³⁺) and P2–A4/A7(Cy5)). We have previously shown⁸ that the use of luminescence resonance energy transfer (LRET)¹² versus 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), an ~30–50-fold increase of FRET signal is observed (not shown).

The phosphorylation of P2 peptide with PKA resulted in a drastic decrease of P2 peptide binding to the antibody, as demonstrated by the native gel electrophoresis mobility shift assay (see Figure 5A). The addition of the antibody to the unphosphorylated P2 resulted in the formation of a complex with greatly

reduced electrophoretic mobility. When the same concentration of the antibody was added to the P2 peptide that was previously incubated with PKA and ATP, no complex with reduced mobility could be detected. Therefore, we hypothesized that we should be able to use the dissociation of the complex between the antibody and P2-oligonucleotide conjugates that are 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(Eu^3+) and P2-A4/A7(Cy5) with increasing amounts of PKA in the presence or absence of ATP. LRET signals were normalized to the signal obtained in the absence of PKA (100%).

**Figure 5.** Application of epitope peptide-based immunosensor used to detect 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%).

**Effect of Serum on the Assay.** Because 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 that contained varying amounts of serum (see Figure 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 of serum in 20 µL of assay mix) was a FRET signal observed that differed significantly from the signal observed in the absence of any serum added (see Figure 6). The larger change in the FRET signal that was 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 a very significant autofluorescence at 485-nm excitation. To improve the function of the sensors in serum-containing samples, different sets of fluorescence probes could be used to avoid exciting at 485 nm. Nevertheless, the data shown in Figure 6 demonstrate that complex samples such as serum do not interfere with the operation of the assay.

**CONCLUSIONS**

The data presented here for the antibiotin antibody model, the two troponin peptides, and the corresponding antibodies validated the sensor design that has been illustrated in Figure 1. The sensors can be used for quantitative determination (if appropriate standards are available) or semiquantitative 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, homogeneous assay that can detect this antibody, according to the design shown in Figure 1A, should be possible to develop. Such sensors that detect specific antibodies could be used in research and also for the diagnosis of infectious and autoimmune diseases. The 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., the concentration of anti-HIV antibodies in the serum of infected patients is at least in the range of a few hundred nanomolar. Thus, a serum that has been diluted by hundred-fold or more 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., serum concentrations of autoantibodies in rheumatoid diseases are in the range of hundreds to a few thousand nanomolar (again, well within the range of our sensors).

The second application that we envision is the use of a competitive variant of the assay (see Figure 1B) to detect the protein that contains the corresponding peptide epitope. Many antibodies are raised using synthetic peptides derived from the target proteins. If the epitope that corresponds 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 homogeneous format. Furthermore, in comparison to a molecular pincer assay that has been described previously, which utilizes a pair of oligonucleotide-labeled antibodies, only a single unmodified antibody is required for the competitive assay that is depicted in Figure 1B. The competitive assay could detect troponin at concentrations of \( \sim 10 \text{ ng/mL} \) (400 pM). This concentration is \( \sim 10 \) times higher, compared to the value determined using our direct molecular pincer assay (\( \sim 1 \text{ ng/mL} \); 40 pM). While the limit of detection (LOD) and sensitivity of peptide-based sensors could be likely improved by optimizing fluorophores for FRET signaling, because of the competitive nature of the assay, it is unlikely that the LODs that are as good as those observed with a molecular pincer assay will be obtained. This could limit the applicability of the competitive assay for protein targets that require a very high sensitivity of detection. For example, normal levels of cardiac troponin I in serum are \( < 1 \text{ ng/mL} \) and can reach hundreds of ng/mL in a patient with acute myocardial infarction. Typical commercial ELISA for troponin has detection limits of 1 ng/mL or better. Therefore, epitope-peptide-based sensors could not be used to detect troponin I at its lower concentration range in serum. There will be protein targets in which the concentrations in the serum will be within the range measurable by the sensors. For example, physiological levels of C-reactive protein, which is a general inflammation marker, are 68–8200 ng/mL, which would be within the range measurable by the sensors. Therefore, practical applicability of the sensor for protein target detection will be defined (and limited) by the LOD and sensitivity requirements.

The third application that we envision is for the detection of enzymatic activities that are involved in post-translational modifications of the proteins. One obvious example of such application is for the detection of protein kinase activities, as demonstrated by the data presented for PKA phosphorylation of the troponin peptide (see Figure 5). There is great interest in general kinase assays and specific kinase assays 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 eliminates either its interaction with the antibody raised against an unmodified peptide or for which there is an antibody available specific for the modified version of the peptide. Because 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 the detection of enzymatic activities involved in post-translational modifications of the proteins seems to be the most promising of their potential practical applications.

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