

Aptamer Beacons for the Direct Detection of Proteins

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We have designed a new class of molecules, which we term aptamer beacons, for detecting a wide range of ligands. Similar to molecular beacons, aptamer beacons can adopt two or more conformations, one of which allows ligand binding. A fluorescence-quenching pair is used to report changes in conformation induced by ligand binding. An anti-thrombin aptamer was engineered into an aptamer beacon by adding nucleotides to the 5'-end which are complementary to nucleotides at the 3'-end of the aptamer. In the absence of thrombin, the added nucleotides will form a duplex with the 3'-end, forcing the aptamer beacon into a stem-loop structure. In the presence of thrombin, the aptamer beacon forms the ligand-binding structure. This conformational change causes a change in the distance between a fluorophore attached to the 5'-end and a quencher attached to the 3'-end. Aptamer beacon can be a sensitive tool for detecting proteins and other chemical compounds. © 2001

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Antibodies have been extensively used to detect protein targets, but, so far, it has proven difficult to adapt antibodies to chip-based formats for proteome analysis. In particular, large-scale biosensor arrays necessitate (a) the development of sensors that can directly transduce molecular recognition into an easily acquired signal and (b) methods for the high-throughput production of biosensors. The simplest antibody-based method for protein detection, ELISA, does not directly signal the presence of applied analytes, but instead requires several time-consuming capture, wash, and

detection steps. Also, at least two antibodies that detect nonoverlapping epitopes are required for each protein target. Even with the nascent development of high-throughput methods for antibody discovery (1, 2), it is unclear how multiple, distinct biosensors will be generated for each protein target.

Aptamers are oligonucleotides that have been selected for specific binding to a variety of molecular targets, ranging from small organics to proteins (3–5). These nucleic acid-binding species can consist of RNA, DNA, or modified nucleotides and are typically 15 to 60 nucleotides long. Their binding affinities range from K_d 's of 1 pM to 1 μ M, with most in the 1–10 nM interval. Aptamers are selected using a relatively rapid *in vitro* selection process and can be inexpensively synthesized. Most importantly, aptamers can be engineered using standard nucleic acid techniques to incorporate radioisotope, fluorescent, or other reporters.

While labeled aptamers can be used as antibody mimics (for example, in sandwich assays, ELISAs, and Western blots (6–8), their most powerful application is as reagents that can directly transduce molecular recognition into optical signals. For example, fluorescently labeled, antiadenosine aptamers have been designed that can transduce adenosine concentrations into increases in fluorescence intensity (9), while fluorescently labeled anti-thrombin aptamers have been designed that can transduce thrombin concentrations into changes in fluorescence anisotropy (10). However, a detailed knowledge of aptamer structure underlies both of these demonstrations, and the design requirements would obviate the application of these methods to large numbers of targets.

In contrast, it has been shown that molecular beacons can be readily designed to directly detect and quantitate different nucleic acid targets (11). In the absence of a sequence target, beacons are designed to form a stem-loop structure with a fluorophore on one end of the stem and a quencher on the other end. In this structure, the fluorophore is quenched by energy

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transfer to the quencher in close proximity. The loop sequence contains a sequence that is complementary to the target nucleic acid. In the presence of the target, a duplex is formed between the molecular beacon and the target nucleic acid. When this occurs, the stem is broken, separating the fluorophore from the quencher. Consequently, the fluorophore is no longer quenched and fluorescence is restored.

If molecular beacon techniques could be generalized to protein detection it might prove possible to more readily develop proteomics chips. The use of molecular beacons for the detection of nonspecific interactions between proteins and nucleic acids has recently been demonstrated (12, 13). In this paper, we describe how molecular beacon techniques can be adapted to aptamers that specifically bind protein targets.

MATERIALS AND METHODS

Materials. All the oligonucleotides, including aptamer beacons, were purchased from Integrated DNA Technology (Coralville, IA). Aptamer beacons (labeled G15DxxMB) were synthesized by coupling fluorescein at the 5'-end and a DABCYL-group at the 3'-end. G15D5dF is G15D5d with 5'-fluorescein, but without any quencher group on the 3'-end. They are G15D, GGTTGGTGTGGTTGG; G15D4d, CCAAGGTTGGTGTGGTTGG; G15D5d, CCAACGGTTGGTGTGGTTGG; G15D6d, CCAACCGTGGTGTGGTTGG; G15D5nd, TTTTGGTGGTGTGGTTGG; G15D5ng, CCAACGGT-GGTGTGGTTGG; G15D5drev, CCAACACACCAAGTTGG. Thrombin, factor IX, and factor Xa were purchased from Enzyme Research Lab.

Circular dichroism spectra. CD spectra were obtained using a Jasco Model 800 (Easton, MD) spectrophotometer using an 800- μ l cuvette with a 1-cm path length at room temperature. Spectra were obtained from 310 to 210 nm at a scan speed of 0.5 nm/s. The aptamer concentration in all measurements was 10 μ M. Buffer or salts were added to the sample and measurements were started within 10 s.

Thrombin-binding assay using a fluorescence spectrometer. The aptamers were diluted to a concentration of 10 μ M in 10 mM Tris-HCl, pH 7.5, heated to 99°C for 3 min and cooled to room temperature prior to the experiment. This process is used to dissociate any intermolecular G-quartet-mediated structures that may have formed. Experiments were performed using 5–40 nM of aptamer in 2 ml. All of the fluorescence intensity measurements were performed at 25°C with an excitation wavelength of 495 nm, and the emission was monitored at its peak—516 nm.

Titration with an oligonucleotide containing the complementary sequence. G15D5dMB at 5 μ M was mixed with various concentrations of G15D5drev in TE. The mixtures were heated at 99°C for 3 min and then incu-

bated at 50°C for 10 min before cooling down to room temperature. The fluorescence intensity was measured at an aptamer beacon concentration of 40 nM.

RESULTS AND DISCUSSION

Aptamer Beacon Design

We hypothesized that additional sequences could be added to an aptamer that would form a stable stem-loop and destabilize the native, binding structure. Analogous to molecular beacons, the aptamer beacon would then exist in a quenched stem-loop structure in the absence of a target molecule. Formation of the aptamer beacon:target molecule complex would alter the equilibrium between quenched, nonnative and unquenched, native structures, concomitantly generating a change in observed fluorescence intensity (Fig. 1).

An antithrombin aptamer that had been previously selected from a random sequence pool (14) and thoroughly studied (15–19) was chosen as a model system for our design approach. The unique structure of the antithrombin aptamer, G15D, had previously been established by NMR analysis and X-ray crystallography (20–22). This G-rich aptamer forms a G-quartet that provides a backbone structure for thrombin binding and the two connecting TT loops make direct contact with thrombin. We reasoned that the addition of several nucleotides to the 5' end of the anti-thrombin aptamer would allow the aptamer to form an alternate conformation that would destroy the G-quartet structure. The addition of thrombin to a solution containing the aptamer would strongly shift the equilibrium concentrations of the two structures toward the thrombin-binding conformer (Fig. 1B). A fluorescence-quenching pair added to the 5'- and 3'-ends of the engineered stem-loop would signal any protein-dependent conformational changes. Several aptamer beacons that differed in the length of the introduced stem structure were initially designed (Fig. 1C).

CD Measurements

We wanted to assess the conformational equilibria of the oligonucleotides themselves in the absence of any additional reporter molecules. To do this, we synthesized the aptamer beacon prototypes G15D4d, G15D5d, and G15D6d along with the control G15D. CD spectroscopy was used to provide a sensitive measurement of the secondary structures of these oligonucleotides. Prior measurements of the CD spectrum of G15D have shown that there is a characteristic peak at 293 nm for G-quartet formation in the presence of K⁺ ions (23, 24). In contrast, duplex formation d(GT)–d(CA) is characterized by a set of peaks at 265, 275, and 285 nm, resulting in a broad plateau between 265 and 285 nm (25).

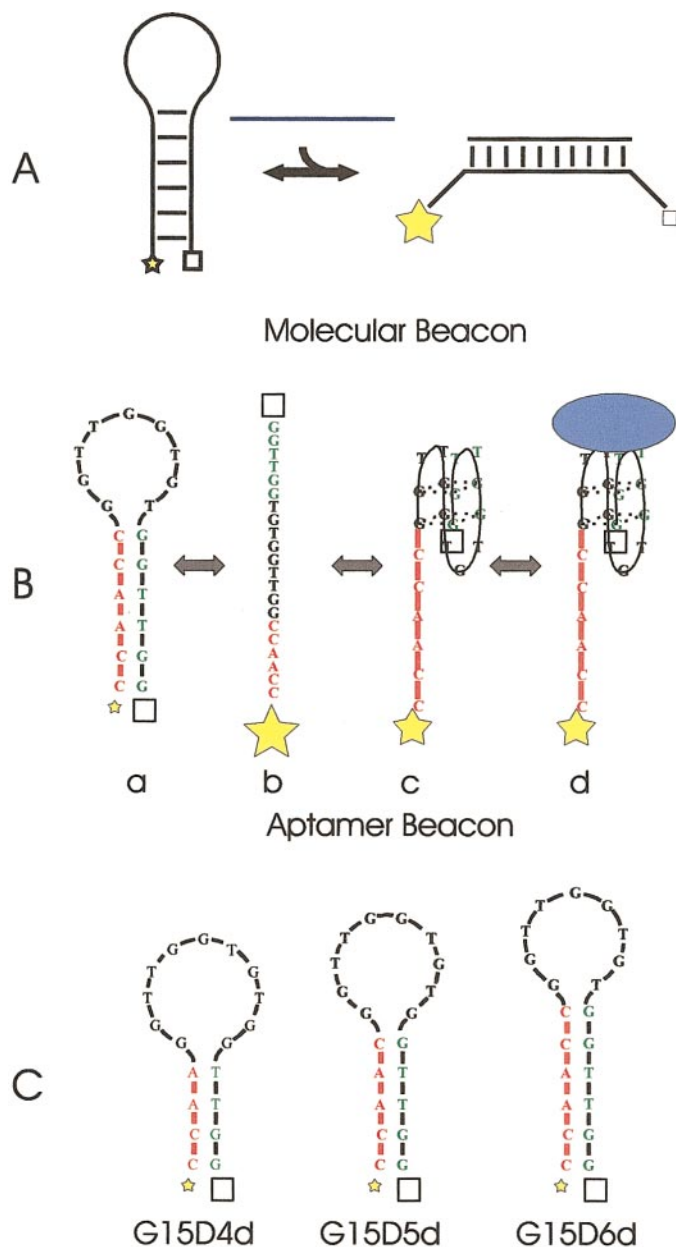


FIG. 1. (A) Molecular beacon—schematic drawing of mechanism of signaling by molecular beacons. (B) Aptamer beacon—schematic drawing of mechanism of signaling by thrombin aptamer beacon. (a) Thrombin bound to aptamer beacon. (b) Aptamer in G-quartet conformation which allows thrombin binding. (c) Unfolded conformation. (d) Aptamer beacon in quenched stem-loop conformation. Yellow stars represent the fluorophore, and the white square represents the quencher. The emission intensity of the fluorophore is represented by the size of the star, increases with the distance between the fluorophore and the quencher. (C) Predicted secondary structures of three aptamer beacons with different stem lengths.

The observed CD spectra of G15D in TE buffer, in TE + 10 mM KCl and in TE + 10 mM MgCl₂ is shown in Fig. 2. As expected, the well-characterized peak at 293 nm was seen in the presence of K⁺ ions. In the

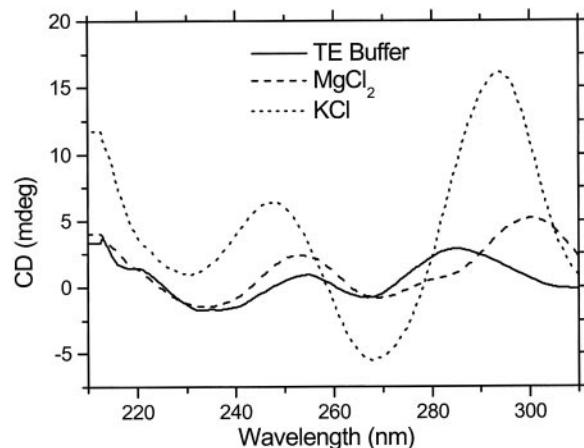


FIG. 2. CD spectrum of G15D as a function of cationic species.

presence of Mg²⁺ ions, the G-quartet peak was shifted slightly to 300 nm, a result that is also consistent with previous observations (26).

The CD spectra of the oligonucleotide G15D5d were then measured in Tris buffer and in Tris + 5 mM MgCl₂. These spectra, shown in Fig. 3, indicate that Mg²⁺ promoted the formation of the stem-loop conformer. Subsequent additions of KCl (Fig. 3) led to the formation of the G-quartet conformer as evidenced by the appearance of the peak at 290 nm. Subsequent addition of Mg²⁺ caused the equilibrium to be shifted back toward the duplex form (data not shown). Similar results were obtained with the oligonucleotide G15D4d. However, the oligonucleotide G15D6d only formed the duplex in the presence of either K⁺ or Mg²⁺ ions.

To confirm the identity of these peaks, two additional oligonucleotides were synthesized. The first, G15Dnd, should be incapable of assuming a duplex conformer.

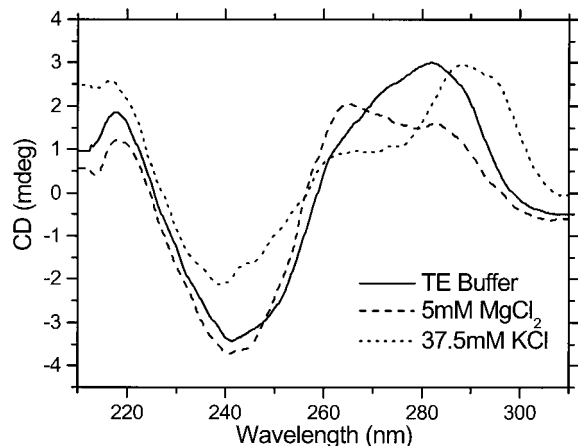


FIG. 3. CD spectrum of G15D5d in TE buffer, after addition of 5 mM MgCl₂, and after subsequent addition of 37.5 mM KCl.

The spectrum for this oligonucleotide showed a peak at 293 nm in the presence of K^+ and at 300 nm in the presence of Mg^{2+} , consistent with G-quartet formation. The second, G15Dng, should be incapable of forming the G-quartet structure. In this case, addition of either K^+ or Mg^{2+} results in peaks at 265, 275, and 285 nm, consistent with duplex formation.

For aptamer beacons to be useful as biosensors, the transitions between the different conformers should be relatively dynamic. For G15D5d, the addition of 1 mM Mg^{2+} quickly (within 10 s) mediates the formation of the duplex, as monitored at 260 nm. Subsequent addition of 20 mM K^+ just as quickly results in the formation of the G-quartet, as monitored at 293 nm.

Predicted Free Energies

One of the difficulties with previous design strategies for signaling aptamers was that they were largely empirical. For example, a number of adenosine-sensing aptamers were originally designed, yet only one worked and the successful design could not be predicted in advance (9). In order to work toward generalized rules for the design of thrombin aptamer beacons we attempted to predict the equilibrium concentrations of G-quartet and duplex conformers. The T_m of the G-quartet structure in 25 mM KCl has been previously measured (27) to be 46.4°C. The Zuker DNA mfold server (28) was used to calculate the free energies of duplex structures as a function of stem length and salt concentration (29). In 10 mM Mg^{2+} the predicted T_m are G15D6d, 64.9°C; G15D5d, 53.9°C; G15D4d, 35.6°C. In 25 mM Na^+ the predicted T_m are G16D6d, 52.8°C; G15D5d, 43.0°C; G15D4d, 25.8°C.

Assuming (a) that the free energy of the G-quartet in 10 mM $MgCl_2$ will be less than that in KCl, and (b) that free energy of the duplex in KCl will be equal to that of NaCl, these calculations predict the experimental results. First, they suggest that the conformations of the oligonucleotides should be switched by the ionic conditions. For example, G15D5d should form the stem-loop conformer in the presence of Mg^{2+} (the T_m of the stem loop in Mg^{2+} is greater than the T_m of G-quartet), but should assume the G-quartet conformer in the presence of K^+ (the T_m of G-quartet is greater than the T_m of stem loop in KCl). Second, the calculations predict that the G15D6d should always assume the stem-loop conformer. Finally, they predict that G15D4d should form the G-quartet structure in KCl. They do make a prediction on the state of G15D4d in $MgCl_2$ since we only have an upper limit on the stability of the G-quartet in $MgCl_2$.

Protein-Dependent Aptamer Beacons

The oligonucleotides G15D4d, G15D5d, and G15D6d were converted into their corresponding aptamer bea-

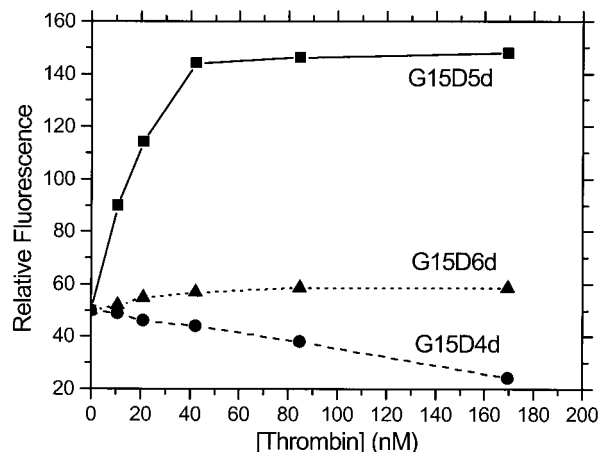


FIG. 4. Aptamer beacon fluorescence emission in TE buffer as a function of the thrombin concentration.

cons by synthetically appending fluorescein to their 5'-ends and DABCYL to their 3'-ends. The aptamer beacons G15D4dMB, G15D5dMB, and G15D6dMB were then evaluated for their responses to varying thrombin concentrations (0–120 nM) in TE buffer. Binding curves for each of the aptamer beacons are shown in Fig. 4. Each of the beacons followed thrombin concentration and could potentially be used to quantify the concentration of protein in solution.

The aptamer beacon G15D5dMB showed the greatest response, an approximately 2.5-fold increase in fluorescence at saturating thrombin concentrations, and was used for further experiments. In comparison, when G15D5dMB was used as a standard molecular beacon, the observed change in fluorescence was approximately 8-fold. These results are consistent with a closer approach of fluorescein and the quencher in the G-quartet than in a fully extended helix. The thrombin: aptamer beacon complex had an apparent K_d of approximately 10 nM.

In order to discern whether the aptamer beacon underwent the programmed conformational change, CD spectra were again examined. However, unlike the G15D5d oligonucleotide, the G15D5dMB aptamer beacon did not appear to form a G-quartet structure in the presence of either K^+ or Mg^{2+} . Instead both cations stabilized similar stem-loop forms. This is most likely due to stabilization of the stem loop by interaction of the fluorescence-quenching pair.

The effect of cation concentration on the fluorescence of G15D5dMB was systematically examined. The aptamer beacon was equilibrated in 10 mM Tris-HCl buffer (pH 7.5) and fluorescence emission was measured with increasing concentration of $MgCl_2$ and KCl. Titration with $MgCl_2$ showed a half-maximum effective concentration of 50 μ M (Fig. 5), while titration with KCl showed a half maximum effective concentration of

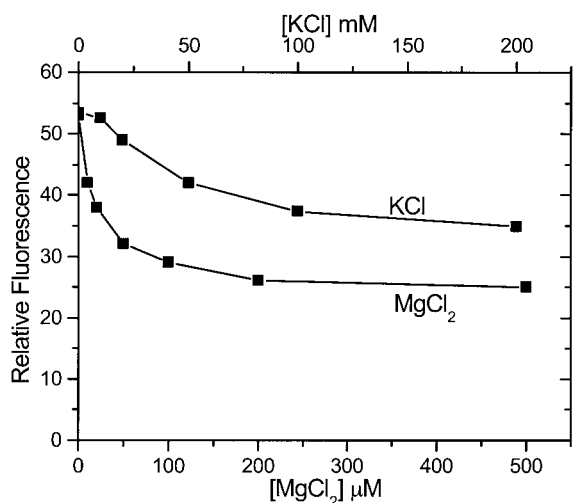


FIG. 5. Fluorescence emission of G15D5dMB in TE as a function of MgCl_2 and KCl concentration.

50 mM (Fig. 5). The observed decreases in emission are likely due to the stabilization effect of the duplex conformer. However, the relative fluorescence at saturating conditions of K^+ was higher than in the presence of saturating Mg^{2+} . Furthermore, adding K^+ to the Mg^{2+} form raised the fluorescence to the K^+ -only level. These findings are consistent with the CD results and suggest that there are two similar, but distinct, duplex conformers.

Taken together, these results suggest that G15D5dMB exists in a duplex form in the presence of either K^+ or Mg^{2+} . However, the observed increase in G15D5dMB fluorescence in the presence of thrombin suggests that the aptamer beacon is in a conformational equilibrium. Since thrombin should only bind to the G-quartet form, the simplest explanation of these data is that thrombin traps the aptamer beacon in the G-quartet form. To eliminate the possibility that thrombin binding was simply causing a change in the chemical environment of the fluorophore, an aptamer which had a 5' fluorescein label, but no 3' DABCYL quencher, was synthesized. This aptamer, G15D5dF, showed essentially no change in fluorescence intensity upon the addition of thrombin.

While the creation of aptamer beacons that increase in fluorescence intensity in the presence of cognate proteins should prove most useful for biosensor applications, it should also be possible to create aptamer beacons that decrease in fluorescence intensity. To this end, we created a modified version of G15D5dMB that was no longer able to form the stem-loop structure. In this aptamer, G15D5ndMB, the 5'-end consists of the sequence TTTTT. Referring to Fig. 1, because structure D can no longer form, the addition of thrombin to a solution containing the aptamer should now change the equilibrium among structures A, B, and C, with

additional thrombin driving the equilibrium state toward structure A. In this assay, which was carried out in TE buffer, the fluorescence decreased with the addition of thrombin with an apparent K_d of approximately 10 nM, consistent with the proposed mechanism.

Binding Specificity

To confirm the binding specificity of G15D5dMB, we used factors IX and Xa. Factors IX and Xa are plasma serine proteases, as is thrombin, and are approximately 37% identical to thrombin in their catalytic domain. Neither factor IX nor factor Xa modulated the fluorescence of G15D5dMB. We also assayed G15D5dMB with the single-strand binding proteins SSB and lactate dehydrogenase. As has previously been reported (13), the addition of these nonspecific nucleic acid-binding proteins led to increases in fluorescence as high as 15-fold (data not shown). However, no saturation was observed within the concentration range that was assayed (up to 100 nM), indicating that the affinities of these proteins for the aptamer beacon are much lower than the affinity of thrombin for the aptamer beacon.

Applications to Protein Detection

The design method that was devised for the preparation of aptamer beacons that detect thrombin can potentially be applied to any aptamer, RNA, or DNA. In short, residues that are important for protein binding can be tied up in an unproductive, terminal stem structure that in turn juxtaposes an appended fluorescent label and quencher. The stability of the unproductive stem structures can be predicted in advance and can be altered to accommodate proteins with different binding affinities by modifying the composition or length of the stem region. Binding of a target protein should stabilize the native structure of the aptamer, changing the distance between the fluorophore and the quencher, resulting in an easily detectable change in fluorescence intensity. Since virtually any aptamer can potentially be directly converted to an aptamer beacon, it may be possible to quickly create numerous biosensors for proteomics applications using high-throughput, automated selection techniques (30). Aptamers have previously been immobilized without significant loss of activity, and it should similarly be possible to immobilize multiple aptamer beacons to generate chip arrays that directly signal the presence of individual proteins.

Our results also suggest limitations of the use of aptamer beacons. First, there are a number of proteins (e.g., ssDNA-binding protein, lactate dehydrogenase) that bind nonspecifically to single-stranded DNA and

that may therefore cause nonspecific increases in fluorescence intensity. The addition of unlabeled, nonspecific DNAs to an assay mixture can mitigate this problem. Second, metal ions can have a profound effect on nucleic acid conformation and signaling. Ion-specific effects on signaling can be minimized by calibrating the aptamer beacon signal in the detection buffer, or by equilibration of samples in a defined buffer prior to analysis.

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