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Forced Intercalation (FIT)-Aptamers

Sasha B. Ebrahimi^{†,‡,§}, Devleena Samanta^{‡,‡,§}, Ho Fung Cheng^{‡,‡}, Levy I. Nathan^{‡,‡}, Chad A. Mirkin^{*,†,‡,‡}

[†]Department of Chemical and Biological Engineering, Northwestern University, Evanston, Illinois 60208, United States

[‡]Department of Chemistry, Northwestern University, Evanston, Illinois 60208, United States

[‡]International Institute for Nanotechnology, Northwestern University, Evanston, Illinois 60208, United States

Abstract

Aptamers are oligonucleotide sequences that can be evolved to bind to various analytes of interest. Here, we present a general design strategy that transduces an aptamer-target binding event into a fluorescence readout via the use of a viscosity-sensitive dye. Target binding to the aptamer leads to forced intercalation (FIT) of the dye between oligonucleotide base pairs, increasing its fluorescence by up to 20-fold. Specifically, we demonstrate that FIT-apptamers can report target presence through intramolecular conformational changes, sandwich assays, and target-templated reassociation of split-apptamers, showing that the most common aptamer-target binding modes can be coupled to a FIT-based readout. This strategy also can be used to detect the formation of a metallo-base pair within a duplexed strand and is therefore attractive for screening for metal-mediated base pairing events. Importantly, FIT-apptamers reduce false-positive signals typically associated with fluorophore-quencher based systems, quantitatively outperform FRET-based probes by providing up to 15-fold higher signal to background ratios, and allow rapid and highly sensitive target detection (nanomolar range) in complex media such as human serum. Taken together, FIT-apptamers are a new class of signaling aptamers which contain a single modification, yet can be used to detect a broad range of targets.

Aptamers, oligonucleotide sequences that can be evolved to bind to analytes with high sensitivity and specificity, have recently found widespread use as effective therapeutic and diagnostic tools.^{1–4} To be used as a tool for detection, the binding of an aptamer to its target must result in a signaling event that can be monitored as a readout for target presence. Fluorescence-based techniques have emerged as popular readout platforms due to their simplicity, low-cost, high-throughput, and ability to multiplex.^{5–15} For example, several strategies have been designed wherein target binding to an aptamer labeled with a

*Corresponding Author: chadnano@northwestern.edu.

§S.B.E. and D.S. contributed equally.

ASSOCIATED CONTENT

Supporting Information

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Experimental methods, additional discussions, and figures (PDF)

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fluorophore-quencher pair induces a structural change that separates the fluorophore and the quencher (e.g., structure-switching signaling aptamers,⁶ aptamer beacons,⁸ aptamer switch probes⁹). Alternatively, constructs that bring a pair of dyes into close proximity upon target binding to elicit a fluorescence signal by Förster resonance energy transfer (FRET) are also commonly employed.¹⁰ More recently, Spinach aptamers and variants thereof have been developed that change structure after aptamer-target complexation, allowing a small molecule fluorophore to bind to the Spinach region in the sensing unit and yield fluorescence turn-on.^{11–13}

While these methods constitute a powerful means to detect targets of interest, they also suffer from limitations. Strategies that rely on partial blocking of the aptamer site (i.e., structure-switching aptamers, aptamer beacons) retard aptamer-target binding kinetics, increasing the time required to obtain a readout.¹⁶ Systems based on fluorophore/quencher pairs are prone to false-positive signals in complex media and cells due in part to nuclease degradation.¹⁷ Moreover, strategies based on FRET are generally associated with low signal-to-noise ratios.¹⁸ Platforms like Spinach require long sequences to be appended to aptamers, making their folding and, therefore, efficacy difficult to predict in complex milieu.¹⁹

In this communication, we present a fundamentally new design strategy for interfacing aptamers with a readout event via viscosity-sensitive fluorophores. The Seitz group has shown that dyes of the thiazole orange family can be covalently attached to mRNA recognition sequences to create “duplex-sensitive” fluorescence turn-on probes.^{20–23} The fluorescence enhancement stems from the restricted rotation of the dye around its methine bridge upon forced intercalation (FIT) in the oligonucleotide duplex. Notably, these probes avoid false-positive signals because their turn-on does not rely on proximity between a fluorophore and a quencher. We hypothesized that by strategically placing the dye in an aptamer sequence such that structural changes of the aptamer upon ligand binding hinders the dye’s internal rotation, a new class of false-positive resistant signaling aptamers can be designed (Figure 1). Additionally, we reasoned that these “FIT-aptamers” would respond faster compared to probes relying on partial blocking of the aptamer site and require only a single modification unlike Spinach-based platforms.¹²

To evaluate the feasibility of realizing FIT-aptamers, we first chose a previously reported DNA sequence (Table S1), known to recognize Hg^{2+} , as an example of an aptamer that binds to its target through an intramolecular conformational change.¹⁶ We used the aptamer sequence as a single-stranded probe and the FIT-dye quinoline blue (D) as a nucleobase surrogate. We considered that this aptamer adopts a hairpin-like structure in the presence of Hg^{2+} due to the Hg^{2+} -mediated bridging of thymine (T) bases (T- Hg^{2+} -T).^{16,24} Therefore, we hypothesized that if a base sandwiched between two Ts in the aptamer sequence was replaced with D, forced intercalation of D between the metallo-base pairs (bps) would turn on its fluorescence. The FIT-aptamer (HgA1) was synthesized by substituting the fourth base from the 3' end of the sequence with an amino-modifier to which D-carboxylate was conjugated via carbodiimide cross-linking chemistry (Figures S2–S3). HgA1 was then titrated with Hg^{2+} in a buffered solution. The fluorescence enhancement factor (I_f/I_0), defined as the ratio of the fluorescence in the presence of target (signal, I_f) to the initial fluorescence (background, I_0), increases with increasing concentrations of Hg^{2+} and reaches

a maximum value of ~ 8 (Figure S3). Significantly, when a short complementary sequence (Figure S3C) is used to partially block the aptamer akin to a structure-switching signaling aptamer,¹⁶ the response time of the probe is 5 times slower (Figure S3D), showing that single-stranded probes such as FIT-aptamers enable faster target detection.

Based on these results, we speculated that this system would enable one to create a fast, highly sensitive, and extremely simple Hg^{2+} probe. Therefore, we proceeded to use a T_{14} sequence in which the fourth base from the 5' end was replaced with D (HgA2, Figure 2A). Remarkably, addition of aqueous Hg^{2+} (250 nM) to HgA2 results in an ~ 20 -fold fluorescence enhancement (Figure 2B, C). In sharp contrast, a traditional FRET-based approach provides a signal-to-background ratio of 1.3 (Figure S5). Based on the calibration curve of HgA2, the limit of detection (LOD) of the FIT-aptamer assay is 1.8 nM, well below the 10 nM toxicity limit for Hg^{2+} in drinking water.²⁵ Importantly, challenging the probe with a panel of 15 different metal ions (Ba^{2+} , Cd^{2+} , Zn^{2+} , Ni^{2+} , Co^{2+} , Ca^{2+} , Cu^{2+} , Mg^{2+} , Mn^{2+} , Fe^{2+} , Fe^{3+} , K^{+} , Pb^{2+} , Ag^{+} , and Hg^{2+}) shows that HgA2 is highly selective for Hg^{2+} (Figure 2D). A small turn on (1.9 ± 0.6) is detectable only in the case of Ag^{+} addition. Using a similar strategy, Ag^{+} ions can be detected via the formation of C- Ag^{+} -C bps using a cytosine-rich strand (Figure S6). This is the first use of forced intercalation to measure the formation of metal-mediated nontraditional bps.

While the above examples show that FIT-aptamers can report metallo-bp formation through global conformational changes, we hypothesized that the sensitivity of FIT-probes to single bp mismatches²⁰ can be utilized to sense metallo-bp formation in a preformed DNA duplex. Current methods for finding metallo-bps rely primarily on single crystal X-ray diffraction studies,²⁶ which necessitate challenging DNA crystallization, or NMR,²⁴ which requires a sufficiently large number of metal binding events to distinguish resonances of metal-bound nucleobases from the inherent signal of other bases in a strand. To assess whether FIT probes can be used as a simple alternative to allow rapid detection in solution, we synthesized six different 21-mer DNA sequences: three oligonucleotides containing TDT, TDA, and ADA regions, respectively, and their complements. The sequences containing D were added to the normal sequences in all combinations to form duplexes with zero T-T, one T-T, or two T-T mismatches directly adjacent to D. We observed that the addition of Hg^{2+} increased the fluorescence only in the duplexes where T- Hg^{2+} -T bases could be formed adjacent to the dye, showing that D can report local conformational changes and, moreover, that this information can be used to determine the identity of the metallo-bp formed (Figure 3, Figure S12). This ability of “local probing” is not possible with FRET or fluorophore/quencher-based techniques and can be potentially useful for screening new metallo-bps, studying local structural changes in DNA and RNA during various biological processes,^{27–29} or identifying drug binding sites on DNA and RNA.^{30–33}

We next investigated the possibility of designing FIT-aptamers that undergo more complex structural transitions upon target binding by studying the i-motif as an example. The i-motif is a cytosine-rich “proton aptamer” that adopts a quadruplex structure at acidic pH due to the formation of C- H^{+} -C bonds.^{34,35} We replaced the ninth base of the i-motif with D (I-mD, Figure 4A). Circular dichroism spectra confirm that the presence of D does not impede i-motif formation (Figure 4B). Lowering the pH from 7.0 to 5.6 results in a 5-fold

fluorescence enhancement (Figure 4C). Importantly, the fluorescence of HgA2 does not significantly change within this pH range, confirming that the enhancement is due to the forced intercalation of D in the i-motif structure as opposed to the dye's inherent pH-sensitivity. Taken together, these results suggest that a FIT-based strategy could be adapted to detect various analytes that cause conformational changes in aptamers.

As a measure of versatility, we further explored how FIT-aptamers can be designed in other detection contexts. We considered that sandwich assays for detecting proteins based on antibodies are popular due to increased specificity.^{36–38} Therefore, we examined systems that require the binding of two aptamers to generate a signal output.³⁹ As a model system, we used thrombin, since there are known aptamers that bind to two distinct sites.^{40,41} By applying the FIT strategy, the two aptamer sequences were appended with spacer groups and short complementary sequences (Figure 5A), one of which (THR1D) was modified with D. The independent binding of these two aptamers (THR1D and THR2) to thrombin brings the complementary sequences into proximity, increasing their local concentration and allowing them to hybridize. In buffer, we observed a 5-fold fluorescence enhancement upon target binding (Figure 5B). Negative controls involving proteins other than thrombin generated negligible signal (Figure 5C). The LOD was 1.4 nM. In comparison, a FRET-based method only yields a 1.4-fold enhancement (Figure S9). For FIT-aptamers to be useful, it is important to be able to use them in complex media at disease relevant target concentrations. As a clinically applicable model, we tested the potential for detecting thrombin in human serum using THR1D and THR2. Our results (Figure S10) show a limit of detection of 6.8 nM in whole serum. This value is below the 10 nM thrombin concentration in serum that is associated with blood clot formation,^{42,43} demonstrating assay utility in medical diagnostic relevant media.

As a structurally similar but distinct detection mode, we also investigated how split-aptamers can be interfaced with a FIT-based strategy. In this case, the aptamer sequence is divided into two fragments such that the presence of the target templates their reassociation.⁴⁴ Utilizing thrombin, we validated that FIT-based split-aptamers can be realized (Figure S11). This observation suggests that the FIT-aptamer approach can be used for detecting analytes that do not have well-characterized aptamer binding sites or do not support two epitope binding.

In summary, we have described a new class of signaling aptamers based on forced intercalation. We have shown that highly sensitive FIT-aptamers can be designed for the most common aptamer-target binding modes, exemplifying that FIT-aptamers constitute a new paradigm for simple, versatile, and reliable detection. FIT-aptamers provide key advantages over state-of-the-art fluorescence-based signaling aptamers. For example, they are kinetically superior to strategies that require partial blocking of the aptamer site and show higher signal-to-background ratios than traditional FRET-based techniques. Furthermore, a FIT-based strategy enables the probing of local target-induced conformational changes, a powerful capability that is not possible with conventional fluorophore/quencher or FRET-based methodologies. The ability to detect thrombin in human serum bodes well for extending the FIT-aptamer strategy to a wide variety of biological detection scenarios.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
FIT-aptamers: Aptamers modified with a visco-sensitive dye (quinoline blue) fluoresce upon target binding due to target-induced conformational changes.

Figure 2.

FIT-aptamer for Hg^{2+} (HgA2). (A) Design scheme. (B) Fluorescence enhancement (I_f/I_0) vs $[\text{Hg}^{2+}]$ added. Black spheres denote experimentally observed values. Red dashed line indicates theoretical fit to a Hill equation. (C) Fluorescence spectra (ex. 560 nm) with increasing $[\text{Hg}^{2+}]$. (D) Selectivity of the probe.

Figure 3. Detecting metallo-bps within a duplexed strand. (A) Design scheme with T-Hg²⁺-T as an example. (B) Fluorescence enhancement when Hg²⁺ is added to DNA duplexes with 0, 1, and 2 T-T mismatches adjacent to the dye.

Figure 4.

FIT-aptamer (I-mD) for pH sensing. (A) Design scheme. Only bases involved in i-motif formation are shown for clarity. (B) Circular dichroism spectra showing i-motif formation at lower pH. (C) Fluorescence enhancement (I_f/I_0) vs pH. Red dashed line indicates theoretical fit to a sigmoidal curve.

Figure 5. FIT-aptamers for two epitope binding. (A) Design scheme. (B) Fluorescence enhancement vs concentration of thrombin denoted as [THR]. (C) Selectivity of the probe.