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Strategies for Creating Structure-Switching Aptamers

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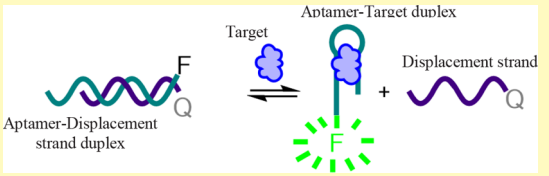
ABSTRACT: Aptamer biosensor that can switch its structure upon target binding offers a powerful strategy for molecular detection. However, the process of converting an aptamer into a "structure-switching" biosensor is challenging and often relies on trial-and-error without established design principles. In this Sensor Issues, we examine a variety of design approaches for incorporating structure-switching functionality into existing aptamers, and provide thermodynamic analyses to highlight the variables that most strongly influence their performance. Finally, we also describe emerging efforts for incorporating the structure-switching functionality directly into the aptamer selection process.



KEYWORDS: *aptamers, biosensors, structure-switching, thermodynamic analysis, displacement strand, fluorescence assay*

The enzyme-linked immunosorbent assay (ELISA)[1] is among the most widely used formats for molecular detection in both basic and clinical research. This strategy employs a pair of antibodies to report the presence of a given target in a sensitive and highly specific fashion. However, other promising biosensor formats have also emerged, including assays that employ only a single affinity reagent that undergoes a conformational change upon binding to its target. This structure-switching event can subsequently be transduced into a variety of readouts, including fluorescent[2] or electrochemical[3] signals. These biosensors offer a simpler alternative to ELISAs, and offer the potential for retaining specificity: in the presence of nonspecific targets, no conformational change takes place, and no signal is generated. Evolved through an in vitro selection process called systematic evolution of ligands via exponential enrichment (SELEX),[4,5] nucleic acid-based aptamers are particularly advantageous for such biosensors because they are chemically synthesized, offering excellent reproducibility, and they are far simpler to engineer and chemically modify than protein affinity reagents. Several reviews have been published on the subject of how aptamer sensors can be made.[6−9] In this article, we survey a variety of different approaches that have been explored to date for incorporating structure-switching functionality into existing aptamer molecules, with thermodynamic analyses of each strategy to study the variables that most strongly influence their performance. Finally, we also describe emerging aptamer selection strategies in which the structure-switching functionality is built-in to the selection process.

POST-SELECTION DESIGN FOR SWITCHING

The majority of aptamer-based fluorescence assays described to date have employed a "post-selection" design approach. This strategy is attractive because it does not require an entirely new aptamer selection procedure, but instead entails engineering existing aptamers to introduce switching functionality. This allows users to draw upon existing aptamers that have previously been discovered and validated in terms of affinity and specificity. However, this engineering process can be challenging, as the optimal thermodynamic balance between bound and unbound states must often be optimized through an iterative process of sequence design and experimentation. Aptamer sensors designed via this approach typically fall into three broad categories: aptamer beacons, split aptamer sensors and strand-displacement sensors.

Aptamer Beacons. Traditional molecular beacons were developed as a tool for fluorescent detection of specific DNA sequences.[10] These constructs comprise hybridization probes end-labeled with a fluorophore and quencher. In the absence of target, the probe forms a hairpin structure, quenching the fluorophore; hybridization with a complementary sequence induces a conformation change that increases the distance between the fluorophore and quencher, resulting in an increase in fluorescence. Inspired by this design, Ellington and co-workers designed the first aptamer beacon based on an existing thrombin-binding aptamer.[11,12] They observed that target binding in this aptamer was enabled by a G-quartet motif, and the authors sought to disrupt this structure by introducing terminal complementary regions that form a stem-loop configuration as shown in Figure 1A. In the absence of thrombin, the aptamer beacon assumes this loop structure, producing minimal fluorescence. Thrombin binding causes the aptamer beacon to undergo a conformational change, resulting in an increase in fluorescence signal. Several stem lengths were initially tested to find an optimal design. The resulting beacon was able to detect thrombin at concentrations of less than 10 nM, demonstrating the feasibility of converting an existing aptamer into a molecular switch without decreasing its reported binding affinity of 25 nM.

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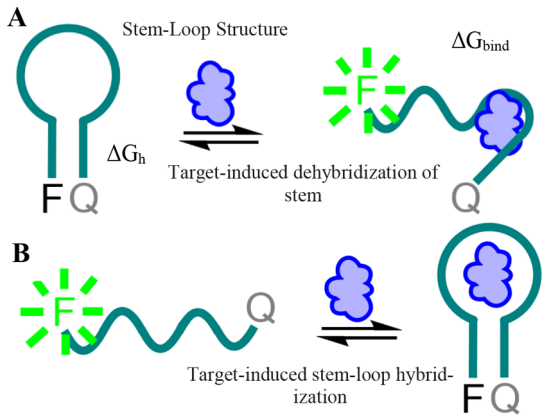

Figure 1. Aptamer beacons. (A) An aptamer with added complementary terminal sections labeled with fluorophore and quencher assumes a stem-loop structure. Target binding leads to a conformational change, denaturing the double-stranded stem and producing an increase in fluorescence. (B) The reverse aptamer beacon assumes a random orientation in solution, such that the fluorophore remains unquenched and produces a signal. Target binding induces aptamer folding resulting in fluorophore quenching.

Many other groups have subsequently used this approach for the design of aptamer beacons.[13−16] The design process requires carefully balancing the thermodynamic equilibrium between the self-hybridized stem-loop and the target-bound aptamer. In a stem-loop aptamer beacon assay such as the one shown in Figure 1A, the target binding reaction of the hairpin L can be modeled as

$$L \leftrightharpoons R \leftrightharpoons S \xrightarrow{+T} S{\cdot}T$$

where R is the aptamer in a random, unfolded fluorescent conformation, and S is the aptamer in a fluorescent conformation favorable to target binding, which is stabilized by the target T. The stability of the stem region and of the S state are reflected in the equilibrium constants $K_R = \frac{[R]}{[L]}$ and $K_S = \frac{[S]}{[R]}$. Solving for an approximate expression for the equilibrium fluorescence of a system with a high affinity aptamer and large K_S and for which the equilibrium dissociation constant $K_d = \frac{[S][T]}{[S{\cdot}T]}$, we obtain

$$F_{eq}{\cdot}\alpha \approx \kappa[L]_i + (1 - \kappa)[T]_i$$

where $[L]_i$ and $[T]_i$ are the initial concentrations of aptamer and target, respectively, and α is a proportionality constant between the fluorescence readout and fluorophore concentration. Factor κ, given by

$$\kappa = \frac{K_R(1 + K_S)}{1 + K_R(1 + K_S)}$$

shows the effect of the stability of the stem region. For an unstable stem-loop structure, K_R will be nonzero, and $0 < \kappa < 1$. This results in a decrease in target-induced fluorescent signal and an increase in background fluorescence. On the other hand, an excessively stable loop will slow down the target-binding reaction by making the $L \rightarrow S$ conversion stochastically unlikely. Clearly, a balance between the two is required to obtain a sensitive readout with minimal background signal.

An alternate approach presented by Landry and co-workers employs "reverse aptamer beacons" (Figure 1B).[17] They end-labeled a cocaine aptamer with a fluorophore and quencher. In the absence of target, the aptamer was shown to favor a random orientation in solution, and thus exhibited high fluorescence. Incubation with cocaine promoted aptamer folding, resulting in fluorescence quenching and enabling the detection of cocaine at concentrations as low as 10 μM in serum.

Split Aptamers. Existing aptamers can also be divided into two nucleic acid strands that only associate in the presence of a specific target. The two split aptamer fragments can then be labeled with a fluorophore and quencher. In the absence of target, they will remain free in solution and produce a fluorescent signal; incubation with the target promotes assembly of the two fragments, resulting in fluorescence quenching (Figure 2A). Since assembly can be tuned, through careful selection of ionic conditions, to occur only in the presence of target, this approach has the potential to offer greater specificity and lower background than aptamer beacons. Szostak and co-workers demonstrated the first example of a split aptamer for the detection of ATP.[18] Using structure prediction and site-directed mutagenesis, they identified stacked G-quartet structural elements that form in the presence of target, and showed that disruption of these structures hindered binding in a selected aptamer with $K_d \approx 6$ μM. They therefore split the aptamer within a loop region connecting the G-quartet nucleotides. The two resulting sequences assembled through the G-quartet structure in the presence of ATP. Subsequently, other groups successfully applied the split-aptamer technique for the detection of Tat protein[19] cocaine and ATP.[20]

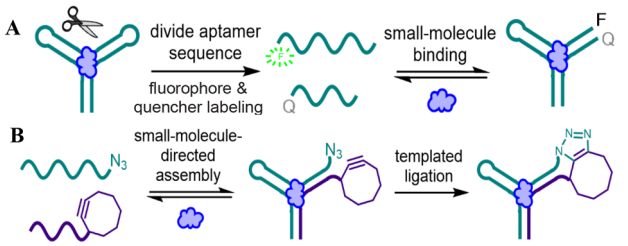

Figure 2. Split aptamer designs. (A) The aptamer is cut in a loop region that is not involved in binding. The two split-aptamer fragments associate only in the presence of the target molecule. (B) Target-induced proximity ligation enables signal amplification, as a single target molecule can induce the permanent assembly of numerous aptamer fragment pairs. This approach also reduces background from target-independent fragment assembly.

The successful design of split aptamer systems depends on a delicate thermodynamic balance to eliminate nonspecific fragment assembly. An assay with split aptamer components $A1$ (fluorophore-labeled) and $A2$ (quencher-labeled), which form a weak complex S stabilized by target T that results in a decrease in fluorescence, can be represented as

$$A1 + A2 \leftrightharpoons S \xrightarrow{+T} S{\cdot}T$$

There may also be a secondary, target-independent interaction between the two strands (U) that does not result in a change in fluorescence. This can be modeled as

$$A1 + A2 \leftrightharpoons U$$

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This undesired interaction has an equilibrium constant defined as

$$K_u = \frac{[U]}{[A1][A2]}$$

Although small, K_u affects the equilibrium of the system. This is easily seen by solving for an approximate expression of the change between initial and equilibrium fluorescence in this assay: $\Delta F = F_{eq} - F_i$. Assuming a very low aptamer K_d and low $K_S = \frac{[S]}{[A1][A2]}$, we find

$$\Delta F{\cdot}\alpha \approx [A1]_i(\gamma - 1) - \gamma[T]_i$$

where $[A1]_i$ and $[T]_i$ are the initial concentrations of A1 and target, respectively. The nonideality factor γ represents the effect of K_u and is given by

$$\gamma = \frac{1 + K_u([A2]_i - [T]_i) + K_u(K_S + K_u)([A2]_i - [T]_i)^2}{1 + (K_S + K_u)([A2]_i - [T]_i)}$$

As the level of target-independent interaction decreases, $K_u \rightarrow 0$, $\gamma \rightarrow 1$, and $\Delta F{\cdot}\alpha = -[T]_i$, and we find a decrease in fluorescence proportional to the concentration of target, which is the desired result. However, if K_u is nonzero and $\gamma > 1$, there will be an increasingly large background signal. Thus, nonspecific fragment assembly can greatly reduce the signal-to-noise ratio of split aptamer systems.

The Heemstra lab addressed this problem by incorporating a covalent proximity ligation event, which exclusively occurs upon target-induced association of the split aptamer fragments (Figure 2B).[21] Using strain-promoted azide–alkyne cycloaddition and rigorous characterization of reaction kinetics, they were able to fine-tune the target-induced assembly to create a robust detection platform allowing for dose-dependent detection of cocaine at concentrations ranging from 10 μM to 1 mM in 20% human blood serum. This system also enables signal amplification, as each target molecule can induce the ligation of several aptamer-fragment pairs.

STRAND-DISPLACEMENT STRATEGIES

The aptamer engineering strategies described above require modification of the target-binding region within the aptamer. The displacement strand structure-switching aptamer (dsSSA) assay offers a useful alternative approach that requires minimal aptamer modification. dsSSAs employ a short displacement strand that is complementary to a region of the aptamer. The aptamer can be labeled with a fluorophore, while the displacement strand is functionalized with a quencher. In the absence of the target, the two strands hybridize, leading to low fluorescence (Figure 3). Alternatively, the aptamer strand can be extended on one end and hybridized with a fluorophore-labeled complementary strand. In the absence of target, this will also be in close proximity of the hybridized quencher strand[22] As with aptamer beacon assays, the thermodynamic stability of this complex must be tuned such that in the presence of target, the aptamer-target complex is preferred and the quenching strand is displaced, leading to dose-dependent fluorescence recovery.

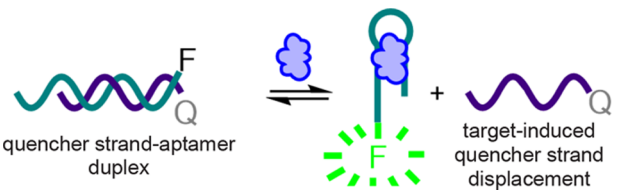

Figure 3. Strand-displacement assay. A fluorophore-tagged aptamer is hybridized with a quencher-tagged complementary DNA strand. Target interaction displaces this strand and promotes aptamer folding, producing a fluorescent signal.

Li and co-workers showed the first proof-of-concept for dsSSA biosensors based on ATP and thrombin aptamers.[23] This approach was hypothesized to be a highly generalizable method for converting virtually any aptamer into a biosensor. This system can be represented by the equilibria

$$A{\cdot}Q \leftrightharpoons A + Q$$

$$A \leftrightharpoons S \xrightarrow{+T} S{\cdot}T$$

where $A{\cdot}Q$ is the fluorescently labeled aptamer hybridized to a quencher-labeled displacement strand, A and Q are the two free species in random conformation, and S is the aptamer in a fluorescent conformation favorable for binding, which is then stabilized by the target T. Defining $K_H^{-1} = \frac{[Q][A]}{[A{\cdot}Q]}$, $K_s = \frac{[S]}{[A]}$, and $K_d = \frac{[S][T]}{[S{\cdot}T]}$, assuming a low K_d and large K_S, we obtain a very similar result to the aptamer beacon system: an approximate expression for the equilibrium fluorescence equal to

$$F_{eq}{\cdot}\alpha \approx \sigma[A{\cdot}Q]_i + (1 - \sigma)[T]_i$$

where $[A{\cdot}Q]_i$ is the initial concentration of the aptamer–quencher complex. Again we note that equilibrium fluorescence is proportional to the initial target concentration. Factor σ, now given by

$$\sigma = \frac{K_H^{-1}(1 + K_S)}{1 + K_H^{-1}(1 + K_S)}$$

shows the effect of the stability of the aptamer–quencher strand complex. For an unstable complex, $0 < \sigma < 1$, which reduces the target-induced fluorescent signal and increases background fluorescence. However, as with aptamer beacons, excessive stability will unreasonably slow down the binding reaction, as the $A{\cdot}Q \rightarrow S$ transition will become unlikely. A suitable thermodynamic equilibrium is therefore required for a sensitive readout with minimal background.

Later, Tan and co-workers expanded upon this technique by introducing the linked strand-displacement paradigm,[24] wherein the competition strand is bound to the aptamer via a PEG linker. This favors more frequent aptamer-displacement-strand interactions than the original assay and decreases the displacement strand length requirement, leading to faster kinetics and a more responsive aptamer probe with lower background.

Strand-Displacement Enabled SELEX. One of the most impactful consequences of the displacement strand assay concept is that they have also enabled the development of aptamer discovery methods that can directly select for aptamers with inherent structure-switching functionality. These typically entail the design of SELEX procedures that require a major conformational change and strand displacement upon target binding to achieve efficient selection of structure-switching aptamers.

In one study, Morse and co-workers demonstrated a structure-switching SELEX method that can simultaneously

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select for specificity, affinity, and binding-induced conformational change, enabling selection of structure-switching RNA aptamers that were readily converted into biosensors. This method employs magnetic beads coupled to a short capture strand that is complementary to the 5′ primer-binding site of a random RNA library (Figure 4A).[25] Only sequences that undergo a large structural rearrangement upon target binding are displaced from the capture strand, released from the bead, and subsequently enriched. Using this method, they were able to directly select for an RNA aptamer biosensor with a K_d of 16 μM for tobramycin. This approach is promising, but is also limited by the requirement that the primer sequence must contribute to the final aptamer structure in order for selection to work. This restricts the range of sequences that can potentially be enriched.

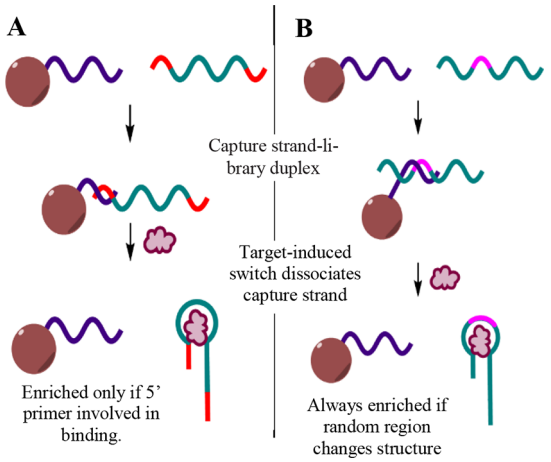

Figure 4. Strategies for direct selection of structure-switching aptamers. (A) In structure-switching SELEX, sequences that undergo a target-induced rearrangement that displaces the docking strand from the 5′ primer-binding site are released from magnetic beads, collected and amplified. This ensures selection for structure-switching activity as well as target affinity. (B) In Capture-SELEX, the docking strand hybridizes within the random region of the aptamer, removing the need for the primer sequence to be involved in folding, and amplifying all sequences that undergo binding-induced structure-switching within the random region.

The capture-SELEX technique addresses this limitation by incorporating the docking sequence complementary to the capture strand within the random region of the library (Figure 4B). As first demonstrated by Li and co-workers,[26] and later by Strehlitz and co-workers,[27] this process could offer a more general approach for the selection of high-quality structure-switching aptamers by allowing for the direct selection of the privileged structure switching architecture. Capture-SELEX was successfully used to select several aptamers for kanamycin A, although the structure-switching functionality of these aptamers was never verified.

A number of other promising direct-selection strategies have been described as well. For example, our lab generated structure-switching aptamers for metal ions using a fluorescence-activated cell sorter with fluorescently labeled displacement strands.[28] Mansy and co-workers designed a selection for RNA aptamers that employed the thiamine pyrophosphate riboswitch to facilitate strand exchange upon target binding.[29] With further improvements in experimental design—including the exploration of novel screening methods that are perhaps not as strictly coupled to the strand-displacement paradigm—such approaches could greatly simplify and accelerate the generation of structure-switching aptamers for a broader range of targets.

CONCLUSION

In this Sensor Issues, we have examined a variety of different design approaches that have been explored for incorporating structure-switching functionality into aptamers, with thermodynamic analyses of each strategy to explore the variables that most strongly influence their performance. We have also described parallel efforts for incorporating the structure-switching functionality directly into the aptamer discovery process. Although not described in this work, the success rate of creating structure-switching aptamers first depends on the availability of high-affinity aptamers. In this regard, recent advancements in non-natural aptamers will greatly aid in expanding the chemical space available for the discovery of high-affinity aptamers. We believe that non-natural aptamers and alternative library designs, combined with innovative aptamer selection strategies may offer the exciting potential for generating novel structure-switching biosensors with sensitivity and specificity performance beyond what is currently achievable.

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Notes

The authors declare no competing financial interest.

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