Aptamer Biosensors

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Riboswitches Based on Kissing Complexes for the Detection of Small Ligands**

Guillaume Durand, Samuele Lisi, Corinne Ravelet, Eric Dausse, Eric Peyrin, and Jean-Jacques Toulmé**

Abstract: *Biosensors derived from aptamers were designed for which folding into a hairpin shape is triggered by binding of the cognate ligand. These aptamers (termed aptaswitches) thus switch between folded and unfolded states in the presence and absence of the ligand, respectively. The apical loop of the folded aptaswitch is recognized by a second hairpin called the aptakiss through loop–loop or kissing interactions, whereas the aptakiss does not bind the unfolded aptaswitch. Therefore, the formation of a kissing complex signals the presence of the ligand. Aptaswitches were designed that enable the detection of GTP and adenosine in a specific and quantitative manner by surface plasmon resonance when using a grafted aptakiss or in solution by anisotropy measurement with a fluorescently labeled aptakiss. This approach is generic and can potentially be extended to the detection of any molecule for which hairpin aptamers have been identified, as long as the apical loop is not involved in ligand binding.*

Aptamers are single-chain nucleic acids obtained through a combinatorial process termed SELEX.[1] They display strong affinity and high specificity for a predetermined target owing to their 3D shape, which results from aptamer intramolecular folding that subsequently leads to optimized intermolecular interactions with the target molecule. They are exquisitely adapted for analytical applications.[2] Previous selection of RNA candidates against RNA hairpins led to stem-loop aptamers in which the loop is complementary to that of the target hairpin, thus generating loop–loop interactions.[3] The stability of such so-called kissing complexes derives from Watson–Crick base pairs in the loop–loop helix, as well as stacking interactions at the junctions between the loop–loop module and the double-stranded stem of each hairpin partner.[4] Intra- and interstrand hydrogen bonds might also contribute to the stability of such complexes. Indeed the binding of the *t*rans-*a*ctivating *r*esponsive (TAR) RNA imperfect stem loop element of human immunodeficiency virus to a hairpin aptamer to generate a 6 base pair (bp) loop–loop helix was characterized by a melting temperature 20 °C higher than that of the complex between TAR and an 8 nucleotide (nt) linear antisense oligomer that gives rise to the same 6 bp duplex.[5] We have exploited the potential of RNA hairpins to discriminate between folded and linear structures for designing aptamer-based sensors.

Riboswitches are RNA modules identified in prokaryotes. They are comprised of a sensor that includes the binding site for a small ligand and they respond to association with this ligand by undergoing a conformational change.[6] The sensor is the functional equivalent of an aptamer and displays similar properties, in particular with regard to specificity. One might develop an aptamer into a molecule that switches between folded and open conformations in the presence and absence of the cognate target, respectively. Several examples of switching aptamers have been described in the literature.[7–9] Their design generally requires tedious trials in order to get a responsive aptamer[7] and/or involves intra- or intermolecular displacement of a complementary strand that decreases the sensitivity of the sensor.[10] Our strategy is based on rationally designed modules (kissing loops) and does not require the displacement of a competitor for signaling but rather includes a signaling partner that contributes to the stability of the complex.

We exploited the formation of kissing complexes for sensing the presence of a ligand that is specifically recognized by a hairpin aptamer. The aptamer is engineered in such a way that the binding of the small molecule shifts its conformation from an unfolded to a folded (hairpin) shape, hence the name aptaswitch. The folded structure is then recognized by a second hairpin that is able to form a kissing complex with the aptaswitch. This second molecule is termed the aptakiss. Therefore the formation of the aptaswitch–aptakiss complex signals the presence of the small molecule (Scheme 1).

We validated this concept with aptamers previously raised against the nucleobase derivatives GTP[11] and adenosine.[12,13] Both of them exhibit a purine-rich central loop that constitutes the ligand binding site (Figure 1). We demonstrated that our strategy can be applied to either RNA or DNA aptamers. As described below, the aptaswitch–aptakiss combination enabled the specific and quantitative detection of the target ligand by either surface plasmon resonance (SPR) or

[*] Dr. G. Durand, E. Dausse, Dr. J.-J. Toulmé
Univ. Bordeaux, IECB, Laboratoire ARNA
2 rue Robert Escarpit, 33607 Pessac (France)
and
Inserm U869, Laboratoire ARNA
146 rue Léo Saignat, 33076 Bordeaux (France)
E-mail: jean-jacques.toulme@inserm.fr
S. Lisi, Dr. C. Ravelet, Prof. E. Peyrin
Univ. Grenoble Alpes
Département de Pharmacochimie Moléculaire
CNRS UMR 5063, 38400 St Martin d'Hères (France)
E-mail: eric.peyrin@ujf-grenoble.fr

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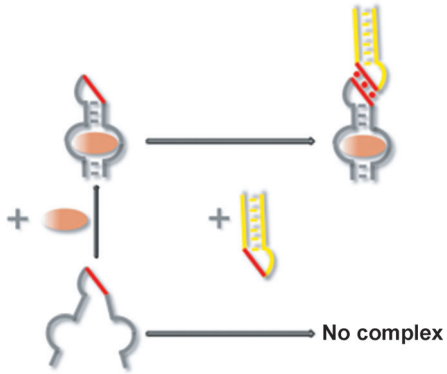

Scheme 1. The aptakiss (yellow) interacts with the folded aptaswitch (top) through loop–loop helix formation (red) but it does not interact with the unfolded structure (bottom). Aptaswitch folding is induced by binding of the cognate ligand (orange).

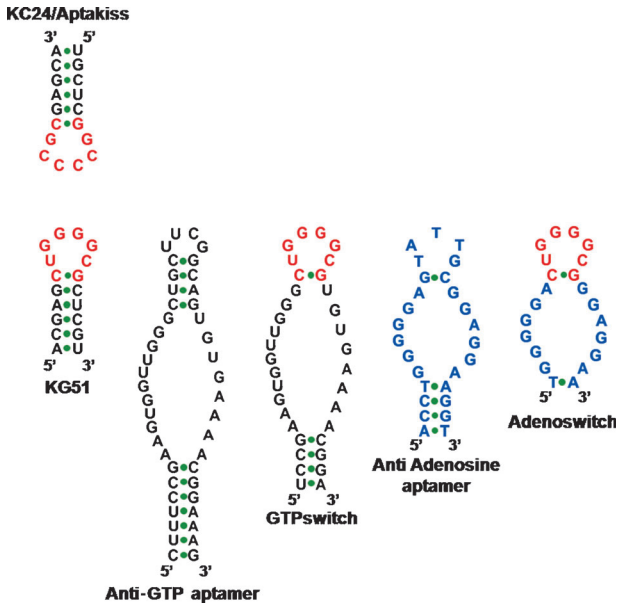

Figure 1. Predicted secondary structures of the aptakiss and aptaswitches. Deoxyribonucleotides are indicated in blue and ribonucleotides in black, except for those that engage in loop–loop interaction, which are shown in red. The sequences of all of the oligonucleotide derivatives used in this study are given in the Table S1 in the Supporting Information.

fluorescence anisotropy through the use of an immobilized or a fluorescently labelled aptakiss, respectively.

We designed our sensors based on KC24-KG51, a RNA–RNA kissing complex previously identified by the group of J. J. Toulmé and characterized by a low K_d value (5 nM at room temperature in 20 mM HEPES buffer pH 7.5, containing 140 mM K^+, 20 mM Na^+, and 10 mM Mg^{++}) as evaluated by SPR (Figure S1 in the Supporting Information). These hairpins potentially form a 6 bp loop–loop helix that includes one GU and five GC pairs (Figure 1). The KC24 hairpin was used as the aptakiss. Aptaswitches were engineered by inserting the KG51 loop sequence 5′CUGGGGCG, which is prone to interaction with the KC24 (aptakiss) loop, into the apical loop of previously described imperfect hairpin aptamers raised against either GTP or adenosine, thereby generating GTPswitch and adenoswitch, respectively (Figure 1). Importantly, the apical loops of the parent aptamers were previously demonstrated not to interact with the respective ligands.[11–13]

In the first case, the stem of the anti-GTP RNA aptamer[11] was truncated down to 4 bp, with the central 27 nt region of the resulting oligonucleotide likely remaining a large nonstructured single-stranded internal loop (Figure 1). This potential GTPswitch did not give rise to a detectable SPR signal when passed over a chip on which the aptakiss was immobilized. By contrast, injection of a preincubated GTPswitch/GTP mixture led to a signal for which the amplitude increased with GTP concentration up to 1 mM (Figure 2 A, B). At 8 μM, a signal of 10 resonance units (RU) was detected under our experimental conditions. This is likely due to recognition by the aptakiss of the structure induced by GTP binding to the GTPswitch. No SPR signal was observed when ATP, which does not bind to the parent aptamer, is substituted for GTP (Figure 2 C). Likewise, no resonance was detected when a point-mutated aptakiss that introduces a G–G mismatch in the loop–loop helix (Table S1 in the Supporting Information) is immobilized on the sensor chip (Figure 2 D). These experiments demonstrate the high specificity of the sensor and underline the role played by ligand–aptaswitch interactions on the one hand and kissing complex formation on the other hand, thus validating our design.

In the second case, the same approach was used except that it resulted in a chimeric aptaswitch since the starting point was a DNA aptamer against adenosine.[12] The 5′CUGGGGCG sequence was substituted for the original apical part of the parent hairpin (Figure 1). The stem of the parent aptamer was then drastically shortened to leave only

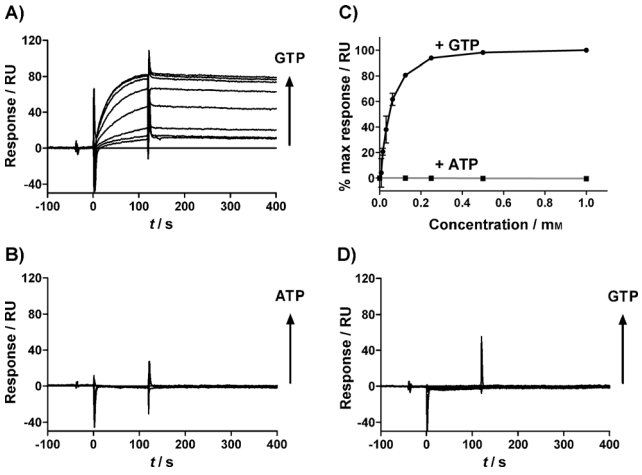

Figure 2. SPR analysis of the GTPswitch/aptakiss complex. GTPswitch (20 μM in 10 mM K_2HPO_4 pH 6.2 containing 200 mM KCl and 10 mM $MgCl_2$) was injected over a chip on which biotinylated aptakiss was immobilized in the presence of increasing concentrations (from 0 to 1 mM) of either GTP (A) or ATP (B). C) The maximum SPR signal obtained was plotted as a function of nucleotide triphosphate concentration; results are expressed as a mean ± SEM of two individuals experiments. D) As a control, GTPswitch in the presence of GTP (from 0 to 1 mM) was injected over a chip functionalized with biotinylated aptakiss^{mut}.

a single potential AT pair at the very bottom of the structure, thus leading to a 21 nt potential adenoswitch. Its properties were then investigated by SPR against biotinylated aptakiss immobilized on the chip. As with the GTPswitch, the amplitude of the signal correlated to the concentration of added adenosine (Figure 3A), with no resonance detected when the adenoswitch was injected alone. At a fixed adenosine concentration, the SPR signal also increased with adenoswitch concentration (Figure 3B), thus indicating that

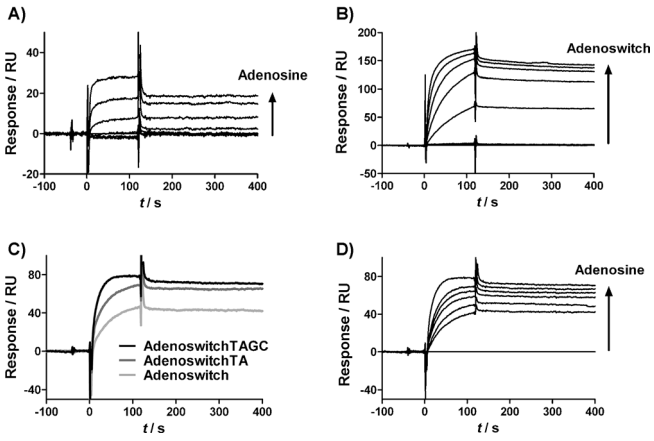

Figure 3. SPR sensorgrams of the adenoswitch/adenosine complex against immobilized biotinylated aptakiss. A) Adenoswitch (5 μM in 10 mM Tris pH 7.5 containing 100 mM NaCl and 10 mM $MgCl_2$) was injected in the presence of increasing amounts (0, 0.125, 0.25, 0.5, 1, 2, 4, or 8 mM) of adenosine. B) A similar experiment under same conditions at 8 mM adenosine with increasing concentrations of adenoswitch (0, 0.08, 0.16, 0.31, 0.63, 1.25, 2.5 or 5 μM). C) SPR sensorgrams of three adenoswitch variants injected at 0.625 μM (in 10 mM Tris pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$) in the presence of 8 mM adenosine against immobilized biotinylated aptatkiss. D) SPR sensorgrams for adenoswitchATGC in the presence of increasing amounts of adenosine (0, 0.125, 0.25, 0.5, 1, 2, 4 or 8 mM).

the adenosine–adenoswitch complex and not the free adenoswitch was the species recognized by the immobilized aptakiss. The sensor comprised of the aptakiss–adenoswitch tandem proved to be specific: no signal was detected when adenosine was substituted by inosine, which is not recognized by the parent aptamer (Figure S2, middle).

The introduction of a point mutation in either the aptakiss or the adenoswitch loop (Table S1) that prevents loop–loop helix formation also resulted in no signal (Figure S2, top and bottom). Interestingly no detection was observed with the aptakiss^{mut}–adenoswitch^{mut} combination (not shown) even though complementarity between the two loops is restored (Table S1). Indeed the stability of kissing complexes is highly dependent on loop sequence and this particular loop–loop duplex is significantly less stable than the parent one (Dausse and Toulmé, unpublished).

A previous NMR study demonstrated stacking of the adenosine ring with purine pairs of the central loop of the aptamer.[13] These interactions actually contribute to shaping the apical part of the adenoswitch into a loop prone to kissing recognition. The sensitivity of the sensor will depend on the equilibrium between the unfolded and folded states of the aptaswitch, that is, on the binding constant of the aptaswitch for its ligand. One might therefore expect that the sensor response will be related to the stability of the adenoswitch hairpin in the absence of the ligand as previously reported for an aptamer-based molecular beacon.[9] To test this hypothesis, we evaluated the properties of adenoswitch variants in which we added one or two base pairs at the bottom of the stem (Table S1). As shown in Figure 3C, the SPR response for a fixed adenosine concentration increased in the order adenoswitch < adenoswitchTA < adenoswitchTAGC, that is, with the number of base pairs in the hairpin stem. Indeed the latter variant allowed the detection of adenosine at a concentration of 0.125 mM (Figure 3D) compared to 2 mM for the parent adenoswitch (Figure 3A). Notably, the binding constant for adenoswitchTAGC and the aptakiss in the presence of saturating adenosine was unchanged compared to the KC24–KG51 complex. The increased sensitivity was not achieved at the expense of specificity: adenoswitchATGC did not recognize inosine and no signal was observed on a chip functionalized with the aptakiss^{mut} (Figure S3).

We further considered the possibility of detecting aptakiss–aptaswitch complexes in solution by taking advantage of fluorescence anisotropy (FA) with a Texas red (TR) 3′-end-conjugated aptakiss as the probe (aptakiss-TR; Table S1). The binding of the aptakiss to the adenoswitch results in increased overall size and consequently in increased FA.[14] In the presence of 10 nM adenoswitch, the FA signal (r) of aptakiss-TR (10 nM) was enhanced when adenosine was added to the reaction mixture. The FA change, $\Delta r = r - r_0$ where r_0 is the anisotropy in absence of ligand, reached ca. 0.015 at an adenosine concentration of 2 mM (Figure S4).

Dose–response curves were then established with the optimized adenoswitchTAGC. As shown in Figure S4, the sensitivity was greatly improved relative to the parent adenoswitch. A half-saturation concentration of 35 μM was obtained with adenoswitchTAGC, close to the 5–10 μM dissociation constant reported for the original aptamer.[12] The FA response was invariant upon adenosine addition when two A residues on the 5′ side of the internal purine loop that are part of the adenosine binding site were exchanged for two G residues (adenoswitchTAGC^{mut2}; Table S1 and Figure S4). As expected, no FA variation was observed upon addition of inosine, which does not bind to the adenosine aptamer. This result confirms that the signal transduction is dependent on adenosine binding to the aptamer domain of the adenoswitch.

Other aptamer-based sensors were previously described for the detection of adenosine. For our aptaswitch-based fluorescence anisotropy assay, the limit of detection for adenosine was estimated to be about 10 μM, in the same range as that commonly reported with fluorescence aptasensing methods (excluding amplification-based biosensors).[9,15] The present sensing format can be considered an original sandwich-like assay for small-ligand detection with unique binding specificity features originating from the double recognition mechanism involved in the formation of the ternary complex. Moreover, such a sandwich-like assay could easily be adapted

to an enzyme-linked immunosorbent assay (ELISA)-type format to achieve signal amplification by using an enzyme-linked aptakiss and surface-immobilized aptaswitch.

In conclusion we engineered sensors based on hairpin aptamers. The structure of the parent aptamers was optimized in such a way that the hairpin shape is adopted exclusively in the presence of the cognate ligand (either GTP or adenosine in the present work). This is relatively easily achieved by shortening the bottom double-stranded stem and introducing a short RNA sequence prone to loop–loop interactions. We demonstrated that the interacting RNA sequence can be introduced in either an RNA or DNA context. We previously demonstrated that a DNA hairpin can engage in loop–loop interactions with an RNA hairpin.[16] We anticipate that DNA-DNA kissing complexes could be identified. One obvious limit to this strategy is the requirement that the apical loop of the hairpin aptamer does not interact with the ligand and that the inserted sequence preserves the functional folding of the aptamer.

Natural kissing loops have been identified in several organisms and have been shown to regulate different biological processes.[17] We recently identified many more potential kissing motifs that are being characterized (Dausse and Toulmé, unpublished). Taking advantage of both natural and artificial repertoires, we could therefore introduce different kissing-prone sequences into different aptamers, thereby generating a series of aptaswitches that could be used simultaneously as long as they could be monitored independently and do not cross-interact. Our strategy could consequently allow multiplexed analysis. Conversely, the same aptakiss can be used for detecting any aptaswitch with a loop that has been appropriately modified with the complementary sequence. Such an aptakiss constitutes a "universal" transducer. The aptakiss–aptaswitch combination appears to be versatile methodology and has a wide potential interest for the design of biosensors.

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