An *in vitro* selection for small molecule induced switching RNA molecules

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Abstract

The selection of RNA and DNA aptamers now has a long history. However, the ability to directly select for conformational changes upon ligand binding has remained elusive. These difficulties have stymied attempts at making small molecule responsive strand displacement circuitry as well as synthetic riboswitches. Herein we present a detailed strand displacement based selection protocol to directly select for RNA molecules with switching activity. The library was based on a previously selected thiamine pyrophosphate riboswitch. The fully in vitro methodology gave sequences that showed strong strand displacement activity in the presence of thiamine pyrophosphate. Further, the selected sequences possessed riboswitch activity similar to that of natural riboswitches. The presented methodology should aid in the design of more complex, environmentally responsive strand displacement circuitry and in the selection of riboswitches responsive to toxic ligands.

Keywords: SELEX, strand displacement, in vitro selection, riboswitch

1. Introduction

Nucleic acid sequences can be designed to fold into desired shapes [1] and to specifically hybridize with target sequences through simple base-pairing rules. In addition to forming static structures, the system can be dynamic in the sense that an oligonucleotide can be designed to displace one strand of a duplex potentially triggering a cascade of events. Such systems have been built to perform autonomous movements, e.g. the DNA walkers [2], rotary DNA devices [3], and DNA oscillators [4], and to act as fuel sources for non-covalent DNA catalysis reactions, as seen in some DNA amplification [5] and nucleated dendritic growth reactions [2]. Further, strand displacement reactions can be built to be responsive to pH [6] and to the presence of specific nucleic acid sequences [7]. However, it is not currently possible to design a priori nucleic acids that are responsive to small molecules. Instead, aptamer domains that have been selected from combinatorial libraries are required to provide sensitivity to specific small molecules. Additionally, the sensing conferred by the aptamer domain needs to be transduced to an output domain, a functionality that cannot be directly selected for with current technologies.

The reason why fusing aptamer and output domains together can work is because aptamers typically experience structural stabilization upon ligand binding often times leading to different base-pairing patterns. This simple effect can be exploited for engineering purposes. For example, ribozyme activity can be regulated by an incorporated aptamer sequence [8], strand displacement can be triggered by small molecule binding to a tethered aptamer domain [9], box shaped origami structures can release their contents in response to ligand binding to fused aptamer sequences [10], and gene expression can similarly be controlled through small molecule binding directly to the mRNA, as in the case of riboswitches. However, the merger of selected aptamers with designed strand displacement sequences has been difficult. Rather than selecting for aptamers that simply bind a specific ligand, a methodology that selects for sequences that undergo the desired structural change upon ligand binding would likely increase the success rate of developing small molecule triggered strand displacement systems.

2. A strand displacement based methodology

Recently, we developed an *in vitro* selection method to identify RNA molecules that interact with specific DNA duplex sequences in a ligand dependent manner [11]. The library is based on a previously selected thiamine pyrophosphate (TPP) responsive riboswitch variant +ThiM#2 [12]. Positions known to inactivate riboswitch activity are randomized. The selection is based on the liberation of the ribosome binding site (RBS) of the RNA upon ligand binding (Fig. 1). In the +ThiM#2 riboswitch, TPP binding causes a conformational change that allows the RBS sequence to become accessible. An accessible RBS is then capable of interacting with the toehold region of duplex DNA conjugated to magnetic beads, thereby resulting in both strand displacement and immobilization. Iterative rounds of selection allow for the isolation of RNA molecules that regulate RBS accessibility in response to ligand binding. In the protocol described herein, the RBS is inaccessible in the absence of the ligand and accessible in the presence of the ligand. In our proof-of-concept selection, the selected sequences are active in strand displacement and riboswitch activity.

There are several advantages to the described strand displacement based method. Rather than selecting for just binding, molecules that undergo the desired conformational change are directly selected. Although here the library is based on a pre-existing riboswitch sequence, it should be

possible to implement this strategy with a fully randomized aptamer domain. In other words, rather than carrying out two separate selections, one for the aptamer domain and another for a transducer domain, as is the norm for the generation of synthetic riboswitches, only one selection would be needed. The protocol is carried out fully *in vitro*, making the method much faster to implement since *in vivo* screening steps are eliminated. The lack of *in vivo* steps also allows for the use of toxic ligands. Additionally, the sequence that base-pairs to the toehold can be easily changed to suit the specific needs of the desired system.

2.1. Assembling the DNA library

The DNA library is based on the ThiM#2 riboswitch [12]. The sequence is chosen because this riboswitch was previously thoroughly characterized [12]. Analogous efforts could use a library not based on a known riboswitch sequence, although this has not been tried yet. The library sequence should contain a transcriptional promoter. If the desired outcome is a riboswitch, then a RBS should be additionally incorporated. Downstream of the RBS element, an initiator strand (*I*) is introduced. This is the region that will mediate the strand displacement reaction and thus must be designed with the reporter sequence in mind. Here, the strand is 26 nucleotides long, avoiding a high GC content that could interfere with strand displacement (Table 1).

2.1.1. The DNA library is assembled by PCR of overlapping oligonucleotides. The length of the oligonucleotides should not exceed 100 bp. The template sequence can be included in a plasmid and modified by introducing randomized positions. For example, here the +ThiM#2 riboswitch sequence is modified by PCR using a reverse primer containing four randomized positions. The forward primer does not carry any modification of the original sequence. 50 ng of template are amplified in a 50 μ L reaction with 0.8 μ M each oligonucleotide and 1.25 U AccuPrime Pfx DNA polymerase (Life Technologies). The cycling protocol follows the manufacturer's instructions for a three-step protocol with a few minor modifications. The initial denaturation step is at 95 °C for 30 s. Annealing is at 55 °C for 30 s, and the extension step is at 68 °C for 1 min/kb of the construct. The amplification is carried out for 25 cycles. Sterile water is preferable to diethyl pyrocarbonate (DEPC) - treated water for the PCR.

2.1.2. PCR products are purified by 2% agarose gel extraction in TAE buffer. For better resolution, a mixture of high resolving agarose can be used. A 1:1 ratio of SeaKem GTG and NuSieve GTG agaroses (Lonza) works well for fragments between 150 and 200 bp. Purification is performed using a commercial kit, such as the Wizard SV gel and PCR clean-up system from Promega. The concentration of DNA is determined by absorbance with a NanoDrop spectrophotometer (Thermo Scientific) and stored at -20 °C.

2.2. RNA library: transcription and purification

RNA switches can control genetic circuits, highly programmable strand displacement reactions, and orthogonal translation [7,13,14]. To select for functional RNA molecules, the DNA library must be converted into a RNA library. The easiest way to do so is to exploit the activity of T7 RNA polymerase. Note that special care needs to be adopted when working with RNA. Since RNA is prone to degrade, solutions for RNA transcription and selection are prepared using DEPC-treated water and then filtered. All the reagents are molecular biology grade and RNase-free. Dedicated stocks should be used when assembling transcription reactions in order to avoid contamination with nucleases or other exogenous material.

2.2.1. A 50 µL transcription reaction is assembled with 2 pmol of purified DNA library as the template. Transcription is performed in T7 RNA polymerase buffer including 40 mM DTT (Sigma-Aldrich Co. LLC.), 5 µg of BSA, 5 mM ribonucleotides, 20 U of human placenta RNase inhibitor, 50 U of yeast inorganic pyrophosphatase, and 150 U of T7 RNA polymerase (New England Biolabs). T7 RNA polymerase buffer (4x) contains 140 mM MgCl₂, 8 mM spermidine (Sigma-Aldrich Co. LLC.), 800 mM HEPES, pH 7.5. Once filtered, aliquots are stored at -20° C. The transcription reaction is incubated for at least 4 h at 37 °C.

2.2.2. The sample is processed by the addition of 0.5 mM CaCl₂ and 2 U DNase I (RNase-free, New England BioLabs) and incubated at 37 °C for 1 h. Subsequently, 37 mM EDTA, pH 8 is added prior to ethanol-precipitation. Briefly, 5 µL of 3 M sodium acetate and 250 µL of cold ethanol are added to the treated reaction and mixed. The sample is placed in a -20 °C freezer for 20 min and then centrifuged at 14800 rpm for 20 min at 4 °C. The supernatant is removed and the RNA pellet is dried on a heat

block for 5 min at 70 °C. The pellet is rehydrated with 2x RNA loading dye and DEPC water in a total volume of 60 µL. The RNA denaturing gel loading dye (2x) contains 7 M urea (Fisher Scientific), 0.1 % (w/v) bromophenol blue (Sigma-Aldrich Co. LLC.) in 1x TBE solution. The pellet rehydration procedure sometimes can be difficult due to the high amount of salt present in the pellet. Initially, DEPC-treated water can be added and vortexed. If the pellet is hard, it may be convenient to warm and vortex the sample. Finally, the addition of the loading buffer may help to dissolve the pellet. Subsequently, the RNA library is denatured by incubation for 5 min at 95 °C.

2.2.3. Denaturing PAGE is carried out with 7 M urea in TBE buffer. Optimum results are obtained using 19:1 of acrylamide–bis acrylamide solution for the gel preparation (Bio-Rad Laboratories). The wells of the comb should be large enough to host ca. 60 µL of solution. The migration is performed in TBE buffer at high voltage (450 V- 500 V) for a short period of time in order to reduce degradation. For this reason, a cooling system is necessary to decrease the temperature of the gel apparatus and to improve RNA migration. A convenient way is to put a small fan in front of the gel plates, if possible. An alternative is to run the gels in a cold room. Initially, the gel is pre-run for 30 min to equilibrate the system before loading the sample. Wells are cleaned with TBE buffer with a syringe before loading the RNA to remove unpolymerized acrylamide.

2.2.4. After the gel is run, the RNA band is visualized by UV shadowing and cut from the gel. The excised band containing the RNA library is then subjected to crush-soak extraction. Briefly, the band is initially crushed with a sterile 1 mL syringe in a micro centrifuge tube. Next, 500 µL TE buffer (1 mM EDTA, 10 mM Tris-HCl, pH 7.5) are added to the sample, which is then left tumbling overnight in an incubator at 37 °C. The sample is centrifuged for 30 s at maximum speed with a bench-top microcentrifuge. The resulting supernatants are then filtered with 0.45 µm ultrafree-MC centrifugal filters (UFC30HV00, Millipore) for 3 min at 12000 g. The recovered supernatant is ethanol-precipitated as above (see section 2.2.2) and resuspended in 30 µL of DEPC-treated water.

2.2.5. Before storing the RNA library, the RNA concentration is checked by absorbance with a NanoDrop spectrophotometer. Typically, the sample will need to be diluted 5 -10 fold in order to get an accurate quantification of the species, since high yields of product are expected. RNA can be

stored at -20 °C for a maximum of six months. However, it is advisable to use the library immediately after purification.

2.3. Reporter design and strand displacement

The selection is based on a strand displacement reaction between RNA and a duplex DNA reporter. The reporter consists of two strands of unequal length. Since the reaction is driven by complementarity, the sequence of the reporter is dependent upon the sequence of the / strand of the library. The / strand of the library should be constant and hybridize with the toehold region of the reporter. In the selection described here, the hybridizing region contains a RBS element. Strand displacement occurs when the RBS is accessible. Hybridization with the toehold allows the displacement of the smaller DNA strand of the reporter. The reporter plays two roles. The reporter is used to isolate active sequences, and the reporter reveals the activity of the library and the selected RNA sequences.

To isolate active sequences, the longer strand of the reporter is modified with a biotin molecule, which is later coupled to streptavidin magnetic beads. In this way, active RNA sequences that displace the shorter DNA strand of the reporter will hybridize to and be retained by the beads. Another version of the reporter is modified with a FRET pair so that the strand displacement reaction can be observed in real-time. This provides a useful readout of the progression of the selection as the activity of the pool of RNA molecules can be monitored throughout the course of the selection.

2.3.1. The design of the reporter is based on the / strand of the library, including the RBS. The reporter consists of two DNA oligonucleotides. The shorter oligonucleotide (called hereafter Rep I) is 26 nucleotides long. The complementary oligonucleotide (Rep B) is 32 nucleotides in length. The toehold region is at the 3' end of Rep B and can interact with the 5' end of the RNA library. The reporter used for the isolation of active sequences should contain a biotin modification at the 5'-end of Rep B. No modification is needed for Rep I. To monitor reactions in real time, a different version of the reporter containing an Iowa Black quencher at the 3' end of Rep I and a fluorescein molecule at the 5' end of Rep B is used. All oligonucleotides should be HPLC purified.

2.3.2. The reporter is assembled by annealing. The lyophilized oligonucleotides are resuspended in sterile water to a concentration of 100 μM . Then, a 100 μL stock of 10 μM solution is prepared containing 2:1 Rep I/ Rep B. The stock is diluted to 1 μM in TNaK buffer with 1 μM oligo (dT)₂₁ in a tube compatible with the available thermocycler. The TNaK buffer (10x) consists of 1.4 M NaCl, 50 mM KCl, 200 mM Tris-HCl, pH 7.5. Once filtered, the TNaK buffer is stored at room temperature. The volume of the annealing reaction is 100 μL , although the volume can be increased or decreased. The annealing is performed in a thermocycler starting with a step at 90 °C for 5 min and followed by slow cooling to 25 °C at a rate of 0.1 °C/s. The annealed oligonucleotide can be stored at -20 °C; however, freshly annealed reporter is preferable. Conversely, the 10 μM stock can be stored at -20 °C and thawed to prepare the reporter each time.

2.3.3. The strand displacement reaction for the selection steps is performed with 50 nM annealed reporter in TNaK buffer containing 1 μM oligo (dT)₂₁, 5 mM MgCl₂, and 100 nM RNA. The volume of the strand displacement reaction for the monitoring of activity by fluorescence is 20 μL . Fluorescence is monitored at the end of each round of selection. Reactions are carried out in a 384-well black plate for small volumes (n. 264705, NUNC) covered with a thin sealing foil (Lightcycler, Roche). The strand displacement reaction is monitored at 37 °C for at least 2 h with a Safire plate reader (TECAN). The excitation and emission wavelengths are of the fluorescein tag, that is, 485 nm and 520 nm, respectively.

2.4. The selection procedure

The selection steps of the protocol make use of the biotin modified version of the reporter. Both negative and positive selection steps are exploited in the same round. In other words, RNA molecules that mediate strand displacement in the absence of ligand are actively removed from the pool prior to the selection of RNA molecules that mediate strand displacement in the presence of ligand. Every round of selection is composed of four steps. The first three steps are negative selections that deplete active sequences in the absence of ligand. The final step is a positive selection where active sequences in the presence of ligand are enriched.

2.4.1. The library is the RNA pool obtained as described in section 2.2. 20 pmol of the RNA pool are exploited for each round. The strand displacement reaction consists of TNaK buffer, 1 μ M oligo (dT)₂₁, 5 mM MgCl₂, 50 nM reporter in a 100 μ L volume. DEPC- treated water should be used while assembling the reaction. RNA is the last element to be added to the solution, since strand displacement starts immediately. The reporter is prepared as described in section 2.3.2 with biotinylated Rep B and unmodified Rep I. Reporter annealing is performed immediately before starting the selection. The annealing reaction volume is at least 100 μ L, and the solution is kept at room temperature while performing the selection protocol. In the first negative selection step, the ligand is not added to the strand displacement reaction. The reaction is incubated at 37 °C for 2 h.

2.4.2. In the meantime, the streptavidin magnetic beads (Dynabeads MyOne Streptavidin C1, Life Technologies) used for RNA isolation are prepared. Magnetic beads are stored at 4 °C and should be equilibrated to room temperature before use. Typically, 5 min is sufficient. The magnetic bead solution should be mixed since the beads tend to aggregate. Then, 20 μ L of beads are washed three times in a 1.5 mL microcentrifuge tube with 150 μ L of BWBT buffer. The BWBT buffer contains 0.2 M NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.40. 0.1% (v/v) Tween 20 is added to the buffer after the solution is filtered. The buffer is stored at room temperature. Finally, beads are washed with 150 μ L of the selection buffer, which consists of TNaK buffer plus 5 mM MgCl₂. The beads are now ready for the selection steps.

2.4.3. The beads are resuspended in the strand displacement reaction from 2.4.1 and incubated at room temperature for 20 min. By magnetic separation (6-Tube Magnetic Separation Rack for 1.5 mL microcentrifuge tube, New England BioLabs), the beads are discarded while the supernatant is transferred to a fresh microcentrifuge tube. This step retains molecules that do not perform strand displacement in the absence of ligand.

2.4.4. 20 μ L of previously annealed reporter (section 2.3.2) and 5 mM MgCl₂ are added to the supernatant isolated from 2.4.3 for the next negative selection step. The procedure is performed as above (sections 2.4.1- 2.4.3), although the strand displacement incubation time at 37 °C is reduced to 1 h. The procedure is then repeated again for a total of three negative selection steps. Notably, the

incubation time of the strand displacement reaction can vary throughout the selection. Decreasing or increasing the incubation time affects the stringency of the selection. For example, decreasing the incubation time during the positive selection steps allows for the isolation of molecules that perform strand displacement more quickly. In the second negative step, the incubation time is reduced, since this passage has already been performed previously, presumably removing the majority of unwanted molecules. Control reactions with the FRET reporter and sequences locked in a conformation with an accessible RBS are complete within 1 h [11].

2.4.5. The supernatant obtained after the third negative selection step is then used for a positive selection in the presence of the ligand. 10 μ L of freshly prepared biotinylated reporter, 5 mM MgCl_2 , and 100 μ M of ligand (TPP) are added. The solution is incubated at 37 $^{\circ}\text{C}$ for 2 h.

2.4.6. In the meantime, 10 μ L of magnetic streptavidin beads are washed and prepared as described above (section 2.4.2). Once resuspended in the strand displacement reaction, the magnetic beads are incubated at room temperature for 20 min. In this positive selection step, molecules that are able to perform strand displacement are isolated. Therefore, the supernatant is discarded. The RNA molecules bound to the beads are washed four times with 150 μ L of selection buffer to remove unspecific binders.

2.4.7. Isolated RNA molecules are amplified by reverse transcription PCR (SuperScript III One-Step RT-PCR System with Platinum *Taq* High Fidelity, Life Technologies). The reaction is assembled on ice in order to prevent initiation, and the protocol follows the manufacturer instructions. Briefly, 50 μ L of reaction are prepared with 2x Reaction mix, 0.5 μ M forward and reverse primers, 1 μ L Platinum *Taq* High Fidelity enzyme mix, and sterile water. The beads are resuspended in the reverse transcription PCR solution after the last magnetic separation. Resuspended beads are transferred to a 200 μ L microcentrifuge tube and placed in a thermocycler. The protocol consists of 55 $^{\circ}\text{C}$ for 30 min for reverse transcription, 94 $^{\circ}\text{C}$ for 2 min to denature the DNA-RNA duplex, 10 cycles of amplification at 94 $^{\circ}\text{C}$ for 15 s, 55 $^{\circ}\text{C}$ for 30s, 68 $^{\circ}\text{C}$ for 30 s, and a final extension at 68 $^{\circ}\text{C}$ for 5 min.

2.4.8. The PCR product is purified from a 2% agarose gel with a Wizard SV gel and PCR clean-up system as described above for the DNA library construction (section 2.1.2). The DNA is quantified by absorbance with a NanoDrop spectrophotometer. This DNA is the result of the first complete round of selection. Subsequent rounds of selection use as an input RNA molecules obtained from transcription of 2 pmol of the DNA obtained at this point.

2.5. Assessment of the selection output

2.5.1. Evaluation of selection progression by strand displacement

One convenient feature of this selection methodology is the ability to rapidly gain insight into the progression of the selection. The fluorophore-quencher reporter can be used to monitor the activity of the pool of RNA molecules after each round of selection, both in terms of background and ligand induced activity (Fig. 2). This information can then be used to adjust the selection conditions. For example, if the activity in the absence of the ligand is high, the length of the negative selection steps could be increased. The selection described here shows that only three rounds of selection are needed to increase the activity of the RNA pool by 120% compared to the initial pool.

2.5.1.1. *Strand displacement assay.* 100 nM RNA obtained at the end of each round are assessed by strand displacement of the FRET modified reporter in the presence and absence of 100 μ M ligand (TPP). Reactions are carried out as described in section 2.3.3 and are in triplicate. The addition of the RNA has to be immediately prior to collecting the data. It is preferable to make a stock solution that is immediately consumed for the preparation of all the reactions, rather than individually mixing each reaction. RNA can be added to multi-well plates simultaneously using a multichannel pipette.

2.5.2. Evaluation of selection progression by sequencing

To identify individual, active molecules rather than just assessing the activity of the pool, sequencing of the resulting DNA at the end of each round of selection is desirable. To do so, PCR product DNA molecules are TA cloned into a convenient vector. Then after transformation of *E. coli* and plasmid isolation, the DNA is sent for sequencing. The *in vivo* steps lengthen the process in comparison to the strand-displacement method described in 2.5.; however, sequencing is the only way to identify specific active sequences. In our selection, we sequenced 10 colonies from the first two rounds of

selection and 20 colonies from the third round. Frequencies of occurrence of individual sequences were calculated from this small set of clones. Enriched sequences were then evaluated for strand displacement activity following the protocol described in section 2.5.1. The data showed that the sequence present at the highest frequency gave a 7.0 ± 0.3 -fold increase in fluorescence in the presence of the ligand and reporter [11]. This sequence showed the greatest activity of all the sequences tested. Deeper insight into the selected pool of nucleic acids could be gained with next generation sequencing technologies.

2.5.2.1. Protocol for cloning and sequencing of selected molecules. DNA obtained after each round of selection is TA cloned with the TA Cloning Kit with the vector pCR2.1 (Life Technologies). 10 ng of DNA are amplified with 2.5 U *Taq* DNA polymerase (New England BioLabs), 0.5 mM dNTPs, 0.2 μ M primers in Standard *Taq* Buffer in a total volume of 50 μ L. An initial melting step at 94 °C for 30 s is used, followed by 20 cycles of 94 °C for 15 s, 55 °C for 15 s, and 68 °C for 30 s, with a final extension step at 68 °C for 5 min. This procedure is necessary since the proofreading activity of Platinum *Taq* High Fidelity DNA polymerase removes the adenine (dA) overhang at the 3' end. DNA is isolated from 2% agarose gel in TAE buffer and purified with the Wizard SV gel and PCR clean-up system. Finally, the insert is ligated in the linearized commercial vector pCR2.1. The protocol is the same as that of the manufacturer. Briefly, two ligation conditions are prepared with a ratio of 1:1 and 1:3 vector:insert in 10 μ L reactions. 5 U of ExpressLink T4 DNA Ligase (Invitrogen) are exploited. Ligations are carried out for 3 h at room temperature. 2 μ L of the ligation reaction are used to transform *E. coli* Top 10 competent cells or similar strain for blue/white screening. Cells are plated on LB medium supplemented with 156 μ M X- Gal (Sigma-Aldrich Co. LLC.) and incubated overnight at 37 °C. White colonies are then isolated and transferred in liquid media for plasmid amplification. Plasmids are recovered with a Wizard Plus SV Minipreps DNA Purification kit (Promega), and the DNA is then sent for sequencing.

2.5.3. Testing riboswitch activity

The presented methodology directly selects for RNA molecules that change conformation in response to ligand binding in a manner that leads to strand displacement. However, the RNA molecules may additionally function as riboswitches if the / strand contains a RBS sequence. By modifying the

construct to include a gene encoding a fluorescent protein behind the selected sequence plus the use of a commercially available *in vitro* transcription-translation kit, riboswitch activity can be directly probed. After three rounds of selection for strand displacement activity, the selected sequences were also capable of functioning as riboswitches. For every sequence tested, protein synthesis was at least 2-fold higher in the presence of the ligand than in the absence of ligand [11].

2.5.3.1. Riboswitch activity assay. The selected sequences are placed in front of a gene encoding the yellow fluorescent protein YPet. Cloning can be done by restriction digestion followed by ligation, Gibson assembly [15], or similar technique. Gibson assembly has the advantage of not introducing additional nucleotides into the construct. YPet is used as a reporter because this fluorescent protein is very bright, photostable, and expresses well *in vitro* [16]. The generated plasmids containing the potential riboswitches can be used directly as templates for transcription-translation or the region of interest can be amplified by PCR. The amplification protocol is described in section 2.1.1. Products are purified with Wizard SV gel and PCR clean-up systems. It is advisable to phenol-chloroform extract the DNA prior to use in the transcription-translation reaction. DNA is stored at -20 °C until use. 250 ng of DNA are exploited for the *in vitro* transcription-translation reaction. Reactions are carried out in a total volume of 20 µL containing the PURE system components (PURExpress *in vitro* protein synthesis kit, New England BioLabs) and 16 U of human placenta RNase inhibitor (New England BioLabs). The ligand is added at 1 mM concentration when needed. Reactions are assembled on ice and then transferred to a 384-well black plate. The plate is sealed with a thin foil. The plate is centrifuged at 1000 rpm for 1 min to remove bubbles that may have formed during pipetting. Reactions are measured for 3 h at 37 °C at the plate reader.

2.5.4. Conclusions

The development of a selection strategy for RNA switches able to trigger a strand displacement reaction through a conformational change induced by ligand binding is presented. The selected RNA sequences additionally show riboswitch activity similar to that of natural riboswitches. The selection scheme should be modifiable for generating RNA-based sensors with different ligand specificities and purposes, including the development of cascading RNA circuitry triggered by the presence of a small molecule.

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DNA Sequences	
+ThiM#2 riboswitch	
5'- ATAAATTAATACGACTCACTATAGGGAGAGGAGGGAATTGTGAGCGGATAACAATTGAATTCAAC CAAACGACTCGGGGTGCCCTTCTGCGTGAAGGCTGAGAAATACCCGTATCACCTGATCTGGATA ATGCCAGCGTAGGGAAGCTATTACAAGAAGATC <u>AGGAG</u> -3'	
Library	
5'- ATAAATTAATACGACTCACTATAGGGAGAGGAGGGAATTGTGAGCGGATAACAATTGAATTCAAC CAAACGACTCGGGGTGCCCTTCTGCGTGAAGGCTGAGAAATACCCGTATCACCTGAT NN GGATA ATGCCAGCGTAGGGAAGCTATTACAAGAN NN ATC <u>AGGAG</u> AAATTAAGTATGGGATCGCACCATCA C-3'	
Primers for library assembly	
Primer FW	5'- ATAAATTAATACGACTCACTATAGGGAGAGGAGGGAATTGTGAGCGGATAACAAT-3'
Primer RV	5'- GTGATGGTGCGATCCCATAGTTAATTTCTCCTGATNNTCTTGTAATAGCTTCCCTAC GCTGGCATTATCCNNATCAGGTGATACGGGTATTT-3'
Reporter strands	
Rep B	5'-/52-Bio/ GTGATGGTGCGATCCCATAGTTAATTTCTCCT-3'
Rep I	5'-AATTAAGTATGGGATCGCACCATCAC-3'

Figure 1. Ligand binding induces a conformational change in the RNA structure thereby triggering a strand displacement reaction. This reaction is driven by the hybridization of a single strand oligonucleotide (RNA) to a nucleic acid duplex (DNA). The protruding strand region, i.e. the toehold (yellow), can interact with the complementary portion of the RNA. This initial interaction leads to the displacement of the shorter strand. The process generates a new molecular species consisting of the original RNA paired with the longer strand of the preexisting duplex. In the selection presented, the toehold region contains a RBS, which becomes accessible in the presence of the ligand. The initiator strand (*I*) is highlighted in cyan on the reporter duplex. The longer strand presents a biotin molecule to facilitate the isolation of strand displacement active sequences during the selection.

Figure 2. Strand displacement activity of the pool of molecules increases during the selection.

An aliquot of the RNA pool after each round of selection is tested with a fluorophore-quencher reporter for strand displacement activity in the presence or absence of 100 μ M TPP. A control reaction is assembled without RNA and TPP. Fluorescence measurements are recorded with a spectrofluorometer at 37 $^{\circ}$ C for 4 h. The reporter strands are modified with fluorescein and Iowa black molecules, respectively.

References

- [1] P.W. Rothemund. Folding DNA to create nanoscale shapes and patterns, *Nature* 440 (2006) 297-302.
- [2] P. Yin; H.M. Choi; C.R. Calvert; N.A. Pierce. Programming biomolecular self-assembly pathways, *Nature* 451 (2008) 318-322.
- [3] H. Yan; X. Zhang; Z. Shen; N.C. Seeman. A robust DNA mechanical device controlled by hybridization topology, *Nature* 415 (2002) 62-65.
- [4] K. Montagne; R. Plasson; Y. Sakai; T. Fujii; Y. Rondelez. Programming an in vitro DNA oscillator using a molecular networking strategy, *Mol. Syst. Biol.* 7 (2011) 466.
- [5] D.Y. Zhang; A.J. Turberfield; B. Yurke; E. Winfree. Engineering entropy-driven reactions and networks catalyzed by DNA, *Science* 318 (2007) 1121-1125.
- [6] A. Amodio; B. Zhao; A. Porchetta; A. Idili; M. Castronovo; C. Fan; F. Ricci. Rational design of pH-controlled DNA strand displacement, *J. Am. Chem. Soc.* 136 (2014) 16469-16472.
- [7] A.A. Green; P.A. Silver; J.J. Collins; P. Yin. Toehold switches: de-novo-designed regulators of gene expression, *Cell* 159 (2014) 925-939.
- [8] M. Wieland; A. Benz; B. Klauser; J.S. Hartig. Artificial ribozyme switches containing natural riboswitch aptamer domains, *Angew. Chem., Int. Ed.* 48 (2009) 2715-2718.
- [9] M. Wieland; A. Benz; J. Haar; K. Halder; J.S. Hartig. Small molecule-triggered assembly of DNA nanoarchitectures, *Chem. Commun.* 46 (2010) 1866-1868.
- [10] E.S. Andersen; M. Dong; M.M. Nielsen; K. Jahn; R. Subramani; W. Mamdouh; M.M. Golas; B. Sander; H. Stark; C.L. Oliveira; J.S. Pedersen; V. Birkedal; F. Besenbacher; K.V. Gothelf; J. Kjems. Self-assembly of a nanoscale DNA box with a controllable lid, *Nature* 459 (2009) 73-76.
- [11] L. Martini; A.J. Meyer; J.W. Ellefson; J.N. Milligan; M. Forlin; A.D. Ellington; S.S. Mansy. In Vitro Selection for Small-Molecule-Triggered Strand Displacement and Riboswitch Activity, *ACS Synth. Biol.* 4 (2015) 1144-1150.
- [12] Y. Nomura; Y. Yokobayashi. Reengineering a natural riboswitch by dual genetic selection, *J. Am. Chem. Soc.* 129 (2007) 13814-13815.
- [13] S. Bhadra; A.D. Ellington. A Spinach molecular beacon triggered by strand displacement, *RNA* 20 (2014) 1183-1194.
- [14] S. Bhadra; A.D. Ellington. Design and application of cotranscriptional non-enzymatic RNA circuits and signal transducers, *Nucleic Acids Res.* 42 (2014) e58.
- [15] D.G. Gibson; L. Young; R.Y. Chuang; J.C. Venter; C.A. Hutchison, 3rd; H.O. Smith. Enzymatic assembly of DNA molecules up to several hundred kilobases, *Nat. Methods* 6 (2009) 343-345.
- [16] R. Lentini; M. Forlin; L. Martini; C. Del Bianco; A.C. Spencer; D. Torino; S.S. Mansy. Fluorescent proteins and in vitro genetic organization for cell-free synthetic biology, *ACS Synth. Biol.* 2 (2013) 482-489.

A fully in vitro strand displacement-based RNA selection is presented.

RNA molecules that undergo a conformational change upon ligand binding are selected.

The resulting RNA sequences can mediate small molecule triggered strand displacement.

This method can be used to identify synthetic riboswitches.