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Tuning the Specificity of a Synthetic Receptor Using a Selected Nucleic Acid Receptor

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Abstract: Because of their relative simplicity, synthetic receptors often lack the selectivity observed for biopolymer receptors, such as aptamers. However, aptamer recognition of ligands is limited by the chemistries inherent in the four canonical nucleotides. Here, we report the design and selection of a ternary complex in which the specificity of a bis-boronic acid synthetic host (**1**) that binds to various carboxylic acids is tuned by a surrounding aptamer. Although, the synthetic receptor alone has higher selectivity for citrate over DL-tartrate, the formation of the aptamer:receptor complex reversed the organic host selectivity to preferentially bind tartrate. The RNA conformation changed upon the introduction of the synthetic host, consistent with an induced-fit mechanism for binding.

Introduction

In nature, there exist numerous examples of molecular recognition between biopolymers such as proteins[1] and nucleic acids,[2] and various small organic ligands. While the strengths of these interactions vary, most are highly specific. Supramolecular chemists have attempted to generate synthetic receptors that have molecular recognition properties similar to those of biopolymers.[3] Even though the rationally designed receptors frequently show high affinity for their ligands,[4] the receptors often fail to discriminate between very structurally similar guests. It seems likely that the receptors have low to medium selectivity because they are small and relatively simple structures.

In contrast, biopolymer receptors with high affinities and selectivities can be generated by directed evolution methods. In particular, nucleic acid receptors (aptamers) can be generated by techniques such as the Systematic Evolution of Ligands by Exponential Enrichment (SELEX). However, the chemistries available to aptamers are for the most part limited to the chemistries of the four canonical nucleotides. For this reason, aptamers frequently cannot bind compounds that are hydrophobic or anionic in character.[5]

We postulated that by combining synthetic receptor design and aptamer selection methods, the specificity of synthetic receptors and the range of compounds targeted by aptamers might be concomitantly improved. To explore our postulate, we choose a synthetic bis-boronic acid receptor (**1**), which is analogous to compound **3** that was known to bind citrate with slightly higher affinity than DL-tartrate.[6] From our previous studies,[7] replacement of the guanidinium moiety with an ammonium does not dramatically effect binding. We then selected an aptamer that could bind to the receptor:tartrate complex. The aptamer was found to tune the selectivity of the synthetic receptor, leading to greater binding of tartrate relative to the similar organic acid, citrate. The ternary complex that is formed is analogous to cofactor-mediated enzymes or catalytic antibodies.[8,9]

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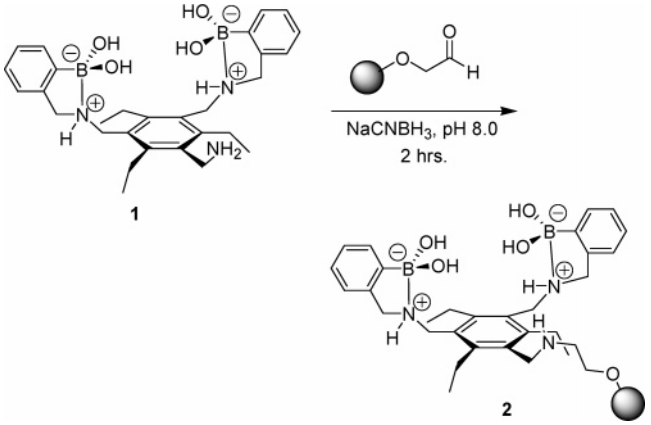
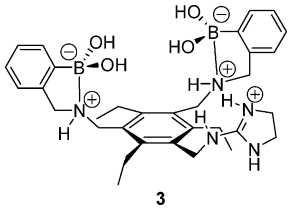


Equation 1: The reductive amination of the bis-boronic acid receptor on glyoxal agarose beads.



Materials and Methods

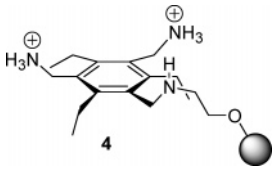
Reagents. The 2% cross-linked glyoxal agarose resin was purchased from Agarose Bead Technologies (ABT, Tampa, FL). The solvents and the buffer salts were purchased from Sigma (St. Louis, MO). The N-50 RNA pool was synthesized on an Expedite 8909 DNA synthesizer (PE Biosystems, Foster City, CA) using synthesis reagents from Glen Research (Sterling, VA). The primers were purchased from Integrated DNA Technologies (Coralville, IA).

Pool Construction. A single stranded DNA pool containing 50 randomized positions (N50: 5′-CATCAGTTAGTCATTACG-N50-ATTGTGAAGTCGTGTCCCTATAGTGAGTCGTATTAGAA-3′) was synthesized according to the previously reported methodology.[9] The pool was PCR amplified using primers 5′-TTCTAATACGACTCACTATAGGGACACGACTTCACAAT-3′ (38.50 primer) and 5′-CATCAGTTAGTCATTACGCTTACG-3′ (24.50 primer), where the underlined residues are part of the T7 RNA polymerase promoter and are not transcribed.

Target Immobilization. The immobilization of the bis-boronic acid receptor (**1**) and its tris-amine precursor were performed as follows. To a 10 mL slurry of glyoxal agarose beads, 50 mL of cyanoborohydride coupling buffer (20 mM sodium phosphate at pH 8.0 and 3 g/L sodium cyanoborohydride) was added. To this mixture, 1 mL of 200 μM target (in 20 mM sodium phosphate buffer) was added. This reaction mixture was incubated for 3 h and the beads were then drained and subsequently washed with the coupling buffer. To block the free aldehyde sites, the resins were then treated with 1 mL of methylamine (in 70% MeOH) in the coupling buffer. After another 3 h of incubation, the resins were drained and washed with the coupling buffer. Further overnight washes were carried out with water to remove any uncoupled amines or reagents from the beads.

In Vitro Selection. The selection was initiated with four pools of RNA. The RNA pools in selection buffer (100 mM tris, 20 mM $MgCl_2$, and 100 mM LiCl at pH 7.6) were initially denatured by heating at 72 °C for two minutes and were cooled to room temperature over 10 min. Methanol (20% final concentration) and DL-tartrate (200 μM final concentration) were then added for a total volume of 300 μL and the mix was incubated with the resin at room temperature. Selection and amplification were otherwise carried out according to standard protocols.[10] In summary, the RNA pool in buffer was incubated with the target loaded resin for 20 min and the resin was washed with the buffer. The bound RNA was then eluted out using an elution buffer (100 mM sodium citrate, 5 M urea, and 2 mM EDTA at pH 7.6). The RNA bound RNA was then ethanol precipitated and the excess urea was removed using a centrisep spin columns (Princeton Separations, Adelphia, NJ). The purified RNA was then amplified through reverse transcription and PCR.

In every round of negative and positive selection, the resin was incubated with the RNA pool and was subsequently washed with selection buffer to remove any unbound and weak binding RNA. The volume of this wash was progressively increased from round one through round thirteen (30 bead volumes to 500 bead volumes). Negative selections were carried out using both tris-amine-loaded agarose resins (**4**), and with agarose resins in which all the glyoxal sites were blocked with methylamine.



The reverse transcription of the RNA pool was carried out using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA), while PCR was carried out using Taq DNA Polymerase (Invitrogen). The transcription was carried out using the Ampliscribe High Yield Transcription Kit (Madison, WI). The round thirteen selected pool was cloned (TA Cloning Kit; Invitrogen) and sequenced using the Dye Terminator Cycle Sequencing Kit (Beckman Coulter, Fullerton, CA) on a CEQ 2000 XL DNA sequencer (Beckman Coulter).

Binding Assays. Following every round of selection, the double-stranded DNA pool was transcribed with [α-^{32}P]UTP (2.0 mCi, 3000 Ci/mmol; Easytides). Binding assays were performed similar to the selection itself, but the resin was only washed with 30 bead volumes of the selection buffer and fractions were collected for analysis. The radioactivity in each fraction was quantified using a scintillation counter. The ratio of bound RNA to unbound RNA was calculated based on the bound RNA to the methylamine loaded resin and to the receptor−ligand loaded resin, and served as a measure of the progress of the selection.

Fluorescent Measurements. The aptamers were 3′ end-labeled using 5-(((2-carbohydrazino) methyl) thiol acetyl) aminofluorescein (Molecular Probes, Eugene, OR), and published conditions.[11] The fluorescein-labeled aptamer was then purified on a 8% denaturing acrylamide gel. All fluorescence measurements were carried out on a PTI Quantamaster QM-4/2003SE spectrofluorimeter (Photon Technology International, Ontario, Canada). The aptamer (10 nM) in selection buffer (480 μL) was incubated at 72 °C for 2 min and was cooled to room temperature over 10 min. The fluorescence response was measured by exciting the samples at 494 nm (the λ_{ex} for fluorescein) and determining emission at 518 nm (the λ_{em} for fluorescein). The response was instantaneous and the signal was steady after 2 min. Precipitation was observed for tartrate in methanol above 1.2 mM concentration, while citrate precipitated above 1.0 mM. Data were only collected at or below these ligand concentrations. The fluorescence emission was then plotted with the incremental addition of analyte. The emission data set was fit to the equation

$$y = 100 + AX/(X+B)$$

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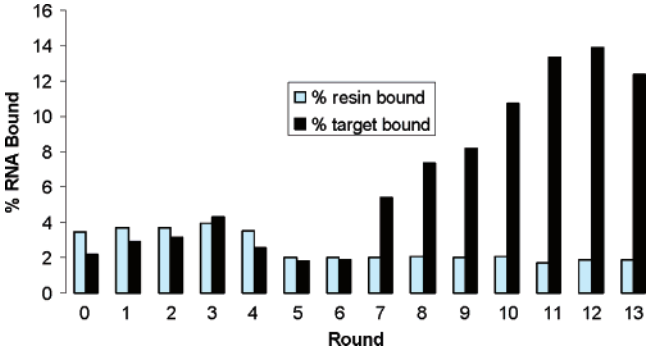

Figure 1. Receptor-binding as a function of the round of selection. The radiolabeled, selected RNA pool was applied to either the resin loaded with methylamine, or the resin with the receptor immobilized. The affinity column was developed with the selection buffer. The % RNA bound was calculated by dividing the amount of bound RNA by the amount of RNA that was washed off.

where y is the percent change in the fluorescence at a given analyte concentration, A is the fluorescence intensity at saturating analyte concentrations, X is the concentration of the analyte, and B is the apparent dissociation constant value.

In-Line Assays. The In-line assays were carried out following both 5′ and 3′ end-labeling of the aptamer. The aptamer was 5′ end-labeled using T4 polynucleotide kinase (Invitrogen) and [γ-^{32}P] ATP (2.0 mCi, 7000 Ci/mmol; ICN Biomedicals) in exchange buffer (Invitrogen), and the radiolabeled RNA was gel purified (6% agarose gel). The aptamer was 3′ end-labeled as described above. The in-line reactions were carried out under selection conditions with 500 pmoles of aptamer in a total volume of 20 μL. The reactions were incubated for various times in the presence of various analytes. Cleavage products were resolved on an 8% agarose gel. The gel was dried and bands were visualized by autoradiography.

Results and Discussion

To generate aptamers that could bind to both a synthetic receptor and the ligand of that receptor, a selection was carried out that targeted the receptor:ligand complex. The synthetic organic receptor (20 μM, **1**) was immobilized on glyoxal agarose beads through reductive amination (eq 1). The remaining aldehyde sites on the resin were blocked with methylamine. A RNA pool (N50) was first passed over a column of an immobilized tris-amine compound (**4**), a precursor to the receptor that was incapable of binding tartrate. This negative selection was carried out to increase the specificity of the selected RNA molecules for the receptor:ligand complex. Then, positive selections were carried out with **1** in the presence of 200 μM tartrate to ensure that the immobilized organic receptor was presented as a complex. The stringency of the selection was progressively increased by increasing the concentration of salt (mainly LiCl) in the wash as well as by increasing the pool-to-target ratio.

The progress of the selection was monitored by incorporating a radiolabel into the RNA pool and determining what fraction of the pool that bound to the column (Figure 1). Starting in round seven, target-binding RNA dominated the pool. After thirteen rounds of selection, there was no further improvement in binding, and the selected RNA pool was cloned and sequenced. Out of 21 sequences, seven distinct aptamer families were identified based on sequence similarities and target-binding abilities; only one sequence was identically repeated (**Clone #5**) (Figure 2).

Figure 2. Aptamer sequences. Only the residues comprising the random region are shown. The names of the individual aptamers are shown to the left of the sequences. Clone #5 was found twice.

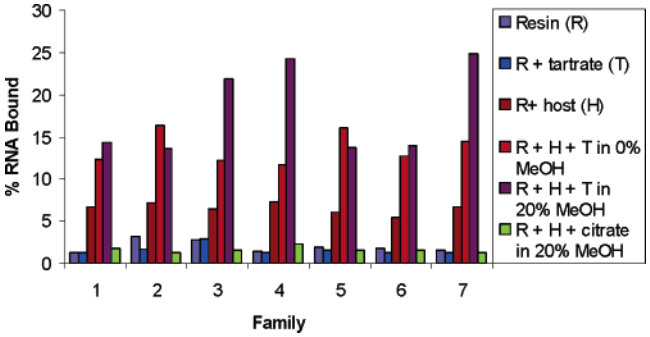

Figure 3. Binding assays. Binding assays with radiolabeled aptamers from each Family (an average of every clone in that family) were carried out as described in Materials and Methods. The fraction of aptamer bound in a standard assay was determined in the presence of methylamine immobilized resin alone, R (by comparing the resin bound RNA to the RNA that was washed off); resin and tartrate in solution, T; resin with the synthetic host immobilized, H; resin with the synthetic host immobilized, tartrate in solution, and either no methanol or 20% (v/v) methanol. Finally, binding assays were carried out in the presence of resin with the synthetic host immobilized, citrate in solution, and 20% methanol. The % RNA bound was calculated as in Figure 1.

The families and their members were analyzed for their ability to bind to the bis-boronic acid receptor in the presence and absence of tartrate. A given RNA aptamer was radiolabeled, passed over an affinity column, washed with fifteen column volumes of buffer, and the fraction of RNA that remained bound was determined by scintillation counting (Figure 3). All of the aptamer families bound to the immobilized receptor, generally bound much better in the presence of tartrate, and could readily distinguish tartrate from citrate.

One novel aspect of the selection was that it was carried out in the presence of 20% methanol, because the receptor **1** has a

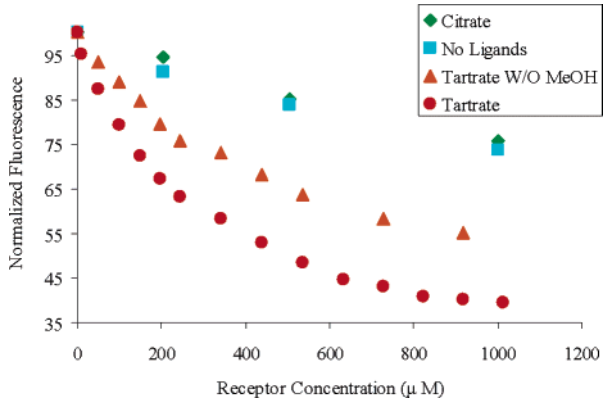

Figure 4. Fluorescent assays with fluorescent Clone #5. The aptamer was labeled with fluorescein as described in Materials and Methods. The receptor was added in increments to the fluorescein tagged RNA under the selection conditions with a constant concentration (10 nM) of analyte (citrate or tartrate). Binding assays with tartrate were carried out in either the presence or absence of 20% methanol.

higher affinity for its organic acid ligands in the presence of methanol.[7] While phage-displayed peptides have previously been selected in organic solvents,[13] and while ribozymes and aptamers have been shown to be active in organic solvents and to sometimes have hydrophobic character,[14] this is one of the first examples of an aptamer selection that has been carried out in the presence of high concentrations of an organic solvent. At the conclusion of the selection, aptamer binding appeared to be dependent on methanol. The optimum methanol concentration for binding was around 20% (v/v). Solvents such as ethanol, DMSO, THF, and DMF were not able to promote significant binding to the receptor:ligand complex. For example, in the presence of 20% ethanol, about 3% of Clone 5 could bind to the resin alone, while 4% could bind to the affinity column. The discovery that aptamers can be selected to function only in the presence of organic solvents is novel, and is consonant with previous studies that showed that ribozyme function could be improved by the addition of organic solvents.[15]

Clone 5 had the highest affinity and specificity for the receptor:ligand complex, and was chosen for further studies. To more precisely determine the binding constant of this aptamer for the receptor and tartrate complex, the aptamer was labeled at its 3′ end with fluorescein,[12] and changes in fluorescence intensity were observed as a function of receptor concentration. Tor and co-workers recently developed a similar system, in which a pyrene-labeled HIV-1 TAR (through a 2′-amino-butyryl linkage) was used to measure the K_d between aminoglycosides and RNA.[17] The results in Figure 4 indicate that the aptamer binds to the free receptor, and that binding is not dependent upon immobilization. Moreover, the ability of the aptamer to respond specifically to tartrate further shows that selected nucleic acids can be readily modified to function as biosensors.

The K_d value for the receptor was extracted by fitting the titration curve (Figure 4). On its own, the bis-boronic acid receptor was only slightly selective for citrate (K_d for tartrate $= 7.1 \times 10^{-6}$ M, K_d for citrate $= 5.5 \times 10^{-6}$ M). However, in the context of the aptamer the specificity of the receptor was dramatically altered. The aptamer showed no change in fluorescence upon titration with tartrate alone. Tartrate was bound with a K_d of 2.1×10^{-4} M, but citrate was bound with a K_d that was below the limit of detection ($<3 \times 10^{-3}$ M) (Table 1). Thus, the discrimination ratio between tartrate and citrate goes from 1.17 (for citrate) in the absence of the aptamer to 14 (for tartrate) in the presence of the aptamer. The simplest explanation for these results is that the aptamer formed a pocket that can more precisely accommodate the receptor:tartrate complex, and that can exclude citrate via steric interactions or charge repulsion.

Table 1. Calculated Dissociation Constants of Aptamer Complexes Formed with the Receptor

analyte	K_d(M)
tartrate	2.1×10^{-4}
tartrate W/O MeOH	5.0×10^{-4}
no ligands	$< 1.3 \times 10^{-3}$
citrate	$< 3.0 \times 10^{-3}$

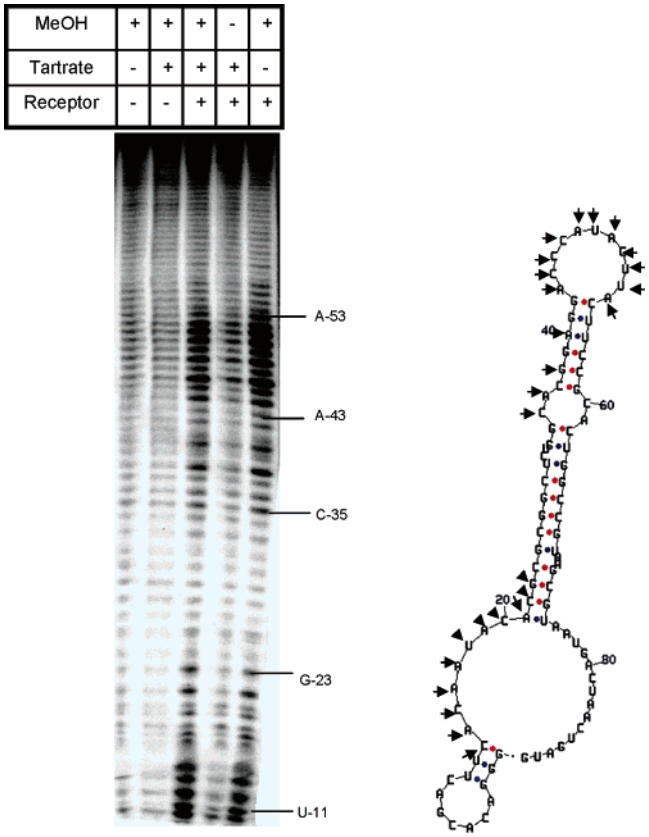

Figure 5. In-line assay with 5′ end-labeled Clone #5. The various additions to the assays were either methanol (20%), tartrate (200 μM), and receptor (20 μM). The location of the individual hydrolysis products was determined using a T1 ladder (not shown here). The positions of enhanced cleavages in the presence of receptor are shown on the predicted aptamer secondary structure.

Given that there is about a 3-fold loss in the affinity of the receptor for tartrate in the context of the aptamer, it can be concluded that at least part of the tartrate-binding energy is used to generate an induced-fit conformation. To characterize the aptamer conformational change upon binding tartrate, In-line assays were performed with radiolabeled aptamer.[18] In-line assays rely upon the enhanced cleavage rates that are observed

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(16) http://biotools.idtdna.com/mfold/
(17) Blount, K. F.; Tor, Y. *Nucleic Acids Res.* **2003**, *31*, 5490−5500.

at neutral pH in single-stranded versus double-stranded regions of structured RNA molecules. In-line assays have previously been used to provide insights into whether portions of aptamers or other functional RNAs change upon ligand-binding.[19] The predicted secondary structures[16] of the receptor-binding aptamers were consistent with their observed digestion patterns (Figure 5). As predicted, the aptamer undergoes a conformational change upon the introduction of the receptor (Lanes 3, 4, and 5). On the other hand, no specific cleavages were observed in the presence of tartrate alone, suggesting that tartrate does not independently bind to the aptamer in the absence of the receptor. When the aptamer was incubated in selection buffer alone for a much longer time, cleavage patterns similar to those observed in the presence of the receptor complex were observed. Taken together, these results verify that the aptamer specifically recognizes the receptor:tartrate complex, and suggest a random kinetic mechanism for binding in which tartrate can bind to the receptor either before or after the receptor complexes with the aptamer. In addition, it seems as though the aptamer undergoes significant folding upon binding the receptor, an observation that is consistent with the hypothesis that the structural reorganization may assist in discrimination against citrate.

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Conclusions

The utility of generating an aptamer:receptor complex for the recognition of small organic molecules is best attested by noting that tartrate is the smallest organic ligand ever recognized by an aptamer. Moreover, since aptamers are negatively charged polyanions, they by and large do not recognize extremely negatively charged ligands, especially small ligands without heterocyclic character. Although the organic receptor lost some affinity for its organic acid ligand in the context of the aptamer, the selectivity was reversed to target the organic acid used during in vitro selection. This work serves as a model for future studies to improve the specificity of synthetic receptors and immediately suggests a novel route to the development of chimeric biosensors for small, otherwise hard-to-detect organic analytes.

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