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Facile Conversion of Aptamers into Sensors Using a 2′-Ribose-Linked Fluorophore

Edward J. Merino and Kevin M. Weeks*

Department of Chemistry, University of North Carolina, Chapel Hill, North Carolina 27599-3290

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Nucleic acids can be efficiently selected in vitro to bind diverse small and macromolecular ligands,[1] and new methods are being developed with the potential for accelerating the in vitro selection process.[2] To use an in vitro selected nucleic acid, termed an aptamer, as a sensor, the ligand-binding event must be detectable via a useful signal. Approaches for creating ligand-sensitive signaling aptamers include (i) post-selection modification of an aptamer[3] and (ii) direct selection for a fluorescence-detectable conformational change.[4] Neither approach allows an existing aptamer with useful ligand-binding properties to be directly converted to a signaling device.

Ideally, fluorescence-detected signaling aptamers should (i) function without the use of other reagents or oligonucleotide partners, (ii) be easy to synthesize and, preferably, contain a single fluorescent modification, (iii) report ligand binding using only steady-state measurements, (iv) function directly in realistic biological environments, and (v) be identifiable via a design strategy that does not require significant pre-existing knowledge about the underlying nucleic acid structure.

We have shown that the electrostatic environment near the 2′-ribose position in nucleic acids is exquisitely sensitive to the extent to which a nucleotide is conformationally constrained.[5] The linkage between chemistry at the 2′-ribose position and local nucleotide flexibility reflects the large influence that the adjacent 3′-phosphodiester backbone group has on the electrostatic environment at the 2′-ribose position.[5b,d] Because aptamers typically bind small molecule ligands via induced fit,[6] we hypothesized that ligand-induced structural reorganization in diverse aptamers might be generically detectable via a 2′-ribose-linked fluorescent probe, due to changes in the proximity of heavy atoms, such as backbone phosphodiester groups and magnesium ions, or changes to the local oxidation potential.[7,8] We focused on the Bodipy-FL fluorophore (**F**, Figure 1) because its fluorescence emission is sensitive to nucleic acid hybridization and sequence.[7]

DNA aptamers have been identified[4b,9] that selectively bind argininamide, AMP, cellobiose, cholic acid, cocaine, tyrosinamide, and ADP and are likely to be chemically stable in realistic biological environments. In this work, we successfully convert the AMP, tyrosinamide, and argininamide aptamers (Figure 2) into sensors. The AMP and argininamide aptamers are structurally well characterized;[10] in contrast, the structure of the tyrosinamide aptamer is unknown[9f] nor can it be predicted using conventional folding algorithms.

Fluorescent aptamer adducts were synthesized by incorporation of a single 2′-amino cytosine residue in each oligodeoxynucleotide (Figure 1), solution phase reaction with an activated ester of **F** with the unique 2′-amine (indicated in bold in Figure 2; Supporting Information), followed by purification of the 2′-amide product by denaturing gel electrophoresis. Aptamer adducts were constructed for all internal cytosine positions in each sequence. Thus, two or three candidate sensors were evaluated for each aptamer. Fluorescently labeled aptamers were irradiated at 492 nm, and emission

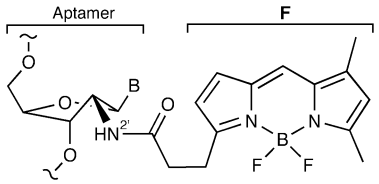

Figure 1. 2′-Ribose-linked fluorophore.

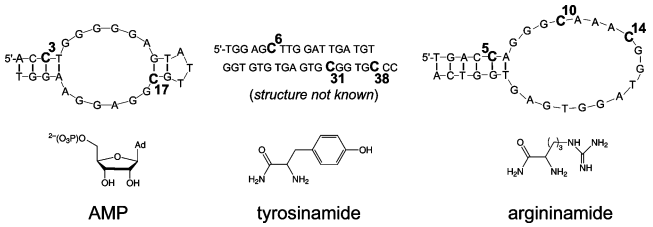

Figure 2. Aptamers converted into sensors. Nucleotides in the AMP, tyrosinamide, and argininamide aptamers individually replaced with 2′-amino cytidine and conjugated to **F** via a 2′-amide linkage are bold.

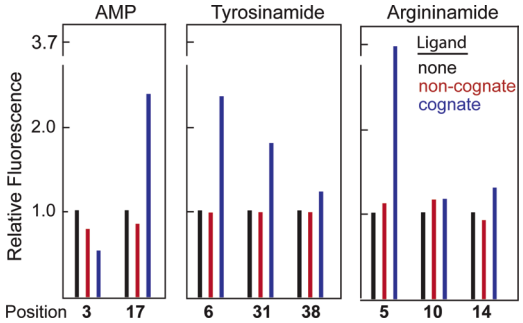

Figure 3. Candidate sensors. Fluorescence intensities are normalized to the no ligand control. Ligand concentrations were 20, 1, and 10 mM for the AMP, tyrosinamide, and argininamide aptamers, respectively.

intensity was recorded from 505 to 565 nm for solutions containing no ligand, a structurally related noncognate ligand, and the cognate ligand (black, red, and blue bars, Figure 3). For the AMP, tyrosinamide, and argininamide aptamers, the noncognate ligands were CMP, alaninamide, and lysinamide, respectively.

Both AMP aptamer adducts are useful sensors. The fluorescence intensity of the 3**F**-AMP and 17**F**-AMP adducts decreased by 40% and increased by 2.4-fold upon binding by AMP, respectively, relative to the no ligand controls (compare black and blue bars, first panel of Figure 3). Both AMP sensors accurately discriminate AMP from CMP (red bars, Figure 3).

Similarly, two of the three tyrosinamide (6**F**-Tyr and 31**F**-Tyr) and one of the three argininamide (5**F**-Arg) **F**-adducts show greater than 40% changes in fluorescence signal upon binding their cognate ligands. Observed fluorescence enhancements for these three sensors span 1.8- to 3.7-fold. In all cases, addition of the noncognate ligand yields no significant change in fluorescence (red bars in Figure 3).

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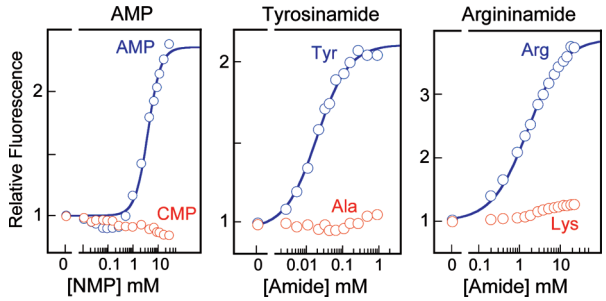

Figure 4. Ligand-binding affinities for aptamer sensors. Cognate and noncognate ligands are shown in blue and red, respectively. Errors from duplicate experiments are comparable to the size of the symbols shown. Binding experiments were performed in 50 mM Hepes, pH 7.5, 100 mM NaCl, and 10 mM $MgCl_2$ (argininamide experiments omitted NaCl and $MgCl_2$).

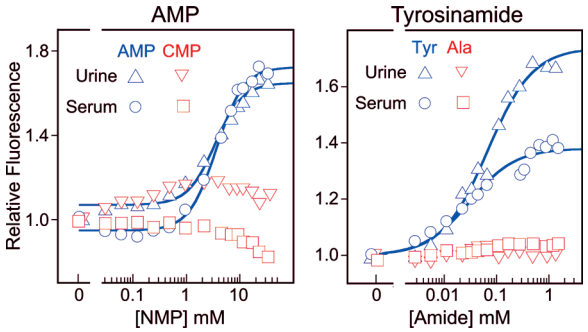

Figure 5. AMP and tyrosinamide sensors function in realistic biological environments. Binding constant determinations were performed in duplicate and are reproducible to ±50% or better.

Thus, of the eight candidate sensors, five yielded effective ligand-sensing devices. Good candidate sites for 2′-derivitization appear to lie at the junctions between base-paired and flexible structures in these aptamers.

We determined ligand-binding affinities in a simple buffer solution for the 17**F**-AMP, 6**F**-Tyr, and 5**F**-Arg sensors (Supporting Information). The AMP aptamer binds 2 equiv of the mononucleotide;[10a] ligand binding by the 17**F**-AMP sensor is well fit assuming cooperative binding with a $K_{1/2}$ value of 3.3 mM and a Hill coefficient of 1.6 (blue line in Figure 4). This $K_{1/2}$ is somewhat higher than the 100−600 μM values typically reported for this aptamer,[5c] likely reflecting destabilization of the aptamer−ligand complex by the 2′-O-adduct. The 6**F**-Tyr sensor binds tyrosinamide with a K_d of 20 μM, essentially identical to the reported value of 40 μM.[9f] The 5**F**-Arg sensor binds argininamide with a K_d of 1.6 mM (Figure 4), again, similar to the independently reported value of 1.2 mM.[10b] All three sensors discriminate against structurally related noncognate ligands (red symbols, Figure 4).

We then examined these reagentless sensors under realistic biological assay conditions. Urine and serum are challenging experimental environments; both absorb approximately 30% of the light at 492 nm, exhibit significant background fluorescence, and comprise uncontrolled high ionic strength solutions. Experiments were performed using either 50% (v/v) unmanipulated human urine or 25% (v/v) fetal bovine serum. Sensor concentrations were increased to 300 nM (Supporting Information). Ligands were incrementally added to the sensor solutions; spectra and data were corrected for (small) volume changes, but are otherwise unaltered.

Both AMP and tyrosinamide sensors function well. Measured $K_{1/2}$ values for 17**F**-AMP sensor in urine and serum are 3.4 and 3.6 mM, respectively, and K_d values for the 6**F**-Tyr sensor are 66 and 30 μM (Figure 5, in blue). For both sensors, binding affinities are identical, within error, to those measured in the simple buffer solution. As expected, binding is selective for the cognate ligands (Figure 5, in red). The argininamide aptamer was originally selected to function at low NaCl concentrations[9a] and, therefore, does not bind its ligand in either urine or serum (data not shown), reflecting an intrinsic feature of this aptamer.

We have converted three nucleic acid aptamers into sensor devices via an extremely straightforward, semi-rational approach. Ligand binding is detected by modulating the local environment at an appropriate fluorescent group, tethered via a 2′-amide linkage (Figure 1). This approach converts arbitrary aptamers into signaling devices at a high success rate (Figure 3). Development of 2′-ribose-linked sensors requires little to no analysis of pre-existing structural information as exemplified, especially, by the tyrosinamide aptamer for which no structural constraints can be inferred using current nucleic acid folding algorithms. Binding behavior and fluorescence signal changes determined during the initial screening process are retained under realistic assay environments (Figures 4 and 5). Environmentally sensitive 2′-ribose-derivatized aptamers represent a major step forward toward the broad goal of creating rationally designed, reagentless, sensors for small molecules.

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Supporting Information Available: Protocols for aptamer−fluorophore conjugation, ligand-binding studies, and representative spectra for each aptamer sensor in buffer, urine, and serum. This material is available free of charge via the Internet at http://pubs.acs.org.

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Supporting Information for
E.J. Merino and K.M. Weeks

Facile Conversion of Aptamers into Sensors using a 2'-Ribose Linked Fluorophore

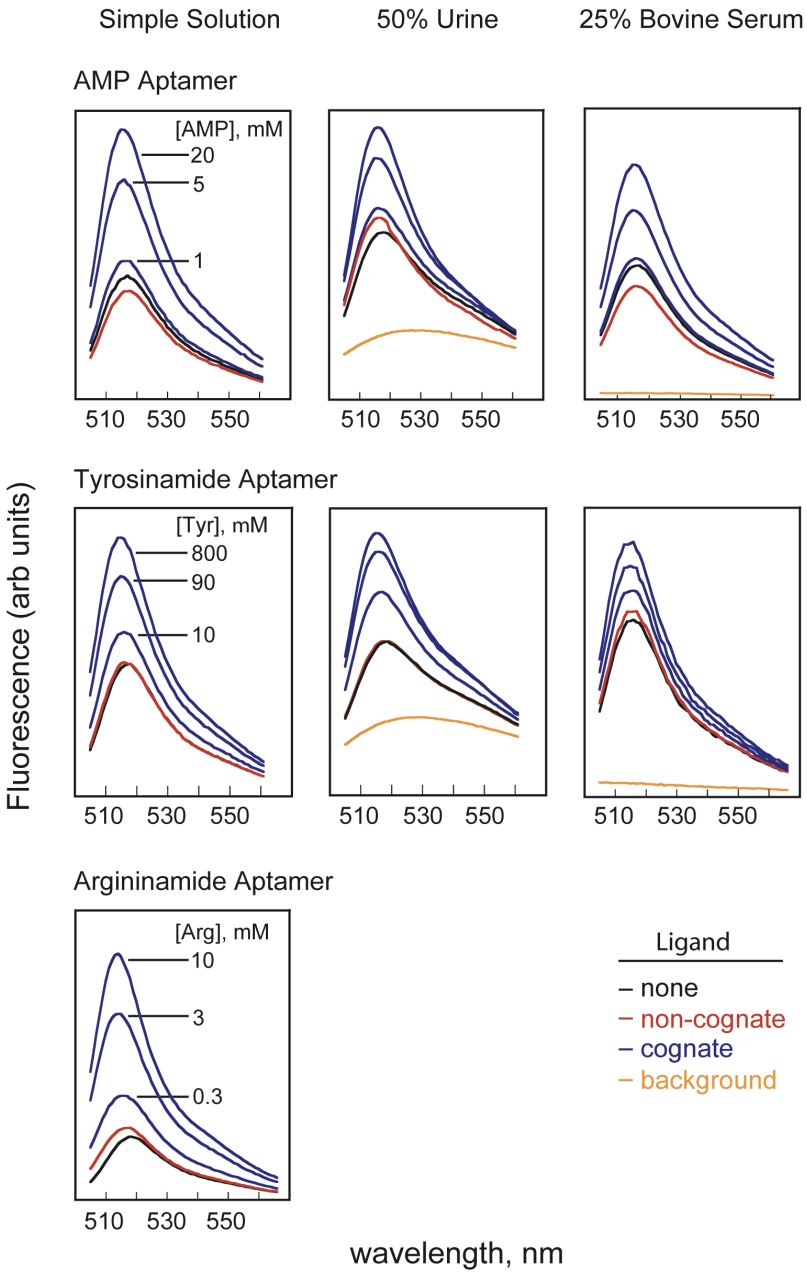
Bodipy fluorophore conjugation of 2'-amine substituted aptamers. Oligodeoxynucleotides containing single 2'-amino cytosine residues (sequences are listed in Figure 2) were purchased from Trilink Biotechnologies (San Diego, CA). 2'-amine substituted cytidine was chosen for synthetic convenience, but the methods described here are expected to work for other nucleotides. Aptamers were 2'-labeled by treating an aptamer solution [10 nmol in 10 μL of 200 mM Hepes (pH 8.0)/25% formamide] with a Bodipy FL-STP ester (100 mM in 10 μL DMSO; Molecular Probes) and incubating for 6 hrs at 50 °C. 380 μL of water were added and the DNA was recovered by ethanol precipitation [add of 0.1 vol 3 M NaCl and 2.5 vol of ethanol; incubate on dry ice for 15 min; centrifuge at 14k rpm in microfuge, 30 min at 4 °C]. The pellet was resuspended in 7 μL water plus 2 vol of 80% (v/v) formamide/1 mM EDTA, heated to 90 °C for 3 min, and placed on ice; the 2'-amide product was purified by denaturing gel electrophoresis [20% (w/v) 29:1 acrylamide:bisacrylamide; 0.75 mm × 31 cm × 38.5 cm; in 90 mM Tris-borate, 7 M urea, 2 mM EDTA; 3.25 hr at 70 W]. Aptamer 2'-adducts were excised from the gel, eluted overnight (4 °C) into 25 mM sodium acetate (pH 5.5)/1 mM EDTA, separated from solid acrylamide by microfiltration (EZ spin columns, Millipore), and concentrated by ethanol precipitation. Aptamers were stored in 2 mM Hepes (pH 7.5). Aptamer 2'-conjugates form at yields of ≥ 60%.

Aptamer Binding Studies. For the AMP and tyrosinamide aptamers, solutions [100 nM aptamer-fluorophore conjugate, 50 mM Hepes (pH 7.5)] were heated to 95 °C for 1.5 min, placed on ice for 2 min, brought to 100 mM NaCl, 10 mM $MgCl_2$, and incubated at 25 °C. For the argininamide aptamer, NaCl and $MgCl_2$ were omitted and experiments were performed at 10 °C. Fluorescence measurements were obtained using a Varian Eclipse Spectrofluorimeter. Excitation was at 492 nm and emission intensity was recorded from 505 to 570 nm at a slit width of 5 nm. Fluorescence measurements used to determine $K_{1/2}$ or K_d values were obtained by averaging the observed fluorescence over 512–520 nm.

All experiments with human samples were performed in accordance with University of North Carolina at Chapel Hill protocols on human subjects. Human urine samples were unmanipulated and untreated. Fetal Bovine Serum (Hyclone, Logan, UT) was untreated and not heat inactivated. Samples containing urine or serum were prepared by folding the aptamer in solutions that contained either 2× or 1.5× of the buffer and ion concentrations given above and then diluted to give final concentrations of 50% (v/v) urine or 25% (v/v) serum. Aptamer concentrations were 300 nM.

Determination of Binding Constants. The DNA-based AMP aptamer binds two equivalents of AMP. All binding data were well fit assuming cooperative binding by AMP: Fluorescence Intensity = $A/(1 + ([ATP]/K_{1/2})^{1.6}) + b$, where b is the observed fluorescence in the

absence of AMP and A is the net amplitude of the fluorescence change. Ligand binding by the tyrosinamide and argininamide aptamers was fit assuming a simple bimolecular equilibrium: Fluorescence Intensity = $A/(1 + [ATP]/K_d) + b$.



Supporting Figure 1. Representative fluorescence spectra for ligand binding by the AMP, tyrosinamide and argininamide aptamers. Non-cognate ligands were CMP, alaninamide and lysinamide, respectively, and were used at the highest concentration of cognate ligand shown in each panel.