Fluorescent Sensors Based on Aptamer Self-Assembly

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In vitro selection of oligonucleotides has made possible the isolation of aptamers with high binding affinity for small molecules, proteins, or even whole cells.[1] Recently, aptamers were demonstrated to rival antibodies in analytical methods based on heterogeneous assays.[2] Aptamers often undergo a conformational change upon ligand binding and, if so, can be converted to fluorescent sensors by either modification with fluorescent oligonucleotide analogs[3] or double-end-labeling with donor and acceptor fluorophores.[4] We describe herein a simple and potentially general approach to the rational construction of sensors[5] based on fluorophore-labeled heterodimeric aptamers that assemble as a function of ligand concentration.

Oligonucleotide aptamers generally consist of a single oligonucleotide chain comprised of two partially complementary domains. These domains are connected through loops, and very often these loops are not essential for ligand binding. We hypothesized that removal of such loop regions would convert the aptamer into subunits that could, nonetheless, reassemble to form the ligand-binding pocket. We were encouraged by a precedent in which an anti-adenosine ribonucleotide triphosphate rATP aptamer consisting of two subunits was still able to bind to an rATP affinity column.[6] We further assumed that, by adjusting the complementarity, we would be able to tune the system to an equilibrium that favors individual subunits over assembled heterodimer but that shifts toward the latter upon the binding of ligand. If each subunit were labeled with a fluorophore, then proximity-dependent communication of the fluorophores could be used to assess the position of the equilibrium and hence the concentration of the ligand. Ligand-dependent self-assembly of aptamers is analogous to the assembly of heavy and light chains of an Fv antibody fragment through ligand binding that was used to develop an "open sandwich fluoroimmunoassay".[7] However, in the case of aptamers, the subunit interactions can be adjusted easily by modifying the length of complementary regions, and therefore the subunit affinity required for ligand-dependent assembly can be attained more predictably.

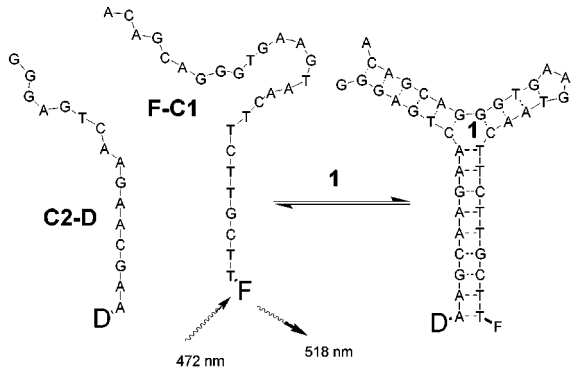
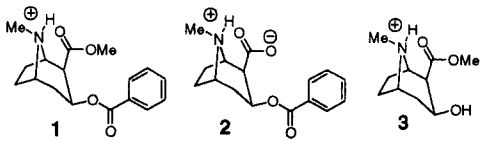


Figure 1. Self-assembly of two aptamer subunits **F-C1** and **C2-D** in the presence of cocaine (**1**): **D**, dabcyl quencher; **F**, 6-FAM; smaller font F quenched 6-FAM. The secondary structure of the aptamer is based on mutational analysis.[10]



In the course of constructing sensors capable of reporting the activity of anticocaine catalytic antibodies,[8] we developed oligodeoxynucleotide aptamers for cocaine (**1**) by standard methods.[1] To construct a hetorodimeric aptamer, we began with a cocaine-binding 39-mer with $K_d = 5\ \mu$M at $c(Mg^{2+}) = 1$ mM and, based on the suggested secondary structure, separated it at a predicted loop into two subunits[9] (apparent K_d of the self-assembled aptamer, ~200 μM by equilibrium gel filtration).[10] We labeled one subunit with a 5′−6-carboxy-fluorescein fluorophore (6-FAM, F in **F-C1**) and the other with a 3′-dabcyl quencher (**C2-D**) (Figure 1). Dabcyl is a universal, nonfluorescent, non-Förster quencher that is used in molecular beacons to follow the intramolecular hybridization of the 5′- and 3′-ends.[11] Dabcyl is characterized by an efficient π-overlap quenching in the hybridized state and little energy transfer quenching in the nonhybridized state. As predicted, in the selection buffer (c(TRIS) = 20 mM, pH = 7.4, c(NaCl) = 140 mM) at the optimum chain concentrations of c(**F-C1**) = 10 nM and c(**C2-D**) = 60 nM and $c(Mg^{2+})$ between 0 and 4 mM, the two subunits behaved as a self-assembling fluorescent cocaine sensor. The sensor reliably reported concentrations of cocaine in the range from 10 to 1250 μM with fluorescein emission at 518 nm ($\lambda_{ex} = 472$) that was quenched to 65% of the initial value (Figure 2). The concentration range can be shifted to 1 μM by employing higher concentrations of Mg^{2+} or by prolonging incubation times.

This sensor showed excellent selectivity for cocaine over its metabolites benzoyl ecgonine (**2**) and ecgonine methyl ester (**3**) (Figure 2). Two important negative controls support a mechanism of action based on ligand-driven association of subunits. First, when **C2** without dabcyl was substituted for **C2-D**, no fluorescence quenching was observed. Second, when **C2-D** was combined with an analogue of **F-C1** incapable of binding cocaine due to substitution of the $N_{12}-N_{20}$ segment d(ATGAAGTGG) with d(AAAAAAAAA), no cocaine-dependent quenching was

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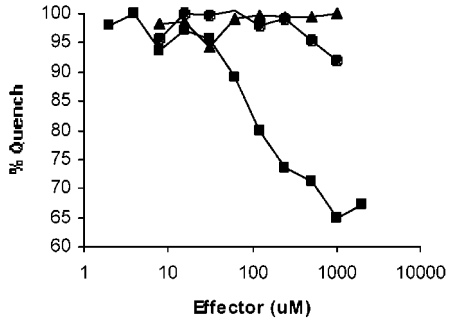

Figure 2. Quenching vs effector concentration curve for (■) cocaine, (▲) ecgonine methyl ester, and (●) benzoyl ecgonine at $c(Mg^{2+}) = 1$ mM, 1 h, room temperature.

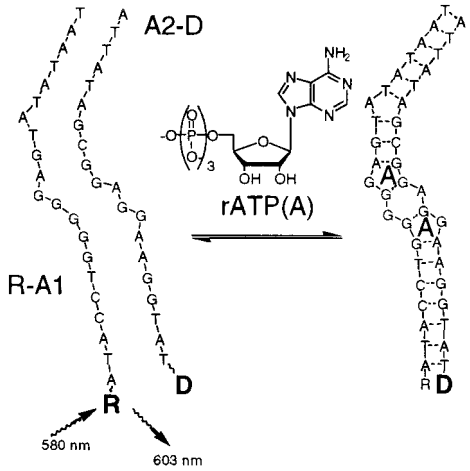

Figure 3. Self-assembly of two aptamer subunits **R-A1** and **A2-D** in the presence of rATP (A); **D**, dabcyl quencher; R, rhodamine; smaller font F quenched rhodamine.

detected. It should be noted that the folding of individual chains probably limits the magnitude of quenching of this sensor. Also, to avoid false positive or negative results in our practical applications, we would need to control confounding fluorescent quenchers and effectors of double-helix formation.

To test the generality of this approach, we also adapted a bipartite aptamer[6] (reported monomeric aptamer $K_d \approx 6$ μM) specific for adenosine ribonucleotide triphosphate (rATP) to generate a fluorescent sensor of its ligand (Figure 3). We labeled the subunits with a 5′-rhodamine (**R** in **R-A1**) and a 3′-dabcyl quencher (**A2-D**). Over the rATP concentration range of 8−2000 μM, at the optimum concentrations of c(**R-A1**) = 10 nM and c(**A2-D**) = 60 nM and $c(Mg^{2+}) = 0.5-5$ mM, this sensor reliably reported rATP concentrations, and the emission at 603 nm ($\lambda_{ex} = 580$) from rhodamine was quenched up to 40% of the initial value (Figure 4). No significant changes in fluorescence were observed upon exposure to other nucleotide triphosphates (Figure 4).

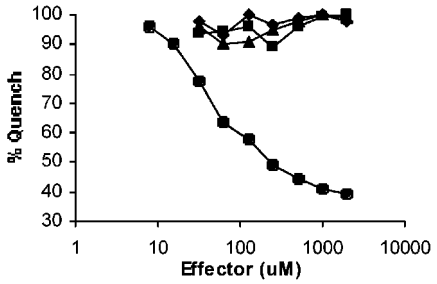

Figure 4. Quenching vs effector concentration curve for (●) rATP, (◆) rUTP, (▲) rGTP, and (■) rCTP at $c(Mg^{2+}) = 2$ mM, 1 h, room temperature.

Finally, the two sensors can be combined to simultaneously report the presence of cocaine and rATP through changes in fluorescence. The fluoresceine-labeled anti-cocaine heterodimer reports at 518 nm, whereas the rhodamine-labeled anti-rATP reports at 603 nm. Within the concentration range from 10 μM to 1 mM rATP and 10−150 μM cocaine, the presence of the rATP does not influence cocaine detection and vice versa. At cocaine concentrations above 150 μM, the sensitivity of the rATP sensor was reduced, because of the chain homology between **R-A1** and the cocaine aptamers.[10] This preliminary demonstration of multiplex reporting capabilities is significant because the current state-of-the-art multicolor detection dye systems would allow for the simultaneous detection of up to six analytes[12] by heterodimeric aptamers.

The ligand-dependent heterodimerization of aptameric subunits has the potential to be a general approach to the construction of fluorescent sensors. Lower magnesium concentrations minimize self-assembly in the absence of ligand, and the low background favors application to microchip sensor arrays.[13] We will report our progress in this area separately.

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Supporting Information Available: Fluorescence spectra of two heterodimeric aptamers in the presence of increasing amounts of ligands (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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