Aptamer-Based Folding Fluorescent Sensor for Cocaine

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Abstract: We adapted in two steps a deoxyribonucleotide-based aptamer to signal the recognition of cocaine: an instability was engineered in one stem of a three-way junction that forms the cocaine-binding pocket and the resulting short stem was end labeled with a fluorophore and a quencher. In the absence of cocaine, two stems are open, but in its presence they close and the three-way junction forms. This major structural change brings fluorophore and quencher together thereby signaling the presence and concentration of ligand. The sensor is selective for cocaine over its metabolites, can operate in serum, and is useful for the screening of cocaine hydrolases.

Introduction

The construction of an optical chemical sensor able to operate in homogeneous solution requires the direct coupling of a molecular recognition process to a change in an optical property. Few general and practical solutions to this challenge are available.[1] Oligonucleotide-based binders, or aptamers, can be selected for affinity to small molecules, biopolymers, surfaces, or even whole cells,[2] and can be used instead of antibodies as recognition elements in heterogeneous assays.[3] Homogeneous assays in which aptamers act as sensors and directly report analyte concentrations are of considerable interest and are actively being sought.[4] In a potentially general approach to stoichiometric noncompetitive sensors, Ellington et al.[5] integrated a fluorophore transducing element into an aptameric recognition element. Residues close to the binding site of adenosine ribonucleotide triphosphate (rATP) were modified with fluorophores and some of the modified aptamers showed increases in fluorescence up to 50% upon ligand binding. This approach has been recently expanded to the in vitro selection of signaling aptamers.[6] In an alternative approach to homogeneous assays, we considered the possibility that an aptamer might be rationally redesigned, such that ligand binding would introduce a substantial change in secondary structure. This change would then be reported through double end-labeling with a fluorophore and a quencher. In the most extreme implementation of this strategy, others and we constructed bipartite aptamers that self-assemble in the presence of their ligands.[7] On the basis of this experience, we hypothesized that a single-chain aptamer might be rationally destabilized such that stem formation would depend on ligand binding. We hoped that this bimolecular recognition event would promote a rapid response time and enable operation in complex media. We now report a double-end labeled aptameric sensor with ligand-induced stem formation that permits optical detection of the ligand through fluorescence quenching.

Results and Discussion

Design of the Sensor. Aptamer **MNS-4.1** is a member of a family of cocaine (**1**) deoxyribonucleotide-based binders constructed in the course of our studies[8] on a new class of peripheral blockers for cocaine overdose and addiction.[9] This aptamer has a dissociation constant of ∼0.4−10 μM. The secondary structure suggested in Figure 1 is based on mutational analysis, including both random and site-specific mutagenesis. The proposed binding pocket is located in the lipophilic cavity of a three-way junction. The accumulation of negative charges and hydrophobic surfaces within the three-way junction necessitates stems of sufficient length and complementarity to stabilize the structure.[10] The short S_3 stem contains two noncanonical base pairs, and by analogy to a previously reported anti-ATP aptamer[11] this stem is likely formed only in the presence of ligand. We hypothesized that an engineered instability in one of the remaining stems of this aptamer would yield a structure dependent on the stabilization provided by ligand binding to form the three-way junction. Thus, we constructed aptamer **MNS-7.9** with a shortened S_1 stem and found that it retained significant, albeit reduced, affinity for cocaine with a K_d of ∼

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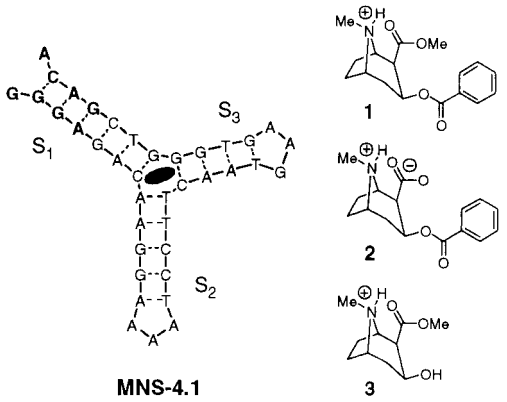

Figure 1. Anti-cocaine aptamer **MNS-4.1** bound to cocaine **1** (black elipsoid). The bold region was cut out to generate **MNS-7.9**. Also shown are cocaine metabolites benzoyl-ecgonine **2** and ecgonine methyl ester **3**.

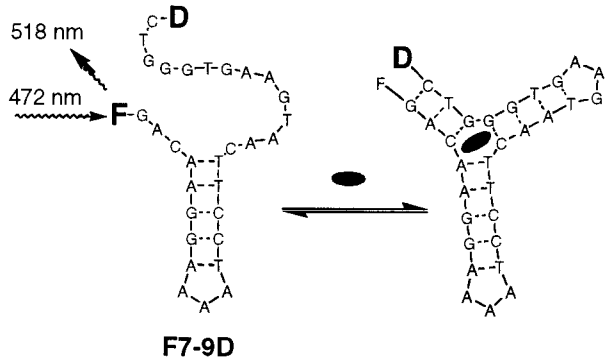

Figure 2. Sensor **F7.9D** signals the presence of cocaine in solution by conformational change that approximates 5′ and 3′ ends ((**F** fluorescein (**4**), F (smaller font) quenched fluorescein, **D** dabcyl (**5**) quencher). The open structure of the aptamer is supported by secondary-structure prediction programs.[13]

20 μM. If **7.9** required cocaine binding to stabilize its three-way junction and close the stem, then double end-labeling of the S_1 stem with a fluorophore and a quencher would result in a sensor analogous to molecular beacons. Fluorescence quenching would report the relative extent of cocaine-dependent folding of **7.9** and, through the appropriate calibration curve, the concentration of cocaine. The potential of the double end-labeling method was demonstrated in the recent report that fluorescence resonance energy transfer can be used to track the conformational changes in tertiary structure of individual RNA molecules induced by binding of ribosomal proteins and by variations in Mg^{2+} concentration.[12]

F7.9D Acts as a Cocaine Sensor. Accordingly, we constructed the double-labeled aptamer **F7.9D** as shown in Figure 2 ($K_d \sim 100$ μM) with a 5′-fluorescein (**F**, **4**) as the fluorophore and a 3′-dabcyl (**D**, **5**) as the quencher (Figure 3). Dabcyl is a universal, non-Förster quencher that is used in molecular beacons to follow the intramolecular interactions of the termini of double-labeled oligonucleotides in numerous practical applications.[14] Efficient π-overlap quenching occurs only in the hybridized state, when the 3′-dabcyl quencher is close to the 5′-fluorophore.

When **F7.9D** was exposed to cocaine in the Mg^{2+}-free tris(hydroxymethyl)aminomethane (TRIS) selection buffer (c(TRIS) = 20 mM, pH 7.4, c(NaCl) = 140 mM, c(KCl) = 5 mM), the aptamer reproducibly behaved as a fluorescent sensor for cocaine in the concentration range from 10 μM to 2.5 mM (Figure 4). Fluorescein emission at 518 nm (λ_{ex} = 472 nm) was quenched to 61% of the baseline value.[15] The aptamer **4.1** was selected to bind cocaine under $c(Mg^{2+}) = 1$ mM, but its analogue **F7.9D** functions well in Mg^{2+}-free buffer. The performance of the sensor decreased somewhat with increasing concentrations of the Mg^{2+} (for cocaine at c = 1 mM, the fluorescence was quenched 9% less efficiently for Mg^{2+} at c = 2 mM than at c = 0). The ability of the sensor to function without Mg^{2+} is important because the influence of small variations in Mg^{2+} can be suppressed by the addition of chelating resins. Values in Figure 4 were obtained over a period of two months in seven independent runs without recalibration, correction, or strict temperature control and the concordance of the data attest to the sturdiness of this sensor under moderately variable experimental conditions. As a negative control we constructed **F7.9**, an aptamer labeled with only the 5′-fluorescein group, and found that fluorescence remained nearly constant over the same concentration range of cocaine. This result confirms the requirement for two chromophores and excludes major participation by some of the alternative quenching mechanisms, such as intermolecular quenching by cocaine, or intramolecular quenching by base-stacking upon change in tertiary conformation. However, we cannot exclude a contribution of some tertiary folding that brings chromophores into proximity. The unresponsiveness of the monofluorophoric aptamer also eliminates the possibility that subtle changes in pH significantly influence fluorescein emission. Substitution of the dabcyl chromophore with the traditional fluorescence resonance energy transfer acceptor tetramethylrhodamine reduced maximum quenching to 70% of the initial value, without a corresponding increase in the tetramethylrhodamine emission. This result is consistent with the proposed mechanism of action for the sensor, i.e., upon folding tetramethylrhodamine appears to act as a π-overlap quencher, albeit less efficiently than dabcyl.

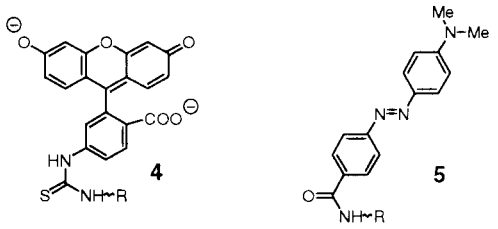

Figure 3. Structures of fluorescein (**4**) and dabcyl (**5**); R represents the position of attachment of tethered oligonucleotides.

We also tested sensor performance with Mg^{2+} concentrations from 0 to 50 mM and NaCl concentrations from 0 to 1000 mM (c(TRIS) = 20 mM, pH 7.4), and under all conditions cocaine caused fluorescence quenching (Supporting Information). Of note, higher salt concentrations were associated with lower background fluorescence and quenching efficacy, consistent with stabilization of the weakened stem. The sensor operated in phosphate buffer (c = 20 mM, pH 7.0), although quenching was not as efficient as in the selection buffer (for 1 mM cocaine

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(15) The basis of this limited response to cocaine is the subject of ongoing investigation. One possibility is that the two-state model (Figure 2) is an oversimplification, and that other conformers or oligomers exist that do not equilibrate to the three-way junction on the time scale of the experiment. It is also possible that the shortened S_1 stem is not fully formed even after the three-way junction is stabilized by cocaine, leading to the less efficient proximity quenching.

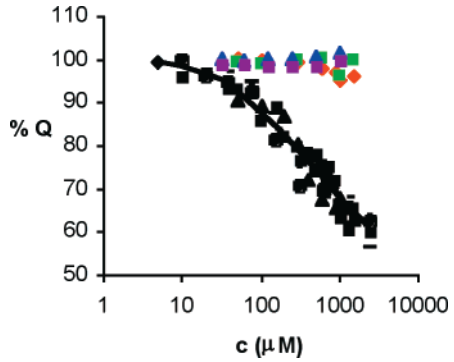

Figure 4. Fluorescence quenching (*Q*, %) vs concentration (*c*) of analytes (in μM): (a) cocaine (**1**, black), seven different runs within two months; (b) benzoyl-ecgonine (**2**, red); (c) ecgonine methyl ester (**3**, green); and (d) **F7**−**9** control vs *c*(**1**) (blue).

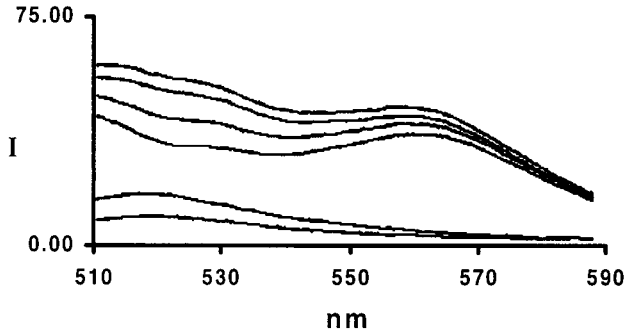

Figure 5. Fluorescence spectra of (from top to bottom) (a) **F7.9D** in serum, (b) **F7.9D** in serum + 100 μM of cocaine, (c) **F7.9D** in serum + 1 mM of cocaine, (d) serum only, (f) **F7.9D** in buffer, and (e) **F7.9D** in buffer + 1 mM of cocaine.

71% vs 85%) and background fluorescence was 60% lower. Apparently, the positively charged TRIS buffer favors the open form of the sensor. In temperature-stable phosphate buffer the sensor operated well up to physiological temperature (37 °C), with stronger quenching (61% vs 85%) and 2-fold higher fluorescence background, consistent with opening of the short stem of the sensor at elevated temperatures. These results indicate that the three-way junction is metastable under a variety of conditions and that specific interactions with cocaine are able to provide additional stabilization under all conditions tested.

The aptameric sensor operated well in one of the most challenging media, serum, in the cocaine concentration range from 10 to 4000 μM (Figure 5 and Supporting Information). Nonspecific interactions with proteins did not hinder interactions with cocaine, and after correction for the background fluorescence of serum, results differed only by a slightly higher quenching efficiency. Although oligonucleotides are prone to degradation by nucleases in serum, **F7.9D** is a double-labeled oligodeoxynucleotide with fast response time and was stable under conditions of brief exposure. For clinical and forensic applications, a cocaine sensor must have picomolar sensitivity for cocaine metabolites and thus the **F7.9D** sensor is limited to high throughput screening and similar in vitro applications (vide infra).

F7.9D Is Selective for Cocaine over Metabolites. The selectivity of the sensor for cocaine over its metabolites was high. **F7.9D** showed no significant change in fluorescence in the presence of benzoyl ecgonine (**2**) and ecgonine methyl ester (**3**) (Figure 4) at concentrations 2 orders of magnitude above the cocaine detection threshold. This selectivity suggested an application in screening assays for cocaine hydrolases. For example, to a reaction mixture of cocaine (0.8 mM) and lipase

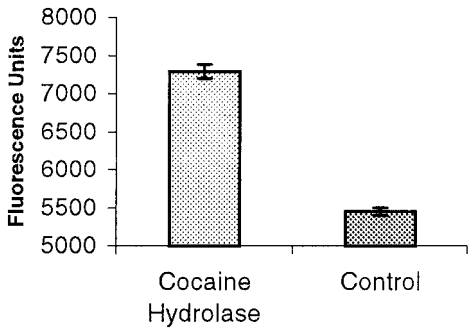

Figure 6. Detection of hydrolase activity of lipase type II from porcine pancreas (lighter bars, left) vs negative controls (darker bars, right) by **F7.9D**. Results were from triplicate runs with standard deviations shown.

type II from porcine pancreas (independently identified as a cocaine hydrolase through catElisa assay; de Prada, unpublished results) addition of **F7.9D** reproducibly showed greater end-point fluorescence than lipase-free control (Figure 6). Cocaine degradation in these experiments was verified by our assay for ^{3}H-cocaine hydrolysis.[9]

The double end-labeled aptamers also provided important thermodynamic and kinetic information about the potential of aptamers to function as stoichiometric peripheral blockers. The demonstrated selectivity of the **F7**−**9D** family for cocaine over metabolites is important for clinical applications, because cocaine metabolites accumulate in vivo. Because fluorescence quenching reaches a maximum within approximately 30 s of the addition of cocaine, a reservoir of circulating aptamers could respond rapidly to blunt cocaine effects under challenging administration conditions.

Selectivity of F7.9D Supports the Proposed Mechanism of Sensor Action and the Binding Pocket Structure. To assess the orientation of cocaine in the binding pocket, we tested the binding of the unnatural (+) antipod of cocaine. If the C.2 carbomethoxy group of cocaine was involved in any base-specific interaction with aptamer, we would expect to see a decrease in fluorescence quenching by the enantiomer, and we would expect similar results in the case of close contacts between C.3−C.4 and C.4−C.5 bonds and hydrophobic surfaces within the three-way junction. We found that the enantiomers were almost indistinguishable in their ability to induce fluorescence quenching. This lack of significant enantiodiferentiation suggests a binding interaction between cocaine and aptamer wherein the planes of symmetry of the tropane ring and the phenyl group of cocaine are aligned with the plane of the three-way junction such that the carbomethoxy group of either antipod projects out of the lipophilic cavity. For the selection of this aptamer, cocaine was attached to the affinity column through the carbomethoxy group and this orientation would seem a plausible consequence of this tether site. We found[8] for **MNS-4.1**, in which the S_1 stem preexists and the three-way junction is already partially formed, that the selectivity of aptamers for metabolites was only severalfold, compared to at least 2 orders of magnitude in **F7.9D**. Thus, the binding elements necessary for inducing formation of stems and for the proper folding of **F7.9D** into a hydrophobic pocket are present in both (−) and (+) cocaine, whereas the metabolites apparently lack a stabilizing group (i.e. the absence of a benzoyl group in **2**), or possess a destabilizing group (i.e. the presence of the carboxylate in **3**) that interferes with formation of the highly negatively charged three-way junction.

Comparison of F7.9D to Other Aptameric Sensors. It is instructive to compare the **F7.9D** folding cocaine sensor to the

previously reported aptameric sensor for rATP and our self-assembling heterodimeric sensors. In contrast to the rATP sensor, our folding cocaine sensor operates optimally without Mg^{2+} and apparently relies primarily on a ligand-dependent formation of secondary structure, as opposed to an induced-fit conformational change. Both systems have high background fluorescence. The two systems have similar practical response ranges (12.5−1500 μM for cocaine vs 20−900[5] μM for ATP) and response magnitudes (up to 60% decrease for cocaine vs 49%[5] or >100%[6] increase for ATP). In the cocaine system the response direction can be reversed by a targeted introduction of a bulge with a fluorescent base in S_1 or by excimer formation.

Unlike folding sensors, the self-assembled sensors are both time- and aptamer-concentration dependent and any confounding effectors of the double helix formation would strongly influence their self-assembly. The folding sensor acts almost instantaneously, an advantage for clinical applications such as the construction of sensors for proteins.

Other reported aptamers could be adapted to serve as folding sensors. For example, we have found that a previously reported anti-rATP aptamer[16] **DH32.39** with a short terminal stem and a significant number of noncanonical base pairs acts as an rATP sensor when double end-labeled. This potential generality suggests an opportunity for multicolor detection. [17]

Conclusions

We engineered anti-cocaine aptamer **MNS-7.9** to obtain a partially folded structure with a ligand-induced binding pocket based on terminal stem-closure. When double-end labeled, aptamer **7.9** could report cocaine concentrations through changes in fluorescence. The sensor operates in the micromolar range with an affinity typical for an aptamer against a small molecule.[18] However, aptamers can bind proteins with nanomolar or even picomolar affinity. The ability of this sensor to operate in serum suggests that this design can provide a new set of analytical tools for clinical chemistry: rapid, homogeneous assays with the potential to simultaneously report the presence or absence of multiple disease-marker proteins within bodily fluids.

Materials and Methods

General. Cocaine hydrochloride and cocaine derivatives (benzoyl ecgonine and ecgonine methyl ester hydrochloride) were obtained as a gift from the National Institute for Drug Abuse. All aptamers were custom-synthesized and HPLC or gel-purified by Integrated DNA Technologies, Iowa, U.S.A, and were used as received. Aptamers were preheated in buffer to 88 °C for 5 min and allowed to cool to room temperature 1 h before use. Serum was obtained as a gift from Long Island Blood Services (Melville, NY) and was sterile-filtered through 25 μm filter before use. All fluorescent spectra were obtained on a Hitachi Instruments Inc. (San Jose, CA) F-2000 fluorescence spectrophotometer with a Hamamatsu xenon lamp.

Procedure. To a solution of aptamer (10 nM, 480 μL) in a selection buffer (c(TRIS) = 20 mM, pH 7.4, c(NaCl) = 140 mM, c(KCl) = 5 mM) or phosphate buffer (c = 20 mM, pH 7, c(NaCl) = 200 mM) or serum at room temperature in a spectrofluorometer cuvette was added a solution of cocaine or derivatives (20 μL). Fluorescence measurements were usually taken within 1 min, after a stable signal was observed (λ_{ex} = 472 nm, λ_{em} = 518 nm).

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Supporting Information Available: Plot of the fluorescence quenching versus concentration of cocaine and a table showing salt and temperature compatibility of F7.9D (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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