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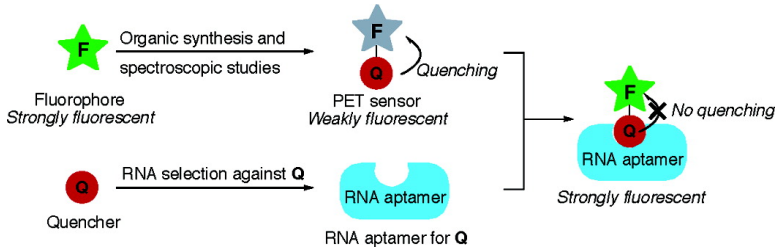

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A Strategy for the Development of Small-Molecule-Based Sensors That Strongly Fluoresce When Bound to a Specific RNA

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Fluorescent imaging is a powerful tool to study biomolecules both in vivo and in vitro. Since the first fluorogenic calcium sensor,[1] chemosensors for specific ions[2] and proteins have been developed by rational approaches.[3] Unlike these imaging methods based on cell-permeable reagents, sequence-specific RNA imaging is currently limited to oligonucleotide- or protein-based approaches,[4,5] suffering from either high background fluorescence, poor permeability, or instability.[6] A more desirable RNA-imaging method would require stable small molecules that permeate cells and fluoresce only in the presence of target RNA. DNA encoding this RNA could be used as a new type of reporter gene, enabling the study of gene expression at the RNA level. It is well-documented that one can find exogenous RNAs that bind to arbitrarily chosen small molecules;[7] the unanswered question is how such RNA binding can be rationally translated into fluorescence induction. In this paper, we describe a broadly applicable strategy for the development of RNA that enhances the fluorescence of a chemosensor.

Many ion sensors have been rationally designed on the basis of the photoinduced electron transfer (PET) process. These PET sensors are modular, consisting of two parts, the fluorophore and the quencher(s), the latter acting as the ligand for the analyte and the fluorescence switch (Figure 1).[8] The fluorescence signal of such a sensor is correlated to both the HOMO energy level of the quencher(s) and the quencher−fluorophore distance. The binding of an analyte to the quencher lowers the HOMO level of the quencher(s) and/or increases the quencher−fluorophore distance. These changes reduce the rate of the PET-based quenching process, thereby enhancing the fluorescence emission. We reasoned that this chemical principle could be applied to the development of RNA chemosensors: if RNA binds to the quencher(s) of a PET sensor and either lowers the HOMO level or increases the quencher−fluorophore distance, the resulting PET suppression would induce fluorescence enhancement (Figure 1). Such an RNA−quencher interaction would be independent of the fluorophore, potentially enabling the rational design of PET sensors based on different combinations of fluorophore and RNA ligand (quencher) modules. To the best of our knowledge, such an approach has never been explored.

Toward the validation of this approach, we previously synthesized and studied the cell-permeable PET sensor **1** (Figure 2).[9,10] This compound contains a dichlorofluorescein moiety as the fluorophore and *N*-(*p*-methoxyphenyl)piperazine (MPP) moieties as the quenchers, exhibiting weak fluorescence ($\Phi = 0.025$). Our previous NMR analysis showed that **1** preferred the closed conformer **1**α (Figure 2) to the open conformer **1**β.[8] We presume that **1**α is weakly fluorescent due to the short distance between the fluorophore and the aniline nitrogen atoms of the quenchers and that **1**β is more fluorescent due to the longer distance. Compound **1** was found to fluoresce more strongly when its MPP moiety was bound to β-cyclodextrin, verifying that the MPP moieties of **1** function as an off−on fluorescence switch, presumably via a conformational change due to steric repulsion between the bound cyclodextrin and the fluorophore.[8] These results suggest that compound **1** could be used as a model sensor to prove the principle shown in Figure 1: if the quencher (MPP) moiety of **1** binds an RNA, the resulting **1**−RNA complex could be more fluorescent than unbound **1**.

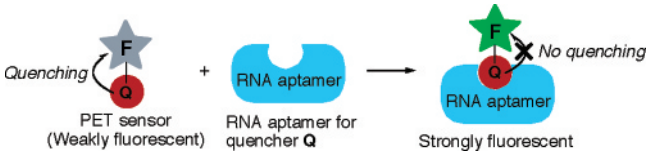

Figure 1. Fluorescence induction by quencher−RNA interaction. PET = photoinduced electron transfer; **F** = fluorophore; **Q** = quencher.

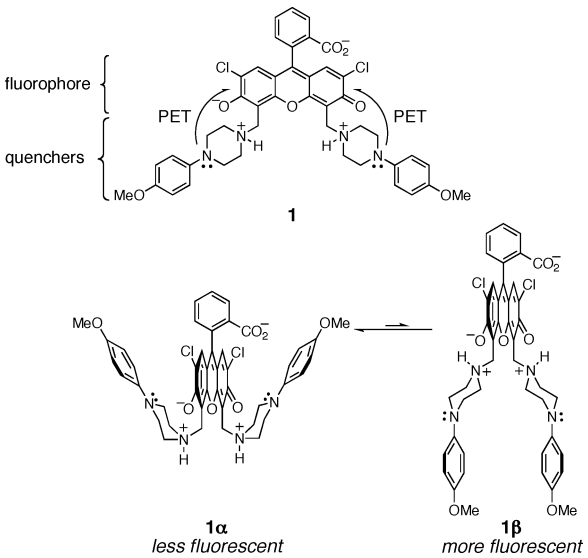

Figure 2. Structure and conformations of the fluorogenic compound **1**.

The crucial question was about how such RNA could be discovered in a broadly applicable manner. To address this question, we turned to in vitro RNA selection. This technique allows for the isolation of RNA aptamers, RNA that bind to specific ligand, from a population of random sequences.[7] Our spectroscopic studies of **1** suggest that interactions with the MPP quencher moiety (such as binding of β-cyclodextrin or RNA) will result in the fluorescence induction of **1**. Thus, we used the MPP moiety alone as bait to find the desired RNA aptamers. If **1** were instead used as bait, the resulting aptamers would presumably bind to the abundant but weakly fluorescent conformer **1**α, shifting the equilibrium further toward **1**α and not inducing a significant fluorescence signal. On the basis of this logic, we synthesized the MPP resin **2** (Figure 3) as bait via the reductive amination of AminoLink Plus Coupling Gel (Pierce) with MPP and $NaBH_3CN$.

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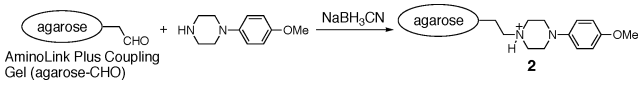

Figure 3. Preparation of affinity column **2** for in vitro RNA selection.

Apt1: 5′ CCUCUGUCCUGAAGCUGCCAGUUCUGACAGGAGUACGG AACUUAAGUGCGUGCUGGAAGCAUGUGCGAGCGAGACCUGCUAGC GUCCGGUGCACAUGUGCACUUGAGCCA 3′

Figure 4. Sequence of an RNA aptamer for MPP. The randomized regions (70 nucleotides) are underlined.

With this affinity column, we proceeded to carry out in vitro selection with an RNA library. Briefly,[11] this library was designed with a 70-base randomized region flanked by constant regions on the 5′- and 3′-ends for enzymatic manipulation. The RNA library, consisting of approximately 10^{13} different sequences, was produced via in vitro transcription from the corresponding chemically synthesized DNA library. In rounds 1−6, the RNA library was incubated directly on MPP resin **2**. After incubation, the resin was washed first with a selection buffer to remove nonbinding RNA, then with a selection buffer containing MPP to specifically elute the binding RNA aptamers. In rounds 6−8, before incubation on resin **2**, the library was passed over the commercial agarose resin to remove any RNA that bound to the agarose portion of **2** instead of the MPP ligand (negative selection). In each round, the eluted RNA aptamers were amplified for the next round of selection with RT-PCR followed by in vitro transcription.

After these eight rounds of selection, the resulting DNA pool was cloned and sequenced. The 18 sequences we determined contained three consensus sequences (**Apt1**, Figure 4; **Apt2** and **Apt3**, Supporting Information). Analysis of these three aptamers using the MFOLD program[12] indicated that they may assume stem-loop structures typical of aptamers that bind to small molecules (see Supporting Information).[13]

The ability of the MPP aptamers to enhance the fluorescence of **1** was determined with fluorescence titration. The fluorescence intensity of **1** at 1 μM was monitored at RNA concentrations up to 100 μM for each aptamer. Although two of the three aptamers did not exhibit significant fluorescence enhancement (2-fold at best; data not shown), to our delight, **Apt1** enhanced the fluorescence signal of **1** by 13-fold at the 100 μM concentration in a concentration-dependent manner (Figure 5). Addition of MPP was found to antagonize this fluorescence induction effect (Supporting Information), suggesting that **Apt1** induces the fluorescence of **1** by the mechanism we originally envisioned (Figure 1). The titration curve implies that the stoichiometry may be 1:1. The substitution of N70 library RNA for **Apt1** did not result in fluorescence enhancement, thus excluding the possibility of enhancement from nonspecific interactions. Although the concentrations of **Apt1** required to observe a change in fluorescence signal are not cellularly relevant, these proof-of-principle studies show that an RNA that binds to a fluorogenic compound through RNA−quencher interactions can restore the fluorescence by suppressing the PET process.

In summary, our results demonstrate that the fluorescence emission of a fluorogenic molecule can be enhanced by an RNA

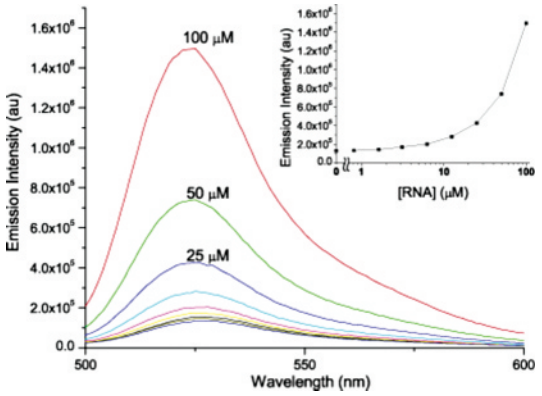

Figure 5. Fluorescence induction of **1** by **Apt1**.

identified through in vitro RNA selection. It is noteworthy that a series of experiments was designed so that the RNA binding in in vitro RNA selection could be directly linked to fluorescence enhancement. Further studies of **Apt1**, other sensors, and the effects of multiple electron donors on fluorescence quenching are underway.

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Supporting Information Available: Synthetic procedures, spectroscopic data, and RNA selection procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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