Light-up properties of complexes between thiazole orange-small molecule conjugates and aptamers

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ABSTRACT

The full understanding of dynamics of cellular processes hinges on the development of efficient and non-invasive labels for intracellular RNA species. Light-up aptamers binding fluorogenic ligands show promise as specific labels for RNA species containing those aptamers. Herein, we took advantage of existing, non-light-up aptamers against small molecules and demonstrated a new class of light-up probes in vitro. We synthesized two conjugates of thiazole orange dye to small molecules (GMP and AMP) and characterized in vitro their interactions with corresponding RNA aptamers. The conjugates preserved specific binding to aptamers while showing several 100-fold increase in fluorescence of the dye (the ‘light-up’ property). In the presence of free small molecules, conjugates can be displaced from aptamers serving also as fluorescent sensors. Our in vitro results provide the proof-of-concept that the small-molecule conjugates with light-up properties can serve as a general approach to label RNA sequences containing aptamers.

INTRODUCTION

The established methods for fluorescent microscopy of intracellular ribonucleic acids (1) rely on either MS2-GFP system (2) or molecular beacons (3). MS2-GFP system suffers from a low signal-to-noise ratio and may be intrusive due to size, while beacons do not readily traverse the cell membranes and are also degraded by nuclease (4).

A different approach, based on small molecule-aptamer pairs with ‘light-up’ properties, has been demonstrated in vitro (4–9), but has yet to be successfully extended to mRNAs in vivo, presumably due to unfavorable characteristics of available fluorophores or insufficient signal-to-noise ratios. Tsien and colleagues were first to report that malachite green in a complex with its RNA aptamer (10) has 2000-fold increase in the quantum yield (5). Sparano and Koide selected the light-up RNA aptamer (10) has 2000-fold increase in the quantum yield (5). Sparano and Koide selected the light-up RNA aptamer against these small molecules, while still achieving an advantage that non-specific light-up probes have well-characterized fluorescent properties (11,12) and have been often already tested in intracellular milieu (13). Our design relied on overcoming the intrinsic lack of specificity of TO through conjugation to small molecules through preferred interactions with a targeted RNA encompassing an aptamer against these small molecules, while still achieving an increase in fluorescence (Scheme 1).

Thiazole orange is a weakly fluorescent dye, which becomes brightly fluorescent (quantum yield up to 0.4, excitation and emission close to fluorescein, $\lambda_{ex} = 480$ nm, $\lambda_{em} = 530$ nm) upon non-specific preferential insertion into double helical nucleic acids ($K_d$ approximately low micromolar, depending on the exact sequence). TO dye has been used in probes targeting nucleic acids, either in conjugates to antisense probes [PNA (14) or DNA (15)] or in conjugates to peptides (16,17) or polyamides (18) for double helical DNA. The common idea behind these approaches was that a sequence-specific targeting module was combined with a non-specific light-up TO dye module, leading to a preferred bivalent binding to a specific sequence coupled with a strong increase in fluorescence.

There are also previous examples of small-molecule-dye conjugates used to study aptamers: for example, Rando’s group has previously taken advantage of environment-sensitive properties of pyrene conjugates to study ligand-aptamer interactions. However, in that approach all states had high fluorescence and the $\lambda_{em}$ is in the UV region (19).

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RNA antagonists, but reported no follow-up observations of light-up properties of these conjugates, albeit fluorescent anisotropy was used for $K_d$ determination (20).

**MATERIALS AND METHODS**

**Materials and instrumentation**

Oligonucleotides were made and DNA/RNAs were free HPLC purified by Integrated DNA Technologies, Inc. (Coralville, IA) and were used as received. TO was obtained from Biochemika (distributed by Sigma-Aldrich Co.). Lambda DNA was purchased from Promega (Madison, WI). RNA from baker's yeast, ATP, GTP, CTP, UTP, AMP and GMP were purchased from Sigma-Aldrich Co. (St Louis, MO).$^1$H NMR (300 MHz, CD$_3$OD) 8.57 (1 H, s), 8.19 (1 H, s), 6.09 (1 H, d, $J = 6.0$ Hz), 4.71–4.75 (1 H, dd, $J = 5.4$ Hz, $J = 6.0$ Hz), 4.38–4.41 (1 H, dd, $J = 3.3$ Hz, $J = 5.2$ Hz), 4.21–4.23 (1 H, m), 4.00–4.03 (2 H, m), 3.37–3.66 (8 H, m), 2.98–3.05 (2 H, dt, $J = 6.0$ Hz, $J = 9.3$ Hz) and 2.80 (2 H, t, $J = 5.1$ Hz). $R_f = 0.65$, silica, 6:1 MeOH/NH$_3$ aq. ESI-MS (positive mode) $m/z = 484$ (M$^+H^+$), 490 (M$^+Li^+$).

**TO-AMP.** AMP-NH-linker-NH$_2$ (12 mg, 0.025 mmol) in 0.40 ml of dimethylformamide (DMF) and 0.30 ml of water were added to a solution of 0.025 mmol TO-OSu in 0.40 ml of DMF. After 14 h, solvent was evaporated and purification was achieved via preparative reverse phase thin layer chromatography (TLC). $R_f = 0.3$, MeOH (4 mg, 16% yield).$^1$H NMR (300 MHz, CD$_3$OD) 8.67 (1 H, d, $J = 9.3$ Hz), 8.56 (1 H, s), 8.46 (1 H, d, $J = 7.2$ Hz), 8.13, (1 H, s), 7.91–8.09 (3 H, m), 7.60–7.80 (3 H, m), 7.42–7.49 (2 H, m), 6.93 (1 H, s), 6.09 (1 H, d, $J = 5.7$ Hz), 4.74 (1 H, t, $J = 10.8$ Hz), 4.65 (2 H, t, $J = 14.7$ Hz), 4.41–4.44 (1 H, m), 4.22–4.28 (1 H, m), 4.04 (3 H, s), 4.02–4.06 (2 H, m), 3.74–3.78 (8 H, m), 3.02–3.11 (2 H, dt, $J = 6.0$ Hz, $J = 9.3$ Hz), 2.20 (2 H, t, $J = 7.2$ Hz), 2.00 (2 H, t, $J = 6.9$ Hz), 1.59 (2 H, m), 1.40–1.35 (2 H, m), 1.20–1.35 (12 H, m) and 0.926 (2 H, t, $J = 4.8$ Hz). $R_f = 0.20$, silica, 6:3:1 dioxane/water/NH$_3$ aq. ESI-MS (positive mode) $m/z = 956$ (M$+Na^+$). HRMS (C$_{46}$H$_{60}$N$_9$O$_9$PS): calcd, 934.4051; found, 934.4019.

**GMP-NH-linker-NH$_2$.** GMP (203 mg, 0.5 mmol) and 2,2’-(ethylenedioxy)bis-ethyamine (0.747 ml, 5.0 mmol) were added to 5 ml of DMSO, followed by PPh$_3$ (655 mg, 2.3 mmol) and 2,2’-dipyridine disulfide (Aldrichirol-2) (660 mg, 3.0 mmol). The mixture was stirred at 40°C for 48 h and at this point the clear solution was observed. The product was isolated by precipitation with LiClO$_4$ in acetone (addition of crude reaction to 20 ml of 2% LiClO$_4$ in acetone, overnight incubation at $-20^\circ$C and centrifugation). The precipitate was resuspended in LiClO$_4$ in acetone (20 ml, 2%) and centrifuged again. The final precipitate was extracted twice with methanol (15 ml). The extracts were combined and solvent evaporated leaving product (70 mg, 60% yield).$^1$H NMR (300 MHz, CD$_3$OD) 8.00 (1 H, s), 5.81 (1 H, d, $J = 6.3$ Hz), 4.70–4.75 (1 H, dd, $J = 4.8$ Hz, $J = 10.2$), 4.32–4.35 (1 H, m), 4.10–4.16 (1 H, m), 3.92–4.03 (2 H, m), 3.35–3.66 (8 H, m), 2.92–3.22 (2 H, dt, $J = 6.6$ Hz, $J = 9.0$ Hz) and 2.74 (2 H, t, $J = 6.0$ Hz). $R_f = 0.8$, silica, MeOH/NH$_3$ aq. ESI-MS (positive mode) $m/z = 492$ (M$^+$.)

**TO-GMP.** A solution of 0.010 mmol TO-OSu in 0.5 ml of dichloromethane was evaporated to a drop of oil in a 1.0 ml Wheaton vial. To this was added GMP-NH-linker-NH$_2$ (26 mg, 0.020 mmol) dissolved in 0.05 ml of DMF and 0.05 ml of 50 mM aq NaHCO$_3$. After 14 h, solvent was evaporated and the residue was purified via preparative reverse phase TLC (3.7 mg, 25% yield).
Fluorescent spectra were taken on a Perkin-Elmer (San Jose, CA) LS-55 luminescence spectrometer. Experiments were performed at the excitation wavelength of 480 nm and emission scan of 510–595 nm. All measurements were performed in 20 mM Tris–HCl (pH 7.4), 140 mM NaCl, 5 mM KCl and 5 mM MgCl2. The dissociation constant \( K_D \) for aptamer with ATP (or GTP) was determined by fluorescence titration of ATP (or GTP) into the complex solution of TO-conjugate and aptamer. The following equation (Supplementary Data) is used for fitting.

\[
[4]_t = \frac{[R]_t - \frac{F}{\varepsilon} - \frac{K_1 F}{\varepsilon[D]_t - F} \left( \frac{K_2[F - F]}{K_1 F} + 1 \right)}{[R]_t - [D]_t}
\]

where \([R]_t\) and \([D]_t\) were the initial concentrations in the mixing solution of aptamer and TO-conjugate. \([4]_t\) was the total concentration of added competitor ATP (or GTP).

RESULTS AND DISCUSSION

The light-up properties of TO-conjugates

The TO-GMP conjugate 1 was synthesized in two steps (Scheme 2 and Supplementary Data) from the previously reported TO-C10 analog (17). We tested several GTP-binding aptamers (21) reported by Szostak’s group with this conjugate (Schemes 2 and 3). The conjugate 1 had negligible fluorescence on its own (lowest spectra in Figure 1a), and showed large (several 100-fold) increase in fluorescence in the presence of various GTP aptamers (Supplementary Data). The strongest fluorescence increase, ~400-fold, was observed with the aptamer GTP Class I (Scheme 3) and we characterized these interactions more closely (Figure 1a). The apparent \( K_D \) of a complex between 1 and this aptamer was approximately 60 nM (Figure 1a, insert), indicating no apparent increase in binding strength over GMP (reported at 23 nM) (21). The substitution of the proposed binding region with the random sequence yielded an aptamer that lost the ability to produce strong fluorescence in the presence of 1 (Supplementary Data).

**Scheme 2.** Synthesis of GMP and AMP-TO conjugate (conjugates were made through an identical procedure and with similar yields).
followed the procedure developed for TO-GMP point mutation that abolishes binding to nucleic acids in the micromolar range (22). The aptamer with a single base pair mutation showed up to 500-fold increase in fluorescence in the presence of the conjugate–aptamer complex.

To confirm that our targeting with conjugates is indeed selective, we tested the cross-reactivity of both TO-AMP and TO-MTP conjugate at increasing concentrations in the presence of varying concentrations of GTP Class I (0, 0.0195, 0.039, 0.078, 0.1563, 0.625 and 2.5 μM, from bottom to top). The insert shows for maximum fluorescence versus aptamer concentration curve fitting (K_d = 60 nM).

The selectivity of TO-conjugates

In order to test the generality of the ‘light-up’ approach to label aptamers, we also synthesized TO-AMP conjugate 2 (Scheme 2). The synthetic process and yields closely followed the procedure developed for TO-GMP. The conjugate again had negligible fluorescence on its own, and showed up to 500-fold increase in fluorescence in the presence of the ATP aptamer ATP-40-1 (22) (Supplementary Data). The apparent dissociation constant of a conjugate–aptamer complex was ~550 nM, indicating a several-fold increase in binding through positive cooperativity between the two binding events [aptamer binds adenosine derivatives in micromolar range (22)]. The aptamer with a single point mutation that abolishes binding [ATP-40-1-G34A, G34 is highly conserved unpaired base (18)] showed no increase in fluorescence beyond what was observed for completely unrelated sequences (Supplementary Data). This result indicates that the binding was small molecule-driven, thus, with a potential for high-selectivity over the unrelated sequences. This conjugate was later used as a fluorescent molecule to study the thermally controlled affinity extraction from aptamer-bound surface on microfluidic device (23). We also synthesized this conjugate with a short linker, and it showed diminished interactions with the aptamer, consistent with bivalent interactions. Finally, we observed diminished interactions when stem without loop was shortened, consistent with intercalation of TO portion in this stem (Supplementary Data).

Scheme 3. Aptamers tested with their conjugates. Black ellipsoid is NMP part of the conjugate, while green fluorescent rectangle is TO-C10 part. In both cases the place of the insertion of TO dyes is shown arbitrarily, although in the case of AMP derivative we have indications based on aptamer modifications (Supplementary Data).

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The selectivity of TO-conjugates

In order to confirm that our targeting with conjugates is indeed selective, we tested the cross-reactivity of both conjugates in the presence of aptamers, and in the presence of unrelated nucleic acids (Supplementary Data). The increase in fluorescence was remarkable only in the presence of targeted aptamers. However, we observed some non-negligible increase in fluorescence with other species as well, indicating that there is residual binding of the TO dye portion to nucleic acids. We tested non-specific responses of λDNA and yeast total RNA in weight had less 50% response than the target aptamer (Supplementary Data). In order to study the selectivity further, we tested TO dye, its C10 analog, and GMP or AMP conjugate at increasing concentrations of GTP Class I aptamer (Figure 1b). As seen in Figure 1b, original TO-dye (with methyl group in place of C10 spacer), at the same concentrations as conjugate, actually yields a bigger fluorescent signal in the high concentration of GTP Class I aptamer (and all other tested nucleic acids as well, not shown), despite somewhat weaker initial binding. In contrast, under the same conditions the TO-dye...
analog with a hydrophobic C\textsubscript{10} spacer used in 1 and 2 shows very low fluorescent response. Mismatched conjugate 2 is even more selective with only negligible interactions with GTP Class 1 aptamer.

These observations are consistent with the mechanism of fluorescent increase for our conjugates: The primary binding event of a small molecule to its aptamer increases the effective concentration (24) of TO dye analogs with otherwise lowered affinity to nucleic acids. In retrospective, the success of our design and the high selectivity of fluorescence increase fortuitously relied on the weakened association between nucleic acids and TO by C\textsubscript{10} modification. Interestingly, both derivatives of Hoechst 33258 (7,8) and dimethylindole red (9) were engineered to suppress nonspecific DNA or RNA binding, and we seem to have accomplished the same characteristic through a fortuitous accident.

The specific displacement of TO-conjugates by small molecules

As expected based on equilibrium considerations, conjugate 1 could be displaced from its complex with aptamer by the excess of GTP, but not ATP, CTP or UTP (Figure 2). Importantly, almost complete displacement indicates the binding is directed by the GMP part of the conjugate. Similarly, conjugate 2 could be displaced from its complex after incubation with either ATP or AMP, at almost identical concentrations, consistent with the reported (22) $K_d$ (Supplementary Data). Other nucleotides were not capable of displacement, indicating that the selectivity of the original aptamer was conserved in its complex. The complete displacement makes complexes of aptamers and their conjugates interesting as displacement sensors for homogeneous detections, with small molecules as analytes. Although our method is synthetically more challenging than simply using off-the-shelf DNA-binding dyes (25–28), it has an advantage of the increased specificity of interactions with aptamers. Because the binding of light-up probes is directed primarily through small molecule ligands, displacement is almost complete, leading to much better signal than in the previous, non-specific designs (26–28). There are many interesting approaches to aptameric sensors (29,30), but there is only one that uses non-covalently modified RNA aptamer to construct light-up probe for the detection of small molecules and is potentially suitable for expressable intracellular sensors (31). With light-up approach described in this work, we can use TO-conjugates to develop light-up probes for the detection of the other small molecules.

We were not just interested in developing yet another set of sensors for purely analytical purposes. Rather, we were looking for indications that, should we eventually be successful in labeling specific mRNA sequences intracellularly, we could also construct them as expressable intracellular sensors for metabolites. Selectivity of binding (for high signal-to-noise ratio) could be further optimized by using multivalent conjugates (32) combined with multipartite modular aptamers (31). Conjugates of other light-up dyes, including those reported to be specific for their cognate aptamers (5,8,9), can be used as well.

In conclusion, we report an apparently general approach (based on two examples) to target aptamers with tightly-bound light-up probes based on TO-dye conjugates to their small molecules. Aptamers binding to small molecules are usually isolated through in vitro selection and amplification procedure that uses affinity selection for small molecule conjugated to the solid phase of affinity column. Conjugation to the TO dye analog instead of an affinity support, may yield light-up aptamer-conjugate couples in all these cases.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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Figure 2. Fluorescence response of TO-GMP (300 nM) containing GTP Class 1 (600 nM) in the presence of varying concentrations of GTP (0, 0.39, 0.78, 1.563, 3.125, 6.25, 12.5, 25, 50, 100 and 200 μM). Small insert shows displacement (% remaining versus concentration) in the presence of GTP, and no response to other NTPs.
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