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Enhance the binding affinity of fluorophore-aptamer pairs for RNA tagging with neomycin conjugation

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Abstract

Fluorogenic sulforhodamine-neomycin conjugates have been designed and synthesized for RNA tagging. Conjugates were fluorescently activated by binding to RNA aptamers and exhibited greater than 250–400 fold enhancement in binding affinity relative to corresponding unconjugated fluorophores.

In recent years, increased attention has been paid to the development of mRNA tagging methods for the in vitro detection of target RNA and its imaging in live cells. A number of mRNA visualization strategies have been reported, such as molecular beacons,¹ and green fluorescent protein (GFP) based systems (e.g. GFP fusion with RNA binding domain or GFP aptamer) for living cell imaging.² One of the potential concerns with the GFP-based method is the large size of the formed protein-RNA complex, which may influence the biochemistry and biology of the target mRNA under study.

A small fluorogenic molecule-based approach is a promising method for specific mRNA tagging since small fluorogenic probes are much smaller and would be amenable to simple co-incubation with target molecules or cells. They can be washed away and re-introduced into cells for repetitive imaging. In this approach, fluorescence of small molecules is initially quenched, but can be recovered upon binding with a specific RNA aptamer. So far several fluorescent probe-RNA aptamer pairs have been developed,³ of which only one (with a K_d of 464 nM) has been successfully applied to live-cell ribosomal 5S RNA imaging.⁴ These probes have failed likely due to very low concentrations of mRNA that normally exists in cells, imposing the requirement for high fluorophore-aptamer binding affinity. Previously we have reported aniline substituted sulforhodamine (ASR) analogues and specific aptamer pairs (Apt10) discovered through a SELEX (Systematic Evolution of Ligands by EXponential enrichment) procedure. ASR dyes were fluorescently quenched by a photoinduced electron transfer (PET) mechanism⁵ and 50–130 fold enhancement in fluorescent signal was achieved after binding with specific aptamers. However, their binding affinities were just at the low micromolar range and insufficient for mRNA imaging in living cells.

In this work, we explored a general strategy to enhance the binding affinity of fluorophores for aptamers by developing fluorogenic ASR-neomycin conjugates. Neomycin, an aminoglycoside known to bind with different RNA structures,⁶ has been conjugated to

pharmacophores, fluorophores, PNA and oligonucleotides for specific recognition or targeting of nucleic acids.^{7,8} Such conjugates generally displayed higher affinity and specificity than those of their parent structures. In our previous study⁵, ASR probes specifically bound to a loop region of Apt10L. Neomycin, however, binds to RNA by non-specific electrostatic interactions. We hypothesize that ASR-neomycin conjugates should have an extended binding region to RNA aptamers and thus display enhanced binding affinity. Herein we report the synthesis of fluorogenic ASR-neomycin conjugates via a Click reaction and characterization of their binding affinity for Apt10 aptamers.

Preparation of azido-neomycin **3** started from a known *N*-Boc and *O*-TIBS (1,3,5-Triisopropylbenzen sulfonyl) derivative of neomycin **2** according to a reported synthetic procedure of Tor *et al.* (Scheme 1).⁸ A substitution with sodium azide followed by deprotection of Boc groups converted compound **2** to azido neomycin **3**. The fluorophore ASR **4** was obtained from sulfofluorescein. ASR **4** was coupled to propargyl amine in order to introduce an alkyne group that would facilitate its conjugation with **3**. Conjugation between compound **3** and ASR derivative **5** proceeded smoothly using Cu (II)-catalysed click chemistry. Two fluorogenic conjugates have been prepared with a different length of linker between neomycin and ASR fluorophores to examine the effect of the linker on the binding affinity.

Both **1a** and **1b** showed very low fluorescence intensity in aqueous buffer solution. However, their fluorescence intensities increased ca. 90-fold in 90% glycerol solution, since such a viscous environment is known to deactivate the PET quenching mechanism (Figure S1). To determine whether neomycin itself could induce the fluorescence activation of ASR compounds by non-specific interactions, various concentrations of neomycin were added to 1 μ M solution of ASR **4** (Figure S2). At low concentrations of neomycin (<1.4 μ M), the fluorescence intensity of **4** increased slightly (ca. 1.5 fold), however, this change was negligible relative to the more than 70–80-fold fluorescence enhancement when ASR **4** bound with Apt10L (40 μ M). Next, we performed a binding competition experiment using neomycin as a competitive Apt10L binder. ASR **4** (1 μ M) was pre-mixed with 10 μ M of Apt10L, then increasing amounts of neomycin were added to this mixture and the fluorescence intensity change was determined (Figure S3). Since the fluorescence intensity of compound **4** showed little change up to 25 μ M of neomycin, non-specific interactions of neomycin with the RNA aptamer did not interfere with the specific binding of the ASR compound.

The binding affinity of neomycin conjugates **1a** and **1b** to Apt10L and Apt10M (Figure S4) were determined by fluorescence titrations. Apt10M is a minimum binding domain (60-mer) of Apt10L (133-mer). We first determined the K_d value and fluorescence recovery of compounds **1a** and **1b** using Apt10L since this aptamer showed the best binding affinity to ASR **4** from our previous results.⁵ The sequence and representative secondary structure of Apt10L and its variants are shown in Figure S4. Job plot analysis showed that the highest intensity was observed at the molar ratio of around 0.3 ([RNA]/[RNA+dye]) for Apt10L and 10M with **1a** (Figure 1a) and for Apt10M with **1b** (Figure 1b), suggesting that the binding stoichiometry between dye and aptamer is 2:1. For **1b** and Apt10L (Figure 1b), the plausible binding seems to have 1.5:1 binding stoichiometry, suggesting that the strong first binding site would be more dominant than weak second binding site at 500 nM of concentration. As a result, the neomycin conjugates **1a** and **1b** have two different binding sites on aptamer, which would provide two different orientations for fluorophore binding. Therefore, K_d and F_{max} for the high affinity binding site (K_{d1} and F_{max1}) were calculated by a specific two site-binding equation.⁹ The fluorescence titration of Apt10L against compound **5a** gave a K_d value of $15.5 \pm 1.7 \mu$ M and the value of F_{max} (maximum fluorescence fold-enhancement at saturation) as 48 ± 3 (Figure 2 and Table 1). The binding affinity (K_d) of Apt10L for

compound **5b** was $99 \pm 33 \mu\text{M}$ and the value of F_{max} was 217 ± 61 ; the different affinity observed with **5a** and **5b** was consistent with our previous study.⁵ However, conjugate **1a** displayed greatly enhanced binding affinity of $0.063 \pm 0.015 \mu\text{M}$ (K_{d1}) for Apt10L with an F_{max1} of 13.9 ± 0.3 . The estimated K_{d} value of compound **1b** for high affinity site showed a more than 400-fold enhanced affinity ($K_{\text{d1}} = 0.24 \pm 0.03 \mu\text{M}$) and F_{max} of 27.9 ± 1.2 . While the F_{max} values of conjugates **1a** and **1b** were lower than those from **5**, the observed fluorescent enhancement of neomycin conjugates was higher than those of corresponding parental ASR derivatives (**5a** and **5b**) at low aptamer concentrations. For example, compounds **5** showed an approximately 3-fold fluorescence enhancement in the presence of $1 \mu\text{M}$ of Apt10L, but conjugates **1a** and **1b** displayed a >15-fold fluorescence recovery under the same conditions. These results further confirm the enhanced affinity of neomycin conjugates for the target RNA aptamer.

We next examined the specificity of neomycin conjugates for the following RNA aptamers: Apt10M, its mutants (Apt10M1 and Apt10M-Lm3; structure of each aptamer was shown in Figure S4.) and a random 130-mer RNA containing 90-mer of randomized sequences (Figure 2c, 2d and Table 2). Conjugate **1a** showed slightly better K_{d} and F_{max} for Apt10L than Apt10M. In comparison, conjugate **1b** showed a ca. 3.8-fold better K_{d} value for Apt10M ($0.063 \pm 0.020 \mu\text{M}$) than for Apt10L ($0.24 \pm 0.03 \mu\text{M}$), suggesting that the long and flexible tetraethylene linker between the neomycin moiety and the fluorophore was more suitable for Apt10M than Apt10L. Binding affinities of **1a** and **1b** for Apt10M were independently measured by a gel mobility shift assay to compare with the fluorescence assay and comparable values were obtained: $0.22 \pm 0.05 \mu\text{M}$ for **1a** and $0.17 \pm 0.07 \mu\text{M}$ for **1b** (Figure S5).

The estimated maximal fluorescence recovery (F_{max}) for mutated aptamers (Apt10M1 and Apt10M-Lm3) (3–5 fold) was much smaller than Apt10L and Apt10M (Table 2). Because of little fluorescence enhancement observed with **1b** and Apt10M1, their K_{d} value cannot be reliably calculated (Table 2), providing further evidence that the linker can enhance the fluorescence activation specificity toward aptamers. Both conjugates showed only ca. 2-fold fluorescence enhancement with random RNA, suggesting that non-specific binding of the neomycin group to RNA structures does not significantly affect the fluorescence activation of the ASR fluorophore. Fluorogenic neomycin conjugates **1a** and **1b** showed large, specific fluorescence enhancement against selected aptamers (Apt10L and 10M). These results show that the neomycin conjugation increases the binding affinity of the conjugates for Apt10L and Apt10M. They may also bind some of the aptamer mutants with enhanced binding affinity since the neomycin interaction is less specific, but the fluorescence activation is highly specific to the binding of the conjugate to the aptamer target. Therefore, the neomycin conjugates display both high binding affinity and high fluorescence turn-on specificity for the aptamer target.

The fluorescence dynamic linear ranges of both **1a** and **1b** were determined for potential applications as an aptamer sensor (Figure S6, Table 1) and as low as 10 nM of RNA aptamer can be detected in solution using a fluorometer. Both showed no toxicity in mammalian cells at $10 \mu\text{M}$ (~100 fold higher than K_{d1}), suggesting their biocompatibility for cellular study (Figure S7).

In previous reports,^{3,5} repeated SELEX and systematic variant and mutation studies have been applied to the optimization of RNA aptamers for improved affinity. This report is the first exploration of the effects of an aminoglycoside (neomycin in this study) conjugation strategy to enhance the binding affinity of fluorogenic probes for aptamers. With this approach, significantly increased binding affinity and high fluorescence activation was achieved without further screening of RNA aptamer sequences.

In summary, we have investigated a bivalent strategy to enhance the binding affinity of activable fluorophores for RNA aptamers through conjugation with neomycin. The fluorogenic conjugates were obtained by facile Cu (II)-catalysed click chemistry and displayed a more than 200-fold increase in binding affinity as compared to unmodified ASR compounds. Moreover, these conjugates showed specific fluorescence activation only upon incubation with selected aptamer. This strategy may be generally applicable for developing high affinity fluorophores for specific RNA aptamers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Job plots to determine the binding stoichiometry between conjugate **1a** with Apt10L and Apt10M (a) and conjugate **1b** with Apt10L and Apt10M (b). FI represents fluorescence intensity. RNA molar ratio is $[RNA]/[RNA+dye]$.

Figure 2. Representative fluorescence titration curves of ASR derivatives **5a** and **5b** (a) and neomycin conjugates **1a** and **1b** (b) with Apt10L. Fluorescence titration curves of conjugates **1a** (c) and **1b** (d) with Apt10M variants (M, M1 and M-Lm3) and random RNA.

Scheme 1.
Synthesis of ASR fluorophore-neomycin conjugates.

Table 1

Estimated K_d , F_{max} and dynamic linear range (DLR) values of ASR fluorophores **4**, **5a**, **5b** and neomycin conjugates **1a** & **1b** with Apt10L (N.D. means not determined).

Compound	K_d (μ M)	F_{max} (fold)	DLR
1a	0.063 ± 0.015	13.9 ± 0.3	11–1000 nM
1b	0.24 ± 0.03	27.9 ± 1.2	16–1000 nM
5a	15.5 ± 1.7	48 ± 3.0	N.D.
5b	99.0 ± 33	217 ± 61	N.D.
4	39.1 ± 7.6	135 ± 15.0	N.D.

Table 2

Estimated K_d and F_{max} values of conjugates **1a** and **1b** with Apt10 variants (M, M1 and M-Lm3) and random RNA (N.C. means not calculable).

Apt10	K_d for 1a (μ M)	F_{max1} for 1a (fold)	K_d for 1b (μ M)	F_{max1} for 1b (fold)
M	0.096 ± 0.026	12.6 ± 1.9	0.063 ± 0.020	11.3 ± 1.7
M1	0.042 ± 0.018	5.6 ± 1.0	N.C.	N.C.
M-Lm3	0.035 ± 0.021	3.7 ± 0.9	0.013 ± 0.011	2.8 ± 0.7
Random RNA	N.C.	N.C.	N.C.	N.C.