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Site-Specific Covalent Labeling of RNA by Enzymatic Transglycosylation

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Supporting Information

ABSTRACT: We demonstrate the site-specific incorporation of nucleobase derivatives bearing fluorophores or affinity labels into a short RNA stem loop recognition motif by exchange of a guanine residue. The RNA-TAG (transglycosylation at guanosine) is carried out by a bacterial (*E. coli*) tRNA guanine transglycosylase (TGT), whose natural substrate is the nitrogenous base $PreQ_1$. Remarkably, we have successfully incorporated large functional groups including biotin, BODIPY, thiazole orange, and Cy7 through a polyethylene glycol linker attached to the exocyclic amine of $PreQ_1$. Larger RNAs, such as mRNA transcripts, can be site-specifically labeled if they possess the 17-nucleotide hairpin recognition motif. The RNA-TAG methodology could facilitate the detection and manipulation of RNA molecules by enabling the direct incorporation of functional artificial nucleobases using a simple hairpin recognition element.

The detection and manipulation of RNA is greatly aided by chemical modification. Therefore, there is tremendous interest in novel methods to site-specifically associate RNA with small molecules such as imaging probes and affinity labels.[1] Conventional methodologies for detecting RNA include the use of antisense probes,[2] aptamers,[3] and fusion proteins that recognize specific RNA secondary structures.[4] Relatively less explored is the use of enzymatic reactions for site-specific RNA labeling.[5] It is known that RNA can be post-transcriptionally modified in numerous ways by specialized enzymes. For instance, there are approximately 85 post-transcriptional modifications of various transfer RNA (tRNA) structures, with the majority of modifications present on the anticodon stem loop.[6] There are several examples of utilizing enzymes to accomplish site-specific covalent modification of RNA.[7] Recent work has also shown that several tRNA-modifying enzymes can covalently attach small analogs bearing either azide or alkyne handles to RNA.[1c,8] Notably, these past approaches have not demonstrated the ability to append large functional molecules directly onto the RNA of interest. Instead they typically rely upon small bio-orthogonal handles, which after undergoing a second chemical reaction can be modified by functional probes such as fluorophores or affinity ligands.

An ideal enzymatic reaction for labeling RNA would involve recognition of a minimal RNA structural motif, would result in irreversible covalent modification, and would be capable of directly incorporating a diverse array of functional molecules (fluorophores, affinity ligands, etc.) in a single step. Here we introduce RNA-TAG (**t**ransglycosylation **a**t **g**uanosine), an enzymatic method to directly append large functional molecules site-specifically to RNA. This method utilizes a bacterial tRNA guanine transglycosylase (TGT) to exchange specific guanine nucleobases with functional derivatives of the bacterial nucleobase $PreQ_1$. By enabling the direct incorporation of functional artificial nucleobases using a simple hairpin recognition element, RNA-TAG should have numerous applications as an RNA labeling tool.

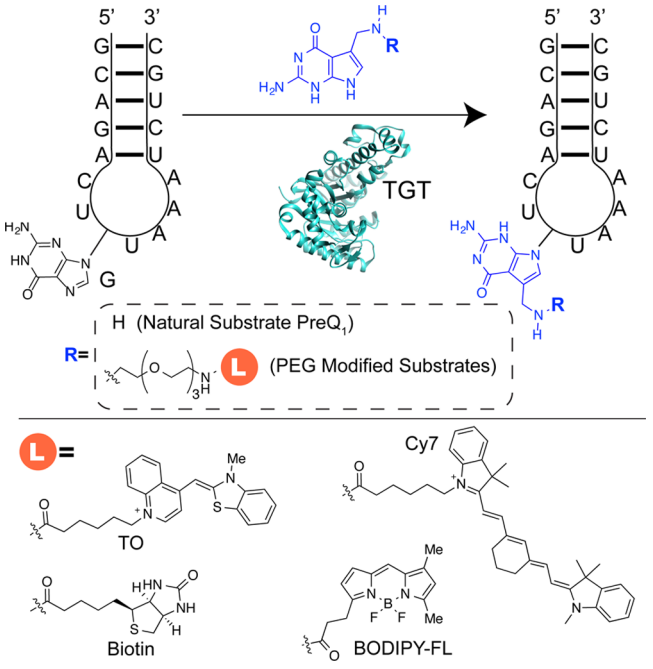

Figure 1. TGT-catalyzed transglycosylation of ECY-A1 minihelix RNA with $PreQ_1$ derivative nucleobases.

TGTs are a well-characterized class of enzymes that are found in archaea, eubacteria, and eukaryotes.[9] While most RNA post-transcriptional modifications occur through derivatization of a genetically encoded nucleoside, TGTs are capable of performing transglycosylation reactions in which a guanine at the wobble position of the anticodon loop is exchanged with 7-deazaguanine derivatives. While eukaryotic TGTs selectively incorporate queuine salvaged from the environment, bacterial TGTs instead incorporate an amine-containing queuine precursor, $PreQ_1$, which is later enzymatically modified to

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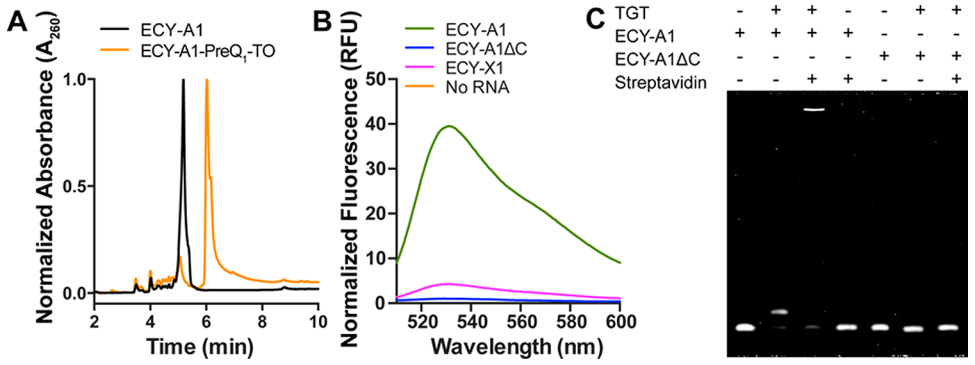

Figure 2. TGT labeling reactions of RNA with $PreQ_1$ derivatives. (A) HPLC trace at 260 nm of ECY-A1 and ECY-A1 modified with $PreQ_1$-TO, indicating covalent modification resulting in a shift of retention time and single TO-labeled product. (B) Fluorescence emission spectra of fluorogenic RNA labeling reaction with $PreQ_1$-TO. Fluorescence normalized to $PreQ_1$-TO in the absence of RNA and TGT enzyme. (C) Biotin gel shift assay illustrating functional biotin covalently linked to ECY-A1. $PreQ_1$-Biotin-modified samples were incubated with streptavidin and analyzed on a 15% denaturing PAGE gel.

yield queuine.[10] Extensive prior studies with the bacterial TGT from *E. coli* revealed a minimal binding domain of a single 17-nucleotide hairpin (ECY-A1) mimicking the anticodon stem loop of the tRNA substrate.[11] Furthermore, complementary studies have shown that the enzyme can recognize extended RNA molecules bearing minimal hairpin recognition elements, including tRNA dimers and mRNA.[12]

Since transglycosylation is an efficient mechanism for the incorporation of highly modified bases into RNA,[13] we speculated that TGT-catalyzed nucleobase exchange could be harnessed to covalently modify RNA site-specifically with synthetic molecules such as fluorophores and affinity ligands. However, although bacterial TGTs have been shown to tolerate various $PreQ_1$ analogs, loss of the exocyclic amine or sterically hindered derivatives can cause a dramatic loss of activity.[14] For instance, several studies have demonstrated that bacterial TGT will not accept queuine as a substrate.[10,15] Indeed, crystal structures of a bacterial TGT with bound RNA and nucleobase substrates reveal several protein contacts with $PreQ_1$, including a key hydrogen bond between the exocyclic amine of $PreQ_1$ and the carbonyl oxygen of a leucine residue.[16] To maintain this important hydrogen bonding interaction in our synthesized $preQ_1$ derivatives, we decided to link functional groups through alkylation of the exocyclic amine. Furthermore, we appended our functional groups via a short oligoethylene glycol spacer, with the goal of extending any large functional groups away from the active site.

To initially test for RNA modification, we utilized the previously reported 17-nucleotide RNA hairpin ECY-A1, which mimics the anticodon arm of $tRNA^{Tyr}$ and has been shown to be recognized as a substrate by bacterial TGT.[11] We incubated ECY-A1 with TGT along with $PreQ_1$ derivatives linked to various fluorophores and affinity ligands such as BODIPY, thiazole orange (TO), Cy7, and biotin (Figure 1). To our delight, we found that all $PreQ_1$ derivatives could be successfully incorporated into the RNA hairpin, as judged by liquid chromatography (LC) retention time shifts and high-resolution mass spectrometry (HRMS) (Figure 2A, Figure S1, and Table S1). It is worth mentioning that the modification worked with large functional groups—in the maximum case, a near-IR cyanine dye of 534 Da molecular weight.

To test for RNA substrate selectivity, we incubated $PreQ_1$-TO, TGT, and RNA hairpin ECY-A1 and compared modifications to alternative RNA hairpins ECY-A1ΔC and ECY-X1 (Table S2). ECY-A1ΔC has the reactive guanine changed to a cytosine, while ECY-X1 maintains the 7-member hairpin loop and UGU recognition element, but shifts the location of the UGU and the neighboring bases. In either case, we were unable to detect incorporation of $PreQ_1$-TO by LC/MS, demonstrating that RNA-TAG is selectively incorporating $PreQ_1$ analogs in place of guanine and that the process is specific to the hairpin recognition element, in agreement with previous studies.[17]

There is significant interest in methods to detect and image RNA.[1] RNA-TAG could be useful for RNA imaging; however, to limit background fluorescence due to an unreacted probe, it would be beneficial if nucleobase exchange was accompanied by an increase in fluorescence intensity. To explore fluorogenic RNA-TAG reactions, we focused our attention on the $PreQ_1$-TO probe. TO probes are known to dramatically increase in fluorescence intensity upon intercalation into double-stranded nucleic acids.[18] While this can lead to nonspecific binding, recent work has demonstrated that TO derivatives bearing functional group handles show decreased nonspecific binding to nucleic acids and, when coupled to small molecules which bind to RNA aptamers, can be used to induce specific fluorogenic responses.[19] As such, we speculated that $PreQ_1$-TO modification of RNA could lead to an increase in fluorescence intensity due to intercalation of the TO probe into the RNA stem, driven by the high local concentration of the covalently bound TO. We tracked the fluorescence intensity of $PreQ_1$-TO before and after covalent incorporation into the RNA hairpin ECY-A1. We found that incorporation of $PreQ_1$-TO led to an approximately 40-fold increase in TO fluorescence intensity, indicating that covalent modification by RNA-TAG likely drives TO-RNA intercalation (Figure 2B).

The ability to use RNA-TAG to introduce a biotin group could have applications for the affinity tagging and pull-down of specific RNAs. While many studies utilize biotinylated RNA, the biotin group is typically incorporated during *in vitro* RNA synthesis.[20] RNA-TAG could be a facile way to covalently incorporate biotin into native RNAs. As a proof of concept, we subjected both ECY-A1 and ECY-A1ΔC to TGT with $PreQ_1$-Biotin and performed a streptavidin gel-shift assay (Figure 2C). We observed a gel shift of ECY-A1 upon binding streptavidin only when the RNA was covalently modified by TGT with $PreQ_1$-Biotin, indicating that the biotin remains functional as an affinity tag, even after incorporation into RNA by TGT.

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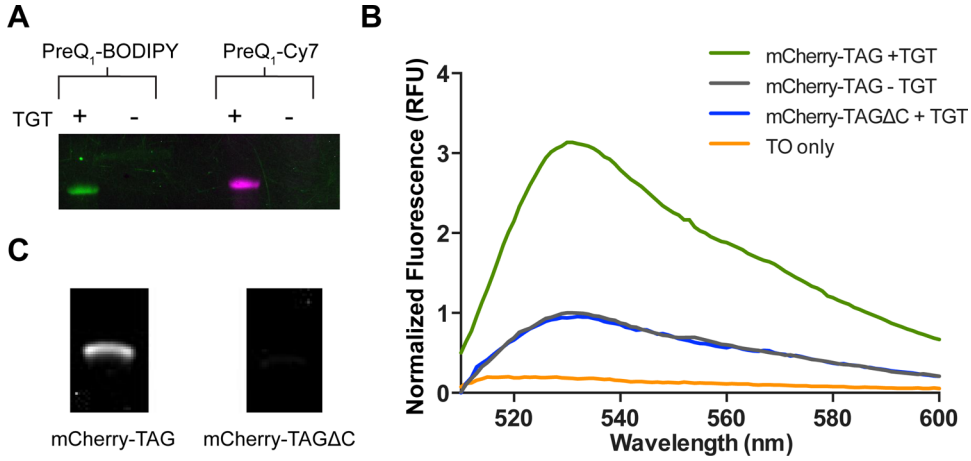

Figure 3. TGT labeling reactions with full-length RNA transcripts containing a recognition sequence in the 3′ UTR. (A) Fluorescent labeling of mCherry-TAG transcript with $PreQ_1$-BODIPY and $PreQ_1$-Cy7 visualized via 4% denaturing PAGE. (B) Fluorescence emission spectra of fluorogenic labeling reaction with $PreQ_1$-TO. Fluorescence normalized to $PreQ_1$-TO with RNA in the absence of TGT enzyme. (C) PAGE analysis of streptavidin pull-down assay. Transcript subjected to TGT reaction conditions with $PreQ_1$-Biotin was subsequently pulled down with streptavidin M-280 Dynabeads. Lanes representing recovered mCherry-TAG and mCherry-TAGΔC are shown.

As our $PreQ_1$-derivatives have structures that deviate significantly from that of natural $PreQ_1$, we next estimated the kinetics of enzymatic incorporation of a fluorophore-linked $PreQ_1$ analog. We capitalized on the fluorogenicity of $PreQ_1$-TO as a facile way to track TGT-catalyzed incorporation of this analog. Monitoring the increase in fluorescence intensity, we determined initial rates and derived an estimated K_m = 9.8 μM and $k_{cat} = 1.6 \times 10^{-3}$ s^{-1} (V_{max} = 1.8 μM s^{-1} mg^{-1}) (Figure S2). Comparatively, $PreQ_1$ has a reported K_m = 0.39 μM and V_{max} = 2.6 μM s^{-1} mg^{-1}, albeit with full-length tRNA.[10,14] Although our substrate has a higher K_m than the natural substrate $PreQ_1$, it does not greatly differ from results found using alternative analogs of $PreQ_1$ with much smaller perturbations in structure.[11,14,17a]

Having demonstrated that RNA-TAG could append unnatural $PreQ_1$ analogs onto minimal RNA hairpins, we next sought to determine if the minimal recognition hairpin could be recognized on much larger RNA molecules such as mRNA transcripts. We inserted the ECY-A1 hairpin within the 3′ UTR of an mRNA transcript coding for the red fluorescent protein mCherry (mCherry-TAG). Transcripts were exposed to $PreQ_1$-BODIPY and $PreQ_1$-Cy7 with and without enzyme. The transcripts were run on a denaturing polyacrylamide gel, and labeling was detected by fluorescence imaging (Figure 3A). When TGT was present, labeling of the transcript took place, as indicated by observation of strong fluorescent bands on the polyacrylamide gel that co-localized with the transcript RNA (Figure S3). The mCherry mRNA transcript contains 11 instances of UGU, making it possible that TGT could modify an off-target sequence. To test this, we mutated the G of the hairpin recognition motif to C (mCherry-TAGΔC) and attempted to incorporate $PreQ_1$-BODIPY. In this case, we observed negligible fluorescence staining of the RNA transcript, demonstrating that labeling is specific to the UGU sequence of our appended hairpin with minimal off-target reactions (Figure S4). To determine if fluorogenic labeling was also possible on *in vitro* transcribed RNA, transcripts were isolated and exposed to $PreQ_1$-TO with or without bacterial TGT. Although background fluorescence was observed due to nonspecific binding of $PreQ_1$-TO to RNA,[19] when TGT was present, we observed a significant increase in fluorescence intensity, which persisted after protease degradation of the enzyme (Figure 3B). Furthermore, this fluorescence increase was reversed upon degradation of the RNA, indicating the probe's ability to serve as a metric for RNA lifetime (Figure S5). We also tested mCherry-TAGΔC for its ability to react with $PreQ_1$-TO. In this case, we observed negligible fluorescence turn-on in the presence of enzyme.

Transcript labeling with $PreQ_1$-Biotin should allow for effective pull-down assays with avidin-based solid support beads. We subjected both mCherry-TAG and mCherry-TAGΔC to TGT labeling with $PreQ_1$-Biotin. The RNA was subsequently treated with streptavidin-linked Dynabeads. The beads were then washed extensively, and the bound RNA was recovered and analyzed using gel electrophoresis. We observed a single RNA band corresponding to the transcript only when mCherry transcript contained the ECY-A1 hairpin in its 3′ UTR (Figures 3C and S6). Additionally, we demonstrated that the mCherry-TAG could be selectively labeled and pulled down in the presence of total CHO RNA extract (Figure S7). This demonstrates that RNA-TAG could have application for the isolation of specific RNA transcripts genetically encoded with the minimal ECY-A1 hairpin.

Given the substrate specificities of bacterial and eukaryotic TGTs, it may be possible to selectively label RNA in eukaryotic cells using RNA-TAG. To test if cellular imaging is possible, we transiently transfected Chinese hamster ovary (CHO) cells with plasmids coding for mCherry-TAG. After verifying transfection by detecting the expression of mCherry by fluorescence microscopy, we fixed and permeabilized the CHO cells and incubated them with 1 μM TGT and 50 μM $PreQ_1$-Cy7. We also performed controls where cells were not administered TGT or transfected with the mCherry-TAG plasmid. We observed significantly greater staining of fixed cells only when they were treated with both TGT and mRNA plasmid. This suggests that endogenous mRNA labeling and imaging is possible using RNA-TAG (Figures 4, S8, and S9).

The RNA-TAG methodology presented herein has been used to fluorogenically label RNA, pull-down RNA from a complex mixture, and image mammalian mRNA transcripts in a fixed cell environment. We believe that site-selective enzymatic transglycosylation will be a powerful tool to covalently modify

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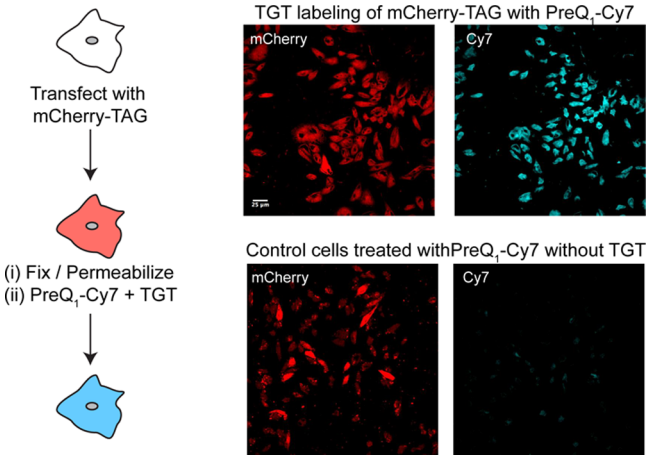

Figure 4. Labeling of mCherry-TAG RNA expressed in CHO cells. CHO cells were transfected with a plasmid expressing mCherry-TAG RNA for 16 h at 37 °C. Cells were fixed, permeabilized, and then treated with $PreQ_1$-Cy7 ± TGT for 4 h at 37 °C, subsequently washed with PBS, and imaged.

native transcripts containing a minimal and encodable 17-nucleotide recognition sequence in a facile, plug-and-play manner.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b07286.

Detailed experimental procedures, additional data controls, and uncut gel images, including Table S1 and Figures S1–S6 (PDF)

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Notes

The authors declare no competing financial interest.

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