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Incorporation Efficiency of 5'-Azido-5'-Deoxyguanosine into 5'-Terminus of RNA for Preparation of Azido-Functionalized RNA

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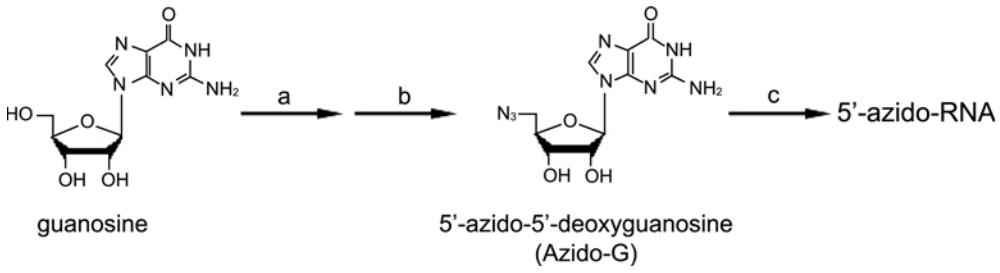
Although the modern biological systems are by large based on DNA genomes and protein enzymes, RNA plays important roles in many fundamental processes in cells, including regulation of protein biosynthesis, RNA splicing, and retroviral replication, with remarkable features.[1] From this point of view, site-specific substitution and derivatization of RNA can provide powerful tools for elucidating RNA structures and functions.[2] The modification of either the 3'- and 5'-termini or an internal position of the oligonucleotides with a primary alkylamine group is a widely used method for introducing additional functional groups to the RNA.[2,3] In particular, several 5'-modifications of RNA molecules such as sulfhydryl modification for functionalizing the 5'-terminus of RNA by a transcription or kinase reaction have been shown to have broad applications in studying RNA structures, mapping RNA-protein interactions, and *in vitro* selection of catalytic RNAs,[4] since a unique functional group incorporated into the RNA can be subsequently conjugated to the desired molecule by a selective chemical reaction. However, there is still a need to develop coupling chemistry with high stability and yield to modify RNA and other biomolecules. In addition, the coupling functional groups are ideally required to be stable under aqueous reaction conditions, and the coupling reaction should be highly chemoselective.[5] In this regard, Cu^{I}-catalyzed azide-alkyne cycloaddition (CuAAC or click chemistry) to form the triazole version of Huisgen's [2+3] cycloaddition family may be the best choice,[6] because this reaction only occurs between alkynyl and azido functional groups with high yield, and because the resulting 1,2,3-triazoles are stable at aqueous conditions and high temperature. Indeed, the azide group is one of the most utilized bioorthogonal chemical tags for biomolecule-conjugate experiments because of its small size and inertness to most components in a biological environment.[7]

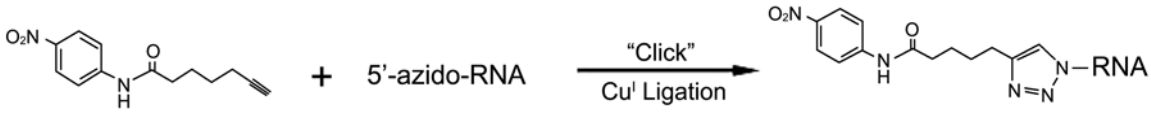
We recently reported a two-step synthetic method for 5'-azido-5'-deoxyguanosine (azido-G) by adapting literature procedures (Scheme 1) and its efficiency for click chemistry using 6-heptynoyl *p*-nitroaniline,[8] in consideration that the click chemistry with rapid reaction between the azide and alkyne groups to form a covalent triazole linkage without cross reacting with other functional groups could be used in bioconjugation chemistry. In the present study, we sought enzymatic methods to incorporate azido groups in RNA and measured incorporation efficiency of azido-G into the 5'-terminus of RNA by *in vitro* transcription using T7 RNA polymerase that requires guanosine to efficiently initiate transcription.[9] This is important in consideration that a terminal azido group is able to be introduced to the 5'-termini of RNA molecules, and that azides are unstable under solid-phase synthesis conditions.

A 97-mer single-stranded DNA containing a T7 promoter at the 3'-end (5'- CAG GAC TGC TCT CAC TCT CAC GCA CCA AGA AGC TGC CAT TGA TCC CGC TGC



Scheme 1. Schematic diagram for two-step synthesis of 5'-azido-5'-deoxyguanosine (azido-G) and for enzymatic preparation of azido-functionalized RNA at 5'-end. Reagents and conditions: (a) $P(Ph)_3$, I_2, imidazole, *N*-methyl-2-pyrrolidinone, 3 h, 71%; (b) NaN_3, DMF (dry), 80 °C, 24 h, 69%; (c) T7 RNA polymerase, azido-G:GTP:ATP:CTP:UTP.

[a]These authors contributed equally to this work.



Scheme 2. Click labeling of azide-functionalized RNA. Azido-G can be used for the click chemistry reaction with 6-heptynoyl *p*-nitroaniline in order to quantitatively monitor click labeling of the azide nucleoside to the alkyne dye.

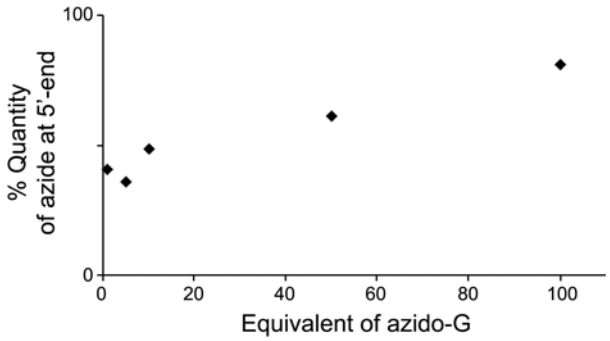


Figure 1. Quantitation of the azido group existent at 5'-end of RNA molecules.

TCA GCA GAT ACT CAG CGG CCC CCC CTA TAG TGA GTC GTA TTA GTC C-3') was used as the template, and the 75-nt 5'-azido-RNA was synthesized by runoff transcription in the presence of azido-G with molar ratios of azido-G:GTP:ATP:CTP:UTP = 1:1:1:1:1, 5:1:1:1:1, 10:1:1:1:1, 50:1:1:1:1, and 100:1:1:1:1, respectively. The resulting azide-functionalized RNA was recovered by ethanol precipitation, and tested for quantitation of the azide group using 6-heptynoyl *p*-nitroaniline (Scheme 2), as previously reported,[8(a)] allowing us to monitor and quantify click labeling of the azide nucleoside existent at the 5'-terminus of RNA to the alkyne dye. Figure 1 shows a gradual increasing graph for the percent quantity of the azide group at 5'-end of RNA molecule. When the ratio of azido-G:GTP was 5:1, approximately 36% of the nascent transcript were initiated with azido-G. The percent of transcripts initiated with azido-G increased to 49%, 62%, and 81% as the azido-G:GTP ratio was varied to 10:1, 50:1, and 100:1, respectively, although 100 times excess of azido-G appeared to slightly inhibit transcription by T7 RNA polymerase (Table 1).

More interestingly, our results demonstrate that click chemistry is compatible with RNA in spite of the intrinsic lability of RNA due to the 2'-vicinal to the internucleotide phosphate, as previously reported.[9] Differently from the literature,[9] RNA degradation was minimized in the present study through stabilization of the Cu^{I} in aqueous buffer with DMSO as cosolvent and no other ligand, suggesting the general possibility of ligandless click chemistry. The ability to utilize click chemistry directly to label RNA would offer an important consideration for handling RNA, providing an orthogonal method that could enable more facile labeling of oligonucleotides and expanding the application of click chemistry approach beyond use with synthetic RNA to any natural RNA. Thus the chemoselective click chemistry method would represent a significant addition to the current repertoire of RNA functionalization tools and enhance the biological detection, study, and delivery of RNA. In addition, since the conditions for click chemistry are orthogonal to the NHS or thiol chemistry that are currently widely used for the post-synthetic labeling of amino- or thiol-modified RNA, our method may be combined for multiple site-specific functionalization of RNA.

Table 1. Percentage of the azido groups existent at 5'-end of RNA molecules. The amount of each RNA obtained from *in vitro* transcription followed by ethanol precipitation or gel extract was measured based on the fact that 40 μg/mL solution of RNA results in A_{260} = 1.000 and the percentage of the azido group existent at 5'-end of each obtained RNA was calculated using 6-heptynoyl *p*-nitroaniline as described in Experimental Section. The total amount of each RNA used for percentage measurement of the azido group existent at 5'-end of RNA is presented in the parenthesis

[Azido-G]:[GTP]:[ATP]:[CTP]:[UTP]	Percentage quantity of azide at 5'-end
1:1:1:1:1	40.7 (7.6 μg)
5:1:1:1:1	36.2 (7.4 μg)
10:1:1:1:1	48.8 (6.6 μg)
50:1:1:1:1	61.5 (7.2 μg)
100:1:1:1:1	81.4 (3.6 μg)

In summary, we report that azido-G, an initiator for the *in vitro* transcription to replace GTP at the 5'-terminus, was used as a substrate for T7 RNA polymerase and enzymatically incorporated at the 5'-terminus of RNA to produce azide-functionalized RNA and that the azide-functionalized RNAs generated by the transcription reaction could be successfully used for click chemistry. With the viability of click chemistry validated on synthetic RNA containing click-reactive azides which can be specifically conjugated with alkyne-containing molecules, our method may provide a useful route to efficiently introduce reporters, such as fluorophores, into the 5'-terminus of RNA *via* a stable triazole-linker, or to tether the oligomer to a solid support.

Experimental Section

General Methods. Unless otherwise noted, reagents were obtained from commercial suppliers and were used without further purification. Depc-treated deionized water was used whenever necessary. All experiments were performed in duplicate.

Azido-G Synthesis. Azido-G was synthesized *via* a two-step reaction (Scheme 1).[8(a)] In brief, a mixture of 5'-deoxy-5'-iodoguanosine (74 mg, 0.19 mmol) and sodium azide (25 mg, 0.38 mmol) in dry DMF (0.5 mL) was stirred at 80 °C

under argon for 20 h. After cooling to room temperature, the solvent was removed under reduced pressure and the residue was stirred in water (1 mL) for 30 min. The resulting solid was collected by filtration and then washed successively with water (0.5 mL), cold ethanol (0.3 mL) and diethyl ether (0.2 mL) before drying *in vacuo* to give 5'-azido-5'-deoxyguanosine as a colorless solid (69%).

***In Vitro* Transcription and Azide Quantitation.** A 97-mer single-stranded DNA containing a T7 promoter at the 3'-end (5'- CAG GAC TGC TCT CAC TCT CAC GCA CCA AGA AGC TGC CAT TGA TCC CGC TGC TCA GCA GAT ACT CAG CGG CCC CCC CTA TAG TGA GTC GTA TTA GTC C-3') was used as the template. Transcription reactions were carried out with 50 units of T7 RNA polymerase in the presence of 0.2 mM each GTP, ATP, CTP, and UTP, 12 μg of DNA template, 2 mM spermidine, 10 mM dithiothreitol, 6 mM $MgCl_2$, and 40 mM Tris buffer (pH 7.9) at 37 °C in a total of 0.2 mL solution. The 75-nt 5'-azido-RNA was synthesized by runoff transcription in the presence of azido-G with a ratio of azido-G:GTP:ATP:CTP:UTP = 1:1:1:1:1, 5:1:1:1:1, 10:1:1:1:1, 50:1:1:1:1, and 100:1:1:1:1, respectively. Each of the azido-labeled RNAs was purified by denaturing 7.5 M urea/8% polyacrylamide gel electrophoresis, and the resulting 5'-azido-RNA was tested for quantitation of the azido group using 6-heptynoyl *p*-nitroaniline, by adapting literature procedures.[6] Briefly, 5'-azido-RNA (0.21 nmol) in 500 μL of water was reacted with a 150-fold excess of 6-heptynoyl *p*-nitroaniline in 25 μL of DMSO at 60 °C until all 5'-azido-RNA was consumed (~72 h), in the presence of $CuSO_4$ (0.21 nmol), and copper wire (1 mg). Unreacted dye was removed by Microcon (YM-10, Millipore). Concentrations of the clicked products were measured by a UV-vis spectrophotometer at 320 nm, to be compared with the concentrations of RNA.

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