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COMMUNICATION

Pin-point chemical modification of RNA with diverse molecules through the functionality transfer reaction and the copper-catalyzed azide–alkyne cycloaddition reaction†

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The internal modification of RNA has been successfully achieved by the functionality transfer reaction (FTR) and following click chemistry with diverse azide compounds. The benefits of the FTR have been demonstrated by its specificity, rapidity, broad applicability, and procedure simplicity.

Knowledge of RNA is being rapidly enlarged such as seen in the study of small RNA, in which a variety of functions of miRNAs are being unrevealed in cell proliferation, cell death and cancer progression, *etc.*[1] Chemically modified nucleic acids play a significant role in genomic research.[2] Specifically modified oligoribonucleotides (ORN) such as with a fluorescent dye and a modified ribose skeleton can be obtained by chemical synthesis.[3] Non-natural nucleosides are randomly incorporated into RNA by the biological processes.[4] Site specifically modified long RNA is obtained by ligation of chemically modified short RNA to long RNA in the presence of the template ODN by enzyme[5] or photochemistry.[6] A catalytic DNA with ligase activity was developed for the internal modification of large RNAs.[7] A simple method for specific modification of RNA at the internal position without the need of enzymes or a multistep procedure might be useful for the study of function and structure of RNA. We have recently developed the original approach based on the efficient functionality-transfer reaction (FTR) within the DNA–RNA duplex.[8] The ODN probe incorporating 6-thioguanosine (G^S) is first functionalized with the 2-methyliden-1,3-diketone transfer group. At the second step, the resulting functionality-transfer ODN (FT-ODN) is hybridized with the target RNA, and the functional group is selectively transferred to the 4-amino group of the cytosine base at pH 7 or the 2-amino group of the guanine base under alkaline conditions (Fig. 1, I to II). Site- and base specificities have been demonstrated using non-complementary sequences.[8b,c] In particular, the FTR under the alkaline conditions or neutral conditions in the presence of $NiCl_2$ proceeds rapidly and selectively to lead to the modification of the 2-amino group of the guanine base (Fig. 1B).[8d]

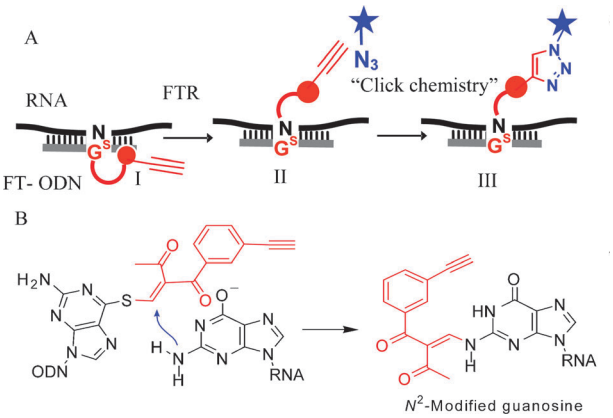

Fig. 1 The functionality transfer reaction (FTR) for modification of the 2-amino group of guanosine of the internal RNA.

These results encouraged us to establish a general method for the internal modification of RNA with a variety of molecules by using a copper(I)-catalyzed cycloaddition reaction ("Click" chemistry) with the acetylene-linked transfer group and the azide compounds.[9,10] We wish to report here that Click-chemistry is applicable to the synthesis of the transfer group for FTR and also the acetylene-modified RNA formed by FTR serves as a scaffold for conjugation with a variety of molecules (Fig. 1, II to III). The benefits of this system have been demonstrated by its specificity, rapidity, broad applicability, and procedure simplicity for the internal RNA modification with varied molecules.

Fig. 2 gives an overview of the method for the internal RNA modification through FTR and the "click chemistry" with a variety of azide compounds. In one approach, the 1,3-diketone transfer group having the acetylene unit of **ODN1** (functional) is transferred to the target **RNA2** and then the resulting **RNA2** (alkyne) serves as a scaffold for further modification by the click chemistry with the diverse azide compounds.‡

In another approach, the diketone derivative **1** is first transformed to the modified compound (**2** or **3**) by the click chemistry, which is then introduced to the thio group of 6-thioguanosine of **ODN1**.

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† Electronic supplementary information (ESI) available: Synthesis of the compounds, and the experimental details for the functionality transfer reaction and the Cu(I)-catalyzed cycloaddition reaction with the RNA substrates. See DOI: 10.1039/c1cc10582e

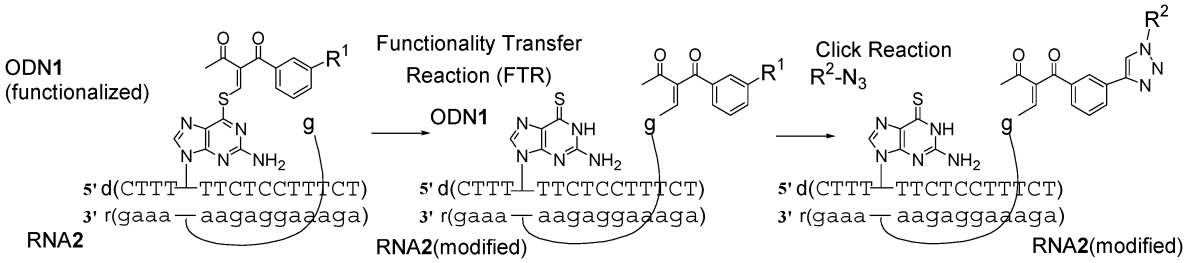
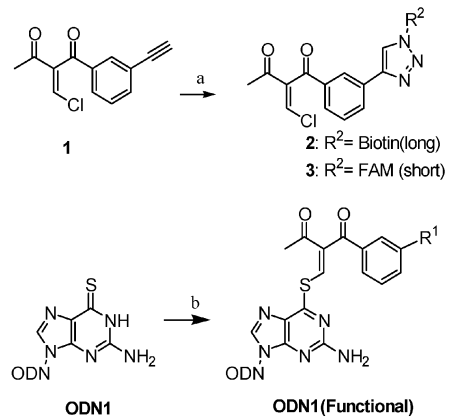


Fig. 2 Functionality transfer reaction for the internal RNA modification: the acetylene transferred to RNA works as a scaffold for labeling with a variety of molecules through the "Click Chemistry".

The biotin (**2**) and the FAM linked diketone derivative (**3**) were synthesized using **1** and the azide compound **8** and **9** in the presence of $CuSO_4$ and TBTA (Scheme 1). The functionalization of **ODN1** was performed by using the diketone derivative (**1**, **2** or **3**) in the carbonate buffer at room temperature at pH 10 for 10 minutes to produce the corresponding **ODN1** (functional) in more than 90% yield based on the HPLC analysis (Fig. S2 and S3, ESI†). The resulting **ODN1** (alkyne, biotin, FAM) was subjected to the functional group transfer reaction with **RNA2** in the carbonate buffer at pH 9.6. The chemical yield of the modified **RNA2** (alkyne, biotin, FAM) was obtained by HPLC analysis (Fig. S2, ESI†). The time courses of the reaction with **ODN1** (alkyne) and **ODN1** (biotin) are compared in Fig. 4. The reaction of **ODN1** (alkyne) for rG was completed within 10 minutes, whereas the reaction for the other bases was almost negligible (Fig. 4A). The reaction of **ODN1** (biotin long) and **ODN1** (FAM short) proceeded similarly at slightly slower reaction rates (Fig. 4B, and Fig. S4 (ESI†)).

We next investigated the second approach based on the click chemistry between the alkyne-modified **RNA2** (alkyne) and the azide compounds shown in Fig. 3. **ODN1** (alkyne) was reacted with **RNA2** (Y = rG) for 10 minutes as described above, and the reaction mixture was used for the next click



Scheme 1 Synthesis of the acetylene-attached diketone derivative (**1**), its FAM, biotin derivative (**2**, **3**), and the functionalized **ODN1**. (a) **8** (biotin long) or **9** (FAM short), $CuSO_4{\cdot}5H_2O$, sodium ascorbate, TBTA, DMSO/H_2O = 9/1, rt, biotin: 48%, FAM: 90%. (b) **ODN1** and **1**, **2** or **3**, in the carbonate buffer for 10 min, rt at pH 10, >90%.

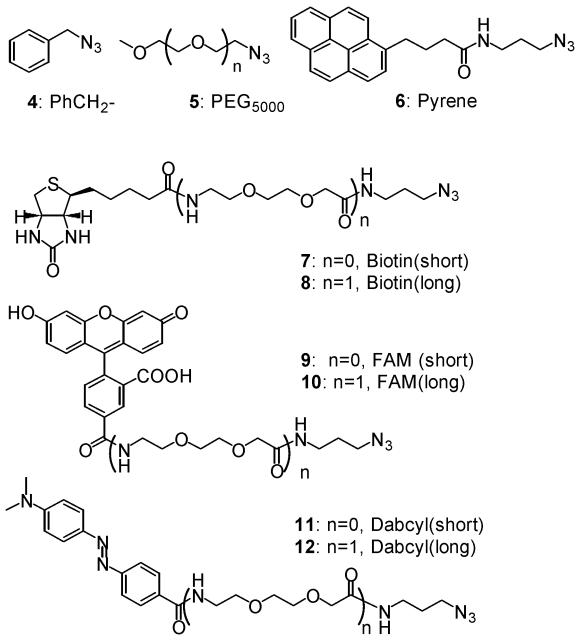


Fig. 3 Representative azide compounds (R^2–N_3) used in this study.

chemistry by using the azide derivatives in the presence of $CuSO_4$. The FTR mixture was mixed with sodium ascorbate, TBTA and the azide compound in DMSO–H_2O solution, and then the cycloaddition reaction was started by the addition of

Table 1 Chemical yield of the modified **RNA2** by the click chemistry[a]

				Yield (%)	
Entry	Azide	Functional group	pH	10 min	50 min
1	4	$PhCH_2$–	9.6	>99	—
2	5	PEG	9.6	>90	>99
3	6	Pyrene	9.6	89	>99
4	7	Biotin (short)	9.6	>99	—
5	8	Biotin (long)	9.6	90	>99
6	9	FAM (short)	9.6	32	69
7	9	FAM (short)	7.3[b]	83	>99
8	10	FAM (long)	7.3[b]	69	>99
9	11	Dabcyl (short)	9.6	31	64
10	12	Dabcyl (short)	7.3[b]	69	97
11	12	Dabcyl (long)	7.3[b]	65	86

[a] The functionality transfer reaction was performed for 10 minutes, then the azide compound (750 μM, DMSO), sodium ascorbate (300 μM), TBTA (300 μM, DMSO), DMSO (DMSO/H_2O = 3/7) and $CuSO_4$ (150 μM) were added to the mixture. The yield was obtained by the HPLC analysis with monitoring UV at 254 nm. [b] pH was adjusted with 1% aqueous AcOH before the click reaction.

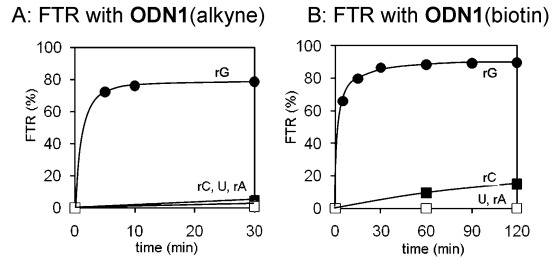


Fig. 4 Time course of the FTR by using **ODN1** (alkyne) (A), and **ODN1** (biotin) (B). A: 1.5 μM of **ODN1** (alkyne), 1.0 μM of the target **RNA2(Y)** in 50 mM carbonate buffer containing 100 mM NaCl at pH 9.6 at 25 °C. B: 15 μM of **ODN1** and 10 μM **RNA2(Y)**. The base represents the base (Y) in the target **RNA2(Y)**.

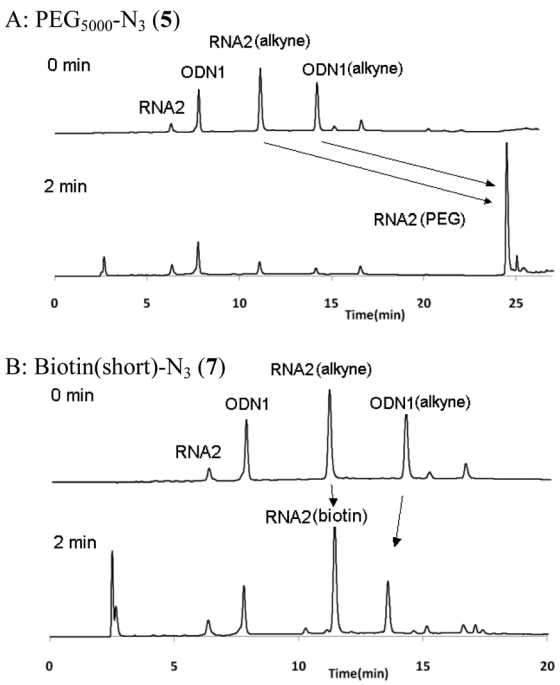


Fig. 5 The HPLC chart changes showing rapid and high-yield modification of RNA by the Cu(I)-catalyzed Click chemistry. (A) The reaction with PEG5000–N_3 (**5**). (B) The reaction with biotin (short)–N_3 (**7**). All oligonucleotides were separated by HPLC under the conditions described in the ESI†, and subjected to MALDI-TOF/MS measurements.

$CuSO_4$. The representative HPLC charts of this modification reaction are shown in Fig. 5. Surprisingly, the reaction with **4**, **5**, **6**, **7** and **8** proceeded very rapidly; the reactions with **4** and **7** nearly completed in 2 min. The transformation yield was obtained by quantification of the peaks, and the results obtained by using all the azide compounds are summarized in Table 1. Non-polar, small molecules produced the cyclo-addition product rapidly in excellent yields (entries 1 and 4). Relatively large compounds are also highly reactive (entries 2, 3 and 5). FAM- and Dabcyl–N_3 showed higher reactivity at pH 7.3 than at pH 9.6. Probably, FAM is less negatively charged at pH 7.3 and less repulsive with the phosphate backbone of RNA. It is also likely that the Dabcyl group is more positively charged at pH 7.3, thereby enable easier access to negatively charged RNA. The azide compounds with the long linker produced lower reaction rates compared to those with the shorter linker, probably because of their steric hindrance. It should be also noted that the biotin group can be easily attached to the internal site of RNA with the high sequence and base selectivity. These results indicated the high potential of the FTR for efficient and selective internal modification of RNA. These results have demonstrated the highly efficient labelling method for the internal position of RNA.

In summary, the internal RNA modification has been successfully achieved by the functionality transfer reaction (FTR) and following "click chemistry" with a variety of azide compounds. The high specificity, rapidity, broad applicability as well as simplicity of the procedure demonstrated in this study emphasize the benefit of this system for the internal modification of RNA. As the functionality transfer reaction can be performed at neural pH in the presence of divalent metal cations,[8d] the method described here is very unique and is expected to be useful in the research of RNA.

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Notes and references

‡ The nomenclature of the ODN and RNA is as follows: **ODN1** (alkyne) represents the structure of **ODN1** functionalized by **1**, in which the name in the parenthesis means the abbreviated name of the substituent R^1. **RNA2** (alkyne) means that the guanine base of **RNA2** is modified by the group with the substituent R^1 = alkyne. The products obtained by the Click chemistry are indicated as **ODN1** (biotin short) or **RNA2** (biotin short), in which the name in the parenthesis means the abbreviated name of the group of R^2 of the azide compound.

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