**Biological Chemistry**

**Click Chemistry Takes a Snapshot of DNA-RNA Hybrid G-Quadruplex in Living Cells**

Takumi Ishizuka and Yan Xu*

Division of Chemistry, Department of Medical Sciences, Faculty of Medicine, University of Miyazaki, 5200 Kihara, Kiyotake, Miyazaki, 889-1692, Japan

In this study, using the click chemistry, we designed and synthesized a “click-light-up” probe to investigate whether DNA-RNA G-quadruplexes exist in living cells. Employing the reporter probe, we found that human telomere DNA and RNA form the hybrid G-quadruplex structure in living cells, providing the in cell evidence for the presence of the DNA-RNA hybrid G-quadruplex.

**RESULTS AND DISCUSSION**

For this purpose, we developed a “click-light-up” reporter strategy to probe DNA-RNA hybrid G-quadruplex formation in living cells. We designed and synthesized a probe (ODN-1, 5′- MGGGTTAGGGTTAGGGT-3′) having the three-repeats 16-mer human telomere DNA and 7-azidocoumarin moiety at its 5′ terminus and another probe with a 5′ alkyne at the 5′ end of a 6-mer RNA (ORN-1) (Figure 1a). Nonfluorescent 7-azidocoumarin was chosen as a profluorophore. The profluorophore has no fluorescence due to the quenching effect from the electron-rich α-nitrogen of the azido group. Substitution at the 7-position of coumarin dyes has a significant influence on their fluorescence properties. In fact, formation of triazole ring at its 7-position by azide-alkyne cycloaddition will eliminate quenching resulting in a strong fluorescence. When the coumarin dye-labeled probe is free in the solution (or in the cell) without G-quadruplex formation, two molecules (azidocoumarin and alkyne at the 5′ end) are separated spatially, and no fluorescence is observed. Formation of the G-quadruplex brings the two molecules at the 5′ ends into close proximity, allowing the azide-alkyne cycloaddition reaction to produce a strong fluorescence by the formation of a triazole ring. The emission color serves as a means to rapidly probe DNA-RNA G-quadruplex formation. The advantage of the structure dependence of an emission production is that the cycloaddition reaction can strongly provide evidence for G-quadruplex formation. A strong interaction by G-quadruplex formation is the only effective way to induce the cycloaddition reaction. The simple molecule colocalization and decoy effects of the oligomers are unable to promote the click reaction by bringing the 5′ alkyne and 5′ azidocoumarin reaction partners into close proximity to one another.

**Conflict of Interest:** no conflicts declared.

* Corresponding Author. Prof. Y. Xu, Division of Chemistry, Department of Medical Sciences, Faculty of Medicine, University of Miyazaki, 5200 Kihara, Kiyotake, Miyazaki, 889-1692, Japan. E-mail: xuyan@med.miyazaki-u.ac.jp

© 2016 by the Authors | JNSCI, published in USA
Figure 1. (a) A “click-light-up” reporter strategy to probe DNA-RNA hybrid G-quadruplex formation. 7-azidocoumarin as a profluorophore was incorporated to 5′ terminus of 16-mer human telomere DNA (ODN-1, 5′-MGGGTTAGGGTTAGGT-3′). Another probe with a 5′ alkyne at the 5′ end of a 6-mer RNA UAGGGU (ORN-1) The profluorophore containing azide moiety is fluorescent inact ive. G-quadruplex formation by DNA and RNA promotes click reaction to trigger the fluorescence signals by formation of triazole ring. This can be used as a reporter to probe DNA-RNA G-quadruplex formation. (b) Fluorescence spectra for oligonucleotide probes. 1, Cu (+) (ex): excitation spectrum; 1, Cu (+) (em): with Cu catalyst for emission spectrum; 2, Cu (-): without Cu catalyst; 3, Cu (+) (em): with Cu catalyst and the 6-mer control ORN-2. (c) Fluorescence image of probes without and with Cu catalyst, and with Cu and the 6-mer control ORN-2, after illumination with a UV lamp (365 nm). (d), MALDI-TOF MS spectrum of the click reaction product of ODN-1 and ORN-1. After completion of the reaction, the click-ligated oligonucleotide was purified by RP-HPLC and analyzed by MALDI-TOF mass spectrometry.

We performed the click reaction between ORN-1 and ODN-1 under the condition of G-quadruplex formation. A clear blue color was observed by the naked eye with addition of Cu catalyst (Figure 1c). The fluorescence spectrum in the presence of Cu catalyst exhibits an emission band at 441 nm (Figure 1b), whereas in the absence of Cu catalyst or using the 6-mer control ORN-2 (without telomeric sequence), no emission was observed. The resulting product of click reaction was characterized by MALDI-TOF MS, revealing that the product is the azido-alkyne cycloaddition product ([M-H] calcd, 7529.9 found. 7528.5) (Figure 1d).

Having established this efficient click reaction on RNA and DNA G-quadruplexes, we applied this method to directly probe DNA-RNA G-quadruplex structures in living cells. (Figure 2). We incubated human HeLa cells with the probes and visualized the live cells by fluorescence microscopy. Cycloaddition reaction was started by adding new cell medium containing Cu catalyst. Without fixing and washing steps for cell, we directly observed the cells by fluorescent microscope. We observed clear the blue fluorescence from catalyst-treated cells (Figure 2c). Negative cells that were not treated with Cu catalyst remained virtually nonfluorescent (Figure 2b). These results demonstrate that the DNA-RNA G-quadruplex structure is present in living cells.

CONCLUSION
In this study, using a “click-light-up” reporter probe, we found that human telomere DNA and RNA form the hybrid G-quadruplex structure in living cells, providing the in vivo evidence for the presence of the DNA-RNA hybrid G-quadruplex in human telomere DNA and RNA. Detection of the click-reaction products directly probes DNA-RNA G-quadruplex structures in complicated solution, whereas traditional methods such as NMR and crystallography may not be suitable. DNA G-quadruplex, RNA G-quadruplex and DNA-RNA hybrid G-quadruplex may exist in equilibrium with one another, and it is often impossible to determine the kind of species present in solution. The click
reaction can trap a particular species or produce a snapshot of the various inter-converting structures that are present in a complex solution or in living cells. The species trapped by the click reaction can be separated and analyzed. G-quadruplex-forming probes that give fluorescence signaling can be used for sensitive monitoring of G-quadruplex formation in living cells without prior separation and after fixing and washing steps. These results provide valuable information for better understanding the structure and function of chromosome end. Such DNA-RNA hybrid G-quadruplex structure may also be a valuable target for anti-cancer agents directed against telomeres.

**EXPERIMENTAL SECTION**

**General Method**

Nucleoside phosphoramidite monomers, 5'-hexynyl phosphoramidite monomer and 5'-bromohexyl phosphoramidite monomer were purchased from Glen Research Co. The oligonucleotide with 5'-alkyne was synthesized with an Applied Biosystems 3400 DNA/RNA synthesizer, and characterized by MALDI-TOF MS. The detailed synthesis methods were described in Supporting Information. The substrate and marker oligonucleotides were commercial products of Sigma Genosys and were purified by the conventional methods before use. Water was deionized by a Millipore water purification system and sterilized by an autoclave immediately before use. Water soluble polyhydroxypropyl-triazole amine, Cu (I) binding ligand, was synthesized by the method reported in the literature with minor modification. The molar ratio of CuSO₄: Ascorbic acid : Ligand, 1:10:7, was constant as previously reported (“Cu catalyst” indicates those mixtures in this paper).

**MALDI-TOF MS**

The MALDI matrix was 3-hydroxypicolinic acid (HPA), 50:50 acetonitrile (ACN)/H₂O, ammonium iron citrate. 1 μL of sample (0.5 mM) was mixed with 1 μL of matrix solution. A spot of 1 μL of the sample-matrix mixture was placed on a stainless steel (384-well) MALDI target plate and allowed to air dry at room temperature. The MALDI-TOFMS spectrum was measured using a matrix-assisted laser desorption/ionization-time-of-flight mass spectrometer (MALDI-TOFMS) on an autoflex III smartbeam mass spectrometer (negative mode).

**Click Reaction on G-quadruplex**

To 50 μL of Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)-methyl]amine ligand (0.7 mM as final concentration), sodium ascorbate (1 mM), and CuSO₄·5H₂O (100 μM) were added sequentially to prepare the “Cu catalyst.” 7-azidocoumarin-contained OND-1 (1 μM) and alkyne-contained ONR-1 (1 μM) were added to the Cu catalyst solution, and the reaction mixture was kept at room temperature for 2 h. After completion of the reaction, click-ligated oligonucleotide was purified by RP-HPLC and analyzed by MALDI-TOF mass spectrometry.

**Fluorescent Measurements**

Fluorescent spectra were measured using a Jasco model FP-6500 spectrofluorometer. The spectra were recorded using a 1-cm path-length cell. For each sample, at least two spectrum scans were accumulated over a wavelength range from 300 to 650 nm. In photography experiments, UV irradiation of 365 nm was achieved with a UV Spot Light Source (Hamamatsu Photonics, 200 W) and UV-D36C filter (Asahi Technoglass).

**Fluorescence Microscopy**

HeLa cells (3×10⁴) were seeded in a 35-mm dish for fluorescence microscopy experiments. Cultures were incubated at 37 °C and 5% CO₂ in DMEM containing 10% FBS and antibiotics. For transfection, probe oligomers (0.4 μM) in was diluted with DMEM without 10% FBS and antibiotics. LipofectAMINE 2000 reagent (Invitrogen) (10 μL) was activated in DMEM and antibiotics by equilibration for 10 min at RT. The probes and activated LipofectAMINE were mixed together, and the lipid complexes were incubated at 37 °C for 20 min. The lipid complexes were directly added to a 35-mm dish containing HeLa cells and mixed gently by rocking after incubation at 37 °C for 16 h. Then the Cu catalyst was added and incubated at 37 °C for 3 h. For imaging the probes, the excitation and absorbance filters were 360/40 and 470/40 nm, respectively.

**Synthesis of 7-Azidocoumarin-labeled Oligonucleotide**

Preparation of 7-azidocoumarin is described in the previous study (15, 23). To incorporate the azido group at the 5' end of C6-aminoalkyl oligonucleotide, 30 nmol of the oligonucleotide in 300 μl of 0.5 M Na₂CO₃/NaHCO₃ buffer (pH 9.2) was incubated for 12 h at room temperature with 10 μmol of succinimidyl-1-7-azidocoumarin in 80 μl of dimethyl sulfoxide. The crude oligonucleotide was purified by reverse-phase HPLC.

**ACKNOWLEDGEMENT**

This work was supported by a Grant-in-Aid for Scientific Research (B) (26288083) and a Grant-in-Aid for Research Activity Start-up (25888019) from the Ministry of Education, Science, Sports, Culture and Technology (Japan). This work was also supported by grants from the Takeda Science Foundation.


