Biological Chemistry

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

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In this study, using the click chemistry, we designed and synthesized a "click-light-up" probe to investigate whether DNA–RNA G-quadruplexs 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.

Click chemistry | Chemical probe | Biological structure | Telomere | G-quadruplex

INTRODUCTION

G-quadruplexes are formed by the stacking of several G-tetrads and are constituted by four backbone strands in which several loops connect these strands. A large number of different structures have been observed in the human telomere DNA (1–3). The different G-quadruplex topologies may be associated with some related aspects: the syn/anti conformation of guanine residues, the relative orientation of the G-quartet core, the types of linking loops, the sequences and lengths of telomere DNA, and the nature of the associated metal cations (3–5). G-quadruplex structures represent a new class of molecular targets for DNA-interactive compounds that may be useful to target telomeres (3–9).

Recently, a breaking finding demonstrated that telomere DNA is transcribed into telomeric repeat-containing RNA in mammalian cells, containing mainly UUAGGG repeats in heterogeneous length (10, 11). This finding raises a crucial question of how telomeric RNA is specifically associated with telomeric DNA in response to chromosome ends regulation and protection. Telomere RNA has been found to localize to the telomere DNA (10, 11), suggesting a possible association between telomere RNA and telomere DNA. We demonstrated that human telomere RNA forms G-quadruplex structures in which the G-rich telomeric RNA sequences are able to form a parallel G-quadruplex (12, 14). Recently, we also demonstrated that telomere RNA G-quadruplex structure plays an important role in providing a protective structure for telomere ends (15).

The two-repeat human telomeric $d(TAGGGT)_2$ sequence (12-mer) has been reported to form a dimeric parallel-stranded G-quadruplex (16). A three-repeat (16-mer) and a single-repeat (6-mer) human telomeric sequence are known to form a (3 + 1) dimeric DNA G-quadruplex (17). RNA and PNA also formed RNA-PNA hybrid G-quadruplex structure (18). These pioneering works led us to suspect that telomere RNA may be bound to telomere DNA by forming an intermolecular DNA-RNA G-quadruplex. DNA-RNA hybrid G-quadruplex structure is technically difficult to study by traditional methods such as NMR and crystallography, since DNA G-quadruplex, RNA G-quadruplex and DNA-RNA hybrid G-quadruplex may coexist as a mixture in solution. For example, a 6-mer RNA and a 16-mer DNA may form three types G-quadruplex structures as mixture in a solution. In fact, we tried to investigate the structure of DNA-RNA hybrid G-quadruplex formed by the 6-mer RNA and 16-mer DNA by NMR. However, the quality of NMR spectrum of the 6-mer RNA and 16-mer DN A was not suitable for detailed structural analysis.

Click chemistry has been applied to biochemical studies (19–21). We have used the method to detect G-quadruplexes in solution (22). Using the click chemistry, we successfully found that a 6-mer human telomere RNA and 16-mer human telomere DNA sequence can form a DNA-RNA hybrid type G-quadruplex structure solution (23). In this study, using the click chemistry, we designed and synthesized a "click-light-up" probe to investigate whether DNA–RNA G-quadruplexs 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 vivo evidence for the presence of the DNA-RNA hybrid G-quadruplex.

RESULTS AND DISCUSSIION

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′-***M***GGGTTAGGGTTAGGGT-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-alkynecycloaddition 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.

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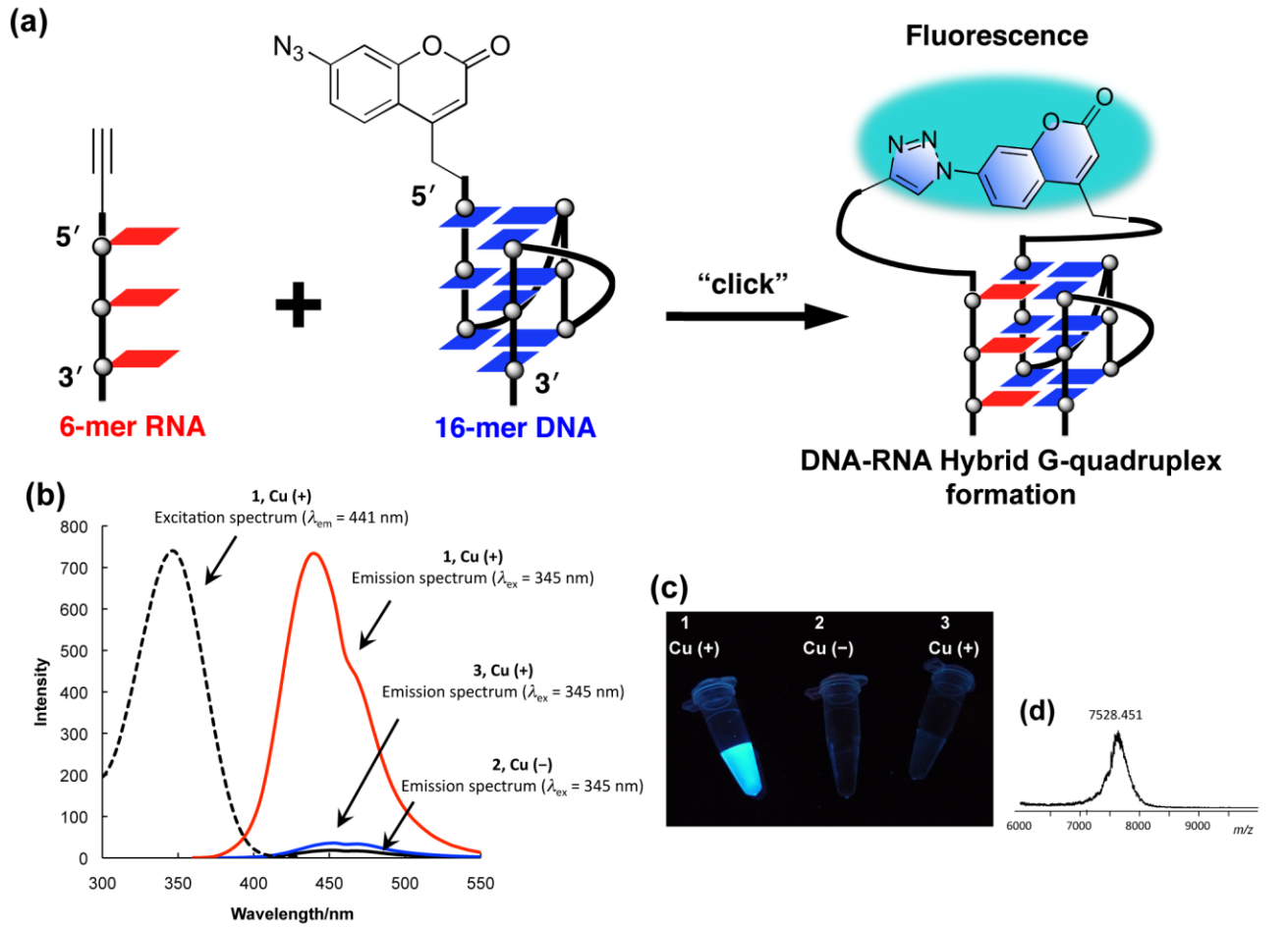

Figure 1. (a) A "click-light-up" reporter strategy to probe DNA-RNA hybrid G-quadruplex formation. 7-azidocoumarin as a profluorophore was incorporated to 5′ terminus of 16-mer human telomere DNA (**ODN-1**, 5′-***M***GGGTTAGGGTTAGGGT-3′). Another probe with a 5′ alkyne at the 5′ end of a 6-mer RNA UAGGGU (**ORN-1**) The profluorophore containing azide moiety is fluoresent inactive. 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-formed 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.

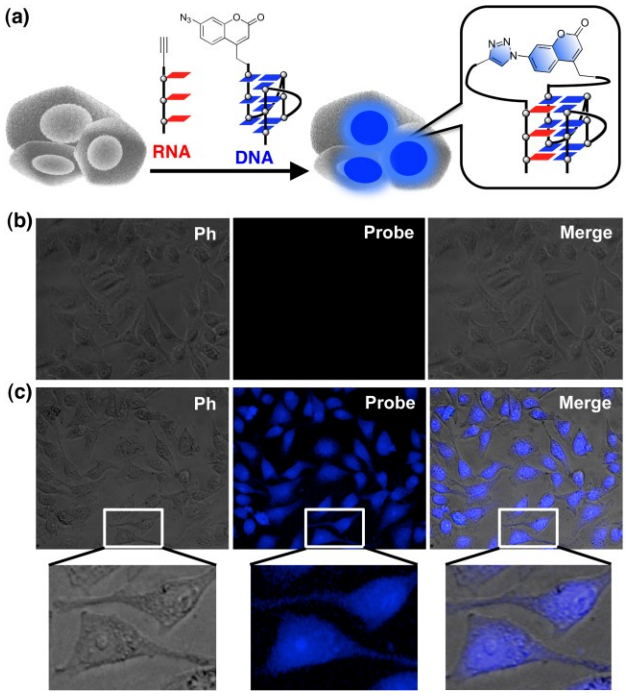

Figure 2. (a) Schematic for detection of DNA-RNA G-quadruplex formation in living cells using oligonucleotide probes. G-quadruplex formation in living cells will induce the fluorescence signals. (b) and (c) Fluorescence microscopy images of live cells. (b) without Cu catalyst, (c) with Cu catalyst, bottom insert in (c) shows expansion of part of (c). ph: phase-contrast imaging.

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 $CuSO_4$: 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)/H_2O, 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-1*H*-1,2,3-triazol-4-yl)-methyl]amine ligand (0.7 mM as final concentration), sodium ascorbate (1 mM), and $CuSO_4 \cdot 5H_2O$ (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 (3×10^4) were seeded in a 35-mm dish for fluorescence microscopy experiments. Cultures were incubated at 37 °C and 5% CO_2 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 Na_2CO_3/$NaHCO_3$ buffer (pH 9.2) was incubated for 12 h at room temperature with 10 μmol of succinimidyl-7-azidocoumarin 6 in 80 μl of dimethyl sulfoxide. The crude oligonucleotide was purified by reverse-phase HPLC.

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