Biological Structures

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Click Chemistry for the Identification of G-Quadruplex Structures: Discovery of a DNA–RNA G-Quadruplex**

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Four-stranded DNA structures formed by guanine-rich sequences are known as G-quadruplexes. They have received much interest over the last decade owing to their remarkable structural features and biological importance.[1] For example, G-quadruplex DNA composed of telomeric sequences plays an important role in telomere maintenance and has become a potential tumor-selective target for chemotherapy.[2] Various studies based on platinum cross-linking, fluorescence resonant energy transfer (FRET), ^{125}I radioprobing, covalent ligation, sedimentation, NMR spectroscopy, and X-ray crystallography, among other techniques, have been carried out to investigate G-quadruplex structures.[3] For example, by using a photochemical method, we detected the diagonal loops in an antiparallel G-quadruplex.[4] Although these approaches gave some structural information, the development of a more effective method based on a chemical reaction for probing G-quadruplex structures would be desirable. Ideally, a chemical reaction to distinguish a complex G-quadruplex structure should be mild, highly selective, almost quantitative, and readily initiated and quenched. Click chemistry, a classification for powerful and selective reactions, may fulfill all the necessary criteria.[5]

Herein, we describe the application of the copper-catalyzed azide–alkyne cycloaddition (CuAAC), the most extensively studied "click reaction",[6] to explore G-quadruplex solution structures. Click chemistry has previously been used to functionalize viruses, proteins, and oligonucleotides, as well as to immobilize DNA on electrode surfaces and chips.[7] A phosphodiester linkage within the loop position of a G-quadruplex has also been used to synthesize circular oligonucleotides for exploring G-quadruplex structure.[8] We now report that a copper-catalyzed cycloaddition occurs in different G-quadruplex structures between azido and alkyne groups located at the 5′ and 3′ ends of the G-quadruplex. The use of this simple "click" method enables the detection of the G-quadruplex structure. Importantly, by using this approach we discovered a DNA–RNA hybrid-type G-quadruplex structure formed from human telomeric DNA and RNA sequences. G-quadruplexes often exist in equilibrium with one another, and it is often impossible to determine the kind of species present in solution. Whereas FRET and other methods, such as NMR spectroscopy, give an averaged signal (depending on the time scale of interconversion in the case of NMR spectroscopy), the click method enables a snapshot to be taken of the existing species in solution. The species trapped by the click reaction can be separated and analyzed. This approach will greatly facilitate G-quandruplex analysis in solution under various conditions.

To determine whether a click reaction could occur on a G-quadruplex, we first employed a parallel dimeric telomere RNA G-quadruplex used in our recent studies (Figure 1a).[9]

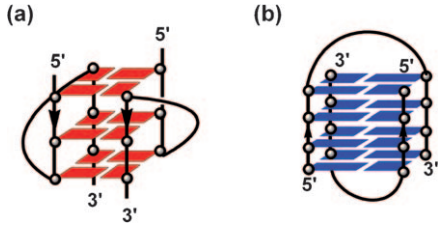

Figure 1. a) The parallel dimeric telomere RNA G-quadruplex formed by 12-mer telomere RNA. b) The antiparallel dimeric G-quadruplex formed by the 12-mer *Oxytricha nova* sequence d(G4T4G4).

We prepared the 12-mer 2′-OMe oligoribonucleotides (ORNs) ORN-1, with a 5′-alkyne, and ORN-2, with a 5′-azido group (Figure 2a; see the Experimental Section for the sequences). Analysis by denaturing gel electrophoresis of the click reaction of ORN-1 and ORN-2 for 1 h at room temperature under the conditions of G-quadruplex formation (200 mM KCl) showed that no reaction occurred without the copper catalyst (Figure 2b, lane 1). In the presence of the copper catalyst, a new band of lower mobility appeared (lanes 2–4). The appearance of this band is attributed to the reaction between the 5′-alkyne of ORN-1 and the 5′-azido group of ORN-2 to form linear ORN-3, which was purified by reversed-phase (RP) HPLC and characterized by MALDI-TOF MS (Figure 2c). To further demonstrate that the click reaction was promoted by G-quadruplex formation, we prepared the 12-mer ORN-4 (rKU_{12}) without the telomeric sequence. No reaction occurred (Figure 2b, lane 5) under the conditions used for the reactions analyzed in lanes 2–4. This result indicates clearly that the formation of the G-quadruplex promotes the click reaction by bringing the 5′-alkyne

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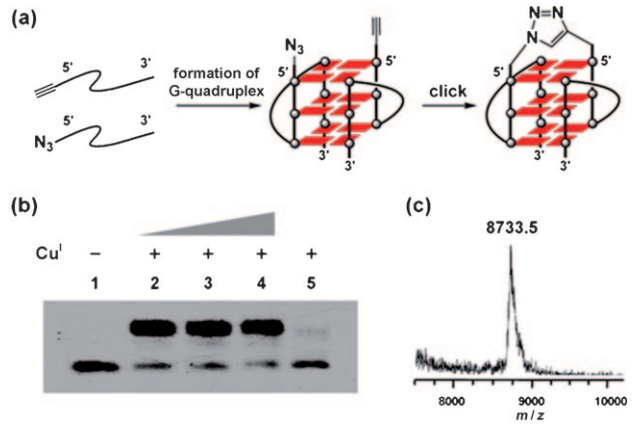

Figure 2. a) Click reaction on a human telomeric RNA G-quadruplex. b) Analysis of the click reaction of ORN-1 and ORN-2 by denaturing gel electrophoresis. Lane 1: without the Cu catalyst. Lanes 2–4: with the Cu catalyst. The molar ratio of ORN to Cu was 1:25 (lane 2), 1:50 (lane 3), and 1:100 (lane 4). ORN-1 and ORN-2 (lower band) are gradually consumed to give ORN-3 (upper band). Lane 5: with the Cu catalyst and with ORN-4 (without the telomere sequence) in place of ORN-2. c) MALDI-TOF MS spectrum of ORN-3 (*m*/*z* 8733.5 $[M-H+Na]^+$).

and 5′-azido reaction partners into close proximity to one another.

We next employed an antiparallel G-quadruplex formed by an *Oxytricha nova* sequence to investigate the possibility of click reactions on different types of G-quadruplex structure (Figure 1b).[10] The 5′-alkyne- and 3′-azido-labeled 12-mer oligodeoxyribonucleotide (ODN) ODN-5 was used (Figure 3a). Without the copper catalyst, no reaction occurred (Figure 3b, lane 1). Interestingly, in the presence of the copper catalyst, two new bands appeared (lane 2). The upper band, with a position between those of the 20-mer and 30-mer oligonucleotide markers, was thought to be a click-reaction product, 24C: a 24-mer circular oligonucleotide. The bottom band in lane 2 was a self-cyclization product,

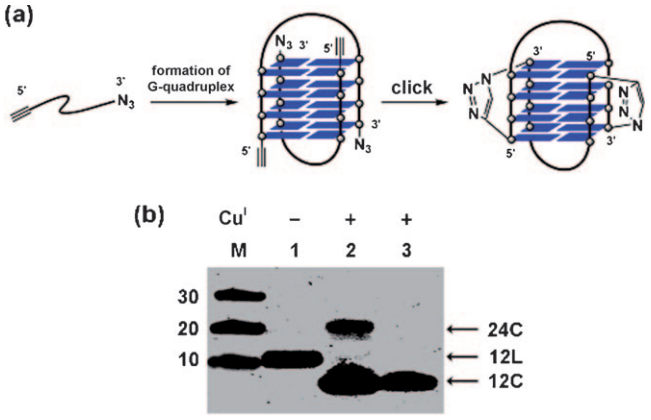

Figure 3. a) Click reaction between a 5′-alkyne and a 3′-azido group on the G-quadruplex of the *Oxytricha nova* sequence. b) Denaturing gel electrophoresis of the click-reaction products. Lane M: 30-, 20-, and 10-mer oligonucleotide markers; lane 1: without the Cu catalyst; lane 2: with the Cu catalyst; lane 3: ODN-6 as a reference. L: linear DNA, C: circular DNA.

12C, which was identified by comparison with the gel mobility of the reference oligonucleotide ODN-6 in lane 3 (Figure 3b). These results suggested that click reactions can occur on DNA and RNA G-quadruplex scaffolds. The click ligation reaction on the G-quadruplex is very mild and straightforward. It was carried out in water at room temperature for a reaction time of only 1 h. The reaction is highly selective and can be initiated simply by adding the copper catalyst and quenched by gel filtration.

Having established this efficient click reaction on RNA and DNA G-quadruplexes, we applied this method to the analysis of G-quadruplex structures. A three-repeat and a single-repeat human telomeric sequence are known to form a (3+1) dimeric DNA G-quadruplex in the presence of NaCl.[11] Although it has been speculated that these two oligonucleotides could form the same structure in a K^+ solution, which would represent physiological conditions more closely, critical evidence is required (Figure 4a).[12] We

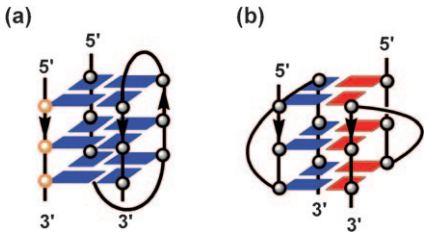

Figure 4. Schematic representation of two possible G-quadruplex structures: a) a (3+1) dimeric DNA G-quadruplex formed by 16-mer and 6-mer human telomeric sequences; b) a DNA–RNA hybrid G-quadruplex formed by 12-mer human telomere DNA and 12-mer human telomere RNA.

used the method developed in the current study to determine whether the (3+1) dimeric DNA G-quadruplex can be formed in the presence of KCl. For this purpose, we synthesized the 5′-alkyne- and 3′-azido-labeled 16-mer oligonucleotide ODN-7, and the 5′-azido- and 3′-alkyne-labeled 6-mer oligonucleotide ODN-8 (Figure 5a; see Schemes S2 and S3 in the Supporting Information). A click reaction between ODN-7 and ODN-8 was performed in the presence of KCl and analyzed by polyacrylamide gel electrophoresis (Figure 5b). When the copper catalyst was added, a new product (lane 2, 22C), likely to be the circular oligonucleotide derived from the two linear precursors, was generated from the click reaction. This result indicates that the formation of a (3+1) dimeric DNA G-quadruplex between ODN-7 and ODN-8 promotes the click reaction to form the circular oligonucleotide.

To obtain further evidence of the formation of the (3+1) dimeric G-quadruplex in KCl solution, we prepared a 22-mer circular ODN containing a photocleavable linker (Figure 6a) from the 6-mer ODN (containing the photocleavable linker) and the 16-mer ODN-7. Upon irradiation with UV light, the 22-mer circular ODN 22C should dissociate to give the 22-mer linear ODN 22L (Figure 6a), whereas a linear ODN would produce two linear ODNs. The photocleavable circular oligonucleotide was irradiated with UV light for 15 min

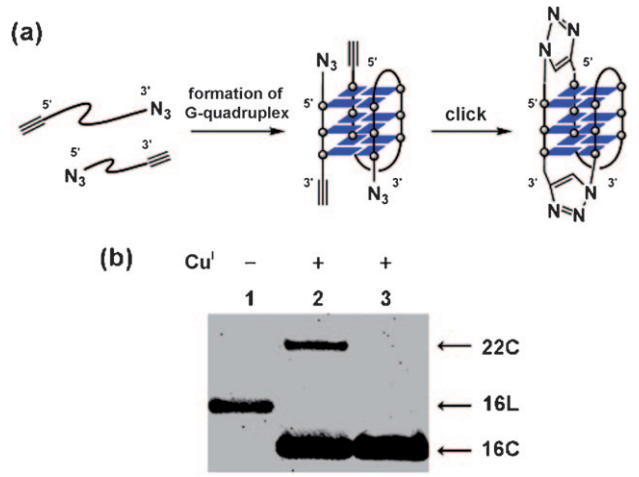

Figure 5. Confirmation of the formation of a (3+1) dimeric DNA G-quadruplex by a click reaction in a solution containing K^+ ions. a) Click reactions between the 5′-alkyne and the 5′-azido group and between the 3′-azido group and the 3′-alkyne lead to a 22-mer circular oligonucleotide. b) Analysis of the click reaction between ODN-7 and ODN-8 by denaturing gel electrophoresis. Lane 1: without the Cu catalyst; lane 2: with the Cu catalyst; lane 3: ODN-9 as a reference.

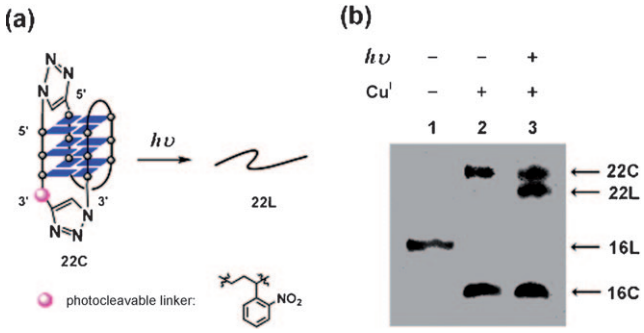

Figure 6. Further evidence for the formation of a (3+1) dimeric DNA G-quadruplex in a solution containing K^+ ions. a) The photocleavable linker in a circular ODN, 22C, is cleaved by UV irradiation to give a linear ODN, 22L. b) Analysis by denaturing gel electrophoresis of the dissociation of the circular oligonucleotide through cleavage by UV irradiation. Lane 1: without the Cu catalyst and without UV irradiation; lane 2: with the Cu catalyst without UV irradiation; lane 3: with the Cu catalyst and UV irradiation. The new band, 22L, in lane 3 is the linear oligonucleotide produced by the cleavage of 22C with UV light.

(Figure 6b). As expected, only one new band (lane 3, 22L) appeared, with a similar mobility to that of the circular ODN 22C. Without UV irradiation, no new band appeared (Figure 6b, lane 2). We could therefore conclude that the product of the click reaction was the circular ODN 22C.

The products of the click reaction were characterized further by RP HPLC and MALDI-TOF MS (see Figure S1 in the Supporting Information). The main two peaks with different retention times (t_R(ODN-9) = 18.1 min; t_R(ODN-10) = 19.5 min) from those of the starting materials (t_R(ODN-7) = 17.6 min; t_R(ODN-8) = 20.8 min) were characterized by MALDI-TOF MS (see Figure S1 in the Supporting Information), which revealed that the peaks corresponded to the circular product ODN-10 (m/z 8050.9 $[M-H+Na]^+$) and the self-cyclization product ODN-9.

Recently, two studies demonstrated that telomeres are transcribed into telomeric repeat-containing RNA in mammalian cells.[13,14] Telomeric RNAs containing mainly UUAGGG repeats of heterogeneous length were detected in different human and rodent cell lines. This discovery of telomere RNA raises the crucial question of how telomeric RNA is specifically associated with telomeric DNA in terms of chromosome-end regulation and protection. Telomere RNAs were found to be localized with the telomere DNA,[13,14] which suggests a possible association between telomere RNA and telomere DNA. We suspect that telomere RNA may bind to telomere DNA through the formation of an intermolecular DNA–RNA G-quadruplex (Figure 4b), although no experimental data have yet been obtained that demonstrate the existence of the DNA–RNA hybrid G-quadruplex structure directly. It is technically difficult to study the DNA–RNA hybrid G-quadruplex structure by traditional methods, such as NMR spectroscopy and X-ray crystallography, since the DNA G-quadruplex, the RNA G-quadruplex, and the DNA–RNA hybrid G-quadruplex may coexist as a mixture in solution. In fact, we have demonstrated that human telomere RNA can form a parallel G-quadruplex structure in the presence of sodium[9] and had difficulty in studying the DNA–RNA hybrid structure by NMR spectroscopy (unpublished data).

We believed that click chemistry might be a useful method for the detection of a DNA–RNA hybrid G-quadruplex. We designed a click reaction in which only the DNA–RNA hybrid G-quadruplex could undergo an azide–alkyne cycloaddition, even in the presence of the corresponding DNA–DNA or RNA–RNA dimeric G-quadruplex (Figure 7a). A 5′-azido ODN, ODN-12, and a 5′-alkyne-labeled ORN, ORN-1, were prepared as substrates for the click reaction. With a copper catalyst, a new band appeared with a mobility shift between that of the 20-mer oligonucleotide marker and that of the 30-mer marker (Figure 7b, lane 1). This band is thought to be the product of a click reaction between the 5′-alkyne-labeled RNA and the 5′-azido DNA in a DNA–RNA hybrid G-quadruplex. Moreover, the 5′-alkyne-labeled 12-mer ORN-4 without the telomere sequence and 5′-azido ODN-12 were subjected to the same reaction conditions; no reaction occurred (lane 2), which suggests that DNA–RNA G-quadruplex formation is required for the click reaction. This result indicates strongly that human telomere 12-mer DNA and 12-mer RNA can form a hybrid-type G-quadruplex.

To the best of our knowledge, it has not been reported previously that a G-quadruplex can act as a scaffold for a click reaction. Reactions of a 5′ alkyne with a 5′ azide, of a 3′ alkyne with a 3′ azide, and of a 5′ alkyne with a 3′ azide can occur in different types of G-quadruplex structures. We used this method to probe the structure of G-quadruplexes and showed that the (3+1) dimeric DNA G-quadruplex can form in the presence of KCl. Most importantly, by using this approach, we discovered a DNA–RNA hybrid-type G-quadruplex structure formed by human telomeric DNA and RNA sequences. The advantage of the click reaction is that the detection of the reaction products identifies the G-quadruplex structure

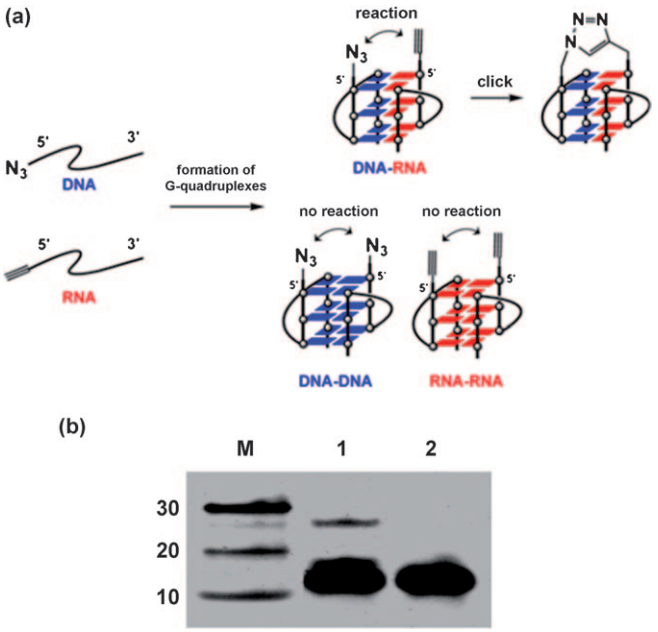

Figure 7. Detection of a DNA–RNA hybrid G-quadruplex by a click reaction. a) Schematic depiction of the detection of the DNA–RNA G-quadruplex. The use of 5′-azido-labeled DNA and 5′-alkyne-labeled RNA may result in a mixture of three types of G-quadruplex. Only the DNA–RNA G-quadruplex brings the alkyne and the azido group into close proximity to give the product of an azide–alkyne cycloaddition. b) Analysis of the click reaction of the 5′-azido ODN and the 5′-alkyne ORN by denaturing gel electrophoresis. Lane M: 30-, 20-, and 10-mer oligonucleotide markers; lane 1: with the Cu catalyst; lane 2: with the Cu catalyst and with ORN-4 in place of ORN-1.

directly in a complex solution, whereas traditional methods, such as NMR spectroscopy and X-ray crystallography, may not be suitable.

Experimental Section

The oligonucleotides used in this study (Table 1) were prepared according to Schemes S1–S3 in the Supporting Information.

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Table 1: Oligonucleotides used in this study.[a]

Name	Sequence
ORN-1	5′-*K*UAGGGUUAGGGU-3′
ORN-2	5′-*Z*UAGGGUUAGGGU-3′
ORN-3	(3′-UGGGAUUGGGAU-5′)*X*(5′-UAGGGUUAGGGU-3′)
ORN-4	5′-*K*UUUUUUUUUUUU-3′
ODN-5	5′-*K*GGGGTTTTGGGG*Z*-3′
ODN-6	oligonucleotide derived from ODN-5 by self-cyclization
ODN-7	5′-*K*GGGTTAGGGTTAGGGT*Z*-3′
ODN-8	5′-*Z*TAGGGT*K*-3′
ODN-9	oligonucleotide derived from ODN-7 by self-cyclization
ODN-10	circular oligonucleotide formed from ODN-7 and ODN-8
ODN-11	5′-*Z*TAGGGT*PK*-3′
ODN-12	5′-*Z*TAGGGTTAGGGT-3′

[a] The ORNs are composed of 2′-OMe RNA. *K* = alkyne, *Z* = azide, *P* = photocleavable linker, *X* = 1,2,3-triazole.

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