Site specific self-cleavage of certain assemblies of G-quadruplex†

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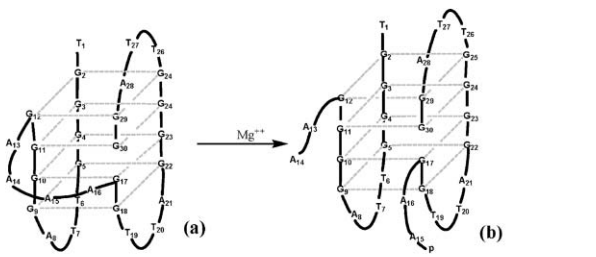
It is demonstrated that certain structural assemblies of G-quadruplex are capable of performing self-cleaving actions in a site specific fashion.

G-quadruplex is a structural organization of DNA composed of two or more stacks of G-quartets in which four guanines are arranged in a square planar array.[1–3] This tetraplex assembly has received considerable attention in the past few years owing to its unique spatial arrangement as well as its great biological and nanotechnological significance.[4–6] It has been suggested, for example, that a G-quadruplex structure could be present in the promoter region of c-myc, in the immunoglobulin switch region and at the ends of telomeres.[7,8] In addition, a self-assembly of guanine-rich oligonucleotides could form rod-shaped cholesteric liquid crystals and act as the scaffold of artificial ion channels and as ion carriers.[9] Moreover, certain deoxyribozymes[10–13] and aptamers[14] are believed to rely on the formation of G-quadruplex for their biological actions. Herein we report that besides the physical and chemical properties reported previously, certain assemblies of G-quadruplex can perform self-cleaving actions in a site specific fashion.

Fig. 1 depicts a schematic diagram of a DNA self-cleavage process uncovered during our recent investigations. A guanine-rich 30-mer oligonucleotide (Oligonucleotide 1 in Fig. 1) was designed previously in our laboratory with the expectation that this oligonucleotide would form an externally looped G-quadruplex assembly (**a** in Fig. 1) under proper conditions. Our initial intention in designing such a guanine-rich oligonucleotide was to examine whether a transesterification reaction could be feasible between the hydroxyl group at its 3′ end and the phosphodiester bond between A_{16} and G_{17} since these functional groups are proximal to each other upon G-quadruplex formation. Instead of observing such a designed transesterification reaction, a self-cleavage reaction of Oligonucleotide 1 at one of the two phosphodiester bonds between A_{14} and A_{15} was observed by chance (Fig. 1).



Oligonucleotide 1: 5′ T_1G_2GGGTTAGGGGAA$_{14}A_{15}$AGGTTAGGGGTTAG$_{29}G_{30}$ 3′ (30-mer)
Fragment 1: 5′ T_1G_2GGGTTAGGGA$_{13}A_{14}$ 3′ (14-mer)
Fragment 2: 5′ p-$A_{15}A_{16}$GGTTAGGGGTTAG$_{29}G_{30}$ 3′ (16-mer)
p: phosphate group

Fig. 1 Schematic representation of a self-cleavage process of Oligonucleotide 1 uncovered in these studies.

Oligonucleotide 1 was accordingly phosphorylated at its 5′ end with [γ-^{32}P] ATP in the presence of T_4 polynucleotide kinase and further purified by polyacrylamide gel electrophoresis and gel filtration chromatography in our studies. In order to allow the formation of proper G-quadruplex assemblies, this guanine-rich oligonucleotide was next incubated at 20 °C in the presence of 5 mM NaCl for 12 h followed by addition of KCl (final concentration 5 mM), and the mixture was then kept at the same temperature for an additional 12 h. The self-cleavage reactions of Oligonucleotide 1 were initiated next by adding a premixed solution of $MgCl_2$ and histidine to the mixture, which was then kept at 34 °C for different periods of time. As shown in Fig. 2, a new fast moving band was observed when such a reaction was allowed to proceed for 2 h (Band 1 in Lane 3). The mobility shift of this new band is close to that of a molecular weight marker of 14-mer (5′ *p-TGGGGTTAGGGGAA 3′, Lane 5), which implied that a cleavage reaction took place between A_{14} and A_{15} of this guanine-rich sequence. In addition, the time dependence of these self-cleavage reactions was examined in our studies. As shown in Fig. 3, the yield of the self-cleavage reactions increased with increasing reaction time and ~50% cleavage of Oligonucleotide 1 could be achieved within ~2 h.

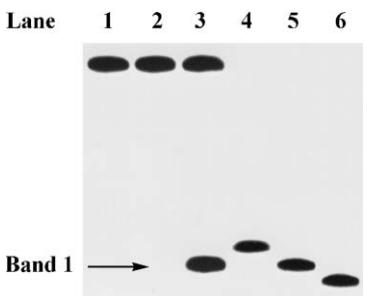


Fig. 2 Polyacrylamide gel electrophoretic analysis of self-cleavage of DNA visualized by autoradiography. Lane 1: Oligonucleotide 1 alone; Lanes 2 to 3: self-cleavage reactions lasting for 0 and 2 h respectively; Lane 4: a 15-mer Oligonucleotide (*p-TGGGGTTAGGGGAAA) alone; Lane 5: a 14-mer (*p-TGGGGTTAGGGGAA) alone; Lane 6: a 13-mer (*p-TGGGGTTAGGGGA) alone. (see ESI for detailed experimental descriptions).

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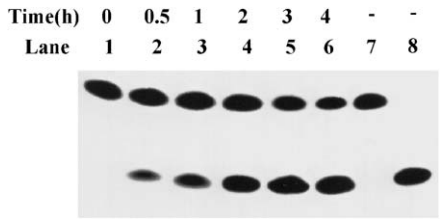

Fig. 3 Time dependence of self-cleavage reaction of Oligonucleotide 1. The same procedures as those for preparing samples loaded in Lane 3 in Fig. 2 were used except that the reactions were stopped at different time intervals. Lane 7: Oligonucleotide 1 alone; Lane 8: a 14-mer (*p-TGGGGTTAGGGGAA) alone. The times of reaction in Lanes 1, 2, 3, 4, 5 and 6 were 0, 0.5, 1, 2, 3 and 4 h respectively.

If a DNA cleavage reaction indeed occurred in the middle of the sequence of Oligonucleotide 1 in our studies, a second fragment of 16-mer should in theory be generated at the same time. In order to visualize the two fragments of 14-mer and 16-mer (Fragment 1 and Fragment 2 shown in Fig. 1) simultaneously, methylene blue staining experiments were conducted next. As shown in Fig. 4, two fast moving bands (Band 1 and Band 2 in Lane 2) were visible from the stained polyacrylamide gel, which displayed the same mobility shifts as those of a 14-mer marker (Lane 6) and a 16-mer marker (Lane 4) respectively. These electrophoretic analysis data are indications that a cleavage reaction indeed took place between A_{14} and A_{15} in the middle of the sequence of Oligonucleotide 1 as shown in Fig. 1.

Oligonucleotide 1 containing radiolabeled phosphorus (^{32}P) between A_{14} and A_{15} (5′ TGGGGTTAGGGGAA-^{32}p-AAGGTTAGGGGTTAGG 3′, internally ^{32}P-labeled Oligonucleotide 1) was next synthesized and examined during our investigations in order to determine which of the two fragments possesses the phosphate group. As shown in Fig. 5, the only observable

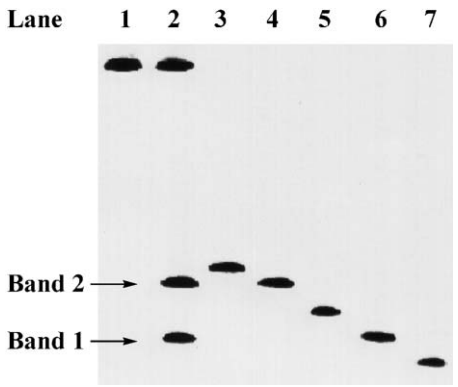

Fig. 4 Polyacrylamide gel electrophoretic analysis of self-cleavage of DNA visualized by methylene blue staining. The same procedures as those for preparing samples loaded in Lane 3 in Fig. 2 were used except that 5′ ^{32}P-labeled Oligonucleotide 1 was replaced with 5′ hydroxyl Oligonucleotide 1 and a methylene blue staining protocol was adopted for visualizing the DNA bands. Lane 1: Oligonucleotide 1 alone; Lane 2: self-cleavage reaction lasting for 2 h; Lane 3: a 17-mer (5′ AAAGGTTAGGGGTTAGG 3′) alone; Lane 4: a 16-mer (5′ AAGGTTAGGGGTTAGG 3′) alone; Lane 5: a 15-mer (5′ TGGGGTTAGGGGAAA 3′) alone; Lane 6: a 14-mer (5′ TGGGGTTAGGGGAA 3′) alone; Lane 7: a 13-mer (5′ TGGGGTTAGGGGA 3′) alone.

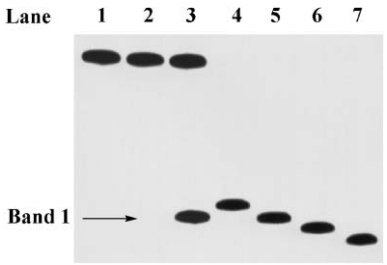

Fig. 5 Polyacrylamide gel electrophoretic analysis of internally ^{32}P-labeled Oligonucleotide 1 (5′ TGGGGTTAGGGGAA-^{32}p-AAGGTTAGGGGTTAGG 3′) in its self-cleavage reactions (see ESI† for detailed description).

self-cleavage product from the internally ^{32}P-labeled Oligonucleotide 1 is a 16-mer fragment (5′ *p-A_{15}AGGTTAGGGGTTAGG$_{30}$ 3′) while not even a trace amount of 14-mer (5′ T_1GGGGTTAGGGGAA$_{14}$-*p 3′) is detectable, which is a sign that the phosphate group goes exclusively with the 16-mer fragment rather than with the 14-mer as illustrated in Fig. 1. In addition, the oligonucleotide fragment in Band 1 in Lane 3 in Fig. 5 was purified and further analyzed through hydrolysis by exonuclease I, an enzyme that digests single-stranded DNA in a 3′ to 5′ direction in a stepwise fashion. As shown in Fig. S1,† the purified ^{32}P-containing oligonucleotide fragment was completely degraded in the presence of the single strand-specific nuclease (Lane 3), which could be taken as an indication that this oligonucleotide fragment (Fragment 2 in Fig. 1) holds a linear structure in its backbone. Based on the above observations, it can be suggested that the self-cleaving reaction of Oligonucleotide 1 took place at one of the phosphodiester bonds near the 3′ end of A_{14} in the middle of its sequence as illustrated in Fig. S2.† Nevertheless, more direct evidence is evidently needed to further verify this suggested mechanism in the future.

With the aim of verifying that a G-quadruplex structure could be formed by Oligonucleotide 1 as anticipated, CD spectroscopic analysis on a solution containing this guanine-rich sequence was also carried out. As shown in Fig. S3,† this Oligonucleotide 1-containing solution displayed a maximum absorption at 295 nm, which is a characteristic sign of the presence of anti-parallel G-quadruplex structures in the mixture.[15] Potassium ion is, on the other hand, known to be one of the preferable monovalent cations for stabilizing G-quadruplex structures of DNA.[1–3] As a comparison, additional self-cleavage reactions of Oligonucleotide 1 were carried out in our studies in which the concentration of potassium ion varied. As shown in Fig. S4,† there was no DNA cleavage detectable when potassium ion was absent from the corresponding reaction mixture (Lane 2). This observation is consistent with the suggestion that formation of stable G-quadruplex is a prerequisite for the self-cleavage reaction of Oligonucleotide 1. Furthermore, the temperature dependence of the self-cleavage reactions of Oligonucleotide 1 was examined during our investigations. It appeared that the self-cleaving reactivity of this oligonucleotide was lost completely when the temperature of the corresponding reaction increased to 45 °C (Lane 7 in Fig. S5†), which could result from the dissociation of a G-quadruplex structure at relatively high temperatures.

Two new guanine-rich oligonucleotides were further designed during our investigations that contain the same sequences as

Table 1 Guanine-rich oligonucleotides that were examined in our studies

(a) Oligonucleotide 1:5′
TGGGGTTAGGGGAAAAGGTTAGGGGTTAGG 3′
(b) Oligonucleotides that contain one and two 'mismatched' guanines.Oligonucleotide 2:5′
TGG**C**GTTAGGGGAAAAGGTTAGGGGTTAGG
3′Oligonucleotide 3:5′
TGG**C**GTTAG**A**GGAAAAGGTTAGGGGTTAGG 3′
(c) Oligonucleotides that contain alternative nucleotides in the loops appearing at the ends of their columnar structures.Oligonucleotide
4:5′ TGGGGTTAGGGGAAAAGG**TTT**GGGGTTAGG
3′Oligonucleotide 5:5′
TGGGGTTAGGGGAAAAGG**TTTT**GGGGTTAGG 3′
(d) Oligonucleotides that contain five and three consecutive adenosines in their side loops.Oligonucleotide 6:5′
TGGGGTTAGGGG**AAAAA**GGTTAGGGGTTA GG
3′Oligonucleotide 7:5′
TGGGGTTAGGGG**AAA**GGTTAGGGGTTAGG 3′

Oligonucleotide 1 except that one or two guanines are replaced with non-guanine nucleotides (Oligonucleotide 2 and Oligonucleotide 3 in Table 1). These two new oligonucleotides are in theory unable to form ordinary G-quadruplex structures due to the presence of 'mismatched' guanine bases. It turned out that neither of these two mismatched sequences displayed a detectable self-cleaving activity under our standard reaction conditions (Lane 4 and Lane 6 in Fig. S6†). However, when alterations of nucleotides in some loops located at the ends of the columnar structure of Oligonucleotide 1 were made, the resulting oligonucleotides (Oligonucleotide 4 and Oligonucleotide 5 in Table 1) still exhibited self-cleaving activity (Lanes 8 and 10 in Fig. S6†). These observations could be indications that Oligonucleotide 1 relies on the formation of a G-quadruplex structure for its self-cleaving activity.

As a comparison with Oligonucleotide 1 which possesses four consecutive adenosines in the middle of its sequence, new G-quadruplex structures containing five and three adenosines in a row (Oligonucleotides 6 and 7 in Table 1) in the loop located on the sides of their columnar structures were also designed and examined. As shown in Fig. S7,† the self-cleavage rate of Oligonucleotide 6 (a five adenosine-containing structure) was close to that of Oligonucleotide 1 (Lane 6) while there was no self-cleavage product observable (Lane 2) when the size of its side loop was reduced to three consecutive adenosines (Oligonucleotide 7). These results suggest that besides the formation of a G-quadruplex assembly, the geometry of the side loop within the tetraplex columnar structure plays certain crucial roles in the site specific self-DNA cleaving processes.

In conclusion, it has been demonstrated in our studies that certain structural assemblies of G-quadruplex (Oligonucleotide 1, Oligonucleotide 4, Oligonucleotide 5 and Oligonucleotide 6) are capable of performing self-cleavage actions in a site specific fashion. It is our hope that the findings presented in this report will inspire further exploration for new chemical and biological properties of G-quadruplex that have not yet been recognized.

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