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Kinetic study of the binding of triplex-forming oligonucleotides containing partial cationic modifications to double-stranded DNA

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ABSTRACT

Several triplex-forming oligonucleotides (TFOs) partially modified with 2′-*O*-(2-aminoethyl)- or 2′-*O*-(2-guanidinoethyl)-nucleotides were synthesized and their association rate constants (k_{on}) with double-stranded DNA were estimated by UV spectrophotometry. Introduction of cationic modifications in the 5′-region of the TFOs significantly increased the k_{on} values compared to that of natural TFO, while no enhancement in the rate of triplex DNA formation was observed when the modifications were in the middle and at the 3′-region. The k_{on} value of a TFO with three adjacent cationic modifications at the 5′-region was found to be 3.4 times larger than that of a natural one. These results provide useful information for overcoming the inherent sluggishness of triplex DNA formation.

Triplex-forming oligonucleotides (TFOs) bind to double-stranded DNA (dsDNA) forming triplex DNA. They can be used as reagents for gene modification, control of gene expression, gene detection etc.[1] However, triplex DNA formation by TFO has some drawbacks. For instance, triplexes are formed by two Hoogsteen hydrogen bonds between T and C in the TFO with AT and GC base pairs in dsDNA, respectively, which is less stable than the Watson–Crick base pairing in the duplex DNA. Moreover, the sequence recognizable by natural TFO is limited to homopurine/homopyrimidine tracts within dsDNA. Several strategies, including introduction of modified nucleotides in the TFO, to stabilize triplex DNA or to expand the recognizable sequence have been reported.[2] These reports mainly deal with the thermodynamic evaluations of triplex DNA formation using melting temperature (T_m), binding/dissociation constant (K_d) etc.

Kinetic studies of triplex formation revealed that the rate of formation of triplex DNA was approximately 10^3 times slower than that of duplex DNA.[3] In 2004, Seidmen and coworkers studied the effects of partial 2′-*O*-(2-aminoethyl) modification within 5′-trimethylpsoralen-conjugated 2′-methoxy-TFOs, on kinetics of triplex DNA formation.[4] Interestingly, they found that an increase in the rate of formation of triplex led to a significant increase in the gene targeting efficacy, which strongly suggests the significance of the evaluation of kinetic parameter of triplex formation.[4,5] However, the kinetic studies are limited to examples of 5′-trimethylpsoralen-conjugated 2′-methoxy-TFOs. Moreover, to the best of our knowledge, there are no other reports where the effects of the number and distribution of modified nucleotides in TFOs were examined in detail.[3,6–8]

Under such background, we considered that it is worth exploring the effects of modifications in TFOs without any conjugation on the rate of triplex DNA formation. Cationic modifications in the TFO are expected to enhance the rate of triplex formation by suppressing the electrostatic repulsion between phosphate backbones of TFO and dsDNA. Therefore, 2′-*O*-(2-aminoethyl)-[6] or 2′-*O*-(2-guanidinoethyl)-nucleotides[9] were chosen as cationic modifications (Fig. 1), and in this study, we aimed at exploration of the rate of triplex DNA formation using their modified TFOs.

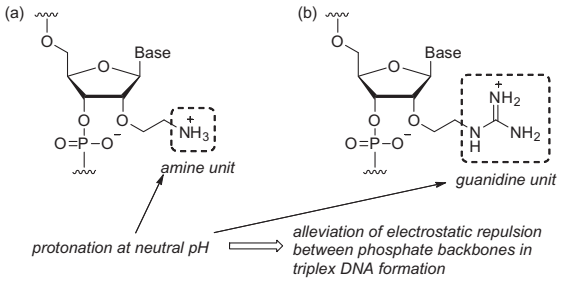

Figure 1. Structures of 2′-*O*-(2-aminoethyl)nucleotide (a) and 2′-*O*-(2-guanidinoethyl)nucleotide (b).

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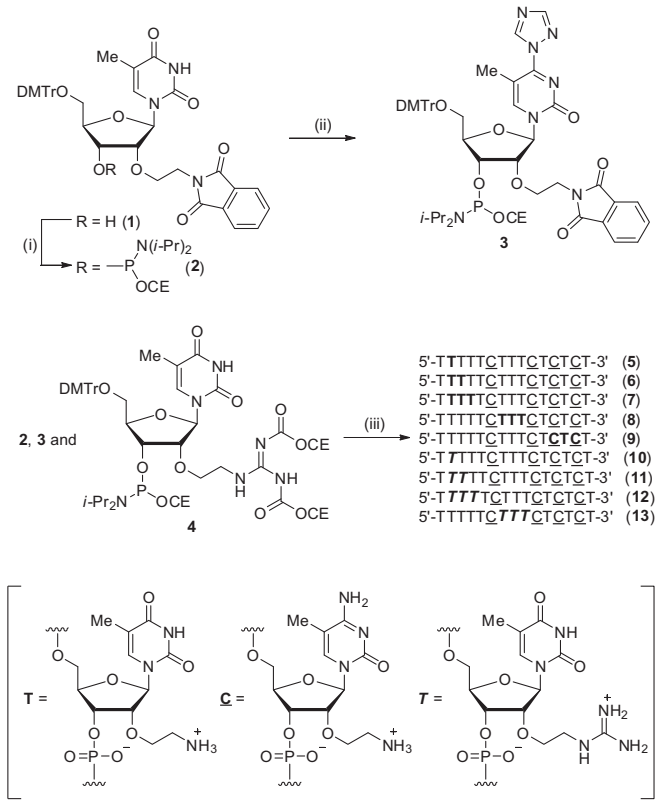

Scheme 1. Reagents and conditions: (i) *i*-$Pr_2NP(Cl)OCH_2CH_2CN$, *i*-Pr_2NEt, CH_2Cl_2, rt, 2 h, 95%; (ii) $POCl_3$, Et_3N, 1,3,4-triazole, MeCN, 0 °C, 1 h, 77%; (iii) DNA synthesis (DMTr = 4,4′-dimethoxytrityl, CE = 2-cyanoethyl, C = 2′-deoxy-5-methylcytidine).

The synthesis of modified TFOs used in this study is shown in Scheme 1. Phosphitylation of the phthalimide-protected compound **1**[10] prepared in reference to an alternative method[11] gave the desired phosphoramidite **2** in 95% yield.[12] The compound **3** which was converted into 2′-*O*-(2-aminoethyl)-5-methylcytidine in TFO synthesis was prepared by commonly used triazolylation.[13] TFOs **5**–**13** with partial modifications were synthesized using phosphoramidites **2** and **3** for 2′-*O*-(2-aminoethyl) modification, and **4**[9] for 2′-*O*-(2-guanidinoethyl) modification on an automated DNA synthesizer, and purified by reversed phase HPLC.[14]

To optimize the conditions for kinetic experiments, thermal stability of triplex formed between natural TFO **14**, 5′-TTTTTC TTTCTCTCT-3′ (C = 2′-deoxy-5-methylcytidine), and dsDNA target **15**, 5′-GGCAAAAAGAAAGAGAGACGC-spacer-GCGTCTCTTTCTTTTT GCC-3′ (spacer = hexaethylene glycol), was examined at various concentrations of KCl and $MgCl_2$ at pH 6.8 (Table 1).[15,16] In general, at a given $MgCl_2$ concentration, the T_m values decreased with increase in KCl concentration (e.g., entries 2, 6 and 9). On the contrary, increase in $MgCl_2$ concentration significantly stabilized the triplex DNA (e.g., entries 8–11), and in the absence of $MgCl_2$ no triplex formation was observed (entries 4 and 11). These findings coincide with that reported by Dervan's group.[17] Eventually, a buffer with 10 mM KCl and 10 mM $MgCl_2$, in which the most stable triplex DNA was formed (T_m = 43 °C, entry 8), was chosen for kinetic experiments.

All kinetic experiments were carried out at 20 °C, where complete triplex formation was observed (Fig. 2).[18] The measurements were made on a UV spectrophotometer equipped with a rapid mixing stopped-flow accessory.[19] This method can observe formation of triplex DNA itself without conjugation to any molecules or solid-supports. As representative results, the decay curves obtained for the triplex formation between TFOs **7**–**9** and **14** and dsDNA **15** are shown in Figure 3. The association rate constants, k_{on}, calculated are summarized in Table 2. As it can be seen from the table, k_{on} values depends on the number of 2′-*O*-(2-aminoethyl) modification at 5′-region of the TFO and increased with increase in the number of modifications (entries 1–4). TFO **7** including three adjacent modifications formed a triplex 3.4 times faster than natural TFO **14**. On the other hand, TFO **8** with three adjacent modifications in the middle showed only a slightly higher k_{on} value than natural **14**, whereas TFO **9** with the modification at the 3′-region, showed a lower k_{on} value than that of **14** (entries 5 and 6). The result of TFO **9** might imply that modification of 5-methylcytidine negatively affects the rate of triplex formation though the further investigation is required to clarify it. Interestingly, although triplexes formed by TFOs **7** and **8** had the same thermal stability (T_m = 54 °C), the k_{on} of TFO **7** is apparently larger than that of TFO **8**. Similar trend in k_{on} and T_m values were observed for the corresponding 2′-*O*-(2-guanidinoethyl) modified TFOs **10**–**13** (entries 7–10).

The kinetic data shown in Table 2 revealed that the rate of triplex formation increased significantly when the cationic modifications were introduced at the 5′-region. Interestingly, this result is in contrast to the report by Seidman's group[4] where they observed an increase in the association rate, even with a single modification at the 3′-region. Although the reason is unclear, the presence of trimethylpsoralen at the 5′-terminus is considered to have an effect in triplex formation.

The mechanism of triplex formation could be explained by nucleation-zipping model.[7] Here, the nucleation step in which a small number of base triads are formed can be assumed to be the rate-limiting step. It is possible that the formation of the

Table 1
Thermal stability of the triplex formed between TFO **14** and dsDNA **15**[a]

Entry	[KCl] (mM)	[$MgCl_2$] (mM)	T_m (°C)
1	100	10	40
2	100	1	35
3	100	0.5	34
4	100	0	nd[b]
5	50	10	41
6	50	1	37
7	50	0.5	35
8	10	10	43
9	10	1	41
10	10	0.5	40
11	10	0	nd[b]

[a] Conditions: Sodium cacodylate buffer (10 mM, pH 6.8), KCl (100, 50 or 10 mM) and $MgCl_2$ (10, 1, 0.5 or 0 mM). The final concentration of each oligonucleotide used was 1.89 μM.
[b] No triplex DNA formation was detected.

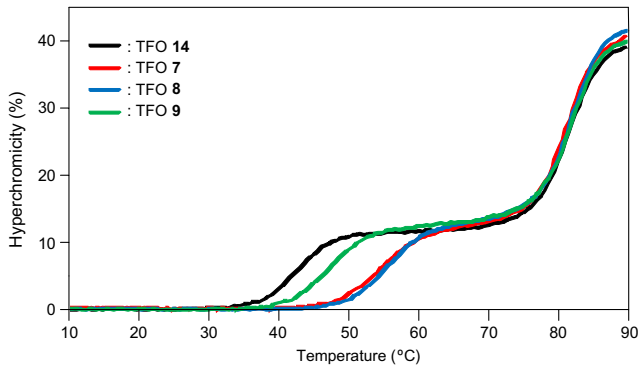

Figure 2. UV-melting curves of representative triplexes formed between TFOs and dsDNA **15**. [Conditions: Sodium cacodylate buffer (10 mM, pH 6.8), KCl (10 mM) and $MgCl_2$ (10 mM). The final concentration of each oligonucleotide used was 1.89 μM.]