Highly Stable Pyrimidine-Motif Triplex Formation at Physiological pH Values by a Bridged Nucleic Acid Analogue**

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Formation of a stable triplex DNA molecule at physiological pH values is a highly desirable phenomenon in molecular biology and medicinal chemistry because of its great importance in regulation of gene expression, site-specific cleavage of DNA, gene mapping and isolation, maintenance of folded chromosome conformations, and gene-targeted mutagenesis. In a pyrimidine-motif triplex DNA, the (homopyrimidine) triplex-forming oligonucleotide (TFO) binds with the homopurine tract of the target duplex DNA in a sequence-specific manner through Hoogsteen hydrogen bonds to form T-A:T and C-G:C triads. However, formation of the C-G:C triad is dependent on the cytosine protonation, which is only favorable at acidic pH values (pK_a = 4.5) and, therefore, homopyrimidine-motif triplexes are extremely unstable at physiological pH values, which severely restricts their biological application.

Although during the past few decades several efforts have been directed to the formation of stable triplex DNA, most of the investigations did not reach a practical level owing to instability of the triplexes at physiological pH values. Recently, our observation concluded that incorporation of a bridged nucleic acid (BNA), such as 2',4'-BNA and 2',4'-BNANC (as LNA), dramatically improved TFO affinity for the target duplex and formed a stable triplex at neutral pH values. However, to our dismay, fully modified TFO failed to bind with double-stranded DNA (dsDNA). The optimum binding ability was found with TFOs composed of alternating BNA and DNA monomers. Ethylene-bridged nucleic acid (ENA), developed by us and Koizumi and co-workers, also exhibited comparable or better triplex-forming ability than that obtained with 2',4'-BNA. Although triplex formation with fully modified ENA was achieved at neutral pH values, partially modified TFOs provided variable results depending on the pH value (when compared with 2',4'-BNA). As a result of our continued investigations of the BNA structure, we report herein a novel BNA molecule, 2',4'-BNA NC, partially and fully modified TFOs formed highly stable triplexes at physiological pH values. Their overall triplex-forming ability is superior to that of ENA and 2',4'-BNA, which is the most widely used BNA (as LNA) for versatile genomic applications.

As shown in Scheme 2, the 2',4'-BNA NC-thymine and 5'-methylcytosine phosphoroamidites 12 and 13, respectively, were synthesized from the nucleoside derivative 1. The acetyl group was removed by aqueous methylvamine and the resultant alcohol 2 was converted to a mesylate 3, which was treated with alkali to give the stereocchemically inverted alcohol 4 in very high yields. Debenzylation of 4 followed by reprotection with a cyclic disilyl group afforded the bicyclic compound 5 in good yield. The 2'-hydroxy group of 5 was transformed to the triflate 6 by the treatment of trifluoromethanesulfonic anhydride in the presence of DMAP and pyridine. The crude 6 was subjected to the S_N2 reaction with N-hydroxypthalimide to yield the phthalimide derivative 7, which was treated with hydrazine to deliver the aminoxime compound 8. Exposure of 8 to phenoxyacetyl chloride in the presence of pyridine provided the desired cyclized product 9 in one step. Deprotection of the silyl groups furnished our target molecule, 2',4'-BNA NC-thymine monomer 10, in excellent yields. Tritylation of the primary hydroxy group of 10 with 4,4'-dimethoxytrityl chloride gave 11. Then, phosphitylation of the secondary hydroxy group of 11 with 2-cyanoethyl-N,N',N'-tetraisopropylphosphorodiamidite yielded the desired thymine phosphoroamidite 12 in a very good yield. On treatment with 1,2,4-triazole in the presence of triethylamine and phosphoryl chloride, compound 12 afforded the triazole derivative 13, which was successfully used as a building block for the 5'-methylcytidine unit of 2',4'-BNA NC. Various TFOs were synthesized from these phos-
phosphoramidites and natural amide building blocks on an automated DNA synthesizer by using a conventional phosphoramidite protocol. By using the usual workup procedure, the phenoxacyetyl group was removed and the triazole group was converted to an amino group to give 2-4,4'-BNANC monomer 10. With the phenoxyacetyl group removed and the triazole group converted to an amino group, the triplex-forming ability of 2,4,-BNA NC-modified TFOs, such as ON-0, ON-1, ON-5, and ON-7, was tested. For the triplex-forming ability of 2,4,-BNA NC-modified TFOs, the results were summarized in Table 1. Modification of the natural DNA–TFO with a single 2,4'-BNA NC

state for duplex dissociation and it might be concluded that triplexes were simultaneously converted to three different single strands.[13] Melting temperatures ($T_m$) of the triplexes formed by 2,4'-BNA NC-modified TFOs were compared with those formed by natural DNA, 2,4'-BNA, and ENA-modified TFOs, and the results are summarized in Table 1. Modification of the natural DNA–TFO with a single 2,4'-BNA NC

Table 1: $T_m$ values of triplexes containing 2,4,-BNA NC (bold red), 2,4'-BNA (bold blue), and ENA (bold black).[a,b]

<table>
<thead>
<tr>
<th>TFO</th>
<th>Sequence (5'-3')</th>
<th>$T_m$ [°C]</th>
<th>$\Delta T_m$ [°C]</th>
<th>$\Delta T_m$/mod</th>
</tr>
</thead>
<tbody>
<tr>
<td>ON-0</td>
<td>TTTTTTTCTTCTCT</td>
<td>33</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ON-4</td>
<td>TTTTTTTCTTCTCT</td>
<td>58</td>
<td>+25</td>
<td>+6.3</td>
</tr>
<tr>
<td>BNA-4</td>
<td>TTTTTTTCTTCTCT</td>
<td>57</td>
<td>+24</td>
<td>+6.0</td>
</tr>
<tr>
<td>ENA-4</td>
<td>TTTTTTTCTTCTCT</td>
<td>57</td>
<td>+24</td>
<td>+6.0</td>
</tr>
<tr>
<td>ON-5</td>
<td>TTTTTTTCTTCTCT</td>
<td>64</td>
<td>+31</td>
<td>+6.2</td>
</tr>
<tr>
<td>BNA-5</td>
<td>TTTTTTTCTTCTCT</td>
<td>65</td>
<td>+32</td>
<td>+6.4</td>
</tr>
<tr>
<td>ENA-5</td>
<td>TTTTTTTCTTCTCT</td>
<td>58</td>
<td>+25</td>
<td>+5.0</td>
</tr>
<tr>
<td>ON-6</td>
<td>TTTTTTTCTTCTCT</td>
<td>78</td>
<td>+45</td>
<td>+6.4</td>
</tr>
<tr>
<td>BNA-6</td>
<td>TTTTTTTCTTCTCT</td>
<td>67</td>
<td>+34</td>
<td>+4.9</td>
</tr>
<tr>
<td>ENA-6</td>
<td>TTTTTTTCTTCTCT</td>
<td>72</td>
<td>+39</td>
<td>+5.6</td>
</tr>
<tr>
<td>ON-7</td>
<td>TTTTTTTCTTCTCT</td>
<td>80</td>
<td>+47</td>
<td>+3.1</td>
</tr>
<tr>
<td>BNA-7</td>
<td>TTTTTTTCTTCTCT</td>
<td>&lt;5</td>
<td>&lt;28</td>
<td>&lt;2</td>
</tr>
</tbody>
</table>

[a] Target duplex: 5'-d(CCTTAAAGAAGCAGATCG)-3'; 5'-d(CGATTTTCTTCTCTACCG)-3'; underlined portion indicates the target site for triplex formation. (b) Conditions: 7 mM Na$_2$HPO$_4$ buffer solution containing 140 mM KCl; strand concentration = 1.5 μM; scan rate 0.5°C min$^{-1}$. $T_m$ = melting temperatures, $\Delta T_m$ = changes in melting temperature, $\Delta T_m$/mod = changes in melting temperature per single modification; °C = 5-methylcytidine.
monomer (ON-1) increased the $T_m$ value by 11°C, which is equal to that of 2'-4'-BNA-modified TFO (BNA-1) and slightly higher than that of the corresponding ENA-modified TFO (ENA-1). Further modifications greatly enhanced the triplex thermal stability. For example, by increasing the number of modifications from one to three, $T_m$ values increased to 60°C ($\Delta T_m = +27°C$) and 59°C ($\Delta T_m = +26°C$) for ON-2 and ON-3, respectively. Therefore, it is noteworthy that both the values of the 2',4'-BNA NC-modified TFOs with either interrupted or continuous 2',4'-BNA NC residues are very high and the same, whereas the $T_m$ value of BNA-3 (52°C) containing three continuous modifications was found to decrease by 7°C compared with that of BNA-2 (59°C). The corresponding ENA–TFOs (ENA-2 and ENA-3) showed lower $T_m$ values than the 2',4'-BNA NC–TFOs. Interestingly, their triplex-forming behavior is in agreement with that of 2',4'-BNA NC, clarifying that continuous six-membered bridged structures are well tolerated by dsDNA. These observations were also consistent with the results of other TFOs (such as, ON-4 and ON-6 versus BNA-4, BNA-6 versus ENA-4 and ENA-6, and ON-5 versus BNA-5) with the exception of ENA-5. In the cases of ON-4-BNA-4/ENA-4 and ON-5/BNA-5 where modifications are located far apart from each other, all the 2',4'-BNA NC– TFO (ON-6) provided $T_m$ values as high as 78°C, which is 11°C and 6°C higher than those provided by the corresponding 2',4'-BNA– and ENA–TFOs (BNA-6 and ENA-6), respectively. Thus, with continuous modifications or with an increased number of modifications, 2',4'-BNA NC– modified TFOs showed higher $T_m$ values than those of 2',4'-BNA– and ENA–modified TFOs. These interesting characteristics of 2',4'-BNA NC prompted us to synthesize a fully modified TFO, ON-7, which formed a very stable triplex with a $T_m$ value as high as 80°C ($\Delta T_m = +47°C$; $\Delta T_m$ per modification $= +3.1°C$). The corresponding 2',4'-BNA-modified TFO, BNA-7, failed to form a triplex.[14,15]

Next, triplex formation was evaluated by an electrophoretic mobility shift assay (EMSA) at pH 6.8 (Figure 2). Each TFO was incubated with the target dsDNA at a ratio of 1:1 at 4°C in 10 mM 2-[4-[(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid buffer solution (HEPES; pH 6.8) and subjected to a 20% polyacrylamide gel electrophoresis at 4°C and room temperature.[16] To confirm the triplex formation, the target duplex without TFO and TFO with excess duplex (TFO/duplex $= 1:2$) were also run together (Figure 1, lanes 1 and 3, respectively). It was found that all the modified TFOs formed stable triplexes at the stoichiometric ratio under the experimental conditions. The natural DNA (ON-0) was unable to form a triplex at room temperature even though it showed triplex formation at 4°C. In contrast, the TFO with only a single 2',4'-BNA NC modification (ON-1) can form a stable triplex at room temperature. These results correlate with the $T_m$ data and it might be expected that extensively modified TFOs would promote stable triplex formation at higher temperatures. The fully modified TFO, ON-7, also formed a stable triplex with a clear and intense band. The electrophoretic mobilities of the triplexes formed by 2',4'-BNA NC–modified TFOs were slightly lower and that of the fully modified TFO (ON-7) was remarkably lower.[17]

The extraordinarily high triplex-forming ability of 2',4'-BNA NC might result from the combined effects of restricted N conformation[5,18] and protonation of the N atom, which might cause electrostatic interactions between the positively charged TFO and the negatively charged phosphodiester linkage of the target duplex.[19,21] Moreover, in contrast with the fully modified BNA–TFOs, which are too rigid in the overall structure,[18,22] the 2',4'-BNA NC–modified TFOs, bearing a six-membered bridged structure like ENA, might possess suitable conformational flexibility in their overall structure, which facilitates stable triplex formation as well. The above facts are the reason for which the 2',4'-BNA NC–modified TFOs act as excellent TFOs for the recognition of the homopurine–homopyrimidine tract of dsDNA. The predominance of 2',4'-BNA NC over ENA and 2',4'-BNA essentially lies with the role of the protonated nitrogen to neutralize the negatively charged phosphate backbone of the purine strand.

In conclusion, we have synthesized a novel bridged nucleic acid analogue, 2',4'-BNA NC, and demonstrated that the TFOs composed of 2',4'-BNA NC formed highly stable pyrimidine-motif triplexes at physiological pH values. The overall triplex-forming ability is higher than that of 2',4'-BNA/LNA- and ENA-modified TFOs. Unlike the 2',4'-BNA-modified TFOs, these TFOs eliminate the requirement of placing alternating DNA monomers for optimum efficacy.[23] More interestingly, fully modified TFOs still formed a highly stable triplex. These promising properties of 2',4'-BNA NC will be helpful for developing oligonucleotide-based technologies for the postgenome era.

**Experimental Section**

UV melting experiments: UV melting experiments were carried out using a Beckman DU-650 spectrometer equipped with a $T_m$ analysis.
accessory. Equimolecular amounts of the target duplex and TFO were dissolved in 7 mM sodium phosphate buffer solution (pH 7.0) containing 140 mM KCl to give a final strand concentration of 1.5 μM. The strands were annealed by heating the samples at 90°C for 5 minutes followed by slow cooling to room temperature. Then the samples were stored at 4°C for 1 h. The melting profile was recorded at 260 nm from 10 to 85°C at a scan rate of 0.5°C/min. The Tm was calculated as the temperature of the half dissociation of the formed triplexes, which is determined by the first derivative of the melting curve.

Full experimental details are described in the Supporting Information.

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[7] This BNA is defined as 2'-β-BNA NC because the bridge between 2'-O and 4'-C is constituted by N and C atoms.


[11] We had to replace the benzyl groups with a cyclic disiloxane group because we experienced cleavage of the N–O bridged structure during denaturation of the corresponding dibenzyl derivative of 9.


[14] Similar results were obtained by using two different BNA–TFOs in our previous investigation. The TFOs also failed to bind with a 30-bp target duplex.[16]

[15] The Tm value of the corresponding ENA–TFO could not be determined because of the difficulties in purification of the TFO.

[16] In the case of room temperature EMSA, the triplexes were warmed to room temperature after incubating at 4°C and kept at room temperature for two hours before applying onto the gel. The gel was run at room temperature at a constant voltage of 70 V.

[17] This type of lower mobility was also found in the case of the recently reported ε-t-LNA-modified triplexes.[17] Variation of triplex mobility was also noted by Roberts and Crothers, see: R. W. Roberts, D. M. Crothers, *Science* 1992, 258, 1463.


[23] Rules for designing BNA–TFOs for optimum triplex-forming ability were described, see: B. W. Sun, B. R. Babu, M. D. Sørensen, K. Zakrzewska, J. Wengel, *Biochemistry* 2004, 43, 4160.