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DNA Recognition by Hybrid Oligoether–Oligodeoxynucleotide Macrocycles**

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The sequence-specific binding of RNA and DNA by synthetic oligonucleotides and analogues is important as a diagnostic tool in the study of naturally occurring polynucleotides and is the subject of intense research as a potential therapeutic strategy as well.^[1–3] Two limitations of natural DNA oligomers in these applications are their low binding affinity^[2] and also their limited stability, due to cleavage by exo- and endonucleases which occur in natural systems.^[4, 5] We report here the construction of circular hybrid molecules which contain two oligonucleotide domains bridged by two oligoethylene glycol chains. These molecules bind with high affinity to complementary strands of RNA and DNA and display exceptional resistance to degradation by nucleases.

Oligoethylene glycol chains have been successfully used as simple linking groups which replace nucleotide units in linear oligonucleotide chains^[6] and in hairpin-shaped oligonucleotides in duplexes and triplexes as well.^[7] We wished to determine whether such spacers could be incorporated into circular structures and what the effect on their recognition properties and stability would be. We have previously shown that circular oligonucleotides can display very strong binding affinity^[8] and high sequence selectivity^[9] for single-stranded DNA and RNA by forming bimolecular triple helices. These macrocycles bind by forming bonds on two sides of a linear target strand, and their unusual recognition characteristics arise from the preorganized circular structure.^[10]

Examination of models^[11] and published data on DNA triple helices^[12] revealed that a group 19–21 Å long would be ideal for bridging the outer pyrimidine strands, and that five-base nucleotide loops might be replaced with penta- or hexaethylene glycol chains. In addition to confirming our model for the binding, such a replacement might lead to favorable properties, such as lower cost and higher synthesis yield (by decreasing the number of nucleotide units), longer lifetimes in biological media, and possibly, improved membrane permeability (by decreasing total negative charge).

The linear precursors to the compounds investigated in this study **1–3** were constructed by automated DNA synthesis methods^[13] (*Experimental Procedure*) with the introduction of the synthetic dimethoxytrityl hexa- or pentaethylene glycol (HEG or PEG) phosphoramidites^[7] at two positions. The oligomers d(A)₁₂ (for **1**) and

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dAAGAAAAGAAAG (for **2** and **3**) were used as templates in cyclization to align the reactive phosphate and hydroxyl ends in an intramolecular esterification reaction in the presence of BrCN, imidazole, and NiCl₂, as reported previously.^[10]

Thermal denaturation studies were carried out to investigate the binding properties of the macrocycles with their complementary 12-base oligonucleotides (Table 1) at a concentration of 3 μ M in a buffer approximating physiological conditions (pH 7.0, 100 mM NaCl, 10 mM MgCl₂). The dissociation curves reveal sharp, two-state transitions, and similar

hyperchromicities for both complexes, with an average value of 28 % at 260 nm. Mixing curves of absorbance (at 260 nm) vs. mole ratio for both complexes confirm a 1 :1 stoichiometry of binding, consistent with a bimolecular triple-helical structure. The complex of compound **1** and d(A)₁₂ has a melting transition (T_m) of 51 °C, which is 14 °C higher than the corresponding Watson–Crick duplex, d(A)₁₂ · d(T)₁₂^[8] (Table 1). Curve-fitting analysis^[14] gives an estimated free energy (37 °C) of – 15 kcal mol^{–1} for the complex of **1** and d(A)₁₂, which is 7 kcal mol^{–1} more favorable than the duplex. Similarly, compound **2** binds its complementary oligonucleotide to give a complex with a T_m of 58 °C ($\Delta G_{37}^\circ = -17 \text{ kcal mol}^{-1}$), compared to 42 °C (–10 kcal mol^{–1}) for the analogous duplex.

Thus, in both cases the binding by the macrocycles is much stronger than that achieved by simple Watson–Crick binding of the same sequences, giving equilibrium binding constants (k_{assoc}) which are approximately five orders of magnitude greater than those of the duplexes at 37 °C. Comparison with the all-DNA analogues containing nucleotide loops reveals that these synthetic hybrid macrocycles bind only slightly less strongly. For example, the analogue of **1** containing five-base loops (sequence -CACAC-) forms a complex with a T_m of 57 °C ($\Delta G_{37}^\circ = -16 \text{ kcal mol}^{-1}$) under identical conditions.^[8] The small decrease of this value upon introduction of the HEG chain may be due to the increased flexibility relative to a more rotationally restricted nucleotide chain. The fact that the effect is small also helps to confirm the model for “circle binding”,^[10] which requires loops mainly for bridging and not for other more specific interactions.

Model studies^[11] and fiber diffraction data^[12] indicate that a bridge between the outer pyrimidine strands at a given base triad in a DNA triple helix must span a distance of approximately 20 Å. Molecular modeling studies indicate that in its extended conformation, HEG-monophosphate has a length of 23 Å, while penta- (PEG) and tetraethylene glycol (TEG) monophosphates have lengths of 19 and 16 Å, respectively. To test this length requirement, we also attempted construction of versions of **2** with these shorter spacers. Melting studies of the complex of the PEG-bridged macrocycle **3** with its template complement show that this complex is somewhat less stable than the analogous HEG complex ($T_m = 53$ °C, 5 °C less than the T_m of the adduct of **2**). Attempts at constructing the TEG circle failed; apparently, this bridge is too short to span the distance required for full complexation of the 12-base template in the cyclization reaction. Thus, it appears that of these three cases the hexaethylene glycol spacer is the best suited for optimum complexation, and the spacer length of 23 Å is consistent with published models for triple-helix structure.^[11]

The primary cause of degradation of standard DNA oligomers in biological applications is a 3'-exonuclease activity found in cells.^[4, 5] Although present in lower activity, endonucleases are also found, which degrade DNA oligomers at internal phosphodiester bonds.^[2] Attempts at cleavage of **1** and **2** by exonucleases confirm that they are completely resistant, due to their circular structures. Interestingly, we find that exonucleases cleave the linear precursors of **1** and **2** only through the first six bases from the end, leaving the oligoether units, which are apparently not recognized by the enzymes.

In order to test susceptibility of such macrocyclic DNA compounds to degradation in a biological medium, we incubated compound **2** and its all-DNA analogue (see Table 2) in fresh human serum at 37 °C and removed aliquots periodically, analyzing the structural integrity by gel electrophoresis (data not shown here). A linear version of this sequence was found to have a half-life for cleavage of 20–30 min, while both circular compounds showed no sign of cleavage after two days. Thus, circular oligomers are highly resistant in this medium, and exonucleases appear to be the dominant cleaving enzymes in serum, consistent with published findings.

To examine specifically the sensitivity to an endonuclease, we studied the cleavage of compound **2** by Nuclease S1, a single-strand-specific DNA-cleaving enzyme. We compared the results to those for the 34-base all-DNA analogue (Table 2). The sensitivity to degradation was tested with oligomers both alone and in the presence of a complementary oligonucleotide, since the resulting secondary structures (such as triple helices) may not be recognized by enzymes and thus confer added resistance.

The experiments show that both types of macrocycles are susceptible to single-strand endonuclease cleavage (Table 2). Both linear and circular sequences showed the same rates of cleavage (data not shown here). Interestingly, we find that both circular oligomers are considerably more resistant to cleavage when bound to their complement. This is evidently due to the fact that they are held in a tight triple-helical secondary structure in the complex. More significant is the finding that whereas the all-DNA compound is still cleaved, albeit at a slower rate, when bound, we find no evidence for any cleavage of the DNA–oligoether macrocycle **2** after 24 hours of incubation. This difference is likely due to the variance in loop structure for the two complexes: the all-DNA compound still presents single-stranded cleavable nucleotides in the complex, while in **2** there is no single-stranded region of DNA exposed to solvent. In the presence of a binding structure, compounds such as **1–3** are unlikely to be substrates for any exo- or endonuclease enzyme, and would likely be stable during diffusion through serum as well. The combination of this stability and the high binding affinity makes such compounds good candidates for use in biological systems.

Experimental Procedure

1 was prepared from the linear precursor 5'-pTTTTTTPpO-(CH₂CH₂O)₆-pTTTTTTTTTTTTpO-(CH₂CH₂O)₆-pTTTTTT; **2** from 5'-pTTTCTTPpO-(CH₂CH₂O)₆-pTTCTTTTCTTTCPpO-(CH₂CH₂O)₆-pCTTCT; and **3** from 5'-pTTTCTTPpO-(CH₂CH₂O)₅-pTTCTTTTCTTTCPpO-(CH₂CH₂O)₅-pCTT-TCT. Reaction conditions were as described previously [10]. The conversion to macrocyclic product was 85–95% (by gel analysis) in both cases, and the circular products were isolated by preparative gel electrophoresis, giving 43% (**1**), 48 % (**2**), and 24% (**3**) isolated yields. The circular structure was confirmed by complete resistance to **T4** polymerase exonuclease cleavage under conditions under which the linear precursors are fully cleaved up to the ether linkage. Dimethoxytrityl-HEG- and -PEG-phosphoramidites were synthesized by the method of Thuong et al. [7]. Coupling yields for these linkers were > 90%. Intact incorporation of HEG and PEG spacers after final deprotection was also confirmed by ¹H NMR and FAB-MS studies on short oligomers having the sequence dTpO-(CH₂CH₂O)_n-pT.

Thermal denaturation studies and curve fitting were carried out as described [10]. Mixing studies were carried out at a total concentration of 6 μM in a buffer containing 100 mM NaCl, 10 mM MgCl_2 , and 10 mM Tris $\cdot$ HCl at pH 7.0. Exonuclease cleavage was carried out at 37 $^\circ\text{C}$ with 20 μM DNA, 6 mM MgCl_2 , 4 mM potassium phosphate (pH 7.5), and 55 units mL^{-1} of enzyme with T4 Polymerase from New England Biolabs. Nuclease S1 was purchased from Pharmacia, and cleavage reactions were carried out at a DNA concentration of 100 μM , 100 U mL^{-1} enzyme, using published buffer conditions [15]. Human serum was prepared by the published procedure [4], and cleavage studies were carried out at a DNA strand concentration of 50 μM at 37 $^\circ\text{C}$ and analyzed by gel electrophoresis.

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Table 1

Melting temperatures (T_m) and free energies of complexation ($-\Delta G_{37}^\circ$) for linear duplexes and complexes of oligonucleotides and circular ether–DNA hybrids.

Complex ^[a]	T_m [°C] ^[b,c]	$-\Delta G_{37}^\circ$ [kcalmol ⁻¹]
	37	8
	51	15
	42	10
	53	15
	58	17

^aHexa- and pentaethylene glycol are abbreviated as HEG and PEG, respectively.

^bThe data are for pH 7.0, 100 mM NaCl, 10 mM MgCl₂, and oligomer concentrations of 3 μM in each strand.

^cPrecision in T_m values is ± 1 °C, and in free energies, $\pm 10\%$.

Table 2

Susceptibility of synthetic circular DNA and DNA–ether compounds to degradation by staphylococcal endonuclease (S1) in the absence and presence of the complementary oligonucleotide sequence.

Oligomer(s) ^[a]	Nuclease cleavage $t_{1/2}$ [min]
1
20
1
>24h

^aHexaethylene glycol loops are abbreviated as HEG.