Oligonucleotide Rings

Small Circular Oligodeoxynucleotides Achieved from Self-Assembling Entities**

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Over the past decade circular oligodeoxynucleotides[1,2] have emerged as useful molecular tools for hybridization, diagnostics, and sequence-specific inhibition of gene expression because their DNA-binding affinity, sequence selectivity, and exonuclease resistance are better than that of the corresponding linear sequences.[3,4] As a result of advances in solid-phase technology[5] circular oligodeoxynucleotides can now be synthesized efficiently and without any sequence restriction. Most importantly, the size of circular oligodeoxynucleotides constructed by these convenient solid-phase methodologies can be varied easily from 2 to 20 nucleotides.[5] Conversely, the template-directed synthesis of circular oligodeoxynucleotides of comparable size has been unachievable so far.[1] With the aim of probing the size limit of circular oligodeoxynucleotides constructed by self-assembly, we recently fabricated some very short ones. Here we report a new type of spatial arrangement of the i-motif,[10] through which the size of the circular oligonucleotides can be reduced to only nine nucleotides, the smallest circular unit ever achieved by means of self-assembling entities of nucleic acids. Unlike the self-assemblies identified previously for fabricating circular oligodeoxynucleotides,[6–8] the characteristics of the newly designed entity of the i-motif are self-recognition at the oligodeoxynucleotide level, rather than complementarity, and the mutual templating that guides the alignment of two separate strands in the formation of dimeric complexes (**b** in Figure 1). The present work accordingly defines a new size limit for circular oligodeoxynucleotides synthesized through self-assembling entities.[4]

The term i-motif describes a structural entity composed of two parallel-stranded DNA duplexes zipped together in an antiparallel orientation and held together by hemiprotonated C–C$^+$ base pairs.[10] Mainly through NMR investigations, it has been established that the same C-rich strands of oligodeoxynucleotides can form intercalated structural entities of the i-motif that differ essentially in their intercalation and loop topologies.[11] Based on this well-established principle for the formation of the i-motif,[11,12] we selected oligodeoxynucleotide pCCTCCCTTC (**a** in Figure 1), anticipating that this sequence would self-assemble into a dimeric complex (**b**) through self-recognition and that the two termini of this linear sequence would be aligned in a head-to-tail fashion through mutual templating. It should be pointed out that the i-motif formed by pCCTCCCTTC could exist in two or more isomeric forms with different intercalation and loop topologies; **b** represents only one of these possible configurations. In addition, certain constraints from the phosphate–sugar backbone must be present within this assembly of the i-motif, since only one or two nucleotides were required to sustain the hairpin loops. Consequently, an equilibrium could exist between the assembled entity **b** and unstructured form **a** of the linear sequence. Once the dimeric complex **b** is generated, covalent linkage between the two open termini would lead to the desired circular oligodeoxynucleotides with a preferred orientation (**c** and **d** in Figure 1).

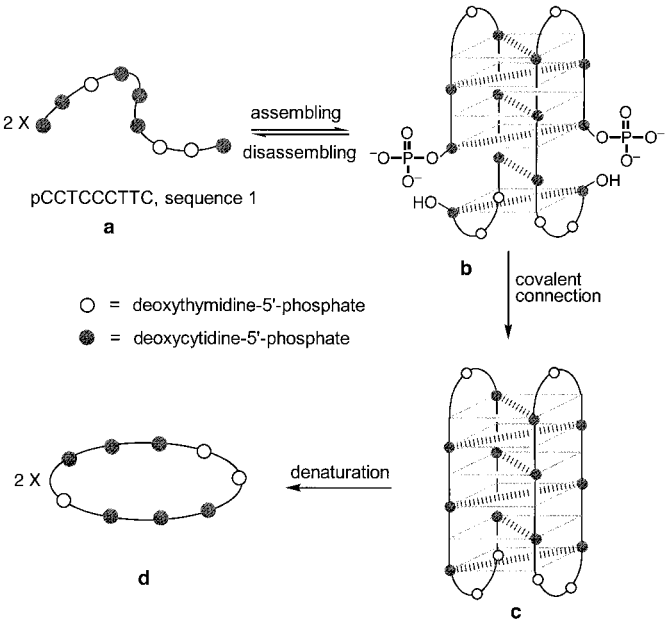

Figure 1. Representation of our strategy for constructing very short circular oligodeoxynucleotides through bimolecular assembly of the i-motif (**b**).

Following the strategy outlined in Figure 1, we first incubated the 9-mer linear sequence pCCTCCCTTC (sequence 1) in pH 5 buffer to generate the desired assembly of the i-motif[11,12] (**a**→**b**). The 5′-terminal phosphate was subsequently activated by the addition of *N*-cyanoimidazole[13] to promote formation of the phosphodiester bonds between the 5′- and 3′-termini of this linear sequence (**b**→**c**). The products of this circularization course were then analyzed by denaturing polyacrylamide gel electrophoresis (PAGE). As apparent on the autoradiograms, a new product was generated in 43 % yield from the circularization reaction (Figure 2a, lane 3), which was deduced by quantifying the radioactivities of both linear and circular products using PhosphoImager SI (Molecular Dynamics). This new product, which has a greater mobility shift than its linear precursor,[9] was identified as the corresponding circular oligodeoxynucleotide

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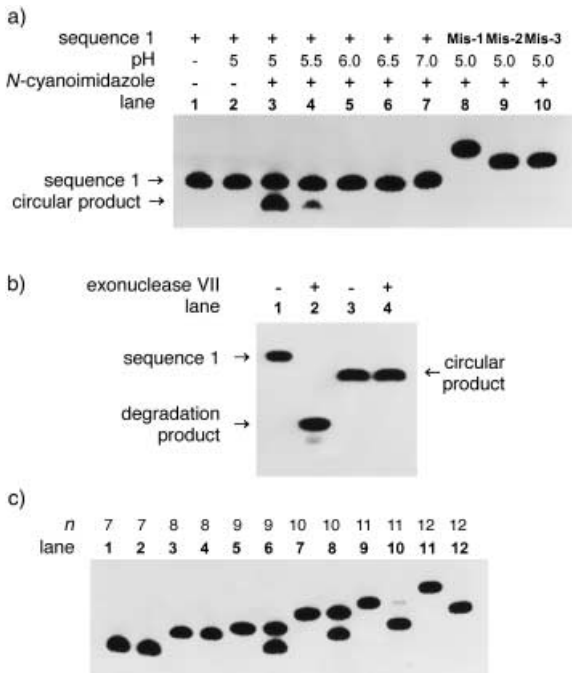

Figure 2. Analysis of the oligodeoxynucleotides obtained in circularization experiments. For details see text and Experimental Section. a) Lanes 1 and 2 are control experiments. b) Treatment with exonuclease VII. Lanes 1 and 3 are control experiments. Lanes 2 and 4 show the different behavior of sequence 1 and the purified (by the "crush-and-soak" method) circular product with the faster mobility shift (see the lower spot in lane 3, Figure 2a).[15] c) Results of experiments to synthesize differently sized circular oligodeoxynucleotides. Lanes 1, 3, 5, 7, 9, and 11: the *n*-mers alone. Lanes 2, 4, 6, 8, 10, and 11: reaction mixtures of the *n*-mers.

⟨CCTCCCTTC⟩. The efficiency of the reaction was found to be strongly pH-dependent. Circularization at pH 5.0 (lane 3), 5.5 (lane 4), and 6.0 (lane 5) proceeded in yields of 43, 17, and 4%, respectively, and at pH 6.5 (lane 6) and 7.0 (lane 7) no product from the reaction was observed. This pH dependency is consistent with the necessity for the formation of base pairs between cytosine and protonated cytosine ($C-C^+$).[10–12] Moreover, we examined the correlation of this circularization course with the structural intactness of the i-motif by using three "mismatched" sequences—pCTACTTGCA (Mis-1), pGCTCCCTTC (Mis-2), and pCCTCCCTTG (Mis-3)—in which one or more cytosines of sequence 1 were replaced with guanine, thymine, and adenine, respectively. According to the rule for the generation of the i-motif,[10,11] these three sequences should be incapable of supporting the correct conformation of the i-motif because they do not have the correct number of cytosines for proper $C-C^+$ base pairing. As expected, none of the sequences of Mis-1 (lane 8), Mis-2 (lane 9), and Mis-3 (lane 10) gave rise to a circular product under the same reaction conditions as described for sequence 1.

With the aim of confirming the circular nature of the phosphate–sugar backbone, we purified the newly formed product (lane 3 in Figure 2b) by PAGE and digested it with exonuclease VII, an exodeoxyribonuclease that hydrolyzes nucleotides from both the 3′- and 5′-ends of single-stranded DNA.[7,14] As expected, no degradation was observed from this digestion reaction (lane 4). As a control, the linear sequence of pCCTCCCTTC was also hydrolyzed under the same reaction conditions, which accordingly gave rise to products of lower molecular weight quantitatively (lane 2). The identified circular product was also digested with exonuclease I and alkaline phosphatase (data not shown). Again, no degradation products were observed from these digestion reactions. These results strongly suggest that the phosphate–sugar backbone of this newly formed product is indeed circular in nature.[7,14]

The bimolecular assembly of the i-motif as an intermediate entity is also suitable for the fabrication of circular oligonucleotides over nine nucleotides in length. For example, when the linear sequences of 10-mer (pCCTTCCCTTC, lane 8 in Figure 2c), 11-mer (pCCTTCCCCTCC, lane 10), and 12-mer (pCCTTCCCCTTCC, lane 12) were incubated under the same conditions as described for sequence 1 (9-mer, lane 6), the corresponding circular oligodeoxynucleotides were generated in yields of 48%, 84%, and 93%, respectively. Conversely, within experimental limits, no circular products were evident when the linear sequences of 7-mer (pCTCCTTC , lane 2) and 8-mer (pCTTCCTTC, lane 4) were incubated under the standard circularization conditions, which implies that these two oligodeoxynucleotides are too short to sustain a steady bimolecular assembly of the i-motif.

In conclusion, by manipulating the dimeric structure of the i-motif, we have synthesized 9- to 12-membered circular oligonucleotides, which are far smaller than the previous smallest circular sequences (20 nucleotides in length)[9] achieved via self-assembling entities. Illustration of the extraordinarily compact conformation of the i-motif (**b** in Figure 1) attainable by nucleic acids may be beneficial to the elucidation of the structures and roles of cytosine-rich sequences in biological processes (e.g. the centromeric region and at the telomeric end).[10]

Experimental Section

Oligodeoxynucleotides were supplied by Operon Technologies, Inc. and phosphorylated at their 5′-ends with T_4 polynucleotide kinase and [γ-^{32}P]ATP. The circularization products were analyzed by 20% PAGE and visualized by autoradiography. The autoradiograms in Figure 2a show the influence of pH and oligonucleotide length on the formation of circular oligodeoxynucleotides. For lane 3, a stock solution of sequence 1 was prepared by incubation of 100 mM 2-(*N*-morpholino)ethanesulfonic acid (MES, pH 5), 50 mM NaCl, and 10 μM sequence 1 at 4°C for 12 h for the formation of bimolecular complexes of the i-motif. The reaction mixture consisting of 5 μM sequence 1 taken from the stock solution, 100 mM MES (pH 5), 25 mM NaCl, 50 mM $NiCl_2$, and 50 mM *N*-cyanoimidazole in a total volume of 20 μL was then incubated at 4°C for 12 h. The reaction was terminated by refrigeration and further analyzed by PAGE. For the experiments in Figure 2b, a reaction mixture of 1 × exonuclease VII buffer (100 mM Tris-HCl, 100 mM potassium phosphate, 16.6 mM ethylenediamine tetraacetic acid, and 20 mM 2-sulfanylethanol, pH 7.9), 10 units of exonuclease VII, and sequence 1 in a total volume of 20 μL was prepared and incubated at 37°C for 2 h. The reaction mixtures for Figure 2c were prepared in the same way as the

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one loaded in Figure 2a, lane 3, except for replacing sequence 1 with oligodeoxynucleotides of different lengths.

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