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An Earring for the Double Helix: Assembly of Topological Links Comprising Duplex DNA and a Circular Oligodeoxynucleotide

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Abstract

Novel DNA nanostructures, locked pseudorotaxane and locked catenane were assembled through topological linkage of a double-stranded target to a circular oligodeoxyribonucleotide (cODN)+. The formation of these supramolecular complexes occurs with remarkable sequence specificity and is accomplished via local opening of duplex DNA by a pair of homopyrimidine bis-PNAs. The obtained cODN label, resembling an earring, forms a true topological link with the linear or closed circular (cc) target DNA and occupies a fixed position along the double helix. The PNA directed assembly described here introduces PNA oligomers into the repertoire of DNA nanotechnological tools.

Introduction

Topological and pseudotopological forms of DNA hold promise for diagnostic and nanotechnological applications. To form such structures, DNA may be targeted sequence-specifically at precise spatial positions. Two approaches in this respect have been reported†. One is the DNA padlock (1,2), which is formed by circularization of a linear oligonucleotide after its hybridization to the complementary sequence on a long single-stranded DNA molecule. Another approach is the sliding clamp (3), a triplex-forming cODN threaded on a duplex DNA fragment. In both systems, pseudotopological links were assembled. Therefore, the cODN tag may travel for considerable distances along the target DNA.

Recently, we designed a PNA-assisted supramolecular DNA assembly, in which a cODN is truly topologically interlocked with dsDNA (see Figure 1), and therefore remains at the site of its formation during various post-assembly manipulations (4). The design became possible due to formation of a new looped structure, the PD-loop, within the DNA double helix (5, 6). Here, we present a more detailed account of the assembly of DNA topological links, and draw future directions for their development and applications.

Results and Discussion

Assembly of Earring Complexes

Figure 1 schematically illustrates a new DNA nanostructure featuring linkage of dsDNA with a cODN. In this structure, resembling an earring, a segment of the cODN appears to be threaded sequence-specifically between complementary strands of dsDNA. Depending on the nature of the targeted dsDNA (linear or cc), the obtained structure is either a locked pseudorotaxane or a locked catenane. The structure retains a true topological link between the cODN and target DNA as long

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as the participating dsDNA macromolecule preserves its native duplex conformation. In this case, the cODN tag will remain correctly positioned along the double helix and will not slip away during various possible post-assembly manipulations on the condition that the openers are bound to the target site. Figure 2 shows the major successive steps of our assembly. At first, we open dsDNA (**I**) at two closely located sites using a pair of cationic bis-PNA openers (5-9). In the resulting complex **II**, both bis-PNA openers are bound to one strand of duplex DNA exposing the opposite strand for hybridization with an ODN (4-6). An ODN is designed in such a manner that its termini (8 -10-nt-long) are complementary to the displaced DNA target and will be in juxtaposition upon hybridization. After hybridization, the ODN is circularized by enzymatic ligation (10). The resulting cODN forms two turns around the displaced DNA strand within a PD-loop structure, and is therefore topologically linked with the target site (**III**).

The yield of product formation was found to be highly dependent on the concentration of added ODN. The maximum yield of **III** was obtained at ODN concentrations between 0.2 and 0.5 μM (Figure 3). While at low ODN concentrations, less PD-loop complexes can be expected, at high ODN concentrations two ODNs may occupy one target site within the PD-loop preventing self-ligation of ODN molecules bound to the target sequence. Thus, even at optimal ODN concentration, prolonged incubations (up to 15 h) resulted in yields of **III** not higher than 75%. However, the yield of **III** can be increased by a sequential ligation procedure, in which an intermediate heating step is placed between two low-temperature ligation steps (see *Experimental Protocol*). In this way, product formation is practically quantitative as assessed by gel electrophoresis (see Figure 4, lane 6). Presumably, at the intermediate heating step ODNs that are bound to, but unlinked with the targeted DNA, are dissociated allowing to reach quantitative ODN circularization at the next, repeated ligation step.

Complex **III** is insensitive to treatment by exonuclease VII, which digests single-stranded DNA from both 3′- and 5′-ends (Figure 4, lane 7). On the other hand, noncircularized ODN was completely degraded (not shown) providing evidence for circularization of the ODN probe (11). In the example shown in Figure 4, the earring label carries a biotin molecule in the linker segment. Therefore, as anticipated, addition of streptavidin to **III** (lane 6) resulted in specific extra retardation due to binding of biotin with streptavidin, while the non-targeted DNA fragment was not affected (lane 8).

Sequence Specificity of the Earring Assembly

In our first experiments described above, we employed plasmid pPL3, which contained an artificially designed insert capable of PD-loop formation (5,6). To demonstrate the generality of the earring assembly, we constructed a plasmid, pHIV, containing a part of the *nef* gene coding region of the HIV-1 virus. The corresponding insert consists of two octapurine stretches separated by 5 bp of mixed pyrimidine-purine sequence. Along with the correct HIV-1 target, several single-mismatched targets were constructed. In them, one base pair was reversed at various positions within the target site in comparison with the correct target sequence (CT; see *Experimental Protocol* and Figure 5 for details). While quantitative earring formation was observed for the correct target (lane III of CT; marked by an arrowhead in the margin), all single-mismatched targets (M1, M2, and M7; the number indicates the position of the mismatch between bound ODN and target strand counting from the 3′-end of the ODN) did not result in any earring complexes (lanes III of M1, M2, and M7). In a separate experiment, the earring assembly reactions were performed with the single-mismatched target fragments in the presence of a 100 bp shorter DNA fragment carrying the correct target and representing an internal control (data not shown). Again, in each case quantitative earring formation was obtained for the correct target, while no product was formed with the incorrect targets.

Note that in two cases (M1 and M2), the mismatches are located between the PNA-binding sites directly at or close to the ligation point. Here, weakening of the ODN-DNA complex together with the intrinsic fidelity of the ligation enzyme (12-14) prevents circularization of the ODN. In case of the target M7, the mismatch is located at the binding site of one bis-PNA opener. Here, the incorrect target can additionally be discriminated at the PNA-binding step (7,15,16). Indeed, only one bis-PNA opener binds to this target. This can be seen by the higher gel mobility of the complexes obtained after incubation with both bis-PNA openers compared with the mobilities for the corresponding complexes formed with the other three targets (lanes II in Figure 5 of M7 vs. CT, M1, and M2).

The results demonstrate the high sequence specificity for the assembly of earring complexes. Due to the requirement of concerted binding of four short probes (two PNA openers and two ODN domains) to the DNA target, single nucleotide differences at various positions within the targeted region should be severely discriminated.

Conclusions and Future Directions

The DNA topological nanostructures assembled by us are substantially different from previously constructed pseudorotaxane-type structures (see Figure 6). Both padlock probe and sliding clamp contain only pseudotopological links and may one-dimensionally slip away from the target during post-assembly manipulations (1-3). A further drawback of the sliding clamp assembly is that only rather short, linear dsDNA fragments can be employed.

On the contrary, in our earring constructions the introduced cODN tag remains at a precise position, as long as both bis-PNA openers are associated with the complex and as long as the participating dsDNA macromolecule retains its native duplex structure. It is yet unclear whether the circular label will remain fixed at the site of its formation after dissociation of the bis-PNA openers. Experiments to clarify this question

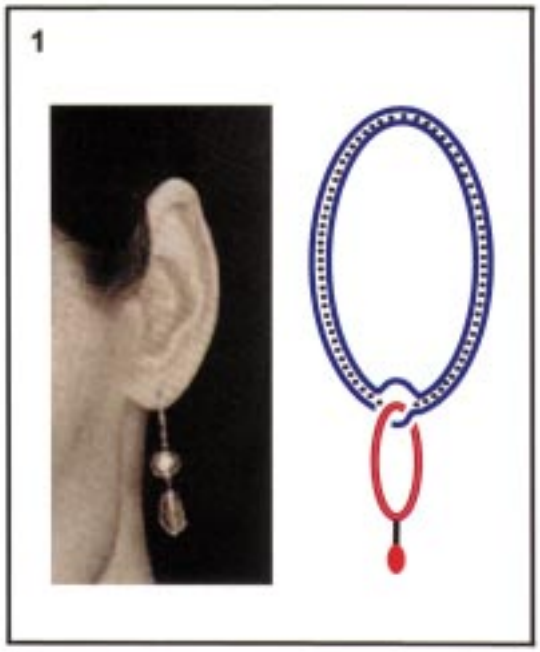

Figure 1: Resemblance of an earring to the assembled DNA complex, in which part of a cODN appears to be threaded between complementary strands of dsDNA at a specific location. The dsDNA may be in a closed circular form leading to a catenated structure, as depicted in this illustration, or in linear form leading to a pseudorotaxane.

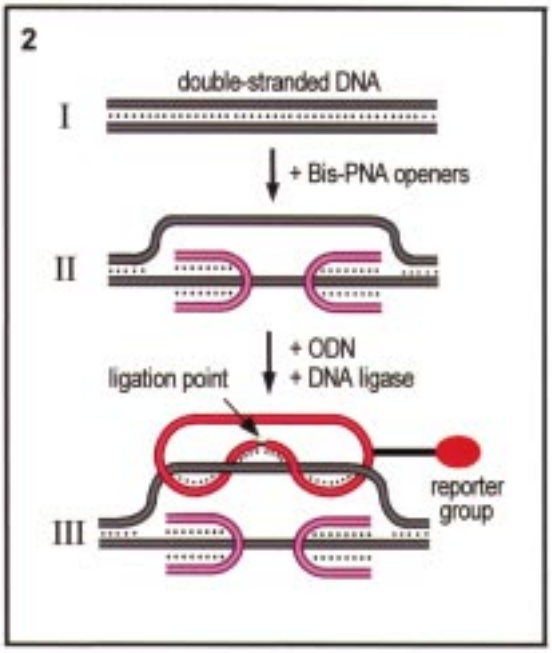

Figure 2: Schematic showing the major steps of earring assembly. First, the target sequence within dsDNA (linear or ccDNA) is opened by a pair of bis-PNAs which bind to two closely located homopurine stretches on the same strand of the duplex target. In our examples these purine stretches comprise 8 nt separated by 3-5 nt of mixed sequence DNA. As a result, structure **II** forms, in which one of two dsDNA strands becomes accessible for hybridization with an ODN to form a PD-loop. The ODN may contain one or more reporter groups, and it is designed in such a way that its termini are complementary to the exposed DNA target and are in juxtaposition upon hybridization. After hybridization, the ODN is circularized within the PD-loop by enzymatic ligation. In our specific examples (shown in Figures 4 and 5) the resulting cODNs form two turns of a double helix with the displaced DNA strand so that they are linked twice (**III**). For simplification, in all other figures complex **III** is illustrated with only one link.

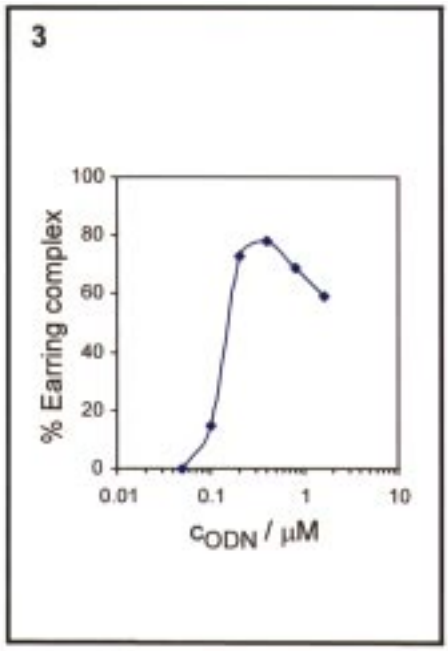

Figure 3: Influence of ODN probe concentrations on the yield of earring complex **III**. Complex **II** (20 nM) was incubated in the presence of 5 units of T4 DNA ligase with various concentrations of ODN at 16°C for 15 h. The reaction samples were then analyzed by electrophoresis in a 10% native polyacrylamide gel. After staining the gel with ethidium bromide, the intensities of bands containing structures **II** and **III** were measured using a CCD camera.

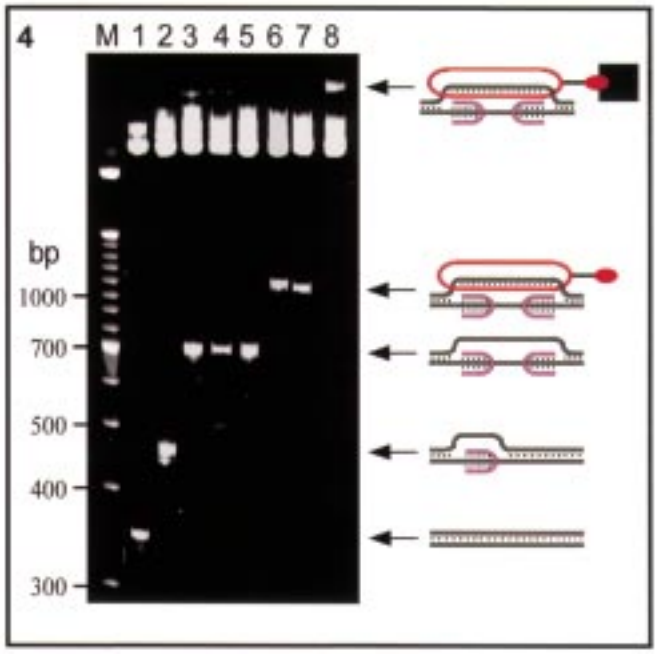

Figure 4: Earring assembly monitored by gel-mobility-shift assay on a native 10% polyacrylamide gel. A 350-bp-long *Pvu*II-*Pvu*II target fragment of plasmid pPL3 (lane 1) was incubated with only one bis-PNA opener (lane 2) or both bis-PNA openers (lane 3). In the presence of both ODN and T4 DNA ligase, quantitative earring formation is observed (lane 6), while the addition of only ligase (lane 4) or only ODN (lane 5) does not lead to a new structure in comparison with complex **II** (lane 3). Lane 7 shows earring complex **III**, as obtained in lane 6, after treatment for 30 min at 37°C with 10 units of exonuclease VII. Lane 8 shows the earring complex after addition of streptavidin to the biotinylated earring label.

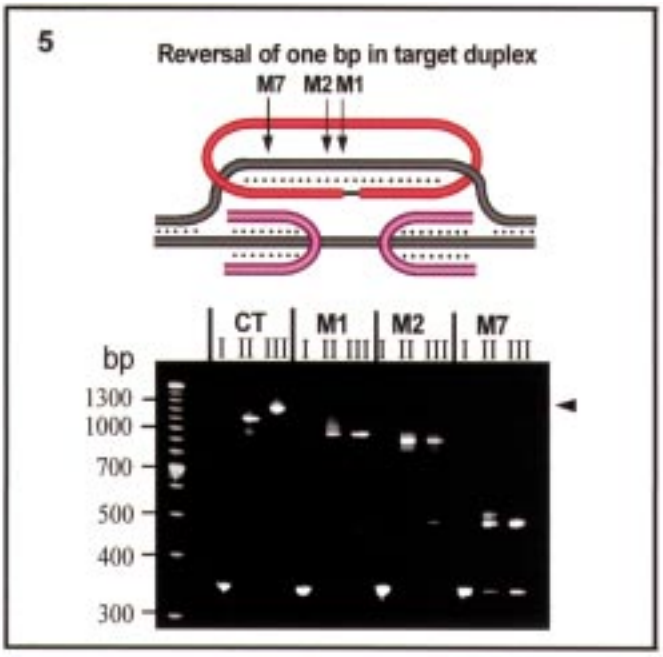

Figure 5: Sequence specificity of the earring assembly monitored by gel-mobility-shift assay on a native 10% polyacrylamide gel. Lanes I correspond to free, 350-bp-long target fragments, one correct target (CT) and three single-mismatched targets (M1, M2, M7). Lanes II shows target fragments after incubation with both bis-PNA openers. Note that for target M7, only one bis-PNA binds to the target site. Lanes III show complexes after addition of ODN and ligase to the complexes obtained in lanes II. Only for the correct target (CT), earring formation is observed (indicated in the margin by an arrowhead).

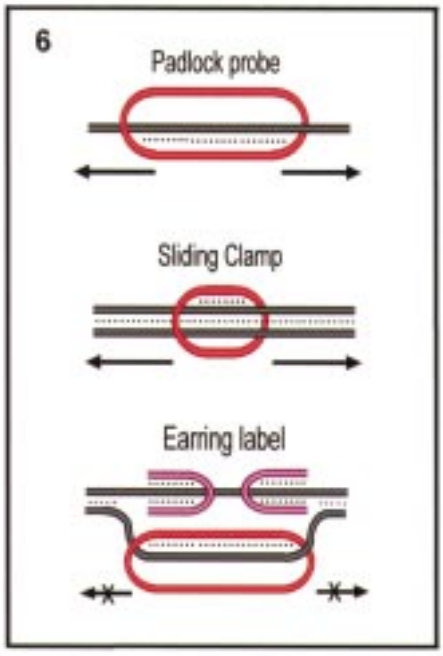

Figure 6: Comparison between three DNA pseudorotaxane-type structures: the padlock probe (1, 2), the sliding clamp (3), and the earring label (4). Note that both padlock probe and sliding clamp may one-dimensionally diffuse on the target DNA during post-assembly manipulations, while preliminary data indicate that the earring label remains at the site of its formation during various handling steps after its assembly.

are currently in progress. In case that sliding movements of the earring tag can somehow be controlled, molecular shuttles or molecular switches made of DNA may be designed. Shuttling within non-DNA rotaxane-type structures that could be driven electrochemically (17,18), by change of pH (19,20), by change of the solvent (21), or by light (22,23) have recently been described, and may lead to molecular machines and motors (24,25). The exploitation of topological interlocked molecular architectures using switching phenomena is still in its early development. However, the potentials of such structures are truly exciting.

The assembly of earring labels can be performed equally well on linear and cc duplex DNA both in solution and within the gel plug. Thus, for instance, targeting of sequences within native chromosomes having topological constraints due to intrinsic looped structures becomes possible. Similar to the PD-loop approach with a linear oligonucleotide probe (5, 6), the earring label may also be used for DNA affinity capture, taking advantage of the higher specificity of the earring complex formation and more stable attachment of a cODN tag to the target.

For the potential use of earring complexes in DNA diagnostics, a high sensitivity of detection is necessary. Several groups have demonstrated that small, single-stranded cODNs can serve as templates for rolling-circle amplification (RCA) by DNA or RNA polymerases, generating many tandemly-linked copies of the complement to the cODN molecule (26-28). Thus it is worthwhile to investigate the possibility to perform RCA on earring complexes. Our preliminary data indicate that RCA is indeed workable on such complexes: ca. 10^6-fold amplification of hybridization signal has so far been achieved in this way. By optimizing the RCA conditions, we expect to increase the amplification even further.

In conclusion, the design presented here introduces PNA oligomers into the repertoire of DNA nanotechnological tools, and opens avenues for various future applications.

Experimental Protocol

Components for Earring Assembly

The components used for the earring assembly, shown in Figures 3 and 4, have been previously described (4). For the assembly shown in Figure 5, the following target sequences were employed: the correct target, pHIV, contained the insert 5′-AGAGGAAGCTACTGGAGGAGA/ 3′-TCTCCTTCGATGACCTCCTCT, and the single-mismatched targets (the reversed base pair is emboldened) contained 5′-AGAGGAAGCT**T**CTGGAGGAGA/3’-TCTCCTTCGA**A**GACCTCCTCT (M1), 5′-AGAGGAAGC**A**ACTGGAGGAGA/3′-TCTCCTTCG**T**TGACCTCCTCT (M2), and 5′-AGAG**C**AAGCTACTGGAGGAGA/3′-TCTC**G**TTCGATGACCTCCTCT (M7). Bis-PNA openers for these targets were $HLys_2$-$TCTC_2TC_2$-$(eg1)_3$-J_2TJ_2TJT-$LysNH_2$ and $HLys_2$-$TCTC_2TC_2$-$(eg1)_3$-J_2TJ_2TJT-$LysNH_2$. The phosphorylated, circularizable ODN probe was 5′-CTGGAGGAGATTTTGTGGTATCGATTCGTCTCTTGAGGAAGCTA.

General Procedure for Earring Assembly

Closed circular or linear, dephosphorylated dsDNA (typically 1-10 μg) is incubated with a pair of bis-PNA openers in 10 mM phosphate buffer (pH 6.8) for 4 h at 37°C. Depending on the binding affinity of the bis-PNAs, a PNA concentration of 0.2 - 4.0 μM was usually required to obtain complex **II** quantitatively. To avoid binding of unbound bis-PNAs with the partially complementary ODN probe, surplus PNA openers were removed by gel filtration (Sephadex G-50). Approximately 20 nM of complex **II** and 400 nM of 5′-phosphorylated ODN were then sequentially incubated with T4 DNA ligase (1-5 units) in 20 μl ofligation buffer (40 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM DTT, 0.5 mM ATP; pH 7.8) for 1.5 h at 16°C, 15 min at 45°C, and 2 h at 16°C. For analysis of the ligation product by electrophoresis, samples containing complex **III** were desalted by gel filtration, extracted by phenol/chloroform/isoamyl alcohol, ethanol precipitated, and redissolved in TE buffer.

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References and Footnotes

[+]Abbreviations and acronyms used: cODN, circular oligodeoxy-ribonucleotide; PNA, peptide nucleic acid; dsDNA, double-stranded DNA, ccDNA, closed circular DNA; PD-loop, complex of dsDNA, PNA openers, and an ODN; RCA, rolling-circle amplification.

[†]Note added in proof. While this manuscript was already in press, one more approach of that kind has been proposed by Escudé *et al*. (29), who formed a padlock structure on duplex DNA via triplex formation. This cODN tag may also move along the double helix.

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