Peptide Nucleic Acids

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Chemodosimeter for Cu^{II} Detection Based on Cyclic Peptide Nucleic Acids**

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DNA and RNA binders (for example, short oligonucleotides or their analogues), whose binding ability is triggered by chemical compounds[1] or physical factors,[2] can be used in the control of gene expression and potentially in analysis. The preparation of such specialized binders includes modification of their backbone, bases, or termini with protecting groups. In the protected state the oligonucleotides do not bind to nucleic acids, while in the presence of a trigger the protecting groups are either removed or modified, thus restoring their binding ability.

Cyclization could be a generally applicable alternative to the existing methods. This statement is based on the following considerations. Oligonucleotides bind complementary nucleic acids to form rigid, rodlike duplexes.[3] Bending of these structures is energetically unfavorable but it occurs in exceptional cases, when certain base sequences are repeated in phase with the DNA helical repeat, for example, poly-A tracts. Therefore, short cyclic oligonucleotides would be expected to have low or no affinity to complementary nucleic acids. Surprisingly, this is not generally the case. For example, cyclic oligonucleotides with specific sequences bind linear nucleic acids in a sequence-specific fashion to form non-canonical base tetrads within four-stranded structures, such as G:C:G:C and G:C:A:T.[4] As a result of this interaction the linear nucleic acids are bent and adopt a looplike conformation. The four-stranded structure is also formed in a solution of cyclic TGCTCGCT[5] and is found in a dimer of HIV-1 RNAs (kissing-loop dimer).[6]

Kool et al. have demonstrated unambiguously that even 12-base cyclic DNAs bind complementary nucleic acids to form duplexes, which are recognized by polymerases. In this case, partial rather than full-length duplexes are formed.[7] The conclusions of Kool and co-workers have been indirectly corroborated by the data of Levy and Ellington,[8] who demonstrated that cyclic DNA–RNA hybrids can be cleaved at a specific site by a DNAzyme. Catalytic activity of the DNAzyme is possible only if the cyclic hybrid binds to the linear single-stranded (ss) DNA part of the DNAzyme. A single report has been published to date, in which it was claimed that cyclization of 20–300-base-long DNAs leads to the loss of their affinity toward the complementary RNAs.[9] Unfortunately, no experimental evidence of this claim has been provided.

Herein, we demonstrate for the first time that cyclization of nucleic acid binders through optimized linkers leads to a complete loss of their binding ability. We used peptide nucleic acids (PNAs) for this optimization, because a) chemical modification of PNAs is straightforward[10] and b) our group has extensive experience in PNA chemistry.[1d,11] PNAs are synthetic analogues of DNAs. Linear PNAs bind complementary nucleic acids with high affinity and specificity.[10] Cyclic PNAs have been prepared before by classical organic synthesis, and were tested as binders of loops in hairpin regions of HIV-1 RNA.[12] No studies of binding of cyclic PNAs to linear nucleic acids have been reported to date.

We also demonstrate a possible application of the optimized cyclic PNAs. In particular, we show that a cyclic PNA, which has a covalent bond sensitive to Cu^{II} ions, can be used as a chemodosimeter for the sensitive and selective detection of Cu^{II}. Cleavage of this cyclic PNA with formation of the linear PNA is dependent on the Cu^{II} concentration (Figure 1). The linear PNA can be detected as a result of its ability to open molecular beacons, which leads to a dramatic increase of the fluorescence intensity. The fluorescence of the majority of reagents for Cu^{II} detection is quenched in the presence of Cu^{II}.[13] Examples of a Cu^{II}-induced increase in fluorescence intensity are rare.[14]

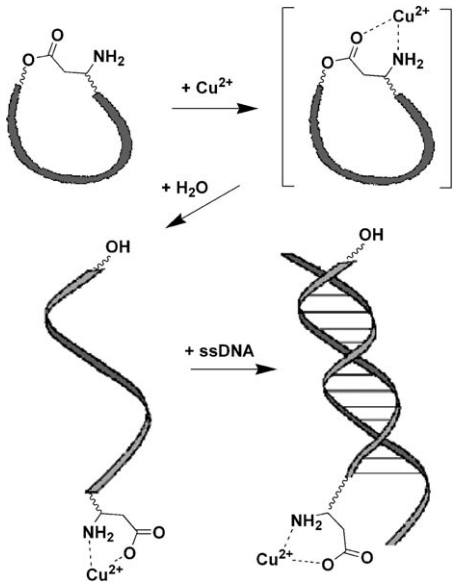

Figure 1. Principle of a chemodosimeter for Cu^{II} detection based on cyclic PNA.

First, we prepared the cyclic PNA **cPNA1** (Scheme 1), which has a 21-atom-long linker between the termini.[15] The β-amino acid ester fragment in this linker can coordinate Cu^{II} through the amino group and the oxygen atom of the carbonyl group to form a stable, six-atom chelating cycle.[16] The

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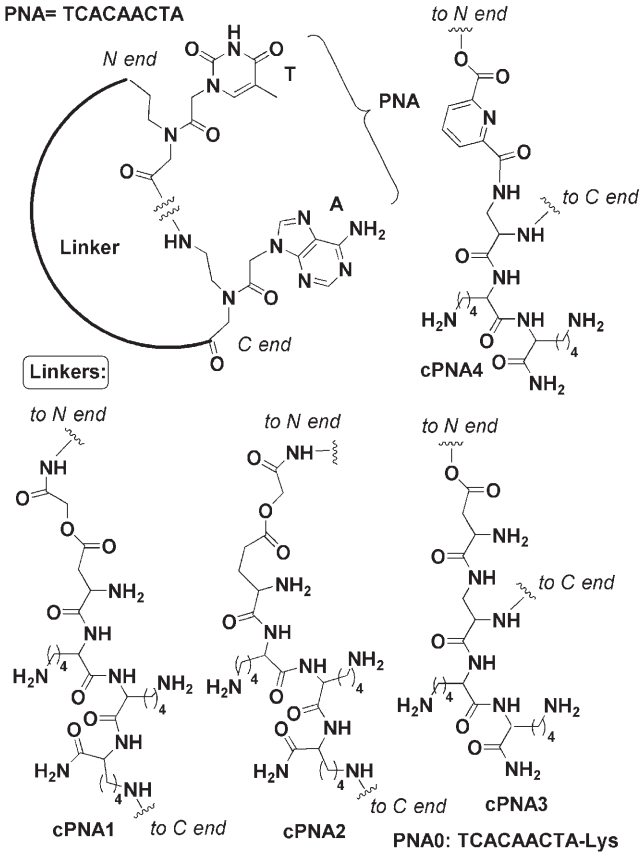

Scheme 1. Structures of PNAs studied.

coordination polarizes the carbonyl group, which facilitates ester hydrolysis. **cPNA1** (1 μM) is fully hydrolyzed 60 min after addition of Cu^{II} (10 equiv). In the absence of the metal ion, **cPNA1** is hydrolyzed much more slowly.[15] To confirm that hydrolysis of **cPNA1** occurs as a result of direct coordination of Cu^{II} to the ester, we prepared an analogue of **cPNA1** (**cPNA2**) containing one additional methylene group between the ester and the metal-anchoring amino group. This minor modification was expected to strongly affect the coordination ability of the ester, as seven-atom chelating cycles are much less stable than the corresponding six-atom chelating cycles. We found that **cPNA2** is not hydrolyzed in the presence of Cu^{II}. Its ester group can be cleaved under harsher conditions, for example, upon incubation with aqueous ammonia (20%).[15]

Binding of **cPNA1** to the complementary DNA was tested using molecular beacon probes **MB1** and **MB2** modified at their termini with Tamra and Dabcyl dyes.[17] The probes differ from each other in their stem length: **MB2** (Tamra-CCTTTAGTTGTGAAAGG-Dabcyl) has one base pair more. Comparable effects have been observed for both compounds. The fluorescence intensity of **MB1** is increased 16-fold after addition of unmodified PNA (1 equiv).[15] Surprisingly, addition of **cPNA1** (1 equiv) to the beacon also leads to a substantial increase in the fluorescence (14-fold).[15] This finding indicates that the DNA affinities of **cPNA1** and **PNA0** are comparable. Analysis of molecular models of **cPNA1**/DNA shows that the linker of **cPNA1** does not destabilize the duplex providing that it has the structure close to A-DNA. Cyclic PNAs with shorter linkers (less than 10 atoms) could not be accommodated within the duplex structure. To test the results of the modeling we prepared **cPNA3** with a nine-atom linker between its termini. The affinity of this compound toward the molecular beacon is significantly weaker than that of **cPNA1**, but is not negligible. The fluorescence of the beacon is increased eightfold after addition of **cPNA3** (1 equiv).[15] This result may indicate the formation of short duplexes of **cPNA3** with the DNA[7] or noncanonical four-stranded structures.[4–6]

To further weaken binding of cyclic PNAs to the complementary DNA, we substituted the aspartic acid residue for a more rigid picolinic acid residue. Analogously to amino acid esters, hydrolysis of picolinic acid esters is catalyzed by Cu^{II}.[18] The fluorescence of an equimolar mixture of **MB2** and **cPNA4** is practically the same as that of the beacon alone (Figure 2), which indicates that **cPNA4** does not bind the DNA under these conditions. This finding is corroborated by gel electrophoresis experiments under native conditions (Figure 3).

To determine the stability constants of PNA/**MB1** we conducted titrations of **MB1** with the PNAs: $\log K = 8.3 \pm 0.2$

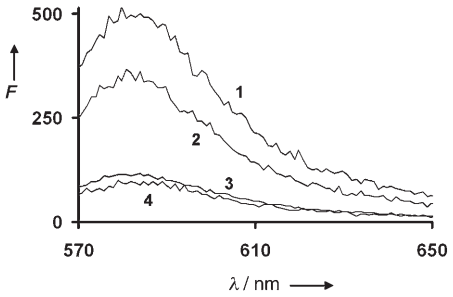

Figure 2. Effect of PNAs (1 equiv) on the fluorescence (*F*) of the molecular beacon **MB2** (100 nM). MOPS, 10 mM; NaCl, 3 mM; EDTA, 2 μM; pH 7. 1) **PNA0** treated with Cu^{II} (10 equiv) for 30 min at 40°C; 2) **cPNA4** treated with Cu^{II} (1 equiv) for 30 min at 40°C; 3) **cPNA4**, no Cu^{II}; 4) no PNA. MOPS = 3-(*N*-morpholino)propanesulfonic acid, EDTA = ethylenediaminetetraacetic acid.

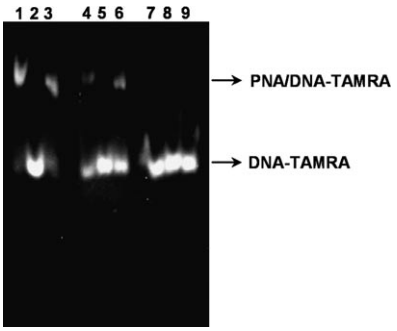

Figure 3. Gel electrophoresis experiment (native conditions). Each sample contained MOPS (10 mM), NaCl (50 mM), and labeled linear DNA: 5′-Tamra TAG TTG TGA (1.7 μM). Lane 1: **PNA0** (3.4 μM); lane 2: **cPNA4** (3.4 μM); lane 3: **cPNA4** (3.4 μM) treated with Cu^{II} (10 equiv) at 40°C for 30 min; lane 4: **PNA0** (1.7 μM); lane 5: **cPNA4** (1.7 μM); lane 6: **cPNA4** (1.7 μM) treated with Cu^{II} (10 equiv) at 40°C for 30 min; lanes 7–9: without PNA.

(**PNA0**/**MB1**), 8.0 ± 0.1 (**cPNA1**/**MB1**), 7.2 ± 0.1 (**cPNA3**/**MB1**).[15] The stability of the **cPNA4**/**MB1** duplex is so low that conditions of PNA binding saturation could not be achieved at reasonable concentrations of the PNA, and its stability constant could not be determined. Hydrolysis of **cPNA1**, **cPNA3**, and **cPNA4** at pH 7 does not take place in the presence of metal ions other than Cu^{II}, namely Zn^{II}, Ni^{II}, $Fe^{II/III}$, Co^{II}, Mn^{II}, Zr^{IV}, Ce^{III}, Ln^{III}, Eu^{III}, or Pr^{III}. Ratios of catalyzed to background hydrolysis rates are decreased in the following order: **cPNA4** (286:1), **cPNA3** (118:1), and **cPNA1** (25:1). As a result of the low affinity of the β-amino acid ester (**cPNA1**, **cPNA3**) for Cu^{II}, a 10 equivalent excess of the metal ion is required for its hydrolysis. In contrast, the metal binding affinity of the picolinic acid ester (**cPNA4**) is high enough to bind the metal ion at lower concentrations. In particular, hydrolysis of **cPNA4** in the presence of only 0.3 equivalents of Cu^{II} is still about five times faster than its background hydrolysis.[15] The absolute amount of Cu^{II} used in the latter experiment was 300 fmol. The Cu^{II} sensitivity can be further increased by incorporation of stronger Cu^{II}-binding ligands within the cyclic PNA (data not shown).

After hydrolysis, **cPNA4** becomes an excellent binder of DNA as well as RNA.[15] In particular, a substantial increase in **MB2** fluorescence after addition of the hydrolyzed PNA (Figure 2) is observed. Linear **PNA0** induces a comparable increase in the fluorescence of **MB2**. Similar effects have been found with **MB1**, which contains a shorter stem region. Both linear PNA and hydrolyzed **cPNA4** form stable duplexes with the linear complementary DNA and RNA under the conditions used for gel electrophoresis experiments (Figure 3).

In summary, we have demonstrated that the binding affinity of cyclic PNAs to nucleic acids can be reduced by variation of the linker between their termini. The Cu^{II} ion acts as an efficient trigger of binding of cyclic PNAs to nucleic acids. This effect can be used for the detection of as little as 300 fmol of Cu^{II}. Our method relies on the modification of the termini rather than the nucleobases or backbone of the probes. Preparation of the cyclic PNAs is fully based on solid-phase synthesis and commercially available starting materials (see Supporting Information). This allows quick tuning of the properties of the cPNAs, which is an important advantage. Specific ester groups can be selectively cleaved by stimuli other than Cu^{II}. For example, Zn^{II}, imidazole derivatives, and UV light all trigger hydrolysis of nitrophenyl esters. These stimuli can potentially be used for activation of the cyclic PNAs in the cell, providing that their substrates are introduced within a linker less than ten atoms long in the cyclic PNA.

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