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Antisense effect of pyrrolidine-based oxy-peptide nucleic acids in *Escherichia coli*

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ABSTRACT

To investigate the antisense effect of a pyrrolidine-based oxy-peptide nucleic acid (POPNA), we carried out the *Lac*Z reporter assay using a 12-mer *trans*-L-POPNA conjugated with a cell-penetrating peptide (antisense reagent). The antisense effect of the conjugated POPNA (inhibition of *Lac*Z activity) was comparable to that shown by a Nielsen-type peptide nucleic acid. Furthermore, the conjugated POPNA could switch the *Lac*Z activity over a wide range of ambient temperatures.

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We have synthesized peptide nucleic acids containing ether linkages and pyrrolidine rings in the backbone (pyrrolidine-based oxy-peptide nucleic acids (POPNAs), Fig. 1).[1–5] Because of the presence of ether linkages in the main chain, the POPNAs are more water-soluble than are Nielsen-type peptide nucleic acids (PNAs, Fig. 1).[6–9] The stability of POPNA/DNA and POPNA/RNA hybrids differs with the configuration of the pyrrolidine rings present in the POPNA. The pyrrolidine ring has two chiral centers, and hence, there are four possible optical isomers (*cis*-L, *trans*-L, *cis*-D, and *trans*-D). Previously, we have shown that among these four configurations, *trans*-L-POPNA and *cis*-L-POPNA form the most stable hybrids with complementary RNA (cRNA) and complementary DNA (cDNA), respectively.[2,4] The *trans*-L-POPNA is expected to be an effective antisense reagent.

Antisense reagents are an attractive new class of antimicrobial agents that can be designed appropriately on the basis of simple rules, so that they effectively inhibit gene expression. Recently, there has been a rapid increase in the number antibiotic-resistant bacteria, and this calls for the development of such new antimicrobial agents. POPNAs are expected to be attractive antimicrobial agents because of their high stability and water solubility and specificity toward DNA and RNA during hybridization.

In this study, we synthesized a *trans*-L-POPNA that is complementary to the start codon region of the chromosomal β-galactosidase (*LacZ*) of *Escherichia coli*. To facilitate internalization by an *E. coli* cell, the POPNA was conjugated to the *C*-terminus of a cell-penetrating peptide (*N*′-KFFKFFKFFK-*C*′).[10–12] We performed the *Lac*Z reporter assay[13,14] to examine the antisense effect of the conjugated POPNA.

First, we synthesized a 12-mer *trans*-L-POPNA conjugated with a cell-penetrating peptide (CPP) by the conventional Fmoc-based solid-phase peptide synthesis (SPPS).[1–5] The chemical structure of the *trans*-L-POPNA-CPP conjugate (POPNA12-CPP) is shown in Figure 2. The sequence is H-CATAGCTGTTTC-Sp2-$(KFF)_3$K-NH_2, where A, T, G, and C indicate adenine, thymine, guanine, and cytosine, respectively. K and F stand for lysine and phenylalanine, respectively. Sp2 (a long spacer consisting of ethylene glycol units) connects the POPNA with the CPP. The *N*- and *C*-termini of the conjugate are a primary amine and a primary amide, respectively. We also synthesized a naked *trans*-L-POPNA (POPNA12; sequence: H-CATAGCTGTTTC-KK-NH_2), a Nielsen-type PNA-CPP conjugate (PNA12-CPP; sequence: H-CATAGCTGTTTC-Sp2-$(KFF)_3$K-NH_2), and a *trans*-L-POPNA-CPP conjugate consisting of a scrambled sequence of POPNA12 (scrambled POPNA12-CPP; sequence: H-ACATGTC GTCTT-Sp2-$(KFF)_3$K-NH_2) as controls. We successfully characterized

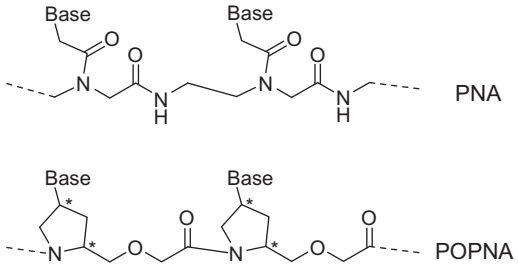

Figure 1. Chemical structures of PNA and POPNA.

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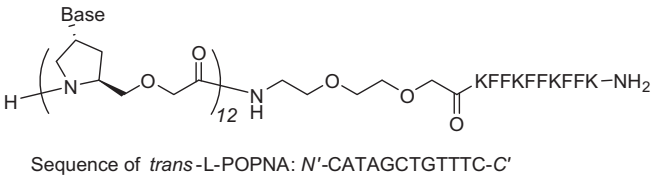

Figure 2. Structure of 12-mer *trans*-L-POPNA conjugated with an cell-penetrating peptide (POPNA12-CPP).

these peptides by MALDI-TOF mass analysis (POPNA12-CPP: calcd $[M+H]^+$ = 4762.20, obsd $[M+H]^+$ = 4762.87; POPNA12: calcd $[M+H]^+$ = 3478.53, obsd $[M+H]^+$ = 3477.90; PNA12-CPP: calcd $[M+H]^+$ = 4774.15, obsd $[M+H]^+$ = 4774.31; scrambled POPNA12-CPP: calcd $[M+H]^+$ = 4762.20, obsd $[M+H]^+$ = 4760.00. See Supplementary data).

To examine the antisense effect of *trans*-L-POPNAs introduced into *E. coli*, we performed the *LacZ* reporter assay for POPNA12-CPP, as per the protocols described below. For the assay, we used a β-galactosidase assay kit (Genlantis, CA, US) containing *o*-nitrophenyl-β-D-galactopyranoside (ONPG) substrate solution, lysis buffer, a standard dilution buffer, and a stop solution. First, *E. coli* K-12 cells were suspended in Mueller–Hinton broth (MHB) (4 mL), and the suspension was shaken and incubated at 37 °C overnight. An aliquot of this cell suspension (up to 6.0 mL) was withdrawn and added to 3.0 μL of 100 mM isopropyl β-D-thiogalactopyranoside (IPTG). The *LacZ* promoter was activated by the addition of IPTG. Four hundred-microliter aliquots of the cell suspension were pipetted into microtubes. A small amount of POPNA12-CPP dissolved in Milli-Q water was then added to the suspensions as prepared at predetermined concentrations (final concentrations of POPNA12-CPP: 0, 0.1, 0.4, 0.8, 1.5, 3.0, and 6 μM). When POPNA12-CPP binds to *Lac*Z, β-galactosidase biosynthesis is inhibited. These cell suspensions were shaken and incubated at 25 °C and 37 °C for 2 h for internalization of POPNA12-CPP by the *E. coli* K-12 cells. Next, 200 μL of each suspension was withdrawn, and the absorbance at 600 nm was measured by using a UV-vis spectrometer (JASCO V-560) to determine the turbidity of the solution. The turbidity values were converted to *E. coli* K-12 survival rates.[15] Then, another 200 μL of each cell suspension was centrifuged to separate the supernatant, and the *E. coli* K-12 cells were harvested. Lysis buffer (50 μL) was added to the *E. coli* K-12 cell pellet, and the mixture was slowly shaken and incubated at room temperature for 10–15 min to disrupt the cell wall. Subsequently, the standard dilution buffer (50 μL) and ONPG (100 μL) were added to the solution. Upon expression in *E. coli* K-12, β-galactosidase hydrolyzed the ONPG substrate to afford *o*-nitrophenol. When the solution became yellow in color, the β-galactosidase reaction was terminated by adding 50 μL of the stop solution. Finally, the absorbance of the solution was measured at 405 nm (A_{405}). This absorbance was due to the presence of *o*-nitrophenol in the solution. The *Lac*Z activity was determined from the β-galactosidase concentration, which in turn was estimated from the A_{405} value. *Lac*Z reporter assays for POPNA12, PNA-CPP, and scrambled POPNA12-CPP were also performed according to the aforementioned protocol.

Figure 3 shows the dependence of the *Lac*Z activity on POPNA12-CAP concentration after incubation for 2 h at 37 °C and 25 °C. The results obtained for the PNA12-CPP control are also shown in Figure 3. From the results, we could confirm that antisense effect of POPNA12-CPP depends on its concentration. The *Lac*Z activity clearly decreased with an increase in the concentrations of POPNA12-CPP and PNA12-CPP. The antisense effect of POPNA12-CPP was temperature-dependent: the antisense effect was observed at a lower temperature (25 °C) but not at a higher temperature (37 °C). On the other hand, the antisense effect of PNA12-CPP was temperature-independent over the tested temperature range. These results suggested that the antisense effect of POPNA12-CPP (gene expression) in *E. coli* can be switched by appropriate temperature control, as the hybrid of the 12-mer *trans*-L-POPNA and cRNA has moderate stability.[16]

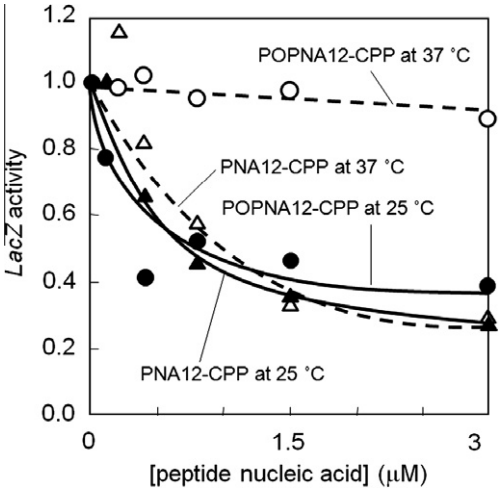

Figure 3. Inhibition of *Lac*Z activity by POPNA12-CPP and PNA12-CPP introduced into *E. coli* K-12 after 2-h incubation at 25 °C and 37 °C.

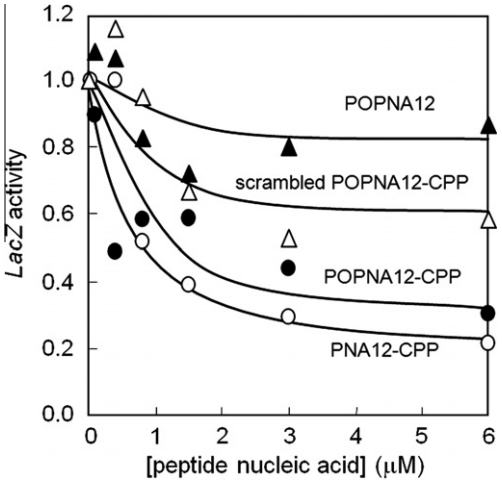

Figure 4. Inhibition of *Lac*Z activity by POPNA12, scrambled POPNA12-CPP, POPNA12-CPP, and PNA12-CPP introduced into *E. coli* K-12 after 5-h incubation at 25 °C.

Figure 4 shows a comparison of the concentration dependence of *Lac*Z activity in the case of POPNA12-CPP, POPNA12, PNA12-CPP, and scrambled POPNA12-CPP after incubation for 5 h at 25 °C. Comparison of the results obtained for *trans*-L-POPNA-CPP and scrambled POPNA12-CPP revealed that the former showed a sequence-specific antisense effect. Further, comparison between the POPNA12 and POPNA12-CPP results indicated that CPP successfully carried *trans*-L-POPNA into the *E. coli* cells.[17] These results also indicated that the antisense effect of POPNA-CPP was equivalent to that of PNA-CPP.

In summary, we successfully synthesized a 12-mer POPNA conjugated with a CPP (antisense reagent). The POPNA-CPP conjugate showed inhibited *LacZ* gene expression in *E. coli*. These results show that the inhibition of *Lac*Z activity is caused by steric hindrance (blocking effect) in the POPNA bound to the target mRNA, as is observed in the case of Nielsen-type PNA. The antisense effect of POPNA could be controlled within a range of ambient temperature. At room temperature, the antisense effect of the synthesized

POPNA was superior to that of the Nielsen-type PNA. The POPNA is readily transferred from the hybrid containing the oligonucleotide to each single strand.[2,4] In other words, the POPNA can mediate all-or-none inhibition of gene expression in the temperature range in which bacteria can survive. The 12-mer POPNA forms a hybrid with RNA at around room temperature. However, the melting temperature of a PNA with the same chain length is much higher; that is, the stability of the PNA/RNA hybrid is too high for it to be effective in controlling the inhibition of gene expression (if the PNA chain length is shortened, the sequence specificity of PNA to RNA will deteriorate). These results are expected to provide important information for the development of a tool that can control gene expression in response to small temperature changes.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.11.034.

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15. It was confirmed that *E. coli* survives under the present conditions.
16. *trans*-L-POPNA forms a hybrid with a cRNA at a moderate T_m. For example, the present POPNA12 forms a hybrid with cRNA at $T_m = 32.0\ ^\circ C$ ([POPNA12] = [RNA] = 5.0 μM in phosphate buffer (100 mM NaCl, 10 mM NaH_2PO_4, and 0.1 mM EDTA; pH 7.0). In the case of Nielsen-type PNA, T_m is greater than 60 °C under the same conditions.
17. In Ref. 5, we investigated the internalization of POPNA into cells by confocal laser scanning microscopy observations. The results showed that as-synthesized POPNA is not internalized into cells.