Peptide Nucleic Acids

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Nucleobase-Modified PNA Suppresses Translation by Forming a Triple Helix with a Hairpin Structure in mRNA In Vitro and in Cells

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Abstract: *Compounds that bind specifically to double-stranded regions of RNA have potential as regulators of structure-based RNA function; however, sequence-selective recognition of double-stranded RNA is challenging. The modification of peptide nucleic acid (PNA) with unnatural nucleobases enables the formation of PNA–RNA triplexes. Herein, we demonstrate that a 9-mer PNA forms a sequence-specific PNA–RNA triplex with a dissociation constant of less than 1 nM at physiological pH. The triplex formed within the 5′ untranslated region of an mRNA reduces the protein expression levels both in vitro and in cells. A single triplet mismatch destabilizes the complex, and in this case, no translation suppression is observed. The triplex-forming PNAs are unique and potent compounds that hold promise as inhibitors of cellular functions that are controlled by double-stranded RNAs, such as RNA interference, RNA editing, and RNA localization mediated by protein–RNA interactions.*

The RNA secondary structure, consisting of Watson–Crick base-paired regions, is a framework of complex tertiary structure and acts as a switching unit for stimuli-responsive conformational transitions[1] and as a target of RNA binding domains of regulatory proteins.[2] In mRNA, the formation of secondary structure changes the patterns of alternative splicing, which produces diverse proteins from one gene.[3] Furthermore, during translation elongation, stable mRNA structures may induce ribosomal frameshifts and regulate co-translational protein folding.[4,5] Intracellular RNA structures are of great interest, and recent advances in chemical-probing technologies have enabled the analysis of RNA structures fundamental for gene regulation.[6] Targeting the double-stranded regions of RNA with artificial molecules therefore has the potential to regulate gene expression.

Triplex-forming oligonucleotides (TFOs) bind in the major groove of the double-stranded regions of both DNA and RNA through formation of sequence-specific Hoogsteen base triplets.[7] Various types of TFOs with chemical modifications in the nucleobase, sugar, and/or backbone have been synthesized; an example is peptide nucleic acid (PNA).[8] Triplex formation on RNA is challenging because TFOs generally bind more tightly to DNA duplexes than to RNA duplexes.[9,10] Furthermore, when the TFO contains natural nucleobases, the formation of triplexes is typically limited to acidic conditions because the pK_a value for protonation of cytosine, which is necessary for formation of the C^+·G-C triplet, is 4.5. Therefore, there are few reports of triplex formation with RNA duplexes under physiological conditions.[9,11]

We recently developed nucleobase-modified PNAs that form PNA–RNA triplexes with stabilities higher than that of the corresponding PNA–DNA triplex.[12,13] Substitution of cytosine with 2-aminopyridine (**M**), which has previously been used for recognition of G-C base pairs on DNA,[14] enabled triplex formation at physiological pH as **M** has a higher pK_a value (pK_a 6.7) than cytosine.[12] Furthermore, the use of 2-pyrimidinone (**P**) enabled recognition of a C-G base pair that is usually unsuitable for triplet formation (Figure 1 a).[13] Herein, we evaluated the use of triplex-forming PNAs modified with unnatural nucleobases for controlling gene expression through stabilization of a double-stranded region in an mRNA.

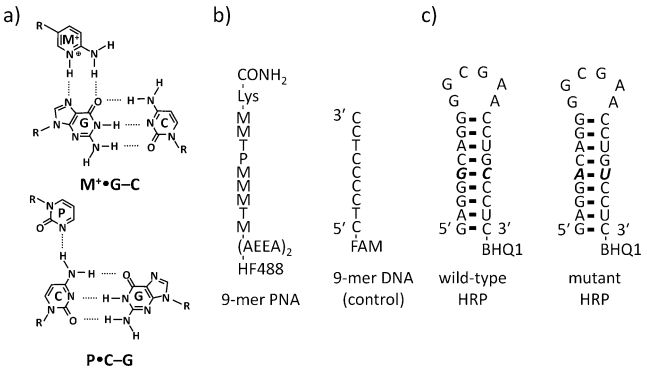

Figure 1. PNA nucleobase modifications and PNA and hairpin RNA sequences. a) Unnatural base triplets consisting of M^+·G-C and P-C-G. b) 9-mer PNA and DNA labeled with HF488 and FAM, respectively. AEEA = [(aminoethoxy)ethoxy]acetic acid, Lys = lysine. c) Wild-type and mutant HRPs. The G-C and A-U base pairs that form the M^+·G-C triplet and mismatched triplet, respectively, are indicated in bold italic.

Stable mRNA structures formed in the 5′-untranslated regions (UTRs) and open reading frames (ORFs) act as roadblocks of the ribosome and suppress protein expres-

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sion.[5,15,16] We designed a 9-mer PNA containing **M** and **P** modified bases to target an RNA hairpin (Figure 1b; see also the Supporting Information, Figure S1). This PNA is shorter than a conventional TFO, which is usually designed to form more than ten base triplets.[9] The PNA was labeled with HiLyte Fluor 488 (HF488). We also designed a control DNA that has a cognate sequence with the 9-mer PNA and was labeled with 6-carboxyfluorescein (FAM; Figure 1b). RNA oligonucleotides that form hairpins cognate to the PNA (wild-type HRP) and with a single mismatch (mutant HRP) were designed and labeled with black hole quencher 1 (BHQ1; Figure 1c).

Various concentrations of the BHQ1-labeled RNAs were mixed with the HF488-labeled PNA or FAM-labeled control DNA at physiological pH (pH 6.8) and salt concentration (100 mM KCl). The fluorescence signals of PNA decreased with increasing HRP concentrations, while those of the control DNA did not change (Figure 2). This result is consistent with protonation of **M** in the PNA, but not of cytosine in the control DNA, at pH 6.8. The fluorescence signal of the PNA decreased at much lower concentrations of the wild-type HRP than that of the mutant HRP. Although PNA and DNA can form a 2:1 strand invasion complex,[17] we have previously shown that PNAs modified with **M** nucleobases form a 1:1 triple helix with hairpin RNAs.[12] Therefore, the values of the equilibrium association constants at 37 °C (K_{A37}) were calculated by fitting to an equation assuming a simple 1:1 binding reaction [Eq. (S1)]. At pH 6.8, the K_{A37} value for the wild-type HRP was $8.75 \times 10^{10}\,M^{-1}$, which is 66 fold higher than that for the mutant HRP (Table 1). Furthermore, the affinities of the 9-mer PNA for single-stranded DNA and RNA that have potential to hybridize to the PNA through Watson–Crick base pairs were approximately 2000-fold lower than for the wild-type HRP (Figure S2). The ability of PNA to specifically bind double-stranded RNA by discriminating a single triplet mismatch at such a high binding affinity is remarkable.

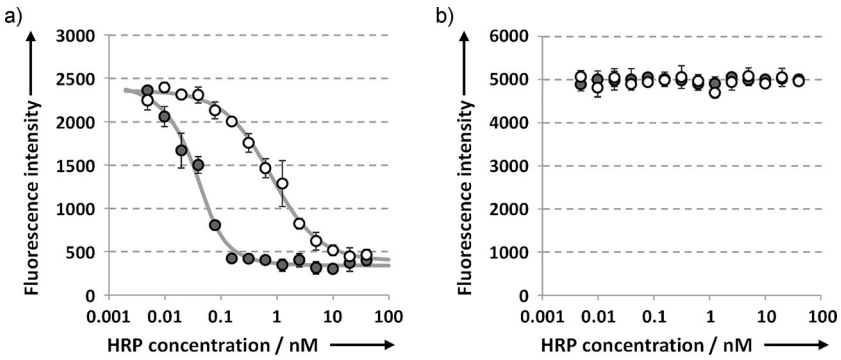

Figure 2. Sequence-specific triplex formation of PNA with RNA. a) HF488-modified 9-mer PNA (0.05 nM) or b) FAM-modified 9-mer DNA (0.05 nM) were mixed with wild-type (●) or mutant (○) hairpin RNA modified with BHQ1 in a buffer solution containing 30 mM HEPES-KOH (pH 6.8), 100 mM KCl, and 0.01% CHAPS. Fluorescence signals were evaluated at 37 °C after 2 h using 490 nm excitation and 530 nm emission. Values are means ± standard deviations of triplicate samples. Gray lines indicate the theoretical signal change obtained by fitting the experimental data.

Table 1: K_{A37} values between 9-mer PNA and HRPs at different pH values in the presence of 100 mM KCl.

pH	K_{A37} [$10^9\,M^{-1}$]	
	wild-type HRP	mutant HRP
6.4	221 ± 74	48.0 ± 7.2
6.6	244 ± 65	18.0 ± 3.0
6.8	87.5 ± 12	1.33 ± 0.29
7.0	37.4 ± 4.0	0.33 ± 0.08
7.2	12.1 ± 1.6	0.07 ± 0.01
7.4	1.52 ± 0.48	n.d.[a]
7.6	0.29 ± 0.04	n.d.[a]

[a] n.d. indicates below detection limit under the experimental conditions.

Triplex formation with the modified PNA should depend on the pH value as **M** must be protonated to form the M^+·G-C triplet (Figure 1a). The K_{A37} values decreased with increasing pH (Table 1; Figure S3). However, the K_{A37} values were considerably higher than those for natural triplexes of 16-mer and longer oligonucleotides,[18] which suggests that PNA is superior at triplex formation under physiological pH conditions. The increase in the K_{A37} values for wild-type HRP saturated below pH 6.6, and the difference in the K_{A37} values between the wild-type and mutant HRPs was less than a factor of five at pH 6.4. The decrease in K_{A37} with increasing KCl concentration was larger for the mutant HRP than for the wild-type HRP (Figure S4). These results suggest that the positive charge of protonated **M** might have promoted nonspecific electrostatic interactions between PNA and HRPs.

To investigate the effect of the triplex formation on translation, reporter mRNAs with the wild-type or mutant HRP sequence in 5′ UTR or ORF were constructed (Figure 3a). The reporter mRNAs encoded *Renilla* luciferase (RLuc) to enable quantification of the protein expression levels by determining the luminescence intensities. Interaction between the PNA and the reporter mRNAs was confirmed by agarose gel electrophoresis (Figure 3b,c). In the absence of a sufficient amount of mRNA, the PNA migrated upwards above the wells owing to its positive charge. Upon addition of mRNA with the wild-type HRP sequence, clear fluorescence signals were observed at two lower positions in the gel (Figure 3b,c). The weaker signals below the main band may be due to polymorphism of the mRNA secondary structures as ethidium bromide staining of the mRNAs showed signals at two positions corresponding to those in Figure 3b and 3c (Figure S5). The ratio of PNA bound to mRNAs with the wild-type HRP sequence was calculated from the fluorescence signals (Figure S6). Saturation of the signal changes was observed at around 50 nM mRNA, which is the same concentration as that of PNA in the mixture. The results suggest that the PNA forms a 1:1 complex with mRNA as was previously found with short hairpin RNAs.[12] In the

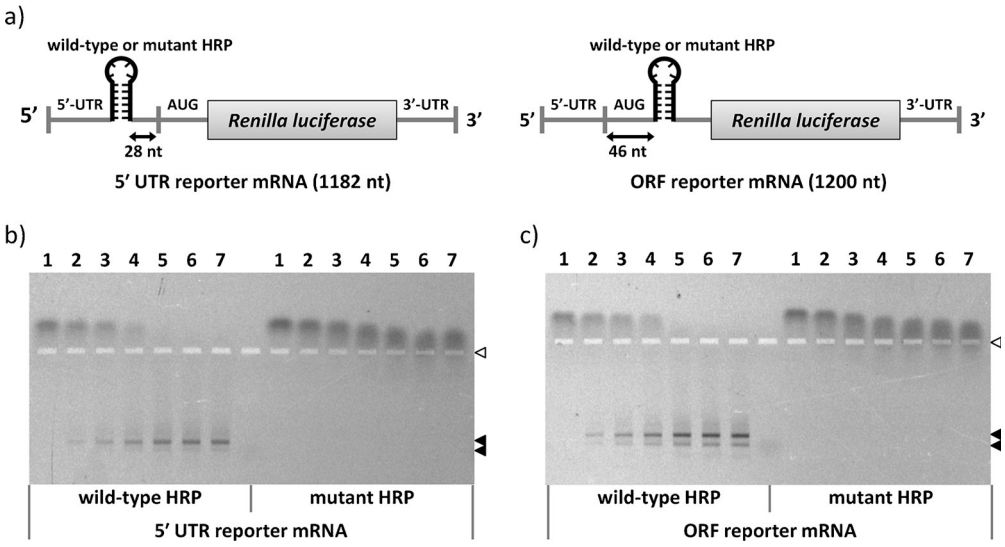

Figure 3. PNA forms a triplex with mRNA containing wild-type HRP. a) Schematic representation of reporter mRNAs with wild-type or mutant HRP sequences in 5′ UTR or ORF; the mRNAs encode *Renilla* luciferase. b, c) PNA (50 nM) was mixed with 0, 10, 20, 30, 50, 75, or 100 nM (lanes 1–7) reporter mRNA with the wild-type or mutant HRP sequence in 5′ UTR (b) or ORF (c). Samples were electrophoresed on 0.7% agarose gels. Fluorescence signals of the PNA were imaged by using 473 nm excitation and 510 nm emission. Filled arrowheads indicate positions of PNA bound to mRNA. Open arrowheads indicate the position where the samples were applied to the gel.

To evaluate the effect of triplex formation on translation, reporter mRNAs were translated in vitro in the presence of various concentrations of the PNA. The relative protein expression levels from mRNA with the wild-type HRP sequence in 5′ UTR linearly decreased with an increase in PNA concentration up to 100 nM, which is the same as the mRNA concentration, and saturated at 20%. Translation of the control mRNA with the mutant HRP was not suppressed even at 200 nM PNA (Figure 4a). The results clearly suggest that the PNA specifically suppressed the translation reaction from the mRNA

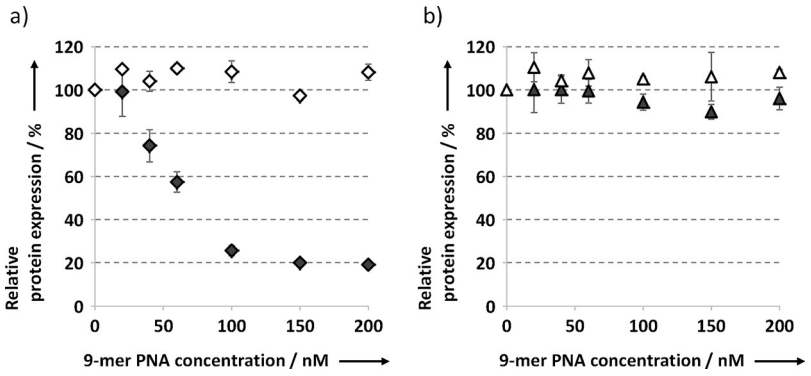

Figure 4. Suppression of protein expression caused by triplex formation on mRNA in vitro. Reporter mRNAs (100 nM) with wild-type (◆/▲) or mutant (◇/△) HRP sequences in 5′ UTR (a) and ORF (b) were translated in the presence of various concentrations of the PNA using a translation solution with rabbit reticulocyte lysate. The PNA and mRNAs were pre-incubated in a buffer solution containing 30 mM HEPES-KOH (pH 6.8), 100 mM KCl, and 0.01% CHAPS at 37°C for 30 min before addition of the translation solution. The luminescence signals of the translated products were measured after 60 min translation reaction and normalized to those in the absence of the PNA. Values are means ± standard deviations of triplicate samples.

presence of mRNA with the mutant hairpin sequence, fluorescence signals were observed only above the wells, whereas the signals became slightly smeared at high concentrations of the mutant mRNA likely because of nonspecific electrostatic interactions. These results clearly suggest that the PNA specifically formed a triplex with the wild-type hairpin region, a sequence of 24 nucleotides within the approximately 1200 nucleotide mRNA.

Triplex formation on the mRNA was also confirmed using a competition assay (Figure S7). Fluorescence signals of the PNA quenched by the BHQ1-modified wild-type HRP increased with increasing concentrations of the mRNA with the wild-type HRP sequence in either the 5′ UTR or in the ORF. When mRNAs with the mutant HRP sequence were used in the competition assay, the fluorescence signals slightly increased at mRNA concentrations of greater than 10 nM. The half maximal inhibitory concentration (IC_{50}) of triplex formation between the PNA and the BHQ1-modified wild-type HRP was 469 ± 45 pM for the mRNA with the wild-type HRP in the 5′ UTR and 892 ± 31 pM for the mRNA with the wild-type HRP sequence in the ORF. These values were similar to or slightly higher than the concentration of the BHQ1-modified wild-type HRP present in the mixture. Therefore, the results suggest that the binding affinities between the PNA and the mRNAs are similar to the affinity between the PNA and a short oligonucleotide hairpin.

with wild-type (but not mutant) HRP in the 5′ UTR, thereby distinguishing a single triplet mismatch. In contrast, the PNA did not suppress translation of mRNAs with either wild-type or mutant HRP sequences in the ORF (Figure 4b), although triplex formation with the wild-type HRP region was confirmed by agarose gel electrophoresis (Figure 3c). Only the small ribosomal subunit scans the 5′ UTR of an mRNA, whereas the ORF is contacted by the mature ribosome, which consists of small and large subunits.[19] Recent studies demonstrated that the mechanical driving force of the mature ribosome is higher than that of the small ribosomal

subunit.[20] Furthermore, the mature ribosome has helicase activity and is less sensitive to the mRNA secondary structure than the small subunit.[16,21] The data presented suggest that the PNA–RNA triplex formed in the ORF was insufficiently stable to suppress the progression of the mature ribosome.

The effect of the PNA on translation was next investigated in human breast cancer (MCF7) cells. A reporter plasmid encoding the reporter mRNA under control of a cytomegalovirus promoter was mixed with a control plasmid (pSV40-FLuc) for expression of firefly luciferase (FLuc) driven by a simian vacuolating virus 40 promoter. The mixtures were transduced into cells with or without HF488-labeled PNA (200 pmol) by electroporation; the PNA concentration in the mixture for electroporation was 9.2 μM. After 24 hours, the cells were imaged by fluorescence microscopy (Figure 5a). The PNAs were distributed evenly in the cells with some brighter spots in the nucleus, which is likely due to nonspecific electrostatic interactions with RNAs in the nucleolus, where the transcription reaction occurs.[22] The protein expression levels of RLuc relative to FLuc were evaluated from their luminescence signals (Figure 5b). Translation of the mRNA with the wild-type HRP sequence in 5′ UTR was reduced to 60% compared to the levels in the absence of PNA. The PNA did not affect expression from the reporter with the mutant HRP sequence in the 5′ UTR. Translation of the mRNA with either the wild-type or the mutant HRP sequence in the ORF was not affected by the PNA as expected from the in vitro experiments (data not shown). These experiments confirm that the 9-mer PNA suppressed translation in cells through formation of a PNA–RNA triplex in the 5′ UTR. The levels of the control firefly luciferase were almost identical in the absence and presence of the PNA, indicating that the PNA does not disrupt translation of non-targeted mRNAs (Figure 5b). The relative protein expression levels depended on the PNA concentration (Figure S8). We expect that the suppression of translation could be further improved by modifications of the PNAs with moieties that enhance the binding affinity and cellular internalization of PNA, such as lysine.[23]

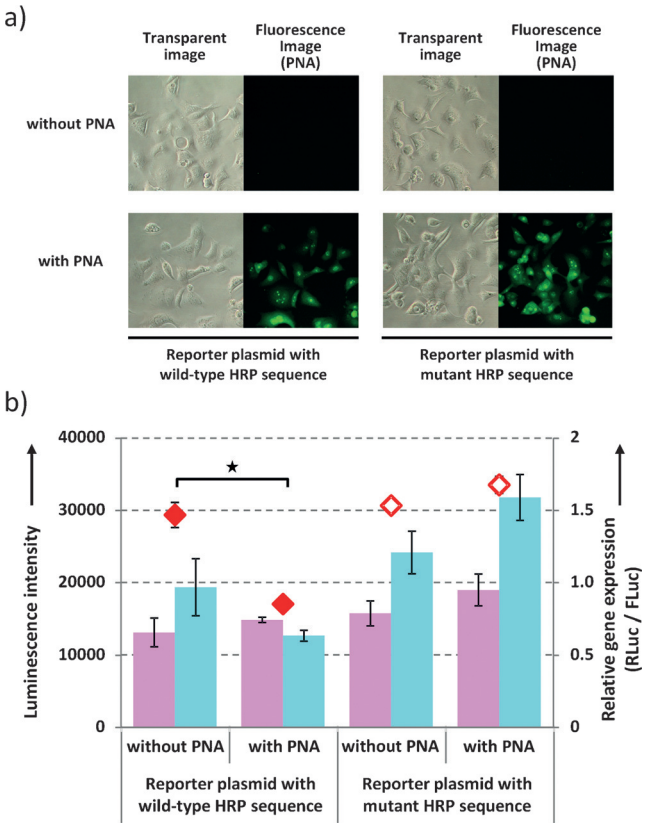

Figure 5. Triplex formation in the 5′ UTR decreases protein expression in cells. a) Phase-contrast image (left) and fluorescence image of the intracellular distribution of HF488-labeled PNA (right) obtained 24 h after transfection. Excitation at 480 nm, emission at 530 nm. b) Luminescence signals from FLuc (pink) and RLuc (blue; left axis). Values of the RLuc signals relative to the FLuc signals are plotted as data points (right axis). Values are means ± standard deviations of triplicate samples. The asterisk indicates a two-tailed *P* value of less than 0.01 as calculated using the Student's *t*-test.

In conclusion, we have demonstrated that PNA modified with unnatural nucleobases forms a highly stable and sequence-selective triple helix with a double-stranded RNA region in the context of long mRNA both in vitro and in cells. Formation of a stable PNA–RNA triple helix in the 5′ UTR of mRNA caused translation suppression; the triplex most likely acts as a roadblock to the small ribosomal subunit. The effect was highly sequence-specific and completely abolished by a single mismatched Hoogsteen triplet, as shown by analysis of the translation of an mRNA containing a mutant RNA hairpin sequence. As the 9-mer PNA requires a double-stranded RNA motif for binding (single-stranded RNA is not a good target), the recognition site is expected to appear rarely in transcriptome sequences. To the best of our knowledge, this is the first demonstration of the biological effects of PNA–RNA triple helix formation in live cells. The binding affinity between the 9-mer PNA and wild-type HRP was higher than the reported affinities of RNA-binding proteins, such as those involved in miRNA processing and post-transcriptional RNA editing.[24] Therefore, it is conceivable that PNA–RNA triple helices may be used to modulate the activity of RNA-binding proteins. Furthermore, the formation of a PNA–RNA triple helix might modulate RNA functions by disrupting RNA conformational transitions that control gene expression.[1,25] Therefore, triple-helix-forming PNAs have potential as regulators of biological activities mediated by RNA.

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