Temperature-dependent Regulation of Antisense Activity Using a DNA/poly(*N*-isopropylacrylamide) Conjugate

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We prepared a novel antisense reagent comprising of oligodeoxynucleotides (ODNs) and a thermo-responsive polymer, poly(*N*-isopropylacrylamide) (PNIPAAm). The conjugate demonstrated stimuli-responsive regulation of gene expression via conformational change of the polymer chain.

Inhibition of disease-related genes represents an effective strategy that might lead to a better understanding of the mechanisms involved in pathogenesis, and also to the development of novel therapeutic approaches.[1,2] Antisense ODNs are designed to hybridize with target mRNA in a sequence-specific manner and inhibit gene expression by preventing translation, either by activation of RNase H or steric blockage of the ribosome complex. A fundamental attraction of the antisense approach is that this method can, theoretically, potentially be applied to any gene product for the treatment of malignant and non-malignant diseases.[3] Indeed, antisense reagents targeting several oncogenes have been reported to specifically inhibit expression of these genes and delay tumor progression in the past decade.[4,5] However, there are still several problems for using antisense technology as a general tool on a clinical basis.[6,7] In fact, ODNs are rapidly degraded in biological fluids and cells by exo- or endonucleases which hydrolyze the phosphodiester linkages. Furthermore, these molecules diffuse poorly across the cell membrane because of their ionic character. To overcome these problems, antisense ODNs have been modified chemically, but none have achieved wide-scale acceptance.[8,9] The development of efficient systems for delivering antisense ODNs to living cells is still being explored worldwide for more practical clinical use of antisense ODNs.

It was shown that polymer modification of antisense ODNs improved the stability against nucleases.[10] Antisense ODNs modified with polyethylene glycol (PEG), the most popular medical polymer, demonstrated excellent resistance to nuclease degradation. Unfortunately, highly hydrophilic PEG-modified ODNs showed poor cellular uptake. We have previously proposed intelligent antisense reagents comprising of phosphodiester-linked ODNs and a thermo-sensitive polymer, PNIPAAm.[11] ODN–PNIPAAm conjugates showed a temperature-induced conformational change at 33 °C under physiological conditions.[11,12] It was shown that PNIPAAm in the globular state has excellent cellular membrane permeability.[13] Furthermore, ODN–PNIPAAm conjugates can control the hybridization properties of the antisense ODN moiety in the conjugate by conformational change of the polymer chain.[11] These properties of the conjugates may be useful for the delivery of antisense reagents. In the present study, we evaluated the sequence specificity of an intelligent antisense reagent. Furthermore, the antisense activity in response to temperature change associated with the phase transition of the grafted PNIPAAm chains on DNA was investigated.

We prepared 3′- and 3′,5′-modified ODN–PNIPAAm conjugates. A synthesized ODN (15-mer or 20-mer) containing the antisense sequence for the ribosomal binding site of the mRNA encoding EGFP was conjugated with PNIPAAm as previously described.[11,12] Briefly, 3′- and 3′,5′-methacryloyl-modified antisense ODNs (TATATCTCCTTCTTA or GGTATATCTCCTTCTTAAAAG) (ribosomal binding site underlined) (0.05 μmol) and NIPAAm (0.2 mmol) were dissolved in 0.94 mL of 10-mM Tris–HCl (pH 8.0). Then, 100 μL of aqueous ammonium persulfate (13 mM) and 40 μL of aqueous *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (2.15 M) were added to the mixture. The resulting mixture was incubated at room temperature for 1 h under a nitrogen atmosphere for copolymerization to obtain the antisense ODN–PNIPAAm conjugate. The ODN–PNIPAAm conjugates showed a temperature-induced conformational change at 33 °C in 10-mM Tris–HCl (pH 7.4) containing 100 mM NaCl.[14] The melting temperature (*T*m) of the duplex formed between the unmodified antisense ODN and its complementary DNA strand (CTTTAAGAAGGAGATATACC) was 47 °C in a buffer containing 10-mM Tris–HCl (pH 7.4). Reference conjugates were prepared using one-base mismatch (GGTATATCTCGTTCTTAAAAG) and scrambled (TTCTCTAGAGGAATCATTTC) sequence ODNs in a similar manner.

The antisense activities of these conjugates were evaluated by a reporter gene assay using the *E. coli* T7 S30 extract system (Promega). Protein expression was performed according to a standard protocol using the EGFP-encoding plasmid pET16EGFP[11] as the transcription template. The reaction was terminated by placing the reaction mixture on ice for 5 min, after which the expression of EGFP was determined by measuring the fluorescence intensity of the solution (excited at 474 nm). Figure 1a shows the inhibition of gene expression by modified- and unmodified-ODNs at 27 °C, which is below the phase transition temperature of the PNIPAAm side chain. The one-base mismatch ODN, and the 15-mer and 20-mer of the full-match antisense ODNs reduced the GFP protein level by 49, 61, and 64%, respectively. In contrast, GFP expression was not affected by the scrambled ODN under the experimental conditions used. 3′- and 3′,5′-modified ODN–PNIPAAm conjugates showed similar sequence specificity to the unmodified ODNs. These results suggest that the DNA moiety of the conjugates retains the original hybridization properties and sequence recognition ability, even when it is grafted into the polymer. On the other hand, PNIPAAm homopolymers had no effect on the gene expression.

At 37 °C, which is above the phase transition temperature of the conjugates, the antisense activities of two conjugates decreased dramatically as shown in Figure 1b. Commonly, hybridization between an antisense ODN and its target mRNA becomes

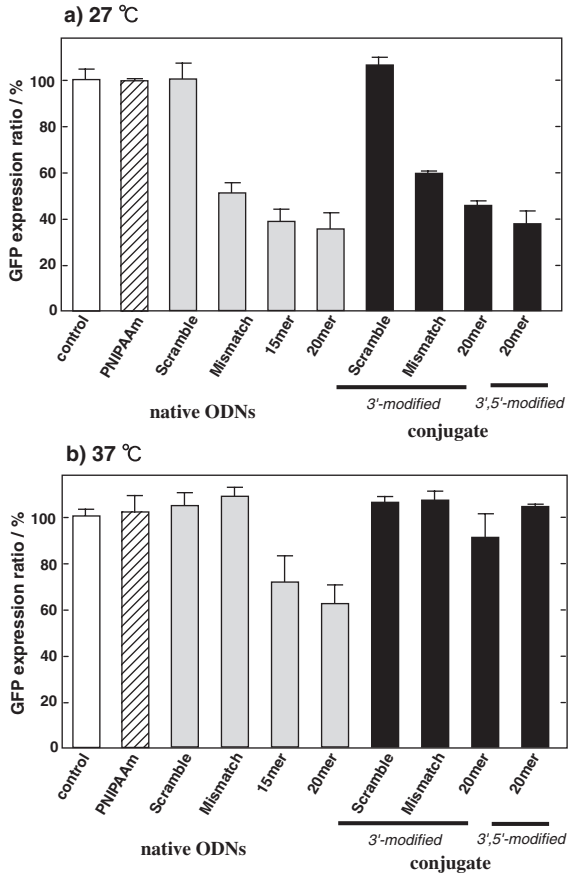

Figure 1. Sequence-specific inhibition of gene expression by antisense ODN–PNIPAAm conjugates. Incubation temperature, 27 °C (a) and 37 °C (b); native and modified ODNs, 2.7 μM; Plasmid pET16EGFP, 5.4 nM; excitation, 474 nm; emission, 504 nm; incubation time, 120 min. T7 S30 extract including pET16EGFP without any polymer and antisense ODN was used as a control.

unstable with increasing incubation temperature, however, the antisense effect of the conjugates was more markedly suppressed than the unmodified ODNs. In particular, the 3′,5′-modified ODN–PNIPAAm conjugate could not inhibit the GFP gene expression at all. This would be caused by the conformational change of the PNIPAAm chain. In a previous report, we confirmed that the hybridization properties of an antisense ODN in a conjugate were controlled by steric hindrance based on the polymer conformation. Thus, it is reasonable that both the conjugates regulate gene expression through hybridization with mRNA depending on the incubation temperature.

To determine whether the intelligent antisense conjugates had potential for clinical use, we tried to regulate the antisense activity continuously by changing the incubation temperature. Reaction mixtures including 5.8 nM of pET16EGFP were kept at 37 °C for 60 min in the presence or absence of 2.7 μM of the 3′,5′-modified ODN–PNIPAAm conjugate, and then incubated at 27 °C. Figure 2 shows the GFP expression profiles under the experimental conditions. The antisense ODN–PNIPAAm conjugate showed no effect on the gene expression at 37 °C. The conjugate, in which the PNIPAAm polymer was in the globule state, suppressed the antisense activity. At 27 °C, the fluorescence intensity of the control experiment kept increasing at the same rate, and then reached a plateau at 120 min. In contrast, the antisense ODN–PNIPAAm conjugate inhibited the gene expression effectively. After decreasing the incubation temperature, the GFP expression in the presence of the conjugate was suppressed by 74% compared with the control experiment. These results shows that the antisense activity of the ODN–PNIPAAm conjugate is regulated rapidly by conformational change of the polymer moiety.

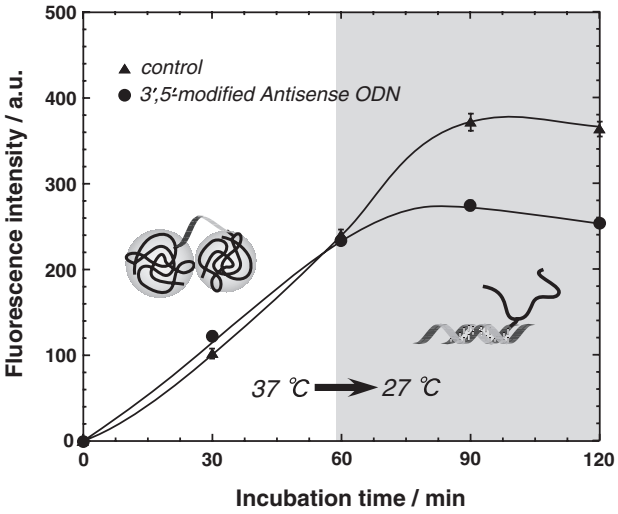

Figure 2. Regulation of antisense activity of an ODN–PNIPAAm conjugate by temperature. The 3′,5′-modified 20mer-antisense ODN was added to an *E. coli* T7 S30 extract at 2.7 μM. Plasmid pET16EGFP, 5.4 nM; excitation, 474 nm; emission, 504 nm. T7 S30 extract including pET16EGFP without the conjugate was used as a control.

In conclusion, we have demonstrated stimuli-responsive regulation of gene expression using an antisense ODN–PNIPAAm conjugate. We observed that the conjugate was transported effectively into mammalian cells, A549 human lung carcinoma cells, at 37 °C without a gene carrier such as lipofectamine (data not shown). In fact, this antisense ODN–PNIPAAm conjugate may achieve efficient gene delivery into cells above the *T*c, and then block target gene expression below the *T*c. These characteristics of the conjugate suggest that it has potential for use in a new antisense ODN delivery system.

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