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Novel DNA/Polymer Conjugate for Intelligent Antisense Reagent with Improved Nuclease Resistance

Masaharu Murata,* Wataru Kaku, Takahisa Anada, Yoshikuni Sato, Takeshi Kano, Mizuo Maeda and Yoshiki Katayama

Department of Applied Chemistry, Graduate School of Engineering, Kyushu University, Fukuoka 812-8581, Japan

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Abstract—Antisense technology provides an effective strategy to inhibit synthesis of the gene product. We prepared a novel antisense reagent comprised of oligodeoxynucleotides (ODN) and a thermo responsive polymer, poly(*N*-isopropylacrylamide) (PNIPAAm). The conjugate inhibited gene expression in a dose-dependent manner. The ODN–PNIPAAm conjugate demonstrated excellent resistance to S1 nuclease. In particular, PNIPAAm-modified antisense ODN at the 3′,5′-ends of the ODN provided complete resistance against nuclease at 37 °C, which is above the phase transition temperature of the PNIPAAm side chain. These characteristics of the conjugate suggest it may have potential for use in a new gene delivery system as part of an antisense strategy.

Antisense therapy works at the genetic level to prevent mutated or overactive genes from directing the synthesis of disease-related proteins.[1–3] Attempts have been made to interfere with gene expression by in situ generation of mRNA from recombinant vectors[4] or by exogenous introduction of synthetic ODN.[5,6] Antisense phosphodiester-linked ODNs have demonstrated inhibition of many viral and cellular gene products, both in vitro and in vivo. However, such ODNs are susceptible to degradation by *exo*- and *endo*-nucleases, which are ubiquitous in serum and in the intracellular milieu. Chemical modification of ODNs to enhance their stability is a typical approach to overcome this obstacle.[7–9] Phosphorothioate-linked ODNs are commonly used, and effectively inhibit the synthesis of the gene product in various cell types.[10,11] Unfortunately, phosphorothioate ODN possesses a relatively low binding affinity for target RNA, which impacts its potency in antisense applications.[12] Thus, the development of new ways to modify and/or protect conventional phosphodiester ODN to improve nuclease resistance is important.

We previously proposed an intelligent antisense reagent comprised of phosphodiester-linked ODN and a thermo sensitive polymer, PNIPAAm.[13] The antisense ODN–PNIPAAm conjugate demonstrated a stimuli-responsible regulation of the hybridization between the conjugate and the target RNAs. This phenomenon was caused by a conformational change in polymer moiety linked to the ODN at the 3′-end and depending on the temperature condition. In the present study, we prepared 3′- and 3′,5′-modified ODN–PNIPAAm conjugates, and then evaluated their antisense activity and nuclease resistance.

A synthesized ODN (20 mer) having the antisense sequence for the ribosomal binding site of the mRNA coding enhanced green fluorescent protein (EGFP) was conjugated with PNIPAAm. The 3′-modified ODN–PNIPAAm conjugate was prepared as described in a previous report.[13–15] The novel antisense reagent 3′,5′-modified ODN–PNIPAAm conjugate was synthesized as follows: 3′,5′-Methacryloyl-modified antisense ODNs (GGTATATC**TCCT**TCTTAAAAG) (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 solution of *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 nitrogen atmosphere for copolymerization to obtain the antisense ODN–PNIPAAm conjugate. The conversions of vinyl-ODNs and NIPAAm were determined to be ca.

*Corresponding author. Tel.: +81-926-424-206; fax: +81-926-423-606; e-mail: muratatcm@mbox.nc.kyushu-u.ac.jp

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93% and 87%, respectively, by HPLC analysis. ODN-PNIPAAm conjugates showed a temperature-induced conformational change at 33 °C in 10 mM Tris–HCl (pH 7.4) containing 100 mM NaCl. The melting temperature (T_m) of the duplex formed between unmodified antisense ODN and its complementary DNA strand (CTTTAAGAAGGAGATATACC) was 47 °C in a buffer containing 10 mM Tris–HCl (pH 7.4) and 100 mM NaCl.

The antisense activities of these conjugates were evaluated using the *Escherichia coli* T7 S30 extract system (Promega). This system can monitor the efficacy of the transcription/translation of DNA sequences cloned in a plasmid vector containing a T7 promoter by providing an extract that contains T7 RNA polymerase for transcription and all the necessary components for translation. T7 RNA polymerase transcription reaction was performed according to standard protocol using plasmid pET16EGFP[13] as a transcription template. The reaction was terminated by placing the reaction mixture on ice for 5 min, after which the expression of GFP was determined by measurement of the fluorescence intensity of the solution (excited at 474 nm). Figure 1 shows the dose-dependent translational repression by 20-mer antisense ODN–PNIPAAm conjugates at 27 °C. The conjugates were added to the solutions by 10-, 100- and 500-fold higher amounts than that for the template, respectively. Fluorescence intensity was clearly decreased with increasing dosage of the conjugates. We observed 63 and 74% reduction in GFP expression after treatment of 2.7 μM of 3′- and 3′,5′-modified ODN–PNIPAAm conjugates, respectively. On the other hand, the PNIPAAm homopolymer had no effect on the GFP gene expression under the same conditions (data not shown). In a previous report, we confirmed that DNA moiety of the conjugate retains the original hybridization property and sequence recognition ability, even if it is grafted into the polymer.[13] Thus, the inhibition of gene expression would be caused by the binding of the antisense ODN to the target mRNA.

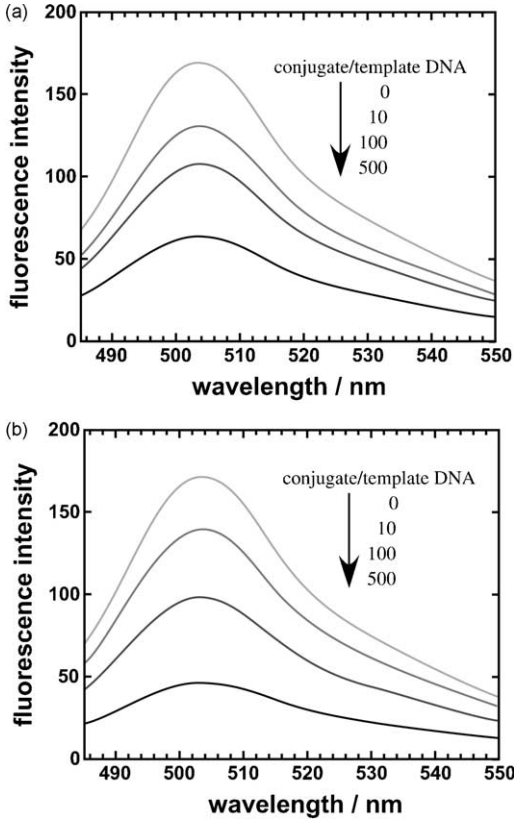

Figure 1. Dose-dependent inhibition of gene expression by antisense ODN–PNIPAAm conjugates: 3′-modified ODN (a) and 3′,5′-modified ODN (b) were added to *E. coli* T7 S30 extract by 0.054, 0.54 and 2.7 μM, respectively.

In order to investigate the stability of DNA moiety in the conjugate, the ODN–PNIPAAm conjugates were treated with endonuclease S1 nuclease. 3′- and 3′,5′-modified ODN–PNIPAAm conjugates (1 nmol each) were dissolved in reaction buffer [30 mM sodium acetate (pH 4.6), 280 mM NaCl, and 10 mM $ZnSO_4$]. 1.5 U of S1 nuclease was added to the solutions (final volume, 500 μL). Half of each conjugate mixture was incubated for 120 min at 27 °C, and the other half of each mixture for 120 min at 37 °C; these temperatures are respectively below and above the phase transition temperature of the conjugates. Figure 2 shows the profiles of nuclease digestion of the DNA as detected by an increase in absorbance at 260 nm caused by nucleobases liberation

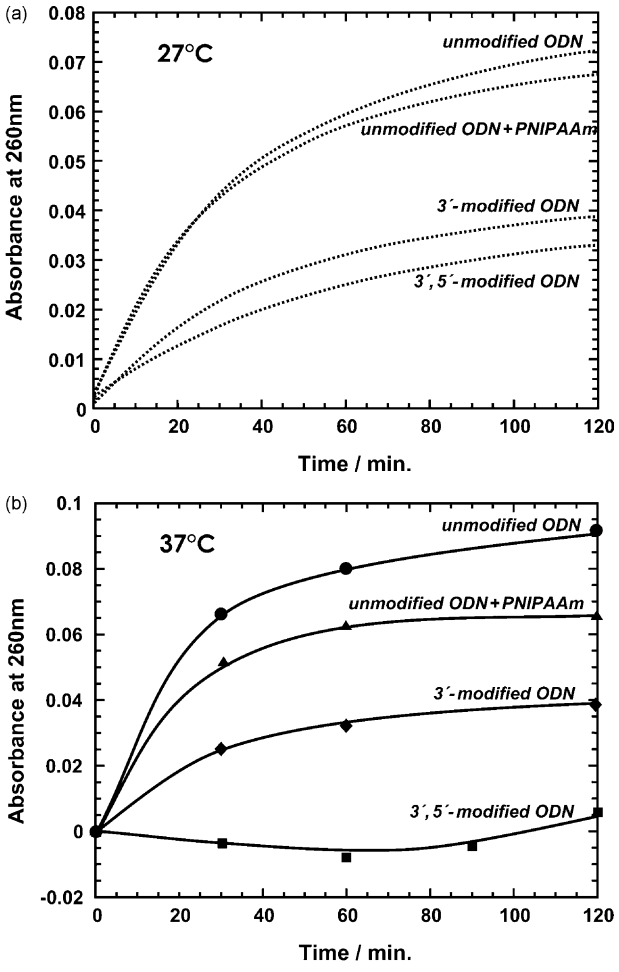

Figure 2. Stability of antisense ODN–PNIPAAm conjugate against S1 nuclease. Incubation temperature, 27 °C (a) and 37 °C (b); ODN, 1 nmol; S1 nuclease, 1.5 U.

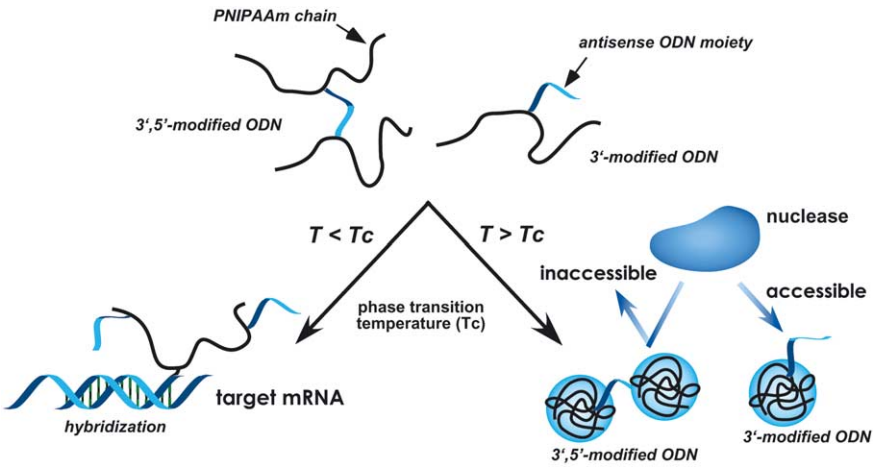

Figure 3. Schematic illustration of stimuli-responsive antisense reagent comprised of oligodeoxynucleotide and poly(*N*-isopropylacrylamide).

from stacking in oligomer.[16] Unmodified ODN was rapidly digested by nuclease regardless of incubation temperature. On the other hand, both conjugates demonstrated excellent resistance to nuclease degradation at 27 °C (Fig. 2a). After incubation the increase in UV absorbance caused by DNA degradation was suppressed by 47% for the 3′-modified conjugate and 54% for the 3′,5′-modified conjugate. In contrast, under the same conditions, the PNIPAAm homopolymer in reaction solution showed essentially no effect against the nuclease digestion of the DNA. These results suggested that the stability of antisense ODN against cellular nuclease attack was effectively improved by polymer modification of the DNA.

At 37 °C, the 3′,5′-modified ODN–PNIPAAm conjugate was completely protected against degradation by the S1 nuclease (Fig. 2b). In contrast, nuclease resistance of the 3′-modified ODN–PNIPAAm conjugate was not significantly influenced by incubation temperature. The reason for this difference in nuclease resistance between the two conjugates is not clear. The most plausible possibility is the difference of accessibility of the nuclease to the grafted ODN. An ODN–PNIPAAm conjugate can reversibly change its conformation from extended to condensed in response to temperature change associated with the phase transition of the grafted PNIPAAm chains on DNA. The PNIPAAm part of the conjugate was in the globule state at 37 °C. Thus, condensed polymer moiety of the conjugate may affect accessibility of the nuclease to the ODN moiety (Fig. 3). In the case of the 3′,5′-modified ODN–PNIPAAm conjugate, the two condensed PNIPAAm parts can inhibit access of nuclease to the ODN moiety more effectively by steric hindrance. On the other hand, the protective effect against the nuclease by the polymer moiety in the 3′-modified conjugate is limited to the PNIPAAm-modified side of the ODN moiety. Therefore, the nucleases may be able to access the DNA from the opposite side.

In conclusion, we evaluated the antisense activity and nuclease stability of an intelligent antisense agent comprised of an antisense ODN and stimuli-responsible polymer PNIPAAm. These properties are key criteria in the search for optimal antisense nucleic acid modifications, though the origins of the various levels of resistance to nuclease degradation conferred by chemical modification of DNA are currently not understood. The ODN–PNIPAAm conjugate could successfully control the nuclease resistance property via conformation change of the polymer chain. It has been shown that PNIPAAm in a globule state has excellent cellular membrane permeability and low toxicity.[17] Further, this strategy can use antisense ODN without chemical modification at the phosphodiester linkage of the ODN, which means the conjugate may retain its binding affinity for the target RNA. These characteristics of the conjugate are promising for its use as an antisense ODN carrier in gene therapy.

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