Thermo responsive DNA/Polymer Conjugate for Intelligent Antisense Strategy

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The conjugate between 20-mer antisense oligodeoxynucleotides (ODN) and thermo-responsive polymer, poly(*N*-isopropylacrylamide) (PNIPAAm), was prepared. Hybridization reaction between the conjugate and their target RNA was modulated by conformational change of polymer moiety depending on temperature condition.

Antisense ODN can specifically bind to their complementary messenger RNA, and thereby block the production of particular proteins at the genetic level.[1,2] Several antisense reagents have currently been evaluated for possible clinical applications.[3,4] However, the intrinsic phosphodiester-linked ODN, that had been used initially, could not be used as in vivo therapeutic agents, because they were degraded rapidly by cellular nucleases. Furthermore, the negatively charged and highly hydrophilic ODN suffers from poor cellular uptake and cell specificity. To overcome these problems, chemical modifications of ODN have been proposed,[5,6] whereas none of them have achieved wide scale acceptance. Development of better delivery systems of antisense into living cells have still been explored worldwide for more practical clinical use of antisense.

We have previously shown that DNA grafted with PNIPAAm can reversibly change its conformation between extended and condensed in response to temperature change.[7,8] This phenomenon may be due to the phase transition of the grafted PNIPAAm chains on DNA. Recently, we discovered that such conjugate forms colloidal nanoparticles by heating over the lower critical solution temperature (LCST) of PNIPAAm unit, when increasing the molar ratio of the ODN in the copolymer.[9,10] Here, we have constructed antisense ODN-PNIPAAm conjugate that is able to bind target mRNA under physiological conditions. It was shown that polymer modification of ODN, mainly using polyethylene glycol (PEG), improved the stability against nuclease.[11] Furthermore, the temperature-induced conformational change of the PNIPAAm chain may increase hydrophobicity of the conjugate and affect to the hybridization ability between the conjugate and the mRNA, thereby regulating gene expression depending on the temperature change (Figure 1).

The DNA fragment encoding the *Aequorea victoria*-enhanced green fluorescence protein (EGFP) was amplified by PCR using pQBI63 (Wako Pure Chemical) as a template. The primers for EGFP that contained *Bam*HI sites are: GGATCCAATGGCTAGCAAAGGAGAA and GGATCCTCAGTTGTACAGTTCATC (restriction sites underlined). The 723-bp fragment produced during the PCR was incorporated into pGEM-T TA cloning vector (Promega). This plasmid was then digested with *Bam*HI and was subcloned into the same site of bacterial expression vector pET16b (Novagen). Correct insertion of the fragment was verified by DNA sequencing. This plasmid was called pET16EGFP.

A synthesized ODN that has the antisense sequence for the ribosomal binding site of the mRNA coding EGFP, was conjugated with PNIPAAm as follows.[9] 3′-Methacryloyl-modified-antisense ODNs (20 mer; GGTATATC**TCCT**TCTTAAAAG) (ribosomal binding site underlined) (0.15 μmol) and NIPAAm (0.3 mmol) were dissolved in 1.72 mL of 10 mM Tris-HCl (pH 8.0). Then 200 μL of aqueous ammonium persulfate (13 mM) and 80 μL of aqueous solution of *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (86 mM) were added to the mixture. The resulting mixture was incubated at room temperature for 1 h under nitrogen atmosphere for copolymerization to obtain antisense ODN-PNIPAAm conjugate. The conversions of vinyl-ODNs and NIPAAm were determined to be ca. 85% and 79%, respectively, by HPLC analysis. Reference conjugates were synthesized using scrambled (TTCTCTAGAGGAATCATTTC) sequence ODNs in similar manner. ODN-PNIPAAm conjugates showed temperature-induced conformational change at 33 °C in 10 mM Tris-HCl (pH7.4) containing 100 mM NaCl.[12] A target RNA for antisense ODN was synthesized by in vitro transcription reaction using RiboMAX™ large scale RNA production system for T7 RNA polymerase (Promega). After removal of template DNA (pET16EGFP) and unincorporated nucleotides, the RNA concentration and purity were estimated by UV spectrometry (long, 1037 nt; Yield, 0.28 mg; $A_{260/280} = 1.77$).

We examined the binding activity of the full-match 20 mer antisense ODN- and scrambled ODN-PNIPAAm conjugates to the target RNA in a buffer containing 10 mM Tris-HCl (pH7.4) and 100 mM NaCl. The conjugates were added to the solutions by 0.5, 1, 2 and 4-fold higher amount for the RNA, respectively. The solutions

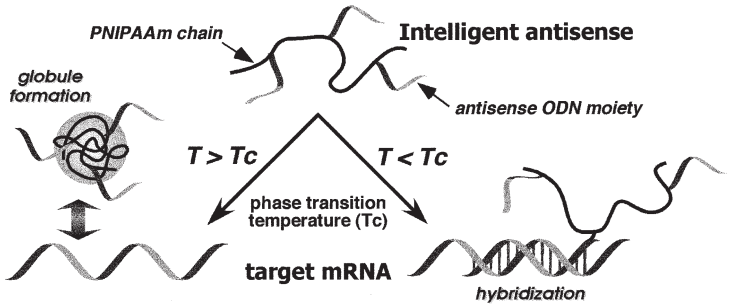

Figure 1. Schematic illustration of stimuli-responsive antisense reagent comprising oligodeoxynucleotide and poly(*N*-isopropylacrylamide).

were incubated for 15 min at 27 °C, and then free RNA in the solution was detected by electrophoretic retardation assay using Agilent 2100 bioanalyzer with RNA 6000 nano chip. As shown in Figure 2, free RNA amount decreased with increasing dosage of antisense ODN-PNIPAAm conjugate. It is most likely due to the complexation between the RNA and the conjugate, because the neutral PNIPAAm unit should suppress the electrophoretic mobility of the RNA hybridized with the conjugate. Remarkably, almost all antisense in the conjugate hybridized with the RNA when the antisense unit and the RNA were mixed at 1:1 molar ratio. On the other hand, scrambled-ODN conjugate had no effect on the mobility of the RNA. These results suggest that DNA moiety of the conjugate retains the original hybridization property and sequence recognition ability, even if it is grafted into the polymer.

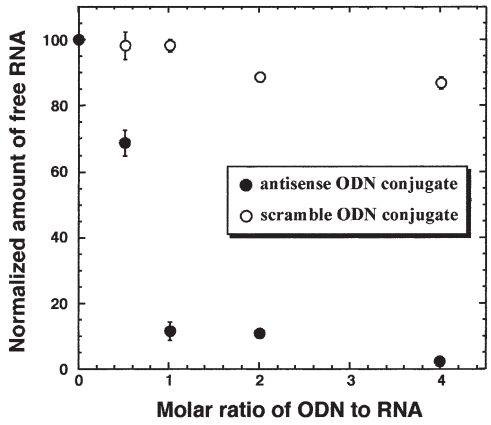

Figure 2. Effect of antisense ODN-PNIPAAm conjugates on electrophoretic mobility of target RNA. ●, antisense 20 mer ODN-PNIAAm conjugate; ○, scramble 20 mer ODN-PNIAAm conjugate. Free RNA (1037 nt) in the solution containing the conjugates and RNA was detected by Agilent 2100 bioanalyzer with RNA 6000 nano chip at 27 °C. Control experiment was performed in the absence of the conjugate. Experimental conditions: 10 mM Tris-HCl (pH7.4); 100 mM NaCl; 1 μM RNA; 0–4 μM conjugate.

In order to investigate the function of PNIPAAm chain in the conjugate, an electrophoretic retardation assay was performed at 27 °C and 37 °C, which are below and above phase transition temperature of the conjugate, respectively. The results of this experiment are summarized in Figure 3. At 37 °C, electrophoretic mobility of the RNA was not significantly influenced in the presence of the antisense ODN-PNIPAAM conjugate. This indicates that there is only very weak interaction between DNA moiety of the conjugate and the RNA in the solution. The melting temperature (*T*m) of the duplex formed between unmodified antisense ODN and its complementary DNA strand (CTTTAAGAAGGAGATATACC) was 47 °C under the same conditions. Since 37 °C is still lower than *T*m, this inhibiting effect of the conjugate against the hybridization at the temperature may be explained by the conformational change of PNIPAAm region. A polymer moiety of the conjugate in a globule state may inhibit the interaction between ODN moiety and their target RNA by steric hindrance. In contrast, free RNA amount was decreased to 11% of control in the presence of antisense ODN conjugate at 27 °C, at which the PNIPAAm part of the conjugates is random coiled under the present solution conditions. On the other hand, neither PNIPAAm homopolymer nor scramble conjugate gave any effect on electrophoretic mobility of the RNA irrespective of the temperature condition. This result indicates that hybridization ability of the antisense ODN in the conjugate can be regulated by the temperature.

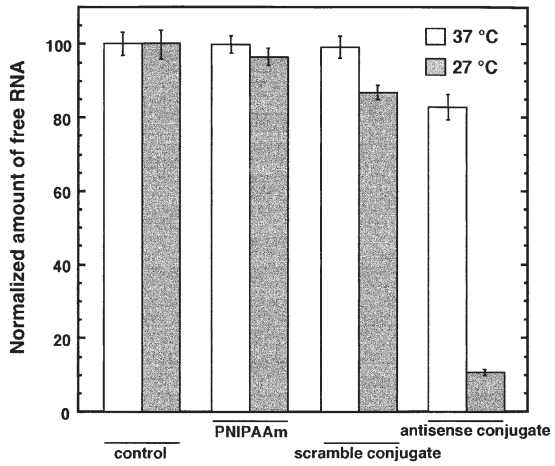

Figure 3. Temperature-dependency of electrophoretic mobility of target RNA in the absence or presence of the conjugates and PNIPAAm homopolymer. Open bar and solid bar indicates the amount of free RNA after incubation at 37 °C and 27 °C, respectively. Control experiment was performed in the absence of the conjugates and PNIPAAm homopolymer. Error bars indicate standard derivations. Experimental conditions: 10 mM Tris-HCl (pH7.4); 100 mM NaCl; 1 μM RNA; 2 μM conjugate.

In conclusion, we have designed an intelligent antisense reagent comprising antisense DNA and thermo-sensitive polymer PNIPAAm. Hybridization properties of antisense ODN in conjugate was successfully controlled by the conformation change of the polymer chain. This is the first report that demonstrates stimuli-responsible regulation of the hybridization using polymer modification. These results suggest that antisense ODN-PNIPAAm conjugate can regulate gene expression depending on the temperature. Furthermore, it is shown that PNIPAAm in a globule state has excellent cellular membrane permeablity.[13] Thus, this antisense ODN-PNIPAAm conjugate may achieve efficient gene delivery into cells above *T*c, and then block target gene expression below *T*c. This feature will make possible to restrict antisense action around the diseased tissue. The present novel strategy on design of antisense reagent will be expanded by choosing another polymer which shows stimuli-responsive behavior.

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