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We report the development of photo-crosslinked siRNA strands modified at each termini with *p*-cyanostilbene. The siRNA was nuclease resistant and retained RNAi activity. We further studied activation mechanism of the covalently-crosslinked siRNA. Interestingly Dicer, which is known to generate siRNA with overhanging 3' ends from precursor siRNA, did not cleave the crosslinked siRNA at all. Our results suggest that activation of the crosslinked siRNAs required cleavage by Argonaute2.

Chemically synthesized oligonucleotides including antisense oligonucleotides, short interfering RNAs (siRNAs), decoy nucleic acids, and DNAzymes are promising clinical agents that sequence-specifically impact target gene expression.[1-4] Digestion by endogenous nucleases limits practical application of oligonucleotide-based therapies. Chemical modification can improve resistance to nuclease digestion. For example, antisense oligonucleotides generally have backbone alterations such as replacement of phosphate oxygen with sulfur, boron, or acetate, modification of 2'-OH of ribose (2'-*O*-methylation, 2'-*O*-allylation), or alteration of ribose structure.[1, 2, 4] These types of modifications were also applied to siRNA.[2, 5-9] Because activation of siRNA involves formation of the RNAi-induced silencing complex (RISC) via interaction and processing by Dicer and Argonaute 2 (Ago2),[10-12] design of chemically modified siRNAs suitable for the RNAi system have been considered.[8, 9, 13, 14]

To increase nuclease resistance, crosslinkings of the siRNA strands at both of termini were suggested.[15-17] Dumbbell-type siRNAs circularized using enzyme-catalyzed ligation reportedly have high stability and RNAi activity.[15, 16] Various chemical crosslinking techniques for oligonucleotides have been developed.[17-21] Such methods could be used as an alternate strategy for ligation. Recently, we demonstrated a DNA photo-crosslinking system using a [2+2] photo-cycloaddition reaction of various stilbene derivatives.[22, 23] This result prompted us to introduce the stilbene derivatives at the termini of siRNA strands. Photo-crosslinking resulted in "termini-free" siRNAs (Figure 1a and b). In the present manuscript, we prepared crosslinked siRNA and evaluated stability and RNAi activity. Furthermore, the activation mechanism of the crosslinked siRNA was investigated.

Firstly, we evaluated the photo-crosslinking properties of the stilbene derivatives in the context of short double-stranded RNAs (dsRNAs). Among reported stilbene derivatives, we selected *p*-stilbazole (B) and *p*-cyanostilbene (S_{CN}) as candidate molecules (Figure S1). We first investigated photo-crosslinking ability of homodimers of these stilbene derivatives introduced into an RNA duplex via a D-threoninol scaffold. Various model duplexes (rX1a/rX1b, rX2a/rX2b, and rX3a/rX3b, Figure S1) having *p*-stilbazoles or *p*-cyanostilbenes at the central base-pairing position were synthesized. UV irradiation of the dsRNAs resulted in decrease of UV absorption around 330 nm, which is assigned to the π–π* absorption band of the stilbene derivatives (Figures S1). The melting temperatures (T_ms) of the dsRNAs greatly increased after photo-irradiation (Figures S1). These data indicated that homodimers of **B** and $\mathbf{S_{CN}}$ in RNA duplex undergo [2+2] photo-cycloaddition. The changes of UV-Vis spectra show that the rates constants of [2+2] photo-cycloaddition in the dsRNA strands of $\mathbf{S_{CN}}$ are larger than those of **B** in all examined dsRNAs (Figure S2). In addition, quenching of photo-dimerization by neighbouring G-C pair was less effective for $\mathbf{S_{CN}}$ than for **B**. We therefore used $\mathbf{S_{CN}}$ for preparation of termini-free siRNA (Figure 1).

To evaluate biological activity of the termini-free siRNA, the sequence used in previous work to target the mPIASy gene was prepared unmodified and modified with $\mathbf{S_{CN}}$ s at all termini (Figure 1).[14, 24, 25] Crosslinking by photo-irradiation was confirmed by a decrease of UV absorption at 330 nm, an increase in T_m value, and the HPLC profile (Figure 1).

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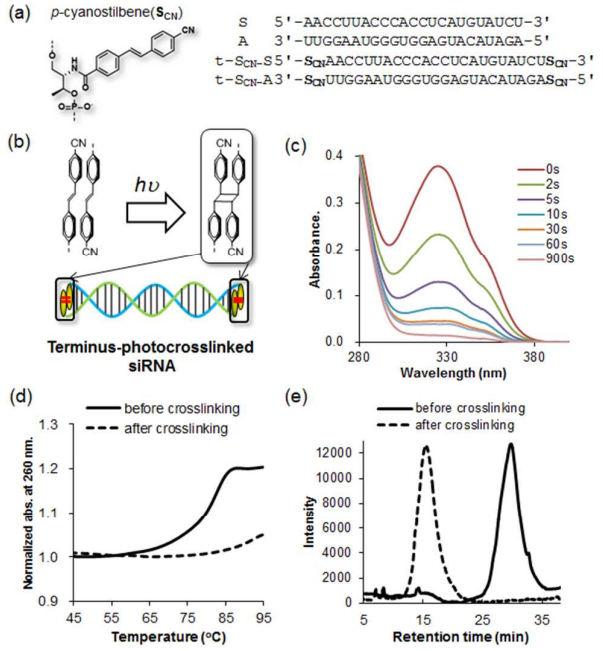

Figure 1. (a, b) Photo-crosslinking of *p*-cyanostilbenes in t-S_{CN}-S/t-S_{CN}-A. UV-vis spectra (c), melting curves (d), and HPLC profiles (e) of each siRNA before and after irradiation at 340 nm are shown.

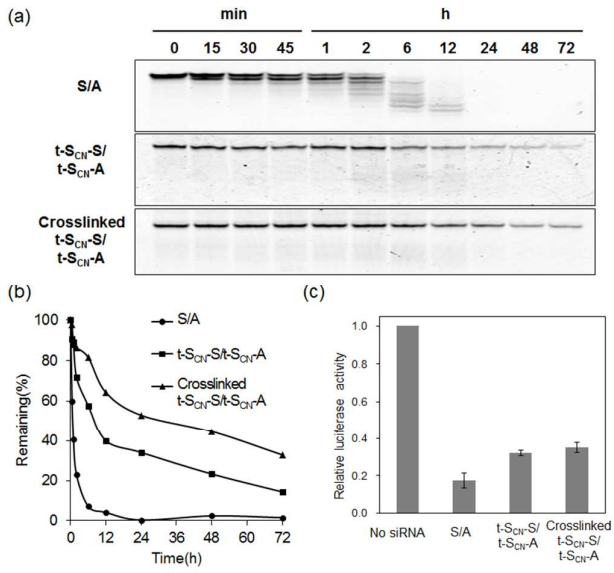

Figure 2. (a)Unmodified siRNA (S/A), uncrosslinked t-S_{CN}-S/t-S_{CN}-A, and crosslinked t-S_{CN}-S/t-S_{CN}-A (1 μM) were incubated with HeLa lysate at 37 °C. (b) Remaining siRNAs were quantified and data are plotted. (c) RNAi activity of the unmodified (S/A) and *p*-cyanostilbene-modified siRNA before and after crosslinking. Reporter plasmids pGL3-Fw was created by inserting the target sequence into the 3′-UTR of the firefly luciferase expression vector, pGL3. The *Renilla* luciferase expression vector was used as an internal control.

Then we examined nuclease resistance of the crosslinked siRNA. For this we incubated the siRNA in HeLa lysate in which exonucleases are highly active.[14] Degradation of native siRNA

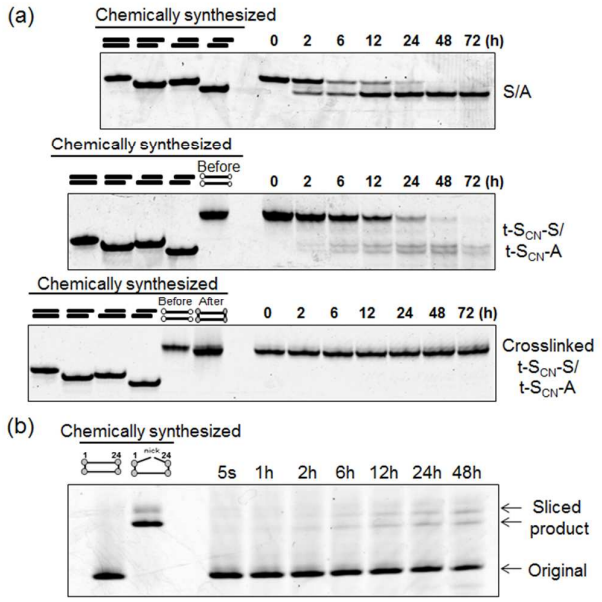

Figure 3. (a) Effect of Dicer on unmodified and *p*-cyanostilbene modified siRNA before and after crosslinking. siRNA (6.0 μM) was incubated with Dicer (0.1 U/μl) at 37 °C. "Standard" lanes contained chemically synthesized siRNAs of indicated structure. (b) Cleavage of crosslinked siRNA by purified recombinant Ago2. Crosslinked t-S_{CN}-S/t-S_{CN}-A (1.0 μM) was incubated with Ago2 (50 μM) at 37 °C. The structures of "Chemically synthesized" siRNAs are indicated schematically. Details are described in supporting information.

(S/A) began immediately upon addition of lysate, and after 6 h of incubation little of the full-length S/A was detected (Figures 2a and b, S/A). Uncrosslinked t-S_{CN}-S/t-S_{CN}-A was more resistant to degradation by endogenous ribonucleases than the unmodified siRNA (Figure 2a and b, t-S_{CN}-S/t-S_{CN}-A). After photo-crosslinking, the siRNA was highly resistant (Figure 2a and b, Crosslinked t-S_{CN}-S/t-S_{CN}-A). Then RNAi activities of t-S_{CN}-S/t-S_{CN}-A before and after photo-dimerization were evaluated using a luciferase reporter assay. We used the firefly luciferase expression vector pGL3 with the target sequence complementary to the antisense strand (pGL3-Fw) for this assay. Figure 2c shows relative luciferase expression in 293 FT cells. Although the **S_{CN}**-modified siRNA exhibited lower RNAi activity than native siRNA as reported in the previous work on crosslinked siRNA,[17] it still kept enough RNAi activities both before and after photo-crosslinking.

Next we examined how crosslinked siRNA was activated in cells. During RNAi process, precursor siRNA is edited by Dicer and resultant siRNA with 3′-overhangs interacts with Ago2. Upon unwinding of the siRNA duplex on Ago2, the sense strand is liberated, leading to activation of RISC.[11, 12, 26] As the crosslinked siRNA efficiently inhibited luciferase expression in our reporter assay, we hypothesized that the ends of strands in the siRNA are digested by Dicer, the enzyme responsible for generation of siRNAs from longer precursors, and resultant siRNA is unwound in RISC. Therefore, we incubated the **S_{CN}**-

modified siRNA before and after crosslinking with Dicer. Upon incubation of unmodified or uncrosslinked siRNA with Dicer, a shorter duplex was produced as shown by migration on 20% non-denaturing PAGE (Figure 3a); this is likely a duplex with 3' overhanging nucleotides as reported previously.[14] Major two products from t-S_{CN}-S/t-S_{CN}-A might be caused by alteration of dicing site due to the $\mathbf{S_{CN}}$ modifications at terminus positions. Interestingly, the crosslinked t-S_{CN}-S/t-S_{CN}-A was not processed by Dicer (Figure 3a, bottom). Previous studies on the mechanism of RISC activation indicated that Dicer first binds to the termini of blunt-ended siRNA and cleaves 5'-terminal nucleotides to afford siRNA with 3' overhanging nucleotides, which is recognized by Ago2 to form RISC.[10-12]

As Dicer does not process the crosslinked siRNA, we tested the ability of Ago2, which has an endonuclease activity,[27] to cleave the crosslinked siRNA. Crosslinked t-S_{CN}-S/t-S_{CN}-A was incubated with recombinant human Ago2, and products were analyzed by denaturing PAGE. New bands were observed above the original one after 6 h of incubation (Figure 3b and S3a).[28] We confirmed that positions of new bands correspond to bands of crosslinked t-S_{CN}-S/t-S_{CN}-A with a nick in t-S_{CN}-S strand by comparison to chemically synthesized oligonucleotide of this structure. These data suggest that the crosslinked t-S_{CN}-S/t-S_{CN}-A was activated by Ago2 in our cell-based reporter assay. Namely, if Dicer does not work for crosslinked siRNA, Ago2 can release the sense strand to activate the crosslinked siRNA as long as it can exert cleavage activity.

In order to validate this hypothesis, we next evaluated relationship between slicing of siRNA by Ago2 and RNAi activity. We prepared two siRNAs with $\mathbf{S_{CN}}$ modifications in the central region (Figure 4a) and conducted a luciferase reporter assay of the siRNAs before and after crosslinking. We evaluated effects on luciferase expression from pGL3-Fw and pGL3-Rv. In pGL3-Rv the region complementary to the sense strands of the siRNAs is inserted in the sense orientation in the 3'-UTR. This construct allowed us to evaluate off-target activity that arises from undesired sense strand incorporation into RISC. Our previous report indicated that insertion of the dye such as azobenzene or fluorophore through D-threoninol scaffold near 5' region of sense strand (pseudo seed region) can reduce off-target effects due to lower selectivity of sense strand in mature RISC.[24, 25] The unmodified siRNA silenced luciferase expression from both the pGL3-Fw vector and the pGL3-Rv vector (Figure 4b).

t-S_{CN}-S/t-S_{CN}-A before and after crosslinking show RNAi activities for pGL3-Fw and pGL3-Rv, indicating that either antisense or sense strand can make activated RISC. In S_{CN}S7/S_{CN}A7, the $\mathbf{S_{CN}}$s modification is located within the pseudo seed region of the sense strand and is outside the seed region of the antisense strand (Figure 4a). The uncrosslinked S_{CN}S7/S_{CN}A7 silenced expression more effectively from pGL3-Fw and there was less off-target activity than observed with the unmodified siRNA (Figure 4b). Interestingly, after interstrand-crosslinking of S_{CN}S7/S_{CN}A7, no off-target activity was observed, and the siRNA retained on-target pGL3-Fw silencing activity (Figure 4b). We also analyzed the RNAi

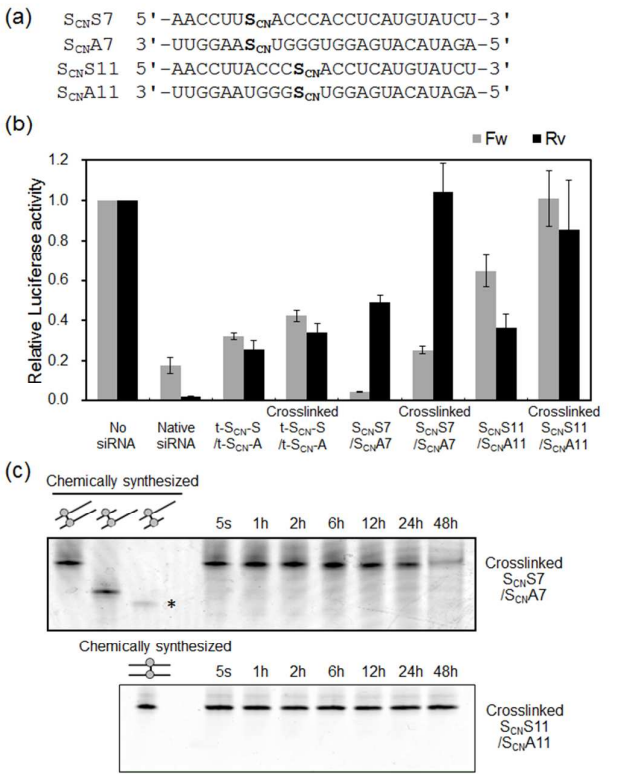

Figure 4. (a) Sequences of internally S_{CN}-modified siRNAs. (b) On- and off-target silencing assay with internally S_{CN}-modified siRNAs before and after crosslinking. Reporter plasmid, pGL3-Fw (Fw) or pGL3-Rv (Rv) and *Renilla* luciferase expression vector as an internal control were co-transfected into 293FT cells with indicated siRNA. (c) Cleavage of the internally S_{CN}-modified siRNAs by purified recombinant Ago2. The structures of "Chemically synthesized" RNAs are indicated schematically. The third RNA from left in upper figure is hard to stain compare to the others, therefore the location is indicated by *asterisk*. Details are described in supporting information

activities of siRNA modified with $\mathbf{S_{CN}}$s in the center (S_{CN}S11/S_{CN}A11), which is predicted to be near the active site of Ago2 in the RISC. Before photo-crosslinking, the S_{CN}S11/S_{CN}A11 siRNA had both on- and off-target activities; however, after photo-crosslinking, almost no silencing of luciferase expression from either pGL3-Fw or pGL3-Rv was observed (Figure 4b). Next, we analyzed the cleavability of these crosslinked siRNAs by Ago2. Upon incubation with purified recombinant Ago2, crosslinked S_{CN}S11/S_{CN}A11 was not cleaved at all, whereas crosslinked S_{CN}S7/S_{CN}A7 was gradually digested (Figure 4c, S3b, and S3c).[29]

On the bases of these data, we hypothesize activation mechanisms of crosslinked siRNAs by Ago2 (Figure S4). An unmodified siRNA can be single stranded in RISC by cleavage and/or unwinding by Ago2 (Figure S4a). Ago2 cleavage of crosslinked S_{CN}S7/S_{CN}A7 was possible because the scission site is at a distance from the crosslinked positions. Cleavage of the S_{CN}S7/S_{CN}A7 sense strand allowed the seed region of antisense strand to be single stranded in RISC and capture the target mRNA (Figure S4b). The lack of off-target silencing with the crosslinked S_{CN}S7/S_{CN}A7 may be due to crosslinking of the siRNA in the pseudo-seed region of sense strand.

Uncrosslinked S_{CN}S11/S_{CN}A11 might be unwound due to structural change of Ago2,[30] but crosslinked S_{CN}S11/S_{CN}A11 was neither cleaved nor unwound in RISC due to the crosslinking, resulting in a complete loss of RNAi activity (Figure S4b). Our data suggest that slicer activity of Ago2 is important in RISC activation. Recently it has been suggested that endonuclease C3PO specifically degrades the nicked passenger (sense) strand.[31, 32] The termini-free t-S_{CN}-S/t-S_{CN}-A is likely first activated by Ago2-mediated slicing and subsequently C3PO-mediated degradation of the sense strand may enhance activity (Figure S4c).

Conclusions

In summary, we successfully demonstrated the [2+2] photo-cycloaddition reaction of **S_{CN}** residues at the termini and at internal positions of RNA duplexes. Photo-crosslinking of **S_{CN}**s at both termini of an siRNA remarkably increased nuclease resistance and the siRNA retained RNAi activity. We also found that crosslinking at pseudo-seed region can reduce the off-target effect induced by intended incorporation of the sense strand in RISC. Our crosslinking studies also showed that cleavage by Dicer is not critical for activation of siRNA but slicing of the sense strand by Ago2 promotes the RISC maturation. These findings contribute to the understanding of the mechanism of the RISC maturation process and provide a new methodology for enhancing nuclease resistance of siRNA.

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