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Disulfide-unit conjugation enables ultrafast cytosolic internalization of antisense DNA and siRNA

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Abstract: Development of intracellular delivery methods for antisense DNA and siRNA is important. Previously reported methods using liposomes or receptor-ligands have a problem that oligonucleotides take several hours or more to reach the cytoplasm due to long-time residence of oligonucleotides at endosomes. In this study, we clarified that oligonucleotides modified with low molecular disulfide units at the terminus reaches the cytoplasm 10 minutes after the administration to cultured cells. This rapid cytoplasmic internalization of disulfide-modified oligonucleotides suggests the existence of an uptake pathway other than endocytosis. In fact, the mechanistic analysis revealed that the modified oligonucleotides are efficiently internalized into the cytoplasm through disulfide exchange reactions with the thiol groups on the cellular surface. Because our approach solves several critical problems with the currently available methods for enhancing cellular uptake, including toxicity, undefined molecular composition, inefficient endosomal escape, and serum stability, this method may be an effective approach in the medicinal application of antisense and RNAi methods.

Antisense DNA and siRNA have been widely used for gene silencing in basic research and in medicinal applications.[1] Effective delivery of the oligonucleotides (ONs) into the cells is important for clinical applications.[2] For this reason, delivery methods for ONs have been extensively studied.[3] A variety of methods using polymeric materials have been investigated and one of the most popular methods utilizes cationic liposomes. [4] However, the material of the cationic liposomes needs to be 5 to 10 times the weight of the ONs.[5] In addition, the liposome and the ONs form a complex, and the molecular composition of this complex cannot be uniformly defined.[6] Therefore, a new method is required based on a well-defined molecular composition that enables intracellular delivery of ONs using low molecular weight material. One strategy is to make ON conjugates with small molecules that are membrane permeable. For example, cell-penetrating peptides (CPPs) are peptide molecules with a molecular weight equal to or less than that of ONs. [7] It has been reported that antisense DNA and siRNA can be introduced into cells by making conjugates of CPPs and ONs. However, since most of the CPPs are cationic, the conjugates show cytotoxicity.[8] Furthermore, the conjugates can form a polyion complex and become insoluble.[9] Conjugates of hydrophobic molecules, such as cholesterol and lipids, and ONs have also been reported,[10] but because they form micelles, these conjugates do not exist as single molecules. ON conjugates with ligands for receptors on the cell surface are also efficiently taken up by the target cells.[11] For example, it has been reported that siRNA conjugated with GalNAc (N-acetyl galactosamine), which is a ligand for the asialoglycoprotein receptor is efficiently taken up into the primary cultured cells expressing this receptor.[12] This strategy is useful for targeting specific cells that express a particular receptor. Finally, most of the methods described above have problems in endosomal escape of ONs.[13] In this study, we attempted to develop a conjugate molecule that universally improves the cellular uptake of ONs. Based on the background summarized above, we designed molecules for ON conjugates that satisfy the following requirements (Fig. 1). (1) Small molecule without charge, (2) Does not aggregate and exists as a single molecule. (3) Does not cause cytotoxicity. It has been reported that peptides with disulfide groups have cell membrane permeability and such a disulfide unit may fulfill the above requirements. Cyclic disulfide-modified peptides and small molecules with cell membrane permeability have also been reported.[14],[15] To our knowledge, there have been no reports of the use of disulfide unit for the intracellular delivery of ONs. Therefore, we synthesized series of ONs with disulfide groups and evaluated their cellular uptake. Then, we found the modification of repeated linear disulfide unit with ONs enables the rapid cytosolic internalization of ONs without the endosomal trap which is triggered by the formation of a disulfide bond with a thiol group of a protein on the cell surface and affords potent gene silencing effects (Fig. 1).

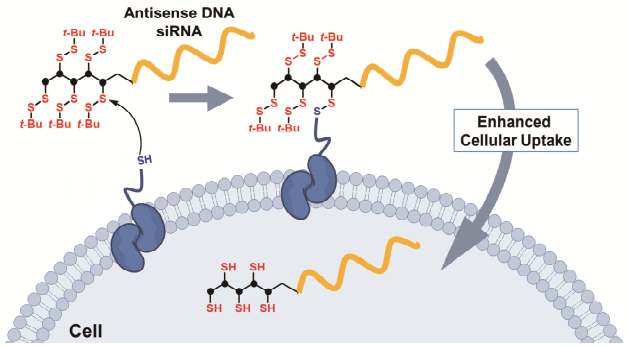

Figure 1. Conjugation with disulfide units enhanced the permeability of oligonucleotides.

We designed antisense DNA and siRNA molecules having di-sulfide groups. Because introduction of the disulfide group within the sequence of the ON strands would impair the

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biological function, we introduced disulfide units at the terminus of the ON strands by phosphoramidite chemistry. Two types of phosphoramidite units with a disulfide group were designed (Fig. 2a, Scheme S1). That is, a linear disulfide (LD) unit amidite LDA having a tertbutyl disulfide group and a cyclic disulfide (CD) unit amidite CDA having a dithiothreitol (DTT) group. These disulfide units can be introduced into any position of the ONs with any number of repeats by an automatic ON synthesizer. In addition, because they have a phosphate triester structure, there is no charge on the units.

Antisense DNA (**ASD**, **1CD**, **5CD**, **10CD**, **1LD**, **5LD**, and **10LD**) was designed by introducing CD or LD units with 1, 5, or 10 repeats at the 5' end of 19 nt phosphorothioate antisense DNA (Fig. 2b). The target sequence of the antisense DNA was the firefly luciferase gene (961–979 site).[16] These antisense DNAs were synthesized by a DNA synthesizer and purified using HPLC and the purity was confirmed by HPLC and MALDI-TOF-MS (Fig. S1, Table S2). First, the micelle-forming ability of LD-modified ONs was examined using pyrene, which is an environment-responsive fluorescent molecule used for estimating the critical micellar concentration (CMC) (Fig. S3). No enhancement of fluorescence intensity was observed for **5LD** or **10LD** at 1 μM and 100 nM. These results suggested that the LD-modified ONs do not form micelles at 1 μM or less, and exist as single molecules. Hence, LD-modified ONs may have desirable properties for clinical applications.

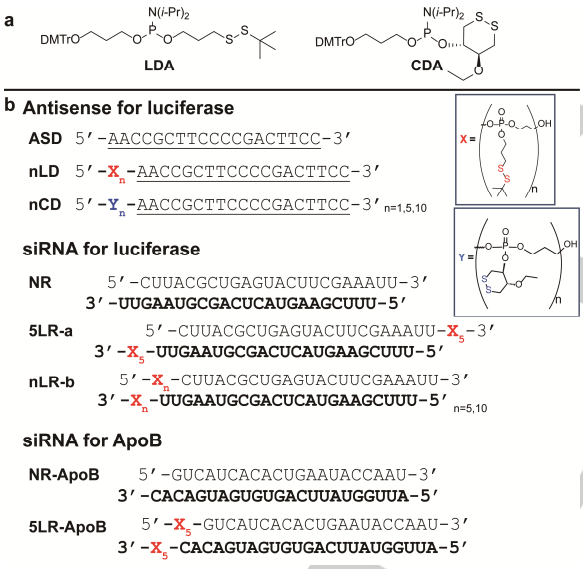

Figure 2. a Structure of disulfide phosphoramidites and **b** sequence of antisense DNA and siRNA. Underlined parts are phosphorothioate DNA. The guide strands of siRNA are shown in bold.

Next, the cellular uptake was evaluated using the antisense DNAs labeled with fluorescein (marked with *, Table S1) by flow cytometric analysis (Fig. 3a). For ONs at concentrations of 1 μM and 100 nM, the **ASD** was hardly up-taken without lipofection, whereas the LD-conjugated DNA **5LD** was significantly up-taken. More efficient uptake was observed as the number of repeats of the LD unit increased (Fig. 3b). The uptake of **5LD** was almost the same compared with the case using Lipofectamine 3000 (Lipo), and the uptake of **10LD** was twice as high. The uptake of **5CD** at 1 μM was approximately 70% of that of **5LD** (Fig. 3a).

The comparable efficiency of cellular uptake between **ASD** and **5LD** was also confirmed in other cell lines including NIH/3T3 and A431, which demonstrated the versatility of the disulfide-based strategy (Fig. S4). The intracellular distribution of **5LD** and **5CD** was analyzed by confocal laser scanning microscopy (CLSM). The image of the ONs at 10 min after lipofection showed that fluorescent-labeled **ASD** (**ASD***) was co-localized mainly with the endosome (Fig. 3c), which indicated the endosome trap of **ASD**. The images of **5LD** and **5CD** at the same time point showed that they were not co-localized with the endosome, but were quickly distributed in the cytoplasm. This implies that the disulfide modified ONs internalize into cells via non-endocytotic pathway. After incubation for 1 h, the uptake of **5LD** and **5CD** was increased and good distribution to the cytoplasm was maintained (Fig. 3d).

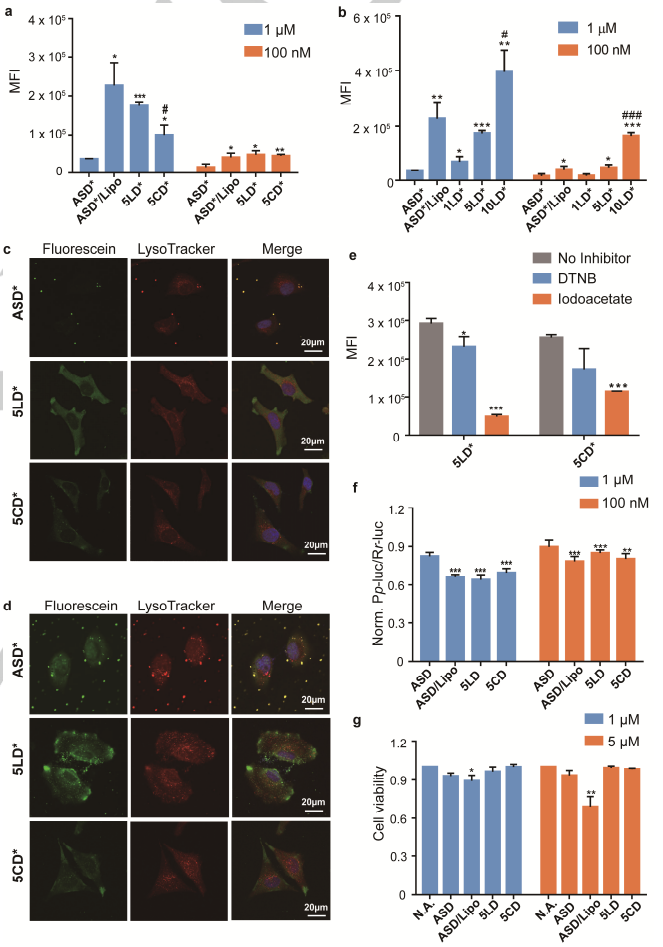

Figure 3. **a-g**; Disulfide unit conjugation enhanced the cellular uptake and antisense effect. **a** The cellular uptake of **5LD***, **5CD***, and **ASD*** measured by flow cytometry. **b** Effect of the repeat number of **LD** on cellular uptake. **c** The intracellular distribution of **5LD*** and **5CD*** compared with **ASD*** transfected by Lipofectamine 3000 after 10 min incubation or **d** after 1 h incubation. **e** Inhibitory assays for cellular uptake of **5LD*** and **5CD*** using DTNB and iodoacetate. **f** Gene silencing effect of **5LD** and **5CD** in dual luciferase assays. The luminescence signals for each sample were normalized to conditions of no administration. **g** Evaluation of cell viability by MTT assays after 24 h treatment. The experiment was performed three times independently. Data are presented as the mean ± SEM, *P < 0.05, **P < 0.01, ***P < 0.001 versus controls; #P < 0.05, ##P < 0.01, ###P < 0.001 versus **ASD*/Lipo**. MFI is the abbreviation for mean fluorescence intensity. Sequence names marked with * means that the ON is fluorescently-labelled.

These results suggested that the designed disulfide unit, not only promoted cellular uptake, but also enables ultrafast cytoplasm distribution, which is desirable for clinical use.

Intracellular uptake experiments were carried out in the presence of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) and sodium iodoacetate[17] to clarify whether disulfide-modified ONs are taken up via disulfide bond formation on the cell membrane surface (Fig. 3e). DTNB inhibited uptake of **5CD** and **5LD** by 7% and 11%, respectively. For iodoacetate, the uptake of both **5LD** (82%) and **5CD** (55%) was significantly inhibited. It was suggested that disulfide bond formation may be involved in the uptake mechanism. Previously, transferrin was suggested to be a membrane protein involved in the disulfide exchange in the similar system for peptide delivery.[15] However, it is difficult so far to mention the responsible membrane protein, since the unusually efficient cytosolic distribution was observed in our system, which suggests the different mechanism to that of the related reported methods. [18]

To evaluate the gene silencing effect, the antisense effects of **5LD** and **5CD** were investigated by dual luciferase assay using a HeLa cell line stably expressing firefly and Renilla luciferase genes. When using ONs at 1 μM or 100 nM concentrations, lipofection, **5LD**, and **5CD** showed comparable inhibitory effects in a concentration-dependent manner (Fig. 3f). In addition, the cytotoxicity of these intracellular delivery methods was evaluated by MTT assays. The results indicated that the lipofection method reduced the cell viability to 90% and 70% at concentrations of 1 and 5 μM, respectively, while **5LD** and **5CD** did not affect the cell viability (Fig. 3g).

Next, the LD unit was introduced into siRNA strands and the cellular uptake and RNAi effects were evaluated. The sequence of the siRNA used targeted the firefly luciferase gene (867–885 site).[16] The introduction of the LD unit to the 5' end of the guide strand was avoided because it is known to be important for RISC (RNA-induced silencing complex) formation. Several types of LD-modified siRNAs were designed as follows; **5LR-a**: five repeated LD units were introduced at both 3' ends of the siRNA strands, **5LR-b**: five repeated LD units were introduced at the 5' end of the passenger strand and the 3' end of the guide strand, **10LR-b**: 10 repeated LD units were introduced at the 5' end of the passenger strand and the 3' end of the guide strand (Fig. 2b, Fig. S2). First, the possibility of micelle formation was checked; fluorescence analysis using pyrene revealed that **5LR-b** and **10LR-b** were monodisperse and did not form micelles at 1 μM or less (Fig. S5). Next, the cellular uptake of these modified siRNAs was evaluated using fluorescently labeled RNAs (marked with *, Table S1). At concentrations of 1 μM and 100 nM, **10LR-b** showed higher intracellular uptake than siRNA administrated via the lipofection method (Fig. 4a). In addition, **5LR-a** and **5LR-b** resulted in cell uptake efficiency of approximately 40% that of lipofection. CLSM imaging analysis showed that after 10 min, **5LR-b** was localized mainly in the cytoplasm, not in the endosome (Fig. 4b), similar to the results for the LD-modified DNA 5LD. In contrast, NR transfected by lipofection was co-localized mainly with the endosome even after 1 h (Fig. 4c). Next, various inhibitors were tested to investigate the cellular uptake mechanism. First, the intracellular uptake of **5LR-b** was significantly inhibited by both DTNB and iodoacetate, which indicated thiols on the cell surface have a critical role for the internalization of **5LR-b** (Fig. 4d). Second, the intracellular uptake of **5LR-b** was not inhibited by three kinds of endocytosis inhibitors, although the uptake of **NR** with lipofection was significantly inhibited (Fig. S6). These inhibition experiments suggested that **5LR** may be directly introduced into the cytoplasm via disulfide bond formation on the cell surface, which indicated the mechanism is different from classical endocytosis internalization. Similarly, to the experiments with antisense DNA, the gene silencing effect of LD-modified siRNA was evaluated using HeLa cells stably expressing the luciferase gene (Fig. 4e).

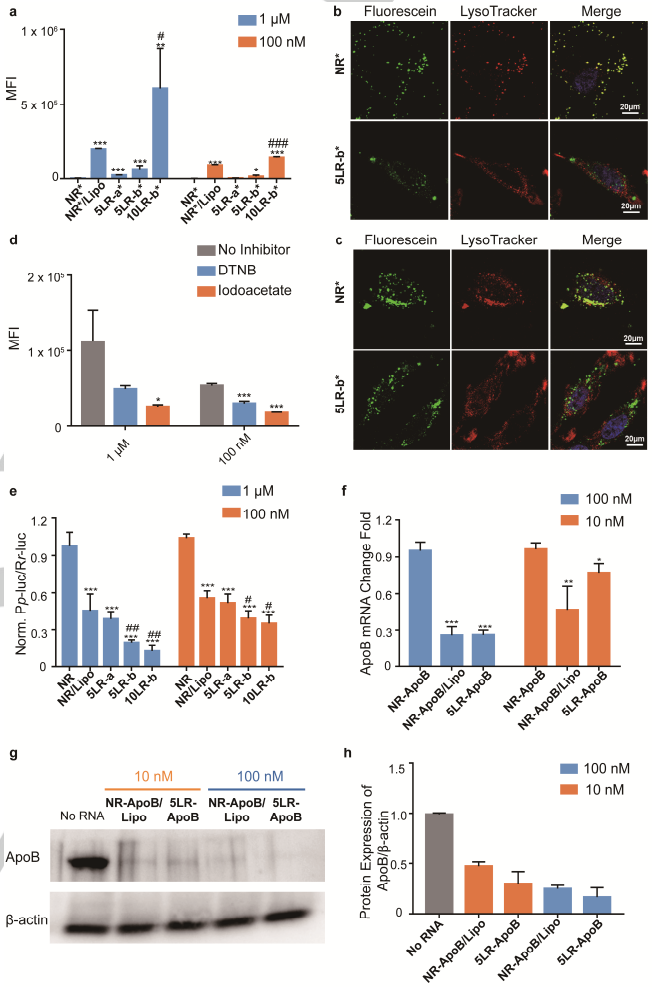

Figure 4. a-h Disulfide unit conjugation enhanced the cellular uptake and knockdown effect of siRNA. **a** The cellular uptake of LD-conjugated siRNA measured by flow cytometry. **b** The intracellular distribution of **5LR-b*** and **NR*** transfected by Lipofectamine 3000 after 10 min incubation or **c** after 1 h incubation. **d** Inhibitory assays for cellular uptake of **5LR-b*** using DTNB and iodoacetate. **e** Gene silencing effect of **5LR-a**, **5LR-b**, and **10LR-b** in dual luciferase assays. The luminescence signals for each sample were normalized to conditions of no administration. **f** The real-time qPCR measurement of the change in ApoB mRNA after 24 h administration of **5LR-ApoB**. The experiment was performed three times independently. Data are presented as the mean ± SEM, *P < 0.05, **P < 0.01, ***P < 0.001 versus controls; #P < 0.05, ##P < 0.01, ###P < 0.001 versus **NR*/Lipo**, **NR/Lipo** or **NR-ApoB/Lipo**. MFI is the abbreviation for mean fluorescence intensity. Sequence names marked with * means that the sequence is fluorescently-labelled. **g** Knock-down effect for ApoB by Western-blotting assay. **h** The quantitative analysis of ApoB protein expression normalized by β-actin. Western blot experiment was performed two times independently.

At both concentrations of 1 μM and 100 nM, **5LR-a** and **5LR-b** showed a higher silencing effect than ON administrated via lipofection. Interestingly, **5LR-b**, which has LD units on the same

sides of the double-stranded RNA, showed a higher silencing effect than **5LR-a**. Therefore, we concluded that **5LR-b** was the optimal molecular design. The stability of **5LR-b** in serum was evaluated (Figs. 5a, b). After 30 min, the canonical siRNA (**NR**) had completely disappeared, whereas 72% of **5LR-b** remained. Since degradation of siRNA in serum is one of major clearance pathways,[19] this result indicated that LD modification would like to be beneficial for *in vivo* applications of siRNA also in terms of biostability. Compared with classical nanoparticle system (e.g. liposomes), the stabilization effect might be weaker, but still many advantages such as efficient cytosolic delivery, low toxicity, and defined molecular composition would surpass this inferiority.

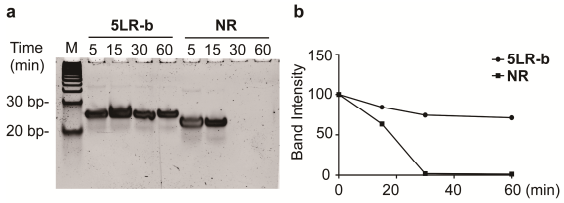

Figure 5. Stability of **5LR-b**. **a** Non-denaturing PAGE analysis of the digestion reaction with 10% human serum at different incubation times. **b** Band intensity analysis of **a**.

Finally, siRNA targeting the endogenous gene ApoB (Apolipoprotein B) was designed and the RNAi effect was evaluated (Figs. 4f-h).[20] Inhibition of ApoB expression lowers the levels of low-density lipoprotein (LDL), enabling the treatment of familial hyper-cholesterolemia. siRNA (**5LR-ApoB**) was designed by introducing five repeats of LD units at the 3' end of the guide strand and the 5' end of the passenger strand of the siRNA (Fig. 2b). The expression level of ApoB mRNA was quantitatively analyzed using real-time PCR after the transfection. When 100-nM siRNA was used, **5LR-ApoB** showed an inhibitory effect of 70% or greater, which was comparable to that of lipofection-mediated siRNA. In addition, comparable silencing effect for ApoB was confirmed by Western-blotting assays (Figs. 4g, h).

In summary, we have successfully developed a new method for universally enhancing the cellular uptake of ONs. The key disulfide moieties can be introduced easily based on phosphoramidite chemistry at any position in the ONs with the desired number of repeats, which widens the range of the molecular design. The disulfide-modified ONs were efficiently distributed in the cytoplasm, where the target mRNA molecules exist, within 10 min, and showed silencing effects superior or comparable to those of lipofection-mediated ONs, notably with no cytotoxicity. Additionally, our new method circumvents critical problems associated with the conventional methods for cellular uptake enhancement and thus should be suitable for clinical applications. Further studies of applications *in vivo* are underway in our group.

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Keywords: cellular uptake • antisense • siRNA • cytosolic delivery • disulfide

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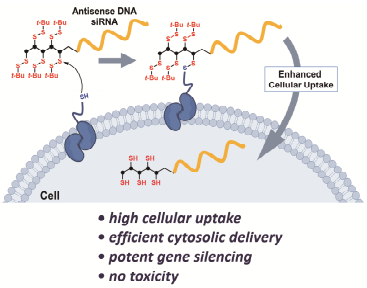
COMMUNICATION

Oligo-disulfide Magic!

Disulfide conjugation with antisense DNA and siRNA enabled efficient and ultrafast cytosolic uptake of these bioactive oligonucleotides without toxicity. The new method represented herein solved the long-standing problems of various oligonucleotide delivery methods and should enhance therapeutic applications of antisense DNA and siRNA.



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Disulfide-unit conjugation enables ultrafast cytosolic internalization of antisense DNA and siRNA