siRNA Modification

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Highly Potent and Stable Capped siRNAs with Picomolar Activity for RNA Interference**

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Small interfering RNAs (siRNAs) can be used to silence gene expression in a sequence-specific manner through a process known as RNA interference (RNAi).[1] Many efforts have been devoted to the application of siRNAs for biological techniques and also as therapeutic agents.[2] Currently, synthetic siRNAs as potential therapeutic molecultes could be applied for clinical purposes. However, serum instability, off-target effects, immunostimulatory activity, and poor delivery into cells are the main barriers to this application.[3] The potential of siRNAs to become a new therapeutic approach has stimulated much interest in chemical modifications to optimize the biological properties of these siRNAs.[4] Improvements in the serum stability and potency of siRNAs can decrease the dosage required, which would be beneficial for mitigating off-target effects and could also modulate immunostimulatory activity and improve drug delivery.

Double-stranded RNAs with different strand lengths or shapes, compared to the canonical 21 nucleotide (nt) siRNA duplexes, have been heavily investigated, including dumbbell-shaped RNAs.[5] Herein, chemical mimics of hairpin-shaped and dumbbell-shaped RNAs (Figure 1) were designed and constructed using a biocompatible thiol–maleimido Michael addition[6] based on a dimeric cross-linker 1,2-bis(maleimido)-ethane (BME; Figure 2a). One or both ends of the double-stranded RNA could easily be capped using BME. The native 21 nt siRNA duplex used in our study was siFL867–885

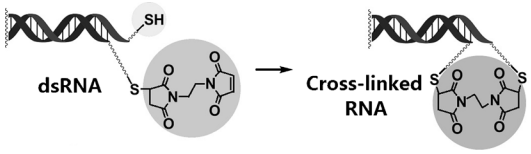

Figure 1. Scheme of the formation of cross-linked RNA through a thiol–maleimido Michael addition.

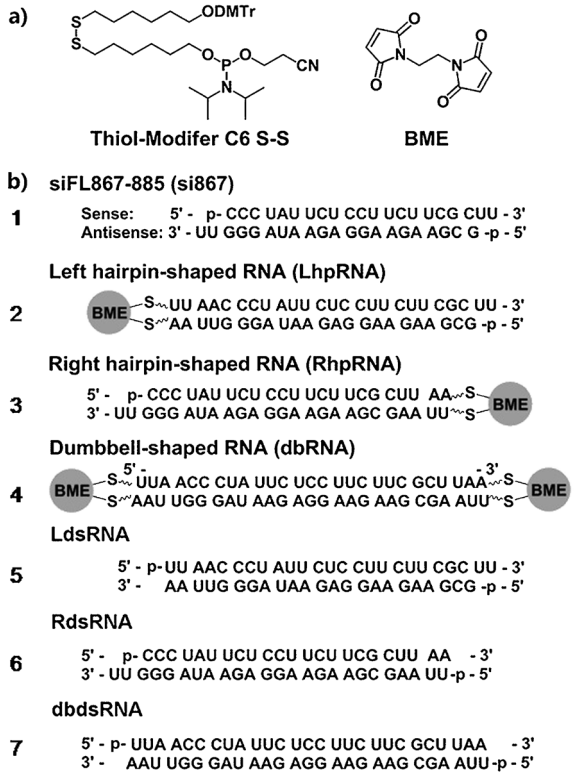

Figure 2. a) Chemical formulas of the Thiol-Modifier C6 S-S and BME. DMTr = dimethoxytrityl. b) Sequences **1**–**7** of native si867, cross-linked RNAs (LhpRNA, RhpRNA, dbRNA), and control linear dsRNAs (LdsRNA, RdsRNA and dbdsRNA).

(si867), which can effectively suppress the expression of a firefly luciferase reporter gene.[7] Modified thiol-containing RNAs were synthesized using a commercial thiol-modifier C6 S-S (Figure 2a) following a standard phosphoramidite elongation cycle for coupling of commercial nucleoside phosphoramidites.[8] Based on the sequence of si867, three cross-linked RNAs (LhpRNA, RhpRNA, and dbRNA) were designed to have four base-pairs in addition to the Dicer cleavage site next to the thiol-modifier C6 S-S (Figure 2b, **1**–**4**). Three corresponding linear dsRNAs (LdsRNA, RdsRNA, and dbdsRNA) were employed as a control (Figure 2b, **5**–**7**).

To construct single-end cross-linked RNAs, both left hairpin-shaped RNAs (LhpRNA) and right hairpin-shaped RNAs (RhpRNA) were closed at their designated ends of the modified dsRNAs using thiol–maleimido Michael addition (Supporting Information, Figure S1). A newly formed band that migrated more slowly than the single-stranded RNA was found by denaturing polyacrylamide gel electrophoresis (PAGE) analysis (Figure S3a). ESI-TOF MS was conducted

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to characterize the molecular weight of the artificially prepared hairpin-shaped RNAs. The mass spectra for both isolated LhpRNA and RhpRNA showed a single major peak at 15944 Da, which matched with the calculated molecular weight of 15945 Da (see Supporting Information). This observation confirmed that capped RNAs could be obtained from two modified RNA strands by Michael addition with a good yield. Encouraged by these results, the thiol–maleimido Michael addition was also used to construct dumbbell-shaped RNA (dbRNA; Figure S2). To avoid intermolecular polymerization, the optimal single-stranded RNA concentration for annealing was discovered to be below 0.5 μM, which showed thiol–maleimido Michael addition only after dsRNA annealing. A newly formed band appeared in the denaturing PAGE analysis (Figure S3b), which was characterized as dumbbell-shaped RNA by ESI-TOF MS, with a single major peak at 18414 Da (calculated value was 18407 Da).

To determine their biological stability, the cross-linked RNAs (LhpRNA, RhpRNA, dbRNA) and their corresponding control RNAs (LdsRNA, RdsRNA, dbdsRNA) were incubated in 50% normal human serum at 37°C (Figure 3). The cross-linked RNAs showed higher stability compared with that of their linear counterpart or native siRNA control. After 48 hours incubation, more than 35% of the cross-linked RNAs remained full-length, while no linear dsRNAs were detected after 24 hours incubation. Similar results were also seen when these RNAs were incubated in 50% fetal bovine serum (Figure S4). The improved serum stability of the cross-linked RNAs could be due to their continuous structures.[5c] Because RNase A is one of the main nucleases responsible for siRNA degradation in serum,[4e,9] a dosage-dependent RNase A digestion of the cross-linked RNAs were also carried out. After one hour of incubation with 1 mg L^{-1} RNase A at 37°C, 80% of dbRNA was still full length, in contrast to only 60% of si867 (Figure S5), which indicated the increased RNase A resistance of the modified RNAs than that of the native siRNAs. These results showed that a capping method for siRNAs using thiol–maleimido cross-linking can significantly improve the stability of siRNAs in biological environments.

The capping of siRNA through thiol-maleimido cross-linking described herein also gave a dramatic increase in the thermal stability of the duplexes. The melting temperatures (T_m) of si867, LhpRNA, RhpRNA, and dbRNA were 65, 79, 79, and > 90°C, respectively, showing that this modification is effective at increasing the thermal stability of the siRNA duplexes (Table S1). The circular dichroism (CD) spectra of si867, LhpRNA, RhpRNA, and dbRNA showed similar patterns for the cross-linked RNAs, which corresponded to a typical A-form structure of duplex RNA (Figure S6).[10]

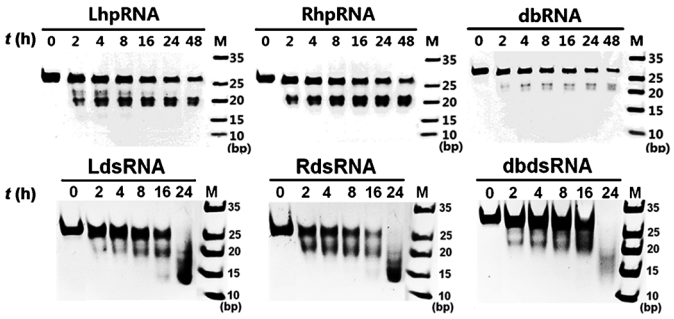

Figure 3. Serum stability of modified RNAs and their corresponding control dsRNAs in 50% normal human serum. The reaction mixtures were analyzed using 15% native PAGE and visualized with SYBR Gold staining. M indicates a double-stranded RNA marker.

Next, the thiol–maleimido capped RNAs were treated with Dicer, an enzyme that activates the RNA interference pathways.[11] The Dicer enzyme BLOCK-iT (0.05 units μL^{-1}) was used to digest cross-linked RNAs and their corresponding control duplexes. As shown in Figure 4, approximately 23–25 nt RNAs were produced from LhpRNA, RhpRNA, and dbRNA by Dicer, which is in accordance with length of the Dicer products of LdsRNA, RdsRNA, and dbdsRNA.[11a,12] Under the same reaction conditions, more than 90% of the cross-linked RNAs and duplex RNAs were digested into smaller fragments after 20 hours of incubation. We concluded that the cross-linked RNAs could be cleaved by the Dicer enzyme to form dsRNAs in vitro.

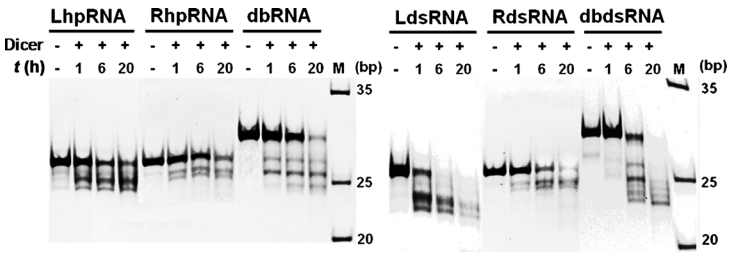

Figure 4. Analysis of the Dicer cleavage reaction of cross-linked RNAs and their corresponding control dsRNAs. Annealed RNAs (2.5 μM) were incubated with BLOCK-iT DICER (0.05 units μL^{-1}). The reaction mixtures were analyzed by 15% native PAGE and visualized with SYBR Gold staining. M indicates a double-stranded RNA marker.

The RNA interference activities of the cross-linked RNAs and their control sequences were measured using a dual-luciferase reporter system.[7] HEK293A cells were co-transfected with dual-reporter plasmids, pRL-TK and pGL3-Rosa, and also the different RNAs with various concentrations. Expression levels of the two luciferase genes were assayed 48 hours after transfection. As shown in Figure 5, the IC_{50} values for modified LhpRNA, RhpRNA, and dbRNA were 131.75 ± 40.32, 6.19 ± 0.18, and 68.72 ± 19.07 pM, respectively; as controls, the IC_{50} values for native si867, LdsRNA, RdsRNA, and dbdsRNA were 32.55 ± 9.01, 29.45 ± 13.33, 15.06 ± 1.79, 98.23 ± 31.59 pM, respectively. The highest suppression was induced by RhpRNA, which was about 2.5-fold more potent than that induced by RdsRNA. Compared to reported IC_{50} values for a 21 nt duplex siRNA,[13] RhpRNA with an IC_{50} value of about 6 pM showed the highest potency for RNAi thus far.

There was a dramatic difference in the RNAi efficiency between LhpRNA and RhpRNA at low concentrations (for example, below 0.1 nM), which may largely be due to the effect of the different structures of the two modifications on the formation of the RISC (RNA-induced silencing complex).

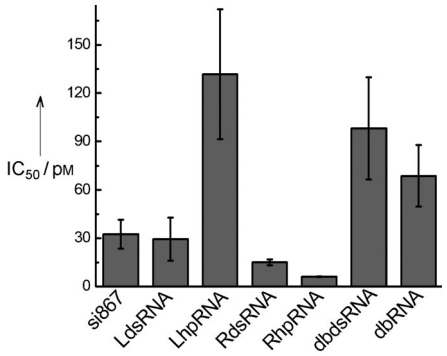

Figure 5. RNA interference with si867, LhpRNA, RhpRNA, dbRNA, and three corresponding control dsRNAs. The luciferase activities of the samples were measured according to the instructions of the dual-luciferase reporter assay kit (Promega, USA). Dosage curves of the RNA silencing effect were fitted by a Boltzmann nonlinear least-squares regression to give the IC_{50} values (Figure S7). The data shown are the mean ± standard deviation of three independent experiments.

3′-modified siRNAs are typically better tolerated by the RNAi enzymes,[14a] while the 5′-phosphate of siRNA, which is anchored in the MID pocket of Ago2, is more important in RISC.[14b] In our study, RhpRNA (with a 3′-overhang on the antisense strand) was much more potent than almost all reported siRNAs, with its IC_{50} value of 6 pM, while LhpRNA and dbRNA exhibited worse RNAi potency compared with that of the native siRNA. Note that RhpRNA has been cleaved by Dicer slower than LhpRNA, so in addition to the factors discussed above, the difference in potency may also be explained by a longer persistence of RhpRNA in the cytosol and thus a longer RNAi effect. These results further indicate that chemical modification of the 3′-end of siRNAs can be used to improve RNAi efficiency and increase RNA stability.

In conclusion, we have demonstrated herein that the capping of siRNAs using a thiol–maleimido cross-linking method resulted in an improvement in both the serum stability and the thermal stability of siRNAs. The capped RNAs can still be recognized and digested by Dicer. RNA interference experiments showed that RhpRNA was a highly efficient modification with an IC_{50} value of 6 pM, more potent than any reported siRNAs. Our results should provide an efficient method to improve siRNA serum stability and potency for RNAi, and further improvements in chemical modifications of siRNAs will provide better siRNA-based biological methods and therapeutic agents.

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