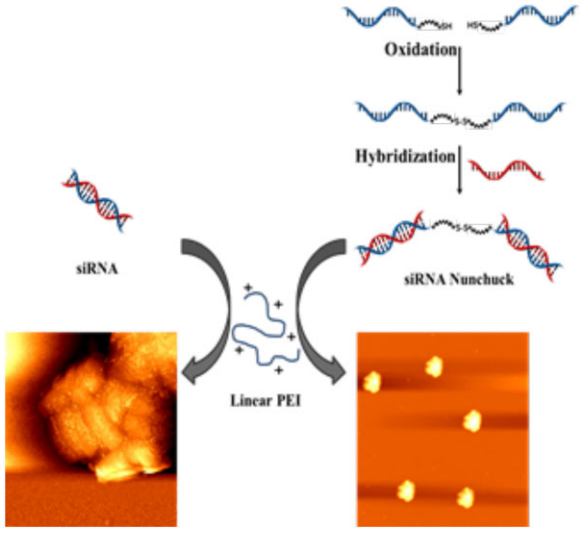
Small Interfering RNA Nunchucks with a Hydrophobic Linker for Efficient Intracellular Delivery[a]

Sung Duk Jo, Jee Seon Kim, Cheol O. Joe, Hyejung Mok,* Yoon Sung Nam*

The high stiffness and low spatial charge density of siRNA limit the effectiveness of the electrostatic condensation of siRNA with cationic polyelectrolytes. Here, a facile method to stabilize nanoscale siRNA/polyelectrolyte complexes by introducing a reductively cleavable alkyl chain to siRNA as a hybrophobic linker of dimeric siRNA conjugates is reported. The increased length of the hydrophobic linker increases the intracellular translocation and gene silencing activity of the dimeric siRNA conjugates when they are complexed with linear polyethylenimine (LPEI). The results suggest that the introduction of a hydrophobic linker in the dimeric siRNA conjugates can facilitate the intracellular delivery of siRNA through effective condensation with cationic polyelectrolytes.



S. D. Jo, Prof. C. O. Joe, Prof. Y. S. Nam
Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Daejeon 305-701, Republic of Korea
E-mail: yoonsung@kaist.ac.kr
Dr. J. S. Kim, Prof. Y. S. Nam
Department of Materials Science and Engineering, Korea Advanced Institute of Science and Technology, Daejeon 305-701, Republic of Korea
Prof. H. Mok
Department of Bioscience and Biotechnology, Konkuk University, Seoul 143-701, Republic of Korea
E-mail: hjmok@konkuk.ac.kr
Prof. Y. S. Nam
KAIST Institutes for NanoCentury and BioCentury, Korea Advanced Institute of Science and Technology, Daejeon 305-701, Republic of Korea

[a]**Supporting Information** is available from the Wiley Online Library or from the author.

1. Introduction

Over the last decade there has been increasing attention on RNA interference (RNAi) as a potent therapeutic approach for a variety of human diseases, including cancers and infectious diseases.[1,2] Small interfering RNA (siRNA) with a length of 20–25 base pairs can suppress specific target genes through the incorporation to RNA-induced silencing complex (RISC) and subsequent degradation of a target mRNA in a sequence-specific manner. Furthermore, the development of new RNA synthesis techniques, which are more effective in terms of cost and quality control, facilitates the use of siRNA as a feasible therapeutic candidate.[3,4] A current bottleneck for the clinical translation of siRNA is inefficient delivery capability.[5] To address this issue, nanoscale polyelectrolyte complexes have been widely investigated; however, this approach has not yet

DOI: 10.1002/mabi.201300291

been successful for siRNA because of the high stiffness of siRNA that leads to the inefficient condensation of siRNA with polyelectrolytes. Note that siRNA behaves as a rigid rod because of the high persistence length (l_p) of double stranded RNA ($l_p = 65–80$ nm), corresponding to about 150 base pairs, which is relatively large compared to that of double stranded DNA ($l_p =$ about 50 nm).[6–8] Therefore, the polyelectrolyte condensation is sterically restricted by the rigid structure of siRNA, making it difficult to reduce the overall size of the siRNA/polyelectrolyte complex.[9,10] Structural instability is another problem of the siRNA/polyelectrolyte complex.[11–13] Electrostatic interaction is relatively weak ($\Delta G^o = 1.4$ kcal mol^{-1}) in physiological conditions and susceptible to the changes of pH and ionic strength.[14] Charged biomolecules in biological fluids can strongly interact with the polyelectrolyte complex, eventually changing the structure of the original siRNA/polyelectrolyte complex. In particular, a relatively short siRNA molecule has a lower level of valence for electrostatic association, which makes the siRNA/polyelectrolyte interaction intrinsically weaker compared to the interaction with a longer DNA chain.[15]

It is basically possible to more effectively condense siRNA into nanoscale polyplexes if cationic polyelectrolytes with highly charged moieties and a high molecular weight are used. However, highly charged, long cationic polyelectrolytes significantly increase nonspecific cytotoxicity, limiting their practical applications.[16] Recently, not only cationic carriers but also inherent siRNA structures have been extensively modified to improve the siRNA delivery efficiency.[17–19] For instance, the incorporation of a flexible moiety to the backbone of siRNA could increase the condensation efficiency of siRNA.[20] In addition, siRNA-based nanostructures with an increased spatial charge density can be fabricated by chemical modification of siRNA.[21,22] Recently, self-multimerized siRNA, also called multimeric siRNA, was reported to enable the compact condensation of siRNA with lowly charged cationic polyelectrolytes by increasing both of the spatial charge density and chain flexibility of siRNA.[21] However, multimeric siRNA is a heterogeneous mixture of siRNA structures including monomer, dimer, trimer, and other oligomer siRNAs, and its preparation method is relatively complicated. For the clinical translation of siRNA, it is essential that siRNA structures are well defined, and their preparation procedures are simple enough to maintain the quality and reproducibility. Recently it was reported that the polyelectrolyte complexes of dimeric siRNA conjugates with linear polyethylenimine (LPEI) showed significantly higher gene silencing efficiency than monomeric siRNA molecules.[23] The dimeric conjugates are a promising siRNA structure for therapeutic applications because it has a higher spatial charge density compared to monomeric siRNA while possessing a homogeneous and well-defined structure compared to multimeric siRNA. However, the effects of the spacer properties (e.g., hydrophobicity and flexibility) in the dimeric siRNA conjugates on the efficiency of intracellular delivery and gene silencing have not yet carefully been examined though the type of a spacer can significantly change the condensation efficiency of the dimeric siRNA conjugates with cationic polyelectrolytes.[24]

In this study, we investigated the effects of a hydrophobic spacer in dimeric siRNA structures on the condensation and cellular uptake of the siRNA/polyelectrolyte complexes. Double-stranded dimeric siRNA conjugates were prepared by linking two single-stranded sense siRNA oligomers modified with the spacers of three different lengths of alkyl groups (three, six, and twelve carbons) and a terminal thiol group at the 3'-end via a disulfide bond. The prepared dimeric sense siRNA conjugates were sequentially hybridized with two single-stranded antisense siRNA oligomers to produce double stranded dimeric siRNA conjugates. LPEI was used as a cationic polyelectrolyte carrier to prepare nanoscale siRNA polyplexes because of its relatively less cytotoxic property, though it has a poor gene silencing efficiency with monomeric siRNA. The cellular uptake and gene silencing activities of the dimeric siRNA conjugates were directly compared with those of monomeric siRNA.

2. Experimental Section

2.1. Preparation of Dimeric siRNA Conjugates

All siRNA samples were purchased from Bioneer Co. (Daejeon, Republic of Korea). The sequences of green fluorescent protein (GFP) targeted siRNA were 5'-GCA AGC UGA CCC UGA AGU UdTdT-3' (sense) and 5'-AAC UUC AGG GUG AGC UUG CdTdT-3' (antisense). To synthesize single stranded dimeric siRNA conjugates, 50 nmol of single stranded sense strand with different spacers (C_3, C_6, and C_{12}) modified with a thiol group at 3' end were incubated in phosphate-buffered saline (PBS, pH = 7.4, 2.97×10^{-3} M sodium phosphate, 1.06×10^{-3} M potassium phosphate, and 155.17×10^{-3} M sodium chloride) in the presence of 25 vol.% dimethyl sulfoxide (DMSO) at room temperature. After 12 h incubation, the resulting products were lyophilized. Double stranded dimeric siRNA conjugates were prepared by annealing single stranded sense dimers with monomeric single-stranded antisense strands. The prepared siRNAs were analyzed by 12% polyacrylamide gel electrophoresis (PAGE) in TAE (40×10^{-3} M tris-acetate and 1×10^{-3} M EDTA) buffer. The gel was than stained with ethidium bromide and visualized using a GelDoc-IT TS imaging system (UVP, USA). The concentrations of siRNA and dimeric siRNA conjugates were determined using a UV-Vis Nanodrop (Thermo Scientific, Wilmington, DE, USA).

2.2. Synthesis of Cy5.5-labeled LPEI

Cy5.5-labeled LPEI was synthesized as previously reported.[18] LPEI (17 mg, 7 μmol) was dissolved in 1 mL deionized water, and the pH

was adjusted to 7.2 by adding a trace amount of 0.1 N HCl. After the evaporation of water under a reduced pressure, the resulting product was dissolved in 1.5 mL methanol, and the solution was purged with argon for 5 min. To the solution was added 10 vol% methylthiirane (5.3 μL, 7 μmol) and reacted for 24 h at 50 °C. The reaction mixture was evaporated under a reduced pressure and kept under argon atmosphere. The product of thiol-grafted LPEI (7.25 mg, 0.29 μmol) was re-dissolved in PBS at pH = 7.2 at a concentration of 1 mg mL^{-1}. Cy5.5-maleimide (1 mg, 0.87 μmol, GE Healthcare) in dimethyl sulfoxide (DMSO, 1 mL) was added and reacted for 12 h at room temperature. The reaction was then purified with dialysis (molecular-weight cut off = 1 000) against deionized water at room temperature for 1 d in the dark.

2.3. Characterization of Dimeric siRNA/LPEI Polyplexes

siRNAs were complexed with LPEI (MW = 25 kDa) at a nitrogen/phosphate (N/P) ratio of 20 at room temperature for 15 min. The hydrodynamic diameters and zeta potentials of the complexes were measured using dynamic light scattering (DLS) (ELSZ-1000, Otsuka Electronics, Japan) in triplicate at 25 °C. For atomic force microscopy (AFM) analysis, 50 μL of the complex solution was placed on the surface of a freshly cleaved mica and dried using nitrogen gas. The morphologies of the siRNA/LPEI polyplexes were analyzed AFM (PSIA XE-100, Santa Clara, CA, USA) in a non-contact mode.

2.4. Cellular Uptake

GFP-expressing MDA-MB-435 breast carcinoma cells, denoted "MDA-MB-GFP", were maintained in Dulbecco's modified Eagle' medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin at 37 °C in 5% CO_2 atmosphere. MDA-MB-GFP cells were plated on a 4-well chamber slide at a density of 2×10^5 cells per well for 24 h prior to transfection. The cells were treated with the siRNA/LPEI polyplexes with the Cy5.5-labled LPEI at an N/P ratio of 20 for 2 h in serum-free medium. The cells were washed with PBS and fixed with 3.7% formaldehyde solution for 20 min at room temperature. After mounting with a Vectashield medium (Vector Laboratories, Burlingame, Canada), the cells treated with the siRNA/LPEI polyplexes were visualized using confocal laser scanning microscopy (LSM 510, Carl Zeiss, Germany). To determine the amount of siRNA quantitatively, Taqman Small RNA assay was performed as follows. MDA-MB-GFP cells were plated on a 6-well plates at a density of 2×10^5 cells per well for 24 h prior to transfection. siRNAs complexed with LPEI at an N/P ratio 20 in PBS were transfected to the cells in serum-free medium for 5 h. After washing with PBS several times, the total RNA was extracted by the TRI reagent (Ambion, USA) according to the manufacturer's protocol. The extracted RNA was converted into cDNA with specially designed primers provided by the Taqman Small RNA assay kit (Applied Biosystems, USA) and applied to real-time polymerase chain reaction (PCR) (iCycler iQ, Bio-Rad, USA) according to the manufacturer's instructions.

2.5. Gene Silencing Analysis

MDA-MB-GFP cells were seeded on 12 well plates at a density of 1×10^5 cells per well for 24 h prior to transfection. The dimeric siRNA conjugates (2 μg) were complexed with LPEI (5.34 μg) at an N/P ratio of 20 in PBS and transfected to the cells in a serum-free medium (2 μg siRNA · mL^{-1}). After 5 h incubation, the culture medium was replaced with a fresh medium containing 10% fetal bovine serum (FBS) and further incubated for 48 h. To evaluate the extent of GFP expression, the cells were washed with phosphate-buffered saline (PBS) and lysed with 1% Triton-X 100 in PBS. After centrifugation to remove cell debris, the supernatant was analyzed with a spectrofluorophotometer (RF-5301PC, Shimadzu, Japan) with an excitation wavelength at 488 nm and an emission wavelength at 509 nm, respectively. For visualization of gene silencing effect, MDA-MB-GFP cells were seeded on a 4-well chamber slide at a density of 1×10^5 cells per well. After 24 h incubation, monomeric and dimeric siRNA polyplexes formulated with LPEI 25 kDa at an N/P ratio of 20 were treated to the cell in serum-free medium for 5 h and the medium was replaced by a fresh medium. After 48 h incubation, the cells were washed with PBS and fixed with 3.7% formaldehyde solution for 20 min. The sample was mounted with Vectashield medium containing 4',6-diamidine-2-phenylindole (DAPI) and observed on a confocal laser scanning microscope.

2.6. Cell Viability Assay

MDA-MB-GFP cells were seeded on 12 well plates at a density of 1×10^5 cells per well for 24 h prior to transfection. The siRNAs were complexed with LPEI at an N/P ratio of 20 in PBS and transfected to the cells in a serum-free medium for 5 h. The culture medium was replaced with a fresh medium containing 10% FBS and further incubated for 48 h. The cell viability was determined by treating the cells with a cell counting kit-8 (CCK-8, Dojindo laboratories, Japan) solution according to the manufacturer's protocol. The absorbance was measured at 450 nm using a microplate reader (Bio-Rad, Model 550, CA, USA). In addition, total protein amounts in the cells were analyzed after transfection as a measure of cell viability using BCA assay (Pierce BCA Protein Assay Kit, Pierce, USA) according to the manufacturer's instructions.

2.7. Statistical Analysis

Statistical significance was determined by the two-tailed Student's *t*-test with a confidence level of 95%.

3. Results and Discussion

3.1. Synthesis of Dimeric siRNA Conjugates

To strengthen the polyelectrolyte complexes by hydrophobic interaction, alkyl spacers with different lengths were introduced to the dimeric siRNA conjugates, as shown in Figure 1a. The 3′ ends of single-stranded sense siRNAs were modified with different lengths of alkyl chains

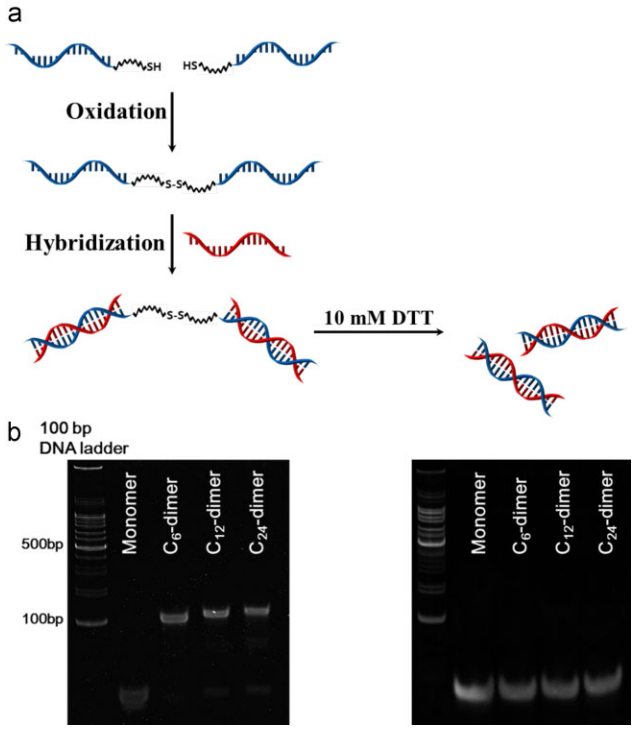


Figure 1. a) Schematic illustration for the preparation of three dimeric siRNA conjugates (C_6-dimer, C_{12}-dimer, and C_{24}-dimer) with different alkyl chain spacers. b) PAGE analysis of the dimeric siRNA conjugates in the absence (left panel) and presence (right panel) of 10^{-2} M DTT. Note that the DNA size marker used here does not represent the precise size of siRNA because of the different electrophoretic migration rate of DNA and RNA.

(C_3, C_6, and C_{12}) having a terminal thiol group at the end. The disulfide linkage in the dimeric conjugates can increase the chain flexibility, which is also critically important for effective condensation of siRNA.[21,23] The thiol groups at the 3' ends of single-stranded sense siRNA were oxidized by DMSO to prepare three types of dimeric siRNA conjugates via the C_3, C_6, and C_{12} alkyl chains spacers. The dimeric conjugates were annealed with complementary antisense siRNA molecules to produce double-strand dimeric siRNA. Dimeric siRNA conjugates linked by the C_3, C_6, and C_{12} alkyl chain spacers were denoted as "C_6-dimer"', "C_{12}-dimer"', and "C_{24}-dimer"', respectively. The calculated lengths of the alkyl spacers were 1.1, 1.8, and 3.4 nm for the C_6-dimer, C_{12}-dimer, and C_{24}-dimer, respectively. Those lengths were obtained using the Abalone molecular modeling software (Agile Molecule) in a ball-and-stick mode. The alkyl chain was assumed to be in a stretched form with its carbon and hydrogen atoms in the tetrahedral conformation with a bond angle of 109.5° and a torsional angle of 180°. The distance between the first and last carbon atoms was calculated, so the actual spacer length would be smaller because of chain bending and folding.

3.2. Characterization of Dimeric siRNA Conjugates

The prepared siRNA dimers were analyzed by gel electrophoresis (Figure 1b). The dimeric siRNA conjugates showed much retarded gel migration compared to monomeric siRNA because of twofold increased molecular weight. In addition, the C_{24}-dimers evidently migrated slowly compared to the C_6- and C_{12}-dimers, indicating that the dimeric siRNA conjugates with a longer spacer exhibited more significant retardation than those with shorter spacers. In a previous study, the introduction of flexible molecules into DNA could mediate different electrophoretic mobilities.[25–27] Accordingly, a slight band shift between the dimeric siRNA conjugates might be attributed to the different flexibility of the internal spacers, where a disulfide bond exists, in the dimeric structures. For the facile dissociation of the dimeric siRNA conjugates into monomeric siRNAs in the cytoplasm, the dimeric siRNA conjugates were linked via disulfide bonds. To examine whether monomeric siRNAs could be generated from the dimeric siRNA conjugates in a reductive condition, the PAGE analysis of dimeric siRNA conjugates was carried out after 10^{-2} M dithiothreitol (DTT) treatment. The most dimeric siRNA conjugates were cleaved into monomeric siRNA molecule in 1 h as shown in right panel of Figure 1b. This result indicates that the siRNA dimeric conjugates could be dissociated to biologically active monomeric siRNA molecule in the intracellular reductive environment where the concentration of glutathione (GSH) is about 10^{-2} M.[28–30]

3.3. siRNA-Polyelectrolyte Complexation

To form nano/sized polyelectrolyte complexes, the monomeric siRNA and dimeric siRNAs conjugates were electrostatically complexed with LPEI (25 kDa) at an N/P ratio of 20. Their morphological characteristics and sizes were examined by using DLS and AFM as shown in Figure 2. The dimeric siRNA conjugates formed nanoscale polyelectrolyte complexes with LPEI, while the monomeric siRNA only showed much large aggregates. The hydrodynamic size of the monomeric siRNA/LPEI complexes determined by DLS was about 74 272 nm with a polydispersity index (PDI) of 1.467, although no consistent data were obtained in repeated experiments presumably because of the unstable and larger aggregates polyplex formations. The mean hydrodynamic diameters of the polyplexes with the dimeric siRNA conjugates were very similar to each other; 253.8 ± 6.2 nm for C_6-dimer, 255.9 ± 3.5 nm for C_{12}-dimer, and 252.6 ± 8.7 nm for C_{24}-dimer, respectively. AFM image shows irregular large aggregates of the monomeric siRNA/LPEI complexes while all of the dimeric-siRNA polyplexes show well-dispersed small particles of 200–300 nm in diameter (Figure 2). The zeta potentials of the dimeric siRNA conjugates/LPEI complexes were also determined:

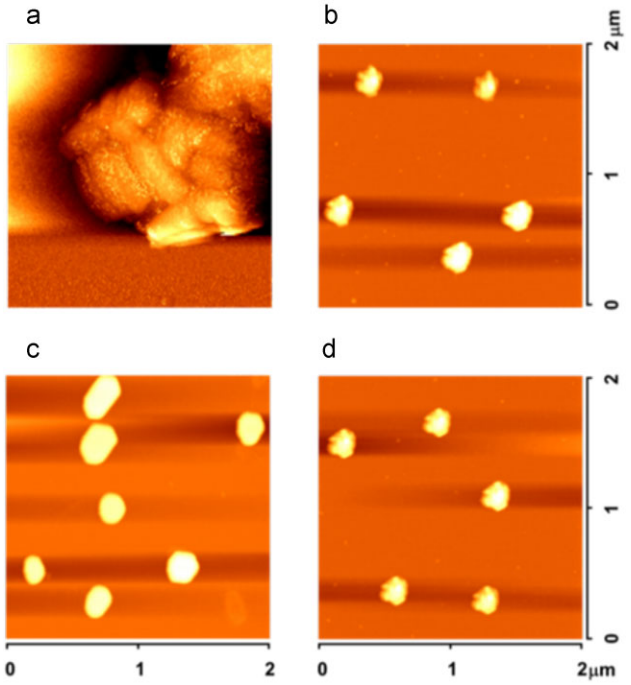


Figure 2. AFM images of the monomeric and dimeric siRNA polyplexes with LPEI at an N/P ratio of 20: a) monomer, b) C_6-dimer, c) C_{12}-dimer, and d) C_{24}-dimer, respectively.

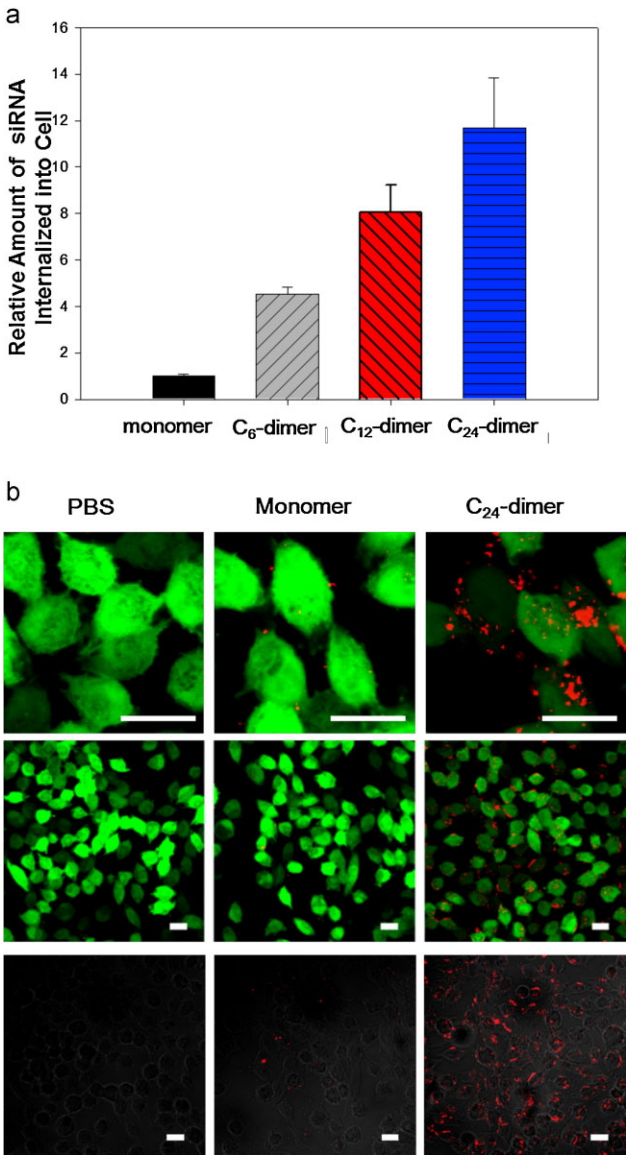


Figure 3. a) Quantitative analysis of intracellular siRNA amount measured by PCR based Taqman Small RNA assay. b) Confocal microscopic images to visualize the cellular uptake of the monomeric and dimeric siRNA polyplexes with Cy5.5-LPEI at an N/P ratio of 20 in GFP-expressing MDA-MB-435 cells. Scale bar = 20 μm.

19.35 ± 0.67 for C_6-dimer, 19.28 ± 0.55 for C_{12}-dimer, and 18.77 ± 0.60 for C_{24}-dimer, respectively. It is likely that the positively charged surface of the polyplexes can facilitate the internalization of the polyplexes by endocytosis. It is conceivable that the increased spatial charge density of the dimeric siRNA conjugates could play a critical role in the condensation with LPEI to generate the compact polyplex structures. In addition, the bivalent binding structure and hydrophobic interactions among the spacer moieties with flexible disulfide bonds of the dimeric siRNA conjugates could significantly increase the condensation efficiency with cationic polyelectrolytes.[31]

3.4. Cellular Uptake

To investigate whether the internal spacers in the dimeric siRNA conjugates have an impact on the cellular uptake of the polyplexes, the relative intracellular amounts of siRNA were quantitatively measured by reverse transcription with custom-made primers and real-time PCR after complexation with LPEI. Figure 3a shows that the relative extent of internalized siRNA was gradually increased with the increased length of spacers in the dimeric structures. The relative amount of siRNA internalized into the cell by the C_6-, C_{12}-, and C_{24}-dimers were higher by 4.5, 8.0, and 11.6-fold compared with that of the monomeric siRNAs. This result might be attributed to the increased compactness and hydrophobic property in the C_{24}-dimer/LPEI complexes, which could mediate more efficient translocation into the cytoplasm. It is likely that the hydrocarbon spacer in the dimeric siRNA conjugates could serve as a hydrophobic block of amphiphilic copolymers, enabling the formation of much more compact polyplexes and facilitating membrane translocation. The peripheral hydrophobic moiety of the complexes can interact more efficiently with

the cell membrane.[32] The external hydrophobic moiety may facilitate the internalization and translocation of the complexes. In addition, the length of the hydrophobic spacer could increase the compactness of the dimeric siRNA/LPEI polyplexes because of the hydrophobic interactions induced by the hydrocarbon spacers. Such increased hydrophobicity can generate non-polar region within the polyplexes, which also positively contributes to the increased electrostatic condensation of siRNA with LPEI through the decreased dielectric constant of the local domains within the polyplexes. Although fully understanding the underlying mechanism on the contribution of the hydrophobic spacer on structural changes of polyplexes is not provided, it is clear that the smaller size of the dimeric siRNA/LPEI polyplexes compared to the monomeric siRNA counterpart indicates the formation of more compact and stable polyplexes based on the dimeric siRNA conjugates. Several works have attempted to investigate the effect of lipid and hydrophobic modification of cationic carriers in gene delivery.[33] They found that proper modification resulted in better complex stabilities and transfection efficiencies. However, substitution on cationic carrier can change the carrier's structure and properties which often has negative effects on the stability of complexes and gene transfections. One way of circumventing these problems is the hydrophobic modification of siRNAs rather than the cationic carriers. The in vitro cellular uptake of the monomeric and dimeric siRNA/LPEI polyplexes were also visualized using a breast carcinoma cell line (MDA-MB-GFP). Figure 3b shows the confocal microscopic images of the cells treated with the siRNA/LPEI polyplexes prepared using fluorescently labeled LPEI. As expected, the dimeric siRNA/LPEI polyplexes showed much more efficient cellular uptake compared to the monomeric siRNA/LPEI polyplexes.

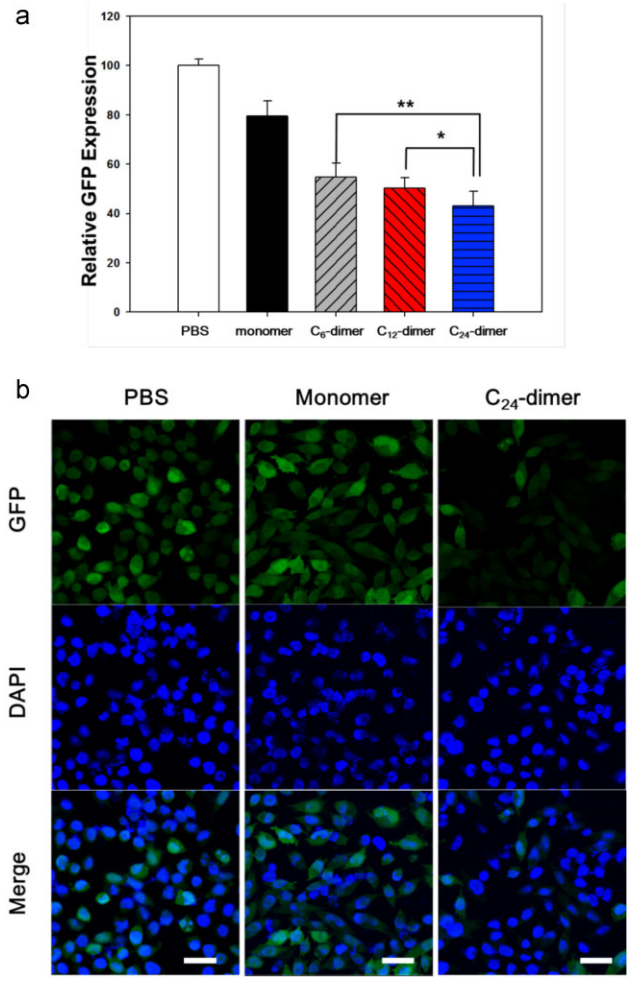


Figure 4. a) Gene suppression levels by the monomeric and dimeric siRNA polyplexes with LPEI at an N/P ratio of 20 in GFP-expressing MDA-MB-435 cells. * $p < 0.01$, ** $p < 0.0005$. b) Confocal microscopic images of MDA-MB-435 cells treated with the monomeric and dimeric siRNA polyplexes with LPEI at an N/P ratio of 20. Scale bar = 40 μm.

3.5. Gene Silencing Efficiency

The gene silencing efficiency of the siRNA/LPEI polyplexes was examined by transfecting the polyplexes into MDA-MB-GFP cells (Figure 4a). The dimeric siRNA/LPEI polyplexes exhibited far greater GFP gene silencing efficiency compared to the monomeric siRNA/LPEI polyplexes. The gene silencing increased with the increased length of the hydrophobic spacer: the extents of gene suppression by the dimeric siRNA conjugates with C_6, C_{12}, and C_{24} spacers were 54.7 ± 5.7, 50.2 ± 4.3, and $43.1 \pm 5.9\%$, respectively. The siRNA/LPEI complexes did not show any significant cytotoxicity, suggesting that the gene down efficiencies of the complexes were originated from specific RNAi effects rather than non-specific cytotoxicity (Figure S1, Supporting Information). The increased gene silencing effect seems to be induced by the increased level of cellular uptake of the siRNA/LPEI complexes. Confocal microscopy also revealed that the cells treated with the dimeric siRNA/LPEI polyplexes showed faint GFP fluorescence induced by efficient gene silencing (Figure 4b). It was reported that hydrophobically modified cationic carriers enhanced transfection efficiency by stabilizing polyplexes and enhancing translocation of the cell membrane.[34–36] Our finding is that the hydrophobic spacers of the dimeric siRNA conjugates could contribute to the stabilization of the polyplexes and facilitate the cellular uptake, thereby enhancing the transfection efficiency. Furthermore, the optimization of the dimeric siRNA conjugates can be achieved by employing diverse spacers with different flexibility and hydrophobicity, both of which can affect the amphiphilicity of the dimeric siRNA conjugates.

4. Conclusion

This study has demonstrated that the cellular uptake and gene silencing efficiency of the dimeric siRNA conjugates can be highly affected by the length of a hydrocarbon spacer between two identical siRNA monomers. Simple conjugation of two identical siRNAs using alkyl chain spacers effectively facilitated the polyelectrolyte condensation of siRNA, forming a more compact nanoscale structures presumably due to increased charge density and chain flexibility. All of the dimeric siRNA conjugates with the hydrophobic spacers exhibited much greater cellular uptake and gene silencing compared to monomeric siRNA. In particular, the dimeric siRNA conjugates with a longer spacer showed significantly greater efficiencies of intracellular translocation and gene silencing. This study suggests that the dimeric siRNA conjugates with a hydrophobic linker can be used as a promising platform to improve the therapeutic efficacy of siRNA.

Acknowledgements: This work was supported by the National Research Foundation of Korea (NRF) Grant funded by the Korean Government (MSIP) (2012M3A7B4049802).

Received: June 13, 2013; Revised: July 22, 2013; Published online: September 16, 2013; DOI: 10.1002/mabi.201300291

Keywords: conjugated polymers; drug delivery systems; gene silencing; siRNAs; structure-property relations

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