Fusion peptide-mediated siRNA delivery using self-assembled nano-complex

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Abstract

Gene therapy using siRNA can be a new potent strategy to treat many incurable diseases, including cancer and viral infection, at the genetic level. Therapies using siRNA essentially need an efficient and safe method of siRNA delivery into cells while maintaining the stability of the siRNA. Here, we designed new fusion peptides of SPACE and oligo arginine. Fusion peptides formed uniform self-assembled nano-complexes without additional reactions. Moreover, siRNAs were stable in nano-complexes for four days in 10% fetal bovine serum. Cellular uptake efficiency of each complex was similar or higher than that of commercialized available Lipofectamine 2000. GAPDH-siRNA/peptide complex knock GAPDH mRNA down similar to that mediated by Lipofectamine 2000. The increase of arginine residues in fusion peptides induced higher siRNA stability, which enhanced GAPDH knockdown. Co-localization and cellular internalization of siRNA/S-R15 complexes were verified peripherally around the nucleus. The endocytosis pathway of the siRNA/S-R15 complex was identified via lipid-raft mediated endocytosis. Besides, the biosafety of each fusion peptide was proven under a particular concentration. Therefore, a safe and stable self-assembled complex could be expected to deliver siRNA into cells efficiently for the treatment of many diseases.

1. Introduction

RNA interference (RNAi) has been demonstrated as a promising gene therapy approach that regulates the expression of specific genes (Deng et al., 2014; Elbashir et al., 2001; Whitehead et al., 2009). As an RNAi mediator, short-interfering RNA (siRNA) is a double-stranded molecule composed of about 21-23 nucleotides, designed as a sequence complementary to the target mRNA. The exogenously penetrated siRNAs activate RNA-induced silencing complexes (RISC) in the cytoplasm and result in outstanding selective mRNA inhibition with low cytotoxicity. Therefore, gene therapy using siRNA can be a new potent strategy to treat cancer, viral infectious diseases, and local diseases at the genetic level. However, a significant barrier to siRNA delivery is a low uptake efficiency into the cell membranes composed of phospholipid bilayers due to its hydrophilic nature (Sarett et al., 2015; Wang et al., 2010). Moreover, the siRNA is vulnerable to degradation by large amounts of nucleases present in the cytoplasm or interstitial fluid (Lee et al., 2013). Therefore, it is essential to develop an efficient and safe delivery method of siRNA with stability.

Until now, several methods to deliver siRNA have been developed. These techniques could be categorized into physical and chemical methods (Pathak et al., 2009). First, physical methods could deliver siRNAs using the specialized equipment, including microinjector, gene gun, electroporator, sonoporator, laser, and magnetofector (Sinn et al., 2005). Physical methods have limitations for various applications due to the need for special equipment, non-specificity of siRNA delivery, and instability of delivered siRNA. Next, chemical methods could deliver siRNAs using carriers capable of interacting with siRNAs and transferring siRNA into cells. Types of carriers could become lipoplex, polyplex, dendrimer, peptide, and various nanoparticles

(Akhtar, 2006; Levine et al., 2013; Yin et al., 2014; Zhou et al., 2017). These carriers could enhance the stability of siRNAs as well as delivery efficiency. On the other hand, chemical methods have shortages of limited delivery efficiency and potential toxicity of chemicals. Therefore, an ideal siRNA delivery method requires enhanced delivery efficiency, biosafety, and siRNA stability.

Recently, peptides have been intensively studied as an attractive siRNA carrier owing to their structural and functional versatility, potential biocompatibility, and targeting ability to cells. Primarily, cell-penetrating peptides (CPPs) have been known to penetrate the cell membrane effectively. The TAT sequence was originated from the Tat protein of human immunodeficiency virus (HIV) (Vives et al., 1997). Positively-charged oligo arginine can assist cellular internalization by forming a hydrogen bond with the sulfate of the cell membrane and phosphate group of the nucleic acid (Koren and Torchilin, 2012; Sarett et al., 2016; Tang et al., 2013; Zeller et al., 2015). The histidine-rich peptide was confirmed using the efficient delivery of siRNA (Langlet-Bertin et al., 2010). Besides, the development of the phage display technique enables us to find new-type cell-penetrating peptides. For example, the skin permeating and cell entering (SPACE) peptide has a superior ability to facilitate the penetration of conjugated cargoes into epidermis and dermis (Hsu and Mitragotri, 2011). However, single peptide showed limited delivery efficiency, and some peptides such as SPACE need the additional conjugation reaction. Therefore, for a facile and useful siRNA carrier, it needs the method with enhanced delivery efficiency without additional reaction.

Herein, we designed novel fusion peptides and investigated their potential as a siRNA delivery carrier. The three fusion peptides were composed of SPACE and cationic oligo arginine (R7, R11, and R15) linked by GCG sequence (Mitchell et al., 2000). The self-assembled complex was identified between each peptide and siRNA without any conjugation. Moreover, each complex was characterized in terms of size, zeta-potential, and siRNA stability. Cellular uptake efficiency of each complex was measured using FACS and fluorescence microscopy. Intracellular co-localization or dissociation of the complex was analyzed using a confocal microscope. The complex-mediated GAPDH knockdown was assessed using mRNA expression level. In addition, the internalization pathway of the siRNA/S-R15 complex was analyzed using FACS and endocytosis inhibitors. Furthermore, the biosafety of each complex was checked using a cytotoxicity test of human dermal fibroblast cells.

2. Materials and methods

2.1. Materials

AccuTarget GAPDH positive control siRNA was provided from Bioneer Co. (Daejeon, South Korea), and Cy3-labeled siRNA was synthesized from GenePharma Co., Ltd (Shanghai, China) in duplex form and purified by HPLC. All single and fusion peptides were synthesized by GL Biochem Ltd. (Shanghai, China) with more than 95% purity. Hank's balanced salt solution (HBSS) was procured from Life Technologies (CA, USA). Agarose and 10,000X TopRed Nucleic Acid Gel Stain were purchased from GenomicBase (Seoul, South Korea). Tris (Glentham Life Sciences Ltd., Corsham, UK), acetic acid (glacial) (Merck, Darmstadt, Germany), and EDTA (GenomicBase, Seoul, South Korea) were used for 1X TAE buffer. Heparin sodium salt (from porcine intestinal mucosa) was purchased from Sigma-Aldrich (MO, USA). 6X DNA loading dye was procured from Biofact Co., Ltd (Daejeon, South Korea). For cell culture, Dulbecco's Modified Eagle's Medium (DMEM; Corning, MA, USA), fetal bovine serum (FBS; PAN Biotech, Bavaria, Germany) and penicillin-streptomycin (Life Technologies, CA, USA) were used. Opti-MEM and 0.25% trypsin-EDTA (1X) were obtained from Thermo Fisher Scientific (MA, USA). Lipofectamine 2000 reagent was purchased from Invitrogen (CA, USA). Hoechst 33342 (Invitrogen, CA, USA), Flamma® 496 Phalloidin (BioActs, Incheon, South Korea) and SiR-actin kit (Cytoskeleton, Inc., CO, USA) were used for fluorescent labeling. 10% formalin solution (Sigma-Aldrich, MO, USA), Triton X-100 (Bio-Rad Laboratories, Inc., CA, USA) and bovine serum albumin (BSA; Generay Biotech Co., Ltd., Shanghai, China) were used for fluorescence imaging. Nuclease-free water was purchased from Integrated DNA Technologies, Inc. (IA, USA). Tri-RNA reagent (Favorgen biotech co., Kaohsiung, Taiwan), chloroform (Sigma-Aldrich, MO, USA), isopropanol (Molecular biology grade; Fisher Scientific, NH, USA), and absolute ethanol (Molecular biology grade; Fisher Scientific) were used for RNA extraction. ReverTra Ace® qPCR RT Master Mix with gDNA Remover

kit and THUNDERBIRD® SYBR® qPCR Mix (TOYOBO Co., Ltd., Osaka, Japan) was procured for cDNA synthesis and quantitative real-time PCR. CytoTox 96® Non-radioactive cytotoxicity assay kit was obtained from Promega (WI, USA). Chlorpromazine hydrochloride, methyl-β-cyclodextrin, cytochalasin D (from *Zygosporium mansonii*), and filipin III (from*Streptomyces filipinensis*) endocytosis inhibitors were purchased from Merck (Darmstadt, Germany) for the mechanism study. All cell culture flasks and plates were purchased from NEST Biotechnology Co., Ltd (Wuxi, China).

2.2. Preparation of siRNA/peptide complexes

GAPDH-siRNA was dissolved in HBSS as 1 μM, and all peptides were dissolved in HBSS or distilled water for 1-2 mg/mL. The sequences of all peptides were summarized in Table 1. The fusion peptides designed in this study have a GCG linker between SPACE and oligo arginine peptides. Fusion peptides and siRNAs in HBSS buffer formed the self-assembled complexes under incubation at room temperature (25°C) for 30 minutes with appropriate nitrogen/phosphate (N/P) ratio (Fig. 1). The N/P ratio was derived using the molar ratio of amine groups in the cationic peptides to those of phosphate groups in the RNA.

2.3. Gel retardation assay

The formation of siRNA/peptide complexes was confirmed by gel retardation assay. Total 10 μL complexes of 10 pmol siRNA and each peptide were self-assembled with a range of N/P ratios (1:1, 5:1, 10:1, 20:1, 30:1, 40:1, 50:1, and 100:1) as mentioned above. After adding 6X loading dye, total 12 μL complexes were loaded into 2% agarose gel (w/v) prepared in 1X TAE buffer (40 mM tris, 20 mM acetic acid, 1 mM EDTA, pH=8.6) with 10,000X TopRed Nucleic Acid Gel Stain for visualization. Gel running was performed at 25 VA for 30 minutes using the Mupid-2plus electrophoresis system (Optima Inc., Tokyo, Japan). The electrophoretic mobility shift of complexes was taken pictures by ChemiDoc XRS+ System (Bio-Rad, CA, USA).

2.4. Size and zeta potential measurement

Size and zeta potential of complexes were measured by dynamic light scattering (DLS). Based on the results of the previous gel retardation assay, a 20:1 N/P ratio was determined for the rest of the experiments due to the stable complex formation of all fusion peptides. 200 pmol siRNA and each peptide were self-assembled at a 20:1 N/P ratio, as described above. After 30 minutes, the complexes were diluted with HBSS to a final siRNA concentration of 200 nM. Then, the 200 nM solution was filtered with a 0.45 μm syringe filter (GVS, Bologna, Italy). After vortexing for 30 seconds, 1 mL of the complexes was loaded into a cuvette (Ratiolab, Dreieich, Germany) to measure the size and disposable folded capillary cell (Malvern Panalytical Ltd., Malvern, UK) to measure the zeta potential. The size and zeta potential of complexes were measured using a Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK).

2.5. Stability of siRNA/peptide complexes in serum

The stability of siRNA in complexes was confirmed using the agarose gel electrophoresis. 100 pmol of siRNAs and peptides (20:1 N/P ratio) were self-assembled for 30 minutes at room temperature. Then, the complexes in 10% (v/v) FBS were incubated at 37°C, and 20 μL of each sample was collected at 24, 48, 72, and 96 hours. On the other hand, the complex in 50% (v/v) FBS was incubated at 37°C, and 20 μL sample was collected at 4, 8, 12, 24, and 48 hours. The siRNAs were dissociated from complexes using incubation at 37°C for 30 minutes after the addition of 4 μL of 1 mg/mL heparin. After mixing the 6X loading dye to each sample, 24 μL samples were loaded into 2% agarose gel with 1X TAE buffer in Mupid-2plus. Gel running was performed for 30 minutes at 25 VA. The remaining siRNA was analyzed by the gel documentation system LSG 1000 (iNtRON Biotechnology, Seongnam, South Korea).

2.6. Cellular uptake efficiency using flow cytometry

Human cervical cancer HeLa, human dermal fibroblasts neonatal (HDFn), and immortal keratinocyte cell line HaCaT were cultured in DMEM medium supplemented with 10% FBS and 1% penicillin-streptomycin at 37°C in 5% CO_2 humidified incubator (Esco Micro Pte. Ltd., Changi, Singapore). 3.0×10^5 cells were added to each well of a 6-well plate and incubated at 37°C in a 5% CO_2 incubator overnight. After the complex

formation of the final 200 nM Cy3-labeled IL10-siRNA and peptides (20:1 N/P ratio) in serum-reduced Opti-MEM, 325 μL of the complex was added to each well and incubated for 4 hours at 37°C in 5% CO_2 incubator. The wells were washed twice with 1 mL of pre-warmed phosphate-buffered saline (PBS). After 200 μL treatment of 0.25% trypsin-EDTA for 2 minutes, 2 mL of fresh DMEM medium was added. The suspended cells were centrifuged at 360 × g for 5 minutes. After the supernatant was removed, cells were washed with PBS twice under the same conditions. The final cell pellet was resuspended in ice-cold PBS and analyzed using a flow cytometer (Gallios; Beckman Coulter, CA, USA).

2.7. Observation of cellular uptake using fluorescence microscopy

HeLa cells were seeded into a 24-well plate at the number of 1.0×10^5 cells per well and incubated overnight at 37°C in a 5% CO_2 incubator. After the complex formation of the final 200 nM Cy3-labeled IL10-siRNA and peptides (20:1 N/P ratio), 160 μL of complexes were applied to the cells in Opti-MEM for 4 hours. After washing twice with 200 μL of pre-warmed PBS, the cells were fixed using 200 μL of 10% formalin solution for 10 minutes. Then, cells were serially treated with 200 μL of 0.1% Triton X-100 in PBS (0.1% PBST) for 10 minutes, then 200 μL of 2% BSA in 0.1% PBST at room temperature for 30 minutes. Cells were incubated in the Hoechst 33342 dye solution for 10 minutes under the absence of light. After washing twice with 200 μL of pre-warmed PBS, the cells were incubated in the Phalloidin dye solution at room temperature for 1 hour. After washing twice with 200 μL of pre-warmed PBS, the cells were observed at 200× magnification by a fluorescence microscope (Ti-E; Nikon, Tokyo, Japan).

2.8. Cellular internalization observed using confocal microscopy

The cellular internalization of siRNA/peptide complex was investigated using confocal images. HeLa cells of 2.0×10^4 were incubated in a 35 mm confocal dish (SPL Life Sciences Co., Ltd., Pocheon, South Korea) for 24 hours. After 30-minute incubation of final 50 nM Cy3-labeled siRNA and fluorescein (FITC)-labeled S-R15 (20:1 N/P ratio), the complex was applied to the cells for 4 hours. The nucleus and actin were stained using 5 μg/mL Hoechst 33342 and 100 nM SiR-actin kit, respectively. The intracellular localization and co-localization of siRNA and S-R15 were confirmed using fluorescence and confocal microscope (Ti2; Nikon, Tokyo, Japan). Each of them was analyzed at the single-molecule level using super-resolution radial fluctuation (SRRF). Bright-field and fluorescence images were acquired at 900× magnification. Fluorescence images of Cy3, FITC, and SiR-actin were merged using Image J software (Schneider et al., 2012).

2.9. Quantitative RT-PCR

GAPDH mRNA expression reduced by complex was checked using quantitative real-time PCR. HeLa cells were seeded into a 24-well plate at a density of 1.0×10^5 cells per well. After overnight incubation at 37°C in a 5% CO_2 incubator, 160 μL complex with the final 200 nM siRNA at 20:1 N/P ratio was applied to the cells in Opti-MEM for 5 hours. As a positive control, Lipofectamine 2000 reagent was used according to the provided protocols. After incubation for 5 hours, the media were replaced with 500 μL of fresh supplemented DMEM and incubated further for 19 hours. After washing three times with nuclease-free water, the total RNA of the cells was purified using Tri-RNA reagent following the manufacturer's protocol. The concentration and purity of purified RNA were measured using the Take3 plate as a nanodrop mode of a microplate reader (Epoch 2; BioTek Instruments, Inc., VT, USA). 100 ng of RNA was reversely transcribed to cDNA using ReverTra Ace® qPCR RT Master Mix with a gDNA Remover kit according to the manufacturer's protocol. Used GAPDH-specific primer sequences were forward primer 5'-GTCTCCTCTGACTTCAACAGCG-3' and reverse primer: 5'-ACCACCCTGTTGCTGTAGCCAA-3'. After mixing 10 ng of cDNA, primers and THUNDERBIRD® SYBR® qPCR Mix according to the manufacturer's protocol, PCR reaction was performed following to the three-step cycle (Pre-denaturation; 95°C for 60 seconds, Denaturation; 95°C for 15 seconds, Annealing; 55°C for 30 seconds, Extension; 72°C for 60 seconds). GAPDH mRNA expression was analyzed using the QuantStudio 3 real-time PCR system (Applied Biosystems, CA, USA). The GAPDH mRNA expression was normalized by β-actin mRNA expression. The relative expression level was calculated using the ΔΔCt method.

2.10. Endocytosis pathway study of complex

In a 6-well plate, HeLa cells were seeded at a density of 4.0×10^5 cells per well. After replacing the medium with Opti-MEM, cells were treated with each inhibitor for 30 minutes. Inhibitors were Chlorpromazine (10 μg/mL), Methyl-β-cyclodextrin (5 mM), Cytochalasin D (1 μM), and Filipin III (1 μg/mL) (Dutta and Donaldson, 2012). The complex of final 200 nM Cy3-labeled siRNA and FITC-labeled S-R15 was self-assembled as described above. After adding 325 μL of complex to inhibitor-treated cells, cells were incubated for 4 hours at 37°C in a 5% CO_2 incubator. Cells with the fluorescence of Cy3 and FITC were analyzed using flow cytometry, as mentioned in 2.6.

2.11. Cytotoxicity of fusion peptides

The cytotoxicity of fusion peptides was assessed using CytoTox 96® non-radioactive cytotoxicity assay according to the manufacturer's protocol. Human dermal fibroblasts neonatal (HDFn) cells were cultured in DMEM medium supplemented with 10% FBS and 1% penicillin-streptomycin at 37°C in 5% CO_2 humidified incubator (Esco Micro Pte. Ltd., Changi, Singapore). HDFn cells were seeded on a 96-well plate at a density of 8.0×10^3cells per well. After overnight incubation, serially-diluted fusion peptides were added to cells at concentrations of 1, 0.5, 0.25, 0.125 and 0.0625 mg/mL. After incubation for 5 hours, 50 μL aliquot of each well was transferred to each empty well of a 96-well plate. Then, 50 μL of CytoTox 96® reagent was added to each well. Cells were incubated for 30 minutes at room temperature under light-free conditions. After adding 50 μL of stop solution, absorbance was measured at 490 nm using a microplate reader.

2.12. Statistical analysis

The quantitative data were presented as mean ± standard deviation. Statistical significance of differences was evaluated using ap -value less than 0.05, 0.01, and 0.001 calculated by a t-test.

3. Results

3.1. Confirmation and characterization of siRNA/peptide complexes

The formation of siRNA/peptide complexes at different N/P ratios was confirmed by electrophoretic mobility shift (Fig. 2A). As a result, R11, S-R7, S-R11, and S-R15 retarded the siRNA band. As fusion peptides, S-R7, S-R11, and S-R15 showed complete retardation of siRNA at ratios over 20:1, 10:1, and 40:1, respectively. Partial retardation was observed with R11, a single peptide, based on the blur siRNA band at ratios over 30:1. On the other hand, SPACE and TAT did not retard the siRNA band at all for all N/P ratios. Because TAT, a single peptide, did not form a condensed complex with siRNA, other peptides except for TAT peptide were used for the following experiments.

The size and zeta potential of the complex were measured three times using dynamic light scattering (Fig. 2B). Complexes using SPACE, R11, S-R7, S-R11, and S-R15 had average sizes of 647.7, 413.7, 327.1, 457.0, and 287.0 nm, respectively. Complexes using SPACE, R11, S-R7, S-R11, and S-R15 had average polydispersity indexes (PdI) of 0.89, 0.23, 0.22, 0.23, and 0.04, respectively. Complexes using SPACE, R11, S-R7, S-R11, and S-R15 had average zeta potentials of -28.33, -11.77, 5.23, 6.00, and 6.08, respectively.

The stability of siRNA under complexes was assessed using serum incubation (Fig. 2C). First, each complex was incubated with 10% FBS to simulate a cell culture condition. Interestingly, complexes using S-R15 and S-R11 maintained the siRNA stability until 96 hours. On the other hand, siRNA bands of other complexes gradually disappeared over time. Decomposition rates in 10% FBS were S-R15, S-R11, R11, siRNA only, S-R7, and SPACE in increasing order. Second, each complex was incubated with 50% FBS to simulate extreme decomposition conditions. Interestingly, the S-R15 complex only maintained siRNA stability until 48 hours. On the other hand, siRNA bands of other complexes gradually disappeared and completely disappeared at 24 hours. Decomposition rates in 50% FBS were S-R15, S-R11, R11, S-R7, siRNA only, and SPACE in increasing order. In summary, S-R15 showed the best siRNA stability under complex in both conditions.

3.2. *In vitro* cellular uptake evaluation

Cellular uptake efficiencies of each complex were evaluated using flow cytometry and HeLa, HDFn, and HaCaT cells (Fig. 3). Using Cy3-labeled IL10-siRNA, fluorescent cells with siRNA only and each condi-

tion exhibited in red and green populations, respectively. The percentage represented the population of fluorescent-positive cells over total cells. Complexes using S-R7, S-R11, and S-R15 showed the high cellular uptake efficiencies of 95.59%, 85.24%, and 78.18% in HeLa cells (Fig. 3A), 99.75, 99.79 and 99.55% in HDFn cells (Fig. 3B), and 95.46%, 79.07% and 99.99% in HaCaT cells (Fig. 3C), respectively. These efficiencies were higher than 71.34% in HeLa cells, 86.95% in HDFn cells, and 79.79% in HaCaT cells treated by Lipofectamine 2000 as a commercialized positive control. The complex using R11, a single peptide, showed cellular uptake efficiency of 92.58% in HeLa cells, 88.48% in HDFn cells, and 99.82% in HaCaT cells. In contrast, the complex using SPACE showed negligible cellular uptake efficiency in HeLa cells (4.51%), HDFn cells (0.25%), and HaCaT cells (0.19%).

Cellular uptake of each complex was observed using a fluorescence microscope and HeLa cells (Fig. 4A). Images represented Cy3-labeled siRNA of orange fluorescence, the nucleus of blue fluorescence, and actin filament of green fluorescence. Orange fluorescence inside of cells was observed in the images of Lipofectamine 2000 (the second row), R11 (the fourth row), S-R7 (the fifth row), S-R11 (the sixth row), and S-R15 (the seventh row) in Fig. 4A. Complexes using fusion peptides showed orange spots in the cytosol. On the other hand, Lipofectamine 2000 showed dispersed orange fluorescence within the cytosol, and an R11 complex showed orange fluorescence spots and spread within the cytosol. In contrast, siRNA only and SPACE complex did not show any orange fluorescence in the first and third rows of Fig. 4A.

In addition, co-localization and cellular internalization of the siRNA/peptide complex were confirmed using a confocal microscope with super-resolution at the single-molecule level (Fig. 4B&C). The confocal images represented Cy3-modified siRNA of magenta fluorescence and FITC-modified S-R15 peptide of green fluorescence (Fig. 4B). The arrowed spot of the merged image indicated the co-localization of siRNA and S-R15 peptide complex with white fluorescence. The Z-stack image of confocal microscopy represented intracellular localization of Cy3-labeled IL10-siRNA and FITC-labeled S-R15 complex in HeLa cells (Fig. 4C). The arrowed spot of white fluorescence was located in the cytoplasm around the nucleus.

siRNA delivered by complex knocked down GAPDH mRNA expression in HeLa and HaCaT cells using quantitative RT-PCR analysis (Fig. 5). In the HeLa cells, complex using S-R15 reduced relative GAPDH mRNA expression of 38.7% compared to that of siRNA only. This knockdown percentage was significantly different from that of SPACE (p -value = 0.011) and comparable to 35.3% using Lipofectamine 2000. Complexes using R11, S-R7, and S-R11 knocked down GAPDH mRNA expression to 52.6, 56.8, and 51.3%, respectively. Moreover, the complex using SPACE induced the least knockdown of 72.8%. In the HaCaT cells, the 49.8% knockdown using S-R15 was comparable to 40.8% of Lipofectamine 2000 without a statistically significant difference. These results indicated that these complexes could knock down mRNA expression in different cell types, including cancer cell and skin keratinocyte.

3.3. Mechanism study of complex uptake

The intracellular delivery mechanism of the S-R15 complex was analyzed using endocytosis inhibitors and flow cytometry (Fig. 6). Graphs showed cell distributions without an inhibitor of the red line and with an inhibitor of the green line, respectively. The images of the first row represented the penetrating inhibition of FITC-labeled S-R15 (Fig. 6). When the reference point was taken at 89.46% in red distribution, the cell population decreased to 53.14% in clathrin-meditated endocytosis inhibitor chlorpromazine and 68.33% in lipid raft-mediated endocytosis inhibitor methyl-β-cyclodextrin. In contrast, the cell populations showed no decrease in the cases of phagocytosis/micropinocytosis inhibitor cytochalasin D and caveolae-meditated endocytosis inhibitor Filipin III. Thus, the penetration of FITC-labeled S-R15 was inhibited by clathrin-mediated endocytosis dominantly and lipid raft-mediated endocytosis secondly.

The images of the second row represented the penetrating inhibition of Cy3-labeled siRNA. When the reference point was set at 89.26% in red distribution, the cell population decreased to 68.20% prominently in lipid raft-mediated endocytosis inhibitor methyl-β-cyclodextrin. In contrast, the other cell populations showed no decrease, except for methyl-β-cyclodextrin. Therefore, the permeation of Cy3-labeled siRNA was inhibited by lipid raft-mediated endocytosis dominantly. In summary, both experiments using FITC-labeled

S-R15 and Cy3-labeled siRNA showed consistency of inhibition by lipid raft-mediated endocytosis inhibitor methyl-β-cyclodextrin. Besides, penetration of FITC-labeled S-R15 was inhibited by the clathrin-mediated endocytosis.

3.4. Biocompatibility of fusion peptides

The biocompatibility of each fusion peptide was verified using a lactate dehydrogenase (LDH) assay and HDFn cells (Fig. 7). Each LDH activity was normalized with the LDH activity using a lysis buffer as 100%. Normalized LDH activity of each fusion peptide does not show any significant cytotoxicity depending on various concentrations compared to the negative control. Therefore, fusion peptides were biocompatible to apply to the cells under a concentration of 200 μg/mL.

4. Discussion

Novel fusion peptides were designed using SPACE and oligo-arginine and evaluated for siRNA delivery with a self-assembled complex. The newly-designed fusion peptides successfully formed a self-assembled complex stably with siRNAs via electrostatic interaction. All fusion peptide complex enhanced siRNA penetration similar to or better than that of commercialized Lipofectamine 2000. Among fusion peptides, the S-R15 complex induced the highest knockdown of GAPDH mRNA expression comparable to that of commercialized Lipofectamine 2000. This mRNA knockdown by S-R15 could be affected by uniform small size and stability of complex because the stability of complex increased as arginine residues increase. Co-localization and cellular internalization of siRNA/S-R15 complexes were verified peripherally around the nucleus. The primary penetration mechanism of the S-R15 complex was identified as lipid raft-mediated endocytosis. Also, each fusion peptide was biocompatible to human dermal fibroblast cells under a concentration of 200 μg/mL.

First, R11 and fusion peptides formed a self-assembled and condensed complex with siRNAs based on complete retardation of the siRNA band. In contrast, SPACE and TAT did not form a condensed complex with siRNA based on almost no retardation of the siRNA band. These results coincided with the result using oligo arginine (Kim et al., 2010) and indicated that a positively charged region with high-density was crucial to forming a complex with siRNA. Using amino acid analysis via the peptide 2.0 web (www.peptide2.com), percentages of positive basic residues were R11 of 100%, SPACE peptide of 9.1% among 11 amino acids, TAT peptide of 88.9% basic residue among nine amino acids. As a result, successive positive charge and longer length of R11 was supposed to interact with negatively charged siRNAs sufficient to form a stable complex. For fusion peptides, percentages of positive basic residues were S-R7 of 38.1%, S-R11 of 48%, and S-R15 of 55.2%. The successive and longer positive charge of the peptide could increase the electrostatic interaction with the negative charge of siRNA and result in charge neutralization. Moreover, the long length of fusion peptides was favorable to form and maintain condensed complexes with siRNA via van der Waals or hydrophobic interactions.

Interestingly, the S-R15 peptide formed the smallest and uniform self-assembled complex with weakly positive zeta potential based on the light scattering analysis. Other fusion peptide complexes had small and medium-size, respectively, with acceptable PdI and weak positive zeta potential. Weakly positive zeta potential of three fusion peptide complexes could mean that positively charged fusion peptides covered the surface of the complex with siRNA. Furthermore, slightly positive zeta potential and uniform size could become favorable to cellular internalization of the complex (Kim et al., 2003). Also, the R11 complex had a medium size, acceptable PdI, and negative zeta potential. Its negative zeta potential could mean that R11 peptide did not completely shield the negative charge of siRNA on the complex surface. In contrast, the SPACE peptide appeared to fail to form a condensed complex based on the large size, high polydispersity index, and highly negative zeta potential. Consequently, the strategy of fusion peptides enabled to form self-assembled complexes of the proper size, PdI, and weakly positive zeta potential, which could be expected to enhance the cellular internalization.

S-R15 formed the most stable complex, which might show increased stability of cellular uptake. The stability of the S-R15 complex could be affected by charge neutralization and cohesive strength caused by the electrostatic interaction between peptide and siRNA (Tai and Gao, 2017). That is, long and successive positive

charge residues of peptides could shield negative charge of siRNA effectively as well as interact with other siRNAs. Therefore, due to long positive charged residues, S-R15 could neutralize the negative charge of siRNA and interact with other siRNAs most effectively. Besides, SPACE part of S-R11 enhanced complex stability compared to that of R11 peptide. This enhanced stability might be affected by hydrophobic and van der Waals interactions.

Co-localized particles and dissociated spread of siRNA/S-R15 complexes were distributed peripherally around the nucleus. Co-localization of the complex was identified via white fluorescence merged between siRNA of magenta fluorescence and S-R15 of green fluorescence. The co-localized siRNA/S-R15 complexes were distributed peripherally around the nucleus in particle-like forms within the cytoplasm. Particle-like forms might represent endosomes containing complexes. This co-localization supported the complex formation. On the other hand, the spread of magenta fluorescence might indicate siRNAs dissociated from complexes after the endosomal escape. These results coincided with those of previous reports (Chiu et al., 2004; Kim et al., 2010; Wang et al., 2014; Wang et al., 2007).

GAPDH mRNA knockdown by the S-R15 complex showed the highest reduction among the three fusion peptides. This result could be affected by the synergistic effect of cellular uptake efficiency and complex stability. That is, high cellular uptake efficiency and higher stability of the S-R15 complex could enable prolonged cellular uptake compared to those of other complexes. Resultantly, prolonged cellular uptake could enhance the knockdown of the GAPDH mRNA expression. As a result, the increase of arginine length enhanced complex stability. Enhance complex stability could affect increased efficiencies of cellular uptake and mRNA knockdown.

The endocytosis pathway of S-R15 peptide agrees with not the micropinocytosis of SPACE peptide (Hsu and Mitragotri, 2011) but the clathrin-mediated endocytosis of arginine-rich peptides (Schmidt et al., 2010). This result indicated that S-R15 peptide could dominantly bind a specific cell surface receptor resulting in the clustering formed by the assembly of clathrin (Schmidt et al., 2010). On the other hand, the endocytosis pathway of the siRNA/S-R15 complex was different from that of S-R15 peptide only. The siRNA/S-R15 complex associated with the cell membrane and then became trapped in lipid raft. The possible reasons could be the large size of the S-R15 complex and weak binding to a specific cell surface receptor caused by strong interactions between oligo-arginine and siRNA. This result coincides with the endocytosis pathway of arginine-rich peptide fusion proteins or large cargo (Jones et al., 2005).

Conclusion

Novel fusion peptides could successfully enhance siRNA penetration into cells via a self-assembled complex and resultantly induced mRNA knockdown similar to or better than those of commercialized Lipofectamine 2000. The efficiency of mRNA knockdown was synergistically affected by high cellular uptake efficiency, and higher complex stability improved by the increase of arginine length. Co-localization and cellular internalization of siRNA/S-R15 complexes were confirmed peripherally around the nucleus. The primary endocytosis pathway of the siRNA/S-R15 complex was identified as lipid raft-mediated endocytosis. Also, the biosafety of each fusion peptide was confirmed by the cytotoxicity test. Therefore, the safe, stable, and straightforward complex strategy was expected to become a potent and efficient gene delivery platform to treat various target diseases through gene therapy.

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Conflict of interest

The authors declare no financial or commercial conflict of interest.

Figure Legends

Figure 1. Scheme of intracellular delivery using siRNA/fusion peptide complexes. The complex could be self-assembled via electrostatic interaction between negatively charged siRNA and positively charged arginine-rich region of fusion peptides as well as hydrophobic interaction between charge-shielded siRNA and hydrophobic SPACE region of fusion peptides. The complex penetrated cellular membrane via endocytosis pathway. siRNA escaped from endosomes knocked down the specific gene through RISC formation.

Figure 2. Characterization of siRNA/peptide self-assembled complexes. (A) Complex formation was checked using a gel retardation assay. The 21-nt siRNA was mixed with each peptide: SPACE, R11, TAT, S-R7, S-R11, and S-R15 at N/P ratios of 1:1, 5:1, 10:1, 20:1, 30:1, 40:1, 50:1, and 100:1. After a 30-minute incubation, complexes mixed with a 6X loading dye were loaded into 2% (w/v) agarose gel stained with TopRed. Gel electrophoresis was run in TAE buffer at 25 VA for 30 minutes. The gel was visualized through a ChemiDoc XRS+ System. The brightness and contrast of each picture were adjusted. (B) Size and zeta potential of complexes were measured using dynamic light scattering. The 21-nt siRNA of 200 nM final concentration was incubated for 30 minutes with each peptide: SPACE, R11, S-R7, S-R11, and S-R15. After filtration and vortexing, each complex was loaded in the cell and analyzed through Nano ZS. Bars represented the average ± standard deviation. P-value was calculated using a t-test compared to that of SPACE (**; $p<0.01$. independent repeat=3). (C) siRNA stability was tested in serum. In the left pictures, each 21-nt siRNA/peptide complex was incubated in 10% FBS for 24, 48, 72, and 96 hours. In the right pictures, each complex was incubated in 50% FBS for 4, 8, 12, 24, and 48 hours. Then, samples mixed with a 6X loading dye were loaded into 2% (w/v) agarose gel stained with TopRed. Gel electrophoresis was run in TAE buffer at 25 VA for 25-30 minutes. The gel was visualized through a gel documentation system. The brightness and contrast of each picture were adjusted.

Figure 3. Cellular uptake evaluation of siRNA/peptide complexes into (A) HeLa, (B) HDFn, and (C) HaCaT cells using flow cytometry. 265 μL complex of the final 200 nM Cy3-labeled IL10-siRNAs was delivered into each 3.0×10^5 cells in a 6-well plate via PC: Lipofectamine 2000 as a commercialized positive control, SPACE, R11, S-R7, S-R11, and S-R15 (20:1 N/P ratio) for 4 hours. Fluorescent cells of siRNA only and each condition exhibited in red and green populations, respectively. The population of fluorescent-positive cells was expressed as a percentage.

Figure 4. Cellular uptake evaluation of siRNA/peptide complexes into HeLa cells using fluorescence and confocal microscopes. (A) Cellular uptake of Cy3-labeled IL10-siRNA/peptide complexes using a fluorescence microscope. 132 μL complex of the final 200 nM Cy3-labeled IL10-siRNAs were delivered into each 1.0×10^5 HeLa cells in a 24-well plate via PC: Lipofectamine 2000 as a commercialized positive control, SPACE, R11, S-R7, S-R11, and S-R15 (20:1 N/P ratio) for 4 hours. Nucleus and actin filaments were labeled using Hoechst 33342 (blue) and Phalloidin (green), respectively. The complex was observed in orange color at 200x magnification (scale bar=100 μm). (B) Single-molecule images of siRNA/S-R15 complex using a super-resolution radial fluctuation. 2.0×10^4 cells of HeLa were incubated in a 35 mm confocal dish. 20 μL complex of the final 50 nM of siRNA and S-R15 (20:1 N/P ratio) was applied to the cells for 4 hours. Cy3-labeled IL10-siRNA and FITC-labeled S-R15 peptide were observed in magenta and green, respectively, at 900× magnification (scale bar=1 μm). Actin filaments were labeled using a SiR-actin kit (red). Fluorescence images of Cy3, FITC, and SiR-actin were merged using Image J software. Co-localization of the complex was visualized with arrowed white spot-like areas in a merged image (scale bar=10 μm). (C) Cellular internalization of siRNA/S-R15 complex using a Z-stack image. Actin filaments were labeled using a SiR-actin kit (red). The arrowed white spot-like areas demonstrated co-localization of siRNA and peptide in the cytoplasm at 900× magnification (scale bar=5 μm). Right, and bottom images showed a cross-sectional z-axis image of the arrowed white spot.

Figure 5. Target GAPDH mRNA knockdown in HeLa and HaCaT by siRNA/peptide complexes using quantitative RT-PCR. 132 μL complex of the final 200 nM GAPDH-siRNAs was delivered into each 1.0×10^5 HeLa cells in a 24-well plate using Lipofectamine 2000 as a commercialized positive control, SPACE, R11, S-R7, S-R11, and S-R15 (20:1 N/P ratio) for 5 hours. 100 ng of total RNAs isolated from cells was reverse-transcribed into cDNA. 10 ng cDNA was used for PCR reaction with GAPDH-specific forward and reverse

primers. Relative mRNA expression levels were calculated using the ΔΔCt method based on the housekeeping β-actin expression level. The relative expression levels of GAPDH mRNA were normalized by mRNA expression of siRNA only. The data represented mean ± standard deviation (*; p<0.05, **; p<0.01 and independent n[?]3).

Figure 6. Endocytosis pathway identification of the siRNA/S-R15 complex into HeLa cells using flow cytometry and various chemical inhibitors. HeLa cells of $4.0x10^5$ were pretreated with each endocytosis inhibitor (10 μg/mL of chlorpromazine, 5 mM of methyl-β-cyclodextrin, 1 μM of cytochalasin D, and 1 μg/mL of filipin III) for 30 minutes. 150 μL complex of the final 100 nM Cy3-labeled siRNA at 20:1 N/P ratio was delivered into the cells for 4 hours. Each fluorescent cell population was counted using detached cells via FITC (upper row) and Cy3 (lower row) intensities, respectively. Fluorescent cell population w/o and w/ each inhibitor exhibited in red and green, respectively. The reduced population of fluorescent-positive cells was expressed as a percentage.

Figure 7. Cytotoxicity test of three fusion peptides using a lactate dehydrogenase (LDH) assay. Released LDH activities were measured from 8.0×10^3 HDFn cells under each fusion peptide at different concentrations in a 96-well plate using a cytotoxicity kit. LDH activity of the cells treated with each peptide was normalized with the LDH activity without peptide. The data represented the mean ± standard deviation (independent repeat=3). P-value was calculated using a t-test.

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