Intracellular siRNA delivery with novel spermine based surfactants

XU RongZuo¹, WANG XuLi¹ & LU Zheng-Rong^{2*}

¹Departments of Pharmaceutics and Pharmaceutical Chemistry, University of Utah, Salt Lake City, UT 84108, USA;

²Department of Biomedical Engineering, Case Western Reserve University, Cleveland, OH 44106, USA

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The design and development of safe and effective multifunctional siRNA delivery systems are critical for clinical application of RNAi therapeutics. Here we evaluated eight new spermine-based surfactant multifunctional carriers for siRNA delivery. These carriers complexed with siRNA forming stable compact nanoparticles with sizes around 100 nm. The multifunctional carriers mediated higher intracellular siRNA transfection than Lipofectamine-2000. The siRNA nanoparticles of the multifunctional carriers exhibited low cytotoxicity as shown by MTT assay. Three of the eight multifunctional carriers showed higher silencing efficiency than Lipofectamine-2000 in both U87-Luc cells and CHO-GFP cells. SKAHCO showed the highest siRNA delivery efficiency among the carriers. It resulted in $84.6 \pm 5.5\%$ silencing of luciferase activity in U87-Luc cells, much higher than that ($62.8 \pm 3.4\%$) from Lipofectamine-2000. In conclusion, the spermine based multifunctional carriers are promising for highly efficient intracellular siRNA delivery.

RNAi, siRNA delivery systems, surfactant, nanoparticles, multifunctional carriers

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RNA interference (RNAi) is an emerging and promising therapeutic modality [1,2]. RNAi involves the degradation of an mRNA complementary to a small interfering RNA (siRNA) by a RNA-induced silencing complex (RISC) in mammalian cells. Any gene related to a human disease can be down-regulated by RNAi [3]. Clinical application of RNAi requires effective *in vivo* introduction of siRNA into the cells of interest, which still remains as the main challenge. siRNA can be encoded into plasmid DNA to deliver it to target cells with a viral or nonviral vector, but it is not a desirable approach [4,5]. Direct delivery of siRNA into cells is advantageous over siRNA encoding viral or plasmid DNA nonviral nanoparticles. siRNA can readily be synthesized by chemical methods in a large scale and high purity. It can also be chemically modified to improve its stability and efficacy [6–8]. Direct siRNA delivery avoids the complicated transcription procedure of a plasmid DNA and can induce RNAi activity in an efficient and controllable

manner [3].

We have recently developed pH-sensitive polymerizable surfactants as multifunctional carriers for nucleic acid delivery [9–11]. These carriers can form stable complexes with plasmid DNA or siRNA with sizes in the range of 100–200 nm. The carriers are composed of a pH-sensitive amino head group, two distant lipid tails and two cysteinyl residues. With a packing parameter larger than 1, these carriers could transform into fusogenic inverse hexagonal conformation at acidic pH [12–14]. The protonation of the amino head group at pH lower than 6 could enhance the carriers' positive charge densities and their membrane disruption abilities due to pH-sensitive amphiphilicity [15,16]. The thiol groups of the cysteinyl residues can be polymerized through autooxidation to form disulfide bonds to further stabilize the nanoparticles. These functions enable the carriers to facilitate endosomal escape of the nanoparticles and to release nucleic acids into cytosol via reduction of the disulfide bonds in the nanoparticles by cytosolic glutathione. The multifunctional carriers have demonstrated high intra-

*Corresponding author (email: zx1125@case.edu)

cellular and *in vivo* siRNA delivery efficiency, resulting in high intracellular gene silencing efficiency and therapeutic efficacy in a mouse tumor model [10,11,17,18].

In a previous study, we reported a new series of multifunctional carriers based on spermine with modified structures and simplified synthetic procedure for the delivery of nucleic acids [19]. Spermine is a natural oligoamine that can condense nucleic acids via charge complexation [20,21]. The new multifunctional carriers demonstrated the ability of forming stable nanoparticles with plasmid DNA at low concentrations, the capability of efficient intracellular transfection of plasmid DNA and low cytotoxicity. In this study, we further investigated the effectiveness of these carriers for intracellular siRNA delivery. The nanoparticle formation between siRNA and the carriers was evaluated by dynamic light scattering. Intracellular siRNA delivery efficiency of these carriers was determined in CHO-GFP and U87-Luc cells with Lipofectamine-2000 as a control. The structure-activity relationship of these carriers for siRNA delivery was also evaluated.

1 Materials and methods

1.1 Materials

N-(2,3-dioleoyloxy-1-propyl)trimethylammonium methyl sulfate (DOTAP), bovine serum albumin (BSA), and 2,5-diphenyl-3-(4,5-dimethyl-2-thiazolyl)tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The preparation of spermine based multifunctional carriers, SKCO, SKHCO, SHKCO, SHHKCO, SKACO, SKAHCO, SHKACO and SHHKACO, was reported in a previous literature [19]. The anti-Luc siRNA (sense sequence: 5'-CUU-ACGCUGAGUACUUCGAdTdT-3'; antisense sequence: 5'-UCGAAGUACUCAGCGUAAAdTdT-3') and anti-GFP siRNA (sense sequence: 5'-GCAAGCUGACCCUGAAGUUCAU-3'; antisense: 5'-GAACUUCAGGGUCAGCUUGCCG-3') were purchased from Qiagen (Valencia, CA, USA). The human glioma U87 MG cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The U87 cells constantly expressing luciferase were a gift from Dr. Randy Jansen of the University of Utah. CHO-EGFP cells expressing a green fluorescent protein were kindly provided by Dr. Charles Roth of Rutgers University.

1.2 Particle size measurement

The siRNA nanoparticles were prepared by mixing siRNA solution with spermine based surfactants at predetermined *N/P* ratios and incubated for 30 min. The size of the nanoparticles was analyzed using a Brookhaven Instruments BI-200SM equipped with a 5 mW helium-neon laser with output at 633 nm. Measurements were performed at room temperature in triplicates. The effective diameters of the nanoparticles were computed using the software provided

by the manufacturer of the instrument.

1.3 Surfactant mediated intracellular siRNA uptake

Approximately 3×10^5 U87 cells per well were plated in 12-well plates. After 24 h, the nanoparticles, which were prepared by mixing 1 μ g AlexaFluor 488 labeled all negative siRNA (Qiagen) with each of multifunctional carriers at an *N/P* ratio of 12 or Lipofectamine-2000 for 30 min, were added into each well and incubated for 4 h at 37°C in 1 mL serum free medium. The medium was removed and the cells were washed twice with PBS and then trypsinized. The cells were collected and fixed by 4% paraformaldehyde in PBS for 20 min. The samples were analyzed using a FACSCalibur flow cytometer (BD Bioscience) with FL-1 channel focused on the green fluorescence of AlexaFluor 488. Results were analyzed using Cell-Quest (BD Bioscience) software.

1.4 Surfactants mediated silencing of luciferase with siRNA

U87-Luc cells were seeded in 96 well plates at a density of 5×10^3 cells/well in DMEM medium containing 10% FBS, G418 (300 μ g/mL), streptomycin (100 μ g/mL) and penicillin (100 units/mL) 24 h prior to transfection. Anti-Luc siRNA was complexed with multifunctional carriers at an *N/P* ratio of 12 or Lipofectamine-2000 for 30 min and then incubated with cells at a siRNA concentration of 50 nmol/L for 4 h at 37°C in serum free DMEM medium. The medium was then replaced with complete DMEM medium and incubated for an additional 44 h. Cells were washed with pre-warmed PBS, and treated with 200 μ L/well lysis buffer followed by a freeze-thaw cycle. Luciferase activity in cell lysates was then measured by a luciferase assay kit (Promega, Madison, WI, USA) on a luminometer (Dynex Tech., Chantilly, VA, USA). The gene silencing efficiency was normalized against the luciferase activity of untreated cells.

1.5 Surfactants mediated GFP silencing with siRNA

CHO-GFP cells were cultured in F12-K medium. Cells were seeded into 12-well plates at the density of 2×10^5 cells per well and further cultured overnight. Nanoparticles formed by mixing 1.0 μ g anti-GFP siRNA with multifunctional carriers at an *N/P* ratio of 12 or Lipofectamine-2000 for 30 min at room temperature. The formed nanoparticles were then added to CHO-GFP cells in serum free F-12K medium at a siRNA concentration of 34 nmol/L. After 4 h incubation at 37°C, the medium was replaced with fresh complete F12-K medium and cultured for an additional 20 h. Cells were then washed with pre-warmed PBS, harvested through trypsinization, and fixed in cold PBS containing 4% paraformaldehyde for 20 min. Samples were analyzed using a FACSCalibur flow cytometer with FL-1 channel focused on green fluorescence. Results were analyzed using Cell-Quest software.

1.6 Cytotoxicity study of siRNA/multifunctional carrier nanoparticles

The cytotoxicity of siRNA/multifunctional carrier nanoparticles was evaluated by an MTT assay in U87 cells. Briefly, U87 cells were plated in 96-well plates with 1×10^4 cells per well and cultured overnight. Anti-Luc siRNA (10 pmol) was mixed with multifunctional carriers at an *N/P* ratio of 12 or Lipofectamine-2000 at a predetermined dose and incubated at room temperature for 30 min to form nanoparticles. The formed nanoparticles were then added into serum-free MEM medium to reach a final concentration of 50 nmol/L siRNA and incubated with U87 cells for 4 h. The medium was then replaced with complete MEM medium and continued culturing for an additional 20 h. The medium was removed and cells were digested by 200 μ L DMSO. The absorption was read at 570 nm using a plate reader (Model 680, Bio-Rad, Hercules, CA, USA). The relative cell viability was calculated by the equation: $([\text{Abs}]_{\text{sample}} - [\text{Abs}]_{\text{blank}}) / ([\text{Abs}]_{\text{control}} - [\text{Abs}]_{\text{blank}}) \times 100\%$.

2 Results and discussion

The chemical structures of the spermine-based surfactants,

SKCO, SKHCO, SHKCO, SHHKCO, SKACO, SKAHCO, SHKACO and SHHKACO, are shown in Figure 1. All of spermine based multifunctional carriers formed stable nanoparticles with siRNA at low concentrations. The size of nanoparticles at *N/P* ratio 12 was in the range of 85.5 nm (SKHCO) and 120.6 nm (SKAHCO) as determined by dynamic light scattering (Figure 2). The combination of charge complexation of siRNA and the spermine head group, hydrophobic condensation of oleoyl lipid tails and oxidation of the dithiols resulted in the formation of compact siRNA nanoparticles.

The spermine based multifunctional carriers mediated high intracellular delivery efficiency of AlexaFluor 488 labeled siRNA. Figure 3 shows flow cytometric diagrams of the uptake of the labeled siRNA in U87 cells mediated by various carriers at an *N/P* ratio of 12. The multifunctional carriers resulted in approximately 91% to 96% siRNA transfection in U87 cells, while Lipofectamine-2000 only transfected 73% of cells under the same condition. It appears that the carriers SKHCO, SKCO and SHHKCO with a lysine "Y" joint were lightly more effective intracellular siRNA delivery than the corresponding SKAKCO, SKACO and SHHKACO with a lysyl- β -alanine joint.

Figure 4 shows the efficiency of silencing luciferase expression in U87-Luc cells by an anti-Luc siRNA mediated

Figure 1 Chemical structures of the spermine based lipid surfactants.

Figure 2 The size of the siRNA nanoparticles with the spermine based multifunctional carriers at an N/P ratio of 12 ($n=3$).

by the carriers. SKHCO, SHHKCO, SKAHCO, SKACO and SHKACO resulted in higher luciferase silencing efficiency than Lipofectamine-2000. Among these carriers, SKAHCO showed the highest gene silencing efficiency ($84.6 \pm 5.5\%$) at 50 nmol/L siRNA and N/P ratio of 12. The gene silencing efficiency mediated by the carriers was further determined in CHO-GFP cells with an anti-GFP siRNA. Figure 5 shows flow cytometric diagrams of CHO-GFP cells after the treatment with the nanoparticles of an anti-GFP siRNA and various carriers. SKAHCO at N/P ratio 12 again mediated the highest GFP silencing efficiency (61%). SHKACO (49%), SKHCO (49%) and SKACO (51%) also resulted in better

Figure 3 Flow cytometric diagrams showing cellular uptake of siRNA mediated by the multifunctional carriers in the U937 cells with Lipofectamine 2000 (Lipo-2000) as a control.

Figure 4 Silencing efficiency of luciferase expression in U87-Luc cells mediated by the nanoparticles of an anti-Luc siRNA (50 nmol/L) with the multifunctional carriers. The relative gene silencing efficiency was normalized to untreated cells.

GFP silencing than Lipofectamine-2000 (40%).

Figure 6 shows the viability of U87 cells treated with the siRNA nanoparticles of the spermine based siRNA nanoparticles at *N/P* of 12. The MTT assay showed that the siRNA nanoparticles of the carriers resulted in low cytotoxicity at 50 nmol/L siRNA. The SKAHCO/siRNA nanoparticles with the highest gene silencing efficiency caused little cytotoxicity. The cytotoxicity of the siRNA nanoparticles of the multifunctional carriers was comparable with that of the siRNA complexes of Lipofectamine-2000.

The use of RNAi to treat human diseases requires the delivery of therapeutic siRNA into target cells. The design and development of safe and effective siRNA delivery system is a key step towards clinical application of RNAi therapeutics. The delivery systems should be able to protect siRNA from

Figure 5 Flow cytometric diagrams showing GFP silencing in CHO-GFP cells mediated by the nanoparticles of an anti-GFP siRNA (100 nmol/L) with the multifunctional carriers.

Figure 6 Cytotoxicity of the siRNA nanoparticles with the multifunctional carriers at an *N/P* ratio of 12 in U87 cells. siRNA concentration was 50 nmol/L. Lipofectamine 2000 (Lipo-2000) was used as control.

enzymatic degradation during delivery process [22], to deliver siRNA into target cells and to release it into cytosol. In this study, we evaluated the efficacy of eight new spermine based surfactants as the multifunctional carriers for siRNA delivery. These carriers were able to form stable and compact nanoparticles with siRNA and resulted in high intracellular siRNA uptake. Six carriers contained one or two histidine residuals. The imidazole group in histidine had a pK_a around 6.5 and was expected to facilitate endosomal escape of the siRNA nanoparticles at endosomal pH for enhanced gene silencing. However, SHHKACO with two histidine residues mediated the lowest gene silencing efficiency in both U87-Luc and CHO-GFP cells among the carriers. The other histidine containing carriers did not have any advantage over the carriers without histidine, SKACO and SKCO. The carriers SKACO, SKAHCO, SHKACO and SHHKACO had a more symmetric location of lipid tails than SKCO, SKHCO, SHKCO and SHHKCO. It appears that the location of the lipid tails did not have a significant impact on intracellular siRNA uptake and gene silencing efficiency mediated by the surfactants. Nevertheless, three carriers, SKAHCO, SKHCO and SKACO, were more effective than Lipofectamine-2000 in silencing the expression of both luciferase and GFP. Overall, SKACO demonstrated the highest intracellular plasmid DNA delivery efficiency as shown in the previous study [19] and SKAHCO showed the highest siRNA delivery efficiency.

3 Conclusion

The spermine based surfactants formed stable nanoparticles with siRNA at an *N/P* ratio of 12. The nanoparticles had relatively low cytotoxicity and resulted in high intracellular uptake of AlexaFluor488-labeled siRNA. Five of these carriers possessed a higher overall luciferase activity knock-down efficiency than Lipofectamine-200 when delivering

anti-Luc siRNA. Four of these multifunctional carriers showed a higher GFP silencing efficiency than Lipofectamine-2000. Among these tested carriers, SKAHCO demonstrated the highest intracellular siRNA delivery efficiency. It has a great potential for safe and effective siRNA delivery.

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