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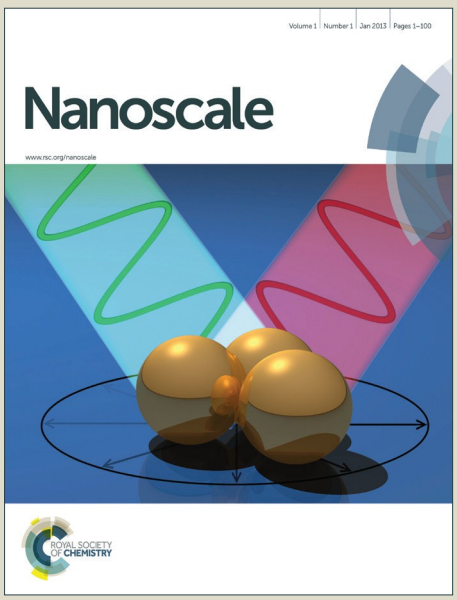

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Effective cytoplasmic release of siRNA from liposomal carriers by controlling the electrostatic interaction of siRNA with a charge-invertible peptide, in response to cytoplasmic pH†

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Condensing siRNA with cationic polymers is a major strategy used in the development of siRNA carriers that can avoid degradation by nucleases and achieve effective delivery of siRNA into the cytoplasm. However, ineffective release of siRNA from such condensed forms into the cytoplasm is a limiting step for induction of RNAi effects, and can be attributed to tight condensation of siRNA with the cationic polymers, due to potent electrostatic interactions. Here, we report that siRNA condensed with slightly acidic pH-sensitive peptide (SAPSP), whose total charge is inverted from positive to negative in response to cytoplasmic pH, is effectively released via electrostatic repulsion of siRNA with negatively charged SAPSP at cytoplasmic pH (7.4). The condensed complex of siRNA and positively-charged SAPSP at acidic pH (siRNA/SAPSP) was found to result in almost complete release of siRNA upon charge inversion of SAPSP at pH 7.4, with the resultant negatively-charged SAPSP having no undesirable interactions with endogenous mRNA. Moreover, liposomes encapsulating siRNA/SAPSP demonstrated knockdown efficiencies comparable to those of commercially available siRNA carriers. Taken together, SAPSP may be very useful as a siRNA condenser, as it facilitates effective cytoplasmic release of siRNA, and subsequent induction of specific RNAi effects.

Introduction

With the increase in identification of disease-associated genes, it is expected that nucleic acid medicines (NAMs), such as small interfering RNA (siRNA) and antisense oligonucleotides, may serve as alternatives to monoclonal antibodies and low-molecular-weight drugs, for specific molecular targets. [1-3] NAMs exhibit higher specificity to target molecules than low-molecular-weight drugs, and can be chemically synthesized on a large scale, unlike monoclonal antibodies. [4,5] Thus, NAMs have the potential to overcome the shortcomings of conventional therapies. To realize the clinical application of NAMs as common drugs for specific molecular targets, however, there are two major obstacles that need to be overcome, namely that NAMs are easily biodegraded by nucleases, [6] and that anionic hydrophilic macromolecules NAMs are often difficult to deliver into the cytoplasm. [7,8] Development of appropriate carriers for effective delivery of NAMs is, therefore, a rational strategy for overcoming these obstacles.

Since the RNAi effect was first reported by Fire et al. in 1998, siRNA has been extensively studied as the most likely NAM candidate for potential clinical application. [9] siRNA is short-chain double-stranded RNA that can specifically cleave target mRNA as the trigger for integration into the RNA-induced silencing complex (RISC) in the cytoplasm. [10,11] Induction of the RNAi effect, therefore, requires that siRNA be taken up by target cells and delivered into the cytoplasm. Cationic polymers and liposomes have previously been utilized to overcome inferior cytoplasmic delivery of siRNA, as both systems can easily be complexed with anionic siRNA via electrostatic interactions. [12-14] Although such carrier systems exhibit a positive surface charge necessary for effective cellular entry, and show effective cytoplasmic delivery of siRNA, the release of free siRNA into the cytoplasm is a rate-limiting step for induction of their RNAi effects.

It is known that the representative cationic polymer polyethylenimine (PEI) can deliver siRNA into the cytoplasm, and escapes endosomes by the “proton sponge” effect following cellular uptake via the endocytic pathway. [15,16] However, the strong electrostatic interactions between siRNA and PEI-based carriers results in a low release efficiency of free siRNA into the cytoplasm. [17] Lipoplex, a complex comprised of cationic liposome and siRNA, exhibits a higher siRNA release efficiency compared with PEI, due to the comparatively low charge density. [18] Despite this advantage, it is necessary to improve the ineffective bio-distribution of lipoplex that results from interaction of the cationic surface charge with biogenic substances, and to avoid degradation of the surface siRNA. [19,20] Liposome-based carriers, on the other hand, in which siRNA is encapsulated into the liposome, are a useful alternative, as the surface of the carrier can be easily modified with polyethylene glycol and cell-penetrating peptides, to control bio-distribution and intracellular trafficking, respectively. [21-23] To encapsulate siRNA into

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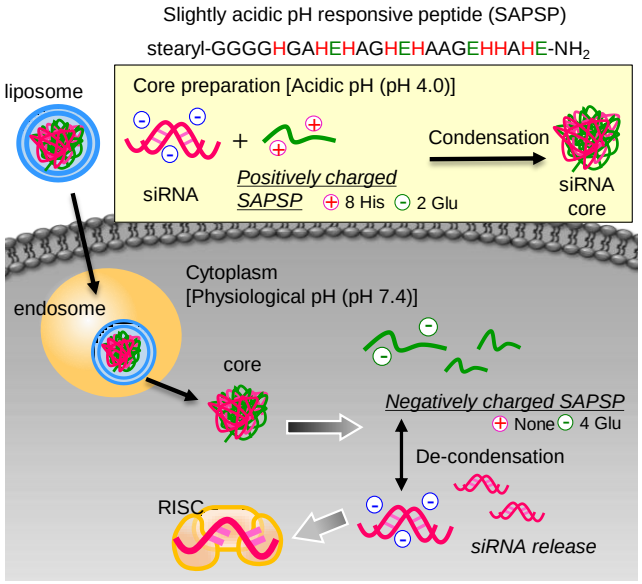

Fig. 1 Conceptual diagram of the cytosolic release of siRNA from cores prepared with slightly acidic pH-sensitive peptide (SAPSP). The total charge of SAPSP reversibly alternates between positive and negative upon protonation/deprotonation of histidine residues in response to acidic/physiological pH. Positively-charged SAPSP at slightly acidic pH (pH 4.0) is condensed with negatively-charged siRNA (SAPSP core) via electrostatic interactions. When SAPSP cores are delivered into the cytoplasm, free siRNA is released from the core by electrostatic repulsion between siRNA and negatively-charged SAPSP at physiological pH (pH 7.4).

liposomes with high efficiency, the core, which is comprised of siRNA electrostatically condensed with cationic polymers and peptides, is first prepared, followed by electrostatically covering the core with a lipid membrane.[22,23] The surface charge of the nano-sized core can be either positive or negative, depending on the lipid composition, and is a useful property for encapsulation into liposomes.[22,23] Owing to the tight interaction of siRNA with cationic polymers or peptides in conventional cores, the inferior release efficiency of free siRNA following cytoplasmic delivery of the cores hampers the development of siRNA carriers that are capable of inducing a potent RNAi effect.

To enhance the cytosolic release of siRNA from the core, we focused on the slightly acidic pH-sensitive charge-invertible peptide SAPSP as a condenser for preparation of the core. SAPSP, which is comprised of histidine (His) and glutamic acid (Glu) residues,[24] exhibits higher sensitivity to pH change than conventional pH-sensitive devices.[25-29] To design the peptide to exhibit high sensitivity to pH, Glu residues were placed as neighbors to the His residues in the peptide sequence to increase the pKa value of His via stabilization of its protonated form.[24] The total charge of SAPSP at acidic pH is positive due to protonation of the His residues, while the total charge is inverted to negative at physiological pH via deprotonation of the His residues, as shown in Fig. 1. Our hypothesis in the present study is that cores prepared by condensing siRNA with positively-charged SAPSP at acidic pH can effectively release free siRNA via electrostatic repulsion between siRNA and negatively-charged SAPSP in response to physiological pH, following delivery of the cores into the cytoplasm compared with conventional pH-sensitive siRNA condensers that do not exhibit charge-invertible properties (Fig. 1). To prove our hypothesis, we prepared cores comprised of siRNA and SAPSP (SAPSP core) and examined the pH-sensitive release of free siRNA from the SAPSP cores compared with cores prepared using stearylated octaarginine (stearyl-R8), which is a positively-charged peptide under both acidic and neutral conditions. Moreover, we determined the release of free siRNA from SAPSP cores following delivery into the cytoplasm via liposome carriers.

Experimental

Materials

3-sn-phosphatidic acid (PA) and Hoechst 33342 were purchased from Sigma Aldrich (St. Louis, MO, USA). 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). Rabbit Reticulocyte Lysate System (Nuclease Treated), luciferase control RNA and the luciferase assay system were purchased from Promega (Madison, WI, USA). The mouse melanoma B16-F1 cell line was obtained from DS Pharma Biomedical Co., Ltd (Osaka, Japan). SYBR Gold was purchased from Invitrogen (Carlsbad, CA, USA). Ribonuclease (RNase) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Stearylated octaarginine (stearyl-R8), stearylated slightly acidic pH sensitive peptide (SAPSP), stearylated NBD-R8 (R8-GC-NBD-NH_2) and stearylated NBD-SAPSP (SAPSP-GC-NBD-NH_2) were synthesized by SCRUM (Tokyo, Japan). Anti-luciferase siRNA (21-mer, 5'-GCGCUGCUGGUGCCAACCCTT-3', 5'-GGGUUGGCACCAGCAGCGCTT-3'), negative control siRNA (21-mer, 5'-UAUUGCGUCUGUACACUCATT-3', 5'-UGAGUGUACAGACGCAAUATT-3') and Alexa546-labeled siRNA (21-mer, 5'Alexa546-UAUUGCGUCUGUACACUCATT-3', 5'Alexa546-UGAGUGUACAGACGCAAUATT-3') were synthesized by Invitrogen (Carlsbad, CA, USA).

Preparation of SAPSP and STR-R8 cores

siRNA, SAPSP and STR-R8 were diluted in 10 mM HEPES buffer (pH 4.0). To prepare the siRNA/SAPSP (SAPSP core) and siRNA/STR-R8 (STR-R8 core) complexes, the siRNA solution was mixed with the SAPSP and STR-R8 solutions, respectively, and was continuously vortexed at a nitrogen/phosphate (N/P) ratio of 5.5. The particle size and surface charge of the nanoparticles were determined by a Zetasizer Nano (Malvern Instruments Ltd., Worcestershire, UK).

Preparation of R8-modified liposomes (R8-lipo) encapsulating siRNA cores

siRNA cores were encapsulated into R8-lipo according to previous reports, with minor modifications.[30] A lipid film comprised of DOPE/PA (7:2) was hydrated with 10 mM HEPES buffer (pH 4.0), followed by sonication for 20 min using a probe-type sonicator (Qsonica, CT, USA) to prepare small unilamellar vesicles (SUVs). The SUVs were mixed with siRNA cores at a volume ratio of 2:1 (lipo). The surface of lipo was modified with STR-R8 (10 mol %), by

incubating at room temperature for 30 min (R8-lipo). The final lipid concentration was 0.67 mM.

siRNA transfection assay

B16-F1 cells stably expressing luciferase (B16-F1-luc) were seeded at 2×10^3 cells/well on a 96 well plate. Following a 24 h incubation period, the medium was exchanged with serum-free DMEM, and the cells were treated with siRNA-encapsulated R8-lipo, with a siRNA concentration of 50 nM. Following incubation at 37°C for 3 h, the medium was removed, and the cells were washed, followed by addition of DMEM containing 10% FBS. After an additional incubation period of 9 h at 37°C, the transfected cells were lysed with Reporter Lysis Buffer (Promega, Madison, WI, USA), and allowed to react with the luciferase assay substrate. The relative light units (RLU) were measured with a luminometer (Luminescenser-PSN; Atto, Tokyo). The total protein concentration was determined using a BCA Protein Assay Kit (Thermo Scientific). Luciferase activity is reported as RLU/mg protein and the gene knockdown efficiency was calculated by the following formula: gene knockdown efficiency (%) = [1- (R_{luc} / R_{cont})] × 100, where R_{luc} and R_{cont} are the luciferase activities in cells transfected with anti-luciferase siRNA and the negative control siRNA, respectively.

Ethidium bromide (EtBr) exclusion assay

To evaluate the degree of SAPSP/siRNA or STR-R8/siRNA condensation, EtBr solution was mixed with SAPSP or STR-R8 cores at a weight ratio of 0.49:1.0 (EtBr:siRNA) in 10 mM HEPES buffer prepared at pH 7.4 or pH 4.0. Following incubation for 10 min, the fluorescence intensity of EtBr was measured by PLATE manager Infinite M200 (Tecan) at an excitation wavelength of 520 nm and an emission wavelength of 590 nm.

Agarose gel electrophoresis

To evaluate the release of siRNA from the cores, each core containing 0.3 μg of siRNA was diluted with phosphate buffer (pH 7.4) or citric acid-phosphate buffer (pH 4.0) containing 5% glycerol to prepare the sample for electrophoresis. Electrophoresis was performed using 1% agarose gel in 50 mM phosphate buffer (pH 7.4) or 50 mM citric acid-phosphate buffer (pH 4.0) at 100 V for 1 h. Gels were then stained with SYBR Gold (Invitrogen, Carlsbad, CA, USA) for 30 min, and images were captured using a STAGE-1000 imaging system (AMZ System Science, Osaka, Japan). Additionally, to evaluate the resistance of SAPSP cores against degradation by nucleases, free siRNA and SAPSP cores were incubated with or without 10 μg/mL RNase at 37°C for 2 h. Following incubation, the reaction mixtures were then incubated with 5 μg of heparin on ice for 10 min to de-condense the SAPSP cores. Samples containing 5% glycerol were then electrophoresed using 2% agarose gel in 0.5 × Tris-Borate-EDTA (TBE) buffer at 100 V for 20 min, followed by detection of siRNA as described above.

Time-lapse imaging of intracellular siRNA release by confocal laser scanning microscopy (CLSM)

SAPSP cores comprised of Alexa546-labeled siRNA and NBD-labeled SAPSP were encapsulated into R8-lipo (at a lipid concentration of 0.55 mM), as described above. B16-F1 cells were cultured on a 0.002% PLL-coated 35 mm glass-bottom dish (IWAKI) at a density of 2×10^5 cells/dish in DMEM containing 10% FBS. After 24 h, the cells were washed with PBS (-) and treated with R8-lipo in serum-free DMEM at 37°C for 15 min. Following removal of the liposomes, the cells were washed three times with PBS (-) containing 20 units/mL of heparin sodium, followed by incubation with 25 μM of Hoechst 33342 for 10 min to stain the cellular nuclei. The culture medium was then exchanged with fresh DMEM containing 10% FBS after washing with PBS (-). Time-lapse images were acquired every 3 min over 1.5 h with a Nikon A1 CLSM (Nikon Instruments Inc., Melville, USA) equipped with an oil-immersion objective lens (Plan Apo VC 60x 1.4 N.A.). Conditions were maintained at 37°C and 5% CO_2 for all imaging experiments. The spectral properties of the fluorescent dyes used in this study are as follows: Hoechst 33342: excitation: 355 nm, emission: 465 nm; NBD: excitation: 460 nm, emission: 535 nm; Alexa546: excitation: 556 nm, emission: 573 nm.

***In vivo* imaging**

B16-F1-luc cell-bearing mice were prepared according to our previous report. [31] The cell suspension of B16-F1-luc was mixed with Matrigel Matrix (Corning Inc., NY, USA) at a volume ratio of 5:1 to a final density of 2×10^7 cells/mL. A 100 μL suspension was injected under the skin of seven-week-old nude female mice (BALB/c Slc-nu/nu). When a tumor volume of >70 mm^3 was achieved, R8-lipo encapsulating SAPSP or STR-R8 cores was administrated into the center of the tumor using a microsyringe at a siRNA dose of 1.08 μg/mouse. Tumor volumes were estimated according to the formula: $T_{vol} = (length) \times (width)^2 \times 0.5$. To obtain the luminescent images, VivoGlo Luciferin (Promega, Madison, WI, USA) was administered by intraperitoneal injection 15 min before image acquisition. Luminescence images were acquired using an IVIS Lumina XR imaging system (PerkinElmer Inc., Waltham, MA, USA) equipped with an open luminescence filter with an exposure time of 20 sec. The radiance (photons/second/cm^2/steradian) of the region of interest (ROI) was quantified using the Living Image software (PerkinElmer Inc.). The relative luciferase activity of the tumor was calculated using the following formula: relative luciferase activity = ($R24/T_{vol}24$)/($R0/T_{vol}0$), where $R24$ and $R0$ are the radiance values obtained 24 h after and before the intratumoral sample injection, respectively, and $T_{vol}24$ and $T_{vol}0$ are the tumor volumes observed 24 hour after and before intratumoral injection, respectively. All animal experiments were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Kyoto Pharmaceutical University.

***In vitro* translation assay**

The *in vitro* translation assay was performed using a Rabbit Reticulocyte Lysate System according to previous reports. [32] Briefly, luciferase mRNA (1 μg) was incubated with either SAPSP or STR-R8 cores containing anti-luciferase siRNA (1 μg) for 10 min on ice,

followed by reaction with rabbit reticulocyte lysate according to the manufacturer's instructions. Luciferase activity, indicating *in vitro* translation, was measured as described above.

Statistical analysis

Statistical significance was determined by ANOVA and Student's t-test. *P* values <0.05 were considered to be significant.

Results

Condensation of siRNA with SAPSP to prepare siRNA core-encapsulated liposome-based carriers

To develop liposome-based siRNA carriers capable of releasing siRNA into the cytoplasm, we prepared siRNA cores by condensing siRNA with slightly acidic pH-sensitive peptide (SAPSP). As SAPSP is a charge-invertible peptide, which exhibits a positive charge at acidic pH and a negative charge at neutral pH, [24] it is expected to form a complex with siRNA (SAPSP cores) at acidic pH via electrostatic interactions (Fig. 1), and effectively release free siRNA from the core complex in response to cytosolic pH, due to electrostatic repulsion between the siRNA and negatively-charged SAPSP (Fig. 1). To encapsulate the siRNA cores exhibiting positive surface charges into anionic liposomes for *in vivo* applications, positively-charged SAPSP (pH 4.0) was mixed with siRNA at various nitrogen/phosphate (N/P) ratios. When SAPSP was mixed with siRNA at an N/P ratio of 5.5, the particle sizes of the resultant SAPSP cores were less than 100 nm, and their surface charges were greater than +20 mV (Figs. 2a and b). These results suggest that SAPSP cores exhibit physicochemical properties that are conducive to encapsulation into anionic liposomes, i.e. small particle sizes and positive surface charges. The degree of condensation of SAPSP cores was evaluated using an EtBr exclusion assay. When free siRNA was mixed with EtBr, the fluorescence intensity increased as a result of the intercalation of EtBr into the siRNA (Fig. 2c). By contrast, the fluorescence intensities of SAPSP cores prepared at an N/P ratio of 5.5 were significantly reduced, owing to the exclusion of EtBr from the SAPSP cores (Fig. 2c). These results indicate that SAPSP is complexed with siRNA with a high degree of condensation within the SAPSP cores. Based on the results shown in Figs. 2a-c, we determined the optimum N/P ratio to prepare SAPSP cores to be 5.5.

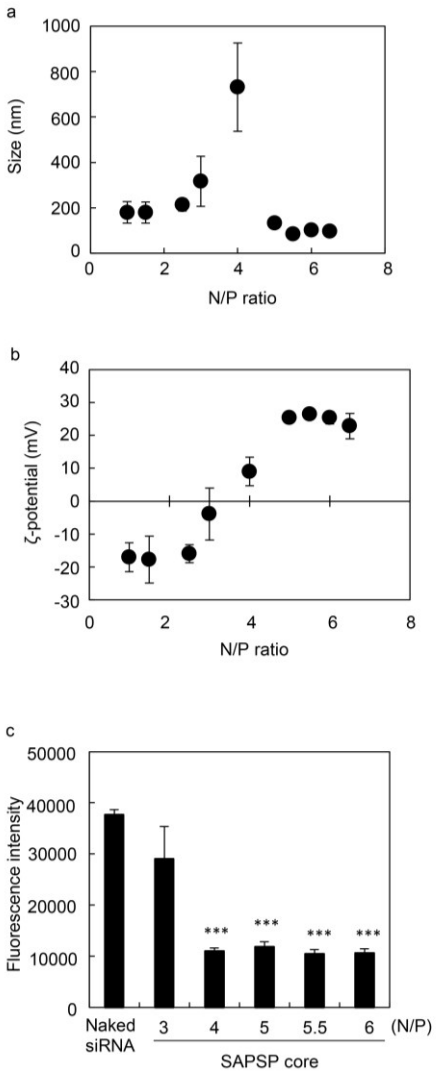

Fig. 2 Characterization of SAPSP cores prepared at various N/P ratios at pH 4.0. The particle size (a) and ζ-potential (b) of SAPSP cores were measured by Zetasizer Nano. Data are mean ± S.D. n=3. (c) The degree of siRNA condensation was evaluated using an EtBr exclusion assay. Data are mean fluorescence intensity ± S.D. ***P<0.001 vs free siRNA. n=3.

Release of siRNA from SAPSP cores in response to pH change

We compared the release of siRNA from SAPSP cores prepared at an N/P ratio of 5.5 in response to cytoplasmic pH (pH 7.4) with that of cores comprised of siRNA and stearyl-octaarginine (STR-R8 cores). STR-R8 is a positively-charged peptide at both acid and neutral pH, and has been typically used for the preparation of siRNA cores encapsulated within liposome-based carriers.[23] The STR-R8 cores used in this study had physicochemical properties similar to those of the SAPSP cores (Table 1). As shown in Fig. 3a, the reduced fluorescence intensity observed for EtBr in the SAPSP cores at pH 4.0 was recovered in response to a pH change from pH 4.0 to 7.4, to levels similar to those of free siRNA, suggesting that SAPSP cores could release free siRNA at cytoplasmic pH (pH 7.4). In contrast, STR-R8 cores were found to exhibit a significantly lower fluorescence intensity than free siRNA, even when the pH was changed from 4.0 to 7.4. Moreover, the fluorescence intensity of the STR-R8 cores was significantly lower than that of the SAPSP cores at pH 4.0. It is, therefore, suggested that the release efficiency

Table 1 Physicochemical properties of SAPSP and STR-R8 cores at pH 4.0.

	Size (nm)	PDI	Zeta-potential (mV)
SAPSP cores	90±9	0.23±0.06	22±6
STR-R8 cores	87±9	0.28±0.04	17±3

Data are expressed as mean ± S.D. (n=4-6).

of siRNA from STR-R8 cores was lower than that of the SAPSP cores owing to a more tightly condensed siRNA complex in the STR-R8 cores. Cytoplasmic pH-sensitive release of siRNA from the SAPSP cores was further confirmed by agarose gel electrophoresis. As shown in Fig. 3b, the siRNA band was not observed in either the SAPSP or STR-R8 cores at pH 4.0 due to the positive surface charges under those conditions, which does not allow for electronic migration. Upon changing the pH to 7.4, SAPSP cores showed an electrophoretic mobility similar to that of free siRNA, while STR-R8 cores showed no migration upon changing the pH. These results suggest that free siRNA was released from SAPSP cores in response to cytoplasmic pH, consistent with Fig. 3a.

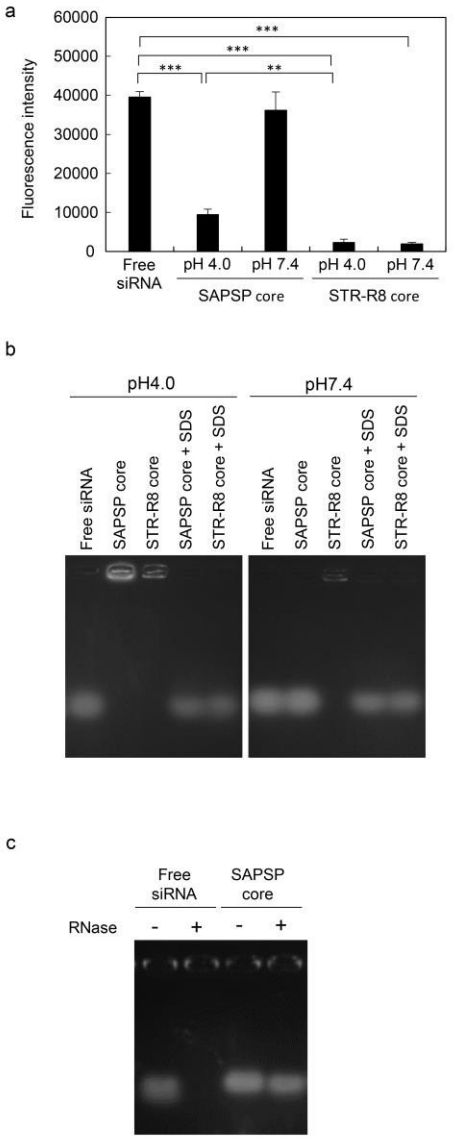

Fig. 3 Release of siRNA from SAPSP cores in response to pH change. (a) Evaluation of siRNA release by fluorescence recovery of EtBr. SAPSP and STR-R8 cores were incubated with EtBr in HEPES buffer prepared at pH 7.4 or pH 4.0 for 10 min, and the fluorescence intensities at 590 nm were measured. Data are mean fluorescence intensity ± S.D. n=3, ***P<0.001 vs free siRNA. (b) Typical image of agarose gel electrophoresis. Free siRNA, SAPSP cores, and STR-R8 cores with or without 1% SDS were electrophoresed in buffer prepared at the indicated pH; siRNA was visualized by staining with SYBR Gold. (c) Evaluation of the resistance of SAPSP cores against degradation by nuclease by agarose gel electrophoresis. Free siRNA and SAPSP cores treated with or without nuclease were electrophoresed in 0.5 × TBE buffer.

To determine the transition of condensation/de-condensation via the control of electrostatic interactions, we examined the effect of counter-anions on the release of siRNA from each of the cores. When SAPSP and STR-R8 cores were de-condensed by the addition of sodium dodecyl sulfate (SDS), used as a counter-anion, migration of siRNA was observed, regardless of a change in pH (Fig. 3b). De-condensation of both cores was also induced by the addition of the non-surfactant counter-anion heparin (Fig. S1). These results suggest that SAPSP is condensed with siRNA at pH 4.0 via electrostatic interactions between negatively charged SAPSP and siRNA, similar to STR-R8. Upon increasing the pH to 7.4, SAPSP cores were de-condensed via electrostatic repulsion, triggered by inversion of the total charge of SAPSP from positive to negative. To determine whether SAPSP cores could protect siRNA against degradation by RNase, agarose gel electrophoresis at 7.4 was performed following incubation of SAPSP cores or free siRNA with RNase at pH 4.0. As shown in Fig. 3c, siRNA condensed with SAPSP was protected from degradation by RNase, while free siRNA, on the other hand, was completely degraded.

Observation of cytoplasmic siRNA release from SAPSP cores by time-lapse imaging

To examine the cytoplasmic release of siRNA from SAPSP cores in living cells by CLSM observation, SAPSP cores dually-labeled with fluorescent dyes were prepared by mixing NBD- labeled SAPSP (NBD-SAPSP) with Alexa Fluor 546- labeled siRNA (Alexa546-siRNA). SAPSP cores were encapsulated into anionic liposomes modified with octaarginine (R8) for delivery into the cytosol.[30] The anionic liposomes used in this study were R8-modified liposomes (R8-lipo) with a dual-layered membrane comprised of fusiogenic lipid DOPE and anionic lipid PA (Table2). Compared with other conventional liposomes, R8-lipo, which was previously developed by Akita et al. , effectively delivers its cargo into the cytosol as a result of its dual-layered membrane and fusiogenic activity. [30] In this study, we used R8-lipo as an efficient cytosolic delivery system of siRNA cores to obtain direct evidence of cytosolic siRNA release from the cores. As shown in Fig. 4, Alexa546-labeld siRNA (observed as dot-like

Table 2 Physicochemical properties of R8-lipo encapsulating SAPSP or STR-R8 cores.

	Size (nm)	PDI	Zeta potential (mV)
lipo (SAPSP)	193±54	0.30±0.06	-15±1
R8-lipo (SAPSP)	262±55	0.19±0.03	21±3
lipo (STR-R8)	216±40	0.31±0.12	-16±2
R8-lipo (STR-R8)	225±38	0.23±0.07	27±11

Data are expressed as mean ± S.D. (n=5-7)

fluorescent signals) was co-localized with NBD-STR-R8 and NBD-SAPSP (shown as yellow dot-like signals) in the cells immediately (0min) after R8-lipo mediated transfection, suggesting that the condensed siRNA/STR-R8 and siRNA/SAPSP complexes were effectively delivered into the cells immediately after transfection. Following delivery of the STR-R8 cores into the cells, Alexa546-labeled siRNA was only observed as dot-like fluorescent signals, even after an elapsed time of 30 min, suggesting that siRNA was not effectively released from the STR-R8 cores (Figure 4 and Movie S2). Time-lapse images of the cytoplasmic release of Alex546-labeled siRNA from the SAPSP cores, on the other hand, show the expansion of red fluorescence signals within the cells with increasing the incubation time (above 6 min) (Figure 4 and Movie S1). These results demonstrate that intracellular siRNA was more effectively diffused upon de-condensation of the siRNA/SAPSP complexes compared with the siRNA/STR-R8 complexes. Taken together, these results suggest that free siRNA was released from the SAPSP cores in response to cytoplasmic pH, following R8-lipo-mediated delivery of SAPSP cores into the cytoplasm.

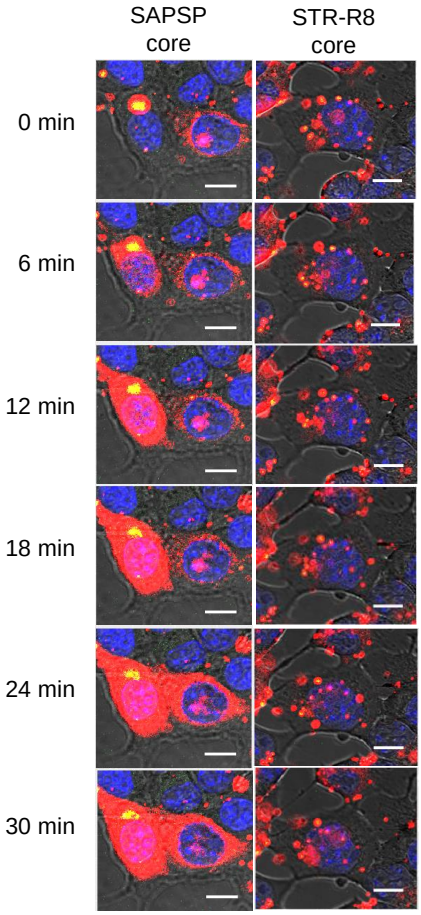

Fig. 4 Time-lapse images of cytoplasmic release of siRNA from SAPSP cores (left) and STR-R8 cores (right). B16-F1 cells were treated with R8-lipo encapsulating STR-R8 cores and SAPSP cores. Time-lapse images were acquired at intervals of 6 min by CLSM. The red, green and blue signals indicate Alexa546-labeled siRNA, NBD-STR-R8 or NBD-SAPSP and nuclei, respectively. Scale bars: 10 µm.

RNAi effect of SAPSP cores encapsulated within liposome-based carriers in cells stably expressing the luciferase gene

To understand the contribution of the potent cytoplasmic siRNA release from SAPSP cores to the RNAi effect, we compared the knockdown efficiencies of SAPSP and STR-R8 cores encapsulated within R8-lipo. The particle size and surface charge of R8-lipo encapsulating STR-R8 cores, which exhibit low de-condensation efficiencies, were comparable to those of R8-lipo encapsulating SAPSP cores (Table 2). As shown in Fig. 5, SAPSP cores encapsulated within R8-lipo showed a knockdown efficiency twice as high as that of STR-R8 cores encapsulated within R8-lipo, with the efficiency of encapsulated SAPSP cores being comparable to that of Lipofectamine 2000, a commercially available transfection reagent. These results suggest that the increase of free siRNA released from SAPSP cores in response to cytoplasmic pH, indeed, contributes to the enhancement of the RNAi effect.

***In vivo* knockdown efficiency of R8-lipo-encapsulated SAPSP cores**

To obtain *in vivo* evidence regarding the high RNAi effect of SAPSP core, we compared the knockdown effects of R8-lipo-encapsulated SAPSP and STR-R8 cores administered intratumorally into mice bearing B16-F1 cells stably expressing the luciferase gene. As shown in Figs. 6a and b, the administration of SAPSP and STR-R8 cores containing control siRNA (Cont) showed luciferase activities comparable to that of PBS (-), used as a negative control, indicating that R8-lipo-encapsulated SAPSP and STR-R8 cores did not affect the luciferase activity within the tumor. When cores containing anti-luciferase siRNA (Luc) were administered, SAPSP cores (Luc) showed significantly lower luciferase activity than SAPSP cores (Cont), while no significant difference was observed between the STR-R8 cores (Luc) and STR-R8 cores (Cont). Similar to the results obtained following intratumoral injection, intravenous injection of SAPSP cores (Luc) tended to show lower luciferase activity than that of SAPSP cores (Cont), although no significant difference was

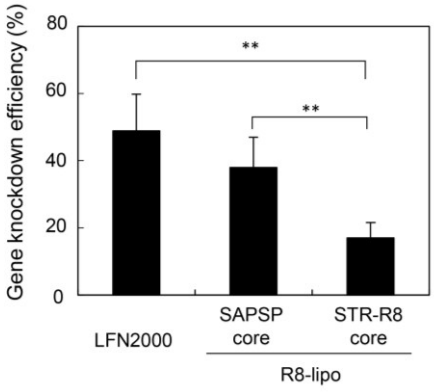

Fig. 5 *In vitro* knockdown efficiency of R8-lipo encapsulating SAPSP cores. B16-F1-Luc cells were treated with R8-lipo encapsulating SAPSP or STR-R8 cores at 50 nM anti-luc siRNA in serum-free DMEM. Following incubation for 10 h, luciferase activity was measured. Lipofectamine 2000 (LFN2000) was used as a positive control. Values and bars represent mean knockdown efficiencies and S.D., respectively. n=4, $^{**}P<0.01$.

observed (Fig. S2). Taken together, these results suggest that SAPSP cores, which exhibit high de-condensation efficiencies, can enhance the *in vivo* RNAi effect.

Interaction of SAPSP with intracellular nucleic acids

It is known that when cationic polymers and nanoparticles are delivered into the cytoplasm, electrostatic interactions between these cationic substances and intracellular nucleic acids, such as host mRNA and exogenous siRNA, lead to non-specific RNAi effects and undesirable inhibition of host translation.[32] SAPSP is expected to avoid such unwanted electrostatic interactions, as the total charge of the peptide is inverted from positive to negative following cytoplasmic delivery. We investigated potential undesirable interactions associated with both SAPSP and STR-R8 cores using an *in vitro* translation assay comprised of rabbit reticulocyte lysate, luciferase mRNA and either SAPSP or STR-R8 cores, followed by measurement of luciferase activity. If the cores interact with the applied luciferase mRNA electrostatically, low levels of luciferase activity will be observed due to inhibition of mRNA-based translation. Anti-luciferase siRNA was used for this evaluation. As shown in Fig. 7, SAPSP cores showed luciferase activity comparable to that of the control and free siRNA. On the other hand, luciferase activity was significantly inhibited by addition of STR-R8 core, owing to potent electrostatic interactions between mRNA and positively-charged STR-R8 at cytoplasmic pH. Therefore, the total negative charge of SAPSP following cytoplasmic delivery makes it useful for siRNA condensation, whereby it helps in avoiding undesirable interactions with both host and exogenous nucleic acids, and results in effective cytoplasmic release of free siRNA from the core.

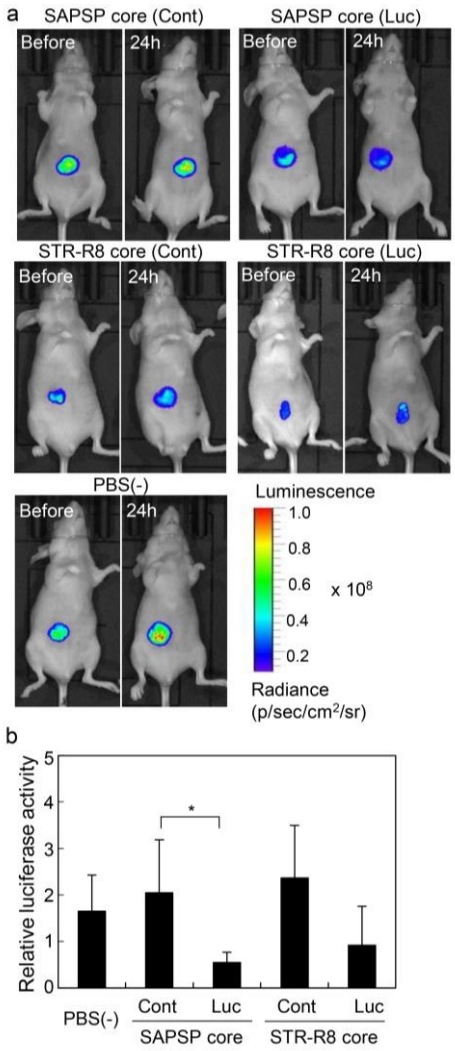

Fig. 6 *In vivo* knockdown efficiency of R8-lipo encapsulating SAPSP cores. B16-F1-Luc cell-bearing mice were treated with R8-lipo encapsulating SAPSP or STR-R8 cores containing either negative control siRNA (Cont) or anti-luciferase siRNA (Luc). Luciferase activities in the tumor-bearing mice were measured with an IVIS Lumina XR imaging system before and 24 hr after injection, and the radiances were estimated using the Living Imaging software before and 24 hr after injection. The PBS (-)-administered mice were used as the negative control group. (a) Representative images of luciferase activity in mice that were administered the indicated samples. (b) Relative luciferase activities in the tumors of mice 24 hr after intratumoral injection, compared with those prior to injection. Data are mean ± S.D. n=5, **P*<0.05.

Discussion

To overcome limitations associated with the *in vivo* application of conventional liposome-based siRNA carriers, we developed a novel transfection method capable of achieving effective cytoplasmic release of free siRNA via encapsulation of a core comprised of siRNA and the cytoplasmic pH-sensitive charge-invertible peptide SAPSP into liposomes (Fig. 1). As shown in Figs. 2a and b, we successfully prepared cores approximately 100 nm in size with sufficient positive surface charge by mixing positively-charged SAPSP at pH 4.0 with siRNA at an N/P ratio of 5.5. It is suggested that mixtures prepared at an N/P ratio of 4.0 result in aggregation

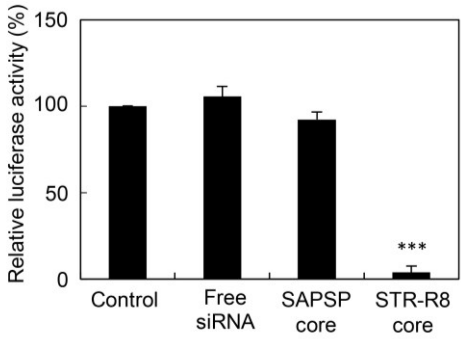

Fig. 7 Effect of SAPSP and STR-R8 cores on *in vitro* translation. mRNA coding luciferase (1 μg) was incubated with free siRNA, SAPSP cores or STR-R8 cores (1 μg of anti-Luc siRNA). *In vitro* translation was carried out using a Rabbit Reticulocyte Lysate System. Translation efficiencies were determined by luciferase activity. Results are reported as percentages relative to mRNA (Control). Data are mean ± S.D. n=3, ****P*<0.001.

of the core due to the decreased electrostatic repulsion among the core particles. When linear 22 kD PEI, used as a representative condenser, is mixed with siRNA at an N/P ratio of 8.0, PEI-based cores exhibit surface charges and particle sizes comparable to those of SAPSP cores, as previously reported by Grayson et al.[33] Comparison of the N/P ratios necessary for the preparation of nano-sized and positively-charged cores suggests that SAPSP is superior to PEI as a condenser for siRNA. Moreover, SAPSP cores prepared at N/P ratios of 5.5 were found to have been condensed tightly enough to exclude EtBr (Fig. 2c), resulting in protection of siRNA against degradation by RNase (Fig. 3c).

When the extent of condensation of SAPSP and STR-R8 cores prepared at pH 4.0 was compared, SAPSP cores showed significantly lower EtBr exclusion than STR-R8 cores (Fig. 3a), even though both cores have similar physicochemical properties (Table 1). These results suggest that SAPSP cores exhibit a more loose structure than STR-R8 cores. Considering the difference in condensation potency between SAPSP and STR-R8 from their positive charge densities (based on their respective peptide sequences), STR-R8, which contains eight consecutive positively-charged arginine residues, has a higher positive charge density than SAPSP, which contains glutamic acid and histidine residues that are negatively and positively charged at pH 4.0, respectively (Fig. 1). In contrast to STR-R8, whose potent cohesive forces result from electrostatic interactions, SAPSP is suggested to be relaxationally-condensed with siRNA. As shown in Figs. 3a and b, siRNA was almost completely released from SAPSP cores upon a change in pH from 4.0 to 7.4, but was not released from the STR-R8 cores containing arginine residues that are positively-charged under both pH conditions, suggesting that effective release of siRNA from SAPSP cores is triggered by electrostatic repulsion between siRNA and negatively-charged SAPSP at pH 7.4, in accordance with the concept depicted in Fig. 1. As described above, it is assumed that histidine is a key amino acid residue for effective cytoplasmic release of siRNA via prompt transition between condensation and de-condensation using a pH-sensitive charge-invertible peptide. Toriyabe et al. reported that siRNA cores comprised of stearylated octahistidine (STR-H8) can release free siRNA into the cytoplasm via weakened electrostatic siRNA/STR-H8 interactions upon deprotonation of histidine residues in response to cytoplasmic pH, leading to enhancement of the RNAi effect.[34] Although STR-H8 can be strongly condensed with siRNA, similar to SAPSP at acidic pH, the release efficiency of siRNA from STR-H8 is about 40%.[34] It is estimated that STR-H8, which is comprised of eight consecutive positively-charged histidine residues at acidic pH, exhibits a higher positive charge density than SAPSP. This charge density-based potent cohesive force inherent in the STR-H8 peptide sequence may hamper effective cytoplasmic release of siRNA. Moreover, the mechanism of siRNA release from STR-H8 is based on weakened electrostatic interactions resulting from deprotonation of histidine residues in response to cytoplasmic pH. The more effective release of siRNA mediated by SAPSP, on the other hand, is attributed to electrostatic repulsion between SAPSP and siRNA at cytoplasmic pH, due to the anionic charge of the glutamic acid residues, as well as the loss of the cationic charge on the histidine residues.

Release of free siRNA from SAPSP cores was also observed in cultured cells immediately after R8-lipo mediated delivery (Fig. 4). Moreover, SAPSP cores exhibited knockdown efficiencies that were more than twice as high as those of STR-R8 (Fig. 5). Considering the cytoplasmic delivery processes of SAPSP and STR-R8 cores, which are mediated by the outer layers of R8-lipo, it is suggested that both cores exhibit similar efficiencies with regard to intracellular trafficking, such as cellular uptake and endosomal escape, so that effective release of free siRNA from the SAPSP cores can be attributed to the potent RNAi effect observed. Moreover, we confirmed that intratumoral injection of R8-lipo encapsulating SAPSP cores (Luc) showed *in vivo* knock down effect (Fig. 6), being consistent with the results of *in vitro* experiments.

Other advantages associated with the use of SAPSP as a siRNA condenser include the ability to avoid undesired interactions with cytosolic mRNA, as well as effective siRNA release. It has been previously reported that a lipoplex containing cationic lipids electrostatically interacted with mRNA upon examination using an *in vitro* translation assay.[32] Based on this finding, one can assume that positively-charged substances that exist in the cytoplasm result in non-specific interactions with exogenous siRNA and endogenous mRNA, leading to inefficient induction of the RNAi effect and undesired cytotoxicity, respectively. As shown in Fig. 7, cores comprised of cationic STR-R8, regardless of pH, significantly inhibited *in vitro* translation due to electrostatic interactions between STR-R8 and mRNA added to the assay, while SAPSP cores with a negative charge at pH 7.4 showed no effect, avoiding interactions with the added mRNA. As shown in Figure 6, no significant differences in knockdown effects were observed between STR-R8 cores containing negative control siRNA (Cont) and STR-R8 cores containing anti-luc siRNA (Luc), suggesting that STR-R8 induces some non-specific RNAi effects in the cytoplasm. With regard to the intracellular fate of the siRNA condensers following endosomal escape of the siRNA core, SAPSP can avoid non-specific electrostatic interactions with endogenous mRNA owing to its negative charge at cytoplasmic pH, and may therefore be the more useful condenser. Thus, encapsulation of SAPSP cores into liposomes comprised of anionic lipids leads to the development of siRNA carriers that exhibit the ability to induce a potent RNAi effects, without undesired cytotoxicity.

Conclusions

The findings reported in the present study demonstrate that the charge-invertible peptide SAPSP is a prominent siRNA condenser, useful for the development of liposome-based carriers encapsulating siRNA cores. SAPSP cores can release free siRNA in response to cytoplasmic pH, resulting in enhancement of the RNAi effect. Moreover, inversion of the overall charge of SAPSP from positive to negative following delivery into the cytoplasm leads to enhancement of siRNA functionality, and has no undesired cytotoxicity, avoiding non-specific interactions with endogenous mRNA, unlike conventional cationic materials used for the preparation of siRNA carriers. SAPSP cores having different physicochemical properties can be easily prepared by mixing with siRNA at

various N/P ratios, which can also apply to other conventional siRNA carriers. Thus, SAPSP cores are expected to be essential for the development of siRNA carriers exhibiting potent *in vivo* RNAi effects.

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