Introduction of stearyl moieties into a biocompatible cationic polyaspartamide derivative, PAsp(DET), with endosomal escaping function for enhanced siRNA-mediated gene knockdown

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Abstract

Applications of siRNA for cancer therapy have been spotlighted in recent years, but the rational design of efficient siRNA delivery carriers is still controversial, especially because of possible toxicity of the carrier components. Previously, a cationic polyaspartamide derivative, poly{N-[N-(2-aminoethyl)-2-aminoethyl]aspartamide} (PAsp(DET)), was reported to exert high transfection efficacy for plasmid DNA with negligible cytotoxicity. However, its direct application for siRNA delivery was fairly limited due to the unstable polymer/siRNA complex formation. In this study, to overcome such instability, stearic acid as a hydrophobic moiety was conjugated to the side chain of PAsp(DET) with various substitution degrees. The stearyl introduction contributed not only to siRNA complex formation with higher association numbers but also to complex stabilization. The obtained stearyl PAsp(DET)/siRNA complex significantly accomplished more efficient endogenous gene (BCL-2 and VEGF) knockdown *in vitro* against the human pancreatic adenocarcinoma (Panc-1) cells than did the unmodified PAsp(DET) complex and commercially available reagents, probably due to the facilitated cellular internalization. This finding suggests that the hydrophobic PAsp(DET)-mediated siRNA delivery is a promising platform for *in vivo* siRNA delivery.

Keywords: RNAi, siRNA delivery, polyplex, stearylation, polyaspartamide

1. Introduction

RNA interference (RNAi) is a powerful regulatory mechanism of the target mRNA degradation induced by the complementary RNA strand. Because of the selectivity of the targeted mRNA degradation as well as the potential of silencing almost all the endogenous genes, small interfering

RNA (siRNA) has been highlighted as an attractive choice for future therapeutics. The advent of siRNA opened a new era of therapy for cancers, autoimmune diseases, and dominant genetic disorders by the selective control of disease-related gene expression [1].

In spite of its promise, the *in vivo* therapeutic application of siRNA needs to overcome several biological hurdles [2]. Naked siRNA is easily degraded by nucleases in the blood-stream and also is cleared by the kidneys within several minutes [3]. The negative charge of the siRNA inhibits its interaction with negatively charged plasma membrane for the internalization into cells. Even though some portions of siRNAs could be internalized into cells, the internalized siRNA would be degraded in lysosome. Hence, a carrier system to protect siRNA from these external circumstances and to allow it to escape the endosome efficiently before the lysosomal degradation is required for effective siRNA therapeutics.

Various siRNA carriers such as lipid-based carriers (lipoplexes) [4] and polycation-based carriers (polyplexes) [5] have been developed to fulfill such requirements, including cellular uptake and endosomal escape of siRNA. For example, polyethylenimine (PEI) has been widely studied for siRNA delivery as well as plasmid DNA delivery due to its powerful endosomal escape ability, based on the proton sponge hypothesis [5, 6]. Despite the partial success of such delivery vehicles, toxicity issues derived from the carrier components limit practical applications. Thus, the design of a carrier for efficient siRNA delivery with limited cytotoxicity is of great importance. We previously reported that the cationic polyaspartamide derivative, poly{*N*-[*N*-(2-aminoethyl)-2-aminoethyl]aspartamide} (PAsp(DET)), possessed pH-sensitive endosome destabilizing activity [7]. PAsp(DET) can destabilize the cellular membrane only at the endosomal pH, probably because the mono-protonated form of 1, 2-diaminoethane moiety in the PAsp(DET) side chain at neutral pH is converted to the di-protonated form at the acidic pH condition in the endosome. PAsp(DET)-based polyplexes with plasmid DNA have remarkable transfection efficiency without marked cytotoxicity in various cultured cells, including primary cells [8, 9]. Also, our recent studies have revealed the *in vivo* utilities of PAsp(DET) polyplexes through several animal experiments: (i) local transfection into a rabbit's clamped carotid artery with neointima via intra-arterial injection [10] and into mouse lungs via intra-tracheal injection [11], (ii) systemic delivery into subcutaneous pancreatic tumors via intravenous injection into a mouse tail vein [12], and (iii) transfection into a mouse skull via the regulated release from a calcium phosphate cement scaffold [13].

Although PAsp(DET) polyplexes showed significant success in delivering plasmid DNA into cells, their direct application to siRNA delivery was appreciably limited due to the instability of the polymer/siRNA complexes under physiological conditions. One of the reasons for such lower stability of the PAsp(DET)/siRNA complex is explained by the short and rigid structure of siRNA (21 base pairs long) compared to plasmid DNA (over 3000 base pairs long). The condensation of a single plasmid DNA by polycations provides the polyplex with a stable core, whereas such

condensation does not occur with much shorter siRNAs [14]. Therefore, an additional association force is needed for stable siRNA complex formation.

As for an additional association force, hydrophobic interaction is a promising candidate for stabilization of siRNA complex based on the polyion complex formation. Indeed, hydrophobic group-modified polycations, such as poly(*N*-methyldietheneamine sebacate) (PMDS), PEI, and oligo-arginine, were tested for siRNA delivery, resulting in the formation of stable complexes [15, 16, 17]. Here, we report the development of hydrophobic polycations with high complex stability as well as low cytotoxicity by using PAsp(DET) as the backbone polycation and stearyl groups as a hydrophobic moiety. To optimize the interaction between hydrophobic polycations and siRNA, stearyl PAsp(DET) was synthesized with varying substitution degrees and characterized from the view-point of siRNA complex stability and RNAi activity in cultured cells. Furthermore, the efficient RNAi obtained from stearyl PAsp(DET)/siRNA complexes motivated us to investigate the transfection mechanism, which revealed cellular internalization and intracellular trafficking to be key steps.

2. Materials and methods

2.1. Materials

β -Benzyl-L-aspartate *N*-carboxy-anhydride (BLA-NCA) was synthesized according to Fuchs's method [18]. *N,N*-Dimethylformamide (DMF), dichloromethane (DCM), *n*-butylamine, diethylenetriamine (DET), methanol (MeOH), *N*-hydroxysuccinimide (NHS), and *N*-methyl-2-pyrrolidone (NMP) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Poly(L-lysine) hydrobromide ($M_w = 15,000$ -30,000), Dulbecco's modified eagle's medium (DMEM), DMEM without L-glutamine and phenol red, diisopropylethylamine (DIPEA), stearic acid, and 0.4% trypan blue solution were purchased from Sigma-Aldrich Co. (St. Louis, Mo). 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide, hydrochloride (EDC) was purchased from Dojindo (Kumamoto, Japan). DMF, *n*-butylamine, DET, and DIPEA were distilled with the conventional methods before use. The human pancreatic adenocarcinoma cell line, Panc-1, was obtained from the American Type Culture Collection (Manassas, VA). The luciferase-expressing mouse melanoma cell line, B16F10-Luc was purchased from Caliper LifeScience (Hopkinton, MA). Fetal bovine serum (FBS) was purchased from Dainippon Sumitomo Parma Co., Ltd. (Osaka, Japan). Lipofectamine 2000 and ExGen500 were purchased from Invitrogen (Carlsbad, CA) and Fermentas (Ontario, Canada), respectively. Firefly luciferase siRNA (sense: 5'-CUU ACG CUG AGU ACU UCG AdTdT-3'; antisense: 5'-UCG AAG UAC UCA GCG UAA GdTdT-3'), Cy5-labeled firefly luciferase siRNA, Cy3-labeled firefly luciferase siRNA, Enhanced Green Fluorescence Protein (EGFP) siRNA (sense: 5'-GCA GCA CGA CUU CUU CAA GdTdT-3'; antisense: 5'-CUU GAA GAA GUC GUG CUG CdTdT-3'), BCL-2 (human BCL-2, M13994) siRNA (sense: 5'-CAG GAC CUC GCC GCU GCA GAC-3'; antisense: 3'-CGG UCC UGG AGC GGC GAC GUC UG-5' [19]),

and VEGF (human VEGF, NM_001025366) siRNA (sense: 5'-GGA GUA CCC UGA UGA GAU CdTdT-3'; antisense: 5'-GAU CUC AUC AGG GUA CUC CdTdT-3') were synthesized by Hokkaido System Science Co., Ltd. (Hokkaido, Japan).

2.2 Synthesis

Synthesis methods are available in [Supplement Data](#).

2.3. Preparation and characterization of siRNA complex with stearyl PAsp(DET)

Polycations were dissolved in 10 mM HEPES buffer (pH 7.3) or 50% ethanol solution (ethanol/10 mM HEPES buffer, 1:1 v/v) and then mixed with 20 μ M siRNA solution (10 mM HEPES buffer, pH 7.3) to form siRNA complexes (5 μ M of siRNA) at the desired N/P ratio. Complex size and zeta potential were determined using a Zetasizer (Malvern Instruments, Worcestershire, U.K.) with a He-Ne Laser (λ =633 nm) for the incident beam at a detection angle of 173° and a temperature of 25 °C. The size measurement was performed in a low-volume quartz cuvette (ZEN2112, Malvern Instruments, volume 12 μ L). The data obtained from the rate of decay in the photon correlation function were analyzed by the cumulant method and the corresponding hydrodynamic diameter of the complexes was then calculated by the Stokes-Einstein equation. For zeta potential measurements, each complex solution was placed in a folded capillary cell (Malvern Instruments). Zeta potential was calculated from the measured electrophoretic mobility using the Smoluchowski equation.

2.4. Diffusion coefficient measurement by fluorescence correlation spectroscopy (FCS)

FCS experiments were performed using a Confocor3 module (Carl Zeiss, Jena, Germany) equipped with a Zeiss C-Apochromat 40X water objective. A HeNe laser (543 nm) was used for Cy3-labeled siRNA excitation and emission was filtered through a 560-615 nm band pass filter. Samples were placed into 8-well Lab-Tek chambered borosilicate cover-glass (Nalge Nunc International, Rochester, NY) and measured at room temperature. siRNA stock was prepared to contain 1% Cy3-labeled siRNA concentration, and each analysis of naked Cy3-siRNA, PAsp(DET)/Cy3-siRNA, stearyl PAsp(DET)/Cy3-siRNA complexes (5 μ M siRNA, N/P 5.0), and Rhodamine 6G as a reference in 10 mM HEPES buffer (pH 7.3) consisted of 10 measurements with a sampling time of 20 sec. The measured autocorrelation curves were fitted with the Zeiss Confocor3 software package to obtain the diffusion coefficient, D .

Stability of siRNA complexes in the cell culture condition was evaluated in DMEM without L-glutamine and phenol red containing 10% FBS (DMEM/FBS). siRNA stock was prepared to contain 2% Cy3-labeled siRNA concentration, and each sample of naked Cy3-siRNA, PAsp(DET)/Cy3-siRNA, stearyl PAsp(DET)/Cy3-siRNA complexes (5 μ M siRNA, N/P 5.0) was diluted 10 times with DMEM/FBS and incubated at designated period before measurements.

2.5. Endogenous luciferase gene knockdown in B16F10-Luc

Luciferase-expressing B16F10 cells were seeded into a 96-well plate at a density of 5,000 cells/well in DMEM containing 10% FBS. Firefly luciferase and EGFP siRNA complexes were transfected at 100 nM siRNA. After 48 hr incubation, the media was exchanged and the cells were incubated for another 24 hours. Luciferase gene knockdown was measured using the Luciferase Assay System (Promega) in a luminescence microplate reader (Mithras LB 940, Berthold technologies, Bad Wildbad, Germany).

2.6. BCL-2 endogenous gene knockdown by real-time PCR

Panc-1 cells were seeded into a 6-well plate at a density of 100,000 cells/well in DMEM containing 10% FBS. BCL-2 siRNA complexes were transfected at 100 nM siRNA. After 48-hour incubation, total RNA was collected with an RNeasy Mini kit (Qiagen). Reverse transcription PCR was carried out using a TAKARA PrimeScript RT reagent kit and real-time PCR was measured using a QuantiTect SYBR Green PCR kit (Qiagen) with an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). GAPDH was used as an internal control for the real-time PCR amplification. The mRNAs of BCL-2 and GAPDH were amplified with TAKARA BCL-2 and GAPDH primers (Primer Set ID HA032557 and HA067812, respectively). All procedures followed the manufacturer's protocol.

2.7. VEGF endogenous gene knockdown in real-time PCR

Endogenous VEGF gene suppression in Panc-1 cells was evaluated using the same procedure as BCL-2 gene knockdown with actin used as the internal control. VEGF was amplified using 5'- AGT GGT CCC AGG CTG CAC -3' as the forward primer and 5'- TCC ATG AAC TTC ACC ACT TCG T -3' as the reverse primer. Actin was amplified using 5'- CCT GGC ACC CAG CAC AAT G -3' as the forward primer and 5'- CGC CGAT CCA CAC GGA GTA C -3' as the reverse primer. All experiments were performed in triplicate and data was normalized to actin expression.

2.8. Flow cytometer measurement

B16F10 cells were seeded into a 6-well plate at a density of 100,000 cells/well in DMEM containing 10% FBS. Cy5-labeled siRNA complexes were prepared for each polymer (N/P 5.0) and transfected at 100 nM siRNA. After 3-hour incubation, the media was removed and the cells were washed with 0.5 mL of PBS. The cells were treated with a trypsin-EDTA solution for two minutes and suspended in PBS. The intracellular uptake of the siRNA complexes was measured using a BDTM LSR II flow cytometer (BD Biosciences).

2.9. Confocal laser scanning microscope (CLSM)

Panc-1 cells were seeded into a 35-mm glass-based dish (Iwaki, Tokyo, Japan) at a density of 50,000 cells/well in DMEM containing 10% FBS. The Cy3-labeled siRNA complex of each polymer (N/P 5.0) was transfected at 100 nM siRNA. After the designated incubation period, each dish was observed using a CLSM (ZEISS LSM 510, Carl Zeiss, Oberlochen, Germany) equipped with a C-Apochromat 63X objective (Carl Zeiss). Late-endosomes/lysosomes were stained by LysoTracker

Green (Molecular Probes, Eugene, OR) for 1.5 hours and nuclei were stained with Hoechst 33342 (Dojindo, Japan) for 10 minutes before each observation. 0.4% trypan blue solution was added to media just before observation to quench extracellular-bound Cy3-labeled siRNA [20]. The excitation wavelengths used were 488 nm (Ar laser) for LysoTracker, 543 nm (He-Ne laser) for Cy3-labeled siRNA, and 710 nm (MaiTai laser; two photon excitation; Spectra Physics, Mountain View, CA) for Hoechst 33342.

Statistical colocalization was calculated from Spearman's rank correlation coefficient [21];

$$\rho_s = 1 - 6 \sum \frac{d_i^2}{n(n^2 - 1)}$$

, where a set of n pairs of numbers (x_i, y_i) means the values recorded from the red and green channels of a image, and d_i is the difference between the rank position in the (x_i, y_i) data pair. Because Spearman's rank correlation coefficient can remove the effects of brightness difference between two images, it is suitable for excluding the parameter for the amount of intracellular uptake of Cy3-labeled siRNA.

2.10. Cell viability assay

Cell viability was determined using a Cell Counting Kit-8 (Dojindo, Japan). Panc-1 cells were seeded into a 24-well plate at a density of 20,000 cells/well in DMEM containing 10% FBS. After overnight incubation, siRNA complexes were introduced and cells were then incubated for another 48 hours. The growth medium was exchanged with fresh media (500 μ l) containing the manufacturer's reagent (50 μ l) and the cells were then incubated for 1.5 hours. The absorbance of the media was measured at 450 nm using a microplate reader (Biorad).

3. Results and Discussion

3.1 Synthesis of stearyl PAsp(DET) and stearyl PLL

Stearyl PAsp(DET) was synthesized by amide bond formation between *N*-succinimidyl octadecanoate and the primary amine of PAsp(DET) (Scheme 1). The feed ratio was defined as the relative molar ratio of *N*-succinimidyl octadecanoate to primary amines of PAsp(DET) in the reaction mixture. The resulting introduction rate of the stearyl amide was calculated from the peak intensity ratio between the methyl protons of stearyl moieties (CH_3 -, δ =1.19 ppm) and β -protons of aspartate groups in PAsp(DET) ($-CH_2$ -, δ =3.16 ppm) in the 1H NMR spectra (data not shown). Three different substituted stearyl PAsp(DET)s (12%, 19%, and 32%) were prepared by varying the feed ratio.

As a control polycation without endosomal disrupting ability, 23% stearyl PLL was synthesized in the same fashion by amide bond formation between *N*-succinimidyl octadecanoate and primary amines of PLL. The feed ratio was calculated from the relative molar ratio of *N*-succinimidyl octadecanoate to primary amines of PLL. The resulting stearyl substitution rate was obtained from the peak intensity ratio between the methyl protons of stearyl moieties and ϵ -protons

of the lysine groups ($-CH_2-$, $\delta=3.04-3.17$ ppm) in the 1H NMR spectra (data not shown). A series of the hydrophobic polycations were abbreviated as PAsp(DET)-ST12, PAsp(DET)-ST19, PAsp(DET)-ST32 for 12%, 19%, 32% stearyl PAsp(DET), respectively, and as PLL-ST23 for 23% stearyl PLL.

Scheme 1. Synthetic routes of *N*-succinimidyl octadecanoate (**2**), stearyl PAsp(DET) (**4**), and stearyl poly(L-lysine) (**6**).

3.2 Formation of siRNA complex with stearyl PAsp(DET)

Formation of siRNA complexes with stearyl PAsp(DET) was evaluated by gel electrophoresis at various N/P ratios. The N/P ratio is defined as the molar ratio of the total amines in PAsp(DET) (N) to the phosphates in siRNA (P). Note that stearyl introduction concurrently reduces the number of primary amines (N) of PAsp(DET) with increasing degree of substitution. Thus, the total amines in stearyl PAsp(DET) do not include the primary amines of PAsp(DET) which were converted to amide bonds after stearyl introduction. Because the 1, 2-diaminoethane side chain in PAsp(DET) takes a monoprotonated form at pH 7.3 [7], The free siRNA band disappears above the stoichiometric N/P ratio of complete siRNA formation (N/P=2) (Fig S1). However, smear bands were detected at higher molecular weight positions at N/P=2.5~4.5. Thus N/P $\geq$ 5 was selected for further experiments because all of the PAsp(DET) and stearyl PAsp(DET) derivatives showed the disappearance of the free siRNA and smear band, indicating that all the siRNA molecules were associated with polycations.

The size and zeta potential of siRNA complexes with stearyl PAsp(DET) in 10 mM HEPES buffer (pH 7.3) were determined by dynamic light scattering (DLS) and electrophoretic mobility measurements, respectively, and the results are summarized in Table 1. Stearyl PAsp(DET) complexes (N/P 5 ~ N/P 10) ranged in size from 120 nm to 200 nm with a polydispersity index (PDI) of less than 0.2. Zeta potentials of the siRNA complexes showed relatively constant values (+30 mV ~ +40 mV) irrespective of the stearylation degree. On the other hand, the siRNA complex formed with unmodified PAsp(DET) exhibited appreciably low scattered light intensity close to the background of the solvent. In this regard, complex formation was further evaluated by FCS with Cy3-labeled siRNA, which allows determination of the diffusion coefficient of a complex in a very small volume (~femto L) [22, 23]. All the stearyl PAsp(DET) complexes had a one order of magnitude lower diffusion coefficient than naked siRNA and unmodified PAsp(DET) complex (Table 2). Considering that the diffusion coefficient is reversely correlated to the hydrodynamic diameter of the complexes, with the assumption that siRNA complexes are spherical, the obtained result confirmed that stearyl introduction would contribute to the formation of larger complexes with higher association numbers, compared to the unmodified PAsp(DET). It is worth noting that the diffusion coefficients of the PAsp(DET)-ST12, ST19, and ST32 complexes measured by FCS are

roughly correlated to those measured by DLS (3.27 ± 0.13 , 2.79 ± 0.32 , and $2.59 \pm 0.26 \mu\text{m}^2/\text{sec}$).

Table 1
Size and zeta potential of stearyl PAsp(DET)/siRNA complexes

Stearyl introduction ratio	N/P	Stearyl ratio	Zeta Potential (mV)
10%	1	100:1	20.50 ± 0.1
	10	100:10	20.50 ± 0.1
	100	100:100	20.50 ± 0.1
19%	1	100:1	20.50 ± 0.1
	10	100:10	20.50 ± 0.1
	100	100:100	20.50 ± 0.1
32%	1	100:1	20.50 ± 0.1
	10	100:10	20.50 ± 0.1
	100	100:100	20.50 ± 0.1

Table 2
Diffusion coefficient of stearyl PAsp(DET)/siRNA complexes

Complex	Diffusion coefficient ($\mu\text{m}^2/\text{sec}$) ± S.D.
Naked siRNA	50.20 ± 0.18
Unmodified PAsp(DET)	20.50 ± 0.14
PAsp(DET)-ST12	2.17 ± 0.03
PAsp(DET)-ST19	1.89 ± 0.03
PAsp(DET)-ST32	1.46 ± 0.02

The stability of stearyl PAsp(DET)/siRNA complex was investigated in cell culture conditions. The diffusion coefficient was monitored over time by FCS to estimate the dissociation tendency of siRNA complexes in DMEM containing 10% FBS (Fig 1). The diffusion coefficient of unmodified PAsp(DET)/siRNA complex was increased to that of naked siRNA with increased incubation period, indicating that the unmodified PAsp(DET)/siRNA complex was completely dissociated in a 3-hour incubation period. In contrast, stearyl PAsp(DET)/siRNA complexes maintained their initial diffusion coefficient for a 3-hour incubation period. This result indicates the higher stability of stearyl PAsp(DET) complexes than the unmodified PAsp(DET) complex in the serum-containing media. These results reveal that the stearyl PAsp(DET) complex tolerates the cell culture condition better than does the unmodified PAsp(DET) complex, allowing for improved cellular internalization.

Fig. 1. Time-dependent change in diffusion coefficients of siRNA complexes in the cell culture medium measured by FCS. Curves show naked siRNA(●), unmodified PAsp(DET)/siRNA (○), PAsp(DET)-ST12 (▼), PAsp(DET)-ST19 (▲), and PAsp(DET)-ST32 (■) complexes.

3.3 Gene expression inhibition by stearyl PAsp(DET)/siRNA complexes

To determine the optimal stearylization degree for efficient gene knockdown, the luciferase gene knockdown ability of stearyl PAsp(DET)/siRNA complexes was measured in luciferase-expressing B16F10 cells (Fig 2). siRNA complexes were prepared with stearyl PAsp(DET) comprising different stearyl introduction ratios at various N/P ratios. The luminescence intensity of non-treated cells was set as 1.0, and EGFP siRNA was used as a negative control. Unmodified PAsp(DET) complex showed almost no luciferase gene knockdown at the tested N/Ps. However, stearyl introduction significantly enhanced the gene suppression efficacy. Especially, the PAsp(DET)-ST19 showed 60% gene suppression at the N/P ratios of 7.5 and 10. Apparently, stearyl introduction had an optimal stearylization ratio in the gene suppression tendency. Considering that all the stearyl PAsp(DET) (12%, 19%, and 32%) had almost the same cellular uptake in 24-hour incubation by

flow cytometer measurement (data not shown), 19% stearoyl introduction was the optimal ratio, possibly because of the excellent balance between siRNA release via the intracellular dissociation and sufficient stability of the siRNA complex in the serum-containing media (Fig 1). It is worth mentioning that none of the tested complexes showed significant gene knockdown when prepared containing EGFP siRNA, suggesting low cytotoxicity and minimal off-target effects of PAsp(DET)-based complexes.

Fig. 2. Luciferase gene knockdown by siRNA complexes of stearoyl PAsp(DET) with different substitution degrees in the B16F10-Luc cell. EGFP siRNA (black) was used as a control for luciferase siRNA (gray). The siRNA complexes were treated at 100 nM concentration for 72-hour incubation. Results are expressed as the mean $\pm$ S.D. (n=4).

Based on this result, the PAsp(DET)-ST19 complex was further examined for the knockdown ability of a therapeutic gene. VEGF, a well-known regulator of angiogenesis, was selected as the target gene for siRNA knockdown [24]. The endogenous VEGF gene knockdown efficacy of the PAsp(DET)-ST19 complex in Panc-1 cells was compared with two commercially available transfection reagents, Lipofectamine2000 and the linear polyethylenimine-based reagent ExGen500. VEGF mRNA levels were evaluated by real-time PCR (Fig 3A). The PAsp(DET)-ST19/siRNA complex exhibited around 60% VEGF mRNA reduction, similar to Lipofectamine2000 and much higher than ExGen500 (15% reduction). Furthermore, the amount of VEGF protein secreted from Panc-1 cells was also measured by immunoassay techniques. The VEGF protein production in cells treated with the PAsp(DET)-ST19 complex was also reduced to approximately 50% (Fig S2).

Another therapeutic gene, anti-apoptotic BCL-2, was also selected to evaluate the potential for future siRNA therapeutics in cancer treatments. The BCL-2 protein has been a common cause of tumor genesis and chemotherapy resistance in various types of tumors [25]. Anti-cancer drug treatment with the reduction of BCL-2 protein expression has been one of the approaches to induce apoptosis in cancer cells [26]. The BCL-2 siRNA sequence used here was selected from a previously published work that indicated this sequence showed high *in vivo* gene knockdown efficacy [19]. In this experiment, the PAsp(DET)-ST19 was compared with 23% stearoyl PLL (PLL-ST23) to confirm the backbone superiority for siRNA delivery because PLL is a polycation with poor endosomal escape ability (Fig 3B). After 48 hours of incubation, the BCL-2 mRNA level was evaluated by real-time PCR. The PLL-ST23 showed almost no endogenous gene knockdown or slightly increased BCL-2 expression at the examined N/Ps. On the other hand, gene knockdown was significantly improved with the PAsp(DET)-ST19 complex, with mRNA expression reduced over 60%. The obtained result suggests that the endosomal escaping ability of PAsp(DET) might be

crucial for efficient gene knockdown.

Fig. 3. Endogenous gene knockdown by various siRNA complexes. (A) Comparison of stearyl PAsp(DET) with ExGen500 and Lipofectamine2000 for endogenous VEGF knockdown by real-time PCR. Each sample was transfected in the Panc-1 cells at 100 nM siRNA concentration for 48 hours. The VEGF mRNA amount was normalized with actin mRNA as an internal control. (B) Comparison of stearyl PAsp(DET) with stearyl PLL for anti-apoptotic BCL-2 endogenous gene knockdown measured by real-time PCR. Each sample was transfected in the Panc-1 cells for 48 hours. The BCL-2 mRNA amount was normalized with GAPDH mRNA as an internal control. In both experiments, luciferase siRNA was used as a control. Results are expressed as the mean $\pm$ S.D. (n=4).

3.4 Cellular uptake and intracellular trafficking of stearyl PAsp(DET)

The transfection results revealed that stearyl introduction into a PAsp(DET) polymer backbone led to improved siRNA delivery. To elucidate the reason for this improvement, the cellular uptake and intracellular trafficking of the stearyl PAsp(DET)/siRNA complex were monitored by flow cytometry and CLSM using fluorescence dye-labeled siRNA. B16F10 cells were incubated with naked Cy5-labeled siRNA, Cy5-labeled siRNA complexes of unmodified PAsp(DET) and PAsp(DET)-ST19 for three hours and analyzed by flow cytometer (Fig 4). Stearyl introduction clearly improved the intracellular uptake of siRNA complexes formed with PAsp(DET)-ST19. The uptake of PAsp(DET)-ST19 complex (mean fluorescence 2.3×10^3) was significantly higher than those of unmodified PAsp(DET) complex (8.7×10^2) and naked siRNA (7.4×10^2). This result is probably due to the higher stability of siRNA complexes with stearyl groups in the cell culture condition (Fig 1). The uptake of unmodified PAsp(DET) complex was shown to be similar to that of naked siRNA, indicating that cellular uptake of unstable siRNA complex is not efficient. On the other hand, the cellular uptake of PAsp(DET)-ST19 and PLL-ST23 was compared in Panc-1 cells. Although the PLL-ST23 complex showed no gene knockdown in Fig 3B, it (1.3×10^4) was internalized more efficiently than the PAsp(DET)-ST19 complex (1.0×10^4) (data not shown),

indicating that steps following internalization, such as endosome escape, are crucial for siRNA-mediated gene knockdown.

Fig. 4. Cellular uptake of the siRNA complexes in B16F10 cells. Each peak represents the cells treated by naked siRNA (A), unmodified PAsp(DET) (B), and PAsp(DET)-ST19 (C) siRNA complexes (N/P 5.0) from the left

The endosome escape behavior of the PAsp(DET)-ST19 complex was compared with that of the PLL-ST23 complex by CLSM observation in Panc-1 cells (Fig 5). The siRNA was labeled with Cy3 (red), and late-endosomes and lysosomes were stained with LysoTracker Green (green). Nuclei were stained with Hoechst 33342 (blue). Cy3-labeled siRNA colocalized in late-endosomes and lysosomes should be shown as yellow, and endosomal escape of the siRNA would induce the recovery of red color from yellow over time. After 3-hour incubation, Cy3-labeled siRNA of the PLL-ST23 complex was observed mainly in the cell interior with yellow fluorescence and localized in small confined spaces (Fig 5A), indicating that the majority of Cy3-labeled siRNA was trapped in late-endosomes/lysosomes and only a small portion of the complex escaped from the organelles. Even after 24-hour incubation (Fig 5B), very little red fluorescence was observed in the cytoplasm, whereas large yellow regions were still observed, indicating that the major stearyl PLL complexes were still trapped in the late-endosome/lysosome compartments. In contrast, the PAsp(DET)-ST19 complex exhibited some red fluorescence surrounding yellow late-endosomes/lysosomes even after only 3-hour incubation (Fig 5C). The observation in 24-hour incubation (Fig 5D) showed that red and green fluorescence were separated in the cytoplasm with almost no yellow fluorescence, demonstrating the ability of the stearyl PAsp(DET) to decrease the amount of siRNA molecules trapped in the late-endosomes/lysosomes.

Fig. 5. CLSM images showing intracellular distribution of the siRNA complexes in Panc-1 cells. Panels A and B represent the cells incubated with PLL-ST23 complexes (N/P 5.0) after 3-hour and 24-hour incubations, respectively. Panels C and D represent the cells incubated with PAsp(DET)-ST19 complexes (N/P 5.0) after 3-hour and 24-hour incubations, respectively. Cy3-labeled siRNA and LysoTracker (a late-endosome and lysosome marker) are shown in red and green, respectively. The nucleus was stained with Hoechst 33342 in blue.

The detailed colocalization profile was further analyzed using ImageJ software, which calculates Spearman's rank correlation coefficient from the red and green channels of the confocal

images [21]. The coefficient value ranges from -1 to 1, where 0 indicates that there is no discernable correlation and -1 and +1 mean strong negative and positive correlations, respectively. The correlation coefficient value for the PLL-ST23 complex of the 24-hour incubation was 0.72, which means there was a strong positive correlation between the distribution of red and green fluorescence. In contrast, the correlation coefficient value of the PAsp(DET)-ST19 complex after 24-hour incubation was calculated to be 0.23, indicating little discernable correlation between Cy3-siRNA and late-endosomes/lysosomes. The obtained correlation values quantitatively confirm that stearyl PAsp(DET) significantly decreased the amount of siRNA complexes trapped in late-endosomes/lysosomes. This stronger endosome escape ability is likely responsible for the enhanced siRNA transfection compared to stearyl PLL. The outstanding endosome escape ability of stearyl PAsp(DET) is considered to be mainly due to the pH-dependent protonation of *N*'-(2-aminoethyl)-2-aminoethyl groups in the PAsp(DET) side chain [7]. Consequently, the enhanced gene suppression efficacy of the PAsp(DET)-ST19 appears to be based on the higher cellular uptake and efficient endosomal escape (Fig 4).

3.5 Cell viability of stearyl PAsp(DET)

Low cytotoxicity as well as high knockdown efficacy is critical factors for the development of an effective siRNA carrier. Therefore, cell viability after introduction of stearyl PAsp(DET)/siRNA complexes was measured by the MTT assay in Panc-1 cells (Fig 6). The obtained cell viability was over 90% for each stearyl PAsp(DET) complex, similar to ExGen500 and higher than Lipofectamine 2000 (approximately 80% cell viability), indicating the minimal cytotoxicity of the stearyl PAsp(DET) complexes in this experimental condition.

Fig. 6. Viability of Panc-1 cells treated with each siRNA complex under the same experimental condition as the real-time PCR. Results are expressed as the mean $\pm$ S.D. (n=4).

4. Conclusion

In this study, we demonstrated that stearyl introduction onto PAsp(DET) side chains led to the formation of a siRNA complex with improved *in vitro* RNAi activity. Stearyl introduction significantly enhanced the cellular uptake of siRNA due to complex stabilization via hydrophobic interaction. Additionally, the PAsp(DET) polycation backbone contributed to the excellent endosomal escape of siRNA complexes. Ultimately, the PAsp(DET)-ST19 complexes formed at N/Ps of 7.5 ~ 10 exhibited the best combination of high RNAi activity with low cytotoxicity of the tested samples, including commercially available reagents. The stearyl PAsp(DET)/siRNA complex is a promising candidate for therapeutic siRNA delivery, a result of rational carrier design aimed to overcome specific subcellular barriers.

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References

- [1] L. Aagaard, J.J. Rossi, RNAi therapeutics: Principles, prospects and challenges, *Adv. Drug Deliv. Rev.* 59 (2007) 75-86.
- [2] D.R. Corey, Chemical modification: the key to clinical application of RNA interference?, *J. Clin. Invest.* 117 (2007) 3615-3622.
- [3] D.A. Braasch, Z. Paroo, A. Constantinescu, G. Ren, O.K. Öz, R.P. Mason, D.R. Corey, Biodistribution of phosphodiester and phosphorothioate siRNA, *Bioorg. Med. Chem. Lett.* 14 (2004) 1139-1143.
- [4] A. Akinc, A. Zumbuehl, M. Goldberg, E.S. Leshchiner, V. Busini, N. Hossain, S.A. Bacallado, D.N. Nguyen, J. Fuller, R. Alvarez, A. Borodovsky, T. Borland, R. Constien, A. de Fougères, J.R. Dorkin, K.N. Jayaprakash, M. Jayaraman, M. John, V. Kotliansky, M. Manoharan, L. Nechev, J. Qin, T. Racie, D. Raitcheva, K.G. Rajeev, D.W.Y. Sah, J. Soutschek, I. Toudjarska, H.-P. Vornlocher, T.S. Zimmermann, R. Langer, D.G. Anderson, A combinatorial library of lipid-like materials for delivery of RNAi therapeutics, *Nat. Biotechnol.* 26 (2008) 561-569.
- [5] H. de Martimprey, C. Vauthier, C. Malvy, P. Couvreur, Polymer nanocarriers for the delivery of small fragments of nucleic acids: Oligonucleotides and siRNA, *Eur. J. Pharm. Biopharm.* 71 (2009) 490-504.
- [6] M. Neu, D. Fischer, T. Kissel, Recent advances in rational gene transfer vector design based on poly(ethylene imine) and its derivatives, *J. Gene Med.* 7 (2005) 992-1009.
- [7] K. Miyata, M. Oba, M. Nakanishi, S. Fukushima, Y. Yamasaki, H. Koyama, N. Nishiyama, K. Kataoka, Polyplexes from poly(aspartamide) bearing 1,2-diaminoethane side chains induce pH-selective, endosomal membrane destabilization with amplified transfection and negligible cytotoxicity, *J. Am. Chem. Soc.* 130 (2008) 16287-16294.
- [8] N. Kanayama, S. Fukushima, N. Nishiyama, K. Itaka, W. -D. Jang, K. Miyata, Y. Yamasaki, U. -I. Chung, K. Kataoka, A PEG-based biocompatible block cationomer with high buffering capacity for the construction of polyplex micelles showing efficient gene transfer toward primary cells, *ChemMedChem* 1 (2006) 439-444.
- [9] K. Masago, K. Itaka, N. Nishiyama, U. -I. Chung, K. Kataoka, Gene delivery with biocompatible cationic polymer: pharmacogenomic analysis on cell bioactivity, *Biomaterials* 28 (2007) 5169-5179.
- [10] D. Akagi, M. Oba, H. Koyama, N. Nishiyama, S. Fukushima, T. Miyata, H. Nagawa, K. Kayaoka, Biocompatible micellar nanovectors achieve efficient gene transfer to vascular lesions without cytotoxicity and thrombus formation, *Gene Ther.* 14 (2007) 1029-1038.

- [11] M. Harada-Shiba, I. Takamisawa, K. Miyata, T. Ishii, N. Nishiyama, K. Itaka, K. Kangawa, F. Yoshihara, Y. Asada, K. Hatakeyama, N. Nagaya, K. Kataoka, Intratracheal gene transfer of adrenomedullin using polyplex nanomicelles attenuates monocrotaline-induced pulmonary hypertension in rats, *Mol. Ther.* 17 (2009) 1180-1186.
- [12] K. Miyata, M. Oba, M. R. Kano, S. Fukushima, Y. Vachutinsky, M. Han, H. Koyama, K. Miyazono, N. Nishiyama, K. Kataoka, Polyplex micelles from triblock copolymers composed of tandemly aligned segments with biocompatible, endosomal escaping, and DNA-condensing functions for systemic gene delivery to pancreatic tumor tissue, *Pharm. Res.* 25 (2008) 2924-2936.
- [13] K. Itaka, S. Ohba, K. Miyata, H. Kawaguchi, K. Nakamura, T. Takato, U. -I. Chung, K. Kataoka, Bone regeneration by regulated in vivo gene transfer using biocompatible polyplex nanomicelles, *Mol. Ther.* 15 (2007) 1655-1662.
- [14] S.C. de Smedt, J. Demeester, W.E. Hennink, Cationic polymer based gene delivery systems, *Pharm. Res.* 17 (2000) 113-126.
- [15] Y. Wang, S. Gao, W.-H. Ye, H.S. Yoon, Y.-Y. Yang, Co-delivery of drugs and DNA from cationic core-shell nanoparticles self-assembled from a biodegradable copolymer, *Nat. Mater.* 5 (2006) 791-796.
- [16] A. Alshamsan, A. Haddadi, V. Incani, J. Samuel, A. Lavasanifar, H. Uludag, Formulation and delivery of siRNA by oleic acid and stearic acid modified polyethylenimine, *Mol. Pharm.* 6 (2009) 121-133.
- [17] W. J. Kim, L. V. Christensen, S. Jo, J. W. Yockman, J. H. Jeong, Y. -H. Kim, S. W. Kim, Cholesteryl oligoarginine delivering vascular endothelial growth factor siRNA effectively inhibits tumor growth in colon adenocarcinoma, *Mol. Ther.* 14 (2006) 343-350.
- [18] A. Harada, K. Kataoka, Formation of polyion complex micelles in an aqueous milieu from a pair of oppositely-charged block copolymers with poly(ethylene glycol) segments, *Macromolecules* 28 (1995) 5294-5299.
- [19] M. Ocker, D. Neureiter, M. Lueders, S. Zopf, M. Ganslmayer, E.G. Hahn, C. Herold, D. Schuppan, Variants of bcl-2 specific siRNA for silencing antiapoptotic bcl-2 in pancreatic cancer, *Gut* 54 (2005) 1298-1308.
- [20] K. de Bruin, N. Ruthardt, K. von Gersdorff, R. Bausinger, E. Wagner, M. Ogris, C. Bräuchle, Cellular dynamics of EGF receptor-targeted synthetic viruses, *Mol. Ther.* 15 (2007) 1297-1305.
- [21] A.P. French, S. Mills, R. Swarup, M.J. Bennett, T.P. Pridmore, Colocalization of fluorescent markers in confocal microscope images of plant cells, *Nature Protocols* 3 (2008) 619-628.
- [22] M. Meyer, A. Philipp, R. Oskuee, C. Schmidt, E. Wagner, Breathing life into polycations: functionalization with pH-responsive endosomolytic peptides and polyethylene glycol enables siRNA delivery, *J. Am. Chem. Soc.* 130 (2008) 3272-3273.
- [23] J. DeRouchey, C. Schmidt, G. F. Walker, C. Koch, C. Plank, E. Wagner, J. O. Rädler,

Monomolecular assembly of siRNA and poly(ethylene glycol)-peptide copolymers, *Biomacromolecules* 9 (2008) 724-732.

[24] L.M. Ellis, D.J. Hicklin, VEGF-targeted therapy: mechanisms of anti-tumour activity, *Nat. Rev. Cancer* 8 (2008) 579-591.

[25] D.S. Ziegler, A.L. Kung, Therapeutic targeting of apoptosis pathways in cancer, *Curr. Opin. Oncol.* 20 (2008) 97-103.

[26] J. George, N.L. Banik, S.K. Ray, Bcl-2 siRNA Augments Taxol Mediated Apoptotic Death in Human Glioblastoma U138MG and U251MG Cells, *Neurochem. Res.* 34 (2009) 66-78.

Figure Legends

Scheme 1. Synthetic routes of *N*-succinimidyl octadecanoate (**2**), stearoyl PAsp(DET) (**4**), and stearoyl poly(L-lysine) (**6**).

Fig. 1. Time-dependent change in diffusion coefficients of siRNA complexes in the cell culture medium measured by FCS. Curves show naked siRNA(●), unmodified PAsp(DET)/siRNA (○), PAsp(DET)-ST12 (▼), PAsp(DET)-ST19 (△), and PAsp(DET)-ST32 (■) complexes.

Fig. 2. Luciferase gene knockdown by siRNA complexes of stearoyl PAsp(DET) with different substitution degrees in the B16F10-Luc cell. EGFP siRNA (black) was used as a control for luciferase siRNA (gray). The siRNA complexes were treated at 100 nM concentration for 72-hour incubation. Results are expressed as the mean $\pm$ S.D. (n=4).

Fig. 3. Endogenous gene knockdown by various siRNA complexes. (A) Comparison of stearoyl PAsp(DET) with ExGen500 and Lipofectamine2000 for endogenous VEGF knockdown by real-time PCR. Each sample was transfected in the Panc-1 cells at 100 nM siRNA concentration for 48 hours. The VEGF mRNA amount was normalized with actin mRNA as an internal control. (B) Comparison of stearoyl PAsp(DET) with stearoyl PLL for anti-apoptotic BCL-2 endogenous gene knockdown measured by real-time PCR. Each sample was transfected in the Panc-1 cells for 48 hours. The BCL-2 mRNA amount was normalized with GAPDH mRNA as an internal control. In both experiments, luciferase siRNA was used as a control. Results are expressed as the mean $\pm$ S.D. (n=4).

Fig. 4. Cellular uptake of the siRNA complexes in B16F10 cells. Each peak represents the cells treated by naked siRNA (A), unmodified PAsp(DET) (B), and PAsp(DET)-ST19 (C) siRNA complexes (N/P 5.0) from the left.

Fig. 5. CLSM images showing intracellular distribution of the siRNA complexes in Panc-1 cells. Panels A and B represent the cells incubated with PLL-ST23 complexes (N/P 5.0) after 3-hour and 24-hour incubations, respectively. Panels C and D represent the cells incubated with PAsp(DET)-

ST19 complexes (N/P 5.0) after 3-hour and 24-hour incubations, respectively. Cy3-labeled siRNA and LysoTracker (a late-endosome and lysosome marker) are shown in red and green, respectively. The nucleus was stained with Hoechst 33342 in blue.

Fig. 6. Viability of Panc-1 cells treated with each siRNA complex under the same experimental condition as the real-time PCR. Results are expressed as the mean $\pm$ S.D. (n=4).

Scheme 1

Table 1**Size and zeta potential of stearoyl PAsp(DET)/siRNA complexes**

Stearoyl Introduction Rate	N/P	Size (nm) ± S.D	Zeta Potential (mV) ± S.D
12%	5	150±6	39.7±8.0
	7.5	136±2	39.0±8.5
	10	122±5	37.4±9.7
19%	5	196±1	29.5±6.1
	7.5	134±4	36.8±5.2
	10	129±1	36.0±5.3
	15	141±1	38.2±6.6
32%	5	187±24	32.0±5.0
	7.5	177±45	34.5±5.1
	10	192±23	35.5±5.0

Table 2

Diffusion coefficient of stearyl PAsp(DET)/Cy3-siRNA complexes

	Diffusion coefficient ($\mu\text{m}^2/\text{sec}$) $\pm$ S.D
Naked Cy3-siRNA	56.26 $\pm$ 0.18
Unmodified PAsp(DET)	33.88 $\pm$ 0.14
PAsp(DET)-ST12	2.17 $\pm$ 0.03
PAsp(DET)-ST19	1.89 $\pm$ 0.03
PAsp(DET)-ST32	1.46 $\pm$ 0.02

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6