1 Actively-targeted Polyion Complex Micelles Stabilized by Cholesterol
2 and Disulfide Cross-linking for Systemic Delivery of siRNA to Solid
3 Tumors

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ABSTRACT

For small interfering RNA (siRNA)-based cancer therapies, we report an actively-targeted and stabilized polyion complex micelle designed to improve tumor accumulation and cancer cell uptake of siRNA following systemic administration. Improvement in micelle stability was achieved using two stabilization mechanisms; covalent disulfide cross-linking and non-covalent hydrophobic interactions. The polymer component was designed to provide disulfide cross-linking and cancer cell-targeting cyclic RGD peptide ligands, while cholesterol-modified siRNA (Chol-siRNA) provided additional hydrophobic stabilization to the micelle structure. Dynamic light scattering confirmed formation of nano-sized disulfide cross-linked micelles (<50 nm in diameter) with a narrow size distribution. Improved stability of Chol-siRNA-loaded micelles (Chol-siRNA micelles) was demonstrated by resistance to both the dilution in serum-containing medium and counter polyion exchange with dextran sulfate, compared to control micelles prepared with Chol-free siRNA (Chol-free micelles). Improved stability resulted in prolonged blood circulation time of Chol-siRNA micelles compared to Chol-free micelles. Furthermore, introduction of cRGD ligands onto Chol-siRNA micelles significantly facilitated accumulation of siRNA in a subcutaneous cervical

1 cancer model following systemic administration. Ultimately, systemically administered
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6 2 cRGD/Chol-siRNA micelles exhibited significant gene silencing activity in the tumor,
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10 3 presumably due to their active targeting ability combined with the enhanced stability
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13 4 through both hydrophobic interactions of cholesterol and disulfide cross-linking.
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18 6 **Keywords:** siRNA delivery; polyion complex micelle; active targeting; cyclic RGD
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1. Introduction

Small interfering RNA (siRNA) inhibits expression of genes by a sequence-specific gene silencing effect, known as RNA interference (RNAi) [1–3]. This property has generated much interest for development of siRNA drugs that inhibit production of proteins associated with disease. However, low bioavailability of siRNA has hampered its translation into clinical use. Efforts to improve the efficacy of siRNA drugs have led to development of many types of siRNA-loaded nanoparticles to overcome biological hurdles associated with siRNA delivery, e.g., enzymatic degradation, accumulation in non-target organs/tissues and inefficient cellular uptake [4–6]. In particular, the ability to target specific cells has proven to be highly effective for enhanced accumulation of nanoparticles in solid tumors through systemic administration and has also been shown to facilitate cellular/subcellular delivery of siRNA [7–16]. Thus, a variety of ligand molecules that bind to specific receptors on cancer cells have been installed on the surface of nanoparticles [7–16]. In order to take full advantage of such targeting ligands, however, maintaining the nanoparticle structure in circulation is essential; targeting ligands can cooperatively function when distributed on the nanoparticle surface, allowing for avidity through multisite binding [13,15,17,18]. Therefore, a highly effective siRNA delivery

1 system should result from incorporating cellular surface-targeting ability to a
2 nanoparticle platform resistant to destabilization (or dissociation), thus maximizing the
3 ligand binding effect.

4 A promising platform for systemic siRNA delivery into solid tumors is the polyion
5 complex (PIC) micelle, constructed with block copolymers of poly(ethylene glycol)
6 (PEG) and a polycation as an siRNA binding segment [13,16,19–22]. Charge
7 neutralization between siRNA and the polycationic segment of the block copolymer in
8 aqueous solution enables formation of PIC micelles, in which the siRNA-loaded PIC core
9 is surrounded by a nonionic and hydrophilic PEG shell. This core-shell structure results in
10 enhanced colloidal stability and reduced nonspecific interactions with charged
11 biomacromolecules. To further increase micelle stability for *in vivo* delivery, several
12 stabilizing approaches via hydrophobic interactions [16,23] or disulfide cross-links
13 [13,24–26] have been investigated so far. Disulfide cross-links are noteworthy as they
14 impart reversible stability to the micelle core upon cleavage (reduction) in the cell interior
15 in response to the increased glutathione concentration, which is 100–1000 times higher
16 than that in the cell exterior [27,28]. Reversible micelle stability is an important feature
17 for nucleic acid delivery vehicles, since siRNA release into the cytoplasm is required to

1 access the RNAi pathway.

2 Meanwhile, our previous studies revealed that siRNA micelles could be disrupted
3 even with disulfide cross-linking in the core, leading to undesirable release of siRNA
4 payloads [26]. These results suggest that the cross-linking within the micelle core may be
5 highly localized, incapable of stabilizing the whole core structure. Thus, an additional
6 stabilizing mechanism may further reinforce the cross-linked siRNA micelle structure,
7 leading to longer blood circulation and enhanced tumor accumulation. Herein,
8 cholesterol-conjugated siRNA (Chol-siRNA) [29] was utilized to stabilize micelle core
9 structures in addition to disulfide cross-linking. Hydrophobized siRNAs are expected to
10 suppress micelle disruption and subsequent leakage of siRNA due to hydrophobic
11 associations of cholesterol groups [16,30]. Therefore, the combined use of a thiolated
12 block copolymer and Chol-siRNA creates a stable, yet reversible, platform for improved
13 systemic siRNA delivery.

14 In this work, we employed a functional block copolymer comprising PEG segment
15 installed with cyclo-Arg-Gly-Asp (cRGD) peptide as the tumor-targeting hydrophilic
16 block [13,16,31] and poly(L-lysine) (PLL) segment modified with
17 dithiobispropionimidate (DTBP) as the cationic block [26]. DTBP modification was

chosen for generating a single and stable side chain structure comprising an amidine and thiol functionality, making polyionic pairs/hydrogen bonds with siRNA phosphates in addition to disulfide cross-linking [26]. After examining the contribution of to micelle stability, the targeting ability of cRGD ligand was verified utilizing a luciferase-expressing cervical cancer (HeLa-Luc) cell line. Finally, the *in vivo* siRNA delivery efficacy of the actively-targeted/stabilized micelles was evaluated by luciferase gene silencing activity in the murine subcutaneous tumors after systemic administration, demonstrating strong potential for tumor-targeted systemic siRNA delivery.

2. Materials and methods

2.1. Materials

D₂O (99.9%), tetramethylsilane (TMS, 99.5%), boric acid, trizma base and Dulbecco's modified Eagle's medium (DMEM) were purchased from Sigma Aldrich (St. Louis, MO) and used without further purification. Dithiothreitol (DTT, molecular biology grade DNase and RNase free), ethylenediamine tetraacetic acid disodium salt dihydrate (EDTA, 99.5%) and ethidium bromide solution were supplied by Wako Pure Chemical Industries (Osaka, Japan). Dimethyl-3,3'-dithiobispropionimidate/2HCl

(DTBP/HCl) and slide-a-lyzer dialysis cassettes (MWCO 3,500 Da) were purchased from Thermo Scientific (Rockford, IL). Sterile HEPES (1 M, pH 7.3) was purchased from Amresco (Solon, OH). Agarose L03 TAKARA was purchased from Takara Bio Inc (Shiga, Japan) and used for gel electrophoresis. Cell Counting Kit 8 (CCK-8) was purchased from Dojindo Laboratories (Kumamoto, Japan). Cyclo-[RGDfK(C- ϵ -Acp)] (cRGD) peptide was synthesized by Peptide Institute Inc. (Osaka, Japan). A series of siRNAs were synthesized by Hokkaido System Science Co., Ltd. (Hokkaido, Japan) and their sequences were as follows; (1) firefly GL3 luciferase (siLuc): 5'-CUU ACG CUG AGU ACU UCG AdTdT-3' (sense), 5'-UCG AAG UAC UCA GCG UAA GdTdT-3' (antisense); (2) control scramble sequence (siScr): 5'-UUC UCC GAA CGU GUC ACG UdTdT-3' (sense), 5'-ACG UGA CAC GUU CGG AGA AdTdT-3' (antisense). Cy5 dye and Chol moiety were introduced to the 5'-end of the antisense strand and the sense strand, respectively.

2.2. Synthesis of cyclic RGD peptide-poly(ethylene glycol)-block-poly(L-lysine) (cRGD-PEG-PLL)

cRGD-PEG-PLL and methoxy-PEG-PLL (termed PEG-PLL) block copolymers (molecular weight (MW) of PEG: 12,000 Da; degree of polymerization (DP) of PLL

segment: approximately 45) were synthesized as previously described [13,19,31]. The cRGD peptide was introduced to the PEG terminus of acetal-PEG-PLL through the thiazolidine ring formation between the N-terminal cysteine of cRGD and the aldehyde group generated in acetal-PEG-PLL after incubation at acidic pH [31]. The obtained polymer (yield: 112 mg, 86%) was characterized at 22 °C by ¹H NMR (JEOL ECS-400, JEOL, Tokyo, Japan). The amount of cRGD conjugated to the polymer was estimated from ¹H NMR spectrum based on the peak intensity ratio of phenyl protons in cRGD peptide (D-Phe, δ = 7.2–7.4 ppm) to ethylene protons in PEG ($-CH_2CH_2-$, δ = 3.6–3.8 ppm) (Fig. S1). The cRGD introduction rate was calculated to be approximately 70%.

2.3. Synthesis of cyclic RGD peptide-poly(ethylene glycol)-block-poly(L-lysine modified with 1-(3-mercaptopropyl)amidine) (cRGD-PEG-PLL(MPA))

cRGD-PEG-PLL(MPA) was synthesized by introducing MPA moieties into the lysine primary amines in cRGD-PEG-PLL using DTBP, as shown in Scheme 1 [26]. Briefly, cRGD-PEG-PLL (50 mg, 0.1 mmol amines) was dissolved in 100 mM borate buffer (pH 9.0) (10 mL), followed by the addition of DTBP/HCl (69 mg, 0.2 mmol). The reaction solution was stirred at 25 °C for 45 min and then dialyzed (MWCO 3,500 Da) against 10 mM PBS (pH 6.0) for 2 h and distilled water for 2 h. The dialyzed

1 solution was lyophilized after filtration (yield: 53.9 mg, 80%). The substitution degree
2 of MPA groups in the obtained polymer was determined from the ^1H NMR spectrum
3 based on the peak intensity ratio of β , γ and δ methylene protons in PLL side chains
4 ($-(\text{CH}_2)_3-$, $\delta = 1.3\text{--}1.9$ ppm) to mercaptoethyl protons in MPA moieties ($\text{HS}-(\text{CH}_2)_2-$, δ
5 $= 2.7\text{--}2.9$ ppm) (Fig. 1), demonstrating quantitative introduction ($>97\%$). In a similar
6 manner, PEG-PLL(MPA) without cRGD peptide was synthesized by the reaction of
7 PEG-PLL with DTBP (Scheme 1). For PEG-PLL(MPA), PEG-PLL (300 mg, 0.71
8 mmol amines) and DTBP/HCl (439 mg, 1.4 mol) were used (yield: 299 mg, 73%).
9 Quantitative introduction of MPA group ($>99\%$) was also confirmed by ^1H NMR
10 analysis (Fig. S2).

11 2.4. Polyionic complexation of block copolymers with siRNA

12 cRGD-PEG-PLL(MPA) (or PEG-PLL(MPA)) block copolymer was dissolved in 10
13 mM HEPES buffer (pH 7.4) and incubated with 100 mM DTT at 25 °C for 15 min for
14 disulfide reduction. The reduced polymer solution was mixed with siRNA dissolved in
15 the same buffer (15 μM siRNA) at different molar charge ratios of the block copolymer
16 to siRNA, i.e., amidines and primary amines in the block copolymer/phosphates in
17 siRNA. To proceed disulfide cross-linking in the PIC core, PIC solutions (10 μM

siRNA) were dialyzed (MWCO 3,500 Da) against 5 mM HEPES (pH 7.4) containing 0.5% (v/v) DMSO for 2 days and 5 mM HEPES (pH 7.4) for 2 days. Dialyzed PIC solutions were filtered (0.22 μ m) before characterization. In a similar manner, control PICs without Chol moieties were also prepared with PEG-PLL(MPA) and siRNA. For *in vivo* experiments, micelle solutions were dispersed in 5 mM HEPES (pH 7.4) and made isotonic by addition of 1.5 M NaCl to a final concentration of 150 mM.

2.5. Static and dynamic light scattering (SLS and DLS) analyses

SLS and DLS measurements were performed with a ZetaSizer Nano ZS instrument (Malvern Instruments Ltd., Worcestershire, UK) equipped with a He-Ne laser ($\lambda = 633$ nm) as the incident beam. All measurements were made at 25 °C and a detection angle of 173°. PIC samples (10 μ M siRNA, 18 μ L) dispersed in 5 mM HEPES buffer (pH 7.4) were loaded into a low-volume cuvette (Zen 2112) for each analysis. Scattered light intensity (SLI) was measured to monitor micelle formation, using a constant attenuator setting in the instrument. Cumulant size, polydispersity index (PDI), and size distribution (intensity-weighted) histogram were calculated based on the autocorrelation function of samples, with automated attenuator adjustment and multiple scans (typically 12–30 scans) for optimal accuracy.

2.6. Fluorescence correlation spectroscopy (FCS) measurement

Diffusion time of fluorescently-labeled micelles (or naked siRNA) was measured by FCS using samples prepared with Cy5-labeled siRNA (Cy5-siRNA). Samples containing 10 μ M Cy5-siRNA were diluted with PBS containing 10% FBS, followed by 1 h incubation. Then, the samples were placed into an 8-well Lab-Tek chambered borosilicate cover-glass (Nalge Nunc International, Rochester, NY) and measured with a combination system of ConfoCor3 module and LSM510 equipped C-Apochromat 40 $\times$, N.A. 1.2 water immersion objective (Carl Zeiss, Oberkochen, Germany) (sampling time: 10 s, repeating time: 10). A He-Ne laser (633nm) was used for excitation of Cy5-siRNA and emission was filtered through the corresponding band-pass filter. The measured autocorrelation curves were fitted with the Zeiss Confocor3 software package to obtain the corresponding diffusion time. The obtained diffusion time was converted to the corresponding hydrodynamic diameter by the Stokes-Einstein equation.

2.7. Gel electrophoresis

Agarose gel was prepared by adding agarose (0.7 g) to a TBE buffer (70 mL) prepared with trizma base (10.8 g), boric acid (5.5 g) and EDTA/2Na (74 mg) in pure water (1 L). The mixture was heated to dissolve the agarose powder and then allowed to

1 cool down before adding ethidium bromide (1 μ L). Micelle solutions (2 μ L) with or
2 without DTT (200 mM DTT) were mixed with dextran sulfate solutions (2 μ L). The
3 concentrations of dextran sulfate groups were set at 0, 1.0, 1.5, 2.0 and 3.0 times
4 equivalent to the siRNA phosphates. After incubation for 2 h at 25 °C, each sample was
5 loaded on the gel and electrophoresed (100 V, 30 min) in TBE running buffer. After
6 electrophoresis, gel images were captured by a Typhoon 9410 instrument equipped with
7 a 532 nm laser. Also, micelle samples were subjected to the electrophoresis (50 V, 30
8 min) after incubation with different amounts of anionic lipid, DOPS
9 (1,2-dioleoyl-sn-glycero-3-phospho-L-serine sodium salt), for 8 h at 25 °C.

10 2.8. Cellular uptake

11 Luciferase-expressing human cervical cancer cells, HeLa-Luc (Caliper LifeScience,
12 Hopkinton, MA), were seeded into 6-well plates (100,000 cells/well) and incubated in
13 DMEM containing 10% fetal bovine serum (FBS) (Dainippon Sumitomo Pharma Co.
14 Osaka, Japan) (1.5 mL) for 24 h. The culture media were then exchanged with fresh
15 media containing micelle samples prepared with Cy5-siRNA (siRNA concentration:
16 333 nM). Cells were incubated in the presence of micelles for 75 min at 37 °C and then
17 further incubated in the absence of micelles for 8 h after medium exchange. The cells

1 were harvested by trypsinization, followed by flow cytometric analyses using a BD
2 LSR II instrument (BD Biosciences, San Jose, CA) and BD FACS Diva software (BD
3 Biosciences).

4 2.9. *In vitro* luciferase assay

5 HeLa-Luc cells were seeded in 35 mm dishes (25,000 cells/dish) and incubated in
6 DMEM containing 10% FBS (2 mL) for 24 h. Then, the culture media were exchanged
7 with fresh media (2 mL) containing 100 μ M luciferin (Summit Pharmaceutical
8 International, Tokyo, Japan) and micelle samples (200 nM siRNA). For each analysis,
9 control samples were prepared by treating cells with 5 mM HEPES buffer (pH 7.4).
10 Samples were placed into a Kronos real-time photon countable incubator (ATTO Corp.,
11 Tokyo, Japan) at 37 °C and 5% CO₂ and the luminescence intensity (counts) was
12 measured periodically over 50 h. Relative luminescence intensity was determined by
13 normalizing the average luminescence intensity of treated samples (n = 4) to the
14 average luminescence intensity of control samples (n = 4).

15 2.10. Cytotoxicity assay

16 HeLa-Luc cells were seeded in 24-well plates (20,000 cells/well) and incubated in
17 DMEM containing 10% FBS (0.5 mL) for 24 h. Thereafter, the culture media were

1 replaced with fresh media containing micelle samples at varying siRNA concentrations
2 and cells were further incubated for 48 h. Then, CCK-8 solution (1 μ L/10 μ L medium)
3 was added and samples were further incubated at 37 °C for 1 h. Cell viability was
4 determined from the absorbance of extracellular media at 450 nm, which was measured
5 using a BIO-RAD 680 microplate reader (Bio-Rad, CA). All data are expressed relative
6 to untreated control cells (n = 6).

7 *2.11. Blood circulation behavior*

8 All animal experimental procedures were performed in accordance with the
9 policies of the Animal Ethics Committee of The University of Tokyo. The blood
10 circulation property of siRNA-loaded micelles was determined by IVRT-CLSM
11 observation of live mice (BALB/c nude, female, 8 weeks old) (Charles River
12 laboratories, Tokyo, Japan) using a Nikon A1R CLSM system attached to an upright
13 ECLIPSE FN1 (Nikon Corp., Tokyo, Japan) equipped with a 20 $\times$, 640 nm diode laser,
14 and a band-pass emission filter of 700/75 nm, as previously described [32]. The mice
15 were anesthetized with 2–3% isoflurane (Abbott Japan Co., Ltd., Tokyo, Japan) using a
16 Univenter 400 anaesthesia unit (Univentor Ltd., Zejtun, Malta) and placed onto the
17 temperature-controlled microscope stage. Blood vessels in the ear-lobe dermis were

1 observed after tail vein injection of micelles prepared with Cy5-siRNA (3.6 nmol
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6 2 Cy5-siRNA/mouse, n = 3), as they were clearly observed without surgery (thus
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9 3 non-invasive) and were unaffected by the cardiac beat (thus steadily observed). The
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13 4 obtained images were analyzed by selecting regions of interest (ROIs) within blood
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16 5 vessels for determining the average fluorescence intensity at each time point. To
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20 6 produce blood circulation profiles shown in Fig. 5A, the vein fluorescence intensities
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23 7 were expressed as a relative value to the highest (1.0) and the lowest (0) obtained
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27 8 through the observation. In addition, the time point for the highest fluorescence
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31 9 intensity was defined as $t = 0$.

10 2.12. Tumor accumulation

11 HeLa-Luc cells (2.6×10^6 cells) were injected under the skin of mice for
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13 preparation of donor tumors. After 2 weeks, the donor tumors were excised and cut into
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15 pieces, then transplanted under the skin of host mice. After 8 days, the subcutaneous
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17 tumor-bearing mice were subjected to tail vein injection of micelles prepared with
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15 Cy5-siRNA (1.8 nmol Cy5-siRNA/mouse, n = 4–5). After 4 h, they were sacrificed, and
16 tumors (and major organs) were excised, followed by fluorescence quantification using
17 an IVIS instrument equipped with Living Image software. Total photon counts were

1 normalized to the illumination time and the sample area as follows: fluorescence
2 intensity = total photons/[illumination time (s) × area (cm²)]. Further normalization was
3 performed by dividing the fluorescence intensity from the tumors treated with RGD-free
4 controls.

5 2.13. *In vivo* luciferase assay

6 Subcutaneous HeLa-Luc tumor models were prepared by *in vivo* passage of tumor
7 fragments, as described in the former section. After 8 days, the tumor-bearing mice
8 were subjected to the tail vein injection of micelle samples (1.8 nmol
9 siRNA/mouse/injection, n = 5) at 48 h, 38 h and 24 h before measurement. A luciferin
10 solution (50 mM, 200 μL) was injected intraperitoneally 25 min prior to measurement
11 to obtain the stable luminescence intensity from tumors. The luminescence intensity
12 (counts) was recorded using an IVIS instrument under the same condition for each
13 image (acquisition time: 5 sec, binning: small, f/stop: 1, and field of view: D). Tumor
14 volumes were determined by manual measurement with a caliper and calculated using
15 the following equation: volume = $ab^2/2$, where a is the long axis and b is the short axis
16 measured. The luminescence intensity obtained from sample-treated tumors was
17 normalized to the tumor volume and further to that from buffer-treated control tumors:

1 relative luminescence intensity = total photons of sample / sample-treated tumor volume
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6 2 (cm³) / total photons of buffer control / control-treated tumor volume (cm³).
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9 2.14. Data analysis

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12 4 The experimental data were analyzed by Student's *t*-test. *p* <0.05 was considered
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21 3. Results and discussion

22 3.1. Synthesis of block copolymers and their characterizations

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27 8 In order to create actively-targeted and stabilized PIC micelles, a functional block
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34 10 copolymer was synthesized to comprise a targeting ligand, cationic charges, and free
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38 11 thiol groups (Scheme 1). The cRGD peptide was utilized as the ligand for tumor
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41 12 targeting through specific binding to $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins, which are overexpressed
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45 13 on various cancer cells [33,34]. Also, DTBP was selected as the thiolation reagent
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48 14 because a cationic amidine group is concurrently introduced following reaction with
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52 15 lysine amines, preserving the polymer ability to form ion pairs with siRNA phosphates
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55 16 [26]. First, the cRGD peptide was conjugated to acetal-PEG-PLL by thiazolidine ring
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59 17 formation between the aldehyde generated on the PEG terminus at low pH and the
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1 N-terminal cysteine residue contained on the cRGD peptide [31]. Next,
2 cRGD-PEG-PLL (or PEG-PLL as a non-targeted control) was reacted with DTBP under
3 basic conditions to generate cRGD-PEG-PLL(MPA) (or PEG-PLL(MPA)) bearing the
4 desired amidine and thiol functionalities in PLL side chains. Successful introduction of
5 cRGD (71%) and MPA modification of amines (97%) was confirmed by ¹H NMR
6 analysis of the cRGD-PEG-PLL(MPA) reaction product (Fig. 1). Similarly, quantitative
7 reaction of DTBP with lysine amines in PEG-PLL lacking cRGD (>99%) was also
8 confirmed (Fig. S2).

3.2. Preparation and characterization of Chol-siRNA micelles

9 Micelle formation behavior with Chol-siRNA was investigated in terms of SLI, size
10 and PDI by light scattering measurements (SLS and DLS). SLI values were utilized for
11 confirming whether i) the signal intensity (or S/N ratio) of samples was suitable for
12 fitting and ii) the polymer/siRNA mixtures formed multimolecular assemblies (i.e.
13 micelles) prior to fitting. The size and the PDI were then determined by appropriate
14 fitting. PEG-PLL(MPA) was mixed with Chol-siRNA or Chol-free siRNA as a control
15 at varying residual molar charge ratios of the block copolymer to siRNA (+/- ratio). SLI
16 values observed for polymer/Chol-siRNA mixture increased sharply in +/- ratios from 0

to 1.2 (Fig. 2A), similar to those for Chol-free siRNA mixture, indicating formation of PIC micelles due to the charge-neutralization between the polymer and siRNA. At $\pm = 1.4$, Chol-siRNA micelles were 43 ± 2 nm ($n = 3$) in cumulant diameter, and exhibited the lowest level of PDI (Fig. S3) as well as the highest SLI in the charge ratios tested. This slightly shifted $\pm$ ratio from the charge-stoichiometric point is presumably due to the fact that complete ion-pair formation within PICs can be sterically hindered in block copolymers [26,35]. The intensity-weighted histograms at this mixing ratio clearly show the similar size distributions between Chol-siRNA and Chol-free micelles (Fig. 2B). These results confirm that the Chol moiety did not alter the micelle formation behavior between PEG-PLL(MPA) and siRNA at $\pm < 1.4$. Note that a remarkable difference was observed between the two micelle formulations in their SLI values at $\pm > 1.4$. The Chol-free siRNA mixture showed a drastic decrease in SLI, possibly due to the binding of a charge-excess amount of block copolymers to siRNA, generating electrostatic repulsion between PICs, and thus impeding their self-assemble into micelle structures [26]. In contrast, the Chol-siRNA mixture greatly resisted such decrease in SLI, indicating a stronger association force of Chol-siRNA PICs that can overcome electrostatic repulsion [30]. Meanwhile, the effect of MPA moieties in PEG-PLL(MPA)

was additionally examined on the polyion complexation with Chol-siRNA by comparing with non-thiolated PEG-PLL. The PEG-PLL/Chol-siRNA mixtures showed the PDI of >0.18 in the tested mixing ratios from 0 to 4 (Fig. S3). These results demonstrate the crucial role of MPA moieties for the uniform micelle formation between Chol-siRNA and PEG-PLL(MPA); a possible explanation for the role of MPA moiety is that a higher degree of MPA moiety, i.e., thiol and guanidino groups, in the polycationic side chain could allow stronger intermolecular interactions with each other and/or siRNA through hydrogen bonding, dipole-dipole interactions, or Van der Waals force, directed toward more uniform and stable micelle formation. Altogether, siRNA micelles prepared at $\pm = 1.4$ were used for subsequent experiments owing to the similarity in size between the two formulations as well as the sample homogeneity (lowest PDI). It should be further noted that the size distribution histograms were similar between non-targeted Chol-siRNA micelles prepared with PEG-PLL(MPA) and targeted Chol-siRNA micelles prepared with cRGD-PEG-PLL(MPA) at $\pm = 1.4$ (Fig. S4), suggesting that the cRGD peptide does not affect PIC formation with Chol-siRNA.

3.3. Stability of Chol-siRNA micelles

To confirm the stabilizing effect of cholesterol, the sizes of Chol-siRNA micelles

1 and Chol-free micelles were determined under diluted conditions in 10%
2 FBS-containing PBS by FCS measurement, which allows monitoring of the
3 hydrodynamic size (or diffusion coefficient) of fluorescently labeled nanoparticles even
4 in the presence of abundant serum proteins [16]. Indeed, this measurement determined
5 the diffusion coefficients of Cy5-siRNA or Chol/Cy5-siRNA molecules complexed with
6 the polymers, followed by conversion to hydrodynamic diameters based on the
7 Stokes-Einstein equation (Fig. 3A). Whereas the original size of Chol-free micelles (ca.
8 40 nm) was maintained at 200 nM Cy5-siRNA, further dilution below 20 nM
9 Cy5-siRNA dramatically decreased the micelle size, indicating disruption of Chol-free
10 micelles upon dilution in the presence of FBS. It should be noted that the size of
11 disrupted micelles (ca. 20 nm) was significantly larger than that of naked Cy5-siRNA
12 (ca. 5 nm), suggesting that the micelles might be fragmented into small PIC fractions
13 with a low association number of Cy5-siRNAs and block copolymers upon dilution,
14 possibly due to limited intermolecular disulfide cross-linking within the micelle core. In
15 contrast, Chol-siRNA micelles maintained their original size after dilution with 10%
16 FBS, demonstrating the stabilizing effect of Chol moieties for maintaining micelle
17 structure integrity.

Next, to further investigate micelle destabilization due to disulfide reduction as well as the stabilizing effect of Chol moieties, agarose gel electrophoresis of micelle samples was performed after incubation with or without DTT in the presence of varying concentrations of dextran sulfate (MW = 5,000). This strong polyanion was used for mimicking the negatively charged extracellular matrixes, as a recent study reported that those polyanionic components play a crucial role for the destabilization of blood circulating siRNA PICs [36]. The stabilizing effect of Chol moiety was again observed upon analysis of siRNA release induced by the counter polyanions. Chol-siRNA micelles required larger amounts of dextran sulfate for the appearance of the released siRNA band, compared to Chol-free micelles (Fig. 3B and 3C). Furthermore, the decreased stability under the reductive conditions was confirmed for Chol-siRNA micelles, as the incubation under 100 mM DTT apparently decreased the amount of dextran sulfate required to release free siRNA from micelles (Fig. 3B and 3C). The similar facilitated siRNA release profiles were also observed for Chol-siRNA micelles under a cytoplasm-mimicking condition (10 mM DTT and 150 mM NaCl) in comparison with a cell exterior-mimicking condition (10 μ M DTT and 150 mM NaCl) (Fig. S5A and S5B). Nevertheless, the modest difference between non-reductive and

1 reductive conditions implies the significant, yet restricted contribution of disulfide
2 cross-linking to the resistance of micelles against a strong polyanion. Note that the
3 stabilizing effect of disulfide cross-linking was more evident under milder conditions
4 where micelle samples were incubated with an anionic lipid molecule (DOPS). In this
5 experiment, micelle sensitivity to reductive conditions was clearly observed even for
6 Chol-free micelles (Fig. S5C and S5D).

7 3.4. *In vitro* efficacy of actively-targeted/stabilized micelles

8 To verify the biological effect of cRGD ligand as well as the stabilizing effect of
9 Chol-siRNA, cellular uptake efficiencies of siRNA micelle formulations were compared
10 by flow cytometric analyses. In this experiment, the fluorescence intensity of HeLa-Luc
11 cells was determined after their treatment with micelles prepared with Cy5-siRNA.

12 Note that HeLa-Luc cells were chosen as a target cell line because they overexpress
13 integrins, especially $\alpha_v\beta_5$, on their cellular surface (Fig. S6) [31,37], and are thus
14 appropriate for assessing the cRGD effect on cellular uptake of siRNA. Significantly
15 higher fluorescence intensity was observed for cRGD-installed micelles, i.e.,
16 RGD(+)/Chol(-) and RGD(+)/Chol(+) micelles, in comparison with control micelles
17 without cRGD, i.e., RGD(-)/Chol(-) and RGD(-)/Chol(+) micelles (Fig. 4A). These

1 results highlight the effect of cRGD on the enhanced cellular uptake of siRNA micelles.

2 In the absence of cRGD ligand, the cells incubated with RGD(-)/Chol(+) micelles
3 showed modestly higher fluorescence intensity than those incubated with
4 RGD(-)/Chol(-) micelles, indicating that Chol-siRNA facilitated the cellular uptake of
5 RGD(-) micelles. Apparently, the Chol-siRNA was more effective for improving the
6 cellular uptake of non-targeted RGD(-) micelles, compared to the targeted RGD(+)
7 micelles. These different effects of Chol-siRNA can be explained from the standpoint of
8 cellular uptake rate of micelles; the targeted RGD(+) micelles may be internalized more
9 rapidly and less affected by micelle stability in cell culture conditions, compared to the
10 non-targeted RGD(-) micelles.

11 Next, *in vitro* gene silencing ability of the actively-targeted/stabilized
12 (RGD(+)/Chol(+)) micelles was evaluated by comparing with those of the other micelle
13 formulations. siRNA for luciferase gene, i.e., siLuc, was selected for this
14 luminescence-based gene silencing assay, which allows quantitative determination of
15 gene silencing by comparing the luminescence intensity among samples. Fig. 4B shows
16 the relative luminescence intensity of cells treated with each siRNA micelle (or naked
17 siRNA) at 200 nM siRNA after 50 h incubation. The observed *in vitro* gene silencing

activity is in good agreement with the results from the cellular uptake study (Fig. 4A); as cRGD-installed, RGD(+)/Chol(+) and RGD(+)/Chol(-) micelles achieved the most efficient luciferase gene silencing, followed by the RGD(-)/Chol(+) micelles and the RGD(-)/Chol(-) micelles. Actively-targeted micelles prepared with siScr induced no significant decrease in relative luminescence intensity, confirming the sequence-specific gene silencing activity of actively-targeted micelles. Consequently, the cRGD ligands installed on the micelle surface enhanced *in vitro* gene silencing activity of siRNA micelles regardless of the Chol moiety conjugated to siRNA. This is consistent with the result seen in Fig. 3A; the Chol-free micelles could avoid the rapid dissociation in the 10% serum-containing PBS under 200 nM siRNA corresponding to the transfection condition, presumably enabling ligand-mediated rapid cellular uptake. It should be noted in this regard that the Chol-free siRNA micelles prepared with cRGD-PEG-PLL without thiol (or other stabilizing) moieties induced no gene silencing under similar conditions as we reported previously [13], indicating the impact of disulfide cross-linking on gene silencing efficiency.

Cell viability following treatment with siRNA micelles was also examined in order to exclude the possibility of cytotoxic effects on the gene silencing activity of siRNA

micelles. RGD(+)/Chol(−) micelles and RGD(+)/Chol(+) micelles, which showed the highest gene silencing activity, were subjected to a cell viability assay using the commercially available CCK-8 kit, based on a water soluble tetrazolium salt (WST-8). Neither micelle formulation induced significant cytotoxicity even at a high concentration of siRNA (1,000 nM) (Fig. S7), indicating negligible cytotoxic effects of siRNA micelles at concentrations used for the gene silencing studies. Considering that homopolymer PLLs with a high molecular weight (e.g. 27 kDa) are known to induce significant cytotoxicity due to their cationic charges [38], this negligible cytotoxicity of the siRNA micelles might be the result that they were composed of a relatively shorter PLL segment and equipped with a PEG outer layer that masked the charged component.

3.5. *In vivo* efficacy of actively-targeted/stabilized micelles

Since enhanced micelle stability and active targeting ability were both demonstrated under *in vitro* conditions (Fig. 3, 4A and 4B), the *in vivo* behaviors of actively-targeted/stabilized micelles were investigated following systemic administration. The longevity of siRNA micelles in the bloodstream is a critical factor for tumor accumulation of macromolecules. Prolonged circulation results in increased

1 contact opportunity for actively-targeted micelles with the cellular surface receptors in
2 tumor tissues.

3 First, the blood circulation property of fluorescently-labeled micelles (prepared
4 with Cy5-siRNA) was evaluated by IVRT-CLSM, which can continuously monitor the
5 fluorescence intensity of Cy5-siRNA (or its micelles) circulating in the blood of mice
6 immediately after intravenous injection (Fig. S8A). Fluorescence intensities determined
7 from ROIs selected within the vein were plotted against time to compare blood retention
8 profiles of different micelle formulations (Fig. 5A). In this regard, non-targeted micelles
9 were utilized for simply validating the effect of Chol-siRNA encapsulated within the
10 micelles on blood retention. Chol-free micelles were rapidly eliminated from circulation,
11 similar to naked siRNA, and consequently their blood half-life ($T_{1/2}$) was within 5 min.
12 In sharp contrast, Chol-siRNA micelles showed significantly longer blood retention
13 time ($T_{1/2} = >20$ min, $p < 0.05$) than Chol-free micelles. This prolonged blood circulation
14 observed for Chol-siRNA micelles is in good agreement with the results demonstrating
15 higher stability against dilution with serum-containing PBS (Fig. 3A) and improved
16 resistance to polyanion exchange with polysulfates (Fig. 3C). It is also worth
17 mentioning that the elimination of Chol-siRNA micelles from the bloodstream followed

1 a single exponential decay (or a one-compartment model in pharmacokinetics) (Fig.
2 S8B), suggesting that the Chol-siRNA micelles were eliminated mainly from the kidney
3 without being distributed into peripheral tissues. This is supported by the result that the
4 Chol-siRNA micelles (or the siRNA payloads) were mainly accumulated in the kidney
5 after systemic administration (Fig. S9). Note that the blood retention time of naked
6 Chol-siRNA was modestly longer than those of naked Chol-free siRNA. This may be
7 due to interaction of Chol-siRNA with lipoproteins in the bloodstream leading to
8 compromised renal filtration [39].

9 Next, the tumor-targeting ability of cRGD-installed micelles was investigated by
10 measuring their accumulation in subcutaneous HeLa-Luc tumors. Fluorescently-labeled
11 micelles were administered by tail vein injection and tumors were excised after 4 h
12 followed by measurement of the fluorescence intensity of each tumor mass with an IVIS
13 instrument. While there was almost no difference in fluorescence intensity between
14 Chol-free micelles with and without cRGD, significantly higher fluorescence intensity
15 was observed for Chol-siRNA micelles equipped with cRGD, compared to those
16 without cRGD (Fig. 5B). These results indicate that the cRGD ligand enabled more
17 efficient tumor accumulation of the highly stabilized Chol-siRNA micelles following

1 systemic administration, presumably due to the enhanced avidity of cRGD ligands to
2 $\alpha_v\beta_3/\alpha_v\beta_5$ integrin receptors on cancerous cells and also tumor-associated endothelial
3 cells [13,33,34]. Intratumoral distribution of RGD(+)/Chol(+) micelles was further
4 examined by continuous CLSM observation of the subcutaneous tumor tissue after
5 systemic administration. The CLSM image captured at 80 min after injection displays
6 massive distribution of the micelles in the tumor tissue through the blood vessels (Fig.
7 S10). Importantly, there were no significant differences in healthy organ/tissue
8 accumulation between non-targeted and actively-targeted micelles ($p > 0.05$) (Fig. S9),
9 demonstrating tumor-selective targeting of RGD(+)/Chol(+) micelles.

10 Finally, the *in vivo* gene silencing activity of siRNA micelles was investigated
11 through luciferase gene silencing (luminescence measurement) in subcutaneous
12 HeLa-Luc tumors, similar to the luminescence-based assay used for *in vitro* experiments.
13 At 48 h after the initial injection of samples (total 3 intravenous injections), luciferin
14 solution was intraperitoneally injected into mice, followed by measurement of the
15 luminescence intensity in the tumor tissues with an IVIS instrument (Fig. 5C and Fig.
16 S11). Non-targeted RGD(-)/Chol(-) micelles did not decrease tumor luminescence
17 intensity, whereas the actively-targeted, RGD(+)/Chol(-) and RGD(+)/Chol(+) micelles

1 did reduce tumor luminescence intensities compared to buffer-treated controls. In particular, the actively-targeted/stabilized, RGD(+)/Chol(+) micelles achieved significant decrease in the luminescence intensity ($p < 0.05$ for buffer-treated controls). It should be noted that the RGD(+)/Chol(+) micelles carrying siScr as a control sequence caused no decrease in the luminescence intensity, demonstrating sequence-specific gene silencing (i.e. RNAi) activity of the actively-targeted/stabilized micelles. In addition, it was also confirmed that all the tested micelles did not induce significant changes in the body weight of tumor-bearing mice (Table S1). In total, actively-targeted and stabilized micelles were more effective in delivering intact (thus active) siRNA to the cytoplasm of tumor cells following systemic administration. The present study particularly focused on the separate functionalization of the macromolecular components, i.e., PEG-PLL and siRNA, for construction of the multifunctional formulation, i.e., actively-targeted/stabilized micelles. This approach permitted the facile functionalization based on a simple chemistry, which is in contrast to the previously developed block copolymer modified with 2-iminothiolane, where two functional groups, open chain and closed ring structures, are equilibrated in the side chain of PLL [13].

4. Conclusions

Actively-targeted and stabilized PIC micelles were constructed with Chol-siRNA and PEG-PLL comprising the cRGD ligand at the PEG terminus and thiol (and amidine) functionality in PLL side chains, for systemic siRNA delivery to solid tumors. The Chol modification of siRNA allowed the production of PIC micelles at wider mixing ratios above the charge-stoichiometric point and dramatically stabilized the micelle structure, resulting in the enhanced blood circulation property of siRNA micelles. Further, the active targeting ability of the cRGD ligand was proven by enhanced cellular uptake *in vitro* and also enhanced tumor accumulation *in vivo* following systemic administration. Ultimately, the synergistic effect of active targetability and improved stability enabled significant sequence-specific gene silencing in the subcutaneous tumor tissue following systemic administration of siRNA micelles. The results obtained in this study highlight the importance of additional stabilizing mechanisms in PIC micelle systems, and that stabilization can be achieved from both the polymer component and the siRNA component used. Here, Chol-conjugation to siRNA reinforced the limited effect of disulfide cross-linking, thus improving the active targetability of nanoparticulate

1 formulations for systemic transport of siRNA into tumor tissues.

2

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Figure captions

Scheme 1. Synthesis schemes of cRGD-PEG-PLL(MPA) and PEG-PLL(MPA).

Figure 1. ^1H NMR spectrum of cRGD-PEG-PLL(MPA), recorded in D_2O (10 mg/mL) at 25 °C.

Figure 2. Light scattering behavior of PEG-PLL(MPA) polymer and siRNA mixtures in 10 mM HEPES buffer (pH 7.4) at 25 °C. Open circles: Chol-free siRNA, closed circles: Chol-siRNA. A) SLI of mixtures at different $+/-$ ratios. B) Intensity-weighted histograms of mixtures at $+/- = 1.4$.

Figure 3. Micelle stability assays. A) Change in size of Chol-free/Cy5-siRNA micelles and Chol/Cy5-siRNA micelles associated with dilution in 10% FBS-containing PBS. The diffusion time was measured by FCS and then the hydrodynamic diameter was calculated from the diffusion time (or diffusion coefficient) by the Stokes-Einstein equation. Each micelle was incubated for 1 h before measurements. Data represent the average value $\pm$ standard deviation (sampling time = 10 s, repetition time = 10). B and C) Agarose gel electrophoresis of siRNA micelles prepared with B) Chol-free siRNA and C) Chol-siRNA (0.3 μg) incubated with different concentrations of dextran sulfate under 0 or 100 mM DTT for 2 h. S/P ratio was defined as the molar ratio of sulfate groups in dextran sulfate to phosphate groups in siRNA.

Figure 4. A) Cellular uptake of Cy5-siRNA micelles in cultured HeLa-Luc cells, evaluated by flow cytometry. Cells were incubated with micelles for 75 min before medium change, and then further incubated for 8 h at 37 °C. Data represent the average value $\pm$ standard deviation ($n = 4$, *: $p < 0.05$). B) Gene silencing activity

of naked siRNAs and siRNA micelles at 200 nM siRNA in cultured HeLa-Luc cells. Luminescence intensity was measured after 50 h incubation with siRNA samples and normalized to the value of nontreated control cells. Data represent the average value $\pm$ standard deviation ($n = 4$, *: $p < 0.05$).

Figure 5. *In vivo* performance of siRNA micelles. A) Blood circulation profiles, determined by IVRT-CLSM after intravenous injection (3.6 nmol siRNA/mouse) into BALB/c nude mice (open triangle: naked Chol-free/Cy5-siRNA, closed triangle: naked Chol/Cy5-siRNA, open circle: Chol-free/Cy5-siRNA micelles and closed circle: Chol/Cy5-siRNA micelles). Data represent the average value ($n = 3$). B) Relative accumulation of actively-targeted siRNA micelles to non-targeted controls in subcutaneous HeLa-Luc tumors (1.8 nmol siRNA/mouse) 4 h after intravenous injection into BALB/c nude mice. Fluorescence intensity from excised tumors was measured with an IVIS instrument, followed by the normalization as described in the section 2.12. Data represent the average value $\pm$ standard deviation ($n = 4$ or 5, *: $p < 0.05$). C) Luciferase gene silencing activity of siRNA micelles in subcutaneous HeLa-Luc tumors. siRNA micelles were intravenously injected (1.8 nmol siRNA/mouse/injection) at 48, 38 and 24 h prior to measurement of the luminescence intensity from the excised tumors. The obtained luminescence intensity was normalized to that from the buffer-treated control tumors and the tumor volume. Data represent the average value $\pm$ standard deviation ($n = 5$, *: $p < 0.05$).

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Figure 3.

Figure 4.

Figure 5.