

Figure 2. Amount of exposed amine in PEG-PAsp(DET-PMM) and PEG-PAsp(DET-ACO) after incubation at A) pH 5.5 and B) pH 7.4. The amount of exposed amine was determined from standard curves prepared with glycine solutions in an acetate buffer (pH 5.5) and a phosphate buffer (pH 7.4).

followed by lysosomal degradation.^[2,22] Thus, they need to escape from those vesicular compartments to the cytoplasm before degrading for exerting gene silencing effect. Here, a smart block copolymer of biocompatible PEG and endosome-disrupting PAsp(DET-PMM) was synthesized by introducing a PMM moiety into primary amines in PAsp(DET) side chains through the amide bond formation (Figure 1 and Scheme S1, Supporting Information). It is reported that PMM amide is cleaved more rapidly than ACO amide in acidic conditions, probably because the pK_a value of carboxylates in dimethylmaleamylate derivatives is higher than that in citraconylate derivatives.^[21] Thus, PAsp(DET-PMM) is expected to be more rapidly converted to the parent polycation PAsp(DET) in acidic endosomes compared with PAsp(DET-ACO), for facilitating the endosome disruption (Figure S1, Supporting Information). The successful synthesis of PEG-PAsp(DET-PMM) was confirmed from the size exclusion chromatogram (Figure S2, Supporting Information) and ^1H NMR spectrum (Figure S3 and Table S1, Supporting Information).

The charge-conversion functionality of PEG-PAsp(DET-PMM) was compared with that of PEG-PAsp(DET-ACO) by determining the amount of amines generated from the CCP segments at pH 7.4 and 5.5 (Figure 2). Obviously, PEG-PAsp(DET-PMM) exerted higher conversion rate than PEG-PAsp(DET-ACO) over time at both pHs of 5.5 and 7.4, demonstrating more rapid hydrolysis of the PMM amide bonds. Also, the accelerated hydrolysis at the lower pH of 5.5 was demonstrated for both CCP segments. Nevertheless, the considerable increase in the conversion ratios even at pH 7.4 is likely to induce destabilization of hybrid micelles under extracellular neutral conditions. Thus, the stability of hybrid micelles in serum-containing medium was further examined as described below.

The newly synthesized PEG-CCP, PEG-PAsp(DET-PMM), was applied for the preparation of CaP hybrid

micelles loaded with siRNA and then characterized by DLS. The obtained DLS (volume-weighted) histogram of siRNA-loaded hybrid micelles prepared with PEG-PAsp(DET-PMM) (PMM micelles) displays a hydrodynamic diameter of ≈ 70 nm (Figure S4, Supporting Information), associated with a narrow size distribution (polydispersity index = 0.1), similar to hybrid micelles prepared with PEG-PAsp(DET-ACO) (ACO micelles). Note that the micelle formation was not observed in the absence of calcium and phosphate ions under the similar condition because of the hydrophilic nature of PEG-CCPs. In sharp contrast, non-PEGylated CaP precipitates showed much larger size ($\approx 1 \mu\text{m}$) (data

not shown). These results demonstrate that the block copolymers with PEG were essential for the sub-100 nm nanoparticle formation due to enhanced colloidal stability based on the PEG shell.

The carrier stability under cell culture conditions is a prerequisite for efficient cellular uptake of siRNA. In our previous study, ACO micelles were confirmed to stably entrap siRNA in 10% fetal bovine serum (FBS)-containing Dulbecco's modified Eagle's medium (DMEM) for a relatively short incubation time of 4 h.^[17] Nevertheless, the stable entrapment of siRNA in the micelles for longer incubation time (e.g., 24 h) is crucial from the standpoint of systemic delivery. Thus, the stability of hybrid micelles in the DMEM containing 10% FBS was investigated over 24 h at 37 °C by fluorescence correlation spectroscopy.^[17,23] By assuming the spherical shape of hybrid micelles,^[17,18] the obtained diffusion coefficients of hybrid micelles incorporating Alexa647-siLuc were converted to the corresponding hydrodynamic diameters based on the Stokes-Einstein equation (Figure S5, Supporting Information), and then, normalized to the value of naked siRNA. Both hybrid micelles maintained their initial size during 24 h incubation, indicating the stable encapsulation of siRNA within the hybrid micelles in the serum-containing medium. These results strongly suggest that PEG-PAsp(DET-PMM) and PEG-PAsp(DET-ACO) should be stably bound to CaP core without hydrolysis of maleic acid amides in the serum-containing medium at pH 7.4.

Next, the cellular internalization behavior of hybrid micelles was examined by a flow cytometer. Luciferase-expressing human ovarian cancer (SKOV3-Luc) cells were incubated with Cy5-siLuc-loaded hybrid micelles for 6 and 24 h, followed by the flow cytometric analysis (Figure 3A and Figure S6, Supporting Information). The significant cellular uptake of Cy5-siLuc was confirmed for all three hybrid micelles, i.e., PMM micelles, ACO micelles,

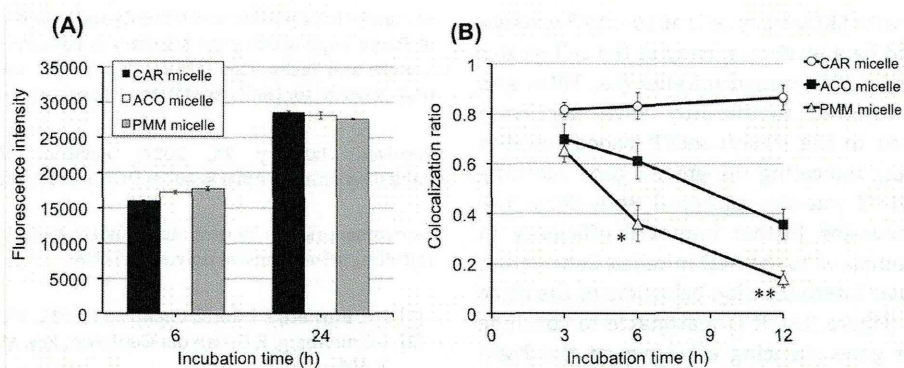


Figure 3. A) Cellular uptake efficiency of Cys-siLuc delivered by hybrid micelles in SKOV3-Luc cells after 6 h incubation and 24 h incubation (100×10^{-9} M siRNA). Results are expressed as mean \pm SEM ($n = 3$). (B) Time-dependent change in colocalization ratio of Alexa647-siLuc (red) with CellLight Late Endosomes-GFP (green) in SKOV3-Luc cells (200×10^{-9} M siRNA). The data are represented as mean \pm SEM obtained from 14 cells (* $p < 0.05$, ** $p < 0.01$).

and the micelles prepared with PEG-PAsp(DET-CAR) as a non-charge-conversion control polymer (nonCCP), in 6 h incubation. The uptake was further increased by prolonging the incubation time to 24 h. Importantly, almost the same cellular internalization profiles were observed among the three micelles, presumably because they have similar PEGylated shell structures. Next, the intracellular trafficking of hybrid micelles was investigated as a critical step following the cellular internalization. In particular, the colocalization of hybrid micelles (or the siRNA payload) with the late endosomes was focused to verify the endosome-escaping functionality of PEG-PAsp(DET-PMM) in comparison with PEG-PAsp(DET-ACO) and PEG-PAsp(DET-CAR). SKOV3-Luc cells were incubated with each hybrid micelle prepared with Alexa647-siLuc,

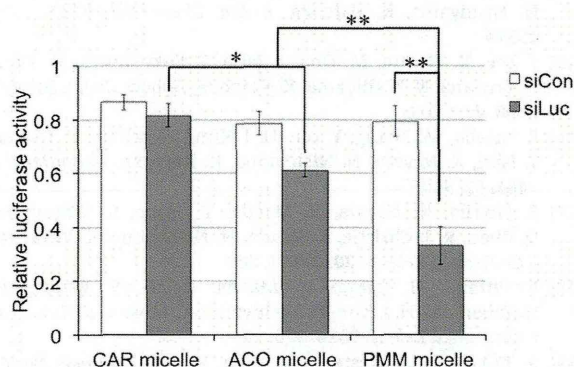


Figure 4. Luciferase gene silencing efficiency of CAR micelles, ACO micelles, and PMM micelles in SKOV3-Luc cells. The cells were incubated with each micelle incorporating siLuc (target sequence) or siCon (control sequence) at 50×10^{-9} M siRNA for 48 h, followed by a conventional luciferase assay. The obtained luminescence intensities from cell lysates were normalized to that from non-treated control cells. Results are shown as mean \pm SEM ($n = 6$, * $p < 0.05$, ** $p < 0.01$).

and then, the transfection medium containing the hybrid micelles was exchanged with the fresh one without micelle samples, followed by additional incubation before confocal laser scanning microscope (CLSM) imaging. The intracellular Alexa647-siLuc was shown red and also the endosomal membrane was stained with CellLight Late Endosomes-GFP (green), thereby their colocalization points within the cells should be shown yellow in the merged CLSM images (Figure S7, Supporting Information). Then, the colocalization ratio of Alexa647-siLuc with the stained late endosomes at the designated time points was calculated by pixel counting for each hybrid micelle (Figure 3B), as described in Supporting Information. The CCP-integrated micelles, i.e., PMM and ACO micelles, showed time-dependent decreases in the colocalization ratio from 3 to 12 h, whereas the colocalization ratio of the nonCCP-integrated, CAR micelles was not significantly altered. This result strongly suggests the facilitated endosomal escape of Alexa647-siLuc by the CCPs integrated into the hybrid micelles. Further, the colocalization ratio of PMM micelles was significantly lower than that of ACO micelles, demonstrating stronger endosome-escaping functionality of the CCP integrated with PAsp(DET-PMM). On the contrary, the apparently high colocalization ratios of CAR micelles indicate that the hybrid micelles without CCPs could not effectively induce the endosome disruption under the tested condition. These results are consistent with the polymer design concept that more rapid conversion of CCPs to the endosome-disrupting polycation PAsp(DET) at an endosomal acidic pH of 5.5 should enable more efficient endosomal escape of hybrid micelles.

The gene silencing efficiency of hybrid micelles was determined to elucidate the effect of endosome-escaping functionality of CCPs on the ultimate biological activity of siRNA. The hybrid micelles prepared with a target sequence of siRNA (siLuc) or a control sequence (siCon)

were incubated with SKOV3-Luc cells at 50×10^{-9} M siRNA for 48 h, followed by a luciferase assay of the cell lysates (Figure 4). The siLuc/CCP-loaded micelles (i.e., PMM and ACO micelles) achieved significantly lower luciferase activity compared to the siLuc/nonCCP-loaded micelles (i.e., CAR micelles), indicating the greater gene silencing efficiency of hybrid micelles equipped with CCPs. The PMM micelles revealed further improved efficiency in gene silencing compared to the ACO micelles. Considering the similar cellular internalization behaviors of the three hybrid micelles (Figure 3A), it is reasonable to conclude that the greater gene silencing efficiency of the PMM hybrid micelles is mainly due to their improved capability of translocating siRNA payloads from endosomal compartment to cytosol based on their prominent charge-conversion functionality (Figure 3B). It should be noted that the significant difference in relative luciferase activity was observed between siLuc-loaded and siCon-loaded micelles and also that the PMM micelles did not affect the viability of SKOV3-Luc cells under the same condition as the gene silencing assay (Figure S8, Supporting Information). These results confirm the sequence-specific gene silencing effect of siLuc-loaded hybrid micelles.

4. Conclusions

In this study, PEG–PAsp(DET–PMM) was synthesized by utilizing PMM amide, which was more sensitive to acid hydrolysis compared to the previously developed ACO amide. The obtained PEG–PAsp(DET–PMM) successfully formed siRNA-loaded hybrid micelles having the size of ≈ 70 nm. The PMM micelles were stable in the 10% FBS-containing medium at least for 24 h, leading to the efficient cellular uptake of siRNA in cultured SKOV3-Luc cells, similar to ACO micelles. Ultimately, the PMM micelles achieved the greater gene silencing activity in the cells, compared to ACO micelles, presumably due to the more efficient endosomal escape of the siRNA payload by PEG–PAsp(DET–PMM). These results demonstrate that the fine-tuning of endosome-disrupting polymers improves the delivery efficacy of siRNA nanocarriers for enhanced gene silencing activity.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

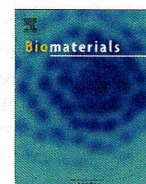
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Actively-targeted polyion complex micelles stabilized by cholesterol and disulfide cross-linking for systemic delivery of siRNA to solid tumors



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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 cancer model following systemic administration. Ultimately, systemically administered cRGD/Chol-siRNA micelles exhibited significant gene silencing activity in the tumor, presumably due to their active targeting ability combined with the enhanced stability through both hydrophobic interactions of cholesterol and disulfide cross-linking.

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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

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

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

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

In this work, we employed a functional block copolymer comprising PEG segment installed with cyclo-Arg-Gly-Asp (cRGD) peptide as the tumor-targeting hydrophilic block [13,16,31] and poly(L-lysine) (PLL) segment modified with 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 Chol-siRNA 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, trimaza 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 3500 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- Σ -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 CCG 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 (–CH₂CH₂–, 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))

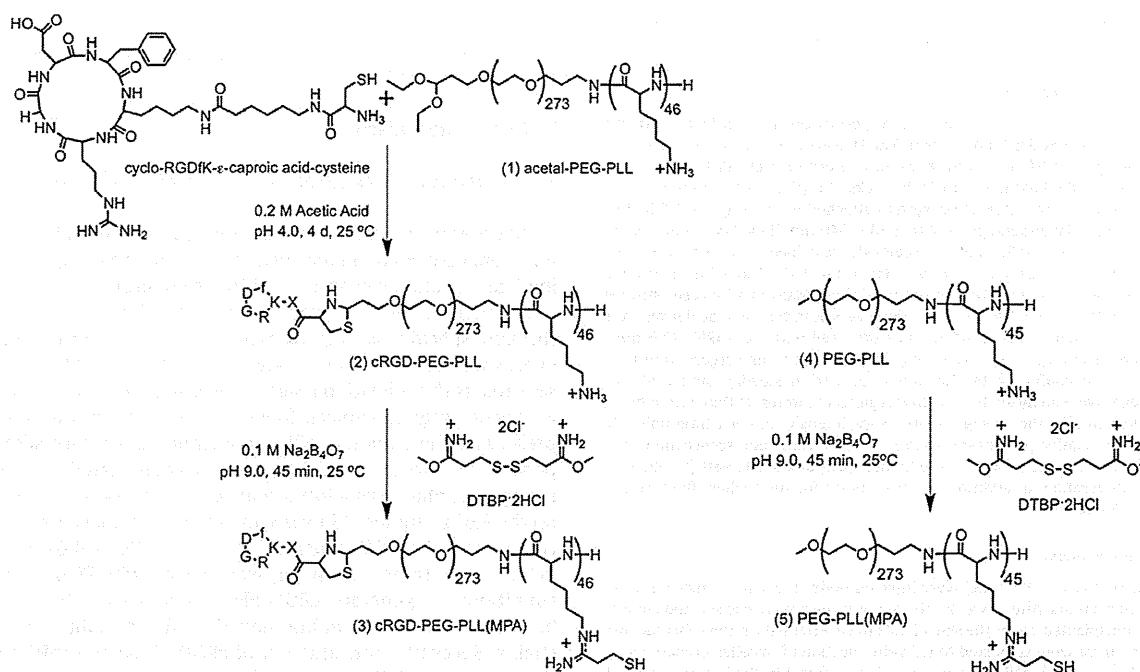
cRGD-PEG-PLL(MPA) was synthesized by introducing MPA moieties into the lysine primary amines in cRGD-PEG-PLL using DTBP, as shown in Scheme 1 [26]. Briefly, cRGD-PEG-PLL (50 mg, 0.1 mmol amines) was dissolved in 100 mM borate buffer (pH 9.0) (10 mL), followed by the addition of DTBP/HCl (69 mg, 0.2 mmol). The reaction solution was stirred at 25 °C for 45 min and then dialyzed (MWCO 3500 Da) against 10 mM PBS (pH 6.0) for 2 h and distilled water for 2 h. The dialyzed solution was lyophilized after filtration (yield: 53.9 mg, 80%). The substitution degree of MPA groups in the obtained polymer was determined from the ¹H NMR spectrum based on the peak intensity ratio of β , γ and δ methylene protons in PLL side chains (–(CH₂)₃–, δ = 1.3–1.9 ppm) to mercaptoethyl protons in MPA moieties (HS–(CH₂)₂–, δ = 2.7–2.9 ppm) (Fig. 1), demonstrating quantitative introduction (>97%). In a similar manner, PEG-PLL(MPA) without cRGD peptide was synthesized by the reaction of PEG-PLL with DTBP (Scheme 1). For PEG-PLL(MPA), PEG-PLL (300 mg, 0.71 mmol amines) and DTBP/HCl (439 mg, 1.4 mol) were used (yield: 299 mg, 73%). Quantitative introduction of MPA group (>99%) was also confirmed by ¹H NMR analysis (Fig. S2).

2.4. Polyionic complexation of block copolymers with siRNA

cRGD-PEG-PLL(MPA) (or PEG-PLL(MPA)) block copolymer was dissolved in 10 mM HEPES buffer (pH 7.4) and incubated with 100 mM DTT at 25 °C for 15 min for disulfide reduction. The reduced polymer solution was mixed with siRNA dissolved in the same buffer (15 μ M siRNA) at different molar charge ratios of the block copolymer to siRNA, i.e., amidines and primary amines in the block copolymer/phosphates in siRNA. To proceed disulfide cross-linking in the PIC core, PIC solutions (10 μ M siRNA) were dialyzed (MWCO 3500 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 (λ = 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.



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

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 μM 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×, N.A. 1.2 water immersion objective (Carl Zeiss, Oberkochen, Germany) (sampling time: 10 s, repeating time: 10). A He–Ne laser (633 nm) 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 cool down before adding ethidium bromide (1 μL). Micelle solutions (2 μL) with or without DTT (200 mM DTT) were mixed with dextran sulfate solutions (2 μL). The concentrations of dextran sulfate groups were set at 0, 1.0, 1.5, 2.0 and 3.0 times equivalent to the siRNA phosphates. After incubation for 2 h at 25 °C, each sample was loaded on the gel and electrophoresed (100 V, 30 min) in TBE running buffer. After electrophoresis, gel images were captured by a Typhoon 9410 instrument equipped with a 532 nm laser. Also, micelle samples were subjected to the electrophoresis (50 V, 30 min) after incubation with different amounts of anionic lipid, DOPS (1,2-dioleoyl-sn-glycero-3-phospho-L-serine sodium salt), for 8 h at 25 °C.

2.8. Cellular uptake

Luciferase-expressing human cervical cancer cells, HeLa-Luc (Caliper Life-Science, Hopkinton, MA), were seeded into 6-well plates (100,000 cells/well) and incubated in DMEM containing 10% fetal bovine serum (FBS) (Dainippon Sumitomo Pharma Co. Osaka, Japan) (1.5 mL) for 24 h. The culture media were then exchanged with fresh media containing micelle samples prepared with Cy5-siRNA (siRNA concentration: 333 nM). Cells were incubated in the presence of micelles for 75 min at 37 °C and then further incubated in the absence of micelles for 8 h after medium exchange. The cells were harvested by trypsinization, followed by flow cytometric analyses using a BD LSR II instrument (BD Biosciences, San Jose, CA) and BD FACS Diva software (BD Biosciences).

2.9. In vitro luciferase assay

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

2.10. Cytotoxicity assay

HeLa-Luc cells were seeded in 24-well plates (20,000 cells/well) and incubated in DMEM containing 10% FBS (0.5 mL) for 24 h. Thereafter, the culture media were replaced with fresh media containing micelle samples at varying siRNA concentrations and cells were further incubated for 48 h. Then, CCK-8 solution (1 μL/10 μL medium) was added and samples were further incubated at 37 °C for 1 h. Cell viability was determined from the absorbance of extracellular media at 450 nm,

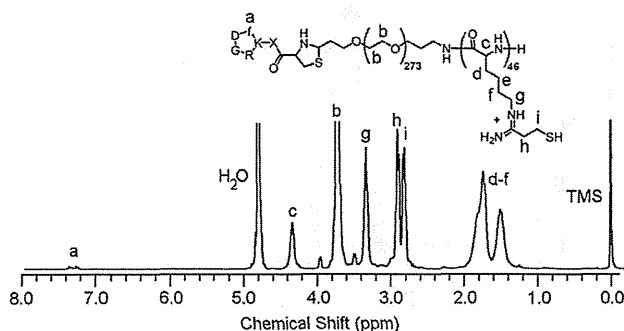


Fig. 1. ¹H NMR spectrum of cRGD-PEG-PLL(MPA), recorded in D₂O (10 mg/mL) at 25 °C.

which was measured using a BIO-RAD 680 microplate reader (Bio-Rad, CA). All data are expressed relative to untreated control cells ($n = 6$).

2.11. Blood circulation behavior

All animal experimental procedures were performed in accordance with the policies of the Animal Ethics Committee of The University of Tokyo. The blood circulation property of siRNA-loaded micelles was determined by IVRT-CLSM observation of live mice (BALB/c nude, female, 8 weeks old) (Charles River laboratories, Tokyo, Japan) using a Nikon A1R CLSM system attached to an upright ECLIPSE FN1 (Nikon Corp., Tokyo, Japan) equipped with a 20 \times , 640 nm diode laser, and a band-pass emission filter of 700/75 nm, as previously described [32]. The mice were anesthetized with 2–3% isoflurane (Abbott Japan Co., Ltd., Tokyo, Japan) using a Univentor 400 anesthesia unit (Univentor Ltd., Zejtun, Malta) and placed onto the temperature-controlled microscope stage. Blood vessels in the ear-lobe dermis were observed after tail vein injection of micelles prepared with Cy5-siRNA (3.6 nmol Cy5-siRNA/mouse, $n = 3$), as they were clearly observed without surgery (thus non-invasive) and were unaffected by the cardiac beat (thus steadily observed). The obtained images were analyzed by selecting regions of interest (ROIs) within blood vessels for determining the average fluorescence intensity at each time point. To produce blood circulation profiles shown in Fig. 5A, the vein fluorescence intensities were expressed as a relative value to the highest (1.0) and the lowest (0) obtained through the observation. In addition, the time point for the highest fluorescence intensity was defined as $t = 0$.

2.12. Tumor accumulation

HeLa-Luc cells (2.6×10^6 cells) were injected under the skin of mice for preparation of donor tumors. After 2 weeks, the donor tumors were excised and cut into pieces, then transplanted under the skin of host mice. After 8 days, the subcutaneous tumor-bearing mice were subjected to tail vein injection of micelles prepared with Cy5-siRNA (1.8 nmol Cy5-siRNA/mouse, $n = 4-5$). After 4 h, they were sacrificed, and tumors (and major organs) were excised, followed by fluorescence quantification using an IVIS instrument equipped with Living Image software. Total photon counts were normalized to the illumination time and the sample area as follows: fluorescence intensity = total photons/[illumination time (s) \times area (cm²)]. Further normalization was performed by dividing the fluorescence intensity from the tumors treated with RGD-free controls.

2.13. In vivo luciferase assay

Subcutaneous HeLa-Luc tumor models were prepared by *in vivo* passage of tumor fragments, as described in the former section. After 8 days, the tumor-bearing mice were subjected to the tail vein injection of micelle samples (1.8 nmol siRNA/mouse/injection, $n = 5$) at 48 h, 38 h and 24 h before measurement. A luciferin solution (50 mM, 200 μ L) was injected intraperitoneally 25 min prior to measurement to obtain the stable luminescence intensity from tumors. The luminescence intensity (counts) was recorded using an IVIS instrument under the same condition for each image (acquisition time: 5 s, binning: small, f/stop: 1, and field of view: D). Tumor volumes were determined by manual measurement with a caliper and calculated using the following equation: volume = $ab^2/2$, where a is the long axis and b is the short axis measured. The luminescence intensity obtained from sample-treated tumors was normalized to the tumor volume and further to that from buffer-treated control tumors: relative luminescence intensity = total photons of sample/sample-treated tumor volume (cm³)/total photons of buffer control/control-treated tumor volume (cm³).

2.14. Data analysis

The experimental data were analyzed by Student's *t*-test. $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Synthesis of block copolymers and their characterizations

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

3.2. Preparation and characterization of Chol-siRNA micelles

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

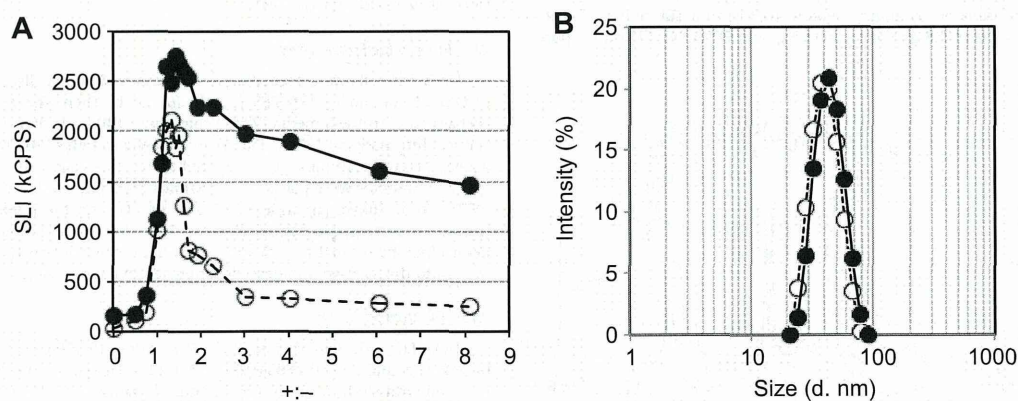


Fig. 2. Light scattering behavior of PEG-PLL(MPA) polymer and siRNA mixtures in 10 mM HEPES buffer (pH 7.4) at 25 °C. Open circles: Chol-free siRNA, closed circles: Chol-siRNA. A) SLI of mixtures at different +:– ratios. B) Intensity-weighted histograms of mixtures at +:– = 1.4.

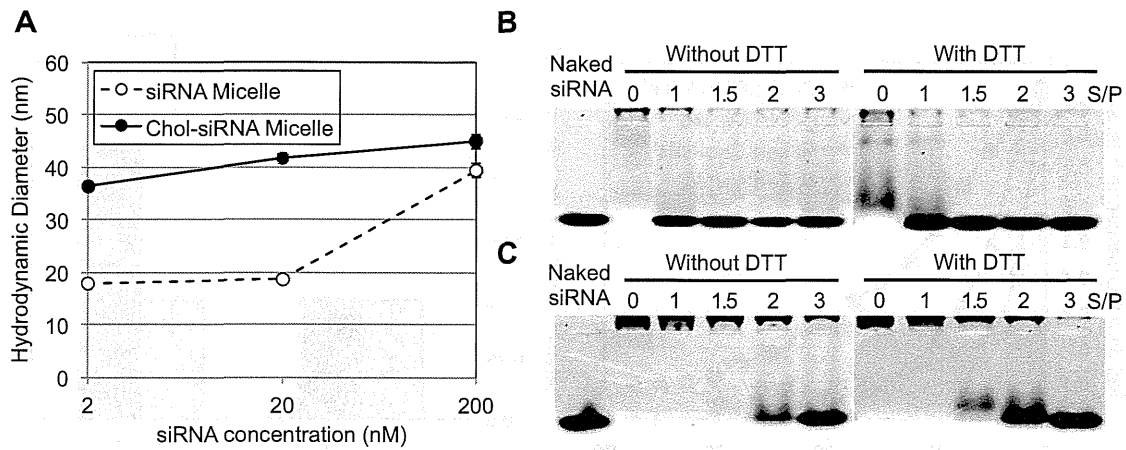


Fig. 3. Micelle stability assays. A) Change in size of Chol-free/Cy5-siRNA micelles and Chol/Cy5-siRNA micelles associated with dilution in 10% FBS-containing PBS. The diffusion time was measured by FCS and then the hydrodynamic diameter was calculated from the diffusion time (or diffusion coefficient) 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 μ g) 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.

$+:- = 1.4$, 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 $+:-$ 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 $+:- < 1.4$. Note that a remarkable difference was observed between the two micelle formulations in their SLI values at $+:- > 1.4$. 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-assembly 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,

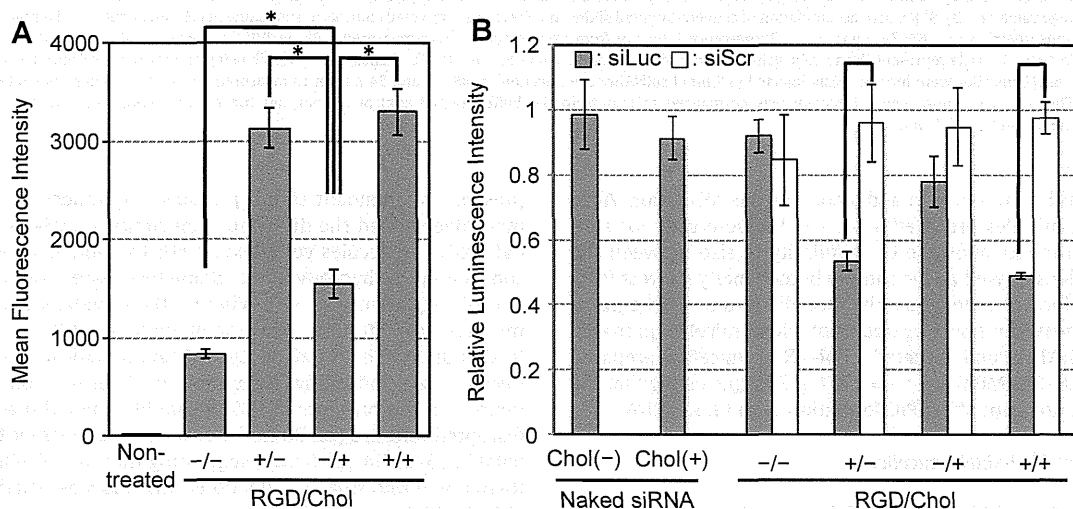


Fig. 4. A) Cellular uptake of Cy5-siRNA micelles in cultured HeLa-Luc cells, evaluated by flow cytometry. Cells were incubated with micelles for 75 min before medium change, and then further incubated for 8 h at 37 °C. Data represent the average value \pm standard deviation ($n = 4$, *; $p < 0.05$).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

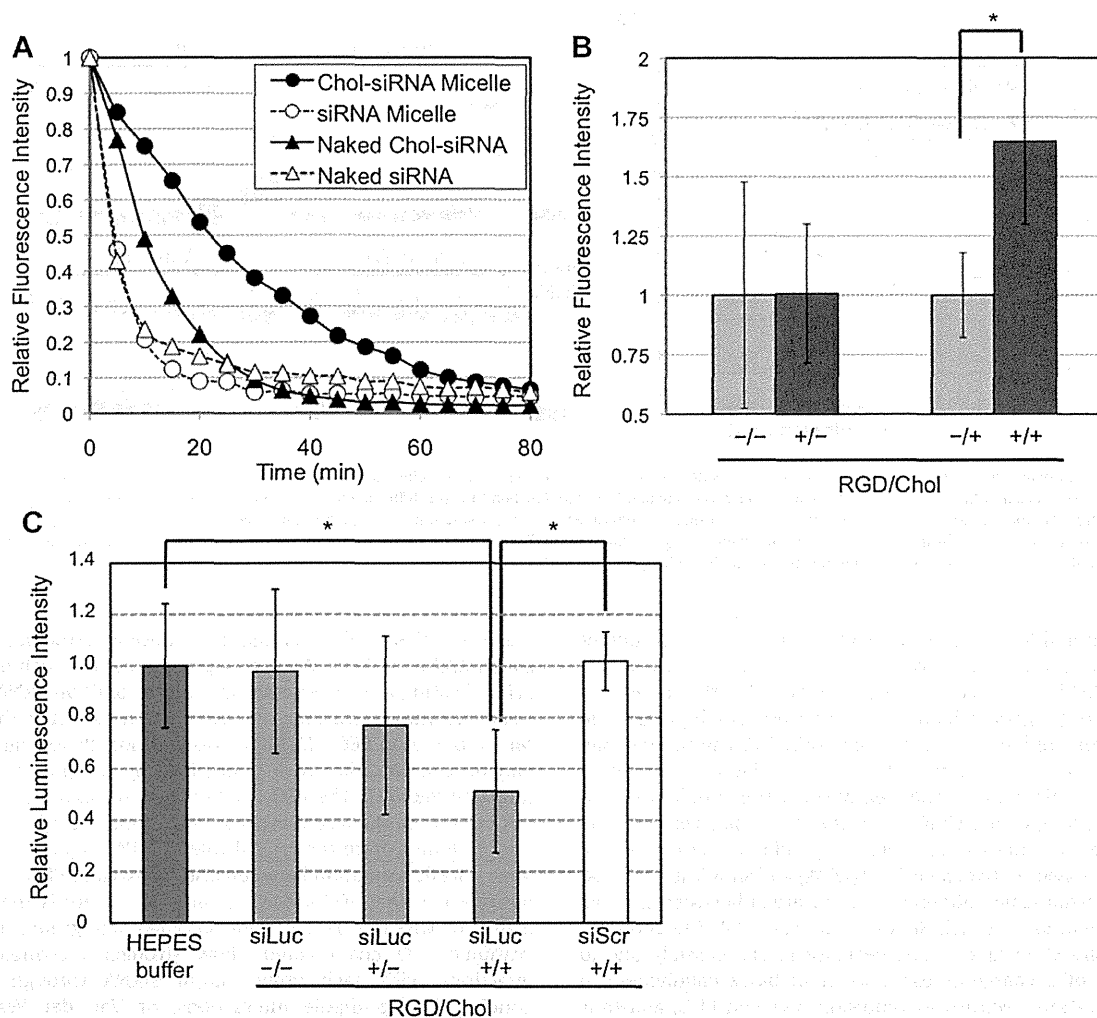


Fig. 5. *In vivo* performance of siRNA micelles. A) Blood circulation profiles, determined by IVRT-CLSM after intravenous injection (3.6 nmol siRNA/mouse) into BALB/c nude mice (open triangle: naked Chol-free/Cy5-siRNA, closed triangle: naked Chol/Cy5-siRNA, open circle: Chol-free/Cy5-siRNA micelles 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 ($n = 5$, *; $p < 0.05$).

directed toward more uniform and stable micelle formation. Altogether, siRNA micelles prepared at $+:- = 1.4$ 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 $+:- = 1.4$ (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 and Chol-free micelles were determined under diluted conditions in 10% FBS-containing PBS by FCS measurement, which allows monitoring of the hydrodynamic size (or diffusion coefficient) of fluorescently labeled nanoparticles even in the

presence of abundant serum proteins [16]. Indeed, this measurement determined the diffusion coefficients of Cy5-siRNA or Chol/Cy5-siRNA molecules complexed with the polymers, followed by conversion to hydrodynamic diameters based on the Stokes-Einstein equation (Fig. 3A). Whereas the original size of Chol-free micelles (ca. 40 nm) was maintained at 200 nM Cy5-siRNA, further dilution below 20 nM Cy5-siRNA dramatically decreased the micelle size, indicating disruption of Chol-free micelles upon dilution in the presence of FBS. It should be noted that the size of disrupted micelles (ca. 20 nm) was significantly larger than that of naked Cy5-siRNA (ca. 5 nm), suggesting that the micelles might be fragmented into small PIC fractions with a low association number of Cy5-siRNAs and block copolymers upon dilution, possibly due to limited intermolecular disulfide cross-linking within the micelle core. In contrast, Chol-siRNA micelles maintained their original size after dilution with 10% FBS, demonstrating the stabilizing effect of Chol moieties for maintaining micelle 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 = 5000). 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 C). 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 C). 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 B). Nevertheless, the modest difference between non-reductive and reductive conditions implies the significant, yet restricted contribution of disulfide cross-linking to the resistance of micelles against a strong polyanion. Note that the stabilizing effect of disulfide cross-linking was more evident under milder conditions where micelle samples were incubated with an anionic lipid molecule (DOPS). In this experiment, micelle sensitivity to reductive conditions was clearly observed even for Chol-free micelles (Fig. S5C and D).

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

To verify the biological effect of cRGD ligand as well as the stabilizing effect of Chol-siRNA, cellular uptake efficiencies of siRNA micelle formulations were compared by flow cytometric analyses. In this experiment, the fluorescence intensity of HeLa-Luc cells was determined after their treatment with micelles prepared with Cy5-siRNA. Note that HeLa-Luc cells were chosen as a target cell line because they overexpress integrins, especially $\alpha_v\beta_5$, on their cellular surface (Fig. S6) [31,37], and are thus appropriate for assessing the cRGD effect on cellular uptake of siRNA. Significantly higher fluorescence intensity was observed for cRGD-installed micelles, i.e., RGD(+)/Chol(-) and RGD(+)/Chol(+) micelles, in comparison with control micelles without cRGD, i.e., RGD(-)/Chol(-) and RGD(-)/Chol(+) micelles (Fig. 4A). These results highlight the effect of cRGD on the enhanced cellular uptake of siRNA micelles. In the absence of cRGD ligand, the cells incubated with RGD(-)/Chol(+) micelles showed modestly higher fluorescence intensity than those incubated with RGD(-)/Chol(-) micelles, indicating that Chol-siRNA facilitated the cellular uptake of RGD(-) micelles. Apparently, the Chol-siRNA was more effective for improving the cellular uptake of non-targeted RGD(-) micelles, compared to the targeted RGD(+) micelles. These different effects of Chol-siRNA can be explained from the standpoint of cellular uptake rate of micelles; the targeted RGD(+) micelles may be internalized more rapidly and less affected by micelle stability in cell culture conditions, compared to the non-targeted RGD(-) micelles.

Next, *in vitro* gene silencing ability of the actively-targeted/stabilized (RGD(+)/Chol(+)) micelles was evaluated by comparing with those of the other micelle formulations. siRNA for luciferase gene, i.e., siLuc, was selected for this luminescence-based gene silencing assay, which allows quantitative determination of gene silencing by comparing the luminescence intensity among samples. Fig. 4B shows the relative luminescence intensity of cells treated with each siRNA micelle (or naked 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 (1000 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 (Figs. 3 and 4A and B), 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 contact opportunity for actively-targeted micelles with the cellular surface receptors in tumor tissues.

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