Optimized rod length of polyplex micelles for maximizing transfection efficiency and their performance in systemic gene therapy against stroma-rich pancreatic tumors

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Abstract

Poly(ethylene glycol) (PEG) modification onto a gene delivery carrier for systemic application results in a trade-off between prolonged blood circulation and promoted transfection because high PEG shielding is advantageous in prolonging blood retention, while it is disadvantageous with regard to obtaining efficient transfection owing to hampered cellular uptake. To tackle this challenging issue, the present investigation focused on the structure of polyplex micelles (PMs) obtained from PEG-poly(L-lysine) (PEG-PLys) block copolymers characterized as rod-shaped structures to seek the most appreciable formulation. Comprehensive investigations conducted with particular focus on stability, PEG crowdedness, and rod length, controlled by varying PLys segment length, clarified the effect of these structural features, with particular emphasis on rod length as a critical parameter in promoting cellular uptake. PMs with rod length regulated below the critical threshold length of 200 nm fully exploited the benefits of cross-linking and the cyclic RGD ligand, consequently, exhibiting remarkable transfection efficiency comparable with that of ExGen 500 and Lipofectamine[®] LTX with PLUS[™] even though PMs were PEG shielded. The identified PMs exhibited significant antitumor efficacy in systemic treatment of pancreatic adenocarcinoma, whereas PMs with rod length above 200 nm exhibited negligible antitumor efficacy despite a superior blood circulation property, thereby highlighting the significance of controlling the rod length of PMs to promote gene transduction.

1. Introduction

Significant advances in the development of smart nano-devices constructed by exploiting material-based molecular design concepts have dramatically improved medical diagnosis and treatment [1-4]. One of their intriguing application is gene therapy, which has emerged as a potential tool for treating intractable diseases [5,6] by means of transducing therapeutic proteins, because this needs an appropriate delivery system that transports inherently fragile DNA in biological milieu to pathological sites and enters into the cells for expressing therapeutic proteins [7,8]. To establish this therapeutic concept, through by systemic administration, the property of blood retention for increasing delivery efficiency to the targeted sites is an essential prerequisite along with gene transfection capability [7,8]. The primary property of a gene delivery system for systemic application is to securely package DNA within the delivery system and to inhibit nonspecific interactions with biological components, including opsonins, in addition to nuclease tolerability [9]. In view of these requirements, we have developed a poly(ethylene glycol) (PEG)-shielded gene delivery system, polyplex micelles (PMs). As a promising formulation constructed on the basis of the self-assembly of PEG-polycation block copolymers and plasmid DNA (pDNA) [10,11]. Following this electrostatic complexation, pDNA is packaged into a distinct rod-shaped bundle composed of pDNA folded several times [12] as the core compartment and the PEG chains surrounding it as the protective shell compartment. This characteristic structure of PMs enables systemic applications [13]. However, the blood retention property of the firstly developed PMs was unsatisfactory to obtain a sufficient therapeutic effect via systemic administration; thus, modifications were required to reinforce PM stability in the bloodstream. For this purpose, the introduction of a disulfide cross-linking into the core compartment [14] for preventing disassembly was an effective strategy, as evidenced by the cross-linked PEG-poly(L-lysine) (PEG-PLys) block copolymer-based PMs indeed exhibiting prolonged blood circulation [15,16].

Besides the cross-linking, the PEG compartment also substantially contributes in promoting blood circulation. A recent study highlighting the role of the PEG compartment described the importance of increasing PEG crowdedness in prolonging the blood retention period: higher PEG crowdedness on PMs prepared from a lower degree of polymerization (DP) of the PLys segment of PEG-PLys led to a better blood circulation property [17]. Moreover, PMs of lower PLys DP exhibited increased transcription

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3 efficiency [18]. These studies suggested that cross-linked PMs prepared from lower PLys
4 DP would be a suitable formulation for systemic application. However, with regard to the
5 transfection activity, highly PEG-shielded PMs may in turn be disadvantageous because
6 the reduced affinity of these PMs with the cellular membrane may result in precluded
7 cellular uptake. In addition, we found that PLys DP affects the folding number of pDNA
8 [18] within the PM core; PMs prepared from lower PLys DP result in a longer rod length
9 by less folded pDNA. Indeed, PMs prepared from PLys DP 20 displayed rod length
10 ranging several hundred nanometers, while those prepared from PLys DP 70 displayed
11 rod length around 70 nm [17,18]. Such long PMs with several hundred nanometer-sized
12 particles may unlikely be uptake efficiently compared with that of short PMs [19].
13 Furthermore, a relatively lower binding affinity of PEG-PLys of lower PLys DP to pDNA
14 than that of PEG-PLys of higher PLys DP [20] may decrease the structural stability of
15 PMs, thereby influencing transfection efficiency. Although such impaired stability and
16 unfavorable character for cellular uptake may be improved by applying cross-linking [14]
17 and the ligand molecules [21-24], the inherent structural features of PMs prepared from
18 lower PLys DP may suggest their disadvantageous property in obtaining efficient
19 transfection. Eventually, a concern may arise that the achievement of PMs arriving at
20 target sites by the end of a long systemic journey may result in precluded transfection.
21 These arguments raise the need of a comprehensive study taking each structural feature
22 into consideration for obtaining an ultimate PM formulation. Therefore, the present study
23 aimed to seek the most appreciable formulation to maximize the transfection efficiency
24 on a basis of precise structural control of PMs by varying PLys DP, with particular focus
25 on structural stability [20], PEG crowdedness [17], and rod length [17,18], for completely
26 utilizing the benefits of cross-linking [14-16] and the cyclic RGD (Arg-Gly-Asp)
27 (cRGD) peptide as a ligand for specific integrin-mediated uptake [21-24].
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47 The present study clarified the effect of the abovementioned structural features on
48 cellular uptake and revealed the presence of critical rod length for efficient cellular
49 uptake, consequently identifying the most cross-linking and the cRGD ligand upon
50 systemic application to provide stability and tumor homing, respectively, the PM
51 formulation was challenged to systemic treatment of one of the most intractable solid
52 tumor, pancreatic adenocarcinoma, with stroma-rich characteristics, thereby validating
53 the significance of careful control of PM structure, particularly rod length, for promoting
54 systemic gene transfection.
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2. Materials and Methods

2.1. Materials

α -Methoxy- ω -amino-PEG (MeO-PEG) and α -acetal- ω -amino-PEG (acetal-PEG) of M_w 21 kDa were obtained from NOF Co., Ltd. (Tokyo, Japan). N^{ϵ} -trifluoroacetyl-L-lysine N -carboxyanhydride [Lys(TFA)-NCA] was synthesized as reported previously [25]. N -succinimidyl 3-(2-pyridyldithio)-propionate (SPDP) and Slide-a-lyzer dialysis cassettes ($MWCO = 3.5$ kDa) were purchased from Thermo Scientific (Rockford, IL). Cyclo[RGDfK(C- ϵ -Acp)] (cRGD) peptide (ϵ -Acp: 6-aminocaproic acid) was purchased from Peptide Institute Inc. (Osaka, Japan). Luciferase (Luc)-encoding gene-inserted pCAcc vector having the CAG promoter (pCAG-Luc2: 6,477 bp) was obtained from RIKEN Gene Bank (Tsukuba, Japan). pDNA encoding human soluble form of vascular endothelial growth factor receptor-1 (VEGFR-1) (or soluble fms-like tyrosine kinase-1: sFlt-1) with pCAcc vector having the CAG promoter (pCAG-sFlt-1: 7,186 bp) was constructed according to a previously described method [15]. Both pCAG-Luc2 and pCAG-sFlt-1 were transformed into competent *Escherichia coli* DH5 α cells and amplified, following which endotoxin-free pDNAs were obtained using the NucleoBond[®] Xtra Maxi EF Kit (Macherey-Nagel GmbH & Co., Germany). The other pDNAs, pKF18 (2,204 bp) and pAUR316 (11,613 bp), were purchased from Takara Bio Inc. (Otsu, Japan). pDNAs were labeled with Cy5 using the Label IT[®] Tracker[™] Intracellular Nucleic Acid Localization Kit obtained from Mirus Bio Corp. (Madison, WI) according to the manufacturer's instructions and used for studying cellular uptake. Dulbecco's modified eagle's medium (DMEM) and Dulbecco's phosphate-buffered saline (DPBS) were purchased from Sigma-Aldrich Co. (Madison, WI). Fetal bovine serum (FBS) was purchased from Dainippon Sumitomo Parma Co., Ltd. (Osaka, Japan). Cell culture lysis buffer and Luciferase Assay System Kit were purchased from Promega Co. (Madison, WI). Micro BCA[™] Protein Assay Reagent Kit was purchased from Pierce Co., Inc. (Rockford, IL). ExGen 500 (linear 22-kDa polyethylenimine) and Lipofectamine[®] LTX with PLUS[™] reagent were purchased from MBI Fermentas Co. (Burlington, ON, Canada) and Invitrogen Life Technologies Corp. (Carlsbad, CA), respectively. Rat monoclonal antibody against mouse platelet endothelial cell adhesion molecule-1 (PECAM-1) was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Alexa 488-conjugated (A11006),

Alexa 647-conjugated (A21247) goat anti-rat, and Alexa 488-conjugated (A11008) goat antirabbit IgG (H + L) secondary antibodies were obtained from Invitrogen Molecular Probes (Eugene, OR). Rabbit monoclonal antibody against mouse VEGF receptor-1 [Y103] (ab32152) was purchased from Abcam (Tokyo, Japan). Human epithelial ovarian carcinoma cells (HeLa) and human pancreatic adenocarcinoma-BxPC3 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). BALB/c-nu/nu mice (female, 5-weeks old) were purchased from Charles River Laboratories (Tokyo, Japan). All animal experimental procedures were performed in compliance with the Guidelines for the Care and Use of Laboratory Animals as stated by the Animal Committee of the University of Tokyo.

2.2. Synthesis of PEG-block copolymers

2.2.1. Synthesis of PEG-PLys

A series of MeO-PEG-PLys block copolymers with varying PLys DP were synthesized according a previously reported procedure [18]. In brief, Lys(TFA)-NCA was conjugated onto the ω -NH₂ terminal group of MeO-PEG via ring-opening polymerization in *N,N*-dimethylformamide containing 1 M thiourea. It should be noted that 21-kDa PEG was used in the present study instead of 12-kDa PEG [21,22] and 17-kDa PEG used previously [16,23] because we found that the cRGD ligand effect was particularly pronounced for PMs with high PEG shielding [23,24]. The molecular weight distribution (M_w/M_n) of a series of MeO-PEG-PLys(TFA) was determined to be ≤ 1.05 (Table 1) from gel permeation chromatography (GPC) equipped with TOSOH HLC-8220 calibrated with varying M_w of commercial PEG standards. Following this, MeO-PEG-PLys(TFA) was dissolved in methanol containing 1 N NaOH to remove protective TFA groups at 35°C for 6 h. PLys DP was determined to be 20, 42, and 69 by comparing the peak intensity ratio of the methylene protons of PEG [(CH₂)₂O, δ = 3.7 ppm] to the β , γ , and δ -methylene protons of lysine [(CH₂)₃, δ = 1.3–1.9 ppm] units from the ¹H-NMR spectra, respectively. A series of acetal-PEG-PLys with varying PLys DP was also synthesized similarly. M_w/M_n of a series of acetal-PEG-PLys(TFA) with varying PLys DP was determined to be ≤ 1.06 from GPC (Table 1). PLys DP was determined to be 21, 41, and 69 from the ¹H-NMR spectra.

2.2.2. Synthesis of thiolated PEG-PLys [PEG-PLys(SH)]

PEG-PLys(SH) polymers were synthesized by conjugating pyridyldithiopropionyl (PDP) groups onto the ϵ -amino groups of lysine units of the PLys segment of PEG-PLys using the heterobifunctional reagent *N*-succinimidyl 3-(2-pyridyldithio) propionate (SPDP), as reported previously [14]. In brief, MeO-PEG-PLys composed of 21-kDa PEG and PLys 69 (50 mg, 1.55 μ mol) was dissolved in 2 ml of *N*-methyl-2-pyrrolidone (NMP) supplemented with 5 wt% LiCl and reacted with SPDP (5 mg, 16 μ mol) pre-dissolved in NMP containing *N,N*-diisopropylethylamine (10 mol excess against SPDP) at room temperature (RT). After 4 h of stirring, the reaction was terminated by precipitation into diethyl ether. The precipitated polymer was then dissolved in 0.01 N HCl, dialyzed against the distilled water, and lyophilized to obtain MeO-PEG-PLys(PDP). A series of acetal-PEG-PLys(PDP) with varying PLys DP was also synthesized similarly and further used for cRGD ligand conjugation. The introduction ratio of thiol groups was determined from the ^1H -NMR spectrum recorded in DCl containing D₂O (pD = 2.4) at 25°C by the peak intensity ratio of the β , γ , and δ -methylene protons of lysine [(CH₂)₃, δ = 1.3–1.9 ppm] units to the pyridyl protons of the 3-(2-pyridyldithio) propionyl group (C₅H₄N, δ = 7.2–8.3 ppm). The degree of thiol group substitution was determined to be 10%–13% for all polymers (Table 1). PEG-PLys(SH) was obtained by treating PEG-PLys(PDP) with dithiothreitol (DTT).

2.2.3. Synthesis of cRGD-PEG-PLys and cRGD-PEG-PLys(SH)

The cyclo[RGDfK(C- ϵ -Acp)] (cRGD) peptide ligand was conjugated onto the α -end of acetal-PEG-PLys through the formation of a thiazolidine ring between the *N*-terminal cysteine of the cRGD peptide and the aldehyde group generated in acetal-PEG-PLys following incubation at acidic pH, as reported previously [21]. In brief, acetal-PEG-PLys of PLys 69 (50 mg, 1.55 μ mol) was dissolved in 2 ml of 10 mM HEPES buffer (pH 7.4) and dialyzed against 0.01 N HCl (pH 2.0) to yield aldehyde from the acetal group. The pH of the dialyzed solution was adjusted to 5.0 by 0.01 N NaOH, following which the pre-DTT-treated cRGD (1.5 mg, 1.62 μ mol) solution was added dropwise while stirring. After stirring overnight at 25°C, the polymer solution was dialyzed against HEPES with 150 mM NaCl, followed by distilled water, and then lyophilized to obtain cRGD-PEG-PLys. A series of cRGD-PEG-PLys(SH) with varying PLys DP was also synthesized similarly, as described above, with pre-DTT treatment.

The percentage of cRGD conjugation was determined by the peak intensity ratio of benzyl protons (D-Phenyl alanine, f. D-Phe; $\delta = 7.3\text{--}7.4$ ppm) of the cRGD peptide to the methylene protons of PEG ($\delta = 3.7$ ppm) from the ^1H -NMR spectra, and the conjugation ratio was calculated to be 80%–92% for all polymers (Table 1).

2.3. Preparation of PMs

A series of PEG-PLys with varying PLys DP and pDNA were separately dissolved in 10 mM HEPES (pH 7.4). Noncross-linked PMs (non-CPMs) were simply prepared by fast mixing 1-unit volume of the PEG-PLys solution with 2-unit volume of pDNA solution at the N/P ratio of 2. The N/P ratio is defined as the residual molar ratio of amine (N) groups of PEG-PLys to the phosphate (P) groups of pDNA. It should be noted that cross-linked PMs (CPMs) were prepared from PEG-PLys(SH) polymers as reported previously [14]. In brief, a series of PEG-PLys(SH) with varying PLys DP was dissolved in 100 mM DTT containing 10 mM HEPES (pH 7.4) and incubated for 4 h at RT to cleave any undesired preliminary formation of disulfide bonds. PMs were then prepared at the N/P ratio of 2 by fast mixing the pre-DTT treated PEG-PLys(SH) solution with the pDNA solution. After overnight incubation at RT, PM solutions were transferred to dialysis cassettes and dialyzed against 0.5% dimethyl sulfoxide (DMSO) containing HEPES for a day, followed by additional dialysis against only HEPES at RT for 2 days to remove DTT and DMSO from the PM solutions. DMSO added to the dialysis buffer facilitates the formation of disulfide cross-links between PEG-PLys(SH) polymers because it is an oxidizing agent for thiol (SH) groups [26]. The CPM solutions were recovered from dialysis cassettes and checked for pDNA concentration in each micelle solution by measuring UV absorption at 260 nm using NanoDrop ND-1000 (NanoDrop Technologies Inc., Rockland, DE) to confirm that the process of dialysis did not affect the concentration of pDNA of PMs. PMs without and with cross-linking were abbreviated as PEG-PLys and PEG-PLys(SH), respectively. CPMs equipped with the cRGD ligand were abbreviated as R-PEG-PLys(SH).

2.4. Preparation of transfection complexes from ExGen 500 and Lipofectamine[®] LTX with PLUS[™]

Polyplexes based on ExGen 500 were formulated according to manufacturer's protocol at the recommended N/P ratio of 6. In brief, 3.3 μl of ExGen 500 was added to

100 µl of pCAG-Luc2 DNA (10 ng/µl) and immediately vortexed for 10 s. After 30 min of incubation at RT, the polyplex solution containing 1 µg of pDNA was directly added to the cells in DMEM containing 10% FBS to evaluate their transfection efficacy. In addition, lipoplexes based on Lipofectamine[®] LTX with PLUS[™] were prepared according to the manufacturer's protocol. In brief, 1 µl of PLUS[™] reagent containing 50 µl of Opti-MEM[®] medium with 1 µg of pCAG-Luc2 DNA and 5 µl of Lipofectamine[®] LTX containing 50 µl of Opti-MEM[®] were prepared simultaneously. Lipoplexes were obtained by mixing these two solutions at a 1:1 v/v ratio. After 5 min of incubation at RT, 1 µg of pDNA containing the lipoplex solution was directly added to the cells in DMEM containing 10% FBS to evaluate their transfection efficacy.

2.5. Transmission electron microscopy (TEM) characterization

The morphology of PMs was observed using the H-7000 TEM machine (Hitachi Ltd., Tokyo, Japan) operated at an acceleration voltage of 75 kV, as reported previously [18]. In brief, copper TEM grids (Nisshin EM Corp., Japan) containing carbon-coated collodion membrane were glow-discharged for 30 s using an Eiko IB-3 ion coater (Eiko Engineering Co. Ltd., Japan) for hydrophilization. The hydrophilized grids were immersed into uranyl acetate (UA) (2% w/v)-treated PM solutions for 30 s to achieve effective staining. The sample-deposited grids were blotted onto the filter paper to remove excess solution, followed by air-drying for 30 min. Following this, the grids were transferred to the TEM machine for imaging. Rod lengths were obtained by measuring the major axis length of folded pDNA (appeared as rod-shaped structures) from the TEM images using Image J 1.45 software (National Institutes of Health, USA), and more than 100-rod structures were measured to plot the rod length distribution of each PM. It should be noted that the PEG shell of PMs was not observed owing to low affinity of UA.

2.6. Cellular uptake of PMs by flow cytometry

HeLa cells were seeded onto 12-well culture plates (50,000 cells/well) with 500 µl of DMEM containing 10% FBS and 1% antibiotics (penicillin and streptomycin) and incubated in a humidified atmosphere supplemented with 5% CO₂ at 37°C. After 24 h of incubation, the medium was replaced with fresh medium. The cells were then treated with PM solutions (1 µg of Cy5-labeled pDNA/well) and incubated for 24 h. The medium was removed, cells were then washed three times with ice-cold DPBS to remove extracellular

fluorescence, and cells were detached by trypsin–EDTA treatment. Trypsinized cells were then harvested from the cell culture plate and resuspended in 1 ml of ice-cold DPBS. After filtering the cells through a nylon mesh, the cells were analyzed by a BD™ LSR II flow cytometer equipped with FACS-Diva™ software (BD Biosciences, Franklin Lakes, NJ) to measure the fluorescence intensity of internalized pDNA. The obtained data were expressed as the mean fluorescence intensity from three independent samples ($n = 3$).

2.7. Evaluation of in vitro transfection efficiency

HeLa cells were seeded onto 24-well culture plates (20,000 cells/well) with 400 μ l of DMEM containing 10% FBS and 1% antibiotics (penicillin and streptomycin) and incubated for 24 h in a humidified atmosphere supplemented with 5% CO₂ at 37°C. After exchanging the medium with a fresh one, 1 μ g of pCAG-Luc2 DNA containing PM solutions was added to each well. After 24 h of incubation, the medium was exchanged with 400 μ l of fresh medium, followed by another 24 h of incubation. The cells were then washed three times with 400 μ l of ice-cold DPBS and lysed by 150 μ l of cell culture lysis buffer at 37°C for 15 min. Immediately, 20 μ l of the cell lysate was transferred to a 96-well luminometry plate, followed by the addition of 100 μ l of Luciferase Assay Reagent (Promega, Madison, WI) to each well, and allowed to react for 15 min. The Luc expression was then measured for 10 s from the photoluminescence intensity using Mithras LB 940 (Berthold Technologies, Bad Wildbad, Germany). The amount of total protein in the cell lysate was quantified using the Micro BCA™ Protein Assay Kit (Pierce, Rockford, IL), and the obtained Luc activity was normalized against the corresponding amount of total protein in the cell lysates. The transfection efficiency of polyplexes from ExGen 500 and lipoplexes from Lipofectamine® LTX with PLUS™ was simultaneously evaluated with the same concentration of pCAG-Luc2 DNA (1 μ g of pDNA/well). The data were expressed as relative light units (RLU) per mg of protein (RLU/mg protein) ($n = 6$).

2.8. Evaluation of transfection-mediated cytotoxicity

HeLa cells were plated onto 24-well culture dishes (20,000 cells/well) in 400 μ l DMEM containing 10% FBS and 1% antibiotics (penicillin and streptomycin) and incubated in a humidified atmosphere with 5% CO₂ at 37°C. After 24 h of incubation, the medium was replaced with 400 μ l of fresh medium, followed by the addition of

CPMs of R-PEG-PLys69(SH), polyplexes of ExGen 500, and lipoplexes of Lipofectamine[®] LTX with PLUS[™] solutions equivalent to 1 µg of pDNA/well. After 24 h of incubation, the medium was replaced with fresh medium, followed by another 24 h of incubation. The cells were washed three times with ice-cold DPBS, followed by the addition of 200 µl of fresh medium. Cell viability was assessed on the basis of 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-8) reduction to WST-8 formazan by the dehydrogenase activity of viable cells using the Cell Counting Kit-8 (CCK-8) (Dojindo, Kumamoto, Japan) according to manufacturer's instructions. In brief, 20 µl of the CCK-8 reagent was added to each well and allowed to develop orange-colored WST-8 formazan for 2 h. The UV absorbance of WST-8 formazan in each well was quantified at 450 nm using a microplate reader (Model 680, Bio-Rad, UK). The cell toxicity was expressed as the percentage of cell viability normalized against control cells treated with 10 mM HEPES (pH 7.4) ($n = 6$).

2.9. In vivo tumor growth suppression

BxPC3 cells (100 µl of 1×10^7 cells/ml in DPBS) derived from human pancreatic adenocarcinoma were subcutaneously inoculated into BALB/c-nu/nu mice (female, 6-weeks old) to develop xenografts and allowed to grow until they reached a proliferative phase. Once the tumor volume reached approximately 75 mm³, PMs loading either pCAG-sFlt-1 or pCAG-Luc2 DNA (20 µg in 200 µl of 10 mM HEPES containing 150 mM NaCl) were intravenously injected into the tail vein three times at a 4-day interval (day 0, 4, and 8). It should be noted that PM loading pCAG-Luc2 DNA containing the nontherapeutic gene was used as a control. The tumor size was measured over time using a digital vernier caliper across its longest longitudinal diameter (a) and longest transverse diameter (b), and the tumor volume (V) was calculated using the following equation: $V = 0.5 ab^2$. The measurement of tumor size was terminated on day 27 in compliance with the guidelines for animal experiments because the tumors of the control group showed ulcer characteristics. The relative tumor volume (V/V_0) was calculated against the tumor volume at day 0 (V_0) ($n = 4$). Day 0 was the first day of PM injection for treatment.

2.10. Quantification of sFlt-1 protein expression at the tumor site

PMs loading pCAG-sFlt-1 DNA (20 µg in 200 µl of 10 mM HEPES containing 150 mM NaCl) were intravenously injected once into the tumor-bearing mice via the tail vein. The mice were sacrificed after 48 h of injection and xenografted tumors were excised, placed into tissue-Tek-OCT, frozen at -80°C for 2 days, and sectioned into 10-µm-thick slices with a rotary microtome maintained in a cryostat at -20°C, following which they were transferred onto a microscopic slide. The expressed sFlt-1 protein was immunofluorescent-stained by rabbit monoclonal antibody against mouse VEGF receptor-1 [Y103] (ab32152) (Abcam, Tokyo, Japan), followed by incubation with Alexa Fluor 488-conjugated goat anti-rabbit IgG (H + L) secondary antibody (A11008) (Invitrogen, Carlsbad, CA). VECs of the tumor vasculature were immunofluorescent-stained by rat monoclonal antibody against mouse PECAM-1, followed by incubation with Alexa Fluor 647-conjugated goat anti-rat IgG (H + L) secondary antibody (A21247) (Invitrogen, Carlsbad, CA). The nuclei were fluorescent-stained with Hoechst 33342 (H3570) (Invitrogen, Carlsbad, CA). The immunofluorescent-stained tumor sections were analyzed for fluorescence using a confocal laser scanning microscope (CLSM), LSM 780 (Carl Zeiss, Oberlochen, Germany). The sFlt-1 gene expression was quantified by measuring the integrated density of Alexa-488 fluorescence per image with 6 images for each sample from the CLSM images using Image J 1.45 software.

2.11. Quantification of vascular density in the tumors

PMs loading pCAG-sFlt-1 DNA (20 µg in 200 µl of 10 mM HEPES containing 150 mM NaCl) were intravenously administered to the tumor-bearing mice via the tail vein three times at a 4-day interval (day 0, 4, and 8). After 20 days, the mice were sacrificed and xenografted tumors were excised, placed into tissue-Tek-OCT, frozen at -80°C for 2 days, and sectioned into 10-µm-thick slices with a rotary microtome maintained in a cryostat at -20°C, followed which they were transferred onto a microscopic slide. VECs of the tumor vasculature were immunofluorescent-stained by rat monoclonal antibody against mouse PECAM-1, followed by incubation with Alexa Fluor 488-conjugated goat anti-rat IgG (H + L) secondary antibody (A11006) (Invitrogen, Carlsbad, CA). The immunofluorescent-stained tumor cryosections were analyzed for fluorescence by LSM 780. The vascular density was quantified by measuring the

percentage area of PECAM-1-positive green pixels per image with 6 images for each sample from the CLSM images using Image J 1.45 software.

3. Results and Discussion

3.1. Cellular uptake of PMs without core cross-linking

The effect of PLys DP (20, 42, and 69) on cellular uptake was initially examined. PMs prepared from a series of PEG-PLys with varying PLys DP and fluorescent-labeled pCAG-Luc2 DNA (6,477 bp) were used to evaluate the cellular uptake efficiency of HeLa cells by measuring the intracellular fluorescence intensity using flow cytometry. As presented in Fig. 1a, cellular uptake was highly dependent on PLys DP of PEG-PLys; higher cellular uptake was observed for higher PLys DP, whereas lower cellular uptake was observed for lower PLys DP, in accordance with our previous report on PMs with PLys DP ranging from 7 to 48 [27]. In order to explore the underlying effect of PLys DP on cellular uptake, the stability of PMs in the culture medium and polyanionic environment was examined because these two factors may interfere the level of cellular uptake efficiency because of following reasons: the culture medium supplemented with 10% FBS includes nucleases [28] and the cell membrane contains negatively charged extracellular glycosaminoglycans (GAGs) [29,30], which can potentially destabilize PMs through polyion exchange reactions. The stability in the culture medium was evaluated by observing the intactness of pDNA through quantitative real-time polymerase chain reaction (qRT-PCR). pDNA within PMs showed almost no digestion regardless of PLys DP, whereas naked pDNA was rapidly digested (Fig. S1). This observation indicated that PMs were stable enough in the culture medium regardless of PLys DP, thereby implying another possibility occurring on the cell membrane. It should be noted that previous reports suggested that GAGs, particularly heparan sulfate (HS), could destabilize electrostatically driven self-assembled polymeric and liposomal gene delivery systems [31,32]. Thus, the tolerability of HS by PMs was evaluated by observing the release of pDNA on agarose gel electrophoresis, with a note that HS is the most abundant [29,30] and sulfated (0.9 sulfate per repeating disaccharide unit) [33] GAG among all other extracellular GAGs present on the cell membrane. Indeed, HS dissociated PMs regardless of PLys DP at high concentrations (6 mg/ml). Noteworthy was that at mid-range concentrations (1 to 4 mg/ml), there is a trend showing that the tolerability was progressively increased with

the increase in PLys DP. The highest PLys DP showed better tolerability than the lowest PLys DP (Fig. S2a). This difference in tolerability may be accountable for the trend of cellular uptake of different PLys DP (Fig. 1a), such that less stable PMs of PEG-PLys20 were susceptible to disassembly at the cellular membrane, thereby resulting in lower cellular uptake. The possibility of HS as a barrier for cellular uptake was examined by further evaluation using HeLa cells treated with heparanase, a HS digestive enzyme, which can selectively remove extracellular HS [34]. The amount of uptake was indeed increased in heparanase-treated cells compared to that of cells without heparanase treatment (Fig. S3); yet there was only a limited increase in cellular uptake for PMs of PEG-PLys20.

3.2. Cellular uptake of CPMs

To further explore the precluded cellular uptake of PMs of PEG-PLys20, disulfide cross-linking was introduced in the core of PMs to stabilize the structure according to our previous procedure [14]. Thiol groups were introduced onto ϵ -NH₂ groups of lysine units of a series of PEG-PLys using the *N*-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) reagent to obtain PEG-PLys(SH) for preparing CPMs. Here, the thiolation degree (Table 1) was chosen to be 10% on the basis of previous optimization in the *in vitro* gene expression assay [15]. It should be noted that the disulfide cross-links retain environmental responsiveness, providing remarkable stability in the extracellular milieu, whereas CPMs are liable to be cleaved in the intracellular reductive environment responding to high glutathione (10 mM) concentration, thereby triggering the release of encapsulated pDNA [14,35]. As anticipated, tolerability against HS treatment was significantly improved for all CPMs regardless of PLys DP, and no pDNA release from CPMs was observed in an electrophoretogram (Fig. S2b) even at the highest HS concentration (6 mg/ml) able to dissociate PMs without cross-linking (non-CPMs). Eventually, cellular uptake was significantly promoted by the introduction of disulfide cross-linking in all PLys DP (Fig. 1b). Nevertheless, the trend of PLys DP dependence persisted even after cross-linking. The CPMs of PEG-PLys20(SH) still exhibited significantly precluded cellular uptake compared with other PLys DP regardless of remarkably improved stability against HS, as shown by no release of pDNA in the electrophoretogram (Fig.

S2b). Therefore, there should be other factors apart from an unfavorable dissociation that limit cellular uptake.

3.3. Effect of PEG density of PMs on their cellular uptake

We then focused on the different PEG density of these PMs with varying PLys DP; PMs with lower PLys DP are characterized by higher PEG density, whereas those with higher PLys DP are characterized by lower PEG density [17]. Accordingly, it is assumed that higher PEG density of PEG-PLys20 may account for lower cellular uptake as a result of reduced interactions with the cell membrane [36,37]. To clarify this assumption, PEG density of CPMs with different PLys DP needed to be quantitatively determined. Upon this demonstration, the binding number of tethered PEG to pDNA should be known [17]. However, its direct estimation was technically difficult for PEG-PLys(SH) within CPMs because Alexa Fluor-labeled polymers may interact with the dialysis membrane during the preparation process of CPMs; therefore, the binding number of block copolymers to pDNA may not be accurately quantified. Hence, we inferred the PEG density of CPMs from that of non-CPMs, considering that pDNA within PMs is packaged into a distinct rod-shaped structure (see Fig. S4 and Fig. S5 for representative structures) with specific rod length distribution based on the quantized folding scheme [12], and PMs with similar rod length distribution were assumed to retain similar PEG density because the rod length is a function of PEG density [17]. Thus, the rod length distributions of CPMs and non-CPMs of respective PLys DP were examined and found to exhibit almost a similar distribution (Fig. 2a-f). Accordingly, it may be reasonable to consider that the PEG density of CPMs of particular PLys DP was similar to that of non-CPMs. Moreover, direct PEG height observation from cryogenic TEM (cryo-TEM) indicated a similar PEG height for CPMs and non-CPMs at corresponding PLys DP (Table S1, Fig. S6a for representative cryo-TEM images), further supporting their similar PEG density. Thus, the PEG density of non-CPMs was calculated for different PLys DP using a previously reported method [17] and inferred to CPMs of corresponding PLys DP (Table S2), giving the values of 0.072, 0.054, and 0.045 chains/nm² for PLys 20, 42, and 69, respectively. Indeed, PEG density analysis clarified that CPMs of lower PLys DP retained higher PEG density and thereby likely contributed to lower cellular uptake efficiency.

3.4. Effect of rod length of PMs on their cellular uptake

In addition, there is another issue to be considered with regard to cellular uptake. PLys DP modulation affects not only PEG density [17] but also rod length distribution [17,18]. CPMs of lower PLys DP exhibited a longer rod length distribution (Fig. 2d, Fig. S4d for representative TEM images), whereas those of higher PLys DP exhibited a shorter rod length distribution (Fig. 2f, Fig. S4f for representative TEM images). The longer rod length may also be a problem for efficient cellular uptake because it has been claimed that the process of each endocytosis has an upper size limit with regard to the diameter of the endocytic vesicles [38]. Because HeLa cells are generally considered to be nonphagocytic [39], the possibility of phagocytosis could be excluded. Then, the following four possible endocytic pathways should be considered: macropinocytosis and clathrin-dependent, caveolae-dependent, and clathrin/caveolae-independent endocytosis, with the upper size restriction of the endocytic vesicles being 5 μm [40], 200 nm [41,42], 80 nm [43], and 90 nm [38], respectively. With regard to the rod length distribution for CPMs of PEG-PLys20(SH) loading pCAG-Luc2 (Fig. 2d), most of the rod length fraction was found to be above 200 nm, leaving a very small fraction below 200 nm. Taking this observation into consideration, limited cellular uptake of CPMs of PEG-PLys20(SH) may be reasonable because most of the fraction has to rely on only macropinocytosis. On the other hand, CPMs of PEG-PLys69(SH) (Fig. 2f) presenting their entire fraction below 200 nm may be taken up through all four endocytic pathways. This analysis clearly pointed out that the rod length of PMs may also be accountable for determining cellular uptake behavior (Fig. 1b).

To gain further insight on structural parameters (PEG density and rod length) contributing to cellular uptake, CPMs with different rod length distribution but retaining comparable PEG density were prepared using fixed DP of PLys 20 of PEG-PLys(SH) (which provided the highest PEG density among the series of PLys DP) and three different pDNAs with varying lengths (pKF18: 2,204 bp, pCAG-Luc2: 6,477 bp, and pAUR316: 11,613 bp). As evidenced by the rod length distribution (Fig. 2g-i, Fig. S5 a-c for representative TEM images), CPMs prepared from different pDNAs showed different rod length distribution, and it was found that pKF18 DNA retained a shorter rod length distribution, with a major fraction being below 200 nm (Fig. 2g), whereas pAUR316 DNA retained longer rod length distribution, with the entire fraction being above 200 nm (Fig. 2i). The similar PEG density of these CPMs of differently sized

pDNAs was suggested by the similar folding number pattern (Fig. S8) because the folding number determines the rod length in the quantized folding scheme of pDNA [18] (Table S3) and is a function of PEG density [17]. The cellular uptake efficiency of these CPMs (PEG-PLys20(SH)) showed substantial rod length dependence (Fig. 1c); shorter rod CPMs of pKF18 DNA (mostly below 200 nm) were more readily internalized into the cells, whereas longer rod CPMs of pAUR316 DNA (entirely above 200 nm) exhibited limited uptake. These observations on CPMs with comparable PEG density indicate that the rod length of PM obviously plays a crucial role in cellular uptake efficiency. It should be noted that PMs of a longer rod length were indeed capable of being taken up, as also reported for micrometer-sized rod-shaped filomicelles [44]; however, their efficiency was limited. Particularly interest was that the critical rod length was consistent with the well-documented restriction size of clathrin-dependent endocytic vesicles (200 nm) [41,42].

In order to further promote cellular uptake efficiency, use of specific ligand is an effective strategy [21-24]. We applied cRGD peptide ligands on a series of PMs and examined the cellular uptake on HeLa cells, characterized by the overexpression of cRGD-specific $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin receptors [21]. The promoted cellular uptake was obtained for all series of CPMs regardless of PLys DP (Fig. 1d) and pDNA size (Fig. 1c), which confirmed the significance of the effect of cRGD conjugation to promote cellular uptake. However, CPMs of PEG-PLys21(SH) with cRGD (R-PEG-PLys21(SH)) remained lower cellular uptake than those of other PLys DP with cRGD (Fig. 1d). This observation confirmed that rod length is an important structural parameter that should be primarily considered to achieve high cellular uptake.

It would be interesting to examine whether rod length dependence is also applicable to other cell lines. Accordingly, we examined the cellular uptake efficiency of CPMs with and without the cRGD ligand using BxPC3 cells (epithelial cells) [45] (Fig. S9a) and human umbilical vein endothelial cells (HUVECs) [46] (endothelial cells) (Fig. S9b), both characterized by the overexpression of integrins binding to cRGD. We observed a quite consistent trend of PLys DP and cRGD effects, as observed in HeLa cells. Interestingly, CPMs of PEG-PLys20(SH) again exhibited substantially limited cellular uptake, validating the critical rod length for efficient cellular uptake and further demonstrating that the rod length of PMs should be regulated below 200 nm to induce efficient cellular uptake.

3.5. Transfection efficiency of PMs

Responding to the cellular uptake behavior, transfection efficiency was evaluated in HeLa cells using the reporter gene Luc (pCAG-Luc2 DNA). As shown in Fig. 3a, the trend of transfection efficiency appeared to be consistent with that of cellular uptake. PMs modified with cross-linking showed significantly higher gene expression efficiency than PMs without cross-linking. PMs with the cRGD ligand also exhibited higher gene expression efficiency than those without the cRGD ligand. Synergistically, PMs modified with both cross-linking and the cRGD ligand showed significantly augmented gene expression efficiency. Ultimately, with the rod length requirement of below 200 nm, cRGD-installed CPMs of PEG-PLys69(SH) [R-PEG-PLys69(SH)] exhibited the highest transfection efficiency among the other examined PMs. To evaluate the ability of R-PEG-PLys69(SH), the transfection efficiency was compared with that of well-acknowledged commercially available transfection reagents, cationic-polymer based ExGen 500 and cationic-lipid based Lipofectamine[®] LTX with PLUS[™], at optimized preparations recommended by the manufacturer. Notably, the transfection efficiency of R-PEG-PLys69(SH) was almost comparable with that of the commercial transfection reagents (Fig. 3b) despite the fact that PM was covered with PEG palisade. Moreover, PMs do not possess any specific endosomal escape function such as the well-known proton sponge effect of ExGen 500 [47] and endosomolytic effect of Lipofectamine[®] LTX with PLUS[™] [48]. In addition to the enhancement of cellular uptake by the use of the cRGD ligand, an alternative intracellular trafficking pathway to the nucleus should be involved because we observed that cRGD-installed PMs showed rapid accumulation in proximity to the nucleus [21,23], achieving such a high transfection efficiency. PMs also presented advantage with regard to cytotoxicity because the R-PEG-PLys69(SH) formulation did not show any transfection-mediated cytotoxicity, whereas ExGen 500 and Lipofectamine[®] LTX with PLUS[™] showed a significant decrease in cell viability (Fig. 3c) evaluated at an equivalent dose used for the transfection study (1 µg of pDNA/well), further validating the high potency of this PM formulation in terms of the safety concern.

3.6. Systemic treatment of xenografted pancreatic tumors by PMs loaded with *sFlt-1*-encoding pDNA

Given the advanced functions of the R-PEG-PLys69(SH) formulation, including systemic application as a PEG palisade for providing stealth effect, core cross-linking for avoiding unfavorable disassembly in blood circulation, and a cRGD ligand as a tumor-targeting moiety, this formulation was challenged to treat pancreatic tumor, one of the most intractable solid tumors, in mice with subcutaneous xenografts of BxPC3 human pancreatic adenocarcinoma. It should be noted that BxPC3 xenografts are characterized by hypovascularity and reduced vascular permeability due to thick pericyte coverage around the tumor blood vessels [49,50]. In addition, the tumor nest is surrounded by a fibroblast-rich stromal matrix [49-52], thereby limiting the access of therapeutic agents to the tumor cells. With this regard, tumor vascular endothelial cells (VECs) were targeted by noting that VECs are featured by overexpression of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin receptors [53]. As a strategy for suppressing tumor growth, an antiangiogenic approach was chosen using a gene encoding a potent antiangiogenic exogenous protein, sFlt-1, which captures vascular endothelial growth factor (VEGF), exerting an antiangiogenic effect [54,55]. The therapeutic activity of PMs loading either pCAG-sFlt-1 or pCAG-Luc2 DNA (control), followed by intravenous injection, was evaluated by the tumor growth inhibition study. As presented in Fig. 4, compared with the control group, significant suppression of tumor growth was observed in the sFlt-1-treated group, thereby validating the feasibility of R-PEG-PLys69(SH) as a formulation for systemic gene therapy. To confirm that the obtained tumor growth inhibition was due to sFlt-1 protein expression at the tumor site, tumor cryosections were histologically analyzed and the level of sFlt-1 expression was quantified with an antibody that binds to sFlt-1 and inherently expressed VEGFR-1. The immunofluorescent-stained images showed remarkable sFlt-1 expression at the tumor site (Fig. 5c). Notably, most of the sFlt-1 protein was found in the region of the tumor stroma adjacent to VECs. Presumably, the secreted sFlt-1 protein from transfected cells captured VEGF and limited the binding to VEGFR-1, thereby preventing endothelial cell proliferation and consequently inhibiting neovasculature formation (antiangiogenesis). Indeed, the inhibition of neovascularization was confirmed by the reduced vascular density of tumor treated with sFlt-1 loaded R-PEG-PLys69(SH) in comparison with that of the control group (HEPES) (Fig. 5h), as shown by immunofluorescent staining of VECs using PECAM-1 (Fig. 5e,g,h). These evaluations confirmed that the obtained antitumor activity was certainly attributed to

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3 antiangiogenesis by the expressed sFlt-1 protein, which ultimately led to the
4 suppression of tumor growth. Along with the immunofluorescent staining method, we
5 previously confirmed that the sFlt-1 protein was specifically found at the tumor site and
6 not in any other normal organs by enzyme-linked immunosorbent assay (ELISA) for the
7 similar CPM system [15]. Notably, CPMs of lower PLys DP exhibited higher PEG
8 density, thus can be expected for better blood circulation. Accordingly, effective
9 antitumor efficacy can be readily anticipated for R-PEG-PLys21(SH) because PMs have
10 an increased chance of accumulating at the targeted tumor site. However, this
11 formulation did not exhibit significant antitumor efficacy (Fig. 4), although
12 PEG-PLys20(SH) exhibited a better blood retention time than PEG-PLys69(SH) (Fig.
13 S10). Most likely, the inferior cellular uptake for CPMs of R-PEG-PLys21(SH) due to
14 the longer rod length, as observed in the *in vitro* study (Fig. 1c), may result in limited
15 transfection of the targeted cells. It should be noted that CPMs of PEG-PLys20(SH)
16 loading pCAG-sFlt-1 DNA (7186 bp) exhibited most of the rod length fraction above
17 200 nm, while those of PEG-PLys69(SH) exhibited most of the rod length fraction
18 below 200 nm (Fig. S11). Indeed, neither sFlt-1 expression (Fig. 5b) nor decreased
19 vascular density (Fig. 5f) was observed for R-PEG-PLys21(SH) at the tumor site. This
20 result validated the significance of regulating the rod length of PMs for promoting *in*
21 *vivo* gene transfection upon systemic administration, concurrently encouraging
22 subsequent research to develop an ideal systemic gene delivery system with prolonged
23 blood circulation capacity on the basis of this recognized PM formulation.

4. Conclusions

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42 Aiming to capture the correlation between structural features of PMs and
43 biological performance, we intensively studied the effect of PLys DP on cellular uptake,
44 integrating previously approved strategies such as disulfide cross-linking and use of the
45 cRGD moiety as a ligand for improving cellular uptake efficiency. The structural
46 features of PMs such as stability of assembly and PEG density affected cellular uptake
47 efficiency; however, rod length was found to be the most significant parameter affecting
48 cellular uptake. With a critical rod length of 200 nm, which is consistent with the
49 well-documented upper size limit of clathrin-dependent endocytic vesicles, PMs with a
50 longer rod showed limited uptake, whereas those with a shorter rod showed efficient
51 uptake. In particular, coupling with cross-linking and the cRGD ligand substantially
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3 elevated the cellular uptake. Consequently, the PM formulation satisfying all these
4 parameters, i.e., R-PEG-PLys69(SH), showed promoted transfection efficiency almost
5 comparable with that of ExGen 500 and Lipofectamine[®] LTX with PLUS[™],
6 well-known transfection reagents, despite the fact that PMs did not have any specific
7 endosomal escape function and were also covered by PEG. Upon systemic
8 administration, the identified R-PEG-PLys69(SH), with advanced features for systemic
9 tumor therapy by virtue of PEG shielding, core cross-linking, and the cRGD ligand,
10 exhibited significant antitumor efficacy against a stroma-rich pancreatic tumor model
11 through an antiangiogenic effect. Furthermore, CPMs of R-PEG-PLys21(SH) with most
12 of the rod length fraction above 200 nm resulted in no significant antitumor efficacy
13 even if this formulation showed a better blood retention profile than PLys 69, approving
14 a critical role of rod length even in *in vivo* gene transfection. Therefore, the present
15 study clearly identified the importance of regulating the length of rod-shaped PMs to
16 obtain efficient gene transfection not only in cultured cell lines but also in systemic
17 applications.
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Table 1. Chemical characterization of a series of PEG-PLys with varying PLys DP

Block copolymers	PLys DP	M _w /M _n ^a	Conjugation ratio of SH in Lys (%) ^b	Conjugation ratio of cRGD to PEG-PLys (%) ^c
PEG-PLys20	20	1.04	0	0
PEG-PLys42	42	1.04	0	0
PEG-PLys69	69	1.05	0	0
PEG-PLys20(SH)	20	1.04	10	0
PEG-PLys42(SH)	42	1.04	12	0
PEG-PLys69(SH)	69	1.05	12	0
R-PEG-PLys21	21	1.03	0	92
R-PEG-PLys41	41	1.05	0	84
R-PEG-PLys69	69	1.06	0	84
R-PEG-PLys21(SH)	21	1.03	12	85
R-PEG-PLys41(SH)	41	1.05	13	80
R-PEG-PLys69(SH)	69	1.06	13	83

^aDetermined from GPC equipped with the TOSOH HLC-8220 column calibrated with commercial PEG standards.

^bIntroducing ratio of thiol (SH) groups against ϵ -NH₂ groups of lysine units of either MeO PEG-PLys or acetal-PEG-PLys determined from ¹H-NMR spectra.

^cConjugation ratio of cRGD ligand onto α -end of either acetal-PEG-PLys or acetal-PEG-PLys(SH) determined from ¹H-NMR spectra.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Fig. 1. Effect of different structural features of PMs on cellular uptake efficiency in HeLa cells. (a) PLys DP dependence for PMs of pCAG-Luc2 DNA. (b) PLys DP and cross-linking dependence for PMs of pCAG-Luc2 DNA. (c) Rod length and cRGD ligand dependence for CPMs [PEG-PLys20(SH)] of differently sized pDNAs. (d) PLys DP and cRGD ligand dependence for CPMs of pCAG-Luc2 DNA. Note that PLys 20 in Fig. 1d represents either PEG-PLys20(SH) or R-PEG-PLys21(SH) and PLys 42 represents either PEG-PLys42(SH) or R-PEG-PLys41(SH) (mean $\pm$ SD, $n = 3$, two-tailed Student's t-test, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$).

Fig. 2. Rod length distribution of PMs obtained by measuring the major axis length of the rod structures using TEM images. (a-c): Non-CPMs of pCAG-Luc2 (6,477 bp); (a) PEG-PLys20, (b) PEG-PLys42, and (c) PEG-PLys69. (d-f): CPMs of pCAG-Luc2; (d) PEG-PLys20(SH), (e) PEG-PLys42(SH), and (f) PEG-PLys69(SH). (g-i): CPMs of differently sized pDNAs with fixed PEG-PLys20(SH); (g) pKF18 (2,204 bp), (h) pCAG-Luc2 (6,477 bp), and (i) pAUR316 (11,613 bp). Representative TEM images of (a-f) and (g-i) are shown in Fig. S4 and Fig. S5, respectively. Dotted vertical lines represent the theoretical rod lengths expected on the basis of the quantized folding scheme at each folding number (n).

Fig. 3. Transfection efficiency and transfection-mediated cytotoxicity of PMs. (a) Transfection efficiency of both non-CPMs and CPMs of varying PLys DP with and without the cRGD ligand. Note that PLys 20 in Fig. 3a represents either PEG-PLys20(SH) or R-PEG-PLys21(SH) and PLys 42 represents either PEG-PLys42(SH) or R-PEG-PLys41(SH). (b) Transfection efficiency of R-PEG-PLys69(SH) compared with that of the commercial transfection reagents ExGen 500 and Lipofectamine[®] LTX with PLUS[™]. (c) Transfection-mediated cytotoxicity of R-PEG-PLys69(SH) compared with that of commercial reagents. Cells treated with HEPES were used as a control in all the experiments. pDNA encoding the luciferase gene (pCAG-Luc2) was used for all the experiments (mean $\pm$ SD, $n = 6$, two-tailed Student's t-test, $*p < 0.05$, $***p < 0.001$, $****p < 0.0001$).

Fig. 4. Antitumor activity of cRGD-conjugated CPMs (R-CPMs) loading either pCAG-sFlt-1 or pCAG-Luc2 pDNA in subcutaneous BxPC3 tumor-bearing mice via

intravenous administration. Mice injected with HEPES and CPMs of R-PEG-PLys69(SH) loading Luc2-expressing pDNA were used as control groups. R-CPMs were administered on days 0, 4, and 8 (indicated by arrows). Data points marked with asterisks show statistical significance for CPMs of R-PEG-PLys69(SH) loading sFlt-1-injecting group relative to not only control groups but also R-PEG-PLys21(SH) (mean $\pm$ SEM, $n = 4$, two-tailed Student's t-test, $*p < 0.05$, $**p < 0.01$).

Fig. 5. Histological examination of BxPC3 tumor tissue after intravenous administration of R-CPMs containing sFlt-1 pDNA. Top row: Representative CLSM images of sFlt-1 protein expression in tumor cryosections of (a) HEPES (control), (b) R-PEG-PLys21(SH), and (c) R-PEG-PLys69(SH). Green: expressed sFlt-1 (or inherent VEGFR-1); red: vascular endothelial cells; blue: nucleus. (d) Integrated density of sFlt-1/VEGFR-1-positive fluorescence quantified from CLSM images (mean $\pm$ SD, $n = 6$, two-tailed Student's t- test, $**p < 0.01$). Bottom row: Representative CLSM images of immunofluorescent-stained VECs (green) of the tumor vasculature using PECAM-1 in tumor cryosections of (e) HEPES (control), (f) R-PEG-PLys21(SH), (g) R-PEG-PLys69(SH), and (h) percentage area of PECAM-1-positive region quantified from CLSM images (mean $\pm$ SD, $n = 6$, two-tailed Student's t-test, $**p < 0.01$).