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Targeted Gene Delivery by Polyplex Micelles with Crowded PEG Palisade and cRGD Moiety for Systemic Treatment of Pancreatic Tumors

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ABSTRACT Adequate retention in systemic circulation is the preliminary requirement for systemic gene delivery to afford high bioavailability into the targeted site. Polyplex micelle formulated through self-assembly of oppositely-charged poly(ethylene glycol) (PEG)-polycation block copolymer and plasmid DNA has gained tempting perspective upon its advantageous core-shell architecture, where outer hydrophilic PEG shell offers superior stealth behaviors. Aiming to promote these potential characters toward systemic applications, we strategically introduced hydrophobic cholesteryl moiety at the ω -terminus of block copolymer, anticipating to promote not only the stability of polyplex structure but also the tethered PEG crowdedness. Moreover, M_w of PEG in the PEGylated polyplex micelle was elongated up to 20 kDa for expecting further enhancement in PEG crowdedness. Furthermore, cyclic RGD peptide as ligand molecule to integrin receptors was installed at the distal end of PEG in order for facilitating targeted delivery to the tumor site as well as promoting cellular uptake and intracellular trafficking behaviors. Thus constructed cRGD conjugated polyplex micelle with the elevated PEG shielding was challenged to a modeled intractable pancreatic cancer in mice, achieving potent tumor growth suppression by efficient gene expression of antiangiogenic protein (sFlt-1) at the tumor site.

1. Introduction:

Significant advance in nanotechnology-based biomaterials has inspired to develop smart nano-devices to seek the breakthrough of medical care, highlighted as nanomedicine. These medical nano-devices, which are fabricated according to versatile chemistry-based engineering to achieve specific functionalities, have dramatically improved medical diagnosis and treatment [1-4]. One of intriguing example is a gene delivery carrier, which is defined as nanoparticles embedded with genomic materials for targeted delivery to the pathological site and exert functional protein expression with the consequence of restoring normal conditions [5]. To realize this concept of gene therapy through systemic administration, the stabilities of gene delivery carriers in blood circulation so as to increase delivery efficiency to the targeted sites are of crucial importance for ultimate therapeutic potency. In order to attain adequate longevity in the harsh biological environment, gene delivery carrier must circumvent an ensemble of predefined biological interferences including enzymatic degradation, protein adsorption and opsonization followed by reticuloendothelial (RES) system capture [6], thereby development of a gene delivery carrier with stealth surface character along with substantial structural stabilities is imperative for systemic use. In view of these requirements, we developed a promising polyplex micelle by self-assemble of block copolymer poly(ethylene glycol) (PEG)-poly{N'-[N-(2-aminoethyl)-2-aminoethyl]aspartamide} [PAsp(DET)] and plasmid DNA (pDNA) through electrostatic interaction, which has demonstrated considerable therapeutic potential with respect to both safety and efficiency concerns, relying largely on the tempting features of efficient transfection activity and biodegradable nature from PAsp(DET) [7-10]. The PEG in the block copolymer also plays a crucial role in the polyplex micelle as a protective palisade, which diminishes non-specific interactions with biological components, accounting for stealthiness in the biological condition [6, 11-15]. Nevertheless, the systemic use of this system was limited by its insufficient retention in the blood circulation [16], thereby motivating adaptation of this system with strengthened PEG palisade to obtain improved stealthiness for persistence in the bloodstream. Very recently, we established method to quantify PEG crowdedness for polyplex micelles, and recognized importance of increasing crowdedness of PEG palisade in avoiding blood clearance related to RES capture, which is initiated by opsonin adsorption [17]. Prolonged blood retention was observed for polyplex micelle possessing crowded PEG shell characterized as squeezed conformation, whereas those

micelles possessing PEG shell characterized by mushroom conformation were cleared from blood compartment at the early stage, validating a worthy strategy of modulation of PEG to high crowdedness for prolonging blood retention. In designing polyplex micelle with elevated PEG crowdedness, energetic description in regulating structure of polyplex micelle should present a rational strategy. To this point, we have clarified DNA condensation is a counterbalance between compaction force from minimization of surface energy by reducing contact area of pDNA/polycation complex and water molecules, and anti-compaction force from PEG steric repulsion, including osmotic pressure from crowded PEG palisade and entropic elasticity derived from restricted conformation and additionally rigidity of folded DNA bundle [17]. Learning from this description, hydrophobic cholesteryl moiety was introduced at the ω -terminus of the block copolymer with the intention of increasing hydrophobicity of complex core. We expected the increased hydrophobicity provides additional compaction force to condense DNA more, which can prevail PEG steric repulsion, eventually promoting PEG for further crowding. Apparently, the increased hydrophobicity of complex is also expected to stabilize the complex structure, so that preventing polyplex micelle from dissociation in the biological condition. Furthermore, in view of a previous finding that the PEG shielding effect was more pronounced with the use of longer PEG (17 kDa rather than 12 kDa), as evidenced by reduced affinity to the cellular membrane [18], 20-kDa PEG was introduced into a PEG-PAsp(DET)-cholesteryl system. To counter the reduced cellular uptake resulting from enhanced PEG shielding by virtue of above-mentioned strategies, we installed a cyclic RGD (Arg-Gly-Asp) peptide (cRGD) at the distal end of PEG. This modification was expected to enhance cellular uptake while prompting the accumulation of polyplex micelle into tumor angiogenic endothelial cells and malignant tumor cells, which feature with the overexpression of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins as cRGD receptors on the cell surface [19-21]. We note that a previous study has indicated that the benefits of ligand conjugation were particularly pronounced for polyplex micelles with high PEG shielding, for which non-specific interaction was minimized so that ligand efficacy elicited and eventually afforded efficient gene expression activity in the transfected cells. In this regard, the polyplex micelle with 20-kDa PEG may hold tempting perspective in fully exploiting benefits of ligand-integrin-mediated affinity to produce high gene expression activity after systemic administration. The current research intends to highlight the impact of PEG elongation upon cholesteryl-stabilized polyplex micelle to pursue prolonged circulation and to emphasize the benefits of strategically utilizing ligand cRGD peptide to promote active tumor targeting

abilities. Ultimately, we attempted to test the systemic utility of the proposed system for treatment of modeled pancreatic adenocarcinoma in mice using BxPC3 cells, which is fibrotic and also hard to be treated with conventional chemotherapy [22].

2. Materials and methods

2.1. Materials

α -Methoxy- ω -amino-PEG (M_w 12,000 and 20,000) and α -acetal- ω -amino-PEG (M_w 20,000) were obtained from NOF Co., Ltd. (Tokyo, Japan). β -Benzyl-L-aspartate N-carboxyanhydride (BLA-NCA) was obtained from Chuo Kaseihin Co., Inc. (Tokyo, Japan). Diethylenetriamine (DET), cholesterol, n-butylamine, and trifluoroacetic acid were purchased from Wako Pure Chemical Industries, Ltd. 4-Dimethylaminopyridine (DMAP) and dicyclohexylcarbodiimide (DCC) were purchased from Aldrich Chemical Co. Ltd. (Milwaukee, WI). Other chemicals were used as received. The cRGDFK- ϵ -caproic acid-cysteine (cRGD peptide) was purchased from Peptide Institute Inc. (Osaka, Japan). Alexa680 succinimidyl ester was a product of Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from Dainippon Sumitomo Parma Co., Ltd. (Osaka, Japan). pDNAs, pBR322 (4,361 bp) and pGL3 control vector (5,256 bp) were purchased from Takara Bio Inc. (Otsu, Japan). The pDNA encoding luciferase with a CAG promoter provided by RIKEN Gene Bank (Tsukuba, Japan) was amplified in competent DH5 α *Escherichia coli* and purified with a QIAGEN HiSpeed Plasmid MaxiKit (Germantown, MD). Cell culture lysis buffer and luciferase Assay System Kit was purchased from Promega Co. (Madison, WI). The Micro BCATM Protein Assay Reagent Kit was purchased from Pierce Co., Inc. (Rockford, IL). The pDNA encoding a soluble form of vascular endothelial growth factor (VEGF) receptor-1 (sFlt-1) was provided by Prof. Masabumi Shibuya at Jobu University, and was prepared as previously reported [16]. Dulbecco's modified Eagle's medium (DMEM) was purchased from Sigma-Aldrich (St. Louis, MO). For cellular uptake and intracellular distribution assay, pDNA was labeled with Cy5 using a Label IT Nucleic Acid Labeling Kit from Mirus Bio Corporation (Madison, WI) according to the manufacturer's protocol. Human pancreatic cancer BxPC3 cells were obtained from the American Type Culture Collection (Manassas, VA). BALB/c nude mice (female, 5 weeks old) were purchased from Charles River Laboratories

(Tokyo, Japan). All animal experimental protocols followed the guidelines of the Animal Committee of the University of Tokyo.

2.2. Polymer synthesis

The procedures for synthesis of block copolymers, PEG-PAsp(DET)-cholesteryl and cRGD-PEG-PAsp(DET)-cholesteryl are presented in Scheme S1. Cholesteryl bearing a reactive carboxyl group was prepared from substitution of 3-hydroxyl group in cholesterol to a primary amine, followed by reaction with succinic anhydride (Detailed procedure is described in Supporting Information). The precursor block copolymer PEG-poly(β -benzyl-L-aspartate) (PEG-PBLA) was prepared according to a ring-opening polymerization scheme as previously reported [7]. The polymerization of monomer BLA-NCA was initiated from the ω -NH₂ terminal group of α -methoxy- ω -amino-PEG to obtain PEG-PBLA. The terminal amino group of PBLA segment was then coupled with the carboxyl group of cholesteryl in the presence of DCC (10 eq) and DMAP (2 eq) in DMF for an overnight reaction. The resulting polymer was purified by precipitation into mixture of chilled diethyl ether and isopropanol (v:v = 2:1) thrice and lyophilized from benzene to obtain PEG-PBLA-cholesteryl. Then, an aminolysis reaction was performed to introduce diaminoethane moiety into the side chain of PBLA for PEG-PAsp(DET)-cholesteryl. For synthesis of cRGD-PEG-PAsp(DET)-cholesteryl, acetal-PEG-NH₂ was used as a starting material for polymerization, followed by a similar synthetic procedure to prepare acetal-PEG-PAsp(DET)-cholesteryl with only minor modifications in the purification process after aminolysis. The reaction mixture was added dropwise to a chilled mixture of diethyl ether and ethanol (v:v = 7:3, also containing acetic acid to neutralize DET) for precipitation to obtain the crude product of acetal-PEG-PAsp(DET)-cholesteryl. The precipitated product was further purified by sequential vacuum drying and dialysis against acetate buffer for thrice and deionized water for thrice at 4°C. The proposed purification procedures were applied to prevent the conversion of the acetal to be reactive. The acetal-PEG-PAsp(DET)-cholesteryl product was dissolved in water and the solution was adjusted to pH 2 using HCl for conversion of acetal group to be reactive with cRGD peptide (10 eq to the block copolymer). Note that cRGD peptide was prior dissolved in NaHCO₃ buffer (0.1 M, pH 7.4) containing dithiothreitol (10 eq to peptide) to cleave the potential disulfide bond between the cysteine termini. After 1 h of incubation, cRGD peptide solution was

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4 added into the acetal-PEG-PAsp(DET)-cholesteryl block copolymer solution, followed by adjusting pH to 5 for
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6 overnight reaction at 4°C. The final product cRGD-PEG-PAsp(DET)-cholesteryl was obtained after dialysis
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8 against 1 M NaCl solution (thrice) and dialysis against deionized water (thrice) at 4°C. The dialyzed product
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10 solution was lyophilized to yield the final product as a chloride salt form. The prepared polymers were
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12 determined to have a narrow unimodal molecular weight distribution by gel permeation chromatography
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14 (Supporting Information Fig. S3). The polymerization degree of the PAsp(DET) segment, efficiencies of
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16 cholesteryl and cRGD conjugation was determined from the ¹H-NMR spectra in D₂O at 25°C (Table 1).
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21 *2.3. Preparation of polyplex micelles*

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24 Synthesized block copolymers were dissolved in 10 mM HEPES buffer (pH 7.4) as stock solutions.
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26 Aliquot of polymer solution was added to pDNA solution for complexation at varying N/P ratios (molar ratio
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28 of amino groups in polymer to phosphate groups in pDNA), followed by overnight incubation at 4°C. The final
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30 concentration of pDNA in all the samples was adjusted to 33.3 µg/mL for *in vitro* experiments and 100 µg/mL
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32 for *in vivo* experiments. Note that all the pre-experimental procedures involving with polymer solution or
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34 complex solution were strictly carried out at low temperature, under 4°C refrigeration or in an ice bath to
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36 avoid polymer fragmentation [9].
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40 *2.4. Cellular uptake*

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43 Cellular uptake efficiency was evaluated by flow cytometry (BD LSR II, BD, Franklin Lakes, NJ). Cy5-
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45 labeled pDNA was used to prepare a group of polyplex micelles. HeLa cells were seeded on a 6-well culture
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47 plate (100,000 cells/well) and incubated for 24 h in 2 mL of DMEM containing 10% FBS in a humidified
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49 atmosphere with 5% CO₂ at 37°C. The medium was replaced with fresh medium, followed by addition of 150
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51 µL polyplex micelle solution (33.3 µg pDNA/mL) into each well. After 24 h incubation, the cells were washed
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53 thrice with PBS to remove extracellular Cy5 fluorescence. After detachment of cells from the culture plate
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55 with trypsin, the cells were harvested and re-suspended in PBS for flow cytometry measurement.
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60 *2.5. In vitro gene expression*

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HeLa cells were seeded on 24-well culture plates (20,000 cells/ well) and incubated for 24 h in 400 μ L of DMEM containing 10% FBS in a humidified atmosphere with 5% CO₂ at 37°C. The medium was replaced with 400 μ L of fresh medium, followed by addition of 30 μ L each polyplex micelle solution (1 μ g pDNA/well). After 24 h of incubation, the medium was replaced with 400 μ L fresh DMEM, followed by another 24 h of incubation. The cells were washed with 400 μ L of PBS, and lysed in 150 μ L of the cell culture lysis buffer. The luciferase activity of the lysates was evaluated from the photoluminescence intensity using Mithras LB 940 (Berthold Technologies, Bad Wildbad, Germany). The measured luciferase activity was normalized according to corresponding amount of proteins in the lysates determined by the Micro BCA™ Protein Assay Reagent Kit.

2.6. Intracellular distribution

The endosome entrapment ratio was determined by evaluating colocalization degree of pDNA and endosomes by confocal laser scanning microscopy (CLSM). In brief, Cy5-labeled pDNA was used to prepare a class of polyplex micelles at N/P 8. HeLa cells (50,000 cells) were seeded on 35-mm cell culture dishes and incubated for 24 h in 1 mL of DMEM containing 10% FBS. The medium was replaced with fresh medium, followed by the addition of 75 μ L of polyplex micelle solution (33.3 μ g pDNA/mL) into each cell culture dishes. After 24 h of incubation, the medium was removed and the cells were rinsed thrice with PBS prior to the imaging. The intracellular distribution of each polyplex micelle was observed by CLSM after staining acidic late endosomes and lysosomes with LysoTracker Green (Molecular Probes, Eugene, OR) and nuclei with Hoechst 33342 (Dojindo Laboratories, Kumamoto, Japan). The CLSM observation was performed using LSM 510 (Carl Zeiss, Germany) with a 63 $\times$ objective (C-Apochromat, Carl Zeiss, Germany) at excitation wavelengths of 488 nm (Ar laser), 633 nm (He-Ne laser), and 710 nm (MaiTai laser for 2-photon imaging) for LysoTracker Green (green), Cy5 (red), and Hoechst 33342 (blue), respectively. The colocalization ratio was calculated as previously described [10] according to the formula:

$$\text{Colocalization ratio} = \text{number of yellow pixels} / \text{number of yellow and red pixels},$$

where yellow corresponds to the pDNA that is trapped in late endosome/lysosomes, and red corresponds to pDNA in the cytosol.

2.7. Morphology observation

Morphology observation was performed with transmission electron microscopy (TEM) measurement by an H-7000 electron microscope (Hitachi, Tokyo, Japan) operated at 75 kV acceleration voltages for insight on the morphology of polyplex micelles containing pBR322 pDNA at N/P 8. Copper TEM grids with carbon-coated collodion film were glow-discharged for 10 s using an Eiko IB-3 ion coater (Eiko Engineering Co. Ltd., Japan). The grids were dipped into the desired polyplex micelle solution, which was premixed with uranyl acetate (UA) solution (2% (w/v)) for 30 s to stain the DNA. The sample grids were blotted by filter paper to remove excess complex solution, followed by air-drying for 30 min. Note that the PEG shell is invisible under TEM owing to its low affinity with UA. Thus, the DNA in the complex was selectively visualized without interference from PEG moieties surrounding pDNA strands. The obtained TEM image was further analyzed with ImageJ 1.44 (National Institutes of Health) to quantify the length of the major axis in each sample, and more than 100 individual polyplex micelles were measured to obtain rod length distribution.

2.8. Binding numbers of block copolymers to pDNA in polyplex micelles

The binding behaviors of block copolymers to pDNA in each polyplex micelle were investigated according to an ultracentrifuge method [23]. As reported previously, ultracentrifugation of polyplex micelle solution allows selective sedimentation of polyplex micelles, while unbound free polymers remain in the supernatant. In consequence, the binding fraction of polymer can be determined by subtracting free polymer (remaining in the supernatant after sedimentation) from the total fed polymer. To quantify the associating number of block copolymers in the polyplex micelle, Alexa-680 labeled block copolymer (labeling procedures according to protocol provided by the manufacturer, conjugation efficiency 0.5 Alexa-680 molecules per block copolymer) was used to prepare a class of polyplex micelle at N/P 8. After overnight incubation at 4°C, 500 μ L of each polyplex micelle solution was placed into thickwall polycarbonate tube, 343776 (Beckman Coulter, Inc., Fullerton, CA) and subjected to ultracentrifugation (Optima TLX, Beckman Coulter, Inc., Fullerton, CA) equipped with TLA-120.1 rotor for 3 h under 50,000 $\times g$ for complete sedimentation of polyplex micelles, while the unbound free polymer remained in the upper supernatant. The supernatant containing free block

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4 copolymer was collected for absorbance measurement by a UV-Vis spectrometry (ND-3300, NanoDrop,
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6 Wilmington, NC) at a wavelength of 680 nm. The concentration of free block copolymer in the supernatant
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8 was determined from the obtained absorbance according to a calibration curve from Alexa680-labeled block
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10 copolymer solutions.
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14 *2.9. Stability in blood circulation*

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17 Polyplex micelles prepared from CAG-Luc2 were intravenously administered into blood stream. After
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19 an appropriate time (5 min, 10 min, 15 min and 30 min), the blood samples were collected and the remaining
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21 pDNA in the blood was purified and extracted with a DNeasy blood and tissue kit (QIAGEN, Germantown, MD)
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23 according to the protocol provided by the manufacture. Quantification of pDNA was obtained by real time
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25 polymerase chain reaction (RT-PCR) measurement by using the ABI 7500 Fast Real-time RT-PCR System
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27 (Applied Biosystems, Foster City, CA) with forward primer TGCAAAAGATCCTCAACGTG and reverse primer
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29 AATGGGAAGTCACGAAGGTG. The remaining pDNA in the blood stream was expressed as the percentage of
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36 *2.10. Tumor suppression efficacy*

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39 BALB/c nude mice were inoculated subcutaneously with BxPC3 cells derived from human pancreatic
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41 adenocarcinoma (1×10^7 cells in 100 μ L of PBS). Tumors were allowed to grow for 10 days until proliferative
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43 phase (tumor size was approximately 75 mm³). Then 200 μ L of each polyplex micelle solution [20 μ g soluble
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45 fms-like kinase-1 (sFlt-1) pDNA/mouse] in 10 mM HEPES buffer (pH 7.4) with 150 mM NaCl was
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47 intravenously injected through the tail vein thrice on days 0, 4 and 8. Tumor size was measured every two or
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49 three days with a digital vernier caliper across its longest (a) and shortest diameters (b), and its volume (V)
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51 was calculated according to the formula $V = 0.5ab^2$. The tumor size measurement was discontinued when the
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53 tumors in the control group showed ulcer characteristics according to the guideline for animal experiments.
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55 Tumor progression was evaluated in terms of relative tumor volume to day 0 (day for the first polyplex
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57 micelle injection), n = 6.
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2.11. Vascular density

Each polyplex micelle loading sFlt-1 (20 µg of pDNA in 200 µL HEPES buffer containing 150 mM NaCl) was intravenously injected into the BxPC3 tumor-bearing mice through the tail vein on days 0 and 4. Mice were sacrificed on day 6, and the tumors were excised, frozen in dry-iced acetone, and sectioned into 20 µm thick slices with a cryostat. Vascular endothelial cells (VECs) were immunostained with rat monoclonal antibody antiplatelet endothelial cell adhesion molecule-1 (PECAM-1) (BD Pharmingen, Franklin Lakes, NJ), followed by incubation with Alexa Fluor 488-conjugated secondary antibody. The immunostained sections were observed with CLSM780 (Carl Zeiss, Germany). The vascular density was quantified by counting the percentage area of PECAM-1-positive pixels per image with 12 images per sample.

2.12. sFlt-1 expression at the tumor site

Each polyplex micelle loading sFlt-1 (20 µg of pDNA in 200 µL HEPES buffer containing 150 mM NaCl) was intravenously injected into the BxPC3 tumor-bearing mice *via* the tail vein. Mice were sacrificed at 48 h after injection. The tumors were excised, frozen in dry-iced acetone, and sectioned into 10 µm thick slices with a cryostat. VECs were immunostained using the antibodies anti-mouse PECAM-1 (BD Pharmingen, San Diego, CA) and anti-human and mouse VEGFR1 (ab32152, Abcam Japan, Tokyo, Japan). The sections immunostained was observed with CLSM780 (Carl Zeiss, Germany). The sFlt-1 gene expression was quantified by counting the integral fluorescence intensity of ab32152-positive pixels per image with six images per sample.

2.13. Tumor accumulation

Mice bearing BxPC3 tumor with similar tumor volume of approximate 700 mm³ were randomly selected for each cohort (n = 9). Polyplex micelles of 200 µL of solution prepared from Cy5-labeled pDNA were intravenously injected into the blood stream through the tail vein to quantify the tumor accumulation of polyplex micelles. Mice were sacrificed 24 h after injection. The tumor tissue was excised. The lysed solution of tumor tissue was dispensed into 96-well opaque plate and the corresponding Cy5 fluorescence was

measured by IVIS® Lumina II (Caliper Life Sciences, Hopkinton, MA). The results were expressed as total photon counts normalized by tumor weight.

2.14. Statistical analysis

The p values were determined by the Student's t-test using a two-tailed distribution and two-sample un-equal variance with the T. Test function of Microsoft Excel. The p values of less than 0.05 were considered as statistically significant.

3. Results and discussion

3.1. Polymer synthesis and characterizations of polyplex micelles

PEG-PAsp(DET)-cholesteryl was synthesized by conjugation of cholesteryl group to precursor PEG-PBLA), followed by aminolysis reaction with DET. For the synthesis of cRGD-PEG-PAsp(DET)-cholesteryl, acetal-PEG-NH₂ was used as the starting material for polymerization according to a similar synthetic procedures, yielding acetal-PEG-PAsp(DET)-cholesteryl. Then the acetal group in this product was activated at acidic pH for reaction with the cRGD peptide to yield cRGD-PEG-PAsp(DET)-cholesteryl (Supporting Information, Scheme S1). The resulting block copolymers were characterized by ¹H-NMR and GPC measurement (Supporting Information Fig. S1, S2 and S3) and the chemical descriptions are summarized in Table 1.

For preparation of polyplex micelles, each polymer product listed in Table 1 was dissolved in 10 mM HEPES buffer (pH 7.4), followed by mixing with pDNA solution at varying N/P ratios. The abbreviations for each polyplex micelle are listed in Table 1. The sizes of the polyplex micelles were measured by dynamic light scattering (DLS) measurement and all the samples presented cumulant diameters ranging from 80 nm to 100 nm with unimodal size distributions of a low polydispersity index (PDI) of ~ 0.1 (Supporting Information, Table S1).

3.2. In vitro evaluation for polyplex micelles

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4 For enhancing PEG shielding to confer prolonged blood retention on polyplex micelles, we attempted
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6 to increase the M_w of PEG in the block copolymer from previously used 12 kDa to 20 kDa. To evaluate its
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8 effect on enhanced PEG shielding, we first evaluated cellular uptake activity. Given that the
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10 enhanced PEG shielding tends to reduce interactions with biological structures (e.g. cell membrane
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12 so as for reduced cell uptake activity [18]), the cellular uptake can be an indicator for PEG shielding.
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14 Herein, pDNA labeled with fluorescence dye was used to prepare polyplex micelles, and the cellular uptake
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16 efficiency in HeLa cells was quantified by measurement of the fluorescence intensity of internalized pDNA by
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18 flow cytometry. The result (Fig. 1a) confirmed a low cellular uptake profile for PEG20C, indicating enhanced
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20 PEG shielding. In contrast, the higher cellular uptake for PEG12C implied insufficient PEG shielding.
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24 To address the dilemma of PEG shielding for the reduced cellular uptake, a ligand molecule (cRGD
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26 peptides) was strategically conjugated at the distal end of PEG segment in the block copolymer intended for
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28 promoting specific ligand-integrin-mediated uptake. Our previous study showed that the benefits of ligand
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30 conjugation were particularly pronounced for the polyplex micelles with high PEG shielding, where non-
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32 specific interaction was minimized so that cRGD-integrin mediated affinity elicited and mediated efficient
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34 gene expression activity in the transfected cells [18]. We accordingly expected that R-PEG20C with its high
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36 PEG shielding effect would offer the prospect of fully exploiting benefits of ligand-integrin mediated affinity.
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38 Because HeLa cells with overexpression of cRGD specific integrins ($\alpha_v\beta_3$ and $\alpha_v\beta_5$) on the cell surface [18],
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40 cellular uptake and subsequent gene expression of polyplex micelles were examined to verify the cRGD effect.
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42 R-PEG20C showed a marked increase in contrast to low uptake level of PEG20C (Fig. 1a), indicating the
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44 benefit of cRGD in promoting cellular uptake.
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47 Elevated gene expression was also observed in reporter gene expression evaluation, where gene
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49 expression level of R-PEG20C exhibited one order of magnitude higher than that of PEG20C (Fig. 1b). It is
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51 noteworthy that this higher expression was observed despite the lower cellular uptake activities shown by R-
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53 PEG20C compared with PEG12C (Fig. 1a). To this regard, gene expression activity was normalized by cellular
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55 uptake to yield transfection efficiency (Fig. 1c). R-PEG20C exhibited markedly higher transfection efficiency
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57 than PEG12C and PEG20C, in particular R-PEG20C at an N/P ratio of 8 presented the highest transfection
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59 efficiency (N/P 8 was accordingly chosen for hereafter biophysical evaluation and therapeutic trials). The
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high transfection efficiency of R-PEG20C implied that the cRGD motif may confer benefits not only for cellular uptake but also for the post-endocytosis process (intracellular trafficking). For the ligand-free polyplex micelle system [cRGD(-)], the internalized polyplex micelles after endocytosis are subjected to endosome entrapment, eventually resulting in enzymatic degradation if they cannot afford adequate facilities to retrieve the entrapped polyplex micelles from late endosome. In this respect, the colocalization of polyplex micelle with digestive late endosome and lysosome was quantified for each sample. It should be noted that the cationic segment of PAsp(DET) affords escape capacity from acidic endosome entrapment: PAsp(DET) featured as the functional flanking ethylenediamine moiety in the side chain of N-substituted polyaspartamide (PAsp), exhibits distinctive two-step protonation behavior in response to a pH gradient, where the protonation of ethylenediamine is stimulated from single protonation (pH 7.4) to double protonation (90% at pH 5) [8]. This acid-responsive trait of PAsp(DET) results in a selective endosome membrane destabilization function, consequently led to liberation of embedded polyplex micelles from endosome entrapment to the cytosol. To gain clear insight on intracellular trafficking of polyplex micelles, CLSM observation was carried out to distinguish their intracellular distribution (Fig. 2). Herein, pDNA was labeled with Cy5 (red) and digestive late endosome/lysosomes were labeled by LysoTracker Green (green). Therefore, intracellular yellow pixels (colocalization) represent entrapment of polyplex micelles in the late-endosome/lysosome compartment, whereas intracellular red pixels (non-colocalization) indicate their presence in the cytosol. Accordingly, a lower colocalization degree indicates lower entrapment of polyplex micelles in the acidic digestive compartment. Fig. 2d reveals that the samples of PEG12C and PEG20C showed a colocalization ratio of approximately 60%, confirming efficient endosome escape capacity presumably by virtue of the functionalities of PAsp(DET). It is noteworthy that colocalization ratios of R-PEG20C appeared to be much lower than others. To this issue, the previous study suggested possibility of alternative pathway for intracellular trafficking after cRGD-mediated uptake, featured with rapid migration to the nucleus proximity in the cytosol in clear comparison of cRGD(-) micelle [24]. Thus, R-PEG20C may follow a favorable intracellular trafficking pathway avoiding entrapment in digestive compartments and thereby mediating dramatically high gene expression efficiency.

Although the cellular uptake level of PEG20C was much lower than PEG12C, the normalized transfection efficiency of PEG20C was higher than PEG12C. In view of their comparable endosome escape

activity, we suspected that the pDNA encapsulated within PEG12C may have been damaged to some extent, whereas it was more protected in PEG20C. To investigate this hypothesis, the tolerance of polyplex micelle to DNase I digestion was evaluated by quantifying the remaining percentage of intact functional DNA (including supercoiled, open-circular and linear DNA, the transcriptionally active forms), revealing better tolerance by PEG20C than by PEG12C (Supporting Information Fig. S4). This superiority may be explained by the higher shielding effect of PEG20C than that of PEG12C, such that the accessibility of digestive enzyme to encapsulated pDNA is reduced, permitting the transport of more intact DNA into the nucleus.

3.3. PEG shielding and blood retention property

As described above, improved PEG shielding for polyplex micelle was achieved by elongation of the PEG segment from 12 kDa to 20 kDa. To characterize quantitatively the PEG surface shielding for polyplex micelles, we calculated the PEG density for polyplex micelles of PEG20C and PEG12C. To evaluate the cholesteryl effect on formulating the polyplex micelle, we included a control PEG12 sample without the cholesteryl moiety. For estimation of PEG density, the surface area of the complex core, representing the area available for PEG tethering, was the first requirement to be determined because the PEG density is expressed as the number of tethered PEG chains per unit area available for PEG (the core surface area). With this purpose, the morphology of polyplex micelles was characterized by transmission electron microscopy (TEM) measurement. Note that pDNA in polyplex micelles were selectively observed in the TEM image owing to stronger affinity of uranyl acetate (staining agent) to DNA than PEG. All polyplex micelles appeared as uniform rod-shaped particles (Fig. 3), suggesting that pDNA is packaged into the rod-shaped bundle through a regular folding behavior [25]. Assuming the bundle to be a cylindrical structure, the surface area of the complex core (Table 2) can be calculated by the surface area of the cylinder side plus the area of both of its ends (a detailed calculation was described in Supporting Information). The number of tethered PEG chains in the polyplex micelle is another requirement for determining the tethered PEG density, which was determined from ultracentrifuge analysis as previously reported (Table 2) [23]. The average PEG tethering density $\langle \sigma \rangle$ was then calculated in terms of the number of PEG chains per unit area (chains/nm²) (Table 2). Considering that different M_w of PEG (12 kDa and 20 kDa) were used in this PEG density analysis, the normalized PEG

density, namely reduced tethering PEG density (RTD), defined as the number of chains that occupy an area covered by an unperturbed polymer chain, was used to characterize PEG tethering density,

$$RTD = \sigma \pi R_g^2$$

where the R_g (radius of gyration of an unperturbed PEG chain) of 12-kDa and 20-kDa PEG were calculated to be 4.7 nm and 6.3 nm, respectively [26, 27].

The quantified PEG density in RTD (Table 2) displayed the order PEG12 < PEG12C < PEG20C, which corroborating the effect of cholesteryl conjugation and PEG elongation from 12 kDa to 20 kDa on elevating PEG density in terms of RTD.

Noteworthy is that cholesteryl conjugation (in comparison of PEG12 and PEG12C) appears to promote a markedly increased number of block copolymers bound to pDNA (Table 2). As for PEG12 formulation, approximately 160 chains of PEG12 polymer were bound to each pDNA (Table 2). Given that the polymerization degree of the PAsp(DET) segment of PEG12 polymer is 56 (Table 1) and two amino groups reside in one ethylenediamine unit, these 160 block copolymers accounts for a total of 16,960 ($= 2 \times 160 \times 56$) amino groups. With 53% of amino groups of PAsp(DET) presumed to be positively charged at pH 7.4 [8], these 16,960 amino groups would account for 8,989 ($= 16960 \times 0.53$) positive charges at pH 7.4, coinciding reasonably well with the number of negative charge from phosphate groups in one pBR322 (pBR322: 8,722 negative charges from 4,361 bp). The charge ratio (+/-) of PEG12 formulation is thus calculated to be 1.03 (8,989/8,722), suggesting a formulation following stoichiometric charge ratio.

In contrast to PEG12, cholesteryl-conjugated PEG12C and PEG20C polymers exhibited considerably larger numbers of block copolymers associating with pDNA. This difference is possibly due to the hydrophobic nature of the cholesteryl moiety. Presumably, block copolymers with hydrophobic cholesteryl conjugation prefer to incorporate into the hydrophobic complex core, rather than staying in aqueous solution, so that a larger number of block copolymers, exceeding the stoichiometric charge ratio, may bind to pDNA. The cholesteryl conjugation also affected the packaging structure of pDNA in the polyplex micelle. The rod length of PEG12C was observed to be shortened from 78.6 nm to 55.8 nm compared with non-cholesteryl PEG12 polyplex micelles (Fig. 3d and e). Presumably the hydrophobic cholesteryl moiety associated with the complex core caused surface energy to increase, and the developed additional compaction force stimulated DNA to more condense for minimizing contact area to water molecules. Eventually, the reduced PEG tethering

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4 area could contribute to increase crowdedness of PEG palisade. These results verified worthy strategy of
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6 using cholesteryl conjugation to compact packaged DNA more and promote PEG to be further crowding.
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9 In addition, PEG molecular weight also affected the pDNA packaging structure, as found that PEG20C
10 (Fig. 3f) exhibited a longer rod particle than PEG12C (Fig. 3e). It is possible that the steric repulsion resulting
11 from the higher PEG crowding of PEG20C prevents a form as compact as that of PEG12C. Moreover, the
12 polyplex micelle formulated from 20-kDa PEG showed a slightly smaller number of block copolymers bound
13 to pDNA than the micelle of PEG12C (Table 2). Presumably, the larger steric repulsion of 20-kDa PEG may
14 limit further association to the polyplex micelles likewise 12-kDa PEG, implying that the PEG in PEG20C is
15 already in a substantially crowded state. In summary, the synergistic effect of cholesteryl conjugation was
16 identified; facilitating a larger number of polymers bound to pDNA (over-stoichiometry) and inducing more
17 condensation of pDNA, which eventually resulted in an elevated tethered PEG density. Elongation of the PEG
18 from 12 kDa to 20 kDa also contributed to increased PEG crowdedness. Noteworthy is the crowdedness of
19 PEG palisade predicted from the RTD value (Table 2). According to our previous study on correlation of RTD
20 and PEG conformation [16], the PEG conformation of PEG12 adopted mushroom with overlapped by
21 neighboring PEG chains, whereas that of PEG12C adopted a squeezed conformation. The RTD of PEG20C
22 indicated the PEG conformation adopting further squeezed, even possibly to scalable brush region [17]. As we
23 demonstrated in previous work, PEG modulation to a squeezed conformation increased blood circulation as
24 compared to that of mushroom conformation [17], it is anticipated PEG20C with the elevated PEG
25 crowdedness should afford better stealth function.
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43 The effect of enhanced PEG crowdedness on the blood retention of polyplex micelles was then
44 evaluated. (Note that the blood circulation profile of PEG12 without cholesteryl moiety was not determined
45 because we had previously shown its low blood retention after systemic administration [16].) In brief,
46 following intravenous administration of polyplex micelles into the bloodstream, the blood was collected at
47 appropriate time points and the remaining pDNA in the blood was quantified according to RT-PCR
48 measurement. Note that the method based on PCR more directly estimates the blood retention because the
49 PCR detects the quantity of intact pDNA (at least primer region). As shown in Fig. 4a, PEG20C showed
50 appreciably increased retention life in the blood circulation compared with PEG12C, which validated PEG
51 elongation as an effective strategy for prolonging blood circulation. The promotion of PEG shielding appeared
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to be particularly pronounced at the early stage of circulation. Extrapolation of the decay curve to the starting time (0 min) for PEG20C profiles provided approximately 100%, which was contrasted to approximately 10% for PEG12C, suggesting that polyplex micelles of PEG20C, managed to overcome the rapid elimination from the circulation following injection. Most likely, the elevated PEG crowdedness of PEG20C afforded this stealth function to escape from the rapid blood elimination, possibly RES capture initiated by opsonization. It should be noted that tethered PEG with a scalable brush conformation has reported to entirely prohibit large biomolecules (> 10 nm) adsorption [29, 30], such as opsonins, supporting reasonability of above scheme. However, despite accomplishment to avoid the rapid blood clearance by PEG20C, the retention was not sustained. Instead, the profiles decreased showing similar decay curve with PEG12C, implying that the polyplex micelles were eliminated by the same mode irrespective of the PEG crowdedness. Possibly, the PEG shielding of PEG20C was still inadequate to afford as an effective barrier to prevent relatively small biomolecules, such as nucleases with approximate size of several nanometers [30]. The polyplex micelle received DNase attack for digestion of the encapsulated DNA, resulting in the destabilization of polyplex structure to become more susceptible to dissociation particularly when passing anionic abundant sites or structures, e.g. by heparan sulfate in glomerular basement membrane [31]. Therefore, further efforts will be needed to develop a strategy to resolve this issue for pursuing persistence in the bloodstream. Yet, the effort to promote PEG crowdedness by cholesteryl conjugation, which offered synergistic effect on increase of PEG density in the form of increased tethering PEG chains on more compacted polyplex core, together with PEG elongation, resulted in an appreciable crowded palisade, possibly, scalable brush region, eventually affording a stealth character to escape the rapid elimination after the injection. Of note, further evaluation of blood circulation profile of R-PEG20C confirmed its prolonged retention activities comparable to PEG20C (Fig. S5), thus portending potential systemic use for tumor treatment.

3.4. Systemic treatment of pancreatic tumors

Given that polyplex micelles of R-PEG20C appeared to retain potency for systemic application with enhanced blood circulation property and transfection ability, we attempted to test their therapeutic effect in

the treatment of subcutaneous xenografts of pancreatic adenocarcinoma. The pancreatic cancer is one of the most intractable solid tumors [32], and it is worth noting that BxPC3 xenografts share the histopathologic characteristics of the human pancreatic adenocarcinoma: The xenografts have fibrotic parts, and tumor vasculature and nests of tumor cells embedded in the fibrotic parts [22, 33]. It is also noteworthy in this model that all of 1) endothelial cells in tumor blood vessels, 2) fibroblast cells in tumor stroma and 3) tumor cells *per se* overexpress $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin receptors, which are recognition motifs for the cRGD peptide, so all of these cells in the model can be the targets for cRGD micelles. On the other hand, the characteristic features of pancreatic tumor, as mentioned above as common characteristics in humans and BxPC3 xenografts, which are reduced vascular permeability due to pericyte coverage of tumor vessels and thick fibrosis, may be an obstacle to deliver massive amount of therapeutic gene to the whole pancreatic tumor cells. Regarding to this, we attempted to apply an antiangiogenic approach, aiming to suppress tumor growth through inhibition of neo-vasculature formation [34-36]. As a pDNA payload for a tumor gene therapy test, we selected a secreted protein, soluble sFlt-1, which exerts antiangiogenesis by trapping angiogenic molecules (VEGF) [37]. From the result of maximal cell transfection efficiency at N/P 8 (Fig. 1c), R-PEG20C prepared at N/P 8 was evaluated. As shown in Fig. 4b, PEG20C without a ligand molecule [cRGD(-)] (N/P 8) displayed an inhibitory effect on tumor growth compared with control groups, moreover R-PEG20C with ligand molecule [cRGD(+)] exerted a significantly more potent tumor suppression effect than PEG20C, supporting the strategy of ligand conjugation for promoting systemic antitumor efficacy.

To confirm that the observed antitumor efficacy was due to loaded sFlt-1 pDNA expression at the tumor site, we immunostained the lightly fixed cyro-section of tumor tissue with an antibody which binds to both membrane-bound Flt-1, or VEGF receptor 1 (VEGFR1), and sFlt-1/sVEGFR1. The expression of total VEGFR1/Flt-1 (green) was remarkably more in the mice administered with PEG20C and R-PEG20C as compared to the control sample (Fig. 5). In particular, R-PEG20C displayed higher gene expression than with PEG20C, concurrently in good agreement with inhibitory effect on tumor growth. Noteworthy, the location of the expressed sFlt-1 was found to be enriched in the tumor stroma adjacent to the vascular endothelial cells (red), rather than in the tumor nest (cell nucleus stained in blue). In view of the function of VEGF molecule to stimulate growth of and to maintain existence of neo-vasculature, it is reasonable that the expressed sFlt-1 secreted from the transfected cells may be found to localize there as a consequence of entrapping VEGF

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4 molecules. Furthermore, to investigate the benefits of the cRGD motif for targeted accumulation of polyplex
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6 micelles into the tumor site, polyplex micelles of PEG20C and R-PEG20C prepared from pDNA labeled with
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8 Cy5 fluorescent dye were intravenously administered into the bloodstream. The tumor accumulation of
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10 polyplex micelles was quantified by measurement of the fluorescence intensity of Cy5 in homogenized tumor
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12 tissue (Fig. 4c). Apparently, R-PEG20C exhibited markedly higher tumor accumulation, approximately five
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14 times higher than that of PEG20C. Finally, to confirm the antiangiogenic effect of loaded sFlt-1 gene
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16 expression from R-PEG20C, vascular endothelial cells were immunostained with PECAM-1. The quantified
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18 vascular density (Fig. 6) of tumors treated by R-PEG20C was significantly lower than that of the control
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20 sample (** $p < 0.01$). Overall, these results confirmed that the accumulation of R-PEG20C into a targeted
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22 tumor site facilitated marked loaded antiangiogenic gene expression at the targeted tumor site, resulting in
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24 an antiangiogenic effect for inhibition on neo-vasculature growth and eventually suppressing tumor growth.
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27 The investigations above approved that polyplex micelle with improved PEG shielding and ligand
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29 molecule conjugation led to active targeted accumulation into a tumor site, along with the promotion of
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31 cellular uptake and favorable intracellular trafficking, produced pronounced gene expression in the targeted
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33 cells and elicited a potent therapeutic effect for antitumor treatment through the antiangiogenic pathway.
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35 This result validates the feasibility of ligand molecule (cRGD peptide) conjugation with our proposed polyplex
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37 micelle having an improved blood retention profile for potential use in systemic targeted delivery to a desired
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39 site. It particularly provides important implications for versatile use in the treatment of diverse diseases by
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41 assigning approximate ligands onto the surface of the polyplex micelle for systemic gene therapy.
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45 46 **4. Conclusions**

47 Aiming for developing gene delivery carrier for systemic use, promotion of PEG shielding and
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49 utilization of ligand molecules for tumor targeting was proposed on PEG-PAsp(DET) based polyplex micelles.
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51 Increased retention in blood circulation was successfully achieved by virtue of enhanced PEG shielding
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53 through conjugation of a cholesteryl group and with the use of a longer PEG, 20 kDa. Noteworthy was the
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55 strategic use of cholesteryl conjugation for enhanced PEG shielding: it increased the number of tethering PEG
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57 chains and compacted the polyplex micelle core more, synergistically increasing PEG density. Further
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59 elongation of PEG from 12 kDa to 20 kDa induced PEG for further crowding, which may afford significant
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4 contribution in avoiding rapid blood clearance mode. Furthermore, ligand conjugation onto the polyplex
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6 micelle surface contributed to both improved accumulation at the tumor site and enhanced gene expression
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8 of antiangiogenic protein through facilitated cellular uptake and intracellular trafficking. The proposed
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10 polyplex micelle with these features ultimately demonstrated significant tumor growth suppression as a
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12 result of its antiangiogenic effect. The results validate the systemic usage of this system for tumor-targeted
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14 gene therapy, thus endows tremendous future perspective of developing the system for broad utilities by
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16 choosing appropriate therapeutic genes and targeting moieties.
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Figure Caption

Fig 1. Cellular uptake and gene expression activity of polyplex micelles at varying N/P ratios in HeLa cells.

(a): Cellular uptake (mean $\pm$ SEM, n = 3). (b): Gene expression activity (mean $\pm$ SEM, n = 4). (c): Normalized transfection efficiency obtained from dividing gene expression activity by cellular uptake activity.

Fig 2. Confocal laser scanning microscopy (CLSM) observation for intracellular trafficking. (a): PEG12C; (b): PEG20C; and (c): R-PEG20C. Blue: nucleus; Green: late endosome or lysosome; Red: pDNA. Endosome entrapment was determined by quantifying colocalization ratio of pDNA and late endosome/lysosome as summarized in (d) (mean $\pm$ SEM, n = 10). The scale bars represent 20 μ m in all CLSM images.

Fig 3. Morphology and rod length distribution of polyplex micelles according to TEM observation.

Representative TEM images of polyplex micelles, (a): PEG12; (b): PEG12C; and (c): PEG20C. Scale bar: 100 nm. Rod length distribution of polyplex micelles analyzed according to acquired TEM images, (d): PEG12; (e): PEG12C; and (f): PEG20C, n > 100.

Fig 4. *In vivo* evaluation after systemic administration of polyplex micelles. (a): PEG length effect on retention in the blood circulation determined by RT-PCR. (b): Antitumor activity in terms of tumor size (mean $\pm$ SEM, n = 3). The polyplex micelle was intravenously administered through the tail vein for three times on days 0, 4 and 8. Data points marked with asterisks are statistically significance of PEG20C vs HEPES and R-PEG20C vs PEG20C. (* p < 0.05 and ** p < 0.01; Student's t-test). (c): Accumulations of polyplex micelles in the tumor 24 h after intravenous injection of polyplex micelles loading Cy5-labeled DNA into BxPC3-tumor bearing mice. The fluorescence intensity was expressed as photon counts normalized by tumor weight (*** p < 0.005; Student's t-test).

Fig 5. Expression of sFlt-1 protein in tumor tissue after systemic injection of polyplex micelles. (a): HEPES buffer as a control; (b): PEG20C; and (c): R-PEG20C. Blue: nucleus; Red: vascular endothelial cells immunostained positive with PECAM1. Green: expressed sFlt-1 (or inherent VEGFR1). The scale bars

represent 200 μm in all CLSM images. (d) Integral intensity of Flt-1/VEGFR1-positive region (green) were quantified from the images (mean $\pm$ SEM, $n = 4$; $*p < 0.05$ and $**p < 0.01$, Student's t-test).

Fig 6. Vasculature of sFlt-1 treated tumors observed by CLSM of immunostaining PECAM-1-positive-vascular endothelial cells in the BxPC3 tumor tissue. (a): HEPES and (b): R-PEG20C loading sFlt-1 pDNA. The scale bars represent 100 μm in all CLSM images. (c): Areas of PECAM-1-positive region (green) quantified from CLSM images (mean $\pm$ SEM, $n = 8$; $**p < 0.01$, Student's t-test).

Table 1. A series of synthesized block copolymers

Polymers	PEG M_w	PAsp(DET) DP*	Abbreviation	Cholesteryl group [%]	cRGD [%]	M_w/M_n^{**}
PEG ₂₇₂ -PAsp(DET) ₅₃	12,000	53	PEG12	0	0	1.05
PEG ₂₇₂ -PAsp(DET) ₆₁ - cholesteryl	12,000	61	PEG12C	87	0	1.04
PEG ₄₅₄ -PAsp(DET) ₆₀ - cholesteryl	20,000	60	PEG20C	89	0	1.07
cRGD-PEG ₄₅₄ - PAsp(DET) ₅₇ -cholesteryl	20,000	57	R-PEG20C	86	39	1.06

DP*: polymerization degree of PAsp(DET) segment

M_w/M_n^{**} was determined from the precursor of each block copolymer before aminolysis according to calibration with differing M_w of PEG as standards

Table 2. Characterizations of polyplex micelles for reduced tethered PEG density estimation

	Aver. Rod length [nm]	Binding number of block copolymers per DNA	Binding [+/-]*	Density [chains / nm ²]	RTD**
PEG12	78.6	160.5	1.03	0.0391	2.68
PEG12C	55.8	298.0	2.17	0.0836	5.74
PEG20C	75.5	238.9	1.77	0.0592	7.39

Binding [+/-]*: molar ratio of positive charge from bound block copolymers to negative charge from pDNA.

RTD**: reduced tethering PEG density.

Figure1

Figure2

Figure3

Figure 4

Figure5

Figure6