Polyplex Micelle Installing Intracellular Self-processing Functionalities without Free Cationomers for Safe and Efficient Systemic Gene Therapy through Tumor Vasculature Targeting

Qixian Chen,^a Kensuke Osada,^{b,g*} Zhishen Ge,^{a,h} Satoshi Uchida,^c Theofilus A. Tockary,^{a,d} Anjaneyulu Dirisala,^{b,d} Akitsugu Matsui,^c Kazuko Toh,^{c,d} Kaori M. Takeda,^a Xueying Liu,^{c,d} Takahiro Nomoto,^e Tekihiko Ishii,^b Makoto Oba,^f Yu Matsumoto,^c Kazunori Kataoka^{a,b,c,d*}

^a*Department of Materials Engineering, Graduate School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan*

^b*Department of Bioengineering, Graduate School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan*

^c*Division of Clinical Biotechnology, Center for Disease Biology and Integrative Medicine, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan*

^d*Innovation Center of NanoMedicine (iCONM), Institute of Industrial Promotion – KAWASAKI, 3-25-14 Tonomachi, Kawasaki-ku, Kawasaki 210-0821, Japan*

^ePolymer Chemistry Division, Laboratory for Chemistry and Life Science, Tokyo Institute of Technology, R1-11, 4259 Nagatsuta, Midori-ku, Yokohama 226-8503, Japan.

^fDepartment of Molecular Medicinal Sciences, Division of Pharmaceutical Chemistry, School of Pharmaceutical Sciences, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki 852-8521, Japan

^gJapan Science and Technology Agency, PRESTO, Japan,

^hCAS Key Laboratory of Soft Matter Chemistry, Department of Polymer Science and Engineering, University of Science and Technology of China, Hefei, 230026, China

Corresponding Author

*Address correspondence to osada@bmw.t.u-tokyo.ac.jp (K. Osada) and kataoka@bmw.t.u-tokyo.ac.jp (K. Kataoka)

Keywords: polyplex micelle; toxicity; endosome escape; systemic administration; tumor vasculature targeting; tumor gene therapy

Abstract

Both efficiency and safety profiles are crucial for promotion of gene delivery systems towards practical applications. A promising template system was previously developed based on block cationomer of poly(ethylene glycol) (PEG)-*b*-poly{N'-[N-(2-aminoethyl)-2-aminoethyl]aspartamide}-cholesteryl [PEG-PAsp(DET)-cholesteryl] with strategies of ligand conjugation at the α -terminus for specific affinity to the targeted cells and cholesteryl conjugation at the ω -terminus for structural stabilization to obtain systemic retention. Aiming for advocating this formulation towards practical applications, in the current study, the binding profile of this polymer to plasmid DNA (pDNA) was carefully studied to address an issue of toxicity origin. Quantification of free polymer composition confirmed that the toxicity mainly results from unbound polymer and polyplex micelle itself has negligible toxicity. This evaluation allowed for identifying an optimal condition to prepare safe polyplex micelles for systemic application that possess maximal polymer-binding but exclude free polymers. The identified polyplex micelles then faced a drawback of limited transfection efficiency due to the absence of free polymer, which is an acknowledged tendency found in various synthetic gene carriers. Thus, series of functional components was strategically compiled to improve the transfection efficiency such as attachment of cyclic(Arg-Gly-Asp) (cRGD) peptide as a ligand onto the polyplex micelles to facilitate cellular uptake, use of endosome membrane disruptive cationomer of PAsp(DET) for facilitating endosome escape along with use of the conjugated cholesteryl group to amplify the effect of PAsp(DET) on membrane disruption, so as to obtain efficient transfection. The mechanistic investigation respecting the appreciated pH dependent protonation behavior of PAsp(DET) permitted to depict an intriguing scenario how the block cationomers manage to escape from the endosome entrapment in response to the pH gradient. Subsequent

systemic application to the pancreatic tumor demonstrated a capability of vascular targeting mediated by the cRGD ligand, which was directly confirmed based on *in situ* confocal laser scanning microscopy observation. Encouraging this result, the vascular targeting to transfect a secretable anti-angiogenic gene was attempted to treat the intractable pancreatic tumor with anticipation that the strategy could circumvent the intrinsic physiological barriers derived from hypovascular and fibrotic characters. The obtained therapeutic efficiency demonstrates promising utilities of the proposed formulation as a safe systemic gene delivery carrier in practical use.

1. Introduction

The tremendous advantages of gene therapy have led to decades-long development of novel therapeutic modalities in the quest for a breakthrough in the treatment of intractable diseases [1]. To move the concept of gene therapy towards practical applications, aside from essential safety concern, systemic gene delivery carriers should equip a range of functionalities to overcome predefined obstacles, including stable packaging of therapeutic DNA into nano-sized particle, stealth surface coating of the packaged DNA [e.g. poly(ethylene glycol) surface modification: PEGylation] to protect fragile DNA for sufficient retention in the bloodstream, adequate structural stabilities to avoid unfavorable dissociation, effective targeting activities for high bioavailability to the pathogenic site, efficient cellular uptake, favorable intracellular trafficking by prompt escape from endosome entrapment, and DNA has to be susceptible to transcription for finally achieving efficient transfection [2-4]. To address these issues, we had previously developed a feasible polyplex micelle based on the self-assembly of a multifunctional block

cationer, PEG_{20kDa}-poly{N'-[N-(2-aminoethyl)-2-aminoethyl]aspartamide}-cholesteryl [PEG-PAsp(DET)-cholesteryl] (PEG20C), and plasmid DNA (pDNA), which was characterized with its substantial colloidal stability due to polyion complex core stabilized by hydrophobic cholesteryl residue and superb PEG shielding profile, capable of addressing the inadequate blood retention of ordinary PEG-PAsp(DET), so as to become applicable to systemic applications [5]. Moreover, conjugation of ligand molecule, cyclic(Arg-Gly-Asp) (cRGD) peptide, at the distal end of the PEG segment has granted targeted accumulation of polyplex micelles into the tumor site and facilitate efficient gene expression in the targeted cells, eventually exerting potent anti-tumor efficacy [5]. Particular noteworthy was the strategic use of cholesteryl moiety for enhanced PEG shielding. As opposed to a charge-stoichiometric polyplex micelle from ordinary PEG-PAsp(DET), the cholesteryl-functionalized block cationer, PEG-PAsp(DET)-cholesteryl, appeared to induce the binding of a markedly larger number of block cationers to the pDNA, thus exceeding the typical stoichiometric charge ratio [5,6]. Previously, N/P ratio (defined as the residual molar ratio of the amino groups from block cationer to the phosphate groups of pDNA) of 8 was chosen for preparation of polyplex micelles to take advantage of cholesteryl-mediated enhanced polymer binding behavior for the sake of obtaining a high crowdedness of PEGylation for systemic circulation [5]. Nevertheless, one concern remained: a small fraction of free polymer remained in the complex solution at the N/P ratio of 8 despite the cholesteryl group facilitated polymer binding to pDNA [5]. Considering free cationers can electrostatically interact with charged biological substances or structures, the existence of free cationers may elicit potential toxic concern. Therefore, even though PEG-PAsp(DET) has a biodegradable nature that limits its potential toxicity [7,8], the existence of free cationers may restrict its further applications in clinical trials. In this respect, polyplex micelles

that exhibit both maximal polymer binding for shielding and thus prolonged blood retention and the absence of free polymers for minimal potential toxicity would be the ideal formulation for a safe and efficient systemic gene delivery system. Then, there is an issue that has to be solved. A number of studies have claimed that free polymers tend to facilitate *in vitro* transfection [9,10], in other words, transfection efficiency results limited without aids of free polymer. Indeed, the transfection efficiency of polyplex micelles from PEG20C also exhibited an N/P ratio dependence [5]. Therefore, a critical task is to develop polyplex micelles that can prevail obstacles presented by cell transfection without aids of free polymers.

To achieve this task, the present study demonstrated the following scheme to improve the potential of the aforementioned polymer for use in practical applications: 1) as safety issue is the primary issue, we attempted to discriminate the toxicity of free polymers from polyplex micelles so as to identify the desirable safe formulation; 2) the functional components were rationally integrated aiming to promote efficiencies of cellular uptake, endosome escape, and transcription for enhancing transfection activity of the identified safe formulation without aids of free polymers; and 3) the ultimate system was translated into *in vivo* to test its efficacy for systemic treatment of intractable pancreatic tumors. First, precise investigation of polymer binding behaviors was conducted so as for confirmation of the toxicity resulting from free polymers rather than polyplex micelles. This attempt could lead to identification of the ideal conditions to prepare polyplex micelles that do not contain free polymers, but have maximized binding number of polymers to ensure high PEG shielding. Second, potency of the compiled functional components, i.e., PAsp(DET) as an endosome membrane disruptive cationomer and the conjugated cholesteryl group as its enhancer, on endosome escape was specifically investigated based on the appreciated pH dependent protonation behavior of PAsp(DET) to rationalize the molecular

mechanism of their superb endosome escape functionality. Ultimately, the identified formulation characterized with its excellent safety profile by eliminating presence of free polymers and efficient transfection activity by deploying diverse functional units, was translated into systemic treatment of intractable pancreatic tumor through vasculature targeting by utilizing cRGD with an aim of circumventing its intrinsic physiological barrier by hypovascular and fibrotic characters.

2. Materials and method

2.1. Materials, cells, and animals

β -Benzyl-L-aspartate *N*-carboxyanhydride (BLA-NCA) was obtained from Chuo Kaseihin Co., Inc. (Tokyo, Japan). α -Methoxy- ω -amino-PEG (M_w 20K) and α -acetal- ω -amino-PEG (M_w 20K) were obtained from NOF Co., Ltd. (Tokyo, Japan). Dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP) were purchased from Aldrich Chemical Co. Ltd. (Milwaukee, WI). Diethylenetriamine (DET), *n*-butylamine, trifluoroacetic acid and cholesterol were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). Other chemicals were used as received unless specifically noted. The cRGDfK- ϵ -caproic acid-cysteine (cRGD peptide) was purchased from Peptide Institute Inc. (Osaka, Japan). Alexa546 and Alexa680 succinimidyl esters were products of Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from Dainippon Sumitomo Pharma 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 were

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 reported previously [6]. pDNA was labeled with Cy5 or Cy3 using a Label IT Nucleic Acid Labeling Kit from Mirus Bio Corporation (Madison, WI) according to the manufacturer's protocol. Human pancreatic adenocarcinoma-BxPC3 cells, human epithelial ovarian carcinoma cells (Hela) and hepatocarcinoma cells (Huh-7) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). BxPC3-GFP cells were obtained from AntiCancer Japan (Chiba, Japan). Balb/c nude mice (female, 5 or 7 weeks old) were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan). All animal experimental protocols were conducted according to 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), PEG-PAsp(DET)-cholesteryl and cRGD-PEG-PAsp(DET)-cholesteryl were described in the previous report.⁵ The polymerization degree of the PAsp(DET) segment, efficiencies of cholesteryl and cRGD conjugation were determined from the ¹H-NMR spectra in D₂O at 25 °C.

2.3 Polyplex micelle preparation

The obtained polymers, PEG-PAsp(DET), PEG-PAsp(DET)-cholesteryl and cRGD-PEG-PAsp(DET)-cholesteryl composed with same PEG segment (M_w : 20 kDa) and similar PAsp(DET) segment (degree of polymerization: approximate 60) was used to prepare polyplex micelle solution, and sequentially referred to be PEG20, PEG20C and R-PEG20, respectively. In brief, the polymer powder was dissolved in 10 mM HEPES buffer (pH 7.4) at a concentration

of 5 mg/mL as stock solution. Aliquot of stock solution was taken and diluted to whatever appropriate concentration to produce the correct volume of polyplex called for by the subsequent experiments. It was also necessary to consider the target feed N/P ratios when carrying out complexation. The polymer solution was then added to vortexing pDNA solution dissolved in 10 mM pH 7.4 HEPES buffer (50 µg/mL) via one-shot mixing to achieve a targeted feed N/P ratio. The solution was then incubated for 24 h at 4 °C. The final concentration was adjusted to 33.3 µg/mL.

2.4. Cell viability

HeLa cells, HuH-7 cells or HUVECs were seeded in 24-well culture plates (20,000 cells/well) and incubated overnight in 400 µL DMEM supplemented with 10% FBS (or MCDB131 containing 10% FBS and 10 ng/mL b-FGF for HUVEC). The medium was replaced with 400 µL of fresh medium, followed by addition of 30 µL of polyplex micelle solution (1 µg pDNA/well) or polymer solution into each well. After 24 h incubation at 37 °C, the medium was changed to 400 µL of fresh medium, followed by another 24 h incubation. The cells were washed with 400 µL, and cell viability was determined using the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) according to the manufacturer's protocol. Each well with 200 µL fresh medium was added with 20 µL Cell Counting Kit-8 Agent. After 2 h reaction at 37 °C, the absorbance at 450 nm of the formazan in each well was quantified from a microplate reader (Model 680, Bio-rad, UK). The cell viability in each well was calculated and presented as a percentage of control wells without any addition.

2.5. 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 [5]. As reported previously,

ultracentrifugation of polyplex micelle solution allows selective sedimentation of polyplex micelles, while the free polymers remain in the supernatant. In consequence, the binding fraction of polymers can be determined by subtracting free polymers (remaining in the supernatant after sedimentation) from the total feed polymer. To quantify the associating number of block copolymers in the polyplex micelle, the block copolymers were labeled with Alexa680 succinimidyl ester using the protocol provided by the manufacturer (conjugation efficiency: 0.5 Alexa680 molecules per block copolymer), and the labeled block copolymer was used to prepare a class of polyplex micelles at varying feed N/P ratios in 10 mM HEPES buffer containing 150 mM NaCl (pH 7.4). After overnight incubation at 4 °C, 500 μ L of each polyplex micelle solution was placed into thick-wall polycarbonate tube, 343776 (Beckman Coulter, Inc., Fullerton, CA) and subjected to ultracentrifugation (Optima TLX, Beckman Coulter, Inc.) equipped with TLA-120.1 rotor for 3 h under $50,000 \times g$ for complete sedimentation of polyplex micelles, while the free polymers were confirmed to be in the upper supernatant. The supernatant containing free block copolymers was collected for absorbance measurement by a UV-Vis spectrometry (ND-3300, NanoDrop, Wilmington, NC) at a wavelength of 680 nm. The concentration of free block copolymers in the supernatant was determined from the obtained absorbance according to a calibration curve from Alexa680-labeled block copolymer solutions.

2.6. Cell membrane destabilization activity

The membrane destabilizing ability was assessed by measuring intracellular membrane-impermeable YOYO PRO1 from the cells contacting with PEG20 and PEG20C. HUVECs (5,000 cells/well) were plated on 96-well plates and incubated overnight in 100 μ L of MCDB131 containing 10% FBS and 10 ng/mL b-FGF. The cell culture medium was changed to 100 μ L of the PEG20, PEG20C polymer solutions, or polyplex micelle solutions in 20 mM HEPES buffer

(pH 7.4) or 20 mM MES buffer (pH 5.5) with 150 mM NaCl together with extra component of YOYO PRO1 (1 μ M). After the incubation at 37 °C for 30 min, the fluorescent intensity of YOYO PRO1 in each nucleus (stained by 2.5 μ g/ml Hoechst 33342) was quantitatively evaluated using a fluorescence microscope equipped with image-analysis software (IN Cell Analyzer 1000: GE Healthcare UK Ltd., Buckinghamshire, England).

2.7. Cellular uptake

Cellular uptake efficiency was evaluated by flow cytometry (BD LSR II, BD, Franklin Lakes, NJ). Cy5-labeled pDNA were used to prepare two groups of PEG20C and R-PEG20C polyplex micelles at varying N/P ratios. HUVECs were seeded on collagen-coated 6-well culture plate (100,000 cells/well) and incubated overnight in 2 mL of MCDB131 containing 10% FBS and 10 ng/mL b-FGF in a humidified atmosphere with 5% CO₂ at 37 °C. The medium was replaced with fresh medium, followed by addition of 150 μ L polyplex micelle solution (33.3 μ g pDNA/mL) into each well. At 24 h post incubation, the cells were washed 3 times with PBS to remove extracellular Cy5 fluorescence. After cell detachment by trypsin treatment, the cells were harvested and re-suspended in PBS for flow cytometry measurement.

2.8. In vitro Transfection efficiency

HUVECs were seeded on collagen-coated 24-well culture plates (20,000 cells/ well) and incubated 24 h in 400 μ L of MCDB131 containing 10% FBS and 10 ng/mL b-FGF 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 incubation, the medium was replaced with 400 μ L fresh medium, followed by another 24 h 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 obtained luciferase activity was normalized according to corresponding amount of proteins in the lysates determined by the Micro BCATM Protein Assay Reagent Kit.

2.9. Systemic toxicity

Polyplex micelles at varying N/P ratio were intravenously injected into the tail vein of Balb/c mice (female, 7-week-old) at the pDNA dosage of 20 µg/ mouse. The blood was collected at 4 h post-injection from inferior vena cava to determine the plasma level of glutamate oxaloacetate transaminase (GOT), glutamic pyruvic transaminase (GPT), and lactate dehydrogenase (LDH) with DRI-CHEM 7000i (Fuji Film, Tokyo, Japan).

2.10. Release of polymer in endosome by confocal laser scanning microscopy (CLSM)

HeLa cells (5,000 cells) were seeded on 35-mm cell culture dishes and incubated overnight in 1 mL of DMEM containing 10% FBS. The medium was replaced with fresh medium, followed by addition of 30 µL of Celllight late-endosome GFP to selectively label endosome membrane for 48 h incubation. Aiming to visualize the released polymers, PEG20C labeled by Alexa546 was used to prepare polyplex micelle at N/P ratio of 4. Then, polyplex micelle was added to HeLa cells for 6 h incubation. The medium was removed and the cells were rinsed 3 times with PBS. The cell nuclei were further stained with Hoechst 33342 (Dojindo Laboratories, Kumamoto, Japan). The CLSM observation was performed using LSM 780 (Carl Zeiss, Oberkochen, Germany) with a 63× objective (C-Apochromat, Carl Zeiss) at excitation wavelengths of 488 nm (Ar laser) for GFP or potential FRET from couple of GFP/Alexa546, and 405 nm (diode laser) for Hoechst 33342.

2.11. Intravital real time confocal laser scanning microscopy (IVRTCLSM) observation

To investigate the cRGD effect for tumor accumulation efficacy of polyplex micelles, PEG20C and R-PEG20C polyplex micelle solutions were co-injected intravenously into bloodstream of Balb/c nude mice bearing xenografted BxPC3-GFP tumor through the tail vein. PEG20C polyplex micelles were prepared from Cy5-labeled pDNA and PEG20C, while R-PEG20C micelles were prepared from Cy3-labeled pDNA and R-PEG20C, followed by overnight incubation under dark at 4 °C. BxPC3-GFP cells were used to prepare subcutaneous tumors at dorsal part of the Balb/c nude mice, which allows identification of tumor nest. Prior to observation, mice were anesthetized and the tumor was exposed by a series of dorsal cuts in the skin surrounding the tumor to create a hinged fold chamber with the tumor attached to the skin, while leaving blood vessels feeding the tumor intact. The exposed tumor was mounted under a coverslip and imaged using the IVRTCLSM equipped with a 40 × objective. Just before the injection, the aforementioned polyplex micelle solutions, Cy5-PEG20C and Cy3-R-PEG20C, were mixed. GFP, Cy3 and Cy5 signals were detected simultaneously using 488, 561 and 640 nm excitation lasers and band-pass emission filters of 525/50, 576/40 and 700/75 nm, respectively. Images and video were captured for a total of 18 h lapse with 5 min interval. The accumulation efficiency of PEG20C or R-PEG20C was calculated:

$$E = F_{12h}/F_{1min}$$

where E is referred to accumulation of PEG20C or R-PEG20C, F_{12h} is referred to the entire fluorescence intensity of PEG20C or R-PEG20C in the captured tumor region at 12 h post injection, and F_{1min} is referred to the entire fluorescence intensity of PEG20C or R-PEG20C in the captured tumor region at 1 min post injection.

2.12. Tumor Suppression Efficacy

Balb/c nude mice were inoculated subcutaneously with BxPC3 cells derived from human pancreatic adenocarcinoma (10^7 cells in 100 μ L of PBS). Tumors were allowed to grow for 10 days until proliferative phase (tumor size was approximately 75 mm³). Then 200 μ L of polyplex micelle solution (20 μ g sFlt-1 pDNA) in 10 mM HEPES buffer with 150 mM NaCl (pH 7.4) was intravenously injected through the tail vein 5 times (on days 0, 4, 8, 12, and 16). Tumor size was measured every two or three days with a digital vernier caliper across its longest (a) and shortest diameters (b), and its volume (V) was calculated according to the formula $V = 0.5 \times ab^2$. The tumor size measurement was discontinued when the tumors in the control group showed distinct ulcer characteristics according to the guideline for animal experiments. Tumor progression was express as the relative volume of the tumors to that at day 0 (day for the first polyplex micelle injection). Six mice were used for each group.

2.13. Vascular Density

Each polyplex micelle loading pDNA encoding sFlt-1 (20 μ g of pDNA in 200 μ L of 10 mM HEPES buffer containing 150 mM NaCl (pH 7.4)) was intravenously injected into the BxPC3 tumor-bearing mice through the tail vein on days 0, 4, 8, 12 and 16. Mice were sacrificed on day 20, and the tumors were excised, frozen in dry-iced acetone, and sectioned into 20 μ m thick slices with a cryostat. Vascular endothelial cells (VECs) were immunostained with anti-platelet endothelial cell adhesion molecule-1 (PECAM-1) rat monoclonal antibody (BD Pharmingen, Franklin Lakes, NJ), followed by incubation with anti-rat Alexa Fluor 488-conjugated secondary antibody. The immunostained sections were observed using LSM780. The vascular density was quantified by counting the percentage area of PECAM-1-positive pixels per image with 12 images per sample.

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. Identification of an optimal polyplex micelle formulation for a safe gene delivery system

The series of block cationomers used in the present study was synthesized according to the previously described procedures [5], and the chemical descriptions for each block copolymer are summarized with abbreviations in Table 1 (chemical structures were provided in Fig. S1). To address the issue of potential toxic concerns of the free polymers existing in the polyplex micelle solution, the toxicities of polyplex micelles prepared from PEG20C at varying N/P ratios were examined with polyplex micelles prepared from PEG20 and polyplexes prepared from PEI as commercially available standard to work as control samples. In contrast to the severe toxicity induced by PEI polyplexes, no distinct cytotoxicity was observed for PEG20C polyplex micelles in both Huh-7 cells and HeLa cells (Fig. 1a and b), confirming advantageous low cytotoxicity characteristics of PAsp(DET). Cytotoxicity was further assessed for toxic-sensitive HUVECs to discriminate potential cytotoxicity of polymers. Note that HUVECs are derived from the endothelium of veins, thus the use of these cells seems more relevant to examine cytotoxicity of polymers upon aiming for systemic application. [7, 11, 12] Non-cholesteryl PEG20 polyplex micelles did not show significant toxicity, whereas the PEG20C polyplex micelles showed toxicity in the high N/P ratio range (N/P ratio ≥ 16 , Fig. 1c) in which the free polymers are thought to be present in the polyplex micelle solution. Presumably, the cholesteryl group attached to the PEG20C polymer may facilitate association with cells since it is a component of

cell membrane; thus, the disruptive potency of the tethered polycation to the cellular membrane may eventually be intensified and elicited toxicities. To examine this possibility, the strength of membrane destabilizing capacity was assessed using intracellular membrane-impermeable YOYO PRO1 [7, 13-15], with the similar uses as LDH assay, by measuring its fluorescence from the cells exposed to the PEG20 polymer and the PEG20C polymer. Here, HUVECs were only used for this evaluation since other cells were insensitive in cytotoxicity (Fig. 1a, b). The subsequent result verified PEG20C polymer presented marked potency to provoke loss of cell membrane integrity in high amine concentration while PEG20 polymer presented minimal loss of cell membrane integrity (Fig. 2), appealing the safety feature of PEG20 polymer [13].

Table 1. A series of synthesized block cationomers

Polymers	PEG M_w	PAsp(DET) DP*	Abbreviation	Cholesteryl group [%]	cRGD [%]	M_w/M_n^{**}
PEG ₄₅₄ -PAsp(DET) ₅₅	20,000	55	PEG20	0	0	1.05
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	87	1.06

DP*: polymerization degree of the PAsp(DET) segment

M_w/M_n^{**} was determined from the precursor of each block copolymer before aminolysis based on a calibration curve using PEG standards of differing M_w

Fig. 1. Cytotoxicity profiles of polyplex micelles from PEG20C and control polyplexes from PEG20 and PEI at varying N/P ratios. a): HeLa cells; b): Huh-7 cells; and c): HUVECs. The results are expressed as mean $\pm$ S.D. (n = 4).

Fig. 2. Evaluation of the cell membrane integrity in the presence of polymers (PEG20 and PEG20C) at varying concentrations at pH 7.4 based on a YOYO PRO-1 assay using an INCELL Analyzer. The results are expressed as mean $\pm$ S.D. (n = 4).

To determine the observed cytotoxicities attributable to the presence of free polymers in the polyplex micelle solution from PEG20C rather than polyplex micelle itself, the binding profile of PEG-PAsp(DET)-cholesteryl to pDNA was investigated using the ultracentrifugation method as previously reported [14]. For clarification, the total number of polymers used to complex with pDNA and the actual binding number of polymers to pDNA were expressed as feed N/P and bind N/P, respectively. In accordance with the electrostatic neutralization for the control group of PEG20, entire feed polymers were complexed with pDNA (pBR322: 4,361 bp) until feed N/P $\leq$ 2, followed by a constant value of polymer binding (140 chains of polymers per pDNA, bind N/P $\approx$ 2) irrespective of the increased feed N/P ratio (Fig. 3a). Given that 53% of amino groups were protonated at pH 7.4 for PAsp(DET) [7], this bind N/P ratio of 2 suggests formation of PEG20 polyplex micelles based on stoichiometric charge ratio [charge ratio (+/-) = (binding number of polymers $\times$ DP $\times$ 0.53 / 2 $\times$ 4361) = 1.06]. On the contrary, for the samples from PEG20C, the range that allows entire polymer binding was extended to the feed N/P ratio of 4 (263 chains of polymers per pDNA at feed N/P ratio of 4) (Fig. 3a). In the range of feed N/P $\geq$ 4, polymers progressively bind to pDNA with increased feed N/Ps (Fig. 3a). In light of PEG20 formulation with basis of charge neutralization, it may be reasonable to assume that the extra polymer binding of PEG20C might be as a consequence of cholesteryl conjugation. Presumably, the conjugated cholesteryl group at the block cationomers preferred to incorporate into the hydrophobic polyplex core rather than staying in aqueous solution. To understand this increased binding of PEG20C that exceeded the typical charge stoichiometry (bind N/P ratio of 2), the binding behavior was analyzed by assuming a scheme of cholesteryl-mediated polymer adsorption onto a charge-neutralized polyplex as the feature of the increased binding of PEG20C with increasing of feed N/Ps in the region above N/P 2 reminds an adsorption profile. Here, the Freundlich

adsorption model [15], which describes a phenomenon of solute molecules adsorbing onto the surface of substrate in the solution, was applied. Pertaining to the Freundlich adsorption, the number of adsorbed molecules (A) on the surface of the substrate depends on the concentration of solute molecules (C) in the solution, which is expressed as the Freundlich adsorption isotherm:

$$A = k \times C^b \quad (1)$$

where k and b are constants.

For analysis of PEG20C, the adsorbed polymers $(N/P - 2)_{bind}$ and the concentration in the solution $(N/P - 2)_{feed}$, were used for A and C , respectively, then the equation (1) became:

$$(N/P - 2)_{bind} = k \times (N/P - 2)_{feed}^b \quad (2)$$

Therefore,

$$\ln(N/P - 2)_{bind} = \ln(k) + b \times \ln(N/P - 2)_{feed} \quad (3)$$

Thus, $\ln(N/P - 2)_{bind}$ was plotted against $\ln(N/P - 2)_{feed}$ as shown in Fig. 3b. To our interests, the data points fitted well to a linear function, validating the applicability of the Freundlich adsorption model for describing the binding behaviors of PEG20C over the range of charge stoichiometry (Fig. 3b). Nevertheless, the data points at high feed N/P ratios [$\ln(N/P - 2)_{feed} = 2.3$ or 2.6 , corresponding to feed N/P = 12 or 16, respectively] seemed to deviate from the linear profile predicted by the Freundlich adsorption isotherm (Fig. 3b). Taking the previous estimation of PEG crowdedness for polyplex micelles prepared at feed N/P ratio of 8 suggested that the PEG chains adopted a squeezed conformation or might enter the scalable brush regime [5,16], it is predicted that the tethered PEG chains of polyplex micelles prepared at feed N/P ratios of 12 and 16 are substantially crowded. Therefore, further polymer binding is likely to be restricted due to the strong steric hindrance caused by the increased PEG tethering crowdedness.

Moreover, the charge repulsion that results from excessive polymer binding over the charge stoichiometric ratio may restrict further binding at these high feed N/P ratios. Consequently, the incorporation of PEG20C into the polyplex micelle exceeding the charge stoichiometry in feed N/P ratios ranging from 2 to 8 could be consistently understood as the adsorption of polymers onto the charge-neutral polyplex core, which profiles well follows the Freundlich adsorption model.

Fig. 3. Binding profiles of the block copolymers PEG20 and PEG20C in polyplex micelles at varying N/P ratios. a): Binding of polymer per pBR322 at varying feed polymer shown in N/P ratios. The solid line represents the one-to-one proportion, which indicates all feed polymers are used to form polyplex micelles; b): Binding profile of PEG20C assuming the Freundlich adsorption model. Plots in the range of N/P ratio 2.5 to 8 were fitted by a linear function (solid line; $R = 0.99$).

The investigation of the binding profile revealed the presence of a growing fraction of free polymers at high feed N/P ratios. To examine the correlation between the toxicities of polyplex

micelle solution and the fraction of free polymers, the cell viability was evaluated as a function of the amount of free polymers determined by the ultracentrifuge measurement (Fig. 3) against HUVECs. For PEG20C, cell viability decreased with an increase of the free polymer amount (Fig. 4, Open circle). Then, the evaluated cell viability for PEG20C polyplex micelles (Fig. 1c) was plotted with respect to free polymer amount coexisted in the polyplex micelles (Fig. 4 closed circle), demonstrated close correlation with that for free polymer. This conformity indicated that the cytotoxicity was largely derived from the quantity of free polymers, and the toxicity from polyplex micelles themselves was limited. This is also evidenced in the negligible toxicity for the polyplex micelles that did not contain free polymers. These observations affirmed the necessity of eliminating the free polymers for pursuing safety profile. Of note, cytotoxicity was still low for PEG20 polymer and its micelle formulation even at high N/P ratio (Fig.4), affirming the safety feature of PEG20 polymer [13].

Fig. 4. Viability of HUVECs as a function of free polymers with or without polyplex micelles. Quantity of free polymers was determined from Fig. 3 and represented in N/P ratio. Viabilities for polyplex micelles were replotting of Fig. 1c. The results are expressed as mean $\pm$ S.D. (n = 4).

To further verify the toxicity profile of the free polymers, the *in vivo* toxicities were evaluated at varying N/P ratios after intravenous (i.v.) injection. As markers, intracellular glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) proteins, which are mainly found in liver cells were chosen to evaluate liver impairment; and lactate dehydrogenase (LDH) protein was used to evaluate the tissue impairment induced by the overall systemic toxicity. Here, the polyplex micelle solutions prepared at varying feed N/P ratios were i.v. injected into Balb/c mice, and the blood was collected at 4 h post-injection. The concentration of each marker molecule in the blood samples was quantified by enzyme-linked immunosorbent assay (ELISA) measurements. The evaluation showed a quite limited toxicity for the polyplex micelles prepared at low N/P ratios ≤ 8 , whereas significant systemic toxicity appeared at high feed N/P ratios, such as the feed N/P ratios of 16 and 32 (Fig. 5). It should be noted that the *in vivo* toxicity evaluations demonstrated at the longer period of 24 h post-injection for polyplex micelles of N/P ratio 4 and 8 also did not present significant toxicity (Fig. S2). This *in vivo* observation along with the *in vitro* cytotoxicity evaluations verified that the free polymers caused toxicity and concomitantly verified the appreciable safety of the polyplex micelles themselves. Furthermore, the negligible toxicity of polyplex micelles at N/P ratios below 8 implied that occurrence of dissolution of the PEG20C polymers from the polyplex micelles may be limited even in the blood circulation despite the polyplex micelles contain over-stoichiometric composition.

These investigations ultimately identified polyplex micelles from PEG20C at feed N/P ratio of 4 as the ideal formulation for safe systemic application respecting their characteristic feature of no existence of free polymer and maximal polymer binding.

Fig. 5. Systemic toxicity of polyplex micelle after 4 h of the intravenous injection in terms of liver damage and tissue damage. GOT and GPT specifically existing in liver cells represents as indicator of liver damage. LDH abundantly existing in diverse tissue cells represents as indicator of overall systemic toxicity. a): GOT; b): GPT; and c): LDH. The results are expressed as mean $\pm$ S.D. (n = 4).

3.2. Promotion of the Identified Safe Polyplex Micelle for Efficient Transfection

It is well acknowledged that high transfection efficiency tend to be obtained at high N/P ratios [10]. The transfection efficiency of polyplex micelles based on PEG20C also followed this general trend, as indicated by studies using HeLa and Huh-7 cells [5,6], and eventually, the transfection efficiency of PEG20C polyplex micelles at N/P ratio of 4 possessing no free polymer was limited. The N/P ratio dependence of the transfection efficiency was also observed for toxic-sensitive HUVECs, although the efficiency was decreased at higher N/P ratios [Fig. S3]. Most likely, the presence of the free polymers resulted in toxicities at high N/P ratios. Thus, this again affirmed polyplex micelle that does not contain free polymers is desirable in the safety aspect. To this end, a necessary task is to manage the low transfection efficiency of the identified

polyplex micelle prepared at N/P ratio of 4. To serve this purpose, we employed integration of several functional components in to the polyplex micelles to promote cellular uptake efficiency and intracellular trafficking process for enhancement of transfection efficiency. For efficient cellular uptake, ligand molecule of cRGD peptide was attached onto the polyplex micelle surface (cRGD conjugation at the distal end of PEG; R-PEG20C) with the aim of harnessing its specific interaction with its receptors ($\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins), which are specifically overexpressed on the membrane of tumor cells and angiogenic cells [17-22]. Meanwhile, the cRGD functionalization can anticipate high tumor-targeted accumulation after the systemic application [5]. A subsequent study was conducted for the samples of PEG20C and R-PEG20C in angiogenic HUVECs with the aim of validating the role of cRGD functionalization in cellular uptake efficiency, and thereby the transfection efficiency. The results of both the cellular uptake and transfection evaluations demonstrated the efficient activity of R-PEG20C at an N/P ratio of 4 (Fig. 6), so as to address its drawback of the limited cellular uptake and transfection efficiency of PEG20C.

Fig. 6. Cellular uptake activities (a) and gene expression (b) of PEG20C and R-PEG20C at N/P ratio of 4 in HUVECs. The results are expressed as mean $\pm$ S.D. (n = 4).

As for the endosome escape function, it is noted that the PAsp(DET) has an intriguing functionality that could aid the escape of polyplex micelles from endosome entrapment after endocytosis. This polycation has been validated to have appreciable endosome escape function by virtue of two-step protonation with different pK_a amines [7]. Further research on utilizing the PEG-PAsp(DET)-cholesteryl block copolymer has verified its high potency in pronouncing the escape activity of a polyplex micelle from endosome entrapment [6]. Taking consideration of the distinct characteristics of PAsp(DET) and the cholesteryl moiety, it may be possible to expect the following scenario as molecular mechanism to the demonstrated endosome escape capability of PEG-PAsp(DET)-cholesteryl polyplex micelles: (i) along the pH decrease from pH 7.4 to pH 5.5 after endocytosis, the polyplex micelles would experience a charge unbalance due to the promoted protonation of PAsp(DET) [53% at pH 7.4 and 85% at pH 5.5] [7], thereby resulting in excess positive charge arising in the polyplex micelles with the eventual release of some of the polymers from the polyplex micelles; (ii) the PEG20C polymers that are secreted from the polyplex micelles are presumed to associate with the endosome membrane as cholesteryl has strong affinity for the membrane; (iii) the PEG20C polymers that associated with the membrane would markedly destabilize the endosome membrane due to the promoted protonation of PAsp(DET), consequently resulting in efficient escape from endosome entrapment and liberation of PEG20C into the cytosol; (iv) in the cytosolic pH of 7.4, the remaining PEG20C polymers in the polyplex formulations would recover the initial mono-protonation status, which gives rise to a loosening of pDNA packaging as the polyplex micelles lack the sufficient number of polymers to neutralize pDNA. Ultimately, this polyplex micelle would be pleasant status for undergoing of transcription.

Fig. 7. Endosome escape scenario of PEG20C at an N/P ratio of 4. a): Quantification of the binding numbers of polymers per pBR322 pDNA. Polyplex micelles from PEG20C were prepared at N/P ratio of 4 in 10 mM HEPES buffer (pH 7.4), followed by incubation at pH 7.4 or 5.5. The binding number of polymer to pDNA was estimated by ultracentrifuge measurement. The results are expressed as mean $\pm$ S.D. ($n = 3$); b): Observation of PEG20C polymer release from polyplex micelles in the late endosomes and association with endosome membranes. Endosome membranes were tagged with GFP (shown in green). Nuclei were stained using Hoechst 33342 (shown in blue). The attachment of released PEG20C polymers to endosome membranes was observed as red signal due to FRET of GFP-endosome membrane/Alexa546-

polymer. c): Evaluation of the membrane destabilization activities of the PEG20C polymer for incubation pH of 7.4 or 5.5 in HUVECs using an INCELL Analyzer, and the membrane impermeable YOYO PRO-1 fluorescent dye as a probe to assess the cell integrity. The results are expressed as mean $\pm$ S.D. (n = 4); d) Evaluation of the membrane destabilization activities of PEG20C polyplex micelles at an N/P ratio of 4 at incubation pH of 7.4 or 5.5 in HUVEC by INCELL Analyzer, and the membrane-impermeable YOYO PRO-1 fluorescent dye as a probe to assess cell integrity. The results are expressed as mean $\pm$ S.D. (n = 4).

To verify the feasibility of the expected scenario, firstly, we examined the capability of the scenario (i); whether PEG20C polymers have been released from the polyplex micelles in response to a pH decrease from the physiological pH 7.4 to the endosomal pH 5.5. The binding number of PEG20C block polymers was evaluated for polyplex micelles at an N/P ratio of 4 prepared in 10 mM HEPES buffer containing 150 mM NaCl (pH 7.4), followed by post incubation at pH 5.5. The ultracentrifugation measurements revealed that approximately 46 chains of polymers were released from the polyplex micelles when the incubation pH was decreased from pH 7.4 to pH 5.5 (Fig. 7a). To examine the release was the result of the promoted protonation profile of PAsp(DET) in response to pH change, amount of potential released polymer was then estimated from the protonation degree. As determined, the total binding of 263 chains ($\text{bind}_{\text{total}}$) of PEG20C at pH 7.4 can be presumed to comprise electrostatic mediated association ($\text{bind}_{\text{elec}}$: 140 chains as determined from PEG20) and additional cholesteryl mediated association (bind_{add} : 123 chains, calculated as $\text{bind}_{\text{total}} - \text{bind}_{\text{elec}}$). The number of electrostatic-mediated binding events may decrease at pH 5.5 due to the increased protonation (0.85) [7]. The prediction indicates that 87 chains [$140 \times (0.53/0.85)$] will bind to pDNA and thus 53 chains can

be released, given the assumption that the cholesteryl-mediated association is not influenced by pH change. The close correspondence of the estimated value to the observed value corroborated the reasonability of the pH-responsive polymer release due to the increased protonation profile of PEG20C.

The released PEG20C is expected to subsequently associate with endosome membrane [scenario (ii)]. This association was directly captured by a high resolution *in situ* confocal laser scanning microscopy (CLSM) observation, where Alexa 567-conjugated PEG20C polymers were used to prepare polyplex micelles, and a late endosome membrane was selectively tagged with GFP. In this observation, the released polymers that associated with the endosome membrane could be distinguished using the FRET signal measured by excitation of GFP (at a wavelength of 488 nm). It should be noted that the complexed polymers in the polyplex micelles cannot be detected under a same CLSM setting in spite of the polyplex micelles approaching the endosome membranes because the thickness of PEG shell, which has been determined to be over 13 nm [5], exceeds the distance limitation of FRET (1-9 nm) between Alexa 567 and GFP [23]. Additional experiments were conducted to demonstrate that the observed FRET signal was from the released polymers, e.g., the FRET emission range was determined by emission fingerprinting with Lambda stacks (Fig. S4) and the lack of FRET emission was confirmed for the condition in which the late endosome membrane was not stained (Fig. S5). As shown in Fig. 7b, late endosomes were observed as well-defined circles (green) by exciting GFP at wavelength of 488 nm. Moreover, FRET signal (red) was found along the endosome membrane, which is observed as complete circles or arc of circles (Fig. 7b). This FRET-based observation provided solid evidence to confirm that polymers were released from the polyplex micelles in endosomes and subsequently associated with endosome membranes.

In the scenario (iii), the elevated charged density in response to acidic pH (85% at pH 5.5) could be presumed to result in enhanced destabilization of endosome membranes once the released PEG20C polymers associated with the endosome membranes, giving rise to promoting endosome escape. To verify this possibility, the cellular membrane integrity was evaluated for the PEG20C polymer at pH 7.4 and pH 5.5 in HUVECs. PEG20C showed a distinct pH-dependent cell membrane destabilization profile where PEG20C exerted pronounced higher destabilization activity to cause loss of cell membrane at pH 5.5 than pH 7.4 (Fig. 7c). Furthermore, to demonstrate that this PEG20C polyplex micelle prepared at N/P ratio of 4 was able to specifically disrupt membranes at the acidic endosome pH, we incubated this polyplex micelle with HUVECs at pH 7.4 and 5.5, respectively. The plasma membrane destabilization efficiency was evaluated using a YOYO-PRO1 assay. No loss of cell membrane integrity was observed for the PEG20C polyplex micelles under pH 7.4 (Fig. 7d), which is consistent with the result that the entire cationic PAsp(DET) segment and cholesteryl moiety were masked beneath the PEG shell. By contrast, a substantial loss of cell membrane integrity was observed for the PEG20C polyplex micelle at an N/P ratio of 4 under pH 5.5 (Fig. 7d), suggesting release of membrane destabilization agent from the polyplex micelle (note that HUVECs were pre-determined to be robust when incubated at pH 5.5).

These results verified the validity of the expected scenario to facilitate intracellular trafficking for pronouncing transfection by liberations from the endosome entrapment as well as from the tight packaging for promoted transcription. Noteworthy is the molecular mechanism of the PEG20C system to deal with the predefined obstacles within the intracellular trafficking by sensing the chemical signal of pH change at the each step of intracellular environment and

actively modulate the character of chain, which self-processing behavior may be resembling the intracellular trafficking behaviors of natural vectors such as an adenovirus [24].

3.3 Tumor Vasculature-Targeted Delivery of an Anti-angiogenic Gene for the Systemic Treatment of a Pancreatic Tumor

The identified polyplex micelles exhibiting an appreciable safety profile and efficient transfection activity were translated into systemic trial to evaluate its ability to treat an intractable pancreatic tumor. Pancreatic tumors have the formidable features that a 3-years survival rate is lower than 5% despite the application of diverse therapeutic modes [25-29], and thus its successful treatment gives a significant impact. The pancreatic tumor was featured with its characteristic histology of poor vascularity, covered by tight pericyte alignment, and separated tumor nest from the vasculature by thick fibrosis [30,31]. These features are in stark contrast to the characteristics of hypervascular tumors (e.g., colon cancers) and thus cause tremendous obstacles for nanocarrier-based delivery into the tumor. The solid pancreatic tumor xenografted by subcutaneous inoculation of BxPC3 cells into Balb/c nude mice possesses the aforementioned hypovascularity and poor vasculature-penetration features, which well-reproduce histological characteristics of the real pancreatic tumor of human [30]. A previous study claims that these critical characteristics of pancreatic tumors restrict the accumulation and penetration of nanocarriers larger than 50 nm [31]. According to size characterization, the proposed polyplex micelle showed uniform rod structure with its average rod-length of approximately 90 nm determined by TEM images (Fig. S6) and 89 nm determined by dynamic light scattering (DLS) measurement [5]. Thus, it may be difficult to anticipate this relative large-sized nanostructure respect to the critical size can accumulate and penetrate into pancreatic tumor. To address this

critical issue, we focused overexpression of $\alpha v\beta_3$ and $\alpha v\beta_5$ integrins, as specific receptor for cRGD, in the vascular endothelial cells (VECs), pericytes, and attempted tumor vascular targeting to transfect functional proteins that secret to the tumor nest for achieving therapeutic effect. It should be noted that PEG20C polyplex micelles could successfully avoid the early elimination mode after the injection, namely first pass effect, because the blood circulation profiles followed the one-compartment model and their extrapolation in semi-logarithmic plot to time 0 pointed out approximately 100 % of remaining pDNA (Fig. S7). Importantly, the profiles suggested that the conjugation of cRGD ligands onto the PEG20C polyplex micelles (R-PEG20C) did not substantially undermine the circulation capability (Fig. S7). Thus, the strategy of the tumor vascular targeting by the R-PEG20C polyplex micelles could be expected. Note that the cRGD conjugation did not affect the zeta-potential (Table S1). The capability of tumor targeting was indeed confirmed through the biodistribution study examined for spleen, liver, kidney, intestine, lung and tumor (Fig. S8). Subsequently, the capability of vascular targeting of those micelles was investigated by directly observing their accumulations within the tumor tissue using intravital real-time CLSM. Herein, BxPC3-GFP cells (tumor nest as blue) were used as the inoculum for the tumor model, and pDNA labeled with various fluorescent dyes was assigned to prepare PEG20C (Cy5, red) and R-PEG20C (Cy3, green), with feed N/P ratios of 4 for both types of micelles. Prior to the intravenous injection through the tail of BxPC3-GFP tumor-bearing mice, the prepared polyplex micelle solutions were mixed in equal volumes. For the immediate image of the intra-tumor distribution after injection (Fig. 8a), the micelle mixture was observed in the bloodstream as a merged yellow-orange color, indicating the coexistence of R-PEG20C (green) and PEG20C (red). At 12 h post-injection, there were no remaining polyplex micelles circulating in the bloodstream as judged from the captured images (Fig. 8b, see Movie

in Supporting Information). Furthermore, the blood circulation profile determined from RT-PCR method (Fig. S7) predicted that polyplex micelles have disappeared from blood stream at 12 h post-injection. Therefore, the fluorescence in the tumor site reflects the accumulated amount of polyplex micelles at the tumor. Apparently, PEG20C polyplex micelles (red) resulted in limited accumulation at the tumor site (Fig. 8b). By contrast, R-PEG20C polyplex micelles (green) were found at high levels in the tumor site, such as at the periphery of the tumor nest. The accumulation efficiency was estimated by taking the ratio of the fluorescence intensity in the observed tumor region (the entire image) at 12 h to the original fluorescence intensity at 1 min as an index of the accumulated amount respect to the initially appeared amount in the captured tumor site. The high accumulation efficiency of R-PEG20C (Fig. 8c) corroborated the tumor targeting capability by the cRGD conjugation, which was verified by the biodistribution study (Fig. S8). To our interest, the R-PEG20C (green) was specifically accumulated in the tumor vasculature region, as suggested by the merged images of intra-tumor distribution taken at 12 h (Fig. 8d, green) and of the blood vessel taken at 1 min (Fig. 8d, white). (The preferential accumulation activity of the targeted polyplex micelles was shown in a Movie in Supporting Information.) Notably, neither R-PEG20C nor PEG20C polyplex micelles were found in the tumor nests, which further validated the difficulty of direct delivery due to the characteristic histological features of the pancreatic tumor.

Fig. 8 Intra-tumor distribution of R-PEG20C micelles (green) and PEG20C (red) for the intravenous co-injection into Balb/c nude BxPC3-GFP tumor-bearing mice; the tumor nest is indicated in blue. a): One minute post intravenous injection; b): 12 h post intravenous injection. Scale bar: 200 μ m. The quantified data (accumulation efficiency) are summarized in a bar graph c). d): Detailed identification of the distribution of polyplex micelles at the tumor site. The pattern of blood flow (white) obtained at 1 min post injection was superimposed on the image obtained at 12 h for insight into the intra-tumor distribution of the polyplex micelle. A representative region was selected and is indicated by the marked square in Fig. 8b.

Upon confirming capability of the vasculature targeting, an anti-angiogenic gene therapy was demonstrated using pDNA encoding soluble fms-like tyrosine kinase-1 (sFlt-1) as a payload [32,33]. Note that sFlt-1 is the soluble VEGF receptor-1 and thus able to inhibit VEGF signaling by binding to VEGF molecules so as to stop transducing signals into cells, ultimately inhibiting endothelial cell proliferation and migration (anti-angiogenesis) [34]. A subsequent antitumor efficacy evaluation confirmed potent tumor growth suppression effect of the polyplex micelle based on R-PEG20C (Fig. 9a). Moreover, the tumor vascular density of R-PEG20C micelle-treated mice was significantly lower than that of other groups, thus confirming that the obtained anti-tumor efficacy could be attributed to the antiangiogenic effect (Fig. 9b,c). Notably, R-PEG20C with a CAG-Luc gene cargo did not show a noticeable vascular density reduction and therapeutic efficacy compared to those of the control group, further suggested that the obtained anti-tumor efficacy was the result of the loaded antiangiogenic gene. A consistent observation was obtained in our previous report on R-PEG20C at an N/P ratio of 8; expressed sFlt-1 was enriched in the vasculature vicinal region and tumor stroma [5], supporting the secret of the transfected sFlt-1 protein to the tumor. The obtained anti-tumor efficacy proved an intriguing therapeutic strategy capable of circumventing the histological barriers, even though direct delivery is not accomplished, by choosing appropriate gene encoding secreted functional protein and targeting to the cells surrounding the tumors for treating stroma-rich intractable cancer. Moreover, the devised formulation characterized by the absence of free polymers and thus minimized systemic toxicity is envisioned to present as a safe and efficient gene delivery system for translation into practical applications.

Fig. 9 Anti-tumor efficacies by systemic administration of polyplex micelles containing the anti-angiogenic gene sFlt-1 or the non-therapeutic LUC gene as a control. a): Anti-tumor efficacy of polyplex micelles in terms of tumor size measurement. The polyplex micelle was intravenously injected into Balb/c nude BxPC3 tumor-bearing mice through the tail vein at days 0, 4, 8, 12 and 16. (** $p < 0.01$; Student's t-test). The results are expressed as mean $\pm$ S.D. ($n = 6$); b) Vasculature density of tumor tissue with treatment of polyplex micelles. Scale bar: 200 μ m. c) The quantified vascular density is summarized in a bar graph. (** $p < 0.01$; Student's t-test). The results are expressed as mean $\pm$ S.D. ($n = 12$).

4. Conclusions

The present study reported development of a safe and efficient synthetic delivery system for systemic gene therapy. The investigation of polymer binding profile to pDNA allowed for distinguishing the toxicity contribution. It was determined that the toxicity was mainly caused by the free polymers and the toxicity of polyplex micelles itself was indeed minimal. Accordingly,

the polyplex micelle prepared at N/P ratio of 4, characterized with absence of free polymer but with maximal polymer binding, was identified as a safe template. Yet, a critical issue concerning to the limited transfection efficiency had to be solved to utilize this tempting formulation because the acknowledged effect of the free polymer on promoting transfection efficiency had been no more available. To this end, several functional components were compiled into the polyplex micelles for gaining pronounced gene transfection efficiency by themselves without aids from free polymers, that include cRGD conjugation at the distal end of PEG block for promoted cellular uptake, and synergistic use of functional PAsp(DET) cationer and cholesteryl conjugation at the distal end of PAsp(DET) block for facilitated endosome escape. A particular interesting was the active functions of the block cationer on facilitating intracellular trafficking, which were based on the distinct two-step protonation profile of PAsp(DET) in response to pH gradient. The investigation could clarify the molecular mechanism of the functions, which permitted to depict an intriguing scenario; (i) polyplex micelles release a fraction of block cationers in response to pH decrease in the endosomes, (ii) the conjugated cholesteryl group facilitates association of these released block cationers with the endosome membrane, (iii) the high membrane disruptive potency of the double-protonated PAsp(DET) amplified by the assist of the cholesteryl group synergistically causes disruption of the endosome membrane, (iv) the liberated polyplex micelles from endosome entrapment to cytoplasm experience the neutral pH environment. Then, the remaining PEG20C polymers in the polyplex formulations recover the initial mono-protonation status, which gives rise to a loosening of pDNA packaging as the polyplex micelles lack the sufficient number of polymers to neutralize pDNA. Ultimately, pDNA is liberated from the tight packaging and becomes rather pleasant form for receiving transcription. By these smart self-processing functions, the polyplex micelles eventually

demonstrated efficient transfection, despite the absence of the free polymer assist. The subsequent *in vivo* study under the *in situ* CLSM observation verified the cRGD ligand-mediated targeting to the vasculature of pancreatic tumor. The ultimate significant anti-tumor efficacy to the intractable stroma-rich pancreatic tumor based on an anti-angiogenic approach approved the significant potency of this safe and efficient systemic delivery carrier for wide applications in treatment of intractable diseases.

Supporting Information

The polymer cytotoxicity, N/P ratio dependent cellular uptake and transfection, validation of FRET by CLSM, TEM observation for polyplex micelles, and real-time observation of intercellular distribution of polyplex micelles by IVRTCLSM are provided.

Present Address

Qixian Chen: Department of Chemistry, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, MA, 02139, USA; David H. Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, MA, 02139-4307, USA; Center for Cancer Research, Massachusetts General Hospital, 55 Fruit St, Boston, MA 02114, USA.

Zhishen Ge: CAS Key Laboratory of Soft Matter Chemistry, Department of Polymer Science and Engineering, University of Science and Technology of China, Hefei, 230026, China.

Notes

Conflict of Interest: The authors declare no competing financial interest.

ACKNOWLEDGMENT

This work was financially supported by the Center of Innovation (COI) and Precursory Research for Embryonic Science and Technology (PRESTO) from the Japan Science and Technology Corporation (JST), and by the Japan Society for the Promotion of Science (JSPS) through its Funding Program for Specially Promoted Research (Grant Number 25000006) and Core to Core Program for A. Advanced Research Networks. Q. C. acknowledges a fellowship from Ministry of Education, Science, Sports and Culture, Japan (MEXT). The authors are grateful to Dr. H. Kim and Dr. H. Wu for their intellectual contributions to the discussion.

REFERENCES:

- 1 U. Lachelt, E. Wagner E. Nucleic acid therapeutics using polyplexes: a journey of 50 years (and beyond), *Chem. Rev.* 115 (2015) 11043–11078.
- 2 F. Mingozzi, K.A. High. Therapeutic *in vivo* gene transfer for genetic disease using AAV: progress and challenges, *Nature Rev. Genet.* 12 (2011) 341–355.
- 3 L. Naldini. *Ex vivo* gene transfer and correction for cell-based therapies, *Nature Rev. Genet.* 12 (2011) 301–315.
- 4 Y. Lee, K. Kataoka. Delivery of Nucleic Acid Drugs, *Adv. Polym. Sci.* 249 (2012) 95–134.

- 5 Z. Ge, Q. Chen, K. Osada, X. Liu, T.A. Tockary, S. Uchida, et al., Targeted gene delivery by polyplex micelles with crowded PEG palisade and cRGD moiety for systemic treatment of pancreatic tumors, *Biomaterials* 35 (2014) 3416–3426.
- 6 M. Oba, K. Miyata, K. Osada, R.J. Christie, M. Sanjoh, W. Li, et al., Polyplex micelles prepared from ω -cholesteryl PEG-polycation block copolymers for systemic gene delivery, *Biomaterials* 32 (2011) 652–663.
- 7 K. Miyata, M. Oba, M. Nakanishi, S. Fukushima, Y. Yamasaki, H. Koyama, et al., Polyplexes from poly(aspartamide) bearing 1,2-diaminoethane side chains induce pH-selective, endosomal membrane destabilization with amplified transfection and negligible cytotoxicity, *J. Am. Chem. Soc.* 130 (2008) 16287–16294.
- 8 K. Itaka, T. Ishii, Y. Hasegawa, K. Kataoka. Biodegradable polyamino acid-based polycations as safe and effective gene carrier minimizing cumulative toxicity, *Biomaterials* 31 (2010) 3707–3714.
- 9 Y. Yue, F. Jin, R. Deng, J. Cai, Y. Chen, M.C. Lin, et al., Revisit complexation between DNA and polyethylenimine — Effect of uncomplexed chains free in the solution mixture on gene transfection, *J Control Release* 155 (2011) 67–76.
- 10 S. Boeckle, K. von Gersdorff, S. van der Piepen, C. Culmsee, F. Wagner, M. Ogris. Purification of polyethylenimine polyplexes highlights the role of free polycations in gene transfer, *J. Gene Med.* 6 (2004) 1102–1111.

- 11 M. Sanjoh, K. Miyata, R.J. Christie, T. Ishii, Y. Maeda, F. Pittella, et. al., Dual environment-responsive polyplex carriers for enhanced intracellular delivery of plasmid DNA, *Biomacromolecules*, 13, 11 (2012) 3641–3649.
- 12 J. Blechinger, A.T. Bauer, A.A. Torrano, C. Gorzelanny, C. Bräuchle, and S.W. Schneider, Uptake kinetics and nanotoxicity of silica nanoparticles are cell type dependent, *Small*, 9, 23 (2013) 3970–3980.
- 13 S. Uchida, K. Itaka, Q. Chen, K. Osada, T. Ishii, M.A. Shibata, et al, PEGylated polyplex with optimized PEG shielding enhances gene introduction in lungs by minimizing inflammatory responses. *Mol. Ther.* 20 (2012) 1196–1203.
- 14 Q. Chen, K. Osada, T. Ishii, M. Oba, S. Uchida, T.A. Tockary, et al., Homo-cationer integration into PEGylated polyplex micelle from block-cationer for systemic antiangiogenic gene therapy for fibrotic pancreatic tumors, *Biomaterials* 33 (2012) 4722–4730.
- 15 R.J. Umpleby, S.C. Baxter, M. Bode, J.K. Berch, R.N. Shah, K.D. Shimizu. Application of the Freundlich adsorption isotherm in the characterization of molecularly imprinted polymers, *Analytica. Chimica. Acta.* 435 (2001) 35–42.
- 16 T.A. Tockary, K. Osada, Q. Chen, K. Machitani, A. Dirisala, S. Uchida, et al., Tethered PEG crowdedness determining shape and blood circulation profile of polyplex micelle gene carriers, *Macromolecules* 46 (2013) 6585–6592.
- 17 F. Danhier, A.L. Breton, V. Preat. RGD-based strategies to target $\alpha(v)\beta(3)$ integrin in cancer therapy and diagnosis, *Mol. Pharmaceut.* 9 (2012) 2961–2973.

- 18 R. Hwang, J.V. Varner. The role of integrins in tumor angiogenesis, *Hematol. Oncol. Clin. North Am.* 18 (2004) 991–1006.
- 19 H. Kagaya, M. Oba, Y. Miura, H. Koyama, T. Ishii, T. Shimada, et al., Impact of polyplex micelles installed with cyclic RGD peptide as ligand on gene delivery to vascular lesions, *Gene Ther.* 19 (2012) 61– 69.
- 20 S. Takayama, S. Ishii, S. Ikeda, S. Masamura, M. Doi, M. Kitajima. The relationship between bone metastasis from human breast cancer and integrin $\alpha(v)\beta3$ expression, *Anticancer Res.* 25 (2005) 79– 83.
- 21 K.A. Furger, A.L. Allan, S.M. Wilson, C. Hota, S.A. Vantyghem, C.O. Postenka, et al., $\beta3$ integrin expression increases breast carcinoma cell responsiveness to the malignancy-enhancing effects of osteopontin, *Mol. Cancer Res.* 1 (2003) 810–819.
- 22 L. Vellon, J.A. Menendez, H. Liu, R. Lupu. Up-regulation of $\alpha v \beta 3$ integrin expression is a novel molecular response to chemotherapy-induced cell damage in a heregulin-dependent manner, *Differentiation* 75 (2007) 819–30.
- 23 G.H. Patterson, D.W. Piston, B.G. Barisas. Förster distances between green fluorescent protein pairs, *Anal. Biochem.* 284 (2000) 438–440.
- 24 B. Tsai. Penetration of nonenveloped viruses into the cytoplasm, *Annu. Rev. Cell Dev. Biol.* 23 (2007) 23–43.
- 25 A. Mancuso, F. Calabrò, C.N. Sternberg. Current therapies and advances in the treatment of pancreatic cancer, *Crit. Rev. Oncol. Hematol.* 58 (2006) 231–241.
- 26 R. Freelove, A.D. Walling. Pancreatic cancer: diagnosis and management, *Am. Fam. Physician* 73 (2006) 485–492.

- 27 F. Huguet, N. Girard, C.S. Guerche. Chemoradiotherapy in the management of locally advanced pancreatic carcinoma: a qualitative systematic review, *J. Clin. Oncol.* 27 (2009) 2269–2277.
- 28 G. Klautke, T.B. Brunner. Radiotherapy in pancreatic cancer, *Strahlenther. Onkol.* 184 (2008) 557–564.
- 29 D.S. Zuckerman, D.P. Ryan. Adjuvant therapy for pancreatic cancer: a review, *Cancer* 112 (2008) 243–249
- 30 M.R. Kano, Y. Bae, C. Iwata, Y. Morishita, M. Yashiro, M. Oka, et al., Improvement of cancer-targeting therapy, using nanocarriers for intractable solid tumors by inhibition of TGF- β signaling, *Proc. Natl. Acad. Sci. USA* 104 (2007) 3460–3465.
- 31 H. Cabral, Y. Matsumoto, K. Mizuno, Q. Chen, M. Murakami, M. Kimura, et al., Accumulation of sub-100nm polymeric micelles in poorly permeable tumours depends on size, *Nat. Nanotech.* 12 (2011) 815–823.
- 32 S. Yamaguchi, K. Iwata, M. Shibuya. Soluble Flt-1 (soluble VEGFR-1), a potent natural antiangiogenic molecule in mammals, is phylogenetically conserved in avians, *Biochem. Biophys. Res. Commun.* 291 (2002) 554–559.
- 33 M. Oba, Y. Vachutinsky, K. Miyata, M.R. Kano, S. Ikeda, N. Nishiyama, et al., Antiangiogenic gene therapy of solid tumor by systemic injection of polyplex micelles loading plasmid DNA encoding soluble Flt-1, *Mol. Pharm.* 7 (2010) 501–509.
- 34 P. Carmeliet, R.K. Jain. Angiogenesis in cancer and other diseases, *Nature* 407 (2000) 249–257.