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

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ABSTRACT

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

1. Introduction

Significant advances in the development of smart nano-devices constructed by exploiting material-based molecular design concepts have dramatically improved medical diagnosis and treatment [1–4]. One of their intriguing application is gene therapy, which has emerged as a potential tool for treating intractable diseases [5,6] by means of transducing therapeutic proteins, because this needs an appropriate delivery system that transports inherently fragile DNA in biological milieu to pathological sites and enters into the cells for expressing therapeutic proteins [7,8]. To establish this therapeutic concept, through systemic administration, the property of blood retention for increasing delivery efficiency to the targeted sites is an essential prerequisite along with gene transfection capability [7,8]. The primary property of a gene delivery system for systemic application is to securely package DNA

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within the delivery system and to inhibit nonspecific interactions with biological components, including opsonins, in addition to nuclease tolerability [9]. In view of these requirements, we have developed a poly(ethylene glycol) (PEG)-shielded gene delivery system, polyplex micelles (PMs), as a promising formulation constructed on the basis of the self-assembly of PEG–polycation block copolymers and plasmid DNA (pDNA) [10,11]. Following this electrostatic complexation, pDNA is packaged into a distinct rod-shaped bundle composed of pDNA folded several times [12] as the core compartment and the PEG chains surrounding it as the protective shell compartment. This characteristic structure of PMs enables systemic applications [13]. However, the blood retention property of the firstly developed PMs was unsatisfactory to obtain a sufficient therapeutic effect via systemic administration; thus, modifications were required to reinforce PM stability in the bloodstream. For this purpose, the introduction of a disulfide cross-linking into the core compartment [14] for preventing disassembly was an effective strategy, as evidenced by the cross-linked PEG–poly(L-lysine) (PEG–PLys) block copolymer-based PMs indeed exhibiting prolonged blood circulation [15,16].

Besides the cross-linking, the PEG compartment also substantially contributes in promoting blood circulation. A recent study highlighting the role of the PEG compartment described the importance of increasing PEG crowdedness in prolonging the blood retention period: higher PEG crowdedness on PMs prepared from a lower degree of polymerization (DP) of the PLys segment of PEG–PLys led to a better blood circulation property [17]. Moreover, PMs of lower PLys DP exhibited increased transcription efficiency [18]. These studies suggested that cross-linked PMs prepared from lower PLys DP would be a suitable formulation for systemic application. However, with regard to the transfection activity, highly PEG-shielded PMs may in turn be disadvantageous because the reduced affinity of these PMs with the cellular membrane may result in precluded cellular uptake. In addition, we found that PLys DP affects the folding number of pDNA [18] within the PM core; PMs prepared from lower PLys DP result in a longer rod length by less folded pDNA. Indeed, PMs prepared from PLys DP 20 displayed rod length ranging several hundred nanometers, while those prepared from PLys DP 70 displayed rod length around 70 nm [17,18]. Such long PMs with several hundred nanometer-sized particles may unlikely be uptake efficiently compared with that of short PMs [19]. Furthermore, a relatively lower binding affinity of PEG–PLys of lower PLys DP to pDNA than that of PEG–PLys of higher PLys DP [20] may decrease the structural stability of PMs, thereby influencing transfection efficiency. Although such impaired stability and unfavorable character for cellular uptake may be improved by applying cross-linking [14] and the ligand molecules [21–24], the inherent structural features of PMs prepared from lower PLys DP may suggest their disadvantageous property in obtaining efficient transfection. Eventually, a concern may arise that the achievement of PMs arriving at target sites by the end of a long systemic journey may result in precluded transfection. These arguments raise the need of a comprehensive study taking each structural feature into consideration for obtaining an ultimate PM formulation. Therefore, the present study aimed to seek the most appreciable formulation to maximize the transfection efficiency on a basis of precise structural control of PMs by varying PLys DP, with particular focus on structural stability [20], PEG crowdedness [17], and rod length [17,18], for completely utilizing the benefits of cross-linking [14–16] and the cyclic RGD (Arg–Gly–Asp) (cRGD) peptide as a ligand for specific integrin-mediated uptake [21–24].

The present study clarified the effect of the abovementioned structural features on cellular uptake and revealed the presence of critical rod length for efficient cellular uptake, consequently identifying the most appreciable PM formulation to promote transfection efficiency. Taking the advantages of the cross-linking and the cRGD ligand upon systemic application to provide stability and tumor homing, respectively, the PM formulation was challenged to systemic treatment of one of the most intractable solid tumor, pancreatic adenocarcinoma, with stroma-rich characteristics, thereby validating the significance of careful control of PM structure, particularly rod length, for promoting systemic gene transfection.

Table 1
Chemical characterization of a series of PEG–PLys with varying PLys DP.

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

[a] Determined from GPC equipped with the TOSOH HLC-8220 column calibrated with commercial PEG standards.
[b] Introducing ratio of thiol (SH) groups against ε-NH_2 groups of lysine units of either MeO PEG–PLys or acetal–PEG–PLys determined from ^{1}H NMR spectra.
[c] Conjugation ratio of cRGD ligand onto α-end of either acetal–PEG–PLys or acetal–PEG–PLys(SH) determined from ^{1}H NMR spectra.

2. Materials and methods

2.1. Materials

α-Methoxy-ω-amino-PEG (MeO–PEG) and α-acetal-ω-amino-PEG (acetal–PEG) of M_w 21 kDa were obtained from NOF Co., Ltd. (Tokyo, Japan). N^{ε}-trifluoroacetyl-L-lysine *N*-carboxyanhydride [Lys(TFA)–NCA] was synthesized as reported previously [25]. *N*-succinimidyl 3-(2-pyridyldithio)-propionate (SPDP) and Slide-a-lyzer dialysis cassettes (MWCO = 3.5 kDa) were purchased from Thermo Scientific (Rockford, IL). Cyclo[RGDfK(C-ε-Acp)] (cRGD) peptide (ε-Acp: 6-aminocaproic acid) was purchased from Peptide Institute Inc. (Osaka, Japan). Luciferase (Luc)-encoding gene-inserted pCAcc vector having the CAG promoter (pCAG–Luc2: 6477 bp) was obtained from RIKEN Gene Bank (Tsukuba, Japan). pDNA encoding human soluble form of vascular endothelial growth factor receptor-1 (VEGFR-1) (or soluble fms-like tyrosine kinase-1: sFlt-1) with pCAcc vector having the CAG promoter (pCAG–sFlt-1: 7186 bp) was constructed according to a previously described method [15]. Both pCAG–Luc2 and pCAG–sFlt-1 were transformed into competent *Escherichia coli* DH5α cells and amplified, following which endotoxin-free pDNAs were obtained using the NucleoBond® Xtra Maxi EF Kit (Macherey–Nagel GmbH & Co., Germany). The other pDNAs, pKF18 (2204 bp) and pAUR316 (11,613 bp), were purchased from Takara Bio Inc. (Otsu, Japan). pDNAs were labeled with Cy5 using the Label IT® Tracker™ Intracellular Nucleic Acid Localization Kit obtained from Mirus Bio Corp. (Madison, WI) according to the manufacturer's instructions and used for studying cellular uptake. Dulbecco's modified eagle's medium (DMEM) and Dulbecco's phosphate-buffered saline (DPBS) were purchased from Sigma–Aldrich Co. (Madison, WI). Fetal bovine serum (FBS) was purchased from Dainippon Sumitomo Parma Co., Ltd. (Osaka, Japan). Cell culture lysis buffer and Luciferase Assay System Kit were purchased from Promega Co. (Madison, WI). Micro BCA™ Protein Assay Reagent Kit was purchased from Pierce Co., Inc. (Rockford, IL). ExGen 500 (linear 22-kDa polyethylenimine) and Lipofectamine® LTX with PLUS™ reagent were purchased from MBI Fermentas Co. (Burlington, ON, Canada) and Invitrogen Life Technologies Corp. (Carlsbad, CA), respectively. Rat monoclonal antibody against mouse platelet endothelial cell adhesion molecule-1 (PECAM-1) was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Alexa 488-conjugated (A11006), Alexa 647-conjugated (A21247) goat anti-rat, and Alexa 488-conjugated (A11008) goat anti-rabbit IgG (H + L) secondary antibodies were obtained from Invitrogen Molecular Probes (Eugene, OR). Rabbit monoclonal antibody against mouse VEGF receptor-1 [Y103] (ab32152) was purchased from Abcam (Tokyo, Japan). Human epithelial ovarian carcinoma cells (HeLa) and human pancreatic adenocarcinoma-BxPC3 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). BALB/c-nu/nu mice (female, 5-weeks old) were purchased from Charles River Laboratories (Tokyo, Japan). All animal experimental procedures were performed in