博士論文(要約)

Optimization of structural features of polyplex micelles to promote transfection efficiency for systemic gene therapy against intractable solid tumors

(難治性固形がんに対する遺伝子発現促進に基づく
全身投与型遺伝子内包高分子ミセルの構造最適化)

ディリサラ アンジャネユル

Gene therapy emerged as a potential tool for treatment of both congenital and acquired diseases including intractable cancers by means of transducing therapeutic proteins. To realize this concept of gene therapy through systemic application, a gene delivery system with stealth surface is required to circumvent biological interferences including nuclease attack, opsonin adsorption followed by reticuloendothelial system (RES) capture. In view of these requirements, Kataoka et al. have developed poly(ethylene glycol) (PEG) shielded gene delivery system as promising formulation based on electrostatic self-assembly of PEG-polycation block copolymers and plasmid DNA (pDNA) to form polyplex micelle (PM), where pDNA is packaged into rod-shaped bundle as core and PEG as shell. The PEG shell effectively prevents interactions with biological interferences capable for systemic applications; nevertheless, the blood retention of this prototype PM was unsatisfactory to obtain sufficient therapeutic activity, thus demanded modifications to reinforce PM stability in bloodstream. For this purpose, environment-responsive disulfide cross-links were introduced into the core of PMs (cross-linked PMs: CPMs) (10% thiolation degree was used based on previous optimization) fabricated from PEG-poly(L-lysine) (PEG-PLys) for preventing unfavorable dissociation. Indeed CPMs exhibited better blood circulation than that of PMs without cross-linking (non-cross-linked PMs: non-CPMs). Besides core cross-linking, the role of PEG shell in achieving promoted blood circulation was demonstrated, where higher PEG crowdedness was found to be settle on PMs prepared from lower degree (DP) of PLys segment of PEG-PLys certainly exhibited better blood circulation property. Moreover, PMs of lower PLys DP exhibited promoted transcription efficiency. These studies suggested that applying cross-linking into the PMs prepared from lower PLys DP would be a suitable formulation for systemic applications. However, upon considering transfection activity, the highly PEG crowded PMs may in turn be disadvantageous because the reduced affinity of these PMs with cellular membrane may result in precluded cellular uptake. Additionally, it was found that PLys DP affects the rod-length of PMs: lower PLys DP resulted in longer rod-lengths (several 100-nm) while higher PLys DP resulted in shorter rod-lengths (≈70-nm). Likely, these longer rod PMs may be unfavorable to obtain efficient cellular uptake because each endocytic pathway has upper size limit with regard to their vesicle diameter. Furthermore, relatively lower binding affinity of lower PLys DP to pDNA than that of higher PLys DP might decrease structural stability of the PMs thus may influence transfection efficiency. Even though such impaired stability and unfavorable high PEG shielding for cellular uptake may be overcome by applying the cross-linking and the ligand molecules, respectively, yet longer rod-length of PMs 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 long systemic journey may result in precluded transfection. These arguments raise a need of comprehensive study taking each structural feature into consideration for attaining an ultimate PM formulation. In view of this necessity, this study aimed to seek the most appreciable formulation for elevating transfection efficiency on basis of precise structural control of PMs by varying PLys DP with particular focus on stability, PEG crowdedness, and rod-length, to fully utilize the benefits of the cross-linking and cyclic RGD (Arg–Gly–Asp) (cRGD) peptide as ligand for specific integrin-mediated uptake. As the cRGD ligand effect was particularly pronounced for the PMs with high PEG shielding, 21 kDa PEG was used in this study instead of previously used 12 kDa PEG and 17 kDa PEG. The present study devoted to clarify the effect of above-mentioned structural features on cellular uptake behavior, revealed the presence of critical rod-length for efficient cellular uptake, consequently identifying the PM with maximized *in vitro* transfection efficiency, and its feasibility for systemic gene therapy against pancreatic adenocarcinoma, one of the most intractable solid tumors.

Initially, cross-linking was applied to lower PLys DP of PEG-PLys with an anticipation to achieve prolonged blood circulation along with higher PLys DP. Indeed, CPMs of lower PLys DP

presented prolonged blood circulation than that of higher PLys DP. In order to understand this effect, PEG density (σ) should be calculated as it determines the extent of opsonin adsorption and thus RES elimination from bloodstream. However, direct estimation of σ is technically difficult for CPMs. In this case, rod-length distribution of non-CPMs and CPMs of corresponding PLys DP should be similar, because rod-length is a function of σ. Thus, the rod-length distributions of CPMs and non-CPMs of respective PLys DP were examined from TEM images and confirmed that they presented almost similar distribution. Accordingly, it may be reasonable to consider that the σ of CPMs of corresponding PLys DP was similar to that of non-CPMs. σ of non-CPMs was thus inferred to CPMs of corresponding PLys DP, giving 0.072, 0.054, and 0.045 chains/nm2 for PLys 20, 42, and 69, respectively, suggesting that lower PLys DP indeed retained higher σ. Furthermore, PEG height (*H*) observation from cryo-TEM approved similar PEG height for CPMs and non-CPMs at corresponding PLys DP, directly supporting their similar σ. From σ analysis, it is likely that PMs of PLys 69 (lower σ) retain mushroom PEG while PMs of PLys 20 (higher σ) retain squeezed PEG. Indeed, PMs of PLys 69 retain *H* from cryo-TEM is 13.1 ± 1.3 nm, which numerically agrees with theoretical *H* of PEG mushroom (= 2*Rg* = 13.0 nm for PEG Mw 21 kDa) while PLys 20 exhibited higher value than mushroom *H* (*H* from cryo-TEM = 17.8 ± 3.0 nm), suggesting PEG chains were in squeezed conformation. Possibly, improved blood circulation of PMs of PLys 20 might be due to eliminating RES-mediated clearance because squeezed PEG experience less opsonization while PMs of PLys 69 with mushroom PEG were more susceptible to opsonization, thus removed by RES.

Prolonged circulation time was accomplished for CPMs prepared from PLys 20. However, it is necessary to see their feasibility for transfection efficiency. In this regard, cellular uptake was initially examined for non-CPMs and compared to CPMs. CPMs exhibited higher cellular uptake than that of non-CPMs irrespective PLys DP, yet CPMs of PLys 20 exhibited limited cellular uptake compared to other PLys DP, suggesting that there should be other issues that limit the cellular uptake aside from unfavorable dissociation. Possibly, higher σ of PLys 20 may account for lower cellular uptake as a result of diminished interactions with cell membrane. Additionally, there is another issue to be considered for cellular uptake is rod-lengh as PLys DP modulates not only σ but also rod-length distribution. The CPMs of lower PLys DP presented longer rod-length distribution while that of higher PLys DP presented shorter rod length distribution. The longer rod-length may be a problem for efficient cellular uptake because the process of each endocytosis has upper size limit with regard to their vesicle diameter. Since HeLa cells are generally considered as non-phagocytic cells, the possibility of phagocytosis can be excluded. Then, the following four possible endocytic pathways could be considered: macropinocytosis, clathrin-, caveolae-dependent, and clathrin/caveolae-independent endocytosis with upper size limit of endocytic vesicles to be 5 μm, 200 nm, 80 nm, and 90 nm, respectively. Note that pDNA within PMs is packaged into rod-shaped structure with specific rod-length distribution based on characteristic quantized folding. Referring the rod-length distribution obtained from TEM images, CPMs of PLys 20 loading pCAG-Luc2, majority of rod-length fraction was found to be above 200 nm, leaving a very small fraction below 200 nm. Taking this observation into consideration, limited cellular uptake of CPMs of PLys 20 may be reasonable because most of the fraction has to rely on only macropinocytosis. On the other hand, CPMs of PLys 69 presenting their entire fraction below 200 nm may take all four endocytic pathways. This analysis clearly pointed out that the rod-length of PMs was also accountable for determining cellular uptake behavior. In order to gain further insight on structural feature contributing for the cellular uptake behavior among σ and rod-length, CPMs with different rod-length distribution but retaining comparable σ were prepared by using fixed PLys 20 (which provided highest σ) and three differently sized pDNAs (pKF18: 2204 bp, pCAG-Luc2: 6477 bp, and pAUR316: 11613 bp). As evidenced from the rod length distribution, it was found

that pKF18 DNA retained shorter rod length distribution with major fraction below 200 nm while pAUR316 DNA retained longer rod length distribution with entire fraction above 200 nm. The similar PEG density of these CPMs of differently sized pDNAs was suggested from similar folding number pattern as the folding number determines the rod length in the quantized folding scheme of pDNA and it is a function of PEG density. The cellular uptake efficiency of these CPMs showed substantial rod-length dependence; shorter rod CPMs of pKF18 DNA appeared to be more readily internalized into the cells while longer rod CPMs of pAUR316 DNA exhibited limited uptake, suggesting that the rod-length of PMs obviously played crucial contribution in determining cellular uptake efficiency. Particular interest was that the critical rod-length found to be 200 nm for efficient cellular uptake. In order to further promoting cellular uptake, cRGD peptide ligands were installed onto PMs and the cellular uptake was examined against HeLa cells, characterized with over expression of cRGD-specific $\alpha v\beta 3$ and $\alpha v\beta 5$ integrin receptors. The promoted cellular uptake was obtained for all series of CPMs regardless of PLys DP and pDNA size approving significant effect of cRGD conjugation to promote the cellular uptake. Despite, CPMs of PEG-PLys21(SH) equipped with cRGD (R-PEG-PLys21(SH)), still remained lower cellular uptake than that of other PLys DP with cRGD. This observation again confirmed that the rod length is an important structural parameter to be primarily considered to achieve high cellular uptake. Responding to cellular uptake study, transfection efficiency was evaluated using reporter gene Luc (pCAG-Luc2 DNA). CPMs showed significantly elevated gene expression efficiency than that of non-CPMs. PMs with cRGD ligand also exhibitedhigher gene expression efficiency than that of non-cRGD counterparts. Synergistically, PMs modified with both cross-linking and cRGD ligand significantly augmented gene expression efficiency. Ultimately, after meeting the critical rod-length requirement to be below 200 nm, the cRGD installed CPMs of PEG-PLys69(SH) (R-PEG-PLys69(SH)) exhibited the highest transfection efficiency among other examined PMs and also presented comparable transfection efficiency as like commercial transfection reagents: ExGen 500 and Lipofectamine® LTX with PLUS™, despite these PMs are covered with PEG palisade and do not possess any specific endosomal escape function.

The PM formulation with maximized *in vitro* transfection efficiency i.e., R-PEG-PLys69(SH) was challenged to see its feasibility to treat BxPC3-human pancreatic adenocarcinoma based on antiangiogenic approach using a gene encoding for potent antiangiogenic exogenous protein, soluble fms-like tyrosine kinase-1 (sFlt-1) via trapping vascular endothelial growth factor (VEGF). The therapeutic activity of PMs loading either sFlt-1 or Luc pDNA (control) followed by intravenous injection was evaluated by tumor growth inhibition study. Significant suppression of tumor growth was achieved for sFlt-1 treated group compared to the control group, validating the feasibility of R-PEG-PLys69(SH) as a formulation for systemic gene therapy. The resulted antitumor activity was confirmed by remarkable sFlt-1 protein expression at tumor site. Presumably, the secreted sFlt-1 captured the VEGF and limited the access to bind with VEGF receptor-1, preventing the endothelial cell proliferation, consequently inhibited neovasculature formation (antiangiogenesis). Indeed, the inhibition of neovascularization was confirmed by reduced vascular density of tumor treated with sFlt-1 loaded R-PEG-PLys69(SH) than that of control group (HEPES). It is noteworthy that PMs of PEG-PLys21(SH) presented higher σ and exhibited prolonged blood circulation. Accordingly, effective antitumor efficacy can be readily anticipated for R-PEG-PLys21(SH) by virtue of an increased chances of the PMs to be accumulated at targeted tumor site. However, this formulation did not show antitumor efficacy even though PEG-PLys21(SH) showed better blood circulation than PEG-PLys69(SH). Most likely, the inferior cellular uptake for PMs of R-PEG-PLys21(SH) due to their longer rod-length character as found in *in vitro* study may resulted in limited transfection at the targeted cells. Indeed, neither sFlt-1 expression nor decreased vascular density was observed for R-PEG-PLys21(SH) at the

tumor site. This result again validated the significance of regulation of rod-length of PMs around 200 nm in order to promote *in vivo* gene transfection upon systemic administration, concurrently suggesting subsequent research direction to attain ideal systemic gene delivery system, conjoined with prolonged blood circulation capacity on R-PEG-PLys69(SH) formulation.

Taking above-mentioned message into consideration, a CPM formulation satisfying prolonged blood circulation with high transfection efficiency was further developed using high Mw 80 kDa PEG and PLys 69. High Mw 80 kDa PEG was used to pursue high PEG shielding by increasing PEG height of the PM surface and thus anticipating better blood circulation. Indeed, extended blood circulation period was attained for PMs of 80 kDa PEG compared to PMs of 21 kDa PEG with the same PLys DP of 69. PLys 69 was used to obtain shorter rod-lengths, as it packages pDNA with high folding number based on quantized folding. Indeed, entire rod-length fraction was found to be below 200 nm and consequently resulting in high transfection efficiency. Therefore, further development by equipping appropriate ligand molecules onto well-defined PM with high PEG crowdedness for prolonged blood circulation and appropriate packaging size for efficient transfection efficiency should confer tempting perspective to accomplish potent therapeutic efficacy towards targeted systemic gene therapy.

In summary, this study clarifies the role of structural features of PMs such as stability, σ, and rod-length on cellular uptake, however most significant feature affecting cellular uptake was found to be rod-length. With a critical rod-length around 200 nm, PMs of longer rod showed limited uptake while PMs of shorter rod showed efficient uptake. In particularly, coupling with cross-linking and cRGD ligand substantially improved the cellular uptake. Consequently, the PM formulation satisfying all these features, i.e., R-PEG-PLys69(SH), showed promoted transfection efficiency almost comparable to commercial transfection agents, despite the PMs do not have any specific endosomal escape function and also covered by PEG. The identified R-PEG-PLys69(SH), exhibited potent tumor growth suppression as a result of induced antiangiogenesis. Noteworthy, the CPMs of PEG-PLys21(SH), presented most of their rod-lengths above 200 nm, resulted in negligible antitumor efficacy even they showed better blood retention profile than that of PEG-PLys69(SH), approving critical role of rod-length even at *in vivo* gene transfection. Finally, prolonged blood circulation was further conjoined to the CPMs of PEG-PLys69(SH) using high Mw 80 kDa PEG. This well-defined CPM formulation featured with prolonged blood circulation and high transfection efficiency may serve as potential platform for versatile use in the treatment of variety of intractable diseases by assigning specific ligands onto the surface of PMs for targeted systemic gene therapy.