博士論文（要約）

論文題目　Development of polyplex micelles for systemic gene therapy of intractable solid tumors

（難治固形がんに対する遺伝子治療のための全身投与型遺伝子内包高分子ミセルの開発）

氏　名：　陳　麒先　(Qixian Chen)

Development of non-viral gene delivery carriers has been highlighted with respect to their advantages in low host immunogenicity and large-scale manufacturing. Cationic gene carriers, which are formulated through electrostatic self-assembly of anionic plasmid DNA (pDNA) and cationic materials (e.g. polycations, cationic lipids), have emerged as a tempting gene delivery modality in view of their tremendous potential to circumvent ensemble of predefined biological barriers via versatile chemistry-based engineering. The principle design criteria in view of the barriers encountered in delivery of exogenous gene to the targeted cells include the abilities of protecting encapsulated pDNA from enzymatic degradation, preventing undesired non-specific interactions in the biological environment, readily being internalized into the targeted cells, retrieving from endosome entrapment, trafficking in the cytoplasm, and localizing into nucleus as well as pDNA releasing. To these required principles, Kataoka et. al. have developed a multi-functional catiomer, poly{*N*'-[*N*-(2-aminoethyl)-2-aminoehtyl]aspartamide}PAsp(DET). This PAsp(DET) catiomer featured as the flanking ethylenediamine moiety in the side chain of N-substituted polyaspartamide (PAsp), displayed distinctive two-step protonation behavior in response to pH gradient, where the protonation of ethylenediamine is facilitated in acidic pH. Interestingly, this acid-responsive trait elicits a selective endosome membrane destabilization function. Furthermore, PAsp(DET) will not provoke cumulative cytotoxic concern due to its appreciable biodegradable nature, consequently allowing safe and efficient gene expression in the targeted cells. These advantageous features of PAsp (DET) entitled its potential use in fabrication of safe and efficient gene delivery carriers for therapeutic use. Recent efforts in utilizing this system have demonstrated appreciable therapeutic outcomes particularly for local administration. Nevertheless, to promote it towards broad systemic use where gene carriers were administered into a harsh environment and subjected to diverse biological impairing (e.g. dissociation, enzymatic degradation, opsonization and captured by reticuloendothelial system: RES), it is imperative for gene delivery carriers to provide stealth surface to avoid non-specific interactions in blood circulation. For this purpose, surface modification of polyion complex with poly(ethylene glycol) (PEG) (PEGylation) was developed via complexation of block catiomer PEG-*b*-PAsp(DET) with pDNA, where single pDNA can be packaged into nanosized core covered by the hydrophilic and biocompatible PEG corona. With merits of this PEG shielding shell, non-specific interactions with biological components can be minimized and allow for well dispersing in the blood fluid, leading to potential use in systemic delivery. Nevertheless, systemic use of this system was still limited with respect to its insufficient delivery efficiency to the targeted site and cell-transfecting activity in the targeted site. Aiming for promoting systemic efficacy of this system as well as taking note of safety concern, several strategies were proposed and demonstrated in this thesis:

1: Enhance cell-transfecting activity for improved gene expression in the targeted cells. Since the therapeutic outcome relied on the level of protein expression in the targeted cells, the cell-transfecting activity extends significance for ultimate therapeutic potency. In this respect, my first strategy was emphasized on enhancing transfection activity of polyplex micelle based on integration of PAsp(DET) (H) characterized by high transfection activity, into PEG-*b*-PAsp(DET) (B) formulated polyplex micelle (BHPM), to pursue enhanced gene expression in the targeted sites via systemic administration. Note that direct use of H is not applicable to systemic application with respect to its readily aggregation in blood circulation and followed RES clearance. Hence, the proposed BHPM may hold promise to achieve both merits of PEG shielding from B for stealthily blood circulation and high cell-transfecting activity from H for improved gene expression at the targeted cells.

2: Prolong blood circulation and install targeting molecule for improved delivery efficiency in the targeted site. On the other hand, the longevity of gene delivery carrier in blood circulation so as to increase delivery efficiency to the targeted sites is also of crucial importance for ultimate therapeutic potency. In this respect, the second strategy was motivated to prolong systemic retention of polyplex micelle. Hydrophobic cholesteryl moiety was strategically introduced at ω-terminus of block copolymer, anticipating for promotion of not only stability of polyplex structure but also the tethered PEG crowdedness to gain better stealth function. Moreover, PEG molecular weight was attempted to elongate for further enhancement for PEG crowdedness. To address the dilemma that increased PEG shielding may result in reduced cellular uptake, ligand molecule (cRGD peptides) was strategically conjugated onto the polyplex micelle surface intended for enhanced cellular uptake and also promoting accumulation of polyplex micelles into tumor cells (overexpressed $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrins as cRGD specific receptors on the cell surface).

The therapeutic performance of the polyplex micelles prepared according to aforementioned strategies was examined in treatment of one of most intractable solid tumor constructed from a human pancreatic adenocarcinoma BxPC3. Note that the characteristic features of pancreatic tumor with low vascularization, reduced permeability due to pericyte coverage of blood vessels and thick fibrosis, massive delivery of therapeutic gene to the whole pancreatic tumor cells would be difficult to accomplish. In this regard, I attempted to utilize anti-angiogenic approach, where tumor growth is conceptually to be inhibited through destruction of neo-vasculature formation. Herein, I selected secreted protein, soluble fms-like tyrosine kinase-1 (sFlt-1), which exerts anti-angiogenesis via trapping angiogenic molecules (e.g. vascular endothelial growth factors: VEGF) as payload of pDNA for tumor gene therapy test.

1. Enhance cell-transfecting activity for improved gene expression in the targeted cells: In light of dramatic high-transfecting activity of homo-PAsp(DET) (H), H was attempted to integrate into PEG-*b*-PAsp(DET) (B) formulated polyplex micelle (BHPM) with the aim of enhancing cell transfection efficiency for polyplex micelle. For preparation of BHPM, mixture of B and H solutions at varying B/H ratios (residual molar ratio of amino groups in B and H) was added to pDNA solution for complexation at varying N/P ratios (residual molar ratio of total amino groups in B and H to phosphate groups in pDNA). Physical characterizations confirmed nanosized formulation (cumulant diameters ranging from 60 nm to 100 nm) with unimodal size distributions of low PDI from 0.1 to 0.2 were obtained in all B/H series. Ultracentrifuge measurement revealed the binding compositions of B and H in BHPMs remain fairly consistent with the fed B/H ratios and all the BHPMs were formulated exclusively according to stoichiometric charge ratio. To see the biological impact of H integration, transfection activities of BHPMs at varying B/H ratio were evaluated and revealed potent stimulation of H integration in enhancing cell-transfecting activity of polyplex micelles. Noteworthy was this enhancement attributable to facilitated cellular uptake and promoted endosome escape capacity. Furthermore, with regard to the safety concern, cytotoxic profiles of BHPMs were evaluated to identify the most promising B/H ratio, allowing for select BHPM of B/H = 70/30, N/P = 8 as the best formulation for translation into *in vivo* application to test its feasibility in systemic gene therapy. Polyplex micelles loaded by antiangiogenic sFlt-1 gene were intravenously injected into mice bearing pancreatic adenocarcinoma BxPC3 tumor. Subsequent

investigations revealed that sFlt-1 expression in the tumor site appeared remarkably high in BHPM treated mice. In consistency, vascular density of tumors treated with BHPM was significantly lowered and ultimately resulted in tumor growth suppression by BHPM. These results approved feasibility of BHPM for enhanced transfection and potential use in systemic application, which could have important implications on strategic use of H in non-viral gene carrier design.

As demonstrated, the H integration was confirmed to be effective strategy on enhancing cell-transfecting activity of polyplex micelle, nevertheless, this strategy is concomitant with reduced binding number of B, which results in lowered PEG tethering crowdedness, and thus undermines its stealthiness in blood circulation. The principal design criteria for systemic gene delivery carrier are stealthy retention abilities in the blood circulation so as to avoid non-specific interactions (e.g. protein adsorption, blood cell adhesion and RES capturing) and arrive at the targeted site. Regarding to this, my second strategy was emphasized on promoting PEG tethering crowdedness to minimize non-specific interactions for prolonged systemic retention.

Prolong blood circulation and install targeting molecule for improved delivery efficiency in the targeted site: The primary issue is to promote PEG shielding for prolonged blood circulation of polyplex micelle. To serve this purpose, hydrophobic cholesteryl moiety was strategically introduced onto ω-terminus of block copolymer, anticipating for promotion of both stability of polyplex structure and tethered PEG crowdedness. Subsequent investigation identified synergistic effect of cholesteryl conjugation: facilitating a larger number of polymers bound to pDNA (over-stoichiometry) and inducing more condensation of pDNA, which eventually resulted in elevated tethered PEG density. Furthermore, elongation of PEG from 12 kDa to 20 kDa also contributed for increased PEG crowdedness. Towards systemic treatment for intractable pancreatic tumor, ligand molecule cRGD peptide was installed onto micelle surface in order for facilitating targeted delivery to the tumor site as well as promoting cellular uptake in virtue of cRGD-integrin affinity. Of note, *in vitro* evaluation approved benefits of cRGD conjugation for enhanced cellular uptake and favorable intracellular trafficking behaviors, so that accounting for remarkably high transfection activity. Systemic evaluations approved improved accumulation of cRGD micelles into the tumor site, together with its high transfection activity, thus resulted in efficient gene expression of anti-angiogenic protein (sFlt-1) in the tumor site. Ultimately, potent tumor growth suppression was achieved as a result of induced anti-angiogenesis on tumor vasculature. The obtained result validated systemic usage of this system for tumor-targeted gene therapy, thus endows tremendous future perspective of developing the system for broad utilities by choosing appropriate therapeutic genes and targeting moieties.

With respect to notably efficiency of polyplex micelle based on strategy 2, it is intriguing to promote this system towards practical clinical trial, therefore the safety profile of proposed polyplex micelle as should be carefully investigated. Note that previous polyplex micelle prepared at N/P ratio of 8 contains fraction of unbound free polymer, which may potentially interact with charged blood components after systemic administration. In this respect, polyplex micelle formulation including no unbound polymer is preferable for practical applications. Subsequent investigations uncovered the underlying binding principles for cholesteryl-conjugated block copolymer, and identify the appreciable N/P ratio range for preparation of polyplex micelle without free

polymers. Taken into consideration of efficiency and PEG shielding effect, most appreciable polyplex micelle with superb safety profile was distinguished, which exhibited comparable potent tumor suppression efficacy as that of N/P 8. The obtained results validated safety of constructed polyplex micelle as efficient gene delivery carrier, enable active targeted delivery into tumor site via systemic administration and abundant therapeutic protein expression in the targeted cells, therefore encourages for clinical trial to demonstrate the potentials of this polyplex micelle for treatment of intractable diseases according to its unique therapeutic mechanism.

I have developed two strategies in promoting PEG-*b*-PAsp(DET) based polyplex micelle towards systemic gene therapy, and both have achieved appreciable therapeutic outcomes in treatment of pancreatic tumors. The first strategy proposed to integrate H characterized with its high cell-transfecting activity into B based polyplex micelle to pursue enhancement of transfection efficiency for PEGylated polyplex micelle, leadingly conducing to elevated therapeutic potency. The second strategy works on chemical design of block copolymer to prolong blood retention of polyplex micelle via increasing PEG tethering crowdedness. By virtue of cholesteryl conjugation and use of longer PEG, enhanced PEG shielding was obtained and has gain prolonged retention in blood circulation. Noteworthy was dual effect of the cholesteryl conjugation on increased PEG tethering density: increases of tethering PEG chains and more compacted core, synergistically increased PEG density, demonstrating beneficial to strategic use of hydrophobic moiety on improved PEG shielding. With aids of cRGD conjugation, a targeted polyplex micelle with crowded PEG palisade was established as active targeting gene delivery carrier for systemic anti-angiogenic tumor treatment. Furthermore, the targeted polyplex micelle was further developed to exclude unbound polymer in order to minimize potential toxic concern. Ultimately, the pure polyplex micelle armed with ensemble of intriguing functionalities, such as substantial blood retention, active tumor targeting, high cell transfecting activity, and appreciable biodegradability of block copolymer, demonstrated as an ideal gene delivery carrier applicable to clinical trial. It is worthy of intensive efforts to accelerate translation of this laboratory achievement to revive the concept of gene therapy as available tool for treatment of most intractable diseases.