論 文 の 内 容 の 要 旨

論文題目　Molecular design of amphiphilic polyaspartamide derivatives with hydrophobic moieties for efficient nucleic acids delivery
（核酸送達効率の向上を目指した疎水性官能基導入ポリアスパルタミド誘導体の分子設計）

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Nucleic acid drugs have been attracting much attention as promising biopharmaceuticals because they can regulate the target gene expression in target cells. Antisense oligonucleotide (ASO), small interfering RNA (siRNA), and *in vitro* transcribed messenger RNA (IVT mRNA) are representatives of nucleic acid drugs. Whereas ASO and siRNA can inhibit target gene expression *via* complementary interaction to target mRNA, IVT mRNA can produce coded-protein in the cytoplasm of the target cells. However, *in vivo* transfection efficiency of naked nucleic acids is extremely low due to its high susceptibility to degradation by ribonucleases and the inefficient cellular uptake caused by the electrostatic repulsion against the negatively charged cytoplasmic membrane. To improve the bioavailability of naked nucleic acids, various delivery technologies have been developed. Among the technologies, nonviral delivery vehicles, such as lipid nanoparticles (LNPs) and polymeric nanoparticles (polyplexes), have been considerably developed for the protection of mRNA from nucleases and their rapid internalization into target cells. Indeed, siRNA- and mRNA-loaded LNPs have been approved for the medicine of hereditary liver diseases and COVID-19 vaccinations by the FDA. Polyplexes also have great potential because of the ease of their preparation in aqueous buffers and high functionalization by fine-tuning the component of polymer chemical structure. Various polymer designs (functionalization) can elicit enhanced polyplex stability in the extracellular milieu, increased endosomal escape, and high biodegradability, leading to efficient nucleic acid delivery. Among the functionalized polymers, amphiphilic polyaspartamide derivatives (PAsp(DET/R)s) with diethylenetriamine (DET) and hydrophobicity (R) moieties showed efficient mRNA delivery. Cationic DET moiety endows two functionalities, mRNA binding and endosomal escape that is achieved by changing the protonated form from the mono-protonated state to the di-protonated state at endosomal acidic pH, destabilizing the

endosomal membrane. Also, the derivatives with DET moieties have high degradability *via* main chain cleavage, allowing lower toxicity and enhanced release of drug payloads. R moieties stabilized mRNA-loaded nanoparticles in an aqueous milieu *via* hydrophobic interactions. The previous study revealed that the mRNA expression efficiencies in cultured cells were nicely correlated with the hydrophobicity (log$D_{7.3}$; octanol–water (or buffer at pH 7.3) distribution coefficient) of nanoparticle-forming PAsp(DET/R)s. In particular, PAsp(DET/R)s with log$D_{7.3}$ larger than −2.4 exhibited appreciably higher mRNA expression efficiencies compared with the counterparts with log$D_{7.3}$ lower than –2.4. Ultimately, a polyaspartamide derivative comprising cyclohexylethyl (CHE) moieties, PAsp(DET/CHE), accomplished the highest transfection efficiency, presumably due to the optimal hydrophobicity for the polyplexes stability in the extracellular milieu and the mRNA releasability in the cell cytoplasm. Also, it achieved the IVT-mRNA-mediated gene editing in the brain *via* local injection into the mouse. However, the availabilities of PAsp(DET/R)s were unrevealed for other nucleic acids delivery and systemic mRNA delivery. In this thesis, the author aimed at optimizing the molecular design of polyaspartamide derivatives for efficient antisense oligonucleotide delivery and systemic mRNA delivery.

First, the author investigated the impact of R moieties on *in vitro* ASO transfection efficiency for the optimization of R moiety for efficient ASO delivery. Before the evaluation of *in vitro* ASO transfection efficiency, the ASO-loaded polyplex formation was confirmed and these showed equivalent hydrodynamic size and zeta-potential were confirmed, regardless of varying R moieties. In the comparison of *in vitro* ASO transfection efficiency among the polyplexes, it was revealed that the ASO transfection efficiency of PAsp(DET/R)s depends on R moieties. The presence of a threshold for efficient ASO expression of polyplexes was observed in log$D_{7.4}$ of PAsp(DET/R)s at around –2.4. The derivatives with higher log$D_{7.4}$ than the threshold value elicited considerably higher ASO delivery than the counterpart with lower log$D_{7.4}$. This was proved to be due to enhanced cellular uptake of ASO payloads, caused by higher polyplex stability. From the result, the author found out the optimized R moieties for efficient ASO delivery. Further, for the translation into *in vivo* ASO delivery *via* local injection, the accumulation level of ASO was examined after intratracheal administration of ASO-loaded polyplex and naked ASO. As a result, polyplex-based delivery showed higher ASO accumulation in the lungs than naked ASO, presumably due to the enhanced retention efficiency derived from the increased size. It shows the potential of the polyplex for effective ASO transfection in the lungs *via* intratracheal injection.

Next, the author aimed at improving systemic mRNA delivery *via* polyplex by synthesizing and comparing PAsp(DET/R)s. Based on that PAsp(DET/CHE) exhibited the highest *in vitro* mRNA delivery efficiency, a series of PAsp(DET/R)s were newly synthesized to have slightly different alicyclic (R) moieties from CHE moiety. Moreover, a new series of PAsp(DET/R)s were prepared to have a lower degree of polymerization (~20) than PAsp(DET/R) library in a previous study, to reduce the systemic toxicity derived from cationic components. Whereas all PAsp(DET/R)s possess equivalent introduction

rates of alicyclic moieties, they have varying log$D_{7.3}$ based on different R structures. All polyplexes exhibited higher *in vitro* mRNA delivery efficiencies, which is comparable to commercially available Lipofectamine MessengerMAX (LipoM) control, except for the polyplex (CHM-polyplex) formed by the cyclohexylmethyl (CHM)-installed derivative (PAsp(DET/CHM)). This is because that PAsp(DET/CHM) has lower log$D_{7.3}$, resulting in limited polyplex stability and cellular uptake. To investigate the effect of R moieties on systemic mRNA delivery, the mRNA expression level of each mouse organ was evaluated after intravenous administration of mRNA-loaded polyplexes. The polyplexes with log$D_{7.3}$ between –1.8 and –1.3 elicited efficient systemic mRNA delivery into the lungs. Whereas the cyclohexylbuthyl (CHB)-installed derivative (PAsp(DET/CHB)) (log$D_{7.3}$ = –1.3) showed higher *in vitro* mRNA delivery, it exhibited limited *in vivo* expression levels in the organs, indicating the optimized log$D_{7.3}$ range for systemic mRNA delivery is narrower than that for *in vitro* delivery. Although the reason for inconsistency between *in vivo* and *in vitro* expression profiles of PAsp(DET/CHB) was not revealed, it is presumed that excessive hydrophobicity interfered with mRNA expression *in vivo*. Meanwhile, LipoM exhibited *in vivo* mRNA expression mainly in the spleen. To investigate the lung-preferential mRNA expression of polyplex, two kinds of biodistribution assays were performed by determining the fluorescence intensity derived from Cy5-labeled mRNA and quantifying the amount of intact mRNA by qRT-PCR. Whereas the *in vivo* mRNA expression profiles in mouse organs were inconsistent with the fluorescence intensity-based biodistribution, they were apparently consistent with the biodistribution of intact mRNA measured by qRT-PCR. The inconsistency between fluorescence intensity-based biodistribution and *in vivo* mRNA expression profiles might be due to the overestimation derived from inactive (degraded) mRNA. This study demonstrates the potential of PAsp(DET/R)s with fine-tuned R moieties for polyplex-based mRNA delivery to the therapeutic applications in the lungs.

In this thesis, the author developed PAsp(DET/R)s for efficient nucleic acid, *i.e.*, ASO and mRNA delivery by fine-tuning R moieties and demonstrated the therapeutic potential of the optimized polyplexes for lung-targeted mRNA delivery *via* intravenous administration.