審査結果の要旨

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Messenger RNA (mRNA) is a promising therapeutic oligonucleotide with several advantages over plasmid DNA (pDNA), including no insertional mutagenesis and translation in non-dividing cells. However, therapeutic outcome is limited due to rapid degradation by nucleases and poor cellular uptake. Thus, mRNA carriers that can overcome the aforesaid issues are key for developing mRNA-based therapies. Polyion complex (PIC) micelles comprising block copolymers with polycation segments are promising nanocarriers for mRNA delivery. These micelles can load the mRNA in their core *via* electrostatic interaction with the polycation blocks, having the potential for controlling the *in vivo* biodistribution while protecting mRNA from the harsh biological environments. Recent observations have suggested that the rigidity of the charged blocks is critical in the association and stability of PICs between block ionomers and nucleic acids. However, the effects of the flexibility of the block ionomers on the assembly and performance of mRNA-loaded micelles remains unexplored.

In this dissertation, I developed novel mRNA delivery systems based on PIC micelles with controlled rigidity of the backbone of the polycation segment by using a block copolymers consisting of poly(ethylene glycol) and the flexible block ionomers with poly(ether) backbones, which were synthesized by efficient one-pot reactions with optimized catalyst. Moreover, for developing a safe and effective carrier for *in vivo* application, I prepared flexible block ionomers conjugating a set of amino acids based on the interaction between protein and oligonucleotides determined by bioinformatics. These amino acids were conjugated to a flexible segment poly(glycerol) *via* ester bonds, which can be gradually hydrolyzed to reduce the cytotoxicity. Furthermore, an active targeted system using phosphocoline (PC) as the ligand moiety, which is directed to the phospholipid transfer protein (PLTP) overexpressed in various kinds of cancer, *e.g.* pancreatic cancer, was developed for enhancing the intracellular delivery and the mRNA translation.

In Chapter 2, with the flexible poly(ether)-based block copolymers synthesized by one-pot reactions, I prepared block ionomers having primary amines to self-assemble mRNA-loaded PIC micelles. The micelles prepared from PEG-poly(glycidyl butylamine) (PEG-PGBA) were approximately 50 nm in diameter, which is comparable to the size of the relatively more rigid block ionomer PEG-poly(L-lysine) (PEG-PLL) formulation. Isothermal calorimetry (ITC) measurements clarified the loss of enthalpy and the gain of entropy in the case of PEG-PGBA formulations, indicating that the flexible PGBA chains decreased the contact area between block ionomers and nucleic acids and increased the release of free water during ionic pair formation to increase the free energy of the formulated PIC micelles. This high stability resulted in better protection of encapsulated mRNA against both polyanion exchange and enzymatic degradation to show high gene expression in cultured cells. These *in vitro* observations were correlated with the high gene expression in the lungs, as well as with the prolonged blood circulation time of intact mRNA in mice. These results indicated that the flexible polycation segment can enhance the stability of mRNA for *in vivo* applications.

In Chapter 3, I synthesized hydrolysable polymers by conjugating functional amino acids *via* ester bonds. In this case, I used leucine with hydrophobic isobutyl groups, tryptophan with indole groups and tyrosine with phenolic alcohols, which interact with bases in mRNA, based on the informatics of protein-nucleic acids interactions. The polymers showed the relatively rapid degradation of the ester bonds to reduce the number of primary amine in the block ionomers, resulting in low cytotoxicity against cultured cells. After preparing mRNA-loaded PIC micelles with these degradable block ionomers, the stability against polyanion exchange and enzymatic degradation were examined. All formulations showed better protection of mRNA than PIC micelles prepared by control block ionomers, in this case, glycine-conjugated polymers. This enhanced stability resulted in high gene expression both *in vitro* and *in vivo* after pulmonary administration. For systemic delivery of mRNA, the bioavailability of mRNA-loaded PIC micelles in mice was investigated. Accordingly, the micelles prepared with the polymers having tryptophan moieties show the longest blood circulation. These results indicated that introducing bio-inspired ligands promoting the interaction with nucleic acids improved the stability of the PIC and biological performance of the micelles.

In Chapter 4, I functionalized the surface of PIC micelles with PC ligands for improving the cellular uptake of the micelles. To investigate the effect of ligands on the uptake by cancer cells, I prepared empty PIC micelles with irreversibly crosslinked core for stabilization in *in vitro* conditions. The PC-installed micelles showed comparable size (50 nm) with PIC micelles having no ligands. In cultured pancreatic cancer BxPC3 cells, PC micelles showed faster uptake than the control micelles without ligand. By doing a competition

experiment with free PC molecules, I clarified that the PC molecules on the surface of PIC micelles promoted the cellular uptake, as free PC inhibited the cellular uptake of the PC-installed micelles. Moreover, an inhibition assay was conducted to check the interaction between PC micelles and PLTP by using the PLTP inhibitor thimersal. Thus, thiomersal inhibited the uptake of PC, demonstrating that PLTP mediated the uptake of PC micelles in the pancreatic cancer cells. After the installation of PC ligands on the mRNA-loaded PIC micelles prepared by tryptophan-conjugated polymers, the gene expression was examined *in vitro*. The PC-installed mRNA-loaded micelles showed higher gene expression than the micelles without ligands. These results indicate that the ligand/receptor pair of PC and PLTP is a promising target for enhancing the delivery against intractable pancreatic tumors, which can promote the internalization of several formulations, including our novel mRNA-loaded PIC micelles.

As a corollary, in Chapter 5, I reported a new synthesis route for PEG-poly(amino acids) (PEG-PAAs), including PEG-poly(γ-benzyl-L-glutamate) (PEG-PBLG) and PEG-poly(ε-tetrafluoroaceticacids-L-lysine) (PEG-PLL-TFA), by doing one-pot polymerization of epoxides and N-carboxyanhydride (NCA) of the specific amino acids by using 1,1,3,3-tetramethylguanidine (TMG) as the catalyst. This catalyst proceeded the polymerization of both ethylene oxide and NCAs to show comparable molecular weight distribution to conventional NCA polymerization initiated with PEG macroinitiators. TMG also promoted faster polymerization kinetics than the conventional NCA polymerization. Besides, the obtained PEG-PAAs present an ester bond between the PEG and PAA segment that can be hydrolyzed in physiological conditions for improving the biocompatibility. The ester bond did not affect the preparation of polymeric micelles based on the new PEG-PAAs, nor their stability in physiological conditions. Moreover, TMG catalyzed the co-polymerization of a different epoxy monomer, epichlorohydrin, to obtain flexible PEG-poly(epichlorohydrin) (PEG-PECH) with sharp molecular weight distribution. However, in the case of the precursor of the flexible poly(glycidyl butylamine) segment (PEG-PGBA), more reactive catalyst triisobutylalminum was adapted to polymerize the epoxy monomer, 1,2-epoxy-5-hexene. These results indicate that the careful selection of catalyst based on the reactivity was important to polymerize epoxides and NCAs without unfavorable side reactions.

Finally in Chapter 6, I provided the conclusion and future perspectives of my works. There I highlighted the development of novel PIC micelles stably encapsulating mRNA by engineering block ionomers with flexible poly(ether) backbone, which were prepared by one-pot polymerizations in the presence of catalyst. These micelles showed high resistant to degradation by nucleases and polyanions, which lead to enhanced *in vitro* translation, high gene expression in lungs and prolonged blood circulation in mice. Introducing amino acids to the poly(glycerol) backbone *via* ester bonds, further improved the stability against polyanions, significantly increasing the bioavailability. Also, in physiological conditions, the polymers with amino acids degraded to safe poly(glycerol). In addition, PC was identified as a ligand molecule to promote cellular uptake, and by functionalizing micelles with PC ligands, the gene expression was augmented in cultured cells. In the future, I will investigate the possibility of our novel flexible block ionomers to encapsulate different type of nucleic acids, including small interfering RNA (siRNA) and the set of guide RNA and mRNA encoding Cas9 protein for genome editing. Moreover, to further functionalize the mRNA-loaded PIC micelles, I will conjugate more amino acids to construct polypeptide pendants in the side chain of poly(ether) segments. This polymer could also be used for synthesizing polypeptides in liquid phase by cutting the degradable ester bonds between polymer backbone and polypeptides. In addition, the accumulation in tumors and the gene expression of PC-installed micelles with mRNA will be investigated to check the capability of our system for *in vivo* applications.

These findings will have great significance not only for the delivery of nucleic acids and other drugs by using novel poly(ether) polymers, but also for several materials and bioengineering applications. For example, the one-pot synthesis strategy developed in this work fits the good manufacturing practice (GMP) for PEG-PAAs, such as PEG-poly(glutamate), which is the building block of polymeric micelles under the clinical trials. Moreover, the effect of flexibility of ionizable segments on the high stability of complex may be extrapolated to lipidic materials toward the development of novel LNPs for siRNA and mRNA delivery. In addition, the possibility to use hydrophilic PC ligands with betain structure not only for nanomedicine delivery, but also for modifying low and middle molecular compounds for enhancing their selectivity. We expect that the systems developed in this thesis will make significant impact in various research and industry fields, including chemistry, biology and medicine.

According to the reviewers, this thesis is eligible for applying a diploma of Ph. D. (engineering).