論文の内容の要旨

Design and Evaluation of Environment-Responsive Block Copolymer Micelle Directed toward Enhanced siRNA Delivery
(siRNAデリバリーのための環境応答性高分子ミセルの創製)

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This dissertation provides a comprehensive analysis of structure-activity relationship of cationic-hydrophilic block copolymers as a building block of polymeric micelle for siRNA delivery. Our work should make a significant contribution toward the development of siRNA-based therapy, because the delivery system is believed to govern the feasibility of therapeutic siRNA as reviewed in chapter 1. Since the discovery of siRNA in 2001, methods based on RNAi machinery have been utilized to silence specific target genes, as a research tool to elucidate the gene function and also for the prevention of undesired disease gene expression. Some studies have already progressed to clinical trials, but almost all cases utilize local administration, an application limited to readily accessible tissues such as those found in the ocular and respiratory systems. Indeed, it is the unfavorable pharmaceutical property of siRNA that prevents the extensive practical application of RNAi based therapy. For example, siRNAs are known to rapidly degrade into non-effective fragments in the RNase-rich physiological environment. Additionally, systemically administered naked siRNAs are rapidly eliminated from circulation, with plasma-half lives reported to be as short as 0.03 hour in mice and 0.1 hour in rats. It is clear that an innovative carrier system for the systemic administration is required for further development of siRNA as a universal drug. Fundamental design and evaluation of a siRNA carrier, which could be developed into a system for therapeutic use, was a specific aim of this work.

An incorporation of siRNA into block copolymer micelle is of great interest for the improvement of pharmaceutical property of siRNA. However, little is known about the molecular design of a block copolymer that is suitable for siRNA delivery, because the short and rigid siRNA molecule was almost a new entity as a cargo molecule of block copolymer micelle. In order to explore the requirement of molecular structure for the successful siRNA transfection, we began with the screening of siRNA transfection efficacy of block copolymers with various compositions. In this regard, PEG-*b*-PLL and its derivatives (e.g. PEG-*b*-P[Asp(DET)/Lys]), which possess ethylene diamine structure with lowered basicity, were evaluated as a carrier for siRNA delivery in chapter 2. Cationic segment of block copolymer is vital for the interaction with anionic nucleic acids: the protonated state of primary amine in Lys residue is quite stable with the p*K*a of nearly 10 in aqueous solution, allowing the stable complexation with the anionic phosphodiester in siRNA. On the contrary, ethylene diamine structure of Asp(DET) residue is less cationic than primary amine, hence the substitution of Lys for Asp(DET) may decrease the stability of the complex with siRNA. The looser complexation appears to be disadvantageous for siRNA delivery,

however, Asp(DET) has been successfully enhanced plasmid DNA transfection by facilitating the endosomal escape of the plasmid DNA complex. Endosomal escape is believed to be based on the ethylene diamine structure which allows the molecule to act as a 'proton sponge' that buffers a proton influx during endosome acidification, leading to endosome swelling and disruption. Thus, block copolymers have been successfully optimized those structures for plasmid DNA transfection by utilizing a hybrid cationic segment of Lys and Asp(DET) to balance the plasmid DNA binding force and proton sponge effect. Given the similar requirement of stable complexation and endosomal escape for siRNA transfection, same strategy was considered to be adoptable for the development of siRNA carrier. As a result, siRNA was successfully complexed with all of PEG-*b*-PLL and its derivatives examined, and obtained remarkable resistance against enzymatic degradation upon the complexation with PEG-*b*-PLL, however, none of these block copolymers showed significant improvement in transfection efficacy. Even though the cationic moieties of block copolymers were substituted with PAsp(DET), these copolymers exhibited rather weak transfection efficacy than PEG-*b*-PLL and PEI. Furthermore, any attempts to compensate the instability of PAsp(DET)/siRNA complex by increasing Lys/Asp(DET) ratio or adding a hydrophobic segment (PLL(Z)) hardly improved transfection efficacy. It was suspected that the common PEG shell of block copolymer/siRNA complexes hindered the cellular uptake of them, however, homo PAsp(DET) still showed poor efficacy in siRNA transfection. One explanation for the paradoxical results between plasmid DNA and siRNA might be the difference in the intracellular destinations of each nucleic acid: siRNA has been supposed to be recruited into RNAi machinery in cytoplasm, while plasmid DNA should be transported into nucleus, where siRNA can not mediate RNAi. In this regard, PAsp(DET) derivatives, which may achieve highly-efficient plasmid DNA transfection by enhancing the nuclear transport of those cargo molecule, could have unfavorably distributed siRNAs to nucleus. Furthermore, siRNA may require to be in its intact form to be active in cytosol, because the enzymatic molecular recognitions of siRNA and target mRNA occur at each stage in RNAi process. Hence, the recruitment of siRNA into RNAi machinery might be susceptible to the hindrance associated with the surrounding cationic copolymers, which does not interfere the successful transfection in the case of plasmid DNA. Based on these considerations about the difference between plasmid DNA and siRNA, ideal siRNA carrier may well require a distinct development in the structure of block copolymer.

In chapter 3, we shifted our focus to nano-scaled structuring of block copolymers and siRNA as a fundamental step of the development of siRNA carrier. Although the successful complexation of siRNA with block copolymers were indicated by gel retardation analysis and EtBr assay in chapter 2, it provided no information on the resulting structure of the block copolymer:siRNA complexes. Knowing the detailed structure of the block copolymer complex was considered to be important, because block copolymers have been demonstrated to form not a simple but a variety of higher-ordered structure (e.g. micelle and PICsome) depending on the copolymer composition and mixing condition of different types of copolymers. Thus, siRNA may well be structured into various types of higher ordered assembly, whose property may affect the transfection efficacy, upon the complexation with block copolymers. As a mean to observe the

nano-scaled structuring, we utilized light scattering techniques: static light scattering (SLS) for confirming the existence of large assembly and dynamic light scattering (DLS) for the size distribution analysis. Indeed, SLS analysis of PEG-*b*-PLL/siRNA complexes as a function of N/P revealed a distinct assembling behavior which was not known before. That is, PEG-*b*-PLL and siRNA formed higher-ordered structuring at precisely N/P=1.2, while no large assemblies were observed at any other N/P. Furthermore, size distribution analysis of the PEG-*b*-PLL/siRNA complex suggested the formation of gigantic higher-ordered structuring but not the typical PIC micelle, and the complex was so sensitive to ionic strength that it was almost diminished even at 150 mM HCl. Although the relationship between this unprecedented assembling behavior and poor siRNA transfection efficacy of PEG-*b*-PLL has not been clarified yet, we could conclude that PEG-*b*-PLL does not form PIC micelle with siRNA at physiological condition.

It was assumed that the incorporation of siRNA into PIC micelle is more difficult than that of plasmid DNA or oligo DNA, because the length of siRNA is less than one hundredth the length of plasmid DNA and its degree of conformational freedom is likely more restricted than single-stranded oligo DNA. Therefore, refinement of the block copolymer structure was considered to be necessary to support the micellar assembly with short and rigid siRNAs. While increased stability is needed to allow micelle formation, micelle was also required to promptly release siRNA in the cytoplasm of the target cell for recruitment of siRNAs into RNAi. Thus, ideal PIC micelle for siRNA delivery must meet the following rather conflicting requirements: stability of PIC micelles in extracellular media and efficient release of free siRNAs from the carrier in the cytoplasm of target cells following internalization. In order to meet the requirements, PEG-*b*-PLL was reacted with 2-iminothiolane to obtain PEG-*b*-(PLL-IM), with a portion of lysine residues bearing both mercaptopropyl and amidine groups. Thiol groups were utilized to form disulfide cross-links in a core of the micelle to confer environment-responsive stability. The disulfide cross-links are stable under non-reductive physiological conditions helping to maintain micellar structure, while they degraded in reductive conditions following core destabilization. Indeed, the core-shell type PIC micelle with a disulfide cross-linked core was prepared through the assembly of PEG-*b*-(PLL-IM) and siRNAs at a characteristic optimum mixing ratio. The PIC micelles showed spherical shape of approximately 70 nm in diameter with narrow distribution. Micellar structure was successfully maintained at physiological ionic strength, but was feasibly disrupted under reductive conditions due to the cleavage of disulfide cross-links, which is desirable for release of siRNAs in the intracellular reductive environment. In fact, the environment-responsive PIC micelles achieved 100-fold higher siRNA transfection efficiency compared to non-crosslinked PICs prepared using PEG-*b*-PLL, which were not stable at physiological ionic strength. Furthermore, PIC assemblies formed with PEG-*b*-(PLL-IM) at non-optimum ratios failed to show micellar structure and also failed in siRNA transfection. Most importantly, environment-responsive PIC micelles successfully prolonged the retention time of siRNA in blood circulation. These findings show that PIC micelle can be used as carriers for therapeutic siRNA, and that stable micellar structure is a crucial factor for efficient siRNA delivery.

In conclusion, we successfully developed the disulfide cross-like micelle as a new siRNA delivery

system, which was derived from a comprehensive analysis of structure-activity relationships explored so far. The discovery of unprecedented assembling behavior of block copolymers and siRNA would provide a universal basis for all polymer formulation that is relevant to siRNA delivery. With all the incremental improvements that are being made to the development therapeutic siRNA around the world, RNAi-based drug would be available in the very near future.