博士論文（要約）

Control of pDNA packaging structures within polyplex micelle into rod- or toroid- shape to exploit their biological activities

（遺伝子内包高分子ミセルの生理活性向上のためのロッド型およびトロイド型への pDNA 収容構造制御）

李　艶　敏

1. Introduction

Controlling biological macromolecules to possess particular ordered structure is a crucial objective to pursue maximal activity with the aim of performing this functionality in practical applications. Moreover, the knowledge from these studies could provide important information to infer native structures for these macromolecules. pDNA with micrometers in contour length is a fascinating target to challenge this objective with respect of utility as gene-delivery system to establish the intriguing therapeutic concept of gene therapy, because the controlled packaging of the encapsulated pDNA in gene carriers is presumed to correlate with their biological performance. Furthermore, a study that reveals the relationship between the packaging structure of pDNA and its biological activity may further our understanding of the principle of chromatin formation in genome packaging. The packaging of DNA is generally achieved by complexation with cationic compounds to neutralize the negative charge of DNA, in which process DNA subsequently experiences a large volume transition from the expanded coil to approximately 100 nm-sized compact form, known as DNA condensation. Morphological investigations have revealed that pDNA is often condensed into the characteristic structures of rods and donuts (toroids). These structures usually involve multiple DNA molecules and are observed to coexist among multiple DNA associated aggregates. So far, the preparation of each distinct structure from a single molecule of pDNA has not yet been achieved in a selective manner. Therefore, successful demonstration of the selective preparation of each structure would provide tremendous impact to approach the above-mentioned objectives. To challenge this, two problems must be solved: the occurrence of spontaneously formed secondary association to induce aggregation and the regulation of the obscure process of DNA packaging in a controllable manner. To address these problems, we focused on 1) block catiomers comprising an additional neutral hydrophilic poly(ethylene glycol) (PEG) block segment to the DNA condensing cationic segment and 2) using varying ionic strengths to control the condensation process by regulating electrostatic interactions. As demonstrated previously, block catiomers allow a single pDNA molecule to be packaged into the core compartment of spontaneously formed polyplex micelles (PMs), in which the tethering PEG shell compartment inhibits inter-complex secondary associations that form aggregates. NaCl was used to regulate the kinetics of the DNA packaging process by modulating the electrostatic interactions between DNA and block catiomers. Based on these two methodologies, I aimed to control the selection of rod-like or toroidal structures in pDNA packaging and explored the potential biological activities of each structure as a gene delivery system.

2. Selectively packaging pDNA within polyplex micelles into rod shape or toroid shape via varying NaCl concentrations

The block catiomer of PEG-*b*-poly[*N*'-[*N*-(2-aminoethyl)-2-aminoehtyl]aspartamide] [PEG-PAsp(DET)], which has been validated as safe and efficient for use as a gene-delivery carrier, was complexed with pDNA in solutions that both contained identical NaCl in range from 0 mM to 2,000 mM in 10 mM HEPES (pH 7.4) at a fixed N/P ratio of 3. The prepared PMs were observed using TEM to examine the effect of the salt concentration on the packaging structures of pDNA. Selective packaging of pDNA into rod- or toroid-shaped nanostructure within PM was achieved by modulating NaCl concentration for complexation. The specific salt concentration for complexation was identified to be 0 mM NaCl for selective rod formation (95%) and 600 mM NaCl for selective toroid formation (90%). The average rod length increased by raising the NaCl concentration and long rod structures, maybe better described as “strings,” were found at relative high NaCl concentrations (> 1,000 mM). However, the size of the toroidal structures did not show remarkable salt concentration dependence with the average circumference in the range of 100 to 200 nm. The relative invariance of toroidal circumference to NaCl may suggest the energetic favorability of this packing pattern in the toroidal structure. Analysis of the rod/string length or toroid circumference could predict detailed packaging fashion of pDNA within PMs. Following Osada et al (J. Am. Chem. Soc. 2010, 132, 12343-12348) report, the rod structure prepared at 0 mM NaCl with average long axis length of 57 nm describes the rod as a bundle consisting of a pDNA folded 10 times involving 22 double-stranded DNA segments packed in orthogonal cross section. By taking the toroid is formed by circumferential winding around a circle, i.e., spooling, the toroid structure prepared at 600 mM NaCl with average circumference of 175 nm predicted the spooling number to be 6, containing 7 double-stranded DNA segments packed in orthogonal cross section of the spooled toroid. This number is critical to form a hexagon, which implies that DNA chains inherently prefer a hexagonal packing structure as found in genomic DNA packing in bacteriophages.

3. Mechanistic investigation of NaCl effect on structure formation

The impact of NaCl to conduce to distinct packaging structure was elucidated with the aim of understanding the underlying principles for structural development. First, the change of the DNA helical structure caused by NaCl can be excluded with evidence by identical circular dichroism (CD) spectra of naked pDNA ranging of 0

- 2,000 mM NaCl. Hence, focus was placed on the interaction between block catiomers and pDNA as a function of salt concentration. It has been acknowledged that salt markedly affected electrostatic interaction as a result of charge shielding. To affirm this effect, the electrophoretic mobility was measured for naked pDNA as a function of NaCl concentration to reflect the electrostatic "strength" of pDNA. The magnitude of the electrophoretic mobility decreased as the NaCl concentration increased from 0 mM to 600 mM, and followed by a plateau when the NaCl concentration exceeded 600 mM. This profile suggested the electrostatic interaction could play significant contribution up to 600 mM NaCl, while its potency decreasing as NaCl concentration continue to increase. Furthermore, complexation behavior was also examined by studying the number of block catiomers bound to pDNA through the ultracentrifuge method. At 0 mM NaCl, 144 block catiomers bind to each pDNA, which corresponded to a charge ratio of block catiomer to pDNA of 1.07. This result indicated that complex formation was driven by charge neutralization, as supported by the neutral ζ-potential of this PM. The binding number appeared to decrease by raising NaCl concentration, and finally almost no block catiomers bound to pDNA at 2,000 mM NaCl, as consistent with the observation of non-packaged DNA structures at this salt concentration. It is noted that the binding number slightly dropped at 600-700 mM NaCl concentration range, which coincided with the shift of the electrophoretic mobility to plateau. Moreover, the ethidium bromide (EtBr) exclusion assay, often used to measure the degree of DNA condensation, also verified a significant change in the degree of condensation at this NaCl concentration range. Taking all these results into considerations, the salt concentration of 600-700 mM may be regarded as a critical condition for electrostatic-driven complexation. Below 600 mM, electrostatic interaction is presumably the main contributor to the association of polyelectrolytes (pDNA and block catiomers) in which the liberation of counter-ions to gain entropy may drive the complexation. In contrast, at NaCl concentrations above 700 mM, the gain in entropy from the liberation of counter-ions may not be expected to drive complexation due to the presence of abundant ions in the bulk media. Then, enthalpy favorability between the charge-shielded DNA and block catiomers may serve as a primary interaction mode for complexation, even though complexation was not significantly promoted as the binding number was far below the charge stoichiometry. A fine balance of interactive potency between pDNA and block catiomers may intricately commit in the complexation and play a critical role in determining the pathway to develop the most favored structure at each condition, where a NaCl concentration of 0 or 600 mM could be identified as the most favorable condition for the formation of rod-like or toroidal structures, respectively.

4. Kinetics of structure formation process and thermodynamic status of each formed structure

It is important to verify kinetics of structure formation process in presence of each optimized NaCl concentration and their energetic status of formed structures of rod, toroid and string. Packaging process is initiated by polyion complexation followed by compaction. Taking the effect of salt, complexation would proceed faster in absence of salt as compared to presence of salt. Then it is presumed that the rods are formed through faster process relative to toroids and strings. Indeed, polyion complexation behavior monitored by circular dichroism (CD) time course in presence of 0 mM, 600 mM and 1,500 mM NaCl validated the above presumed rate; CD change had completed faster for rods compared to toroids and strings. Given the general tendency of structure formation that slower process yields energetically more favorable structure compared to faster process, it could be regarded that rods are more kinetically favorable structure, in other words, toroids and strings are more thermodynamically favored structures. Indeed, by changing the salt concentration of the formed structure through dialysis, the rod to toroid and toroid to string transitions occurred, but the toroid to rod transition did not occur, while strings did not change their structures. These structural transitions may reflect the energy status of each structure, likely string < toroid < rod in order of increasing free energy.

5. Toroid: Superior biological function

The stepwise dialysis allowed for preparing the rod and toroid structures, which were prepared at each optimized condition, at the identical physiological NaCl concentration (150 mM) with maintaining the frequency and size. This enabled further investigation into the relevance of structures and their functions. Transcription efficiency, examined from a cell-free transcription assay, revealed that the toroid mediated a two-fold increase in transcription activity compared to the rod. Previously, Osada et al (J. Am. Chem. Soc. 2010, 132, 12343-12348) clarified that the double-stranded structure locally dissociates at the rod ends for folding back. Then, DNA double-stranded structure within the toroid is predicted to be intact because there is no sharp folding. This difference may influence the ability of the transcription machinery to traverse DNA strands during transcription. To investigate this scenario, the intactness of double-stranded DNA in packaged structures was examined at 150 mM NaCl using the S1 nuclease, an enzyme that specifically cleaves single-stranded DNA. Interestingly, clear bands corresponding to intact pDNA were observed for the toroid structure but were not observed for the rod structure. This result revealed a difference in status of the double-stranded DNA structure

within the rod and toroid structures. It may be possible to expect the transcription machinery to continuously and infinitely slide along the DNA in the toroid structure, while such motion may be interrupted in the rod structure, probably at the rod ends. To further explore the potency of the toroidal structure, an in vivo gene transfer trial was investigated in skeletal muscle cells via intravenous injection with the aid of a transient tourniquet. In this administration method, it could be anticipated the obtained increased transcription efficiency can result in an improved outcome, as we previously demonstrated; accordingly, the advantageous characters of the toroid structure may readily be elicited in this application. As observed in the in vivo imaging system (IVIS) for luciferase expression, the toroid structure exhibited significantly higher ($p<0.0001$) transgene expression than the rod structure, thus demonstrating the superior function of the toroid structure in vivo.

6. Stabilization of toroid structure by cross-linking for promoted *in vitro* gene expression

Toroid-shaped PM formulated through electrostatic self-assembly of pDNA and block catiomer PEG-PAsp(DET) was validated to exhibit superior biological functions including in vitro transcription efficiency and *in vivo g*ene transduction efficiency compared to the rod-shaped structure. However, there was almost no observable performance in *in vitro t*ransfection and limited cellular uptake. Probably, polyplex micelle was susceptiable to dissociation before entering cell. The stability in extracellular environment and capacity to penetrate cell membrane still remain to be a challenge for systematic application because of enzymatic degradation and non-specific interactions in biological environment. Therefore, a strategy is imperative to keep the stability of PM structure in the extracytoplasmic environment, while inducing effective release of encapsulated pDNA from the packaged formation upon their trafficking into the cytoplasm. A promising approach to achieve this goal is to cross-link the PM with thiol-cleavable cross linker to reinforce PM stability. In this end, water-soluble 3,3'-dithiobis(sulfosuccinimidyl propionate) (DTSSP) might be a favorable candidate because it possesses activated bifunctional N-hydroxysuccinimide esters, which selectively reacts with free primary amine groups, resulting in the formation of stable amide linkages. Moreover, the disulfide bond in its spacer arm is cleavable in intracelluar reductive environment, thus allowing the dissociation of cross-linked PM (CPM) after entering the cell. Upon introduction of DTSSP, the stability of advantageous toroid-shaped PM against counter polyanion exchange was increased substantially. It could be anticipated that the enhanced stabilization of cross-linked toroid structure can result in an improved outcome in *in vitro* gene transfection assay in Hela cells. Indeed, almost no observable *in vitro* transfection was found as for non-CPM system regardless of rod or toroid structure, whereas CPM system at fixed D/N ratio of 0.3 exhibited superior efficiency in *in vitro* transfection. It should be emphasized that the cross-linked toroid structure showed 3-fold higher gene transfection efficiency than cross-linked rod structure at this ratio. The promoted cellular uptake from toroid-shaped CPM was demonstrated, which may contribute to the enhanced *in vitro* transfection efficiency. These promising results encouraged us to explore the appreciable toroid-shaped CPM in further biological applications, such as blood circulation and tumor suppression.

7. Conclusions

Selective packaging of single pDNA molecule into distinct rod-like or toroidal structure was achieved for the first time by modulating the NaCl concentration of polyion complexation at 0 mM or 600 mM, respectively. Stepwise dialysis permitted the retention of these two specific structures within a wide range of NaCl concentrations, which additionally enabled the direct comparison of these structures and their biological functions. Compared to rod structured PM, the toroidal structure was revealed to have remarkably higher transcription efficiency via cell-free transcription assay as well as *in vivo* gene transfer efficacy via delivery of pDNA into intracellular compartment directly. To improve the stability of PM in extracellular environment, thiol-cleavable cross-linker DTSSP was introduced. Consequently, cross-linked toroid PM exhibited significant *in vitro* gene transfection efficiency performed with Hela cells compared to its rod counterpart, expanding the utility of this potent toroid structure in gene therapy via systemic route. Finally, the advantageous toroidal structure, which resembles the viral genomic packaging structure and was specifically formed at NaCl concentration identical to that of seawater, inspire us to further investigation not only to promote gene therapy mediated by this novel virus-like gene-delivery system but also to determine the mechanism of genomic packaging in nature.