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NOTE

Production of small nano-sized particles by complex formation between polycations and linearized plasmid DNA at a low pH

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We report on the technical advance of linearized pDNA ($pDNA_{linear}$) above the circular one ($pDNA_{circ}$) for preparation of small-sized DNA/polycation complexes (DPC) at a low pH. Also, the resistance of the DPC formed with $pDNA_{linear}$ against poly-L-asparagine indicates that effective ion-pairing occurred between the $pDNA_{linear}$ and polycations.

[**Key words:** Compaction; Polycation; Plasmid DNA; Linearized DNA; pH]

A lipid envelope-type lipid nanoparticle (LNP) in which plasmid DNA (pDNA) is encapsulated, is a promising carrier for *in vitro* and *in vivo* gene delivery (1–4). For the effective packaging of the pDNA into a lipid envelope, it is generally compacted via the use of various polycations [i.e., polyethyleneimine (2), poly-L-lysine (3) and protamine (1,4)] as a first process of the preparation of LNPs. Therefore, the physicochemical characteristics of the pDNA/polycation complex (DPC) are a critical factor that determines the feature and function of an LNP.

For the development of a gene delivery system that can be delivered via *i.v.* administration, the sizes and the amount of the cationic components of the pDNA-loaded nanoparticle must be taken into the consideration. As to the sizes, previous studies demonstrated that smaller sized particles (approximately 50 nm) accumulate to hepatocytes more frequently than larger sized particles (approximately 150 nm) (5,6), most likely because smaller particles are able to penetrate through the endothelial fenestrae in liver sinusoids more easily (5,7). Also, recent studies have demonstrated that smaller sized particles (approximately 30 nm) spread more extensively in tumor tissue then larger sized ones (>70 nm) (8). Regarding the amount of the cationic component, high dosing levels of cationic polymer cause apoptosis to the cells (9,10). Furthermore, a previous quantitative comparison of the intracellular trafficking between adenovirus and cationic non-viral vectors revealed that cationic materials can have adverse effects after entering a cell; blocking transcription and translation via electrostatic interactions with the pDNA cargo and mRNA (11,12). These collective data indicate that a technology that permits pDNA to be compacted to the smallest size with a minimum nitrogen/phosphate (N/P) ratio of polycations would be highly desirable as a method for preparing a core particle of the LNP.

In the present study, the emphasis was placed on the topology of pDNA on the size of the particles after compaction with a polycation having a low N/P ratio. In the case of the non-viral approach, the nanoparticle generally carries a circular pDNA ($pDNA_{circ}$), a closed-end molecule that forms a coiled-coil structure. However, double-stranded DNA is recognized as a rigid polymer with a persistent length of ~50 nm (corresponding to the 160–170 bp) (13). Therefore, the bending of the DNA chain is intrinsically limited within the sub-micron range. Taking these points into consideration, we assumed that the poor flexibility of closed-end pDNA would limit the ease of formation of a small particle when compacted with a polycation, especially at a low N/P-ratio, a condition where the driving force toward the condensation is weak. This assumption is also supported by the fact that the huge sized genomes of a large variety of viruses (i.e., adenovirus: ~36 kbps) and phages (i.e., λ-phage: 48 kbps) are naturally in a linearized form, and are well compacted into the capsid with a size of <100 nm without excess cationic charges. In this report, we provide technical information related to the preparation of small pDNA/polycation core particles that contain linearized pDNA ($pDNA_{linear}$).

As a model polycation, we first used protamine sulfate salmon milt (Calbiochem, Ishikari, Japan), a spermatozoal peptide widely used as a DNA condenser (1,4,14). For the preparation of the $pDNA_{linear}$, $pDNA_{circ}$ (5.1 kbps) was cleaved at a single position by the restriction enzyme (Ase I). Complete digestion was confirmed by 1% agarose gel electrophoresis, and the product was then extracted by phenol/chloroform mixture for deproteination. Finally, pDNA was purified from the deproteinized supernatant by ethanol precipitation, followed by washing with 70% ethanol. Previous studies demonstrated that the compaction of pDNA in high-salt solutions (i.e., 150 mM NaCl or PBS) resulted in the formation of relatively large DPC particles (>1 μm) (15). In addition, we

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previously reported that the ξ-potential of the DNA/polycation particles was usually converted from negative to positive when the N/P ratio was increased to more than 1.6–1.8 (3). Therefore, in the present study, the $pDNA_{circ}$ and $pDNA_{linear}$ was compacted with protamine in a low-salt buffer (10 mM HEPES) at N/P-ratio of 0.8, 1.0 and 1.2, where the excess polycation would be expected to be minimal. For the preparation of the pDNA/protamine complex, pDNA solutions (0.1 mg/ml) were first prepared in 10 mM HEPES buffer (pH 7.4). DNA particles at N/P ratio of 0.8, 1.0 and 1.2 were prepared by dropping 125 μl of the protamine solution (0.052, 0.065 and 0.078 mg/ml, respectively) into the 125 μl of DNA solution (0.1 mg/ml) with vortexing. The diameter and ξ-potential of the DPCs were determined using an electrophoretic light-scattering spectrophotometer (Zetasizer; Malvern Instruments Ltd., Malvern, WR, UK).

As a result, the ξ-potentials of the resulting DPC particles prepared with $pDNA_{circ}$ ($_{circ}DPC_{prot}$) and $pDNA_{linear}$ ($_{linear}DPC_{prot}$) were negatively charged (<approximately −30 mV), and were comparable or slightly higher in $_{linear}DPC_{prot}$ (Fig. 1A). Unexpectedly, the $_{linear}DPC_{prot}$ molecules were marginally larger than of the $_{circ}DPC_{prot}$ particles (Fig. 1B). In contrast, when pDNA was compacted in a low pH buffer (10 mM HEPES, pH 5.3), the results were opposite: the size of the $_{linear}DPC_{prot}$ was significantly smaller than that for the $_{circ}DPC_{prot}$, especially when the sample was prepared at a lower N/P ratio (80.9 ± 7.1 nm vs 52.3 ± 5.2 nm at N/P = 0.8; Fig. 1D), while the ξ-potentials were comparable or slightly higher in $_{linear}DPC_{prot}$ (Fig. 1C). The formation of a smaller particle in $_{linear}DPC_{prot}$ was also achieved when pDNA was compacted at a higher charge ratio (N/P = 2.0 or 4.0).

To further address the mechanism for the smaller particle formation in $_{linear}DPC_{prot}$, the decondensation of pDNA from the DPCs was evaluated by agarose gel electrophoresis after treatment with poly-L-asparagine (pAsp) (Fig. 1E). pAsp were applied to the 10 μl of DPC solutions (containing 150 ng pDNA) at indicated concentrations. Loading buffer (2 μl, 50% sucrose in distilled water) was further applied, and then pDNA was analyzed by gel electrophoresis using 1% (w/v) agarose, followed by staining with ethidium bromide. Equivalent amounts of $pDNA_{circ}$ and $pDNA_{linear}$ (150 ng) were also applied to the electrophoresis gel (Fig. 1E, lanes 1 and 6, respectively). In the absence of the pAsp treatment, the migration of pDNA was negligible for both the $_{circ}DPC_{prot}$ and $_{linear}DPC_{prot}$ (Fig. 1E, lanes 2 and 7, respectively), indicating that nearly all of the pDNA was compacted with protamine even under a low N/P ratio. However, significant differences were observed in the decondensation profiles in response to the pAsp treatment; the migration of $pDNA_{circ}$ was clearly observed as the result of treatment with a 1 mg/ml solution of pAsp (Fig. 1E, lane 3), while the $pDNA_{linear}$ only partially migrated, even when the $_{linear}DPC_{prot}$ was treated with a 4 mg/ml solution of pAsp (Fig. 1E, lanes 8–10). It is most plausible that the decondensation occurs when protamine replaces its anionic counterpart from pDNA to pAsp. Therefore, the poor decondensation in $_{linear}DPC_{prot}$ indicates that multivalent ion-pairing between DNA and protamine is more effectively achieved in the case of $pDNA_{linear}$ in comparison with $pDNA_{circ}$. A hypothetical model for this mechanism is illustrated in Fig. 2. Since the $pDNA_{circ}$ spontaneously forms a coiled-coil structure, the molecule has only limited flexibility. Therefore, spatial mismatching of the ion-pairing

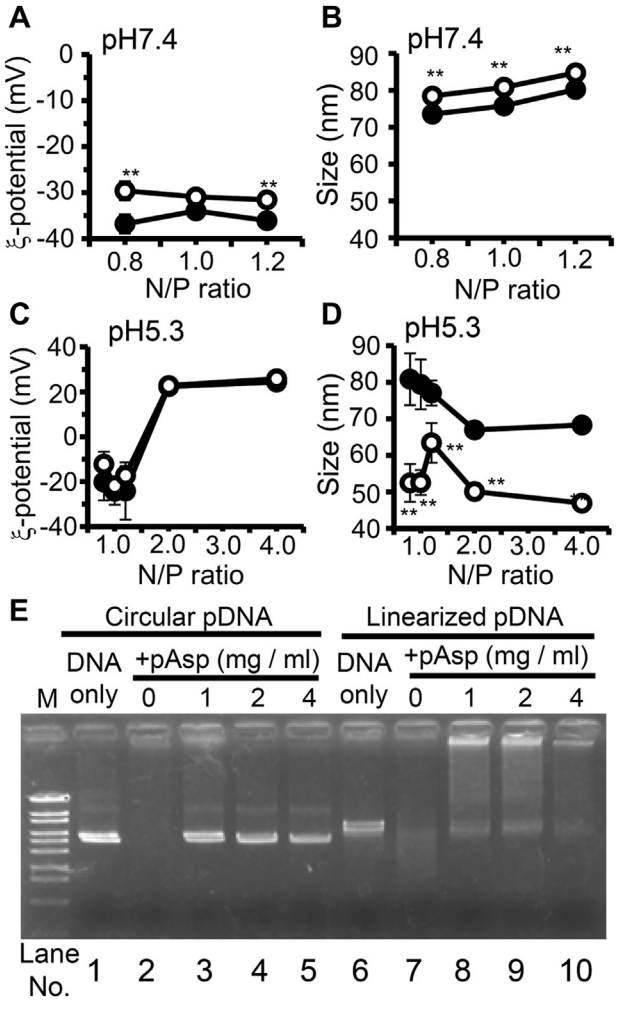

FIG. 1. Physicochemical characters of $_{circ}DPC_{prot}$ and $_{linear}DPC_{prot}$ formed with various N/P ratios. The ξ-potentials (A, C) and sizes (B, D) of $_{circ}DPC_{prot}$ (closed circles) and $_{linear}DPC_{prot}$ (opened circles) prepared in the neutral (A, B) or acidic (C, D) buffer were determined using an electrophoretic light-scattering spectrophotometer. Error bars represent the standard deviations for 3 independent experiments. The asterisks represent a significant difference, determined by one-way analysis of variance (ANOVA), followed by the Student's *t*-test ($^{*}p < 0.05$, $^{**}p < 0.01$) at each N/P ratios. (E) Agarose gel electrophoresis of $_{circ}DPC_{prot}$ and $_{linear}DPC_{prot}$ after the treatment with pAsp. The $_{circ}DPC_{prot}$ (lanes 2–5) and $_{linear}DPC_{prot}$ (lanes 7–10) containing 150 ng pDNA were incubated with or without pAsp for 5 min. Naked pDNAs (150 ng; $pDNA_{circ}$ and $pDNA_{linear}$) were also applied in lanes 1 and 6, respectively. The samples were analyzed by gel electrophoresis thorough 1% (w/v) agarose, followed by staining with ethidium bromide. Lane M, marker (λ-Sty I).

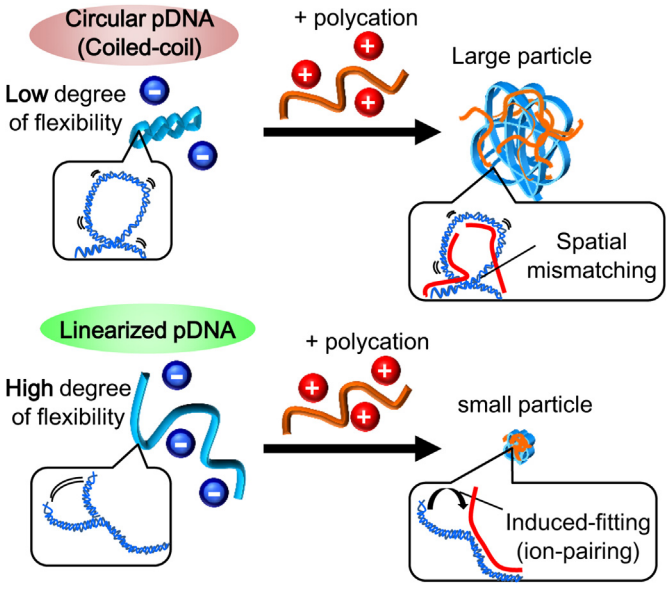

FIG. 2. Schematic diagram illustrating a hypothetical model for the formation of smaller sized DPC molecules using $pDNA_{linear}$.