FOCUS REVIEW

Versatile DNA folding structures organized by cationic block copolymers

Kensuke Osada[1]

Abstract

DNA folding induced by polyion complexion with cationic polyelectrolytes (or catiomers) is attracting remarkable attention in the context of nucleosome formation during genome packaging and gene vector preparation. The application of block catiomers, in contrast to homocatiomers, is attractive because the spontaneously formed polyplex micellar structure can suppress the occurrence of secondary aggregation, allowing the folding of a single DNA molecule. Here, DNA is folded to form several higher-order structures, including rod-shaped, globular, and ring-shaped (toroid) structures. This review discusses the origin of this versatile higher-order structure formation by addressing the conditions and potential mechanisms underlying when and how DNA is organized into these structures upon complexation with block catiomers.

Introduction

DNA organizes multihierarchically ordered structures in the nucleus. This process is initiated by the association of DNA with histones, which ultimately leads to chromosome formation. The dynamic motion of these higher-order structures plays a crucial role in the regulation of DNA functions. To understand this intriguing regulatory mechanism, it is imperative to understand the first step of the higher-order structure formation. The basis of this mechanism is directly attributable to the formation of a polyion complex (PIC) between negatively charged DNA and positively charged histone tails that contain abundant lysine units. Therefore, PICs obtained from plasmid DNA (pDNA) and catiomers, such as poly(L-lysine) (PLys), spermine, spermidine, and cobalt hexamine have often been used as a potential model [1–3]. In addition to this fundamental aspect, the PICs of DNA have been highlighted in the field of nonviral vector development for gene therapy [4, 5]. In principle, PIC formation is driven by an increase in the entropy of the system associated with the release of adsorbed counterions into the bulk solution [6]. Ultimately, DNA undergoes a large volume transition called DNA condensation from extended coil structure to approximately 100 nm compact particles. pDNA forms versatile higher-order structures by complexation with catiomers to form polyplexes such as globular, rod-shaped, or ring-shaped (toroid) structures [3, 7–9]. These structures are observed when pDNA is complexed with an excessive concentration of catiomers. However, polyplexes undergo aggregation when complexation is conducted under a charge stoichiometric condition, hampering extensive investigation into the formation of these structures. At this step, the introduction of a neutral hydrophilic block such as poly(ethylene glycol) (PEG) to construct block or graft catiomers is effective because block catiomers prevent the formation of secondary aggregation even under the charge stoichiometric condition and allow the condensation of a single DNA molecule within a polyplex micelle (PM) [8, 10–13]. Consequently, the distinct higher-order structures are exclusively obtained in PMs. PMs are prepared from a variety of block catiomers, typically consist of PEG, poly(N-(2-hydroxypropyl) methacrylamide) (PHPMA) [10], poly[2-(methacryloyloxy) ethylphosphorylcholine] (PMPC) [13], or poly(2-ethyl-2-oxazoline) (PEtOx) [14] as the representative hydrophilic neutral polymer and PLys [8, 15–19], poly(trimethyl-lammonioethyl methacrylate chloride) (PTMAEM) [10], poly(amidoamine) (PAA) [20], poly(dimethylaminoethyl methacrylate) (PDMAEMA) [13, 21], poly{N-[N-(2-aminoethyl)-2-aminoethyl]aspartamide} [PAsp(DET)] [22–24],

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or polyphosphoramidate (PPA) [25] as the representative catiomer. These PMs ubiquitously form rod-shaped, globular, and toroid structures, suggesting that pDNA tends to undergo condensation through folding, collapsing, and spooling processes, respectively. As these structures typically coexist, it appears that pDNA undergoes any of these processes, while the fraction of these structures varies based on the polymer species. However, the conditions and mechanisms underlying when and how pDNA undergoes this structural polymorphism remain unclear. Therefore, the production and regulation of these structures remain uncontrollable. This review attempts to clarify the conditions, regulatory factors, and mechanisms of the structural versatility of DNA based on our investigations to gain insight into the organization of multihierarchically ordered structures of DNA.

DNA folding in PMs

The typical structures of folded pDNA within PMs are shown in Fig. 1. There are several issues to be considered regarding pDNA folding in PMs. First, let us consider the difference in the size scale between pDNA and block catiomers. The typical pDNAs are several thousand base pairs (bp) in length, several million daltons (Da) in molecular weight (MW), and a few micrometers in contour length. In contrast, a typical block copolymer is several tens of kDa in MW and a few nanometers (nm) in hydrodynamic diameter. Therefore, pDNA is an extremely large molecule relative to a block catiomer. Theoretically, during complexation, when a block catiomer that retains 50 positive charges is applied to a 5000 bp pDNA, 200 block catiomer molecules are required to compensate the negative charges of pDNA. Second, the concentration of pDNA solution must be in a diluted condition to ensure single DNA folding. The micellar formation must be completed prior to the collision of charge-neutralized DNA with its neighbors, as the translational motion causes the secondary aggregation of DNA. It has been demonstrated that aggregation occurs when the complexation is conducted at a pDNA concentration of ≥350 mg/L [26]. This concentration corresponds to the concentration at which the pDNA molecules overlap with each other in solution (indicated as C*). Third, DNA behaves as a semiflexible chain owing to the intrinsic rigidity of its double-stranded structure with a persistence length of 50 nm. This rigidity limits the possible conformation that a DNA molecule can undergo and in turn affects the ultimate structure formation. Finally, it is important to consider the presence of PEG in the PM formation. DNA folding involves a two-step process comprising the association of the block catiomer with pDNA followed by volume transition to produce the compact form. The presence of PEG interferes with the course of the
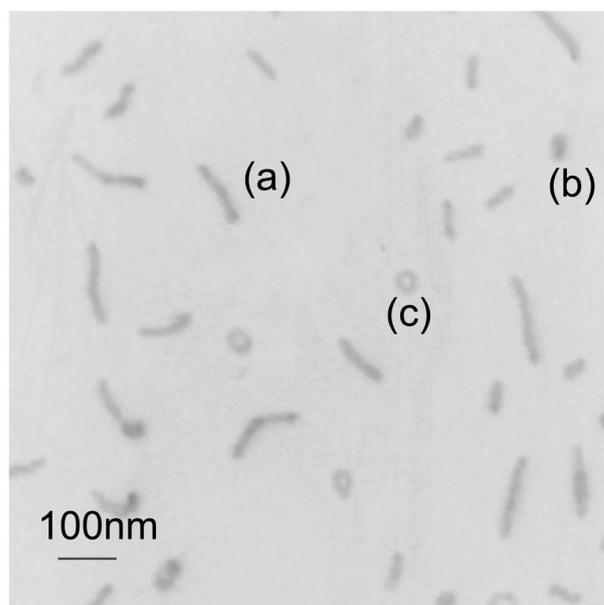
Fig. 1 Versatile structures of pDNA folding within polyplex micelles observed using transmission electron microscopy. a Rod-shaped, b globular, and c toroid structures

second step because the compaction causes the PEG chains to settle within the PM shells. This process reduces the possible conformations that PEG chains can take and thus is unfavorable due to the decrease in the conformational entropy of the PEG chain. Therefore, PEG is assumed to play an inhibitory role during the folding process in addition to its primary role to simply solubilize the polyplex within the aqueous medium. These issues collectively influence pDNA folding and ultimately structure formation.

Rod-shaped and globular structures

Rod-shaped and globular structures are the major structures found in PMs. However, their fraction appears to vary depending on the polymers and the condition of complexation. This issue has been addressed through systematic study based on various PEG-b-PLys/pDNA complexes obtained from PEGs with different MWs and with PLys at multiple degrees of polymerization (DP). These parameters, namely, the MW of PEG and DP of PLys, affect the size and quantity, respectively, of the associated PEG in a polyplex [26] and ultimately alter the fraction of rod-shaped or globular structures and the length of the rod-shaped structures. The structural analysis performed by modulating these parameters has clarified the critical condition for the formation of rod-shaped or globular structures. This condition involves PEG crowding around the pDNA before it undergoes condensation, i.e., a precondensed state. It was observed that the rod-shaped structures were preferentially formed when the tethered PEG chains on a polyplex were

Fig. 2 Critical condition for rod-folding or globular-collapsing. pDNA is folded into rod-shaped structures when poly(ethylene glycol) (PEG) chains on pDNA prior to condensation are sufficiently dense to overlap between neighbors (**a**); pDNA is collapsed into globular structures when PEG chains are not overlapped (**b**). DNA strands, PEG chains, and cationic segments are indicated in red lines, green lines, and blue lines, respectively
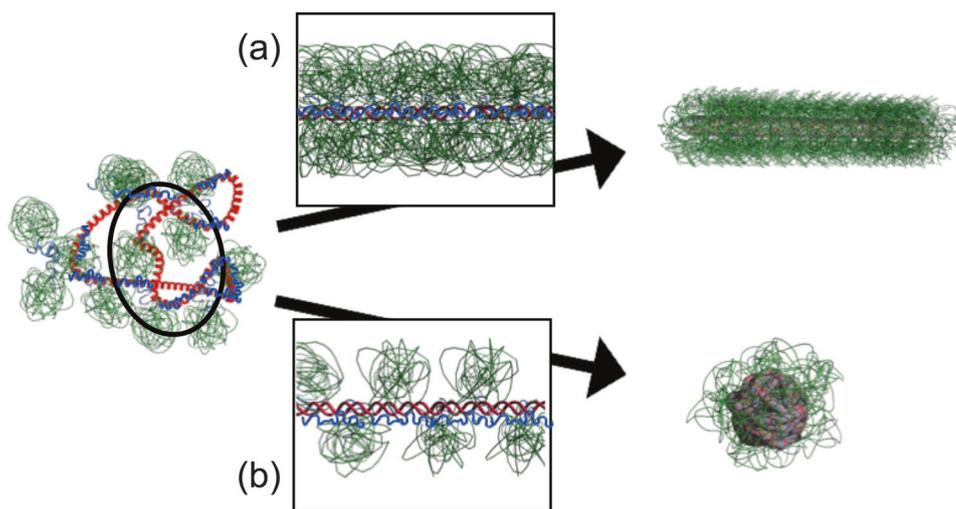
sufficiently dense to allow mutual overlap, while globular structures were preferentially formed when the PEG chains were not overlapped (Fig. 2). These results indicate that PEG interference plays a crucial role in regulating the formation of rod-shaped or globular structures. For example, the critical condition for PEG-*b*-PLys block catiomers with 12 kDa MW PEG was identified as PLys at a DP of 100. The PEG-*b*-PLys with PLys at DP < 100 and >100 produced mainly rod-shaped and globular structures, respectively. Notably, this critical condition is applicable to other PMs. The structural studies regarding PMs obtained using PHPMA$_{18k}$-*b*-PTMAEM$_{170}$ [10], PEG$_{3k}$-*b*-DMAEMA$_{100}$ [21], and PMPC$_{9k}$-*b*-PDMAEMA$_{100}$ indicated the formation of a globular shape (the subscript values in the hydrophilic segment denote the MW in kDa, and those in the cationic segment denote the DP). Note that these block catiomers commonly exhibit a relatively low fraction of hydrophilic segments with respect to the cationic segments. Therefore, the presence of hydrophilic segments in these block catiomers is considered to cause a limited impact on the DNA condensation, thereby enabling pDNA to follow the collapsing pathway rather than the folding pathway.

Regulatory factors of the length of rod-shaped structures

Rod-shaped structures are found in various lengths ranging from approximately 10–100 nm depending on the block catiomers used in the process. Studies performed by altering the fraction of PEG and DP in the cationic segment have indicated a tendency for increased rod length with either an increase in the MW of PEG [19, 26] or a decrease in the DP in the cationic segment [13, 26, 27]. This variation of rod length is attributable to the presence of PEG. In the rod-shaped structures, the polyplex core tends to decrease the

length to minimize the contact area with water. However, the rigidity of the folded DNA bundle resists this tendency. Moreover, the decrease in rod length causes the PEG chains to settle within the decreased volume of the shell, resulting in more crowding of PEG in the shell, and thereby decreases the conformational entropy of the PEG chains. Furthermore, the increase in PEG density increases the osmotic pressure derived from the PEG. Therefore, the presence of PEG suppresses the tendency of the polyplex core to decrease the rod length. Based on these aspects, rod-shaped structures are described in terms of free energies during DNA compaction ($dF_{compaction, DNA} = Gldl - dE_{surface}$) and PEG repulsion ($dF_{anti-compaction, PEG} = \Pi(dV_{occ,PEG}) - T(dS_{conf,PEG})$) [28]. Here, G, l, $E_{surface}$, Π, $V_{occ,PEG}$, T, and $S_{conf,PEG}$ represent the modulus of rigidity of the bundled DNA core, rod length, interfacial free energy developed on the polyplex core, PEG osmotic pressure, number-average occupied volume of PEG, temperature, and conformational entropy of PEG, respectively. This scheme illustrates the variation in the rod length as follows: the increase in PEG steric repulsion obtained by either an increase in the number or size of PEG present in the shell (which is obtained by using PLys at low DP or PEG with high MW, respectively) elongates the rod length. On the other hand, a longer rod length is associated with more unfavorable interfacial free energy than the short-rod length. Therefore, the long-rod length retains elevated PEG crowding to compensate the energy balance than the short rod length. This scheme is consistent with the observed rod length variation and its PEG crowding, e.g., PM prepared from PEG$_{12k}$-PLys$_{70}$ and PEG$_{12k}$-PLys$_{20}$ produces rod-shaped structures with 60–150 nm length as a major fraction and PEG of mushroom conformation and with 100–300 nm length as a major fraction and PEG of upward squeezed conformation, respectively [28]. Overall, the presence of

dense PEG sustains the rod-shaped structures. The rationality of this scheme was further supported by an investigation using block catiomer with acid cleavable acetal linker, PEG_{12k}-acetal-$PLys_{19}$. This study determined that the original rod-shaped structures were ultimately changed to globular structures after the detachment of PEG chains from PMs [29], indicating that PEG sustains the rod-shaped structure. The energy balance scheme consistently explains the variation in rod length observed in the other PM systems. For example, PMs prepared from cocomplexation of PEG-b-PAsp(DET) and homo-PAsp(DET) exhibit a decrease in its rod length by decreasing the PEG fraction [24], PMs containing thermoresponsive polymers such as poly(N-isopropylacrylamide) (PNIPAM) [30] or poly(2-n-propyl-2-oxazoline) (PnPrOx) [14] exhibit a decrease in rod length by elevating the temperature above the lower-critical solution temperatures to decrease the volume, PMs prepared from the triblock copolymer PEG_{12k}-b-$PAsp(DET)_{34}$-b-$PLys_{52}$ exhibit a decrease in rod length after extinguishing the ionic osmotic pressure of PAsp(DET) segment settled in the middle compartment of the PMs by its complexation with negatively charged phthalocyanine-loaded dendrimers [31], and PMs prepared from PEG_{10k}-b-PPA exhibit an alteration in its rod length by the regulation of solvent polarity [25].

Quantized folding of pDNA in PMs

Rod-shaped PMs exhibit an intriguing distribution pattern in length. The rod-shaped structures in PMs formed from PEG_{12k}-b-$PLys_{17}$ and pDNA with 4361 bp (pBR322) present a discrete distribution pattern centered at 610, 305, 190, 150, 115, 100, and 75 nm (Fig. 3). These lengths correspond to 1, 1/2, 1/3, 1/4, 1/5, 1/6, and 1/8 of the 610 nm length. The 610 nm length coincides with the length of a structure formed by the collapse of the circular DNA along the diameter and the contribution of the superhelicity. Based on this observation, a specific folding scheme is proposed, namely, pDNA is folded n-times (f_n) to form a bundle that results in a quantized length of 1/2 ($n + 1$) of the pDNA original contour length [32]. This "quantized folding scheme" is applicable to various PMs irrespective of pDNAs [27] and polymers [14, 27, 33].

Ring-spooling process

Toroid structures, formed from DNA spooling, have gained attention owing to their relevance in the packaging mechanism of viruses [34–37]. Toroid structures are constantly found in PMs irrespective of polymers; however, their fraction typically remains low despite modulating the effect of PEG. To this issue, a selective preparation method was reported to produce toroid structures. This study

indicated that NaCl exhibited a significant effect on the yield of the toroid structures. The PMs prepared from PEG-b-PAsp (DET) predominantly produced rod-shaped structures in the absence of NaCl (95% fraction). In contrast, toroid structures were predominantly (90% fraction) formed when PMs were prepared in the presence of 600 mM NaCl (Fig. 4) [38]. However, the further increase in NaCl concentration resulted in a decrease in the fraction of the toroid structures and an increase in the fraction of the rod-shaped structures. Notably, the 600 mM NaCl concentration coincides with that of seawater. The selective toroid formation was previously reported by performing DNA complexation with low-molecular weight polycations, namely, hexamine cobalt and spermidine [39, 40] and producing a thick toroid structures composed of multiple DNA molecules as a result of the incorporation of surrounding DNA molecules into the preformed ring. In contrast to this toroid, the one in PMs might highlight the significance in its single DNA molecule, as the mechanism of spooling a single DNA molecule packaged within the PEG shell simulates that of the typical structure of virus [34–37].

Double-stranded DNA in rod-shaped, globular, and toroid structures

As previously discussed, pDNA forms various structures by undergoing folding, collapsing, or spooling of its strand. At this point, a basic question arises regarding rod-shaped and globular structure formation. Considering that DNA behaves as a semiflexible chain due to the intrinsic rigidity of the double-helix structure expressed by the persistence length of 50 nm, the sharp flection of DNA at the end of the rod-shaped structures and also the mechanism underlying the formation globular structures with a size less than the persistence length remains unclear. A clue to solving this
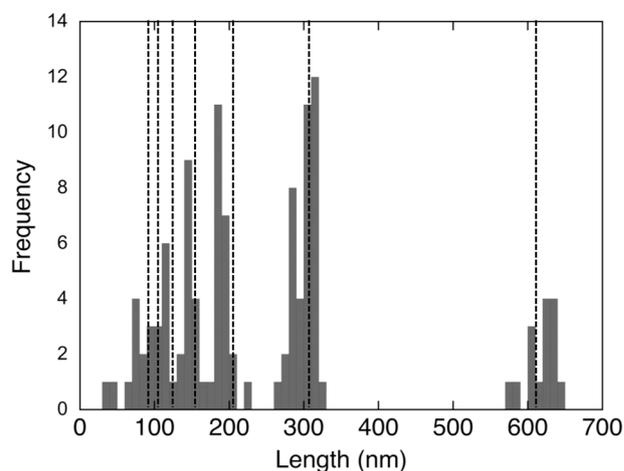
Fig. 3 Discrete rod length distribution of folded pDNA within polyplex micelles prepared from PEG_{12k}-b-$PLys_{17}$ and pBR322

Fig. 4 Selective formation of pDNA into rod-folding (**a**) or toroid-spooling (**b**) structures by conducting complexation in the absence (**a**) or presence (**b**) of 600 mM NaCl
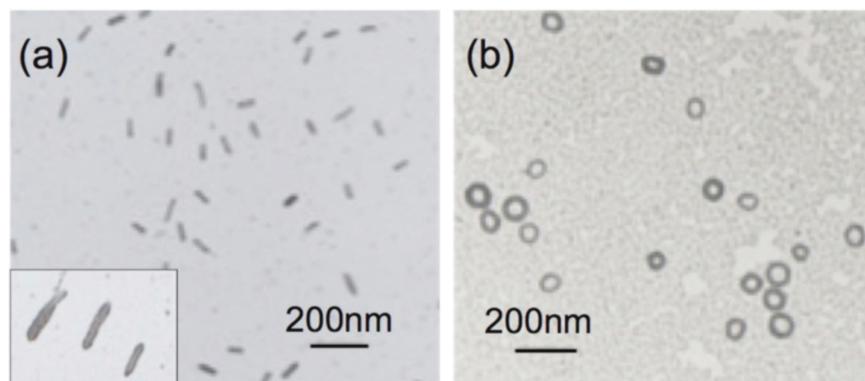
question was obtained through investigations using S1 nuclease, a single-strand DNA specific nuclease. These studies indicated that the rod-shaped PM yielded specific fragments consisting of $1/2m$ multiples of the original pDNA length (m denotes integer numbers) when S1 nuclease was applied [27, 32]. The DNA fragments corresponded to the multiples of rod length observed in TEM. The fragmentation was consistent under the assumption that the double-strand DNA was open at the end of the rod-shaped structures. Notably, this specific fragmentation pattern concomitantly explains the occurrence of a quantized folding scheme of pDNA in the rod-shaped structures. Based on these observations, a folding mechanism was proposed in which DNA is folded by the local dissociation of the double-strand and the flexible nature of the single-strand permits the DNA backfolding. In the case of globular structures, samples treated with S1 nuclease exhibited a smear-like pattern on the gel electropherogram demonstrating that the double-strands were markedly dissociated into the single strands, which indicated that the formation of globular structures was possible owing to the flexibility of the single-stranded DNA [26]. Moreover, the smear-like pattern, instead of complete digestion, indicated that the double-stranded structure partially remained in the globular structures. This remaining double-stranded DNA might represent the exact shape of globular structures that often appears as an irregular shape, distinct from a complete sphere [26]. With respect to the toroid structures, the S1 nuclease did not digest the DNA in toroid structures, indicating that double-stranded DNA is intact in this structure [38].

In general, a rigid chain forms a ring-spooled toroid structures but cannot form rod-shaped and globular structures. In this regard, the toroid formation of pDNA in PMs is reasonable, while the formation of rod-shaped and globular structures is unreasonable. This issue can be rationally explained by recognizing the capability of DNA to alter its stiffness through opening the double-stranded structure, and this capability might be a reason for the production of these versatile structures.

Summary

pDNA forms versatile higher-order structures upon PIC with block catiomers through folding, collapsing, and spooling of its strand to form rod-shaped, globular, and toroid structures, respectively. Mechanistic investigations have revealed that the presence of PEG and interactive potency for PIC formation play essential roles in regulating the formation of these versatile structures. The knowledge of underlying physical mechanisms might ultimately improve our understanding of the formation of hierarchically ordered structures in the nucleosomes and also the operating principles governing how DNA functionality emerges. With respect to the practical aspect, the underlying mechanisms provide a solid strategy to prepare structured nonviral gene vectors, as the structural features, such as the size, PEG crowding, and the folding structure of pDNA, collectively affect the efficiency of delivery processes in addition to gene expression efficiency as described in a previous report [41].

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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