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Tadpole-shaped Gene Carrier with Distinct Phase Segregation in A Ternary Polymeric Micelle†

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Distinct tadpole-shaped nanostructure characterized with spherical head and extended shaft was identified in a single plasmid DNA (pDNA)-based polymeric micelle. The tadpole-shaped structure was constructed by adding anionic chondroitin sulfate (CS) to the rod-shaped polyplex micelle containing single pDNA molecule packaged by PEG-polycation block copolymer through their electrostatic self-assembly. The complex consequently developed a novel structure composing segregated domains of CS-rich inflated head and CS-poor folded DNA tail. Hence, this tadpole structure can be regarded as distinct phase segregation occurred in a single polymeric micelle containing pDNA.

Supramolecular self-assembly has garnered numerous utilities in illustrating structural and functional diversity for fabrication of nanoscale materials [1-3]. The underlying principle in molecular self-assembly is autonomous organization of components, more particularly, subsequence to molecular interactions (e.g. ionic, hydrophobic, hydrogen and coordination bonds), immiscible components in terms of their inherent natures would segregate into distinct compartments to minimize interfacial contacts between incompatible components and ultimately achieve thermodynamically favorable structures [4,5]. Learning from this principle, block copolymers consisting of two distinctive segments, one segment responsible for binding to cargo molecules (e.g. drugs, nucleic acids, imaging agents, etc) to form internal reservoir and the other segment [e.g. poly(ethylene glycol) (PEG)] to formulate external shielding shell, have been extensively used in construction of nanodevices with pharmaceutical and medical interests [6,7]. In particular, DNA loaded nanodevices are of immense interest due to not only the therapeutic utilities as gene delivery carriers but also the structural curiosity of the resulting supramolecular assembly [8]. A single molecule of plasmid DNA (pDNA), subsequence to electrostatic interactions with the polycation segment of PEG-polycation block copolymer, could be compartmented into the dehydrated core to segregate with the hydrated PEG shell to form polyplex micelle. The ultimate structure of the polyplex micelle was clarified to be as a consequence of fine-balance between DNA compacting impetus to reduce the interfacial surface energy of immiscible polycation/DNA interior and the tethered hydrated PEG exterior, and counteracting factor given by the steric repulsion of the tethered PEG crowdedness [9]. In addition, as a result of inherent rigidity of double strand of DNA (dsDNA, whose persistence length approximate 50 nm [10]), the encapsulated pDNA was packaged into rod-shaped structure via regular self-folding DNA strands for alignment as DNA bundle [11]. Recently, in the process of study to improve efficiency and safety of the polyplex micelles as a gene delivery carrier, we discovered an unprecedented structure, observed as tadpole-shape, which was different from the ever-found rod-shaped polyplex micelle. The tadpole-shaped structure was observed in addition of anionic chondroitin sulfate (CS) to a standard rod-shaped polyplex micelle prepared from PEG-poly[N-[N'-(2-aminoethyl)-2-aminoethyl]aspartamide] [PAsp(DET)] block copolymer and pDNA [12]. CS was added to the polyplex micelle solution with aims of neutralizing unbound free polymers to minimize their potential toxic issues [12]. Note that the binding number of PEG-PAsp(DET) follows distinct charge stoichiometric ratio [13], [e.g. 143 PEG-PAsp(DET) chains to pBR322 (4,361 bp)], thus added polymers exceeding this amount readily remain as free polymers in the complex solution. CS is a primary component of cartilage thus appreciably biocompatible, characterized as an unbranched polysaccharide of variable length containing two alternating monosaccharides with carboxyl and sulfonyl groups.

In this study, to highlight the impacts of CS on the structural characteristics of PEG-PAsp(DET)/pDNA polyplex micelle, we attempted to gain the detailed insight on the unique tadpole-shaped structure and explore the possible evolving scenario of structure transformation from rod to tadpole as a result of CS addition. Here, another two polysaccharide-based polyanions, hyaluronic acid (HA) and dextran sulfate (DS) (chemical structures in Fig. 1a) were used to compare with CS in regard to the interaction with the polyplex micelles. Note that HA and DS possess similarity in their structure to CS but variety in their 'strength as a polyanion' in terms of the differences in their functional charged groups. HA containing only carboxylic groups whereas DS containing only sulfonic groups, as opposed to CS possessing alternative carboxylic group and sulfonic group per repeating unit. Note that three possible occurrences could be predicted when the polyanions were added to the PEG-PAsp(DET)/pDNA polyplex micelle solution in view of their various anionic potencies: (i) bind only to free PEG-PAsp(DET),

having no interaction with the PEG-PAsp(DET)/pDNA complexes, (ii) bind to the PEG-PAsp(DET)/pDNA complexes to form a ternary formation, or (iii) bind to the PEG-PAsp(DET)/pDNA complexes followed immediately by releasing of pDNA through polyion exchange reaction.

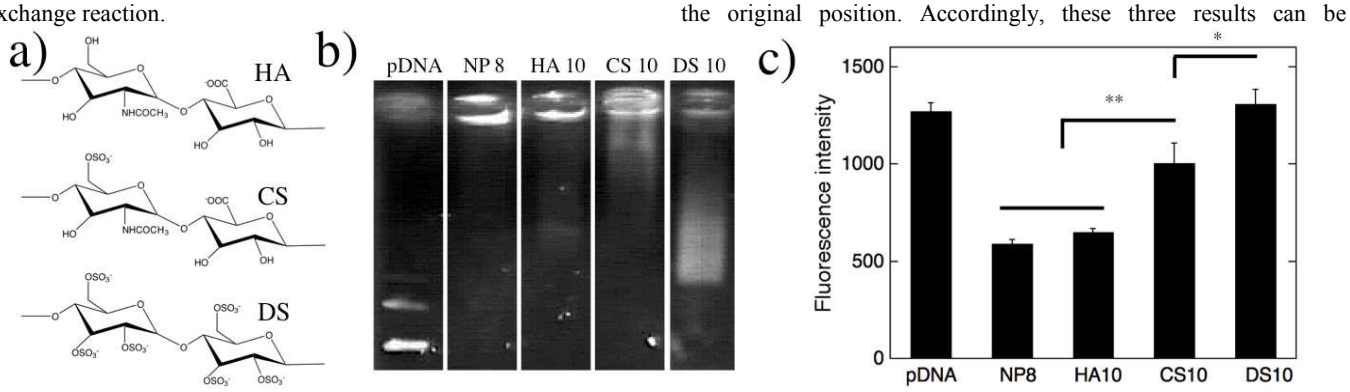

Fig. 1 Effect of diverse anionic polysaccharides on the standard polyplex micelle (prepared at N/P ratio of 8, thus abbreviated as NP 8). a): The chemical structures of a single unit of anionic polysaccharides HA, CS, and DS. b): Gel electrophoresis of PEG-PAsp(DET)/pDNA complex at N/P ratio of 8 with added polyanions at A/P ratio of 10. The samples were abbreviated to follow a form of species + A/P ratio. c): EtBr exclusion assay to evaluate pDNA condensation degree. ($^{*}p < 0.05$, $^{**}p < 0.01$; Student's t-test).

Standard polyplex micelle composing of pBR322 pDNA (4,361 bp) and PEG-PAsp(DET) was prepared at N/P ratio [defined as the molar ratio of amino groups from PEG-PAsp(DET) and phosphate groups in pDNA] of 8. With the aim of neutralizing the unbound free polymers, an excess of CS pertinent to free polymers at A/P ratios (defined as the molar ratio of sulfonic and carboxyl groups in CS to phosphate groups in pDNA) of 10 was added to the complex solution.

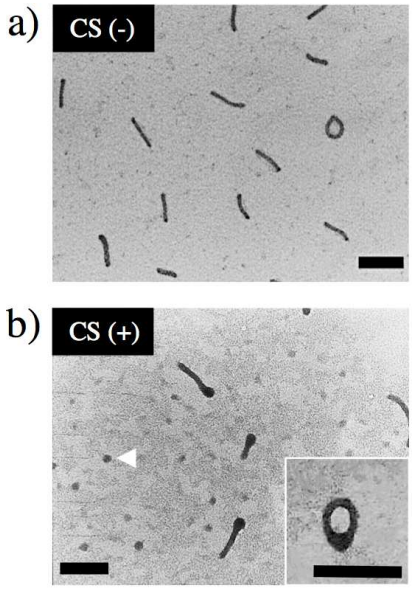

Fig. 2 TEM observation of morphology of the standard PEG-PAsp(DET)/pDNA polyplex micelle and PEG-PAsp(DET)/pDNA/CS tadpole micelle. a): PEG-PAsp(DET)/pDNA polyplex micelle at N/P ratio of 8; and b): PEG-PAsp(DET)/pDNA/CS tadpole micelle at N/P ratio of 8 and A/P ratio of 10. Scale bar: 100 nm.

To start with, gel electrophoresis was carried out in order to obtain general estimation for above predictions. The standard polyplex micelle appeared to be essentially shielded from the electric potential and remained static in the electric field (Fig. 1b). The sample with added HA retained its initial position, similar to the standard polyplex micelle. The DS added sample exhibited to be somewhat smear, suggesting decomplexation behavior of polyplex micelle. In contrast, the CS added sample appeared to slightly extravasate from the original position. Accordingly, these three results can be evaluated as HA having no effect on the complexes, DS initiating dissociation, and CS inducing some kind of intermediate effect.

An ethidium bromide (EtBr) exclusion assay was further conducted with the intention of quantifying the electrophoretic data. The EtBr assay (Fig. 1c) directly supported the gel electrophoresis data that HA and DS resembled the standard complex and naked pDNA, respectively whereas CS presented an intermediate effect. Considering both the gel electrophoresis and EtBr data, it can be conjectured that HA yielded negligible interactions with PEG-PAsp(DET)/pDNA complexes, DS prompted strong interactions with PEG-PAsp(DET)/pDNA complexes resulting in dissociation of complexes, yet CS not only interacted with PEG-PAsp(DET)/pDNA complexes but also might induce some change of polyplex micelles rather than entire dissociation. Note that PEG possessed mushroom conformation not tethered as squeezed or scalable brush conformation as analysed in a previous study [9], thus the translocation of CS through the PEG shell to interact with polyplex core may be possible to occur. Nevertheless, the characterization of this intermediate effect from CS addition was difficult to describe prior to direct structural observation.

In this regard, the morphology of CS-added sample was characterized by transmission electron microscopy (TEM) measurement. Note that pDNA in polyplex micelles were selectively observed in the TEM images owing to stronger affinity of uranyl acetate (staining agent) to DNA than PEG. Our previous studies have explicitly elucidated the detailed structure of the standard polyplex micelle, where pDNA was regularly folded into rod-like DNA bundle [Fig. 2a] beneath the PEG shell [9]. Unprecedentedly, TEM observation revealed the CS-added sample was observed to form a novel structure [Fig. 2b]. This novel structure is characterized with a round sphere tied with a long shaft in high frequency (approximate 95%). In view of this distinctive spherical head (average 18.1 $\pm$ 2.1 nm in diameter) and long tail (average 80.2 $\pm$ 19.3 nm in rod length), thus it can be best described as a 'tadpole micelle' [Fig. 2b, CS(+)]. In view of this distinctive spherical head and long tail, thus it can be best described as a 'tadpole micelle' [Fig. 2b, CS(+)]. In addition, TEM images were also taken of samples added with HA and DS (Fig. S1), which confirmed that the tadpole structure was indeed unique to CS, where HA-added sample appeared to preserve the rod-shaped structure as the standard

polyplex micelle and decondensed structure for DS-added sample, as consistently suggested by gel electrophoresis and EtBr assay. The unique formation of tadpole-shaped micelle only by CS addition is presumably a result of a moderate anionic strength of CS (CS possessing alternative carboxyl and sulfate groups in contrast to HA possessing only carboxyl groups and DS possessing only sulfate groups). Referring to the fact that dextran sulfate (DS) possessing higher sulfated degree to CS is a widely used agent to evaluate stabilities of polyplex systems against dissociation in terms of polyion exchange reaction, it is possible that the additive charged CS component could interact with the standard rod-shaped polyplex micelle to develop a ternary polyplex micelle. Most likely, anionic CS could interact with the core of the standard polyplex micelle due to the slight positive-charge of the standard polyplex micelle as evidenced in zeta-potential measurement (Table S1), through translocation of PEG shell characterized to have mushroom conformation [13]. This process may be driven by entropy gain from releasing counter ions by electrostatic complexation. This speculation is consistent with the observation that the zeta potential shifted from slight positive to slight negative by addition of CS to the standard polyplex micelles (Table S1). Note that aside from presence of tadpole structures, some uniform-sized spherical structures, not present in the solutions of the standard polyplex micelle, were observed in CS-added sample (indicated by arrow head in Fig. 2b). These structures were most probably the result of the added polyanions binding to free PEG-PAsp(DET) polymers. Indeed, TEM morphology for the sample of CS/PEG-PAsp(DET) complex revealed similar spherical structures resembling the background spherical structures in CS-added sample (with comparable size) (Fig. S1).

Nevertheless, this result alone cannot exclude a possibility of the obtained high S/P ratio in the head of tadpole was formed by simply attaching the spherical complex of CS/PEG-PAsp(DET) to the end of the standard rod micelle. However, if this is the case, we could anticipate occurrence of double-headed tadpole (a rod-shaped shaft tied with spheres at its both ends) and tadpole-shaped micelle also for HA-added sample. In fact, two-headed micelles did exist in CS-added sample (Fig. S2), however, they were extremely rare: only two double-headed tadpole micelles have been observed out of the hundreds of complexes that have been imaged. Moreover, no tadpole micelle was found in HA-added sample, though the spherical structures were found in the background probably as a result of the interaction between HA and free PEG-PAsp(DET) (Fig. S1). Based on these results, it may be reasonable to consider that this tadpole formation was developed from direct association of CS with the pDNA/PEG-PAsp(DET), unlike the preferential binding of spherical CS/PEG-PAsp(DET) complex to the standard polyplex micelle. Presumably, the associations of CS to the pDNA/PEG-PAsp(DET) complex core may initially occur at random, but this would create numerous energetically unfavorable boundaries between pDNA/PEG-PAsp(DET) and pDNA/PEG-PAsp(DET)/CS phases. To reduce unfavorable interface of these domains, rearrangement of CS may subsequently occur for attaining the thermodynamically more favorable structure, consequently may lead to the initially distributed domains accumulating into two-separated phases, namely phase segregation into two distinct phases in single nanoparticle [14]. Furthermore, the separated CS accumulation would be preferably located in one single rod end rather than both rod ends with respect to minimize interfacial free energy, accordingly giving rise to vast majority of single-headed tadpole micelle structures. It is noteworthy

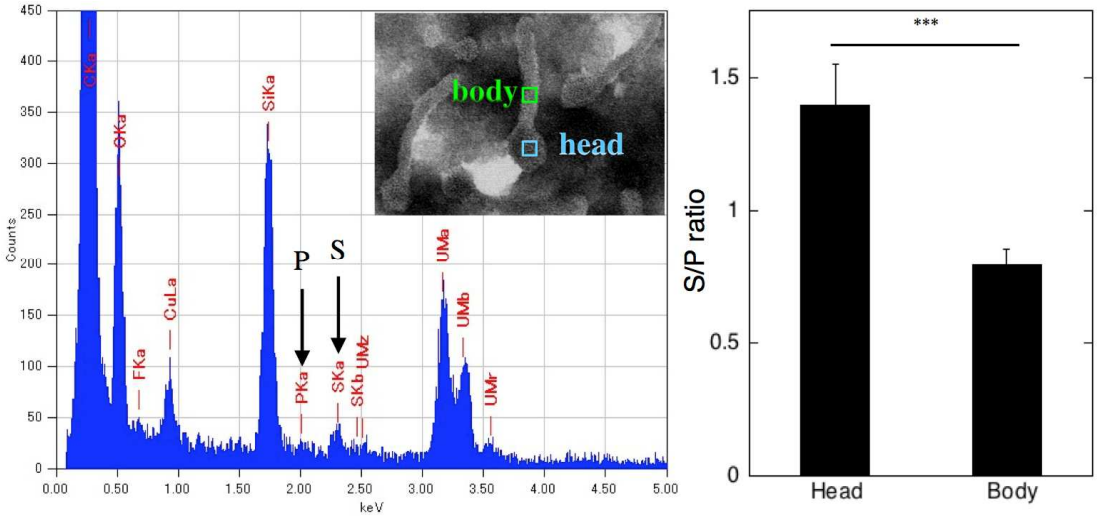

Fig. 3 Distributions of chondroitin sulfate in tadpole polyplex micelle in terms of quantification of S/P ratio by EDS. a): a representative EDS spectrum to characterize element quantification in the head of tadpole. b): The quantified S/P ratio in the head and body region of the tadpole micelle, where a total of 8 tadpole was used in this quantitative investigation. ($^{***}p < 0.001$; Student's t-test).

To directly confirm the possibility of CS integration into polyplex micelle, energy-dispersive X-ray spectroscopy (EDS) equipped in TEM [JEM-2100F (JOEL)] was utilized in order to determine the presence and the distribution of CS in this tadpole polyplex micelle relative to the packaged pDNA via a signal intensity ratio of sulfur to phosphorous (S/P). Note that EDS allows direct element quantification by means of the characteristic X-ray emission for targeted element from sample exposure to an electron beam. To our interests, the resulting S/P ratios showed significantly higher for the head domain than the tail of the tadpole micelle (Fig. 3), suggesting that CS is preferentially localized at the head of the tadpole micelle.

that this tadpole-shaped structure was observed to preserve even with extended incubation for 72 h (Fig. S3), thus it is consistent to assume the observed tadpole-shaped structure had already reached a thermodynamic favorable structure. Moreover, there exists a small fraction of toroid-shaped structures in the standard polyplex micelle aside from rod-shaped structures, and we subsequently observed these toroid structure developed into possessing a single inflated domain in the toroid structures after CS addition (inset in Fig. 2b). This observation additionally suggested that tadpole micelle was not formed by preferential binding of spherical CS/PEG-PAsp(DET)

complex to the standard polyplex micelle, but consequence of segregation of CS phase into distinct area.

In light of CS preferentially concentrated at one end of the rod, significant ionic osmotic pressure could be readily developed at this CS-localized end and give rise to inflated formation as swollen state of the tadpole head. Considering dsDNA at rod end was locally dissociated into ssDNA for folding [6], it is reasonable to assume further unzipping of dsDNA into ssDNA in the tadpole head as a result of the ionic osmotic swelling. Following this process, the quantity of ssDNA should be increased in the tadpole-shaped sample as compared to the standard polyplex micelle. To examine this assumption, a fluorescence dye SYBR green II, generating significant higher quantum yield to ssDNA (0.54) than dsDNA (0.36), was employed as an indicator for estimation of ssDNA evolution. Indeed, higher fluorescence intensity was observed for CS-added sample than standard polyplex micelles, suggesting further dissociation into ssDNA in the tadpole structure. This is consistent with our assumption and implied the possibility of unzipping dsDNA into ssDNA as a consequence of osmotic swelling in the CS-accumulated end (Fig. 4). The above investigations implied the overall scenario of tadpole-shaped polyplex micelle formation: charged CS initially bound to PEG-PAsp(DET)/pDNA polyplex micelle at random but concomitantly elicited numerous boundary between pDNA/PEG-PAsp(DET) and pDNA/PEG-PAsp(DET)/CS phases. To minimize these unfavorable interfaces, CS tended to translocate and accumulate into a distinct area (one end of the rod). Then, substantial osmotic pressure was developed due to this CS localization, which consequently led to inflation of CS-localized rod end as tadpole head and unzipping of dsDNA to evolve ssDNA in this folding end. To this end, the novel tadpole-shaped structure can be understood by assuming the scheme of phase segregation, which could be regarded as phase separation occurring in a single pDNA molecule, with the two distinct phases existing simultaneously of swollen phase as tadpole head, and compacted DNA bundle as tadpole tail.

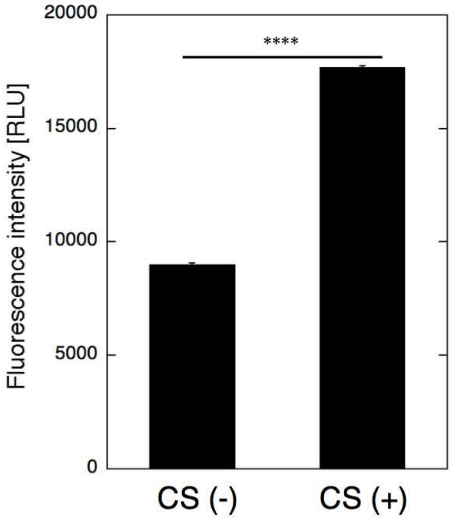

Fig. 4 Estimation of ssDNA in the standard polyplex micelle [CS (-)] and CS added tadpole sample [CS (+)] by SYBR green II assay. ($^{****}p < 0.0001$; Student's t-test).

In conclusion, CS, as opposed to the other polyanions with similar structures, is unique to provide structural change on the PEG-PAsp(DET)/pDNA polyplex micelle system, prompting a transition from a standard rod-shaped structure into a novel tadpole-shape structure. This was a stark contrast to DS inducing complete dissociation and HA limited to no effect. Noteworthy was that the added CS is preferred to be localized at one end of the rod to form the head of the tadpole resulting in a distinct phase boundary between the head and tail of the complex, which is most likely to be the result of an attempt at minimizing interfacial free energy by reducing the unfavorable interface area. Eventually, the tadpole-shaped polyplex micelle with distinct two-phase domains was explicitly characterized, implying coexistence of both CS-rich swollen head region and CS-poor DNA packed bundle body region.

Of note, the proposed tadpole-shaped system has not only addressed the toxic issues, e.g. substantially reducing *in vitro* cytotoxicity, *in vivo* tissue damage, and inflammatory response, by virtue of using CS to eliminate unbound free polycations (potentially provoke toxic outcome due to readily interaction with charged biological components and structures) by complexation, but also validated high *in vivo* efficiency for intratracheal gene transfection [12]. These results suggest its advantage in utility as a safe and efficient gene delivery carrier. Moreover, this novel tadpole-shaped structure appeals to the reminiscence of structure of bacteriophages, M13, characterized as rod-shaped structure where functional residues mediating cell infection (gene 3 protein) was specifically accumulating at one end of the rod structure [15]. Then, the unique structure of the tadpole-shaped polyplex micelles with the concentrated CS at the tadpole head may allow for directing cells expressing CS receptors such as cells of central nerve system including neurons [16]. In this respect, our identified tadpole-shaped gene carrier should endow notable implications in mimicking natural viral vectors by conjugation of appropriate functional moieties onto the head to ignite evolution of standard polyplex micelles to active structural-intelligent delivery carriers.

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Notes and references

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Tadpole-shaped nanostructure characterized as phase segregation occurred in a single pDNA-containing polymeric micelle.