Electronic Supplementary Material (ESI) for Soft Matter.

Supporting Information

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

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Materials and methods

Materials

α-Methoxy-ω-amino-poly(ethylene glycol) (M_w 12,000) was purchased from Nippon Oil and Fats Co., Ltd. (Tokyo, Japan). *β*-Benzyl-L-aspartate *N*-carboxyanhydride (BLA-NCA) was purchased from Chuo Kaseihin Co., Inc. (Tokyo, Japan). Diethylenetriamine (DET), *N*, *N*-dimethylformamide (DMF), n-butylamine, dichloromethane, benzene, and trifluoroacetic acid were obtained from Wako Pure Chemical Industries, Ltd (Osaka, Japan). The pDNA pBR322 (4,361 bp) was purchased from Takara Bio Inc. (Otsu, Japan). Chondroitin sulfate sodium (CS), dextran sulfate sodium (DS), and hyaluronic acid (HA) were purchased from Sigma-Aldrich (St. Louis, MO). SYBR Green II was purchased from Life Technologies (Carlsbad, CA).

Polymer Synthesis

Block copolymer PEG-PAsp(DET) was synthesized according to a ring-opening polymerization scheme as previously reported [S1]. In brief, the polymerization of monomer BLA-NCA was initiated from the ω-NH_2 terminal group of α-methoxy-ω-amino-PEG (M_n 12,000) to yield PEG-PBLA, followed by aminolysis reaction to introduce diethylenetriamine into the side chain of PBLA. The prepared block polymer was determined to have a narrow unimodal molecular weight distribution (M_w/M_n = 1.05) according to measurement from gel permeation chromatography. The polymerization degree of PAsp(DET) segment was determined to be 61 from the peak intensity ratio of the methylene protons in PEG (-OCH_2CH_2-, δ = 3.7 ppm) to the methylene groups in the bis-ethylamine of PAsp(DET) [-NH_2(CH_2)$_2$NH(CH_2)$_2$NH-, δ = 3.1 - 3.5 ppm] located in the side chain in the ^{1}H-NMR spectrum in D_2O at 25 °C, thus the synthetic PAsp(DET) segment was calculated to have a M_n of 16.7 kDa. The block copolymer PEG-PAsp(DET) has a total M_n of 28.7 kDa.

Preparation of Standard Polyplex Micelle:

PEG-PAsp(DET) powder stock was dissolved in 10 mM HEPES buffer (pH 7.4) at a concentration of 5 mg/mL as a stock solution, which could be frozen for future use. An aliquot of stock solution was taken and diluted to whatever

appropriate concentration to produce the correct volume of polyplex called for by the subsequent experiments. The polymer solution was then added to, via one-shot mixing as to reduce equilibration time, and vortexed with pDNA solution dissolved in 10 mM pH 7.4 HEPES buffer (50 μg/mL) achieving an N/P ratio of 8, the ratio utilized for all following experiments. The solution was then incubated for 24 h at 4 °C with aim of complete complexation and the final concentration was adjusted to 33.3 μg/mL. It should be noted that all steps were carried out at relative low temperature as possible, either at 4 °C refrigerator or an ice bath, in view of the degradable nature of PEG-PAsp(DET) at room temperature.

Preparation of Polyanion Containing Polyplex Micelle:

The primary focus of this research is to investigate the physiochemical changes that occur to the PEG-PAsp(DET)/pDNA micelle upon the addition of chondroitin sulfate to the polyplex micelle solution. However, the results must be confirmed to be unique to chondroitin sulfate and not a general effect standard to all polyanions, thus dextran sulfate and hyaluronic acid were also utilized. The chemical structures of three polyanions are shown in Fig. 1a. PEG-PAsp(DET)/pDNA micelles at N/P ratio of 8 were created by the method described above. Furthermore, the desired polyanion, chondroitin sulfate, dextran sulfate, or hyaluronic acid, which was added to the polyplex micelle solution, followed by vortexing. The polyanion was added to create A/P ratio of 10 that were utilized for all subsequent experiments. A/P ratio, similar to N/P ratio, is the molar ratio between the total sulfonic acid and carboxyl acid from anionic species to the phosphate group from pDNA. After vortexing, the samples were incubated for 24 h at 4 °C.

Gel electrophoresis:

The reaction of the polyplex micelle with differing anionic species was estimated from the release of pDNA from the polyplex micelle. Aliquot of differing anionic species (e.g. CS, HA and DS) at charge ratio A/P of 10 was added to the standard polyplex micelle solution for overnight incubation at 4 °C. Each sample solution containing 167 ng of pDNA was electrophoresed through a 0.9 wt% agarose gel in a running buffer of (3.3 mM Tris-acetic acid + 1.7 mM sodium acetate + 1 mM EDTA2Na, pH 7.4). The migration of pDNA in the gel were visualized by soaking the gel into distilled with containing EtBr (0.5 mg/L).

Ethidium Bromide Exclusion Assay:

The impact of the polyanion addition on the degree of pDNA condensation of original PEG-PAsp(DET)/pDNA micelle was estimated from the reduction in fluorescence intensity of ethidium bromide (EtBr) due to the exclusion from DNA. Polyplex micelle solutions (33.3 μg of pDNA/m) prepared at N/P ratio of 8 and allowed to incubate for 1h at 4 °C. EtBr was then added at a ratio of 1.66 μ of 100 μg/mL EtBr per 10μl 50 ng/μl pDNA. Finally, the desired polyion was added at A/P ratio of 10. The solutions were incubated at ambient temperature overnight. Fluorescence measurement of sample solution was carried out using NanoDrop (ND-3300, Wilmington, NC), and each measurement was repeated three times. Excitation (Ex) and emission (Em) wavelengths were 355 and 590 nm, respectively. The obtained results were expressed as relative fluorescence intensity. The fluorescence of pDNA solution with EtBr was set at 100%, and the measurement was conduct against a background of EtBr without pDNA.

Transmission Electron Microscopy (TEM):

The morphology of polyplex micelles was directly observed by transmission electron microscopy (TEM). Micelles were prepared as aforementioned, and then the pDNA within polyplex micelles was stained by uranyl acetate (10 μL of a 2% w/v solution) for 30 s. A carbon-coated 400 mesh Cu grid (Nisshin EM), which was subjected to prior hydrophilizing treatment, was then immersed into the micelle/uranyl acetate mixture, allowing both sides of the grid fairly saturated. Furthermore, the grid was taken for air-drying on a piece of filter paper and then transferred to an H-7000 TEM (Hitachi Ltd, Tokyo, Japan) for observation. Note that TEM observation was taken at an optical magnification 50,000 or 80,000 times with an acceleration voltage of 75 kV.

Energy Dispersive X-ray Spectroscopy:

Energy Dispersive X-ray Spectroscopy (EDS) was utilized in conjunction with STEM in order to identify the location of CS in the tadpole micelles. Given that when electromagnetic radiation strikes an atom to release an electron, and in order to compensate for this, an electron from a higher orbital, thus of higher energy, drops down to fill the gap, the electron releases energy in the form of an X-ray that is characteristic of that element.

SYBR Green II assay:

Solution containing SYBR Green II was added to the standard polyplex micelle or the CS-added complex solution for reaction, where the concentration of pDNA was adjusted 20 ng/μl and the concentration of SYBR Green II was adjusted to 1/10,000 relative to the purchased concentration. After overnight incubation in dark, quantification of ssDNA was conducted by measuring fluorescence intensity of each sample at Ex/Em of 492/520.

TEM observation:

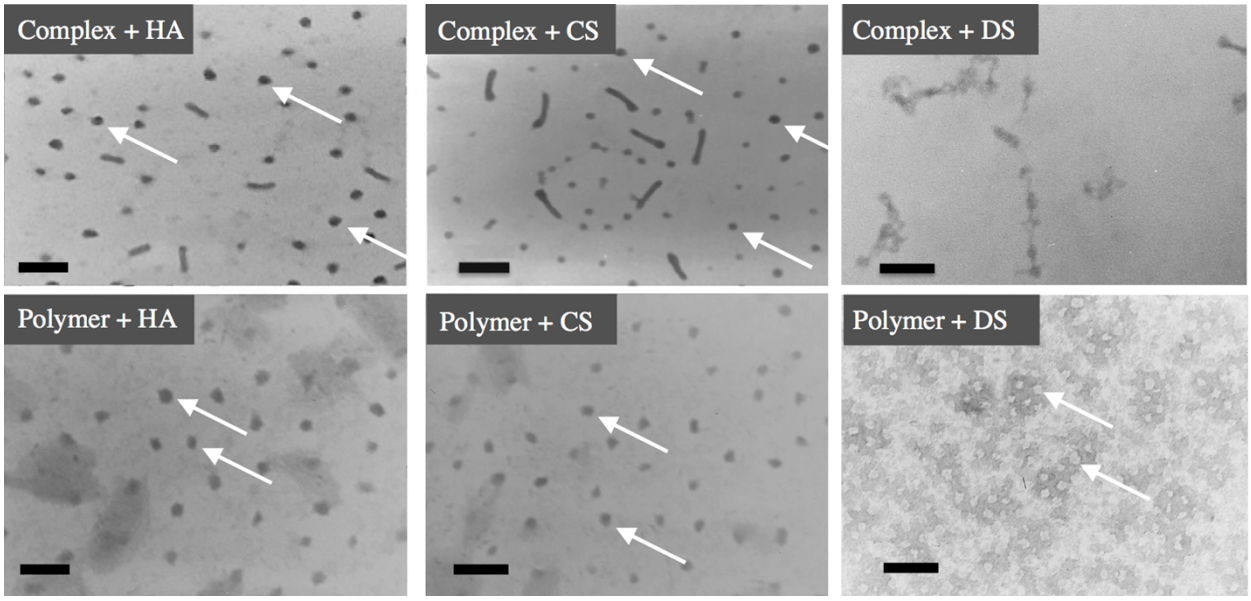

Fig. S1 TEM images demonstrating uniqueness of CS when compared to other similar polyanions. Top Left: PEG-PAsp(DET)/pDNA complexes with HA prepared at N/P = 8 and A/P = 10. Bottom Left: TEM image of HA/PEG-PAsp(DET) complexes. Top middle: PEG-PAsp(DET)/pDNA complexes with CS prepared at N/P = 8 and A/P = 10. Bottom Middle: TEM image of CS/PEG-PAsp(DET) complexes. Top Right: PEG-PAsp(DET)/pDNA complexes with DS prepared at N/P = 8 and A/P = 10. Bottom Right: TEM image of DS/PEG-PAsp(DET) complexes. Scale bar: 100 nm. Spherical structures (denoted by arrows) are observed for HA and CS added samples (upper left and middle images). Since similar spherical structures are observed for the complexes prepared from PEG-PAsp(DET) and polyanions such as HA, CS, and DS (bottom images)), these spherical structures found in HA or CS added PAsp(DET)/pDNA complexes may be as a result of complexation of excess free PEG-PAsp(DET) and polyanions. Note that DS might cause decondensation of pDNA since no typical rod-shaped polyplex micelle structures were found.

The structures in all TEM images were observed as positive staining expect for the bottom right image observed as negative staining.

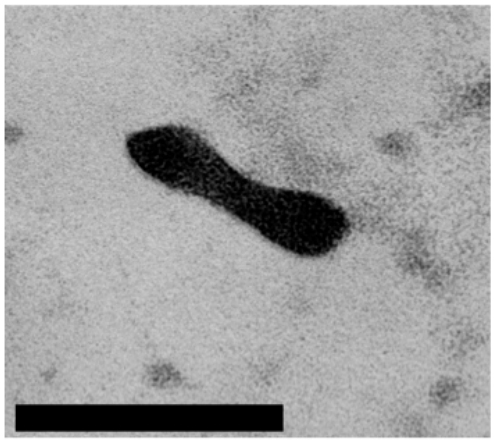

Fig. S2 Occurrence of two-headed tadpole micelle in TEM images from PEG-PAsp(DET)/pDNA/CS system. Scale bar: 100 nm.

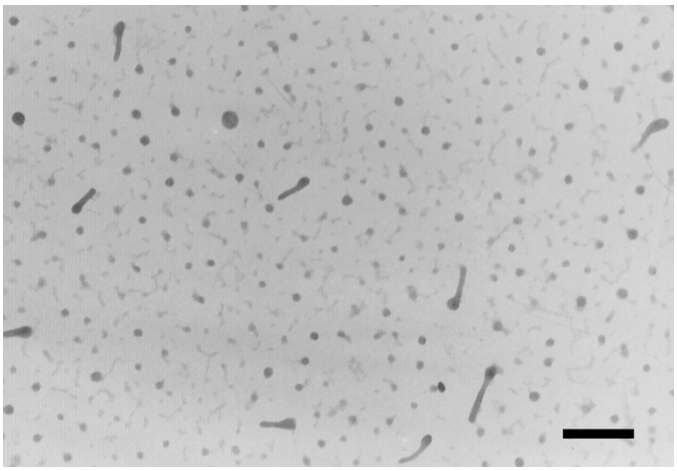

Fig. S3 TEM observation of samples with CS addition for extended incubation of 72 h, scale bar: 100 nm.

Zeta potential measurement:

Table S1. Zeta potential of standard polyplex micelle and polyplex micelle with CS addition.

Samples	CS (-)	CS (+)
Zeta-potential	+4.3 mV	-3.9 mV

1 N. Kanayama, S. Fukushima, N. Nishiyama, K. Itaka, W. Jang, K. Miyata, K. Miyata, Y. Yamasaki, U. Chung, K. Kataoka, A PEG-based biocompatible block catiomer with high buffering capacity for the construction of polyplex micelles showing efficient gene transfer toward primary cells. *Chem. Med. Chem.* 2006, **1**, 439–444.