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Effects of the nanostructure of dendrimer/DNA complexes on their endocytosis and gene expression

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ABSTRACT

Cationic dendrimers constitute a potential nonviral vector for gene therapy due to their ability of forming electrostatic complexes with DNA (dendriplexes). However, the supramolecular structure of dendriplexes and its impact on the cellular uptake and gene transfection remain largely unknown. Using synchrotron small angle X-ray scattering, here we show that DNA in complexes with poly(amidoamine) (PAMAM) G4 dendrimers exhibited three distinct packaging states modulated by the degree of their protonation (dp). Our structure characterization suggests that the nanostructure of DNA in dendriplexes transformed from square-packed straightened chains (dp/0.1) to hexagonally-packed superhelices (dp/0.3) and eventually to a beads-on-string configuration (dp/0.6 and dp/0.9). The transfection efficiency in HT1080 cells significantly enhanced when the dp value was increased from 0.1 to 0.3. This enhancement was due to a higher positive surface charge of dendriplexes formed at higher dp, which facilitated adherence of test dendriplexes to the negatively charged cell membranes for the subsequent endocytosis. Although the surface charge of dendriplexes still increased accordingly, further increase of the dendrimer dp value to 0.9 reduced the transfection efficiency. This unexpected suppression of transfection may be attributed to the wrapping of DNA around dendrimers that frustrates the interaction between dendrimer and cholesterol in the membrane raft via the caveola-mediated endocytosis. These results can be used for the rational design of dendrimer-based gene delivery devices.

1. Introduction

Numerous materials have been studied as potential vectors for gene delivery with varying results [1]. Dendrimers constitute a unique class of hyperbranched macromolecules composed of layers of monomer units radiating from a central core; each complete grafting cycle is called a generation [2,3]. Although dendrimers are less efficient than viral vectors, they have the potential for use in gene therapy and other therapeutic applications due to their safety and lack of immunogenicity [4]. Because of its relatively high transfection efficiency, polyamidoamine (PAMAM) is the most commonly used dendrimers for gene delivery [5]. The amine groups of PAMAM dendrimers can be positively charged through proton transfer in acidic aqueous media. PAMAM dendrimers and DNA form complexes via electrostatic interactions between their protonated amine groups and the negatively charged phosphate groups of nucleic acids [6].

Dendrimers are reported to be internalized into cells by endocytosis [7]. The surface charge of dendrimers may influence their cellular uptake and subsequent transfection efficiency [8]. In this study, we prepared different degrees of protonation (dp, i.e., the number fraction of protonated amine groups) of PAMAM dendrimers and then complex with DNA to form dendriplexes in distinct nanostructures. Understanding the nanostructures of dendriplexes and their effects on the endocytosis and transfection efficiency is essential for the rational design of dendrimer-based gene delivery devices.

The size and zeta potential (surface charge) of the prepared dendriplexes together with their morphology and internal structures were examined by dynamic light scattering (DLS), transmission electron microscopy (TEM) and small angle X-ray scattering (SAXS), respectively. The potential of transfection efficiency of test dendriplexes was evaluated by luminance spectrometry and flow cytometry, while their internalization effectiveness was examined using a confocal laser scanning microscope (CLSM) and a flow cytometer.

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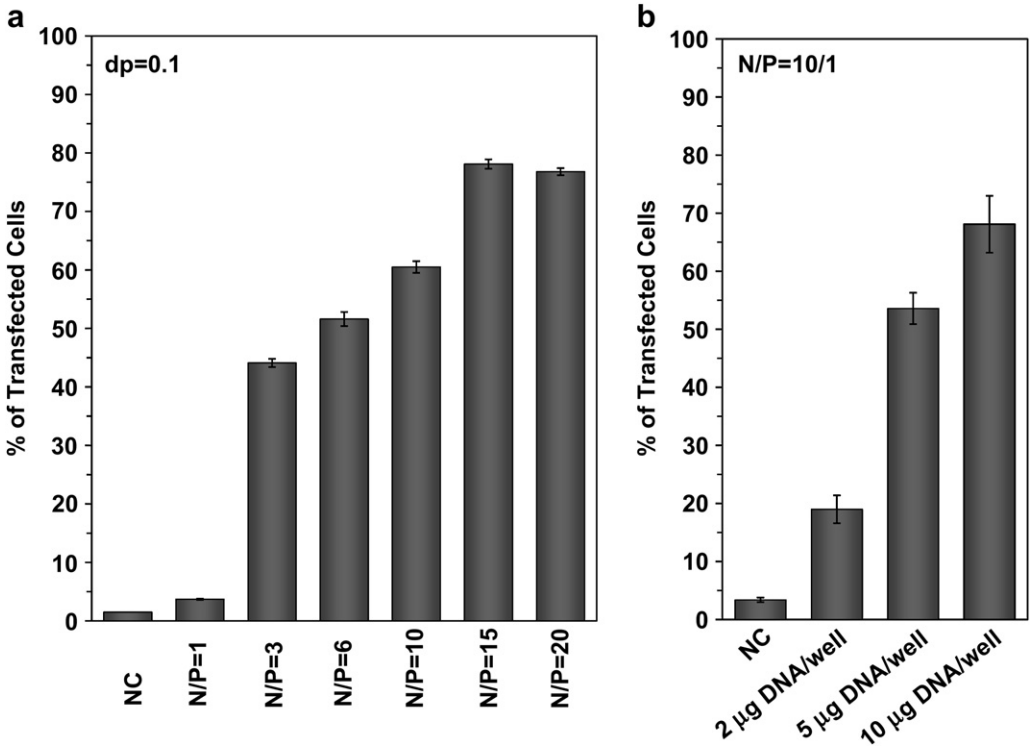

Fig. 1. (a) Percentages of cells that were transfected with dendriplexes (dp/0.1) prepared at different N/P ratios, analyzed by flow cytometry ($n = 3$); (b) percentages of cells that were transfected with dendriplexes prepared at an N/P ratio of 10/1 with different amounts of DNA/well used (N/P = 10), analyzed by flow cytometry ($n = 3$). NC: negative control (the group without any treatment).

2. Materials and methods

2.1. Plasmid DNA

The plasmid DNAs used in the study were pEGFP-N2 (4.7 kb, coding an enhanced green fluorescence protein reporter gene, Clontech, Palo Alto, CA, USA) and pGL4.13 (4.6 kb, coding a firefly luciferase reporter gene, Promega, Madison, WI, USA). pEGFP-N2 and pGL4.13 were amplified using DH5α and purified by Qiagen Plasmid Mega Kit (Valencia, CA, USA) according to the manufacturer's instructions. The purity of plasmids was analyzed by gel electrophoresis (0.8% agarose), while their concentration was measured by UV absorption at 260 nm (Jasco, Tokyo, Japan). pEGFP-N2 and pGL4.13 were linearized using *Eco*O109I and *Bam*HI (New England Biolabs, Ipswich, MA, USA), respectively, and subsequently purified by Gene-Spin™ 1-4-3 DNA Purification Kit-V[3] (Protech Technology Enterprise, Taipei, Taiwan).

2.2. Preparation of test dendriplexes

PAMAM G4 dendrimers with a diaminobutane core in methanol solution were acquired from Dendritic Nanotechnologies (Mount Pleasant, MI, USA). After thoroughly drying, the solid was redissolved in distilled water to produce a 0.2% (w/v) stock solution [6]. The solutions were stored at 4 °C until use. Protonated dendrimers with different average degrees of protonation were prepared by adding predetermined amounts of 0.1 N HCl into the aqueous solutions. The values of dp were then determined from the pH (measured by an ISTEK Model 720P pH meter) of the protonated dendrimer solutions [9], where the pH values of the solutions of dp/0.1, dp/0.3, dp/0.6 and dp/0.9 dendrimers were 9.1, 8.0, 5.3 and 3.4, respectively. For the preparation of PAMAM/DNA dendriplexes suspension (700 μl) in distinct nanostructures, 32.5 μg of linear pEGFP-N2 (or pGL4.13) was individually added to 222.5 μg of aqueous dendrimers with different dp values and then thoroughly mixed for 30–60 s using a vortexer and left for at least 1 h at room temperature. The molar ratio of the amine groups (N) of dendrimers to the phosphate groups (P) of DNA was 1, 3, 6, 10, 15 or 20.

2.3. Characterization of test dendriplexes

The loading efficiency of DNA in each studied group was measured by the PicoGreen assay [10,11]. Briefly, 200 μl of the PicoGreen reagent (diluted 200-fold, Molecular Probes, Carlsbad, CA, USA) was mixed with the same volume of a blank solution (TE buffer, 10 mM Tris-Cl, pH 7.5, 1 mM EDTA) or dendriplex solutions prepared at various N/P ratios. After 2 min incubation, each solution was added to 1.6 ml of TE buffer in a test tube and then analyzed using an FL-2500 Fluorescence Spectrofluorometer (Hitachi, Japan). Results were represented as relative fluorescence (%) to DNA control.

The retardation of DNA in dendriplexes prepared at different dp values was evaluated by electrophoresis. The size and zeta potential of test dendriplexes were investigated using DLS (Zetasizer 3000HS, Malvern Instruments Ltd., Worcestershire, UK). The morphology of test dendriplexes was examined by TEM (JEOL, Tokyo, Japan) [12].

2.4. SAXS experiments

The internal structure of test dendriplexes was probed by SAXS. For the SAXS measurements, the dendriplexes in the form of aqueous suspensions prepared using DNA and dendrimers with dp values of 0.1, 0.3, 0.6 and 0.9 were individually introduced into the sample cell comprising two ultralene windows. The SAXS experiments were performed at room temperature using Beamline BL17A1 at the National Synchrotron Radiation Research Center (NSRRC), Hsin-Chu, Taiwan. The wavelength (λ) of the X-ray was 1.333 Å and a two-dimensional MAR image plate with $100 \times 100\ \mu m^2$ pixel resolution was used to collect the scattering intensity data. The sample-to-detector distance and flat-field correction were calibrated by the mixture of silver behenate and Si powders. The intensity profile was output as the plot of the scattering intensity (I) vs. the scattering vector, $q = 4\pi/\lambda \sin(\theta/2)$ (θ = scattering angle) [13].

2.5. In vitro transfection

HT1080 (human fibrosarcoma) cells were cultured in a supplemented cell medium (Dulbecco's Modified Eagle's Medium, DMEM) with 1% penicillin-streptomycin and 10% fetal bovine serum (FBS). Cells were seeded on 12-well plates at 2×10^5 cells/well overnight and then transfected at 50–80% confluency [14]. Prior to

Table 1
Sizes and zeta potentials of test dendriplexes prepared at a defined N/P ratio of 10/1 and various dp values (or charge densities, $n = 5$).

N/P Ratio = 10/1	Size (nm)	Zeta Potential (mV)
dp/0.1 dendriplexes	149.2 ± 1.7	38.9 ± 0.5
dp/0.3 dendriplexes	151.3 ± 1.6	47.8 ± 0.6
dp/0.6 dendriplexes	161.3 ± 1.2	47.3 ± 1.6
dp/0.9 dendriplexes	163.6 ± 2.5	51.1 ± 1.7