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Mechanisms of cellular uptake and intracellular trafficking with chitosan/DNA/poly(γ-glutamic acid) complexes as a gene delivery vector

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ABSTRACT

Chitosan (CS)-based complexes have been considered as a vector for DNA delivery; nonetheless, their transfection efficiency is relatively low. An approach by incorporating poly(γ-glutamic acid) (γ-PGA) in CS/DNA complexes was developed in our previous study to enhance their gene expression level; however, the detailed mechanisms remain to be understood. The study was designed to investigate the mechanisms in cellular uptake and intracellular trafficking of CS/DNA/γ-PGA complexes. The results of our molecular dynamic simulations suggest that after forming complexes with CS, γ-PGA displays a free γ-glutamic acid in its N-terminal end and thus may be recognized by γ-glutamyl transpeptidase in the cell membrane, resulting in a significant increase in their cellular uptake. In the endocytosis inhibition study, we found that the internalization of CS/DNA complexes took place via macropinocytosis and caveolae-mediated pathway; by incorporating γ-PGA in complexes, both uptake pathways were further enhanced but the caveolae-mediated pathway played a major role. TEM was used to gain directly understanding of the internalization mechanism of test complexes and confirmed our findings obtained in the inhibition experiments. After internalization, a less percentage of co-localization of CS/DNA/γ-PGA complexes with lysosomes was observed when compared with their CS/DNA counterparts. A greater cellular uptake together with a less entry into lysosomes might thus explain the promotion of transfection efficiency of CS/DNA/γ-PGA complexes. Knowledge of these mechanisms involving CS-based complexes containing γ-PGA is critical for the development of an efficient vector for DNA transfection.

1. Introduction

Chitosan (CS), a cationic polysaccharide, is biodegradable, non-toxic and tissue compatible [1,2]. It has the potential to condense anionic DNA into a compact structure (CS/DNA complexes) through electrostatic interactions and has been considered as a non-viral vector for gene delivery [3,4]. Although advantageous for DNA packing and protection, CS-based complexes may lead to difficulties in DNA release once arriving at the site of action intracellularly, thus limiting their transfection efficiency. To overcome this problem, an approach that can modify the internal structure of CS/DNA complexes by incorporating a negatively charged poly(γ-glutamic acid) (γ-PGA) was developed in our previous study [5].

Analysis of the internal structure of CS/DNA/γ-PGA complexes by small angle X-ray scattering (SAXS) revealed that CS formed complexes with DNA and γ-PGA separately and yielded two types of domains, leading to the formation of "compounded nanoparticles" [5]. With this unique internal structure, the compounded nanoparticles might disintegrate into a number of even smaller subparticles after cellular internalization, thus improving the dissociation capacity of CS and DNA and enhancing the efficacy of gene expression [5]. In addition to improving the release of DNA intracellularly, the incorporation of γ-PGA in CS/DNA complexes markedly increased their cellular uptake. Similar observations were also reported by Kurosaki et al. [6], using cationic complexes coated with γ-PGA. However, the detailed mechanisms in endocytosis and intracellular routing of complexes incorporating with γ-PGA remain to be understood. Understanding the role of test complexes on their cellular uptake and intracellular fate is essential for the rational design of non-viral delivery devices.

The study was therefore designed to investigate the potential internalization mechanism of CS/DNA complexes with or without the incorporation of γ-PGA, using transmission electron microscopy (TEM) and the inhibitors specific to various endocytotic

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pathways. The role that γ-PGA may play in the cellular uptake of test complexes was modeling by molecular dynamic (MD) simulations. The intracellular routing of test complexes was observed by a confocal laser scanning microscope (CLSM). Additionally, test complexes were characterized using dynamic light scattering (DLS), and their efficacy in gene expression was determined by luminance spectrometry and flow cytometry.

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).

2.2. Preparation of test complexes

The charge ratio (N/P/C) of test complexes was expressed as the ratio of moles of the amino groups (N) on CS to the phosphate groups (P) on DNA and the carboxyl groups (C) on γ-PGA. Test complexes at N/P/C molar ratios of 10/1/0 (CS/DNA complexes) and 10/1/4 (CS/DNA/γ-PGA complexes) were prepared by an ionic-gelation method [7]. Briefly, an aqueous DNA (pEGFP-N2 or pGL4.13, 33 μg) was mixed with an aqueous γ-PGA (20 kDa, 0 or 51.2 μg, Vedan, Taichung, Taiwan) with a final volume of 100 μl. Test complexes were obtained upon addition of the mixed solution, using a pipette, into an aqueous CS (15 kDa, with a degree of deacetylation of 85%, 0.2 μg/μl, 100 μl, pH 6.0, Challenge Bioproducts, Taichung, Taiwan) and then thoroughly mixed for 30–60 s by vortex and left for at least 1 h at room temperature. The morphology of the obtained complexes was examined by TEM (JEOL, Tokyo, Japan) [8].

2.3. In vitro transfection

HT1080 (human fibrosarcoma) cells were cultured in DMEM media supplemented with 2.2 g/l sodium bicarbonate and 10% fetal bovine serum (FBS). Cells were subcultured according to ATCC recommendations without using any antibiotics. For transfection, cells were seeded on 12-well plates at 2×10^5 cells/well and transfected the next day at 50–80% confluency. Prior to transfection, the media were removed and cells were rinsed twice with transfection media (DMEM without FBS, pH 6.0). Cells were replenished with 0.6 ml transfection media containing test complexes at a concentration of 2 μg DNA/well.

At 2 h post transfection, the transfection media containing test complexes were removed, the cells rinsed twice with transfection media and refilled with FBS-containing media until analysis at 48 h after transfection. Cells were then observed under a fluorescence microscope (Carl Zeiss Optical, Chester, VA, USA) to monitor any morphological changes and to obtain an estimate of the transfection efficiency. Cells transfected with Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) were used as a positive control and those without any treatment were used as a negative control. Transfection efficiencies were presented by two numeric indicators: percentage of cells transfected and gene expression level [9].

2.4. Percentage of cells transfected

The percentage of cells transfected was quantitatively assessed at 48 h after transfection by flow cytometry. Cells were detached by 0.025% trypsin–EDTA. Cell suspensions were then transferred to microtubes, fixed by 4% paraformaldehyde and determined the transfection efficiency by a flow cytometer (Beckman Coulter, Fullerton, CA, USA) equipped with a 488 nm argon laser for excitation. For each sample, 10,000 events were collected and fluorescence was detected. Signals were amplified in logarithmic mode for fluorescence to determine the EGFP-positive events by a standard gating technique. The percentage of positive events was calculated as the events within the gate divided by the total number of events, excluding cell debris.

2.5. Gene expression level

The gene expression levels of cells were assayed by quantifying the expressions of EGFP or luciferase. The expression level of EGFP was quantified by comparing mean fluorescence of 2×10^5 cells. Briefly, cells were treated with test complexes containing pEGFP-N2. After 48 h, cells were detached and analyzed by flow cytometry as described in Section 2.4.

For the expression of luciferase, cells were plated on 24-well plates (with a seeding density of 1×10^5 cells) and transfected as described in Section 2.3 with the exception that 1 μg pGL4.13 was used. The cells transfected were lysed by 100 μl of passive lysis buffer (Promega). The cell lysate was transferred into a 1.5 ml microtube, while the cell debris was separated by centrifugation (14,000 rpm, 5 min). Subsequently, a 100 μl of the luciferase assay reagent (Promega) was added to a 20 μl of the supernatant and the relative luminescence of the sample was determined by a microplate luminometer (Berthold Technologies, Bad Wildbad, Germany) and normalized to the total cell protein concentration by the Bradford method.

2.6. Fluorescent complex preparation and flow-cytometry analysis

Cy3-labeled CS (Cy3-CS) and FITC-labeled CS (FITC-CS) were synthesized as per the methods described in the literature [10,11]. To remove the unconjugated Cy3 and FITC, the synthesized Cy3-CS and FITC-CS were dialyzed in the dark against deionized (DI) water and replaced on a daily basis until no fluorescence was detected in the supernatant. The resultant Cy3-CS and FITC-CS were lyophilized in a freeze dryer. Cy3- and FITC-labeled complexes were then prepared as described in Section 2.2.

To quantify the cellular uptake of test complexes, cells were plated on 12-well plates and transfected with FITC-labeled complexes at a concentration of 2 μg DNA/well for 2 h. After transfection, cells were detached by 0.025% trypsin–EDTA and transferred to microtubes. Subsequently, cells were resuspended in phosphate buffered saline (PBS) containing 1 mM EDTA and fixed in 4% paraformaldehyde. Finally, the cells were analyzed by flow cytometry as described in Section 2.4.

2.7. MD simulations

MD simulations of the self-assembly of CS and γ-PGA in complexation were performed by an MD method [12]. MD simulations were accomplished with the program NAMD [13] using parameters adapted from the CHARMM 27 force field [14]. The models were minimized to remove unfavorable contacts, brought to 310 K by velocity rescaling and equilibrated for 1 ns. Before any MD trajectory was run, 40 ps of energy minimization were performed to relax the conformational and structural tensions. This minimum structure was the starting point for the MD simulations. For this purpose, the molecule was embedded into a cubic simulation box of 80 Å. A cutoff distance of 12 Å was employed for the nonbonded and electrostatic interactions. The heating process was performed from 0 to 310 K through Langevin damping with a coefficient of 10 ps^{-1}. A time step of 2 fs was employed for rescaling the temperature. After 20 ps heating to 310 K, equilibration trajectories of 1 ns were recorded, which provided the data for the structural and thermodynamic evaluations. The equations of motion were integrated with the Shake algorithm with a time step of 1 fs. Figures displaying atomistic pictures of molecules with hydrogen bondings were generated using UCSF Chimera [15].

2.8. Endocytosis inhibition

To study the effect of various inhibitors on the uptake of test complexes, cells were pre-incubated with the following inhibitors individually at concentrations which were not toxic to the cells: 10 μg/ml of chlorpromazine [16], 50 nM wortamannin [17], 5 μg/ml cytochalasin D [18], 5 μg/ml filipin [19] or 200 μM genistein [19,20]; the MTT assay [16] was employed to confirm their toxicity. In the study, the group without any treatment was used as a background in the flow cytometry analysis, while the groups in the presence of test complexes but without inhibitor treatment were used as controls and their fluorescence intensities were expressed as 100%. Following pre-incubation for 30 min, the inhibitor solutions were removed, and the freshly prepared test complexes (FITC-labeled) in media containing inhibitors at the same concentrations were added and further incubated for 2 h. Subsequently, cells were washed three times with PBS, collected according to the methods described above and analyzed by flow cytometry.

2.9. Examination of internalization of test complexes by TEM

To directly observe the mechanism of cellular internalization, cells were incubated with test complexes at 37 °C. After washing three times with PBS, cells were fixed for 30 min at room temperature in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4. The cells were rinsed twice in the same buffer with 6.8% sucrose and subsequently postfixed in 1% OsO_4. After rinsing followed by dehydration in graded alcohol series, the cells were embedded in Spurr resin and polymerized at 70 °C overnight. Ultrathin sections were then cut with a diamond knife and loaded onto TEM grids. The sections were examined by a Philips CM10 electron microscope at accelerating voltage of 120 kV and micrographs were taken [21].

2.10. Intracellular trafficking

To study the intracellular trafficking, cells were treated with the Cy3-labeled test complexes in the serum-free medium. After incubation for 1.5 h, cells were washed twice with the pre-warmed PBS and then treated with 50 nM Lysotracker (HCK-123, Invitrogene, Carlsbad, CA, USA) for 30 min at 37 °C following the supplier's protocol and examined using CLSM (TCS SL, Leica, Germany).