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Combination of Poly(ethylenimine) and Chitosan Induces High Gene Transfection Efficiency and Low Cytotoxicity

Qing-Qing Zhao,[1] Jin-Liang Chen,[1] Min Han,[1] Wen-Quan Liang,[1]
Yasuhiko Tabata,[2] and Jian-Qing Gao[1]*

College of Pharmaceutical Sciences, Zhejiang University, Yuhangtang Road 388, Hangzhou 310058, China[1]
and Department of Biomaterials, Field of Tissue Engineering, Institute for Frontier Medical Sciences,
Kyoto University, 53 Kawanara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan[2]

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In this study, the cytotoxicity and transfection efficiency of using chitosan/DNA complex combined with poly(ethylenimine) (PEI) were investigated. The combination of PEI with the chitosan/DNA complex markedly enhanced the gene expression of HeLa cells to 1000-fold of that induced by chitosan alone. PEI's cytotoxicity was considerably decreased upon combination with the chitosan/DNA complex. Furthermore, the PEI/chitosan/DNA could maintain the gene expression efficiency in the presence of serum.

[**Key words:** poly(ethylenimine), chitosan, cytotoxicity, transfection, serum]

Gene therapy is a promising approach for the treatment of a wide range of diseases because it results in the production of bioactive agents or the cessation of abnormal functions in the cell (1, 2). However, the growing potential of gene therapy for both genetically based and infectious diseases cannot be realized unless the issue of gene delivery is resolved (3). The development of efficient and safe gene transfer systems could be one of the most important factors for successful gene therapy. Non-viral vectors have attracted much attention because of their potential advantages such as ease of synthesis, cell/tissue targeting, low immune response, and unrestricted plasmid size (4, 5). Among the nonviral vectors, cationic polymers and cationic lipids have gained increasing attention because they can easily self-assemble polyelecrolyte complexes between plasmid DNA and cationic polymers, and also, mediate transfection via condensing DNA into nanoparticles, protecting DNA from enzymatic degradation, and facilitating the cell uptake and endolysosomal escape (6–8).

Chitosan is considered to be a good candidate for a gene delivery carrier, because it is a biocompatible, biodegradable, and low-toxicity material with high cationic potential (9). However, this material has a significant limitation, namely, low transfection efficiency. One of the primary causes of poor gene delivery is the inefficient release of vectors from endosomes into the cytoplasm. On the other hand, poly(ethylenimine) (PEI) has been revealed to be the most effective nonviral vector based on cationic polymers owing to its high pH buffering capacity that is believed to enhance the exit of vectors from the endosomal compartment (10). Recently, low-molecular-weight PEI has been combined with cationic or anionic liposomes, and proved to be effective in the delivery of the genes into cells owing to the synergistic advantages of both PEI and cationic liposomes (11, 12). Our previous study also demonstrated that polycation liposomes modified with PEI-Chol showed significantly low cytotoxicity and equivalent transfection efficiency to Lipofectamine™ 2000 (13). However, sometimes the high toxicity of PEI limits its application in gene therapy. Therefore, the combination of chitosan and PEI might be a promising approach for enhancing tranfection efficiency while decreasing the cytotoxicity at the same time.

In this study, a chitosan/DNA complex was induced to self-assemble in distilled water by mixing DNA plasmid with an appropriate polymer solution at charge ratios of 0.5, 1, 2, 4, and 8. The charge ratios (N/P) were expressed as the ratios of moles of the amine groups of chitosan to those of the phosphate groups of DNA. The DNA combination ability of chitosan was studied by gel retardation assay. After the addition of chitosan at an N/P ratio of 4, the DNA band almost disappeared (data not shown). This loss in fluorescence can be attributed to the polycation/DNA complex, which could result in the displacement of the intercalated ethidium bromide in the DNA (14). Complexes with an N/P ratio of 4 were observed using a transmission electron microscope (Fig. 1), which indicated a round morphology of the nanoparticle . The mean size was also measured and it was approximately 100 nm (data not shown). Subsequently, PEI solution (MW 25 K, N/P ratio: 10) was added to the complex-dispersed media before adding to the cells. A preliminary study indicated that PEI might coat the surface of the chitosan/DNA complex through ionic interaction (15).

The potential cytotoxicity of PEI after combination with the chitosan/DNA complex was then investigated in HeLa cells by MTT assay. The results showed no cytotoxicity in HeLa cells treated with either the chitosan/DNA complex or PEI/chitosan/DNA complex (Fig. 2). In contrast, the PEI/DNA complex showed clear cytotoxicity toward the HeLa cells.

* Corresponding author. e-mail: gaojianqing1029@yahoo.com.cn
phone: +86-571-88208437 fax: +86-571-88208436

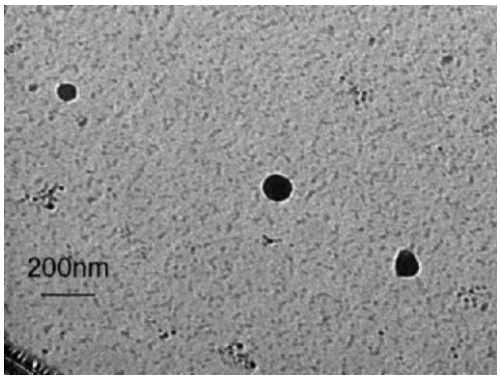

FIG. 1. Morphology of PEI/chitosan/DNA complex (chitosan/DNA : N/P=4, PEI/DNA N/P=10) as observed by transmission electron microscope (×65,000). Bar: 200 nm.

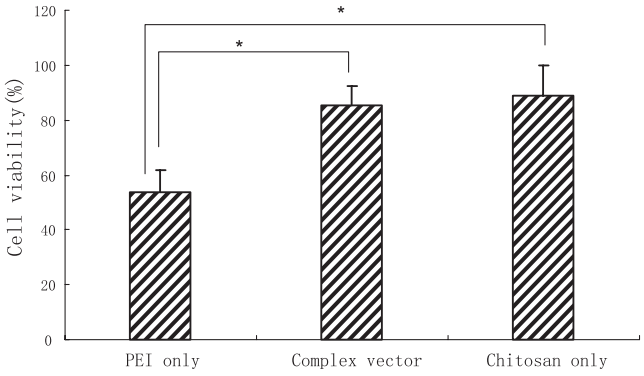

FIG. 2. Viability of HeLa cells treated with PEI/DNA, PEI/chitosan/DNA and chitosan/DNA as determined by MTT assay. The N/P ratio of chitosan/DNAis 4 and that of PEI/DNA is 10. DNA's concentration is 0.2 μg/well (96-well plate). * $p<0.05$ indicates significant difference.

From this study, it was found that the chitosan/DNA complex combined with PEI is much less cytotoxicity than the PEI/DNA complex even though the same amount of PEI was used in both experiments. Although the exact mechanism still remains unclear, it is considered that the interaction of chitosan nanoparticles with PEI might increase the safety of PEI. There are also several approaches have been reported which could reduce the cytotoxicity of PEI. Our previous study demonstrated that PEI linked with cholesterol and develop liposome modified with this polymer decreased the cytotoxicity (13). Another study using galactosylated chitosan (GC) derivatives as hepatocyte-targeting gene carriers, forming the GC/DNA complex, also showed that GC combined with PEI resulted in a much low cytotoxicity than the PEI/DNA complex and it is considered that the steric hindrance and the charge shielding effect are the main reasons for the decrease in cytotoxicity (15).

Next, the tranfection efficiency was evaluated. As shown in Fig. 3, chitosan itself was not good for transfecting HeLa cells. The luciferase activity of cells transfected using chitosan was very low. However, the transfection efficiency markedly increased when PEI was combined with the chitosan/DNA complexes, increasing to 1000-fold of that induced using chitosan alone and almost the same as that induced

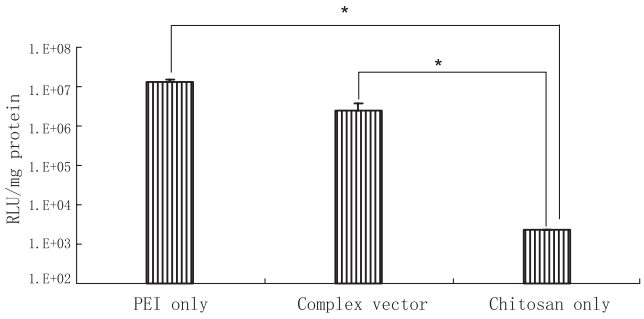

FIG. 3. Transfection efficiency using PEI/DNA, PEI/chitosan/DNA and chitosan/DNA in HeLa cells as determined by luciferase activity. HeLa cells were seeded in a 24-well plate at a density of 1×10^5 cells/well, and cultured for 18 h before the transfection. The chitosan/DNA complex was prepared by mixing 1 μg of PGL3-control with appropriate amounts of chitosan at N/P ratios of 4, followed by incubation 30 min at room temperature. PEI (N/P=10, weight per 1 μg of DNA) was added to the complex-dispersed solution and the final volume was adjusted to 500 μl using serum-free DMEM medium. All the solutions were filtered through a 0.22 μm aseptic filter before mixing, and all solution were of the same volume. The vector/DNA complex was added to the 24-well plate, followed by incubation for 6 h at 37°C under a 5% CO_2 atmosphere. Then, 1 ml of serum-free medium was replaced with fresh medium containing serum, followed by incubation for 24 h at 37°C under a 5% CO_2 atmosphere. The luciferase assay was carried out according to the manufacture's instruction (Promega, Madison, WI, USA). All the experiments were carried out triplicate to ascertain the reproducibility of results. The DNA's concentration was 1 μg/well (24-well plate). ** $p<0.01$ indicates significant difference.

using PEI 25 K. A key cellular barrier affecting the transfection efficiency of nonviral gene vectors is the inefficient release of endosomally trapped DNA into the cytosol (16). The use of chitosan for gene delivery is limited due to the low transfection efficiency and the difficulty in transfecting into a variety of cell types. It is considered that PEI can escape from the endosome through the proton-sponge mechanism and facilitate gene entry into the nucleus (17, 18). Therefore, the addition of PEI enhanced the escape of the chitosan/DNA nanoparticle from the acidic endosome and subsequently induced the high gene transfection efficiency. A previous study also showed that bafilomycin, which can inhibit the endo-/lysosomal proton pump, decreases the PEI-mediated transfection, suggesting the synergism between PEI and chitosan (19).

The effect of PEI on the transfection using the chitosan complex was also observed by fluorescence microscopy. Chitosan/DNA transfection complexes containing the GFP expression plasmid were constructed and used for the transfection of HeLa cells. There were no GFP transfected cells found among the HeLa cells transfected using the chitosan/DNA complexes (Fig. 4A). However, when PEI was added to the chitosan/DNA complexes, the number of green fluorescent cells increased markedly (Fig. 4B) and it was almost the same as that of cells transfected using the PEI/DNA complexes (Fig. 4C). Furthermore, to evaluate the effect of serum on transfection, the transfection efficiency using PEI/chitosan/DNA in the absence or presence of serum was investigated. As shown in Fig. 3, the transfection efficiency using PEI/chitosan/DNA was almost the same, even slightly increased in the presence of serum (Fig. 3D, E). These results have also been confirmed in A549 malignant cells