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Stability of Ionic Complexes Prepared from Plasmid DNA and Self-Aggregated Chitosan Nanoparticles

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Introduction

Gene therapy has recently attracted considerable attention as a potential strategy to alleviate the symptoms of and prevent the occurrence of particular diseases.[1] In this approach, genes are transferred into cells, typically mediated by either viral or non-viral vectors. Several viral vectors, such as retroviruses and adenoviruses, have been extensively used.[2] However, the safety issues of using viruses have limited their uses in clinical applications, likely due to their toxicity, immunogenicity, and inflammatory responses in the body.[3,4] Non-viral vectors, including cationic polymers, have been extensively investigated as an alternative delivery carrier of genes that could circumvent the limitations of using viral vectors. For example, simple mixing of DNA with poly-L-lysine or DEAE-dextran enables the formation of ionic complexes that can mediate the transfer of the DNA into the nuclei of target cells. Various cationic polymers such as poly(ethylene glycol)-*b*-poly-L-lysine, poly-*N*-(2-hydroxypropyl) methylacrylamide-*b*-poly(trimethylammonioethyl methacrylate chloride), poly(ethylene imine), and cationic cascade polymers have been synthesized and their efficacy as novel gene delivery carriers has been tested.[5-9]

We previously reported that deoxycholic acid-modified chitosan can form stable nanoparticles in a physiological buffer solution, and their various physicochemical characteristics have been investigated using a fluorometer and light scattering method.[10,11] The self-aggregated chitosan nanoparticles were found to form ionic complexes with plasmid DNA, and their complex formation behavior was controlled by the degree of substitution, pH of the medium, and charge ratio, which were closely related to the transfection efficiency of COS-1 cells *in vitro*.[12,13] In this study we report a novel screening method on the stability of DNA/chitosan self-aggregate complexes, and compare with that of complexes formed between DNA and commercially available liposome. The stability of the DNA/chitosan self-aggregate complexes was tested using anionic polymers that may mimic the negatively charged macromolecules existing in the body and can cause exchange reactions while DNA is being delivered into the body.

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Experimental

Materials. Chitosan (Samchully, $M_v=7\times10^4$, degree of deacetylation=80%) was hydrophobically modified with deoxycholic acid (Sigma) in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, Sigma) as previously reported.[10] In brief, deoxycholic acid in methanol was added to a 1% chitosan solution, followed by the dropwise addition of EDC under stirring at room temperature. After 24 h the reaction mixture was poured into a methanol/ammonia solution (7/3, v/v). The precipitates were filtered off, washed thoroughly with distilled water, methanol, and ether, and then followed by drying in a vacuum. The degree of substitution, defined as the number of deoxycholic acid groups per 100 anhydroglucose units of chitosan, was determined by elemental analysis. The deoxycholic acid-modified chitosan was then suspended in a phosphate buffered saline (PBS) solution (pH 7.2) at 37 °C for 48 h, followed by ultrasonication (30 W, three times for 2 min) using a pulse function (pulse on, 5.0 sec; pulse off, 1.0 sec) to prepare a clear micellar solution. The plasmid pCMV-CAT, encoding chloramphenicol acetyltransferase (5.8 kb), was grown in *E. coli*, extracted by the alkali lysis technique, and purified using a QIAGEN® Plasmid Midi kit. The purity of the plasmid, consisting of supercoiled and open circular forms, was checked by electrophoresis on a 1.0% agarose gel, and the concentration of DNA was determined by measuring the ratio of the UV absorbance values at 260 and 280 nm.

Complex Formation between Chitosan Self-Aggregates and Plasmid DNA. Chitosan self-aggregate/DNA complexes were prepared by the addition of a DNA solution to a solution of chitosan self-aggregates at room temperature. Complex formation was confirmed by electrophoresis on a 1.0% agarose gel with tris-acetate (TAE) running buffer at 100 V for 30 min. UV transillumination of the gel was employed with ethidium bromide to visualize DNA. The stability of the complexes was tested by ionic exchange reactions using anionic polymers such as poly(L-aspartic acid) (Sigma, $M_w=1.1\times10^4$) and heparin (Sigma, $M_w=6.0\times10^3$). The anionic polymers were mixed with DNA/chitosan self-aggregate complexes and incubated for 2 h at room temperature, and the DNA released from the complexes was confirmed by electrophoresis on agarose gels. Lipofectamine® (GIBCO), a frequently used and commercialized non-viral gene carrier, was also used to form complexes with the plasmid DNA and mixed with the anionic polymers, in

order to compare the stability with DNA/chitosan self-aggregate complexes.

Results and Discussion

Colloidally stable self-aggregated nanoparticles in a physiological buffer solution can be obtained by hydrophobic modification of chitosan with deoxycholic acid. Deoxycholic acid was covalently coupled to an amino group of chitosan using water-soluble carbodiimide. The degree of substitution of deoxycholic acid was determined to be 5 per 100 anhydroglucose units of chitosan by elemental analysis. Deoxycholic acid-modified chitosan formed self-aggregates in aqueous media with a mean diameter of less than 200 nm, which can be controlled by the degree of substitution of hydrophobic groups to the chitosan backbone and pH and ionic strength of the medium.[10] When DNA is mixed with self-aggregates, electrostatic interactions mainly drive the formation of complexes. The migration of DNA on an agarose gel was delayed because of the charge neutralization and/or an increase in the molecular size of the complexes. The charge ratio (+/-) was defined as the ratio between the number of amino groups in chitosan self-aggregates and the number of phosphate groups in DNA. When the charge ratio was greater than 4/1, the migration of DNA was completely delayed. The characteristics of DNA/chitosan self-aggregate complexes varied depending on the charge ratio and pH of the medium. However, the formation and stability of the complexes were not significantly influenced by incubation time.[14]

Reversibility of the complex formation between chitosan self-aggregates and plasmid DNA was investigated by monitoring exchange reactions with anionic polymers, which may suggest a useful means for testing the stability of the complexes.[15-17] Increasing amounts of anionic polymers, such as poly(L-aspartic acid) (Figure 1(a)) or heparin (Figure 2(a)), were added to the complexes and incubated for 2 h at room temperature. The plasmid DNA was slightly dissociated from self-aggregate/DNA complexes by the addition of excess amounts of anionic polymers (lanes 6 and 7, Figures 1(b) and 2(b)). A larger amount of DNA was dissociated from the complexes formed between the commercially-available cationic liposomes (i.e., Lipofectamine®) and plasmid DNA than the amount of DNA dissociated from DNA/chitosan self-aggregate complexes (lanes 10-12, Figure 1(b); lanes 11-12, Figure 2(b)). This clearly indicates that more compact and stable complexes can be formed between plasmid DNA and chitosan self-aggregates, as compared with those formed between plasmid DNA and Lipofectamine®.

When a solution of DNA is heated above a characteristic temperature, its native structure collapses and its two complementary strands separate and form a random coil conformation. This process, called "denaturation" or "melting" of DNA, is accompanied by changes in the physical properties of DNA, such as an increase in viscosity. When DNA denatures, its UV absorbance increases at all wavelengths. This phenomenon, known as the "hyperchromic effect," results

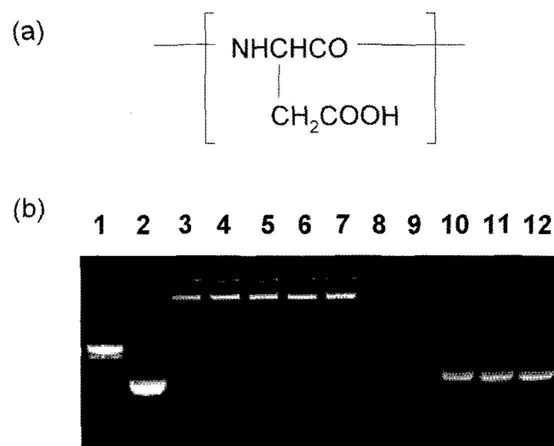

Figure 1. (a) Chemical structure of poly(L-aspartic acid). (b) Migration of polycation/DNA complexes in the presence of excess amounts of poly(L-aspartic acid) incubated for 2 h at room temperature. DNA was mixed with chitosan self-aggregates (lanes 3-7) or with Lipofectamine® (lanes 8-12) at a fixed charge ratio (+/-) of 4/1. Lane 1, DNA molecular weight marker II; lane 2, DNA only; lanes 3 and 8, complex only; lanes 4 and 9, 5; lanes 5 and 10, 10; lanes 6 and 11, 50; lanes 7 and 12, 100 equivalent of poly(L-aspartic acid) to plasmid DNA.

Figure 2. (a) Chemical structure of heparin. (b) Migration of polycation/DNA complexes in the presence of excess amounts of heparin incubated for 2 h at room temperature. DNA was mixed with chitosan self-aggregates (lanes 3-7) or with Lipofectamine® (lanes 8-12) at a fixed charge ratio (+/-) of 4/1. Lane 1, DNA molecular weight marker II; lane 2, DNA only; lanes 3 and 8, complex only; lanes 4 and 9, 5; lanes 5 and 10, 10; lanes 6 and 11, 50; lanes 7 and 12, 100 equivalent of heparin to plasmid DNA.

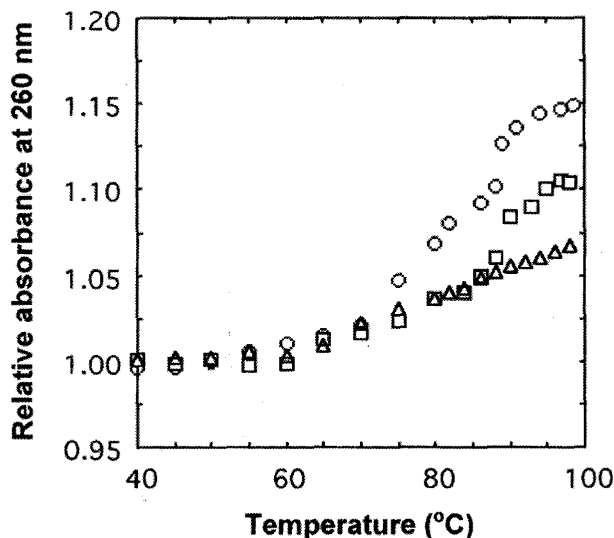

Figure 3. Effect of complex formation between DNA and chitosan self-aggregates on the thermal denaturation of the DNA (○ , DNA only; □ , +/- = 1/1; △ , +/- = 4/1).

from the disruption of the electronic interaction among nearby bases.[18,19] When the plasmid DNA was mixed with chitosan self-aggregates and formed ionic complexes, its thermal denaturation appeared to be retarded and its melting transition shifted to a temperature higher than that of intact DNA (Figure 3). If a solution of denatured DNA is annealed at around 25 °C for a sufficient length of time, the denatured DNA completely "renatures" as the complementary strands find another ones and form base-paired structures.[18] However, residual hyperchromicity may exist because of incomplete base pairing between the complementary strands.[20] The residual hyperchromicity of DNA formed complexes with chitosan self-aggregates was larger than that of DNA alone (Table I). This may have been due to the disruption of base-pairing by the chitosan self-aggregates during the annealing process.

Conclusions

The stability of the ionic complexes formed between plasmid DNA and chitosan self-aggregates was investigated by testing the reversibility of the complex formation using anionic polymers. Complexes formed between plasmid DNA

Table I. Residual Hyperchromicity of DNA after Annealing[a]

Sample	Residual Hyperchromicity (%)
DNA only	1.1 ± 0.3
+/- = 1/1	6.8 ± 0.1
+/- = 4/1	8.0 ± 0.5

[a]DNA was annealed at room temperature after denaturation.

and chitosan self-aggregates appeared to be far more stable against anionic exchange reactions, compared with complexes formed between plasmid DNA and Lipofectamine®, although both complexes had the same charge ratios. This approach to testing the stability of DNA/polycation complexes may be critical in designing novel non-viral gene delivery systems.

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