Nano-structured Chitosan Self-aggregates as a Drug Delivery Carrier

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ABSTRACT

Colloidal delivery systems including polymeric self-aggregates have been widely investigated to date, as these formulations may reduce unwanted side effects and may improve the therapeutic effects. Various characteristics of self-aggregates prepared from deoxycholic acid-modified chitosan were investigated by fluorescence spectroscopic and dynamic light scattering methods, and found to be controlled by the degree of substitution of hydrophobic groups, and pH and ionic strength of the medium. Chitosan self-aggregates can form complexes with plasmid DNA, and can be used to transfect COS-1 cells in vitro. Adriamycin can be also entrapped into chitosan self-aggregates and released in a sustained manner in vitro. These self-aggregates may find potential applications as a delivery vehicle of genes and anti-cancer drugs.

Keywords: chitosan, self-aggregates, DNA, adriamycin.

1 INTRODUCTION

A number of novel delivery systems have been developed including the entrapment of drugs, proteins, antigens or genes in small vesicles or within polymeric matrices. The importance of water-soluble polymers has been increasingly grown for biological and medical applications, including delivery of bioactive molecules. One of the particularly interesting classes of water-soluble polymers is a polymeric amphiphile. Polymeric amphiphiles can self-organize in aqueous media due to the hydrophobic interaction, however, still isotropically soluble. Self-assembly of polymeric amphiphiles in aqueous media including block copolymer micelles or self-aggregates of hydrophobically modified polymers has been widely used for delivery of bioactive agents [1].

Gene therapy is one recent and exciting approach, designed to introduce genetic materials into appropriate cells to alleviate symptoms and/or prevent the occurrence of particular diseases. Several viral vectors such as retroviruses and adenoviruses have been extensively employed. However, their safety in many clinical uses has been issued due to their toxicity, immunogenicity, and inflammatory responses [2]. Thus, non-viral vectors have found numerous advantages as an alternative delivery vehicle of genes, regardless of their low transfection efficiency. Non-viral delivery systems include direct DNA injection, microencapsulation, cationic liposomes, and cationic polymers [3, 4].

Chitosan is one important natural polymer, which is known to be biocompatible, biodegradable, and non-toxic in many clinical trials. In this study, chitosan was hydrophobically modified with deoxycholic acid, a main component of bile acid (Figure 1). We have previously reported that deoxycholic acid-modified chitosan can form self-aggregated nanoparticles in a physiological buffer solution, and their various physicochemical characteristics have been investigated by using a fluorometer and light scattering method [5]. In this study, we hypothesized complex formation behavior between plasmid DNA and chitosan self-aggregates can be significantly affected by the charge ratio, pH, and incubation time. We also tested the efficacy of chitosan self-aggregates as a novel delivery vehicle of adriamycin (ADR), a widely utilized anti-cancer drug molecule to date.

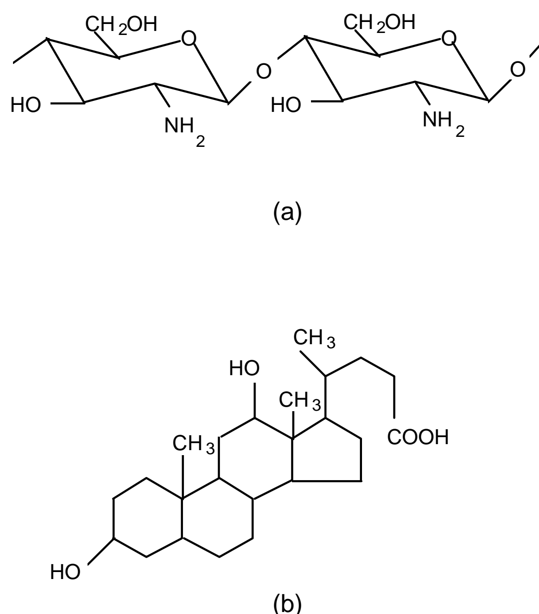

Figure 1: Chemical structure of (a) a repeating unit of chitosan and (b) deoxycholic acid.

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2 EXPERMENTAL METHODS

2.1 Materials

Biomedical grade chitosan (M_v = 7 × 10^4, degree of deacetylation = 80 %) was supplied from Samchully Pharm. Co. (Seoul, Korea). Deoxycholic acid (>99% purity) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were purchased from Sigma (St. Louis, MO).

The plasmid pCMV-CAT encoding chloramphenicol acetyltransferase (Figure 2) was grown in *E. coli*, extracted by the alkali lysis technique, and purified by using a QIAGEN® 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 an UV absorbance at 260 nm. Adriamycin was obtained from Chongkeundang Co. (Seoul, Korea) and used as received. The water was purified by distillation, deionization, and reverse osmosis (MilliQ Plus).

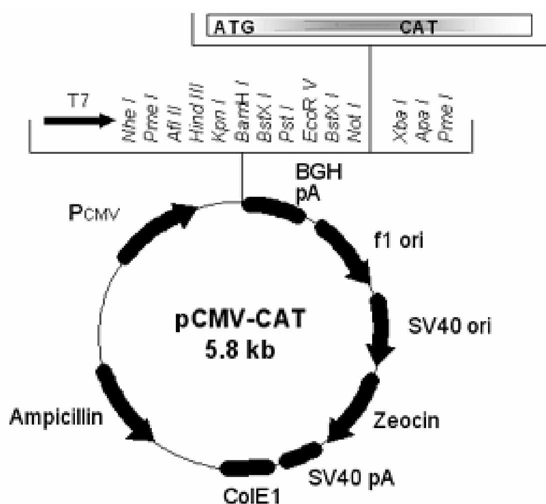

Figure 2: Features of pCMV-CAT vector.

2.2 Methods

Deoxycholic acid was coupled to chitosan by an EDC-mediated reaction. In brief, chitosan was dissolved in 1% acetic acid solution and deoxycholic acid was added, followed by the dropwise addition of EDC at room temperature. After 24 h, the modified chitosan was precipitated by methanol/ammonia solution (7/3, v/v), filtered off, washed thoroughly, and then dried in a vacuum at room temperature. The number of deoxycholic acid groups per 100 anhydroglucose residues of chitosan was determined by elemental analysis. The modified chitosan was suspended in phosphate-buffered saline (PBS) solution (pH 7.2) at 37 ^oC for 48 h, and sonicated using a probe type sonifier (Sigma Ultrasonic Processor, GEX-600) at 30 W for 2 min. The sonication was repeated 3 times to get an optically clear solution using pulse function (pulse on, 5.0 sec; pulse off, 1.0 sec).

Dynamic light scattering experiments were carried out with an argon ion laser system (Lexel Laser Model 95) tuned at 488 nm. The scattering angle was varied from 30 to 135°. The intensity autocorrelation was measured at a scattering angle of 90° with a Brookhaven BI-9000AT digital autocorrelator at 25±0.1 ^oC. A nonlinear regularized inverse Laplace transformation technique (CONTIN) was used to obtain the distribution of decay function. Mean diameter was evaluated by the Stokes-Einstein equation.

Steady-state fluorescence spectra were recorded on an ISS K2 multi-frequency phase and modulation fluorometer (ISS, Champaign, IL). Samples were excited using a 300 W Xenon arc lamp (ILC Technology, Sunnyvale, CA). For the measurement of the emission intensity of pyrene, the slit openings for excitation and emission were set at 1.0 mm and 0.5 mm, respectively. The excitation wavelength (λ_{ex}) was 336 nm, and the spectra were accumulated with an integration time of 5 s/1 nm and used to determine the critical aggregation concentration (cac) of chitosan self-aggregates. Fluorescence intensity of ADR was also measured by an ISS K2 fluorometer. The excitation wavelength (λ_{ex}) and the emission wavelength (λ_{em}) were set at 470 and 595 nm, respectively. Atomic force microscopic (AFM) images were obtained by an Autoprobe CP system (Park Science, Sunnyvale, CA) using the contact mode under ambient condition. A silicon nitride tip on a cantilever with spring constant of 0.12 N/m was used.

The complex formation of self-aggregates with plasmid DNA (pCMV-CAT, 5.8 kb) was monitored by the gel retardation assay. Electrophoresis was done on a 1.0 % agarose gel with TAE running buffer at 100 V for 20 min. DNA was visualized with ethidium bromide. Chitosan self-aggregate/ DNA complexes were added to COS-1 cells seeded at a density of 1.5 × 10^5 per 35-mm culture plate and grown in DMEM (Dulbecco's modified Eagle's medium) containing 10 % fetal bovine serum at 37 ^oC under a 5 % CO_2 atmosphere. The culture media was replaced with fresh serum-containing media after 24 hr of mixing, and incubated for additional 48 hr at 37 ^oC under a 5 % CO_2 atmosphere. After incubation, the cells were removed from the culture plate, washed with 0.25 M Tris-HCl (pH 8.0), and followed by the determination of CAT activities.

3 RESULTS AND DISCUSSION

Owing to preference for the formation of free energy-minimized structure, the deoxycholic acid-modified chitosan formed self-aggregated structures with the inner core of hydrophobic segments and the outer shell of hydrophilic segments. Critical aggregation concentration (cac), which is the threshold concentration of self-aggregates formation by intra- and/or intermolecular association, was determined from the change of intensity ratio (I_{336}/I_{333}) of pyrene in the presence of polymeric

amphiphiles. The cac values of deoxycholic acid-modified chitosans are lower than the critical micelle concentration (cmc) of low molecular weight surfactants (e.g., 2.3 mg/mL for sodium dodecyl sulfate in water; 1.0 mg/mL for deoxycholic acid in water) [6]. The lower cac values of the modified chitosans as compared with low molecular weight surfactants may be one of the important characteristics of polymeric amphiphiles, as small amounts of the chitosan derivatives can form self-aggregates and maintain the stability in dilute conditions. The increasing hydrophobicity of the chitosan derivatives by introduction of large amount of deoxycholic acid moieties further reduces the cac values (Figure 3). Chitosan self-aggregates had the mean diameter of less than 200 nm, decreasing with an increase of the DS.

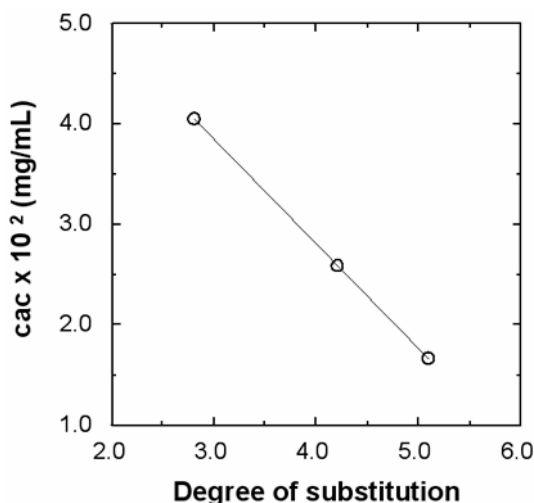

Figure 3: Effect of degree of substitution on the critical aggregation concentration of self-aggregates of chitosan derivatives in PBS solution (pH 7.2).

We next tested the efficacy of chitosan self-aggregates as a gene delivery carrier. It was expected that chitosan self-aggregates could form complexes with DNA, as chitosan is a cationic polyelectrolyte. Migration of DNA was retarded during agarose gel electrophoresis because of the charge neutralization of the complexes, and excess amounts of self-aggregates were required to complete retardation of DNA (Figure 4). The charge ratio (+/-) was defined as a 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, migration of DNA was completely retarded. We next measured the size of the complexes by dynamic light scattering method. The DNA/self-aggregate complexes in PBS solution (pH 7.2) had the mean diameter of 350 nm with unimodal size distribution, which is bigger than that of chitosan self-aggregates only. Interestingly no remarkable change in the size distribution of complex particles was observed.

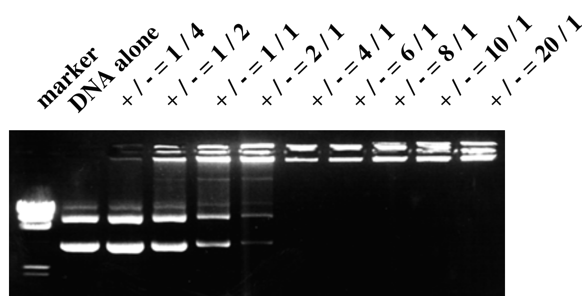

Figure 4: Gel retardation assay for self-aggregate/DNA complexes in PBS solution (pH 7.2).

The characteristics of DNA/chitosan self-aggregate complexes were varied depending on the charge ratio and pH of the medium. However, formation and stability of the complexes were not influenced by incubation time. An efficient transfection of COS-1 cells was achieved by self-aggregates/DNA complexes, compared with using DNA only, indicating potential applications of chitosan self-aggregates as a gene delivery vehicle (Figure 5).

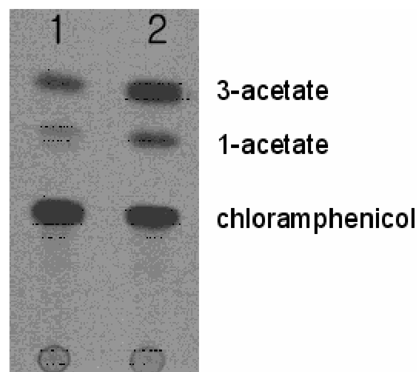

Figure 5: CAT assay of gene expression in COS-1 cells. Lane 1, DNA only; lane 2, DNA complexed with self-aggregates of chitosan derivatives (+/- = 4/1)

We next investigated the potential application of chitosan self-aggregates as a delivery vehicle of adriamycin (ADR), a widely used anti-cancer drug molecule. We chose adriamycin (ADR) as a model drug, not only due to easy method of characterization but also its wide clinical utilization. It was hypothesized that deoxycholic acid-modified chitosan can form self-assembled structures with multiple cores in aqueous media (Figure 5). These cores can act as a reservoir of hydrophobic drugs. ADR was physically entrapped inside the self-aggregates, and the amount of entrapped ADR reached up to 16.5 wt% of chitosan self-aggregates, suggesting loading efficiency of 49.6 % (w/w), which was slowly released from the self-aggregates in PBS solution (pH 7.2). The AFM images indicated spherical shape of self-aggregates after drug loading (Figure 6).

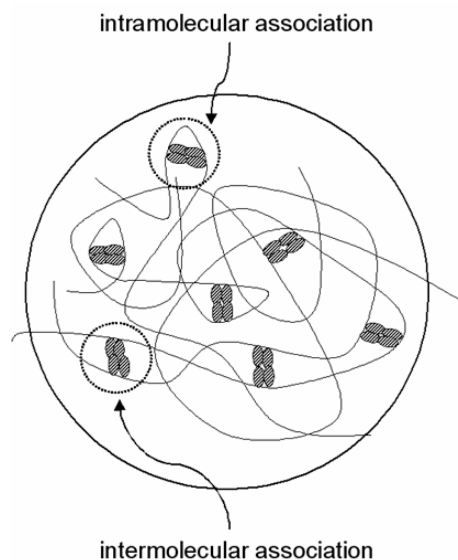

Figure 6: Internal structure of self-aggregates prepared from hydrophobically modified chitosan in aqueous media.

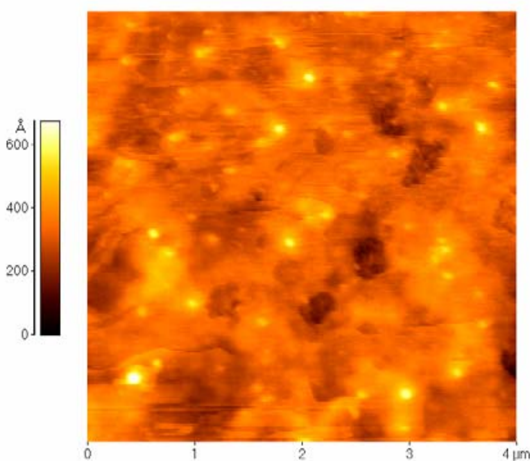

Figure 7: Atomic force microscopic image of ADR-loaded chitosan self-aggregates (X-Y scan, 4 × 4 µm scale).

4 CONCLUSIONS

Deoxycholic acid-modified chitosan forms self-aggregates that are stable in a physiological buffer solution. Their mean diameter is less than 200 nm, depending on the degree of substitution of deoxycholic acid, and pH and ionic strength of the medium. These chitosan self-aggregates may be useful in delivery of either genes using cationic characteristic of chitosan or anticancer drugs that can be physically entrapped into the multiple core of the self-aggregates, even in vivo as well as in vitro due to the enzymatically degradable properties of chitosan.

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