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Physical Properties and Stability of Plasmid DNA-Loaded Chitosan-TPP Nanoparticle

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Abstract

Chitosan (CS) is a biodegradable natural polymer that has shown potential for gene delivery. Although a number of in vitro studies showed that chitosan and its derivatives have emerged as promising vehicles for efficient non-viral gene and plasmid DNA (pDNA) vaccine delivery, the stability of chitosan/plasmid nanoparticle remain insufficient. In the present study, ionically crosslinked chitosan nanoparticles were formulated with plasmid DNA using the ionic gelation technique with sodium tripolyphospate (TPP) as a crosslinking agent. We investigate the stability of chitosan/pDNA nanoparticles which was synthesized by this method. Optimization study showed that chitosan to TPP ratios of 1:0.4(w/w) results in the reproducible formation of nanoparticles with good production yields. SEM and DLS analyses revealed a circular shape of the CS/TPP nanoparticles with an average size diameter of 173 nm. The zeta potential of the nanoparticles was + 10.8 mv. In vitro study of pDNA release from CS/TPP nanoparticles revealed no DNA release following incubation of chitosan/pDNA nanoparticles for up to 1 month, in mediums of PBS and acetic acid at pH 4 and pH 7.4. According to the results, ionically crosslinked CS/TPP nanoparticles have the potential to be used as a biocompatible non-viral gene delivery system with strong stability.

Keywords: Chitosan, Gene delivery, Nanoparticles, Ionic gelation

1 Introduction

Although macrobiomolecules have great potential as therapeutic agents, but the potential has yet to be completely exploited and have been addressed through the development of proper nanocarriers. As surface functionalization of nanoparticle are involved in medical applications, structural materials, catalysts, as well as in cleaning and purification systems. In this context, nanoparticles have emerged as one of the most exciting tools, due to the increased surface-to-volume ratio, which enable the encapsulated molecules to retain their biological activity, from the production steps to the final release.(1-16) Chitosan (CS) is an interesting natural linear chain poly-amino saccharide composed of D-glucosamine residues linked by β (1→4) glycosidic bonds. Its desirable features such as non-toxicity, biodegradation, and biocompatibility as well as have a highly chemically reactive structure render it highly useful for the pharmaceutical application. A number of therapeutic effects of CS have been reported, including wound healing (17), anticancer (18), promotion of hemostasis and epidermal cell growth (19). These properties have attracted interest in the application of this substance in biomedical various fields, such as drug delivery and targeting (20), wound dressing (21), and tissue engineering (22).

A number of in vitro studies showed that chitosan and its derivatives have emerged as promising vehicles for efficient non-viral gene and plasmid DNA (pDNA) vaccine delivery (23, 24). In addition to the fact that CS has a high positive charge and low toxicity, it also has a high mucus binding affinity, which makes it able

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to increase the penetration of large molecules across a mucosal surface. Today, it has been proven that chitosan-based delivery systems can be useful for nasal delivery of siRNA and gene interference in the lung mucosa (25). As a non-viral vector for gene delivery, chitosan nanoparticles (CNPs) have many advantages because viral systems may make it possible to make recombinant recombination and oncogenic effects and immunological reactions leading to potentially serious complications.

CNPs are usually made up by ionic gelation method, which describes the crosslinking reaction of CS with sodium tripolyphosphate (TPP). This technique involves the addition of a crosslinking agent, i.e. TPP, into the aqueous phase containing chitosan, thus leading to the formation of chitosan nanogels (26, 27). This technique has been previously adapted for the encapsulation of peptides and proteins (28). As compared to the other methods used for DNA association, the nanoparticle formation is not only determined by the electrostatic interactions between chitosan and DNA but simultaneously also by physical entrapment upon the ionic crosslinking induced by TPP. This process results in the controlled gelation of chitosan in the form of spherical, homogeneous and compact nanoparticles, characteristics that are expected to benefit the performance of the system both in vitro and in vivo (29-33). It has advantages of not necessitating sonication and organic solvents for its preparation, therefore minimizing possible damage to DNA during complexation. Although the experimental evidence suggests that in this strategy chitosan can easily bind or encapsulate DNA and protect it eff ectively from DNases, but the stability of chitosan/plasmid nanoparticle still needs to be elucidated. The aim of this study was to investigate the stability of chitosan/pDNA nanoparticles which was synthesized by the method of ionic gelation. The physical–chemical properties of chitosan nanoparticles were optimized by studying the influence of several key parameters including CS concentration, CS molecular weight (i.e. 125 kDa vs. 300 kDa), CS/TPP polymer ratio, and DNA loading.

2 Materials and methods

2.1 Materials

Chitosan with 75-85% deacetylation degree and medium molecular weight was purchased from Sigma-Alderich. Plasmid DNA (pDNA) encoding green fluorescent protein (PKScGFP). tripolyphosphate (TPP) was obtained from Sigma–Aldrich (Madrid, Spain). One kBp DNA ladder was obtained from Merck Millipore Bioscience. All other solvents and chemicals were of the highest grade commercially available.

2.2 Preparation and physicochemical characterization of CS/TPP nanoparticles

After optimization, ionically crosslinked nanoparticles based on medium molecular weight CS were formulated with plasmid DNA (pDNA). For this formulation, the ionic gelation technique was used. Chitosan solution is prepared at the concentration of 0.05% (W/V) in acetate buffer solution (pH 4.5). The sample was stirred overnight and then filtered. The TPP is dissolved in double-distilled water at a concentration of 0.20 mg/mL. For formation of chitosan-TPP nanoparticles containing plasmid in their matrix, following procedure was performed: I) the solution of plasmid is added to the previously prepared solution TPP (0.20 mg/mL), II) nanoparticles were formed instantaneously upon the dropwise addition of a volume of plasmid/TPP solution to a volume of chitosan solution (1:0.4) under magnetic stirring at room temperature. Chitosan nanoparticles were recovered by ultracentrifugation (Hettich®, 200R, Tuttlingen, Germany) 12,000 rpm, at 10 ºC for 20 min. All these steps are shown in schematic Figure 1. The particle size distribution and zeta potential were obtained by the DLS technique, using a Zetasizer Nano ZS 3600(Malvern Instruments). The morphology of the nanoparticles was examined via scanning electron microscope (KYKYEM3200) at an operating voltage of 25 kV.

1.1 Loading capacity of CS/TPP nanoparticles

Encapsulation efficiencies of pDNA were calculated from the amount of non-encapsulated material recovered in the supernatant samples collected upon centrifugation of the nanoparticles (10000 RPM, 100C, 20 min). The amount of recovered DNA was determined by spectrophotometer (Biochrom®, U.K). Additionally, the association of DNA to the nanoparticles was also determined by gel electrophoresis assays (1% agarose containing safe nucleic acid stain, Safe-green®, 80 V, 40 min).

1.1 In vitro release of DNA

In vitro release of pDNA was determined by incubating the nanoparticles in acetate buffer and phosphate buffered saline (PBS) (pH 4 and 7.4), (37 ºC, horizontal shaking). At time intervals of 1 day and 1, 2, 3, 4 weeks, individual samples were isolated by centrifugation (10,000 RPM, 20 min). The supernatant of samples was analyzed by agarose gel electrophoresis using un-encapsulated PKScGFP plasmid as the positive control sample.

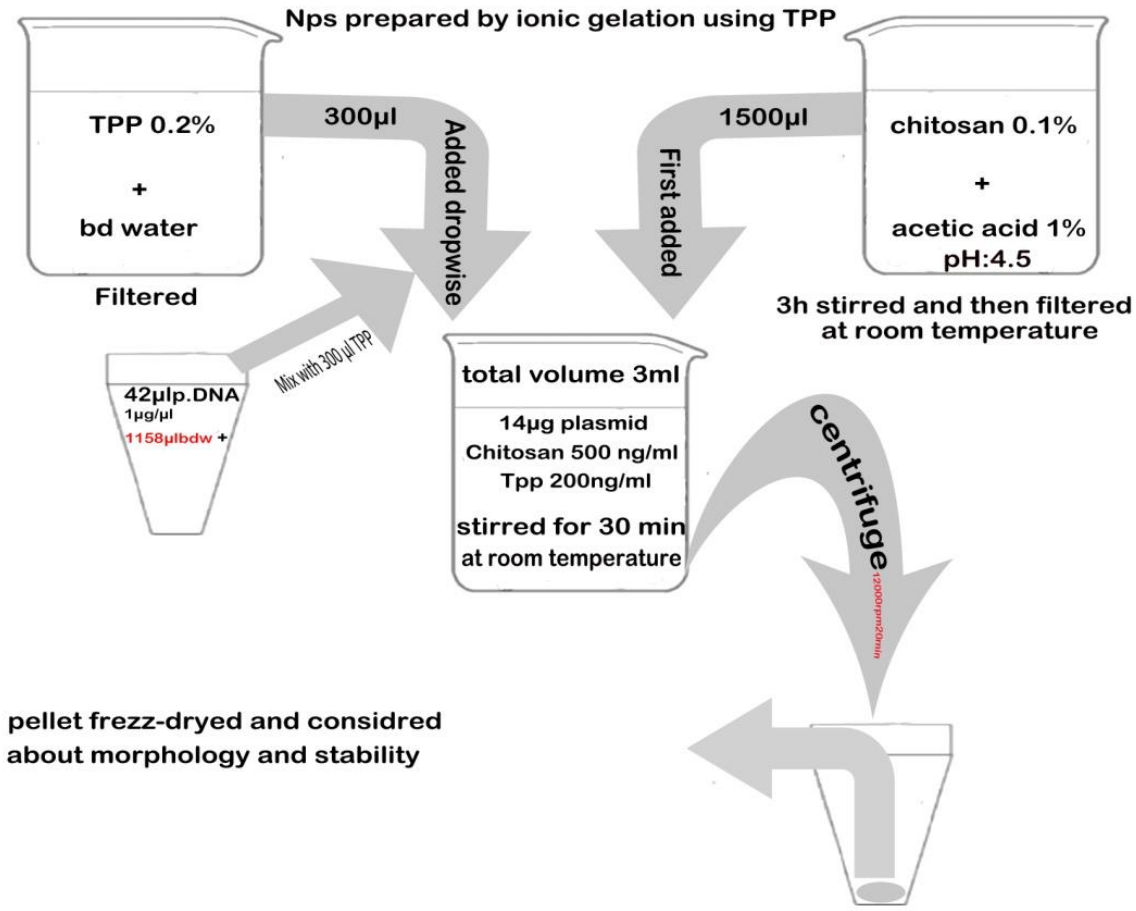

Figure 1: Schematic figure of chitosan/pDNA nanoparticle synthesis by ionic gelation method

3 Results and discussion

3.1 Formation and physicochemical characterization of CS/pDNA nanoparticles

Initial studies aiming at the optimization of the nanoparticle formation indicated that chitosan to TPP ratios of 1:0.4(w/w) results in the reproducible formation of nanoparticles with good production yields. The average size of the nanoparticles prepared of medium molecular weight chitosan (MMW CS/TPP) was 173 nm. Polydispersity Index (P.I.) of nanoparticles was 0/292 (Table1). Also, Zeta potential of chitosan/pDNA was +10.8 mV (Figure 2). The morphology and structure of CNPs are demonstrated by SEM. The results showed that the majority of CNPs were circular in shape with an only limited degree of aggregation (Figure 3).

Table1: Physical–chemical characteristics of CS/TPP nanoparticles loaded with pDNA (i.e. pKScGFP).

Size (nm)	P.I.	ζ Potential (mV)	E.E. (%)
173	0.292	+10.8	100

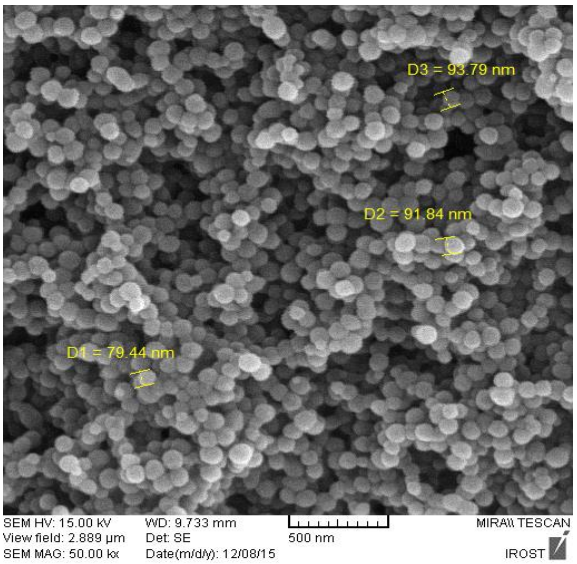

Figure 3: SEM micrograph of synthesized chitosan/pDNA nanoparticles

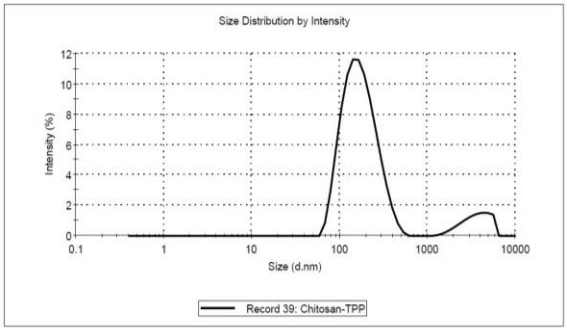

Figure 2: Particle size distribution of the CS/pDNA nanoparticles

3.2 Entrapment of plasmid DNA

According to research conducted by Carrillo et al (34), for loading process, 14µg/ml of plasmid DNA was included in TPP solution prior to nanoparticle formation. In this study, chitosan showed high efficiency for the encapsulation of pDNA, reaching almost 100% (Table 1). Previous studies have shown that the entrapment of the pDNA within the CS/TPP nanoparticles has several advantages over absorbance of pDNA onto preformed CNPs, such as the more effective protection of pDNA from decomposition when administered In vivo, the easier surface modification of nanoparticles to improve their interaction with biological surfaces, and more controllable of the pDNA release process.

3.3 In vitro release of pDNA from CS/TPP nanoparticles

In order to study the stability of chitosan/pDNA nanoparticle and the pDNA release properties of CS/TPP nanoparticles, they were incubated in 2 type of release media with 2 different pH values (acetate buffer pH 4 and pH 7.4 and phosphate buffered saline (PBS) pH 4 and 7.4), and assayed in agarose gel retardation assays. The results showed no DNA release following incubation of chitosan/pDNA nanoparticles for up to 1 month, in all of types incubation medium (Fig. 4a and b). These results indicate that pDNA is very firmly associated with CS/TPP nanoparticles. However, plasmid DNA could be released from the nanoparticles following incubation in lysozyme solution (Fig 5).

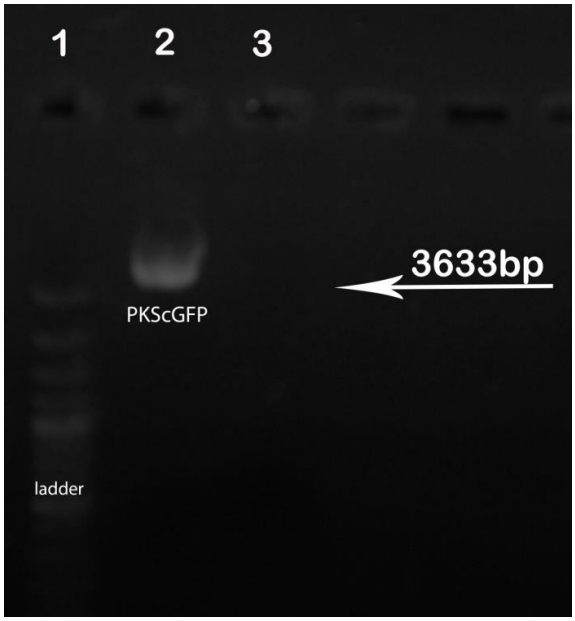

Figure 5: Agarose gel assays following incubation of CS/pDNA nanoparticles with lysozyme

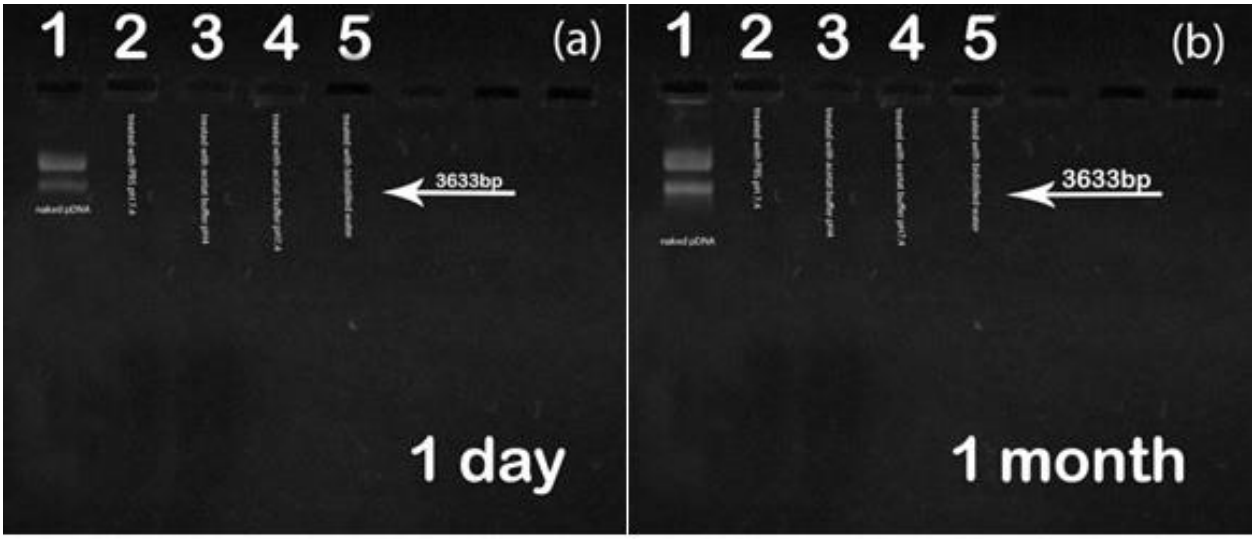

Figure 4: *In vitro* pDNA release studies from CS/pDNA nanoparticles incubated for (a) 1 day and (b) 1month. The studies were performed at pH 4 and 7.4 acetate buffer and PBS at 37 ◦C.(Line 1: naked pDNA, Line 2: CNP treated with PBS pH 7.4, Line 2: CNP treated with acetate buffer pH 4, Line 4: CNP treated with acetate buffer pH 7.4, Line 5: CNP treated with distilled water)

4 Conclusion

In this investigation, we have successfully synthesized ionically crosslinked CS/TPP nanoparticles as an interesting delivery system for nucleic acids such as plasmid DNA. Nanoparticles are spherical in shape with a mean particle size of 173 nm. Due to strong electrostatic interaction, the chitosan/pDNA nanoparticles are highly stable and no pDNA was released after one month in nanoparticles treated with a solution of PBS and acetate buffer in pH 4 and 7.4. This study demonstrated that chitosan–pDNA nanoparticles had great potential in designing novel non-viral gene delivery systems.

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