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Original Article

# Chitosan nanoparticles as non-viral gene delivery systems: Determination of loading efficiency

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ABSTRACT

Chitosan has been studied for use in particle delivery systems for therapeutic purposes, since one of its most important applications is as a non-viral vector in gene therapy. Due to its positive charge, it is capable of forming DNA complexes (polyplexes) obtained through several methods and with the property of protecting nucleic acids. Two methods for obtaining the nanoparticles of chitosan-nucleic acids are reported in this study: simple complexation (of depolymerized chitosan or of different chitosan salts with plasmid) and ionic gelation (by adsorption of plasmid in the nanoparticles or by encapsulation of plasmid into nanoparticles). The determination of the loading efficiency of chitosan nanoparticles with the plasmid is carried out by electrophoretic mobility of the samples on agarose gel. Furthermore, the nanoparticles have been characterized according to their morphology, size and surface charge using AFM, TEM, laser diffraction and dynamic light scattering techniques. The polyplexes obtained have been found to be spherical and nanometric in size (between 100–230 nm) with a zeta potential between 37 and 48 mV. Positive results have been obtained by agarose gel electrophoresis for all studied cases: a concentration of between 20 and 30 μg/mL of chitosan salts is required while for the remaining chitosan samples studied, 100% loading efficiency does not occur until a concentration equal to 100 μg/mL (regardless of previous depolymerisation and the method performed). Chitosan–plasmid nanocapsules have been obtained at the polymer concentrations worked with (between 0.025 and 0.2%).



## 1. Introduction

Gene therapy is attracting attention in the medical, pharmaceutical and biotechnology sectors due to its application for the treatment of diseases for which there is currently no effective conventional therapy. It is known as the set of techniques, which transfer DNA or RNA fragments into specific cells, using vectors and to modulate the expression or suppression of biosynthesis of certain altered proteins, thus, reversing the disorder or genetic alteration [1,2]. Vectors are the systems used to facilitate the transport and entry of genetic material into cells for therapeutic purposes, however, having a suitable vector that provides safety and efficiency becomes a major problem in gene therapy. A variety of vectors have been examined for the release of genetic material and are divided into two categories: viral vectors (modified viruses that retain the ability to infect cells but not of replicating) and non-viral vectors based on chemical or physical methods [3]. Non-viral vectors are being extensively studied and applied as stable transfection systems with low toxicity and are not immunogenic because they can bind to nucleic acids and form nanoparticles that are capable of delivering genetic material to cells [4–11]. Cationic nanoparticles have the property to act as vectors in gene therapy forming complexes known as polyplexes or lipoplexes (depending on the nature of the cationic systems), which are capable of directing the genetic material to target cells [12–16]. These complexes are formed due to the fact that the negatively loaded groups of nucleic acids interact electrostatically with the cationic groups of the nanoparticle to condense it. Thus, a structure is created that envelops the nucleic acid and serves as a protective barrier against nucleases or macrophages, thereby, reducing its elimination from the body. The polymers and cationic lipids, in addition to protecting the genetic material from degradation, condense and package it for release into cells, facilitating

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interaction with cell membranes and their endosomal release [6,17,18]. Among the polymers of natural origin, one of the best known and studied is chitosan. It is a linear cationic polymer composed of D-glucosamine and *N*-acetyl-D-glucosamine units linked by β-(1-4) glycosidic bonds and derived from chitin by a thermoakaline deacetylation process. Chitin is an abundant biopolymer in nature as it is found in the exoskeleton of crustaceans and insects and cell walls of certain fungi and bacteria [19–21]. One of the most important applications of chitosan is as a non-viral vector in gene therapy and for this reason for some years, chitosan has been studied for use in particle delivery systems for therapeutic purposes [22–28]. Mumper et al. [29] were the first to propose the introduction of genes into cells using chitosan as a vector and showing that the chitosan-DNA complexes with mean particle sizes of between 150 and 600 nm in diameter can be formed by mixing a certain proportion of plasmid DNA and chitosan. Following this first study, it was shown that due to its polycationic nature, chitosan is capable of forming colloidal particles and is thus able to trap macromolecules via several mechanisms giving rise to an effective expression and release of nucleic acids both *in vivo* and *in vitro* [30–36]. The positive charge of chitosan enables easy complexing with the negatively loaded DNA and proteins obtaining particles of several nanometers (ranging from 80–500 nm) with the property of protecting the DNA from nuclease action [37–40]. Chitosan nanoparticles can be obtained through several methods using different types of bonds, different arrangements of the polymer chain or by inter- and intramolecular interactions, among others [31,41–43]. The ionic gelation method is one of the most used methods for obtaining chitosan nanoparticles and that receives the most attention, particularly by polyelectrolyte sodium tripolyphosphate (TPP). TPP is useful in the preparation of such chitosan nanoparticles as it exhibits no toxicity and its ability to bind with the polymer [44–49]. The self-assembly of polycations and polyanions leads to the formation of the complexes due to the link between the TPP tripolyphosphoric groups and the chitosan amino groups [42,45,50–53]. While chitosan at physiological pH is partially protonated, the TPP is covalently bound to the chitosan amino groups providing a structural change and better protonation [54]. The most noteworthy advantages of this method are that it is not necessary to apply heat to the sample, no organic solvents are used, and the methodology carried out is not aggressive to the nanoparticles. Furthermore, it has been shown that the ionic gelation method for obtaining nanoparticles is flexible and easily controllable in its application as a protein delivery system [55]. The aim of this work is to develop cationic polymeric nanoparticles from chitosan polymer by simple complexation or ionic gelation, which are able to bind to nucleic acids for further study as genetic material transport and release systems.

## 2. Materials and methods

### 2.1. Material

Low molecular weight chitosan (Sigma-Aldrich®, MA, USA), sodium tripolyphosphate (TPP) (Panreac Química®, Barcelona, Spain), sodium nitrite (Panreac®, Barcelona, Spain), bidistilled water (MilliQ®, Millipore®, MA, USA) acetate buffer solution, four different marketed salts of chitosan hydrochloride and glutamate CL113 (110 kDa), CL213 (270 kDa), G113 (160 kDa), G213 (470 kDa), (Protasan® UP, Novamatrix®, FMC, Norway), sodium hydroxide (Fagron®, Barcelona, Spain), acetic acid (Panreac®, Barcelona, Spain), sodium nitrite (Panreac®, Barcelona, Spain). The following were used for agarose gel electrophoresis: Agarose D-1 Medium EEO (Pronadisa®, Spain), Tris base, acetic acid, EDTA and ethidium bromide. For the charge controller, bromophenol blue, xylene cyanol FF and glycerol. The molecular weight marker used is 1 Kb DNA ladder from New England BioLabs®. The DNA used in the binding experiments is plasmid encoding *C*-ter and *N*-tert-ter regions of the TCERG-1 (or CA150) transcriptional elongation factor [56] of about 7 Kb purified with kits by Qiagen® (Hilden, Germany).

### 2.2. Methods

#### 2.2.1. Methods of obtaining poliplexes

Two methods were carried out for obtaining the nanoparticles of chitosan-nucleic acids (polyplexes): simple complexation (by electrostatic interaction) or ionic gelation. A distinction is made in this latter method between nanoparticles that are obtained by adsorption of the plasmid on the surface of the nanoparticle or by encapsulation of nucleic acids inside of chitosan nanoparticles.

#### 2.2.2. Simple complexation

Simple complexation for obtaining polyplexes was carried out using the method previously adapted and proposed by Katas and Alpar [33], based on the addition of the polymer solution directly to the solution containing the plasmid. After incubation by stirring the sample, chitosan–plasmid complexes form spontaneously. In this section, a distinction is made according to whether the polymer has previously been depolymerized (Sigma-Aldrich®) or different marketed chitosan salts are used (Protasan® UP, Novamatrix®).

*2.2.2.1. Simple complexation of depolymerized chitosan with plasmid.* To carry out chitosan depolymerisation, chitosan (Sigma) in 0.2% acetate buffer solution was dissolved and then the sample was filtered. Having obtained the solution of depolymerized chitosan with sodium nitrite (3.5 mg/mL) after 1 h of agitation (3:1 of chitosan:sodium nitrite respectively), the pH is adjusted (Crison 2000 microph®, Spain) to a value pH of 9, causing precipitation of the polymer particles that are recovered by ultracentrifugation (10 °C, 12,000 rpm, 20 min) (OrtoAlresa® Digicen 20-R, Spain). Once the chitosan particles are obtained, a process of lyophilization is carried out (Telstar® L-3 freeze dryer, Barcelona, Spain). The lyophilized sample was used to prepare a series of concentrations of between 25 and 300 μg/ml of lyophilized chitosan resuspended in bidistilled water after stirring, to which the same volume of plasmid (20 μg/mL) as chitosan solution is added. The samples are incubated by mixing them (Stuart® SSL4 see-saw rockers, UK) at room temperature for 30 min to form chitosan–plasmid complexes.

*2.2.2.2. Simple complexation of different chitosan salts with plasmid.* Simple complexation of the four different types of chitosan (hydrochloride: CL113, CL213 and glutamate: G113, G213) is carried out by preparing the same series of concentrations as in the preceding paragraph. The chitosan salts are previously dissolved in bidistilled water at concentrations of between 25 μg/mL and 300 μg/mL and the plasmid solution (20 μg/mL) is prepared in an equal volume to that of chitosan. The plasmid solution is added dropwise to the chitosan solution. After 30 min of incubation at room temperature (Stuart® SSL4 see-saw rockers, UK), polyplexes are obtained.

#### 2.2.3. Ionic gelation

Via ionic gelation, chitosan nanoparticles are obtained spontaneously by electrostatic interactions with a polyanion. Chitosan nanoparticles are prepared according to the described modified ionic gelation method [41]. The chitosan solution is prepared at a concentration of 0.2% in acetate buffer solution (pH < 6.5). The