



Contents lists available at ScienceDirect

Colloids and Surfaces A: Physicochemical and Engineering Aspects

journal homepage: www.elsevier.com/locate/colsurfa

Elaboration of chitosan nanoparticles: Favorable impact of a mild thermal treatment to obtain finely divided, spherical, and colloidally stable objects



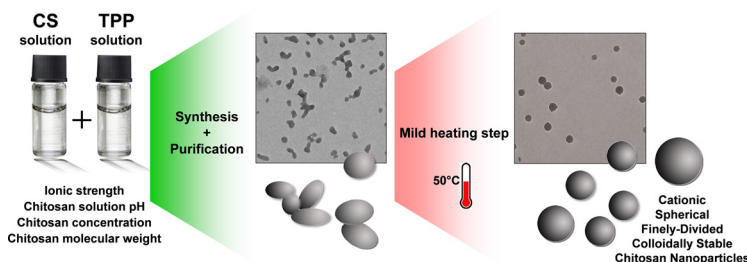
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HIGHLIGHTS

- Chitosan nanoparticles were synthesized by an ionic gelation method.
- The main physicochemical parameters involved in the synthesis were studied.
- Aggregates were successfully reduced by a heat treatment of the colloidal suspension.
- Nanoparticle sphericity was drastically increased through a mild heating.
- Optimized nanoparticles were stable at least 135 days at 20 °C (pH 4.5).

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:

Received 3 April 2014

Received in revised form 12 June 2014

Accepted 15 June 2014

Available online 20 June 2014

Keywords:

Chitosan

Nanoparticle

Morphology

Ionic gelation

Physicochemical characterization

ABSTRACT

The elaboration of chitosan (CS) nanoparticles *via* an ionic gelation process using sodium tripolyphosphate (TPP) as cross-linking agent was thoroughly studied in order to develop colloidally stable, spherical CS nanoparticles with a reproducible sub-micrometer size, and a narrow size distribution. To this end, the most relevant parameters involved in the synthesis such as CS chains concentration and molecular weight as well as ionic strength and pH of CS initial solutions were investigated and optimized. The physicochemical characterization of resulting CS nanoparticles was carried out in terms of size, size distribution, and surface charge by quasi-elastic light scattering (QELS), nanoparticle tracking analysis (NTA), and zeta potential measurements. Morphological characterization using classical and cryogenic transmission electron microscopy (TEM and cryo-TEM) was then performed revealing a high number of aggregates mixed to individualized nanoparticles with drop-like and irregular shapes. These limitations were overcome by applying a specific and optimized thermal treatment which appeared to significantly reduce the aggregate number, and to restructure the CS nanoparticles into highly spherical objects. Based on all these findings, reproducible, cationic (zeta potential = $+37 \pm 5$ mV), stable (at least 4 months at 20 °C), spherical, and mainly individualized CS nanoparticles of 100 ± 30 nm (determined from cryo-TEM images) were obtained.

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1. Introduction

Chitosan (CS) is a linear polysaccharide obtained by deacetylation of chitin and composed of randomly distributed β -(1-4) linked D-glucosamine and N-acetyl-D-glucosamine units. CS has received

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many attentions because of its advantageous biological properties such as biodegradability, biocompatibility, relative non-toxicity, as well as bioactivity [1]. In addition, thanks to its remarkable physicochemical properties, a large variety of CS-based materials such as hydrogels, films, fibers, membranes, sponges, threads, or particles have been designed [2]. Among them, CS nanoparticles have been employed as vectors of proteins [3], anti-cancer agents [4,5], genes or antigens delivery [6,7]. With this significant attractiveness of CS nanoparticles, various elaboration methods have been developed like ionic gelation [8], emulsion/reticulation [9], precipitation/coacervation [10], and polyelectrolyte complexation [11]. Ionic gelation method has been thoroughly examined in order to obtain reproducible and nanometer sized particles with a narrow size distribution, as well as a highly positive surface charge [12]. This simple method, described for the first time by Calvo et al. in 1997, consists in a one-shot addition of a CS solution to a solution of a polyanion as the sodium tripolyphosphate (TPP) [8]. TPP is a widely spread and non-toxic cross-linking agent, replacing glutaraldehyde which is usually used despite its cell toxicity [13]. In the ionic gelation process, the nanoparticles are spontaneously formed within a few minutes by the establishment of several electrostatic interactions between CS chains and TPP molecules. Another advantage of this synthesis is that it occurs in a total aqueous environment, avoiding the use of organic solvents. Numerous studies have discussed the influence of physicochemical parameters involved in this synthesis to obtain reproducible CS nanoparticles with a narrow size distribution [14–17]. However, to our knowledge, no work has been reported on considerations about the nanoparticle morphology which can significantly influence the biological fate of drug vectors once administered in the blood circulation [18]. For example, Zhang et al. [19] have shown that changing polystyrene nanoparticles from 3D spherical shape to 2D disk shape promotes their cell surface binding but with a significant decrease of cell uptake. Dasgupta et al. [20] have very recently demonstrated by theoretical calculations, based on the curvature energy of lipid membranes combined with the particle/membrane adhesion energy, that the cell uptake of spherical nanoparticles is much higher than ellipsoidal nanoparticles, leading to an improved efficiency of spherical nanoparticles for the intra-cellular drug delivery.

The aim of work presented herein is to elaborate reproducible spherical CS nanoparticles with a narrow size distribution and a satisfactory colloidal stability by optimizing different physicochemical parameters, and synthesis steps. Firstly, the influence of concentration and molecular weight of chitosan, as well as pH and ionic strength of biopolymer initial solutions was examined in terms of object size characteristics. Then, a supplementary and simple step was set up in this work, and inserted in the synthesis process to more control the individualization and sphericity of nanoparticles. The particle individualization was estimated by using nanoparticle tracking analysis (NTA) technique based on a particle-by-particle measurement approach (less biased by the larger particles within a sample). Concerning the sphericity characterization, TEM, and more particularly, cryo-TEM experiments were carried out on these CS/TPP nanoparticles for the first time to our knowledge. This technique has the great advantage of measuring the “real” morphology of colloids thanks to a very fast freezing of sample avoiding the artifacts of drying effects encountered in classical TEM.

2. Materials and methods

2.1. Materials

Chitosan (CS) obtained from squid pens was provided by Mah-tani Chitosan Pvt. Ltd. India from batch 114 with a degree of

acetylation (DA) of 2.5% determined by ^1H NMR, a weight-average molecular weight (M_w) of $395,000 \pm 3300 \text{ g mol}^{-1}$, and a dispersity (M_w/M_n or \mathcal{D}) value of 1.770 ± 0.043 determined by a system based on size-exclusion chromatography, as detailed in the below CS characterization section. Sodium tripolyphosphate (TPP, purity >98.0%), glacial acetic acid (99.98%) and glycerol (99.9%) were purchased from Sigma–Aldrich, and deionized water was provided by Aguettant (Versol[®], water for irrigation).

2.2. Chitosan preparation.

Chitosan was previously purified before any use, as described elsewhere [9]. Briefly, the polymer was dissolved in deionized water with acetic acid at a stoichiometric quantity versus the CS amino groups (acetic acid/CS mass ratio=0.35). The solution was filtered through membranes (Millipore) with a decreasing pore size (from 3 to 0.45 μm), followed by a precipitation with an ammonium hydroxide solution. The resulting precipitate was washed with deionized water until neutrality before freeze-drying. Two other CS chain weight-average molecular weights ($M_w = 16,800$ and $163,000 \text{ g mol}^{-1}$) were obtained via a nitrous deamination process previously described by Allan and Peron [21,22]. This process enables to obtain very small reproducible CS chain lengths with low dispersities. To this end, CS was dissolved at 0.5% (w/v) in a 0.2 M acetic acid/0.1 M sodium acetate buffer pH 4.5, and 1 mL of a sodium nitrite solution (0.15 M) was added to the CS solution in order to obtain a final nitrite/glucosamine unit molar ratio of 0.5. The reaction was carried out under magnetic stirring for 5 min and 120 min to get CS weight-average molecular weights of $163,000 \text{ g mol}^{-1}$, and $16,800 \text{ g mol}^{-1}$, respectively. Each resulting CS was recovered by precipitation with an ammonium hydroxide solution, followed by several washings with deionized water until neutrality, and finally, by a freeze-drying step.

2.3. Chitosan characterization.

The weight-average molecular weight (M_w) and the dispersity (\mathcal{D}) of chitosan chains were measured by size-exclusion chromatography (SEC) system (TSKgel G2500PW and G6000PW columns, inner diameter = 7.8 mm, length = 300 mm) coupled online with a refractive index (RI) detector (Optilab T-rEX differential refractometer, Wyatt Technology), and a multi-angle laser light scattering (MALLS) detector (Dawn EOS, Wyatt Technology) equipped with a laser operating at $\lambda = 690 \text{ nm}$. A degassed and filtered on 0.1 μm 0.2 M acetic acid/0.15 M ammonium acetate buffer (pH 4.5) was used as eluent, with a flow rate of 0.5 mL min^{-1} . A CS refractive index increment value, dn/dc , of 0.198 mL g^{-1} in the same buffer as the eluent was selected according to the CS DA used in this study [23].

Concerning the CS solution viscosity measurements, the Newtonian viscosity of CS solutions was determined using an AR2000 rheometer (TA instruments, C.A.) equipped with a couette geometry (TA instrument C.A.). Steady sweep measurements were carried out at 25°C by applying shear rate from 10 to 1000 s^{-1} with 10 points per decade.

2.4. Synthesis of CS/TPP nanoparticles

The CS/TPP nanoparticles were prepared at $20 \pm 2^\circ\text{C}$ via an ionic gelation process described for the first time by Calvo et al. [8]. The different physicochemical parameters involved in the nanoparticle synthesis process are presented in Table 1.

The first step of the synthesis is the dissolution of CS of various molecular weights in water with an acetic acid concentration of

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