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Role of proton balance in formation of self-assembled chitosan nanoparticles

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ABSTRACT

Researchers have explored the ability of chitosan to form nanoparticles, to suit varying applications, ranging from wound-healing to gene delivery. Ionic gelation is a widely used method for formulating chitosan nanoparticles, where self-assembly plays a crucial role. This self-assembly is initially promoted by hydrophilic-hydrophobic parity amongst individual chitosan residues, along with electrostatic and Van der Waals interactions with the cross-linker. However, until now the intrinsic ability of chitosan to self-assemble is not widely studied; hence, we investigate the self-assembly of chitosan, based on proton balance between its protonated and deprotonated residues, to promote facile nanoparticle synthesis. This is one of the first reports that highlights subtle but critical influence of proton balance in the chitosan polymer on the formation of chitosan nanoparticles.

1. Introduction

Chitosan has varied applications in diverse areas due to its biocompatibility, biodegradability, polycationic nature and non-toxic properties [1,2]. The versatile properties of this polymer have been explored for delivering labile therapeutic molecules like proteins or nucleic acids or development of bioplastics for sustainable applications [3,4]. Chitosan is derived by deacetylation of chitin and consists of a blend of acetylated and deacetylated glycopyranose units. Solubility of chitosan requires acidic media, as amine groups are protonated in this condition [5]. Protonation of amine groups creates electrostatic repulsion within the individual polymer chains reducing the intra- and inter- hydrogen bonding, weakening the attractive forces within the inter-chain residues, thereby promoting solubility. Hence solubility is determined by its protonation in solution, which in turn is associated with its degree of deacetylation (DD). However, reports state that at high protonation, *i.e.*, at pH below 3.5, depolymerisation of chitosan takes place, which is not observed at pH above 4.5 [6]. Further, at a high protonation state, the polymer behaves as "rigid rods" [7], with high repulsive forces acting between the polymer chains. These forces suppress the intra and inter-hydrogen bonds, thus creating a strain on the polymer chains and resisting their native curvature. Consequently, the chains break to relax the strain and attain a stable state, eventually resulting in depolymerisation of the polymer. A study by Krishnan et al. reiterates the involvement of protonation in depolymerisation of chitosan into its low molecular weight oligosaccharides [8]. Thus, solution behaviour and depolymerization of chitosan are strongly influenced by the DD, persistence length (L_p) and molecular weight (M_w) of the polymer [5,9,10]. Therefore, it is vital to equilibrate the degree of protonation of this polymer to enable its reproducible utility in diverse applications.

Protonation of chitosan is an attribute of pH; consequently, the degree of protonation can be inferred from the pH of chitosan solution. Thus, while formulating chitosan nanoparticles (Chnps), we observed that modulation in pH had a significant influence on the formulated particles. At pH 5.5, nanoparticles of desired size were achieved along with a homogenous particle distribution. These nanoparticles were prepared by ionic gelation method, which involved anionic sodium tripolyphosphate (Na-TPP) to cross-link the positively charged chitosan polymer, through electrostatic interactions [11,12]. Although formation of nanoparticles is a function of self-assembly, it was observed that the nanoparticles could be formed only at the pH 5.5 and a higher or lower pH disturbed the particle characteristics. Thus the self-assembly of Chnps was

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governed by protons, through a balance between hydrophobic and hydrophilic forces of the milieu.

Chitosan chains possess an intrinsic property to self-assemble among themselves because of a balance amongst the various forces (hydrophobic forces, electrostatic and van der Waal's interaction and hydrogen bonding) arising out of protonation of its residues [13,14]. In this intrinsic self-assembled state, isolated chitosan residues possess optimal surface charge to cross-link with Na-TPP, without being affected due to electrostatic repulsion amongst protonated domains or due to short range attractive forces that lead to formation of ideal nanoparticles (size and distribution) [15]. However, since chitosan possesses random sequence of acetylated and deacetylated residues, the degree of protonation may vary and hence affect the pH of self-assembly. Thus a detailed knowledge about proton balance is essential for the mechanistic understanding of self-assembled Chnps, to aid a stable and reproducible formulation, which is the focus of this investigation.

In this study, umbrella sampling (US) was employed for computationally calculating the free energy change of a system to study self-assembly of Chnps. US utilizes a biased potential that is harmonic in nature, hence providing energy required for a molecule to cross the energy barrier [16]. This harmonic, biased potential is applied along a reaction coordinate, such as the distance between the centre of mass (COM). Subsequently unbiasing of the obtained results gives the potential of mean force (PMF), which is basically the potential that gives an average force over all the configurations of a given system [17]. According to Zhang et al., the PMF arises out of two types of interactions, electrostatic and excluded-volume type [18]. Hence, an attempt was made to characterize the interactions that prevail amongst chitosan residues, by studying the change in their PMF profiles, under different protonation conditions that replicate the effect of pH on self-assembly of Chnps.

2. Materials and method

2.1. Materials

Chitosan (Mw ~150–200 kDa, DD ~95%) was purchased from Hi Media Laboratories Pvt. Ltd., Mumbai, India. Sodium tripolyphosphate pentabasic (Na-TPP) and sodium hydroxide (NaOH) were procured from Sigma–Aldrich Co. LLC, Germany. Deionized, double-distilled water (Milli-Q Plus system, Millipore, Bedford, MA, USA) was used in all the experiments.

2.2. *Investigating* in situ *formation of chitosan nanoparticles*

2.2.1. Preparation of Chnps

Chnps were prepared by the ionic gelation method [19–21]. Solutions of chitosan and Na-TPP were prepared at the concentration of 1 mg/mL. Chitosan solution was prepared in 1% (v/v) acetic acid. Na-TPP solution was added to chitosan solution, followed by stirring the mixture at 300–500 rpm for 1 h, at 25° C. Formation of nanoparticles was confirmed from opalescence of the mixture at the end of 1 h. Throughout the investigation, the ratio of chitosan to sodium tripolyphosphate (Na-TPP) was maintained at 5:1 (v/v), as optimal nanoparticles, with regards to size, pDI and surface charge was obtained using this ratio [12,19].

2.2.2. Formation of Chnps at varying pH conditions

Chnps were formulated at different pH conditions (native pH *i.e.*, 2.89, 4.09, 5.6), using the method as stated in Section 2.2.1. Briefly, chitosan was solubilized in 1% (v/v) of acetic acid (1 mg/mL), followed by attaining the desired pH through addition of 1N NaOH. Thereafter, the solution was stirred for some time to attain equilibrium, before adding Na-TPP solution (1 mg/mL) to formulate the nanoparticles.

2.2.3. Characterization of Chnps

2.2.3.1. Dynamic light scattering (DLS). The size (hydrodynamic diameter) and surface charge of Chnps was characterized using DLS, on a Zetasizer Nano ZSP (Malvern Instruments, UK). This instrument works on the principle of dual DLS system, where it uses forward scatter at 90° and non-invasive back-scatter (NIBS) at 173°, which makes the system highly sensitive in detecting molecular size or aggregation behaviour of polymers and proteins. Thus, principally the system is able to distinguish nanoparticles from merely entangled polymer structures. All the measurements were carried out at 25 °C, in triplicates.

2.2.3.2. Cryo-transmission electron microscopy (Cryo-TEM). The morphology of nanoparticles was studied using Cryo-TEM; (Philips CM120 transmission electron microscope) for visualising the supra-molecular complex structures. Cryo-TEM was performed according to a procedure reported by Danino et al. and Danino [22,23]. Briefly, samples were prepared at a controlled temperature of 25 °C and 100% humidity, in the Controlled Environment Vitrification System (CEVS). A small drop of each test sample (Chnps formed at different pH) was placed on a perforated carbon film, supported on a TEM copper grid that was held using tweezers. Thinning and removing excess solution with a piece of filter paper produced a liquid sample film of ~100–250 nm in thickness. Then, the specimen was plunged into a reservoir of liquid ethane and cooled with liquid nitrogen to ensure vitrification. Vitrified samples were stored in liquid nitrogen at −195 °C, until examination under the microscope. During imaging, a vitrified specimen was mounted onto a Gatan 626 cryogenic sample holder, cooled with liquid nitrogen to below −170 °C and maintained at this temperature during the entire investigation. All samples were investigated under a low electron dose, in an FEI T12 G2 TEM, operated at 120 kV. Images were recorded on a Gatan US1000 2k × 2k high-resolution cooled CCD camera, using Digital Micrograph software.

2.3. *Investigating* in silico *formation of chitosan nanoparticles*

2.3.1. Investigating interaction between chitosan chains

A prototypical model of a 10-mer chitosan was generated using the software Chimera version 1.8 [24]. The variation in the state of protonation was brought about by randomly inserting *N*-acetylated glucosamine units (CHA) and protonated glucosamine units (CHP) within the sequence. A total of 5 systems were thus created, with degrees of protonation (DPr) as 0, 40, 50, 60 and 100%. Ideally, 0% and 100% system does not exist in reality and these systems were assumed for experimental feasibility. A model of polymer was created by inserting two chitosan chains, with centre of mass (COM) separation of 5 nm, in a periodic box of 6.560 nm × 4.362 nm × 12 nm. These systems were then subjected to umbrella sampling (Section 2.3.3).

2.3.2. Investigating interaction between chitosan and Na-TPP

Nanoparticle formation was studied by evaluating the interaction between chitosan and Na-TPP. The chitosan polymer (10-mer, varying degree of protonation) and Na-TPP were subjected to umbrella sampling simulation. This interaction was investigated with chitosan having different degrees of protonation (DPr) *viz.*, 0, 40, 50, 60, 100%. A periodic box of dimension 3.280 nm, 2.181 nm, 2.477 nm was optimized. Further, the systems were equilibrated and PMF's were generated (Section 2.3.3).