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Preparation, physical–chemical and biological characterization of chitosan nanoparticles loaded with lysozyme

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ABSTRACT

A commercially available chitosan (CS) was employed in the formulation of nanoparticles loaded with lysozyme (LZ) as antimicrobial protein drug model. Due to the variability of commercially available batches of chitosans and to the strict dependence of their physical and biological properties to the molecular weight (M_w) and deacetylation degree (DD) of the material, the CS was fully characterized resulting in weight-average molecular weight of 108,120 g/mol and DD of 92%. LZ-loaded nanoparticles (LZ-NPs) of 150 nm diameter were prepared by inotropic gelation. The nanoparticles were effectively preserving the antibacterial activity of the loaded enzyme, which was slowly released over 3 weeks *in vitro* and remained active toward *Staphylococcus epidermidis* up to 5 days of incubation. Beyond the intrinsic antibacterial activity of CS and LZ, the LZ-NPs evidenced a sustained antibacterial activity that resulted in about 2 log reduction of the number of viable *S. epidermidis* compared to plain CS nanoparticles. Furthermore, the LZ-NPs showed a full *in vitro* cytocompatibility toward murine fibroblasts and, in addition to the potential antimicrobial applications of the developed system, the proposed study could serve as an optimal model for development of CS nanoparticles carrying antimicrobial peptides for biomedical applications.

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1. Introduction

Chitosan (CS) is a linear copolymer of β-(1–4) linked 2-acetamido-2-deoxy-β-D-glucopyranose and 2-amino-2-deoxy-β-D-glucopyranose. It is obtained by deacetylation of its parent polymer Chitin, the second most abundant natural polymer in nature after cellulose, found in the structure of a wide number of invertebrates (crustaceans' exoskeleton, insects' cuticles) and in the cell walls of fungi. Due to its natural origin, CS can not be defined as a unique chemical structure but as a family of polymers, namely chitosans, which present a high variability in their chemical and physical properties. This variability is related not only to the origin of the pristine Chitin, but also to the method adopted for their preparation. Chitosans, with the main structural differences being represented by the relative proportions of *N*-acetyl-D-glucosamine/D-glucosamine residues and the molecular weight (M_w), provide specific chemical and physical features affecting their biological properties. Chitosans are used in various fields of applications comprising food, biomedicine and agriculture, among others [1]. The bactericidal action of chitosans is well known and it has been widely investigated especially in the recent years. The mechanism of the bactericidal effect of chitosans has been attributed to an electrostatic interaction between NH_3^+ groups of CS and phosphoryl groups of phospholipid components and lipopolysaccharides (LPS) of bacterial cell membranes, which increases the permeability, forms pores, and ultimately disrupts the bacterial cell membranes, with the release of cellular contents [2,3]. The bactericidal effect has also been correlated to the structural properties of chitosans [4–8], leading also to the determination of alternative bactericidal mechanisms as the blocking of the RNA transcription by direct interaction of DNA with low M_w chitosans penetrated into the bacterial cell [9]. Bactericidal effects have been described also for chitosan constructs such as, membranes [10], and composites [11] (*e.g.* with silver [12,13], and copper [14]). Furthermore, several studies report on the use of CS for the formulation of micro/nanoparticles as drug delivery systems, either alone [15] or combined into polyelectrolyte complexes (PEC) with alginate [16] or poly(γ)-glutamic acid (PGA) [17]. The latter PEC form has also been applied for the loading of lysozyme (LZ) for bactericidal purpose [18]. LZ is a 14.3 kDa

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enzyme, which catalyzes the hydrolysis of β-1,4-linkages between *N*-acetylmuramic acid and *N*-acetyl-D-glucosamine residues in a peptidoglycan [19]. It is a relatively small protein (129 aa) with an optimal pH in the range 6–9, an isoelectric point near 9.2 and antimicrobial, antiviral, antitumor and immune-modulatory activities. Besides being a good model protein for its structural and enzymatic properties [20,21], LZ received attention also for its use as food preservative and in the cosmetic field.

In the present work, a commercially available CS has been employed in the formulation of nanoparticles loaded with LZ as antimicrobial protein drug model. Due to the variability of commercially available batches of CS and to the strict dependence of their physical and biological properties to the structural features (M_w and deacetylation degree—DD) of the material, this study was conducted starting from the characterization of the employed material, followed by the determination of the physical and biological properties of the developed CS–LZ nanosystem (LZ-NPs), with particular attention to its enhanced bactericidal effect compared to the unloaded CS nanoparticles (CS-NPs). It is our convincement that this approach should be followed especially when CS is applied for the development of bactericidal constructs, for reproducibility aims and scientific significance of the data collected. To our knowledge, this is the first time that a CS–LZ nanoparticulate system is investigated starting from the determination of the most important material properties to correctly identify the CS used.

2. Materials and methods

2.1. Materials

CS (weight-average molecular weight M_w 50–190 kDa and DD 75–85%), sodium tripolyphosphate (TPP) LZ from chicken egg white (~100,000 U/mg), D-(+)-glucosamine hydrochloride (GlcN) purity 99%, *N*-acetyl-D-glucosamine (GlcNAc) purity 99%, and potassium bromide (KBr) FTIR grade, were all purchased from Sigma-Aldrich, Milan, Italy. Pullulan (Molecular weight 5.8–853k) was obtained from Showa Denko K.K., Tokyo, Japan. Acetic acid analytical grade was obtained from Carlo Erba, Milan, Italy. Deionized water (Milli-Q, ddH_2O) was used throughout the experiments. Cell line 3T3/BALB-C Clone A31 mouse embryo fibroblast (CCL163) was purchased from American Type Culture Collection (ATCC, LGC standards, Milan, Italy) and propagated as indicated by the supplier; Dulbecco's Modified Eagles Medium (DMEM), 0.01 M pH 7.4 Dulbecco's Phosphate Buffer Saline without Ca^{2+} and Mg^{2+} (DPBS), bovine calf serum (BCS), glutamine and antibiotics (penicillin/streptomycin) were purchased from GIBCO/Brl, Monza, Italy; Cell proliferation reagent WST-1 was provided by Roche diagnostic, Milan, Italy.

The *S. epidermidis* strain used in the study was purchased from the American Type Culture Collection (*S. epidermidis* ATCC 35984). For preparation of stock cultures, the bacterial strain was grown in Tryptone soya broth (TSB) (Oxoid Basingstoke, United Kingdom) at 37 °C until mid-log phase, subdivided in aliquots, and kept frozen at −80 °C until future use. For colony forming units (CFU) count serially diluted bacterial suspensions were plated on Tryptone soya agar (TSA) (Oxoid, Basingstoke, United Kingdom) and incubated for 48 h at 37 °C.

2.2. Characterization of chitosan

The Gel Permeation Chromatography (GPC) apparatus consisted of a Waters™ 600 Controller equipped with a Waters 410 Differential Refractometer and a Waters ™ 600 Pump, managed by ChromNAV software (Jasco Europe, Lecco, Italy). An Ultrahydrogel™ guard column and two Ultrahydrogel™ linear 6–13 μm columns (7.8 × 300 mm) (Waters, Milford, USA) were employed. The eluent was 0.5 M sodium acetate buffer and the flow rate was maintained at 1 ml/min. Column temperature was kept at 40 °C in Jones oven 7971 (Jones, USA). Pullulan standards (Polymer Laboratories, UK) were used to obtain a calibration curve. CS samples were dissolved in the eluent at a concentration of 2 mg/ml.

Fourier transform infrared spectroscopy (FTIR) measurements were carried out by a Perkin-Elmer Spectrum One spectrophotometer (Perkin-Elmer, Monza, Italy). Absorbance spectra of 1.5 wt% CS pellets were taken as an average of 32 scans with 2 cm^{-1} resolution in the frequency range 4000–400 cm^{-1}. Prior to analysis, CS and KBr were dried at 60 °C for 2 h.

Ultraviolet spectra were recorded in the range 200–250 nm using a Jasco V-530 UV/V spectrophotometer. Calibration curves for GlcN and GlcNAc were drawn through a linear regression between the concentration and first derivative UV signal arising from each one at 205 nm (0.6–2 mM, R^2 = 0.9921 and 0.02–0.3 mM, R^2 = 0.9904, respectively). A solution of acetic acid 0.01 M was used as blank. Accurately weighed (10 mg) CS samples were dissolved in 1 ml of acetic acid 0.1 M and diluted 10-fold with distilled water to obtain a final acetic acid concentration of 0.01 M. CS was not dissolved directly in acetic acid 0.01 M since it would be difficult to get a complete dissolution of the sample in a reasonable short time.

Thermogravimetric analysis (TGA) evaluations were performed using a Mettler TA 4000 System instrument (Mettler Toledo, Milan, Italy) consisting of TGA-50 furnace with a M3 microbalance, and $STAR^e$ software 9.01 (Mettler Toledo). Samples of *ca.* 5 mg were scanned at 10 °C/min from 25 to 700 °C, under 300 ml/min flow rate of nitrogen.

Differential scanning calorimetry (DSC) measurements were performed using a Mettler DSC-882 instrument. The DSC curves were performed using 8 mg samples under nitrogen atmosphere on aluminium pans. The scanning rate was 5 °C/min in the range 25–500 °C.

2.3. Preparation of blank or lysozyme-loaded nanoparticles

Several nanoparticles (NPs) formulations were prepared using a simple ionic gelation process [22] (Table 1). Briefly, CS was dissolved in 1% (v/v) acetic acid (1 mg/ml) and TPP was dissolved in water (0.5–1 mg/ml); for LZ-NPs, 2.5 mg of the enzyme were added to the CS solution. NPs formed spontaneously upon addition of 2 ml of TPP aqueous solution to 5 ml of CS solution under magnetic stirring; the mixture was stirred at room temperature for 2 h. NP suspensions were purified by centrifugation in ALC® (Milan, Italy) PK121R centrifuge at 8500 rpm for 60 min, at 4 °C.

2.4. Characterization of NPs

2.4.1. Physical–chemical characterizations

The size distribution of the developed NPs was measured by mean of dynamic light scattering (DLS) (Coulter LS230 Laser Diffraction Particle Size Analyzer, Beckman Coulter, Nyon, Switzerland). The Zeta-potential of the developed formulations was evaluated using a Beckman-Coulter Delsa™ Nano C, at 25 °C and aqueous solutions pH 6.6. The morphology of the nanoparticles was assessed through Scanning Transmission Electron Microscopy (STEM) by using a GEMINI® Multi-Mode STEM (Carl Zeiss Microscopy GmbH). Samples were diluted in ethanol (1/200) and directly air dried on the grid, before the analysis.

2.4.2. Evaluation of LZ loading capacity of NPs

The loading content and encapsulation efficiency of the NPs were determined as previously described [23]. Briefly, LZ-NPs were collected by centrifugation at 8500 rpm 4 °C for 60 min; the amount of free LZ in the supernatant was measured recording

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