Journal of Controlled Release 157 (2012) 383–390

Contents lists available at SciVerse ScienceDirect

Journal of Controlled Release

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

# Microencapsulated chitosan nanoparticles for pulmonary protein delivery: In vivo evaluation of insulin-loaded formulations

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ARTICLE INFO

*Article history:*
Received 1 May 2011
Accepted 5 August 2011
Available online 12 August 2011

*Keywords:*
Chitosan nanoparticles
Dry powders
In vivo administration
Lung distribution
Pulmonary protein delivery

ABSTRACT

This work presents a new dry powder system consisting of microencapsulated protein-loaded chitosan nanoparticles (CS NPs). The developed system was evaluated in vivo in rats in order to investigate its potential to transport insulin (INS), a model protein, to the deep lung, where it is absorbed into systemic circulation. The INS-loaded CS NPs were prepared by ionotropic gelation and characterized for morphology, size, zeta potential, association efficiency and loading capacity. Afterwards, the NPs were co-spray dried with mannitol resulting in a dry powder with adequate aerodynamic properties for deposition in deep lungs. The assessment of the plasmatic glucose levels following intratracheal administration to rats revealed that the microencapsulated INS-loaded CS NPs induced a more pronounced and prolonged hypoglycemic effect compared to the controls. Accordingly, the developed system constitutes a promising alternative to systemically deliver therapeutic macromolecules to the lungs, but it can also be used to provide a local effect.


*To Prof. José Luis Vila-Jato, in memoriam*

## 1. Introduction

Therapeutic macromolecules (i.e. peptides and proteins) are prone to intestinal enzymatic degradation and exhibit poor membrane permeability due to their hydrophilicity and large size. This is the explanation for their usual administration as injectable formulations, which causes patient's pain and discomfort. Thus, these molecules are good candidates for non-invasive administration through mucosal routes, such as the pulmonary. Lungs are ideally suited for this purpose as they are characterized by large absorptive surface area, high vascularization and thin blood–alveolar barrier which, together, facilitate macromolecule transport into systemic circulation [1]. When compared to the oral route, lungs have become more enticing for drug delivery due to the absence of hepatic first pass-effect and low enzymatic activity [2]. Besides, the possible targeted drug delivery to the lungs is an attractive therapeutic approach that may result in reduced administered dose, as well as reduced drug side effects [3]. Different peptides and proteins have been successfully explored for pulmonary administration, such as insulin [1,4,5], calcitonin [6], human growth hormone [7], rhDNase [8] and interferon-alpha [9]. Moreover, inhalation aerosols offer significant potential for pulmonary administration of macromolecules for either local or systemic effect [5,10]. From the formulations available for inhalation, dry powders are usually preferred, as they have demonstrated the most suitable behavior in terms of stability and bioavailability of active ingredient, compared to the liquid counterparts.

Despite the advantages mentioned above, the lungs present several challenges to drug delivery which include airway geometry and humidity, pulmonary epithelium and the specific defense mechanisms, including the mucociliary escalator, as well as the macrophagic and enzymatic activities [11–13]. In addition, the presence of lung disease might also affect therapeutic outcomes. These physiological barriers are known to interfere with inhalation therapy and, therefore, adequate drug carriers are required to overcome the imposed limitations, enhancing aerosolization properties and delivery and, consequently, drug bioavailability. To this end, many formulation strategies have been pursued, such as the use of protease inhibitors, macrophage activity suppressors, surfactants, as well as mucoadhesive and permeation enhancing polymers [6,7,14,15].

Polymeric nanoparticles (NPs) have been an exciting approach for lung delivery due to their ability to enter intracellular compartments and escape macrophages phagocytosis. Furthermore, they provide the possibility of achieving high drug loading capacity, sustained release, enhanced drug stability and absorption, as well as targeted deposition [3]. Chitosan (CS) is attractive for transmucosal drug delivery, e.g. pulmonary administration, owing to its reported low toxicity, biodegradability, biocompatibility [16] and mucoadhesivity, as well as enhancement of macromolecules permeation [17], thus being extensively employed in the development of micro- and nanocarriers. Furthermore, it has also been used as a dispersibility enhancer for dry powders [18]. We developed CS-based NPs, using an ionotropic

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doi:10.1016/j.jconrel.2011.08.008

gelation technique [19], that show a great potential for transmucosal delivery of macromolecules [20]. However, NPs are impractical for pulmonary administration due to the fact that their direct delivery poses many challenges, including formulation instability due to particle–particle interactions and poor delivery efficiency due to exhalation of low-inertia NPs [21]. We proposed, thereby, the microencapsulation of these NPs through a spray drying technique and using a sugar, in order to obtain a dry powder [22]. Microencapsulation improved NPs handling and aerosolization performance, achieved by enhanced stability and acquisition of aerodynamic properties which result definitively in efficient lung delivery [22,23]. Interestingly, these new powders have been shown to be biocompatible with pulmonary cell lines [16]. For deep lung deposition, dry powders are mainly required to bear adequate aerodynamic characteristics, e.g. the aerodynamic diameter which must be less than 5 μm [24]. Besides, micro- and nano-particles based delivery can provide control over drug release profile and targeting at the tissue and cellular levels.

In the current study, we investigate in vivo the potential of aerosol delivery of microencapsulated peptide-loaded CS NPs, which are in the dry powder form. We expect that this spray dried powder is able to deliver the NPs which, then, mediate the macromolecule absorption, resulting in a systemic effect.

## 2. Materials and methods

### 2.1. Materials

Two types of CS in the form of hydrochloride salt and differing in the Mw (Protasan® 213Cl and Protasan® 113Cl) were purchased from Pronova Biopolymer, A.S. (Norway). Their main characteristics are summarized in Table 1. Bovine insulin (INS) (Mw = 5.7 kDa), fluorescein isothiocyanate albumin (FITC-BSA) (Mw = 67 kDa), pentasodium tripolyphosphate (TPP), glycerol, D-mannitol, phosphate buffered saline tablets (PBS) pH 7.4 and heparin were supplied by Sigma Chemicals Co. (USA). Trifluoroacetic acid (TFA) HPLC grade (≥99.9%) was purchased from Sigma-Aldrich Chemie (Steinheim, Germany). Acetonitrile HPLC grade (≥99.9%) was from Merck KgaA (Darmstadt, Germany). Millipore filters (0.22 μm filters, Millipore Type GV membranes) (Millipore Ibérica, Madrid, Spain) were used to filter HPLC solutions. Ultrapure water (MilliQ plus, Millipore Ibérica, Spain) was used throughout. All other chemicals were reagent grade.

### 2.2. Preparation of chitosan nanoparticles

CS/TPP NPs (CS NPs) were prepared by ionotropic gelation [19]. Two types of CS, 213 and 113, were dissolved in purified water to obtain solutions of 1 mg/mL (w/v) and 0.83 mg/mL (w/v), respectively. Insulin (INS) was dissolved in 0.01 M NaOH to have concentrations of 1.5, 1.8 and 2 mg/mL, to be mixed afterwards with equal volumes of 1 mg/mL TPP to obtain the anionic solution. CS/TPP/INS NPs (INS-loaded CS NPs) were spontaneously formed upon incorporation of 1.2 mL or 1 mL of the anionic solution (INS + TPP) onto 3 mL of CS 213 and 113, resulting in a final NPs suspension of 4 or 4.2 mL. Reaction was maintained under mild magnetic stirring for 10 min. CS NPs were isolated for further analysis by centrifugation on a 10 μL glycerol layer (16,000×*g*, 45 min, 15 °C), and then re-suspended in 100 μL of purified water after discarding the supernatants. The procedure for NPs preparation was optimized in terms of formulation pH, stirring rate, centrifugation time and amount of glycerol.

Table 2 summarizes the ratios and concentrations used for each NPs formulation. The theoretical INS content based on CS was 30% (w/w) for NPs made of CS 213 and 40% (w/w) for NPs made of CS 113.

**Table 1**
Physicochemical characteristics of chitosan samples (Protasan® HCl).

| Property | Chitosan® 113Cl | Chitosan® 213Cl |
|---|---|---|
| Molecular weight, Mw (KDa) | <150 | 150–400 |
| Viscosity (mpa.s, 25 °C) | <20 | 20–200 |
| Deacetylation degree (%) | 75–90 | 75–90 |
| Key | CS 113 | CS 213 |

### 2.3. Determination of nanoparticles production yield

The NPs production yield was calculated by gravimetry. Fixed volumes of NPs suspensions were centrifuged (16,000×*g*, 45 min, 15 °C) and sediments of NPs were freeze-dried over 48 h (24 h set at −34 °C and gradual ascent until 20 °C), using a Labconco Freeze Dryer (Labconco, USA) (n = 6). The process yield was calculated as follows:

$$\text{Process Yield (\%)} = \frac{\text{NPs weight}}{\text{Total solids(CS + TPP + INS) weight}} \times 100$$

### 2.4. Characterization of nanoparticles morphology, size and zeta potential

The morphological examination of NPs was performed by transmission electron microscopy (TEM) (CM 12 Philips, Eindhoven, Netherlands). The samples were stained with 2% phosphotungstic acid and placed on copper grids with Formvar® films for viewing by TEM.

Sizes and zeta potentials were measured on freshly prepared samples by photon correlation spectroscopy and laser Doppler anemometry, respectively, using a Zetasizer® Nano-ZS (Malvern instruments, Malvern, UK). For the particle size analysis, each sample of isolated NPs was diluted to the appropriate concentration with ultrapure water. Then, analysis was performed at 25 °C for 3 min at a detection angle of 173°. For the determination of zeta potential or electrophoretic mobility, isolated NPs were diluted with 0.1 mM KCl and placed in an electrophoretic cell, where a potential of ±150 mV was established. Size and zeta potential of each formulation were analyzed in triplicate (n = 3).

### 2.5. Determination of protein association efficiency and protein-loading capacity of nanoparticles

The association efficiency refers to the amount of protein associated with formed NPs, expressed as the percentage of the total amount of protein added in the process; whereas, loading capacity is defined as the percentage by weight of the associated protein in the resultant NPs. To determine these parameters, NPs were separated by centrifugation (16,000×*g*, 45 min, 15 °C) from the aqueous preparation medium containing the non-associated protein. The amount of free INS was determined in the supernatant by HPLC assay (see below). Each sample was assayed in triplicate (n = 3). The protein association efficiency and NPs loading capacity were calculated as follows:

$$\text{Association Efficiency (\%)} = \frac{\text{Total INS amount} - \text{Free INS amount}}{\text{Total INS amount}} \times 100$$

$$\text{Loading Capacity (\%)} = \frac{\text{Total INS amount} - \text{Free INS amount}}{\text{NPs weight}} \times 100$$

### 2.6. High Performance Liquid Chromatography (HPLC) assay

Free INS in the supernatant was measured by means of a previously described and modified method [26,27]; using a HPLC system Agilent 1100 series (Santa Clara. CA, USA) equipped with a pump, a diode array detector, a column (C18 monomeric 120A