Inhalable chitosan microparticles as tools in tuberculosis therapy

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Summary: Lung tuberculosis (TB) represents approximately 80% of total cases and, therefore, the lung has been explored as an effective route for the delivery of drugs in the ambit of pulmonary TB. The pulmonary delivery of antitubercular drugs in a carrier system capable of reaching the alveoli, being recognized and phagocytosed by alveolar macrophages (mycobacterium hosts), would be a significant improvement to current oral drug regimens. Chitosan (CS) is a polysaccharide composed of *N*-acetylglucosamine and D-glucosamine residues, the former being recognised by macrophages and possibly potentiating phagocytosis. This work aimed at producing chitosan microparticles (CS MP) containing two first-line antitubercular drugs, isoniazid (INH) and rifabutin (RFB). A polymeric solution containing 10% (w/w) of INH and 5% (w/w) RFB (weight respective to CS), was spray-dried and the resulting microparticles evaluated as dry powder inhalation targeting alveolar macrophages. Spray-dried CS MP with theoretically adequate properties for deep lung delivery (aerodynamic diameter of 1.98 µm) were produced, efficiently associating isoniazid (INH) and rifabutin (RFB) – 73% and 97%, respectively – in combination. The effect of drug-loaded CS MP on the viability of two cell lines representative of the environment of relevance in pulmonary TB were assessed and absence of toxicity was observed in human alveolar epithelium (A549) and macrophage-differentiated (THP-1) cells. Human macrophage-differentiated THP-1 cells and rat alveolar macrophages NR8383 were exposed to fluorescein-labelled CS MP to assess the ability of CS MP to be taken up by alveolar macrophages. The analysis was performed by flow cytometry and CS MP evidenced strong ability to be captured by macrophages (percentage of phagocytosis >98%). Overall, the obtained data gave positive indications on the potential of the proposed system for an application as inhalable tuberculosis therapy.

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Introduction: Although tuberculosis is a curable condition, it remains a major global health problem. The lung has been explored as an effective route for the delivery of drugs in the ambit of respiratory diseases, allowing direct targeting of the affected organ and possibly reducing systemic drug toxicity, which is a special advantage in diseases involving long-term treatments, such as TB. The approach in this case involves direct delivery of antibiotics to the infection site, thus possibly decreasing severe systemic side effects, such as hepatotoxicity and nephrotoxicity, and reducing the period of TB treatment, which are main reasons for patient incompliance. However, several limitations of pulmonary delivery have to be considered as well, mainly related with airway structure and specific defense mechanisms, such as the mucociliary clearance. Overcoming these limitations demands the design of aerodynamically suitable carriers that are capable of reaching the alveoli. Additionally, further benefit may be attained if the carriers can be recognised and phagocytosed by alveolar macrophages (mycobacterium hosts), an effect that can be mediated by a composition of the carriers favouring recognition by macrophage surface receptors (1,2). In this context, this work aimed at using CS to produce MP that efficiently associate a combination of the first-line antitubercular drugs INH and RFB, for an application in pulmonary tuberculosis therapy.

Materials and Methods: CS MP were successfully produced by spray-drying (Buchi mini-spray dryer, B-290) a 2% (w/v) aqueous solution of CS (116 KDa; Sigma-Aldrich), with or without drugs – 10% (w/w) INH (3), and 2% (w/w) RFB (w/w respective to CS). Given the well-known potency of RFB as anti-TB agent (4) and after verifying the strong cell toxicity induced by RFB (data not shown), a concentration of 2% (w/w) was chosen for this drug. The formulation was denominated CS/INH/RFB = 10/1/0.2 (w/w). Briefly, the polymer was solubilized in a mixture of acetic acid/ethanol (10/1) at a concentration of 2% (w/v). Both drugs were separately ground in a porcelain mortar and solubilized in acetic acid (INH) or ethanol (RFB), prior to incorporation into CS dispersion previously prepared. In the case of unloaded CS MP, the polymer was solubilized in a mixture of acetic acid/ethanol (10/1) at a concentration of 2% (w/v) without drug association. The resulting dispersion was left under stirring for 1 h and then spray-dried. The spray-drying operating parameters were optimized as follows: inlet temperature 160 ± 1 ºC, aspirator 80%, feed flow 1.3 mL/min, and spray flow rate 473 L/h. MP were characterized regarding surface morphology, Feret's diameter and real density. Aerodynamic diameter (D_{aer}) was theoretically calculated based on the Feret's diameter and the real density (D_{aer} = Feret's diameter × (real density/f)$^{0.5}$; where f represents the shape factor of MP, which in this case is 1. In order to determine the drug association efficiency, 30 mg of drug-loaded CS MP were solubilized in 10 mL of HCl 0.1M, under magnetic stirring for about 20 min. After dissolution, aliquots (1 mL) were filtered (0.45 µm) and the drug content was quantified by UV-Vis spectrophotometry (Pharmaspec UV-1700, Shimadazu) at 268.5 nm (INH, aliquot diluted 1:10) and 500 nm (RFB). Calibration curves for each drug were previously established by solubilizing the drug at different concentrations in solutions of unloaded-CS MP produced in the same medium (HCl 0.1M).

The cytotoxic evaluation of CS MP was performed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) assay in A549 and macrophage differentiated THP-1 cells. CS, as well as unloaded and drug-loaded CS MP were tested at concentrations of 0.1, 0.5, and 1 mg/mL. Cell culture medium (CCM) and sodium dodecyl sulphate (SDS, Sigma-Aldrich) at a concentration of 2% (w/v) were tested as positive and negative controls of cell viability, respectively. CCM for A549 cells was Dulbecco's modified Eagle's (DMEM) medium and RPMI 1640 medium for THP-1 cells. Cells were seeded in 96 well plates and exposed to the samples for 3 or 24 h (37 ºC, humidified 5% CO_2/95% atmospheric air). Macrophage ability to uptake unloaded-CS MP was assessed by flow cytometry (FacScalibur cell analyser, BD Biosciences). Briefly, CS was labelled with fluorescein sodium salt activated by N-(3-dimethylaminopropyl)-N1-ethylcarbodiimide hydrochloride (EDAC), at pH 4. The stained solution was stirred for 12 h at room temperature and then dialyzed against water. Several washings with purified water removed nonspecific staining. The fluorescent chitosan was freeze-dried and solutions prepared with this polymer were then spray-dried under the same conditions described for unloaded-CS MP. NR8383 cells (rat alveolar macrophages) and macrophage-differentiated (by phorbol myristate acetate) THP-1 cells were exposed to fluorescently-labelled CS MP, and after 2 h of incubation, cells were scraped and centrifuged (1500 rpm, 2 min) in 2 mL of PBS.3% FBS. The cycle of resuspension in PBS.3% FBS and centrifugation was repeated thrice. Cells were re-suspended in 1 mL of PBS.3% FBS, transferred to cytometry tubes (BD Biosciences) and the phagocytosis determined by flow cytometry. A total of 10,000 events were counted within a gated region and the data was presented as mean fluorescence (FL) intensity. The number of cells associated with fluorescence was considered the definition for uptake.

Results and Discussion: The properties of dry powders play an important role in the development of inhalable formulations, as deep lung deposition depends mainly on characteristics like particle size, shape, and density (5). Morphological analysis by SEM showed that spray-dried CS MP are spherical, having a wrinkled surface that becomes smoother after drug incorporation (Figure 1). The observed morphologies are similar to those reported in other works involving spray-dried CS MP (6,7).

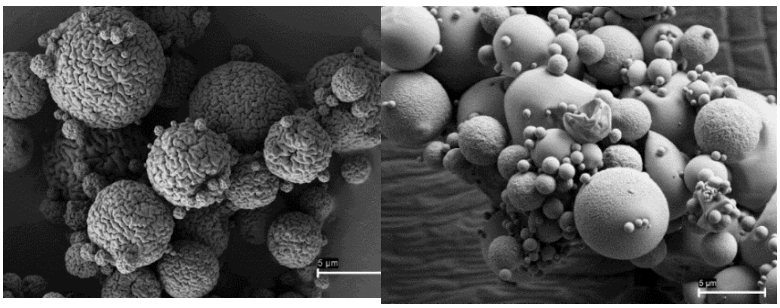

Figure 1 – Scanning electron microscopy microphotographs of unloaded (left) and drug-loaded (right) chitosan microparticles.

Spray-drying CS yielded up to 80% of CS MP with mean size ranging from 1.5 µm to 1.9 µm (Table 1). Although some microparticles presented diameters over 2 µm, the size of the majority lies between 1 and 2 µm. As expected, the incorporation of drugs had no effect on size, given the relatively low loading. Likewise, no significant differences were observed in real densities (1.39 g/cm^3 for unloaded MP and 1.37 g/cm^3 for drug-loaded MP). Real density measurements are considered more accurate than bulk density, since the measured volume of particles excludes the interstitial space between the particles. The real density along with Feret's diameter resulted in a theoretical aerodynamic diameter of 1.98 µm for drug-loaded MP, indicating theoretically suitable properties for deep lung delivery (8). Association efficiency and loading capacity of the developed CS MP were determined (Table 1). INH and RFB in combination were efficiently associated to CS MP, complying with the combined therapeutic regimen of TB, as recommended by WHO (9). INH was associated with an efficiency of 73%, resulting in a loading capacity around 7.5%. In turn, a higher association efficiency (97%) was observed for RFB, resulting in a loading capacity of 1.9%.

Table 1 – Drug association efficiency, loading capacity, Feret's diameter, real density and theoretical aerodynamic diameter of chitosan-based microparticles (mean ± SD, n = 3).

PROPERTIES	Unloaded CS MP	Drug-loaded CS MP (CS/INH/RFB = 10/1/0.2; w/w)
Drug association efficiency (%)	-	73 ± 1 (INH) 97 ± 2 (RFB)
Loading capacity (%)	-	7.4 ± 0.1 (INH) 1.9 ± 0.1 (RFB)
Feret's diameter (µm)	1.53 ± 0.86	1.86 ± 0.98
Real density (g/cm^3)	1.39 ± 0.02	1.37 ± 0.04
Aerodynamic diameter (µm)	1.84 ± 0.12	1.98 ± 0.17

CS is one of the most studied natural polymers and its biocompatibility and biodegradability have been reported in the context of several delivery routes, including the pulmonary (5). To the best of our knowledge spray-dried CS MP loaded with a combination of INH and RFB are not described in the literature. We have tested the effect of CS/INH/RFB MP on the viability of two cell lines representative of the environment of relevance in pulmonary TB therapy, an alveolar epithelial line (A549 cells) and a line representing macrophages (macrophage-differentiated THP-1 cells). A solution of CS and a suspension of unloaded CS MP were tested as controls. As depicted in Figure 2, no cytotoxic effect was observed towards macrophage-like cells upon exposure to concentrations up to 1 mg/mL for 24 h. A similar response was obtained in A549 cells (Figure 3). The cell viability level of 70% (indicated with a dashed line) was considered the threshold beyond which a cytotoxic effect was assumed to occur, as designated by ISO 10993-5 (10).

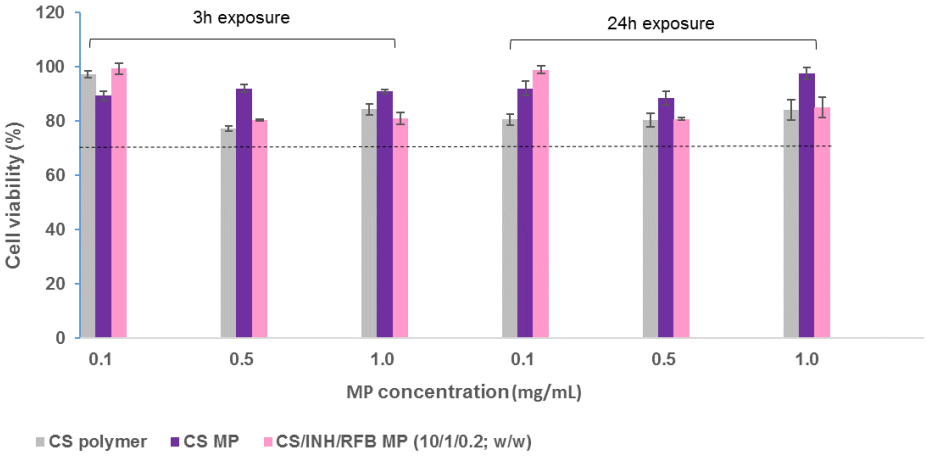

Figure 2 – Macrophage-differentiated THP-1 cell viability after 3 h and 24 h of exposure to chitosan, unloaded (CS MP) and drug-loaded (CS/INH/RFB = 10/1/0.2, w/w) chitosan microparticles. Results are expressed as mean ± SEM (n = 3; six replicates per experiment at each concentration). Dashed line represents 70% cell viability.

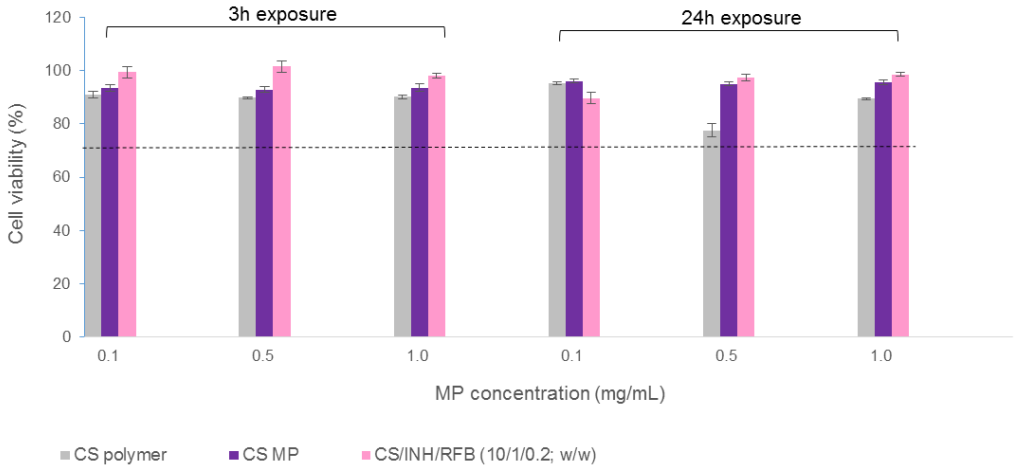

Figure 3 – Alveolar epithelial line (A549 cells) cell viability after 3 h and 24 h of exposure to chitosan, unloaded (CS MP) and drug-loaded (CS/INH/RFB = 10/1/0.2, w/w) chitosan microparticles. Results are expressed as mean ± SEM (n = 3; six replicates per experiment at each concentration). Dashed line represents 70% cell viability.

Inhaled antitubercular therapies are desired to specifically target alveolar macrophages infected with TB bacilli (11). In order to assess the ability of CS MP to be taken up by alveolar macrophages, the polymer was labelled with fluorescein (fluorescein sodium salt was activated by *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (EDAC), producing fluorescent-CS). This was then spray-dried and fluorescent unloaded-CS MP uptake was evaluated on human macrophage-differentiated THP-1 cells and rat alveolar macrophages NR8383. Microparticles were incubated with macrophages for 2 h, as it is reported that about 50-75% of deposited particles are phagocytosed within this period (12). The analysis was performed by flow cytometry and cells exhibiting fluorescence were assumed to have phagocytosed MP. Cells not exposed to fluorescence-labelled MP (control incubated with CCM) showed a certain degree of auto-fluorescence, while cells exposed to fluorescent-CS MP evidenced a stronger increase of the fluorescence signal.

The percentage of CS MP taken up by macrophages was very high in both cases, 98.1 ± 1.8% for human macrophage-differentiated THP-1 cells and 99.9 ± 0.1 % for rat alveolar macrophages NR8383. Two concentrations of CS MP were tested (80 and 240 μg/cm^2) and a dose-dependent uptake was not observed. These results suggest a high affinity of macrophages for CS MP which can be explained by the molecular composition of the polymer. In spite of a natural ability of macrophages for the phagocytosis of particulate matter, a preference for certain particles/materials is observed, depending on the affinity for macrophage surface receptors. In fact, CS is described to interact with macrophage receptors by means of both the toll-like receptors (TLR-4) and the mannose receptor (13). However, further investigation on the ability for preferential macrophage capture remains to be done by a comparison with a polymer devoid of units/residues potentially recognized by macrophage receptors.

Conclusion: Spray-dried inhalable powders consisting of chitosan microparticles loaded with a combination of isoniazid and rifabutin were efficiently produced for pulmonary administration. The delivery of these inhalable carriers using an adequate inhaler, could replace the conventional oral delivery of antitubercular drugs. The developed MP evidenced theoretically adequate aerodynamic properties for deep lung delivery. Drug-loaded MP had no effect on cell viability, as indicated by a metabolic assay performed on macrophage-like cells (THP-1) and rat alveolar macrophage cells (NR8383). Furthermore, CS MP evidenced strong ability to be captured by macrophages, upon 2 h incubation with macrophages. Local administration of these MP would possibly target alveolar macrophages hosting mycobacteria, allowing less frequent administration of lower drug doses and reducing major side effects at systemic level, thus contributing to therapeutic effectiveness.

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