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Research paper

Microspheres containing lipid/chitosan nanoparticles complexes for pulmonary delivery of therapeutic proteins

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Abstract

Chitosan/tripolyphosphate nanoparticles have already been demonstrated to promote peptide absorption through several mucosal surfaces. We have recently developed a new drug delivery system consisting of complexes formed between preformed chitosan/tripolyphosphate nanoparticles and phospholipids, named as lipid/chitosan nanoparticles (L/CS-NP) complexes. The aim of this work was to microencapsulate these protein-loaded L/CS-NP complexes by spray-drying, using mannitol as excipient to produce microspheres with adequate properties for pulmonary delivery. Results show that the obtained microspheres are spherical and present appropriate aerodynamic characteristics for lung delivery (aerodynamic diameters around 2–3 μm and low apparent tap density of 0.4–0.5 g/cm^3). The physicochemical properties of the L/CS-NP complexes are affected by the phospholipids composition. Phospholipids provide a controlled release of the encapsulated protein (insulin), which was successfully associated to the system (68%). The complexes can be easily recovered from the mannitol microspheres upon incubation in aqueous medium, maintaining their morphology and physicochemical characteristics. Therefore, this work demonstrates that protein-loaded L/CS-NP complexes can be efficiently microencapsulated, resulting in microspheres with adequate properties to provide a deep inhalation pattern. Furthermore, they are expected to release their payload (the complexes and, consequently, the encapsulated macromolecule) after contacting with the lung aqueous environment.

Keywords: Chitosan nanoparticles; Dry powders; Microspheres; Phospholipids; Pulmonary delivery; Spray-drying

1. Introduction

The pulmonary administration of therapeutic macromolecules is currently receiving increased attention, and the design of adequate carriers appears as the limiting factor to succeed. In this respect, microspheres have been proposed, since they can be tailored to exhibit appropriate aerodynamic properties [1] and they should possess a very narrow range of aerodynamic diameter, usually accepted to vary between 1 and 5 μm, although some authors restrict this range to 2–3 μm [1–4].

Liposomes have been presented as an interesting alternative for administration of biomolecules through several mucosal surfaces [5], since they are versatile and tend to be relatively innocuous (produced with natural and biodegradable compounds), and also provide protection to the encapsulated material [3,6–8]. Their organised structure (an aqueous core encapsulated within one or more phospholipid bilayers) permits the association of drugs to both the aqueous and lipid phase and drug release can usually be controlled, depending on the bilayers number and composition [3,6]. In order to achieve an improved controlled release, the incorporation of a drug-loaded vesicle inside a second vesicle, the encapsulation of particulate matter inside lipid vesicles, or even the adsorption of lipid bilayers onto polyelectrolyte-coated capsules have been reported [9–13]. Moreover, we have recently reported the preparation of, a new drug delivery

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system consisting of lipid/chitosan nanoparticles (L/CS-NP) complexes, intended for gastric delivery as a first approach that permitted the protection of chitosan nanoparticles from the acidic gastric environment [14]. Similar structures have been reported as promising carriers in biotechnology, as drug, antigen or gene delivery systems [15–18].

The application of liposomes has been suggested for sustained lung release of several drugs and their interaction with the endogenous phospholipids was proposed as a contribution to the prolonged retention of peptides within the lung. Furthermore, enhanced drug absorption provided by phospholipids similar to those composing the pulmonary surfactant was also reported, although the mechanism of absorption enhancement is still unknown [19–21]. One of the major problems concerning the pulmonary administration of particulate systems is the rapid capture by the alveolar macrophages [3,22], a process known to be affected by several factors such as particle size, surface properties, composition and local concentration [23–25]. In this respect, the macrophagic capture of PLGA microparticles upon interaction of microparticles with alveolar macrophages in culture was reported to be reduced by the inclusion of phosphatidylcholine, -serine and -ethanolamine in the formulation [26].

Nanoparticles, which can be produced with a wide variety of polymers and nanotechnologies [27,28], have also been recently proposed as delivery systems for peptides and proteins through the pulmonary route [29–32]. In this respect, chitosan is a very attractive polysaccharide due to its reported low toxicity, biodegradability and mucoadhesivity [33–35]. In fact, chitosan has been demonstrated to induce low or absent toxicity in cell lines representative of the pulmonary route (16HBE14o- and Calu-3) [36,37]. Our group has introduced the preparation of chitosan/tripolyphosphate (CS/TPP) nanoparticles by an extremely mild and rapid ionotropic gelation procedure between chitosan and its counterion TPP [38], which show an excellent capacity for protein association (as high as 95%), as well as an improvement of peptide absorption through several epithelia, such as the nasal, ocular and intestinal [39–42]. Furthermore, we recently reported the production of microspheres as carriers for protein-loaded chitosan nanoparticles to the lung, with the aim of improving their aerosolisation patterns. These nanoparticle-loaded microspheres, obtained by spray-drying a suspension of nanoparticles in mannitol, exhibited adequate aerodynamic properties for lung delivery [32] and demonstrated to be biocompatible with respiratory epithelial cell layers (Calu-3 and A549) [43].

The spray-drying of liposomes has been reported to not compromise their stability [44], and a work on the spray-drying of solid lipid nanoparticles demonstrated that the presence of carbohydrates like mannitol, lactose and trehalose provided an increased stability to the spray-dried product, because the sugar layer around the particles prevented the lipids coalescence [45].

In this work, the production of microspheres containing lipid/chitosan nanoparticles complexes, intended for the pulmonary administration of macromolecules, using a spray-drying technique is reported. For this purpose, mannitol, which is known for its non-toxic and degradable properties [2], was chosen as microencapsulation excipient and insulin as the model protein. Microspheres, aerodynamic properties were characterised, as well as their ability to deliver *in vitro* the lipid/nanoparticles complexes. Moreover, the effect of different lipid compositions on the complexes physicochemical characteristics and on the nanoparticles release profile was investigated.

2. Materials and methods

2.1. Chemicals

Chitosan (CS) in the form of hydrochloride salt (Protasan® 213 Cl, deacetylation degree: 86%, viscosity: 95 mPa) was purchased from FMC Biopolymer AS (Norway). Dipalmitoylphosphatidylcholine (DPPC) and dimyristoylphosphatidyl glycerol (DMPG) were supplied by Lipoid (Germany). Pentasodium tripolyphosphate (TPP), glycerol, D-mannitol (Ma), phosphate buffered saline tablets (PBS) pH 7.4 and bovine insulin were supplied by Sigma Chemicals (USA). Ultrapure water (MilliQ Plus, Millipore Ibérica, Spain) was used throughout. All other chemicals were of reagent grade.

2.2. Preparation of chitosan nanoparticles (CS-NP)

CS-NP were prepared according to the procedure developed by our group, based on the ionotropic gelation of CS with TPP, in which the positively charged amino groups of CS interact with the negatively charged TPP [38]. Briefly, CS and TPP were dissolved in purified water in order to obtain solutions of 1 mg/mL and 0.42 mg/mL, respectively. The spontaneous formation of nanoparticles occurred upon incorporation of 1.2 mL of the TPP solution in 3 mL of the CS solution (final CS/TPP ratio of 6:1 (w/w)), under mild magnetic stirring (Plate A-13 Serie D, SBS, USA) at room temperature.

The insulin loaded CS-NP were obtained following the protein dissolution in NaOH 0.01 M (0.9 mg insulin/0.6 mL NaOH) and its consequent incorporation in the TPP solution (pH 11.6; 0.6 mL TPP solution + 0.6 mL insulin solution). The insulin concentration in the TPP solution was calculated in order to obtain CS-NP with a theoretical content of 30% (w/w) insulin respective to CS.

CS-NP were concentrated by centrifugation at 16,000*g* on a 10 μL glycerol bed for 30 min at 15 °C (Beckman Avanti 30, Beckman, USA). The supernatants were discarded and nanoparticles were re-suspended in 100 μL of purified water.

CS-NP were also prepared on a large scale, adding 12 mL of the TPP solution to 30 mL of the CS solution and maintaining the stirring conditions. They were

centrifuged at 10,000*g* and the re-suspension conditions were proportionally adapted. CS-NP produced in large scale were used to prepare all the microspheres of lipid/nanoparticles (L/CS-NP) complexes, with the low scale being used only to characterise nanoparticles.

2.3. Determination of CS-NP production yield

The CS-NP production yield was calculated by gravimetry. Fixed volumes of nanoparticle suspensions were centrifuged (16,000*g*, 30 min, 15 °C) and sediments were freeze-dried for 24 h at −34 °C, followed by a gradual ascent until 20 °C, using a Labconco Freeze Dryer (Labconco, USA) ($n = 3$).

The process yield (P.Y.) was calculated as follows:

$$\text{P.Y.}(\%) = \frac{\text{CS-NP weight}}{\text{total solids (CS + TPP + insulin) weight}} \times 100 \quad (1)$$

2.4. Characterisation of CS-NP

Measurements of CS-NP size and zeta potential were performed by photon correlation spectroscopy and laser Doppler anemometry, respectively, using a Zetasizer® 3000 HS (Malvern Instruments, Malvern, UK). For the particle size analysis, each sample was diluted to the appropriate concentration with filtered (0.2 μm filters Millex®-GN, Millipore Iberica, Spain) ultrapure water. Each analysis lasted 180 s and was performed at 25 °C with a detection angle of 90°. For the determination of the electrophoretic mobility, samples were diluted with KCl 0.1 mM and placed in the electrophoretic cell, where a potential of ±150 mV was established. Three batches of each formulation were analysed in triplicate ($n = 3$).

2.5. Determination of insulin loading capacity

The CS-NP association efficiency was determined upon separation of nanoparticles from the aqueous preparation medium containing the non-associated protein by centrifugation (16,000*g*, 30 min, 15 °C). The amount of free insulin was determined in the supernatant by the MicroBCA protein assay (Pierce, USA), measuring the absorbances by spectrophotometry (Shimadzu UV–Visible Spectrophotometer UV-1603, Japan) at 562 nm. A calibration curve was made using the supernatant of unloaded CS-NP. Each sample was assayed in triplicate ($n = 3$). The CS-NP protein loading capacity (L.C.) and association efficiency (A.E.) were calculated as follows:

$$\text{L.C.}(\%) = \frac{\text{total insulin amount} - \text{free insulin amount}}{\text{CS-NP weight}} \times 100 \quad (2)$$

$$\text{A.E.}(\%) = \frac{\text{total insulin amount} - \text{free insulin amount}}{\text{total insulin amount}} \times 100 \quad (3)$$

2.6. Preparation of lipid/chitosan nanoparticle (L/CS-NP) complexes

The L/CS-NP complexes were prepared by adding a suspension of previously prepared CS-NP to a dry lipid film, following a procedure previously developed by our group [14]. Briefly, DPPC or a mixture of DPPC and DMPG (10:1 molar ratio) was dissolved in 20 mL of chloroform, obtaining a 0.3 mM lipid concentration, and 50 mg of glass beads was added to increase the surface area available to form the film. The organic solvent was then removed by evaporation under reduced pressure on a rotary evaporator (Buchi® R-114, Buchi, Switzerland) at approximately 55 °C, for 3 h on a nitrogen atmosphere, leading to the formation of a thin film of dry lipid [46–48]. This film was then hydrated for 30 min with a suspension of the CS-NP (unloaded or insulin loaded), forming L/CS-NP systems of ratio 3:1 (w/w). Immediately afterwards, the complexes were filtered under vacuum to allow for their separation from the glass beads. Control vesicles were produced under the same conditions, using water as the hydrating phase.

2.7. Characterisation of the L/CS-NP complexes

The morphological examination of the complexes was conducted by optical (Olympus BH-2, Japan) and transmission electron microscopy (TEM) (CM 12 Philips, The Netherlands). For TEM observation, samples were stained with 2% (w/v) phosphotungstic acid and placed on copper grids, previously covered with Formvar® films.

The size of the control vesicles and L/CS-NP complexes was determined by the Coulter counter method (Coulter® Multisizer II, Coulter Electronics, England), equipped with a tube with an orifice aperture of 50 μm. To perform the measurements, 20 μL of the previously obtained suspension of complexes was dispersed in 100 mL of the electrolyte Isoton II (filtered, phosphate-buffered saline solution PBS). The instrument was previously calibrated using Isoton II and monodisperse latex microspheres of 13 μm, both supplied by Coulter ($n = 3$). Zeta potential measurements were performed by laser Doppler anemometry, using the Zetasizer®. Samples were diluted with KCl 0.1 mM and placed in the electrophoretic cell, where a potential of ±150 mV was established. Three batches of each formulation were analysed in triplicate ($n = 3$).

2.8. Preparation of dry powders containing L/CS-NP complexes

Dry powders containing the L/CS-NP complexes were obtained by a spray-drying technique, as previously described for the microencapsulation of nanoparticles

[32]. Briefly, an aqueous solution of mannitol was added to the previously obtained suspension of L/CS-NP complexes, in order to achieve a theoretical mannitol/complexes ratio of 80/20 (w/w), and a final solids content of 2.1% (w/v). The chosen carbohydrate/complexes ratio was the optimum used to prepare microspheres containing mannitol and nanoparticles, as reported before, the resultant microspheres presenting adequate aerodynamic properties for pulmonary delivery [32]. The spray-drying process was performed using a laboratory-scale spray-dryer (Büchi® Mini Spray Dryer, B-290, Buchi, Switzerland), with the following conditions: a two fluids external mixing 0.7 mm nozzle was used, feed rate was 2.5 mL/min, inlet and outlet temperatures were 120 ± 2 °C and 85 ± 2 °C, respectively. The air flow rate and the aspirator were constant at 400 Nl/h and 80%, respectively. Microspheres were collected using a spatula and were stored in a dessicator at room temperature until use.

2.9. Determination of spray-drying process yield

The spray-drying process yield (P.Y.) was calculated by gravimetry, comparing the total solids weight of the spray suspension with the resultant powder weight after spray-drying, as follows ($n = 3$):

$$\text{P.Y.}(\%) = \frac{\text{microspheres weight}}{\text{suspension total solids (L/CS-NP + mannitol) weight}} \times 100 \quad (4)$$

2.10. Morphological analysis of microspheres

Microspheres were visualised using a scanning electron microscope (SEM, Leo 435VP, UK). Dry powders were placed onto metal plates and a 200-nm thick gold palladium film was sputter coated on the samples (High Resolution Sputter Coater SC7640, Termo VG Scientific, UK) before viewing. The particle size was determined as Feret's diameter (distance between two tangents on opposite sides of the particles) and was directly determined with an optical microscope (Olympus BH-2, Japan), where a 300 particles measurement ($n = 300$) was estimated as the mean.

2.11. Determination of microspheres density

Real density was determined using a Helium Pycnometer (Micropycnometer, Quanta Chrome, model MPY-2, USA) ($n = 3$). Apparent tap density was obtained by measuring the volume of a known weight of powder in a 10 mL test tube after mechanical tapping (30 tap/min, Tecnociencia, Spain). After registration of the initial volume, the test tube was submitted to tapping until constant volume was achieved, according to a previously described method [49] ($n = 3$).

2.12. Evaluation of aerodynamic properties

Aerodynamic diameters were obtained using a TSI Aerosizer® LD equipped with an Aerodisperser® (Amherst Process Instrument, Inc., Amherst, MA, USA), where the measuring principle is based on the measurement of the particles time of flight in an air stream ($n = 3$), according to the following equation:

$$C_d \frac{\pi d^2}{4} \rho_a \frac{(V_a - V_p)}{2} = 1/6 \pi d^3 \rho_p \frac{dV_p}{dt}, \quad (5)$$

where C_d, drag coefficient; d, particle diameter; ρ_a, density of air; V_a, velocity of air; V_p, velocity of particle and ρ_p, density of particle.

2.13. Recovery of L/CS-NP complexes from dry powder (complexes-loaded microspheres) in aqueous medium

In order to recover the initial L/CS-NP complexes from the dry powders, approximately 50 mg of the microspheres was incubated in 3 mL of PBS, pH 7.4, for 90 min, under mild magnetic stirring (Plate A-13 Serie D, SBS, USA), at room temperature. Thereafter, the recovered complexes morphology, size and zeta potential were analyzed by TEM (CM 12 Philips, Eindhoven, The Netherlands), Coulter counter method (Coulter® Multisizer II, Coulter Electronics, England) and laser Doppler anemometry (Zetasizer® 3000 HS, Malvern Instruments, Malvern, UK), respectively ($n = 3$).

2.14. In vitro release studies of insulin from CS-NP, L/CS-NP complexes and dry powders (complexes-loaded microspheres)

The release of insulin was determined by incubating the different formulations (CS-NP, L/CS-NP complexes and complexes-loaded microspheres) in 5 mL of pH 7.4 phosphate buffer (0.21 mg CS-NP/mL, 0.83 mg complexes/mL, 4.18 mg microspheres/mL), with mild horizontal shaking (Promax 1020, Heidolph, Germany) at 37 °C. A further release study using a physical mixture of insulin and unloaded L/CS-NP complexes was performed. This was used as a control in order to evaluate the occurrence of adsorption phenomena.

At appropriate time intervals (15, 30, 60 and 90 min) individual samples were filtered (0.22 μm filters Millex®-GV, low protein binding, Millipore Ibérica, Spain) and the amount of protein released evaluated in the supernatants by the MicroBCA protein assay (Pierce, USA) measuring the absorbances by spectrophotometry (Shimadzu UV–Visible Spectrophotometer UV-1603, Japan) at 562 nm ($n = 3$).

2.15. Statistical analysis

The *t*-test and the one-way analysis of variance (ANOVA) with the pairwise multiple comparison proce-

dures (Student–Newman–Keuls method) were performed to compare two or multiple groups, respectively. All analyses were carried out using the SigmaStat statistical program (Version 3, Systat Software, USA) and differences were considered to be significant at a level of $P < 0.05$.

3. Results and discussion

In this work, a dry powder integrating a combination of three systems was prepared, comprised of microspheres containing protein-loaded lipid/chitosan nanoparticle (L/CS-NP) complexes intended for pulmonary administration. Furthermore, the system was aerodynamically characterised *in vitro* and its ability to deliver insulin, used as the model therapeutic protein, was investigated.

3.1. Preparation and characterisation of chitosan nanoparticles (CS-NP)

CS-NP were obtained with CS and TPP (CS/TPP = 6:1, w/w), using the ionotropic gelation method described in the methodology section. As shown in Table 1, unloaded CS-NP present a size around 430 nm and a positive zeta potential of approximately +44 mV. Insulin was associated to the nanosystem with efficiency of 68%, achieving a particle loading of 36%. As can be observed in the referred Table, the production yield increased significantly ($P < 0.05$) from 22% to 39% with the incorporation of insulin in the CS-NP, which can be easily explained by the nanoparticles formation mechanism, as previously reported [40]. Not only TPP, but also insulin interacts with the CS amino groups, leading to the production of more nanoparticles. The incorporation of insulin in the CS-NP did not have a pronounced effect on particle size, but led to a significant decrease ($P < 0.05$) in the zeta potential, reaching a value around +34 mV. Taking into account that insulin is dissolved in NaOH 0.01 M, with basic pH (approx. 11), the protein is above its isoelectric point (p*I* 5.3) resulting in a negative charge. As a consequence, the association of insulin with the positively charged CS-NP is favored, decreasing the zeta potential value.

Data depicted in Table 1 correspond to CS-NP prepared using the low scale conditions (3 and 1.2 mL of CS and TPP, respectively). The scaling-up of nanoparticles production, using 30 mL of CS and 12 mL of TPP, resulted in CS-NP with similar characteristics, providing another advantage of the ionic gelation as a method of preparing these structures. Unloaded CS-NP obtained in the large scale presented a size of 461 ± 17 nm and a zeta potential of +43.6 ± 0.3 mV.

3.2. Preparation and characterisation of the L/CS-NP complexes

The L/CS-NP complexes were prepared using two phospholipids (DPPC and DMPG), which are endogenous to the lung and principal constituents of the pulmonary surfactant [20]. Our systems were produced using only DPPC or a combination of DPPC and DMPG (DPPC–DMPG = 10:1), in an attempt to approximately respect the proportions of phospholipids existing in the alveolar surfactant. It is well known that phospholipids compose 80–90% of the surfactant, of which approximately 80% is phosphatidylcholine and 5–10% phosphatidylglycerol [20,50]. Moreover, it has been reported that an ideal mixture of phospholipids occurs when combining those whose hydrocarbon chains differ by only two carbon atoms [51], as happens with DPPC and DMPG, which have 16 and 14 carbon atoms, respectively.

Fig. 1 displays the TEM microphotographs of representative fresh L/CS-NP complexes. As expected, the displayed images seem to indicate that, for both assayed formulations and therefore independently of the lipid constitution, there were not only CS-NP entrapped in the phospholipid vesicles, which can be seen as dense black zones, but also isolated CS-NP (signalled with arrows in the figure). Similar images were previously observed by Jain et al., who produced chitosan nanoparticles encapsulated in soya lecithin vesicles intended for oral immunisation, in order to overcome the stability problem of unmodified particles in the acidic pH of the stomach [15].

Fig. 2 schematically shows the possible alternatives that we consider for the formation of the complexes by the method used in this study. As can be seen, our hypothesis is that CS-NP can be either completely coated by the phospholipids or a part of the CS-NP is surrounded by a phospholipid layer, whereas the rest is located at the complexes surface, thus not being coated by the lipid film.

As shown in Table 2, the two formulations of control vesicles (prepared using water as hydrating solution) display a similar size around 2 μm, sizes being adjusted to a lognormal distribution. Both types of vesicles present a negative zeta potential, but in the case of those composed of DPPC, the value is close to neutrality (−7 mV), while vesicles containing DMPG display a significantly stronger negative surface ($P < 0.05$), with a potential around

Table 1
Process yields and physicochemical properties of unloaded and insulin-loaded chitosan/tripolyphosphate nanoparticles (CS-NP) (mean ± SD, $n = 3$)

Nanoparticles formulation	Process yield (%)	Size (nm)	Zeta potential (mV)	Association efficiency (%)	Loading capacity (%)
Unloaded	22 ± 0	427 ± 22	+ 43.7 ± 2.5	–	–
Insulin-loaded	39 ± 1	443 ± 30	+ 34.6 ± 0.6	68 ± 4	36 ± 2

Process yield (%) = [nanoparticles weight/total solids weight] × 100.
Association efficiency (%) = [(total insulin amount − free insulin)/total insulin amount] × 100.
Loading capacity (%) = [(total insulin amount − free insulin)/nanoparticles weight] × 100.

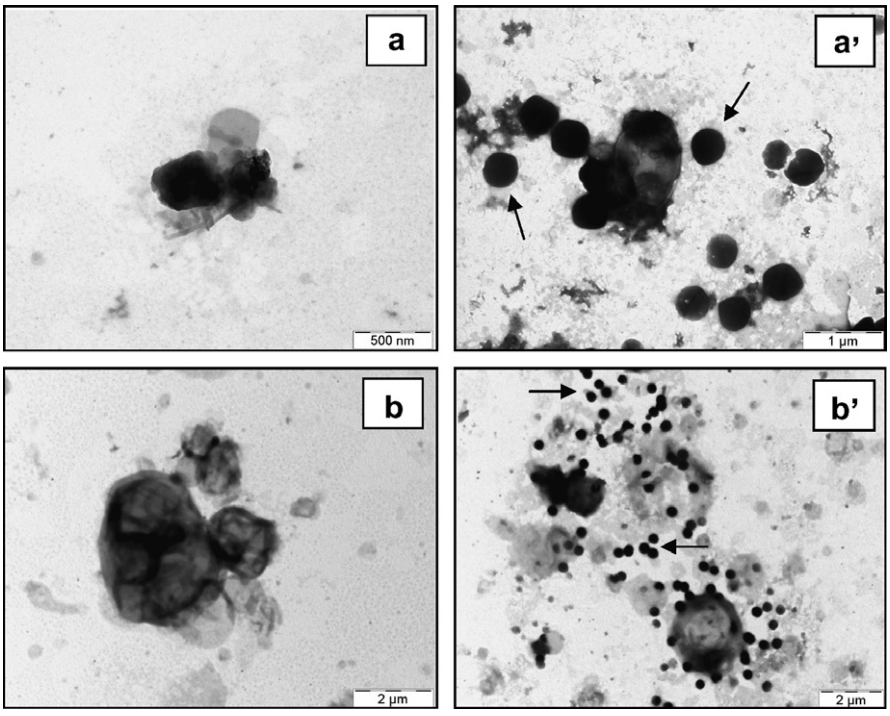

Fig. 1. TEM microphotographs of freshly prepared lipid/nanoparticles (L/CS-NP) complexes (lipid/nanoparticles = 3:1, nanoparticles CS/TPP = 6:1), lipidic fraction composed of: (a, a′) DPPC; (b, b′) DPPC–DMPG = (10:1). Arrows signal isolated nanoparticles.

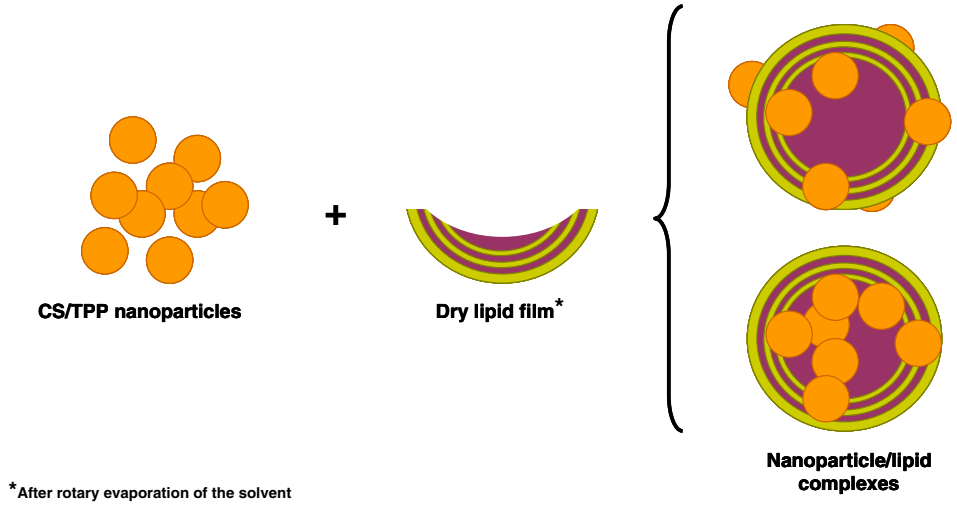

Fig. 2. Schematic representation of the mechanism of formation of lipid/nanoparticles (L/CS-NP) complexes.

Table 2
Physicochemical properties of control vesicles and lipid/nanoparticles (L/CS-NP) complexes (mean ± SD, $n = 3$)

System	Size (μm)	Zeta potential (mV)
Control DPPC vesicles	2.2 ± 1.8	−7.1 ± 4.6
DPPC/CS-NP (unloaded) complexes	1.8 ± 1.8	0.2 ± 1.9
Control DPPC–DMPG vesicles	1.8 ± 1.7	−54.0 ± 4.2
DPPC–DMPG/CS-NP (unloaded) complexes	2.5 ± 1.6	−36.2 ± 1.6

CS, chitosan; DPPC, dipalmitoylphosphatidylcholine; DMPG, dimyristoylphosphatidyl glycerol; NP, nanoparticles; DPPC–DMPG = 10:1.

−54 mV. These results were to be expected since it has been reported that DPPC is a neutral phospholipid and DMPG presents a negative charge [52]. In fact, the slight negative zeta potential of DPPC vesicles was previously mentioned as a consequence of the position of the phospholipid polar head, which may vary with the ionic strength and temperature [53].

The hydration of the dried lipid film with the CS-NP suspension did not lead to a significant size alteration for both formulations, when compared to the respective control vesicles. However, a slight increase could be detected

in the complexes containing both lipids, which present a final size of approximately 2.5 μm, which could be indicative of a more complete phospholipidic coating. Obviously, when comparing the size of both complexes to that of the CS-NP, there is a significant difference ($P < 0.05$) evidencing the lipidic coating over the CS-NP.

Although size measurements did not allow for a great differentiation between the two formulations of complexes, zeta potential evaluation did provide further information on their composition and structure. When only DPPC was present in the lipid film composition, the incorporation of the positively charged CS-NP led to a slight, but not significant, modification in the zeta potential values, which varied from −7 mV in control vesicles to 0 mV in L/CS-NP complexes. However, both values are significantly lower ($P < 0.05$) in comparison to that of the CS-NP (+44 mV). On the other hand, when the lipid composition included also DMPG, the zeta potential varied significantly ($P < 0.05$) from −54 mV in control vesicles to −36 mV in L/CS-NP complexes. When compared to the zeta potential evidenced by the CS-NP, a complete inversion pattern is observed ($P < 0.05$), suggesting the production of a more effective phospholipid coating on the nanoparticles. The results found in our work could be understood as a reflex of the strong negative charge presented by the lipid film containing DPPC and DMPG, which enables a more intense interaction with the positive CS-NP, when compared to the film composed of DPPC. However, further characterisation of the complexes surface should be conducted in order to confirm this hypothesis. Furthermore, the lipidic coating will probably take place as a bilayer, because the interaction between solid cores and phospholipids has been reported to have a strong ordering effect on the phospholipid molecules, as was demonstrated using NMR techniques that were sensitive to lipid organisation [54].

Similar results were also found by Carvalho et al., when coating CS-NP with a phospholipids mixture to enable protection against the stomach acidic pH [14]. This interaction pattern was reported as well by Moya et al., upon adsorption of lipid bilayers onto polyelectrolyte-coated capsules, the strong positive zeta potential of the polyallylamine capsules (+40 mV) changing to −40 mV upon coating with dipalmitoyldiphosphatidic acid (negatively charged phospholipid). According to these authors, the zeta potential changes evidenced the lipid adsorption [12]. It was also reported that the successful coating of polystyrene amidine microspheres with a cationic lipid (dioctadecyldimethylammonium) was confirmed by several studies, including the zeta potential determination, which revealed an inversion from −40 mV to +20 mV [17].

It is important to note that the size and zeta potential of the lipid/nanoparticles complexes can be modified and adapted to the specific aim of their application, using different lipidic compositions and/or by modifying the procedure of preparation of the complexes [15,55].

3.3. Microspheres morphological and aerodynamic characterisation

The production of CS-NP-loaded mannitol microspheres was previously described. From all the assayed formulations, we found that the one comprising of mannitol/nanoparticles = 80/20 and solids content = 2.1% was the most suitable for the purpose of carrying CS-NP to the lung, because of its morphological, physical and aerodynamic characteristics [32]. Moreover, the characterisation of the internal structure of these microspheres using confocal microscopy allowed us to demonstrate that CS-NP were homogeneously dispersed through the whole microparticle [56]. Taking into account these considerations, we hypothesised that the microencapsulation of L/CS-NP complexes in mannitol microspheres by spray-drying, using the same mannitol/encapsulated system ratio, would result in the production of microspheres with adequate properties for pulmonary delivery as well.

Concerns about spray-drying phospholipids were not a problem, considering the knowledge that their stability is not compromised after this procedure, due to the short exposure to heat [44,57]. The spray-drying of insulin was not a concern either, since studies exist reporting that its spray-drying at an inlet temperature of 160 °C, with resultant outlet temperature of 100 °C, caused insignificant degradation of the protein around 0.5% [58].

Microspheres were obtained with yields of approximately 50%. As can be observed in the SEM microphotographs depicted in Fig. 3, the spray-drying technique led to the production of well defined microspheres with spherical shape, not being aggregated. Although morphology represents an important parameter in particles intended for inhalation purposes, essentially considering aggregation, which may interfere with the flowing properties, aerodynamic characteristics are the limiting parameters to succeed in pulmonary delivery. The aerodynamic diameter, which is a combination of the particle size and density, influences the dispersion and sedimentation patterns [3,59] and, as has been previously commented, should vary between 1 and 5 μm to allow an optimal lung administration [2,3]. Physical and aerodynamic properties of produced microspheres are depicted in Table 3. The Feret diameters were approximately 3 μm, apparent tap densities were low and significantly different ($P < 0.05$) for both formulations (containing DPPC and DPPC–DMPG), varying between 0.4 and 0.5 g/cm^3 and real densities were around 1.4 g/cm^3. These properties rendered aerodynamic diameters (assessed with an Aerosizer®) between 2.1 and 2.7 μm, which are theoretically adequate for pulmonary administration; results being similar to those previously found [32], possibly due to the production of microspheres using the same ratio between mannitol and the microencapsulated system (CS-NP in the previous work and L/CS-NP complexes in the present work), with the same solids content.

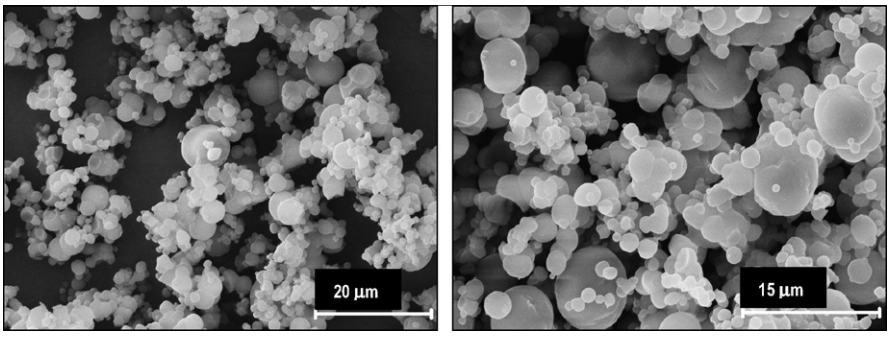

Fig. 3. SEM microphotograph of representative microspheres prepared with mannitol/DPPC–DMPG complexes (theoretical mannitol/complexes ratio = 80/20, solids content = 2.1%, w/v).

Table 3
Physical and aerodynamic properties of microspheres (mannitol/complexes = 80/20, solids content = 2.1%) (mean ± SD, $n = 3$)

Complexes formulation	Feret diameter (μm)	Real density (g/cm^3)	Apparent density (g/cm^3)	Aerodynamic diameter (μm)
DPPC	2.6 ± 1.1	1.44 ± 0.06	0.41 ± 0.04	2.11 ± 0.04
DPPC–DMPG	3.2 ± 1.3	1.42 ± 0.02	0.50 ± 0.01	2.67 ± 0.08

Feret diameters (μm) (distances between two tangents on opposite sides of the particle) were determined by optical microscopy. Real and apparent densities (tap densities) (g/cm^3) were assayed by helium picnometry and by a tapping procedure, respectively. Aerodynamic diameters were obtained using an Aerosizer®.

3.4. Recovery of L/CS-NP complexes from microspheres in aqueous medium

In the current study, we investigated the ability of the obtained complexes-loaded microspheres to deliver the L/CS-NP complexes following incubation in PBS pH 7.4. This pH was chosen in an attempt to perform the assay in a pH close to that of the airway surface liquid, which is approximately 7 [60,61]. We observed that after the microspheres incubation in aqueous medium under low stirring, mannitol dissolved, resulting in a suspension of the complexes. Therefore, as it was previously reported for nanoparticle-loaded microspheres [32], L/CS-NP complexes could be easily recovered from microspheres. Fig. 4 depicts microphotographs of recovered complexes of the formulation comprising DPPC–DMPG, which we consider to be representative. When comparing these microphotographs with those collected in Fig. 1, the presence of nanoparticles entrapped in the phospholipid vesicles and isolated nanoparticles can still be observed, indicating that complexes morphology has not changed. Therefore, we can confirm that the spray-drying process does not have a negative effect on the complexes morphology. As shown in Table 4, the complexes size remained similar after the spray-drying process and zeta potential only changed significantly ($P < 0.05$) after the recovery process in the formulation containing both lipids, decreasing in about 4 mV. Even though, the observed change was minimum, not compromising the fundamental objectives of this work.

Therefore, from the results of this study we could hypothesise that after reaching the deep lung, mannitol will dissolve in the lung lining fluid, releasing the L/CS-NP complexes previously encapsulated in the microspheres.

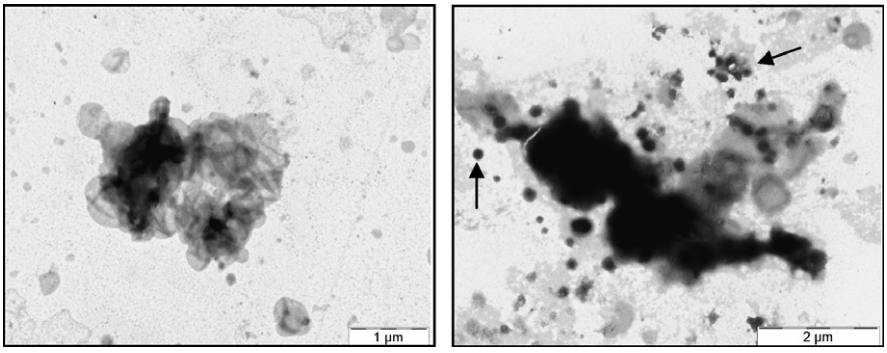

Fig. 4. TEM microphotographs of representative recovered lipid/nanoparticles (L/CS-NP) complexes (lipid/nanoparticles = 3:1, DPPC–DMPG = 10:1, CS/TPP = 6:1). Arrows signal isolated nanoparticles.

Table 4
Zeta potential values of lipid/nanoparticles (L/CS-NP) complexes, fresh and after recovery from microspheres (mannitol/complexes theoretical ratio = 80/20, solids content of 2.1%; mean ± SD, $n = 3$)

L/CS-NP complexes formulation	Size (μm)	Zeta potential (mV)
DPPC/CS-NP, freshly prepared	1.8 ± 1.8	0.2 ± 1.9
DPPC/CS-NP, after recovery	1.4 ± 1.9	−0.6 ± 1.2
DPPC–DMPG/CS-NP, freshly prepared	2.5 ± 1.6	−36.2 ± 1.6
DPPC–DMPG/CS-NP, after recovery	2.2 ± 1.1	−40.7 ± 1.5

3.5. In vitro release studies

Considering the previous information on the structure of the L/CS-NP complexes, it was expected that these would release insulin in a more controlled manner, in comparison to the nanoparticles, because of the extra barrier (the lipid layer) the protein has to overcome before release. Fig. 5 depicts the release profiles of insulin from CS-NP, fresh L/CS-NP complexes containing either DPPC–DMPG or only DPPC, and microspheres containing L/CS-NP complexes composed of DPPC and DMPG in PBS pH 7.4 at 37 °C. The microspheres formulation containing L/CS-NP complexes with both lipids (DPPC and DMPG) was selected to perform the insulin release studies from the microspheres, since it is considered to be representative of both formulations. As expected, the insulin release from CS-NP was very rapid, exhibiting the typical initial burst effect, and at 15 min the maximum amount of insulin was already delivered (80%). As concluded in previous works, this *in vitro* release behaviour suggests that the interaction between CS and insulin is very weak, allowing the insulin release from the CS-NP by a dissociation mechanism [39]. The insulin release profile displayed by the two L/CS-NP formulations assayed is significantly different ($P < 0.05$) to that of CS-NP. Moreover, there is also a significant difference ($P < 0.05$) between the release profiles of both formulations. Actually, they present a slight initial burst effect, followed by a very slight increase in the protein release until 90 min; the formulation containing only DPPC releasing 43% of the insulin content at 15 min, while the one containing both lipids delivers 30% in the same period of time.

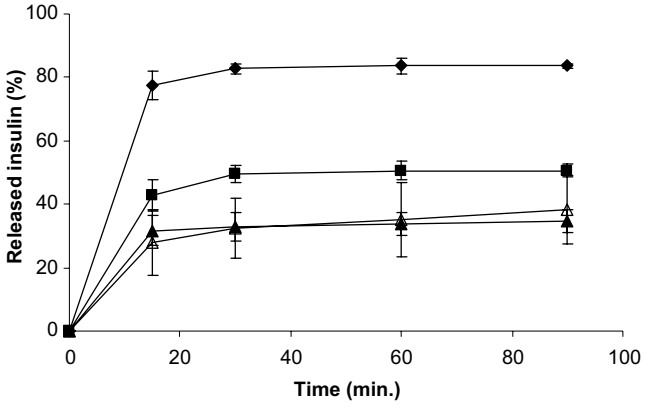

Fig. 5. Release profiles of insulin from (◆) chitosan nanoparticles (CS-NP; CS/TPP = 6:1), (■) DPPC/CS-NP complexes (lipid/nanoparticles = 3:1, CS/TPP = 6:1), (▲) DPPC–DMPG/CS-NP complexes (DPPC–DMPG = 10:1, lipid/nanoparticles = 3:1, CS/TPP = 6:1) and (△) microspheres containing DPPC–DMPG/CS-NP complexes (mannitol/complexes = 80/20, lipid/nanoparticles = 3:1, DPPC–DMPG = 10:1, CS/TPP = 6:1), in PBS, pH 7.4, at 37 °C (insulin = 30% w/w based on CS; mean ± SD, $n = 3$).

The difference found in the release profile presented by both L/CS-NP complexes formulations is undoubtedly a result of the different interaction between the CS-NP and the phospholipids of each formulation of complexes. In fact, the formulation we hypothesised in presenting a stronger interaction between CS-NP and phospholipids (the one containing the phospholipid with the negative charge – DMPG), due to the attraction between the opposite surface charges, gives rise to a much slower and controlled release of the protein, compared to the formulation comprised of solely DPPC. Taking into account the immediate release from the CS-NP, the difference found in the insulin release profile of the complexes compared to the CS-NP is certainly a result of the presence of the phospholipid bilayers, which have to be crossed by the protein. Moreover, in the formulation with DMPG, the protein takes more time to be released, possibly due to the formation of a more homogeneous lipid coating. Therefore, these results not only corroborate our predictions on the differences between both formulations, but also reinforce the theory of a more effective lipidic coating in the formulation containing DMPG, providing a higher control of the therapeutic molecule release. Similar results were previously found in other works [11,14]. In fact, studies with L/CS-NP complexes demonstrated a slow insulin release when a negatively charged phospholipid was included in the system [14]. Furthermore, McPhail et al. compared the release behaviour of carboxyfluorescein from polymeric vesicles and from polymeric vesicles in phospholipid vesicles (vesicle-in-vesicle system formed with a chitosan derivative enclosed in phospholipid vesicles) in PBS, pH 7.4, finding that the release from the encapsulated vesicles was much more controlled [11]. The incubation of unloaded complexes with an insulin solution (data not shown) enabled the detection of released insulin between 92% and 100%. Therefore, we consider that the controlled release showed by the L/CS-NP complexes formulations cannot be attributed to adsorption phenomena.

Finally, concerning the L/CS-NP complexes-loaded microspheres, it can be confirmed that, as expected, mannitol does not influence the protein release profile, allowing the immediate delivery of the complexes, as previously reported [32]. Therefore, mannitol would act only as an inert carrier of the L/CS-NP complexes.

As it was demonstrated, the lipidic coating enabled the modification of the insulin release profile from the chitosan nanoparticles. Furthermore, the encapsulation of a solid core (nanoparticles) inside the lipid vesicles is an excellent

tool not only in providing an adequate loading, but also in improving the release profile and the stability of the encapsulated molecule [15].

4. Conclusions

The present work demonstrates that the proposed technologies are appropriate to obtain complexes between phospholipids and preformed nanoparticles, as well as to produce microspheres containing the referred complexes, which exhibit suitable properties for pulmonary delivery. The phospholipids composition greatly affects the complexes physicochemical characteristics, suggesting that a stronger lipid/ nanoparticles interaction occurs when a negatively charged phospholipid is incorporated in the lipid film, consequently resulting in a more efficient lipidic coating of the chitosan nanoparticles. The complexes were efficiently recovered from the microspheres following their incubation in aqueous medium, without significant changes in their properties. The presence of phospholipids is determinant in controlling the release of the encapsulated insulin, particularly when both lipids, DPPC and DMPG, are present. Moreover, the microencapsulation process does not have any effect on the insulin release profile. As a general conclusion, and taking all these results into account, we believe that the developed system has an ample potential for systemic delivery of therapeutic macromolecules by the pulmonary route.

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