Dry powder formulation for pulmonary infections: Ciprofloxacin loaded in chitosan sub-micron particles generated by electrospray

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Abstract

Electrospray was used as a one-step technique to generate inhalable ciprofloxacin-loaded chitosan sub-micron particles with potential use in the treatment of pulmonary infections. The effect of operating parameters was studied and the preparation method optimized. The final sizes of ciprofloxacin-loaded particles were 386.1 ± 248.5 nm and 501.1 ± 276.3 nm for high and low molecular weight chitosan, respectively. The high surface charge of the particles formed, around +45 mV, enhances their mucoadhesive properties. The particles were biocompatible with alveolar cell line (A549), and showed a high antimicrobial activity against two of the most common respiratory pathogens *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

Keywords*:* Chitosan; Electrospray; Ciprofloxacin; Pulmonary administration; Dry powder; Antimicrobial.

1. Introduction

Resistance to antibiotics is a serious health problem today, caused by their widespread and sometimes un-appropriate use. This resulted in a decrease of their efficacy, and in the appearance of resistant microorganisms (Alqahtani et al., 2019).

In the last decade, direct pulmonary delivery of antibiotics has raised great interest as an alternative administration route (Douafer, Andrieu, Wafo, & Brunel, 2020). Administration by inhalation in the case of pulmonary infections would allow immediate localization of drugs at the action site with a lower dose of antibiotic, thus reducing side effects and significantly improving patients quality of life (Ling et al., 2019). However, the application of inhaled therapy faces important problems. In particular, delivering the drug at the target site in the respiratory tract is highly challenging. Particle size is considered the main factor in determining the location where the particle will be deposited (Thorley & Tetley, 2013). In this sense, micro and nanosized carriers present important advantages for pulmonary drug delivery such as better pharmacokinetics, controlled release, and reduced uptake by alveolar macrophages, making them suitable candidates for drug delivery in the lungs (De Boer, Gjaltema, Hagedoorn, & Frijlink, 2015; Tu et al., 2015). When alveoli are the target, particles with aerodynamic diameters between 0.02–0.05 μm are expected to have the highest alveolar deposition fraction (~50%) (ICPR, 1994). However, this size (20-50 nm) is extremely difficult to aerosolize as such, since particles in this range tend to aggregate into higher size clusters. A second size range suitable for alveolar deposition is 2 –5 μm (~10% of deposition fraction). Interestingly, in both size ranges a large fraction would be deposited outside the alveoli (around 40% for 20-50 nm particles and close to 70% in the 2-5 micron range, respectively) (ICPR, 1994). This opens up another possibility, which is the use of particles in the 200-400 nm (0.2-0.4 micron) range. In this case, a high fraction of the aerosol (ca. 85%) would be expelled again, but most of the rest would deposit in the alveolar region, giving a very significant alveolar deposition yield (ca.~8-10%) with very little load in other pulmonary areas. Thus, a particle size around 300 nm could represent an excellent compromise between alveolar deposition and avoidance of non-selective deposition elsewhere.

A wide variety of polymers is used in pulmonary administration including synthetic such as PLGA (Gaspar et al., 2016) or of natural origin such as chitosan (Gaspar et al., 2015) and alginate (Möbus, Katrin, Siepmann, Jürgen & Bodmeier, 2012). Their structures in

any case have to allow biodegradation, in most cases through hydrolytic pathways. In this work we used chitosan, a natural polymer derived from deacetylation of chitin, it is formed by D-glucosamide and N-acetylglucosamine units (Grenha, Seijo, & Remuñán-López, 2005). Chitosan is biodegradable, biocompatible, has low toxicity (LD_{50} of 16 g/kg body) and is positively charged thanks to its amino groups (Tawfik & El-Masry, 2021).

Electrospray represents one interesting alternative to produce micro – and nanoparticles by submitting the polymer solution to electric field. When the field reaches a critical value, repulsive forces between particles overcome the surface tension of solution a spray is generated and particles can be obtained as a dry powder after solvent removal (Boda, Li, & Xie, 2018). The particle morphology is influenced by the speed of the drying process (Pawar, Thakkar, & Misra, 2018). Importantly, electrospraying avoids freeze-drying and the use of cryoprotectants such mannitol or lactose in the drying process.

Chitosan has often been employed to produce fibers (Qasim et al., 2018) and less frequently particles (Gómez-Mascaraque, Sanchez, & López-Rubio, 2016) by electrospray. Chitosan is a polymer soluble in acid environments, an important feature, given the strong role of solvents in electrospraying. Trifluoroacetic acid (TFA) is a strong acid with a low boiling point (71.8 °C) (Torres-Giner, Ocio, & Lagaron, 2008). It is able to dissolve chitosan thanks to the formation of salts with its amino groups and lowers surface tension with respect to acetic acid (Ardila, Ajji, Heuzey, & Ajji, 2018). Furthermore, the addition of dichloromethane (DCM) decreases the dielectric constant and the conductivity of the mixture, helping the formation of particles rather than fibers (Torres-Giner et al., 2008).

The fundamental hypothesis of this study was that particles of a biodegradable polymer (chitosan) loaded with antibiotic could be produced by electrospray in a single step process, yielding inhalable particles of a size suitable to target alveoli. This could have a strong potential in the treatment of respiratory infections by direct pulmonary delivery of dry powder formulations. We have chosen ciprofloxacin as the antibiotic used in this study. It belongs to fluoroquinolones group and it is active against a wide spectrum of gram (+) and gram (-) bacteria, acting by inhibition of the activity of DNA gyrase and topoisomerase IV (Pignatello et al., 2018). It is effective against a large number of microorganisms that have been associated with infections in the respiratory system, such

as *Staphylococcus aureus* or *Pseudomonas aeruginosa* (Osman, Kan, Awad, Mortada, El-Shamy & Alpar, 2013). Nowadays, a single commercial formulation of ciprofloxacin exists as a dry powder inhaler (DPI) by Bayer AG (Berlin, Germany). Particles of ciprofloxacin are produced by spray–drying and they combine the novel PulmoSphere™ technology (Novartis) with T-326 inhaler for their administration. This formulation has demonstrated a decrease in the number of exacerbations in patients with respiratory bacterial infections (McShane et al., 2018).

Our study presents both a different production method and a novel formulation using ciprofloxacin as an antibiotic for potential use in pulmonary administration against pulmonary infections by *S. aureus* and *P. aeruginosa*. The use of chitosan would lend the electrosprayed particles a positive surface charge which is advantageous for their interaction with the mucus layer in the lungs or with bacteria surfaces that are negatively charged (Ma, Garrido-Maestu, & Jeong, 2017). In addition, the formulation used in this work avoids the use of excipients. Last but not least, the electrospray method allows to obtain particles with a controlled size (Moreno et al., 2018). Thus, this one-step production technique yielded drug-loaded particles with a narrow distribution approaching a size that represents, as shown above, an excellent choice for alveolar deposition.

2. Materials and Methods

2.1 Materials

Reagents, high molecular weight chitosan (HMW_CS) (deacetylation degree: ≥75%; viscosity: 800–2000 cP 1wt.% in 1% acetic acid), low molecular weight chitosan (LMW_CS) (deacetylation degree: 75–85%; viscosity 20–300 cP 1wt.% in 1% acetic acid), phosphate buffered saline tablets (PBS) (pH 7.4) and trifluoroacetic acid (TFA) were purchased from Sigma–Aldrich (USA); dichloromethane (DCM; Fisher Chemicals, UK) and ciprofloxacin (1-cyclopropyl-6-fluoro-4-oxo-7-piperazin-1-ylquinoline-3-carboxylic acid, CPX; Fluka analytical, Spain).

Bacteria strains, *Staphylococcus aureus* (ATCC 25923; Ielab, Spain) and *Pseudomonas aeruginosa* (ATCC 10145; Ielab, Spain). Cell line A549 (ATCC–CCL–185) were kindly provided by Dr. P. Martin-Duque and used between passages 25–32.

2.2 Electrospray production of chitosan-based sub-micron particles

Electrospraying was performed in Yflow 2.2D-500 electrospinner (Coaxial Electrospinning Machines/R&D Microencapsulation, Spain). Briefly, electrospraying of different chitosan solutions in a TFA/DCM mixture was conducted at diverse flow rates controlled by a syringe pump. The needle (0.6 mm, inner diameter) was positioned vertically towards the grounded collector plate and connected to the positive electrode and the negative electrode was connected to a stationary collector plate located at a certain distance from the needle tip. All experiments were conducted at RT, (HR)% of 25-50% and under atmospheric pressure. Preliminary experiments allowed the optimization of some operational parameters such as tip-to-collector distance (H) and applied voltage (V). The reference set of conditions for electrospraying CS solutions are H= 10 cm and V: 9-27 kV.

The effect of chitosan MW, flow rate and polymer concentration were studied to evaluate their influence in particle size distribution and to select the best conditions to produce chitosan sub-micron particles (CS SMPs) reducing the appearance of large beads and especially of fibers. This study was carried out with empty (ciprofloxacin-free) chitosan particles. To do that, electrospraying of diverse solution of HMW and LMW chitosan (from 20 mg/mL to 50 mg/mL) in a mixture of TFA/DCM (70% v/v of TFA) was conducted using flow rates between 0.2 mL/h and 1 mL/h. *Table S1* (*Supplementary Information (SI)*) shown different electrospray parameters evaluated.

Once the conditions were optimized to obtain spherical CS SMPs with a suitable size range, CPX was added to the electrospray solution. CPX concentrations from 1 mg/mL to 10 mg/mL were tested. All the other parameters were kept constant.

2.3 Characterization of chitosan sub-micron particles

FTIR analysis of chitosan, raw ciprofloxacin and chitosan SMPs were made using Vertex-70 FTIR spectrophotometer (Brucker, USA). Each spectrum was analysed with a resolution 4 cm^{-1} and 40-scan.

Scanning electron microscopy (SEM) was conducted on a FEI Inspect Field Emission instrument at accelerating voltages between 5-10 kV to obtain the morphology and size distribution of all prepared materials. Samples were sputter-coated with Palladium (Pd).

Zetasizer Nano ZS® (Malvern Instruments, UK) was used to measure the particle size (photon correlation spectroscopy) and Zeta potential (laser doppler anemometry). For the particle size analysis, each sample was diluted in ultrapure water and sonicated before the measurement. Zeta potential value was measured in a mixture of water and 1 mM KCl and placed in the electrophoretic cell.

2.4 Encapsulation efficiency (%EE) and drug loading (%DR)

Drug content was determined by extracting all the ciprofloxacin from the SMPs in acidic medium (0.1M HCl). 1 mg of SMPs loaded with CPX was added to 2 mL of 0.1M HCl. The suspension was stirred at 500 rpm for 48 h at RT. Finally, solution was centrifuged 10 min at 13000 rpm and the supernatant was recovered.

The absorbance of CPX in the supernatant was measured at 277 nm on a spectrophotometer (PerkinElmer Lambda-35) and the concentration of CPX was calculated using its standard curve. %EE and %DR of SMPs were calculated as follows:

$$\%EE = (\text{Measured mg ciprofloxacin}/\text{Theoretical mg ciprofloxacin}) \cdot 100$$

$$\%DR = (\text{Measured mg ciprofloxacin}/\text{mg SMPs}) \cdot 100$$

2.5 In vitro drug release studies

In vitro drug release study was conducted in PBS. Suspensions of 1 mg/mL of CPX-loaded SMPs were incubated at 37 ºC under horizontal shaking at 250 rpm. At fixed times (0, 15, 30, 45 minutes and 1, 2, 4, 6, 8, 24, 48, 72 h), these suspensions were centrifuged (13000 rpm for 10 min, at RT). The CPX content was obtained by measuring the supernatants.

2.6 Bacteria cultures and assays for antimicrobial activity

Antimicrobial activity of chitosan particles and raw ciprofloxacin was evaluated in gram (+) bacteria model, *S. aureus* and gram (-) bacteria model, *P. aeruginosa* by determining of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values.

Both strains were grown for 16 h in TSB in a shaker (Innova® 40, New Brunswick Scientific) with 150 rpm and 37 ºC. Finally, 10^8-10^9 colony-forming units/mL (CFU/mL) were obtained.

The grown bacteria were diluted in TSB to a final concentration of ~10^5 CFU/mL. Different concentrations of CPX-loaded HMW_CS SMPs and raw CPX (0.125, 0.250, 0.375, 0.500, 0.750 and 1 µg/mL) were inoculated with *S. aureus* and CPX-loaded LMW_CS SMPs and raw CPX (0.0625, 0.125, 0.250, 0.500, 1, 2, 4 and 8 µg/mL) with *P. aeruginosa.*

Control A group was bacteria not exposed to any study sample and control B group was bacteria exposed to the volume of 0.1M HCl solution necessary to dissolve raw CPX, both control groups were included to confirm correct growth of the bacteria.

The optical densities of pathogenic bacteria in contact with SMPs and raw drug were measured at 600 nm (Implen^{TM} OD600; ThermoFisher Scientific) to examine bacteria growth. To this end, *S. aureus* and *P. aeruginosa* (~10^5 CFU/mL) were inoculated in each sample. The mixed suspensions were kept in a shaker at 37 ºC at 150 rpm and OD_{600} was measured at selected times up to 24 h.

Agar dilution method allowed to quantify the number of CFU/mL and it was also used to evaluate the antimicrobial activity of CPX-loaded in CS SMPs. The bacteria were diluted to ~10^5 CFU/mL and then inoculated with different samples. Bacteria were kept in a shaker at 37 ºC at 150 rpm during 24 h. After that, bacteria suspensions were diluted in PBS and seeded in Petri plates with TSA at 37 ºC for 24 h. The MBC was determined by testing the concentration that showed no visible bacteria growth (the lowest concentration that kills > 99.95 of the bacteria)

2.8 In vitro cytotoxicity assay on A549 cells

A549 cell viability after exposure to the prepared CS SMPs were used to assess their toxicity, using the Alamar Blue assay. A549 is a human alveolar epithelium cell line, often employed in cytotoxicity studies. The cell culture medium (CCM) was DMEM with FBS (10% v/v) and antimitotic-antibiotic, penicillin (60 µg/mL), streptomycin (100 µg/mL) and amphotericin B (0.25 µg/mL).

A549 cells were seeded into a 96–well plate at a density of 1×10^4 cells/well in 100 µL CCM for 24 h and 48 h in a CO_2 incubator. Cells were allowed to attach for 24 h. The samples were sterilized by UV irradiation at least 30 min before contacting with cells. Raw CPX, CS SMPs and CPX-loaded CS SMPs were dispersed in CCM at different selected concentrations (0.1 µg/mL – 100 µg/mL). The CCM was replaced with the new culture medium containing the particles. Four wells with cells not exposed to any sample were used as controls in each experiment.

After that, the medium was removed and the cells were washed twice with DPBS. Then, the Alamar Blue reagent was added following the manufacturer´s indications (10% v/v; incubation at least 1 h at 37 ºC and 5% CO_2). A microplate reader (Multimode Synergy HT Microplate Reader; Biotek, USA), at λ_{530} nm excitation and λ_{590} nm emission, was used to record the fluorescence displayed. The viability was calculated by linear interpolation of the mean fluorescence values (MFV) from the cells treated with SMPs and raw ciprofloxacin versus the untreated one:

$$\%\ \text{Cell viability} = (\text{MFV of treated cells}/\text{MFV of control cells}) \cdot 100$$

To determine the toxicity threshold, the ISO 10993-5 norm was used (Biological evaluation of medical devices – Part 5: Tests for in vitro cytotoxicity). This norm considers a material as non-cytotoxic when cellular viability is >70%.

3. Results and discussion

3.1 Electrosprayed unloaded chitosan sub-micron particles

The chitosan concentration was varied from 2% w/w to 5% w/w. The feed flow rate could be increased up from 0.2 to 1 mL/h thanks to the higher stability of the Taylor cone with TFA/DCM. A set of 18 different experimental electrospray parameters was tested (*Table S2*). The morphology of electrosprayed materials and particle size obtained are shown in *Tables S3*, *S4* and *Figures S1, S2* in *SI.*

For both molecular weight of chitosan tested (HMW_CS and LMW_CS), it is possible to observe the effect of the chitosan concentration and flow rate on the morphology of the material obtained (*Figure 1*). The main controlling parameter is chitosan concentration and in fact for CS up to around 3 % w/w the fiber formation can be avoided for the whole interval of flow rates investigated (0.2 to 1 mL/h) and irrespective of the MW of chitosan

used. However, it can be seen that, for HMW_CS, in the region of high CS concentrations and flow rates, nanofibers are obtained, almost exclusively.

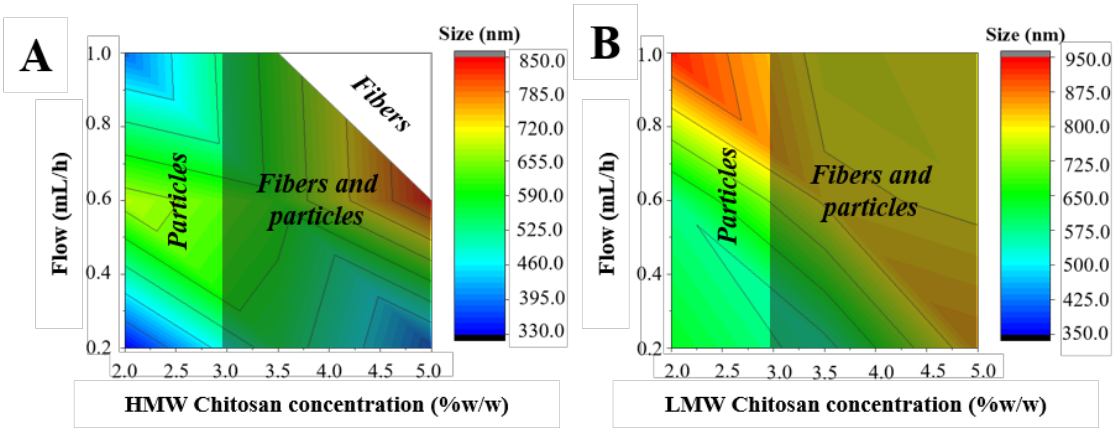


Fig. 1. Flow (mL/h) and polymer concentration (% w/w) influence on particle size diameter for different molecular weight chitosan. (a) HMW chitosan; (b) LMW chitosan.

In both systems, the region of low concentrations of chitosan (~2% w/w) and low flow rates (0.2 mL/h) produced small particles with spherical morphology and narrow size distribution (formulations H02/20 and L02/20 in *SI*). On the other hand, higher chitosan concentrations above 3 % w/w led to a mixture of particles and interconnected fibers as seen in *Figures S1* and *S2*.

In summary, a concentration of chitosan 2% w/w, flow rate of 0.2 mL/h were selected as optimal to produce the particles in this work. This led to fine spherical chitosan particles with a size well that could be inhaled for pulmonary administration. To test the aerosol behaviour of these particles and their aerodynamic diameter, a sample of 10 mg was loaded into a pressure-pulse aerosol generator (Clemente, Lobera, Balas, & Santamaria, 2018) and dispersed while measuring the particle size distribution. The results are shown in *Figure S3*, where it can be seen that the aerosolized particles present a bimodal size distribution with peaks at 73 ± 9 nm, and at 195 ± 80 nm respectively. This is a consequence of the aerosolization process used, that projects the particles through a narrow opening with a high shear stress and may vary with operating conditions, but in any case, these sizes would also be in a similar range regarding alveolar deposition.

3.1.2 Ciprofloxacin-loaded chitosan sub-micron particles

Using the reference set of electrospray parameter, CPX was added to the electrospray solution (concentrations from 1 mg/mL to 10 mg/mL). Spherical, well-formed particles were obtained with increasing concentrations of CPX. Interestingly, the morphology with different CPX concentrations was very similar to that obtained for empty chitosan particles. For this reason, the conditions for the production of SMPs could be extended to a concentration of 5 mg/mL of CPX, enabling a higher drug loading (*Figure 2*).

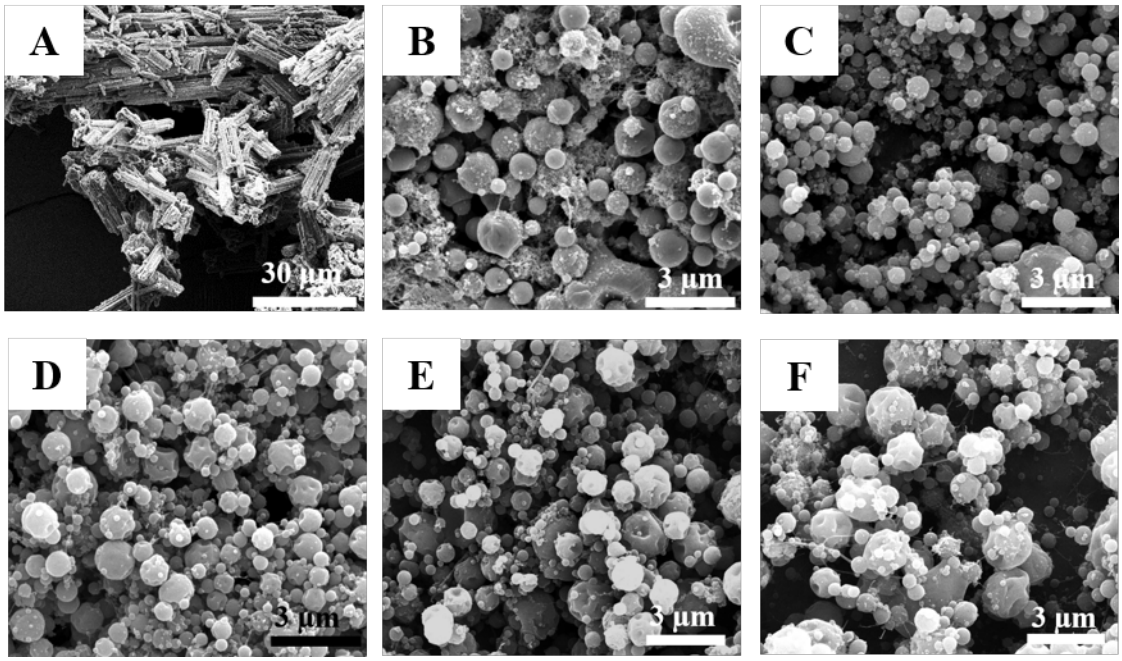
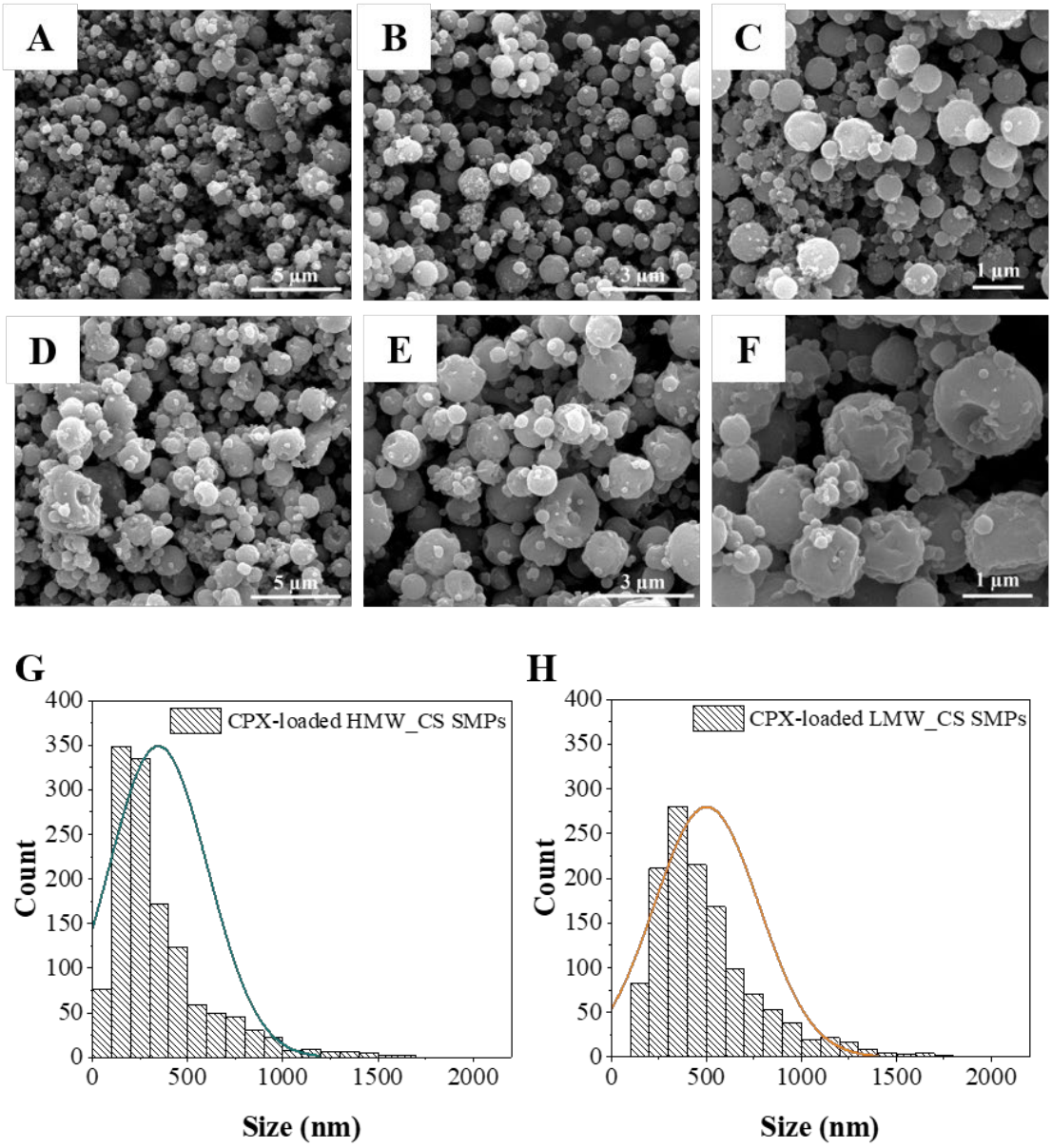


Fig. 2. SEM images (a) Pristine ciprofloxacin; (b-f) Electrosprayed chitosan particles prepared with different concentration of ciprofloxacin; (b) 10 mg/mL; (c) 5 mg/mL; (d) 4 mg/mL; (e) 2 mg/mL; (f) 1 mg/mL.

Using the reference conditions, the electrospraying experiments were repeated with HMW and LMW chitosan. The results are shown in *Figure 3.* As can be seen, LMW_CS always gives rise to larger particles for both empty and loaded SMPs compared to HMW_CS.



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247 Fig. 3. SEM images of chitosan particles. (a - c) CPX-loaded HMW_CS SMPs; (d - e) CPX-
248 loaded LMW_CS SMPs. (g and h) Size distribution particles

249 **3.2 Characteristics and morphology properties of CPX-loaded particles**

250 FTIR chitosan powder spectra showed a characteristic band in region 3400-3200 cm^{-1}
251 attributed to $–NH_2$ and –OH groups, 2920 cm^{-1} (C-H stretching) and amide I (1640 cm^{-1}
252). Raw ciprofloxacin showed a main peak at 1286 due to the C–F bond (Kyzioł, Mazgała,
253 Michna, Regiel-Futyra, & Sebastian, 2017). CS SMPs and CPX-loaded CS SMPs
254 displayed a shifted band from 1640 cm^{-1} to 1660 cm^{-1} corresponding to the presence of
255 chitosan in both SMPs. However, when CPX was loaded the CS SMPs spectrum revealed

a characteristic band at 1286 cm^{-1} (C–F bond), confirming the presence of ciprofloxacin in the loaded particles. Also, chitosan particles presented bands corresponding on one side to N-H stretching (including NH_4^+) at 1660–1520 cm^{-1} and the other to C–O stretching of $C_2F_3O_2^-$ (bands at 1190–890 cm^{-1}) (Torres-Giner et al., 2008). The presence of these active groups (NH_4^+ and $C_2F_3O_2^-$) in CS SMPs could give rise to some antimicrobial activity caused by the particle material itself (*Figure S4* in *SI*).

Table 1 shows the main results from the characterization of the SMPs. Irrespective of the loading, the key factor regarding particle size is the MW of CS used, with considerably larger particles with LMW_CS. Torres–Giner et al. (2008) showed that an increase in the MW of chitosan in a TFA/DCM solution produced an increase in the viscosity and decreased the surface tension. As a consequence, with HMW_CS is easier to obtain fibers and smaller particles, while the formation of larger particles is more likely when LMW_CS is used. Indeed, in the case of LMW the SMPs obtained were almost twice the size of the HMW_CS SMPs. Particles with this size (< 1μm) are normally deposited in the lower respiratory system such as bronchioles and alveoli (Klinger-Strobel et al., 2015; Yhee, Im, & Nho, 2016).

As shown in *Table 1*, both types of SMPs are positively charged due to the amino groups of chitosan. This is an important advantage for biomedical applications since it favours interactions between chitosan particles and surfaces of the lung mucus layer or bacteria, both negatively charged (George & Abraham, 2006). The strong positive surface charge (in excess of +40 mV and in some cases higher than +50 mV) of these SMPs also helps to prevent agglomeration, as shown by the DLS values, giving sizes in solution are less than twice those of individual particles, i.e. the solution is largely made of dispersed individual particles.

Table 1

Properties of chitosan sub-micron particles used in this study.

Sample	SEM	Z potential	DLS	EE	DL
	Mean* ± *SD		***Mean* ± SD**		
	(nm)	*(mV)*	*(nm)*	*(%)*	*(%)*
CPX	-	12.1 ± 4.5	-	-	-
HMW_CS SMPs	333.9 ± 217.9	51.0 ± 11.2	438.0 ± 81.2	-	-

CPX-loaded HMW_CS SMPs	386.1 ± 248.5	42.1 ± 5.2	625.1± 68.6	75.6 ± 10.2	15.8 ± 2.1
LMW CS_SMPs	636.3 ± 312.4	42.6 ± 6.2	596.2 ± 100.7	-	-
CPX-loaded LMW_CS SMPs	501.1 ± 276.3	52.0 ± 10.5	533.3 ± 39.1	70.5 ± 16.5	14.1 ± 3.3

3.4 In vitro release studies: mechanism and kinetics

Many mathematical models have been used to describe and/or design drug delivery systems and to predict the overall release behaviour as a function of time. A realistic model therefore becomes a key tool to tune release patterns in a way that matches the therapeutic regimen of patients.

In this work, the kinetics of ciprofloxacin release from chitosan SMPs were initially fitted to Linder-Lippold (Linder & Lippold, 1995), Ritger-Peppas (Korsmeyer, Meerwall, & Peppas, 1986), and Peppa-Sahlin (Peppas & Sahlin, 1989) empirical models (*Table S5 in SI*). The fitting of the experimental data of ciprofloxacin concentration (m_t) versus time with the above models was not good ($R^2 < 0.900$), in spite of the number of adjustable parameters used (2 or 3 depending on model).

In order to have a more realistic description of kinetics release, we have assumed that the rate of ciprofloxacin released from the chitosan particles, (dm^s_t/dt), is a first order process, and therefore the content of ciprofloxacin in the chitosan particles decays exponentially along time:

$$-dm^s_t/dt = k_R \cdot m^s_t \rightarrow m^s_t = m^s_0 \cdot \exp(-k_R \cdot t)$$

In the above equation m^s_0 represents the initial ciprofloxacin in the chitosan particles and k_R is the kinetic constant of drug release. This parameter depends of the specific interaction developed between the substrate (chitosan) and the drug released (ciprofloxacin), and also on the operational conditions of the kinetic experiments. In fact, this is the only one parameter that is calculated by non-linear regression of the model to the experimental data. The amount of ciprofloxacin released to the solvent can be now calculated directly from the mass balance as follows:

$$m_t = m^s_0 - m^s_t \rightarrow m_t = m^s_0 \cdot (1 - \exp(-k_R \cdot t)); \qquad m^s_0 = m_\infty$$

From this equation it is deduced that the maximum concentration of ciprofloxacin attainable, $m_{\infty,}$ corresponds to the initial amount loaded on the chitosan particles, m^s_0.

On *Table 2* the values of k_R, m_∞ and R^2 are presented for the two samples of chitosan studied. The higher values of R^2 obtained with the one-parameter exponential model, in comparison with the above models suggests the validity of the assumptions made here (curve fittings for the different models are graphically displayed in *Figure S5*).

According to this model, the intrinsic rate of chitosan release from the LMW_CS sample is around 37% higher than of the HMW_CS sample, indicating a lower interaction between the chitosan and the ciprofloxacin on the LMW case. As expected, the estimated values of m_∞ are in agreement with the experimental values of m^s_0 used in each case.

Table 2

Kinetic constant of ciprofloxacin release for the HMW and LMW chitosan samples.

Sample	$k_R \pm SE$ (min^{-1})	$m_\infty \pm SE$ (% mg)	R^2	*ner. points*
CPX-loaded HMW_CS SMPs	0.0303 ± 0.0026	81.237 ± 1.607	0.970	104
CPX-loaded LMW_CS SMPs	0.0416 ± 0.0022	85.938 ± 0.957	0.995	65

Ciprofloxacin release could be enhanced by dissolution of chitosan chains. According to Jayakumar et al., (2010) chitosan structures prepared by electrospray showed a fast and complete dissolution in contact with neutral or weak basic aqueous solutions. This could explain the rapid release observed for CPX in this work. *Figure 4* shows the cumulative drug release (%CDR) for the two kind of chitosan SMPs at 37 ºC as a function of time. A quick release of ciprofloxacin from chitosan SMPs is an advantage when the objective is the treatment of acute bacterial infections, since the antibiotic being released shortly after inhalation.

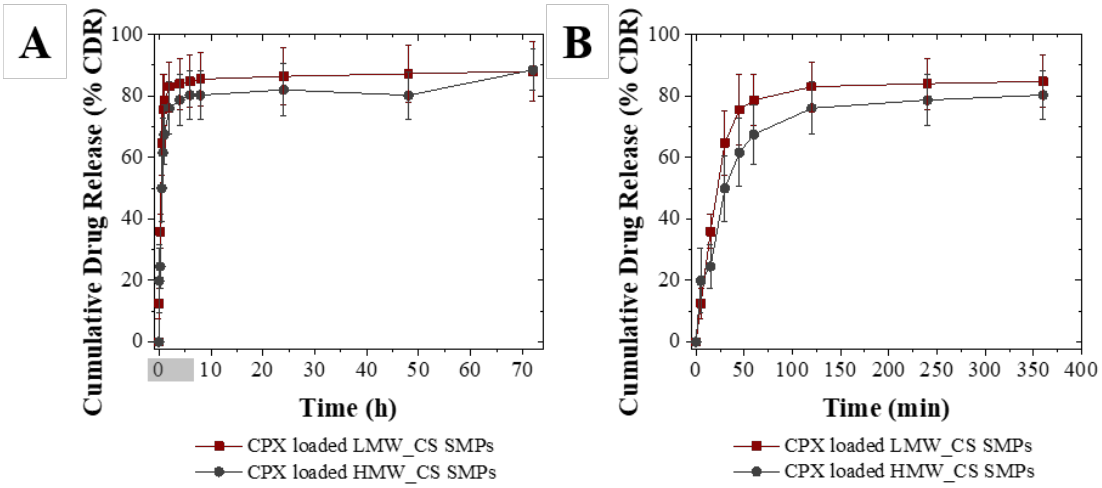

Fig. 4. Cumulative drug release (%CDR) in PBS with respect to total encapsulated drug for two different molecular weight chitosan SMPs (n = 3); (a) %CDR until 72 hours; (b) %CDR until 6 hours.

3.5 Determination of minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC)

The antibacterial activity of chitosan SMPs was tested against two important pathogens (*S. aureus* and *P. aeruginosa*) in lung infections. In the case of gram (+) strains, previous studies showed that the positive charge of chitosan interacts with the negative charge of the bacterial wall, producing a change in the permeability of the wall and facilitates the exit of intracellular components. In contrast, for gram (–) strains chitosan is able to cross the cell membrane and act from the inside, also promoting the exit of cellular material (Verlee, Mincke, & Stevens, 2017).

Growth curves (*Figure S6*) showed that *S. aureus* at 0.250 and 0.375 (MIC) µg/mL displayed a lag phase up to 16 h, indicating that the growth bacteria was inhibited and the log phase was delayed. In the case of *P. aeruginosa,* at 0.250 (MIC) µg/mL the log phase was delayed up to 12 h. However, from 0.500 µg/mL it was not possible to distinguish between the lag and log phases. This result showed that *S. aureus* and *P. aeruginosa* were more susceptible to the exposure to SMPs when compared to raw ciprofloxacin. Those concentrations where a strong decrease in bacterial growth was observed were studied with the agar dilution method. 1 µg/mL (MBC) was the concentration found to inhibit the growth bacteria (*Figure 5*).

348 It can be concluded that ciprofloxacin encapsulated in chitosan SMPs not only retained
349 its antimicrobial activity but also the encapsulated formulations were effective in short
350 periods of time.

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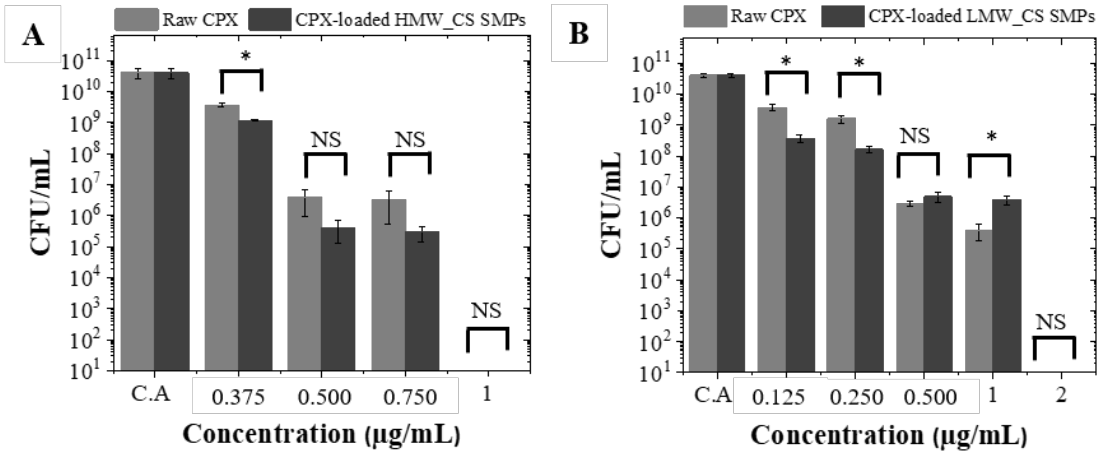

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353 Fig. 5. Antibacterial activity (CFU/mL) of CPX (raw and encapsulated). (a) *S. aureus*; (b) *P.*
354 *aeruginosa* (mean ± SD, n = 3; p* < 0.05; NS, no significant difference).

355 **3.6 Cytotoxicity assay**

356 Finally, a cytotoxicity assay was carried out to study the biocompatibility of CPX-loaded
357 CS SMPs. Raw ciprofloxacin was also tested. Concentration tested were 50 times the
358 MBC (2 µg/mL) found for these SMPs.

359 *Figure 6* shows the cell viability percentages obtained after 24 h and 48 h. It can be seen
360 that, after 24 h of exposure to the different samples, only raw ciprofloxacin showed
361 cytotoxicity at the highest concentration of 100 µg/mL. However, after 48 h, CPX-loaded
362 HMW_CS SMPs and CPX-loaded LMW_CS SMPs were found to be cytotoxic from 10
363 µg/mL, while at the same time of exposure, raw ciprofloxacin showed cytotoxicity at 100
364 µg/mL. However, it must be considered that the limit of toxicity (10 µg/mL), is 10 times
365 of the MBC for *S. aureus* and 5 times of the MBC for *P. aeruginosa.* i.e., for
366 concentrations lower than 10 µg/mL, *S. aureus* and *P. aeruginosa* will still die while the
367 environment would be non–toxic to respiratory cells.

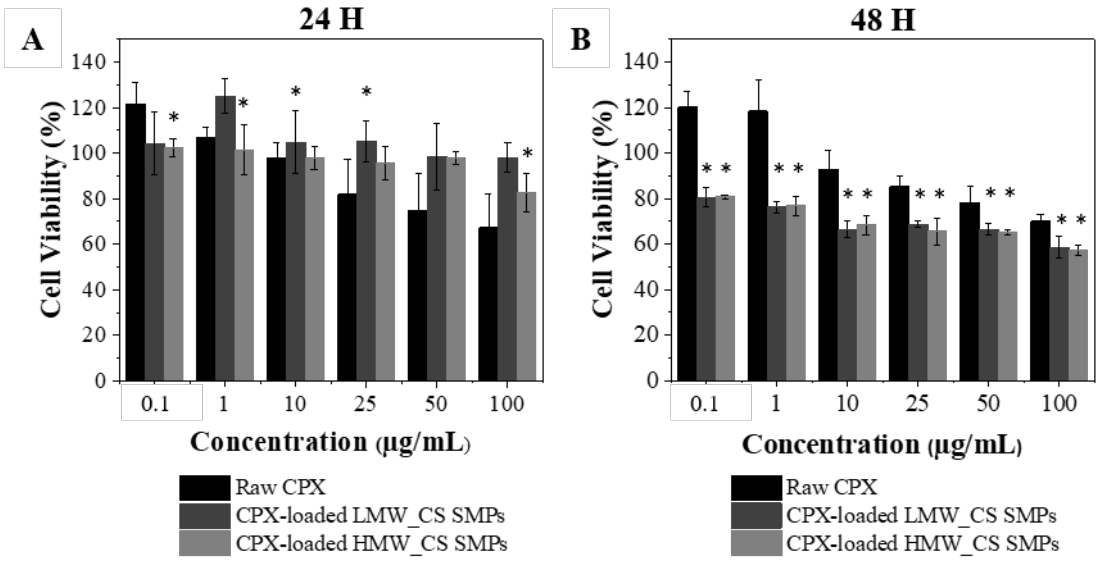

Fig. 6. Cell viability studies of raw CPX and CPX encapsulated in SMPs. Exposure time: (a) 24 h; (b) 48 h. Significant differences ($p < 0.05$) between different samples tested in relation to raw CPX (mean ± SD, n = 3).

4. Conclusions

This study presents a new method to produce dry chitosan SMPs loaded with ciprofloxacin. The particles were produced by electrospray as a dry powder in just one-step and were excipient-free. This method yielded a particle size that would be in principle suitable to reach alveoli, with reduced deposition elsewhere in the lung. The CPX-loaded chitosan SMPs showed potent antimicrobial activity against *S. aureus* and *P. aeruginosa*. CPX release was fast (50% release was achieved in 30 min) and can be characterized as a first order release process. Cytotoxicity assays in A549 human lung epithelial cells showed that ciprofloxacin-containing chitosan particles are safe to be used at the MBC doses. In summary, the results indicate that electrospraying of CS with CPX solutions using TFA/DCM mixtures as solvents yields SMPs of a size suitable for pulmonary delivery by inhalation, with a high drug content (around 15%) and encapsulation efficiency above 75%.

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