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Development of a carrier-free dry powder ofloxacin formulation with enhanced aerosolization properties

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Abstract

Tuberculosis (TB) is a serious infectious disease that affects more than new 10 million patients each year. Many of these cases are resistant to first-line drugs so second-line ones, like fluoroquinolones, need to be incorporated into the therapeutic. Ofloxacin (OF) is a fluoroquinolone which demonstrates high antibiotic activity against the bacteria that causes TB (*M. tuberculosis*). In this work, ionic complexes, composed by hyaluronic acid (HA) and OF, with different neutralization degrees, were prepared and processed by spray drying (SD) to obtain powders for inhalatory administration. Combining a formulation with high neutralization degree, high SD atomization air flowrate and the use of a high-performance collection cyclone, very good process yields were obtained. Carrier-free formulations with a loading of 0.39-0.46 g_{OF}/g_{powder} showed excellent emitted, fine particle, and respirable fractions for capsule loadings of 25 and 100 mg. The ionic complexes demonstrated higher mucoadhesion than pure OF and HA. The best formulation did not affect CALU-3 cell viability up to a dose 6.5 times higher than the MIC_{90} reported to treat multi-drug resistant TB.

Keywords:

Fluoroquinolones, hyaluronic acid, drug-excipient interaction, multi-drug resistant tuberculosis, inhalation, cell viability, mucoadhesive, spray drying

1. Introduction

The inhalatory route is considered a promising non-invasive alternative for drug administration to treat systemic or pulmonary illnesses. For pulmonary diseases, this route delivers the drug directly to the action site minimizing the systemic distribution and side effects. Then, a high concentration of the active pharmaceutical ingredient and rapid onset can be achieved. Consequently, lower doses are often required. Inhalatory route has been used for obstructive illnesses, like chronic obstructive pulmonary diseases (COPD) and asthma, but is also relevant for treatment of infections, like tuberculosis (TB) and those associated with cystic fibrosis[1,2,3,4].

To treat respiratory infections, antibiotics are usually administered by the oral route. Orally administered drugs have problems reaching the lung**s** by systemic distribution. So the delivery of antimicrobials by the inhalatory route has been proposed[3]. However, the performance of inhaled aerosols depends on particle properties like size, morphology and density[5]. For this reason, a rational design of the inhalatory system is required for a successful pharmacotherapy.

Treatments by pulmonary route could be also improved by, for example, increasing drug residence time in the lungs or modifying the active ingredient dissolution rate[6]. In this sense, by combining ionizable drugs with opposite charged polymers (polyelectrolytes) new chemical entities can be obtained with specific and differentiated properties respect to the precursor active ingredient[7]. Different antibiotics have been formulated by using this strategy to be administered by the inhalatory route. Among others, nanoaggregates of nanoparticles containing vancomycin were obtained by ionic interaction with polyacrylic acid where the polyelectrolyte stabilized the nanoaggregates avoiding agglomerations[8]. Cheow and Hadinoto[9] and Kho and Hadinoto[10] produced systems composed by fluoroquinolones and dextran, where the polyelectrolyte allowed obtaining amorphous co-processed formulations. For TB treatment, Zahoor *et al*. produced nanoparticles by combining sodium alginate with rifampicin, isoniazid and pyrazinamide. The polyelectrolyte allowed prolonging the drug residence time and thus reducing the administration frequency[11]. Swai *et al*. produced systems combining poly-lactid-co-glycolic acid or chitosan/alginate with isoniazid[12] and Manca *et al*. obtained microparticles of carrageenan and chitosan carrying rifampicin liposomes[13]. In both reports, the ionic interaction increased the drug residence time.

As previously stated, some inhalatory formulations were developed for TB. It was estimated that just in 2015 there were 10 million new cases of tuberculosis all over the world, killing almost 2 million persons. What is more, 5.5 % of the 2015 new cases were resistant to first-line drugs (mainly rifampicin, isoniazid and pyrazinamide). When drug resistance is detected, second-line drugs are incorporated to the therapeutic and the treatment duration is increased[14,15]. Fluoroquinolones are considered to be an important pharmacological group for alternative TB treatments[16]. In this sense, ofloxacin (OF) possesses high antibiotic activity against *M. tuberculosis*, including those strains resistant to rifampicin.

Then, new materials carrying OF would be of great interest for the pharmacotherapy of resistant TB. In this sense Park *et al*.[3], produced chitosan crosslinked with glutaraldehyde microparticles

carrying OF by water-in-oil emulsification method. The aqueous phase was composed of ofloxacin, chitosan and acetic acid while the oil phase was prepared using dichloromethane, paraffin, surfactants and emulsifiers. The authors focused on the uptake of the microparticles by alveolar macrophages in rat alveolar macrophages cell line and demonstrated that microparticles were uptaken in a higher proportion than pure OF[3]. Hwang *et al*. developed microspheres containing OF and the sodium salt of hyaluronic acid (HANa) by spray drying an ethanolic solution containing these compounds[17]. They administered pure OF, spray-dried OF and spray-dried OF-HANa formulations to rats by oral, intratracheal and intravenous routes. For the intratracheal administration, the authors demonstrated that the OF lung concentration was higher and the OF plasmatic concentration was lower respect to the contents found for the oral and intravenous routes[17]. The OF lung/OF plasma concentrations ratio for the inhalatory administration of the OF-HANa system was about two times higher than the ratio found when pure OF was intratracheally delivered. These authors also found that the OF-HANa microparticles, as reported for the OF-chitosan formulation, were uptaken in a higher proportion than pure OF by alveolar macrophages. Park *et al*. and Hwang *et al*., evaluated the aerosolization properties of the OF-chitosan and OF-HANa powders, respectively, in a twin-impinger when delivered from a dry powder inhaler (DPI) system. The fine particle fraction for sizes lower than 6.4 μm was 43 % and 45 % for OF-HANa and OF-chitosan systems, respectively[3,17].

The previously developed systems carrying OF exhibited attractive biopharmaceutical performance and in vivo macrophages uptake in lab animals[3,17]. However, both contributions used organic solvents for the OF dissolution previous to powder production. In this work, the development of a polyelectrolyte-drug ionic complex constituted by OF-hyaluronic acid (HA) is used as strategy to dissolve OF without using organic solvents, being in this sense an innovative way to produce OF microparticles for inhalatory administration. Then, the objective of this work is the production of OF-HA microparticles by a simple method with optimized aerosolization properties, as a carrier-free DPI. To this aim, a complete physicochemical characterization of the particulate systems was performed to establish relationships between process parameters and product quality. The OF-HA microparticles were obtained by processing aqueous solutions by spray drying and varying the feed formulation (*i.e*., OF/HA ratios) and operating conditions. The process performance (SD yield and air outlet temperature), product properties (OF loading, crystallinity, morphology and particle size), the OF-HA ionic interaction (assessed by Fourier transform infrared spectroscopy and powder X-ray diffraction), in vitro aerosolization performance (using a multistage cascade impactor), mucoadhesion and CALU-3 cell viability were evaluated to study the proposed formulations.

2. Materials and methods

2.1. Materials

Sodium hyaluronan (NaHA, **MW: 1655 kDa**, proanalysis grade, Parafarm, Saporiti, Buenos Aires, Argentina), ofloxacin (pharmaceutical grade, Parafarm, Saporiti, Buenos Aires, Argentina), sulfonic acid resin Amberlite® IR 120 in hydrogen form (proanalysis grade, Sigma–Aldrich, Saint Louis,

United States), sodium hydroxide 0.01 M (proanalysis grade, Anedra, Argentina), hydrochloric acid 10 M (proanalysis grade, Anedra, Buenos Aires, Argentina), potassium bromide (spectroscopic grade, Merck, Darmstadt, Germany), size 3 gelatine capsules (pharmaceutical grade, Parafarm, Saporiti, Buenos Aires, Argentina), lactose monohydrate (pharmaceutical grade, Parafarm, Saporiti, Buenos Aires, Argentina) with particle sizes between 70 and 140 mesh sieves (ASTM), glycerin (pharmaceutical grade, Anedra, Buenos Aires, Argentina), potassium phosphate monobasic (analytical grade, Anedra, Buenos Aires, Argentina), sodium hydroxide (analytical grade, Anedra, Buenos Aires, Argentina) and distilled water were used.

For the assays in cell cultures, CALU-3 cells (ATCC® Cat# HTB55, bronchial human epithelial airway cells) were used. Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicilin-streptomycin, L-Glutamine, non-essential amino acids and trypsine were all from Life Technologies, GIBCO BRL, Rockville, USA. CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay was purchased from Promega, Madison, USA.

2.2. Methods

2.2.1. Preparation of the HA solution

To obtain the HA solution, a 0.5 % w/v aqueous solution of NaHA was prepared and passed through a glass column packed with sulfonic acid resin (previously activated with HCl ≈ 10 M). The HA solution was collected when the pH of the eluted was 3.

2.2.2. Determination of number of acid equivalents

The number of acid equivalents in the HA solution was determined by potentiometric titration using NaOH (0.01 M). The evaluated solution had 2.11×10^{-3} acid equivalents of HA per gram.

2.2.3. Preparation of solutions to be spray dried

Table 1 shows the theoretical composition of solutions prepared to be spray dried. Two aliquots (200 mL each) of the HA solution (obtained as described in Section 2.2.1) were mixed with different amounts of the drug in order to neutralize about 75 and 100 % of the HA available acidic groups. For this, an appropriate mass of the drug, which represents the available acid groups to neutralize was weighted and incorporated under constant magnetic stirring. The theoretical total solid content in these solutions varied from 0.42 to 0.56 % (w/w). The samples were named according to their theoretical composition as $(HA\text{-}OF)_x$, where the subscript x refers to the neutralization degree of HA acidic groups (*i.e.*, x= 75 or 100). The pH of the resulted solutions was measured using a pH meter Orion 410A, Cole Parmer, Vermon Hills, United States.

2.2.4. Spray drying (SD) process

The solutions, prepared as described in Section 2.2.3, were fed in a negative pressure laboratory scale SD equipment (Mini Spray Dryer B-290, BÜCHI, Flawil, Switzerland) with a two-fluid nozzle (cap-orifice diameter: 0.5 mm). Based on exploratory experiments, two sets of process parameters

were selected, which are summarized in Table 2. For comparison purposes, an aqueous solution of pure OF and pure HA were also atomized. The process yield (PY) was calculated as the ratio of the weight of product collected after the spray drying process respect to the initial total solid content.

2.2.5. Powder characterization

- Drug loading: SD powders were dissolved in distilled water and ofloxacin concentration was determined by UV-spectrophotometry (T60, PG instruments, Lutterworth, UK) at 289 nm. The results were reported as gram of drug per gram of powder.
- Fourier Transformed Infrared Spectroscopy (FTIR). Samples (SD materials and raw OF) were studied in 1-1.5 % w/w potassium bromide (KBr) compacts using a FT-IR instrument (Nexus FT, Termonicolet, Maryland, United States). Before analysis, samples and KBr were dried at 105 °C.
- Powder X-ray Diffraction (PXRD). An X-ray powder diffractometer (Philips PW 1710, Philips Industrial & Electro-acoustic Systems Division, Almelo, Netherlands) was used to assess the crystallinity of pure drug and SD powders. Sample diffractograms were obtained under previously reported conditions.[18,19]
- Particle morphology. The morphological characteristics of the pure and co-processed materials were evaluated through Scanning Electron Microscopy (SEM) using an EVO 40-XVP, LEO scanning electron microscope (Oberchoken, Germany).[18,19]
- Particle size. Size distributions were measured by laser diffraction using the dry powder method (LA 950 V2, Horiba, Kyoto, Japan). The spray-dried powders were dispersed in lactose (with a known particle size distribution) in a proportion lactose:sample 4:1 to improve the sample flow from the feed hopper to the measuring cell. The volume average diameter of lactose differs substantially from the average size of the powder obtained by spray drying. As a consequence, bimodal distributions, with two modes perfectly distinguishable were obtained allowing an accurate granulometry measurement. Size is reported as mean volumetric diameter (D_{43}) and distribution width is informed as *span*.[18]
- Drug distribution. The presence of drug in the microparticles was studied by fluorescence confocal microscopy (TCS SP2, LEICA, Wetzlar, Germany) for $(HA\text{-}OF)_{75}$ sample. A small amount of material was placed in a capsule and excited with an argon laser at 488 nm. An immersion objective of 63 X was used.

2.2.6. Aerodynamic characterization

The in vitro aerosolization performance was studied on a Next Generation Impactor (NGI, Copley Scientific, Nottingham, UK[20]) equipped with an induction port (IP) and a pre-separator (PS), filled with 15 mL of water, as previously described[21]. Size 3 gelatin capsules were filled with 25±0.50 mg of SD products and the powder was dispersed using an RS01 high resistance inhaler (Plastiape, Milano, Italy) into the NGI. The air flowrate was fixed at 58.8 L/min in order to reach a pressure drop of 4 kPa as indicated by the USP[22] and 4 L of air passed through the equipment. Drug content in each stage was assessed using a UV-spectrophotometer at 289 nm.

The Emitted Fraction (EF), Fine Particle Fraction (FPF), Respirable Fraction (RF), Mass Median Aerodynamic Diameter (MMAD) and Geometric Standard Deviation (GSD) were determined as follows[23,24,25]:

- EF*:* represents the drug percentage of total drug loaded in the capsule that is effectively released from the capsule and the inhaler;
- FPF: is the percentage of cumulative drug mass with aerodynamic diameters lower than a given size (the cumulative size distribution is built considering the drug mass collected in 1^{st} to 7^{th} NGI stages and the multiple orifice collector (MOC)) relative to the total drug mass recovered from the mouth piece adaptor (MA), PS, IP, 1^{st} to 7^{th} NGI stages and MOC;
- RF: accounts for the cumulative percentage of drug mass with aerodynamic diameters lower than a given size respects to the total drug mass recovered from the capsule, inhaler, MA, PS, IP, 1^{st} to 7^{th} NGI stages and MOC.
- The MMAD was calculated from the drug mass cumulative distribution and is defined as the diameter at which 50 % of the drug is collected in larger particles and the remaining 50 % is collected within smaller particles.
- The GSD, that represents the spread of an aerodynamic particle size distribution, was calculated as $(D_{84}/D_{16})^{1/2}$, where D_{84} and D_{16} represent the diameters at which 84 % and 16 % of the drug mass recovered from the 1^{st} to 7^{th} NGI stages and MOC, respectively.

With the aim to estimate the dose of the best HA-OF product required to treat the multi-drug resistant TB, the formulation was assayed at a higher capsule loading. For this, the size 3 gelatin capsule was filled with 100 mg of powder (**without applying any force or compressing the powder**) and the study was carried out under the same conditions than previously described. EF, FPFs, RFs, MMAD and GSD parameters were calculated.

2.2.7. Cell culture and viability assay

In order to preliminary assess the cytotoxic effect of the microparticles, CALU-3 cell line (derived from human bronchial submucosal glands) was used. CALU-3 cells were cultured in DMEM medium supplemented with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-Glutamine and non-essential amino acids at 37 °C under 5 % CO_2 in humidified incubator, as previously described[26].

For viability assays, CALU-3 cells were seeded in 96-well plates (80,000 cells/well in 100 µL complete DMEM medium) during 24 hours at 37 °C under 5 % CO_2 in humidified incubator. $(AH\text{-}OF)_x$ stock solution (20 mM) was prepared in distilled water. Cells were incubated with serum-free DMEM (considered controls) or with different concentrations of $(AH\text{-}OF)_{100}$ to reach a final concentration of 0.015, 0.03, 0.06, 0.1, 0.3 and 0.6 mM of ofloxacin in serum-free DMEM during 24 more hours. Cell viability was evaluated by using the CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay, according to manufacturer´s instructions. Briefly, after the 24 hours treatment, cells were washed with PBS (pH 7.4) and treated with staining solution containing the

MTS tetrazolium salt. MTS is reduced by mitochondrial oxidases into a formazan product that is soluble in tissue culture. This bioreduction is associated with metabolic activity. Absorbance was recorded at 490 nm using a microplate reader (Benchmark, Bio-Rad, Hercules, USA) and is directly proportional to the number of living cells[27]. Results are expressed in arbitrary units as % of the control condition.

2.2.8. Mucoadhesion assay: Tensile Strength

The mucoadhesion properties of the $(AH\text{-}OF)_x$ sample were studied using a TA Plus texture analyzer (Lloyd Instruments, Godalming, UK) equipped with a 5-kg_f load cell. The technique was adapted from Gallo *et al*[6]. Briefly, 0.1 mL of a mucin solution (3 % in PBS pH 7.4 kept at 37±0.5 °C) was placed over a filter paper (2 cm diameter). The filter paper was attached to a stationary surface and the mucin solution was allowed to stand for 15 minutes. A mobile metallic probe was placed above the stationary surface. A monolayer of microparticles carrying OF were attached to the mobile probe. The mobile probe was lowered, without applying any force, until it soaked in the mucin solution for 3 min. Finally, the probe was raised at withdrawal speed of 0.1 mm/s. The maximum detachment force (MDF) and the total work (TW) were measured using the computer software (Nexygen Plus). The reported results are expressed as the average of six measurements.

2.2.9. Statistical analysis

The significant differences between the mean volumetric diameter, aerodynamic behavior, cell viability and mucoadhesion test were determined by means of one-way ANOVA followed by least significant difference (LSD) test to compare means. Statistical significance was established through the *p*-value: values lower than 0.05 were considered statistically significant

3. Results

3.1. Spray drying: feed composition, feed properties and operating conditions

HA is a natural polysaccharide with anionic groups in its structure (pKa 3[28], Figure 1.a). It has N-acetyl d-glucosamine and β-glucuronic acid as repetitive units. Due to the non-immunogenicity and biocompatibility properties, it has been used for different pharmacological and clinical uses. HA has been proposed for the inhalatory route because increases the bioadhesivity of different therapeutic agents.[29] It is approved in some European countries to reduce bronchial reactivity associated to allergens or pollutants inhalation or caused by physical effort. A combination of HA with hypertonic saline solution is also approved for reducing mucus viscosity in cystic fibrosis patients.[30] Specifically for TB treatments by the inhalatory route, HA is biologically recognized by receptor CD44 in alveolar macrophages. That recognition improves internalization of anti-TB formulations containing both drugs and HA. Besides receptor-mediated phagocytosis, HA can polarize macrophages (leading to an inflammatory response and macrophages activation) and

then improve TB treatments[31]. In fact, HA has been employed to develop inhalatory delivery systems in order to modify dissolution rate and target macrophages for different drugs.[31,32,33,34]

As with others anionic polyelectrolytes, HA acidic groups have the capability to interact with cationic compounds.[18,35] OF is a zwitterionic fluoroquinolonic drug containing both anionic and cationic groups (pKas 5.9 and 8.3[36], Figure 1.b). This drug is slightly soluble in water[37]. The ionic interaction between HA and OF in an aqueous medium would lead to the formation of ionic pairs.

All feed solutions prepared for being processes by SD did not evidence precipitation or phase separation, even when the $(HA\text{-}OF)_{100}$ feed was formulated. For this solution, the OF concentration was around 0.0028 g/mL which is 7.7 % higher than the OF water solubility at 25 °C and pH=7[38]. Considering that for a given temperature the OF water solubility is a strong function of the pH[37], the absence of precipitation can be explained by two combined effects: the acidic resulting pH (around 5, see Table 1) and the improvement of the OF water compatibility due to the ionic interaction with hyaluronic acid[39]. In any case, the use of HA allowed obtaining aqueous solutions for spray drying without the need for organic solvents[3,17].

Table 1 shows the pH of different feed solutions. The HA pure solution showed the lowest pH value within the assayed solutions. As the neutralization of the free HA acidic groups of the polyelectrolyte was increased (from x=75 to x=100), the pH increased from 4 to around 5.

The feed solutions were processed in a spray dryer using the process parameters detailed in Table 2. The resulting SD outlet air temperature (T_{out}) and the process yield (PY) are shown in Table 3. When the set I of the operating conditions was used, the T_{out} was lower than 80 °C for all the formulations. This value is well below the degradation temperatures of OF and HA. In fact, OF thermal degradation starts over 250 °C[40] while hyaluronic acid decomposition occurs above 300 °C[41]. Considering this, thermal degradation during the spray-drying process is not expected. For set I, PYs of co-processed products were around 50 %, satisfactory level for a lab scale equipment[42].

The set II of process parameters increased the atomization air flowrate (aiming to reduce the particle size) respect to the value of set I, and replaced the standard cyclone by a high performance one (from BÜCHI) to improve the collection efficiency. According to Table 3 and for samples with a neutralization degree of 75 and 100 %, the PYs increased more than 35 % when process conditions changed from set I to set II. Simultaneously, the outlet temperature was reduced. So, set II improved the SD global performance for co-processed materials.

3.2. Product characterization

3.2.1. Microparticle composition.

Table 1 also shows the composition of the co-processed powders. As it can be seen, the g_{OF}/g_{powder} ratio of the products is in good agreement with the theoretical composition OF/(HA+OF). Since fluoroquinolones treatments require relative high doses, the high drug load (from 39 to 46 % of the product) in the microparticles is a valuable property of the formulated powders.

300 3.2.2. Fourier transformed Infrared spectroscopy

301 The FT-IR spectra of the SD pure and co-processed materials were obtained and Table 4 shows the
302 position of the characteristic peak wavelengths while in Figure 2 the FT-IR spectra can be seen. A
303 broad band at around 1636 cm^{-1} corresponding to the carboxylic stretching was detected in the
304 pure HA spectra. Other relevant bands for this compound were also found: one ascribed to the
305 stretching of the amide I band (1650 cm^{-1}) and the HC=C double bond stretching (1555 cm^{-1})[43]. The
306 pure OF FT-IR spectra displayed the characteristic bands of ofloxacin: at 2784.17 cm^{-1} appeared
307 the band ascribed to the stretching of the CH_3-N and the band at 1408.5 cm^{-1} was associated to
308 the in-plane deformation of the CH_3-N group. Besides, a peak ascribed to the C=O stretching of the
309 carboxylic acid at 1717.15 cm^{-1} and another peak associated to the C=O ring carbonyl at
310 1621.2 cm^{-1} can also be observed[44].

311 The spectra of the co-processed products showed some changes respect to the pure materials
312 spectra. The two bands ascribed to the OF stretching and in-plane deformation of the CH_3-N group
313 completely disappeared. Simultaneously, two new bands appeared as shoulders at around
314 1600 cm^{-1} and 1400 cm^{-1}. They were ascribed to the C=O asymmetric and symmetric stretching of
315 the carboxylate groups of HA, respectively. These bands were associated to the HA ionization due
316 to the ionic interaction between the OF amine group and the HA carboxylic group.

317 3.2.3. X-ray diffraction

318 Aiming to assess crystalline variations associated to the spray drying process and the interaction
319 between components, X-ray diffractograms of OF (as received from the supplier), spray-dried pure
320 OF and HA and the co-processed products were recorded (Figure 3). According to Figure 3a, pure
321 OF displayed a crystalline structure. The position and relative intensity of the reflections were in
322 good agreement with the ones reported by Peng *et al.*[45]. As it can be seen in Figure 3a, processing
323 OF by spray drying did not affect its crystalline structure as the reflections were placed in the same
324 angular positions that the ones of the raw OF. On the other hand, the spray-dried hyaluronic acid
325 presented an amorphous structure; the baseline is elevated while no peaks can be observed
326 (Figure 3a).

327 Figure 3b shows that the co-processed materials, independently of the neutralization degree and
328 the combination of SD process parameters used, were amorphous. In fact, the reflections
329 corresponding to OF completely disappeared and the baseline is elevated between 15 and 30°,
330 indicating complete crystallinity loss. This phenomenon is an indirect evidence of the interaction
331 between the drug and the polyelectrolyte[46]. The X-ray diffractions of the co-processed materials
332 (set I) demonstrated to be amorphous over five years storage, being then this structure stable for
333 longer periods than shelf-life. The long-term stability of the amorphous state was previously
334 demonstrated for others drug-polyelectrolyte inhalatory systems[19].

335

336 3.2.4. Particle morphology, drug and size distribution

Figures 4 and 5 show SEM micrographs of the drug (as received from the supplier), spray-dried pure OF and HA and the co-processed products. The pure drug without processing exhibited smooth and regular shaped crystals (Figure 4a), in good agreement with the OF morphology described by Duan *et al*.[47] and with the OF crystalline structure demonstrated by X-ray diffraction. When the drug was processed by spray drying, crystal aggregates were observed (Figure 4b). The morphology of SD HA processed is shown in Figure 4c and corresponds to buckling smooth particles, in good agreement with results reported for other SD anionic polyelectrolytes[18,48].

Regarding the co-processed microparticles, Figures 5a to 5d show smooth rounded particles, with one or more concavities. The morphology was similar to the one observed for the pure SD HA. The apparent crystals lack is supported by the amorphous state detected by X-ray diffraction. As it can be seen, neither the process parameters nor the neutralization degree affected microparticles morphology.

Aiming to study if all microparticles obtained by spray drying present OF, Figure 6 shows the laser scanning confocal micrographs. The fluorescence of sample $(HA\text{-}OF)_{100}$ Set I was very high, saturating the image, for this reason is not presented. HA has not fluorescent properties while OF possesses a natural fluorophore given by the 4-keto oxygen and the ionized 3-carboxylic acid group[49]. Transmission image (Figure 6a) represents the whole polymeric microparticles, while in the fluorescent image (Figure 6b) only the OF is shown. As can be seen, all the particles present fluorescence, indicating the presence of the drug in all co-processed powder.

Table 5 shows the mean volumetric diameter (D_{43}), evaluated by laser diffraction for co-processed particles. The particles obtained by using set I showed D_{43} values of 7.31 and 6.90 μm for samples with neutralizations degrees of 75 and 100 %, respectively. On the other hand, the particles obtained using the parameters of set II exhibited D_{43} values of 3.74 and 3.40 μm for $(HA\text{-}OF)_{75}$ and $(HA\text{-}OF)_{100}$ materials, respectively. Differences between samples with different neutralization degree obtained by using the same set of operating conditions were not statistically significant (*p*-value > 0.05). However, the set conditions significantly affected D_{43} values; statistically significant differences were found for samples with the same neutralization degree but processed under different operating conditions (set I and II, *p*-value < 0.05). These results indicate that increasing the atomization air flowrate and using a high-performance cyclone (set II) allowed reducing the D_{43} value, a desired characteristic for inhalatory products. Besides, all materials showed narrow distributions (i.e., *span* values lower than 2[50]).

3.3.1. Aerodynamic behavior

The aerosolization properties for the powders $(HA\text{-}OF)_{75}$ and $(HA\text{-}OF)_{100}$ obtained using the process parameters of sets I and II (see Table 2) were evaluated in an NGI cascade impactor. Table 6 shows the following aerosolization parameters: EF, FPF for different particle sizes (3, 5 and 6.4 μm), RF for particle sizes lower than 3 and 5 μm, MMAD and GSD. To obtain these parameters, a capsule filling of 25 mg was used in order to compare the results here presented with the ones reported by Hwang *et al*[17]., and Park *et al*.[3] These authors used a capsule loading of 30 and 10 mg, respectively.

As can be seen, the aerosolization performance was not affected by the neutralization degree, no statistical differences were found for samples $(HA\text{-}OF)_{75}$ and $(HA\text{-}OF)_{100}$ (p-value > 0.05) for both set of conditions. However, statistically significant differences were found when the same sample was processed under the two different set of conditions (p-value < 0.05). Higher values of FPF and RF and lower MMADs were obtained for Set II. This can be related to the combination of a higher atomization air flow rate and a high-performance cyclone, combination that allows producing and collecting particles with smaller sizes.

As can be seen in Table 6, EF was higher than 90 % in all cases, regardless the neutralization degree or the processing set of conditions. For the OF-chitosan formulation studied by Park *et al*.[3], emitted fractions higher than 90 % and about 81 % with and without using lactose as a carrier were obtained. For the system OF-HA studied by Hwang *et al.*[17], an EF of 94 % was found when a ratio of OF-HA powder:lactose 1:24 was used. The values obtained in this work are very high, considering that samples were not mixtured with carrier particles, and comparable with the EFs reported by Hwang *et al*. and Park *et al*. for mixtures of the co-processed materials and lactose[3,17].

For previously described inhalatory systems carrying OF, FPF for particles smaller than 6.4 μm was obtained by using a twin-impinger impactor. Values reported were 45 % and 43 % for the OF-chitosan and OF-HANa systems, respectively. For the formulations obtained in this work using set I operating conditions, this FPF was similar to the reported by those authors. However, higher values (~ 65 %) than those previously reported were obtained when set II operating conditions were used. Besides, nowadays the USP recommends the use of multi-stage cascade impactors[22] that allows calculating FPFs for different particle sizes (and stablishing, to some extent, in vitro – in vivo correlations[20,51]). For example, for treating pulmonary illnesses, particles should possess aerodynamic diameters lower than 5 μm[52]. As it can be seen in Table 6, FPF < 5 μm is around 58 % and RF < 5 μm is about 54 %. This result indicates that more than the 50 % of the powder loaded in the capsule can reach the lungs even when no carrier is used.

Table 6 also shows the RFs for particles with aerodynamic diameters lower than 3 μm. Although particles with aerodynamic diameters lower 3 μm are associated to systemic administration of drugs by the inhalatory tract, in this work RF<3μm is used as an indicator of the microparticles fraction that can anatomically reach alveolar macrophages because these cells are located within the alveolus[53]. This fraction was around 37 % for particles obtained using set II, *i.e*. about 37 % of the OF dose can reach the deep lung and thus the alveolar macrophages. This fraction was around 2 times higher than the one obtained for set I samples.

The MMAD was around 3.8 and 2.7 μm for particles obtained by using set I and II, respectively (Table 6). In all cases, the GSD value indicated that the aerodynamic particle size distributions were narrow (GSD value lower than 3[54]).

As can be seen, sample $(HA\text{-}OF)_{100}$ (set II) was the best in vitro formulation because it presents smaller aerodynamic diameters and better aerosolization performance than set I; and carries higher OF amounts than the $(HA\text{-}OF)_{75}$ formulation.

3.3.2. HA-OF dose preliminary estimation

In this section, a dose to treat multi-drug resistant-TB was roughly estimated in order to test if $(HA\text{-}OF)_{100}$ (set II) powder could be administered in a conventional capsule-based DPI.

Quinsair™ maketed product delivers a 240 mg dose of nebulized levofloxacin and is approved for treating *Pseudomonas aeruginosa* infection in patients with cystic fibrosis. Reported MIC_{90} values for levofloxacin were 32[55] and 6.25[56] μg/mL against *P. aeruginosa* and *M. tuberculosis*, respectively. Regarding ofloxacin, 32[17,55] μg/mL MIC_{90} value was found against *P. aeruginosa* and *M. tuberculosis*. As MIC_{90} values for treating *P. aeruginosa* infections are similar or higher than the ones needed for treating *M. tuberculosis* with ofloxacin and levofloxacin, it could be assumed that the Quinsair™ dose would be adequate to treat TB by the inhalatory administration.

To calculate the dose for the co-processed materials, besides the analogy supposed in the previous paragraph, the following assumptions are considered:

a) The levofloxacin solution (Quinsair™) for nebulization, that is administered by using a PARI e-flow rapid nebulizer, delivers a respirable delivered dose of 130 mg[57].
b) The presence of hyaluronic acid improves Ofloxacin microparticles pharmacodynamics and targeted indexes by a 50 %[17].

As Ofloxacin is the racemic mixture where only levofloxacin is the active ingredient, 260 mg of OF would be necessary to reach the same respirable dose delivered by Quinsair™. Considering that the presence of hyaluronic acid allows OF dose reduction by a half[17], 130 mg of this drug would be required to be delivered.

To administrate 130 mg of OF, 21 of a size 3 capsules filled with 25 mg of the formulation would be necessary, being this number unsuitable for therapy compliance. However, a size 3 capsule can be filled up to 100 mg of **this** co-processed material[58]. To evaluate the aerodynamic performance of the developed system from a highly loaded capsule, the inhalatory formulation was retested in the NGI equipment in the same conditions that the described in Section 2.2.6. Results are shown in Table 7. As can be seen, EF is almost 90 %. Although FPF and RF values decreased compared to the ones obtained when a 25 mg-loading capsule was used, results are still adequate for inhalatory administration of the formulation. In fact, most commercially available DPIs have a FPF for particles with aerodynamic diameters lower than 5 μm of 10-35 %[25]. The MMAD was lower than 3 and the GSD value indicated that the aerodynamic particle size distribution was narrow[54], as it was found in the assay with 25 mg of capsule loading.

Considering a 100 mg capsule filling (which still presents good aerosolization properties) and a respirable fraction for particles with aerodynamic diameters lower than 5 μm of 42 % (Table 7), 7 capsules would be enough to deliver 130 mg OF dose. Thus, the material has potentiality for the proposed application. In fact, there are commercial DPIs that require up to 10 capsules twice daily to deliver the dose.[59]

Finally, it should be taken into account that the new material reported in this work is in the early stages of development and further studies are needed to determine the required dose, the number of capsules and therapeutic scheme.

3.4. Cell viability

Employing the MTT colorimetric assay, the cytotoxic effect of the sample $(HA\text{-}OF)_{100}$ (set II) on the CALU-3 cell line was evaluated. As can be seen in Figure 7, even at the highest concentration assayed, the cell viability differences between the control and the treatments were not statistically significant (p-value > 0.05).

As mentioned in Section 3.3.2, the highest MIC_{90} dose for multi-drug resistant TB treatments reported is 32 μg/mL[17]. Considering 30 mL volume of pulmonary liquid available for drug dissolution[2], 0.60 mM of ofloxacin (the highest concentration assayed in this work) is around 6.5 times higher than the highest reported MIC_{90}[17]. Even though these results are auspicious, **toxicity studies of high doses of ofloxacin and/or HA in the lung have not been addressed yet. Then, f**urther studies are necessary to assess completely the safety of this new material.

3.5. Mucoadhesion assay: Tensile Strength

In order to increase the mucoadhesivity of formulations, hydrophilic polymers are usually incorporated. In general terms, these polymers possess good stickiness to mucosal membranes. There are several advantages of mucoadhesive like increasing dosage form residence time, reducing the frequency of the drug administration, improving drug targeting, among others[29]. Mucoadhesion is a phenomenon that has not been fully understood; several theories have been proposed but the process is probably the result of combined mechanisms. In this sense, Khutoryanskiy reports that mucoadhesion is a process with sequential steps: a) wetting and swelling, b) developing of physical bonds, c) interpenetrating and entangling, d) developing of chemical bonds[29].

Table 8 shows the maximum detachment force (MDF) and total work of adhesion (TWA) when OF, HA or the $(HA\text{-}OF)_{100}$ (Set II) microparticles were attached to the mobile probe. The test was also carried out soaking the mobile probe without microparticles in the mucin solution. This can be considered as a control test in order to establish the minimum force required for the probe detachment. As expected, this assay showed the lowest MDF and TWA. As can be seen in Table 8, MDF and TWA values for pure OF and mucin were very close. Differences found between these two materials were not statistically different (p-value > 0.05), although they were higher for OF. On the other hand, the pure HA MDF and TWA values were higher and statistically significant compared to mucin and pure OF. What is more, the co-processed microparticles displayed the highest MDF and TWA values in the tensile test. Differences were statistically significant respect to the HA, OF and mucin (p-value < 0.05). **This result suggests that the inclusion of the polyelectrolyte in the co-processed material provides higher mucoadhesion to the respiratory mucosa compared to the free ofloxacin.**[60] **Besides, t**he higher values for the $(HA\text{-}OF)_{100}$ (Set II) compared to the pure HA could be related to the ionization of the carboxylic groups of HA. It has

been reported that carboxylic groups of anionic polymers form hydrogen-bonds with mucin, specifically with oligosaccharides. However, ionized anionic groups of polyelectrolytes repel mucin negatively charged surface and hydrogen bonds cannot be developed[29]. For co-processed materials, the neutralization of the HA anionic groups by OF would prevent repulsion allowing the formation of hydrogen bonds.

According to the results of Li et al.[60], higher mucoadhesiveness can be associated to longer lung residence time and lower systemic exposure. The mucoadhesive properties of the SD co-processed product is particularly interesting in the pharmacotherapy with OF, as the residence time of the drug in the lung could be prolonged, decreasing the microparticle clearance[61].

4. Conclusions

Based on the spray-drying technology, using a feed composed by an aqueous-based solution containing OF and HA (with high neutralization degrees), high atomization air flowrate and the use of a high-performance collection cyclone (set II), powders suitable for inhalatory administration were obtained with very good process yields.

For formulations with neutralization degrees between 75-100 %, the powder OF loading was about 50 % (w/w), being then the formulation appropriate to administrate high OF doses. The ionic interaction between OF and HA allowed stabilizing the amorphous nature of the co-processed products for over five years.

The carrier-free formulation $(HA\text{-}OF)_{100}$ set II showed excellent emitted, fine particle and respirable fractions. In fact, about the 50 % and 37 % of the OF loading in a 25 mg capsule would be able to reach the lungs (respirable fraction for aerodynamic particle size smaller than 5 μm) and the region where alveolar macrophages locate, respectively. The best formulation exhibited higher mucoadhesion than pure OF, property that can increase the drug residence time in the lungs. Comparing the amount of drug that would reach the lung with the required OF to treat TB, the estimated therapeutic dose could be provided by 7 capsules. Besides, the best formulation did not affect CALU-3 cell viability up to a dose 6.5 times higher than the MIC_{90} reported to treat multi-drug resistant TB. Considering the auspicious in vitro results for the developed co-processed product, it is necessary to vigorous assess its stability, pharmacokinetics, and toxicity.

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Table captions

Table 1. Theoretical composition and experimental pH and composition of the feed solutions and powders

Sample	Theoretical values				Experimental values		
	HA (w/w)	OF (w/w)	Total solid content (% w/w)	Relative composition (g_{OF}/g_{powder})	pH	Relative composition (set I) (g_{OF}/g_{powder})	Relative composition (set II) (g_{OF}/g_{powder})
$(HA\text{-}OF)_{75}$	0.28	0.21	49	0.43	4.03	0.40±0.01	0.39±0.01
$(HA\text{-}OF)_{100}$	0.28	0.28	56	0.50	5.08	0.46±0.02	0.46±0.02

Table 2. Process conditions for set I and II

Process conditions	Set	
	I	II
Air inlet temperature (co-current) (°C)	110	110
Feed solution flowrate (mL/min)	3.5	3.5
Atomization air flowrate (L/h)	400	742
Drying air flowrate (m^3/h)	35	35
Cyclone	Standard	High-performance

Table 3. Process yield (PY) and outlet air temperature (T_{out}) for different process parameters and formulations

	Set I		Set II	
Sample	PY (%)	T_{out} (°C)	PY (%)	T_{out} (°C)
$(HA\text{-}OF)_{75}$	53	70	69	58
$(HA\text{-}OF)_{100}$	51	71	74	52

Table 4. Assignments of the FT-IR bands of HA, OF and the co-processed products obtained by spray drying

	Compound					
	Set I				Set II	
Band (cm^{-1})	OF	HA	$(HA\text{-}OF)_{75}$	$(HA\text{-}OF)_{100}$	$(HA\text{-}OF)_{75}$	$(HA\text{-}OF)_{100}$
ν COOH	1717.2	1636.2	1732.9	1729.5	1724.6	1727.6
ν C=O ring	1622.3	-	1622.1	1622.2	1621.1	1621.4
ν CH_3-N	2784.2	-	-	-		
δ CH_3-N	1408.5	-	-	-		
ν amide I band	-	1650.7	1657.0	1658.4	1658.3	1659.6
ν HC=C	-	1555.5	1544.5	1540.2	1544.2	1544.2
ν COO^{-} ^{s} asymmetric	-	-	1608.8	1607.7	1607.6	1607.2
ν COO^{-} ^{s} symmetric	-	-	1416.5	1417.3	1414.7	1415.6

^{s}: the bands were seen as shoulders

ν: stretching band

δ: deformation band

Table 5. Mean volumetric diameters (D_{43}) and particle size distribution *span* values, as measured using laser diffraction

	Set I		Set II	
NGI parameter	$(HA\text{-}OF)_{75}$	$(HA\text{-}OF)_{100}$	$(HA\text{-}OF)_{75}$	$(HA\text{-}OF)_{100}$
D_{43}	7.31±0.46	6.90±0.63	3.10±0.23	3.39±0.31
Span	0.92±0.17	0.92±0.14	1.13±0.17	0.99±0.21

Table 6. Aerosolization performance using 25 mg-capsule loading

	Set I		Set II	
NGI parameter	$(HA\text{-}OF)_{75}$	$(HA\text{-}OF)_{100}$	$(HA\text{-}OF)_{75}$	$(HA\text{-}OF)_{100}$
EF (%)	94.84±0.87	90.82±2.76	93.65±1.40	92.35±2.30
FPF < 6.4µm (%)	49.21±2.09	44.28±1.76	60.14±4.90	67.84±3.20
FPF < 5µm (%)	29.12±1.10	32.68±1.43	56.82±4.27	57.90±0.33
FPF < 3µm (%)	15.87±2.99	20.50±3.32	39.67±2.78	40.89±1.10
RF < 5µm (%)	27.63±1.25	28.90±2.09	52.90±3.41	53.45±1.03
RF < 3µm (%)	15.06±2.85	18.17±3.19	36.95±2.72	38.70±2.26
MMAD (µm)	3.89±0.83	3.68±0.23	2.80±0.28	2.70±0.17
GSD	2.14±0.50	1.96±0.17	1.86±0.09	2.29±0.04

EF: Emitted Fraction; FPF: Fine Particle Fraction; RF: Respirable Fraction; MMAD: Mass Median Aerodynamic Diameter; GSD: Geometric Standard Deviation

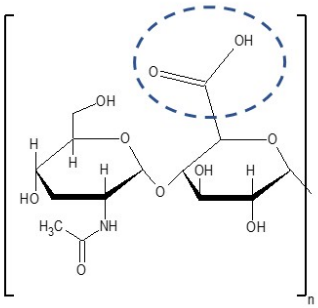
Table 7. Aerosolization performance for co-processed samples using 100 mg-capsule loading

NGI parameter	$(HA\text{-}OF)_{100}$ Set II
EF %	89.18±1.28
FPF < 5µm (%)	48.82±2.06
FPF < 3µm (%)	33.85±2.97
RF < 5µm (%)	42.50±3.09
RF < 3µm (%)	29.63±3.21
MMAD (µm)	2.95±0.33
GSD	2.15±0.44

EF: Emitted Fraction; FPF: Fine Particle Fraction; RF: Respirable Fraction; MMAD: Mass Median Aerodynamic Diameter; GSD: Geometric Standard Deviation

Table 8. Maximum detachment force (MDF) and total work of adhesion (TWA) in the tensile test for powders.

Material	MDF (N)	TWA (J, $x10^{-4}$)
Mucin	0.51±0.04	6.25±1.49
OF: Mucin	0.58±0.05	7.30±1.09
HA: Mucin	0.97±0.12	11.16±1.79
$(HA\text{-}OF)_{100}$ (Set II): Mucin	1.36±0.08	17.91±2.80



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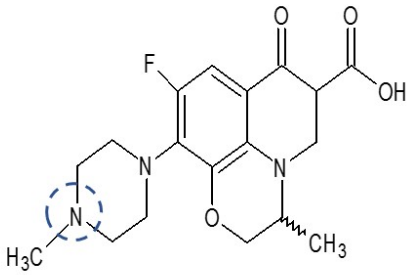
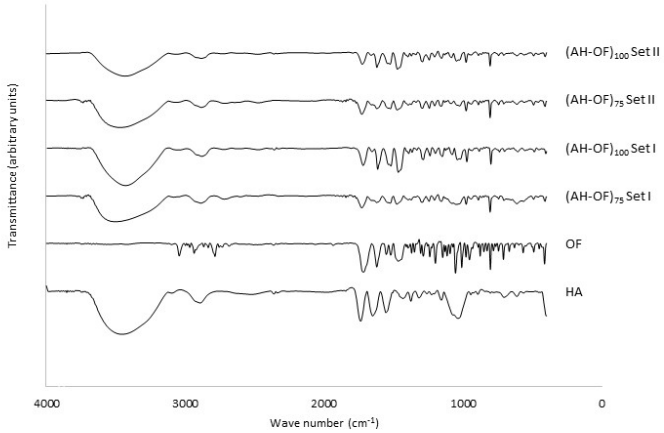
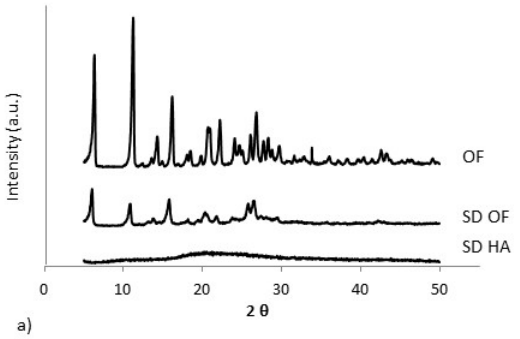
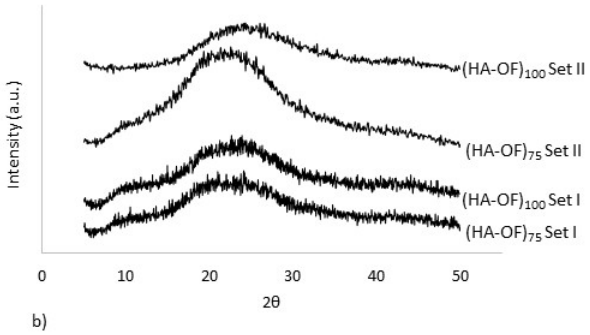


b)

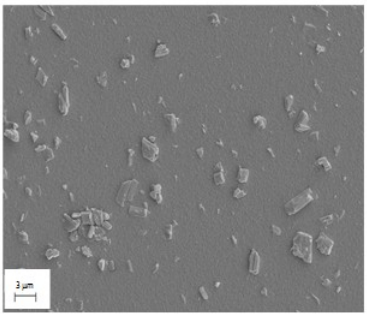

Transmittance (arbitrary units)
(AH-OF)100 Set II
(AH-OF)75 Set II
(AH-OF)100 Set I
(AH-OF)75 Set I
OF
HA
4000
3000
2000
1000
0
Wave number (cm-1)


Intensity (a.u.)
OF
SD OF
SD HA
0
10
20
30
40
50
2 θ

a)


Intensity (a.u.)
(HA-OF)100 Set II
(HA-OF)75 Set II
(HA-OF)100 Set I
(HA-OF)75 Set I
0
10
20
30
40
50
2θ

b)



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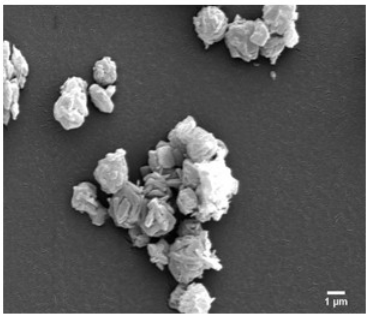
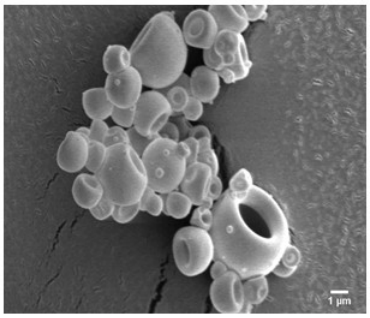
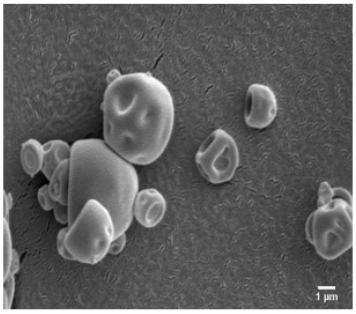


b)



c)



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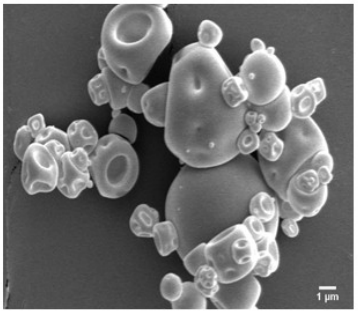

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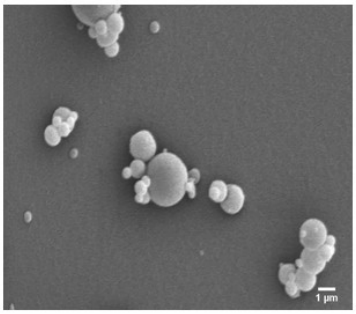

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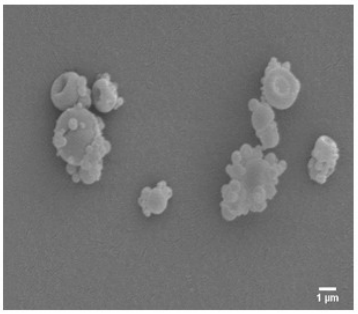
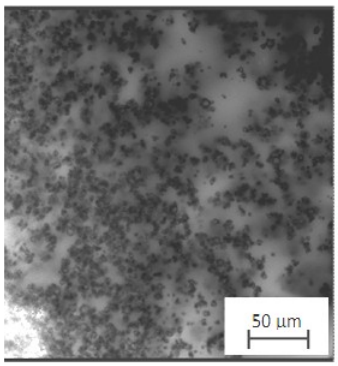


d)



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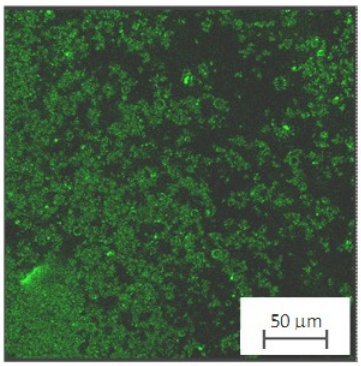

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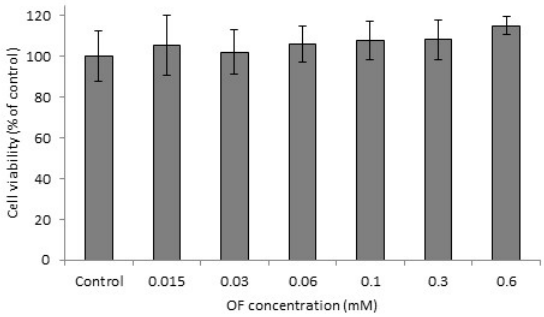
120
100
80
60
40
20
0
Cell viability (% of control)
Control
0.015
0.03
0.06
0.1
0.3
0.6
OF concentration (mM)