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Carrier free indomethacin microparticles for dry powder inhalation

Nazareth Eliana Ceschan[a,b], Verónica Bucalá[a,c], Melina Valeria Mateos[b,d], Hugh David Charles Smyth[e], María Verónica Ramírez-Rigo[a,b,*]

[a] Planta Piloto de Ingeniería Química (PLAPIQUI), CONICET – Universidad Nacional del Sur (UNS), Camino La Carrindanga km 7, 8000 Bahía Blanca, Argentina
[b] Departamento de Biología, Bioquímica y Farmacia, UNS, San Juan 670, 8000 Bahía Blanca, Argentina
[c] Departamento de Ingeniería Química, UNS, Avenida Alem 1253, 8000 Bahía Blanca, Argentina
[d] Instituto de Investigaciones Bioquímicas de Bahía Blanca (INIBIBB), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), 8000 Bahía Blanca, Argentina
[e] College of Pharmacy, The University of Texas at Austin, 2409 West University Avenue, Austin, TX, United States

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ABSTRACT

The present studies were designed to evaluate inhalatory microparticles carrying indomethacin (IN) for potential local (specific and non-specific bronchial inflammatory asthma responses) and systemic treatments (joint inflammation, rheumatoid arthritis and osteoarthritis pain) by optimizing microparticle properties, characterizing their lung deposition, drug release, evaluating cytotoxicity and also pharmacological effect *in vitro*. The acidic groups of IN were complexed with the cationic groups of the polyelectrolyte polylysine in order to increase the drug water compatibility. The polylysine/indomethacin ratio was fixed and the pH was adjusted in different formulations. Microparticles were obtained by spray drying using a relatively high atomization air flowrate (742 L/min) and a high-performance cyclone in order to optimize the production of microparticles with adequate attributes for inhalatory delivery. The produced microparticles exhibited high process yield and IN loading, volumetric mean diameters smaller than 5 μm and narrow particle size distributions. According to demonstrated aerosolization performance, the powders were suitable for inhalatory indomethacin local and systemic treatments. Emitted fraction was higher than 90%, the MMAD was around 3 μm and the GSD lower than 3. The respirable fraction for particles with aerodynamic diameters smaller than 5 μm was around 29% while for particles with aerodynamic diameters smaller than 3 μm the value was around 17%. The addition of lactose as carrier worsened the aerodynamic performance of the microparticles. The developed powdered systems got wet and dissolved quickly and presented higher release rates respect to pure IN in simulated lung physiological conditions. Furthermore, the assays performed in RAW 264.7 cell line showed that the microparticles exhibited the same anti-inflammatory capability as the pure drug. The developed particles did not affect the RAW 264.7 cell viability. In conclusion, a promising powder formulation for DPIs has been developed to treat, locally and systemically, inflammatory diseases.

1. Introduction

Indomethacin (IN) is a non-steroidal anti-inflammatory drug (NSAID) derived from indole-acetic acid. IN is currently approved for the treatment of rheumatoid arthritis, osteoarthritis pain (El-Badry et al., 2009) and demonstrated to be useful for treating specific and non-specific bronchial inflammatory asthma responses, as well as other inflammatory pulmonary and non-pulmonary conditions (Bianco, 2000). However, oral administration of the required IN dose to achieve therapeutic concentrations in the lung is limited due to the high incidence of adverse gastrointestinal effects (Bianco, 2000). Rainsford reported that the gastrointestinal side effects caused by the use of indomethacin accounted for 19–39% of all reported side effects (Rainsford, 1982). These adverse effects lead to treatment discontinuation (Romano et al., 2004).

For this reason, alternative administration routes have been explored for indomethacin, including percutaneous (Ricci et al., 2005), nasal (Karasulu et al., 2008) and pulmonary route (Onischuk et al., 2008). For percutaneous and nasal administration, the addition of permeation enhancers was found to be necessary to achieve adequate systemic concentrations of IN (Ricci et al., 2005; Karasulu et al., 2008). Concerning pulmonary administration, nanoparticles containing pure

* Corresponding author at: Planta Piloto de Ingeniería Química (PLAPIQUI), CONICET-Universidad Nacional del Sur (UNS), Camino La Carrindanga km 7, 8000 Bahía Blanca, Argentina.
E-mail address: vrrigo@plapiqui.edu.ar (M.V. Ramírez-Rigo).

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IN were demonstrated to have adequate systemic drug absorption, without the need of permeation promoters (Onischuk et al., 2008). In previous studies, a relatively complex vaporization/condensation technology was used to produce inhalation particles, and presented limited lung deposition (due to their small sizes). Separately, the US6051566 patent proposed the use of indomethacin via jet nebulization (20 mg/dose) for local treatments in the lung. The formulation and the administration technique successfully allowed the therapy of specific and non-specific bronchial stimuli in asthmatic patients, without presenting adverse effects (Bianco, 2000). Although these contributions are valuable, a Dry Powder Inhalatory system (DPI) to administer IN which provides high dose deposition in the alveolar region and fast drug dissolution rate has not yet been achieved.

In this work, microparticles containing IN were developed to administrate this drug to the airways using a DPI. DPIs have some competitive advantages respect to others inhalatory system and fulfill most of patients and physicians preference. In fact, they are less time-consuming than nebulizers and are easier to use than Metered Dose Inhalers (MDIs) because they are breath-actuated. Also, the DPIs offer high physical, chemical and microbiological stability (Geller, 2005; Roche et al., 2017). The DPI performance is a combination of the particulate-system physicochemical properties, the inhaler design and the physical mechanisms that aerosolize, deagglomerate, disperse and deposit the particles in the lung (Islam and Cleary, 2012).

The use of co-processed materials could greatly improve dry powder formulation performance. For example, biodegradable polymers have been used for a) mucoadhesion capacity, *i.e.* increase of the formulation residence time in lung, or in other terms, decrease the number of daily administrations (Gallo et al., 2017); b) deposition at specific pulmonary target sites (e.g., macrophages or cancerous cells) (Martinelli et al., 2016); c) modified release for the treatment of pulmonary infections (Rivera et al., 2004); d) increased penetration of drugs into target cells (e.g., increased transfection and incorporation of DNA and oligonucleotides in gene therapy) (Colonna et al., 2008), among others. Of particular interest are co-processed materials comprising a polyelectrolyte and an oppositely charged drug to allow the engineering of materials and particles with properties different from the raw materials (Ceschan et al., 2016, 2015, 2014). Particularly, viscous anionic feeds carrying a water soluble drug that led to swellable particulate systems were studied (Ceschan et al., 2016). In order to complement these results, in this article a polyelectrolyte-drug system based on a non-viscous polycation and a low water soluble drug is described.

In a previous work, different polylysine-IN formulations were processed by spray drying to obtain microparticles for inhalatory administration. Among all the studied formulations, a particulate system with relatively high IN load, appropriate aerodynamic particle size (between 1 and 5 μm) and low moisture content emerged as an optimal formulation design within those tested. Using a low-resistance inhaler, the aerodynamic performance was demonstrated to have sufficient efficiency (Ceschan et al., 2015). Based on this foundation, we hypothesized that modulating the spray drying process parameters (operating variables and fed composition) could lead to optimized indomethacin powders for inhalation. In particular, it has been demonstrated that cationic free groups can damage cell membrane (Nafee et al., 2009) and affect respiratory physiology (Gu et al., 2006). In this work the spray drying parameters such as feed composition (by adjusting the pH), the cyclone type and the atomization air flow rate were modified to develop a physiologically compatible inhalation powder to be administrated by a DPI device, either for local or systemic treatments. Powders were also mixed with lactose as an aerodynamic carrier. The developed products were also subjected to *in vitro* biopharmaceutical assays: cell viability, aerosolization, permeation and anti-inflammatory activity tests to study the influence of the pH adjustment and the lactose addition on these experiments.

2. Materials and methods

2.1. Materials

Indomethacin (pharmaceutical grade, Parafarm, Saporiti, Buenos Aires, Argentina), epsilon-polylysine and dextrin (food grade, Purac America, Lincolnshire, United States), hydrochloric acid (analytical grade, Anedra, Buenos Aires, Argentina), lactose monohydrate 140-70 ASTM Mesh (pharmaceutical grade, Parafarm, Saporiti, Buenos Aires, Argentina), potassium phosphate monobasic (analytical grade, Anedra, Buenos Aires, Argentina), sodium hydroxide (analytical grade, Anedra, Buenos Aires, Argentina), size 3 gelatine capsules (pharmaceutical grade, Parafarm, Saporiti, Buenos Aires, Argentina), glycerin (pharmaceutical grade, Anedra, Buenos Aires, Argentina) were used.

The polylysine used in this work was epsilon-polylysine which was supplied as a mixture of polylysine:dextrin (PL:DX 1:1). Dextrin allows improving flow properties (Lee et al., 2001) and storage stability (Huybrechts, 2006). This compound, which is highly water soluble, was previously used as biocompatible excipient in pulmonary formulations (Alsaadi et al., 2012). Its association with polylysine was proposed to improve the PL conformational structure stability (Huybrechts, 2006). PL is a cationic polyelectrolyte with the capability to interact with acidic compounds, like IN.

For the assays in cell cultures, mouse macrophage cell line RAW 264.7 (ATCC® TIB-71®), Dulbecco's Modified Eagle's Medium (DMEM) and antibiotic-antimycotic (Anti-Anti 100X) were provided by Gibco (Life Technologies, United States). Fetal bovine serum (FBS) was from Natocor (Córdoba, Argentina). Polyvinylidene fluoride (PVDF) membranes were obtained from Millipore (Bedford, United States). UltraCruz# Autoradiography, polyclonal horse radish peroxidase (HRP)-conjugated goat anti-rabbit IgG, polyclonal HRP-conjugated goat anti-mouse IgG were obtained from Santa Cruz Biotechnology, Inc. (California, United States). Mouse monoclonal anti-α Tubulin (DM1-A) was from EMD/Biosciences (San Diego, United States). Rabbit polyclonal antibody anti- cyclooxygenase-2 (COX-2) was from Cayman Chemical (Michigan, United States). Bovine serum albumin (BSA), *Klebsiella pneumoniae* lipopolysaccharide (LPS), dimethyl sulfoxide (DMSO), RIPA lyses buffer [10 mM Tris-HCl (pH 7.4), 15 mM NaCl, 1% Triton X-100, 5 mM NaF, 1 mM Na_2VO_4 and the complete protease inhibitor cocktail], TTBS buffer [20 mM Tris–HCl (pH 7.4), 100 mM NaCl and 0.1% (w/v) Tween 20] were obtained from Sigma-Aldrich (Saint Luis, United States). All these reagents were analytical grade. Distilled water was also employed.

2.2. Methods

2.2.1. Liquid feed preparation and spray drying (SD)

In previous work, liquid formulations (fed to the spray drier) with different PL:DX/IN ratios were studied to identify the influence of the feed composition on the powder properties (Ceschan et al., 2015). Among all the formulations studied in that contribution, those with a 50% of neutralization degree of the PL amino groups emerged as the most promising and thus attractive for further characterization, as they exhibited high process yield, low residual moisture and small particle size. In this work, based on the best powder presented by Ceschan et al. (2015), some selected process parameters were modified to obtain improved powders with enhanced properties (adequate particle size for systemic treatments and physiologically compatibility with cell tissues). To this purpose, two liquid formulations were prepared: a) the best formulation found by Ceschan et al. (2015), which was named $(PL\ IN)_{50}$:DX (where the subscript 50 represents the degree of neutralization of the PL amino groups by indomethacin) and b) a formulation with the same composition than a), for which the solution pH was adjusted to almost 7 by adding HCl (denoted as $(PL\ IN)_{50}$:DXCl).

The composition of the spray-dried solutions is detailed in Table 1. In all cases, the sprayed volume was 200 mL. The Table 1 includes the

Table 1
Theoretical and experimental composition and pH of solutions for spray drying.

Sample	Theoretical formulation					Experimental solution properties	
	PL:DX (g)	IN (g)	HCl (g)	Expected composition		Composition (mg_{IN}/mL)	pH
				g_{IN}/g_{solid}	mg_{IN}/mL		
$(PL\text{-}IN)_{50}$:DX	2.50	0.68	–	0.21	3.40	3.25 ± 0.11	8.55 ± 0.05
$(PL\text{-}IN)_{50}$:DXCl	2.51	0.64	0.15	0.19	3.20	3.14 ± 0.15	7.65 ± 0.21

expected dry powder composition (based on the weight of compounds) and the experimental composition of the microparticles carrying IN, (evaluated by UV, UV-160A, Spectrophotometer, Shimadzu, Burladingen, Germany) at 319.5 nm. Also, Table 1 shows the pH of the solutions measured using a pH meter Orion 410A (Cole Parmer, Vermon Hills, United States).

The liquid formulations were fed in a SD lab-equipment under constant magnetic stirring in a negative pressure lab-scale SD unit (Mini Spray Dryer B-290, BÜCHI, Flawil, Switzerland). A two-fluid nozzle with an orifice diameter of 0.5 mm was used. The SD operating conditions were: air inlet temperature (co-current flow) of 140 °C, drying air flowrate of 35 m^3/h, liquid feed flowrate of 6 mL/min and an atomization air flowrate of 742 L/min. A high performance cyclone was selected due to its high fines collection efficiency (Ceschan et al., 2016).

The process yield was calculated as the ratio of product weight collected in the cyclone collection vessel to the initial amount of solids used to prepare the liquid feeds. The collected powder was stored in a desiccator for further characterization. Samples were named as the liquid feeds, *i.e.* $(PL\ IN)_{50}$:DX and $(PL\ IN)_{50}$:DXCl.

2.2.2. Powders characterization

Residual moisture, composition, morphology, particle size and flow properties were determined. Moisture content was quantified after the SD process and was determined in a halogen moisture analyzer (MB45, Ohaus, Pine Brook, United States). Around 500 mg of sample was heated up to 105 °C until the weight change was less than 1 mg in 60 s. Drug loading was determined by dissolving 20 mg of powder in water and then drug concentration was measured by UV spectrophotometry at 319.5 nm. The drug loading was calculated as gram of drug per gram of powder. Particle size distribution of the SD products was analysed by laser diffraction (LA 950V2, Horiba, Kyoto, Japan, dry powder method) using a technique previously reported (Ceschan et al., 2014). Size is reported as mean volumetric diameter (D_{43}) and the distribution width is informed as *span* (Eq. (1)).

$$Span = \frac{(D_{90} - D_{10})}{D_{50}} \quad (1)$$

D_{10}, D_{50} and D_{90} are the volumetric diameters where the 10%, 50% and 90% of the population lies below each value, respectively. A distribution can be considered relatively narrow if the *span* value is less than 2 (Ceschan et al., 2014). Particles morphology of the spray dried materials was evaluated by using a Scanning Electron Microscope (SEM) (EVO 40-XVP, LEO, Oberchoken, Germany). Samples were metalized with a 300 nm gold layer using a sputter coater (PELCO 91000, Ted Pella, California, United States) before analyzing them. Bulk and tap density determination was performed. A 10 cm^3 graduated cylinder was filled with 1 g of sample and used to determine the powders bulk (D_{bulk}) and tap (D_{tap}) densities as previously described (Ceschan et al., 2016). Both densities were used to calculate the Carr Index (*CI*, Eq. (2)) (USP 30–NF 25, 2007):

$$CI = (D_{tap} - D_{bulk})100/D_{tap} \quad (2)$$

The powder flow properties were classified according to the USP criteria (USP 30–NF 25, 2007).

2.2.3. Blends of SD powders with lactose carrier particles

Because of the small size of inhalable particles, these systems usually possess poor flow. To improve the flow properties, the particles carrying the active ingredient can be mixed with carrier particles with larger mean sizes. Among other excipients, lactose has been widely used because its non-toxicity and biocompatibility (Pilcer et al., 2012).

SD powders ($(PL\text{-}IN)_{50}$:DX and $(PL\text{-}IN)_{50}$:DXCl) were mixed with lactose using a Turbula® orbital mixer (Glen Mills, Maywood, United States) in a 1:3 ratio by geometric dilution at 46 RPM for 20 min. Content uniformity in the blends was evaluated in 5 random samples (15 mg each) by dissolving them in water and measuring the absorbance by UV spectrophotometry at 319.5 nm. For these mixtures, flow properties were determined as described in Section 2.2.2. The blends were stored in a desiccator.

2.2.4. In vitro aerosolization properties

The *in vitro* aerosolization performance of the SD powders was evaluated in a Next Generation Impactor (NGI, Copley Scientific, Nottingham, UK). The description of the unit can be found elsewhere (Ceschan et al., 2016; Coplay-Scientific, 2012).

In a previous work, the aerodynamic performance of microparticles carrying IN was assayed by using a low resistance inhaler (Ceschan et al., 2015). Low-resistance DPI inhalers display a higher dose variability and a lower dose deposition as the disaggregation of particles and aerosolization performance is less efficient (Dal Negro, 2015). In this work, a RS01 higher resistance inhaler (Plastiape, Milano, Italy) was used to disperse the powders into the NGI. In this kind of inhalers, an adequate particle dispersion can be achieved even at lower flow rates (Dal Negro, 2015). Size 3 gelatin capsules were filled with 15 ± 0.50 mg of SD powders or with the appropriate amount of SD powder:lactose carrier mixture containing 15 mg of SD powder. The selected pressure drop (ΔP) across the NGI, according to the USP (USP 30–NF 25, 2007), was 4 KPa. The air flowrate for this pressure drop was 58.8 L/min (USP 30–NF 25, 2007). The NGI was run enough time to allow 4 L of air pass through the equipment. The aerodynamic cut-off diameters for each stage of the impactor were calculated following the guidelines given by the USP (USP 30–NF 25, 2007), and are given in Table 2.

To avoid particle re-entrainment or bouncing, the NGI stages were coated with glycerol. The pre-separator (PS) was loaded with 15 mL of water in order to recover the deposited drug. The drug deposited in all the NGI components was collected by rinsing each part also with an

Table 2
Cut-off diameters of the different NGI stages at 58.8 L/min flowrate.

NGI stage (S)	Cut-off diameters (μm)
1	8.15
2	4.51
3	2.85
4	1.68
5	0.95
6	0.56
7	0.34
MOC	0.14

adequate amount of water. Drug content was assessed using a UV-spectrophotometer at 319.5 nm. The experiments were performed in triplicate.

The Emitted Fraction (EF), Fine Particle Fraction (FPF), Respirable Fraction (RF), Mass Median Aerodynamic Diameter (MMAD) and Geometric Standard Deviation (GSD) were determined (Ceschan et al., 2016; Donovan and Smyth, 2010). EF represents the drug percentage of total drug loaded in the capsule that is effectively released from the capsule and the inhaler; FPF is defined here as the percentage of cumulative drug mass with aerodynamic diameters lower than a given value (usually 3 or 5 μm) respects to the total drug mass recovered from the mouth piece adaptor (MPA), PS, induction port (IP), NGI 1–7 stages and multiple orifice collector (MOC) (Donovan and Smyth, 2010; Du et al., 2014); RF accounts for the cumulative percentage of drug mass with aerodynamic diameters lower than a given value (usually 3 or 5 μm) respects to the total drug mass recovered from the capsule, inhaler, MA, PS, IP, NGI 1 to 7 stages and MOC (Donovan and Smyth, 2010; Du et al., 2014).

The mass median aerodynamic diameter (MMAD) was calculated from a drug mass cumulative distribution (built considering the drug mass collected in NGI 1 to 7 stages and MOC) 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 (Wang et al., 2014).

The geometric standard deviation (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 is recovered from the NGI 1 to 7 stages and MOC, respectively. Particle aerodynamic size distribution is considered narrow if GSD is lower than 3 (Razavi Rohani et al., 2014).

2.2.5. Drug release experiments

The drug release assays from powders were performed in vertical Franz Cells at 37 °C, where the receptor and donor compartments were delimited by a dialysis cellulose membrane (Sigma, 14,000 Da cut-off molecular weight). Phosphate buffered saline (PBS) at pH 7.4 was used to simulate lung physiological conditions (Mezzena et al., 2009). The receptor compartment (60 mL) was filled with PBS at pH 7.4 degasified medium and maintained under magnetic stirring. 12.5 mg of raw IN (D_{43}: 5.48 μm) or the adequate amount of the SD samples ($(PL\text{-}IN)_{50}$:DX and $(PL\text{-}IN)_{50}$:DXCl) to provide 12.5 mg of IN were added to the donor compartment. 2 mL samples were withdrawn from the receptor compartment at constant intervals up to 6 h and replaced with fresh medium. IN content in the samples was assayed by UV spectrophotometry at 319.5 nm. The experiments were performed in triplicate.

The IN release profiles from the two samples were compared by using the similarity factor *f2*, defined as follows (Ong et al., 2011)

$$f2 = 50log\left\{\left[1+\left(\frac{1}{n}\right)\sum_{t=1}^{n}(R_t-T_t)^2\right]^{-0.5}\right\}100 \quad (3)$$

where n represents the number of experimental points, R_t and T_t are the drug release percentage at time t from the reference (pure IN) and the SD powders, respectively. Formulations were considered similar if the *f2* value is higher than 50 (Ong et al., 2011).

2.2.6. Anti-inflammatory test. RAW 264.7 cell culture and treatment

Mouse macrophage cell line RAW 264.7 was cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum and antibiotic-antimycotic at 37 °C under 5% CO_2 in humidified incubator, as previously described (Mateos et al., 2014). Cells were grown on plastic 35 mm diameter culture dishes and were serum-starved for 2 h prior to experimental procedures. RAW 264.7 cells were pre-incubated with solutions containing 10 μM of indomethacin or the appropriate amount of $(PL\text{-}IN)_{50}$:DXCl microparticles (to reach a final concentration of 10 μM of indomethacin) or pure PL:DX solution for 1 h at 37 °C. The cells were then treated with 10 μg/mL of *Klebsiella pneumoniae* lipopolysaccharide in serum-free DMEM or sterile ultra-pure water (control conditions) for 24 h. LPS stock (4 mg/mL) was prepared in sterile ultra-pure water and IN stock (70 mM) was prepared in dimethyl sulfoxide (DMSO). DMSO, vehicle of IN, was added to all conditions to achieve a final concentration of 0.015%.

2.2.7. Western blot (WB) analyses

Lung diseases such as asthma are often associated with airway inflammation, caused by the action of different stimuli (Tamaoki et al., 2000). In the inflammatory response diverse cellular mediators derived from metabolites of cyclooxygenase enzymes (COXs) are involved. COXs are activated by responding to the activity of macrophages (immune system cells implicated in inflammatory processes) (Tamaoki et al., 2000; Badesso et al., 2014). IN order to study the anti-inflammatory activity of the $(PL\text{-}IN)_{50}$:DXCl microparticles, RAW 264.7 cells were grown on plastic 35 mm diameter culture dishes and after the treatment described in Section 2.2.6, the medium was removed, cells were washed three times with phosphate buffer saline (PBS) and scraped off with 80 μL ice-cold RIPA lyses buffer. Protein content of total cell lysates was determined by the Bradford method (Bradford, 1976) and samples were denatured with Laemmli sample buffer at 100 °C for 5 min (Laemmli, 1970). 30 μg of the whole-cell lysates proteins were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 10% polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were blocked with 10% BSA in TTBS buffer at room temperature for 2 h and subsequently incubated with anti-COX-2 or anti-α Tubulin primary antibodies overnight at 4 °C. After three washes with TTBS, membranes were exposed to the appropriate HRP-conjugated secondary antibody for 2 h at room temperature. Membranes were washed again three times with TTBS and immunoreactive bands were detected by enhanced chemiluminescence using UltraCruz# Autoradiography Film. Densitometry values of the immunoreactive bands were determined using ImageJ 1.38 software. The molecular weight of bands was determined using the spectra multicolor broad range protein ladder.

The WB analyses shown are representative images of samples from three independent experiments.

2.2.8. Cell viability assay

For this assay, 5000 RAW 264.7 cells were seeded on a 96-well plate and were incubated with DMEM containing $(PL\text{-}IN)_{50}$:DXCl microparticles (to reach a final concentration of 10 μM of indomethacin), pure PL:DX solution or under control condition (DMEM containing 5% fetal bovine serum) for 1 or 4 days . After experimental treatment, cell viability was assessed by MTT reduction assay, as previously described (Mateos et al., 2014). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), a water-soluble tetrazolium salt, is reduced by mitochondrial dehydrogenases of metabolically viable cells to a colored, water-insoluble formazan salt. MTT (5 mg/mL, prepared in phosphate buffer saline) was added to the cell culture medium at a final concentration of 0.5 mg/mL. The culture dish was incubated for 1 h at 37 °C in a 5% CO_2 atmosphere; cells were washed twice with phosphate buffer saline (PBS) and lysed with 0.1 mL of a buffer containing 10% Triton X-100 and 0.1 N HCl in isopropanol. The extent of MTT reduction was measured spectrophotometrically (570 nm absorbance – 650 nm absorbance) using a Multiskan™ 60 microplate spectrophotometer (Thermo Fisher Scientific, Massachusetts, United States). Results are expressed in arbitrary units as % with respect to the control condition.

2.2.9. Statistical analysis

The significant differences between the process yield, particles size, the *in vitro* particle deposition, the anti-inflammatory and cell viability assay were determined by means of one-way ANOVA, followed by Bonferroni's test to compare means. Statistical significance was

established through the *p*-value: values lower than 0.05 were considered statistically significant. Before the analysis, homoscedasticity and normality of ANOVA's assumptions were checked by the Levene test and Standard Kurtosis values, respectively (Villanueva et al., 2000; Nimon, 2012).

3. Results

3.1. Sample characterization

As above mentioned, two aqueous solutions were used to obtain microparticles by spray drying. In them, the PL:DX/IN ratio was maintained constant while the pH was adjusted for one sample to almost 7 by adding HCl. Table 1 shows the theoretical formulation, as well as the measured composition and pH of both water solutions.

According to Table 1, and as expected, the pH of the SD feed decreased when HCl was added to the solution in order to obtain biocompatible powders. As mentioned, the 50% of the PL basic groups were neutralized with the drug, the addition of hydrochloric acid increased the neutralization of the free PL amino groups. As it can be seen in Table 1, the experimental IN composition is in good agreement with the expected one, indicating that IN did not precipitate.

3.2. Spray drying process

The outlet air temperature (T_{out}) is known to affect the stability of labile compounds. In this work, T_{out} was 64.00 ± 4.24 and 70.50 ± 3.18 °C for $(PL\text{-}IN)_{50}$:DX and $(PL\text{-}IN)_{50}$:DXCl, respectively. These temperatures are well below the ones reported (> 80 °C) in a previous work where IN degradation after the SD process was not detected by differential scanning calorimetry and fourier transformed-infrared spectroscopy (Ceschan et al., 2015). The yields were 65.10 ± 2.98% for $(PL\text{-}IN)_{50}$:DX and 69.00 ± 1.36% for $(PL\text{-}IN)_{50}$:DXCl, both very good values for a lab-scale SD unit (Tontul and Topuz, 2017). As mentioned, the formulations were processed by SD maintaining all the process parameters employed in a previous study (air inlet temperature, liquid feed flowrate and drying air flowrate (Ceschan et al., 2015)), except for the atomization air flowrate (that was increased in about 23%, from 601 to 742 L/h) and the use of high performance cyclone instead the standard one. The yields were about 30% higher than the values obtained previously (Ceschan et al., 2015). This could be related to a better efficiency of high performance cyclones to retain smaller particles (produced by higher atomization air flowrates) than the standard ones (Ceschan et al., 2016).

3.3. Product characterization

3.3.1. Powder physicochemical morphological properties

Table 3 summarizes physicochemical properties of the $(PL\text{-}IN)_{50}$:DX and $(PL\text{-}IN)_{50}$:DXCl samples: moisture content, drug loading, particle diameter (expressed as mean volumetric diameter, D_{43}), *span* value, bulk and tap density and Carr Index (*CI*).

Regarding the moisture content, the values were similar for both samples and around 5%. This level of water has been found to allow powder stability suitable for long-term storage (Tontul and Topuz, 2017).

Table 3
Moisture, drug loading, volumetric mean diameter (D_{43}), *span* values, Bulk (D_{bulk}) and Tap (D_{tap}) densities and Carr Index (*CI*).

Sample	$(PL\text{-}IN)_{50}$:DX	$(PL\text{-}IN)_{50}$:DXCl
Moisture content (%)	4.79 ± 0.56	4.92 ± 0.45
Drug loading (g_{IN}/g_{powder})	0.20 ± 0.02	0.18 ± 0.02
D_{43} (μm)	4.99 ± 0.42	4.67 ± 0.08
Span	1.38 ± 0.25	1.50 ± 0.18
D_{bulk} (g/cm^3)	0.23 ± 0.01	0.28 ± 0.01
D_{tap} (g/cm^3)	0.34 ± 0.01	0.39 ± 0.01
CI	32.35 ± 1.29	28.21 ± 3.18

The drug content in the SD powders was determined, after dissolution in water, by UV absorption and was in agreement with the expected composition calculated based on the weighted raw materials (compare Tables 1 and 3).

Particle size distributions (PSDs) were characterized by the mean volumetric diameter (D_{43}) and span. The mean volumetric diameters were similar for both samples, and according to the *span* values both PSDs were narrow (the *span* values were lower than 2). For the sample $(PL\text{-}IN)_{50}$:DX, the D_{43} was 4.99 ± 0.42. This value is around 11% lower than the one previously obtained (Ceschan et al., 2015) when employing a lower atomization air flowrate. This difference was statistically significant (p-value < 0.05). The lower D_{43} value is related to the capability of producing smaller spray droplets (*i.e.* that result in smaller powder particles) by increasing the air flowrate (Ceschan et al., 2016). For sample $(PL\text{-}IN)_{50}$:DXCl, the D_{43} value was 4.67 ± 0.08. The differences between the samples with and without the addition of HCl were not statistically significant (p-value > 0.05), indicating that the pH adjustment did not affect this property.

According to the USP classification, the calculated *CI* values indicated that the samples $(PL\text{-}IN)_{50}$:DX and $(PL\text{-}IN)_{50}$:DXCl had "very poor" (*CI*: 32.35 ± 1.29) and "poor" (*CI*: 28.21 ± 3.18) flow properties, respectively. This result is commonly expected for powders with particle size less than 10 μm. Van der Waals' attractive forces dominate the interactions between particles smaller than 30 μm (Krantz et al., 2009). The poor flowability of cohesive powders may be improved by adding larger carrier particles, like lactose, which may also enhance the aerosolization process (Pilcer et al., 2012). In fact, the *CI* for the microparticles:lactose blends were 22.56 ± 2.28 and 24.62 ± 1.85 for the $(PL\text{-}IN)_{50}$DX and $(PL\text{-}IN)_{50}$DXCl samples, respectively. These values correspond to passable flow properties. The effect of the lactose addition in the *in vitro* aerosolization performance test is discussed below.

Fig. 1 shows the micrographs corresponding to the SD materials $(PL\text{-}IN)_{50}$:DX and $(PL\text{-}IN)_{50}$:DXCl. For both samples, rounded, smooth and aggregated particles were observed. The morphology was similar for both samples, which indicates that, as expected, the addition of HCl did not affect the particle shape.

3.3.2. In vitro aerosolization performance

According to the United States Pharmacopoeia, inhalable products have to be evaluated in cascade impactors, which establish the Aerodynamic Particle Size Distribution (APSD) of the material. This is strongly correlated with *in vivo* deposition and the lung regions where the powders can be deposited. In general, for local treatments, the particles should have aerodynamic diameters less than 5 μm. However, for systemic treatments, aerodynamic diameters even lower have been suggested (≤ 3 μm) to ensure particle deposition at the alveolar membrane (Newman and Chan, 2008).

Table 4 presents the emitted, fine particle and respirable fractions for particles smaller than 5 and 3 μm for samples $(PL\text{-}IN)_{50}$:DX and $(PL\text{-}IN)_{50}$:DXCl. The mass median aerodynamic diameter (MMAD) and the geometric standard deviation (GSD) are also shown in this table.

Both systems exhibited high emitted fractions, higher than 90% (91.09 ± 3.98 and 92.41 ± 1.07 for products $(PL\text{-}IN)_{50}$:DX and $(PL\text{-}IN)_{50}$:DXCl, respectively). Less than the 10% of the powder carrying IN was retained in the capsule and the inhaler. The EF values are above the levels reported for some marketed products (higher than 60%) and were considered adequate for inhalatory administration (Healy et al., 2014).

It is important to note that different definitions of FPF and RF can be found (Beinborn et al., 2012; Li et al., 2014; Newman and Chan, 2008; Pomázi et al., 2013; Razavi Rohani et al., 2014). Therefore, when these fractions were compared for different formulations, special attention in the fractions definition was taken into account.

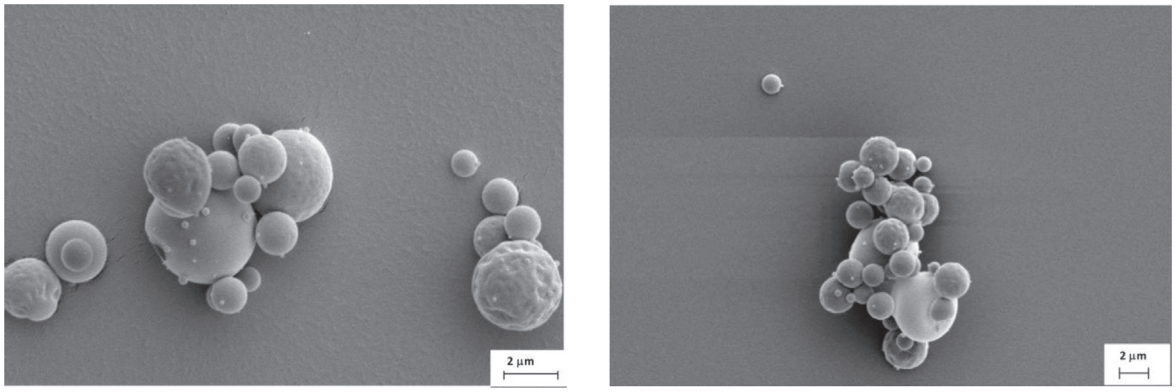

Fig. 1. SEM micrographs for the SD powders (Magnification: 10,000×).

The fine particle and respirable fractions for particles smaller than 3 µm were 18.90 ± 1.14 and 17.10 ± 1.10 for $(PL\text{-}IN)_{50}$:DX, respectively while for $(PL\text{-}IN)_{50}$:DXCl those fractions were 18.50 ± 0.79 and 16.88 ± 0.54. These were comparable to values reported for DPIs which have FPF values between 10 and 35% (Newman and Chan, 2008). No statistically significant differences were found between the two studied systems (*p*-value > 0.05), indicating that the pH adjustment in the feed composition did not affect the aerosolization properties of the powders. The FPFs for particles smaller than 5 µm (aerodynamic diameter) were 32.73 ± 3.70% and 31.26 ± 1.52% for $(PL\text{-}IN)_{50}$:DX and $(PL\text{-}IN)_{50}$:DXCl, respectively. RF values for particles smaller than 5 µm aerodynamic diameter were 29.69 ± 3.36 and 28.53 ± 1.05 for samples $(PL\text{-}IN)_{50}$:DX and $(PL\text{-}IN)_{50}$:DXCl, respectively. Most marketed formulations exhibit RFs between 12 and 40% (Islam and Cleary, 2012). In fact, Demoly et al. reviewed the performance of different commercial DPIs assayed at 4 kPa pressure drop. Half of them exhibited FPFs for particles smaller than 5 µm lower than the values found for the microparticles carrying IN (*i.e.* lower than 30%) while other commercial products possessed FPFs between 30 and 35%, values close to the reported in this work (Demoly et al., 2014).

The particulate systems $(PL\text{-}IN)_{50}$:DX and $(PL\text{-}IN)_{50}$:DXCl exhibited MMAD values of 3.51 ± 0.14 and 3.34 ± 0.08 µm, respectively (see Table 4). These values can be considered adequate for the inhalatory administration of microparticles (Razavi Rohani et al., 2014). Also, the aerodynamic particles distributions can be considered narrow; in fact, the GSD values were lower than 3 (Razavi Rohani et al., 2014).

It has been widely reported that lactose particles used as carriers commonly enhances inhalatory microparticle deagglomeration and thus improves the emission from the inhaler (Cordts and Steckel, 2015). The carrier addition can reduce the cohesive interaction forces between the microparticles, which lead to particle aggregation (Islam and Cleary, 2012; Pilcer et al., 2012). Nevertheless, it has also been reported that although lactose allows particles deagglomeration, the microparticles of the formulation in some cases cannot be detached from the carrier surface (Pilcer and Amighi, 2010). To evaluate the effect of lactose on the aerosolization performance of the tested samples, microparticles carrying IN were mixed with lactose in a 1:3 ratio.

Table 4 also shows the aerosolization performance of the microparticle:lactose mixtures. As can be seen, although the EFs were slightly higher (92.50 ± 2.53 and 93.79 ± 0.47 for samples $(PL\text{-}IN)_{50}$:DX and $(PL\text{-}IN)_{50}$:DXCl lactose mixtures, see Table 4), the differences in EF with or without the carrier were not statistically significant (p-value > 0.05). On the other hand, the FPF and the RF values (for particles smaller than 3 and 5 µm aerodynamic diameter) decreased significantly when blended with the lactose carrier system. In fact, for $(PL\text{-}IN)_{50}$:DX the FPF and RF smaller than 5 µm (aerodynamic diameter) were 15.47 ± 1.08 and 14.30 ± 1.29, respectively. The same fractions were 13.40 ± 1.32 and 12.55 ± 1.22 for the $(PL\text{-}IN)_{50}$:DXCl product blended with lactose (see Table 4). The addition of coarse particles in DPI formulations is often performed to improve drug particle flow dispersion (prevent the formation of stable aggregates). During the blending step, the smaller pharmaceutical particles adhere to the surface of the carrier ones. The mixtures obtained are easier to handle. During inhalation, drug particles need to be dispersed from carrier's surface. However, excessively high adhesive forces could be developed that prevent the drug release from the carrier surfaces. In that cases, the active ingredient particles attached to carrier ones impact on the upper airways. The development of high adhesive forces is related to the presence of active sites of increased energy at carrier surfaces. These active sites decrease drug detachment during particle aerosolization (Grasmeijer et al., 2014; Pilcer et al., 2012; Shur et al., 2013; Tee et al., 2000). Thus, the observed behavior could be related to the development of strong interactions between the microparticles and the lactose (Tee et al., 2000). Further studies are required to understand the developed interactions between the microparticles and the carrier, like the cohesive-adhesive balances forces (Shur et al., 2013).

Fig. 2 compares, for the sample $(PL\text{-}IN)_{50}$:DXCl, the IN mass fractions collected in the different NGI components for formulations with or

Table 4
Aerosolization performance for microparticles carrying IN (with and without lactose addition).

Sample	$(PL\text{-}IN)_{50}$:DX		$(PL\text{-}IN)_{50}$:DXCl	
microparticles:lactose	1:0	1:3	1:0	1:3
EF (%)	91.09 ± 3.98	92.50 ± 2.53	92.41 ± 1.07	93.79 ± 0.47
FPF < 5 µm (%)	32.73 ± 3.70	15.47 ± 1.08	31.26 ± 1.52	13.40 ± 1.32
RF < 5 µm (%)	29.69 ± 3.36	14.30 ± 1.29	28.53 ± 1.05	12.55 ± 1.22
FPF < 3 µm (%)	18.90 ± 1.14	10.74 ± 0.45	18.50 ± 0.79	9.23 ± 1.03
RF < 3 µm (%)	17.10 ± 1.10	9.92 ± 0.50	16.88 ± 0.54	8.64 ± 0.95
MMAD (µm)	3.51 ± 0.14	4.65 ± 1.60	3.34 ± 0.08	5.16 ± 0.47
GSD	2.15 ± 0.22	2.96 ± 0.25	2.09 ± 0.05	2.93 ± 0.22

EF: emitted fraction; FPF: fine particle fraction; RF: respirable fraction; MMAD: mass median aerodynamic diameter; GSD: Geometric Standard Deviation.

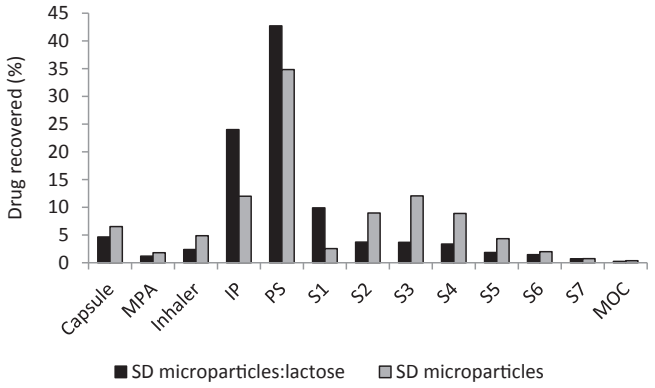

Fig. 2. Drug retention in the different NGI stages and components for the carrier free SD $(PL\text{-}IN)_{50}$:DXCl and the $(PL\text{-}IN)_{50}$:DXCl:lactose mixture. MPA: mouth piece adapter; IP: Induction port; PS: preseparator: S1–S7: NGI stages 1–7; MOC: micro orifice collector.

without lactose. When the SD microparticles:lactose mixtures were assayed, about 67% of the IN was accounted for in the (PS + IP). This fraction was 42% higher than the microparticles formulated without carrier. The retention in the PS and IP may be considered as extrathoracic losses and diminishes the fraction of the formulation that can reach the lung to exert the drug pharmacological effect (Glover et al., 2008). This reduction in aerodynamic performance when bigger carrier particles are mixed with inhalatory powders was previously reported for micronized microparticles of drugs used for lung diseases treatments (Tee et al., 2000; Adi et al., 2006). Also, Thi et al., observed the same performance reduction tendency for terbutaline microparticles obtained by spray drying and micronization (Thi et al., 2008).

The two developed particulate systems without lactose addition had adequate aerosolization properties for both local and systemic treatments (specific and non-specific bronchial inflammatory asthma responses and joint inflammation, rheumatoid arthritis and osteoarthritis pain, respectively). The aerosolization performance was negatively influenced by the carrier addition; however the poor flow properties of the spray dried powders without carrier particles would require the use of capsule filling machines for cohesive powders (Stegemann et al., 2013). The use of particulate systems proposed as carrier free avoids dose dilution and consequently the number of daily administrations.

Onischuk et al. demonstrated that, the inhalatory route for IN (as anti-inflammatory) delivery was more effective than the peroral IN administration (25–200 mg_{IN} daily) for arthritis treatments, even when a dose six orders of magnitude lower than the peroral one was deposited on the deep lung (Onischuk et al., 2008). In order to approximate the dose that would reach the deep lung regions (particles with aerodynamic diameters lower than 3 µm), the following calculations were taken into account. Each capsule was filled with 15 mg of microparticles containing IN. Considering that the composition of the microparticles is *ca.* 0.2 g_{IN}/g_{powder}, 3 mg of IN were loaded in each capsule. Since approximately 18% of the particulate system has aerodynamic diameter smaller than 3 µm, 18% of the 3 mg of IN contained in the capsule would likely reach the alveolar region (about 0.5 mg_{IN}). Considering: a) a daily oral dose of 200 mg_{IN}, b) a dose six order of magnitude lower administered by the pulmonary route has the same therapeutic effect than the peroral administration, then 2×10^{-4} mg_{IN} administrated by pulmonary route would be enough for treating joint inflammation (Onischuk et al., 2008). Therefore, 0.5 mg of drug (delivered from just one capsule to the alveolar membrane) would be, at least, three orders of magnitude higher than the dose that demonstrated anti-inflammatory effect on joint inflammation.

The drug doses for treating pulmonary diseases, administered by the inhalatory route, can be considered 10–20 times lower than the peroral doses (Yazdi and Smyth, 2016). It was also reported that IN administered by nebulization at a dose of 20 mg was effective to treat the bronchial response in asthmatic patients (Bianco, 2000). Considering that up to the 15% of the nebulized dose using a jet nebulizer reaches lung deep regions, 3 mg of IN would be enough to achieve the anti-inflammatory local therapeutic effect. Making the same considerations as described above regarding the microparticles composition and capsule filling, but taking into account that the respirable fraction (lower than 5 µm, without the carrier addition) is around 28%, in each inhalation 0.84 mg of IN would reach lung regions. If the IN local required dose is 3 mg, 2 administrations would be necessary to achieve this dose. Finally, as the systems are proposed as carrier free, a higher amount of microparticles (> 15 mg) can be loaded in the capsule and less capsules would be required to treat the bronchial inflammatory asthma response.

Although *in vivo* assays are required to establish the adequate doses for local/systemic treatments by the inhalatory route, the preliminary calculations would indicate that the treatment could be performed with an acceptable number of daily administrations.

3.3.3. Drug release experiments

The aim of this release assay was to assess the microparticles capability to wet, dissolve and release the drug. Since the polylysine is a macromolecule that cannot pass through the cellulose membrane used in these release experiments, the presence of IN in the receptor compartment indicates that the drug detected was dissociated from the polyelectrolyte-drug complex. This dissociation may occur due to an ionic exchange process with the buffer ions present in the physiological medium in the receptor compartment (phosphate buffer pH 7.4). Fig. 3 shows the cumulative IN release as a function of time for pure IN and SD samples. The IN release rate from the SD particles was much higher than the one exhibited by the pure IN (which is limited by the low drug water solubility (Hancock and Parks, 2000)). This behavior indicated that the SD microparticles (molecular dispersion of IN in a hydrophilic polymeric matrix) were able to wet and dissolve quickly. This is particularly important for a drug with low aqueous solubility, like IN. Once the particles were dissolved the drug was efficiently released by ionic exchange (this process involved the shifting of the ionic equilibrium towards the drug release (Ceschan et al., 2015)).

The *f*2 factor, to compare the $(PL\text{-}IN)_{50}$:DX and $(PL\text{-}IN)_{50}$:DXCl profiles, indicated that both materials have similar tendency to release the drug in phosphate buffer pH 7.4 (*f*2 = 76). Then, the SD feed pH adjustment did not affect the ionic interaction reversibility.

The sample $(PL\text{-}IN)_{50}$:DXCl was selected for further studies because the presence of Cl affected neither the drug release from the ionic complex nor the aerosolization performance in the NGI equipment.

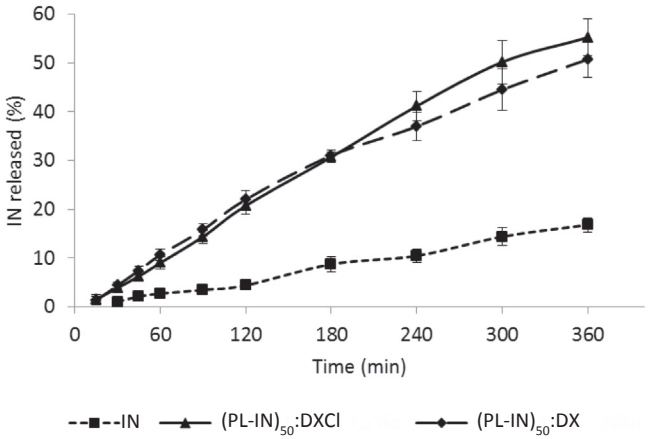

Fig. 3. IN release from the SD materials and the pure drug in phosphate buffer pH 7.4.

3.3.4. Anti-inflammatory effect

It is known that NSAIDs exert their therapeutic effect by inhibiting the COX, enzyme that catalyzes the conversion of arachidonic acid to prostaglandins. COX has two isoforms: cyclooxygenase 1 (COX-1), considered constitutive, and cyclooxygenase 2 (COX-2) inducible in inflamed tissues and whose activation generates inflammation and pain mediators (Badesso et al., 2014). The prostaglandins intervene in the inflammatory response causing vasodilatation, increasing the permeability of the tissues, allowing the leukocyte recruitment and stimulating the pain nerve endings (Enrique-Santos, 2007).

Previous reports have demonstrated that lipopolysaccharide (LPS) treatment increased COX-2 expression in RAW 264.7 cells (Griet et al., 2014) and that 10 μM indomethacin reduced COX-2 expression induced by peptidoglycan in these macrophages (Chen et al., 2006). In order to study the effectiveness of $(PL\text{-}IN)_{50}$:DXCl particles to reduce COX-2 expression in an *in vitro* inflammatory model, RAW 264.7 macrophages were pre-incubated and treated as mentioned in Section 2.2.6.

RAW 264.7 cell line is a line of murine macrophages that has been used in the study of the biochemical mechanisms involved in pulmonary inflammatory processes (Griet et al., 2014). Challenging these cells with bacterial endotoxins (such as lipopolysaccharides and peptidoglycans) enhances the inducible form of COXs (COX-2) expression and allows the evaluation of drugs with anti-inflammatory properties (Griet et al., 2014). Thus, the anti-inflammatory activity of the $(PL\text{-}IN)_{50}$:DXCl sample was tested in the RAW 264.7 cell line and compared to pure drug. It was previously mentioned that the IN inhalatory administration would allow, in addition to the treatment of systemic diseases, the therapeutic of inflammatory diseases located in the lung (Bianco, 2000).

Fig. 4 shows that LPS exposure for 24 h increased COX-2 expression by 350% respect to the control. α-Tubulin content was determined and used as loading control and results are expressed as COX-2/α-Tubulin ratio, in arbitrary units. In cells pre-incubated with $(PL\text{-}IN)_{50}$:DXCl particles, LPS-induced COX-2 expression was reduced by 30% with respect to the LPS treatment. Furthermore, pre-incubation with $(PL\text{-}IN)_{50}$:DXCl solution reduced the LPS-induced COX-2 expression to the same extent than pure IN solution. Pre-incubations performed with

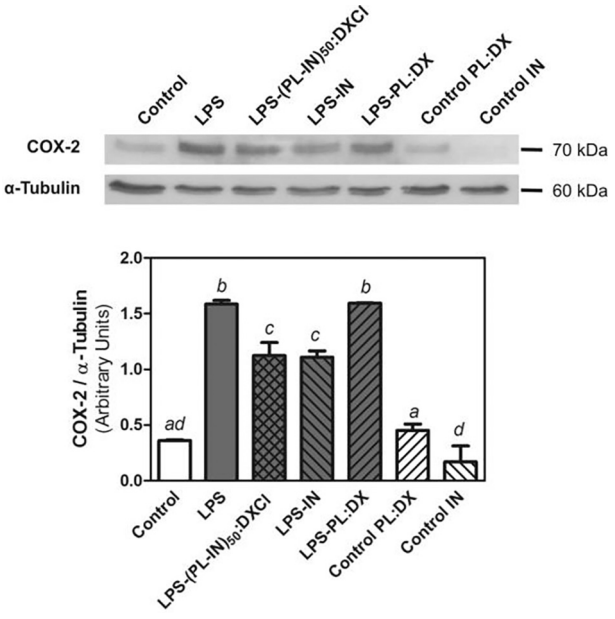

Fig. 4. Incubation of RAW 264.7 cells with the SD microparticles and pure materials. Numbers to the right indicate molecular weights and the bar graph shows the densitometry values of COX-2/α-Tubulin ratio expressed in arbitrary units. Conditions designated with different letters (a–d) present significant differences ($p < 0.05$).

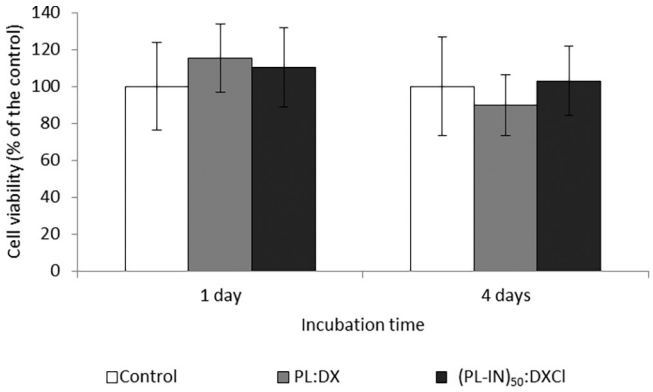

Fig. 5. Cell viability after the incubation (1 and 4 days) of the SD microparticles and the PL:DX material, expressed as % of the control.

PL:DX (control PL:DX) and with IN (control IN) solutions did not show a significant difference in the COX-2 expression respect to the control. These results demonstrate that $(PL\text{-}IN)_{50}$:DXCl solution has the same effect than pure IN solution on COX-2 expression in macrophages exposed to an *in vitro* inflammatory model.

3.3.5. Cytotoxicity assay

The cell line RAW 264.7 was also used to preliminary study the cytotoxic effect of the $(PL\text{-}IN)_{50}$:DXCl sample for local treatments by means of the MTT colorimetric assay. The cytotoxic capacity of the PL:DX was also studied.

Fig. 5 shows the RAW 264.7 cell viability, expressed as percentage of the control absorbance (cells incubated only with growth medium). As can be seen, neither at 1 day nor a 4 days treatment with $(PL\text{-}IN)_{50}$:DXCl or with PL:DX affected cell viability. In all the cases, the differences compared to the control were not statistically significant (p-value > 0.05). This indicates that the $(PL\text{-}IN)_{50}$:DXCl product was not cytotoxic at the concentrations and incubation times assayed in this cell line. Further studies are necessary to assess the cell viability at other concentrations and in different pulmonary cell lines, like the ones representative of systemic treatments.

4. Conclusions

In this work, microparticles carrying indomethacin were developed by spray drying an aqueous-based feed. A set of process parameters were found as adequate to produce microparticles with high process yield and small particle size. Regarding the formulation, samples with and without adjustment of the feed pH were tested. pH was adjusted in order to obtain biocompatible products. It was shown that yield, residual moisture, particle size, morphology and composition of the powders were not affected by the pH adjustment.

The microparticles exhibited aerodynamic properties suitable for treating specific and non-specific bronchial inflammatory asthma responses (locally) and joint inflammation (systemically) by the inhalatory route. In fact, emitted fractions were higher than 90% and fine particle and respirable fractions were within the range of the reported fractions for commercial DPIs. On the other hand, the lactose addition as carrier excipient worsened the microparticles aerosolization properties. Therefore carrier-free IN microparticles are recommended to be administrated by DPIs.

The drug release studies demonstrated that the ionic complexes were reversible, being capable to release the drug by ionic exchange under simulated lung physiological conditions in a higher rate respect to pure IN. Therefore the local or systemic bioavailability of the SD microparticles for inhalatory administration is expected to be higher than the micronized drug.

The microparticles exhibited the same anti-inflammatory capability

than the raw drug on COX-2 expression in macrophages exposed to lipopolysaccharides, indicating that the ionic complex did not exert influence on the therapeutic drug effect. Also, the developed materials did not affect the RAW 264.7 cell viability. Even though *in vivo* studies are required to establish biodistribution and the therapeutic doses, a promising DPI formulation has been developed to treat inflammatory diseases.

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