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PARTICLE ENGINEERING FOR THE PRODUCTION OF RESPIRABLE DRY POWDER FORMULATIONS

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List of abbreviations:

ACI = Andersen Cascade Impactor

API = Active Pharmaceutical Ingredient

BDP = Beclomethasone dipropionate

CAM = Cell adhesion molecule

CF = Cystic Fybrosis

DSC = Differential Scanning Calorimetry

FPD = Fine Particle Dose

FPF = Fine Particle Fraction

FSI = Fast Screening Impactor

GSD = Geometrical Standard Deviation

HA = Sodium Hyaluronate

HCS = High Content Screening

HMW-HA = High molecular weight Hyaluronan

LAMA = Long-Acting Muscarinic Antagonist

LMW-HA = Low molecular weight Hyaluronan

MMAD = Mass Median Aerodynamic Diameter

MTT= 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

Pe = Peclet number

PSD = Particle Size Distribution

SD = Spray-drying

SS = Salbutamol sulphate

TGA = Thermogravimetric analysis

We = Weber number

XRPD = X-Ray Powder Diffraction

ST = Stearylamine

CSA = Cetostearyl alcohol

SA = Stearyl alcohol

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Chapter 1 Introduction

1.1 Dry Powder Inhalers (DPIs)

A great interest in the scientific basis underlying pulmonary drug delivery has recently risen for both local and systemic treatment as the global market for pulmonary technologies reached $32.4 billion in 2013 and is expected to grow to $43.9 billion in 2018, with a compound annual growth rate (CAGR) of 6.2% (http://www.bccresearch.com/market-research/healthcare/pulmonary-drug-delivery-systems-hlc094b.html).

Beside the economic aspects, a key advantage of this route is that it enables delivery of low doses of an aerosolised drug (or combinations of drugs) to its site of action for a localised effect which leads to a fast onset of action while reducing side effects. Another important feature is represented by the possibility to treat systemic diseases; drug deposited in the distal lung (respiratory zone) may be rapidly absorbed due to favourable characteristics such as large surface area (~ 125 m^2), thin epithelial barrier (~ 0.1-0.5 μm) and high vascularization (Dolovich and Dhand, 2011).

Inhaled drug products comprise three main categories: nebulizers, pressurized metered-dose inhalers (pMDIs) and dry powder inhalers (DPIs). DPIs have some advantages over other devices, including relatively high dose delivery, typically no coordination between the patient and the device activation is required (as for MDIs) and no propellants are used. Another advantage lies in the solid state characteristic of the powder which has a greater physicochemical and microbiological stability respect to liquid formulation (Li et al., 2014).

DPIs are an inhalation product which contains and delivers the active medicament as a dry powder of suitable aerodynamic size for the treatment of local and systemic disease states. The inhalation product is composed by the formulation (responsible for the therapeutic effect) and the device (generating the aerosol); this combination is unique and may not be changed with other formulations or devices as the performance would be different.

When a patient inhales through a DPI, the energy derived from his inspiratory effort fluidizes the powdered dose, dispersing the micronized drug particles and eventually, causing its deposition in the deep lung (if the particles have size < 5 μm) where it exerts its intended therapeutic effect (Finlay, 2001). Different devices exploit different de-agglomeration principles using different forces to generate the aerosol. Clearly, the more efficient the force is, the higher the fine particle fraction will be. Friction forces may result in high internal shear forces useful when a soft pellets formulation is considered. For adhesive mixtures the most effective are inertial (e.g., vibratory, centrifugal or impaction) forces, because their magnitude is proportional to the third power of the drug particle diameter (drag and lift forces are proportional only to the first or second power of the diameter). Different technical means can be applied to sustain the action of such forces, such as whirl, circulation or cyclone chambers (Frijlink and De Boer, 2004).

In terms of design, DPI devices are basically either pre-metered dose (powder loaded into a dosage form) or reservoir (Figure 1.1). The first may be divided in single-dose (for example formulation contained in a capsule) or multidose systems (blister loaded with multiple doses). Regarding reservoir devices, a bulk of powder is stored inside the system and they have a mechanism useful to meter a dose prior of patient inhalation.

Figure 1.1. Reservoir device (NextHaler® Chiesi; on the left, www.chiesigroup.com visited on January 2016), single-dose device (RS01®, in the middle, www.tefarco.it visited on December 2015) and multidose device (GyroHaler® Vectura, on the right, www.vectura.com).

An inhaler device is necessary to disperse powders into inhalable aerosols; each device has its own intrinsic resistance to the airflow (measured as the square root of the pressure drop across the device divided by the flow rate through the device) (Clark, A. R., Hollingworth, 1993) affected by the internal geometry. Recent inhaler designs attempt to enhance powder deagglomeration or drug-carrier detachment by increasing air turbulence and particulate collisions, hence for an instance, having a high resistance (Q. (Tony) Zhou et al., 2014). A widespread belief is that high resistance DPIs require a high flow rate or considerable inspiratory effort to be operated correctly, which would be a reason not to prescribe such inhalers for patients with severe Chronic Obstructive Pulmonary Disease, COPD, (Dolovich and Dhand, 2011). However, it has been shown that high flow rate to achieve a pressure drop of 4 kPa through a high resistance device may not be necessary. Indeed, (De Koning et al., 2002) demonstrated that severe COPD patients are capable of generating such pressure drops. A further major advantage of high resistance DPIs is that they reduce the flow rate favouring central and peripheral lung deposition and limiting back throat particle inertial impaction.

Misunderstanding in the correct activation procedure of an inhaler caused a patient's lack of adherence to the therapy as evidenced by (Rau, 2005) so the device should be designed and eventually optimised with the purpose of prevent wrong employment. Extra features can include several ways of signalling to the patient for correct use regarding correct dose activation and inhalation and for the number of doses left in the inhaler (Hoppentocht et al., 2014).

In the last years, several new devices, incorporating the following features have been marketed for clinical use: improvement of aerosol dispersion, development of methods to reduce effort required for inhalation and improvement of delivery efficiency while maintaining portability and ease of use of the inhaler (Dolovich and Dhand, 2011).
A high respirable powder is formed by fine particles, which might face issues in the manipulation and filling operations of inhalers due to their very low flowability. This behaviour is caused by the raising in the specific surface area (following a micronization process for example) and, as a consequent, increasing in the cohesive/adhesive force contributes. Different solutions have been explored:

- **Binary mixtures or adhesive mixture** comprising micronized drug particles and a coarse carrier (traditionally α-lactose monohydrate).

In this way, the Active Pharmaceutical Ingredient, API, is bound to the carrier surface (particle size interval of the carrier between 50 and 200 μm) through weak interparticulate forces (van der Waals, capillary, electrostatic or mechanical forces); during the aerosolization phase, the energy imparted by the patient must overcome the adhesive forces, so that drug microparticles are detached from the surface being able to deposit into the lung. However, the force of adhesion between micronized drug and carrier may

be greater than the energy supplied, thus the drug will remain attached and subsequently swallowed. Controlling, rather than maximising-minimising the interparticulate forces became the new challenge (De Boer et al., 2012). Two factors underlying this concept have been presented: the existence of "active sites" and drug/fine micro-agglomeration. The first is defined as areas on the lactose surface that have a high adhesion potential; such sites may arise from morphological features, surface roughness, amorphous content, large contact surface area, intrinsic surface energy (i.e. based on polymorphism, surface chemistry, etc.), water adsorption and impurities. Nevertheless, other variables affect the overall aerosol performance such as mixing process, drug particle size distribution, drug content and use of "force control agent". The second theory embraces the likelihood of bonds formation among fines (drug-drug and/or drug-excipient). The formation of these micro-agglomerates results in an increase in the effective mass of API and thus decrease in surface area to mass ratio.

Various production techniques have been adopted to modify drug-carrier interaction. For example, altering surface roughness of lactose through controlled crystallization (Zeng et al., 2001) or fluidised bed (Chan et al., 2003). Another approach is to coat with appropriate additives (e.g. magnesium stearate) the lactose surface so to reduce the adhesion between drug particles and lactose surfaces; this case implies a mechanical dry coating process where the mixture experiences very high shear forces and compressive stresses (Zhou and Morton, 2012).

The main disadvantage of this approach is the limit of drug loading; if high drug/carrier ratio is employed a segregation problem may occur (Zhou and Morton, 2012).

- **Soft pellets or particle agglomerates**

Soft pellets or agglomerates (approximate size range of 200 -2000 μm) are constructed via weak attractive forces which are broken up into primary particles through patient inspiratory effort; they typically show appropriate flow properties allowing easy device metering and powder emission. These aggregates may consist of pure micronized drug or mixtures of micronized drug and micronized excipient in accordance with the formulation dose. (Belotti et al. 2015) produced amikacin spray-dried particles and part of this product was agglomerated by tumbling to form soft pellets.

One major drawback is the low mechanical stability which could negatively affect delivered dose uniformity and powder dispersibility (Frijlink and De Boer, 2004).

Finally, the compatibility between device and formulation properties should be considered carefully in DPI's products (Martinelli et al., 2015) as the aerosolising performance might dramatically be affected.

1.2 Particle engineering approach

Recent advances in inhalation research area have given a way in the development of novel particle technologies for respiratory drug formulation. For decades, drug powders for pulmonary delivery have been manufactured by mechanical milling to reduce the particle size required for aerosol formulation to the lungs. Such particles have normally been produced by crystallization, followed by filtering, drying and micronization. This technique carries some drawbacks as the milling process is quite time-consuming and inefficient for soft ductile organic pharmaceuticals, induce electrostatic charges (triboelectrification) and can adversely alter the surface and solid-state properties

generating amorphous domains. Having higher surface energy and containing amorphous regions, these particles are cohesive and thermodynamically unstable, resulting difficult to handle and with a low *in vitro* and *in vivo* performance (Colombo et al., 2013; Rasenack and Müller, 2004; Rehman et al., 2004).

Owing to these limitations, alternative manufacturing methods have been proposed to enhance the performance by optimization of the particulate formulations. Particle engineering is characterized by advances in formulation and/or the particle manufacturing process. This alternative strategy involved the controlled production of drug particles (in pure physical forms or with adjuvants) with desirable attributes such as narrow particle size distribution, improved dispersibility, enhanced drug stability, optimized bioavailability, sustained release and/or specific targeting (Koushik and Kompella, 2004). As the respirability of a dry powder is influenced by the particle size as well as by shape and density factors, the goal is to finely control these parameters improving aerodynamic efficiency (Chow et al., 2007).

The aerodynamic diameter, d_{ae}, is the diameter of a hypothetical spherical particle of unit density that settles in air at the same velocity as the real particle (Eq. (1)):

$$d_{ae} = d_{st}\sqrt{\frac{\rho}{\rho_0}} \qquad (1)$$

where ρ is the density of the real particle, d_{st} is the Stokes diameter, ρ_0 is the density of the spherical particle. An approximation may be made introducing a volume diameter (d_v) instead of Stokes diameter and a correction factor, called dynamic shape factor (χ), resulting in Eq. (2)

$$d_{ae} = d_v\sqrt{\frac{\rho}{\rho_0 \chi}} \qquad (2)$$

Since the respirability of an inhalation powder might be enhanced by particle size or density reduction as well as by shape modification, many techniques have been employed to produce optimized particles such as spray-drying, spray-freeze drying, supercritical fluid micronization, sonocrystallization and particle coating.

Spray-drying (SD) is a very popular technology because of its relative simplicity, availability of large-scale equipment, ease of operation and ability to produce composite materials. The spray drying process consists of four basic steps: nebulization of the feeding liquid, mixing of the liquid with the drying gas, evaporation of the liquid and separation of the dried particles from the gas. The liquid solution or suspension is transported from the container to the nozzle entrance via a pump system. The solvent is mostly aqueous, but even organic solvents are used. Nebulization transforms the liquid stream into fine droplets by applying a force. The high surface to volume ratio favours efficient and rapid drying of the droplets. Several types of nebulization devices are available, depending on the type of energy that is involved: centrifugal energy, pressure energy, kinetic energy and vibrations. Centrifugal forces are generated in a rotary atomizer (disk or wheel type). The most commonly used types of nozzles are kinetic energy or pneumatic nozzles where the fluid stream is broken in small droplets by interaction with a second fluid, which is usually pressurized air (Paudel et al., 2013).

The contact between the liquid spray and the drying gas is mostly established via a co-current design which leads to contact between the droplets and the highest temperature drying gas; of less importance is the counter-current drying procedures.

The main advantage of the spray-drying technique is the ability to manipulate and control a variety of parameters though deep understanding in the correlation of those parameters with the final quality attributes of the product is indispensable.

One of the challenges is derived from the short timescale of the process; indeed, most materials undergo amorphization which can become a stability issue. Besides, processing of macromolecules by spray drying also presents challenges due to potential for degradation as a result of factors such as thermal stress during droplet drying, high shear stress in the nozzle and also because of peptide/protein exposure at the liquid/air interface. A deeper understanding in particle formation process is a prerogative in particle engineering for pulmonary delivery systems. As it is already known, first step involves formation of droplets stemming from nebulization of the starting liquid. The system is held in a low surface energy status through cohesive forces along with effect of the viscosity, which tends to oppose any variation in liquid geometry. Instead, the external forces, such as aerodynamic, centrifugal, and electrostatic forces, act on the liquid surface promoting its disintegration. A dimensionless number called Weber number, We, represents the ratio between fluid's inertia compared to its surface tension Eq. (3).

$$We = \frac{\rho\, d\, v^2}{\sigma} \qquad (3)$$

where ρ is the liquid density, d is the droplet diameter, υ is the liquid velocity and σ is the surface tension.

Then, once droplets have been dispersed, a vaporisation phase takes place. In this step the choice of the solvent (or mixture of solvents) becomes crucial. Vehring and colleagues (Vehring et al., 2007) introduced the Peclet number, Pe, as a dimensionless number to describe the system behaviour during the drying process (Eq. (4)):

$$P_e = \frac{K}{8Di} \qquad (4)$$

Where K represents the evaporation rate and Di is the coefficient of diffusion for component i. The combination of these parameters will determine particle morphology

and the radial distribution of the components in the particle. For Peclet numbers smaller than 1, the diffusional motion of the solutes is fast compared to the radial velocity of the receding droplet surface; solutes will be fairly dispersed with a small surface enrichment. For Pe > 1, the surface moves faster than the dissolved components and this will result in surface enrichment (Vehring, 2008).

From this perspective, porous tobramycin particles (TOBI® Podhaler®) has been produced by emulsion spray drying via the PulmoSphere™ technology to treat Cystic Fibrosis-related pulmonary infections (Geller et al., 2011). Tobramycin is dissolved in the continuous aqueous phase of a submicron o/w emulsion; the resulting feedstock is then nebulized with a twin fluid nozzle into a hot air stream. The micronized particles are spheroidal, porous with a sponge-like morphology (Figure 1.2). Aerosol performance of the PulmoSphere tobramycin formulations was consistent in different temperatures (10–40 °C), relative humidity (10–65%) and airflow rates (40–85 L/min) (Haynes et al., 2010). Improved powder properties are achieved without the addition of carrier particles. Consequently, drug loadings as high as 90–95% w/w are possible (Newhouse et al., 2003). The spheroidal shape and porous surface decrease the area of contact between particles, leading to low particle cohesion.

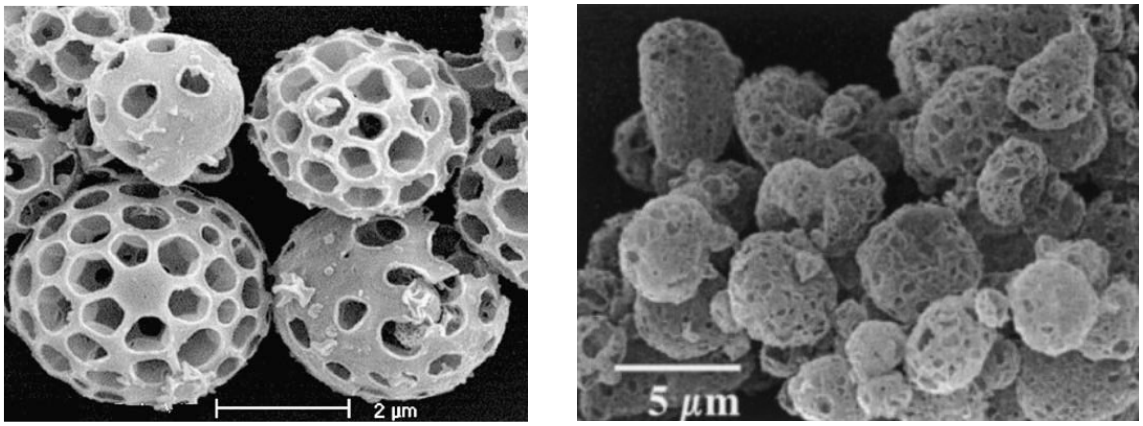

Figure 1.2. Porous particles obtained with the PulmoSphere technology; tobramicin® (left) and budesonide (right) microparticle (Vehring, 2008). Printed with permissions.

The median aerodynamic diameter is lower than 4 μm whereas the median volume diameter is higher than that value. This leads to an increment of the drug bioavailability as a significant number of particles avoid macrophagical phagocytosis. Other drug substances have been produced through this technology like ampicillin and budesonide. Budesonide spray-dried particles (Figure 1.2) whose morphology is engineered to be both hollow and porous applying PulmoSphere technology exhibited excellent flow and dispersion from passive DPIs both *in vitro* and *in vivo* studies (Duddu et al., 2002).

The most advanced options to enhance protein bioavailability is the use of specific excipients aiming at imparting suitable characteristics to the produced particles. In this way, MannKind Corp. has developed a dry powder formulation of insulin through Technosphere technology for use in diabetic patients (AFREZZA™). AFREZZA™ is a drug-device combination product consisting of Technosphere insulin powder pre-metered into single-use dose cartridges with a small and portable device. Fumaryl diketopiperazine (FDKP) constitutes the particle matrix and primary component of the Technosphere® technology; this compound is able to self-assembly via intramolecular hydrogen-bonding depending on the pH conditions. After precipitation through a pH modification, insulin is in a monomeric form (Neumiller and Campbell, 2010). The formed microspheres are freeze-dried in order to produce a powder suitable for inhalation (Figure 1.3). Thus, insulin is more rapidly absorbed in the blood stream with respect to subcutaneous injection, achieving a t_{max} of 15 minutes. Moreover, a significant improvements in bioavailability (30–50%) relative to other forms of inhaled insulin was reported (Angelo et al., 2009).

Finally the Technosphere® platform can be used to deliver assorted APIs from peptides and proteins to inorganic and organic compounds in a wide range of loadings between 0.01% and 90% (Steiner et al., 2000).

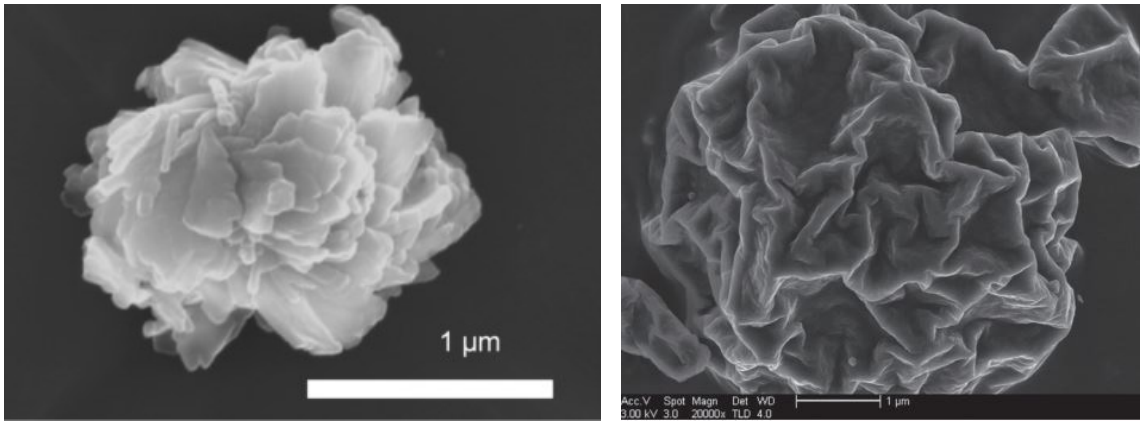

Figure 1.3. Technosphere® microparticles; AFREZZA™ crystalline insulin (left) and amorphous FDKP microparticle (right) (Leone-bay and Baughman, 2010).

Pulmatrix has developed an inhaled dry powder formulation of a long-acting muscarinic antagonist (LAMA) enabled by a non-lactose approach using Pulmatrix iSPERSE™ delivery technology (Figure 1.4, left). Powders have been manufactured by one-step spray-drying process and this platform might carry different APIs such as levofloxacin and insulin (Lawlor et al., 2014; Sung et al., 2011). iSPERSE™ technology claims a high drug payloads per powder volume and large drug molecules in highly dispersible particles, yielding superior drug delivery capabilities compared with conventional dry powder technologies that rely on the use of lactose blending or low-density particles (Babu and Morjaria, 2015).

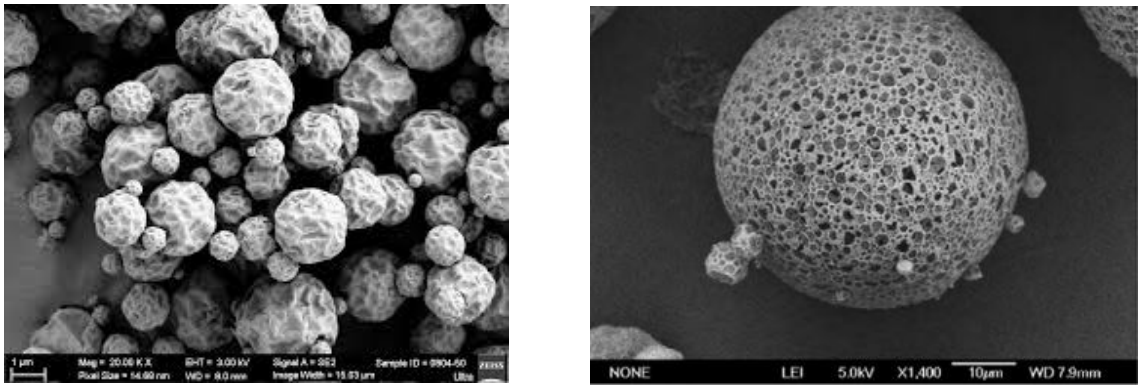

Figure 1.4. Spray-dried iSPERSE particles (left, www.pulmatrix.com visited on December 2015), PLGA-based gas-foamed LPP (right) (Ungaro et al., 2010). Printed with permissions.

(Ungaro et al., 2010) developed PLGA-based gas-foamed large porous particles (LPP) for local and prolonged release of hydrophilic macromolecules in the lungs. LPP were prepared by the double emulsion-solvent evaporation technique using ammonium bicarbonate as porogen (Figure 1.4, right). The addition of a surfactant (1,2-dioleoyl-3-trimethylammonium-propane) was crucial to control encapsulation efficiency and release properties of the developed particles. As compared to excipient-free LPP, lipid-engineered LPP presented favourable *in vitro* and *in vivo* (rodents) aerosolisation properties.

Nektar Therapeutics developed the technology (PulmoSol™) behind the first inhaled insulin – Exubera. An aqueous solution containing recombinant human insulin, mannitol (bulking/stabilising agent), glycine (bulking agent) and sodium citrate (buffering agent) was spray-dried. This technology implemented the construction of insulin particles stabilised in an amorphous glass matrix. Indeed, physical and chemical stability provided 2 years of shelf life at room temperature (Patton and Byron, 2007).

Particle engineering approach may bring to the design of fixed dose combination formulations avoiding the burden associated with complex treatment regimes. Combination of two substances may be produced by spray-drying as in the case of

tobramycin and clarithromycin (in ratio 10:1). Firstly, clarithromycin is dissolved in isopropanol and, thereafter, tobramycin is added and the resulting suspension is processed by high speed homogenisation and spray dried, as described by (Pilcer et al., 2008). As tobramycin is practically insoluble in isopropanol, clarithromycin in solution coats the micronized particles of tobramycin during nebulization. After the process, tobramycin particles appeared a little smaller and consisted of loose and porous agglomerates (Figure 1.5, left), explaining the better de-agglomeration tendency of the spray-dried powder compared to the physical blend (Pilcer et al., 2013).

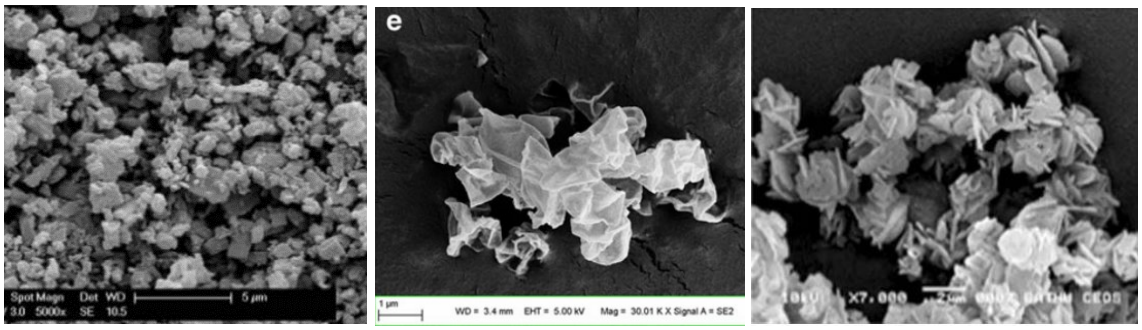

Figure 1.5. Scanning electron microscopy images: on the left tobramycin-clarithromycin particles (Pilcer et al., 2008); in the middle particles derived by co-spray drying colistin and rifampicin (Q. T. Zhou et al., 2014); on the right FP/SX micronized particles obtained by SAX technology (Kaerger and Price, 2004). Printed with permissions.

Co-spray drying has also been employed to produce wrinkled particles of a combination powder containing colistin and rifampicin (Figure 1.5, middle), given that monotherapy of colistin may lead to emergence of bacterial resistance. Synergistic antimicrobial activity, high aerosol efficiency (Fine Particle Fraction > 90% by Aerolizer) and moisture protection (due to the enrichment surface with rifampicin) was obtained (Q. T. Zhou et al., 2014).

An alternative technology is the solution atomization and crystallization by sonication (SAX) process, which produces high-purity, micron-sized and sphere-like crystalline particles in a single-step operation (Kaerger and Price, 2004). The nebulization of a 2%

w/v acetone solution containing fluticasone propionate (FP) and salmeterol xinafoate (SX) (10:1 ratio) caused the formation of droplets that were collected in the non-solvent perfluorodecalin at 5˚C, and exposed to sonic energy using a sonic horn to induce nucleation and crystal growth of the drugs within the supersaturated droplets. The extraction of perfluorodecalin and isolation of combination drug particles containing FP and SX were carried out using supercritical CO_2. The process afforded FP/SX particles with spherical shape and corrugated surface morphology (Figure 1.5 right) while retaining crystalline property. SAX FP/SX particles gave rise to greater and more consistent fine particle delivery in the correct ratio as well as improved formulation stability with respect to a carrier-based formulation (Pitchayajittipong et al., 2009).

Another example is constituted of nanoporous microparticles (NPMPs) containing sodium cromoglicate. This last was dissolved in a solvent mixture (water:methanol:n-butyl acetate) and spray-dried producing almost spherical, crinkled non-porous particles as shown in Figure 1.6, left. A higher fine particle of NPMPs of sodium cromoglicate was highlighted especially for the low density and high specific surface area powders (Nolan et al., 2011).

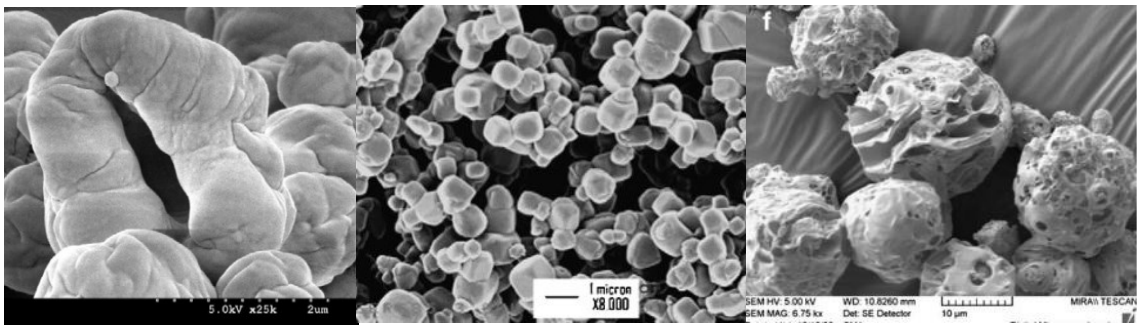

Figure 1.6. SEM images of nanoporous microparticles (NPMPs) of sodium cromoglicate (left) (Nolan et al., 2011, cubic NaCl particles after sonocrystallization (middle) (Abbas et al., 2007) and engineered PTH particles produced by spray freeze-drying (right) (Shoyele et al., 2011). Printed with permissions.

Sonocrystallisation employs ultrasonic radiation to better control the precipitation process; Abbas and co-workers (Abbas et al., 2007) employed this technique in manufacturing NaCl particles suitable for inhalation (Figure 1.6, middle). This technique offers some advantages including smaller and narrower crystal size distribution compared to conventional crystallisation, cost effectiveness of apparatus, the fact that the process can be run at ambient conditions and the reaction vessel involved is of simple geometry making the cleaning process simple for the pharmaceutical requirements. Cavitation causes the creation of voids in the liquid, due to the pressure fluctuations created by ultrasonic waves, which collapsing assist the nucleation phase.

In the study conducted by (Shoyele et al., 2011), inhaled parathyroid hormone (PTH) formulations have been produced by either spray-drying and spray freeze-drying solutions containing trehalose for reducing protein denaturation. Spray-dried PTH powder produced equal aerosol performance compared to the spray freeze-dried PTH powder for inhalation despite the large, porous nature of the spray freeze-dried PTH powder (Figure 1.6, right) and this could be attributed to the surface roughness of the PTH particles produced by spray-drying. *In vitro* bioactivity assay evidenced that the spray dried formulation, rather than the spray freeze-dried one, had a similar bioactivity of the unprocessed PTH.

Spray freeze-drying involves spraying the drug solution into a spray chamber filled with cryogenic liquid (typically liquid nitrogen). The spraying process can be performed beneath (spray-freezing into liquid) or above the surface of the cryogenic liquid. Upon contact with the cryogenic medium, the liquid droplets solidify rapidly and, once the process is completed, the whole content can be lyophilized.

Compared to spray drying, spray freeze-drying process allows achieving production yields of almost 100%; however, it is much less utilised due to its higher complexity, more tedious scale-up and higher costs (Wanning et al., 2015).

Another interesting strategy involves nanoparticles agglomeration (NanoClusters) which minimizes particles cohesion and adhesion. Pulmonary formulations that contain nanoparticles have been explored for some time but some issues obstacle their use; in particular, deposition of discrete aerosolized nanoparticles in the lower airways can be difficult due to the negligible effect of inertial and sedimentation forces (El-Gendy et al., 2010). An optimised wet milling process has been carried out for producing agglomerated budesonide nanoparticles with desirable aerosol properties; in particular, nanoparticles had a high emitted fraction and FPF which indicate suitability for highly efficient delivery and deep lung deposition to improve treatment of peripheral lung diseases such as chronic obstructive pulmonary disease (El-Gendy et al., 2010).

A fluticasone propionate nanosuspension has been prepared using antisolvent precipitation (water) ultrasonic assisted followed by homogenization. During homogenization phase, an albuterol sulphate aqueous solution containing L-leucine (agglomerating agent) has been added. The obtained suspension was lyophilized and the dry agglomerates produced micrometer-sized nanoparticle aerosols with a large fine particle fraction and nanostructure for improving the dissolution rate of poorly water soluble fluticasone (El-Gendy et al., 2011).

Supercritical fluids (SCF) are compressed gases or liquids above their critical pressures and temperatures. CO_2 is the most used SFC since it has low critical pressure (72.9 bar) and temperature (31.1°C). One of the most important physical attributes of SC CO_2 processing is the efficient extraction and separation of organic solvents, which often

enables production of the particles in a pure dry form and also facilitates a clean and recyclable precipitation process at low temperatures. The high diffusivity of SCFs can be utilized for plasticization of polymers and its high compressibility for promoting efficient atomization of solutions or melts (Chow et al., 2007). SCF technologies present advantages such as selective precipitation, impurity separation and control of crystalline forms. Several type of SCF processes exist such as Rapid Expansion Supercritical Solution (RESS), Supercritical Antisolvent precipitation (SAS) and Particles from Gas-Saturated Solution (PGSS).

SAS is based on rapid precipitation when a drug solution in organic solvent is brought into contact with SC CO_2.

The RESS method occurs when a drug is firstly dissolved in the supercritical fluid and then, undergoes a rapid expansion across a heated orifice, causing a reduction in the density of solution. Owing to this change, the solvation power suddenly decreases leading to a precipitation of the drug.

In PGSS process the SF is dissolved in molten solute and the resulting solution fed via an orifice into a chamber to allow a rapid expansion under ambient conditions.

SCF technologies have successfully been used to prepare antiasthmatic dry powders with superior aerodynamic performance related to decreasing surface energy of the particles (Shekunov et al., 2003) and inhaled biopharmaceuticals (Shoyele and Cawthorne, 2006). Innovative techniques such as electrospray/electrospinning are raising interests. Electrospraying and electro-spinning both belong to the class of electrohydrodynamic atomization techniques and are essentially based on the same physical principles. Both techniques make use of a strong electrical potential to drive the nebulization of a liquid into either small droplets or fibres respectively. Droplets and fibres produced then solidify

as the solvent evaporates, typically without the use of an active drying process and result in products ranging between the nano- and micro scale (Figure 1.7). The two techniques can practically be performed on the same device and are relatively simple to setup in a laboratory setting. The main distinction between the two processes is the range in viscosity of the polymer feed solution that is nebulized; A typical electrospraying setup consists of a syringe pump that continuously and precisely drives the liquid feed into the nozzle at low flow rates, a flat-ended capillary nozzle for uniform drop formation and a high voltage power source to provide jet formation. The essential parameters to be controlled include: the applied voltage, the liquid flow rate, the surrounding air temperature and humidity, the surface tension, viscosity and electrical conductivity of the liquid as well as the properties of the materials in the liquid (Bohr et al., 2014). The advantages of electrospraying compared with conventional atomization technologies are the following:

- The droplets are self- dispersing because they are electrically charged and repel each other, hence preventing aggregation during particle formation.
- Smaller droplets below 1 μm can be produced partly due to reduced surface tension and charge-related droplet fission.
- Particles are produced with near-monodisperse size distribution.
- The deposition of particles onto a substrate can be controlled by modifying the electric field limiting particle loss and narrowing the deposition area to a specific region.

The main disadvantage of particle production with electrospraying is its low throughput which is typically a fraction of a gram of dry weight per hour.

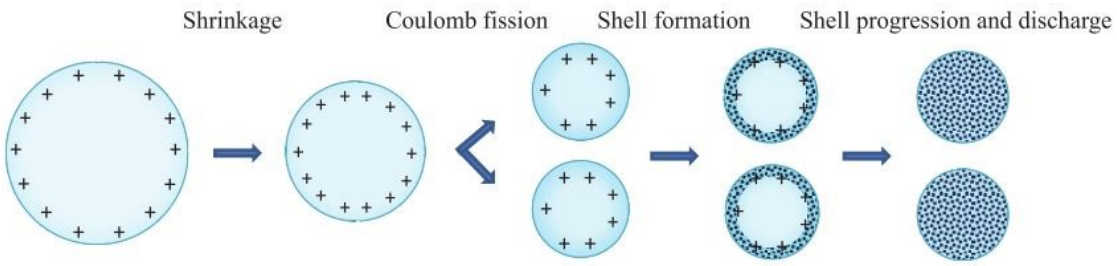

Figure 1.7. Particle formation stages during electrospraying process.

Electrospinning is in essence very similar to electrospraying and utilizes electric forces to break up a liquid but into fibres instead of particles.

1.3 Hyaluronan: physiological role and potential therapeutic applications in the lungs

Sodium hyaluronate (NaHA), hyaluronic acid (HA) and hyaluronan are members of the glycosaminoglicane family and are present in many substrates such as the extracellular matrix and synovial fluids. HA is a linear polysaccharide composed of a repeating disaccharide unit of N-acetyl-D-glucosamine and D-glucuronic acid bound by β 1,4 glycosidic bond (Figure 1.8). The disaccharides are linked by β 1,3 bonds to form the HA chains. Since *in vivo* the polymer exists in an ionized form as polyanion, it is generally referred to as hyaluronan (Lapcík L Jr and et al., 1998).

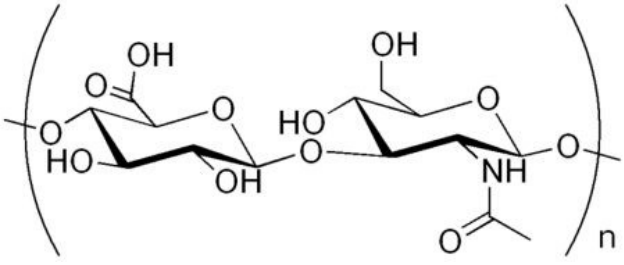

Figure 1.8. Hyaluronic acid disaccharide composed by D-glucuronic acid and N-acetyl-D-glucosamine.

Under physiological conditions, pulmonary HA is involved in various functions as the stabilization of the connective tissue and organization of the extracellular matrix, hydration and water homeostasis (Gerdin and Hällgren, 1997), regulation of the inflammatory response (Cantor et al., 2000), tissue modelling and remodelling (Petrigni and Allegra, 2006) and cell migration and fagocytosis (Turino and Cantor, 2003) as well as it controls CD44 receptor-mediated functions in cell detachment, cancer development, and inflammation (Necas et al., 2008). CD44 receptor belongs to the family of cell adhesion molecules (CAMs) specifically involved in the control of cell behaviour by mediating contact between cells or between cells and the extracellular matrix; this regulation is essential for maintaining tissue integrity (Arpicco et al., 2013). Because of these important functions, they are also involved in pathological conditions including tumour progression and metastasis (Orian-Rousseau, 2010). These receptors bind high molecular weight hyaluronan but can also interact with smaller forms of hyaluronan (Tammi et al., 2002). Consequently, tumor cells expressing CD44 show enhanced binding and internalization of HA so that a higher concentration of HA is taken up by cancer cells, forming a less dense external matrix, thus enhancing invasive ability into other tissues (Jaracz et al., 2005). Recent studies showed that the CD44 protein is over-expressed in many cancer tissues (Arpicco et al., 2013; Zhong et al., 2015) and, particularly, in non-small cell lung cancer (Ko et al., 2011; Quan et al., 2014). These findings encourage researchers to investigate HA as target moieties for active tumor-targeted drug delivery.
On the other side, many studies have been carried out in patient affected by different lung diseases to fully investigate what could be the effect of direct pulmonary administration of hyaluronan. It appears that the molecular weight of this polymer plays different roles with distinctive biological functions. High molecular weight (HMW-HA) is produced

endogenously and is an integral component of the extracellular matrix, synovial fluid, and vitreous humor; recent attention has been focused on the use of exogenously administered HMW-HA in a variety of diseases, including lung disease. In particular, it has been highlighted a reduced consumption and exacerbations in patient with chronic bronchitis treated with subcutaneous injections of HMW HA (Turino and Cantor, 2003); furthermore, a study in humans demonstrated HA efficacy in preventing exercised-induced bronchoconstriction in asthmatic patients (Petrigni and Allegra, 2006). In addition, low levels of HA in peripheral airways had been found in Adult Respiratory Distress Syndrome (ARDS) patient (Hällgren et al., 1989), thus coadministration of surfactant and high molecular weight HA could be helpful to enhance the surface activity as protects surfactants by inactivating substances (Lu et al., 2005).

Lately, HA was shown to prevent elastolysis and limit air-space enlargement in experimental models of emphysema (Cantor, 2007; Cantor et al., 2011) due to the interactions between the polymer chains and the elastic fibers; this further supports the use of HA in patients with pre-existing COPD as already claimed by (Petrigni and Allegra, 2006). In a related observation, it has been noted a significant reduction of HA in the lung of patients with emphysema; thus, exogenous administration of HA could be an indication for its use in emphysema, as well as for its protective function with regard to elastic fibres (Konno et al., 1982).

Finally, a study conducted by (Gavina et al., 2013) showed that nebulized HA was effective in controlling inflammation *in vivo* (in mice with cystic fibrosis) and *in vitro* (in cystic fibrosis airway bronchial epithelial cell lines).

Instead, under certain conditions such as tissue injury and inflammation, HA is more polydisperse, with a preponderance of lower molecular weight forms (LMW-HA) which are known to stimulate the production of a variety of proinflammatory cytokines (Horton et al., 1999). Moreover, LMW-HA induces macrophage and dendritic cell recruitment plus activation of inflammatory genes (Taylor et al., 2004; Termeer et al., 2000). Because of its immunostimulating properties, LMW-HA has been chosen as an adjuvant for novel formulations of the hepatitis B vaccine (HBV) vaccine system (González-Aramundiz et al., 2015; Moon et al., 2015). These studies suggested that HA fragments may play an important role in the mechanism regulating macrophage functions during inflammatory responses. Furthermore, the addition of LMW-HA in the preparation of dendritic cell vaccines against an experimental model of colorectal carcinoma was able to significantly induce an efficient antitumoral effect. LMW-HA can explicate an adjuvant effect inducing dendritic cell migration and activation towards antigen-presenting regions in a tumor vaccination protocol scheme against colorectal carcinoma in mice (Alaniz et al., 2011). (Scheibner et al., 2006) demonstrated that LMW-HA activates the innate immune response via TLR-2 as well as promoting antigen-specific T cell responses *in vivo.*

Aerosolized low molecular weight HA following endotoxin administration significantly increased lung inflammation, whereas pre-treatment with HA had the opposite effect (Nadkarni et al., 2005).

1.4 Formulation strategies to deliver hyaluronan

To date, only two nebulised products have been approved for different therapeutic indications. Hyaneb® (Chiesi Farmaceutici, Italy) is a hypertonic formulation (NaCl 7%

w/v) containing sodium hyaluronate (0.1% w/v; M.W.=500 kDa) utilised to decrease the mucus viscosity in cystic fibrosis patients and in patients with bronchiectasis (Furnari et al., 2012). The polymer has a double function: first, it improves palatability and reduces saline solution side effects, like bronchoconstriction, irritating effect and cough; second, HA extends the desired effect of the saline solution, holding water molecules.

Yabro® (IBSA Farmaceutici, Italy) is a 0.3% w/v HA (M.W. = 800-1000 kDa) solution for nebulization employed to reduce bronchial reactivity induced either by inhalation of substances or by physical effort.

Undeniably, formulating a hyaluronan powder suitable for pulmonary delivery is challenging given that this polymer mainly maintains its hygroscopic property. A dry powder formulation would be advantageous as the possibility of maximizing concentration, a shorter administration time respect to nebulisers as well as a superior physico-chemical and microbiological stability.

Several formulation strategies have been developed since the potential biomedical application of this polysaccharide has been discovered.

A low-viscosity formulation for pulmonary delivery was developed using solutions containing sodium hyaluronate in different molecular weights. Furthermore, the effects of pH and HA concentrations on pulmonary absorption of recombinant human insulin were examined after intratracheal administration in rats. The obtained results demonstrated the superiority of 0.1% w/v sodium hyaluronate solution compared to 0.2% w/v in enhancing insulin bioavailability probably due to the best balance between the absorption-enhancing effect and the diffusion of insulin in the formulation at that given molecular weight (Morimoto et al., 2001).

A novel formulation containing pyrazinamide in association with leucine, ammonium carbonate, hyaluronic acid and dipalmitoylphosphatidylcholine was produced for tuberculosis treatment as a dry powder suitable for pulmonary administration. Large porous particles were manufactured by spray-drying and the powder was stable for more than 4 weeks. The addition of the lasts two excipient was crucial to obtain stable partially crystalline spherical particles (Pham et al., 2013).

Hyaluronic acid and recombinant human insulin were co-spray dried to form a dry powder suitable for inhalation. Insulin and glucose systemic levels were monitored following administration of the microparticles to the lungs of male Beagle dogs. Pharmacokinetic studies highlighted an increase of the mean residence time (>3 fold), AUC/dose (2 fold) and T_{max} (by a factor of 2) with respect to the administration of pure spray-dried insulin (Surendrakumar et al., 2003).

Hyaluronan has also been included in nanoformulation to localize cisplatin preferentially to lymphatic system. HA-Pt conjugates were produced by dissolving the two substances in water, and following dialysis, the solution was concentrated up to 3.5 mg/mL. Compared to conventional Pt i.v. infusion, the HA–Pt lung instillation group had not only higher Pt accumulations in the lung tissues and the draining lung surrounding nodes but also demonstrated a sustained release plasma profile with a reduced peak plasma concentration (Xie et al., 2010).

Another study conducts by (Yang et al., 2013) has demonstrated the possibility of an active tumor targeting through the interaction via CD44 receptor. Nanostructurated lipid carriers carrying paclitaxel were coated with hyaluronic acid through electrostatic

attraction. Pharmacokinetic results showed a prolonged systemic circulation time of paclitaxel as well as accumulation of the drug in tumors, keeping antitumoral effect.

A spray-drying process was employed to prepare hyaluronic acid porous particles with controllable characteristics. Composite particles were dried starting from an aqueous solution of HA and polystyrene latex. Afterwards, particles were recovered and washed with an organic solvent in order to dissolve polystyrene latex. This approach led to a final particles having controllable aerodynamic properties, although safety issues should be considered (Iskandar et al., 2009).

Ofloxacin and hyaluronan were co-spray dried for increasing alveolar macrophages uptake and improving antitubercular efficacy. A 20% ethanol solution was used to solubilised HA and ofloxacin; SD powder was blended with α-lactose monohydrate carrier. Inclusion of HA in the formulation may enhance drug absorption due to the formation of a gel-like layer on the macrophage surface rather than receptor-mediated uptake. Finally, a potential reduction of the drug-treatment period might be expected (Hwang et al., 2008).

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Aim of the project

Products approved for pulmonary drug delivery use only fewer excipients relative to other types of drug delivery formulations. The raise of the number of therapeutic compounds and candidate molecules for lung administration will undoubtedly imply a parallel expansion in the formulation strategies and an increase number of excipients that will be included in inhaled products (Katdare and Chaubal, 2006). In this respect, the use of new excipients will require additional toxicology tests, bearing some risk of potential regulatory delays and product failure due to safety issue (Cipolla and Gonda, 2012).

The aim of this work was the production of highly respirable HA dry powders either as a potential platform for lung drugs delivery or for being administered as such by inhalation. The goal was pursued by building-up a pathway composed of different steps. Specifically, a low molecular weight sodium hyaluronate (HA) has been selected as innovative, biodegradable, biocompatible and nonimmunogenic excipient for lung delivery. This safe polymer was chosen as the main component of engineered particles produced by spray-drying for the optimisation of the aerodynamic properties of the final products.

Firstly, HA was formulated along with model drugs (salbutamol sulphate – hydrophilic drug and beclomethasone dipropionate – lipophilic drug) loaded at the concentration of at least 50 % starting from a polymer solution. Due to the difficulty to make HA microparticles by spray-drying, other biocompatible excipients were added in the formulation in order to improve the yield of the process and the quality of the product. In particular, leucine, tryptophan, arginine and cysteine were investigated.

Secondly, a novel approach to prepare HA powders without drugs, starting from a polymer colloidal suspension was studied. In detail, turbidimetric analysis was adopted

to finely tune the most suitable water:ethanol ratios keeping into consideration different initial HA concentrations. Then, in order to improve the powder aerodynamic characteristics, different types of excipients were tested. In particular, lysine and mannitol were included in the HA powder formulations at 10% w/w. Furthermore, stearylamine, cetostearyl alcohol and stearyl alcohol were investigated in three different percentages (1, 5 and 10% w/w).

All spray-dried powders were characterised in terms of particle size distribution, drug content, morphology and *in vitro* respirability as well as their solid-state properties.

Finally, *in vitro* biocompatibility evaluations by MTT assay and High Content Analysis (HCA) of selected dry powder formulations and the starting raw materials were carried out in cell lines considered relevant to the route of administration, such as A549, Calu-3 and U937 mimicking alveolar and bronchial epithelium and macrophages, respectively. This kind of studies aimed to assess the safety of model excipients and to compare MTT data with HCA method to provide an exemplar screening cascade for such investigations.

Chapter 2 Formulation study and preliminary screening

In the first part of the work, attention was focused on the research of innovative and safe excipient through which manufacturing particles suitable for pulmonary drug delivery. Polymers have been chosen for improving aerodynamic particle property (Sham et al., 2004) and since many concerns arise from the toxicological point of view regarding administration of synthetic and non-biodegradable polymers (Pilcer and Amighi, 2010), glycosaminoglycans were chosen as they are endogenous materials. In the field of drug delivery, sodium hyaluronate has become a carrier of great interest owing to its advantages, such as biodegradability, biocompatibility, high potential drug loading and targeting properties (Mero and Campisi, 2014).

Preliminary formulations were produced dissolving HA and two model drugs (salbutamol sulphate – hydrophilic drug and beclomethasone dipropionate – lipophilic drug) loaded at the concentration of at least 50 % in different water:ethanol mixtures in order to optimise the final product characteristics.

Spray-drying is one step technique used to produce drug microparticles and is become part of particle engineering approach, in controlling particle size and morphology as well as other desirable attributes (Sarrate et al., 2015).

However, production yields and aerodynamic properties were not satisfying and, thus addition of a second excipient was studied. Subsequently, leucine and other aminoacids were employed to improve powder production and performance.

A physical characterisation including size, morphology and aerodynamic characteristics of the spray-dried powders was carried out to evaluate the best candidates for a pulmonary administration.

2.1 Materials and methods

2.1.1 Materials

Sodium hyaluronate (HA) (PrymalHyal 50, average MW=29504 Da) was purchased by Soliance (France). Two APIs have been chosen as model drug: salbutamol sulphate (SS, Fagron, Italy) and beclomethasone dipropionate (BDP, Chiesi Farmaceutici, Italy). L-arginine, L-tryptophan and L-cisteine were supplied by Sigma–Aldrich (Sigma Chemical Co., USA). Potassium dihydrogen phosphate and L-leucine were provided by ACEF (Italy). Hard HPMC capsules size 3 were purchased from Capsugel (France). Medium resistance RS01® inhaler was kindly donated by Plastiape S.p.A. (Italy). All chemicals used were of analytical grade and water was purified by Elix® Essential (Merck Millipore, USA).

2.1.2 Methods

2.1.2.1 Salbutamol sulphate HPLC method

An Agilent 1200 chromatographic system was used for the analysis. Separation was achieved using Supelcosil LC-SCX, 5 µm 4.6 x 250 mm (SUPELCO, Sigma-Aldrich) maintained at 30°C using a column block heater. The mobile phase was a mixture of phosphate buffer (pH=7.0):methanol 40:60 v/v. In detail, for the phosphate buffer, 1 L of purified and degassed water was used to dissolve 6 g of KH_2PO_4. Subsequently, the solution pH was adjusted to 7.0 by adding NaOH 10 M dropwise. The mobile phase flow rate was set at 1.0 mL/min. The injection volume was 50 µL and detection was at 220 nm. ChemStation Agilent software was employed for data acquisition and analysis.

2.1.2.2 Beclomethasone dipropionate HPLC method

BDP was eluted using the same aforementioned chromatographic system. Mobile phase was prepared by mixing acetonitrile and water 60:40 v/v. BDP quantification analysis was performed using Atlantis dC18 column, 3 µm, 150 mm × 3.9 mm (Waters, USA) and adopting the following conditions: flow rate 1.0 mL/min, wavelength 239 nm, and injection volume 50 µL.

2.1.2.3 Spray-drying

Salbutamol sulphate and beclomethasone dipropionate engineered particles were manufactured by spray-drying employing a mini Spray-Dryer Büchi mod. B-290 (Büchi Laboratoriums-Technik, Swiss) with a high performance cyclone in closed-mode connected to the dehumidifier B-296. The feeding solution was prepared by dissolving each excipient in water whereas salbutamol sulphate was added to the final water:ethanol mixture (40:60 v/v) to make drug solution with a concentration of 1% w/v. Instead beclomethasone dipropionate was dissolved in ethanol prior to the addition of water.

The following operative conditions were used and kept constant for all spray-drying processes: inlet temperature 90 °C, spray flow rate 750 L/h, aspirator flow 35 m^3/h, feed rate of 3.0 mL/min and nozzle diameter of 0.7 mm. Spray-dried powders were kept in the collector for at least 24 hours before use in order to reduce electrostatic charges.

2.1.2.4 Particle size distribution by laser diffraction

The particle size distribution of the formulation was measured using a laser light diffractometer Spraytec (Malvern Instruments Ltd, UK) equipped with a 300 mm focal lens, which measures particle size in the range from 0.1 to 900 µm. Samples were prepared by suspending 10 mg of spray-dried powder in 10 mL of Span 85 (0.1 % w/v)

solution in cyclohexane; the dispersion was sonicated for 10 minutes. Particle size distribution was measured in triplicate with an obscuration threshold of 8 % and was expressed in terms of volume diameter at 10^{th} (D_v10), 50^{th} (D_v50) and 90^{th} (D_v90) percentile of the population as well as span value [(D_v90-D_v10)/D_v50].

2.1.2.5 Scanning Electron Microscopy

Scanning electron microscopy, SEM, (Zeiss SUPRA 40, Germany) was employed to investigate particle morphology and surface characteristic of the powders produced by spray-drying. The microscope was operated under high vacuum conditions with an accelerating of 1.5 kV voltage, at different magnifications. Powders were deposited on adhesive black carbon tabs pre-mounted on aluminium stubs and imaged without any metallization process.

2.1.2.6 In vitro deposition study

The aerodynamic size distribution of the spray-dried powders was investigated using an Andersen Cascade Impactor (ACI, Apparatus 3, USP 38, Copley, UK). ACI is an 8-stage cascade impactor, including an Induction Port (or Throat) and a filter, designed for being used at 28.3 L/min; however, through a modified configuration, impaction studies may be run even at 60 L/min.

Approximately 2.0 mg of powder were manually poured into a size 3, hard HPMC capsule. Subsequently one capsule was loaded into the inhaler and aerosolized. A single dose inhaler (RS01) was chosen to disperse and aerosolize the spray-dried powders.
Only one capsule was discharged inside the impactor for each aerodynamic test.
According to current USP guidelines for DPIs (US Pharmacopeial Convention, 2015),the flow rate (60 L/min) generated with a vacuum pump (Erweka VP1000, Germany) during

each experiment was controlled by a Flow Meter DFM 2000 (Copley Scientific, UK) in order to produce a pressure drop of 4 kPa over the inhaler. A Critical Flow Controller TPK (Copley Scientific, UK) was inserted between the pump and the impactor to accurately control the suction time and ensure a stable sonic flow.

The test duration in seconds is defined by the equation T=240/Q where Q is the test flow rate, so that a volume of 4 L of air is withdrawn from the inhaler during the experiment. Therefore, RS01 was activated for 4 seconds. Samples collected in the different plates of the ACI were recovered with a suitable volume of water:methanol mixture (75:25 v/v). Before running the experiment, to ensure efficient particle capture, a thin layer of a solution of 1% w/v Span 85 in cyclohexane was applied on the particle collection surface of each stage. Moreover, a mouthpiece adapter was attached to the end of the induction port to produce an airtight seal between the inhaler mouthpiece and the induction port.

Different aerodynamic parameters were calculated:

- Fine Particle Dose (FPD) is the mass of the aerosolized drug particles with an aerodynamic diameter below 5 μm.
- Fine Particle Fraction % (FPF %) is the percentage of the mass of drug particles with an aerodynamic diameter below 5 μm with respect to the total amount recovered from the system.
- Emitted dose is expressed as the total mass of drug emitted from the inhaler.
- Mass Median Aerodynamic Diameter (MMAD) is defined as the diameter which separates the powder in two populations with equal weight.
- Geometrical Standard Deviation (GSD) is a parameter which indicates how wide a particle size distribution is. Graphically, GSD represents the slope of the

regression line obtained by plotting the cumulative percentage of mass undersize (probability scale) versus the aerodynamic diameter (log scale):

$$\mathrm{GSD} = \sqrt{\frac{\text{Size X}}{\text{Size Y}}} \qquad (5)$$

where size X is the aerodynamic diameter at 84.13% of the population and size Y is the aerodynamic diameter at 15.87% of the particle population (Eq. 5).

2.1.2.7 Statistical analysis

Values were expressed as mean ± SD. Statistical significance of differences was examined using two-tailed unpaired t-test with significance level fixed at p-value = 0.05. Statistical analysis was performed with Microsoft Office Excel 2007 (Microsoft Corp., USA).

2.2 Results and Discussion

2.2.1 Production of spray-dried powders

Preliminary spray-drying tests were performed using water:ethanol mixture (25:75 v/v) containing HA and either BDP or SS (Table 2.I). In all the prepared batches, yields were very low as particles adhered on the cyclone inner wall. The same drawback was experienced by Esposito (Esposito et al., 2005), who sprayed aqueous solutions incorporating HA obtaining microspheres that adhered to the drying chamber.

Table2.I. Preliminary SD tests with 2 different components (HA and drug).

Batch (#)	**HA (%)**	**API (%)**	**Solid content (%w/v)**	**Yield (%)**	**Notes**
a1	100	-	0.1	22.0	Opalescent suspension
a2	16.7	**BDP** 83.3	0.6	6.3	Turbid suspension
a3	16.7	**SS** 83.3	0.6	5.3	The starting suspension became transparent when SS was added

Furthermore, the produced SD powders (batch #a1-a3) were characterised by the presence of strong agglomerates.

Since these first attempts was not satisfying, a second excipient was added in the formulation with the purpose of increasing the production yield and enhancing the powders characteristics.

Leucine (leu) has been chosen as its presence in the list of excipients approved by FDA for inhalation and for its capability to impart favourable technological property and to improve powder quality (Pham et al., 2013; Prota et al., 2011; Son et al., 2013; Vehring, 2008).

Considering the leucine low solubility in water:ethanol (25:75 v/v), the ratio between the two solvents was shifted to 40:60 v/v. Three SD powders were produced (Table 2.II): the first one composed by excipients only (batch #b1), the second containing salbutamol sulphate (batch #b2) and the last one containing BDP (batch #3). For the latter batch, BDP was solubilised in ethanol whereas leu and HA were dissolved in the water phase; then, the two phases were mixed and a suspension was obtained.

Table 2.II. Preliminary SD tests with 3 different components (HA, leu and drug).

Batch	**HA (%)**	**leu (%)**	**API (%)**	**Solid content (%w/v)**	**Yield (%)**	**Drug content (%)**	**Visual characteristic of the solution**
b1	20	80	-	0.5	48.4	-	Clear solution
b2	10	40	SS 50	1.0	69.9	49.55 ± 0.48	Clear solution
b3	12.5	62.5	BDP 25	0.8	48.7	25.32 ± 0.43	Turbid suspension after water and ethanol mixing

These powders were prepared in order to achieve a better understanding of the behaviour of the material undergoing the spray-drying process. Table 2.II showed an increase in the yield and, moreover, powders containing API presented a drug content in accordance with its own theoretical value.

Preliminary aerodynamic assessment was run for batch b2 and b3 using Andersen Cascade Impactor. Both were aerosolised by RS01 device and b2 was easily emitted and dispersed by the airflow; the emitted dose reached 90% and FPF% was at 85%. Whereas for b3 almost 50% of the loaded dose was retained in the device and FPF% was around 26% witnessing poor *in vitro* aerosolisation.

Moreover, particle size distribution of b3 proved to be suitable for pulmonary delivery as the 90% of the particle population was below 2.6 μm with a span value of 1.09, indicating a narrow distribution.

Hence, b2 was chosen as a starting point to carry on the construction of different powders mainly investigating the effect of the type of excipient and its concentration on the powder physical properties. Powders with BDP were not further characterised.

A study was subsequently led exploring formulation variables and the relevant details are reported in Table 2.III. Four set of experiments were carried out using HA and leucine as excipients and SS as the model drug. The effect of the amount of HA and leucine added was investigated in the concentration range of 10-40 % w/w. Firstly, seven SD powders were prepared keeping initially constant HA percentage, varying leucine and SS percentages (batch #1 - #4); afterwards the same thing was made holding the percentage of leucine fixed at 10% (batch #5 - #7).

Subsequently, batch from #8 to #12 were produced with only one excipient in the formulation for investigating the influence of each excipient on the microparticles characteristics.

Finally, (batch #13 - #15) leucine was substituted with other 3 aminoacids selected on the base of their differences on polarity of the side chain: arginine (polar charged aminoacid); tryptophan (apolar aminoacid); cysteine (polar neutral aminoacid).

In all the cases, the solutions obtained before spray-drying were clear and transparent.

Table 2.III. Quali- quantitative composition defined in the first experiment set, process yield and actual drug content in the final dried product (n=3 ± s.d.).

Batch #	HA (%)	leu (%)	SS (%)	Yield (%)	Drug content (%)
1	10	**40**	50	69.9	49.55 ± 0.48
2	10	**30**	60	67.2	57.45 ± 0.92
3	10	**20**	70	67.5	67.78 ± 0.22
4	10	**10**	80	64.2	75.28 ± 0.85
5	**40**	10	50	62.0	49.69 ± 1.86
6	**30**	10	60	64.0	58.21 ± 0.19
7	**20**	10	70	69.2	65.02 ± 1.92
8	**20**	**-**	80	20.0	Batch discarded
9	**10**	**-**	90	38.5	87.76 ± 0.05
10	**-**	**20**	80	75.3	77.94 ± 0.81
11	**-**	**10**	90	65.7	82.91 ± 0.08
12	**-**	**5**	95	43.6	97.04 ± 0.02
13	10	10 (Arg)	80	68.0	76.99 ± 0.18
14	10	10 (Trp)	80	18.0	76.49 ± 0.81
15	10	10 (Cys)	80	17.9	75.66 ± 1.33

Maximum excipient percentage was established to be 50% in order to obtain a relatively high drug loading. Throughout the experiment, high drug loading was considered as a

prerogative. The process yield was always higher than 60 % and powders were electrostatically charged after their production.

As far as the yield of the process was concerned, the results confirmed that the spray-drying was efficient in term of amount of powder produced (around 60-70%) (Table 2.III), despite the small scale of each individual batch. This is particularly true for the powders containing both HA and leucine.

Indeed, difficulty in microparticles production was encountered when HA was sprayed alone given its tendency to bind water molecules (Jin et al., 2010). The yield of batch #8 was not satisfying (20.0%) because the material was stuck on the cyclone. The small amount recovered from the collector appeared as flakes composed by strong microparticles agglomerates thus it was discarded. This result confirms that HA is a polysaccharide difficult to transform into flowable microparticles by spray-dring.

Conversely, batch #10 and #11 containing 20% and 10% of leucine respectively, were produced with a consistent yield (75-65%); poor yield was achieved with 5% of leucine (batch #12) due to the powder adhesion inside the cyclone.

Finally, other three aminoacids were investigated to evaluate which of them could have a positive influence on the physical outcomes of the powder (batch #13-#15).

The yield was very low when triptophan and cysteine were added to the formulation. With the addition of arginine the yield reached 68 % in accordance with previous set.

All the drug content was in agreement with the theoretical concentration of SS and RSD% was always less than 3% indicating that SS was uniformly distributed in the particle collections.

2.2.2 Physical characterization of SD powders

Particle size distribution of SD powders was determined by a laser diffraction apparatus. In Table 2.IV D_v10, D_v50 and D_v90 as well as span values are reported.

Table 2.IV. Particle size parameters of SD powders (mean ± sd; n=3).

	Batch	D_v 10 (µm)	D_v50 (µm)	D_v 90 (µm)	Span
HA + Leu + SS	1	0.91 ± 0.01	1.58 ± 0.01	2.63 ± 0.03	1.09 ± 0.02
	2	1.09 ± 0.01	1.81 ± 0.06	2.98 ± 0.15	1.04 ± 0.04
	3	1.85 ± 0.04	5.55 ± 0.48	13.76 ± 1.22	2.14 ± 0.03
	4	0.88 ± 0.13	1.57 ± 0.18	2.77 ± 0.21	1.30 ± 0.17
	5	1.10 ± 0.08	2.11 ± 0.25	4.10 ± 0.66	1.42 ± 0.10
	6	1.75 ± 0.14	5.07 ± 0.89	12.37 ± 2.63	2.08 ± 0.18
	7	1.26 ± 0.06	2.51 ± 0.23	5.80 ± 0.58	1.40 ± 0.08
HA or Leu + SS	9	95.71 ± 1.58	348.50 ± 2.05	692.10 ± 3.02	1.71 ± 0.60
	10	1.85 ± 0.09	5.90 ± 0.28	19.62 ± 1.08	2.91 ± 0.22
	11	1.96 ± 0.08	7.58 ± 0.72	18.81 ± 1.53	2.24 ± 0.15
	12	1.42 ± 0.07	7.80 ± 0.52	84.46 ± 17.70	8.52 ± 3.77
HA+Arg or Trp or Cys + SS	13	1.43 ± 0.03	3.60 ± 0.25	8.87 ± 1.25	2.05 ± 0.20
	14	1.72 ± 0.16	9.10 ± 1.03	33.27 ± 2.29	3.48 ± 0.17
	15	1.62 ± 0.24	7.58 ± 3.03	39.03 ± 11.92	5.08 ± 0.83

As reported in the Table 2.IV, batch #1, #2, #4, #5 and #7 have a dimensional distribution suitable for lung delivery (ranging from 1.3 µm to 5.8 µm) as well as a low span value, indicating a narrow particle size distribution. These formulations contained both HA and leucine.

When SS was combined with only one excipient (batch #9-#12), the size was higher than 5 μm. In particular, batch #9 had particles with a $D_v50 > 300$ μm hardly compatible with the spray-drying conditions chosen; most likely, the water absorption capacity of the hyaluronan was responsible for the development of capillary forces among microparticles.

Leucine substitution with other selected aminoacids did not afford any enhancement in term of particle size distribution. Nevertheless, arginine provided smaller particle size ($D_v90 = 8.87$ μm) compared to the other two aminoacids. The span value consideration is important as well since the narrower is the particle size distribution, the more accurate the regional lung deposition of the particles is achieved.

Given that the aerodynamic diameter is related to the volume diameter, particles with a dimension below 5 μm could have good respirability. However, this hypothesis must be confirmed carrying out an *in vitro* aerodynamic assessment as the capacity to penetrate into the deep lung is affected also by particle shape and density.

Scanning Electron Microscopy (SEM) is a useful tool to investigate morphological surface characteristics of spray-dried powders. Moreover, SEM was employed to evaluate how different variables (e.g. type of excipient and its concentration) might affect the final particle shape.

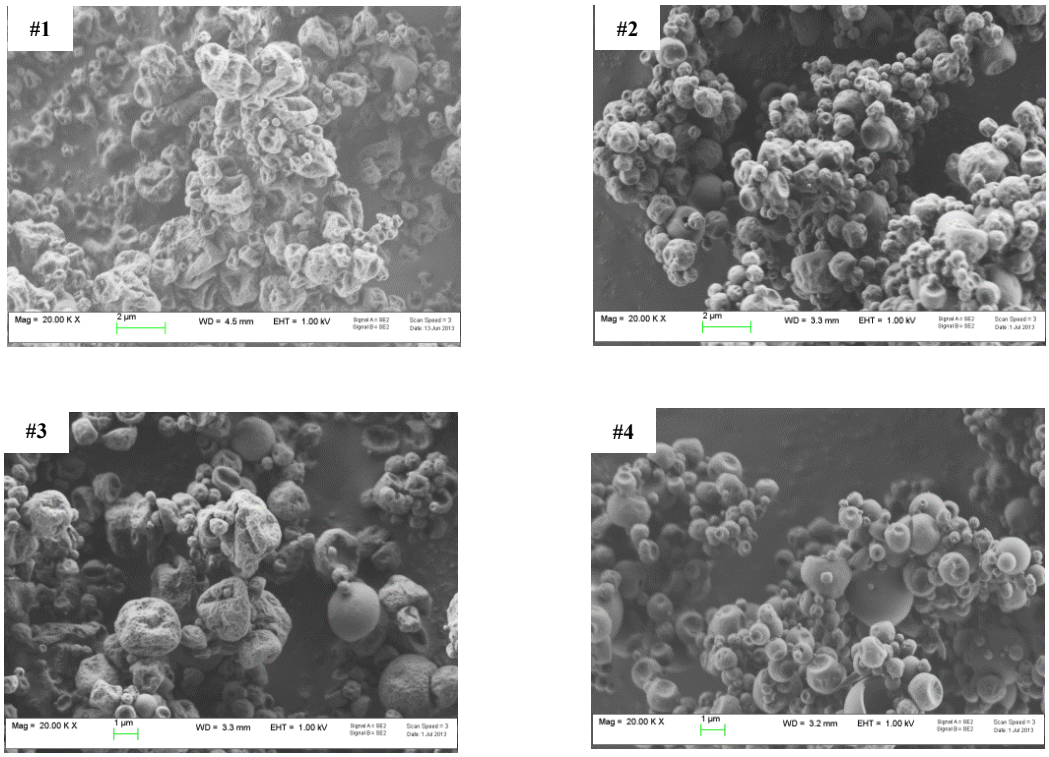

Figure 2.1. SEM images at 20000X magnification of spray-dried powders containing increasing amount of leucine (batch #1-#4).

Figure 2.1 shows the aspect of microparticles obtained with increasing amount of leucine. The pictures evidence the peculiar shape of such particles; indeed, they are spherical with concavities and some nanoparticles are noticed. Increasing the salbutamol sulphate content in the formulation leads to a smoother surface and more porous particles appear. However, it should be underlined that the SS increase was mirrored by a consequent leucine decrease.

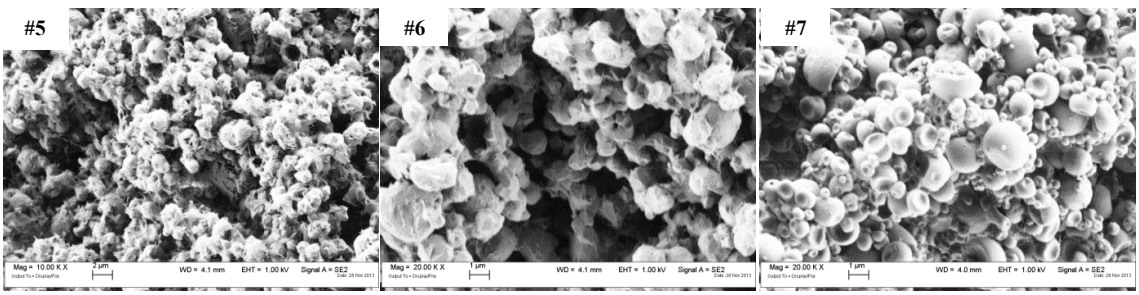

Figure 2.2. SEM images at different magnifications (10000X and 20000X) of spray-dried powders containing increasing amount of HA (batch #5-#7).

On the other hand, wrinkled and bigger particles were obtained when the HA percentage was increased keeping constant the amount of leucine (Figure 2.2). Microparticles having roundish shape were observed when the HA percentage was as low as 20%.

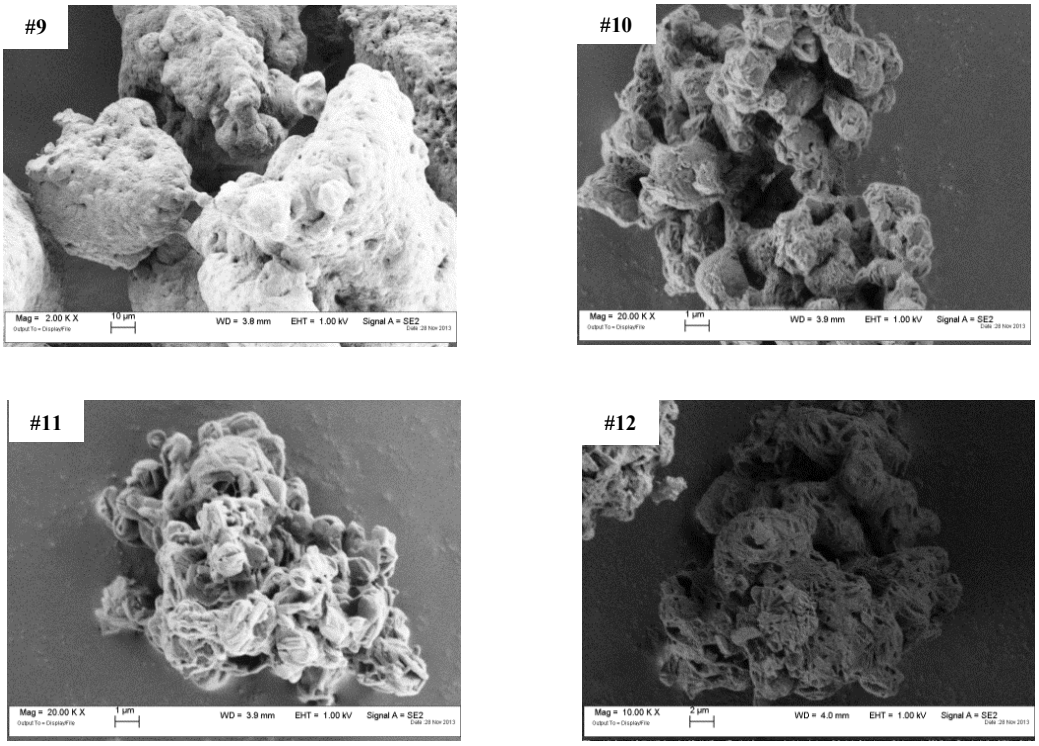

Figure 2.3. SEM images at different magnifications (2000X, 10000X and 20000X) of spray-dried powders containing either HA or leucine (batch #9-#12).

Figure 2.3 shows the aspect of particles containing a binary combination of either SS and HA (10%) or SS and leucine (from 5 to 20%). The particles were bigger as already observed by diffraction analysis data. The presence of leucine as unique excipient led to the formation of smaller particles with respect to the case where the particle contained only HA beside SS. Some holes appear on the surface (batch #11 and #12) which is also more irregular. Particles appeared as fused together and their size were comparable to what come out from laser diffraction analysis.

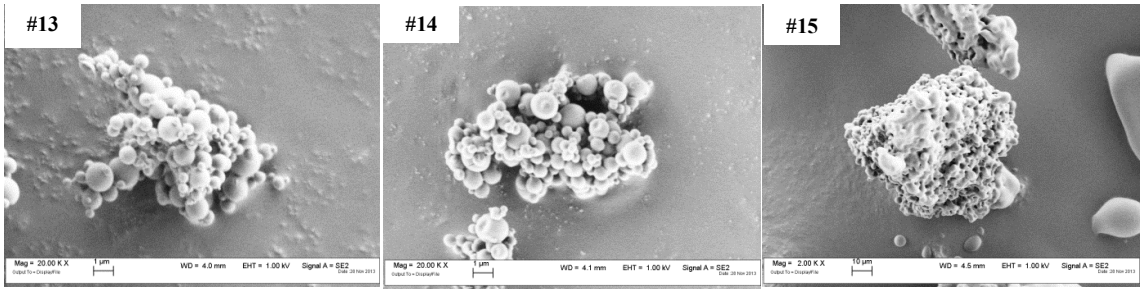

Figure 2.4. SEM images at different magnifications (2000X and 20000X) of spray-dried powders containing HA with arginine, tryptophan and cysteine (batch #13-#15).

The substitution of leucine with either arginine or tryptophan (batch #13 and #14) revealed some similarities; particles exhibited spherical shape with smooth surface and had size of few microns (Figure 2.4). The dimensions reported by laser diffraction were most likely due to strong aggregates which have not been broken into primary particles.

The production of SD powders with cysteine did not fulfil the requirement of micronized particles as there were big agglomerates of microparticles probably stemming by the fusion o of primary particles. In this case, the image relevant to batch #15 showed size agreement with data obtained by laser diffraction.

2.2.3 *In vitro* deposition study

The *in vitro* aerodynamic assessment of spray-dried salbutamol sulphate powders was carried out using an Andersen Cascade Impactor (ACI) which separates particles based on their aerodynamic diameter. From these results, the respirability of an inhaled product could be calculated as a Fine Particle Fraction % (FPF%), that is the fraction of particle with an aerodynamic diameter < 5 μm.

In Table 2.V, aerodynamic parameters included in the USP38 are reported: loaded dose, emitted amount %, Fine Particle Dose, Fine Particle Fraction %, MMAD and GSD. 2 mg

of powders were loaded inside a capsule and one capsule was discharged for each test. Batches produced during the preliminary phase were not investigated.

Table 2.V. Aerodynamic parameters obtained through ACI tests of spray-dried powders (n=3; mean ±s.d.). Loaded amount = 2 mg (salbutamol sulphate content in accordance with its theoretical content for every batch).

Batch	Loaded dose (mg)	Emitted amount (%)	FPD (mg)	FPF (%)	MMAD (µm)	GSD
1	1.18	83.4 ± 1.3	**0.81 ± 0.12**	97.1 ± 0.2	0.65 ± 0.01	2.44 ± 0.04
2	1.32	78.5 ± 2.6	**0.84 ± 0.13**	94.5 ± 0.6	0.94 ± 0.04	2.36 ± 0.04
3	1.57	73.3 ± 7.6	**0.78 ± 0.01**	93.5 ± 1.3	1.42 ± 0.14	2.11 ± 0.01
4	1.69	53.6 ± 2.4	**0.83 ± 0.05**	93.1 ± 0.5	1.53 ± 0.01	2.07 ± 0.01
5	1.05	71.1 ± 5.3	**0.43 ± 0.01**	87.1 ± 6.3	1.62 ± 0.18	2.14 ± 0.17
6	1.25	81.4 ± 1.7	**0.51 ± 0.02**	79.7 ± 2.6	2.46 ± 0.08	1.93 ± 0.05
7	1.36	59.6 ± 13.9	**0.53 ± 0.04**	94.4 ± 2.2	1.48 ± 0.18	1.94 ± 0.14
10	1.52	84.5 ± 9.1	**0.64 ± 0.23**	73.2 ± 27.0	2.36 ± 1.90	1.94 ± 0.14
11	1.69	72.0 ± 2.8	**0.96 ± 0.12**	89.6 ± 2.0	1.45 ± 0.01	2.29 ± 0.17
12	2.01	90.6 ± 1.4	**0.06 ± 0.03**	7.2 ± 3.3	46.45 ± 1.13	14.80 ± 0.67
13	1.52	50.2 ± 0.2	**0.58 ± 0.01**	67.4 ± 4.2	2.68 ± 0.49	3.02 ± 0.16
14	1.56	73.0 ± 15.2	**0.28 ± 0.24**	32.6 ± 27.5	6.92 ± 8.06	5.24 ± 5.78
15	1.46	77.7 ± 1.1	**0.05 ± 0.00**	4.6 ± 0.7	48.85 ± 17.83	13.95 ± 3.80

A first quality parameter related to the powder flow properties and, hence, to its ability to exit the device is the emitted amount. The emitted amount % for each batch is reported in Figure 2.5.

The formulations best performing in terms of emitted amount (>75%) were those of batch #1, #2, #6, #10, #12 and #15. Nevertheless, these lasts two had a good emission but a low respirability (FPF% < 8.0%) owing to the presence of big and hardly dispersible agglomerates. Indeed, enhancement of emission is usually obtained by increasing the particle size.

The standard deviations relevant to formulations 10 and 14 were high since these powders had a wide particle size distribution and 2 mg of powders might not be representative of the particle collection.

All powders tested have demonstrated an emitted amount > 50 %, this observation underlines the fact that the device was capable of efficiently aerosolize the powders and that these lasts were not too cohesive/adhesive.

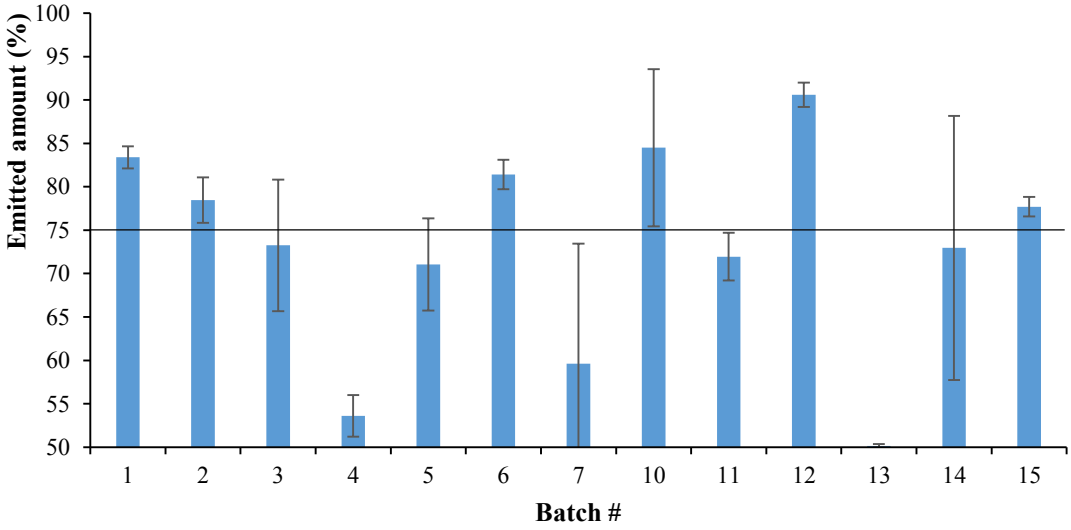

Figure 2.5. Emitted amount % from the device. Horizontal line represents 75% emitted amount considered as the minimum requirement for quality inhaled product.

The consistency of the emitted amount which reaches the patient (delivered dose) as well as and especially the consistency of the fraction of particles with an aerodynamic diameter

less than 5 μm (fine particle dose) have to be considered as indicators for an inhalation product quality.

From the *in vitro* deposition studies becomes evident that powders produced by spray-drying which have the best aerodynamic behaviour (in terms of emitted amount % and FPF %) were batch #1, #2, #6 and #10. The first three powders were formulated with both HA and leucine whereas the last, apart from the drug, contained only 20% of leucine. This finding are in agreement with the data by Aquino who reported a dramatic increase in FPF % when the leucine content rose from 0 to 20% (Aquino et al., 2012).

A clear tendency came out from the first set of experiment (batch #1-#4): as the percentage of SS increased to detriment of leucine content, the FPF% was reduced (from 97.1 to 93.1%).

Moreover, the Fine Particle Dose (FPD) is probably the most significant aerodynamic parameter to look at because it represents the real dose of drug reaching the lung. The parameter takes in consideration the amount loaded in the capsule and the loss of drug occurring during the aerosolization process (Figure 2.6). On the contrary FPF%, calculated as prescribed by the USP Pharmacopoeia (US Pharmacopeial Convention, 2015), represents the respirable fraction of the drug in respect to the emitted dose only. For instance, formulation #7 had a FPF% equal to 94.4 % but only 59% of loaded dose was released by the device.

Figure 2.6 illustrates the highest values of fine particle dose obtained among all the tested formulations.

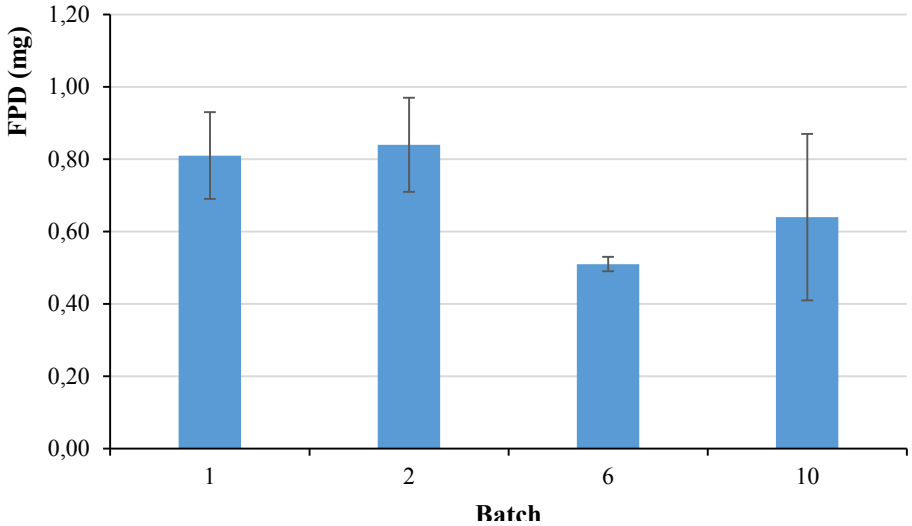

Figure 2.6. FPD obtained from the 4 best performing formulation in terms of emitted amount and fine particle fraction.

FPD values indicated that batch #1 and #2 had an aerodynamic performance significantly superior with respect to #6 ($p<0.05$) whereas compared to #10 there were not significant differences. MMAD indicates the aerodynamic diameter which divides the particle population in two equal parts; for batch #1 and #2 MMAD were below 1µm which would ideally provide higher lung deposition with better penetration into the small airways (Corradi et al., 2014).

Finally, these 3 formulations (#1, #2 and #10) had a narrow aerodynamic particle size distribution since their GSD was below 2.5.

These results suggest that powders containing only HA did not have good aerodynamic properties as they were composed by strong agglomerates. Solely, when HA was formulated with leucine, powders showed improvement in aerodynamic behaviour reaching a FPF% higher than 80%. Among these batches, it has to be underlined that the #10 does not contain HA, so it has little importance for our purpose.

Figure 2.7 reports a comparison among the deposition profiles inside the ACI of the formulations of batch # 1 and 2 that were considered the best respirable formulations produced.

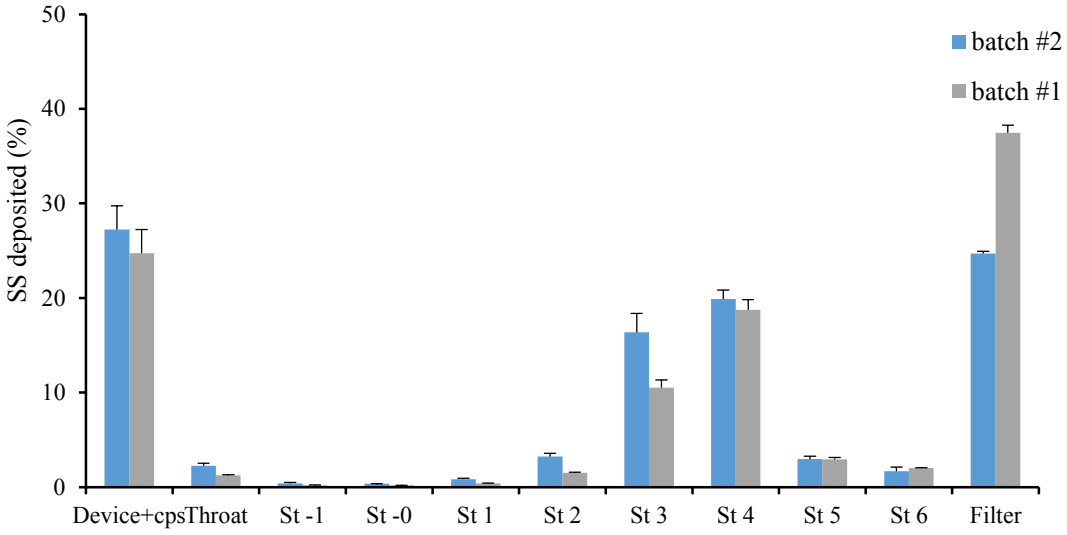

Figure 2.7. Deposition profile of formulations # 1 and 2 using an ACI using RS01 at 60 l/min; n=3.

The amount of drug remained inside the device/capsule was around 25%. In particular, the capsule was empty after the emission of the product, however, some particles were recovered in the RS01 mouthpiece. This aspect could be due to the electrostatic charge of particles and will be further investigated in future studies.

The profiles look very similar in the first stages whereas they are quite different in stage 3 and filter. The peculiarity of batch #1 is the higher fraction of SS deposited inside the filter which reflects the presence of particles with dimension below 0.34 µm.

2.3 Conclusions

Spray-drying technique was a feasible method for the production of microparticles suitable for pulmonary administration. Sodium hyaluronate has been investigated as a biocompatible polymer and has been successfully formulated with a hydrophilic model drug (salbutamol sulphate) in respirable dry powders.

Aminoacids added in the formulation are considered to be safe as pulmonary excipients and are already used to improve aerosolization behaviour of several drugs (Pilcer and Amighi, 2010; Seville et al., 2007; Wang et al., 2009).

In particular, the effect of leucine was primarily studied varying its percentage in dry powder formulations. Leucine aids in improving the powder dispersibility; this effect is attributed to its very quick precipitation/crystallisation at the surface of the evaporating droplets, which results in the creation of a shell enriched in this aminoacid (Feng et al., 2011). In this way, its surfactant properties and relative hydrophobicity affect particle shape and surface property leading to an increase in powder flowability (Boraey et al., 2013). Furthermore, the addition of leucine result in less cohesive particles and a decrease in particle size due to its surfactant behaviour through the reduction in droplet size during atomization (Pilcer and Amighi, 2010). Rabbani and Seville (2005) defined that 10–20% w/w of leucine in spray-dried solutions of ethanol or water give optimal aerosolisation characteristics of powders containing peptides or sodium cromoglycate. Indeed, leucine is capable of coating even large molecules such as polymers when is spray-dried from a water-ethanol co-solvent system adjusted in order to decrease the molecule solubility (Vehring, 2008).

The improvement of particles characteristics due the addition leucine is demonstrated in this work as well. In the spray-drying process HA has to be combined with at least a 20 % of this excipient. Indeed, the best aerodynamic performance in the whole set is highlighted when HA is associated with leucine along with salbutamol sulphate and the percentage of excipients (HA+ leucine) is at least 40%.

Finally, it is possible to conclude that the substitution of leucine with other aminoacids leads to worsening of powder respirability.

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Chapter 3 Novel HA spray-dried powders

As previously stated, hyaluronan has been proposed for many applications in the pharmaceutical field. Among them, it was also proposed as carrier for drug delivery to the lung and more specifically to alveolar macrophages for treating tuberculosis (Hwang et al., 2008). These authors demonstrated the higher *in vitro* uptake of HA-ofloxacin microparticles in comparison to solution or pure drug microparticles. Nevertheless, the inherent poor flowability and tendency to stick of such particles obliged the authors to use an adhesive mixture with lactose in order to be able to dose and aerosolized them.

Given this potential, HA appears to deserve deeper attention with respect to its possible use in particle formation for lung delivery both as such or in combination with drugs in carrier-free formulations.

This second part of the work refers to the production of flowable and highly respirable dry powders of HA and a more extensive analysis on the HA physico-chemical properties. A particle engineering approach based on spray drying technique was employed to produce HA particles and optimize the powder characteristics. A colloidal suspension was obtained from the precipitation of HA after addition of suitable amount of ethanol to an aqueous solution of the polymer; in this way, particles with a size < 1 μm were obtained with potential enhanced deposition to the deep lung (Rabinow, 2004).

However, powders containing only this polysaccharide exhibited a poor aerosolisation behaviour. Thus, different adjuvants were included in the formulation in concentration ≤ 10% by weight, in order to exploit their physico-chemical properties for improving dry powders performance (Bosquillon et al., 2004; Zeng et al., 2001). Firstly, attention was focused on adjuvants already approved for lung administration such as mannitol and

lysine. Subsequently, the class of surfactants (cationic and non-ionic) was investigated aiming at imparting better flowability.

In this section, the formulation development was accomplished keeping a high HA content as a prerogative (at least 90%). Beside its well-known advantages over liquid formulations, HA as dry powder was preferred, because it has been reported that once delivered to the lung in solid state polymer breakdown might be limited (Cantor, 2007). Powders were characterised in terms of particle size distribution, *in vitro* respirability, surface charge, morphology, density and solid-state properties.

3.1 Materials and Methods

3.1.1 Materials

Sodium hyaluronate (HA) (PrymalHyal 50, average MW=29504 Da) was purchased by Soliance (France). Stearylamine (ST), L-lysine were supplied by Sigma–Aldrich (Sigma Chemical Co., USA). Stearyl alcohol (SA) and cetostearyl alcohol (CSA) were provided by ACEF (Italy). A single dose dry powder inhaler, RS01 (Plastiape Spa, Italy), was used to aerosolize sodium hyaluronate powders for the aerodynamic assessment tests. Powder formulations were loaded in size 3 HPMC capsules (Vcaps® DPI, Capsugel, France). All chemicals used were of analytical grade and water was purified by Elix® Essential (Merck Millipore, USA).

3.1.2 Methods

3.1.2.1 HPLC analysis of sodium hyaluronate

The content of sodium hyaluronate in every sample was determined by size exclusion – high performance liquid chromatography (SEC-HPLC) using a BioSep-SEC-s4000, 5 µm 7.8x100 mm (Phenomenex, USA) column. Standard and samples were prepared in purified water. Mobile phase was prepared by dissolving 6.80 g of potassium dihydrogen phosphate (KH_2PO_4) in 1 litre of purified and degassed water and the pH was adjusted to 7.0 by adding 5M potassium hydroxide (KOH). The sample injection volume was 100 µL, flow rate of the mobile phase was 1.0 mL/min and wavelength of detection was 200 nm. Linearity was tested before each analysis in the concentration range between 5 and 500 µg/mL ($R^2 = 0.999$).

3.1.2.2 Turbidimetry

A turbidimetric analysis was employed to study the antisolvent process toward HA in water/ethanol mixtures finalized at obtaining a colloidal suspension to be spray dried. The dispersions were prepared by antisolvent precipitation technique. Turbidimetric experiment were fitted out with a UV-Vis spectrophotometer (Jasco V-530, Japan) in trasmittance mode and using two flow-through cuvettes. Two different wavelengths were selected for these tests (400 nm and 500 nm). Sodium hyaluronate was tested at seven different final concentrations, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75 and 2.0% w/v, in a starting volume of 25 mL (final volume = 50 mL). The starting solutions were set up dissolving hyaluronate raw material in 12.5 mL of water and subsequently adding 12.5 mL of ethanol. After each addition of antisolvent (ethanol) the system was left to equilibrate for 5 minutes under stirring.

Trasmittance value were recorded after any addition of antisolvent to the test solution until a trasmittance below 10% was obtained in comparison to a blank solution (water-ethanol without sodium hyaluronate). The inflection point was calculated for every graph by Microsoft® Excel 2013 software.

3.1.2.3 Spray-drying

Engineered sodium hyaluronate powders were manufactured by spray-drying employing a mini Spray-Dryer Büchi mod. B-290 (Büchi Laboratoriums-Technik, Swiss).

HA was dissolved in purified water at room temperature under stirring at 50 rpm; this solution was then added (3 mL/min) to the required amount of ethanol.

When an adjuvant was incorporated in the formulation, it was added to water or ethanol phase based on its inherent solubility, before making the dispersions. The compositions of HA formulations are reported in Table 3.I.

Table 3.I. Qualitative and quantitative compositions of different HA formulations.

	Adjuvant	**% Ratio HA:Adjuvant**	**Ethanol (% v/v)**	**Water (% v/v)**	**Solid content (% w/v)**
HA_0.83	-	100:0	72.3	27.7	0.83
HA_1.53	-	100:0	69.3	30.7	1.53
HA_2.30	-	100:0	67.2	32.8	2.30
HA_Mann_10	Mannitol	90:10	72.3	27.7	0.92
HA_Lys_10	Lysine	90:10	72.3	27.7	0.92
HA_SteAm_10	Stearylamine	90:10	72.3	27.7	0.92
HA_SteAm_5	Stearylamine	95:5	72.3	27.7	0.88
HA_SteAm_1	Stearylamine	99:1	72.3	27.7	0.84
HA_CetSteAlc_10	Cetostearyl alcohol	90:10	72.3	27.7	0.92
HA_CetSteAlc_5	Cetostearyl alcohol	95:5	72.3	27.7	0.88
HA_CetSteAlc_1	Cetostearyl alcohol	99:1	72.3	27.7	0.84
HA_SteAlc_10	Stearyl alcohol	90:10	72.3	27.7	0.92
HA_SteAlc_5	Stearyl alcohol	95:5	72.3	27.7	0.88
HA_SteAlc_1	Stearyl alcohol	99:1	72.3	27.7	0.84

The prepared dispersions were sprayed using the following process parameters: inlet temperature 90 °C, drying air flow rate 750 L/h, solution feed rate of 3.0 mL/min and nozzle diameter of 0.7 mm Under these conditions an outlet temperature of 45-52 °C was

measured. A dehumidifier B-296 was used to control the air humidity of the system. Spray-dried powders were kept in the collector for at least 24 hours in order to reduce electrostatic charges.

3.1.2.4 Zeta potential measurement

The zeta potential of HA and HA formulations was measured in the same water:ethanol mixtures used for making the suspensions submitted to the spray-drying process. Samples were prepared by suspending the powders at 8.3 mg/mL. Analyses were carried out with a ZetaPlus (Brookhaven Instruments Corporation, USA) particle size and Zeta potential analyser. The collected data were processed by PALS Zeta Potential Analyzer software (Brookhaven Instruments Corporation). The electrophoretic mobility was converted in zeta potential by means of Smoluchowski's equation (Hunter et al., 1981).

3.1.2.5 Particle size distribution by laser diffraction

The particle size distribution of the formulation was determined using a laser light diffractometer Spraytec (Malvern Instruments Ltd, UK) equipped with a 300 mm focal lens, which measures particle size in the range from 0.1 to 900 μm. A software calculated the particle size distribution using Fraunhofer model. Samples were prepared by suspending 10 mg of spray-dried powders in 10 mL of Span 85 (0.1 % w/v) in cyclohexane solution; the suspension was left in an ultrasound bath for 10 minutes before measurement. Particle size distribution was measured in triplicate with an obscuration threshold of 8 %. Data were expressed as in the paragraph 2.1.2.4.

3.1.2.6 Scanning Electron Microscopy

Scanning electron microscopy (SEM, Zeiss SUPRA 40, Oberkochen, Germany) was employed to investigate particle morphology and surface characteristic of the powders

produced by spray-drying. The microscope was operated under high vacuum conditions with an accelerating 1.5 kV voltage, at different magnifications. Powders were deposited on adhesive black carbon tabs pre-mounted on aluminium stubs and imaged without undergoing any metallization process.

3.1.2.7 Fourier Transform-Infrared Spectroscopy (FT-IR)

Infrared spectra were recorded using a FT-IR spectrometer (Jasco 460 Plus, Japan) aiming at investigating the polymer-surfactant interaction. Approximately 2 mg of powder was mixed with 50 mg of KBr and compacted using a hydraulic press operated at 10 atm. Samples were analysed in transmission mode within a range of 4000-400 cm^{-1} with a resolution of 2 cm^{-1}.

3.1.2.8 Solid-state characterization (XRPD, DSC and TGA)

XRPD measurement was carried out at room temperature using a Miniflex X-Ray diffractometer (Rigaku, Japan) to investigate powder cristallinity. Samples were loaded onto a horizontal aluminium sample holder and measured with a slit-detector Cu Kα radiation source (λ = 1.5406 Å, 40 kV voltage, and 44 mA current). The scanning rate of 1.50 °/min over a 2θ range of 2.0–35.0 ° was employed.

The DSC profiles were determined using DSC mod. 821 (Mettler Toledo, USA). For the measurement, 4-5 mg of powder were weighted into a 40 μL aluminium pans sealed and double pierced. The samples were scanned at a heating rate of 10 °C/min under nitrogen flow of 100 mL/min in the temperature range 25-300 °C.

A sample of about 10 mg was placed in a 100 μL alumina pan in the heating zone of the TGA apparatus (TGA/DSC1, Mettler Toledo, USA). The samples were heated at 10 °C

per minute from 25 °C to 300 °C. The mass loss was measured directly by the TGA. Instrument was purged with nitrogen at a constant flow rate (80 mL/min) and data analysis was accomplished using STARe evaluation Software.

3.1.2.9 *In vitro aerodynamic evaluation: Fast Screening Impactor and Andersen Cascade Impactor*

A Fast Screening Impactor (FSI, Copley UK) was selected as an abbreviated impactor that segregates the spray-dried powders into Coarse Particle Mass and Fine Particle Mass. This impactor has some advantages with respect to other full-resolution cascade impactors such as short time for analysis, reduction of analytical errors and allows a rapid screening of new inhaled formulations.

FSI uses the same Throat (T) as the more commonly used Next Generation Impactor (US Pharmacopeial Convention, 2015). It employs a two-stage separation stages: a Coarse Fraction Collector (CFC) that captures particles with an aerodynamic diameter higher than 5 μm and a Fine Fraction Collector (FFC) that collects particles with an aerodynamic diameter lower than 5 μm. The Respirable Fraction % (RF) was calculated by the ratio between the amount of sodium hyaluronate in the FFC and the total amount of hyaluronate recovered. The delivered dose (DD) was quantified by HPLC. The entire system was connected to a vacuum pump (Erweka GmbH, Germany) which created an air flow (60 L/min) drawing the particles over the system.

Approximately 5.0 ± 0.1 mg of powder was manually weighed into a size 3, hard HPMC capsule. Subsequently, one capsule was loaded into the inhaler and aerosolized. Only one capsule was discharged inside the impactor for each aerodynamic test. A single dose inhaler (RS01) was chosen to disperse and aerosolize the spray-dried powders.

According to current USP guidelines, the flow rate used during each tests was adjusted with a Critical Flow Controller TPK (Copley Scientific, Nottingham, UK) in order to produce a pressure drop of 4 kPa over the inhaler (Clark, A. R., Hollingworth, 1993). In particular, 60 L/min were controlled before each experiment by a Flow Meter DFM 2000 (Copley Scientific, Nottingham, UK) in order to produce a pressure drop of 4 kPa.

Furthermore, the test duration in seconds is defined by the equation T=240/Q where Q is the test flow rate, so that a volume of 4 L of air is withdrawn from the inhaler during the experiment. Therefore, RS01 was activated for 4 seconds. Samples were recovered with purified water.

The formulations showed promising aerosolization properties were also tested by Andersen Cascade Impactor (ACI, Copley UK) adjusted for use at a flow rate of 60 L/min in order to characterize the aerodynamic particle size distribution in deeper detail. The cut-off particle aerodynamic diameters at this flow rate for each stage of the impactor were: stage -1 (8.60 μm), stage -0 (6.50 μm), stage 1 (4.40 μm), stage 2 (3.20 μm), stage 3 (2.00 μm), stage 4 (1.10 μm), stage 5 (0.54 μm), stage 6 (0.25 μm); a filter (< 0.25 μm) has been interposed between the stages and the vacuum pump. In order to prevent particles bouncing during the analysis, metal impaction plates were dipped in a cyclohexane solution (containing 1% w/v Span 85). The solvent was evaporated and the ACI assembled; then, 5 mg of powders were weighted in a HPMC capsule and aerosolised with RS01 as described above. The same procedure (see FSI paragraph) was adopted to set-up the whole system before beginning a new test. The measurement of drug deposited throughout the impactor allows the calculation of different deposition parameters. The delivered dose (DD) was the amount of hyaluronate ex-device measured from induction port (IP) to filter (F). The fine particle dose (FPD) was the mass of hyaluronate with

aerodynamic diameter lower than 5 mm; the fine particle fraction (FPF) was the ratio between FPD and DD. The mass median aerodynamic diameter (MMAD) was determined by plotting the cumulative percentage of mass less than stated aerodynamic diameter (probability scale) versus aerodynamic diameter (log scale).

In vitro deposition experiments were made in triplicate.

3.1.2.10 Statistical analysis

The Statistical analysis was performed with Microsoft Office Excel 2007 (Microsoft Corp., USA) employing a two-tailed unpaired t-test and p-values < 0.05 were considered to be significant.

3.2 Results and Discussion

3.2.1 Turbidimetric investigation

Production of HA dried powders form HA water solutions was not feasible since the powder dried in the cyclone of the apparatus and the yield of the production was only around 10%. Hence, the approach adopted to produce HA powders was to dry colloidal solutions of the polymer.

HA was dissolved in purified water and then, ethanol (antisolvent agent) was added dropwise to produce a stable colloidal suspension. The effect of HA concentration and water-ethanol ratio on the HA nanoparticle formation were investigated by turbidimetry.

Figure 3.1 reports the trasmittance of HA solutions of different concentrations as a function of the volume of ethanol added. HA water solutions (in the concentration range 0.5-2.6% w/v) were stable (clear) over a certain volume of added ethanol which depended upon the HA content in the starting solution. For each solution a critical ethanol volume which determined a sudden trasmittance drop (obscuration) was observed. This volume was lower for the higher HA concentration and was calculated from the inflection point of the each trasmittance vs ethanol concentration curve.

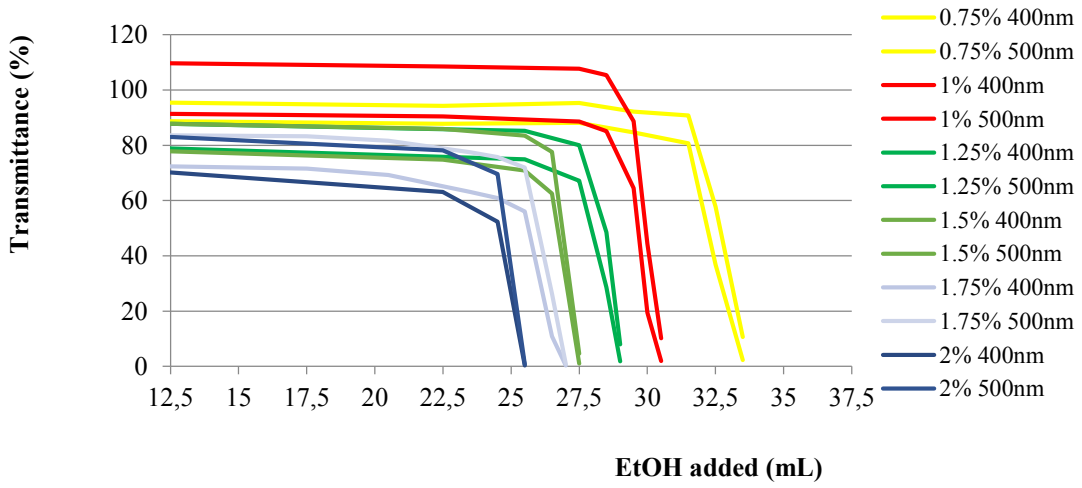


Figure 3.1. Turbidimetric curves on 5 different concentration of HA aqueous solutions at 2 wavelenghts.

These inflection point were used to draw the phase equilibrium curve (Figure 3.2).

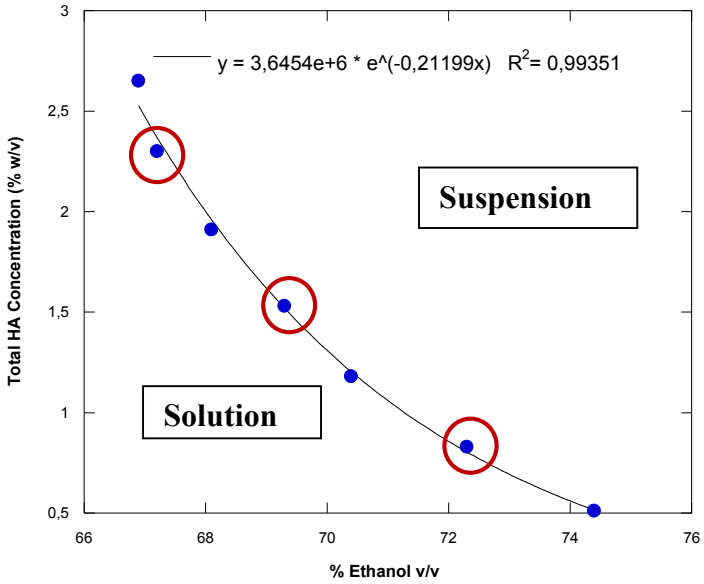


Figure 3.2. Phase equilibrium curve oh HA in ethanol-water solutions.

The graphic of Figure 3.2 shows the existence of two phases (solution and suspension). The curve represents equilibrium conditions of existence of both phases. When hyaluronate concentration was increased, the amount of ethanol required to obtain the turbid white HA dispersion, was lower.

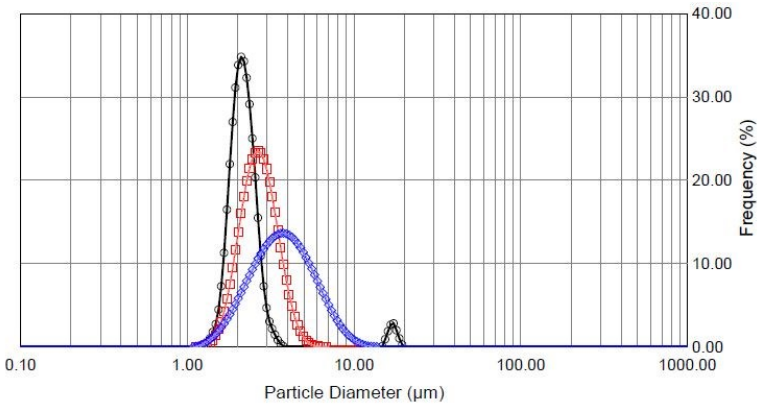
The mathematical function that describes antisolvent behaviour is a hyperbole. In order to prepare a stable colloidal suspension, conditions should be kept closed to the curve: on the left-end side of the graph a limpid solution is obtained, whereas the right-end side is relevant to a particles progressively increasing their size.

The colloidal ternary mixtures lying on the hyperbolic curve (0.83 HA total concentration) was submitted to particle size analysis with ZetaPlus analyser and proved to be around 650 nm in size.

Three colloidal water-ethanol solutions (red encircled), containing HA at the concentration of 0.83% w/v, 1.53% w/v and 2.30% w/v were submitted to spray-drying process (powders coded: HA_0.83, HA_1.53 and HA_2.30).

The three batches of HA SD powder were analysed by laser diffraction in order to determine the particle size distribution as this parameter is directly related to the aerodynamic diameter.

Figure 3.3 depicts the obtained particle size distributions.



	Date-Time	Sample	File	Dx(10)	Dx(50)	Dx(90)
[V]	23 May 2014-1...	HA 0,8...	HA 0,83% 1 1	1.75	2.14	2.71
[V]	10 Mar 2014-1...	HA 1,5...	HA 1,53% 1 1	1.94	2.66	3.70
[V]	31 Mar 2014-1...	HA 2,3...	HA 2,30% 1 1	2.11	3.69	6.40

[V]=Volume [N]=Number

Figure 3.3. Particle size distribution of the 3 SD powders having different HA concentrations in the starting solution.

It can be observed that all the powder had very good particle size distribution. In particular, the particle size as well as the amplitude of the distribution increased with the HA concentration in the starting solution. HA_0.83 showed excellent particle size distibution with D_{10} of 1.75 µm D_{90} around 2.7 µm. This datum permitted to predict good aerodynamic characteristics, thus these particles were selected for further development.

3.2.2 Spray-drying process, drug content and *in vitro* deposition study

In Table 3.II are reported the yield values of the spray-drying process, the HA content in the powders and the aerodynamic parameters obtained aerosolising inside a FSI (emitted dose and respirable fraction).

Table 3.II. Yields of the process, Drug content, Emitted Dose and Respirable Fraction of the spray-dried powders (loaded dose: 5 mg of powder, device RS01 @ 60 L/min; n=3, mean±sd).

	Production Yield (%)	**Drug Content (%)**	**Emitted Dose %**	**Respirable Fraction (%)**
HA_0.83	67.9	94.1 ± 0.5	37.0 ± 2.4	25.4 ± 3.7
HA_1.53	78.0	93.8 ± 1.5	47.3 ± 7.5	24.1 ± 3.8
HA_2.30	83.0	95.2 ± 0.4	40.5 ± 2.9	18.0 ± 4.4
HA_Mann_10	65.0	84.6 ± 0.8	56.2 ± 7.6	27.6 ± 0.6
HA_Lys_10	76.7	84.8 ± 0.8	54.5 ± 5.1	30.9 ± 5.2
HA_SteAm_10	49.0	82.2 ± 2.0	85.2 ± 0.1	45.0 ± 4.1
HA_SteAm_5	55.5	89.4 ± 0.4	76.3 ± 2.9	51.2 ± 2.0
HA_SteAm_1	42.9	94.2 ± 0.8	48.2 ± 3.3	27.0 ± 1.7
HA_CetSteAlc_10	51.5	85.0 ± 0.7	83.3 ± 2.0	34.8 ± 3.6
HA_CetSteAlc_5	59.4	91.4 ± 1.8	71.1 ± 0.5	36.2 ± 1.7
HA_CetSteAlc_1	66.3	95.1 ± 1.2	64.4 ± 1.2	27.2 ± 1.1
HA_SteAlc_10	52.4	85.6 ± 1.0	83.2 ± 0.5	31.6 ± 0.7
HA_SteAlc_5	46.9	90.4 ± 1.8	71.4 ± 0.5	38.4 ± 0.1
HA_SteAlc_1	54.9	95.2 ± 1.3	63.5 ± 0.6	30.0 ± 7.0

Regarding SD powders with HA alone, the yield of the process increased (from 67.9 to 83.0%) by the increasing of hyaluronate concentration. The yield of the process was within acceptable range as it usually varies from 40 to 70%. The drug content was in

agreement with the theoretical content for each SD formulation produced, also taking into account the residual water content (see the following paragraph 3.2.8).

The powders were aerosolized using RS01 device inside the fast screening impactor allowing to separate the particles population in two different fraction: those had an aerodynamic diameter > 5 µm and those < 5 µm.

HA powders without any excipients were very cohesive, presented many agglomerates and had a very poor flowability. They did not show appropriate flow properties as almost a 60% of the loaded powder was retained inside the capsule, the capsule chamber and mouthpiece (Figure 3.4).

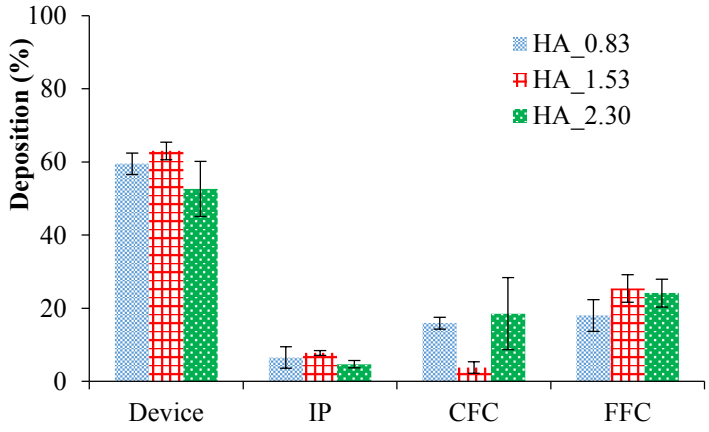


Figure 3.4. Fast Screening deposition obtained aerosolising HA SD powders alone obtained starting from HA colloidal solution with different HA concentration.

HA SD powders showed similar emission from the capsule and microparticles adhered to the internal walls of both capsule and device; furthermore, as it will be mentioned later, SEM images demonstrated the presence of aggregates within the powders. This behaviour is probably due to the high cohesive forces that developed among microparticles as well as adhesive forces between microparticles and components of the device leading to a very low respirable fraction that was between 18 and 25%.

Hence, in order to enhance the flowability property, decrease the particle cohesivity of HA spray-dried powders and increase their respirability, the addition to HA_0.83 of selected excipients at a level of 10% w/w was investigated; specifically mannitol, L-lysine and stearylamine were considered.

Table 3.I and Figure 3.5 report the aerosolization parameters and the FSI deposition data respectively, obtained with these powders in comparison with the relevant pure HA powder. A slight increase in the emitted dose was achieved with mannitol and lysine whereas the RF % resulted statistically unchanged. Much better results were obtained with stearylamine. In all the cases, the emitted dose was higher than 50 %, reaching 85 % when stearylamine was added. Among the selected excipients, stearylamine afforded the highest respirable fraction, improving the microparticles emission and de-aggregation during aerosolization.

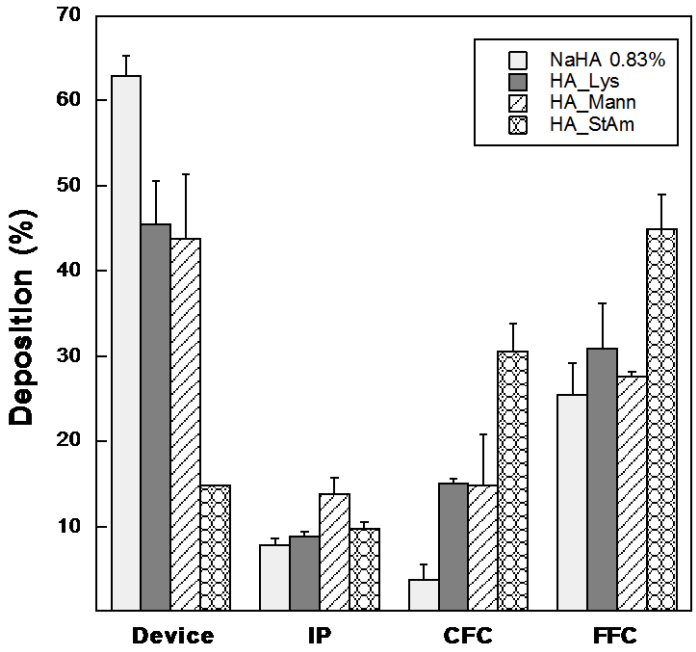


Figure 3.5. Fast Screening deposition obtained aerosolising HA SD alone and HA SD with adjuvants such as lysine, mannitol and stearylamine at 10% w/w.

It has been reported that the addition of a surfactant might enhance the aerodynamic performance of the HA SD powder through a modification of the particle surface characteristics. In particular, this modification should be addressed to the surface active molecule accumulation at the surface during particle formation process which affect particle-particle interaction (Rawat et al., 2008). Therefore, considering that only stearylamine proved to be suitable among the three tested adjuvants, we assumed that its surfactant nature could be considered the specific feature responsible for the observed aerodynamic improvement. Hence, others two surfactants, namely, cetostearyl alcohol and stearyl alcohol, were chosen based on their chemical similarity to stearylamine. Furthermore, stearylamine is a cationic surfactant whereas stearyl and cetostearyl alcohol are nonionic surfactant, thus this aspect was considered for its potential impact on the toxicological and safety issues. Different HA:surfactant ratios were investigated, 90:10, 95:5 and 99:1.

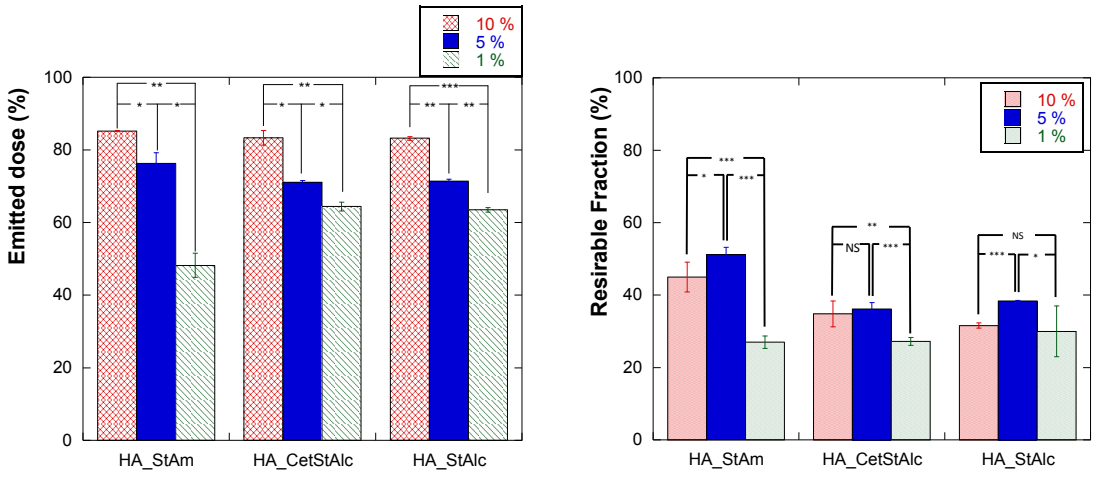


Figure 3.6. Emitted dose (left) and Respirable Fraction % (right) obtained from HA formulations containing different type and concentration of surfactant. *** p< 0.001, ** p<0.01 and * p<0.05.

Figure 3.6 illustrates that even at 1% of surfactant, formulations had a better emission respect to HA pure SD powder ($p<0.05$). An increase in the adjuvant concentration led to an improvement of the emitted dose. Conversely, the best results in term of respirable fraction were achieved using 5% of adjuvant for all the surfactants tested. This apparent paradox could be explain, in agreement to what reported by (Parlati et al., 2009), by hypothesizing that when the amount of surfactant was increased above a certain value it exceeded its Critical Micellar Concentration in the system, thus affording the formation of surfactant aggregates instead of coated HA particles. Formulations with 1% of surfactant showed RF% comparable to those obtained by pure HA SD powders.

3.2.3 Andersen Cascade Impactor

The aerodynamic behaviour of the dry powder formulations which gave the best aerodynamic results, were deeply assessed by performing *in vitro* deposition tests with Andersen Cascade Impactor (ACI). In particular, spray-dried powders with the ratio 95:5 between HA and surfactant, were loaded in capsule and aerosolized using a RS01 at 60 L/min inside the ACI.

Figure 3.7 shows the deposition percentage in the ACI for the three formulations.

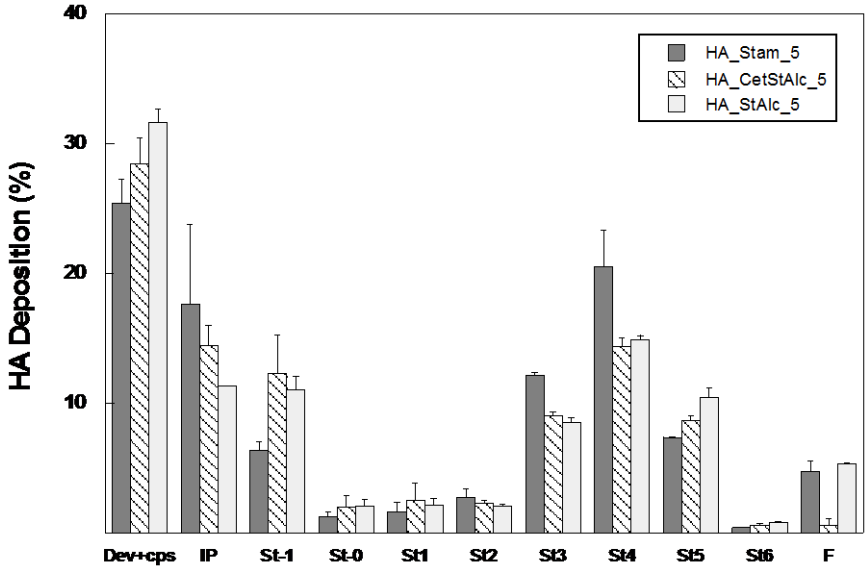


Figure 3.7. Aerodynamic particle size distribution of the spray-dried powders HA:surfactant (95:5) among the stages of the Andersen Cascade Impactor.

It illustrates that, even though HA_SteAm_5 particles impacted in a high percentage in the Induction Port (IP), the *in vitro* deposition of the this powder inside the respirable size stages (from stage 2 to the filter) was higher compared to the powders containing the other two surfactants.

For HA_SteAm_5 and HA_SteAlc_5, a 5% of the HA was recovered on the final filter indicating a significant fraction of ultrafine particles (< 0.25μm).

Table 3.III reports the data obtained with ACI and in Figure 3.7 the drug distribution on the stages is illustrated.

Table 3.III. Emitted Dose %, MMAD, Fine Particle Dose and Fine Particle Fraction, Respirable Fraction of HA: surfactant (ratio 95:5), obtained with the ACI. Loaded amount= 5mg of powder.

	Emitted Dose * **(mg and %)**	**MMAD (μm)**	**FPD** **(mg)**	**FPF** **(%)**	**RF** **(%)**
HA_SteAm_5	3.41± 0.10 76.4%	1.96 ± 0.12	2.05 ± 0.17	66.3 ± 5.6	49.4
HA_CetSteAlc_5	3.38 ± 0.08 73.9%	2.28 ± 0.36	1.76 ± 0.03	59.0 ± 2.5	38.6
HA_SteAlc_5	3.22 ± 0.03 71.2%	2.03 ± 0.13	1.80 ± 0.03	64.0 ± 0.6	43.8

*: determined by weighting the device before and after aerosolization

- FPF = FPD/amount collected in the impactor
- RF= FPD/loaded dose

The mass balance was higher than 80% in all the experiments performed so that a suitable calculation of the aerodynamic parameters might be carried out.

The HA powder formulation with stearylamine (HA_SteAm_5) afforded the best results compared to the powders constituted with the other adjuvants, thus confirming the FSI data. In detail, the Emitted Dose was the highest (reaching 76.4%) due to the better flowability of this powder leading to an easy emission from the device. This behaviour is

probably due to the wrinkled surface of the microparticles as well as the modification of the surface chemistry decreasing the aggregation tendency among them.

The MMAD was the lowest observed in this study confirming the enhanced respirability with respect to powders containing cetostearyl and stearyl alcohol although, despite the lower emitted dose, these powders were properly deaggregated inside the impactor (Figure 3.7).

The results obtained with both the FSI and ACI demonstrated that the presence of a surfactant improve the aerodynamic performance of HA powder; in particular, it is possible to conclude that HA: stearylamine (95:5) had the best aerodynamic performance among all the dry powder formulations tested.

3.2.4 Zeta potential analysis

To understand the surface properties of the prepared HA particles, the surface charge was investigated. As a matter of facts it was hypothesized that the cohesiveness could be due to the strong negative surface charge ascribing to the HA carboxylate group exposure. Thus, the neutral molecule mannitol was selected to modify the surface characteristic by chemical interaction, also considering that it has already been approved for inhalation. L-lysine and stearylamine, positively charge excipients were chosen in an attempt to neutralize the negative charge of HA.

Colloidal solutions of HA were investigated by turbidimetry aiming at making particles with dimensions below the micrometre for improving deposition into the deep lung (Rabinow, 2004). As expected, zeta potential data highlighted that sodium hyaluronate raw material exhibited a negative surface charge (-47.27 mV) to be addressed at the carboxylate group exposition (Table 3.IV).

It was reported that values of at least 50 mV can cause stable dispersions whereas at lower zeta potential values, the particles tend to agglomerate or flocculate (Merkus and Meesters, 2014). Nevertheless, surfactant are well known stabilizing agents for nanosuspensions (Aulton and Taylor, 2013).

Unexpectedly, the stearylamine did not modify the zeta potential value. On the contrary, the addition of mannitol and L-lysine partially neutralized the charge that decreased to -37mV and -27 mV respectively. It is worthy to note that spray-drying technique did not modify the surface charge of the particles, suggesting that the same surface chemical composition was kept throughout the process.

Table 3.IV. Zeta potential of the HA colloidal suspension HA_0.83 before and after spray drying (SD) along with the SD particles produced from HA_0.83 colloidal suspension in the presence of different adjuvants at 10% w/w.

Sample	Zeta potential (mV)
HA_0.83 before SD	-53.43 ± 4.78
HA_0.83	-47.27 ± 2.83
HA_Mann_10	-37.58 ± 3.24
HA_Lys_10	-27.62 ± 5.73
HA_SteAm_10	-42.22 ± 6.23
HA_CetSteAlc_10	-33.60 ± 2.37
HA_SteAlc_10	-38.10 ± 6.74

This data evidenced the interference of cetostearyl and stearyl alcohol with the particle surface characteristics.

From the combination of above reported aerodynamic assessment data and zeta potential data it is possible to conclude that there was not a correlation between the reduction of

the HA charge and the powder cohesiveness and aerodynamic behaviour as the better aerodynamic behaviour was obtained with HA_SteAm_10 that presented a charge not significantly different from that of HA_0.83.

3.2.5 Particle size distribution by laser diffraction

Particle size distribution was measured by laser light diffraction as volume diameter.

The particle size distribution of the HA spray-dried powders containing different adjuvants in different percentages is depicted in Figure 3.8.

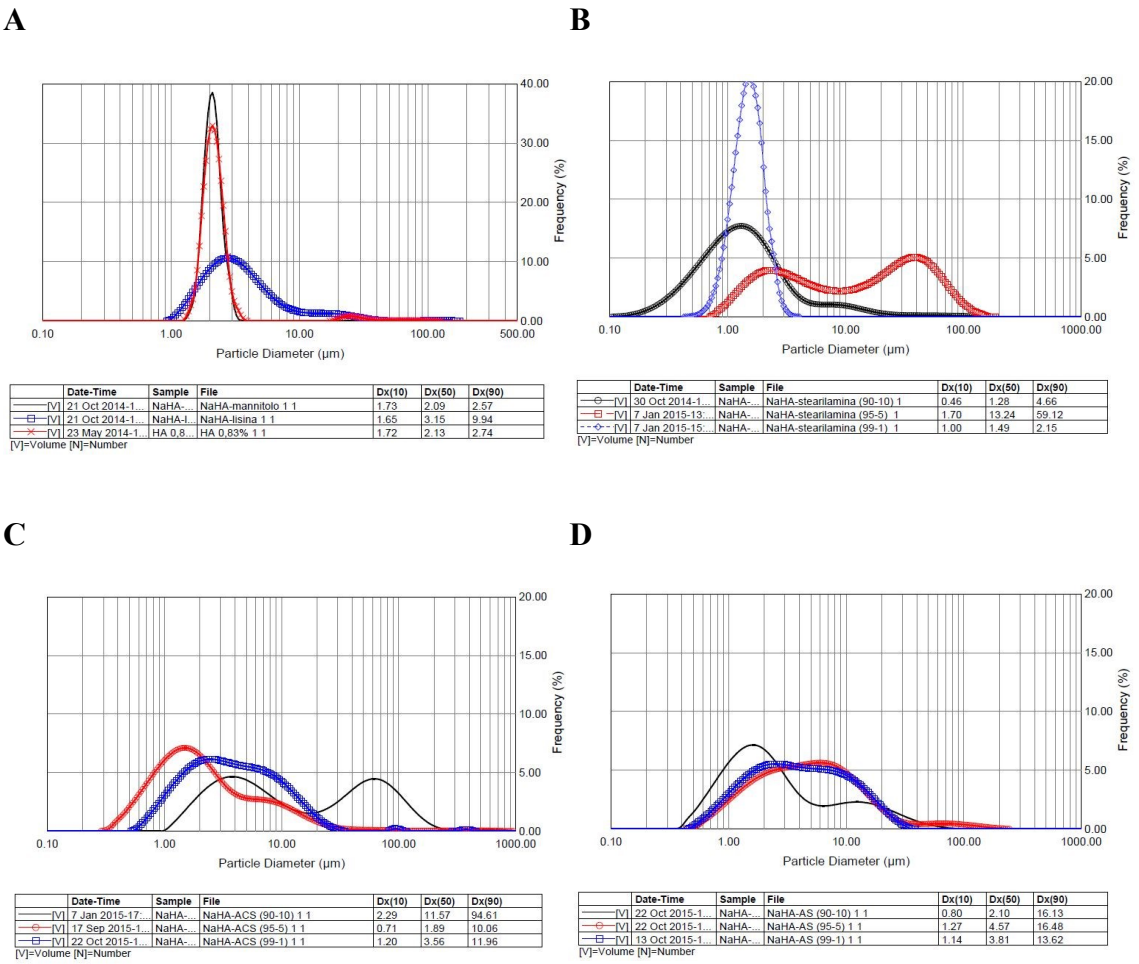


Figure 3.8. Particle size distribution of the SD powders obtained from HA_0.83 dispersions in the presence of different adjuvants in different concentrations. Panel A: HA_0.83 , HA_Mann_10 and HA_Lys_10; Panel B: HA powders containing stearylamine 1-10 % w/w; Panel C: HA powders containing cetostearyl alcohol 1-10 % w/w; panel D: HA powders containing stearyl alcohol 1-10 % w/w.

Analysis of the particle size indicated that the SD powders of HA alone, HA_Mann_10 and HA_Lys_10 had similar unimodal and relatively narrow size distribution (span between 0.4 to 2.6 μm) within a size range suitable for respiratory delivery. Similar, or even narrow profile was obtained with 1% stearylamine whereas, multimodal distributions were observed with the other surfactants and when stearylamine was added to the formulation in higher concentration. As a result, they featured wider distributions. Surprisingly, the formulations showing the broader size distribution and higher median diameter were those best performing in terms of *in vitro* respirability.

This observation underscores once again (Aulton and Taylor, 2013) that, knowledge of primary particle geometric size may not be predictive of pulmonary deposition.

3.2.6 Scanning Electron Microscopy

As stated in paragraph 1.2, the respirability of a powder depends on its aerodynamic diameter which stems from the geometric diameter, the density and the shape of the particle.

To investigate this latter property, scanning electron microscopy (SEM) was used. This technique allows to characterise particle morphology and shape at high resolutions down to nanometres (Shur and Price, 2012).

Figure 3.9 shows that powders containing only HA were constituted by microparticles having a spherical shape with some concavities and a smooth surface. Some nano-sized particles were present. The increase of the HA concentration in the staring solution, led to particle size increased as already illustrated in Figure 3.3, while the morphology was kept similar. These samples showed aggregates which led to very cohesive powders. This

observation allows justifying the poor flowability during the aerodynamic tests shown with FSI (see paragraphs 3.2.2).

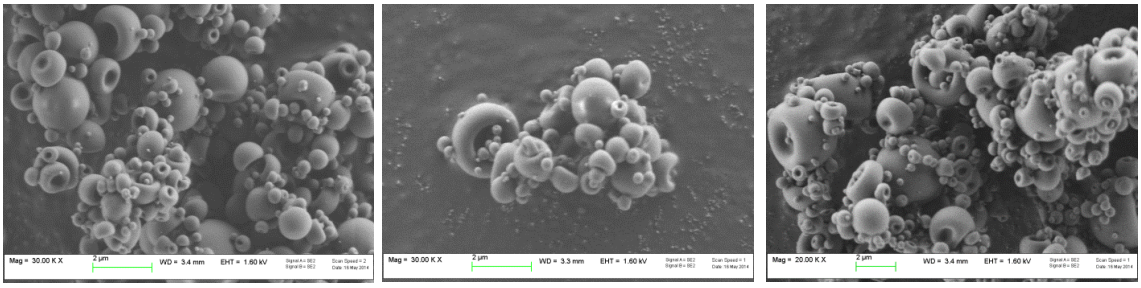


Figure 3.9. SEM images of HA_0.83 (left), HA_1.53 (middle) and HA_2.30 (right).

SEM images of spray-dried powders containing different type of adjuvants exhibited significant differences in morphology. Moreover, the adjuvant increasing concentration contributed to modify the particle shape too.

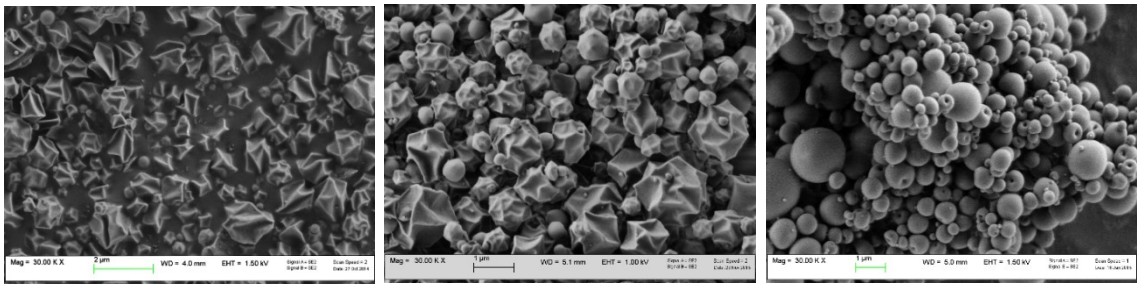


Figure 3.10. SEM images of HA_SteAm_10 (left), HA_SteAm_5 (middle) and HA_SteAm_1 (right).

Powders with stearylamine showed a different shape compared to spray-dried powder containing HA alone (Figure 3.10). Particles had an irregular wrinkled shape according to the amount of excipient added in the formulation. Moreover, the reduction of stearylamine content, led to particles of round shape, similar to that previously observed with HA alone.

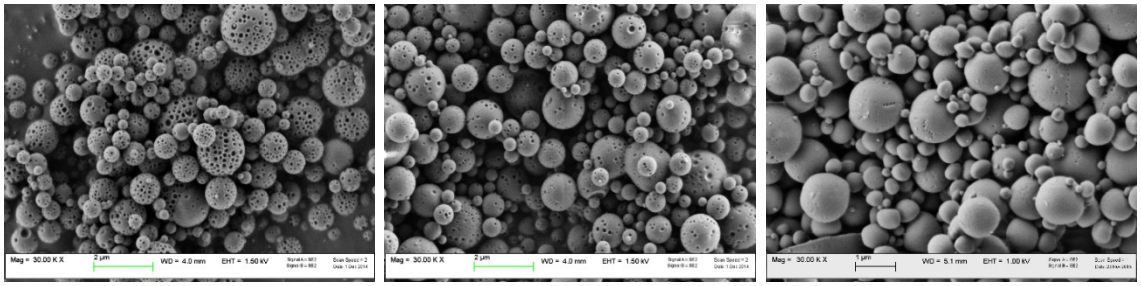


Figure 3.11. SEM images of HA_CetSteAlc_10 (left), HA_CetSteAlc_5 (middle) and HA_CetSteAlc_1 (right).

Powders containing cetostearyl alcohol in different percentages had a roundish shape (Figure 3.11). They become sponge-like with the increase of the adjuvant. Upon the particle surface, some holes were present and their number and size was directly proportional to the cetostearyl concentration. Very similar morphology was highlighted for stearyl alcohol (Figure 3.12).

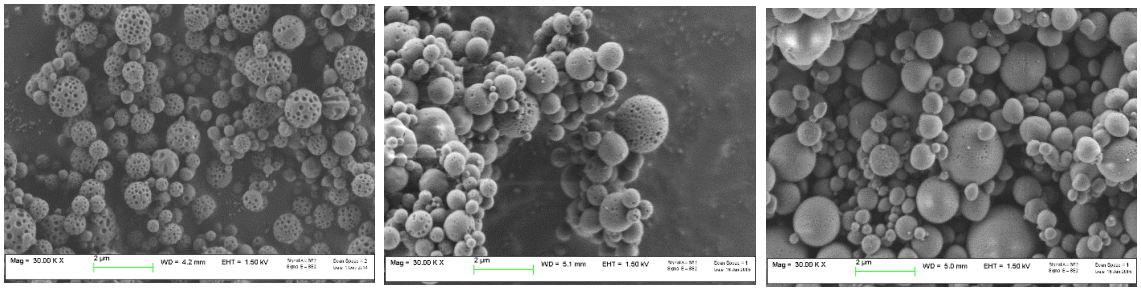


Figure 3.12. SEM images of HA_SteAlc_10 (left), HA_SteAlc_5 (middle) and HA_SteAlc_1 (right).

This behaviour was explained by considering that during particle formation in the spray-drying process as well as the antisolvent precipitation, the surfactants may have the capacity to segregate in different kinetics relative to HA based on the different diffusion coefficient (Columbano et al., 2003).

3.2.7 Spectroscopic Analysis, FT-IR

During the preparation process of the HA dispersions with adjuvants from one common ternary liquid system, molecular interactions between components may be established. These interactions may play a role in the particle formation process as well as on particle morphology development by either changing the orientation of molecules or the local chemical environment, and should manifest themselves as changes in the characteristic Infrared, IR, spectral bands of the interacting molecules in the obtained particles.

Figure 3.13 reports the IR spectrum of HA_0.83 along with that of the powders containing 10 stearylamine or cetostearyl alcohol. HA exhibited a wide absorption peak at 3412.32 cm−1 that can be assigned to hydrogen-bonded O-H and N-H stretching vibrations. Furthermore, the bands at 1617.60 and 1411.89 cm^{-1} can be attributed to the asymmetric (C=O) and symmetric (C-O) stretching modes of the planar carboxyl groups in the hyaluronate (negatively charged form) at 1149 cm^{-1}, 1080 cm^{-1}, 1045 cm^{-1} and 950 cm^{-1} are typical signals for C-O-C groups (Fan et al., 2006; Gilli et al., 1994; Wu, 2012)

The spectra obtained from the powders containing the two surfactants were practically superimposable to that of HA alone. The only difference was the presence of two peaks instead of one broad peak in the region around 2920 cm^{-1} due to the $–CH_3$ and $–CH_2–$ of the long alkyl chain of stearylamine and cetostearyl alcohol.

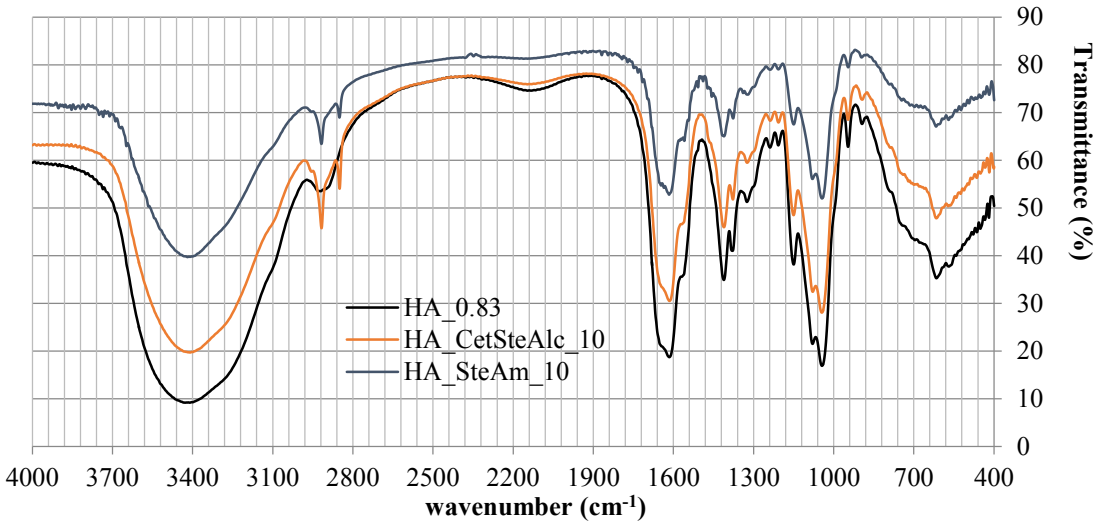


Figure 3.13. IR spectra of HA_0.83, HA_CetSteAlc_10 and HA_SteAm_10.

3.2.8 Solid-state characterization (XRPD, DSC and TGA)

HA raw material was completely amorphous as testified by the halo without peaks exhibited in the X-ray diffractogram. This was furtherly supported by DSC data, which showed only a broad endotherm of moisture evaporation between 40 and 140 °C and no crystallization peaks. Afterwards, no characteristic melting peak was found and the material began to decompose after 220 °C as already reported by (Villetti et al., 2002). In accordance with DSC trace, TGA pointed out a vaporization process (loss of water) from 40 to 180 °C, resulting in a water content of 12.7 % for HA raw material.

Spray-dried powders proved to be amorphous as well as expected for solid materials obtained by rapid solvent evaporation during spray-drying process as well as by antisolvent precipitation from a solution. The water content of all SD formulations ranged from 7.0 to 10.0% by weight.

3.3 Conclusions

A different approach has been taken in this second part of the project with respect to the first one.

SD powders with HA alone showed poor flowability when aerosolised through an inhaler (RS01). Different excipients were chosen for enhancing the aerodynamic performance, investigating their ability to modify particle surface charges. *In vitro* deposition tests (FSI and ACI) show that the presence of a surfactant such as stearylamine, cetostearyl alcohol and stearyl alcohol, is very useful in achieving the better results in terms of emitted dose and respirable fraction compared to powders without adjuvant.

The zeta potential measurements demonstrate that mannitol, lysine, cetostearyl and stearyl alcohol partially neutralised the charge whereas stearylamine did not modify the zeta potential. Thus, the reason why surfactants improve the powder emission and respirability in different magnitude should be searched elsewhere.

SEM images evidence modifications of particle morphology that limited the contact surface and aggregation among the microparticles. Moreover, it is possible to conclude that 5% of surfactants was considered as the optimal concentration to obtain the best HA respirability, especially in the case of stearylamine. Changes in particle shape influence the surface area of contact and along with modification of chemical surface composition can lead to decreasing of cohesion/adhesion forces thus, improving the flowability of the powders and the emission of the powder from the device.

Finally, no interaction between the polymer and the surfactant can be evidenced in the produced particles leading to the conclusion that the two materials segregate during particle formation.

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Chapter 4 In vitro biocompatibility: MTT assay and HCS

4.1 Introduction

Relative to other types of drug delivery formulations, pulmonary drug delivery systems have used only a small number of excipients because either few have been approved by the Food and Drug Administration or not accepted worldwide. However, the field is rapidly expanding and the need for alternatives is getting much more urgent. As stated before, the expansion of the number of therapeutic compounds and candidate molecules (especially protein/peptide) for administration to the lung will lead to an increase in the number of excipients that will be included in approved and marketed products (Katdare and Chaubal, 2006). This drug delivery challenge brings an imperative for safe and effective adjuvants to provide materials with which formulators can design new systems for inhalation (Salem et al., 2009).

This part of the project, focuses on surfactants given that they are receiving much consideration in inhalation field. Surfactants are used both in liquid formulations (suspension or emulsion) and solid formulations. In dry powder formulations, they are able to modify the surface properties of particles reducing the agglomeration tendency of a powder and increase the fine particle fraction (Pilcer and Amighi, 2010). Particularly, the hydrophobic chain of surfactant reduces the absorption of the ubiquitous vapour, leading to a reduction of the aggregation and the adhesion of particles. Moreover, a low concentration lipid coating allows the preparation of powders with few excipients, thereby delivering more active drug to the lungs (Pilcer et al., 2006). (Steckel and Brandes, 2004) suggested that surfactant addition led to low density and porous particles

to enhance delivery of a therapeutic agent to the lung. Finally, some studies reported a protective effect carried out by surface acting agent towards biopharmaceuticals, especially during spray-drying process, excluding proteins from the air-liquid interface and promoting stability (Adler et al., 2000; Shoyele and Cawthorne, 2006).

On the other side, to evaluate the potential of a new excipient, it is not only indispensable to assure the functionality with respect to the formulation, but also to assess the safety profile of such a substance (Scherließ, 2011).

Generally, *in vitro* toxicity studies are used as a screen before pre-clinical *in vivo* investigations, thus cytotoxicity tests are developed along with model cell lines relevant for the intended route of administration (Rodriguez and Melchert, 1998).

MTT assay is one of the most commonly employed methods for the detection of cytotoxicity or cell viability following exposure to potentially toxic substances. This assay is linked with cellular mitochondria function (in particular dehydrogenase activity). In detail, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) is a readily soluble in water salt which is converted to an insoluble purple formazan by dehydrogenase enzymes associated with the endoplasmatic reticulum and the mitochondria (Fotakis and Timbrell, 2006). The formazan product is impermeable to the cell membranes and therefore it accumulates in healthy cells. Only viable cells are able to reduce the MTT into the cellular cytoplasm; the violet crystals are then solubilised through suitable solvents. However, MTT assay is a broad cytotoxicity assay which provides little information about the mechanism of toxicity and is based on an endpoint representative of late-stage toxicity (O'Brien, 2014).

Consequently, to identify multiple endpoints on cell samples, exploring the mechanism of cytotoxicity and cellular processes as well as high-throughput capacity, High Content Screening (HCS) has gained attention as a multiparametric analysis of compound toxicity on individual cells (Wilson et al., 2014). Among its unique capabilities, the HCS provides (a) the ability to measure cell responses at the single cell level, cell subpopulation, and cell population average responses; (b) the measure of specific molecular biomarker features with high specificity and sensitivity using fluorescence detection; (c) measures of multiple biomarker features in the same cells with multiplexing; and (d) measurement of functionally significant spatial and temporal dynamics in cellular responses (Giuliano et al., 2010). In particular, (O'Brien et al., 2006) demonstrated a human hepatotoxicity concordance of results from HCS cytotoxicity assay by analysing cellular responses to a collection of compounds for which clinical data are available. This technology involves probing whole cells with multiple fluorescent dyes which specifically probe different subcellular processes (Buchser et al., 2014).

Whole-well readouts (as those coming from MTT assay) are most commonly used and have the advantage of being fast and simple, and it is straightforward to generate statistical metrics of assay performance and chemical activity. High-content readouts have the advantage, in principle, of providing much more information (e.g. on cell-to-cell heterogeneity in response and on specific cytotoxic mechanisms) but scoring and interpreting the additional information is computationally challenging (Wink et al., 2014).

In vitro biocompatibility of 3 formulations containing surfactants (stearylamine, cetostearyl alcohol and stearyl alcohol) were firstly investigated using human lung carcinoma cell line (A549).

Then, *in vitro* toxicological evaluations regarding 2 non-ionic surfactants (cetostearyl alcohol and stearyl alcohol), one cationic surfactant (stearylamine) and one polysaccharide (sodium hyaluronate) were performed in human-derived cell systems retaining adequate metabolic competency and relevant to the route of administration, in particular A549, a type II alveolar epithelial cell line, Calu-3, an airway epithelial cell line and U937, precursor of human alveolar macrophages. HCS cytotoxicity assay simultaneously measures different parameters such as cell health (number, area and morphology), nucleus area, mitochondrial transmembrane potential and mitochondria area/cell area ratio.

HCA data generated were compared to data obtained using MTT assay under the same experimental conditions. Comparison methods are useful to make predictions about the safety of these molecules, which would be fundamental for the development of new and less harmful surfactants (Inácio et al., 2011).

4.2 Materials and methods

4.2.1 Chemicals and supplies

Sodium hyaluronate (HA) (PrymalHyal 50, average MW=29504 Da) was purchased by Soliance (France). Stearylamine (ST), thiazolyl blue tetrazolium bromide (MTT), sodium dodecyl sulphate (SDS), N,N-dimethylformamyde (DMF), Dulbecco's modified Eagle's medium (DMEM) nutrient mixture F-12 Ham, RPMI-1640, Fetal Bovine Serum (FBS), L-glutamine, non-essential amino acids (100%), penicillin-streptomycin solution (Pen-Strep), trypsin–EDTA solution (2.5 g/l trypsin, 0.5 g/l EDTA), trypan blue solution (0.4%) and phorbol 12-myristate 13-acetate (PMA) were supplied by Sigma–Aldrich (Sigma Chemical Co., USA). Stearyl alcohol (SA) and cetostearyl alcohol (CSA) were provided by ACEF (Italy). Tissue culture flasks (75 and 162 cm^2 with ventilated caps) and 96-well plates were from Costar (through Fisher Scientific, UK). Phosphate buffered saline (PBS) tablets were purchased from Oxoid (UK). Black 96-well plates were obtained by Greiner Bio-One (UK). Mito Tracker® Red, HCS Cell Mask™ Deep Red and Hoechst 33342 were purchased by Molecular Probes (Thermo Fisher Scientific, USA). All chemicals used were of analytical grade.

4.2.2 Cell lines

A549 (human lung carcinoma cell line), Calu-3 (human bronchial lung adenocarcinoma cell line) and U937 (human pro-monocytic cell line) were obtained from the American Type Cell Culture Collection (ATCC, USA) and stored in liquid nitrogen at a local cell bank for distribution as needed. Cells were grown in either 75 cm^2 or 162 cm^2 flasks (Costar Corning, UK) in a humidified 5% CO_2 / 95% atmospheric air incubator at 37°C.

For A549 and U937 cell culture medium was composed by RPMI-1640 supplemented with 10% v/v FBS, 1% v/v L-glutamine and 0.1% v/v Pen-Strep. For Calu-3, Dulbecco's modified Eagle's medium (DMEM) nutrient mixture F-12 Ham was supplemented with 10% v/v Foetal Bovine Serum (FBS), 1% v/v L-glutamine, 1% v/v non-essential amino acids and 0.1% v/v Pen-Strep. Cell counts were determined by hemocytometry before seeding cells to 96-well plates.

4.2.3 MTT assay

The biocompatibility of the excipients can be assessed using the well-known MTT test. Metabolically active cells are able to reduce MTT in formazan through a mitochondrial reductase leading to the formation of purple crystals (Mahto et al., 2010).

For MTT assay, A549 and Calu-3 cells were trypsinated at 80-85% of confluence to recover the cells in suspension from a culture flask, and seeded in 96-well plates at a density of 10,000 and 20,000 cells per well (in 200 µL Cell Culture Media, CCM), respectively; afterwards they were incubated for 24 h to allow the cells to attach and form a monolayer.

U937 cells in suspension were induced to differentiate by exposing them (density of 25,000 cells per well) to 4 nM of phorbol 12-myristate 13-acetate (PMA) in CCM for 48 hours. Thereafter, PMA media was substituted with 200 µL of fresh PMA media in each well for other 48 hours. Finally, cells were incubated for 24 hours with 200 µL of fresh CCM without PMA before adding the test solutions.

Firstly, cytotoxicity was investigated exposing A549 to 3 formulations with surfactants (HA_SteAm_10, HA_CetSteAlc_10 and HA_SteAlc_10) for 4 and 24 h. Afterwards, the biocompatibility of 4 substances (sodium hyaluronate, stearylamine, cetostearyl alcohol

and stearyl alcohol) was tested at a range of concentrations over 24 h on three different cell lines (A549, Calu-3 and U937). Substances were dissolved in a medium:ethanol mixture (99:1 v/v) and incubated at 37°C for at least 1 h before addition to the cells. All materials were tested over 9 different concentrations following a serial quarter log dilution; CCM:ethanol (99:1 v/v) mixture was used as a negative (vehicle) control. After either 4 h or 24h of incubation, cells were washed with PBS and then 200 µL of CCM was added. Finally, 50 µL of MTT solution (2.5 mg/mL in PBS) was added to each well and the plate was incubated for 4 h in a humidified incubator. Subsequently, the CCM was removed and cells were lysed and any formazan crystals generated were solubilised with 100 µL of a surfactant solution comprising 10% SDS in DMF:water (1:1 v/v). Plates were incubated overnight at 37°C before the absorbance of solubilised formazan was measured at 570 nm using a SpectraMax microplate reader (Molecular Devices, UK) The cell viability was expressed as a percentage of negative control (100% metabolic activity). LC_{50} values were calculated as concentration that caused 50% reduction in MTT conversion from the sigmoidal relationship obtained by plotting $\log_{10}$ concentration of adjuvant vs % cellular viability using GraphPad Prism (GraphPad Software, USA). All assays were performed in triplicate.

4.2.4 High Content Analysis (HCA)

Preliminary experiments were performed with each cell type in order to determine optimal reagent concentrations and incubation times to provide sufficient signal strength of each stain while ensuring there was no bleed-through between fluorescent channels as well as optimising cell seeding densities. A549 cells were seeded at 10,000 cells per well in 200 µL CCM using black 96-well plates. The same type of plates was used for Calu-3

and U937 cells (seeding concentration: 30,000 cells/well and 25,000 cells/well, respectively). Samples were prepared as described above for MTT assay and, following the same serial quarter log dilution, substances were incubated for 24 hours. For positive controls, two chemicals with known effects were added in triplicate to each plate to confirm quality of testing for the plate and to determine the maximum responses; Triton-X detergent and FCCP (an uncoupler of mitochondrial oxidative phosphorylation, responsible for disrupting ATP synthesis; 250 μM) were used for maximal effect on nuclear size and permeability, and mitochondrial membrane potential, respectively. Column 1 of each row contained CCM:ethanol (99:1 v/v) but no test substance, and was used as a negative control. After 15 minutes from the addition of positive controls, Mito Tracker® Red (mithocondrial membrane potential) was added to all the wells; cells were incubated for 30 minutes in dark at 37°C. Afterwards, the dye mixture 1 was removed and the cells were washed twice with PBS prior to the addition of 100 μL of fixative agent (4% PFA in 5% sucrose PBS solution) for 15 minutes at room temperature. The cells were washed twice with PBS and 100 μL of dye mixture 2 containing HCS Cell Mask™ Deep Red (cell area and morphology) and Hoechst 33342 (nuclear size and cell number) was added. The plates was incubated overnight at 4°C, washed twice with PBS wrapped in aluminium foil to protect them from light before imaging.

The images were acquired using an IN Cell Analyser 6000 (GE Healthcare) with a 40x objective. Image analysis was performed using IN Cell Developer V1.9.3 (GE Healthcare). Nine fields for each well were analysed to reliably measure different health parameters.

4.3 Results and Discussion

To date, there are no toxicological data available for the employed materials delivered by inhalation.

Hence, optimal seeding density and correct testing concentration were confirmed in preliminary experiments in order to cover a range from 0 to 100% cell viability in the MTT assay (data not shown).

The effect on the metabolic activity of A549 after 4 h and 24 h of exposure to different concentrations of hyaluronate formulations was evaluated by MTT assay and the concentration which produced a 50% reduction in cellular metabolic activity (LC_{50}) was calculated for each formulation (table 4.I).

Figure 4.1 illustrates two plots which are characteristic for 2 time points (4h and 24h).

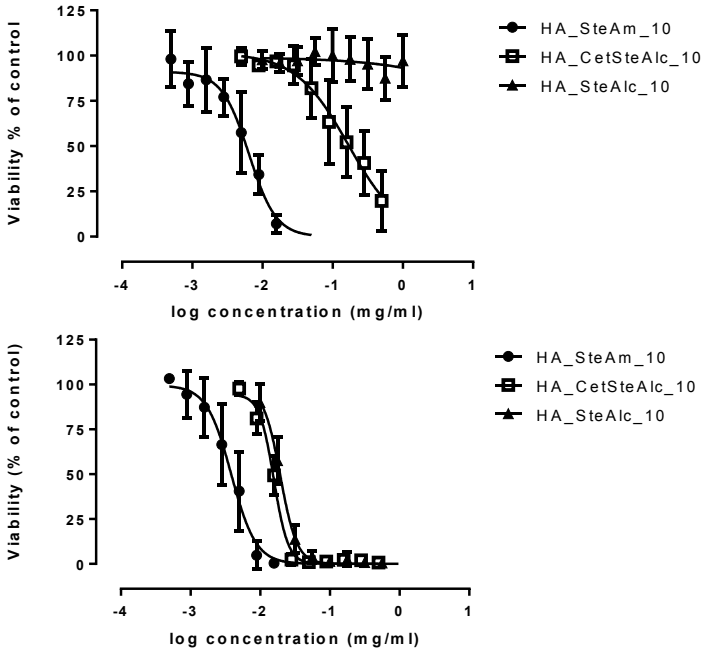

Figure 4.1. A549 cell viability after 4 (top) and 24 h (bottom) exposure to different HA formulations at concentrations between the no observable adverse effect level and complete suppression of cellular metabolism. The data represent mean ± s.d. (n=18; three independent experiments with 6 replicates at each concentration).

A statistically significant concentration- and time-dependent toxic effect is clearly shown for all tested formulations (Figure 4.1 and Table 4.I). This trend is remarkable for cetostearyl alcohol as the LC_{50} after 4 h of incubation is approximately 10 times higher than after 24 h (p-value = 0.034). The LC_{50} for stearyl alcohol was not reached when the A549 were incubated for 4 h (cells viability > 80%) whereas when the incubation time increased (24 h), a sigmoidal curve was achieved and an LC50 could be calculated. Formulation containing 10% of stearylamine was the most toxic whereas that with 10% of stearyl alcohol was the least toxic.

Table 4.I. LC_{50} after 4 and 24 h incubation time of 3 formulations carried out with A549 cell line.

Samples	**LC_{50} (95% Confidence Interval) (µg/mL)**	
	4h incubation	**24h incubation**
HA_SteAm_10	6.43 (5.05-8.18)	3.85 (3.09-4.79)
HA_CetSteAlc_10	167.1 (111.5-250.5)	15.59 (14.42-16.86)
HA_SteAlc_10	N.A.	19.42 (17.17-21.97)

Further MTT experiments were carried out on an expanded panel of lung-relevant cell lines, including A549, Calu-3 and U937 cells, to test the biocompatibility of individual formulation components (sodium hyaluronate, stearylamine, cetostearyl alcohol and stearyl alcohol) over 24 h of exposure. The cell lines selected are all human-derived and reflect different regions of the air-interfaced lung mucosal surface onto which inhaled formulations deposit. A549 is a cell line that retains major characteristics of the type II alveolar epithelium and is extensively used as a model cell for toxicity studies (Foster et

al., 1998; Roggen et al., 2006). Calu-3 cells are derived from human bronchial submucosal glands and has demonstrate many characteristics of the bronchiolar epithelium and have been used for the evaluation of airway injury and response to medical treatments and respiratory therapeutic interventions (Zhu et al., 2010). U937 is a well-characterised human pro-monocytic cell line that can be stimulated to differentiate by phorbol esters, and has previously been employed to test *in vitro* toxicity of surfactants (Jelinek and Klöcking, 1998).

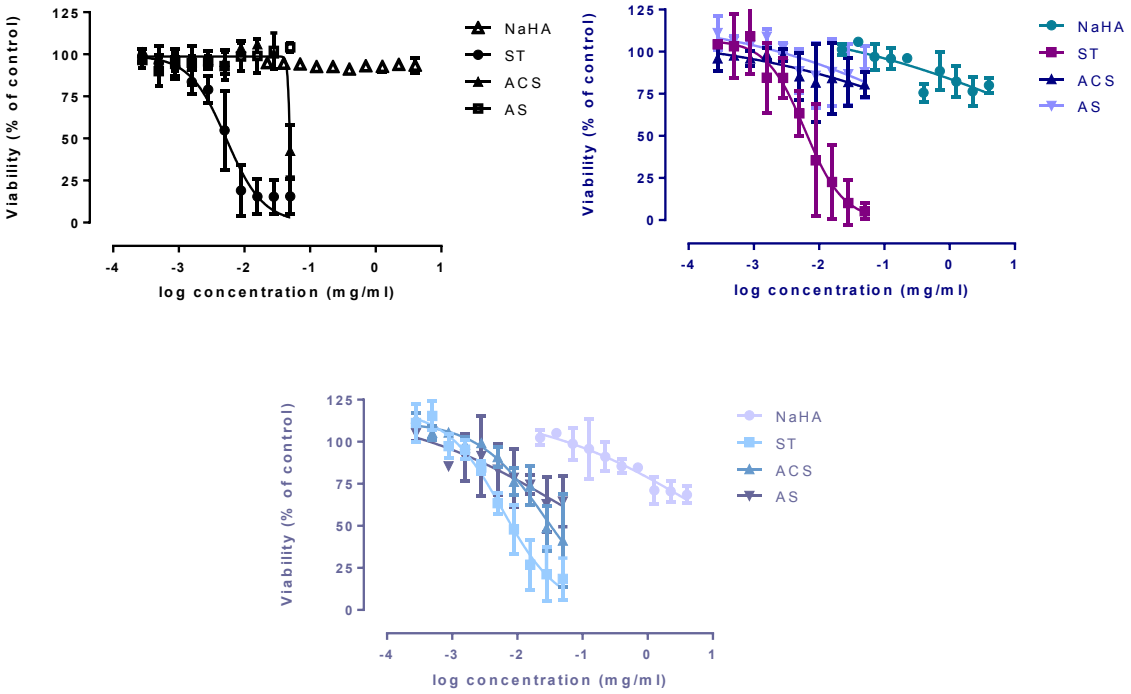

Figure 4.2. Viability of A549 (top left), Calu-3 (top right) and U937 (bottom) cell viability after 24 h of exposure to different substances. The data represent mean ± s.d. (n=18; three independent experiments with 6 replicates at each concentration).

The sensitivity to the surfactant varied between the different cell lines; although stearylamine caused toxic effect irrespective of the cell lines at the same concentration, LC_{50} = 5.32-6.08 µg/mL (Figure 4.2), U937 was more sensitive to cetostearyl alcohol as

the LC_{50} was approximately halved from 49.40 µg/mL (A549) to 25.50 µg/mL (U937). The responsiveness to chemical insults of the cell lines differs according to different functional, structural and compositional characteristics; giving rise to the different absolute LC_{50} values for the different cell lines that were observed in this study.

However, the same rank order for inducing cytotoxity was held across cell types. Stearylamine produced the lowest LC_{50} (5.32-6.08 µg/mL). Sodium hyaluronate had no overt toxic effect even at the highest concentrations used, especially for A549 where no effect at all was seen. The lack of responses to sodium hyaluronate was not surprising since studies have already proved its biocompatibility for drug delivery (Choi et al., 2012; Jansen et al., 2004; Zhong et al., 2015). However, when U937 cells were used a progressive incremental lowering of the assay readout was observed in response to increasing sodium hyaluronate concentration, albeit without reaching 50% loss of viability. This effect could be due to enhanced uptake through a specific interaction via CD44 receptors on macrophages' surface as reported previously (Culty et al., 1992; Hwang et al., 2008). A sigmoidal response-concentration curve was not observed when stearyl alcohol was incubated for 24 h with Calu-3 and U937 cells, and the LC_{50} was not reached. In these cases, the low solubility of surfactants in the medium used for the assay made it unfeasible to furtherly increase the stearyl alcohol concentration.

For comparison with the MTT assay findings, HCS assays were carried out for three surfactants and sodium hyaluronate, keeping constant the concentration range identified through MTT. Dose-related effects on A549, Calu-3 and U937 cells were observed following 24 h exposure (Figure 4.3, 4.4 and 4.5, respectively). These data show that multiple quantitative measurements of cell health can be derived from multiparametric

HCA assessment, such as cell count, nucleus area (Nuc Area), cell area, mitochondrial intensity (Mean Mito Intensity) and mitochondria area/cell area ratio (Mito Area/Cell Area), indicating the richness and multiplexity of data available via this methodology. This provides an opportunity to probe sub-lethal effects of the test agents on the cells.

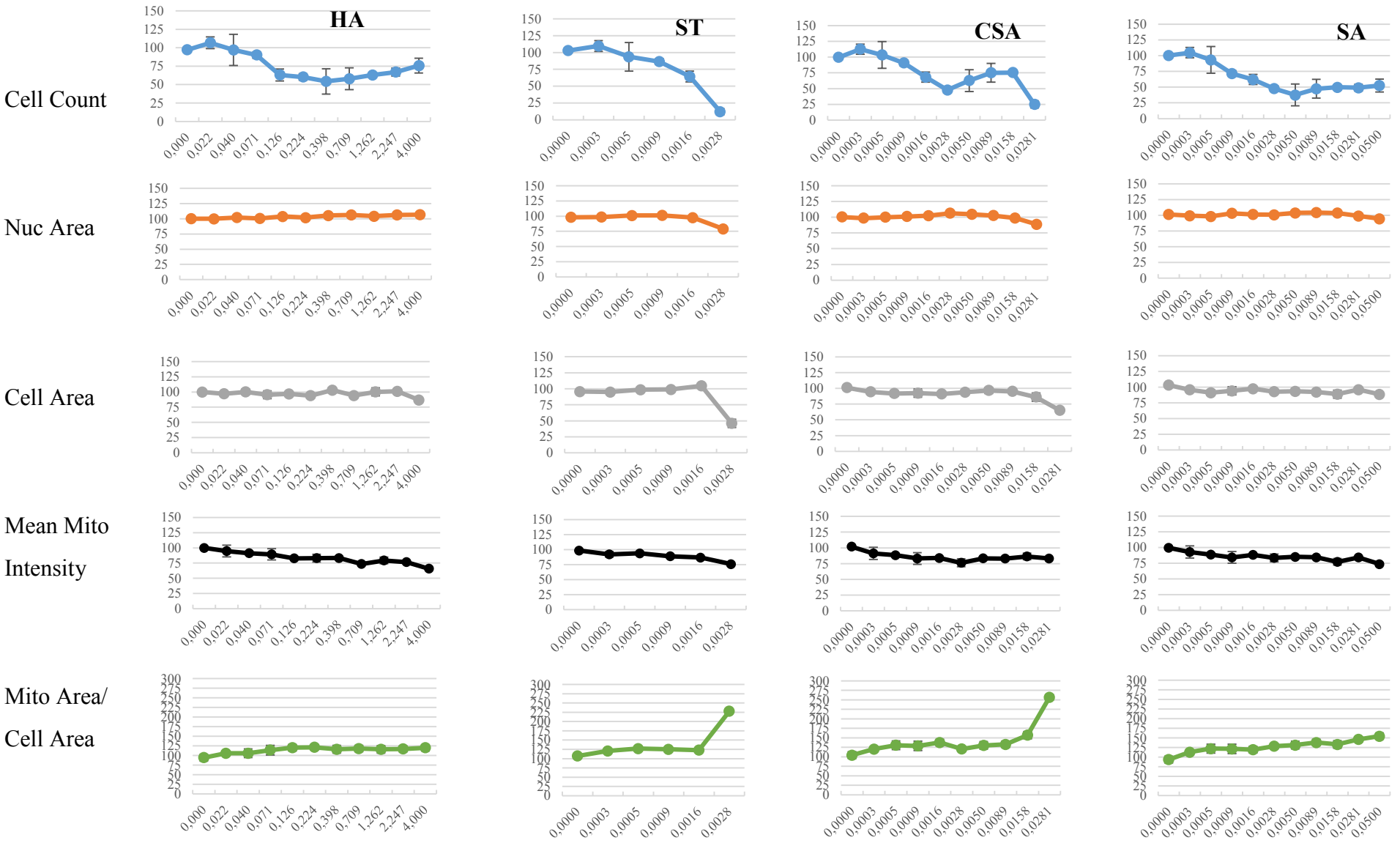

Figure 4.3. High Content Analysis (HCA) experimental results across multiple cell health parameters on A549. Cytotoxic effect of sodium hyaluronate (HA), stearylamine (ST), cetostearyl alcohol (CSA) and stearyl alcohol (SA) after 24 h of exposure. Data are presented as % of vehicle-treated controls and error bars indicate standard deviation (n = 3 wells).

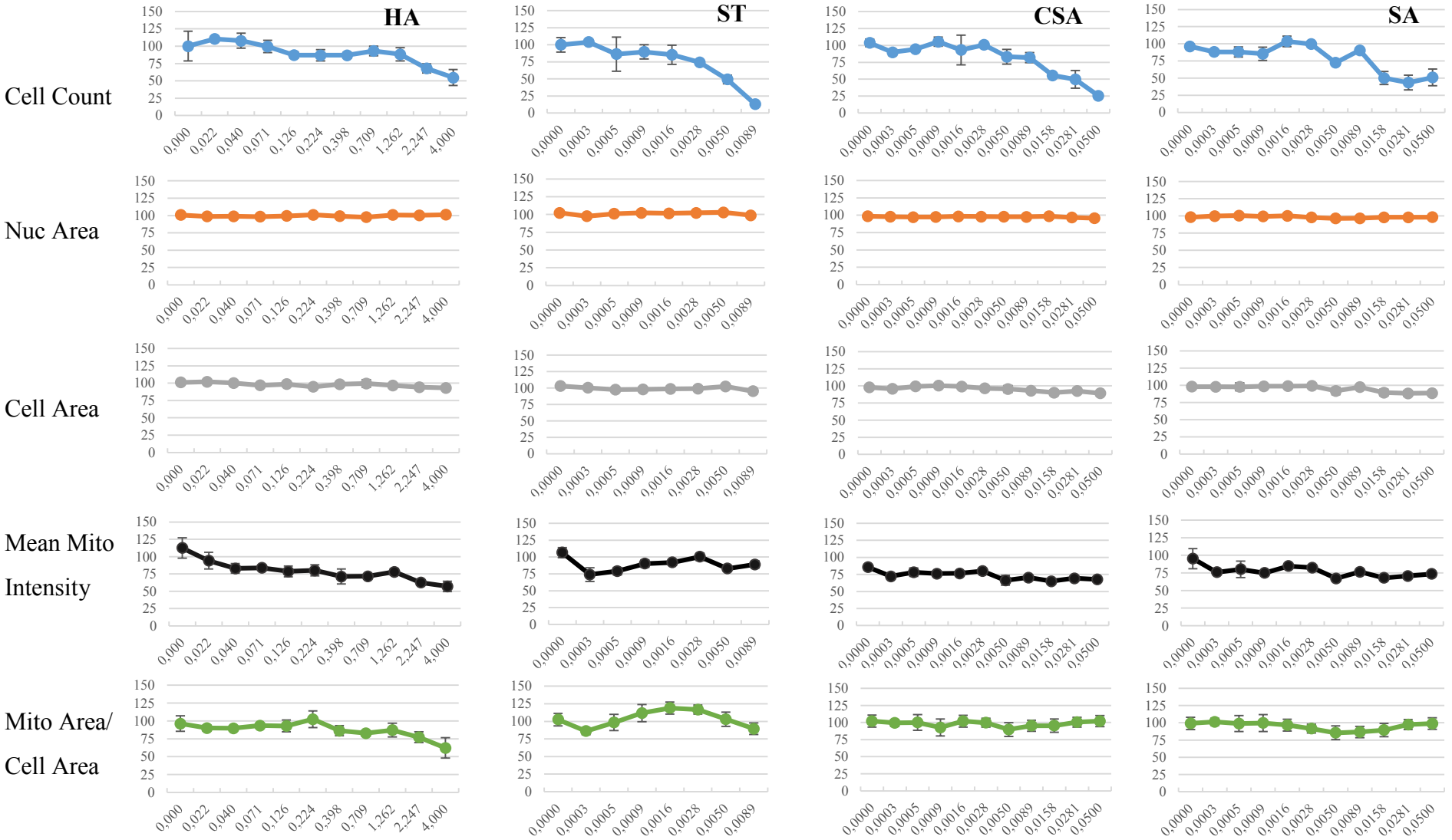

Figure 4.4. High Content Analysis (HCA) experimental results across multiple cell health parameters on Calu-3. Cytotoxic effect of sodium hyaluronate, stearylamine, cetostearyl alcohol and stearyl alcohol after 24 h of exposure. Data are presented as % of vehicle-treated controls and error bars indicate standard deviation (n = 3 wells).

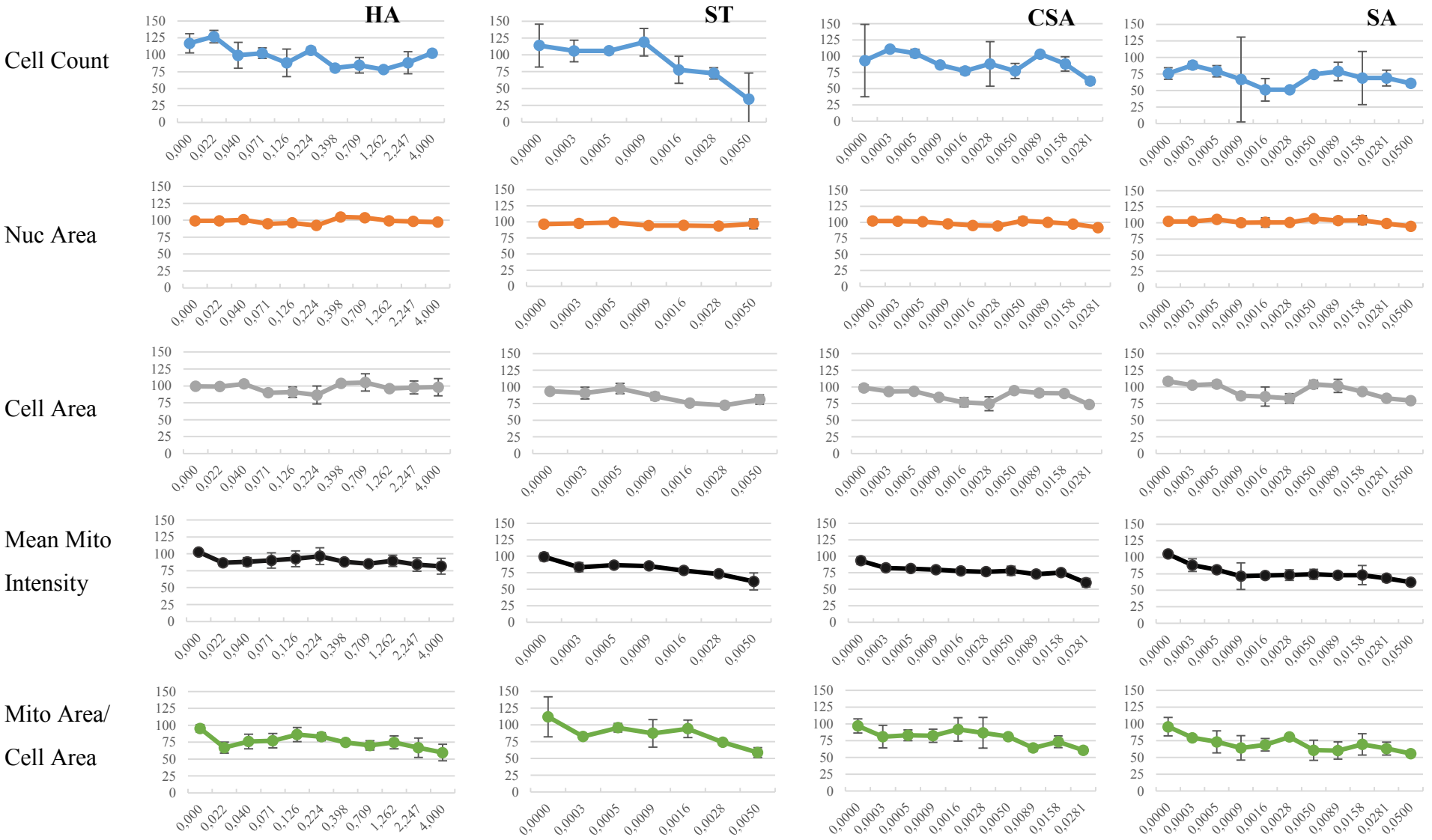

Figure 4.5. High Content Analysis (HCA) experimental results across multiple cell health parameters on U937. Cytotoxic effect of sodium hyaluronate, stearylamine, cetostearyl alcohol and stearyl alcohol after 24 h of exposure. Data are presented as % of vehicle-treated controls and error bars indicate standard deviation (n = 3 wells).

Sodium hyaluronate was found to have no toxic effects as illustrated in Figure 4.2, 4.3, 4.4 and 4.5. Indeed, cell count obtained by HCA was in accordance with MTT data for all the cell lines tested (cell viability > 50%). The small reduction in the cell count (A549 and Calu-3) was mainly due to a mitochondrial effect given that nucleus and cell area parameters were unvaried. U937 were less susceptible as cell count remained above 75% (Figure 4.5).

For stearylamine, a faster reduction in A549 viability was identified compared to the MTT test while cell count for Calu-3 and U937 were in agreement with MTT results. Only at the highest concentration (limited to 2.8 μg/mL), stearylamine caused a 50% reduction in A549 cell area leading to an increase in the ratio between mitochondria and cell area, while nucleus area was partially affected. The destabilization of the membranes structures was already pointed out by (Wang et al., 2006). Interestingly, Calu-3 and U937 differently reacted to this cationic surfactant and the cytotoxicity observed seems to be attributable to a mitochondrial dysfunction.

Regarding cetostearyl alcohol, a slower decline in the A549 cell count was observed compared to MTT assay. The increase in the mitochondrial/cell area ratio certainly demonstrated that cytotoxic effect was focused on plasma membrane (Dimitrijevic et al., 2000; Marianecci et al., 2010). A discrepancy was noted between the MTT data which showed Calu-3 cell viability remained above 75%, and the reduction to 25% in cell count in the HCS assay; moreover, no signs of toxicity towards nuclei, mitochondria or membranes were highlighted. The fluctuations noted for U937 did not allow an accurate data interpretation.

Finally, stearyl alcohol was defined as the most biocompatible surfactant owing to the fact that even when tested at the highest concentration, cell viability did not go below 60% of untreated control for any cell lines (Figure 4.2). The reasons for the low cytotoxicity is only partially understood; indeed a small effect on mitochondria at a non-lethal level in A549 was observed (see Figure 4.2). Calu-3 had the highest tolerability as only minor variations in cell parameters were detected. Regarding U937, the interactions between stearyl alcohol and cell membranes (i.e. plasmatic membrane and mitochondrial membrane) are clearly represents (Figure 4.5).

Generally, Calu-3 cells were thought to be less susceptible to surfactant because their tight junctions which make the basolateral membrane of these cells less accessible to these molecules. Results from (Inácio et al., 2011) confirmed cell tolerability to different surfactants and the toxic effect showed a dependency on the nature of the polar head groups, cationic surfactants being the most toxic. Moreover, the same group established that non-ionic and anionic surfactants showed toxicity at concentrations around their Critical Micellar Concentration (CMC), clearly identifying a toxicity related to destabilisation/destruction of plasma membrane whereas cationic surfactants were toxic at concentrations well below their CMC. The LC_{50} of 6 µg/mL findings for stearylamine (CMC = 0.013 mg/mL (*Committee for Risk Assessment RAC Annex 2 Response to comments document (RCOM) to the Opinion proposing harmonised classification and labelling at Community level of octadecylamine*, 2011)), is consistent with this, indicating that the cytotoxicity does not necessarily involve cell membrane disruption.

4.4 Conclusions

Safety evaluation of new products or ingredients destined for human use is crucial and requires screening during pre-clinical development. Therefore, rapid, sensitive and reliable bioassays are required in order to predict and evaluate the toxicity of these substances. At an early stage, *in vitro* cell based model are used for cytotoxicity testing, for which the MTT assay is widely used and provides a simple and sensitive test (Fotakis and Timbrell, 2006; Scherließ, 2011). However, newer assay formats enable more detailed studies should be carried out and, particularly, the High Content Analysis approach can examine multiple endpoints on cell samples, to explore the mechanism of cytotoxicity and cellular processes at sub-lethal concentrations more extensively than is possible using simpler but cruder single end-points assays. Rigorous screening should utilise a range of different cell lines and *in vitro* endpoints for reliable and informative screening toxicity of chemicals with potential interest to the pharmaceutical industry (Schröterová et al., 2009).

In conclusion, in this part of the project both MTT assays and a multi-parametric HCS cytotoxicity assay were developed and implemented, measuring 6 indicators of cell health, spanning cell area and morphology, mitochondrial membrane potential, nuclear area and cell count on three complementary cell lines relevant for a pulmonary drug delivery. Data support sodium hyaluronate biocompatibility over the entire concentration range tested and demonstrated that the cytotoxicity observed with formulations was due to the presence of surfactants rather than HA. Cell viability in the respiratory cell lines (A549, Calu-3 and U937) decreased in a concentration- and time-dependent manner after addition of the surfactants.

Differences in the sensitivity of model cell lines were observed as expected as these reflect different protective mechanisms of the different cell type as well as cell structural differences (Bačkorová et al., 2011; Schröterová et al., 2009). The weight of evidence provided by the cell lines indicated that rank in surfactant toxicity was consistent with that expected from the molecular composition: stearylamine, cetostearyl alcohol and stearyl alcohol. The agents primarily affect cell membranes by inserting within phospholipid bilayers, thus nuclear regions were not affected by addition of surfactants, although stearylamine produced a limited effect on A549 nuclei. Good accordance was established between MTT and HCA results for the same cell type, even if some disagreement are noted. Traditional cytotoxicity assays have a limited sensitivity that limits their effectiveness in predicting human toxicity. Therefore, complementary endpoint assays based on various mechanisms, as well as comparative analysis of the sensitivity of several cell types, are strongly recommended to increase the reliability of results (Fischer et al., 2003; Schröterová et al., 2009). The richness of data available through multiparametric HCA makes this technique a highly valuable tool for performing detailed and comparable *in vitro* toxicity investigation in different cell lines.

The quantitative ranking in surfactants toxicity can help with selection and concentrations used for the *in vivo* pre-clinical studies that are the necessary next stage for evaluating new dosage forms. The safety profile of the excipients indicates that a limit to exposure is likely to be warranted, but a balance between excipient function (enhancing the respirability of the particles) and safety should be achievable in the formulation.

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Chapter 5 General Conclusions

During this PhD work, the production of respirable dry powder formulations containing sodium hyaluronate was investigated in view of expansion in the number of excipients being included in inhaled products. In particular, this polymer was chosen for its physiological functions in the lung as well as it is considered biocompatible, biodegradable, non-immunogenic. Moreover, there exist a consolidate literature on the effect of hyaluronan as potential therapeutic agent for lung inflammatory disorders and its efficacious role as a drug delivery exploited via the interaction with CD44 receptor expressed on different cell type.

From these perspectives, we produced and optimised flowable and highly respirable HA dry powder through particle engineering approach based on spray-drying technique. Firstly, HA was formulated along with leucine and a hydrophilic model drug (salbutamol sulphate) in order to enhance the aerodynamic performance of the SD powder. From this set of data it was possible to conclude that HA should be associated with a second excipient with adjuvant properties otherwise particles respirability was unsatisfying.

A further approach implied the preparation of a colloidal solution of HA by finely tuning water-ethanol ratios. The advantages obtained after the addition of ethanol originated by the improved yield of the process as well as the remarkable reduction of particle size and distribution. Dry powders suitable for lung delivery were produced by co-spray-drying hyaluronate and surfactants (steraylamine, cetostearyl alcohol and stearyl alcohol). Among them, stearylamine pointed out the highest respirable fraction, affording improved microparticles emission and deaggregation during aerosolization.

Finally, biocompatibility assay of formulations containing surfactants was performed through the well-known MTT test using human lung carcinoma cell line (A549) established that HA was safe over the concentration range tested while the cytotoxic effects are observed in the presence of surface active agent.

Furthermore, MTT and HCS assay carried out on three relevant lung cell lines (A549, Calu-3 and U937) investigating the different level of toxicity of the three surfactants and HA pure materials, highlighted once again that HA was biocompatible although it lowered the cell viability in a concentration-dependent manner due to a partial effect on mitochondrial function.

Surfactants showed the same toxicity rank regardless the cell line involved. In particular, the cytotoxicity decreased from stearylamine to cetostearyl to alcohol stearyl alcohol. As expected from their own chemical structure, surfactants had a toxicity mechanism primarily affecting phospholipidic bilayer even though stearylamine caused a toxic response at the nucleus.

Finally, taking into account both the *in vitro* aerodynamic performance and the *in vitro* cytotoxicity the HA powder prepared with 5% w/w stearyl alcohol was the best performing among the formulation investigated in the present study.

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