In: Gentamicin
Editor: Emilie Kruger
ISBN: 978-1-62808-841-0

Chapter 2

GENTAMICIN AND PARTICLE ENGINEERING: FROM AN OLD MOLECULE TO INNOVATIVE DRUG DELIVERY SYSTEMS

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ABSTRACT

Gentamicin sulfate (GS) is one of the most important antibiotics of the family "Aminoglycosides" worldwide used for its effective bactericidal activities, low bacterial resistance and post-antibiotic effects, and moderate cost. GS, similarly to other members of the "aminoglycosides" family, shows low effectiveness when administered orally, therefore, the antibiotic is usually administered intravenously or intramuscularly. However, due to its pharmacokinetics and biopharmaceutical properties, multiple systemic daily administrations are needed to achieve good antibiotic concentrations; this may cause serious side effects such as ototoxicity and nephrotoxicity which limit its clinical exploitation.

A local administration which can deliver high dose of drug directly to the site of infection, while minimizing systemic exposure, can overcome these limits. In this case, appropriate dosage forms must be

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designed to obtain a local controlled drug release and to solve biopharmaceutical and pharmacokinetic issues that hinder the optimal use of GS in the clinical practice.

In the last few years microtechnologies have been applied as tool to innovate GS delivery. The major drawback encountered when formulating GS microparticles is its high hygroscopicity. In fact, as it is well known, hygroscopicity may modulate the moisture content of microparticles in the final dosage form and it is correlated to chemical or physical instability and poor flowability of the final powder product.

The present chapter briefly describes the critical properties of gentamicin from both a pharmacological and technological point of view.

Particularly, the aim of the chapter is to illustrates "particle engineering" strategies, i.e. spray drying and supercritical fluid techniques, adopted to improve technological properties of GS raw material. A special focus will be on i) the development of dry powders for inhalation, ii) the development of microparticulate powder for topical application in wound care. Both approaches allow to obtain micronized gentamicin powders, easy to handle, stable for long time and suitable for pulmonary and topical administration, respectively.

Keywords: Gentamicin; Particle Engineering; Spray Drying; Dry Powder Inhalers; Cystic Fibrosis; Supercritical Assisted Atomization; Wound bacterial infections

1. INTRODUCTION

Gentamicin sulfate (GS) is an aminoglycoside antibiotic worldwide used for the treatment of severe infections caused by both Gram-positive and, especially, Gram-negative bacteria (Forge and Schacht 2000). The "Amynoglycosides" family includes other active compounds with the same basic chemical structure, such as amikacin, arbekacin, kanamycin, neomycin, netilmicin, paromomycin, streptomycin, rhodostreptomycin, vancomycin, apramycin and tobramycin. Aminoglycosides are very effective bactericidal antibiotics that inhibit protein synthesis (Abdelghany, Quinn et al. 2012) and damage the plasma membrane (Davis 1987) binding to the 30S ribosomal subunit of bacterial cells. GS is one of the most commonly used for its low cost and reliable activity against gram-negative aerobes. This antibiotic exhibits rapid concentration-dependent killing action: increasing concentrations with higher dosages increases both the rate and the extent of bacterial cell death. In addition, GS presents significant post antibiotic effects

demonstrating a persistent suppression of bacterial growth also after short exposure (Gonzalez and Spencer 1998). Like other aminoglycosides, GS shows low effectiveness when administered orally because it is poorly absorbed from the gastrointestinal tract (Balmayor, Baran et al. 2012). Therefore, gentamicin is usually administered intravenously or intramuscularly. However, due to its biopharmaceutical and pharmacokinetics properties (e.g. limited permeability through endothelial cell membranes and short half-life) (Haswani, Nettey et al. 2006; Abdelghany, Quinn et al. 2012), high doses and multiple systemic daily administrations are needed to ensure adequate therapeutic serum concentrations; this may cause serious side effects such as ototoxicity and nephrotoxicity which strongly limit its therapeutic use (Ito, Kusawake et al. 2005; Selimoglu 2007; Balakumar, Rohilla et al. 2010). These problems can be overcome by local administration which can deliver high dose of drug directly to the site of infection, while minimizing systemic exposure (de Jesús Valle, López et al. 2007; Krasko, Golenser et al. 2007).

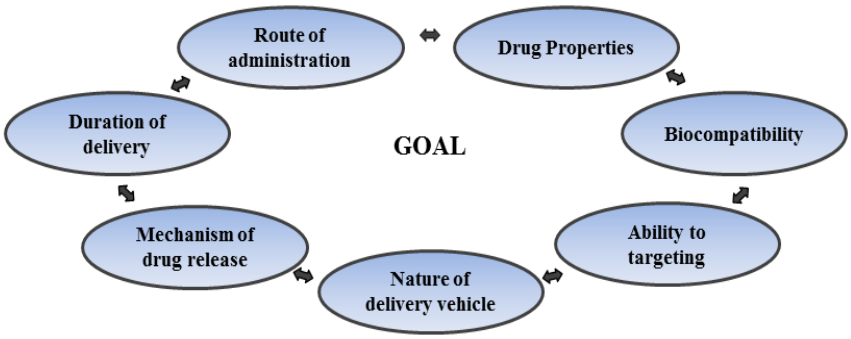
In the past, the conventional approach when dealing with such substances was to modify their chemical structure. Indeed, in the last few years there is a continuous research in the development of processes and techniques that allow to transform active pharmaceutical ingredients (APIs) into new dosage forms providing a solution to the above mentioned problems (Buttini, Colombo et al. 2012).

In fact, the so-called “new drug delivery systems” are able to modify biopharmaceutical and pharmacokinetic properties of the API, to control its release rate, and to obtain a site specific delivery, reducing side effects. All these aspects may increase both the therapeutic efficacy and the safety of known drugs allowing an optimal use in clinical practice (Tiwari, R. et al. 2012).

The design of a controlled drug delivery system requires simultaneous consideration of several factors, such as API properties, route of administration, nature of delivery vehicle, mechanism of drug release, ability of targeting, and biocompatibility (Figure 1), but it is not easy to achieve all these in one system (Coelho, Ferreira et al. 2010). Moreover, reliability and reproducibility of any drug delivery system is the most important factor while designing such a system.

All efforts to enhance therapeutic value of both new and old drugs have been the key driver in particle engineering, a term used to describe particle generation techniques driven by rational design of particle size, morphology and chemical composition (Mack, Horvath et al. 2012). Today, the particle is

no longer seen as a passive carrier, but rather as an essential part of the drug delivery system (Vehring 2008).



Paolino, Sinha et al. 2006.

Figure 1. Design requirement for a drug delivery systems.

The main goal of particle engineering is to incorporate desirable attributes, such as narrow particle-size distribution, improved dispersibility, enhanced drug stability, optimized bioavailability, sustained release and/or precise targeting, into particle while taking into account the specifics of formulation design and drug delivery requirements (Chow, Tong et al. 2007).

This chapter provides a review of two different "particle engineering" strategies, with special focus on spray drying and supercritical fluid technologies, with the aim to develop gentamicin based microparticles for pulmonary administration in cystic fibrosis (CF) or for topical application in wound care.

2. PHARMACEUTICAL PARTICLE ENGINEERING

The key for successfully developing and manufacturing a new dosage form is the study of the relationship between particles properties and final product performance.

Particle shape, size, adhesiveness, morphology, roughness are some of the properties that are usually evaluated. However, investigation on wettability, density, surface chemistry, plasticity, hardness, brittleness, moisture adsorption capability, permeability, and a tendency to gain electrostatic charge are necessary to determine the performance of the final product (Parikh 2011).

Among these, particle size distribution and particle shape usually constitute the critical variables of a pharmaceutical manufacturing process, and also affect quality attributes, such as:

- flow and packing properties, mixing and segregation of powders, rheological characteristics of liquid and semisolid formulations;
- content and dose uniformity and other properties related to the physicochemical stability;
- dissolution rate and bioavailability of APIs;
- drug release rate for sustained and controlled release formulations;
- aerosolization behavior and the corresponding performance of respiratory formulations;
- in-vivo particle distribution and deposition, absorption rate and clearance time (especially for aerosols and different colloidal systems designed for targeted drug delivery) (Parikh 2011).

The possibility to design tailor made particles by "particles engineering" allows to well control and optimize technological and biopharmaceutical properties in order to obtain the desired results.

Particles engineering approaches range from traditional micronization methods to novel and sophisticated Micro- or Nano-encapsulation techniques.

As well known, traditional methods used to produce micrometric particles, such as crushing/milling and crystallization/precipitation, lead to products with a poor control of particle size, shape and morphology (Chow, Tong et al. 2007; Hu, Zhao et al. 2008; Joshi 2011).

Spray drying (SD) and supercritical fluid (SCF) technologies represent new and interesting routes for particle formation, which avoids most of the drawbacks of the traditional processes.

2.1. Spray Drying

Spray drying is a one-step, continuous and scalable drying process which converts liquid feeds (i.e., solutions, suspensions and emulsions) into dry powders. During this process, the liquid feed is first atomized to a spray form (atomization step) that is put immediately into thermal contact with a hot gas, resulting in the rapid evaporation of the droplets (drying step). The dried

particles are then separated from the heated gas by means of a cyclone (separation step) (Pilcer and Amighi 2010).

In the last few years, this technique is gaining more and more attention as approach to form engineered API particles with characteristics that cannot be readily achieved using other manufacturing techniques (Sou, Meeusen et al. 2011). The chemical composition of the solid particulates depends on the content within the feed solution, whereas particle size and morphology are strongly dependent on process parameters such as liquid and gas feed rate, inlet temperature, gas pressure and aspiration (Vehring 2008; Maas, Schaldach et al. 2011). Actually, spray drying is the most commonly used technique to generate inhalable engineered particles (Parlati, Colombo et al. 2009; Wu, Hayes et al. 2013).

2.1.1. Spray Drying And Pulmonary Delivery

Inhalation therapy, highly recommended in pathologies affecting the lung (i.e. asthma, cystic fibrosis, chronic obstructive pulmonary disease), consists of drug administration directly to the lung in form of micronized droplets or solid microparticles, reducing the overall required dose and decreasing systemic exposure to the drug and formulation excipients (Labiris and Dolovich 2003).

The possibility to deliver high doses, the greater stability compared to liquid formulations and the problems of pressurized metered dose inhaler (pMDI) use have recently led research in the direction of formulating dry powder inhalers (DPIs) (Islam; Son and McConville 2008). DPIs is particularly challenging since the preparation of a "respirable" formulation and the selection of an adequate device for metering and aerosolizing the dose are both required. Therefore, formulation and device for inhalation have to be developed together (Friebel, Steckel et al. 2012).

Currently there are essentially four types of DPIs (Islam):

- Single-unit dose (capsule); This inhaler requires the patient to load a single hard gelatin capsule containing the powder formulation into the device before each use. This is a very common type of DPI device currently available on market.
- Single-unit dose (disposable); It is a device containing a pre-metered amount of a single dose that is discarded after use.
- Multi-unit dose (pre-metered unit replaceable set); Multi-unit devices deliver individual doses from pre-metered replaceable blisters, disks, dimples or tubes.

- Multiple dose (reservoir); Multiple dose reservoir inhalers contain a bulk amount of drug powder in the device with a built in mechanism to meter a single dose from the bulk and individual doses are delivered with each actuation.

It is very difficult to compare the performances of different DPIs (which have different design, resistance, mechanism of drug dispersion) without investigating their performances using the same drug formulation and same inspiratory force in a controlled environment. There is no comprehensive information (or data with limited information/access) on the comparative studies based on the performance of various devices. Therefore, it is extremely difficult to make a straightforward comparison on the performances of various devices (Islam). The only certainty is that the effective delivery of drugs from inhaler devices depends not only on the design of the device but also on the drug dry powder formulation.

The biggest issue encountered when formulating a dry powder for inhalation is to guarantee the aerosolization and the deposition at the appropriate site of the respiratory tract. A failure in deposition may result in a failure of efficacy. The aerodynamic behavior of inhaled particles depends on the so-called "aerodynamic diameter" (Dae), a spherical equivalent diameter that derives from the equivalence between the inhaled particle and a sphere of unit density (ρ_0) undergoing sedimentation at the same rate (Eq. 1).

$$Dae = Dv\sqrt{\frac{\rho}{\chi\rho_0}} \quad (1)$$

where Dv is the volume-equivalent diameter, ρ is the particle density and χ is the shape factor (Depreter, Pilcer et al. 2013). Hence, particle geometry, density and volume diameter are the main characteristics to customize since they affect dry powder inhalation performance (Buttini, Colombo et al. 2012). It is generally accepted that particles with an aerodynamic diameter of 1-5 μm (referred to as the "respirable range") tend to deposit in the lungs, while particles larger than 5 μm are trapped in the upper respiratory tract. However, owing to their small size, microparticles are extremely adhesive and cohesive resulting in low dispersibility and, consequently, poor flow properties (Chew and Chan 2002). One way to improve the aerodynamic performance of a spray-dried powder is through the addition of excipients (Shoyele, Sivadas et al. 2011). Many compounds that could enhance drug delivery outcomes also

have the potential to irritate or injure the lungs, so when formulating an inhalation dosage form the structural and functional integrity of respiratory epithelium must be respected (Telko and Hickey 2005). The current excipients approved by the Food and Drug Administration (FDA) for respiratory drug delivery are very limited in number and not accepted world-wide. The array of potential excipients is limited to compounds that are biocompatible to the lung and can easily be metabolized or cleared, like sugars (lactose, mannitol and glucose) and hydrophobic additives (magnesium stearate, DSPC). In the last few years, amino acids (AAs) have been tested as alternative excipients due to their ability to decrease hygroscopicity and improve surface activity and charge density of particles. Different studies have demonstrated that co-spray-drying of few selected amino acids with active compounds provides enhanced aerodynamic properties of the final dry powders (Li, Seville et al. 2005; Seville, Learoyd et al. 2007).

Moreover, as amino acids are endogenous substances, they might not present a major risk of toxicity to the lungs (Pilcer and Amighi 2010). Different amino acids such as arginine, aspartic acid, phenylalanine, threonine and leucine have been tested in dry powder formulations as enhancer of aerodynamic properties, the most noteworthy effects have been observed with leucine (Depreter, Pilcer et al. 2013). For example, selection of appropriate solvent systems and leucine concentration has allowed to produce highly respirable β-oestradiol spray-dried powders (Rabbani and Seville 2005). The influence of leucine amount on powder dispersibility and manufacturability has been reported. A 10–20% (w/w) of leucine in spray-dried ethanol or water solutions gives good aerosolization characteristics to peptides or sodium cromoglycate (Chew, Shekunov et al. 2005; Rabbani and Seville 2005; Padhi, Chougule et al. 2006). It is suggested that addition of leucine results in less cohesive particles and in a decrease of particle size due to the surfactant behavior of leucine, reducing the size of droplets produced during atomization (Vehring 2008). Spray-dried isoleucine has also been shown to improve the aerosol performance and stability of various formulations. Trileucine has also been proven to be an efficient surface active agent able to produce corrugated particles of low cohesivity (Lechuga-Ballesteros, Charan et al. 2008). In this case, the stabilization mechanism seems to be different: as a result of its surface activity, trileucine molecules can orient the hydrophobic groups towards the air at the air/liquid interface during the drying process, providing a hydrophobic surface to the dry particle, thereby contributing to the observed improved aerosol efficiency.

2.1.2. Novel gentamicin DPI (Dry Powder Inhaler) for Cystic Fibrosis Treatment

The aim of our research was to develop inhalable GS powders with satisfying aerodynamic properties and good stability profile by spray drying for the treatment of lung infections in cystic fibrosis (CF). This is the most common lethal genetically inherited disease of the Western World (Shur, Nevell et al. 2008). Pulmonary infections are the major cause of morbidity and mortality in CF, with *Pseudomonas aeruginosa* (Pa) acting as the principal pathogen. The viscous mucus lining the lung of CF patients impairs the mucociliary function, facilitating recurrent and chronic respiratory infections caused mainly by Pa but also by *Haemophilus influenza* and *Bulkolderia cepacia* (Mukhopadhyay, Singh et al. 1996; Ramsey, Pepe et al. 1999).

Treatment of lung disease by antibiotics is an accepted standard in cystic fibrosis cure aimed to reduce decline in lung function and number of hospitalizations (Prayle and Smyth 2010). Various clinical studies on GS inhalation treatment in CF patients chronically infected with Pa have shown that antibiotic solutions for aerosol treatment produce both subjective and objective improvement (Mugabe, Azghani et al. 2005; Abdelghany, Quinn et al. 2012). Interestingly, among aminoglycosides, GS has shown the ability to partially restore the expression of the functional protein CFTR (cystic fibrosis transmembrane conductance regulator) in CF mouse models bearing class I nonsense mutations (Wilschanski, Famini et al. 2000; Clancy, Bebok et al. 2001; Du, Jones et al. 2002; Wilschanski, Yahav et al. 2003). In particular, Du and coll. (Du, Jones et al. 2002) demonstrated that GS was able to induce the expression of a higher CFTR level compared to tobramycin.

Regarding the use of gentamicin sulfate in the treatment of airways infections and class I CFTR mutations, the main problem is its reduced penetration in the endobronchial space after intravenous administration, combined with its high systemic toxicity. Since GS peak sputum concentrations are only 12 to 20% of the peak serum concentrations (Mendelman, Smith et al. 1985) to achieve adequate drug concentrations at the site of action, it is necessary to use large intravenous doses, which may produce serum levels associated with renal and oto-toxicity. These problems can be overcome by the use of aerosolized GS, which can deliver high dose of drug directly to the lungs, while minimizing systemic exposure.

The major drawback encountered when formulating GS microparticles is its high hydrophilia. As its high hydrophilia guarantees a rapid drug solubility and diffusion in the fluids lining the lung, as it may cause high hygroscopicity

and instability, preventing the formulation of a stable and respirable dry powder.

In order to reduce hygroscopicity and to increase powder dispersibility and stability, GS was spray dried alone or with leucine (Leu) as flowability enhancer at different concentrations from water or various hydro-alcoholic solutions (Aquino, Prota et al. 2012).

As reported in Table 1, addition of the organic co-solvent (isopropanol-ISO) into the water feed was extremely helpful in terms of process yield. Differently, Leu addition did not have a linear effect on spray drying yield, especially in hydro-alcoholic solutions.

Table 1. Physical characteristics of spray dried particles: liquid feeds composition, process yield, particle size and bulk density

Sample code	Water/ISO (%v/v)	Leu content (%w/w)	Process yield (%)	d_{50} (μm) and span	Bulk density (g/ml)
G	100% H_2O	0	53.9±1.0	4.06 (1.63)	0.07±0.02
GISO3	7/3	0	85.5±0.7	4.24 (1.97)	0.19±0.02
GISO3-Leu15	7/3	15	82.0±2.1	3.90 (1.62)	0.34±0.01

Optimized process parameters led to micronized powders with d_{50} similar for all batches produced (Table 1), with no evident effect of solvent and Leu content on the particles diameter.

Organic co-solvent and Leu had a massive effect on hygroscopicity, too. In particular, by adding 30% v/v of ISO into the aqueous feed, humidity uptake by GS powders was reduced from 10.5% (water) to 4.8% (water/ISO) after exposure at room conditions. These effects may be explained by the addition of the lower-soluble component (Leu) into the liquid feeds, able to reach the critical concentration for shell formation as the droplet evaporation progresses during spray-drying process (Vehring 2008). Such enrichment in Leu at the particle surface seems to slow down water uptake of hygroscopic drug such as GS and, potentially, increase powder flowability (Shur, Nevell et al. 2008).

Leu effect on spray-dried powders appears clearly, after microscopy studies, as an evident increase in particle corrugation. As an example, SEM pictures of particles dried from 7/3 water/ISO ratio solutions were reported in Figure 2.

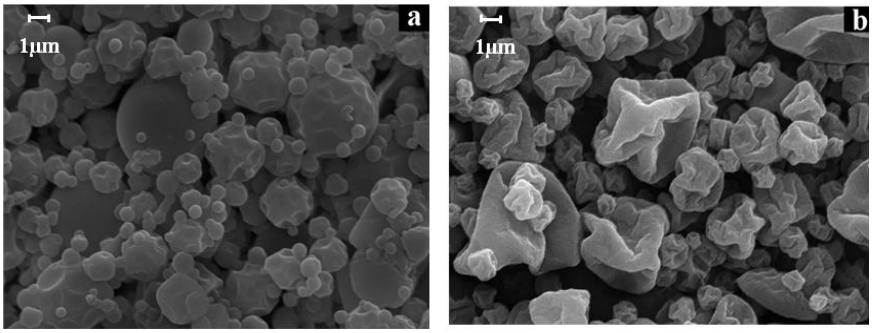


Figure 2. SEM pictures of powders dried from water/IPA 7/3 v/v systems containing: a) GISO3; b) GISO3-Leu15.

As well known, the morphology of spray-dried particles is strongly influenced by the solubility of the components and their initial saturation in the liquid feeds. GS, freely soluble in water, led to the formation of spherical and smooth particles when spray dried alone (Figure 2). According to previous observations (Lechuga-Ballesteros, Charan et al. 2008; Boraey, Hoe et al. 2013), during the co-spray drying process, the saturation of the lower-soluble component (Leu) may increase faster than that of hydrophilic one (GS), due to the preferential evaporation of alcohol and the associated change in the solvent/co-solvent ratio. This led to the formation of a primary solid shell which collapsed, hence corrugated microparticles were formed. As the relative amount of the less soluble component increased, particle corrugation was more and more evident; particles from almost spherical became raisins like or irregularly wrinkled.

By modifying particle shape and corrugation degree, Leu influenced powder bulk density too. In fact, powders processed from hydro-alcoholic systems showed lower bulk density values than those spray-dried from water, whereas Leu inclusion up to 15% (w/w) led to higher density powders (Table 1). As well known, differences in bulk density influence the amount of powder chargeable into the capsules for the inhalation, which shifted from 60 mg for neat GS to 120 mg for GS/15% Leu. The possibility to charge higher amount of drug into the device allows to reduce the number of actuations required, enhancing patient's compliance.

Table 2. Aerodynamic properties of spray-dried powders after single stage glass impinger deposition experiments; device TURBOSPIN, charged with capsules type 2 (mean ± SD of three experiments)

Sample code	ED (%)	FPD (mg)	FPF (%)
G	/	/	/
GISO3	90.9±7.9	7.5±4.9	13.4±8.5
GISO3-Leu15	99.1±0.3	50.4±0.8	49.4±0.8

ED, emitted dose; FPD, fine particle dose; FPF, fine particle fraction.

As aerodynamic properties, it is important to note that neat GS dried from water (G) was a cohesive and sticky material, unable to be aerosolized. Indeed, GS spray drying from hydroalcoholic solvent (e.g. GISO3) reduced powder cohesivity and enabled the aerosolization process; however, the resulting aerodynamic properties were still not satisfying. Only the inclusion of Leu led to the best FPF and FPD values (Aquino, Prota et al. 2012). Among all formulation, GISO3-Leu15 showed very satisfying aerodynamic properties as proven by FPF 49.4% and FPD of 50.4 mg (Table 2), values evaluated by Turbospin® device, a single-unit dose inhaler designed and patented by PH&T for effective drug delivery to the lungs.

Stability tests, performed storing the powders in a climatic chamber for 6 months at 25 ± 2 °C/60 ± 5% RH, were conducted to control over time hygroscopicity and dispersibility of G/Leu systems. After 6 month storage, no variation in powder weight was observed, GS content remained unaltered and no GS degradation product was recorded by HPLC analyses of aged powders.

In order to establish whether the particle engineering has any cytotoxic or cytostatic effect on bronchial epithelial cells (Zabner, Karp et al. 2003; Dechecchi, Nicolis et al. 2008), CuFi1 cells were treated for 24 h with increasing concentrations (from 0.0002 to 2 μM expressed as GS content) of GISO3 or GISO-Leu15 powders in comparison to raw GS. Results showed neither raw GS nor its formulations generally inhibited cells viability as determined by MTT assay (Figure 3b). Only raw GS at concentrations higher than 0.02 μM showed a slight but significant decrease in cell survival. An interesting observation is that an increase in Leu content up to 15%, as in GISO3-Leu15, faintly but not significantly decreased CuFi1 viability at concentration ranging from 0.02 to 0.2 μM ($P<0.05$) (Figure 3b) whereas at 2.0 μM did not. As previously observed in formulations for inhalation containing leucine (Prota, Santoro et al. 2011), this effect seems to be related

to Leu ability to improve cell proliferation and metabolism of bronchial epithelial CF cells.

Furthermore ELISA BrdU immunoassay confirmed that raw GS slightly reduced CF cell growth only at the highest concentration (2 µM, $P<0.01$) (Figure 3a).

Therefore, G/Leu systems had no cytotoxic or cytostatic effect on CF epithelial lung cells (CuFi1 model), at concentrations up to 2 µM.

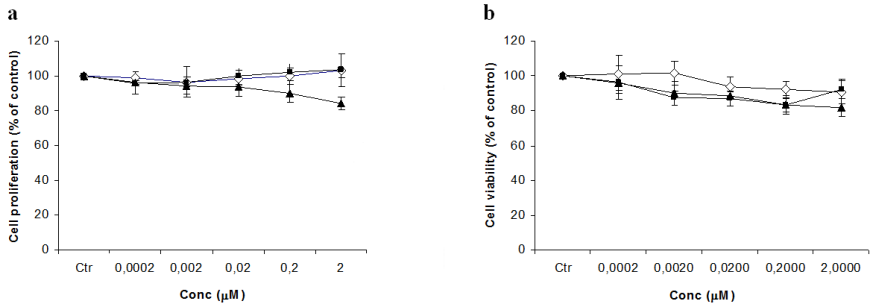


Figure 3. Effect of Gentamicin and its DPI formulations on CuFi1 cell proliferation and viability. Cells were treated for 24 h with: raw Gentamicin (raw GS, ▲), spray-dried Gentamicin (GISO3 ◊) and GS co-sprayed with 15%w/w leucine (GISO3-Leu15 ■) at concentrations from 0.0002 µM to 2 µM. Cell growth (a) was determined using a colorimetric bromodeoxyuridine (BrdU) cell proliferation ELISA kit. Cell viability (b) was determined by MTT assay. All data are shown as mean ± SD of three independent experiments, each done in duplicate (**P*<0.05 and ***P*<0.01 *vs* control).

Finally, to verify the ability of the produced formulations to control *P. aeruginosa* infection, two different microbiological assays were performed. Preliminarily, microsystems were tested by a disc diffusion assay at a concentration corresponding to 5 µg of GS. Results showed that each powder produced an inhibition zone of growth with a diameter of about 2 cm, similar to that observed for GS raw material (Russo, Stigliani et al. 2013). Therefore, neither the spray drying process, nor the presence of the excipient seems to influence the antipseudomonal activity.

However, clinical identification of *P. aeruginosa* often includes identifying the secretion of pigments such as pyocianin (blue-green). In order to evaluate antibiotic activity of the formulations in a model which better reproduces pulmonary environment and, thus, may be clinically relevant, a modified pyocyanin assay was performed in presence of artificial mucus model (AM), developed taking into account CF mucus composition and characteristics (Russo, Stigliani et al. 2013). Results of our study confirmed

that the growth of *P. aeruginosa* was inhibited equally by all formulations and in a manner comparable to neat gentamicin spray dried (G). Therefore, neither the spray drying process, nor the presence of the excipient seems to influence the antipseudomonal activity.

The formulation study of the "old" drug, gentamicin, demonstrate that the engineering process by spray drying, use of water-co-solvent systems as liquid feed and low rate of a safe excipient have enabled to obtain the required significant improvement in therapeutic GS performance. Identifying problems related to GS hygroscopicity and stickiness and reducing them by appropriate operations, led to obtain the specified performance goal, i.e. stable and aerosolizable microparticles in dry powder form for inhalation with an excellent emitted dose and good aerodynamic properties.

Particles engineering has not impact on biological properties of GS; in fact, GS/Leu engineered particles show no cytotoxic or cytostatic effect on bronchial epithelial cells bearing a CFTR F508/F508 mutant genotype and are able to preserve the antibiotic activity against *P. aeruginosa*, even in the presence of mucus. These findings together with the well known GS antibiotic activity and ability to partially restore CFTR expression in class I nonsense mutation, support the use of GS/Leu DPI as a valid alternative to common antibiotics already used in the management of Pa infections.

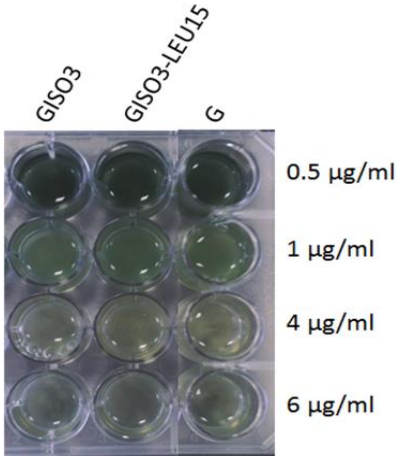


Figure 4. Modified pyocyanin assay on G dry powders. *P. aeruginosa* was cultured for 24h at 37°C in AM; powder concentration tested corresponded to 0.5, 1, 4 and 6 µg/ml of gentamicin used as control. The green color virulence factor pyocyanin decreases with the increase of GS concentrations.

2.2. Supercritical Fluid Technologies

A new approach in particle engineering developed to obtain micro/nanoparticles with desired characteristics is represented by the supercritical fluid (SCF) technology (Pasquali, Bettini et al. 2008; Tabernero, Martín del Valle et al. 2012). A SCF can be defined as any fluid which is at conditions above its critical point. Carbon dioxide, because of its accessible critical point at 31°C and 74 bar, and its low cost and non-toxicity, is the most widely used solvent in many SCF processes (Sihvonen, Järvenpää et al. 1999). Its critical temperature makes SCF suitable for processing heat-labile solutes at conditions close to room temperature.

A SCF can be used as: a solvent, in the rapid expansion of supercritical solutions (RESS); an antisolvent, in the supercritical antisolvent precipitation (SAS); a solute, in the particle from gas saturated solution (PGSS) (Reverchon and Antonacci 2007). Several papers and reviews have been published on pharmaceutical processing using these techniques (Carstensen 2001; Kompella and Koushik 2001; Kakumanu and Bansal 2003; Kayrak, Akman et al. 2003; York, Kompella et al. 2004). Recently, SC-CO_2-assisted spray drying techniques, such as CAN-BD (Carbon Dioxide Assisted Nebulization with a Bubble Dryer®) and SAA (Supercritical Assisted Atomization), have been proposed to produce micro- and nanoparticles of controlled size and distribution (Pasquali, Bettini et al. 2008). These techniques are aerosolization-based methods where supercritical CO_2 is used to assist the nebulization of the feed solution. The mechanism of the process is similar to micronization by spray drying: the SCF and the solution are intimately mixed and sprayed in a drying atmosphere. Claimed advantages of this process include the minimal decomposition of thermally labile drugs, the absence of a high-pressure vessel, and the small size of the produced particles (Charbit, Badens et al. 2004; Pasquali, Bettini et al. 2008).

2.2.1. Supercritical Assisted Atomization

SAA technology is a process based on the solubilization of controlled quantities of SC-CO_2 in liquid feeds containing a solid solute and on the subsequent atomization of the ternary solution through a nozzle. Therefore, SC-CO_2 plays both as co-solute being miscible with the solution to be treated, as well as pneumatic agent to atomize the solution in fine droplets (Reverchon 2002; Reverchon 2007). The solubilization is obtained in a packed bed saturator characterized by a high specific surface and large residence times. The solution formed in the saturator is, then, sent to a thin wall injector and

sprayed into the precipitator at atmospheric pressure in a warm N_2 environment at fixed temperature. A two steps atomization is obtained: the primary droplets produced at the outlet of the injector (pneumatic atomization) are further divided in secondary droplets by CO_2 expansion from the inside of the primary ones (decompressive atomization). Then, the secondary droplets are rapidly dried by warm N_2 causing the micrometric and sub-micrometric particle precipitation. One of the most important aspects of SAA with respect to other SCF-based processes is that not only organic solvents, but also water and aqueous solutions can be used (Reverchon and Antonacci 2006).

Various studies have demonstrated that this technique can be successfully applied to the micronization of some pharmaceutical compounds obtaining particles in the range of 1-3 μm with controlled size distributions (Reverchon, Della Porta et al. 2004; Della Porta, Ercolino et al. 2006; Della Porta and Reverchon 2008). SAA has been also applied to the production of microparticulate drug delivery systems. Particularly, Reverchon et al., have successfully processed by SAA chitosan, hydroxypropyl methylcellulose, and cyclodextrins, obtaining, at the optimal process conditions, well-defined spherical microparticles with controlled drug release properties (Reverchon and Antonacci 2006; Reverchon and Antonacci 2007; Reverchon, Lamberti et al. 2008).

Therefore, selecting the right excipients and optimizing the operating conditions it is possible to tailor the SAA produced particles for different pharmaceutical applications.

2.2.2. Novel Gentamicin Topical Dosage Forms for Treatment of Wound Infections

Infections can be associated to many traumatic occurrences such as skin tears and burns or chronic pathologies or even to post-surgery complications (Baranoski and Ayello 2011). The main goal in treating the various types of wound infections should be to reduce the bacterial load in the wound to a level at which healing processes can take place (Baranoski 2008). Conventional systemic delivery of antibiotics, for both prevention and curing, suffers of the drawbacks of systemic toxicity with associated renal and liver complications, poor penetration into ischemic and necrotic tissue typical of post-traumatic and postoperative tissue and need for hospitalized monitoring (Campton-Johnston and Wilson 2001).

Alternative local delivery of antibiotics by topical administration, or even better by a local delivery device, may consent local control of infection while

minimizing side effects and induced bacterial resistance (Persson, Salvi et al. 2006; Aviv, Berdicevsky et al. 2007; Boateng, Matthews et al. 2008).

A local antibiotic release profile should exhibit a high initial release rate in order to respond to the elevated risk of infection from bacteria introduced during the initial shock, followed by a sustained release at an effective level for inhibiting the occurrence of latent infection (Wu and Grainger 2006). Indeed, the effectiveness of wound controlled release devices is strongly dependent on the rate and manner in which the specific antibiotic is released. If it is released quickly, the entire amount could be released before the infection is arrested. If the release is delayed, infection may set in further, thus making it difficult to manage the wound. Finally, the release of antibiotics at levels below the Minimum Inhibitory Concentration (MIC) must be avoided because it may evoke bacterial resistance at the release site and intensify infectious complications (Gold and Moellering 1996; Aviv, Berdicevsky et al. 2007). Antibiotics of different families have been incorporated in controlled-release medical devices such as gentamicin, vancomycin, tobramycin, cefamandol, cephalothin, carbenicillin, amoxicillin etc. (Stigter, Bezemer et al. 2004; Zilberman and Elsner 2008) and various biodegradable devices have been produced using different processes (Blanco-Prieto, Lecaroz et al. 2002). In an preliminary research (Della Porta, Adami et al. 2010), SAA has been exploited not only as a micronization method but also as a thermal coagulation process for the production of gentamicin/albumin microspheres with slow drug release for the treatment of wound infections. More recently, we have proposed (Aquino, Auriemma et al. 2013), SAA technique for the development of specific controlled release microsystems, made by alginate, pectin and GS. Among natural polymers, two dextrans like alginate and pectin are known as wound dressing materials enhancing the healing process by maintaining optimal moist environment and via a direct effect on wound macrophages. Unlike other polymers, they can adhere to wound site (Fletcher 2005), absorb exudate by changing their physical state into a hydrogel able to cover and preserve an appropriate moisture at the wound bed (Baranoski 2008) while allowing effective oxygen circulation able to increase cells and tissues regeneration and lowering bacterial load (Boateng, Matthews et al. 2008). Moreover, alginate may induce cytokine production by human monocytes via an interaction with mannuronic residues of alginic acid. The pro-inflammatory stimulus is considered particularly useful in the treatment of chronic wounds, when macrophages have not achieved an appropriate differentiation state; the healing process could take advantage of exogenous pro-inflammatory stimuli to which macrophages are receptive (Thomas, Harding et al. 2000).

Therefore, the primary aim of our research was to design microencapsulate GS in such dextrans using SAA with the goal to be directly administered or charged in specific fibers or gels for wound dressing. As well known, the major concern when developing such drug delivery system is to control the technological and biopharmaceutical properties of the final product and to assure biological availability of the drug. Thus, the designed process comprises the study of the effects of i) the selected microencapsulation technique, ii) drug/polymers ratio, iii) operating conditions (i.e. feed composition and process parameters) on particle micromeritics (i.e., morphology, dimensional distribution, solid state of the loaded drug), drug release behavior, and GS antibiotic activity. In this chapter we report the best results obtained (Tables 3-4).

GS/alginate/pectin (GAP 1-4) particles, processed starting from aqueous solutions, were obtained as white powders made by microparticles with good spherical shape and uniform morphology (Figure 5). By contrast, as previously reported (Della Porta et al., 2010), when pure GS aqueous solutions were processed by SAA, pale yellow powder was obtained due to the partial degradation of GS in the precipitator. This observation suggests that the polymer blend consisting of alginate and pectin acts as a protecting agent, covering GS and avoiding its thermal degradation during microencapsulation process. Moreover, the increased stability of the SAA encapsulated GS, may also be due to its selective interaction, as a cationic drug, with mannuronic residues of the alginate (Iannuccelli, Coppi et al. 1996) by forming a polyelectrolyte complex. This hypothesis has been then confirmed by DSC (Differential Scanning Calorimetry) and FT-IR studies (Aquino, Auriemma et al. 2013).

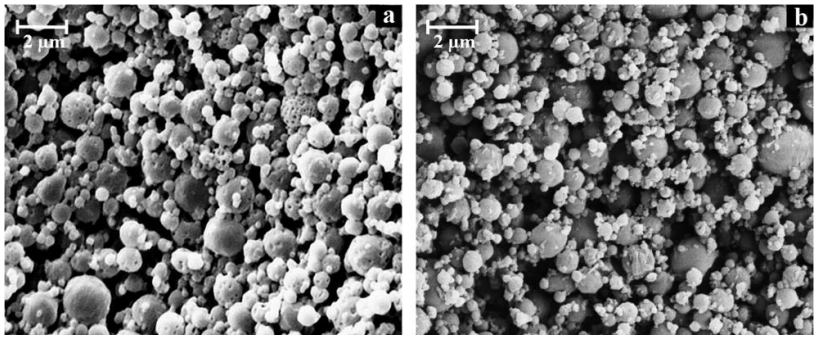


Figure 5. SEM images of GAP microparticles obtained by SAA at different GS/alginate/pectin ratio: GAP1 (a) and GAP4 (b).

Table 3. Composition and particle size distributions data of GAP microparticles produced by SAA at different GS/polymer blend ratio

Sample code	GS/Alginate/Pectin ratio (p/p/p)	Feed (w/v)	Mean diameter (μm)	d_{10} (μm)	d_{50} (μm)	d_{90} (μm)
GAP1	1:1:1	0.5%	2.12	0.89	1.91	3.51
GAP2	1:1:1	1.0%	2.15	0.91	1.82	3.62
GAP3	1:3:1	0.5%	1.71	0.70	1.45	3.09
GAP4	1:3:1	1.0%	1.75	0.73	1.39	3.14

As shown in table 3, GS/polymers ratio affected mean diameter and particle size distribution. Particularly, mean diameter increase from about 1.7 to 2.1 μm, according to the increase in GS/polymers ratio from 1:3:1 (GAP3-GAP4) to 1:1:1 (GAP1-GAP2).

Process yield, drug content, encapsulation efficiency and moisture content of GAP microspheres are reported in table 4. Process yield was satisfying ranging from 65 to 74% and encapsulation efficiency was over 100%. This phenomenon might be dependent on the previously described slight loss of polymers into the saturator, due to the so-called anti-solvent effect. In fact, the addition of carbon dioxide, before the microparticles formation into the precipitation chamber, can induce the precipitation of small quantities of polymer from the feed solution (Reverchon and De Marco 2006).

Table 4. Process yield and properties of GAP microparticles produced by SAA at different GS/polymer blend ratio. Each value represents the mean ± S.D. (n=3)

Sample code	Process yield (%)	Drug content (%)	E.E. (%)	Water content (%)	Drug content* (%)	Water content* (%)
GAP1	74±1.21	35.08±0.36	> 100	5.13±0.24	33.46±0.42	5.86±0.54
GAP2	68±1.09	39.38±0.60	> 100	4.65±0.25	37.89±0.54	5.01±0.42
GAP3	70±1.18	21.24±0.18	> 100	4.98±0.32	19.66±0.21	5.01±0.21
GAP4	65±1.08	29.63±0.09	> 100	4.43±0.21	28.94±0.17	4.56±0.28

*Values registered after 3 months in accelerated storage conditions; e.e. = encapsulation efficiency.

Stability tests, according to ICH accelerated storage conditions, were conducted to verify the stability of GS entrapped in the polymeric matrix by

SAA. As shown in table 4, even after harsh storage conditions, GS content was preserved and only a very slight increase in water uptake was detected for all GAP microparticles, whereas pure GS and SAA processed GS were deliquescent products.

As well known, powders' flow properties may influence the possibility to charge microparticles into fibers or wound dressing material or to spread the powder directly on a wound. Thus, the flowability of powders obtained by SAA was evaluated as ratio between bulk density (ρB) and tapped density (ρT): the lower is this ratio, the lower is flowability. Results indicated that ρB/ρT ratio decreases from 0.50 (GAP2) to 0.45 (GAP3) and 0.41 (GAP 4), in accordance with their reduction in the mean diameter. Only GAP1 ρB/ρT ratio (0.43) was found to be lower than expected; however, this phenomenon can be explained by considering the high surface roughness, partially collapsed and porous structure of GAP1 microparticles (Figure 5a), decreasing flowability of this formulation (Kawashima, Serigano et al. 1998).

The release behavior of the entrapped drug was monitored using vertical Franz-type diffusion cells. Figure 6 reports the permeation curves of both GAP microsystems and, as comparison, the permeation profile of pure GS processed by SAA. As expected for a BCS (Biopharmaceutical Classification System) class III drug, permeation of pure GS is a free diffusion process and total release of the drug is achieved in less than 3 hours. By contrast, GAP microparticles exhibit a prolonged release of the antibiotic following a non-fickian diffusion mechanism (Korsmeyer-Peppas equation with $0.62<n<0.69$) due to the swelling and erosion of the polymers that act as a barrier delaying drug release (Korsmeyer, Gurny et al. 1983).

The complete permeation of GS from GAP microparticles was achieved between 3 and 6 days, according to the increasing polymer blend concentration, while an initial burst effect (till 6 hours) was observed for all formulations. Such intensive release of GS in the first 6 hours of administration could be suitable to prevent infection spreading at the beginning of a local antibiotic therapy.

The antimicrobial activity of the developed formulations against *Staphylococcus aureus* was evaluated by both agar diffusion and time-killing assay (Aquino, Auriemma et al. 2013). Particularly, time-killing assay indicate that, the activity of GS is preserved at 6 days and higher at 12 and 24 days (Figure 7).

In view of these results, it is possible to conclude that the formulation study of the "old" drug, gentamicin, demonstrate that the engineering process by Supercritical Assisted Atomization and use of dextran carriers may

successfully produce dextran based microsystems with high gentamicin content and encapsulation efficiency. Identifying problems related to GS hygroscopicity and instability, and reducing them by appropriate operations led to the improvement in technological properties of the final powder and to a drug prolonged release.

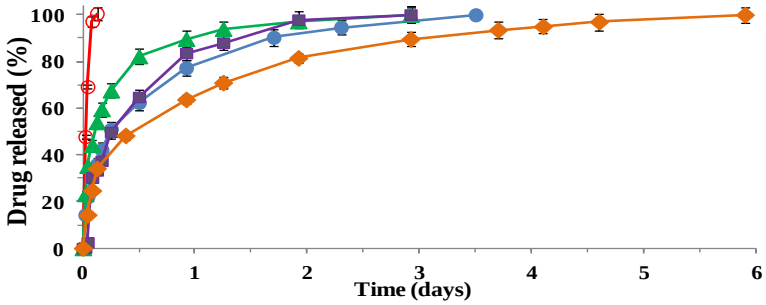


Figure 6. Release profiles of GAP microparticles manufactured by SAA: GAP1 (-●-), GAP2 (-▲-), GAP3 (-■-) and GAP4 (-◆-) in comparison with pure GS produced by SAA (-○-). Mean ± SD; (n=6).

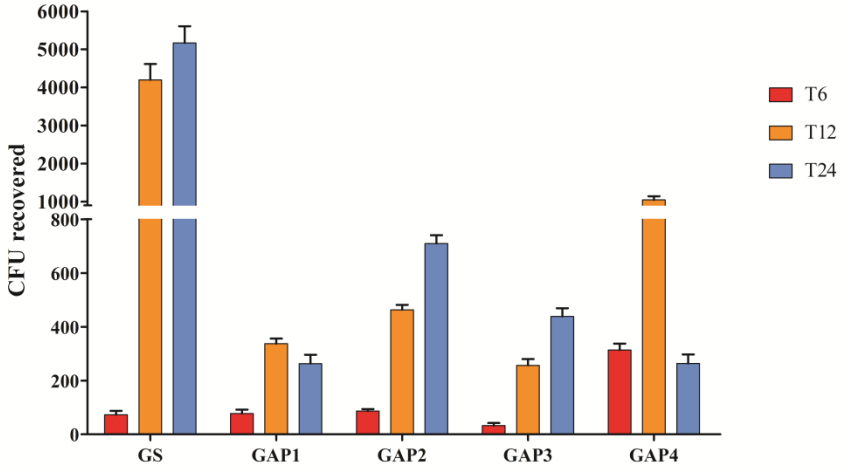


Figure 7. Antimicrobial activity of GAP1-4 against *Staphylococcus aureus* after 6, 12 and 24 days of incubation by time-killing test. GAP1-4 are used at concentrations corresponding to 1.5 mg/ml of gentamicin used as control; CFU recovered = number of *S. aureus colony forming unit*.

Particles engineering also by SAA has not impact on biological properties of GS which activity is retained. The initial burst effect followed by a GS prolonged release suggests that GAP microsystems may be proposed as interesting candidates to be loaded in wound dressing preparations such as fibers and gels or to be administered as self-consistent formulations overstaying wounds.

CONCLUSION

Today, there is a strong need for innovative drug delivery systems able to maximize “new” or “old” drug activity and patient compliance in response to current therapeutic and clinical demands. Particle engineering play a key role in the development of effective, safe and patient-friendly new medicines. In fact, as above discussed, particle engineering approach consists of precisely identify problems of a drug and health need, create and develop a solution that solves the problems or meets the need, defines particle attributes such as narrow particle-size distribution, improved dispersibility, enhanced drug stability, with the goal to optimize bioavailability, obtain a sustained drug release and/or precise targeting. The results presented in this chapter show how particle engineering via two interesting techniques such as Spray Drying and Supercritical Assisted Atomization can be applied to an “old” drug, gentamicin sulfate, to develop new drug delivery systems which meets current therapeutic input and health demands. Accurately planning a product with specified performance goal may led to exploit the potential and re-evaluate the use of an old-generation antibiotic addressing current need of new medicines effective against infections in cystic fibrosis (CF) or chronic wounds. Gentamicin-based microparticles may be developed by a traditional (Spray-drying) or an innovative (SAA) micronization technique and administered by alternative route such as pulmonary or topical application bypassing its well known oto- and nephro-toxicity.

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