Particulate systems for lung delivery of pyrazinamide for tuberculosis treatment

Dinh Duy Pham

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List of Abbreviations

**AB**: Ammonium bicarbonate  
**AC**: Alveolar cell pellet  
**AF**: Alveolar fraction  
**AM**: Amikacin  
**Anti-TB**: Antitubercular  
**ART**: Anti-retroviral treatment  
**AUC**<sub>0-t</sub>: Mean area under the plasma concentration-time curve  
**AUMC**: Areas under the first-moment vs. time curve  
**BALF**: Bronchoalveolar lavage fluid  
**CAP**: Capreomycin  
**CH**: Cholesterol  
**CL**: Clearance  
**C<sub>max</sub>**: Maximal peak plasma concentration  
**DCM**: Dichloromethane  
**DL**: Drug loading  
**DPI**: Dry powder inhalers  
**DPPC**: Dipalmitoyl-sn-glycero-3-phosphatidylcholine  
**DSC**: Differential scanning calorimetry  
**EA**: Ethyl acetate  
**EE**: Encapsulation efficacy  
**EF**: Emitted fraction  
**ETB**: Ethambutol  
**ETH**: Ethionamide  
**Fabs**: The absolute bioavailability  
**FPF**: Fine particle fraction
**GRAS:** Generally recognized as safe

**GSD:** Geometric standard deviation

**HA:** Hyaluronic acid

**HIV:** Human immunodeficiency virus

**HPLC:** High-performance liquid chromatography

**IFN-γ:** Interferon gamma

**INH:** Isoniazid

**IV:** Intravenous administration

**KM:** Kanamycin

**LDH:** Lactate dehydrogenase

**Leu:** DL-Leucine

**LEV:** Levofoxacin

**LPPs:** Large porous particles

**LS:** Liposome suspensions

**MAN:** Mannitol

**MAT:** Mean absorption times

**MDR-TB:** Multi-drug resistant TB

**MIC:** Minimum inhibitory concentration

**MMAD:** Mass median aerodynamic diameters

**MRT:** Mean residence times

**MSLI:** Multi-stage liquid impinger

**NaCl:** Sodium chloride

**NHBE:** Normal human bronchial epithelial cells

**NPs:** Nanoparticles

**PAS:** Para-aminosalicylic acid

**PDR:** Percentage drug retention

**PI:** Polydispersity index

**PK:** Pharmacokinetic parameters
PLA: Poly(L-lactide)
PLGA: Poly(lactide-co-glycolide)
PNAP: Porous nanoparticle-aggregate particle
POA: Pyrazinoic acid
PVA: Poly(vinyl-alcohol)
PZA: Pyrazinamide
RIF: Rifampicin
SAEC: Small airway epithelial cells
SEM: Scanning electron microscopy
SM: Streptomycin
SPC: L-α soybean phosphatidylcholine
T\textsubscript{max}: Time to reach peak concentration
TB: Tuberculosis
Vd: Volume of distribution
W/O/W: Water-in-oil-in-water emulsion
XRPD: X-ray powder diffraction
General Introduction
Tuberculosis (TB) is an infectious disease caused by the bacillus *Mycobacterium tuberculosis* (*M. tuberculosis*). It typically affects the lungs (pulmonary TB) but can affect other sites as well (extrapulmonary TB). The disease is spread in the air when people who are sick with pulmonary TB expel bacteria, for example by coughing. TB remains a major global health problem. It causes ill-health among millions of people each year and ranks as the second leading cause of death from an infectious disease worldwide, after the human immunodeficiency virus (HIV). The latest estimates included in this report are that there were 8.6 million new TB cases in 2012 and 1.3 million TB deaths [1]. Without treatment, TB mortality rates are high which is around 70% died within 10 years [2]. The currently recommended treatment for patients with pulmonary TB is a six-month regimen of four first-line drugs such as isoniazid, rifampicin, ethambutol and pyrazinamide. The success rate of treatment achieve approximately 85% or more [3]. This treatment is effective in both HIV infected and uninfected persons. Treatment failure is mostly related to lack of patient adherence to the drug regimen and to multidrug-resistant tuberculosis (MRT). The treatment of MRT requires second line drugs which are less effective and poorly tolerated. Prevention of resistant tuberculosis needs adequate treatment of each case of tuberculosis and improving of the patient compliance [4].

Pyrazinamide (PZA) is an important first-line antitubercular (anti-TB) drug used in combination with other anti-TB drugs for the treatment of drug susceptible TB and multi-drug resistant TB (MDR-TB) [5]. PZA plays a unique role in shortening *Mycobacterium tuberculosis* (MTB) treatment from the 9–12 months required prior to its introduction to the current standard of 6 months, often referred to as short course chemotherapy [6]. PZA enters *Mycobacterium tuberculosis* in lesions by passive diffusion, is converted to pyrazinoic acid (POA) by pyrazinamidase and is then excreted by a weak efflux pump. The protonated POA diffuses back again into the bacilli under acid conditions and accumulates because the efflux pump is inefficient, causing membrane damage [7]. Therefore PZA is more active against old non-growing bacilli than against young actively growing tubercle bacilli. PZA is usually given at a dose of 20–25 mg/kg daily corresponding to 1.5 to 2g depending of patient weight [8]. Although PZA bioavailability is higher than 90%, a high oral dose over long periods of time leads to hepatotoxicity [6, 9]. PZA lung delivery therefore appears as an interesting alternative to the oral route since it avoids the hepatic first-
pass effect and might help concentrating the drug at its site of action and therefore reduce hepatic side effects.

Pulmonary delivery systems have been widely interested and applied in the treatment of pulmonary-related diseases to reduce side effects of systemic administration and enhance therapeutic effect for targeting delivery. Inhalable dry powders were increasingly developed for the aerosol delivery of anti-TB drugs to lungs. These powders in which the anti-TB drug loaded particles can be delivered directly to the lung have the advantages such as reduction of the dose and frequency administration, resulting in less toxicity and improvement in patient compliance. The particles were designed for targeting the drug to the alveolar macrophage may improve the efficacy and potentially reduce the systemic toxicity of the drug. The high drug concentration localized in the lung may reduce the duration of treatment and prevent the multi-drug resistance (MDR) of TB.

This thesis aims at studying the particulate systems for lung delivery of pyrazinamide for tuberculosis treatment. It is divided into four chapters:

- Chapter I: reviewing the researches related to the field of inhalable formulations in the treatment of tuberculosis.
- Chapter II: the article about «Formulation of pyrazinamide-loaded large porous particles for the pulmonary route: avoiding crystal growth using excipients. »
- Chapter III: the project of article about « In-vivo evaluation of formulation of pyrazinamide-loaded large porous particles for the pulmonary route. »
- Chapter IV: the project of article about « Formulation of Pyrazinamide-loaded Poly(lactic-lactide-co-glycolide) acid nanoparticles: optimization by experimental design. »

References

Chapter I

Formulations for Inhalation in the Treatment of Tuberculosis

Dinh-Duy Pham*, Elias Fattal, Nicolas Tsapis*

Manuscript in preparation
Abstract

Tuberculosis remains a major global health problem which is the second leading cause of death from an infectious disease worldwide, after the human immunodeficiency virus (HIV). Conventional treatment failure is mostly related to lack of patient adherence to the drug regimen and to multidrug-resistant tuberculosis. In this review, we first present recent information of tuberculosis, its pathogenesis and current antitubercular chemotherapies. We then review an interesting strategy to improve first and second line drugs: direct lung delivery of anti-TB drugs using pulmonary delivery systems. In particular we focus on researches performed on inhalable dry powder formulations of antitubercular drugs to target alveolar macrophages where the bacteria develop. Number of studies show that the formulations of anti-TB drugs loaded liposomes, microparticles and nanoparticles in dry powder form can be delivered to the deep lungs for instantaneous and targeted or controlled release. Treatments on infected animals show the significant reduction of viable bacteria number and tissue damage. Therefore, the new formulations are considered to be alternative suitable forms for deliver directly drugs to the lungs.
1. Introduction
Tuberculosis (TB) is an infectious disease caused by the bacillus *Mycobacterium tuberculosis* (*M. tuberculosis*). It typically affects the lungs (pulmonary TB) but can affect other sites as well (extrapulmonary TB). The disease spreads out in the air when people who are sick with pulmonary TB expel bacteria, for example by coughing. TB remains a major global health problem, affecting millions of people each year. It ranks as the second leading cause of death from an infectious disease worldwide, after the human immunodeficiency virus (HIV). The latest estimates are that there were 8.6 million new TB cases in 2012 and 1.3 million TB deaths [1, 2]. Without treatment, TB mortality rates are high, with 70% of infected people dying within 10 years [3]. The currently recommended treatment for patients with pulmonary TB is a six-month regimen of four first-line drugs: namely isoniazid, rifampicin, ethambutol and pyrazinamide. The treatment success rate is at least 85% [4]. This treatment is effective in both HIV-infected and uninfected persons. Treatment failure is mostly related to lack of patient adherence to the drug regimen and to multidrug-resistant tuberculosis (MRT). The treatment of MRT requires second line drugs which are less effective and poorly tolerated. Prevention of resistant tuberculosis needs adequate treatment of each case of tuberculosis and improvement of patient compliance [2].

TB treatment with antitubercular drugs (anti-TB drugs) usually takes a period of 6-9 months, followed by consolidation treatment for a period of 1-2 years, depending of the drug cocktail given. The total duration of treatment may be up to 36 months. With such prolonged duration, the function of the liver and kidneys are overloaded and gradually declining because of the cumulative effects of drugs. Most conventional anti-TB drugs are administered by the oral route, undergo first-pass in the liver followed by metabolism, therefore leading to side-effects. The efficacy of therapy is thus limited by constraints on drug dosage, by adverse drug reactions, especially common among patients concurrently infected with HIV [5], and by inadequate drug distribution in pathological sites. In addition, the recent emergence of resistant strains of TB and the rarity of new anti-TB drugs are threatening to prevent and treat TB in the future. One of the reasons for the emergence of resistant TB strains is the exposure of mycobacteria to sub-therapeutic levels of one or more antibiotics. The conventional therapy by the oral and parenteral routes does not allow to provide therapeutic level of anti-TB drugs to lung lesions containing large numbers of bacteria because these lesions are poorly
vascularized and fortified with thick fibrous tissue. Direct lung delivery of anti-tubercular drugs therefore reveals an interesting strategy to prevent or reduce the spread of tuberculosis and the development of drug-resistant strains.

Pulmonary delivery systems have been widely investigated for the treatment of pulmonary-related diseases to reduce side effects of systemic administration and enhance therapeutic efficacy by delivery the drug directly to its site of action. In particular, inhalable dry powders were developed for the aerosol delivery of anti-TB drugs to lungs. These powders made of anti-TB drug-loaded particles can be delivered directly to the lung. They are designed for targeting the alveolar macrophages where the bacteria develop. Local delivery may lead to high drug concentration localized in the lung may thereby reducing the duration of treatment and preventing multi-drug resistance (MDR). In the present review, after an update on Tuberculosis and its pathogenesis, we describe current anti-TB chemotherapies and their challenges. Finally we review the inhalable dry powder formulations developed during the last 2 decades and their pros and cons.

2. Update on Tuberculosis

2.1. The epidemiology of TB infection

In 2012, the world health organization estimated 8.6 million incident cases of TB and 1.3 million people died of TB. Among these deaths 170 000 arouse from MDR-TB, a relatively high total compared with 450 000 incident cases of MDR-TB. Asia was the region with the highest estimated number of cases (58%), followed by Africa (27%), the Eastern Mediterranean region (8%), Europe (4%) and America (3%). In Fig. 1, the high-income countries including most countries in western Europe, Canada, America, Japan, Australia, New Zealand show the lowest incidence rate (10 cases per 100 000 population). Most of the high-burden countries (HBCs) have rates of around 150-300 cases per 100 000 inhabitants. However, among the HBCs, Brazil and China have markedly lower rates (20-50 cases per 100 000 inhabitants). Mozambique, South Africa and Zimbabwe possess rates above 500 per 100 000 inhabitants. Other countries in the top ten worldwide in terms of incidence rates are mostly situated in Africa. In South Africa and Swaziland, 1 person out of 100 become TB-infected each year [4]. Although,
the incidence of TB fell in 2012, global population growth leads the increase of new cases in absolute numbers [6].

**Figure 1:** Estimated TB incidence rate in 2012 of World Health Organization. Reprinted from Global tuberculosis report 2013 of WHO.

*The global plan to stop TB*

At the beginning of the 21st century, the absolute number of new incident cases of TB continues to rise, despite the fact that TB is a treatable and curable illness. To bridge the gap between what is being done and what could be done, a coalition of partners, including the WHO, established the Stop TB Partnership in 2001. In 2006, this organization launched the ‘Global Plan to Stop TB 2006-2015’ to halt and begin to reverse the TB epidemic by 2015 by reducing 50% relative to 1990 levels of the total number of TB cases and deaths by 2015 [7]. The plan had the following successes: decline in the number of incident cases every year after the rate peaked in 2004; 86% treatment success rate using WHO recommended guidelines; reduction in TB death cases since 2000; death rates compared to 1990 levels are on track to be divided by two in Asia, the Americas and the Eastern Mediterranean [8]. However, 9 million people continue to develop active TB every year and nearly 2 million people die from it. Moreover, among HIV-positive people, 0.4 million people died of TB in 2009. Multidrug-resistant TB and extensively drug-resistant TB (MDR-TB and XDR-TB) continued to
represent major threats to TB control as 440 000 MDR-TB cases emerged and 150 000 deaths were caused by MDR-TB in 2008 [9]. Therefore, TB remains a global health threat and more needs to be done if the plan's targets for 2015 are to be met.

In 2010, the Stop TB Partnership launched the new ‘Global Plan to Stop TB 2011-2015’ from updated the previous Global Plan. The new Plan includes new policy recommendations and costs for anti-retroviral treatment (ART) for HIV-positive patients, more ambitious targets set for fighting MDR-TB, a need for laboratory strengthening and the need for a higher profile research. Without rapid scale-up of TB prevention and treatment cases of MDR-TB would rapidly increase [9].

2.2 Mycobacterium Tuberculosis Pathogenesis

TB is an airborne disease caused by a bacterium: *Mycobacterium tuberculosis* (*M. tuberculosis*), *M. tuberculosis* and seven very close mycobacterial species (*M. bovis, M. africanum, M. microti, M. caprae, M. pinnipedii, M. canettiiand M. mungi*) are known as the *M. tuberculosis* complex [10] and most, but not all, of them may cause disease in humans [11]. Infection occurs when a person inhales droplets containing tubercle bacilli that travel through the upper respiratory tract and bronchi to reach the alveoli of the lungs. The droplets are usually 1-5 μm in diameter and are generated as people suffering from pulmonary or laryngeal TB disease cough, sneeze, shout, or sing [12, 13]. After reaching the alveoli, tubercle bacilli are phagocytosed and accumulated by alveolar macrophages which form Tubercles [14-16]. This may lead to a dormant infection for long time and surviving bacilli may multiply intracellularly to become the source of post primary infection when these foci break down under the conditions of weak host defense mechanisms [13, 17]. The released bacilli are circulating through lymph and bloodstream to different parts of the body including areas where TB disease is more likely to develop, such as the brain, larynx, lymph nodes, lung, spine, bones, or kidneys [13, 18]. Because of bacilli multiplication at these sites, numerous small focii develop throughout the body. This type of wide-spread of infection is known as miliary TB. Within 2-12 weeks, as tubercle bacilli continue to grow in the lungs, macrophages phagocyte them. This stage is characterized by a positive response to tuberculin skin test [12]. The cells form a hard shell (granulomas) that keeps the bacilli contained and under control (TB infection). The tubercle bacilli can remain in the “dormant state” inside macrophages in the granulomas for years [19]. In some people, the tubercle bacilli
overcome the immune system and multiply rapidly, resulting in progression from TB infection to TB disease. Persons who have TB disease are usually infectious and may spread the bacteria to other people. The progression from TB infection to TB disease may occur immediately or many years after infection. Body fluid or tissue from the disease site should be collected for acid-fast bacilli smear and culture. Positive culture for *M. tuberculosis* confirms the diagnosis of TB disease. Patients with pulmonary TB disease usually have a cough and an abnormal chest radiograph, and may be infectious. Although the majority of TB cases are pulmonary, TB can occur in almost any anatomical site or as disseminated disease.

2.3. Current Anti-tuberculosis Chemotherapy

The goals of chemotherapy are to cure patients without relapse, to minimize risk of death and disability, to impede transmission of *M. tuberculosis* to other persons and to prevent the emergence of drug resistance. To achieve these goals, treatment of active TB including a single drug should never be attempted or be added to a failing regimen, the result being development of MDR-TB [20]. As suggested by WHO [6], treatment of TB and drug resistant cases requires a combination of multi-drug regimens over long periods. The initial intensive phase consists of a combination of first-line drugs (three or more) for at least 2 months to reduce the rapidly dividing bacilli load. In the continuation phase, a combination of two or three drugs is used for at least 4 months to sterilize lesions containing fewer and slow-growing bacilli. There are, at present, five ‘first-line’ antituberculosis agents: isoniazid (INH), rifampicin (RIF), pyrazinamide (PZA), ethambutol (ETB) and streptomycin (SM) [21]. For example, table 1 shows the regimen for treatment of incident cases of TB. Recognized ‘second-line’ agents include ethionamide (ETH) or prothionamide, kanamycin (KM) or amikacin (AMK), terizidone/cycloserine (CS), capreomycin (CAP), viomycin and para-aminosalicylic acid (PAS) [22]. Without any formal evaluation, fluoroquinolones (ciprofloxacin, ofloxacin, levofloxacin) have also attained a prominent position in regimens for the treatment of drug resistant tuberculosis; clinical studies are undergoing and may lead to their inclusion in ‘first-line’ regimens [23]. Structures, targets and mechanisms of action of current TB drugs are summarized in Table 2.
**Table 1**: Regimen used for treatment of new smear positive adult patients. Data extracted from [24]

<table>
<thead>
<tr>
<th>Intensive Phase – 2 months</th>
<th>Under 50 kg</th>
<th>Over 50 kg</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>RIF/INH/PZA/ETB</strong></td>
<td>4 tablets/day</td>
<td>5 tablets/day</td>
</tr>
<tr>
<td>Combination tablet 120/60/300/200mg, 5 days per week</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Continuation phase – 4 months</th>
<th>Under 50 kg</th>
<th>Over 50 kg</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>RIF/INH</strong></td>
<td>3 tablets/day</td>
<td>2 tablets/day</td>
</tr>
<tr>
<td>Combination tablet 150/100 mg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Combination tablet 300/150 mg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Drugs</td>
<td>Structure</td>
<td>MIC (µg/mL)</td>
</tr>
<tr>
<td>------------------</td>
<td>-----------</td>
<td>-------------</td>
</tr>
<tr>
<td><strong>First line anti-TB drugs</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| Isoniazid INH    | ![Structure](image1.png) | 0.01-0.2    | -bactericidal activity.  
- inhibition of mycolic acid synthesis by binding tightly to the NADH-dependent enoyl acyl carrier protein (ACP) reductase InhA.  
- other multiple effects on DNA, lipids, carbohydrates and NAD metabolism. | -INH is a pro-drug, requiring oxidative activation by the *M. tuberculosis* catalase peroxide enzyme KatG.  
- Development of INH analogs has not yielded activity against INH-resistant strains. |
| Rifampicin RIF   | ![Structure](image2.png) | 0.05-0.5    | -bactericidal activity.  
- inhibits transcription by binding with high affinity to bacterial DNA-dependent RNA polymerase. | -The precise mechanism, leading to mycobacterial killing, remains unclear. |
| Pyrazinamide PZA | ![Structure](image3.png) | 20-100 At pH 5.5 or 6.0 | -bactericidal activity.  
- disrupts membrane potential and interferes with energy production, necessary for survival of *M. tuberculosis* at an acidic site of infection. | -PZA is a pro-drug, requiring activation to its active form, POA, by the pyrazinamidase enzyme (PZase).  
- POA has an important role to play in new regimens for both susceptible and drug resistant TB. |
| Ethambutol EMB   | ![Structure](image4.png) | 1-5         | -bacteriostatic/bactericidal activity.  
- inhibits cell wall arabinogalactan biosynthesis.  
- other multiple effects on RNA metabolism, transfer of mycolic acids into the cell wall, phospholipid synthesis and spermidine biosynthesis. |                                                                          |
<table>
<thead>
<tr>
<th>Drug</th>
<th>Structure</th>
<th>MIC</th>
<th>Mechanism of Action</th>
<th>Other Notes</th>
</tr>
</thead>
</table>
| Streptomycin (SM) | ![Streptomycin](image) | 2-8 | - Bacteriostatic activity.  
- Binds to the 30S ribosomal subunit, which affects polypeptide synthesis, ultimately resulting in inhibition of translation. | - Less attractive for intensive development against TB due to requirement for parenteral administration. |
| **Second line anti-TB drugs** | | | | |
| Ethionamide (ETH) | ![Ethionamide](image) | 0.6-2.5 | - Bacteriostatic activity.  
- Inhibition of mycolic acid synthesis by binding the ACP reductase InhA. | - ETH, a synthetic compound structurally related to INH, is a pro-drug, requiring activation by the monooxygenase EthA |
| Kanamycin (KM) | ![Kanamycin](image) | 1-8 | - Bactericidal activity.  
- Binding to the bacterial 30S ribosomal subunit and inhibiting RNA-dependent protein synthesis. | - There is cross-resistance between kanamycin and amikacin |
| Cycloserin (CS) | ![Cycloserin](image) | 5-20 | - Bacteriostatic activity.  
- Interrupts peptidoglycan synthesis by inhibiting the d-alanine racemase enzyme (AlrA) | |
| Capreomycin (CAP) | ![Capreomycin](image) | 5-20 | - Bactericidal activity.  
- Inhibits protein synthesis through modification of ribosomal structures at the 16S rRNA. | |
| Paraaminosalicylic acid (PAS) | ![Paraaminosalicylic acid](image) | 1-8 | - Bacteriostatic activity.  
- Inhibits folic acid biosynthesis and uptake of iron. | |
Challenges for the current therapy

Current TB therapy consists in a combination of anti-TB drugs administered by the oral or parenteral route. The oral route is generally the most convenient and usually the safest and least expensive. However, limitations of oral route may include a slower onset of action or potentially significant first-pass effect by the liver. Besides, some drugs are poorly absorbed due to chemical polarity or interference with absorption by ingesta [3]. The parenteral route has the highest bioavailability and is not subject to first-pass metabolism or harsh gastro-intestinal environments. In addition, this administration provides the most control over the actual dose of drug delivered to the body [26]. However, parenteral administration is often perceived as a painful route of administration for which proper treatment adherence can be difficult since it leads to discomfort for the patient and it requires the presence of healthcare workers [27, 28]. It is important to note that both of these conventional administration routes may lead to sub-therapeutic levels of anti-TB drugs at the site of infection due to poor pulmonary distribution of most systemically administered drugs. As a result, drug-resistant strains may appear quickly [29]. Pathologically, although therapeutic drug concentrations are achieved in regions of infection, anti-TB drugs conventionally administered may not penetrate into granulomas which protect mycobacteria. Besides the limitation of the administration routes, the prolongation of treatment is a critical challenge. For anti-TB drugs having narrow therapeutic window and requiring prolonged treatment regimen, patient compliance may be reduced, which may increase the chances of emergence of drug-resistant strains. The extended duration of treatment is also limited by constraints on drug dosage, adverse drug reaction, which is exacerbated in HIV patients, and inadequate drug distribution at the pathological sites [30].

The development of antituberculosis drugs formulations delivered directly to the lungs is a promising avenue to explore. Several groups have investigated direct delivery of anti-TB drugs by nebulization of drug solutions, but also particulate pulmonary drug delivery formulations containing anti-TB agents either in microparticles [31-33], nanoparticles [34] or liposomes [35, 36] or drug powders associated with inhalable excipients [37-39]. This non-invasive route of delivery may also offers additional advantages: i) reduction in dose and frequency to prevent side effect and adverse drug reaction, therefore increasing patient compliance; ii) targeting the drug to the macrophages in the lungs
may improve efficacy and reduce systemic toxicity; iii) giving a high local drug concentration may reduce the duration of treatment and prevent possible multi-drug resistance of TB.

3. Inhalable Antitubercular Drugs

Sacket et al used aminoglycosids solution (streptomycin, kanamycin, amikacin) administered via nebulization for patients with smear-positive pulmonary TB. As a result, aminoglycosides could inhibit bacillary growth in alveoli and prevent transmission, but they could not necessarily affect bacteria inside the macrophages [30]. Condos et al. and Koh et al. have also attempted the aerosolized interferon gamma (IFN-γ), a cytokine involved in the immunological response against mycobacteria, for patients with smears and cultures positive for pulmonary MDR-TB. Patients who received aerosol IFN-g became smear-negative and showed a clear radiological improvement and a reduction in the size of the cavitary lesions [40, 41]. It appears that merely aerosolizing an anti-TB drug may not be sufficient. For efficient bacteria killing, drugs need to be formulated into suitable delivery systems ensuring their rapid uptake into macrophages which harbor the tubercle bacilli.

Many existing anti-TB drugs were considered and suitably formulated for direct delivery to the lungs. They include first-line anti-TB drugs like rifampicin, pyrazinamide and isoniazid, and second-line anti-TB drugs from the groups of aminoglycosides, capreomycin and para-amino salicylic acid. New chemical entities for TB treatment such as TMC207, PA-824, OPC-67683, PNU-100480, AZD-5847, SQ109 and BTZ043 were also considered for pulmonary application against TB [42, 43]. To benefit from lung delivery advantages as well as to overcome some challenges encountered in TB treatment, anti-TB drugs were developed with particulate drug delivery systems for pulmonary administration. Particulate drug carriers include liposomes [44-47], microparticles [37, 48-52] and nanoparticles [53-55]. These inhalable particulate anti-TB drugs have the potential for inhaled therapy. They showed lower drug doses, lower toxicity as well as a lower dosing frequency which were beneficial to patients with pulmonary TB for prolonged treatment.
4. Inhalable Dry Powder Formulations for TB Treatment

Previous research on inhalable anti-TB drugs focused on preparing liquid formulations administrable to the lungs by nebulization. However, liquid formulations as they are typically unstable because of the potential for sedimentation, crystal growth and polymorphism [56]. Therefore, powder formulations seem to be alternative suitable forms for deliver directly drugs to the lungs. They have greater stability than liquid formulation and often do not require the storage at low temperature. They are also packed to protect them from light and humidity, which enhances their stability [57]. Dry powders for inhalation consist of micronized drugs or mixtures of micronized drugs and micronized carriers or formulation into particles of different sizes and micronized carriers, which allows the control of their release from such systems [57, 58]. The blending with micronized carriers, usually lactose or mannitol, in order to be dispersed, and efficiently inhaled. Hence, a considerable number of studies formulating anti-TB drugs into liposomes, microparticles and nanoparticles of different compositions for pulmonary delivery can be presented as follow:

4.1. Liposomal dry powder

Liposomal dry powders for pulmonary delivery include lipospheres and proliposomes. They were formulated with synthetic lipids or phospholipids similar to endogenous lung surfactant. The liposomal dry powders were prepared following 2 step processes. First, drug was encapsulated into liposomes by conventional methods [59]. These liposomes suspensions were then dispersed into the carrier excipients and the mixture was converted into dry powder form by using freeze drying, spray drying, spray freeze drying or supercritical fluid technologies [60]. Liposomal drug dry powder formulations offer the advantages such as: selective localization of drug within the lungs, smaller doses for efficacy, reduced local and systemic toxicity, fewer side effects, high dose carrying capacity, controlled drug release over a prolonged time period, stability and patient compliance [60, 61]. Upon inhalation, drug spontaneously encapsulated into lipid formulations, rehydrated in the lung and then released the drug for a prolonged time [62]. The liposomal dry powders for TB therapy have been recently explored due to their high potential for enhanced lung deposition from which the therapeutic effect index of anti-TB drugs was improved [60, 63, 64]. Liposomes seem to be a feasible system for targeting the lung, they are well tolerated and are non-immunogenic in humans [65-68].
Moreover, they can be phagocytosed by immune cells (e.g., macrophages) and therefore can provide a mean to target antimicrobials and other drug agents to these immune cells [69, 70], where the bacterial infection is located [71]. The fact that tubercle bacilli are phagocytosed and accumulated in lung macrophages means that administration of a drug via liposomal dry powders will be most favorable to allow concentration of the anti-TB drug in the alveolar macrophages [47]. Table 3 presents the recent studies of liposomal dry powders containing anti-TB drugs.

Shah and Misra [72] have prepared liposomal amikacin as a dry powder (AMK-LDPI) and have evaluated the influence of adding fine particles on their in vitro performance. Amikacin loaded liposomes made of hydrogenated soya phosphatidylcholine, cholesterol and saturated soya phosphatidyl glycerol, or stearylamine were prepared by a reverse phase evaporation technique and extruded through 2-μm polycarbonate membranes to a reproducible mean liposomal size of 1.9-2.0 μm. Afterwards they were converted to dry powders by lyophilization using optimized cryoprotectant to achieve maximum percentage drug retention (PDR). Lyophilized liposomes resulted in low fine particle fraction (FPF) value. To optimize FPF, lyophilized liposomes were mixed with sieved Pharmatose 325M (63-90 μm) in the optimum liposome:lactose mass ratio of 1:5 to achieve detachment of liposomal drug from carrier particles. Authors also assessed the effects of adding fines (sieved Sorbolac-400 through no.500) in 5%, 10%, and 15% proportion (wt/wt) and mixing sequence with carrier (63-90 μm) and sieved lyophilized liposomes keeping the final liposome:lactose mass ratio of 1:5 were evaluated. Results showed that the optimum concentration was 10% wt/wt fines. At this level of fines, blending the fines with carrier (63-90 μm) resulted in higher FPF. FPF of AMK-LDPI formulations using Rotahaler as the delivery device at 30, 60, and 90 L/min were found to be 21.85 - 24.6%, 25.9 - 29.2% and 29.5 - 34.2%, respectively. Unfortunately these authors did not perform in vivo experiments.

In 2009, Changsan et al. [63, 71] have encapsulated rifampicin (RIF) into liposomes for delivery to the respiratory tract. RIF-encapsulated liposome suspensions (LS) were obtained from a mixture of L-α soybean phosphatidylcholine and cholesterol using the thin film method and were freeze-dried with mannitol as a cryoprotectant. 200-300nm liposomes were a mixture unilamellar and multilamellar vesicles with 50% RIF encapsulation. Solid-state NMR indicated that RIF was located between the acyl chains
of the phospholipid bilayer and associated with cholesterol molecules. RIF chemical stability was better in the dry powder form in aqueous solution or as a liposomal suspension. The liposome-mannitol powder displayed an MMAD of 3.4 μm and high FPF of 66.8%, suitable for aerosol delivery. Cytotoxicity evaluation of RIF-encapsulated liposomes on normal human bronchial epithelial cells (NHBE), small airway epithelial cells (SAEC) and alveolar macrophages (NR 8383), revealed that cytokines such as IL-1β, TNF-α and nitric oxide were detected at very low level (<50 pg/mL, 100 pg/mL, and <4 μM, respectively). These levels of cytokines would not induce inflammatory effects [73]. RIF liposomes minimum inhibitory concentration on M. bovis was 0.2 μM whereas free RIF MIC was higher (0.8 μM), proving liposomes were more efficient on cell cultures. Once again, no animal study was performed.

Recently, Rojanarat et al. [74-76] have prepared successfully different proliposome powders each containing one of anti-TB drugs such as isoniazid (INH-proliposome), pyrazinamide (PZA-proliposome) and levofloxacin (LEV-proliposome). Anti-TB drugs proliposome powders were obtained as follows: porous mannitol particles (3-5 μm) were suspended in an ethanolic solution containing the anti-TB drug along with L-α soybean phosphatidylcholine (SPC) and cholesterol (CH) before being spray-dried. Mannitol porous particles in these studies play the role of a carrier for efficient lung deposition of liposomes. Since mannitol is very hydrophilic, it is expected to dissolve upon deposition and release liposomes [77]. Encapsulation efficiency ranged between 15 and 45% depending on anti-TB drug hydrophobicity. After reconstitution of proliposomes in water, vesicles in a range from 200 to 1000 nm were observed. Despite these large sizes, vesicles below 600 nm were efficiently taken up by macrophages [78] and larger ones remained outside for extracellular killing of bacilli. The aerosol performance of proliposome powders exhibited mass median aerodynamic diameters (MMAD) from 3 to 5 μm and fine particle fractions (FPF) from 13 to 38%. These powders were tested on respiratory cell lines. Proliposomes did not activate alveolar macrophages (NR8383) to produce inflammatory mediators such as IL-1β, TNF-α and nitric oxide - at a significant level. Proliposomes activity against M. tuberculosis and/or M. bovis were assessed and compared to activities of free drugs. Efficacy against both types of bacteria was similar for free INH and INH proliposomes due to rapid leakage of INH from liposomes. Despite the important burst release of INH, INH proliposomes were more efficient than the free drug on intracellular infection of alveolar macrophages by M. bovis, proving
proliposomes are phagocytosed by macrophages. The efficacy of LEV-proliposomes against *M. bovis* was higher than that of free LEV, whereas similar MICs were obtained against *M. tuberculosis* (0.195 μg/mL). On the other hand, on *M. bovis* infected macrophages, LEV-proliposomes were more efficient than the free drug. PZA and PZA-proliposomes were inactive against both *M. bovis* and *M. tuberculosis*. PZA is known to be inactive on *M. bovis* [79]. The absence of efficacy on *M. tuberculosis* was due to an inadequate pH (7 instead 5.5) than prevents its entrance into the bacteria [80]. *In vivo* repeated dose toxicity study was performed in rats: alanine aminotransferase, aspartate aminotransferase, blood urea nitrogen and creatinine remained within normal biochemical ranges and neither, renal nor liver toxicity were observed after administration of PZA or LEV-proliposome. This experiment was not performed for INH proliposomes.

Although these liposomal dry powder formulations for TB treatment seem promising, they have not been evaluated yet in terms of pharmacokinetics or biodistribution in healthy or diseased animals.
Table 3: Liposomal dry powders contained anti-TB drugs.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Formulation</th>
<th>Method of preparation</th>
<th>Results</th>
<th>Year, Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amikacin</td>
<td>Hydrogenated soybean phosphatidylcholine, Cholesterol</td>
<td>Reverse phase evaporation technique Freeze-drying</td>
<td>AMK-LDPI formulations had good aerodynamic behavior in-vitro.</td>
<td>2004, [72]</td>
</tr>
<tr>
<td></td>
<td>Saturated soybean phosphatidylglycerol or stearylamine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rifampicin</td>
<td>Soybean phosphatidylcholine, Cholesterol, Mannitol.</td>
<td>chloroform film method Freeze-drying</td>
<td>RIF-loaded liposome powders displayed good stability and were suitable for aerosol delivery. Cytotoxicity was detected at very low level.</td>
<td>2009, [63, 71]</td>
</tr>
<tr>
<td>Isoniazid</td>
<td>Soybean phosphatidylcholine, Cholesterol, Microparticulated Mannitol</td>
<td>Spray drying</td>
<td>Proliposome powders showed suitable aerodynamic properties for pulmonary delivery. No toxicity on respiratory-associated cells, no production of inflammatory cytokines</td>
<td>2011, [74]</td>
</tr>
<tr>
<td>Pyrazinamide</td>
<td>Soybean phosphatidylcholine, Cholesterol, Porous mannitol</td>
<td>Spray drying</td>
<td>Proliposome powders were phagocytosed by macrophage cells. INH-proliposome and LEV-proliposome showed anti-mycobacterial activity against intracellular M. bovis and M. tuberculosis, whereas PZA-proliposome was not efficient.</td>
<td>2012, [75]</td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>Soybean phosphatidylcholine, Cholesterol, Porous mannitol</td>
<td>Spray drying</td>
<td></td>
<td>2012, [76]</td>
</tr>
</tbody>
</table>
4.2. Microparticles

Microparticles have been explored as drug delivery carriers for lung administration of drug used in pulmonary disorders like asthma, chronic obstructive pulmonary disease and infectious diseases [81]. Microencapsulation offers the advantage of protecting the drug against pulmonary metabolism, sustained and prolonged drug release. These advantages could be of use for tuberculosis treatment. Microparticulate formulations with extended release would increase patient compliance with long-term therapies by allowing less frequent dosing and would reduce side effects associated with long-term systemic/oral antitubercular therapy [82]. In addition, drug-loaded microparticles could efficiently penetrate to pulmonary alveoli due to their typical mass median aerodynamic diameter of 1–5 μm [83] and then could achieve high intracellular drug concentrations in macrophages [33, 84]. Thereby, they could reduce the duration of treatment and prevent the multi-drug resistance of TB [85].

There are a considerable number of studies formulating anti-TB drugs into microparticles for pulmonary delivery. For anti-TB drugs, these formulations most commonly employ first-line agents such as rifampicin, isoniazid, and pyrazinamide, but the encapsulation of second and third line drugs such as para-aminosalicylic acid, capreomycin, amikacin and PA-824 has also been considered (Table 4). Materials used as excipients range from biodegradable polymers such as polylactide, polyglycolide or poly(lactide-co-glycolide) to lipids, sugars or amino acids.

PLGA microparticles encapsulating rifampicin were prepared by many groups [52], [37], [86] and [87]. O’Hara and Hickey [52] succeeded in preparing RIF-loaded PLGA microspheres with volume median diameters of 2.76 μm and 3.45 μm by spray drying and solvent evaporation, respectively. Suarez et al [37] administered RIF-loaded PLGA microspheres via insufflation or nebulization to guinea pigs, 24 h before aerosol exposure with M. tuberculosis. A dose-effect relationship between RIF-loaded PLGA microspheres and burden of bacteria in the lungs was observed. In addition, guinea pigs treated with RIF-loaded PLGA microspheres presented a significantly smaller number of viable bacteria, reduced inflammation and lung damage than control animals. Suarez et al [51] also evaluated the effect of repeated dosing of the microspheres with a similar experimental approach. Animals treated with single and double doses of RIF-loaded PLGA microspheres exhibited significantly reduced numbers of viable bacteria,
inflammation and lung damage. Besides the evaluation of antimicrobial effect, the
surface characteristics of RIF-loaded PLGA microspheres also play a key role in
predicting particle dispersion, pulmonary deposition [86] and affecting their uptake
efficiency by alveolar macrophage [87]. RIF was also encapsulated into poly(L-lactide)
(PLA) microparticles by spray-drying [88]. RIF-PLA microparticles had a mass median
aerodynamic diameter of 2.22–2.86 μm, with an FPF from 55 to 68%. They did not
exhibit cytotoxicity on J774.A.1 cells. These microparticles were evaluated in vivo in rats
after lung dry powder administration and showed sustained release as compared with
free RIF but similar bioavailability. Alternatively RIF was encapsulated into poly(L-
lactide) (PLA) microparticles by supercritical anti-solvent process as detailed by
Patomchaiviat et al. [89]. The process allowed to obtain discrete microparticles only for
high polymer:drug ratios (9:1 to 6:4). These RIF-PLA microparticles possessed a volume
mean diameter lower than 5 μm and drug content from 3 to 23% by weight. Particles
containing more than 70% PLA by weight showed a sustainable drug release without
initial burst [89]. These studies indicate the potential of RIF-loaded polyester
microspheres delivered by insufflation directly to the lung to treat pulmonary
tuberculosis. Recently, Kundawala et al. have evaluated RIF encapsulation in chitosan
by spray drying [90]. They show that additional excipients such as lactose and leucine
are necessary to yield high values of FPF. These particles however present an
important burst release as compared with polyester microparticles.

Isoniazid (INH) was also formulated into microparticles for sustained delivery to alveolar
macrophages. Sawatdee et al. [91] compared micronized INH formulated with
trehalose, mannose or lactose by physical mixing and spray drying techniques. All INH
dry powder formulations yielded FPF over 60%. Formulations MICs were then assessed
on M. bovis and M. tuberculosis: dry powders were more efficient than standard
isoniazid on M bovis but exhibited similar activity on M tuberculosis. These results might
arise from a difference in INH solubility between crystalline standard INH and spray
dried INH. Kundawal et al [92] have encapsulated INH into chitosan microspheres by
spray drying. Smooth microspheres were obtained with particle size ranging between 4
to 6 μm and with FPF between 55 to 67 %, suitable for lower airways delivery. As
observed with RIF, chitosan microparticles release the drug quite fast (70% within one
hour and 100% within 8 hours). No animal experiments were conducted with these INH-
loaded microparticles.
Some authors have co-encapsulated RIF and INH into inhalable PLA microparticles [49] [33] either by solvent emulsion and evaporation or by spray drying. They show that microparticles are taken up by infected macrophages in a mouse model and induce the activation of innate bactericidal mechanisms that potentiate drug action. Unfortunately a control of drug-free microparticles is missing in the above studies.

Alternatively to RIF, INH was co-encapsulated with rifabutin in PLA microparticles for dry powder inhalation by spray drying [31]. In vitro drug release was rather slow 70% of the payload released in 10 days. Just a few minutes after lung administration, intracellular drug concentration in macrophages were 4-fold higher using microparticles as compared with free drugs. Complete drug kinetics in alveolar macrophages is however missing. Yadav et al [85] showed PLA microparticles sustained high levels of isoniazid and rifabutin in cytosol of macrophages for longer period as compared to free drugs. Microparticles co-localized with intracellular bacteria, and induced a variety of innate bactericidal responses, including induction of free radicals, alteration of mitochondrial membrane potential and apoptosis. It was concluded that inhaled PLA microparticles containing isoniazid and rifabutin can reduce dose frequency and improve the pharmacologic index of the drug combination.

Verma et al (2008) [93] evaluated intracellular concentrations, pharmacokinetics and biodistribution of microparticles containing isoniazid and rifabutin. Microparticles maintained the intracellular concentration of isoniazid for 24h (0.25 μg/10⁶ cells) and rifabutin for 72h (0.5 μg/10⁶ cells). The pharmacokinetic profiles of the two agents consequent to oral dosing, as in conventional drug delivery, are not modified apart from increasing durations of action. The inhalation of these microparticles led to 15 to 20 fold higher drug concentrations in the lung lumen (BAL fluid) than via intravenous route at every time point. Moreover, Chan et al. (2012) produced the micron-sized dry powder by in a single step spray drying process without excipients. Especially, the dry powder contained three first-line anti-TB drugs such as pyrazinamide, rifampicin and isoniazid) in a ratio of approximately (5:2:1). It was obtained a mass median aerodynamic diameter of 3.5 μm and FPF of 45% which indicated excellent aerosol performance. The proportions of the three antibiotics in individual particles were indicated to be 400:150:75 w/w (PZA:RIF:INH). This excipient-free triple antibiotic DPI formulation could be used as a significant enhanced treatment for TB.
The above literature indicates that polymer matrices microparticles have been used in pulmonary drug delivery to control or sustain the release of anti-TB drugs of which polyesters and chitosan have been investigated. Polyester microparticles achieved the sustained drug release without initial burst into the lung and the low drug loading (<25%), whereas chitosan microparticles exhibit burst release and a high drug loading (>45%). Unfortunately, a toxicity evaluation of these microparticles was not carried out completely.

Due to the rising incidence of multidrug-resistant TB (MDR-TB), many researchers developed porous particles containing second-line anti-TB drugs e.g. para-aminosalicylic acid (PAS), capreomycin (CAP) or third-line e.g. PA-824. Tsapis et al (2003) [94] formulated para-aminosalicylic acid (PAS) into inhalable large porous particles for direct delivery into the lungs. The microparticles were produced by spray drying and possessed optimized physical properties for deposition throughout the respiratory tract. After 5 mg of the dried powders was administered to rats via insufflation, PAS concentrations were maintained at therapeutic concentrations in the lung tissue for at least 3h. The measured concentration in the lung lining fluid was thirteen times as much as that in plasma at 15 min but could not be measure at 3h. Other researchers like García-Contreras et al [95] and Fiegel et al [96] have spray-dried a solution containing 80% capreomycin (CAP) and 20% leucine to manufacture large porous particles for inhalation. The particles were obtained a volume diameter of 4.19 μm, a MMAD of 4.99 μm and a FPF of 47.7%. Aerosols loaded with 73% CAP were shown to possess good aerosolization properties and physical-chemical stability for up to 3 months at room temperature. In pharmacokinetic studies, CAP plasma concentrations via IV and insufflation were not different in guinea pigs from 2 to 6h after administration. Capreomycin was also cleared at a lower rate by the pulmonary route than by others, resulting in a significantly longer t1/2. Authors claim that aerosol CAP can lead to high drug concentrations in the lungs but drug concentrations were not measured in the broncho-alveolar lavage. After the high aerosol dose of capreomycin was administrated on TB-infected guinea pigs, their lungs showed significantly less inflammation, bacterial burdens, and percentages of lung tissue affected by granulomas and caseous necrosis (by histopathology) than those for any other treatment. Similarly, Sung et al [97] and Garcia-Contreras et al [39] formulated PA-824, a nitroimidazopyran with great promise in treating both active, latent and MDR-TB, into inhalable porous
particle by spray drying. The particles were 4.14 μm in size and had desirable aerosol properties for pulmonary delivery, as indicated by a MMAD of 4.74 μm and an FPF of 53.3%. These porous particles maintained physical, aerodynamic, and chemical stability at room temperature for 6 months and under refrigerated conditions for more than one year. For the first 12h after administration, plasma concentrations of PA-824 via oral administration and insufflation achieved equivalent values at the same dose. However, PA-824 remained locally in the lungs for 32h in guinea pigs dosed by insufflation, whereas the drug was cleared more rapidly in guinea pigs dosed by oral route. For tuberculosis infected guinea pigs, treatment with aerosolized PA-824 particles appeared to reduce manifestations of disease in the lungs and spleen. Compared to untreated animals, those treated by low and high doses of inhaled PA-824 aerosols exhibited a significant reduction of inflammation, number of viable bacteria and tissue damage. These results suggest that inhalation delivery of second-line anti-TB have considerable potential to provide more effective therapy for MDR-TB.
Table 4: Microparticles contained anti-TB drugs.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Formulation</th>
<th>Method of preparation</th>
<th>Results</th>
<th>Year, Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rifampicin</td>
<td>Poly(lactide-co-glycolide) microspheres</td>
<td>Solvent evaporation, Spray drying</td>
<td>Microspheres showed properties suitable for inhalation therapy.</td>
<td>2000, [52]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Microspheres reduced significantly number of viable bacteria, inflammation and lung damage in guinea.</td>
<td>2001, [37]</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Double doses of microspheres significantly reduced numbers of viable bacteria, inflammation and damage in lung as well as in spleens.</td>
<td>2001, [51]</td>
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<td></td>
<td></td>
<td></td>
<td>Microspheres were shown the killing effect of intracellular <em>M. tuberculosis</em> Kurono and preventing granuloma formation in some lobes.</td>
<td>2006, [98]</td>
</tr>
<tr>
<td>Polylactide microparticles</td>
<td>Supercritical carbon dioxide</td>
<td>Solvent evaporation, Freeze drying</td>
<td>RIF release was accelerated by adsorption of pulmonary surfactant on the particle surface. The changes in surface properties of microspheres affected their uptake efficiency by alveolar macrophage.</td>
<td>2007, [87]</td>
</tr>
<tr>
<td>Poly lactic acid or poly (lactic-co-glycolic acid) microspheres</td>
<td>Spray drying</td>
<td>The effect of parameters of manufacture on microparticle characteristics.</td>
<td>2008, [89]</td>
<td></td>
</tr>
<tr>
<td>Isoniazid</td>
<td>Trehalose, Mannose or Lactose</td>
<td>Physical mixing, Spray drying</td>
<td>Dry powder formulations were more effective against <em>M. tuberculosis</em> than pure isoniazid in the broth microdilution method.</td>
<td>2006, [91]</td>
</tr>
<tr>
<td></td>
<td>Chitosan microsphere</td>
<td>Spray drying</td>
<td>The chitosan microspheres showed physicochemical properties deemed suitable for inhalation therapy.</td>
<td>2011, [92]</td>
</tr>
<tr>
<td>Rifampicin and isoniazid</td>
<td>Poly-D,L-lactide microparticles</td>
<td>Combination of solvent extraction and evaporation.</td>
<td>Inhalatable microparticles could be prepared and were taken up by cultured macrophages. They led to higher intracellular drug concentrations than drug alone.</td>
<td>2001, [49]</td>
</tr>
<tr>
<td>Drug Combination</td>
<td>Formulation Method</td>
<td>Aerosolization Properties</td>
<td>References</td>
<td></td>
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</tr>
<tr>
<td>Isoniazid and rifabutin</td>
<td>Spray-drying</td>
<td>Microparticles induced innate bactericidal response in infected murine macrophages which potentiate drug action on the bacterium and contribute to their high efficacy in experimental tuberculosis.</td>
<td>2007, [33]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Spray-drying</td>
<td>Microparticles with satisfactory aerosol characteristics and sustained the release of drugs for 10 days. Inhalable microparticles targeted macrophages and led to 20 times higher intracellular concentrations than drug solutions.</td>
<td>2007, [31]</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Inhalable microparticles maintained the intracellular and plasma concentration for long time, 15 to 20 fold higher drug concentrations in BAL than via intravenous administration at every time point were obtained.</td>
<td>2008, [93]</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Microparticles led to high levels of drugs in cytosol of macrophages for longer period and induced a variety of innate bactericidal responses.</td>
<td>2009, [85]</td>
<td></td>
</tr>
<tr>
<td>Rifampicin, isoniazid and pyrazinamide</td>
<td>Excipient-free</td>
<td>Spray-dried</td>
<td>Particles were obtained excellent aerosol properties.</td>
<td>2013, [99]</td>
</tr>
<tr>
<td>Para-aminosalicylic acid</td>
<td>Large porous particles made of DPPC</td>
<td>Spray-dried</td>
<td>High drug concentrations in BAL. Therapeutic concentrations in the lung tissue for at least 3 h after insufflation.</td>
<td>2003, [94]</td>
</tr>
<tr>
<td>Capreomycin sulfate</td>
<td>Porous particles made of Leucine</td>
<td>Spray-dried</td>
<td>Good aerosolization and stability properties. Longer half-life in guinea pigs. Particles reduced significantly lung bacterial burden, inflammation, and histopathological damage.</td>
<td>2007, [95, 96]</td>
</tr>
<tr>
<td>PA-824</td>
<td>Porous particles made of Leucine and DPPC</td>
<td>Spray-dried</td>
<td>Good aerosolization and stability properties. Particles remained locally in the lungs for long period post administration. Particles help to reduced significantly bacterial burden and tissue damage in lungs as well as in spleen.</td>
<td>2009, [97]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2010, [39]</td>
<td></td>
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</table>
4.3. Nanoparticles

Besides the advances obtained using microparticles, researchers also considered nanoparticles for antitubercular inhaled therapy (Table 5). Inhalable nanoparticles can achieved a high drug loading [100], a better mucosal cell adherence, hence, enhancing net drug delivery to the lungs [101]. Moreover, nanoparticles were efficiently taken up by alveolar macrophages where M. tuberculosis, reside and subsequently release their payload [102]. Nanoparticles containing anti-TB drugs are usually made from biocompatible and biodegradable materials such as alginate [103], PLGA [53] or solid lipids [55]. These nanoparticles are administered to the lungs by nebulization. Nebulization that consists of finely spraying liquid formulations has several disadvantages for practical implementation in the case of TB such as: long delivery times, low delivery efficiencies, stability of sustained-release formulations in aqueous solution and access to clean water [104]. On the other hand, it is difficult to efficiently deposit individual dry nanoparticles in the deep lung because the majority of the dose is exhaled [105]. To overcome these issues of storing and delivering nanoparticles to the lungs, a particulate form incorporating nanoparticles into micron-scale structures can be used. This type of system was first proposed by Tsapis et al. [106] with non degradable particles. Since then it was adapted to biodegradable systems. Ohashi et al (2009) [107] prepared RIF loaded PLGA nanoparticles agglomerated in mannitol microspheres ((RFP/PLGA)/MAN microspheres) by a four-fluid nozzle spray drier in one step. The mean diameter of RIF loaded PLGA nanoparticles and of (RFP/PLGA)/MAN microspheres were 213 nm and 3.2 μm. Approximately 7% of the (RFP/PLGA)/MAN microspheres were deposited in stages 6–7 of an ACI. Although authors claim this corresponds to a high aerosol performance, it could probably be optimized. In vivo uptake of RIF by alveolar macrophages in rats was evaluated the uptake of RIF from the (RFP/PLGA)/MAN microspheres was higher than of RIF loaded MAN and RIF loaded PLGA microspheres. In vivo imaging showed that PLGA nanoparticles were difficult to be cleared from the lungs, resulting in their retention. Similarly, Sung et al have formulated RIF in a dry powder of ‘porous nanoparticle-aggregate particle’ (PNAP). RIF was encapsulated into PLGA nanoparticles (195 nm) by a solvent evaporation process before being converted to PNAPs by spray drying. PNAPs contained varying amounts of nanoparticles. They had a volume median diameter of 4.2 μm and exhibited properties suitable for deposition in the respiratory tract with FPF of 35.5 - 44.7%. In vitro release showed an initial burst
of approximately 80% RIF content, with the remainder available for release over a period beyond eight hours. Rapid release of RIF led to the early appearance of drug in the plasma in-vivo. However, PNAPs allowed prolonged RIF concentration in the lungs up to eight hours after insufflation. From the pharmacokinetic point of view PNAPS were as efficient as LPPs of Rifampicin.

Recently, Vadakkan et al have developed an amphiphilic lipopolymer system having self-assembling properties to load RIF into inhalable spray-dried microparticles [108]. These particles could be disaggregated into nanomicelles. The generated particles were found to have MMAD in respirable size range (< 3 μm) and FPF of 67.88% with low bulk density (0.194 g/cm³). The controlled delivery of RIF was achieved over a period of 24 hours with initial burst release of 45% within first 2 h. In conclusion, these drug delivery systems have the potential to provide therapeutic advantages for tuberculosis treatment.
**Table 5: Nanoparticles contained anti-TB drugs.**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Formulation</th>
<th>Method of preparation</th>
<th>Results</th>
<th>Year, Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rifampicin</td>
<td>PLGA nanoparticle-containing mannitol microspheres</td>
<td>spray drying</td>
<td>Microspheres showed good <em>in vitro</em> aerosol performance and efficient uptake by alveolar macrophage cells. It was difficult to clear the nano-sized PLG particles, resulting in their retention.</td>
<td>2009, [107]</td>
</tr>
<tr>
<td>Porous PLGA nanoparticle-aggregate</td>
<td>solvent evaporation</td>
<td>spray drying</td>
<td>The PNAP formulations possessed properties suitable for efficient deposition in the lungs. An initial burst of rifampicin with the remainder available for release beyond eight hours was observed. RIF concentrations remained detectable in lung tissue and cells up to and beyond eight hours.</td>
<td>2009, [109]</td>
</tr>
<tr>
<td>Cationic lipopolymeric nanomicelle</td>
<td>Spray drying</td>
<td></td>
<td>Particles were obtained good aerosol performance. The controlled delivery of drug was achieved over a period of 24 hours with initial burst release.</td>
<td>2013, [108]</td>
</tr>
</tbody>
</table>
5. Conclusion

Despite advances in disease management, tuberculosis remains an important health problem worldwide. Poor patient adherence to treatment due to side-effects and treatment extended duration may lead to the emergence of drug resistant strains. Research is therefore evaluating different strategies to improve drug efficacy and reduce treatment duration. Among these strategies, direct delivery of drugs to the lungs seems appealing. In particular, various dry-powders for inhalation such as liposomes-agregates, microparticles and nanoparticles can be delivered to the deep lungs for instantaneous and targeted or controlled release. Treatments on infected animals show significant reduction of viable bacteria number and tissue damage. However, these formulations need to be evaluated in terms of chronic lung toxicity before being considered for clinical studies. Inhalable dry powders might also be considered as an adjunct to conventional therapy to decrease the dosing frequency and duration of TB therapy.

References


Chapter II

Formulation of Pyrazinamide-loaded Large Porous Particles for the Pulmonary Route: Avoiding Crystal Growth Using Excipients

Dinh-Duy Pham*, Elias Fattal, NourEddine Ghermani, Nicolas Guiblin, Nicolas Tsapis*

Published Article
Formulation of pyrazinamide-loaded large porous particles for the pulmonary route: Avoiding crystal growth using excipients

Dinh-Duy Pham, Elias Fattal, NourEddine Ghermani, Nicolas Guiblin, Nicolas Tsapis

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†University of Medicine and Pharmacy, Faculty of Pharmacy, Department of Pharmaceutics, Ho Chi Minh City, Viet Nam
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Article Info
Article History:
Received 12 February 2013
Received in revised form 3 April 2013
Accepted 16 April 2013
Available online 17 April 2013

Keywords:
Pyrazinamide
Pyramethamine
Large porous particle
Lung delivery
Crystallization

Abstract
We have designed a novel formulation of pyrazinamide (PZA), an antitubercular drug within large porous particles intended for deep lung delivery. By simply spray-drying PZA, we have obtained crystalline particles of the β polymorph of PZA that were unstable and not adapted for lung administration. Several excipients were added to the formulation to obtain stable large porous particles with a median size above 6 μm and a low tap density. Although a combination of leucine and ammonium bicarbonate (AB) allowed to reduce tap density and to increase particle size, these excipients were not sufficient to prevent crystalization and promote stability. The addition of hyaluronic acid (HA) in combination with dipalmityloleoylphosphatidylcholine (DPPC) allowed to obtain stable porous crystalline spherical particles adapted for deep lung delivery. The optimized formulation obtained by spray-drying 0.9 g PZA, 0.2 g leucine, 0.4 g HA, 0.2 g DPPC and 2 g AB in a mixture of ethanol-water (70/30, v/v) presents a median size of 5.8 ± 0.1 μm and a tap density around 0.08 ± 0.01 g/cm³. The estimated aerodynamic diameter is around 1.75 μm and the powder is stable for more than 4 weeks of storage.

1. Introduction

Tuberculosis (TB) is a contagious-infectious disease primarily caused by Mycobacterium tuberculosis, an aerobic pathogenic bacterium that infects mainly the lungs (Ducau et al., 2006). World Health Organization estimates that more than one-third of the world population is infected with M. tuberculosis. The disease spreads out when conditions of poverty, malnutrition and limited access to healthcare are met. Therefore, developing countries which have the incidence of TB are the majority of global TB rates (Thaker and Elston, 2008). TB treatments consist in a combination of antituberculogerous drugs to ensure successful outcome and to prevent the development of resistance (Groton, 1995; Mutschler and Derendorf, 1995). Antituberculous drugs in the first-line of defense include: isoniazid, rifampicin, pyrazinamide, streptomycin and ethambutol. The first four drugs possess bactericidal action while ethambutol is bacteriostatic. Isoniazid, rifampicin and streptomycin are pharmacologically active against TB only in the extracellular environment, whereas pyrazinamide is active against a population of non-growing bacteria (Patterson et al., 1998).

Pyrazinamide (PZA) helps shortening TB therapy from 9 months to 12 months to the current 6 months (Mitchison, 1985; Zhang and Mitchison, 2003). Indeed PZA is active against a population of non-growing, persistent tubercule bacilli residing in an acidic pH environment that are not killed by other TB drugs (Hefer and Lindholm-Levy, 1992; Mitchison, 1985; Zhang and Mitchison, 2003). The mechanism of action of PZA is described as followed. When PZA diffuses into the TB bacilli, it is deaminated into pyrazinoic acid (POA), the active moiety. Then, POA is excreted out of bacteria as ammonium pyrazonate. This ammonium salt diffuses more readily than PZA and also allows removing gaseous NH₃ from the bacterial cell (Zhang and Mitchison, 2003). After being excreted, protonated POA diffuses back into the cell. Diffusion, which occurs through the cell wall in an acidic environment, requires no energy. However, intra-cellular POA is only excreted by an inefficient efflux pump which requires energy to function. Moreover, the bacterial metabolism decreases under conditions of dormancy, and less energy is available. Therefore, the fact that the POA accumulates inside the cell and destroys it may be due to destruction of the cell membrane or to other mechanisms (Zhang et al., 2003).
This particularity of PZA, makes it the only anti-TB drug available that kills dormant organisms more effectively than those that are actively metabolized (Hu et al., 2006; Zhang et al., 2002). As a result, PZA is indispensable in any drug combination of current TB treatment regimens.

The recent increase in the emergence of drug-resistant strains of M. tuberculosis and the lack of new anti-TB drugs is threatening the containment of TB. The emergence of drug-resistance arises from mycobacteria exposure to sub-therapeutic levels of one or more antibiotics and problems of patient compliance to long treatments (Mottul et al., 2009; Sacks et al., 2001). A new drug delivery system that would be able to prevent the spread of TB and slow down or prevent the development of drug-resistant strains would therefore be of great help. Lung lesions containing a large number of bacteria are poorly vascularized and are also fortified with thick fibrous tissues, therefore, conventional therapy with oral and parenteral routes may provide sub-therapeutic levels of anti-TB drugs to these highly sequestered organisms. The administration of drugs by the pulmonary route to the lungs would allow to achieve higher drug concentrations in the vicinity of these lesions (Tsapis et al., 2003; Wood and Swanson, 2007). The combination of oral conventional antituberculosis therapy with inhaled anti-TB therapy may achieve effective therapeutic concentration of the drug in lung lesions and treat the resident mycobacteria.

Large porous particles (LPPs), characterized by geometric sizes greater than 5 μm and mass densities lower than 0.4 g/cm³, have been recently introduced for both local and systemic applications by the pulmonary route to the lungs (Edwards et al., 2002; Edwards et al., 1998). A major advantage of LPPs relative to conventional inhaled/parenteral aerosol particles is their aerosolization efficiency (Dunbar et al., 1998; Edwards and Dunbar, 2002). This allows the supply of large drug masses using a simple inhalation device (Edwards, 2002). In addition, these particles have the potential to avoid alveolar macrophage clearance due to their large geometric size (Kawaguchi et al., 1986; Krems and Strauss, 1961; Rudd and Muller, 1992), enabling sustained drug release in the lungs (Vanhever et al., 1998a). These characteristics suggest that inhalation of LPPs may be beneficial in the treatment of tuberculosis (TB) by delivering drug directly to the primary site of infection to achieve therapeutic local drug concentrations with lower systemic exposure than oral dosing. Since the lungs are directly targeted, total body doses are lower, leading to a decrease of potential drug resistance build-up.

In this article, we studied the effect of formulation excipients on characteristics of PZA loaded large porous particles. Dry powders were prepared by spray-drying using generally recognized as safe (GRAS) excipients. The concentration of excipients was investigated in order to optimize the characteristics of spray-dried PZA powders and also to prevent PZA crystallization upon storage. Indeed, PZA crystals do not possess a size distribution that is adapted for lung delivery, being too large and too dense for a correct aerosolization and lung deposition.

2. Materials and methods

2.1. Materials

Pyrazinamide was acquired from Fluka, with specified purity greater than 98%. 1,2-Dipalmitoyl-sn-glyceryo-3-phosphatidylcholine (DPPC) was provided by Corden Pharma (Switzerland) and hyaluronic acid, sodium salt 95% (HA) (MW ~1000 ka) by Acros Organics. Ammonium bicarbonate (AB) and n-lesion (Leu) were provided by Sigma-Aldrich (France). Ethanol absolute in analytical grade was obtained from Carlo Erba Reagents (France). Water was purified using a RIOS/MilliQ system from Millipore (France).

2.2. Large porous particle preparation and yield calculation

Large porous particles were obtained by spray drying using a mini spray dryer Bfchi B-250 (Bacharach, Switzerland) equipped with a 0.7 mm diameter two-fluid nozzle, which operates in a co-current mode. The spray drying parameters such as air flow rate, feed flow rate, inlet temperature and aspiration are reported in Table 1. DPPC was dissolved in 700 mL ethanol. Meanwhile, PZA and Leu were dissolved into 300 mL water. Then, HA was added into the aqueous solution and stirred using a magnetic stir bar for about 1 h until dissolution. Afterwards, AB was dissolved into the aqueous solution and subsequently ethanol solutions and aqueous solutions were mixed immediately prior to atomization. The final concentration of AB in the ethanol/water mixture varied from 0 to 5 g/L. The final solid content of the solution was 2 g/L omitting ammonium bicarbonate since this compound is sublimated during the drying process. Powder samples were stored at room temperature under vacuum in a desiccator immediately after spray drying to limit moisture uptake by samples between production and testing. The yield was calculated as a percentage by dividing the mass of the powder
Fig. 2. Top: DSC thermograms of F2A raw material during the first heating step (a) and after cooling in the room temperature (b'). DSC thermograms of spray-dried F2A during the first heating step (b) and after cooling to the room temperature (b'). Bottom: zoom on solid–solid transitions observed for raw F2A (a,b) and spray-dried F2A (b,c).

2.3. Particle size distribution

Particle size distribution was measured by light diffraction using a Mastersizer 2000 equipped with a Scirocco dry disperser (Malvern Instruments, France) at a dispersing pressure of 1 bar. The refractive index used was 1.5 (real part). Data obtained were expressed in terms of the particle median diameter at 50% of the volume distribution ($D_{50}$). The span of the volume distribution, a measure of the width of the distribution relative to the median diameter, was calculated according to Eq. (1). A large span is indicative of a more heterogeneous size distribution. Values presented are the average of at least 3 determinations, errors bars indicate the standard deviation (S.D.).

\[
\text{Span} = \frac{D_{90} - D_{10}}{D_{50}}
\]  

(1)

2.4. Powder tap density

The powder density was evaluated by tap density measurements using a tapping apparatus (Pharma test PT-TD1). Tap density ($\rho$) was measured in a 10 mL glass measuring cylinder filled with a fixed initial volume of powder around 8 mL. The tap density

Fig. 3. X-ray diffraction patterns of raw F2A (a) corresponding to the α-polymorph and spray-dried F2A (b) corresponding to the β-polymorph.

Fig. 4. Influence of AB concentration on particle median size just after spray-drying (top, left). Size differences are significant as attested by ANOVA (*). Effect of storage time on particle size stability. Size differences are significant as attested by t-test (**).
was determined after 1000 taps form a constant height. Assuming efficient packing, the tap density of monodisperse spheres is approximately a 21% underestimate of the true particle density due to the void spaces between particles. Although polydispersity may reduce the void volume between particles, this is probably counterbalanced by imperfect packing (Vanbever et al, 1998b). Measurements were performed in duplicate.

2.5. Morphology and shape

The morphology and shape of particles composing the powder were examined by scanning electron microscopy (SEM) using a LEO1530 microscope (LEO Electron Microscopy Inc, Thorn-wood, Ny) and operating between 1 kV and 3 kV with a filament current of about 0.5 μA. Powder samples were deposited on a carbon conductive double-sided tape (Europemex, France). They were coated with a palladium-platinum layer of about 4 nm, using a Cressington Sputter-Coater 208HR with a rotary planetary tilt stage, equipped with a MIM-20 thickness controller.

2.6. Differential scanning calorimetry

The thermal properties of the powders were analyzed using differential scanning calorimetry (DSC) (DSC7, PerkinElmer, USA). Thermograms were analyzed using Pyris software. An empty aluminum pan was used as the reference for all measurements. A sample (1–5 mg) of powder was placed in hermetically sealed 40 μl aluminum pan and analyzed. DSC runs were conducted from 30°C to 210°C at a rate of 10°C/min, then samples were quickly cooled down to 30°C and heated again from 30 to 210°C at a rate of 10°C/min. Calibration was achieved using indium (Tmelt = 156.6°C) as well as Zinc (Tmelt = 419.4°C). The onset and peak temperatures and enthalpy of transition (ΔHf) were determined for each peak.

2.7. X-ray powder diffraction (XRPD)

PZA and powder crystallinity were analyzed using X-ray powder diffraction (XRPD). XRPD patterns were measured on a Bruker D2 diffractometer equipped with an XFlash detector in SPMS laboratory – Center of diffraction – Ecole Centrale Paris using Ni-filtered CuKα radiation. Data were collected over an angular range comprised between 5° and 40° (2θ) with a step size of 0.01°, a counting time of 5 s/step.

2.8. Powder stability

Powder stability was assessed by leaving samples to age at room temperature under vacuum in a desiccator (temperature comprised between 15°C and 25°C). Particle size was performed on aged samples at τ 0, 2, and 4 weeks.

2.9. Statistical analysis

An unpaired two-tailed t-test was used to test the differences between the means of two groups. One-way ANOVA was used to test for differences between multiple groups. P-values < 0.05 were considered as statistically significant.

3. Results and discussion

3.1. Characterization of raw pyrazinamide and spray-dried pyrazinamide

Pyrazinamide is quite rigid molecule with only two relevant conformers, having quite different energies, identified in the gas phase (Borba et al, 2010; Cig et al, 2005). Therefore, by contrast with flexible molecules, PZA has no conformational polymorphism (Estevos de Castro et al, 2007; Nanjia, 2008). On the other hand, the different possibilities of intermolecular hydrogen bonds can promote the occurrence of packing polymorphism. In fact, four packing polymorphs of PZA have been identified a, b, c, and d (Takaki et al, 1960). In a recent report, it has been shown that the α polymorph is the most stable while β, γ, and δ polymorphs are prone to transform into the α polymorph. However, upon heating, the α, β, and δ polymorphs give rise to the γ polymorph, with some superheating being observed (Castro et al, 2010).

To investigate the packing polymorphism of PZA after the spray-drying process, DSC, XRPD, and SEM were used to characterize the thermodynamic, morphological and crystalline properties of the raw PZA and spray-dried PZA. Spray-dried PZA was prepared by spray-drying a solution containing 2 g/l PZA in the ethanol-water mixture (70/30, v/v) using spray-drying conditions detailed in Table 1.

Raw PZA exists as needle-shaped crystals (SEM, Fig 1, left). On the other hand, after spray-drying, PZA exists as sheet-like crystals as observed by SEM (Fig 1, right). The spray-dried PZA crystals are not physically stable. Indeed, the particle median size increases from 4.7 ± 0.1 μm to 28.5 ± 0.2 μm (unpaired t-test, P < 0.05) after 4 weeks of storage (Fig 4). In addition, the spray-dried powder
possesses a relatively high tap density of 0.45 ± 0.01 g/cm² that makes it unsuitable for lung delivery (Fig. 5).

DSC thermograms on raw and spray-dried PZA are presented in Fig. 2. Before any thermal treatment, raw PZA presents two endothermic peaks at 153.5 °C and 188.3 °C (Fig. 2, curve a), while after thermal treatment only one endothermic peak at 189.5 °C is visible (Fig. 2, curve b). The peak around 189 °C corresponds to the melting transition as observed by Castro et al. (2010) whereas the transition occurring around 150 °C most probably corresponds to a solid–solid phase transition. Before thermal treatment, the spray-dried PZA thermogram presents three sequential endothermic peaks at 159.3 °C, 151.7 °C (solid–solid phase transitions) and 188.0 °C (melting) (Fig. 2, curve b), whereas after thermal treatment only one endothermic peak at 186.0 °C is visible (melting) (Fig. 2, curve c). These results suggest that spray-dried PZA exists as the α form and can be converted into the α polymorph at 130 °C and then into the γ polymorph at 151 °C upon heating, whereas most probably raw PZA corresponds to the polymorph as observed by Castro et al. (2010).

To identify crystalline forms of raw PZA and spray-dried PZA spray-drying, X-ray diffraction was used (Fig. 3). The X-ray diffraction pattern of raw PZA (Fig. 3a) shows intense peaks at 2θ equal to 7.62°, 13.64°, 15.27°, 15.57°, 17.60°, 20.40°, 22.46°, 24.19°, 26.22°, 27.21° and 35.71°. On the other hand, for spray-dried PZA, the X-ray diffraction pattern (Fig. 3b) shows intense peaks at 2θ equal to 8.81°, 16.11°, 17.17°, 17.69°, 18.09°, 18.76°, 22.13°, 23.51°, 27.44° and 27.55°. Previous studies have demonstrated that these diffraction patterns correspond to the α polymorph for raw PZA and δ polymorph for spray-dried PZA (Castro et al., 2010; Cherukuvada et al., 2010). The X-ray results confirm findings of the DSC experiments. XRPD performed after storage did not show any difference (data not shown).

Taken all together, these results show that spray-dried PZA particles are not suitable for inhalation therapy because of their size and polymorphism instability. For this reason, we reviewed a variety

![Image](https://example.com/figure6.png)  
**Fig. 6.** Top: DSC thermograms of samples (a) PZA = 1.45 mg, (b) PZA = 1.33 mg, (c) PZA = 1.25 mg and (d) PZA = 1.03 mg (Table 2). Bottom: X-ray diffraction patterns of samples (a–d). Arrows on the XRD (b) correspond to peaks arising from leucine crystals and not from PZA.

![Image](https://example.com/figure7.png)  
**Fig. 7.** Influence of leucine concentration on particle median size just after spray-drying (top). Sites are significantly different (ANOVA, p < 0.05). Influence of leucine concentration on particle median size (bottom). Sites with leucine and others (*, t-test < 0.05). Effect of storage time on particle median size (bottom). Sites increase is significantly different (ANOVA, p < 0.05).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Components of formulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>2.0</td>
</tr>
<tr>
<td>b</td>
<td>1.4</td>
</tr>
<tr>
<td>c</td>
<td>1.3</td>
</tr>
<tr>
<td>d</td>
<td>0.9</td>
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of excipients and studied their impact on PZA polymorphism and particle characteristics after spray-drying.

3.2. Effect of ammonium bicarbonate (AB) on characteristics of spray-dried particles

PZA was spray-dried in combination with the process enhancer, AB. PZA is an extremely weak base, which has no acidic group; therefore, it is not possible to react with ammonia, which means AB will behave in the solution as a free molecule and hence the decomposition and removal of AB during spray-drying process is likely to happen. Moreover, previous works have shown that incorporation of this enhancer did not affect the chemical characteristics of spray-dried materials (Nolan et al., 2009; Tajber et al., 2005). Indeed, during spray-drying AB decomposes at 36–60°C into ammonia, carbon dioxide and water vapor. Therefore, given the inlet temperature above 120°C, one can assume that AB is sublimated, leading to porous particles, most probably larger and less dense.

Using 2 g/L PZA in the ethanol–water mixture (70/30, v/v), AB was added at four different final concentrations 1, 2, 3.5 and 5 g/L. As AB concentration increases from 0 to 1 g/L particle median size increases from 4.7 ± 0.1 μm to 6.5 ± 0.5 μm (unpaired t-test, P < 0.05) (Fig. 4, top left). Then as AB concentration is further increased, particle mean size seems to reach a plateau around 5.6 ± 0.6 μm. As expected, addition of AB leads to a decrease of the tap density from 0.44 ± 0.02 g/cm³ to 0.30 ± 0.05 g/cm³ (unpaired t-test and ANOVA, P < 0.05) (Fig. 5). In addition, powder is unstable with increase in median size within 4 weeks. This increase corresponds most probably to Ostwald ripening of crystals upon storage (Vehling, 2008). Indeed SEM images show many needle-shaped crystal agglomerates (Fig. 5). The thermogram and diffractogram of the spray-dried powder of PZA with AB are exhibited in Fig. 6. The DSC thermogram presents two endothermic peaks at 150.0°C and 191.4°C that are similar to those of raw PZA (Fig. 6a). X-ray patterns are characteristic of crystals with peaks at 2θ equal to 7.57°, 13.51°, 15.17°, 15.42°, 17.46°, 20.27°, 23.38°, 24.10°, 26.14°, 27.16° and 35.58°, corresponding to the α-crystalline form but crystals forming the powder are not stable in terms of size and other excipients should be investigated.

3.3. Effect of leucine on characteristics of spray-dried particles

Previous studies have demonstrated that leucine is a useful additive for dry powders designed for pulmonary delivery due to the improvement of the aerosolization properties (Cziz et al., 2011; Gervelas et al., 2007; Lucas et al., 1999; Najafradi et al., 2004; Rabbani and Seville, 2005; Seville et al., 2007). The effect of leucine on the aerodynamic properties is often explained by its distinct hydrophilic and lipophilic regions, which leads to an accumulation of leucine molecules at the air–water interface of the droplet (Lechuga-Ballesteros et al., 2008; Seville et al., 2007; Vehling, 2008).

Firstly, in this experiment, six samples containing PZA in the presence of increasing leucine concentrations: 0, 0.2, 0.4, 0.6, 0.8 and 1 g/L as a dispersivity enhancer and 2 g/L ammonium bicarbonate as a pore-forming agent were prepared to evaluate the effects of leucine on the characteristics of spray-dried particles. Then, 35 samples including the combination of various leucine concentrations: 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 g/L and various AB concentration: 0, 1, 2, 3.5, 5 g/L were prepared to evaluate the interaction of leucine and AB on the particle median size, yield and span.

The first 6 samples were characterized in terms of particle median size, size stability upon storage, tap density, thermal
properties, crystallinity and morphology. Increasing leucine concentration leads to an important reduction of particle median size from 5.4 ± 0.1 μm to 1.3 ± 0.1 μm (ANOVA, P < 0.05) (Fig. 7, top), in agreement with previous results (Lucas et al., 1999; Najafabadi et al., 2004; Rabbani and Seville, 2005; Seville et al., 2007). Incorporation of leucine in the spray-drying process also reduces the tapped density of the collected powders from 0.34 ± 0.04 g/cm² to 0.24 ± 0.03 g/cm² (Fig. 7, unpaired t-test, P < 0.05). Indeed as leucine concentration increases, the surface properties and the morphology of the particles may be affected leading to an increase of porosity and therefore a decrease of tap density as observed before (Cruz et al., 2011; Cervegas et al., 2007; Najafabadi et al., 2004; Rabbani and Seville, 2005). We observe that these powders are not stable with a size increase after 4 weeks of storage. In particular, the median size of the samples prepared with 0.2 g/L and 0.4 g/L leucine increases more than those prepared with 0.6, 0.8 and 1 g/L leucine. These results show that a high concentration of leucine may slow down crystal increase upon time.

SEM images show that particles convert from agglomerated crystals of various sizes (Fig. 8 bottom left) to smaller spherical shaped particle with many pores (Fig. 8, bottom right). Although needle-shaped crystals are still present they seem less numerous as leucine concentration increases. The DSC thermogram of particles prepared with leucine (1.4 g/L PZA + 0.6 g/L Leu + 2 g/L AB) shows two endothermic peaks at 154.7°C and 201.3°C close to those observed for particles prepared with PZA (2 g/L) and AB (2 g/L) (Fig. 8b). However, both peaks are slightly shifted and the second peak (Fig. 8b) is broadened indicating that there is some interaction between PZA with leucine. These results also correlate with the X-ray diffraction patterns of PZA plus leucine (1.4 g/L PZA + 0.6 g/L Leu + 2 g/L AB) with less intense peaks of α-polymorph at 2θ equal to 7.8°, 13.5°, 15.1°, 15.4°, 17.4°, 18.1°, 20.2°, 23.2°, 24.1°, 26.0°, 27.2° and 35.6° and additional peaks at 18.1° and 25.1°. These additional peaks correspond to leucine crystals as assayed by XRPD of the excipient alone (not shown). Altogether these results indicate that although there are some interactions between leucine and PZA, the addition of leucine is not sufficient to inhibit PZA crystallization and prevent crystal growth upon storage.

The next 35 samples were investigated in terms of particle median size, yield and span. Results are presented as 3D-graphs (Fig. 9). Simultaneous variation of the concentration of leucine and AB did not affect tendencies observed above: particle size increases as the concentration AB increases and decreases as the concentration of leucine increases. However, the yield seems to be optimal above 50% for an AB concentration of 2 g/L independently of leucine concentration. Similarly, the span seems the narrow around 2.3 ± 0.3 for an AB concentration of 2 g/L independently of leucine concentration. We therefore decided to fix AB concentration at 2 g/L.
Table 3
Thermodynamic parameters obtained by DSC for raw PZA, spray-dried PZA and spray-dried samples containing various excipients.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Peak 1</th>
<th>Peak 2</th>
<th>Peak 3</th>
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<tbody>
<tr>
<td></td>
<td>Onset(°C)</td>
<td>Peak(°C)</td>
<td>ΔH(J/g)</td>
</tr>
<tr>
<td>Raw PZA (m = 1.68 mg)</td>
<td>146.2</td>
<td>153.5</td>
<td>13.32</td>
</tr>
<tr>
<td>Spray-dried PZA (m = 1.81 mg)</td>
<td>146.2</td>
<td>153.5</td>
<td>13.32</td>
</tr>
<tr>
<td>Sample a (m = 1.45 mg)</td>
<td>146.2</td>
<td>153.5</td>
<td>13.32</td>
</tr>
<tr>
<td>Sample b (m = 1.36 mg)</td>
<td>146.2</td>
<td>153.5</td>
<td>13.32</td>
</tr>
<tr>
<td>Sample c (m = 1.22 mg)</td>
<td>146.2</td>
<td>153.5</td>
<td>13.32</td>
</tr>
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</table>

3.4. Effect of HA and DPPC on characteristics of the recovered spray-dried particles

To overcome crystallization some polymers may be used such as methylcellulose, hydroxypropyl methylcellulose and polyvinyl pyrrolidone (Raghavan et al., 2001). We have chosen to use hyaluronic acid because it is a natural polysaccharide, biocompatible and biodegradable (Kim et al., 2005), well suited for pulmonary route (Surendralaumar et al., 2003). In addition, previous studies had shown that the combination of HA and DPPC, a phospholipid, led to spray-dried powders of low tap density lower and stabilized physical and aerodynamic properties (Gómez Gaete et al., 2008).

We have therefore varied the concentrations of these excipients to find the optimal formulation (Table 3). HA concentration was increased from 0.1 g/L to 0.3 g/L in the presence of 0.2 g/L leucine and 0.1 g/L DPPC to ensure a high yield and a narrow size distribution around 5 μm. Then we have increased DPPC concentration in the presence of 0.6 g/L leucine and 0.2 g/L HA. Results of particle median size and of stability after period of storage are presented in Figs. 10 and 11. Increasing HA concentration leads to an increase of particle median size from 5.0 ± 0.1 μm to 10.5 ± 0.1 μm (ANOVA, P < 0.05), most probably due to an increase of the Peclet number of the solution (Gómez Gaete et al., 2008; Marty and Tsapis, 2000; Tsapis et al., 2002, 2005). Meanwhile, increasing DPPC concentration does not affect particle median size (5.7 ± 0.2 μm). Regarding stability, the presence of HA does not prevent particle median size increase after 4 weeks of storage (Fig. 10). By contrast, samples containing increasing concentrations of DPPC concentration seem to be stable after the same period of storage as no size change occurs (Fig. 11). Stability does not arise from DPPC only but from synergistic effect of high concentrations of leucine and HA. Indeed, samples 2 and 4, which are similar in terms of concentration of HA and DPPC but differ in concentration of leucine, have different behavior upon aging: for the low leucine concentration (sample 2) particle size increases whereas for high leucine concentration (sample 4) particle size seems stable under storage (Table 4).

SEM images of sample 1 show the coexistence of crystals with spherical particles (Fig. 12). As HA concentration is increasing, crystals seem to disappear and one can observe the coexistence of small aggregated spherical particles with large obviously hollow particles. Similar images are obtained for samples 2–6 (Fig. 12). From the tap density point of view, we observe that as DPPC concentration increases, tap density decreases from 0.19 ± 0.04 g/cm³ to 0.09 ± 0.01 g/cm³, however this decrease is not statistically significant (ANOVA, P > 0.05) (Fig. 11). Since samples 1–3 were not stable, we did not evaluate their tap density.

To further characterize the effect of HA and DPPC on powders, DSC and XRFD were performed (Table 2, Fig. 6). DSC thermograms (c) and (d) only exhibit the melting transition of PZA but with a delayed onset (192 °C instead of 188 °C), a broader peak and a reduced enthalpy as compared with samples containing only PZA and AB (with or without leucine). This suggests HA is interacting

Fig. 11. Influence of DPPC concentration on particle median size just after spray-drying and effect of storage time on particle median size, and Influence of DPPC concentration on tap density (Samples 4–6 from Table 3). Differences are non-statistically significant (ANOVA, P = 0.05).

Table 4 Formulations with various concentrations of HA and DPPC.

<table>
<thead>
<tr>
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<td>PZA (g/L)</td>
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</tr>
<tr>
<td>1</td>
<td>1.6</td>
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<tr>
<td>2</td>
<td>1.6</td>
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<td>3</td>
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with PZA preventing solid-solid transitions. Results are similar as DPPC is added. XRPD shed further light on sample structure: diffraction patterns show less intense peaks on a smooth amorphous bump (Fig. 6, curves a and d). Crystalline peak positions are characteristic of the γ polymorph (2θ = 12.8°, 15.2°, 17.0°, 17.5°, 18.1°, 20.4°, 23.6°, 25.1°, 27.0°, 29.9°, 35.3°) with a slight shift. The combination of the excipients and the spray-drying process leads to powders with limited crystallinity. Most probably, due to the high temperature of the spray-drying process, PZA tends to crystallize as the γ polymorph but HA limits the nucleation and growth of PZA crystals, leading to smaller crystalline domains and some disorder in the crystalline structure of γ polymorph. The addition of DPPC gives similar structure. The benefit of DPPC is an increased stability in terms of particle size.

In terms of size and stability, it appears that the optimal formulation would consist of 0.9 g/l PZA; 0.6 g/l L-lysine, 0.2 g/l HA, 0.3 g/l DPPC using 2 g/l AB (sample 5). This formulation yields particle median size of 5.8 ± 0.1 μm with rather spherical morphologies. The tap density is about 0.09 ± 0.01 g/cm² and the stability exceeds 4 weeks of storage. When estimating the aerodynamic diameter using equation \( D_{50} = \frac{D}{\rho R} \), where \( \rho \) is assumed to be the tap density, one finds 1.7 μm. Theoretically, this formulation would be suited for deep lung delivery with efficient deposition in both the alveoli and the bronchi. A complete aerodynamic evaluation in vitro and a pharmacokinetics/biodistribution study will be performed in future work.

4. Conclusions

We have optimized the formulation of PZA as large porous particles intended for deep lung delivery. By simply spray-drying PZA, we have obtained crystalline particles of the γ polymorph of PZA that were unstable and not adapted for deep lung delivery. Several excipients were added to the formulation to obtain stable large porous particles with a median size above 5 μm and a low tap density. Although a combination of leucine and ammonium bicarbonate allowed to reduce tap density and to increase particle size, these excipients were not sufficient to prevent crystallization and promote stability. The addition of hyaluronic acid (HA) in combination with dipalmitylophosphatidylcholine (DPPC) allowed to obtain stable partially crystalline spherical particles adapted for deep lung delivery. The optimized formulation obtained by spray-drying 0.9 g/l PZA; 0.6 g/l L-lysine, 0.2 g/l HA and 0.3 g/l DPPC and 2 g/l AB in a mixture of ethanol-water (70:30, v/v) possesses a median size of 5.8 ± 0.1 μm and a tap density around 0.09 ± 0.01 g/cm². The estimated aerodynamic diameter is around 1.7 μm and the powder is stable for more than 4 weeks of storage. A complete aerodynamic evaluation in vitro using impactors and a pharmacokinetics/biodistribution study will be performed in the future.

Acknowledgments

D. D. Pham fellowship is supported by Project 322, Ministry of Education and Training, Vietnam. Authors thank K. Andrieux, UMR CNRS 8612, Institut Galien Paris-Sud, for help with DSC facility. Institut Galien Paris-Sud is a member of the laboratory of Excellence LERMITI supported by a grant from ANR (ANR-10-LABX-33).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpharm.2013.04.016.

References


Chapter III

In-vivo Evaluation of Pyrazinamide-loaded Large Porous Particles for the Pulmonary Route

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Manuscript in preparation
Abstract

We have improved the aerodynamic properties of pyrazinamide loaded large porous particles (PZA-LPPs). We observe a segregation of the different components occurred during the drying process. To overcome this phenomenon and obtain homogeneous LPPs, spray drying parameters were modified to decrease the drying speed. As a result, we have obtained good aerodynamic properties for lung delivery with a fine particle fraction (FPF) of 40.1 ± 1.0 % and an alveolar fraction (AF) of 29.6 ±3.1 %, a mass median aerodynamic diameter (MMAD$_{aer}$) of 4.1 ± 0.2 μm and a geometric standard deviation (GSD) of 2.16 ± 0.16. Plasma and lung lining fluid concentrations of pyrazinamide were evaluated after intratracheal insufflation of PZA-LPPs into rats and compared to intravenous administration (IV) of a pyrazinamide solution. The in vivo pharmacokinetic evaluation of PZA-LPPs in rats reveals that intratracheal insufflation of PZA-LPPs and IV administration of PZA lead to similar pharmacokinetic parameters proving that PZA-LPPs dissolve fast upon deposition and that PZA crosses efficiently the lung barrier to reach the systemic circulation. The absolute bioavailability of PZA administered by insufflations was of 1. Surprisingly, PZA was cleared faster from the lung lining fluid when administered by intratracheal insufflations than by IV administration. Lung delivery of PZA appears an interesting alternative to oral delivery of the molecule and should now be tested in an infected animal model to evaluate its efficacy against *Mycobacterium tuberculosis*.

Keywords: Tuberculosis, Pyrazinamide, Lung delivery, Aerodynamic properties, Pharmacokinetics.
1. Introduction

Large Porous Particles have been shown to have potential for pulmonary drug delivery. Large porous particles for inhalation are characterized by large geometric diameters (> 5 μm) and low mass densities < 0.4 g/cm³), yielding aerodynamic diameters of approximately 1-5 μm for optimal lung deposition [1, 2]. A significant benefit of LPPs relative to conventional inhaled therapeutic aerosol particles is their aerosolization efficiency [3, 4]. This leads to efficient deposition of large drug masses in the respiratory tract [5-7]. In dry powder inhalation applications for pulmonary drug delivery, the previous studies of [8-10] shown that large porous particles have demonstrated excellent performance. Emitted doses in the range of 90–100% from a passive dry powder inhaler were reported. Total lung deposition and dose variability were clearly superior compared to conventional DPI systems. In addition, the AIR® pulmonary drug delivery system containing a part of large porous particles has entered late stage clinical trials for insulin [11-13] and early human studies for parathyroid hormone [14, 15]. They have been used to formulate human growth hormone [16], heparin [17], and small molecules such as albuterol sulfate [18], paraaminosalicylic acid [19], or levodopa [20].

Multistage cascade impaction is widely used for the characterization of the aerodynamic particle size distribution. This method is described in the European Pharmacopoeias [21]. The popularity of multistage cascade impaction is due to the instrument’s ability to measure the entire mass distribution emitted from the inhaler and the provision of a detailed fractionation of the aerosol drug mass. Multistage cascade impaction are used both in the development and subsequent quality control of marketed inhaler products and the qualification of the clinical batches together with the assessment of add-on devices affecting the size-properties of the emitted aerosol, such as spacers and holding chambers that are used with pressurized meter dose inhalers [22]. Among impactors, The 4-stage multistage liquid impinger (MSLI) is the last widely used cascade impaction for inhaler aerosol evaluations, being a popular choice where collection of the active substance in the eluent for subsequent assay is possible [23]. The particles are captured in a liquid medium rather than on a solid substrate, an advantage in that particle bounce and re-entrainment is avoided, and the liquid can be the eluent medium for recovery and assay of active pharmaceutical ingredients.

In previous study, the in vitro lung deposition estimated from the evaluation of multistage cascade impaction was not correlated with in vivo lung deposition data
obtained from a variety of inhalation drug delivery systems [24]. A number of potential causes for this difference are as following: The geometry of the induction port does not reflect that of the oropharynx [25]. The non-respirable droplets from pressurized metered dose inhalers had the high velocity to distort the aerodynamic particle size distribution [26]; The flow profile used in the cascade impactor may not be representative of the clinical inspiratory flow profile [27-29]; The errors of experimental impactors (bounce, overload, re-entrainment and interstage losses) distort the aerodynamic particle size distribution [30-32]; The cascade impactor only evaluate a fine particle fraction while larger particle fractions depositing in the induction port and pre-separator were not evaluated accurately [33]. Despite these limitations, the combination in vitro and in vivo lung deposition data can make the insights of pulmonary drug delivery more clear.

Drug delivery to the lungs via the inhaled route is the complex process affected by many factors. The particle size and size distribution of the drug particles or droplets, the inhaler device and formulation, breathing patterns of the subject, and airway geometry affect the site of drug deposition in the lungs [34]. For locally acting drugs, the residence time in the vicinity of the site of action restrict the duration of drug release while, for systemically acting drugs, the site of absorption is. In the respiratory tract, there are many different mechanisms to clear the particles deposited. The rate at which a drug is cleared is a function of the dynamic interaction of several factors including the rate of mucociliary clearance, the site of deposition, the physical state of the drug (solid or liquid), drug-release rate from the carrier, and physicochemical properties of the drug such as molecular weight, partition coefficient, and charge [35]. The mucociliary clearance mechanism, based on moving layer of mucus, clear the particles deposited in the conducting airways, whereas alveolar macrophages clear rapidly the particles penetrating down to the respiratory airways by phagocytosis [36]. They clear particles from the alveolar region by transport along the alveolar surface to the mucociliary escalator, translocation to the tracheo-bronchial lymph, or internal enzymatic degradation [35, 37, 38]. Besides, the enzymes present in the lumen surface of the endothelium may metabolize the drugs accumulated in the lung endothelium [9]. The principal barrier to absorption is epithelial cells for systemic delivery of drugs, whereas absorption through this epithelial cell layer is the major clearance mechanism of soluble drugs for local therapy. The proposed routes of absorption include transport via membrane pores, via the intercellular tight junctions by passive diffusion, vesicular transport, active
transport, and drainage to the lymphatics [39-43]. The complex processes can be accurately evaluated only \textit{in vivo}.

In our previous study, we have optimized the formulation of PZA as large porous particles intended for deep lung delivery. So, the purpose of this study was to evaluate the \textit{in vitro} and \textit{in vivo} dose delivery characteristics of optimized formulation of PZA.

2. Materials and Methods

2.1. Materials

Pyrazinamide was obtained from Fluka, with specified purity greater than 99%. 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine (DPPC) was provided by Corden Pharma (Switzerland) and hyaluronic acid, sodium salt 95% (HA) (MW=1000 kDa) by Acros organics. Ammonium bicarbonate (AB), acetazolamide and DL-Leucin (Leu) were provided by Sigma–Aldrich (France). Ethanol absolute in analytical grade was obtained from Carlo Erba Reagents (France). Water was purified using a RIOS/MilliQ system from Millipore (France). HPLC-grade acetonitrile and methanol were purchased from Prolabo (France).

2.2. Particle production via spray drying

Large porous particles were obtained by spray drying using a mini spray dryer Büchi B-290 (Flawil, Switzerland) equipped with a 0.7 mm diameter two-fluid nozzle, which operates in a co-current mode. The formulation chosen was previously optimized in terms of composition [44] to prevent PZA recrystalization and yield stable large porous particles. The spray drying parameters such as air-flow rate, feed-flow rate, inlet temperature and aspiration are reported in Table 1. Briefly, DPPC was dissolved into 700 mL ethanol. Meanwhile, PZA and Leu were dissolved into 300 mL water. Then, HA was added into the aqueous solution and stirred using a magnetic stir bar for about an hour until dissolution. Afterwards, AB was dissolved into the aqueous solution and subsequently ethanolic and aqueous solutions were mixed immediately prior to atomization. The final concentration of AB in the ethanol/water mixture was 2 g/L. The final solid content of the solution was 2 g/L omitting ammonium bicarbonate since this compound is sublimated during the drying process. Powder samples were stored at room temperature under vacuum in a desiccator immediately after spray drying to limit moisture uptake by samples between production and testing. The yield
was calculated as a percentage by dividing the mass of the powder collected by the initial mass of solids in the solution prior to spray drying.

**Table 1:** Operational conditions used for spray drying the initial optimized formulation

<table>
<thead>
<tr>
<th>Spray drying parameter</th>
<th>Operational conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feed-flow rate (FFR, mL/min)</td>
<td>11</td>
</tr>
<tr>
<td>Inlet temperature (T_{inlet}, °C)</td>
<td>160</td>
</tr>
<tr>
<td>Outlet temperature (T_{outlet}, °C)</td>
<td>81</td>
</tr>
<tr>
<td>Drying gas flow rate (m³/h)</td>
<td>38</td>
</tr>
<tr>
<td>Spraying gas flow rate (L/h)</td>
<td>498</td>
</tr>
</tbody>
</table>

**2.3. Characterization of spray-dried powders**

Particle size distribution was measured by light diffraction using a Mastersizer 2000 equipped with a Scirocco dry disperser (Malvern Instruments, France) at a dispersing pressure of 1 bar. The refractive index used was 1.5. Values presented are the average of at least 3 determinations, errors bars indicate the standard deviation (S.D.).

The powder density was evaluated by tap density measurements using a tapping apparatus (Pharma test PT-TD1). Tap density (ρ) was measured in a 10 mL glass measuring cylinder filled with a fixed initial volume of powder around 8 mL. The tap density was determined after 1000 taps form a constant height. Measurements were performed in duplicate.

The morphology and shape of particles composing the powder were examined by scanning electron microscopy (SEM) using a LEO1530 microscope (LEO Electron Microscopy Inc., Thorn-wood, NY) and operating between 1 and 3 kV with a filament current of about 0.5 mA. Powder samples were deposited on a carbon conductive double-sided tape (Euromedex, France). They were coated with a palladium–platinum layer of about 4 nm, using a Cressington sputter-coater 208HR with a rotary planetary-tilt stage, equipped with a MTM-20 thickness controller.

The thermal properties of the powders were analyzed using differential scanning calorimetry (DSC) (DSC7, PerkinElmer, USA). Thermograms were analyzed using Pyris software. An empty aluminum pan was used as the reference for all measurements. A sample (1-5 mg) of powder was placed in hermetically sealed 40 µL aluminum pan and analyzed. DSC runs were conducted from 30 to 210 °C at a rate of 10 °C/min. Calibration was achieved using Indium (T\text{onset} = 156.60 °C) as well as
Zinc ($T_{onset} = 419.47 \, ^\circ\text{C}$). The onset and peak temperatures and enthalpy of transition ($\Delta H$) were determined for each peak.

Powder crystallinity was analyzed using X-ray powder diffraction (XRPD). XRPD patterns were measured on a Bruker D2 diffractometer equipped with a XFlash detector in SPMS laboratory – Centre of diffraction – École Centrale Paris using Ni-filtered CuKα radiation. Data were collected over an angular range comprised between 5 and 40° ($2\theta$) with a step size of 0.01°, a counting time of 5 s/ step.

2.4. Powder stability

Powder stability was assessed by leaving samples to age at room temperature under vacuum in a desiccator (temperature comprised between 15 and 25 °C). Particle size was performed on aged samples at $t = 0$, 2 and 4 weeks.

2.5. Drug Content

The content of PZA in the powder was determined by UV-Visible double beam spectrophotometer (Lambda 25, PerkinElmer, France) with 1 cm matched quartz cells. 20 mg powder was accurately weighed and transferred into 20 mL volumetric flask. It was dissolved properly and diluted up to the mark with ethanol/water (70/30). Then, the solution was diluted to obtain a 10 μg/mL solution. The absorbance of the solutions containing PZA was determined in the UV range 400-200 nm using an ethanol/water (70/30) blank.

The standard curve was constructed by plotting the absorbance of pyrazinamide against the concentration of pyrazinamide which based on the following solutions: 2, 4, 6, 8, 10, 12, 14 and 16 μg/mL of PZA. The slope, y-intercept, and linearity ($R^2$) for standard curve were $y = 0.0664x + 0.0468$ and 0.9999, respectively.

2.6. In-vitro aerodynamic evaluation

The aerodynamic properties of dry powders for inhalation were determined using multi-stage liquid impinger (MSLI, Apparatus C, European Pharmacopoeia 2008, Copley Scientific, Switzerland) through a dry-powder, breath-activated inhaler device (Aerolizer®, Novartis). Prior to testing, 20 mL of the solvent (ethanol/water, 70/30, v/v) was added into each of the four stages of the impinger. 5 mg dry powder was weighed and loaded into a size 4 hydroxypropylmethylcellulose capsules (LGA, France), further placed in the inhaler device. The inhaler device containing a filled capsule was fitted in the induction port. The capsule was then punctured and the powder was delivered at a flow rate of 60 L/min for 4 s to simulate an inspiration. This
process was repeated twice. The amount of powder deposited on the impinger stages, as well as the amount retained by the filter was assayed spectrophotometrically as described below. The solvent of each stage was collected, the filter was rinsed with ethanol/water (70/30, v/v) and the respective volumes were transferred into 50 mL volumetric flasks. Then, the volume was completed with ethanol/water, 70/30 (v/v). Aerodynamic evaluation was performed with normal powder and also a powder fluorescently labeled using 1% of Liss-Rhod-PE before spray-drying (Avanti Polar Lipids, USA). Calibration curves were prepared in the range of 5 – 100 μg mL⁻¹ powder by adding aliquots of the stock solutions of each sample in the 20 mL volumetric flasks. The stock solutions were prepared by dissolving 20 mg of the powder in 20 mL of ethanol/water, 70/30 (v/v). The volume was completed to 20 mL with ethanol/water, 70/30 (v/v). The absorbance values were measured at 268 nm (measuring PZA for normal powder) and at 564 nm (measuring LissRhod-PE for labeled powder).

The emitted fraction (EF) defined as the percent of total loaded powder mass exiting the capsule was determined gravimetrically and calculated using the following equation:

\[ EF = \left( \frac{m_{\text{full}} - m_{\text{empty}}}{m_{\text{powder}}} \right) \times 100 \]

Where \( m_{\text{full}} \) and \( m_{\text{empty}} \) are the masses of the capsule before and after simulating the inhalation and \( m_{\text{powder}} \) the mass of the powder placed in the capsule.

The cumulative mass of powder smaller than the stated size of each stage of the impinger was calculated and plotted on a log probability scale, as percent of total mass recovered in the impactor against the effective cut-off diameter. The cut-off diameter of each individual stage (D) was determined as \( D = D_{60} \cdot (60/Q) \) where \( D_{60} \) is the cut off diameter at a flowrate of 60 l/min, i.e., 13.0, 6.8, 3.1 and 1.7 μm for stages 1 to 4, respectively, and Q is the flowrate employed in the test [21]. The experimental mass median aerodynamic diameter (MMAD_{aer}) of the particles was defined from this graph as the particle size at which the line crosses the 50% mark. And the geometric standard deviation (GSD) as \( \text{GSD} = \sqrt{\text{sizeX}/\text{sizeY}} \) where sizeX is the particle size for which the line crosses the 84% mark and sizeY the 16% mark. The fine particle fraction (FPF) was calculated by interpolation from the same plot as the fraction of powder emitted from the inhaler with an aerodynamic diameter ≤ 5 μm [21]. The alveolar fraction (AF) was calculated by interpolation from the same plot as
the fraction of powder emitted from the inhaler with an aerodynamic diameter $\leq 3.1$ μm.

2.7. **In-vivo studies**

**Animal model**

Experiments have been conducted according to the European rules (86/609/EEC and 2010/63/EU) and the Principles of Laboratory Animal Care and legislation in force in France (Decree No. 2013-118 of February 1, 2013). Male Sprague Dawley rats were obtained from Harlan Laboratory (GANNAT, France). The animals weighed between 250 and 300 g. The animals were in good health upon arrival and remained so until use; no clinical signs of illness were observed at any time. They were housed 4 per cage while on study in accordance to EEC guidelines. The light/dark cycle was 12 h/12 h. The temperature in the animal room was ambient room temperature of approximately 25 °C and the ambient humidity was in the range of approximately 35–60%. Animals were allowed access to food and water ad libitum throughout the duration of the study.

**Drug administration**

40 rats were divided into the following 2 groups (20 rats per group): the first group received LPP-PZA powder by intratracheal insufflation, the second group received a PZA solution (glucose 5% to adjust osmolarity) by intravenous administration. Animals were anaesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg) and then received a PZA dose by intratracheal insufflation of 5 mg LPP-PZA powder, corresponding to 1.65 mg PZA or intravenous injection (IV) of 200 μL solution containing 1.6 mg PZA. For intratracheal insufflation, LPP-PZA powders were weighed out into capsules and stored in desiccators prior to dosing. The LPP-PZA powder was introduced into the lung through the DP-4 dry powder insufflator device (PennCentury, Philadelphia, PA). Once sedation was confirmed, animals were placed onto a modified slant board to optimize visual placement of the insufflation cannula into the trachea. The tip of the insufflation device was placed in the trachea just above the carina and 5 mg of LPP-PZA powder was delivered through the insufflation device by rapidly pushing a 2 cm³ bolus of air through the device. The insufflator was weighed before and after powder filling as well as after administration to determine the actual dose insufflated per rat. For intravenous injection, the isotonic solution of PZA was injected into the jugular vein.
Pharmacokinetic studies

For each rat, blood was taken at 3 different time points via the jugular vein. For each time point, bloods were taken from a minimum of 6 rats. Blood was collected at before PZA administration and 1/4, 1/2, 1, 2, 3, 4, 6, 18 and 24 hours following PZA administration (insufflation or IV). Before taking the blood, the rats were anesthetized by isoflurane inhalation. Approximately 1 ml of blood was taken at each time point. All blood samples were collected in heparinized tubes. Plasma was separated in a tabletop centrifuge (Eppendorf Minispin®) at 10,000 rpm for 10 min. The retrieved plasma was subsequently snap frozen on dry ice and stored at -80 °C until analysis by HPLC. Parameters such as C_{max} (maximal peak plasma concentration), T_{max} (time to reach peak concentration), and AUC_{0-t} (mean area under the plasma concentration-time curve) were obtained from the concentration-time curves. The absolute bioavailability (F_{abs}) was calculated as \( \frac{AUC_{RT} \times \text{dose}_{IV}}{AUC_{RT} \times \text{dose}_{IV}} \times 100 \) and the plasma elimination half-life (t_{1/2}) as \( \ln(2)/k \). The elimination rate constant (k) was estimated by linear regression of the last time points of the log concentration versus time curve. The clearance (CL) was calculated as \( [\text{dose} \times F_{abs}]/\text{AUC} \) and the volume of distribution (Vd) as CL/k [45]. Mean residence times (MRT) were calculated as the ratio between areas under the first-moment vs. time curve (AUMC) and AUC. Mean absorption times (MAT) were calculated by subtracting MRT after IV administration from that after intratracheal administration.

Bronchoalveolar lavage fluid (BALF) and Alveolar cell pellet (AC) collection

BALFs were collected to assess lung lining fluid PZA levels after animals were deeply anaesthetized by isoflurane inhalation, euthanized by cutting the diaphragm, and exsanguinated by cardiac puncture. The trachea was cannulated using an 18 gauge needle adaptor for subsequent injection and retrieval of BALF. Briefly, aliquots of 8 mL of chilled PBS were instilled into lungs after intubating the trachea with a syringe fitted with tracheal cannula, an 18 gauge needle adaptor. The lungs were massaged and the fluid withdrawn immediately and collected in centrifuge tube kept on ice. This process was repeated 3 times for obtaining the most macrophage cells [46]. All lavages were pooled and centrifuged (1500 rpm; 10 min; 4 °C). The supernatant of lavage and the alveolar cell pellet were collected separately, snap-frozen on dry ice, and stored at -80 °C until analysis.
HPLC determination of PZA

Plasma, BALF and alveolar cell pellet PZA concentrations were determined by an HPLC assay described in the literature [47] with minor modifications. Briefly, all samples were analyzed using high-performance liquid chromatography (HPLC) with the following equipment: a Waters model 1525 binary pump and model in-line degasser AF, a model 717 plus autosampler, a model 2487 dual λ absorbance detector, a Dell computer and the Breeze version 3.30 SPA HPLC data management system. Chromatography was performed with a stainless steel Interchrom Spherisorb 5μm ODS2 column (250 mm x 4.6 mm, 5 μm particle size). The mobile phase was 0.02M potassium phosphate buffer – acetonitrile mixture at pH 2.6, with a final acetonitrile concentration of 2%. The flow-rate was set to 1 mL/min. The effluent was monitored at 268 nm with a detection scale of 0.1 or higher as needed. All measurements were performed at room temperature. The retention times for pyrazinamide and acetzolamide were 10.5 and 21.0 min, respectively. While BALF supernatants and AC samples were run for 36 min, plasma was run for 45 min because of late peaks that interfered with the next injection.

Preparation of BALF and Alveolar cell pellet standards and samples:

PBS was chosen as the matrix for standard curves and controls to measure pyrazinamide concentrations in both BALF and AC suspensions. It was ascertained that the slopes, y-intercepts, and linearity of these matrices were similar. Bronchial lavage fluid (around 24 mL) was centrifuged at 400 × g for 10 min, and the cells separated from the supernatant immediately after collection. The cells were resuspended in PBS to yield a tenfold (2 mL PBS) concentration of the original lavage fluid centrifuged. To lyse the cells, the suspension was sonicated at 40% amplitude for 2 min. The drug was extracted as follows: 0.5 mL of spiked PBS (PZA concentrations 0.005, 0.007, 0.010, 0.020, 0.050, 0.100, 0.200, 0.500 μg/mL), or 0.5 mL of diluted BAL supernatant were pipetted into screw cap glass tubes. Twenty microliters of internal standard solution (10 mg/mL acetazolamide in acetonitrile) and 50 μL of 35% hydrochloric acid were then added. After vortexing for 2 min, 4.0 mL of ethyl acetate was added, vortexed for 2 min, and then centrifuged for 10 min at 1000 × g. The organic phase was transferred to a clean tube, and the aqueous phase left in the screwcap tube was extracted again in the same manner. The resulting 8 mL of solvent was pooled, evaporated to dryness under nitrogen, and then reconstituted in 0.2 mL of mobile phase. Fifty microliters were injected into the column. The standard curve was constructed by plotting the peak-area ratios of pyrazinamide to
acetazolamide against the spiked concentration of pyrazinamide (weighted as 1/y where y equals the concentration of pyrazinamide). The standard curve equation was the following: $y = 1.9177x + 0.0441$ ($R^2=0.978$).

**Preparation of plasma standards and samples:**

200 µl of spiked plasma (PZA concentrations: 0.5, 1.0, 2.5, 5.0, 10, 20, 40 and 80 µg/mL) or sample were deproteinated by adding 400 µl internal standard solution (10mg/mL acetazolamide in acetonitrile), vortexing for 30 s, centrifuging at 3000 × g for 10 min (7000 rpm for 10 min eppendorf), decanting into a clean glass tube, and evaporating to dryness under nitrogen. After the addition of 200 µL of mobile phase and vortexing, 50 µL were injected onto the column. The standard curve was constructed by plotting the peak height ratios of pyrazinamide to acetazolamide against the spiked concentration of pyrazinamide (weighted as 1/y where y equals the concentration of pyrazinamide). The standard curve equation was the following: $y = 0.0548x + 0.0204$ ($R^2=0.999$).

**Determination of urea concentration in BALF by colorimetric method**

To determine the urea concentration, a commercially available kit (DIUR-500, BioAssay Systems) based colorimetric method was used in 96-well plates. This kit determines urea directly in biological samples without any pretreatment. For BALF, 50 µL water (blank), 50 µL 5 mg urea/dL (the 50 mg/dL standard diluted in water) and 50 µL samples were deposited in duplicate into separate wells. Then, 200 µL working reagent was added and mixed gently before incubating for 50 min at room temperature. The optical density (OD) at 450nm was then read using a LT-5000MS plate reader (Labtech, France). Urea concentration (mg/dL) of the sample is calculated as: $[\text{Urea}] = [(\text{OD}_{\text{sample}} - \text{OD}_{\text{blank}})/(\text{OD}_{\text{standard}} - \text{OD}_{\text{blank}})] \times n \times [\text{STD}]$ (mg/dL). OD$_{\text{sample}}$, OD$_{\text{blank}}$ and OD$_{\text{standard}}$ are OD values of sample, standard and water, respectively. n is the dilution factor. [STD] = 5 urea standard concentration (mg/dL). The dilution factor is calculated as: dilution factor = [concentration of urea in plasma (mg/dL)]/[concentration of urea in BALF (mg/dL)]. The concentration of urea in plasma we used was 50 mg/dL [48].

**2.8. Statistical analysis**

An unpaired two-tailed t-test was used to test the differences between the means of two groups. A one-way ANOVA was used to test for differences between multiple groups. A two-way ANOVA is an appropriate analysis method for a study with a quantitative outcome and two (or more) categorical explanatory variables. P-values
<0.05 were considered as statistically significant. Correlation analysis used is the correlation coefficient ($r$) which measures the strength of a linear relation between two variables, not the agreement between them. Values between 0.7 and 1.0 (-0.7 and -1.0) indicate a strong positive (negative) linear relationship via a firm linear rule.

3. Results and Discussion

3.1. PZA-loaded LPPs preparation and characterization

In a previous study, PZA-loaded LPPs made of leucine, hyaluronic acid (HA) and dipalmitoylphosphatidylcholine (DPPC) were prepared with good yields by spray-drying using ammonium bicarbonate as a porogen [44]. Whereas spray-drying pure PZA led to agglomerated crystals (not shown) with a tap density of 0.85 ± 0.01 g/cm$^3$, the addition of leucine and ammonium bicarbonate favored the formation of spherical particles with many pores (Fig. 1a), characterized a reduced tap density of 0.25 ± 0.04 g/cm$^3$ (unpaired $t$-test, $P < 0.05$). However, these particles were not stable and recrystallized over time. The addition of HA in combination with DPPC was necessary to limit the nucleation and growth of PZA crystals and yield stable partially crystalline spherical particles (Fig. 1b). The optimized formulation was obtained by spray-drying 0.9 g/L PZA, 0.6 g/L leucine, 0.2 g/L HA and 0.3 g/L DPPC and 2 g/L AB in a mixture of ethanol–water (70/30, v/v), according to conditions detailed in Table 1. The optimized powder possesses a median size of 5.8 ± 0.1 μm and a tap density around 0.09 ± 0.01 g/cm$^3$. The estimated aerodynamic diameter is around 1.75 μm and the powder is stable for more than 4 weeks of storage. Finally, in the optimized formulation, PZA was partially crystalline as the γ polymorph, as confirmed by DSC and X-ray diffraction experiments (Fig. 2)[44].
**Figure 1:** SEM micrographs of spray dried PZA particles containing leucine (a) or leucine added with HA and DPPC (b).
Figure 2: Top: DSC thermograms of raw PZA (dotted line) and the optimized formulation (full line). Bottom: X-Ray diffraction patterns obtained for raw PZA (dotted line) and the optimized formulation (full line).

3.2. In-vitro deposition

Airborne particle deposition in the human respiratory tract is highly dependent of particle aerodynamic diameter distribution [49, 50]. This distribution can be measured in vitro by using impactors as advised by Pharmacopoeia [21]. Impaction results are presented in Figure 3 as percent of initial dose deposited on each stage. Table 2 displays the results obtained after impaction with the optimized powder for EF, FPF, AF, MMAD and GSD. The emitted fraction is high around 99 ± 3%. In spite of high emitted fraction, the FPF and AF values of optimized formulation were only about 30 ± 3% and 20 ± 2%, respectively. The powder has a resulting MMAD of 5.1 ± 0.3 μm, higher than the theoretical one (MMAD\textsubscript{t}) which was 1.75 μm [44]. Indeed, impaction results show that most of the formulation deposits in the induction port of the MSLI (Figure 3). The discrepancy between the theoretical and the experimental MMAD is frequently observed in the literature [8, 51, 52]. Previous study suggest it may be a result from the aggregation of particles during aerosolization [53]. Differences also arise from calculations: the theoretical MMAD is derived from the geometric diameter determined by laser diffraction and the tap density, while the experimental MMAD is obtained from impaction as detailed above [54]. For impaction, moisture may play a
role since the MSLI contains liquid, therefore potentially inducing particle aggregation.

Table 2: Aerodynamic properties of the optimized formulation and marked formulation with fluorescent lipid (mean ± SD, n= 2)

<table>
<thead>
<tr>
<th>Powders</th>
<th>The optimized formulation (unlabeled formulation)</th>
<th>The labeled formulation with fluorescent lipid</th>
</tr>
</thead>
<tbody>
<tr>
<td>EF (%)</td>
<td>99 ± 3</td>
<td>98 ± 1.5</td>
</tr>
<tr>
<td>FPF (%)</td>
<td>30 ± 3</td>
<td>45 ± 3</td>
</tr>
<tr>
<td>AF (%)</td>
<td>20 ± 2</td>
<td>36 ± 4</td>
</tr>
<tr>
<td>MMAD (μm)</td>
<td>5.1 ± 0.3</td>
<td>3.7 ± 0.3</td>
</tr>
<tr>
<td>GSD</td>
<td>2.0± 0.1</td>
<td>2.5 ± 0.6</td>
</tr>
</tbody>
</table>

Figure 3: Comparison of impaction results obtained for the optimized formulation (unlabeled formulation) and its labeled counterpart (Top); for sample 7 and its labeled counterpart (Bottom).
Observing SEM images, we noticed the coexistence of two populations of particles: large particles onto which small ones seem to be adsorbed (Figure 4). We suspected that the composition of these two populations were not identical. We have therefore labeled the optimized formulation using a lipidic fluorescent label, Liss-Rhod-PE, which physico-chemical properties are very different from those of PZA. Impaction was then repeated following the label instead of PZA. Results are presented in Figure 3 and summarized in Table 2. One can notice that the two formulations do not deposit similarly, with the labeled formulation depositing deeper in the impactor. Although the EF is similar, FPF, AF and MMAD_{aer} differ a lot (Table 2). These findings reflect the heterogeneous distribution of components in the particles after spray-drying.

![SEM micrograph](image)

**Figure 4:** SEM micrograph of the optimized powder: A. Large hollow particle; B. smaller independent spherical dense particles.

Many previous studies report that segregation of components in multi-component mixtures could occur during the drying process [55-62]. The segregation was explained as follows. An initial layer of saturated material forms at the outside of the multi-component droplet, as evaporation proceeds, this layer thickens: the solvent moves towards the surface of the droplet, while the solutes move to the center. Solutes movement towards the center of the droplet is governed by their diffusion coefficient, whereas the drying time is set by the solvent used and the gradient of temperature in the spray-dryer. The balance between these two characteristic times is summarized by the Peclet number, defined by the ratio of the mixing time of the
chemicals in the droplet over the drying time of the droplet [63-65]. This parameter however does not take into account the difference of solubility of solutes: a less soluble solute may precipitate at the interface before the other. The surface activity of solutes should also be taken into account. Since in our particular case, the spray-dryer operates at high temperature ($T_{\text{inlet}}=160 \, ^\circ\text{C}$) with a mixture of ethanol-water and a large number of solutes, it is difficult to predict which of them would precipitate first at the interface.

What is known is the following. DPPC, a surfactant, is likely to position at the air-liquid surface of the droplet and to form quickly an initial layer on the surface of droplets during the spray-drying process [52, 66]. HA, a polymer of high molecular weight might not have enough time to diffuse towards the center of droplets during spray-drying. As a result, it could be trapped at the drying front leading to the earlier formation of a hollow shell [67]. Leucine is a rather hydrophobic amino acid with surface activity, driving it at the droplet surface [68, 69]. PZA is very water soluble therefore the inner part of the droplet is probably richer in PZA. Finally, since the droplet temperature is greater than $50^\circ\text{C}$, as AB sublimates it might lead to explosions and satellite droplets probably richer are PZA. However, given the complexity of the system, this remains a hypothesis and further experiments should be carried on to fully investigate the system.

To overcome heterogeneous distribution of components in the particles, while keeping solution composition constant, the spray-drying parameters were modified to decrease the drying speed: the $T_{\text{inlet}}$ was decreased, the drying gas flow rate was reduced, the feed-flow rate was modulated and the spraying-gas flow rate was reduced as described in Table 3. As these parameters were varied, resulting powders were characterized for their geometric diameter and aerodynamic properties (MSLI impaction). Table 4 presents the resulting $T_{\text{outlet}}$, $D_{50}$, yield, drug content, FPF, AF, MMAD$_{ aer}$ and GSD of the different samples. Results show that there are significant differences. The yield increases from 37.1% to 47.3% (unpaired $t$-test, $P < 0.05$), the drug content increases from 11.7% to 34.9% (unpaired $t$-test, $P < 0.05$), the FPF increases from 30.2% to 40.1% (unpaired $t$-test, $P < 0.05$), the AF increases from 19.9% to 29.6% (unpaired $t$-test, $P < 0.05$) and MMAD$_{ aer}$ decreases from 5.1 $\mu$m to 4.1 $\mu$m (unpaired $t$-test, $P < 0.05$).
Table 3: Spray drying parameters modified to optimized the aerodynamic properties of the powder.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Spray drying Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$T_{\text{inlet}}$ (°C)</td>
</tr>
<tr>
<td>1</td>
<td>160</td>
</tr>
<tr>
<td>2</td>
<td>140</td>
</tr>
<tr>
<td>3</td>
<td>120</td>
</tr>
<tr>
<td>4</td>
<td>120</td>
</tr>
<tr>
<td>5</td>
<td>120</td>
</tr>
<tr>
<td>6</td>
<td>140</td>
</tr>
<tr>
<td>7</td>
<td>140</td>
</tr>
</tbody>
</table>

Table 4: Powder characteristics according to the spray drying conditions (mean ± SD, n= 2).

<table>
<thead>
<tr>
<th>Sample</th>
<th>$D_{50}$ (µm)</th>
<th>Yield (%)</th>
<th>Drug Content (%)</th>
<th>FPF (%)</th>
<th>AF (%)</th>
<th>MMAD (µm)</th>
<th>GSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.6 ± 0.3</td>
<td>37.1 ± 5.9</td>
<td>11.7 ± 2.5</td>
<td>30.2 ± 3.2</td>
<td>19.9 ± 2.3</td>
<td>5.1 ± 0.3</td>
<td>1.99 ± 0.06</td>
</tr>
<tr>
<td>2</td>
<td>6.4 ± 1.4</td>
<td>39.1 ± 1.1</td>
<td>26.2 ± 2.0</td>
<td>32.0 ± 7.5</td>
<td>22.8 ± 7.9</td>
<td>4.7 ± 0.8</td>
<td>1.99 ± 0.12</td>
</tr>
<tr>
<td>3</td>
<td>7.0 ± 0.1</td>
<td>48.4 ± 0.3</td>
<td>30.5 ± 3.9</td>
<td>28.4 ± 1.8</td>
<td>17.6 ±0.4</td>
<td>4.9 ± 0.1</td>
<td>1.83 ± 0.06</td>
</tr>
<tr>
<td>4</td>
<td>5.9 ± 0.4</td>
<td>47.6 ± 0.5</td>
<td>34.2 ± 1.6</td>
<td>33.0 ± 2.3</td>
<td>21.2 ± 1.9</td>
<td>4.6 ± 0.1</td>
<td>1.88 ± 0.02</td>
</tr>
<tr>
<td>5</td>
<td>4.7 ± 0.7</td>
<td>42 ± 0.0</td>
<td>27.2 ± 0.6</td>
<td>28.7 ± 1.6</td>
<td>19.0 ± 1.2</td>
<td>4.9 ± 0.1</td>
<td>1.97 ± 0.02</td>
</tr>
<tr>
<td>6</td>
<td>6.5 ± 0.1</td>
<td>42.6 ± 0.0</td>
<td>27.7 ± 2.1</td>
<td>30.7 ± 0.5</td>
<td>19.9 ± 0.8</td>
<td>4.9 ± 0.1</td>
<td>1.86 ± 0.00</td>
</tr>
<tr>
<td>7</td>
<td>5.3 ± 0.1</td>
<td>47.3 ± 0.8</td>
<td>34.9 ± 2.2</td>
<td>40.1 ± 1.0</td>
<td>29.6 ± 3.1</td>
<td>4.1 ± 0.2</td>
<td>2.16 ± 0.16</td>
</tr>
</tbody>
</table>

Correlation analysis was performed between the spray-drying parameters and the dry powders properties. This analysis shows that: i) The drying gas flow rate has a strong influences on FPF and MMAD$_{aer}$ with a negative correlation for FPF ($r = -0.80$) and with a positive correlation for MMAD$_{aer}$ ($r = 0.81$); ii) $T_{\text{outlet}}$ influences yield, drug content and MMAD$_{aer}$ with a negative correlation for yield ($r = -0.84$) and drug content ($r = -0.97$) and with a positive correlation for MMAD$_{aer}$ ($r =0.77$); iii) the yield increase also leads to an increase of drug content ($r = 0.86$) and the FPF increase leads to a decrease of MMAD$_{aer}$ ($r = -0.94$). To summarize, reducing the drying speed will help increasing the yield, the drug content and FPF while reducing the MMAD$_{aer}$. Sample 7 conditions were finally chosen for subsequent in vivo experiments, since this sample exhibits suitable aerodynamic properties for dry powders delivery to the alveoli. Comparing the results in MSLI evaluation reveals that there was slightly significant difference in the deposition on each stage of sample 7 and its fluorescently-labeled counterpart (ANOVA, $P = 0.042 < 0.05$). Sample 7 composition is the following: 0.9 g/L PZA, 0.6 g/L leucine, 0.2 g/L HA and 0.3 g/L DPPC and 2 g/L AB in a mixture of ethanol–water (70/30, v/v), using the following spray-
drying conditions: $T_{\text{inlet}}$ of 140°C, a feed flow rate of 19 mL/min, a spraying gas flow rate of 473 L/h and a drying gas flow rate of 32 m$^3$/h.

3.3. In-vivo pulmonary absorption

The pulmonary absorption of LPP-PZA powder after adjusting the spray-drying conditions was evaluated following administration of 5 mg of PZA powder (corresponding to 1.65 mg PZA) as a single dose via intratracheal insufflations to rats. This pulmonary administration was compared to a control intravenous administration of PZA to calculate the absolute bioavailability of our formulation. The amount of powder actually pushed into the lungs was 70 ± 12 % of the amount placed in the insufflator, as determined by weighing, corresponding to an administered dose by intratracheal administration of 1.16 ± 0.20 mg of PZA. After administration, either by IV or by intratracheal administration, rats did not present apparent signs of discomfort. Mean PZA plasma concentration versus time curves are presented in Figure 5.

![Plasma concentration time profiles of PZA after intratracheal and intravenous administration](image)

**Figure 5:** Plasma concentration time profiles of PZA after intratracheal and intravenous administration (left). Plasma concentration corrected by the actual dose of PZA administered (right).

PZA seems to cross efficiently the lung epithelial barrier, as it is recovered in the plasma at levels very close to those administered intravenously. Key pharmacokinetic parameters obtained from the analysis of PZA plasma concentration versus time curves by the different routes are shown in Table 5. After intratracheal administration, the absorption of PZA from the lung occurs in a short period (MAT= 0.54 h) and plasma PZA concentrations peaked at 30 minutes ($t_{\text{max}}= 0.5$ h) soon after powder was insufflated. In addition, PZA concentrations at 15 minute and 30 minute are very close. The plasma peak concentration ($6.68 \pm 0.91 \mu$g/mL) that we observed in rats is
on the order of magnitude of the minimum inhibitory concentration (MIC = 6.25 μg/mL) of PZA [70]. The AUC and C_{max} corresponding to IV administration exhibit larger values (34.22 ± 3.64 μg.h/ml and 9.57 ± 2.03 μg/ml, respectively) than those corresponding to intratracheal administration (AUC = 25.16 ± 8.56 μg.h/ml; C_{max}= 6.68 ± 0.91 μg/ml). For further comparison between these treatments, AUC and C_{max} were corrected by the actual dose in the respective treatments. Rats received 5 mg PZA-LPP powder by insufflation corresponding to a PZA dose of: 5 x 33% (drug loading) x 70% (amount of powder pushed into the lungs) = 1.16 mg. For IV administration, rats received an injection of 200 μL of a PZA solution corresponding to 1.6 mg PZA. After correction by the actual dose, AUC and C_{max} after IV administration of PZA are not significantly different from those after intratracheal administration (unpaired t-test, P > 0.05) (Fig. 5 right). In addition, t_{1/2} and V_d of both routes of administration do not present any significant difference (unpaired t-test, P > 0.05). The absolute bioavailability (around 1) also reflects the absence of difference between to two routes of administration. PZA crosses efficiently the lung barrier to reach the blood stream.

**Table 5:** Pharmacokinetic parameters obtained after administering PZA by the different routes (mean ± SD, n= 6). NA=not applicable.

<table>
<thead>
<tr>
<th>Pharmacokinetic parameters</th>
<th>IV administration</th>
<th>Intratracheal administration</th>
</tr>
</thead>
<tbody>
<tr>
<td>C_0 or C_{max} (μg/ml)</td>
<td>9.6 ± 2.0</td>
<td>6.7 ± 0.9</td>
</tr>
<tr>
<td>t_{max} (h)</td>
<td>NA</td>
<td>0.5</td>
</tr>
<tr>
<td>AUC (μg.h/ml)</td>
<td>34.2 ± 3.6</td>
<td>25.2 ± 8.6</td>
</tr>
<tr>
<td>CL (ml/h)</td>
<td>47.2 ±5.0</td>
<td>49.8 ± 13.9</td>
</tr>
<tr>
<td>V_d (ml)</td>
<td>151.7 ± 15.4</td>
<td>172.75 ± 26.49</td>
</tr>
<tr>
<td>t_{1/2} (h)</td>
<td>2.2 ± 0.1</td>
<td>2.50 ± 0.47</td>
</tr>
<tr>
<td>MRT</td>
<td>3.2 ± 0.1</td>
<td>3.6 ± 0.7</td>
</tr>
<tr>
<td>MAT</td>
<td>NA</td>
<td>0.54 ± 0.47</td>
</tr>
<tr>
<td>F_{abs}</td>
<td>NA</td>
<td>1.00 ± 0.27</td>
</tr>
</tbody>
</table>

BALF was taken out at different time points after intratracheal or IV administration of PZA. PZA concentration in the BALF as a function of time is presented in (Fig 6). PZA concentration in the BALF quickly decreases for both administration routes, confirming its rapid passage to the blood circulation. Indeed 24 hours after administration PZA concentration in the BALF is below the limit of quantification (LOQ) of 0.007 μg/mL. After correction by the actual dose administered (Figure 6, right), results show that PZA concentrations in the BALF were similar for both administration routes at 15 min and 6 hours (unpaired t-test, P > 0.05). At 30 min and
3 hours, PZA concentrations after intratracheal insufflation were lower than after IV administration (unpaired t-test, $P < 0.05$). This reflects that PZA can cross the lung barrier one way or the other.

![Figure 6: BALF concentration time profiles of PZA after intratracheal and intravenous administration (left). BALF PZA concentration corrected by the actual dose of PZA administered (right).](image)

From PZA concentrations in the BALF, we determined the actual PZA concentrations in the lung lining fluid according to dilution due to lavage based on two methods. One can use the volume of lung lining fluid reported in the literature (80.2 μL) to calculate the dilution factor according to the volume of fluid used for lavage [71]. Alternatively, the concentration of urea in the BAL can be measured as an endogenous marker [72] to determine drug concentrations accurately for each animal independently.

For the former method, since lung lining fluid was diluted into 24 mL of PBS, the dilution factor should be around 300. PZA concentrations in the lung lining fluid calculated from this method are reported in Figure 7 (left). After intratracheal insufflation, PZA concentration in the lung lining fluid at 15 min was $142.15 \pm 40.75$ μg/mL. After IV administration, the actual PZA concentrations in the lung lining fluid were higher than after intratracheal administration for all time points (except 24h below LOQ). These results from the difference of PZA dose actually administered. Indeed, as these concentrations are divided by the actual PZA dose (Figure 7, right), results show that PZA concentrations in the lung lining fluid were similar for both administration routes at 15 min and 6 hours (unpaired t-test, $P > 0.05$). At 30 min and 3 hours, PZA concentrations after intratracheal insufflation were lower than after IV administration (unpaired t-test, $P < 0.05$).
Figure 7: Lung lining fluid PZA concentration time profiles obtained using a constant dilution factor for the lavage (left). Lung lining fluid PZA concentration corrected by the actual dose of PZA administered using a constant dilution factor for the lavage (right).

For the second method, the actual PZA concentration in the lung lining fluid was determined based on the dilution factor of urea (Table 6). One can notice that mean dilution factors are in the 30-90 range for all time points and administration routes except for 30 min rats administered by intratracheal insufflation (dilution factor around 300). The dilution factors are very different for the calculation based on the lung lining fluid volume except from the 30 min dilution factor (IT). The huge difference in dilution factor leads to a strange result with a peak in PZA concentration at 30 min for the intratracheally administered rats (Fig. 8). This increase of PZA concentration from 15 min to 30 min is not logical as local concentration should decrease with time as the drug passes to the blood stream. We believe that the colorimetric method we used for determination of urea concentration might not be precise enough or might suffer from interferences with PZA. Although the first method does not take into account the dilution factor for each individual rat it appears more suitable since it gives more trustable results.

Table 6: Dilution factors due to lavage determined through quantification of urea concentration in the BALF (mean ± SD, n= 6).

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>IV administration</th>
<th>Intratracheal administration</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>47 ± 7</td>
<td>45 ± 10</td>
</tr>
<tr>
<td>0.5</td>
<td>78 ± 12</td>
<td>296 ± 135</td>
</tr>
<tr>
<td>3</td>
<td>43 ± 12</td>
<td>93 ± 22</td>
</tr>
<tr>
<td>6</td>
<td>39 ± 3</td>
<td>63 ± 11</td>
</tr>
<tr>
<td>24</td>
<td>76 ± 30</td>
<td>59 ± 11</td>
</tr>
</tbody>
</table>
**Figure 8:** Lung lining fluid concentration time profiles of PZA obtained using the actual dilution factor after measuring urea concentration in the BLAF for each individual animal.

In the alveolar cell pellet, PZA could only be quantified at 15 and 30 min due to the dilution of the cell pellet and the limit of quantification. There are slight significant difference between both administration routes (Table 7) (ANOVA, \( P = 0.045 < 0.05 \)). PZA most probably diffuses in the alveolar cells from the lung lining fluid and LPPs dissolve too fast to be phagocytosed and yield high alveolar cell concentrations of PZA.

**Table7:** Amount of PZA in alveolar cell pellet versus times (mean ± SD, n= 6).

<table>
<thead>
<tr>
<th>The amount of PZA in AC (μg)</th>
<th>Times</th>
<th>15 min</th>
<th>30 min</th>
<th>3 hour</th>
<th>6 hour</th>
<th>24 hour</th>
</tr>
</thead>
<tbody>
<tr>
<td>IV</td>
<td></td>
<td>0.037 ±</td>
<td>0.060 ±</td>
<td></td>
<td></td>
<td>&lt; LOQ*</td>
</tr>
<tr>
<td>Intratracheal</td>
<td></td>
<td>0.044 ±</td>
<td>0.022 ±</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.015</td>
<td>0.012</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Limit of quantification

Altogether, plasma, BALF and cell pellet results suggest that once PZA-LPPs are deposited in the lungs, PZA is released quickly from excipients to achieve rapid absorption within 15-30 minutes. This ability might arise from the mostly amorphous state of PZA in the powder but also because surface-active compounds are present in the formulation that might favor PZA solubilization. 30 minutes after intratracheal insufflations, PZA concentration in plasma reaches a peak and elimination occurs. It should also be noted that PZA can also pass from the blood to the lung lining fluid. One can notice that PZA concentration in the lung lining fluid decreases more rapidly after intratracheal insufflation than after IV administration. A hypothesis for this finding is that the velocity of diffusion of PZA from lung to blood and the other way differ. Another hypothesis is that some inhaled LPPs are trapped in the mucus and
undergo mucociliary clearance and are finally swallowed [73]: PZA is therefore eliminated from the lungs explaining its quicker decrease than when administered by IV.

4. Conclusion
We have optimized a formulation of pyrazinamide as large porous particles for direct lung delivery by adjusting spray drying parameters to yield homogeneous deposition of particles in the deep lungs. The in vivo pharmacokinetic evaluation of PZA-LPPs in rats reveals that intratracheal insufflation of PZA-LPPs and IV administration of PZA lead to similar pharmacokinetic parameters proving that PZA-LPPs dissolve fast upon deposition and that PZA crosses efficiently the lung barrier to reach the systemic circulation. The absolute bioavailability of PZA administered by insufflations was of 1. Surprisingly, PZA was cleared faster from the lung lining fluid when administered by intratracheal insufflations than by IV administration. Lung delivery of PZA appears an interesting alternative to oral delivery of the molecule and should now be tested in an infected animal model to evaluate its efficacy against Mycobacterium tuberculosis.

Acknowledgments:
D. D. Pham fellowship is supported by Project 322, Ministry of Education and Training, Vietnam. Authors thank N. Guiblin and N.E. Ghermani (SPMS, Ecole Centrale Paris) for help with X-Ray Diffraction, and V. Domergue-Dupont, A. N’Guessan and L. Aragão-Santiago for support with animal experiments. Institut Galien Paris-Sud is a member of the Laboratory of Excellence LERMIIT supported by a grant from ANR (ANR-10-LABX-33).

Reference


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Chapter IV

Pyrazinamide-loaded Poly(lactide-co-glycolide) Nanoparticles: Optimization by Experimental Design

Dinh-Duy Pham*, Elias Fattal, Nicolas Tsapis*

Manuscript in preparation
Abstract

We optimize pyrazinamide-loaded poly(lactide-co-glycolide) nanoparticles to obtain particle size below 200 nm and a polydispersity index (PDI) below 0.2 through Taguchi experimental design. PZA plays a unique role in shortening Mycobacterium tuberculosis (TB) treatment from the 9–12 months required prior to its introduction to the current standard of 6 months, often referred to as short course chemotherapy. Poly(lactide-co-glycolide) (PLGA) nanoparticle based drug delivery systems have been applied for anti-TB drugs with the goal of improving of drug bioavailability, reducing of the dosing frequency and improving patient adherence to prescribed therapy, one of the critical obstacles in the control of TB epidemics. PZA-loaded PLGA nanoparticles were prepared by the double emulsion method. The Taguchi method, a statistical design with an orthogonal array, was implemented to optimize the formulation parameters of PLGA nanoparticles containing PZA. The solvent type (dichloromethane and ethyl acetate), the PZA / PLGA weight ratio and the organic phase / aqueous phase volume ratio were chosen as significant parameters affecting the particle size and PDI. Taguchi method proved to be a quick, valuable tool in optimizing the particle size and PDI of PZA-loaded PLGA nanoparticles. The optimized experimental values for the nanoparticle size and PDI were about 170 nm and < 0.1. Optimized PLGA nanoparticles possessed a zeta potential of about -1 mV, an encapsulation efficacy of 7-8% and a drug loading of 3%. They are suitable to be tested in vivo for TB treatment.

Keywords: nanoparticles, pyrazinamide, optimization, Taguchi, experimental design.
1. Introduction

Pyrazinamide (PZA) is an important first-line antitubercular (anti-TB) drug used in combination with other anti-TB drugs for the treatment of drug susceptible TB and multi-drug resistant TB (MDR-TB) [1]. PZA plays a unique role in shortening Mycobacterium tuberculosis (MTB) treatment from the 9–12 months required prior to its introduction to the current standard of 6 months, often referred to as short course chemotherapy [2]. PZA enters Mycobacterium tuberculosis in lesions by passive diffusion, is converted to pyrazinoic acid (POA) by pyrazinamidase and is then excreted by a weak efflux pump. The protonated POA diffuses back again into the bacilli under acidic conditions and accumulates because the efflux pump is inefficient, causing membrane damage [3]. Therefore PZA is more active against old non-growing bacilli than against young actively growing tubercle bacilli. PZA is usually given at a dose of 20–25 mg/kg daily corresponding to 1.5 to 2 g depending on patient weight [4]. Although PZA bioavailability is higher than 90%, a high oral dose over long periods of time leads to hepatotoxicity [2, 5]. PZA lung delivery therefore appears as an interesting alternative to the oral route since it avoids the hepatic first-pass effect and might help concentrating the drug at its site of action and therefore reduce hepatic side effects.

PZA could be delivery directly to the lungs as a solution by nebulization but it requires drug stability in solution [6]. Dry powder inhalers (DPI) can be considered as they allow to deliver efficiently large amounts of drugs [7], powders cannot enter efficiently macrophages where the bacilli develop due to premature dissolution and large particle sizes. In the recent years, nanoparticle-based drug delivery systems have been applied for anti-TB drugs with the goal of improving of drug bioavailability, reducing the dosing frequency and preventing non adherence to prescribed therapy, a critical obstacle in the control of TB epidemics [8]. Nebulization of nanoparticles encapsulating PZA or administration of these nanoparticles as powders made of Trojan particles [9, 10] could represent an interesting alternative. Indeed, due to their small sizes, nanoparticles can be phagocytosed by macrophages and therefore facilitate drug entrance in these diseased cells. Poly(lactide-co-glycolide) (PLGA) is one of the most common biodegradable and biocompatible polymer used to formulate nanoparticles (NPs). Encapsulation of anti-TB drugs into NPs not only would allow to maintain the drug levels in various tissues for long period of time, but also would reduce adverse effects and would exhibits similar efficacy at reduced dose compared to conventional drug administration in animal models infected with
MTB [11, 12]. Moreover, after being administered via respiratory route, the nanoparticle containing anti-TB drugs showed the higher effect than that obtained after oral administration [12].

PLGA NPs are often formulated using the double emulsion solvent evaporation method to encapsulate water soluble drugs [13]. The physicochemical properties of NPs are affected by various parameters such as drug / polymer ratio, surfactant concentration, volume ratio of organic phase and outer aqueous phase and sonication time to obtain desired NP size with maximum drug entrapment [14, 15]. The effect of the mentioned parameters is complex and optimization of these factors is time and labor consuming. Taguchi method using statistical experimental design represents an easy and rapid strategy to develop an optimal formulation using minimum raw materials [16]. It uses the table of orthogonal arrays and analysis of variance (ANOVA) as analysis tools. ANOVA can estimate the influence of a factor on NP properties. While conventional statistical experimental design determines the optimal conditions based on the measured values of the characteristic properties, Taguchi method determines the experimental conditions having the least variability as the optimum condition [16, 17]. In this study, we investigated parameters affecting the size of PZA-loaded PLGA nanoparticles prepared by the double emulsion solvent evaporation method. Parameters were optimized using the Taguchi method, which provides a comprehensive system for optimizing nanoparticle size by considering equal contributions of variables. The resulting optimized formulation was characterized by evaluating NP size, zeta potential, encapsulation efficacy and drug loading.

2. Materials and Methods

2.1. Materials

Pyrazinamide (PZA) with specified purity greater than 99%, poly(vinyl-alcohol) (PVA) (87–89% hydrolyzed, MW 30,000–70,000) and sodium chloride (NaCl) were obtained from Sigma-Aldrich (France). Poly(D,L-lactide-co-glycolide) (PLGA 75:25) Resomer RG756 was purchased from Boehringer-Ingelheim (Germany). Dichloromethane (DCM) and Ethyl acetate (EA) in analytical grade and acetonitrile in high-performance liquid chromatography (HPLC) grade were all obtained from Carlo Erba Reagents (France). Water was purified using a RIOS/MilliQ system from Millipore (France).
2.2. Preparation of Pyrazinamide-Loaded Nanoparticles

PLGA nanoparticles encapsulating PZA were prepared by double emulsion solvent evaporation method. Briefly, 1 mL of an aqueous drug solution containing 10 mg PZA was first emulsified into 10 mL of evaporated solvent (DCM, EA or mixture of DCM:EA 50:50) containing PLGA (drug/polymer: 1:1 - 1:5 by weight) for 1 min by probe sonication (Digital Sonifier®, Branson, France; 116 W, 20 kHz) over an ice bath. The primary emulsion was poured into 10-200 mL of an aqueous solution including 0.5-2% PVA and 0.5% NaCl. This mixture was then sonicated (300 W, 20 kHz) for 3 min to form the second water-in-oil-in-water emulsion (W/O/W). The secondary emulsion was stirred continuously 6 hours with a magnetic stir bar for complete removal of solvent at room temperature. PZA-loaded PLGA nanoparticles were recovered by centrifugation (40 000 rpm, 30 min), washed twice with water to remove the excess of PVA from the nanoparticles. Finally, the NP suspension was freeze-dried with trehalose (25 mg/mL) as cryoprotectant and stored in the freezer.

2.3. Design of Experiment

Taguchi has developed a method based on “orthogonal array” experiments which reduce experimental variance for the experiment with “optimum settings” of control parameters. Thus the combination of design of experiments with optimization of control parameters to obtain best results is achieved in the Taguchi method [18]. In this study, to identify the best formulations, four important factors that influence the PLGA NP size and polydispersity index were the followings: solvent type, PZA / PLGA weight ratio, organic phase / outer aqueous phase volume ratio and PVA concentration. Three different levels for each parameter (Table 1) and a fractional factorial design, namely, a standard L9 orthogonal array were considered (Table 2).

The effects of the proposed experiments on the responses were then analyzed by the Design Expert software (trial version 8.0.6, Stat-Ease, Minneapolis, USA), to obtain independently the main effects of these factors, followed by the analysis of variance (ANOVA) to determine which factors were statistically significant. The optimum conditions were determined by desirability function.
Table 1: Definition and trial levels of studied variables in Taguchi’s L9 orthogonal array experiment.

<table>
<thead>
<tr>
<th>Studied variables</th>
<th>Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Solvent type</td>
<td>DCM</td>
</tr>
<tr>
<td>PZA / PLGA weight ratio</td>
<td>1:1</td>
</tr>
<tr>
<td>Organic phase / aqueous phase volume ratio</td>
<td>1:1</td>
</tr>
<tr>
<td>PVA concentration (%)</td>
<td>0.5</td>
</tr>
</tbody>
</table>

2.4. Nanoparticle characterization

NP size distribution and Zeta potential

The drug-loaded PLGA NPs were analysed for their size and polydispersity index (PI) using a Zetasizer ZS (Malvern Instruments, France), based on photon correlation spectroscopy. Size measurements were performed in triplicate following a 1/100 v/v dilution of the NP suspension in purified water at 25°C at a fixed angle of 173°. The polydispersity index range was comprised between 0 and 1. Zeta potential was measured using the same instrument with a disposable zeta cuvette at 25°C following a 1/50 v/v dilution in a 1 mM KCl solution. For each sample, the mean diameter/ Zeta potential ± standard deviation of three determinations were calculated applying multimodal analysis.

Encapsulation efficacy (EE%) and Drug loading (DL%) within PLGA nanoparticles

The amount of PZA encapsulated within PLGA nanoparticles was determined by measuring the amount of non-encapsulated PZA in the aqueous solution recovered after ultracentrifugation and washing of the particles. PZA was analyzed by UV spectrophotometer at 268 nm. The drug encapsulation efficiency was expressed as the percentage of drug entrapped with respect to the initial amount, and the drug loading was expressed as the amount of drug entrapped per gram of polymer+drug.

\[
EE\% = \left(\frac{\text{initial amount of drug (mg) - free amount of drug (mg)}}{\text{initial amount of drug (mg)}}\right) \times 100
\]

\[
DL\% = \left(\frac{\text{initial amount of drug (mg) - free amount of drug (mg)}}{\text{total amount of polymer (mg) + encapsulated drug (mg)}}\right) \times 100
\]
3. Results and Discussions

3.1. Taguchi array design and analysis of variance

The Taguchi orthogonal array design is used to identify the optimal conditions and to select the parameters having the principal influence on the average particle size and polydispersity index. Table 2 shows the structure of Taguchi orthogonal array design and the results of measurement of the average particle size and polydispersity index. The experimental results were then analyzed by the Design Expert software to extract independently the main effects of these factors, followed by the analysis of variance (ANOVA) to determine which factors were statistically significant.

<table>
<thead>
<tr>
<th>Table 2: Particle size and Polydispersity index of different PLGA NPs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Runs</td>
</tr>
<tr>
<td>------</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>5</td>
</tr>
<tr>
<td>6</td>
</tr>
<tr>
<td>7</td>
</tr>
<tr>
<td>8</td>
</tr>
<tr>
<td>9</td>
</tr>
</tbody>
</table>

The average particle size and polydispersity index of PLGA nanoparticles were considered as input into the Design Expert software for further analysis. To ensure a good model, test for significance of the regression model and test for significance on individual model coefficients were performed. An ANOVA table is commonly used to summarize the tests performed. Tables 3 and 4 present the ANOVA tables for the Taguchi method.

For the average particle size, Table 3, the value of “Prob > F” for the model is lower than 0.05, which indicates that the model is significant. Solvent type and organic phase / outer aqueous phase volume ratio are significant model terms, whereas PZA / PLGA weight ratio can be considered not significant. This not significant variable can be removed and may result in an improved model. The $R^2$-value calculated is 0.99, reasonably close to 1, which is acceptable. It implies that about 99% of the variability in the data is explained by the model. The predicted $R^2$-value of 0.71 is not as close to the adjusted $R^2$ of 0.94 as one might normally expect. This may indicate a
large block effect or a possible problem with the model and/or data. However, the
difference of both values is not too large (about 0.2), hence, we still used this model
for further analysis. The adjusted $R^2$-value is particularly useful for comparing models
with different number of terms. "adeq. precision" measures the signal to noise ratio. A
ratio greater than 4 is desirable and indicates an adequate signal. Although the PZA / PLGA weight ratio has P-value greater than 0.05, this variable was still kept into the
model because the model becomes significant keeping this parameter.

For the polydispersity index, the original response values were transformed by the
following equation: $y' = (y + k)^\lambda$, where constant $(k) = 0$ and lambda $(\lambda) = 1.82$. The
transformed values were then analyzed similarly as average particle size. In the
ANOVA table (Table 4), the value of “Prob > F” for the model is lower than 0.05,
which indicates that the model is significant. In this case, solvent type, PZA / PLGA
weight ratio and organic phase / outer aqueous phase volume ratio all are significant
model terms. The $R^2$-value calculated is 0.99, reasonably close to 1, which is
acceptable. The predicted $R^2$-value of 0.99 is almost in reasonable agreement with
the adjusted $R^2$ of 0.99. "adeq. precision" greater than 4 is desirable and indicates an
adequate signal.

**Table 3:** ANOVA table for the average particle size ($R^2 = 0.99$; adjusted $R^2 = 0.94$;
adeq precision = 12.13 (> 4)

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of Squares</th>
<th>Degrees of freedom</th>
<th>Mean Square</th>
<th>F-value</th>
<th>$P$-value (Prob &gt; F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>170001.5</td>
<td>6</td>
<td>28333.59</td>
<td>22.5761</td>
<td>0.0430</td>
</tr>
<tr>
<td>A-Solvent type</td>
<td>51347.35</td>
<td>2</td>
<td>25673.67</td>
<td>20.4567</td>
<td>0.0466</td>
</tr>
<tr>
<td>B-PZA / PLGA weight ratio</td>
<td>34441.46</td>
<td>2</td>
<td>17220.73</td>
<td>13.7214</td>
<td>0.0679</td>
</tr>
<tr>
<td>C-Organic phase / aqueous phase volume ratio</td>
<td>84212.7</td>
<td>2</td>
<td>42106.35</td>
<td>33.5501</td>
<td>0.0289</td>
</tr>
<tr>
<td>Residual</td>
<td>2510.056</td>
<td>2</td>
<td>1255.028</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cor Total</td>
<td>172511.6</td>
<td>8</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 4: ANOVA table for the polydispersity index ($R^2 = 0.99$; adjusted $R^2 = 0.99$; adeq precision = 121.07 (> 4))

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of Squares</th>
<th>Degrees of freedom</th>
<th>Mean Square</th>
<th>F-value</th>
<th>$P$-value (Prob &gt; F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>0.1601</td>
<td>6</td>
<td>0.0267</td>
<td>2724.38</td>
<td>0.0004 significant</td>
</tr>
<tr>
<td>A-Solvent type</td>
<td>0.0035</td>
<td>2</td>
<td>0.0018</td>
<td>179.59</td>
<td>0.0055</td>
</tr>
<tr>
<td>B-PZA / PLGA weight ratio</td>
<td>0.1554</td>
<td>2</td>
<td>0.0777</td>
<td>7933.69</td>
<td>0.0001</td>
</tr>
<tr>
<td>C-Organic phase / aqueous phase volume ratio</td>
<td>0.0012</td>
<td>2</td>
<td>0.0006</td>
<td>59.85</td>
<td>0.0164</td>
</tr>
<tr>
<td>Residual</td>
<td>0.0000</td>
<td>2</td>
<td>0.0000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cor Total</td>
<td>0.1601</td>
<td>8</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1 shows the various effects of the independent variables on the average NP size and the polydispersity index. The NP size is found to decrease as DCM is mixed with EA or replaced by EA. NP size exhibits a minimum for the organic phase / outer aqueous phase volume ratio of 1:10. The polydispersity index increases significantly when using a mixture of DCM and EA as well as when the organic phase / outer aqueous phase volume ratio decreases, whereas this index decreases strongly as PZA / PLGA weight ratio decreases.
**Figure 1:** Effects of the independent variables on the average particle size and polydispersity index

Computer optimization process by Design Expert software and a desirability function determined the effect of the levels of independent parameters on the responses. All responses were fitted to the linear model. The constraints of independent variables and the responses are presented in Table 5.
Table 5: Constraints of independent variables and responses

<table>
<thead>
<tr>
<th></th>
<th>Goal</th>
<th>Limited range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent type</td>
<td>in range</td>
<td>DCM – EA</td>
</tr>
<tr>
<td>PZA / PLGA weight ratio</td>
<td>equal to 1:2.5</td>
<td>1:1 – 1.5</td>
</tr>
<tr>
<td>Organic phase / aqueous phase volume ratio</td>
<td>equal to 1:1</td>
<td>1:1 – 1.20</td>
</tr>
<tr>
<td>PVA concentration (%)</td>
<td>Term not in model</td>
<td></td>
</tr>
<tr>
<td>Average particle size (nm)</td>
<td>Minimize</td>
<td>79 – 467</td>
</tr>
<tr>
<td>Polydispersity index</td>
<td>Minimize</td>
<td>0.10 – 0.56</td>
</tr>
</tbody>
</table>

Based on the data of the particle size and polydispersity index and on the ANOVA analysis, Design Expert software proposed optimum solutions for formulating PZA-loaded PLGA nanoparticles (Table 6).

Table 6: Optimum solutions for formulating PZA-loaded PLGA nanoparticles

<table>
<thead>
<tr>
<th>Number</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent type</td>
<td>EA</td>
<td>DCM:EA</td>
<td>EA</td>
<td>DCM</td>
</tr>
<tr>
<td>PZA / PLGA weight ratio</td>
<td>1:2.5</td>
<td>1:2.5</td>
<td>1:5</td>
<td>1:2.5</td>
</tr>
<tr>
<td>Organic phase / aqueous phase volume ratio</td>
<td>1:1</td>
<td>1:1</td>
<td>1:1</td>
<td>1:1</td>
</tr>
<tr>
<td>PVA concentration (%)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Average particle size (nm)</td>
<td>170</td>
<td>225</td>
<td>315</td>
<td>350</td>
</tr>
<tr>
<td>Polydispersity index</td>
<td>0.11</td>
<td>0.20</td>
<td>0.13</td>
<td>0.07</td>
</tr>
<tr>
<td>Desirability</td>
<td>0.87</td>
<td>0.70</td>
<td>0.61</td>
<td>0.55</td>
</tr>
</tbody>
</table>

Optimization was performed by using a desirability function to obtain the levels of investigated factors, which minimized the particle size and polydispersity index (Table 6). The predicted optimal conditions of solution 1 with a desirability factor equal to 87% were chosen to prepare PZA-loaded PLGA nanoparticles since they yield simultaneously the lower PDI and the lower size. The experimental result of average particle size showed an acceptable agreement with the model (Table 7). There is as large difference between the predicted and experimental polydispersity index. However, the experimental polydispersity index is lower than the model, proving that nanoparticles are rather monodisperse.
Table 7: Predicted and experimental results for particle size and polydispersity index of PZA-loaded PLGA nanoparticles

<table>
<thead>
<tr>
<th></th>
<th>Average particle size (nm)</th>
<th>Polydispersity index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prediction</td>
<td>170 ± 35</td>
<td>0.11 ± 0.01</td>
</tr>
<tr>
<td>Experimental</td>
<td>173 ± 4</td>
<td>0.05 ± 0.03</td>
</tr>
<tr>
<td>Error %</td>
<td>1.8</td>
<td>54.5</td>
</tr>
</tbody>
</table>

3.2. Characterization of Nanoparticles

The optimal formulation was then prepared in duplicate and characterized. The physicochemical properties of the optimal formulation of PZA-loaded PLGA NPs are shown in Table 8. The size of the optimized formulations was 173 nm with a polydispersity index lower than 0.1 (Fig 2). This is a suitable size range for macrophage uptake after pulmonary drug delivery [19]. The zeta potential of nanoparticles was found about -1 mV (Fig 2). Particles are therefore very neutral and should interact with lung cells [20, 21]. The encapsulation efficiency of PZA in the optimized formulation was about 8 %, leading to a drug loading of 3.1%, in the range usually found in the literature.

PLGA nanoparticles fabricated by double emulsion solvent evaporation method have been commonly applied for encapsulating hydrophilic molecules [22]. Typically, the size of the PLGA nanoparticles is in the range of 100 to 250 nm. Especially, as the classical solvent (dichloromethane) of this technique was replaced by a partially water-miscible organic solvent (ethyl acetate), the nanoparticle size was improved lower than 200 nm [23]. The zeta potential of PLGA nanoparticles using PVA as stabilizer had often from the slight negative value to neutral value [20, 23]. For encapsulating small hydrophilic molecules in PLGA nanoparticles, the double emulsion solvent evaporation method often suffer from low encapsulation efficiency because of the drug rapid partitioning to the external aqueous phase [23]. However, with the some modification of technique choosing polymer or surfactant, the encapsulation efficiency of PZA can be improved [24, 25].

Table 8: Characteristics of PZA loaded PLGA nanoparticles

<table>
<thead>
<tr>
<th>Lot #</th>
<th>Particle size (nm)</th>
<th>PDI</th>
<th>Zeta potential (mV)</th>
<th>Encapsulation efficacy (%)</th>
<th>Drug loading (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>170 ± 2</td>
<td>0.03 ± 0.01</td>
<td>-1.08 ± 0.14</td>
<td>8.8 ± 4.7</td>
<td>3.4 ± 1.7</td>
</tr>
<tr>
<td>2</td>
<td>175 ± 3</td>
<td>0.07 ± 0.00</td>
<td>-1.10 ± 0.06</td>
<td>7.1 ± 0.8</td>
<td>2.8 ± 0.3</td>
</tr>
</tbody>
</table>
Figure 2: Optimized Nanoparticles Formulation. The top graph represents Particle Size Distribution, the bottom graph represents the Zeta Potential distribution.

4. Conclusion
The Taguchi L9 method appears to be a fast, simple and valuable tool in optimizing the various parameters for the preparation of PZA-loaded PLGA nanoparticles. The organic solvent was found to be the main parameter having significant effects on the particle size and polydispersity index. A good agreement was obtained between the predicted optimized Taguchi method and the experimental data. Further experiments on the physicochemical characteristics of the PLGA nanoparticles to determine their suitability for developing the pulmonary drug delivery will be described in future.
Acknowledgments:

D. D. Pham fellowship is supported by Project 322, Ministry of Education and Training, Vietnam. Institut Galien Paris-Sud is a member of the Laboratory of Excellence LERMIT supported by a grant from ANR (ANR-10-LABX-33).

References


General Discussion and Perspectives
The objective of the thesis work was the formulation of respirable particulate systems containing pyrazinamide intended for deep lung delivery for tuberculosis treatment. The process of work was divided into the following steps: i) Formulation of pyrazinamide-loaded large porous particles for the pulmonary route; ii) In-vitro and in-vivo evaluation of pyrazinamide-loaded large porous particles performances; iii) Preparation of pyrazinamide-loaded poly(lactide-co-glycolide) nanoparticles by design of experiment to include in the formulation of Trojan particles for pulmonary delivery.

In the first part of the thesis, PZA was formulated into large porous particles intended for deep lung delivery. Dry powders were prepared by spray-drying using generally recognized as safe (GRAS) excipients. The concentration of excipients was investigated in order to optimize the characteristics of spray-dried PZA powders and also to prevent PZA crystallization upon storage.

PZA is a quite rigid molecule with only two relevant conformers, having quite different energies [1, 2]. Therefore, PZA has no conformational polymorphism [3, 4]. However, the different possibilities of intermolecular hydrogen bonds can promote the occurrence of packing polymorphisms such as α, β, γ and δ polymorphs [5, 6]. Determination of the polymorphic forms of a drug is an important part of the formulation-development process, because polymorphic forms are not equivalent. Indeed, polymorphs differ by their energy state and thus possess different properties, including stability, solubility, and even bioavailability [7]. To reduce the risk of transformation during processing or storage, the most stable polymorph is typically selected for development, provided its other properties are manageable [8].

In our study, crystalline particles of the δ polymorph of PZA were formed after spray-drying the solution of PZA in a mixture of ethanol water (70/30 v/v). From the thermodynamic point of view, Castro et al. claimed that the δ polymorph is the most stable [9] whereas Cherukuvada et al. found that α polymorph should be preferred taking into account both stability and bioavailability criteria [10]. Our results show that the particle median size of the PZA δ polymorph crystals increased from 4.7 ± 0.1 μm to 26.5 ± 0.2 μm (unpaired t-test, \( P < 0.05 \)) after 4 weeks of storage. Moreover, crystalline particles are typically non-spherical, tend to pack more tightly with a high tapped density [11]. Given their size instability and their tapped density, these crystals were not suitable for lung delivery and there was a need for improving
particle physical properties. For this purpose, we reviewed a variety of excipients and studied their impact on PZA polymorphism and particle characteristics after spray-drying.

Drugs encapsulated into large porous particles that are highly advantageous for respiratory drug delivery are usually in the amorphous state or partly crystalline. It should be noted that the current list of excipients approved by regulatory agencies for respiratory drug delivery is very limited [12]. The array of potential excipients is limited to compounds that are biocompatible or endogenous to the lung and can easily be metabolized or cleared. Currently, inhalable dry powders consisting of combinations of drugs and GRAS excipients showed particularly efficient aerosolization performances [13-16]. Each excipient in the formulation appears necessary for optimal aerosolization properties. For example, dipalmitoylphosphatidylcholine (DPPC) was used to formulate large porous particles by Vanbever et al (1999) [17] as it might facilitate droplet formation in the atomization step of spray drying and might decrease particle surface energy thereby decreasing powder cohesiveness. DPPC also influenced the morphology of the particles: as DPPC content increased particle morphology evolved from torroidal (0% to 30% DPPC) to sponge-like (60% DPPC) and spherical (90% DPPC). DPPC alone could not provide the skeletal structure necessary for the formation of solid particles by spray drying whereas lactose and albumin could [12]. Gomez-Gaete et al. have shown that spray-drying DPPC alone led to aggregated microparticles [18]: addition of hyaluronic acid was needed to yield discrete microparticles. Albumin is also an interesting excipient as it possesses surfactant properties and may limit point to point contacts due to highly indented geometries, reducing interparticle adhesion forces [13].

In our study, we combined 4 excipients not only to generate large porous particles suitable for lung delivery but also to prevent PZA crystallization and stabilize the amorphous form of PZA in the particles. The first excipient was ammonium bicarbonate (AB) which is a porogen often used to prepare large porous particles. During spray drying, AB decomposed at 36 to 60 °C into ammonia, carbon dioxide and water vapor, therefore, leading to porous particles. The addition of AB to the PZA solution led to a decrease of the tap density from 0.44 ± 0.02 g/cm³ to 0.30 ± 0.05 g/cm³ (unpaired t-test and ANOVA, P < 0.05). Moreover as AB is added PZA polymorph exists under the α crystalline form. However, powder was unstable with an increase in median size within 4 weeks of storage. Leucine, a useful additive for
decreasing aggregation of dry powders designed for pulmonary delivery [19-24], was also employed. The effect of leucine on the aerodynamic properties is often explained by its distinct hydrophilic and lipophilic regions, which leads to an accumulation of leucine molecules at the air-solvent interface of the droplet [22, 25, 26]. Incorporation of leucine further favored the reduction of the tapped density from 0.34 ± 0.04 g/cm³ to 0.24 ± 0.03 g/cm³ (unpaired t-test, P < 0.05). Indeed as leucine concentration increases, the surface properties and the morphology of the particles might be affected leading to an increase of porosity and therefore a decrease of tap density as observed before [20, 21, 23, 24]. DSC and XRPD experiments indicated some interactions between leucine and PZA, however the addition of leucine was not sufficient to inhibit PZA crystallization and prevent crystal growth upon storage. To overcome crystallization, hyaluronic acid (HA) was chosen since polymers are known to slow down recrystallization and HA was combined with DPPC [18]. As a result, the presence of HA in combination with high leucine concentration favored the stability of large porous particles. HA limited the nucleation and growth of PZA crystals, leading to smaller crystalline domains and some disorder in the crystalline structure.

Finally, the optimized formulation obtained by spray drying possesses a median size of 5.8 ± 0.1 μm and a tapped density around 0.09 ± 0.01 g/cm³. The estimated aerodynamic diameter was around 1.75 μm and the powder was stable for more than 4 weeks of storage. One of the drawbacks of this optimized powder is its low content in PZA, around 12% (w/w). To prevent crystallization and favor stability, a large amount of excipients was needed when compared with other drugs formulated as powders for inhalation [14, 16]. In the future, it might be of interest to synthetize PZA lipidic prodrugs and incorporate them into DPPC/HA microparticles to obtain a higher drug loading. One might also think of using albumin as an excipient, since albumin is a large molecule, it might prevent the crystallization of PZA.

**In the second part of thesis**, in order to verify the suitability of large porous particles for lung delivery, the optimized formulation was evaluated from an aerodynamic point of view before being administered to rats to determine the pharmacokinetics of PZA and its concentration in the broncho-alveolar lavage.

The aerodynamic diameter is defined as the diameter of a sphere with a unit density. It has the same terminal settling velocity in still air as the particle in consideration [27] and determines the site of deposition in the lungs [28]. The aerodynamic diameter is
routinely measured by sizing techniques that are based on inertial impaction [29]. The test procedures have been guided by the European and US Pharmacopoeias including different types of apparatus - such as the multistage liquid impinge (MSLI), the Andersen cascade impactor (ACI) and the new generation pharmaceutical impactor (NGI). MSLI is a variant in which the particles are captured in a liquid medium rather than on a solid substrate. With this design, the particle bounce and re-entrainment is avoided, and the liquid can be the eluent medium for the recovery and the assay of the active pharmaceutical ingredient. MSLI was used to determine the aerodynamic properties in this thesis. The emitted fraction of the optimized formulation is high around 99 ± 3 % but the fine particle fraction (FPF) and alveolar fraction (AF) values were only about 30 ± 3% and 20 ± 2 %, respectively. The powder has a resulting MMAD of 5.1 ± 0.3 μm, higher than the theoretical one (MMAD) of 1.75 μm which was estimated in the first part of the thesis. Previous studies [30-32] suggest that the discrepancy between the theoretical and the experimental MMADs may be a result from the aggregation of particles during aerosolization. Since the MSLI contains liquid, moisture may play a role in particle aggregation. Besides, a heterogeneous distribution of the different components of the formulation in the particles was observed due to the spray drying process. Segregation of components in multi-component mixtures is the main reason of heterogeneity and has been observed in many studies [33-40]. To overcome this obstacle, while keeping solution composition constant, the spray drying parameters were modified to decrease the drying speed: the T_inlet was decreased, the drying gas flow rate was reduced, the feed-flow rate was modulated and the spraying-gas flow rate was reduced. As a result, the aerodynamic properties for dry powders were improved to be suitable for delivery to the alveoli, as following: FPF increased from 30% to 40% (unpaired t-test, P < 0.05), AF increased from 20% to 30% (unpaired t-test, P < 0.05) and the resulting MMAD decreased from 5.1 μm to 4.1 μm (unpaired t-test, P < 0.05) and the geometric standard deviation (GSD) was around 2.1 ± 0.2 μm.

To improve LPP physical stability and to inhibit recrystalization of PZA, reformulating PZA with other GRAS excipients might be considered. Physical stability of PZA-LPP can be accomplished by designing an amorphous particle with high viscosity [41]. Viscosity in an amorphous glass is characterized by the glass transition temperature. Amorphous glasses possess high viscosity if kept well below the glass transition temperature, which is typically designed to be 40 to 50 °C above the storage temperature. Excipients used for glass stabilization are usually saccharides (glucose,
sorbitol, sucrose, or trehalose), polyols, or organic salts that form glasses of high viscosity. Besides, several polymers were effective crystal growth inhibitors [42]. They have a moderate level of hydrophobicity relative to the drug molecule or contain functional groups that can form specific intermolecular interactions with the crystallizing solid. These interactive forces are likely to promote polymer adsorption onto the surface of the drug crystal to prevent the crystal growth. Therefore, polymers such as: albumin, chitosan and dextran could be investigated to inhibit PZA recrystallization.

Once administered, drug-loaded particles should deposit in the lower airways, followed by drug release from the dosage form, penetration to the site of action or into the general circulation prior to clearance and/or metabolism [43]. These processes depend on many factors such as drug and carrier physico-chemical properties and interactions with the lung environment. Although, cell culture models have been developed in the recent years, many groups prefer to directly evaluate powder for inhalation on animal models. Initial studies of pulmonary drug delivery are performed on small rodents such as mice, rats, and guinea pigs because an assortment of dosing techniques that require a small amount of the drug can be employed, terminal procedures can be easily performed, and large numbers of these animals can be used for statistical validity [44]. Before testing the PZA powder on a TB-infected animal model, we have evaluated it in healthy rats and have measured PZA concentration in the plasma and in the broncho-alveolar lavage as a function of time. 40 male Sprague Dawley rats were used and divided into the following 2 groups (20 rats per group): the first group received LPP-PZA powder by intratracheal insufflation, the second group received a PZA solution by intravenous administration. Rats were selected since their trachea is larger than mice allowing an easier access for insufflation. Different methods exist for administration of aerosol particles which can heavily influence the efficiency of delivery. In order to deliver LPP-PZA to the lung, the Dry Powder Insufflator™ (Penn Century, Philadelphia, PA) was selected.
The device contains a chamber that can be filled manually with a small amount of dry-powder formulation. Once loaded, the insufflator is inserted in the trachea of the animal between vocal cords and the tip of the canula is placed just above the carina as shown in Figure 1. This allows to circumvent oropharyngeal deposition, the dose delivered can be accurately measured, and a range of dose sizes can be delivered. The powder was then dispersed by rapidly pushing a bolus of air through the device using an empty plastic syringe. The dose of drug powder delivered to the lungs can be determined by weighing the device before and after administration. Particle size and distribution of dry powders delivered by insufflation have been reported to be essentially unaffected by passage through the device, as measured by a variety of techniques [15, 45-48]. Once the LPP-PZA deposits in the lungs it can act locally or be absorbed into systemic circulation to yield a systemic effect. PZA concentration in the epithelial lining fluid was measured by HPLC to assess the local efficacy of PZA. To determine the concentration of PZA in the lung lining fluid, broncho-alveolar lavage (BAL) was performed. BAL consist of sampling of the lower respiratory tract fluid by instillation of sterile saline and subsequent aspiration of the fluid [49]. Then, the aspirated fluid is collected and analyzed. For rodents, BAL is usually performed on excised lungs. Typically, lavage volumes are approximately half of the total lung capacity of the section of the lung lavaged, but they can vary for small laboratory animals. Generally, two to four lavages are performed to evaluate drug concentration in BAL. For a total lung lavage, such as that normally performed in a small laboratory animal, a recommended volume is 80% of the volume required to raise intrapulmonary pressure to 30 cm water pressure [49], vary from 8 to 10 mL. The number of lavages depends on the objective of study [50]. If the objective is to
evaluate the cellular portion of the BAL fluid, numerous lavages, sometimes accompanied by gentle massage of the lung, might be used to retrieve the maximal number of cells; if the objective is to evaluate the acellular fraction of the BAL fluid, two to four lavages might be performed to avoid excessive dilution of the biochemical components to be assayed.

For each rat, aliquots of 8 mL of chilled PBS were instilled into lungs and the fluid withdrawn immediately. This process was repeated 3 times for obtaining the most macrophage cells [49]. In order to assess the dilution lung lining fluid during the lavage, two methods can be considered. One can use the volume of epithelial lining fluid reported in the literature (80.2 µL) and can calculate the dilution factor according to the volume of fluid used for lavage. Alternatively, the concentration of urea in the BAL can be measured as an endogenous marker [51] to determine drug concentrations accurately. Indeed, urea concentration should be identical in the plasma and in the lung lining fluid [52]. For the former method, results show that PZA concentrations corrected by dose in the lung lining fluid were similar for both administration routes at 15 min and 6 hours (unpaired t-test, \( P > 0.05 \)). At 30 min and 3 hours, PZA concentrations after intratracheal insufflation were lower than after IV administration (unpaired t-test, \( P < 0.05 \)). For the second method, the huge difference in dilution factor leads to a strange result with a peak in PZA concentration at 30 min for the intratracheally administered rats (Fig. 8, chapter 3.). The increase of PZA concentration from 15 min to 30 min is not logical as local concentration should decrease with time as the drug passes to the blood stream. We believe that the colorimetric method we used for determination of urea concentration might not be precise enough or might suffer from interferences with PZA. Although the first method does not take into account the dilution factor for each individual rat it appears more suitable since it gives more trustable results.

PZA concentrations in plasma were determined to assess the pulmonary absorption, metabolism and clearance of PZA. The blood collection sites are determined based on the volume and frequency of sampling required [43]. For each rat, 1 mL blood was taken at 3 different time points via the jugular vein. For each time point, blood was sampled from a minimum of 6 rats. Blood was collected before PZA administration and 1/4, 1/2, 1, 2, 3, 4, 6, 18 and 24 hours following PZA administration (insufflation or IV). After pulmonary delivery of drugs, the factors such as the site of deposition, clearance mechanisms, drug formulation, dissolution rate, mechanism of absorption and drug metabolism may influence pharmacokinetic parameters (PK) of drug [53].
PK parameters of PZA were calculated using noncompartmental methods. This method is not mechanistic and simply considers the body as a central homogeneous space, where drug input and elimination occur [54]. Drug is sampled in the central space, and the change of drug concentration over time is considered as a statistical distribution curve [55]. Calculation of PK parameters described in materials and methods section of chapter 3 is based on the estimation of the area under the drug concentration–time profile. The results of these parameters by the different routes were shown in Table 5 of chapter 3. On pharmacokinetics curve of intratracheal administration (Figure 5 right, chapter 3) the absorption of PZA from the lung occurs in a short period and plasma PZA concentrations peaked at 30 minutes (t_{max}= 0.5 h) soon after powder was insufflated. This result shows that PZA is released quickly from excipients to achieve rapid absorption within 30 minutes. This ability might due to existence of PZA in powder in the amorphous state and the fact that surface-active compounds present in the formulation (DPPC, Leucine) may favor PZA crossing of the lung barrier. For further comparison between different routes of administration of PZA, AUC and C_{max} after IV administration of PZA were not significantly different from those after intratracheal administration after adjusting AUC and C_{max} by the actual dose administered (unpaired t-test, P > 0.05). The absolute bioavailability (around 1) reflects the absence of difference between to two routes of administration. PZA crosses efficiently the lung barrier to reach the blood stream.

The determination of PZA concentration in the alveolar cell pellet shows that PZA entered alveolar cells by passive diffusion based on gradient concentration. However, the PZA concentration in alveolar cells remains very low as a result from the rapid decline of PZA concentration in the ELF within the first 3 hours as is it crosses the lung barrier and passes to the bloodstream.

The potential toxicity caused by drug administration is one of major concerns of pulmonary drug delivery. Several markers of pulmonary such as lactate dehydrogenase (LDH) release, total protein and macrophage cell counting can be assessed in BAL fluid [56-58]. Lactate dehydrogenase activity and protein levels are biochemical markers of inflammation that are measured in BAL supernatants and indicate tissue injury and alteration of epithelium permeability, respectively. The cell pellet from the BAL can provide the total number of cells withdrawn from the lung airspaces and the cell differential counting can indicate inflammation by the presence of neutrophils. In addition inflammation can be assessed by determining pro-
inflammatory cytokines in BAL supernatant. For chronic treatment both acute and chronic toxicity should be assessed in the future.

From our results, we find that the bioavailability of PZA after lung delivery is 100%, which is very promising as lung delivery could be an alternative to oral delivery of this drug. However, since PZA passes rapidly into the systemic circulation, PZA-LPP might need to be dosed multiple times a day, thereby increasing the risk of non-adherence to drug therapies. In addition, the low percentage of the drug measured in the alveolar cell pellet might not be sufficient to eliminate dormant bacteria. The modulation of PZA release for the powder by selecting other excipients is worth pursuing in the future.

In the final part of thesis, PLGA nanoparticles containing PZA were optimized in terms of particle size and polydispersity by Taguchi methods. The purpose of preparing these nanoparticles is to serve the production of Trojan particles which are large porous particles containing drug-loaded nanoparticles. These particles have the same physical and delivery properties as LPPs, yet, once deposited in the lungs, then should disassociate to yield NPs, with all their attractive features for drug delivery [59].

Statistical experimental design has been applied for optimization of nanoparticle production in various studies [60-62]. It is a good reliable tool to develop better strategies for the optimization of nanoparticle preparation. In this regard, Taguchi approach of orthogonal array experimental design was applied for preparing the PZA-loaded PLGA nanoparticles to yield a particle size ≤ 200 nm. Taguchi experimental design identified the influence of individual factors (solvent type, PZA / PLGA weight ratio, organic phase / outer aqueous phase volume ratio and PVA concentration) over a specific region of interest (levels) on the particle size and polydispersity index (Tables 3 and 4, chapter 4). Then, the relationship of those factors on the particle size and polydispersity index was established. By the above process, the general trends of the influence of the factors towards the particle size and polydispersity index can be predicted and controlled such that a lower or a higher value in a particular influencing factor produces the preferred result (Fig. 1, chapter 4). Thus, the levels of factors, to produce the optimal results, can be predicted (Table 6, chapter 4). Point prediction of the design showed that the particle size of 170 nm and polydispersity index < 0.1 were achieved under optimal experimental conditions.
Finally, characteristics of PZA-loaded PLGA nanoparticles suitable to produce Trojan particles were determined (Table 8 of chapter 4). One drawback of the PZA-loaded nanoparticle formulation is their relatively low PZA content, around 3%, much lower than what is reported in the literature [63]. If these NPs were to be incorporated into the optimized LPPs to yield Trojan particles, it is very likely that PZA concentration in the lung lining fluid would not be very different from that of LPPs only. In vivo experiments should be carried out to validate this hypothesis. Alternative strategies to slow down PZA release from LPPs should be considered in the future.

**Reference**


General Conclusion
We optimized the formulation of PZA as large porous particles and obtained stable partially crystalline spherical particles adapted for deep lung delivery. These particles showed a PK profile after lung administration which was similar to IV administration. However, PZA was cleared faster from the lung lining fluid when administered by intratracheal insufflations than by IV administration. The results indicate that PZA-LPPs can be efficiently delivered to the lungs and would result in high local drug exposure. These particles have considerable potential to provide more effective therapy for tuberculosis. In addition, we also optimized successfully the formulation of PZA-loaded PLGA nanoparticles which have the suitable properties for developing Trojan particles for lung delivery. The advantage in including them into Trojan particles in terms of PK modification needs to be tested.
Summary

Large porous particles containing pyrazinamide, a first-line drug of Tuberculosis treatment, were developed and evaluated from the aerodynamic point of view and in-vivo in rats in terms of pharmacokinetics. In addition, pyrazinamide was also encapsulated into PLGA nanoparticles to develop Trojan particles for lung delivery.

Keywords: Large porous particles, PLGA nanoparticle, Tuberculosis, Pyrazinamide, Pulmonary drug delivery.

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