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Development of Inhalable Dry Powder Antibiotics and On-Bead Transcription Strategies

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DEVELOPMENT OF INHALABLE DRY POWDER ANTIBIOTICS AND ON-BEAD TRANSCRIPTION STRATEGIES

By J'aime Manion

B.A. University of Michigan 2004

A thesis submitted to the Faculty of the Graduate School of the University of Colorado in partial fulfillment of the requirement for the degree of Doctor of Philosophy

Department of Chemistry and Biochemistry

2011
This thesis entitled:

Development of inhalable dry powder antibiotics and on-bead transcription strategies

Written by J’aime Manion

has been approved for the department of the Chemistry and Biochemistry

Date: ______________________________

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Robert E. Sievers, Committee Chair

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Bruce E. Eaton, Committee Member

The final copy of this thesis has been examined by the signatories, and we find the content and the form to meet acceptable presentation standards of scholarly work in the above mentioned discipline.
ABSTRACT

Manion, J’aime

(Ph.D. Chemistry and Biochemistry, Dept. of Chemistry and Biochemistry)

DEVELOPMENT OF INHALABLE DRY POWDER ANTIBIOTICS AND ON-BEAD TRANSCRIPTION STRATEGIES

ADVISOR: Professor Robert E. Sievers

The global resurgence of tuberculosis (TB), the disease caused by Mycobacterium tuberculosis (Mtb) bacilli, has been driven by poverty, the development of drug resistance strains of Mtb, imperfect diagnostic assays, limited access to healthcare, poor healthcare infrastructure in the most highly infected areas, limited vaccine efficacy, lack of new drugs, and -- most profoundly -- the spread of HIV. Within this web of limitations dry powder inhalable antibiotics offer an opportunity to potentially reduce the lengthy treatment times associated with TB treatment. Incomplete tuberculosis treatment is the leading factor in the development of antibiotic resistant strains of Mtb. Inhalable antibiotics, by merit of specific aerodynamic diameters, are designed to target secluded populations of Mtb, often associated with extended treatment duration. The single dose, needle-free delivery strategies proposed here are an attractive treatment alternative for avoiding needle stick injuries and the transmission of blood-borne pathogens.

Techniques incorporating Carbon-dioxide Assisted Nebulization with a Bubble Dryer® (CAN-BD) were used to develop several inhalable antibiotic formulations with desirable inhalation properties. This particle producing technique is versatile for
producing particles from both antibiotic solutions and water-in-oil-in water emulsions. Particle properties such as fine particle fraction, emitted dose, moisture, particle size, and shape were characterized for several antibiotic formulations.

Additionally, inhalable antibiotic microparticle formulations were tested in different dry powder inhalers. One of the inhalers was design enhanced, with a new anti-static innovation, to improve emitted dose. Finally a technique for improving In Vitro Selections, a unique evolutionary method for mediating new particle materials and catalyst, is explored.
Dedication

This thesis is dedicated to the memory of my mom, Michelle Eggleton who passed away this January. My mom was proud of me for everything I have ever done. While I know that this thesis and my defense, regardless of the outcome, would be no different, I wish more than anything that she could be here to see my work come to fruition. She raised my brother; sister and I on her own and went back to school to pursue a master’s degree in special education when we were young. She believed that everyone had a capacity to learn and make the most of their resources. In writing this thesis I thought about her sitting in our garage (the only semi-quiet place at our house) writing her Master’s thesis on a type writer and about how many of her students were at her funeral and I know that parts of her are immortal in the lives and ideas she influenced. I think about my mom so often and there are obvious strengths and accomplishments I admire and then there is a feeling of loss like the way you remember music in a dream where you don’t remember any of the words but you know it was the most profound thing you ever heard. I am so grateful to have had her song in my life and in the moments of my life that I hear a bit of the refrain or the melody I feel so loved and I am truly grateful.
Acknowledgements

I am so grateful to my sweetheart, Steve Jackinsky who has helped me through so many really difficult moments in my life. He has brought Valentine’s Day to the lab, proof read, helped with formatting and a million other things. Most of all he has been his amazing self and going home to him gives me something to look forward to everyday. My family has been amazing and supportive throughout this whole process. I continue to be grateful to have my brother, Corey and my sister, Nikohl as dear friends who make my accomplishments seem greater and my disappointments less severe. My Aunt Cindy has been an inspiration for hard work and dedication and My Aunt Pat has been like a sister who always makes me laugh and feel better. My Grandmother, Geraldine taught me to find creative solutions and to look at life with multiple perspectives. My Dad has helped me to see the detail and beauty in small systems. (Like the LED’s in tail lights) Thank you to my whole family.

I am very lucky to have an amazing network of friends who have gone for therapeutic runs, tea, and trouble shooting lunches. Jess Hattle, Doug Chapnick, Autumn York, Sarah Altshculer, all of you have changed my life. I truly can’t imagine my life without any of you. Thank you so much to Dave McAdams and Jessica Burger who I have had the pleasure of becoming friends with in the Sievers Lab and to Jon Vaught for your friendship and science support. Also to all of the grad students I have taught with over the years I have enjoyed your company and camaraderie. Thank you to the Feldheim lab, especially Carly, Alina, Jamee and Bryan.
I have taught quite a bit over the course of my research and I am very grateful to have worked with Prof. Asirvatham. I have often been inspired by her integrity and the amount of creativity she puts into helping students learn difficult material. Thank you to Prof. Sievers for his patience, and supporting me in my investigations. Dr. Steve Cape has been more helpful and supportive than I will ever be able to describe. Thank you Prof. Bruce Eaton for letting me pursue difficult but exciting projects. Thank you to all of my committee members who have provided me with great feedback and support.
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<td>Mtb</td>
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<td>nC-CO₂</td>
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<td>Palladium</td>
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<td>RNA</td>
<td>Ribonucleic Acid</td>
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<td>SC-CO$_2$</td>
<td>Supercritical Carbon dioxide</td>
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<td>SEM</td>
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<td>Tuberculosis</td>
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<td>TDS</td>
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<td>UTP</td>
<td>Uridine Triphosphate</td>
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<td>UV</td>
<td>Ultra Violet</td>
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<tr>
<td>W/O/W</td>
<td>Water-in-Oil-in-Water</td>
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<tr>
<td>XDR-TB</td>
<td>Extensively Drug Resistant Tuberculosis</td>
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Chapter 1: Inhalable Tuberculosis Antibiotics Introduction

1.1 Background—impact of tuberculosis

Every second someone in the world is infected with the Mycobacterium tuberculosis (Mtb) bacilli. Five to ten percent of those infected, who are not co-infected with the human immunodeficiency virus (HIV), will become sick or infectious at some point in their lives. People co-infected with HIV are much more likely to develop active tuberculosis (TB) due to weakened immune systems that are unable to suppress the TB bacilli (WHO 2010). As infection only requires the inhalation of a few bacilli, each

![Diagram of Mtb droplet nuclei](image1.png)

Figure 1.1: Yellow dots represent droplet nuclei, which contain no more than 3 bacilli and are generated by talking, coughing and sneezing. These nuclei can remain airborne for extended periods of time due to their small size. Droplet nuclei that have a ~5 µm diameter are the most infective. Coughing or talking for 5 minutes generates ~3000 droplet nuclei. Sneezing generates the most droplets and can be spread to individuals 10 feet away (Kenneth Todar 2010). Images are modified from source material at CDC.gov/TB.
person with active TB, left untreated, will infect 10-15 people via talking, coughing, or sneezing (Nicas et al. 2005). (Figure1.1)

The World Health Organization (WHO) estimated that in 2008, 1.3 million people worldwide died of TB. WHO has reported a slow decline in incidence rates of TB infections per capita; however, this decline is offset by population growth. Overall, the number of new TB cases is still increasing globally, illustrated by the increase from 8 million new TB cases reported in 1997 to 9.4 million reported in 2008 (WHO 2008; 2010).

Evidence of TB is found in the spinal cords of Egyptian mummies dating back to 2400 BCE (Zink et al. 2003). There has been effective treatment since 1943, yet TB is still assailing the global population. Poverty, the development of drug resistance strains of Mtb, imperfect diagnostic assays, limited access to healthcare, poor healthcare infrastructure in the most highly infected areas, limited vaccine efficacy, lack of new drugs, and -- most profoundly -- the spread of HIV, have accelerated the global resurgence of TB (Blanchard 1996). Multiple drug resistant TB (MDR-TB) is resistant to at least two of the most potent anti-TB drugs: isoniazid (INH) and rifampicin (RIF). These drugs are considered first-line drugs and are part of the initial course of treatment for TB patients (CDC 2010). Further resistance, to any fluroquinolones, and at least one of three injectable second-line drugs, amikacin, kanamycin, or capreomycin, is considered extensively drug resistant TB (XDR-TB). As XDR-TB is resistant to first-line and second-line drugs, patients are left with less effective, more expensive treatment options that have more adverse effects (WHO 2010). The development of XDR-TB is
more probable in people with HIV or other conditions that weaken the immune system (CDC 2010).

TB is the leading cause of death for people who are co-infected with HIV; Mtb and HIV manifest a deadly synergy responsible for approximately a quarter of all TB-related deaths (CDC 2010; WHO 2009). HIV is the single greatest medical risk factor for developing active TB. Co-infection with HIV and TB increases the risk of developing active TB from 1 in 10 in a lifetime to 1 in 10 during the year after HIV infection (Kaufmann and Schaible 2003). HIV weakens the immune system by targeting CD4 T cells, the very cells responsible for keeping TB infection from progressing to active TB. TB contributes to the survival of HIV by using a cell wall glycolipid to bind the surface receptor of dendritic cells, impairing the activation of protective T cells that would otherwise attack both HIV and TB (Kaufmann and Schaible 2003). Thus a person infected with HIV is more susceptible to TB, the drug resistant variants of TB, and more likely to develop active TB. Due to lack of immune response, TB is not always detectable by a standard TB skin test. Consequently, HIV patients may have undiagnosed TB leading to the further spread of TB and rapid disease progression (CDC 2010). In Africa, HIV is the single most important factor contributing to the increase in TB since 1990 (WHO 2010).

Drug resistance has been reported by all of the countries surveyed by WHO. Extensive drug resistant TB has been reported by 49 countries since 2006 (WHO 2008). While XRD TB data is scarce in many of the countries with the highest incidences of TB, population-based studies from the USA, Republic of Korea, and Latvia showed that 4%, 15% and 19%, respectively, of MDR-TB cases were XDR-TB (WHO 2008). The
increased level of global travel blurs the socio-economical and geographical distinction between developed and developing countries. The spread of TB is a global problem that reaches beyond the inequity of medical treatment as it appears in airports, in schools, in businesses, and hospitals all over the world.

1.2 Background Mycobacterium tuberculosis (Mtb) drug resistance

Drug resistance develops when the treatment of drug-sensitive bacteria is interrupted and the concentration of drugs in the body is insufficient to kill 100% of the bacteria (CDC 2010). Treatment for non-resistant TB consists of a combination of first line antibiotics with different mechanisms of action for six months or longer (Edward A. Nardell 2009). Resistance emerges when patients do not take all their medicines regularly for the required period. Patients may fail to complete treatment because they start to feel better, they forget several doses over the long treatment time (6-9 months for drug non-resistant TB) because doctors and health workers prescribe the wrong treatment regimens, or because the drug supply is unreliable. Of the one third of the world’s population that carries Mtb, the burden of infection is disproportionate in developing countries where drug supplies are not always readily available and where access to treatment is often limited by resource-poor settings (Paul Farmer 2001; WHO 2010). An important aspect of improving compliance and reducing the development of drug resistance is shortening treatment time, thus making compliance more probable.

However, the development of drug resistance cannot be completely accounted for by poor patient compliance, as is illustrated by the 5% possibility of relapse for patients treated for six or more months under the directly observed treatment short-
course (DOTS) program (Girling 1989). Additionally, between 1982 and 1986 a study examining the development of resistance to one or more anti-TB drugs showed that 8.8% and 23% of previously untreated and treated TB patients, respectively, developed resistance (Marian Goble 1993). Resistant strains in untreated patients are likely the result of random mutations occurring due to high bacterial loads. High bacterial loads are probable in resource-poor settings, as it is estimated that HIV negative people are sputum smear positive 1-3 years prior to diagnosis. Given these estimates, a single person with active TB could infect as many as 45 other individuals (Borgdorff 2004; Corbett et al. 2004).

The development of resistant strains of Mtb in patients who were treated with anti-TB drugs can only be partially explained by lack of patient compliance. The development of resistant strains, especially in the case of the DOTS patients, may also be explained by poor drug penetration, and therefore sub-therapeutic concentrations of the drug, into areas of the lungs that have high bacterial loads (Girling 1989; Hiyama et al. 2000). Thus an essential part of preventing the emergence of resistant strains of Mtb is the development of novel methods of delivery that target areas of high bacterial loads.
1.3 Background - Mycobacterium tuberculosis (Mtb), the causative agent of tuberculosis

Humans are the primary hosts to Mtb, the etiologic agent of tuberculosis. Mtb is a non-motile, rod-shaped, gram-positive, acid-fast bacillus with a complex cell envelope. (Figure 1.2) Although TB can manifest as disease at any tissue site in almost every organ in the body (due to oxygen requirement), Mtb, an obligate aerobe, is classically found in the well-aerated upper lobes of the lungs. Further, the lungs represent the main port of entry and an important site of disease manifestation from which Mtb disseminates to other organs (Flynn and Chan 2001). Approximately 80% of all TB disease originates as pulmonary TB (Pandey and Khuller 2005). The Mtb bacterium is an intercellular pathogen. It has a slow generation time of ~24 hours (Kenneth Todar 2010). The slow growth of Mtb bacteria is a major factor that contributes to the virulence of the bacteria, the chronic nature of TB, and the lengthy treatment regimens required to treat the disease (Cole et al. 1998). In the early stages of infection, a person inhales droplet nuclei, which contain minute numbers of bacilli and are generated by infected individuals with active TB by talking, coughing, and sneezing. These nuclei can remain
air-borne for extended periods of time due to their small size. Coughing or talking for 5 minutes generates ~3000 droplet nuclei. Sneezing generates the most droplets and can be spread to individuals 10 feet away. Droplet nuclei that have a ~ 5 µm diameter are the most infective. Further, the mycobacteria are resistant to chemical injury by acids, alkalis and many other disinfectants which kill other bacteria (Lee et al. 1996). The probability of transmission of TB is dependent on several factors including the number of infectious droplets expelled, the effectiveness of ventilation, the duration of exposure, and the virulence of the Mtb strain.

Once the Mtb bacilli have entered the lungs, they target and inhabit the professional antigen presenting cells (APC’s), the alveolar macrophage (AM) and the dendritic cells (DCs) (Wolf et al. 2007). AMs and DCs play an important role in the initiation and maintenance of immune response, and they are part of the innate immunity system that is the first step in a sophisticated adaptive immunity system for combating microorganisms (Hermann and Lagrange 2005). In the case of the AMs, this initial response typically involves phagocytosis followed by acidification, fusion with lysosomes, and destruction by hydrolytic enzymes (Sinai and Joiner 1997). However, Mtb -- protected by a unique cell wall -- takes advantage of the phagocytotic machinery to travel into the macrophage where it subverts AM maturation and innate immune responses from within a vacuole (Nau et al. 2002). By halting the maturation process, the Mtb are spared the typical fate of phagocytosed pathogens and induce ‘alternate activation’, a component of the host-pathogen relationship that favors the bacteria (Kahnert et al. 2006). Mtb infected vacuoles do not acidify or fuse with lysosomes (Armstrong and Hart 1975; Sturgillkoszycki et al. 1994). While these vacuoles fail to
fuse with lysosomes, they remain fusion-competent. This fusion ability allows the Mtb to undergo fusion with vesicles of the early endosomal system (Russell 2001). The vacuoles containing Mtb also contain receptors and class II molecules associated with the transferrin recycling pathway and cell membrane communication. Association with the transferrin recycling pathway allows Mtb to intercept lysosomal constituents directly from the biosynthetic pathway of the host cell (Ulrichs and Kaufmann 2006). The variety of receptors, molecules and pathways available to Mtb within the arrested AM suggests a network of nutrient supply that fuels the multiplication of the bacilli. However, the ingested bacilli cause the infected AM to set off a pro-inflammatory response that leads to the recruitment of mononuclear cells from blood vessels. Eventually, in concert with effector lymphocytes, the infected AMs become the center of granuloma: a focal collection of inflammatory cells designed to prevent the spread and replication of the Mtb (Russell 2001).

Dendritic cells, due to their singular ability to induce primary immune responses and thus establish immunological memory, are unique antigen-presenting cells (APCs) that engulf the Mtb at the site of the infection. The capture of a pathogen induces the immature DCs to migrate to the draining lymphoid organs where, upon maturation, they display peptide-major histocompatibility complexes. These histocompatibility complexes allow selection of rare circulating antigen specific lymphocytes. Once the lymphocytes have been exposed to a pathogen, they cause terminal maturation of DCs, which in turn causes the lymphocytes to differentiate into effector and memory lymphocytes. The memory lymphocytes remain in the peripheral tissue and circulation for future pathogen recognition. The effector lymphocytes migrate and can traverse inflamed epithelia to
reach the injured tissue and infected AMs. The appearance of Mtb-specific lymphocytes occurs about two to three weeks post-infection and is indicative of the containment phase of the disease where bacterial numbers begin to stabilize and rapid bacterial replication slows down. The differentiated lymphocytes recruit an arsenal of immune associated cells and receptors that include cytokine secreting CD4+ T –helper cells, which permit the activation of non-antigen-specific macrophages, eosinophils, and natural killer (NK) cells, antigen specific CD8+ cytotoxic T cells which eventually lyse the infected cells and T cell activated B cells, which migrate and mature into plasma cells that produce antibodies to neutralize the initial pathogen (Banchereau et al. 2000).

The Mtb at the site of capture is attacked by this eradication squad, while the Mtb captured by the dendritic cells and a certain population of bacteria within the AMs resist cellular degradation within the DCs and AMs post-antigen presentation. The environment within DCs deeply contrasts with the communication and nutrient network that fosters growth within AMs. Mtb engulfed by the DCs are stored in vacuoles where cut off from the host-cell recycling pathway including recycling vesicles and exogenous nutrients -- they do not grow and multiply, but rather enter a dormant state that can be maintained for years (Tailleux et al. 2003). Yet, the DCs are incapable of killing the Mtb after capture in the lungs, at the site of infection, and transport to the T lymphocytes (Hermann and Lagrange 2005). Once the pathogen has been presented to the T lymphocytes, the bacteria are contained in small granulomatous lesions of the lung by specific T cells (Hermann and Lagrange 2005).

Granulomas, the hallmark of pulmonary TB, are characterized by a central necrotic core surrounded by concentric layers of macrophages, which differentiate into
epithelioid cells, and/or fuse to form multinucleated Langhans giant cells, foamy macrophages (FMs), and lymphocytes (Ulrichs and Kaufmann 2006). Within the granulomas, the Mtb are contained and prevented from further dissemination by a cellular wall and a fibrotic outer layer. During the containment phase of TB, the granuloma is highly vascularized, and cells are actively recruited to the site of infection. As the disease progresses, the fibrous outer later becomes more pronounced and the number of blood vessels that penetrate the granuloma diminish with concomitant increase in the number of foamy macrophages. In the later stages of the disease, the damaged or necrotic portion of the granuloma becomes hypoxic, a condition associated with non-replicative persistence in Mtb culture (Via et al. 2008). It has been proposed that within the granuloma, dormant or semi-dormant Mtb use the Foamy macrophages, which contain large numbers of lipid-free and lipid-containing vacuoles as reservoir for nutrients and persistence in dormancy (Peyron et al. 2008).

Maintenance of the granulomas results in latent TB, a state of equilibrium in which the host is able to control the infection but not completely eradicate the Mtb. The process is dynamic as suggested anecdotally by recent medical imaging techniques that show metabolically active lesions in humans with latent infection (Hofmeyr et al. 2007), and by the treatment of latent TB. Latent TB is treated with a 9 month course of isoniazid (INH), the efficacy of which is dependent on mycolic acid synthesis during active bacterial replication, implying a range of activity even in the latent bacterial population (Nolan et al. 2005). Poor vascularization in late stage granulomas makes drug penetration by traditional oral or parenteral routes less effective, thus accounting for the long treatment time of latent TB (Muttil et al. 2009).
Granuloma encased bacteria may remain dormant for years, illustrated by the 90% of infected individuals who do not develop primary TB. However, Mtb can be re-activated by immunological compromises such as HIV, malnutrition, tobacco smoke, indoor air pollution, alcoholism, silicosis, insulin-dependent diabetes, renal failure, malignancy, and immune suppressive treatment such as glucocorticoids (Diedrich et al. 2010; Hermann et al. 2006; Lonnroth and Raviglione 2008). Re-activated by immunological compromise, Mtb resume active replication and eventually rupture the granuloma, spilling thousands of infectious bacilli into the airways and causing a bacteria-laden cough (Kaplan et al. 2003).

Mtb shows a range of metabolic activity and occupies a variety of cellular conditions, which further complicates treatment. Mtb may be classified as actively metabolizing and rapidly growing, semi-dormant in an acidic intracellular environment, semi-dormant in a non-acidic intracellular environment, or dormant (Blanchard 1996). The semi-dormant and dormant metabolic states are unique to mycobacterial infections allowing bacteria to remain quiescent for years (Cole et al. 1998). The range of observed metabolic activity is a consequence of the interaction between the host immune response and the virulence of the Mtb strain. Much of the tissue damage and liquefaction of infected parts of the lung are manifestations of the host response rather than inherent properties of the bacteria. Ironically, the liquefied lung material contains fatty acids toxic to macrophages but nutritious to Mtb and thus provides an excellent milieu for bacterial multiplication (Muttil et al. 2009). The peak efficacy of current treatments is largely restricted to actively dividing organisms, which are only a percentage of the infecting bacteria (Russell 2001). For the complete cure of
tuberculosis, all populations of Mtb must be eradicated or potential relapse with resistant bacteria may occur (Blanchard 1996). While patients with the active disease pose the greatest threat for spreading TB, patients with latent infections are the largest reservoir for potential reactivation, and subsequent silent spread, of TB to their close contacts. In fact, the majority of active TB in low-prevalence regions, such as the United States, is from reactivation (47-87%) (Weis et al. 2002).

1.4 Background – TB Treatment Prevention and Control

Many countries administer the live, attenuated Mycobacterium bovis BCG (bacillus Calmette-Guerin) vaccine on the principle that this vaccine has minimal side effects and protects against the more severe forms of the disease (military and meningeal TB) during childhood. However, the BCG vaccine does not protect against the most common form of infection, adult pulmonary TB, and confounds future skin testing (Kaufmann 2000). Further, as treatment of an active TB infection in animal models does not confer protection against challenge, any subsequent vaccine would have to trump the pathogen itself in educating the immune system on recognition and eradication of Mtb (Kaufmann 2000). As protection is not currently possible, treating all persons with TB is the only available option for eradication of the disease.

Conventional chemotherapeutic resolution of active TB with a non-drug resistant strain requires six to nine months of multiple antibiotic treatments. These drugs are typically administered orally. To reduce the rapidly dividing bacillary load, a minimum of three drugs -- typically a combination of isoniazid (INH), rifampicin (RIF), pyrazinamide
(PZA) and an economically dictated fourth drug, either ethambutol (EMB) in the developed world, or often streptomycin in the developing world -- are used in the intensive treatment phase (Edward A. Nardell 2009). In the continuation phase, a minimum of two drugs is used to complete the sterilization of lung lesions, which contain sub-populations of dormant or slow growing bacteria. INH and RIF are particularly important in the initial combination therapy as they are active against both metabolically dynamic and semi-dormant bacteria (Muttil et al. 2009).

Second line drugs are only administered if a patient is not responding to first-line antibiotic treatment or is thought to have a drug resistant strain of Mtb. Typically, the second-line antibiotics are more toxic and less effective than first line antibiotics and are sometimes administered by the parenteral route (Edward A. Nardell 2009). Parenteral administration adds cost and complication to the treatment of tuberculosis as there is a danger of needle re-use and contamination, leading to the transmission of blood born pathogens (Kaufmann 2000; Muttil et al. 2009). Second-line antibiotics consist of aminoglycoside antibiotics, cycloserine, ethionamide, and fluroquinolones. The most common aminoglycoside antibiotics are amikacin, kanamycin and streptomycin. Fluoroquinolone antibiotics include levofloxacin and moxifloxacin. Lastly, capreomycin sulphate is a cyclic polypeptide antibiotic effective against Mtb (Edward A. Nardell 2009; Nolan et al. 2005).

The current best-case scenario for drug susceptible TB is a six to nine month first-line drug regimen. As first-line drugs are delivered orally, therapeutic drug concentrations can only be reached in parts of the body with adequate blood circulation. Bacteria harbored in granulomas often lack a strong blood supply due to loss of
vascularization (Figure 1.3 blue box). Thus, oral drugs may not penetrate the granulomas, resulting in sub-therapeutic doses of antibiotics to this subpopulation of Mtb (Muttil et al. 2009). The difficulty of reaching this population of bacteria is illustrated in the treatment, of mice in which 99% of the bacteria is killed in the first two weeks of drug treatment while three additional months are required to clear remaining 1% of bacteria (Lenaerts et al. 2005; Nueremberger et al. 2004; Tyagi et al. 2005). The standard treatment can be extended for two or more years if the patient fails to respond to initial treatment or develops drug-resistant TB (Nolan et al. 2005; WHO 2010).

The extension of an already long treatment time makes compliance more difficult and unlikely, increasing the probability of resistant Mtb. While treatment of drug susceptible TB costs on average between $10-$1000, drug-resistant TB can cost up to $250,000 to treat, due to the duration and expense of the antibiotics required (Kaufmann 2000). Protracted treatment with more antibiotic combinations is further complicated by dosage limitations, adverse side effects and inadequate drug distribution to the affected sites of pathology (Sacks et al. 2001). New drugs or new methods for delivering existing therapies that shorten the duration of current treatment would greatly improve compliance and thus help prevent the emergence of resistant Mtb.
1.5 Background – TB Treatment—Inhalable Antibiotics

The lungs are the primary site of Mtb infection. Inhalation, the very mode of infection, has logical appeal for delivery of therapeutic drugs. The goal of any novel therapy approach is to improve upon existing treatment. In the case of TB treatment, the major drawbacks of traditional therapy include the length of treatment and the need to use less effective, more expensive parental route second-line antibiotics. Lastly, there is a lack of evidence to support the efficacy of administering drugs via oral or parental routes to achieve adequate drug concentrations in pulmonary blood vessels proximal to the granulomas, so therapeutic levels of drug may diffuse into the fortified granulomas (see Figure 1.3) (Borgdorff 2004; Girling 1989; Muttil et al. 2009). Protration of treatment time in both latent and active TB increases the probability of missed doses or incomplete treatment, leading to resistant strains of Mtb. The use of second-line antibiotics also adds difficulty in needle disposal, prevention of needle re-use and the possibility of transferring blood borne pathogens. Considering the large population of HIV/TB, co-infected individuals, the stigma and potential danger of poorly regulated needle administration poses yet another barrier to treatment completion and prevention of resistant strains of Mtb (WHO 2011). Any treatment that can reduce the length of TB treatment or provide an alternate to needle administration of second-line antibiotics stands to improve TB therapy.
Figure 1.3: The lifecycle of Mtb begins upon inhalation, when the bacilli are phagocytosed by alveolar macrophages. The immune system responds and triggers the infected cells to migrate to the epithelium where monocytes are recruited from circulation and neovascularization of the infected site occurs. The cascade continues as macrophages within the granulomas differentiate to form a variety of cell types. Further stratification occurs as a fibrous cuff of extracellular matrix develops outside the macrophage layer and finally lymphocytes remain on the periphery of this cuff. Granulomas may remain in this enclosed state for years but progress toward disease is characterized by a loss of vascularization, increased necrosis and ultimately the escape of infectious bacilli. Image of lung was modified from The Oxford Illustrated Encyclopedia, Free pictures www.oup.co.uk. Lifecycle of Mtb modified from Refs (Russell et al. 2009) with permission by Nature Publishing Group.
One method of reducing treatment time may be to target the initial Mtb host cells, the AMs, and the granulomas. Hypoxic conditions in the central region of the granuloma, variations in granuloma size, and eventual loss of vascularization pose a challenge to traditional drug administration strategies and are often the Mtb population responsible for resistance and extended treatment times (Lenaerts et al. 2005; Nuermberger et al. 2004; Tyagi et al. 2005). Oral and parental anti-TB drugs can only elevate the circulating concentration of a drug, at most influencing the periphery of granulomas. Even high circulating concentrations, which may have severe systemic side effects, may not be sufficient to penetrate all granulomas, leaving behind potentially resistant bacterial populations (Muttil et al. 2009). The theory that granulomas and the respective bacterial populations within are responsible for extended treatment times is supported by a study that demonstrated that HIV-positive individuals convert to smear negative at a faster rate than HIV-negative patients. HIV/TB co-infected patients are known to lack Mtb lesions, thus there is no barrier for systemic drugs to reach Mtb in the lungs (Telzak et al. 1998).

Particles having an aerodynamic diameter in the 1-3 µm range are suitable for delivery to the deep lung where AMs reside, while nanoparticles approaching 500 nm or larger can be captured by AMs (Edwards et al. 1998). AMs make up the periphery of lung granulomas and may contain engulfed bacteria targeting drug to AMs, which allows for the potential of early eradication for infected AMs and increased penetration of granuloma in the case of non-infected periphery AMs (Muttil et al. 2009). Smaller nanoparticles may be taken up by dendritic cells, which eventually travel to the lymph
nodes, where they may remain or may also migrate to granulomas (Thiele et al. 2003; Tran et al. 2000a; Tran et al. 2000b).

Often nanoparticles, particularly those made with poly(lactic-co-glycolic acid) PLGA, can be used to extend drug release, which takes place by diffusion, swelling, erosion, degradation or by absorbing/attaching the active substance on the surface (Astete and Sabliov 2006). Advantages of nanoparticle drug delivery include the ability to achieve high drug loading, cross permeability barriers, the incorporation of both hydrophilic and hydrophobic drugs, and evoke a better therapeutic response compared to other particulate delivery systems (Muttil et al. 2009). However, due to inertia limitations, non-aggregated nanoparticles tend to be exhaled, preventing deposition of individual nanoparticles into the deep lung (Heyder et al. 1986). To overcome this disadvantage, nanoparticles embedded into the surface of microparticles may have the merits of both micro and nanoparticles allowing aerodynamic targeting to the deep lung and size specific uptake by AMs.

The merits of microparticles were recently highlighted by Misra et al. in a study that showed that intracellular drug concentrations were higher when the drug was administered in microparticle form as opposed to drugs in solution (Verma et al. 2008). Drug-containing microparticles were more efficient than soluble drugs in eliciting a host response to Mtb in THP-1 cells (Yadav and Misra 2007). Further, microparticles studied in THP-1 cells and primary human macrophages illustrated that both drug and non-drug loaded microparticles were capable of clearing bacterial load via different mechanisms. The non-drug loaded microparticles may have activated apoptosis through extensive plasma membrane remodeling (Sansonetti 2006) and perturbed mitochondrial
sufficiency (Duan et al. 2002) thus overcoming anti-apoptotic control mechanisms of the bacteria (Spira et al. 2003). This data implies that microparticles may activate some innate immunity, encouraging synergy between the anti-bacterial properties of the microparticle drugs and the host immune system (Yadav et al. 2010).

While currently no drug or formulation for delivery of antibiotic TB drugs via inhalation has been commercialized for human use, several studies have demonstrated the efficacy of pulmonary-delivered anti-TB drugs in animals, most prominently in the guinea pig (Garcia-Contreras et al. 2006; Nolan et al. 2005; Suarez et al. 2001). From a human perspective, a study in 2001 showed that administration of inhaled aminoglycosides, in combination with traditional therapy, led to faster sputum conversion in drug-susceptible and resistant patients (Sacks et al. 2001).

With an alveolar surface area of about 100 m² in an adult, certain aspects of drug absorption are unique to pulmonary delivery, as compared to oral or parenteral drug administration. Some of the advantages of direct drug delivery to the lung include dose-dependent increase of drug concentrations in local tissue (the primary site of Mtb infection), and diminished side effects due to low plasma drug concentration (Chow et al. 2007). Microparticles and nanoparticles engineered for sustained release may be taken up by macrophages or may act as a repository, releasing drug to nearby blood vessels allowing absorption into circulation and possible re-absorption elsewhere in the tissue, thus treating lesions in other regions of the body (Muttil et al. 2009).

While limitations to inhaled therapy are apparent from diseases such as asthma, chronic obstructive pulmonary disease, and cystic fibrosis, these same diseases have also shown great success in pulmonary therapy especially when combined with other
treatments (Patton 1996). Challenges such as reproducible drug delivery, delivery to obstructed regions of the lung, and potentially poor systemic bioavailability depend on a plethora of modifiable factors, including the ability of the delivery device to generate respirable sized particles, the stability of the drug formulation, and patient specific factors. As there are no approved inhalable anti-TB treatments, these other disease models can only be used to propose therapeutic strategies for TB. Thus, aerosol delivery of micro and nano-particle drug formulations, in combination with conventional therapy, may lead to more effective doses of antibiotics in high bacterial burden areas of the lungs, while conventional therapy could be used to maintain systemic drug concentrations preventing extrapulmonary dissemination. Using inhaled drug strategies to increase the local dose of drugs in and around secluded bacterial populations has potential for reducing resistant strains of Mtb. When viewed together, combination therapy with inhalable antibiotics stands to improve many of the major drawbacks of conventional therapy, such as reducing the high dosing frequency and the overall extended duration of treatment. Thus, pursuing an inhalable treatment component may achieve better patient compliance and less severe-side effects, with reduction in the emergence of drug resistant strains of Mtb.
1.6 References


CDC (2010). Tuberculosis fact sheet, CDC, ed., Division of Tuberculosis Elimination, Atlanta.


Chapter 2: Inhalable Microparticle Tuberculosis Antibiotics: Isoniazid (INH), Kanamycin (KAN), Capreomycin (CAP)

2.1 Introduction

The development of unit-dose, inhalable, antibiotic microparticles for use in primary and combined therapy approaches to treating tuberculosis (TB), multi-drug-resistant (MDR-TB) and extensively drug-resistant TB (XDR-TB) is explored using the gentle drying process of Carbon-dioxide Assisted Nebulization with a Bubble Dryer® (CAN-BD). The antibiotic microparticles are targeted for delivery to the deep lung by optimizing the formulations and particle processing conditions to produce particles with a large fine particle fraction (FPF) below 3.3 µm, the ideal aerodynamic size for reaching the alveolar space and poorly vascularized lesions and granulomas that harbor treatment-elusive bacilli in protective microenvironments. CAN-BD technology is ideally suited for the development of new dry powder inhalable microparticles for needle-free pulmonary delivery strategies due to low processing temperatures, and lack of additional processing steps or precipitation agents. The microparticles produced using this method contain antibiotic particles imbedded in sugars, amino acids, and/or other excipients; and are within a respirable size range (1-5 µm), and have less than 3% residual moisture.
These powder properties are suitable for developing a needle-free delivery system that addresses the Grand Challenges in Global Health Initiative #3. In response to this challenge, we developed unit-dose packaging that preserves powder properties by protecting them from moisture, oxidants and UV exposure as well as a low cost “active” dry powder inhaler, the PuffHaler®, used as a prototype device along with the Aerolizer® to disperse the microparticle antibiotic formulations. Unit-dose dry powder antibiotics have the potential to provide easy-to-use, stable products with improved safety profiles.

TB treatment has many challenges, which include antibiotic product contamination, difficulties in reaching target populations, wastage, the potential for needle-stick injuries, difficulties associated with sharps disposal, and the challenge of reaching high bacterial burden areas of the lung that are cut off from a blood supply by the architecture of granulomas (Horsburgh et al. 2000; Mitragotri 2005; Shakoor et al. 1997; Shin et al. 2004; Taylor et al. 1995; Varmus et al. 2003). Many of these challenges may be overcome with the use of a pulmonary delivery strategy that incorporates unit-dose packaging. CAN-BD was chosen as the method for producing inhalable antibiotic microparticles because of its track record of success in producing a variety of dry, active powders of vaccines and small molecule pharmaceuticals in a size range suitable for pulmonary delivery including Edmonston-Zagreb (EZ) measles vaccine, hepatitis B vaccine, and polyclonal IgG (Cape et al. 2008). CAN-BD processed microparticles have powder properties (size, moisture content, stability and potency) that were developed specifically for pulmonary delivery.
CAN-BD is a unique blend of supercritical/near-critical microparticle forming techniques and spray drying. The CAN-BD process for creating microparticle vaccines and biopharmaceuticals is advantageous because the dense gas is not used to precipitate the solute of interest, but rather it is used as a solvent nebulization enhancer. Unlike many supercritical processes that require the solvent and supercritical or near-critical fluid to be miscible (Jovanovic et al. 2004), CAN-BD is not limited by this requirement, as organic solvents are not necessary to increase the solubility of the dense gas in the antibiotic or biopharmaceutical solution of interest (Cape et al. 2008). The solute can be dissolved in a biologically stabilizing solution such as water with buffers, stabilizers and excipients. Due to the relatively low processing temperatures used in CAN-BD, less decomposition of thermally labile drugs is expected.

The needle-free pulmonary delivery strategy was developed in response to the Grand Challenges in Global Health Initiative #3 (Varmus et al. 2003). This initiative was designed to create new technologies for the administration of vaccines; however, this work expands the application of needle-free pulmonary devices to inhalable antibiotics and TB treatment. Traditional syringe and needle administration is associated with increased risk of infections due to human immunodeficiency virus (HIV) and hepatitis B (HBV), and C (HCV) viruses resulting from needle-stick injuries, inappropriate re-use of needles or syringes, and the challenges of proper needle disposal (Giudice and Campbell 2006; Mitragotri 2005). Needle-free delivery methods have the potential to improve drug delivery safety, while unit-dose packaging may reduce antibiotic wastage, and microbial contamination. The potential therapeutic advantages of inhalable TB treatment are based on the success of aerosol antibiotic drug delivery in cystic fibrosis
patients, the pulmonary route of infection of Mycobacterium tuberculosis (Mtbc), and the large population of HIV and TB co-infected individuals (Borsje et al. 2000; Pandey and Khuller 2005; WHO 2011).

To provide an effective alternative to traditional syringe-based vaccines and biopharmaceuticals, one must develop inhalable vaccine and antibiotic powders with fine particle fractions suitable for pulmonary delivery, having low moisture, high stability, and high potency. Pulmonary delivery of biopharmaceuticals requires specific fine particle fractions and high dispersibility. The aerodynamic particle requirements for pulmonary delivery are particles in the 1 to 5 µm range, preferably with a 1 to 3 µm range for delivering particles to the alveolar or deep lung region (Crowder et al. 2002). Aerodynamic particle size distribution, particle shape and powder dispersion characteristics dictate the performance of inhalation devices (Bosquillon et al. 2001; Crowder et al. 2002). To meet regulatory requirements, measurements of aerodynamic particle size distribution and deposition are usually done using an Andersen Cascade Impactor (ACI) and a Dosage Unit Sampling Apparatus (DUSA) (USP Chapter <601>). ACI experimental results provide fine particle fraction (FPF). The FPF measured is an indication of where the powder is likely to deposit in the lung upon inhalation, and is affected by dispersion of the powder. Results from a DUSA provide emitted dose (ED); ED is the mass percentage of particles leaving the dry powder inhaler (DPI) system and available for inhalation. Particle shape, geometry and surface morphology are examined using a Scanning Electron Microscope (SEM).

The inhalable antibiotic dry powder is formulated with <3% moisture content. Beyond preserving powder aerosolization and deposition properties, stabilization via
residual moisture reduction is an important factor in preserving potency by inhibiting microorganism growth and other water-facilitated degradation (May et al. 1986). To ensure the moisture content remains <3% and that the antibiotics are not exposed to UV, oxidants or other contaminants, the antibiotic dry powder can be single-dose-packaged in tightly sealed aluminum foil-polymer film laminate blister packs. Multiple blister packs can be sealed in a foil overwrap with molecular sieve desiccant. This foil laminate unit-dose blister pack improves the safe use of each dose by mitigating the dangers of UV, oxidant inactivation, and general contamination. Moisture content analysis and the monitoring of crystal structure are important for evaluating powder stability and the success of packaging strategies. Powder moisture content is analyzed using coulometric Karl Fischer titration. Similarly, evaluating the structure of the microparticles using X-ray diffraction (XRD) ensures that the powders remain unchanged (crystalline or amorphous) throughout the storage and usage period.

To illustrate the broad applicability of creating inhalable antibiotics via CAN-BD we chose to investigate a first-line antibiotic, isoniazid (INH) and two second-line antibiotics, capreomycin sulfate and kanamycin sulfate. These antibiotics are structurally diverse. INH is a small molecule pro-drug that at therapeutic levels is bactericidal against both intracellular and extracellular Mtb organisms (Chanwong et al. 2007). Both capreomycin sulfate and kanamycin sulfate are considerably larger molecules that are often used in injection form due to poor absorption through the gastrointestinal tract (Helms 2006). Due to the common injection route of these second-line drugs, inhalable antibiotic options would likely improve safety and eliminate complications associated with disposal of needles, while maintaining absorption. The
Abbreviations, structures and brief mechanisms of action for the antibiotics that were chosen for development into inhalable antibiotics are described in Table 2.1.

<table>
<thead>
<tr>
<th>First Line Antibiotics</th>
<th>Abbreviation</th>
<th>Structure</th>
<th>Mode of Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>isoniazid</td>
<td>INH</td>
<td><img src="chart1.png" alt="INH Structure" /></td>
<td>INH inhibits synthesis of mycolic acid</td>
</tr>
<tr>
<td>kanamycin</td>
<td>KAN</td>
<td><img src="chart2.png" alt="KAN Structure" /></td>
<td>KAN interacts with the bacterial 30S subunit and induces mistranslations and indirectly inhibits translocation during protein synthesis</td>
</tr>
<tr>
<td>capreomycin</td>
<td>CAP</td>
<td><img src="chart3.png" alt="CAP Structure" /></td>
<td>CAP is thought to bind the 70s ribosomal subunit and components of the bacterial cell wall. Results in the production of abnormal proteins</td>
</tr>
</tbody>
</table>

**Table 2.1**: Antibiotics chosen for development into inhalable antibiotic formulations. Structures were created on ACD/ChemSketch 10.0 and are in the public domain. Modes of action adapted from www.drugbank.ca
These antibiotics were also chosen for their impact in potential combination therapy strategies. Combination strategies are essential for preventing resistance to any individual antibiotic as resistance mutations occur independently and spontaneously the more antibiotics in a regimen the lower the probability of Mtb developing resistance. Also because different antibiotics target different aspects of Mtb growth and metabolism combining antibiotics is essential for synergistic destruction of Mtb in different environments and metabolic states (David 1970). However, as the antibiotics in a treatment regimen are increased, the side effects and possible cross-reactions with other medications especially in the case of TB-HIV patients also increase. Thus, inhalable strategies that may reduce systemic side effects of these antibiotics are ideal for improving multi-drug regimens. Increasing the targeted dose in the lung airspace and targeting bacteria in protective microenvironments such as those in granulomas may prevent the development of MDR-TB by ensuring that threshold doses of antibiotics reach bacteria populations that are difficult to target by traditional methods.

Lastly, the dry powder dispersion and emitted dose characteristics have been studied by administration of antibiotic powder using low cost dry powder inhalers (DPIs) (e.g., the PuffHaler®, and the Aerolizer®). The new PuffHaler is an active DPI, with a disposable mask suitable for use with infants or test animal subjects such as cotton rats (Kisich et al. 2011), and rhesus macaques (Lin et al. 2011). The Aerolizer is a well-established, commercial passive DPI, which requires the subject to inhale on command to generate powder dispersion. Both the Aerolizer and the PuffHaler provide alternatives, for different age groups, to traditional subcutaneous injections or oral administration.
The results from these two test devices illustrate the importance of the inhalation device in aerosolization and delivery efficacy.

Of particular importance in the device and testing design, the PuffHaler incorporates POLYBATCH® VLA 55 UK, anti-static material, within the polyethylene film used for the reservoir. Prior to this innovation, an earlier tested non-conductive polymer film in the PuffHaler reservoir retained an unacceptable amount of powder; the emitted dose out of the PuffHaler was approximately 20%. After switching to the POLYBATCH® VLA 55 UK polyethylene film the emitted dose increased to 50% with a placebo powder. The emitted dose for several of the antibiotic formulations is approximately 60%. The anti-static agent is FDA-approved for use in food-grade polyethylene films up to 10% by weight for most foodstuffs. It is present in the polyethylene film material used to make the PuffHaler reservoir at a concentration of about 3%. POLYBATCH is incorporated into the surface of the polyethylene film and the anti-static properties of the film are the result of the formation of a polar layer that absorbs water from the atmosphere. The polar layer is conductive, and dissipates charges on the surface of the film, reducing resistance and charge decay time. This antistatic polymer was chosen due to its ability to maintain antistatic properties at relatively high humidity, an important characteristic for any future DPI development specifically for inhalable antibiotics, which may be used in non-climate controlled settings. Figure 2.1 shows the PuffHaler.
2.2 Materials and Methods

The CAN-BD process and equipment schematic is shown in Figure 2.2. CAN-BD has been described previously in more detail (Cape et al. 2008). In this process, the drug or vaccine is dissolved, or suspended in a solvent (usually water, but it can also be a water-organic mixture, or a pure organic solvent). Both the
drug solution and the nebulization fluid are pumped through 1/16 in. outer diameter (OD) stainless steel tubing into the inlets of a tee (Alltech 0.25 mm bore stainless steel tee (p/n 30771)) at 83 bars. A pair of either Isco screw piston pumps for the liquid CO2 or conventional HPLC pumps for the drug solution were used. Once in the tee, the solution or suspension and the liquid, near critical, CO2 are mixed intimately. The mixture of dense gas and drug solution flows from the tee through a 75 µm inner diameter, 10 cm-long, fused silica restrictor (Polymicro TSP075375) and forms a wet aerosol plume upon the rapid expansion of the compressed CO2 into a glass drying chamber maintained at or near atmospheric pressure. Particles are formed when the decompressed plume containing micro-bubbles and micro-droplets is rapidly dried by warm nitrogen (50-65 °C) flowing through the glass-drying chamber at 30 L/min. Particles are collected downstream of the drying chamber with a single 0.45 µm dead-end filter to achieve collection of smaller amounts of powder. Alternatively, a double filter apparatus that has two 0.45 µm filters on either side of a glass chamber can be used for larger batches of powder. The top filter is back-pulsed at regularly timed intervals to release powder onto the bottom filter, where the powder, optionally, undergoes brief additional drying with warm nitrogen flowing at 30 mL/min through the bottom filter at the end of the primary processing.

PuffHaler reservoirs were manufactured from POLYBATCH VLA 55 UK anti-static polyethylene film bags purchased from Uline (Pleasant Prairie, WI). These anti-static bags were formed into PuffHaler reservoirs with the desired dimensions and volume by following a folding and cutting protocol and using a heat sealer to introduce new permanent seams to hold the desired reservoir configuration.
The Fine Particle Fraction (FPF) and Emitted Dose (ED) were determined according to USP Chapter <601> using an Andersen Cascade Impactor (Westech, Marietta, Georgia) and a Dosage Unit Sampling Apparatus (DUSA), respectively. Tests were conducted at relative humidities below 15%. An aliquot of powder was dispensed volumetrically by the Omnidose TT (Harro Hoefliger GmbH, Almersbach, Germany) into aluminum-foil polymeric laminate blisters. The blisters were loaded and thermally sealed in a glove box maintained at relative humidities below 15%. The mass of the powder was then gravimetrically verified. For testing with the Aerolizer (manufactured by Novartis Pharma AG for Schering Corp., Kenilworth, NJ), gelatin capsules were loaded with sample at relative humidities below 15%. Blisters remained sealed until peeling back the lid immediately before use. The powders were then dispersed into the reservoir before being sampled through a mask by the ACI or DUSA. In these procedures, the powder aerosol in the reservoir is drawn into the ACI or DUSA at 28.3 L/min or 60 L/min. In the ACI procedure, particles are separated by aerodynamic diameter on sequential stages and gravimetrically analyzed to quantitate the FPF (Rebits et al. 2007). The ED is gravimetrically determined by analyzing a single filter in the DUSA. When particles are dispersed from the foil laminate blister pack in the inhaler, a portion of the particles flows with the gas stream, while some remain adhered to the walls of the blister pack and on the walls of the other inhaler parts. The DUSA filter captures only what is actually emitted from the reservoir. As an alternative, total organic carbon (TOC) analysis, which is insensitive to moisture, can be used for quantitation of FPF and ED (Rebits et al. 2007).
Scanning electron microscopy (SEM) was performed on TB drug powders with a JEOL model #JSM-7401 field emission scanning microscope (FESEM) operating between 5 and 10 kV with a filament current of about 0.5 mA. Powder samples were deposited on carbon conductive double-sided tape, and then most of each sample was tapped off to leave a thin layer of microparticles. Samples were coated with a gold layer using a sputter coater operated for 30 seconds at a sputtering current of 40 mA.

The moisture content of the powders was determined by extraction of the water into methanol and measuring the concentration by the Karl Fischer coulometric titration method (Denver Instruments, Model 260 and 275KF). The protocol in USP Chapter <921> was followed and tests were conducted at humidity below 15% RH.

Crystallinity of the powders was assessed using powder X-ray diffraction (pXRD) scanning from 5 ° – 45 ° at a wavelength of 1.54 Angstroms on a Scintag PAD-5 X-ray diffractometer in continuous-scan mode. The step size was 0.02 and the scan rate was 2 ° per minute.

2.3 Results and Discussion

To help combat TB, a disease that kills 1.3 million people a year, we are developing unit-dose, inhalable, antibiotic microparticles for use as a primary and combined therapy approach to treating TB and multiple drug resistant TB (MDR-TB) (Namgyal 2006). Lack of patient compliance during lengthy treatment regimens is a major contributing factor in the development of MDR-TB (Horsburgh et al. 2000; Shin et al. 2004). In developing countries, because inhalable dry powders can be administered
by health-care workers with minimal training and weigh less than combined antibiotics
and water-for-injections, they are transport-friendly and may reach more remote patients,
making compliance more probable. Moreover, as CAN-BD antibiotic powders are also
needle-free, inhaled use improves safety for both administrators and patients by
eliminating needle-sticks and blood-borne infections from needle re-use. These
antibiotic powders have fine particle fractions from the PuffHaler as great as 21.6% less
than 3.3 µm aerodynamic diameter, which are ideal for alveolar deposition. By
depositing antibiotics in the deep lung, one may be able to reach TB lesions that lack a
strong blood supply, and may also increase the targeted dose to the lung airspace and
tissue while reducing systemic side effects and, potentially, the length of treatment.
Using inhalable antibiotics in combination with more traditional treatment may provide a
two-pronged attack strategy, from inhalation as well as the blood side, for MDR-TB.
Failure to kill all bacterial populations often results in the development of resistant
strains (Nuermberger et al. 2004; Tyagi et al. 2005; Wolf et al. 2007). Increasing the
targeted dose in the lung airspace and targeting bacteria in protective
microenvironments such as those in granulomas may prevent the development of MDR-
TB by ensuring that threshold doses of antibiotics reach bacteria populations that are
difficult to target by traditional methods through only the blood (parenteral or oral
delivery).
2.4 Characterization of Inhalable Antibiotic Formulations

2.4.1 Excipients for Better Powder Properties and Finding the Ideal Total Dissolved Solids (TDS) for Efficient CAN-BD Processing and Desired Powder Properties

Considering excipients for the formulation of the three chosen antibiotics the aim was to get the best dispersion, fine particle fraction (FPF), emitted dose (ED) and the smallest amount of moisture without compromising drug concentrations. Single excipient strategies were explored so as to maintain a high concentration of drug. Of the single excipients tested, the one that improved the FPF of all three antibiotics was L-leucine, which was chosen as the only other component of the inhalable antibiotic formulations. Other excipients that were tested included DL-leucine, and several other amino acids, which in small test runs did not show promise for improvement of powder performance. The use of L-leucine for improved dispersion and increased FPF via reduced interparticle interactions in spray-dried formulations is widely supported by the literature (Chew et al. 2005; Learoyd et al. 2009; Lechuga-Ballesteros et al. 2008; Li et al. 2005; Minne et al. 2008; Pilcer and Amighi 2010; Raula et al. 2007; Seville et al. 2007). Once L-leucine was chosen as an excipient, the correct proportion was identified by using ACI to characterize the FPF of several test formulas with different proportions of drug to L-leucine. Additionally, the ideal total dissolved solids (TDS) of each drug were determined by the individual drug solubility in water, FPF characteristics and processing conditions. Specifically higher fine particle fractions often require a slower flow rate, which in some cases was deemed impractical for future scale up. Also, in the case of capreomycin and kanamycin, higher TDS resulted in powders with poor FPF.
Table 2.2 details the FPF results of several different proportions of capreomycin to L-leucine as measured using the Aerolizer operated at 28.3 L/min, which was a flow rate adopted for comparison to other projects in the lab.

All drug formulations consist of 80% antibiotic and 20% L-leucine. The capreomycin and isoniazid formulation contain 5% TDS. The kanamycin formulations contain 5.62% TDS due to greater combined solubility of kanamycin and L-leucine. For all three drugs, capreomycin, kanamycin and isoniazid, 80% antibiotic: 20% L-leucine and between 5 and 5.62% TDS produced inhalable powders with the highest FPFs.

Table 2.2:

<table>
<thead>
<tr>
<th>Percent Fine Particle Fraction</th>
<th>&lt; 3.3 μm</th>
<th>&lt; 5.8 μm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capreomycin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10% TDS 100% capreomycin</td>
<td>1.32</td>
<td>1.81</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>1.62</td>
<td>2.47</td>
</tr>
<tr>
<td>5% TDS 95% capreomycin 5% L-leucine</td>
<td>8.94</td>
<td>17</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>3.29</td>
<td>6.24</td>
</tr>
<tr>
<td>5% TDS 90% capreomycin 10% L-leucine</td>
<td>15.42</td>
<td>28.88</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>3.44</td>
<td>6.58</td>
</tr>
<tr>
<td>5% TDS 80% capreomycin 20% L-leucine</td>
<td>32.99</td>
<td>58.86</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>4.47</td>
<td>8.8</td>
</tr>
</tbody>
</table>

Table 2.2: A comparison of different proportions of capreomycin to leucine showing that the highest FPF was achieved with 5% TDS, 80% capreomycin and 20% L-leucine.

Figure 2.3 shows the ACI results for fine particle fraction (FPF) and Emitted Dose (ED) using the PuffHaler to disperse capreomycin, isoniazid and kanamycin microparticles from foil laminate blister packs. Capreomycin, isoniazid, and kanamycin
were processed at the University of Colorado, Boulder, and packaged into foil laminate blister packs at Aktiv-Dry LLC. The antibiotic microparticles were analyzed at Aktiv-Dry LLC for fine particle fraction (FPF) and emitted dose (ED). The goal of the antibiotic formulations is to have a large ED and a large FPF <3.3 µm. While isoniazid has a lower FPF <5.8 µm than the other antibiotic formulations, the FPF <3.3 µm is comparable. The formulation of isoniazid is still being further developed with an emphasis on the improvement of its homogeneity, FPF and ED.

Figure 2.3: The Fine Particle Fractions and Emitted Doses for capreomycin, kanamycin and isoniazid from the PuffHaler at 28.3 L/min.
2.4.2 Effect of Device Flow Rate on Powder Properties

The FPF is dependent on both the device and the flow rate. To examine device effects, the FPFs of the drug formulations were measured with administration from both devices: the Aerolizer and the PuffHaler at 28.3 L/min. The results described in Figure 2.4 illustrate that the antibiotic formulations have higher FPFs when dispersed from the Aerolizer.

![Figure 2.4: A comparison of the Fine Particle Fractions for capreomycin, kanamycin and isoniazid from the two devices: Aerolizer and PuffHaler at 28.3 L/min](image-url)
Initially, the 28.3 L/min flow rate was adopted for comparability to other projects in the lab, in which 28.3 L/min was used as the standard test flow rate. As the tuberculosis antibiotics are likely to benefit a broad range of patients, with a variety of inspiration capacities, the antibiotic formulations were also tested using the Aerolizer as a dispersion device at 60 L/min. The Aerolizer was chosen as the test device for comparing flow rates because the PuffHaler was specifically designed to be used at the lower 28.3 L/min flow rate, whereas the Aerolizer is designed for use at 60 L/min. The

![Figure 2.5: Comparison of percent FPFs < 3.3 µm when the flow rate is increased from 28.3 L/min to 60 L/min.](image)

Figure 2.5: Comparison of percent FPFs < 3.3 µm when the flow rate is increased from 28.3 L/min to 60 L/min.
The collapsible reservoir of the PuffHaler does not favor improved dispersion at higher flow rates due to particle trapping. The three drug formulations were compared to examine how flow rate affects FPF, specifically the FPF < 3.3 µm. The results in Figure 2.5 illustrate that at rates due to particle trapping, the three drug formulations were compared to examine a higher flow rate of 60 L/min, FPF < 3.3 µm increases for all drug formulations, indicating that the higher energy inspiration is capable of breaking apart agglomerates of smaller particles.

The higher FPF < 3.3 µm is supported by SEM images of the antibiotic formulations, which show agglomerates of smaller particles. The higher energy of the 60 L/min flow rate is capable of dispersing these agglomerates so that the particles can reach the ACI stages as individual particles, thus increasing the FPF. In Figure 2.6, the FESEM images of the three antibiotic formulations illustrate the smaller particles in larger agglomerates.

Figure 2.6: FESEM images of the current formulations of a) Kanamycin b) Capreomycin c) Isoniazid.
2.4.3 Particle Shape and Morphology

The shape and morphology of capreomycin, isoniazid and kanamycin were investigated using SEM. As the capreomycin and kanamycin particles get larger, they tend to have a crumpled morphology. The smaller particles are more spherical. The SEM of isoniazid shows textured spheres, crystals, and some cube-like particles in a range of sizes. However, the amorphous textured spheres are the dominant particle morphology.

2.4.4 Moisture Content and Crystallinity of Antibiotic Formulations

The moisture contents of the capreomycin, kanamycin and isoniazid formulations indicated the consistency of drying. The capreomycin formulation contained 1.52% moisture with a standard deviation of 0.10%. The kanamycin formulation contained 2.70% moisture with a standard deviation of 0.42%. Lastly, the isoniazid formulation contained 1.79% moisture with a standard deviation of 0.24%.

The crystallinity of capreomycin, kanamycin and isoniazid microparticles formulated with 20% L-leucine were analyzed using pXRD. Capreomycin and kanamycin formulated microparticles are amorphous in character while the isoniazid microparticles show significant crystallinity.

2.4.5 Retention of Activity Post CAN-BD
Minimal inhibitory concentrations (MIC) in liquid medium using standardized Bactec-460 technology have previously been used to establish retention of antibiotic activity post CAN-BD processing. To date CAN-BD processed formulations of capreomycin, amikacin, ciprofloxacin-HCl, rifampin and moxifloxacin-HCl resulted in respirable antibiotics with 100 % retention of activity, within error, against M. tuberculosis. Specifically, amikacin, capreomycin, ciprofloxacin, moxifloxacin, and rifampin retained, respectively, 104% +/- 7%, 95% +/- 7%, 93% +/- 20%, 81% +/- 43% and 109% +/-34% activity (Sievers et al. 2006).

2.5 Conclusions

CAN-BD is a powerful supercritical/ near-critical fluid spray-drying technique for creating and drying microparticles of antibiotics formulations. Allied with protective unit-dose packaging and a low cost dry powder inhaler, CAN-BD microparticle processing can make a strong impact on shifting the tuberculosis treatment paradigm toward a needle-free solution. The above work illustrates the feasibility of creating inhalable treatment particles in a suitable aerodynamic size range for inhalation therapy of tuberculosis. By exploiting the aerodynamic properties of the inhalable antibiotic formulations, particles can be targeted to the deep lung where granulomas harboring hard to reach bacterial populations extend treatment times and increase the potential for the development of drug resistance. Beyond aerodynamic properties, the inhalable antibiotic microparticle formulations perform well in different DPIs and contain low moisture contents, which are helpful for maintaining the integrity of the antibiotics. The desirable powder properties of these anti-TB antibiotics, combined with the important...
packaging technologies developed for the inhalable measles vaccine dry powder project, may pave the way for new pulmonary treatments that reduce treatment time as well as and the dangers associated with traditional syringe delivery, while also increasing coverage to high risk populations.
2.6 References


Chapter 3: Exploring the creation of particles from water-in-oil-in-water emulsions using Carbon dioxide Assisted Nebulization with a Bubble Dryer® (CAN-BD)

3.1 Introduction

CAN-BD processed water-in-oil-in-water (w/o/w) emulsions were explored as a method for producing both microparticle biopolymers with potentially tunable controlled release properties and to determine whether CAN-BD can be used for the extraction of the organic phase of w/o/w emulsions and formation of dry microparticles. Time-release properties of w/o/w emulsions are particularly valuable in the treatment of TB as fewer doses of drug may increase patient compliance by reducing the inconvenience of frequent directly observed treatment (DOTS) (Langer 1998). While polymer composition and weight also contribute to controlled release, properties such as drug release, drug diffusion, polymer degradation, and erosion in biopolymer microparticles are most significantly impacted by particle size (Berkland et al. 2003; Berkland et al. 2007).

W/o/w emulsions are a multiple emulsion system in which the internal and external aqueous phases are separated by an oil/organic layer. The w/o/w system consists of internal aqueous drops surrounded by a surfactant film, surrounded by an aqueous layer with a surfactant boundary film. Typical of drug preparations, the drug is dissolved in the inner aqueous phase, a polymer such as poly (lactic-co-glycolic acid) (PLGA) is dissolved in a volatile organic solvent, such as dichloromethane (DCM), ethyl acetate (EA) or acetone (A) forms the oily/organic phase, and an emulsifier is dissolved
in the outer aqueous phase (Florence and Whitehill 1982; 1985). Upon evaporation of the volatile organic solvent from the w/o/w emulsion, solid drug-loaded microspheres are formed via precipitation of the polymer. The microspheres are prepared using modifications of a few basic processes: solvent evaporation or extraction of emulsions, phase separation and spray-drying. Drawbacks of these processes include the use of elevated temperatures, reduced pressure, large amounts of a second solvent, and residual solvent or coacervating agents left in the final microsphere product (Chattopadhyay et al. 2006).

One of the most prevalent methods for producing micro and nanoparticles from w/o/w emulsions is solvent evaporation (SE) used to remove the disperse phase of the w/o/w emulsion (Feczko et al. 2008). In this method there is a fast and slow evaporation phase. In the fast phase, 90% of the polymer solvent is removed and the nascent particles are defined by the size of the emulsion droplets in the parent emulsion. In the second phase of evaporation, the size of the dispersed droplets increases due to the coalescence of droplets. The degree of coalescence is dependent on the ability of the polymer to adsorb in the interface between the oil and water phase of the parent emulsion. The adsorption is reduced by the use of surfactant to stabilize the oil-water boundary. Also coalescence is amplified in polymers with surface-active properties, which enhance interfacial adsorption (Vauthier and Bouchemal 2009). The main disadvantage of this technique is the long evaporation time, which can be many hours and can encourage particle aggregation (Li et al. 2008). DCM is routinely used in the production of these w/o/w emulsions due to its low water solubility and subsequent ability to maintain the w/o/w barrier. However, DCM is a toxic, potentially carcinogenic,
chlorinated solvent and health and environmental concerns have been raised with respect to residual solvent left behind in the particles (Chattopadhyay et al. 2006; Sherratt et al. 2002).

In the work described within this chapter supercritical (SC-\(\text{CO}_2\)) or near critical carbon dioxide (nC-\(\text{CO}_2\)) is used to extract the volatile organic phase of two different types of w/o/w emulsions. In all of the emulsions the biodegradable polymer poly-lactic-co-glycolic acid (PLGA) was used as the biopolymer for controlled release. PLGA, was chosen for its history of safe use in biodegradable sutures and because, via hydrolysis, PLGA degrades into lactic and glycolic acid, metabolites of the Krebs cycle (Anderson and Shive 1997). Due to concerns about the safety of residual solvent, ethyl acetate, which displays a less severe toxicological profile then dichloromethane, was chosen as the organic solvent. Polyvinyl alcohol (PVA) was used as an emulsifier for the stabilization of the boundary surface of multiple oil drops and outer water by surfactant properties and by increasing the viscosity of the water so that emulsified multiple drops are hindered from interflowing (Feczko et al. 2008).

Previous work has demonstrated the use of SC-\(\text{CO}_2\) as an anti-solvent, which resulted in strong particle agglomeration and swelling particles (Lee et al. 2008; Wang et al. 2005). However, the CAN-BD approach is different in that rather than relying on the gas stream and nozzle to produce and atomize microparticles of a particular size, the w/o/w emulsion, as modified by dissolution of CO2 in the emulsion and anti-solvent behavior during the nebulization of the micro-droplets, will dictate the properties of the particles. Upon contact with the organic layer of the pre-formed w/o/w emulsion, the SC-\(\text{CO}_2\) is expected to cause rapid solvent elimination and polymer (PLGA) precipitation.
The efficient nature of solvent elimination is expected to retard the migration of the drug encapsulated in the internal aqueous phase to the external aqueous phase.

3.2 Methods: Techniques for preparing w/o/w emulsions

Several methods for spray drying water oil water emulsions w/o/w have been tested by the Sievers Group using Carbon Dioxide Assisted Nebulization with a Bubble Dryer® (CAN-BD) to aid in precipitation of the polymer and evaporate organic solvents while drying the subsequent microparticles. Protocol one, was adapted from (Lamprecht et al. 1999). The inner water phase consisted of 500 mg of isoniazid in 10 mL of phosphate buffered saline (PBS) or distilled water. The 500 mg of isoniazid is 0.24 % by mass of the entire w/o/w emulsion, which is a total of 210 mL including all three phases. The organic phase consists of 500 mg of poly(lactic/glycolic) acid (PLGA, lactide:glycolide ratio: 50:50, $M_w$: 38,000-54,000, Sigma-Aldrich, St Louis, MO) dissolved in 40 mL of ethyl acetate (EA). The first internal water-in-oil (w/o) emulsion is created by homogenizing the isoniazid/water phase and the EA organic or “oily” phase at 5000 RPM for 3 minutes. A Silverson® L5M-A homogenizer was used for all experiments involving homogenization. The external water phase contains 2% polyvinyl alcohol (PVA, $M_w$: 30,000-70,000, 87%-89% hydrolyzed, Sigma-Aldrich, St Louis, MO) w/v, with respect to the external water phase of 200 mL. To create the w/o/w emulsion, the 10 mL of the previously described w/o emulsion is poured into the external aqueous PVA phase in a glass beaker. The beaker containing the w/o and the aqueous PVA phase is placed in an ice bucket and the contents are homogenized at 8000 RPM for 3 minutes. After homogenization, the w/o/w volume expands from 210 mL to 450 mL with
two phases, a foam phase and a liquid phase. The foam phase has been dried, and evaluated by SEM and does not contain any significant concentration of PLGA/PVA/INH micro-emulsion particles. The liquid phase, containing the homogenized w/o/w solution is poured into the product solution cylinder of the CAN-BD, where the emulsion is pumped, with a flow rate of 0.5 mL/min to a tee, where the w/o/w emulsion and near critical, CO2 are mixed. The mixture of dense gas and the w/o/w emulsion flows from the tee through a fused silica restrictor and forms a wet aerosol plume upon the rapid expansion of the compressed CO2 into a glass drying chamber maintained at or near atmospheric pressure. Particles are formed when the decompressed plume containing micro-bubbles and micro-droplets is rapidly dried by warm nitrogen (60 °C) flowing through the glass-drying chamber at 30 L/min. Protocol two was used for particles unrelated to this study.

Additional particles of a similar size range and morphology were produced using protocol three, which was adapted from (Song et al. 2006). In protocol three, 100 mg of PLGA (lactide:glycolide: 75:25, M_w: 66,000-107,000, Sigma-Aldrich, St Louis, MO) was dissolved in 10 mL of EA. The organic phase is added to 20 mL of an aqueous phase containing the stabilizer PVA. The PVA aqueous phase was 1% w/v with respect to the 20 mL water phase. The mixture was allowed to come to saturation and is then emulsified, on ice, for one minute with a probe tip sonicator (Sonicator Ultrasonic, Processor XL, Misonix Inc.) operating at setting six and between 1-10% intensity. While sonicating, the external aqueous phase, which consists of 80 mL of water and dissolved drug, is added to the w/o emulsion created by the sonication of the PLGA/EA phase and the water PVA phase. After sonication, 30 mL of the w/o/w emulsion was processed via
CAN-BD at 0.5 mL/min, 30 L/min N2, 60°C and between 83 and 87 bar. The particles made using protocol three used bovine serum albumin (BSA) as a model protein, and INH as a target TB drug. Modifications of this protocol include CAN-BD processing modifications such as a processing temperature below the glass transition of PLGA (40-60°C) and a lower flow rate (Della Porta et al. 2010; Della Porta and Reverchon 2008). These modifications were made to narrow the size range of the particles.

3.3 Results and Discussion

The microspheres produced using protocol one are shown in Figure 3.1. In all of the formulations in Figure 3.1, INH was used as the encapsulated drug. Isoniazid was chosen due to its prevalent use as a second line antibiotic and due to its formulation challenges. Difficulties such as low water solubility and poor bioavailability make INH an excellent candidate for new methods of drug encapsulation and delivery. In Figure 3.1 variations on protocol one were compared. The first variation considered is how salt influences the particles as salts are often used to stabilize protein drugs. One goal of this experiment is to determine how robust CAN-BD is as a general solvent extraction and particle forming method for w/o/w emulsions.

To test the effect of salt on the CAN-BD extraction process phosphate buffered saline (PBS) was employed as the drug solvent (see Figure 3.1a). In Figure 3.1b the same formulation was used, replacing the PBS with water. The salt does not impact the size, shape or polydispersity of the particles formed with the CAN-BD process. In Figure 3.1c the effect of decreasing the surfactant PVA was examined. While PVA is one of the most commonly used polymer surfactants, it is well known that it remains on the surface...
of the particles and is very difficult to remove (Carrio et al. 1995). Reducing PVA is desirable for the reduction of any PVA-specific reactions to the particles. Again the decrease in PVA did not affect the size, shape or polydispersity of the particles.

Figure 3.1: SEM images of dry powder antibiotic formulations a) 0.25% INH, 0.25% PLGA, 1.98% PVA (W/O/W: PBS, Ethyl acetate, Water) b) 0.25% INH, 0.25% PLGA, 1.98% PVA (W/O/W: Water, Ethyl acetate, Water) c) 0.25% INH, 0.25% PLGA, 0.99% PVA (W/O/W: Water, Ethyl acetate, Water).
In further testing CAN-BD and protocol one, particles made using CAN-BD as the extraction method were compared with particles made with protocol one, but using solvent evaporation with stirring for 6 hrs. The evaporation method was followed by centrifugation at 3000 RPM for 5 minutes and three washing steps were carried out at 624XG for 5 minutes. The washing steps consisted of removing the supernatant from the pelleted particles, re-suspending the particles in 500 µl of 18 water and centrifuging for 5 minutes at 624XG. Figure 3.2a shows the particles made with CAN-BD, while Figure 3.2b shows the particles made via solvent evaporation with stirring and centrifugation. The particles made via solvent evaporation are more ordered, an artifact of the washing and centrifugation process; however, the overall size and size distribution of the particles is very similar.

Figure 3.2: a) 0.25% INH, 0.25% PLGA, 0.99% PVA (W/O/W: Water, Ethyl acetate, Water) made using CAN-BD for solvent removal and polymer precipitation b) 0.25% INH, 0.25% PLGA, 0.99% PVA (W/O/W: Water, Ethyl acetate, Water) made using solvent evaporation with stirring and subsequent washing and centrifugation steps.
One additional disadvantage of the solvent evaporation technique is that the washing process causes shearing of some of the particles. This is apparent in Figure 3.3 where particles created via solvent evaporation are shown after washing in Figure 3.3a, and before the washing procedure in Figure 3.3b. While the before washing picture is at higher magnification than the after washing pictures, a visual survey of the before sample did not display any of these half particles.

Figure 3.3: Both a) and b) are the same formulation: 0.25% INH, 0.25% PLGA, 0.99% PVA (W/O/W: Water, Ethyl acetate, Water) a) after washing and centrifugation steps b) before the washing and centrifugation steps
To further explore the versatility of CAN-BD in solvent evaporation, particle precipitation, and drying, method three was explored, as a novel method for producing w/o/w emulsions aimed at smaller particle sizes with less polydispersity. Protocol three uses a 75:25 ratio of lactide to glycolide and protocol one uses a 50:50 ratio. Changing the lactide to glycolide ratio was shown to decrease particle size and narrow the particle size range (Belbella et al. 1996; Zambaux et al. 1998). Figure 3.4 illustrates that particles made using protocol three are similar in shape to the particles produced using protocol one, but more polarized to either larger particles or smaller particles. Additionally, egg-shaped particles and particles in the process of coalescing were observed see 3.4b.

The merging particle observation led to the idea that either the w/o/w emulsion was not stable prior to CAN-BD processing or the CAN-BD processing conditions are responsible for the merging behavior. A key difference between protocol one and three is the ratio of lactide to glycolide. This ratio also dictates the glass transition temperature for PLGA, which is between 40-60 °C, depending on the ratio of lactide to glycolide. The CAN-BD was operated at 60 °C for both protocol one and three despite the difference in ratio of lactide to glycolide. Additionally, smaller more uniform particles were made with protocol three using the solvent evaporation method. These particles and the ratio-dependent glass transition temperature led to testing the latter hypothesis. Protocol three was repeated with the lower processing temperature of 40 °C. Figure 3.5 contains images from the lower processing temperature experiments. Evident from these pictures is that the experimental modification did not improve the particle boundary definition, as the merging particles are still present.
Upon data review, particle merging had previously been observed in particles made using protocol one that were stored via refrigeration for 1-2 days, and then subsequently processed. This implies that the w/o/w emulsions tested with CAN-BD were not stable. These emulsions were improved by increasing the concentration of

Figure 3.4: a) Particles made with protocol 3 consisting of 0.5% INH, 0.1% PLGA, 1% PVA (W/O/W: Water, Ethyl acetate, Water) b) Is the same formulation as a) but highlights some of the merging of particles observed (see red arrows) and the egg-shaped particles that result from these mergers (see red box) with this method

Upon data review, particle merging had previously been observed in particles made using protocol one that were stored via refrigeration for 1-2 days, and then subsequently processed. This implies that the w/o/w emulsions tested with CAN-BD were not stable. These emulsions were improved by increasing the concentration of
PVA consequently improving the boundary and stability of the initial droplets produced via homogenization or sonication.

After examining several modifications of procedure one and procedure three, CAN-BD is an effective and gentle method for removing the organic solvent from w/o/w emulsions. Further, the CAN-BD method produces particles that are comparable to those made from more conventional methods such as solvent evaporation without particle shearing. The CAN-BD method offers additional improvement processing speed. While size control was not achieved with this method, it may be possible to obtain a narrower size distribution of particles by producing a more stable initial w/o/w emulsion. One possible method for improving stability is to increase the surfactant concentration.
3.4 References


Chapter 4: The Perpetual Library: Limitations of on-bead T7 transcription

4.1 Introduction: The use of T7 transcription in RNA in vitro evolution experiments

RNA in vitro selection is used to explore the functional capabilities of RNA and has successfully been used as a technique for the discovery of novel RNA sequences that can mediate new material synthesis, have high binding affinities, or catalytic activity (Beaudry and Joyce 1992; Ellington and Szostak 1990; Hager et al. 1996; Lorsch and Szostak 1994; Robertson and Joyce 1990; Tuerk and Gold 1990). The principal of RNA selection is based on “survival” or successful adaptation to a selective pressure on a molecular level. The starting point of an in vitro evolution experiment is a large pool or library of synthetic molecules with varying sequences of the four-nucleotide subunits of RNA. The library contains regions of short sequences that are necessary for molecular replication, and the remainder of the molecule is a random sequence of a particular length. The starting pool is created by synthesizing a random library of single stranded DNA followed by two cycle PCR to convert the single stranded library into a double stranded library. Finally T7 transcription converts the double stranded DNA into the RNA library. After library generation, the RNA pool is subjected to a selection pressure by standard biological methods. The surviving RNAs are then physically separated and purified from the non-functional RNAs. These functional RNA sequences are then reverse transcribed into DNA, which is PCR amplified. Upon continuous selection of RNA sequences that reproducibly fold into intricate 3-D structures to mediate the selected property, families of talented RNAs are converged upon (Ellington and Szostak
1990; Robertson and Joyce 1990; Tuerk and Gold 1990). The selection process is schematically described in Figure 4.1.

Figure 4.1: A generic scheme for an in-vitro evolution experiment.
After each round of selection the amplified DNA from the functional RNA pool must be transcribed into RNA for the next round. This step often involves a lengthy and time consuming gel purification process that results in losses in the RNA pool. Further, if any contamination or unexpected result occurs in the evolution process, the round, or several rounds, must be repeated requiring extensive re-synthesis and purification efforts. To reduce the numerous purification steps and consequent loss of RNA material, the perpetual library was designed. Amplifying the initial single stranded template with a biotinylated 3’ primer, and then capturing the post PCR double stranded template on streptavidin beads, from which transcription proceeds releasing the desired RNA starting material into the supernatant, generates the perpetual library. With this technique, described in Figure 4.2, each round of the selection can theoretically be preserved in double stranded DNA form. Thus if a round or a whole selection needs to be repeated, the same initial double stranded DNA library can be used to generate the initial and subsequent RNA selection pools.
Figure 4.2: Schematic of the generation of the Perpetual Library. Transcription bubble adapted with permission from The American Association for the Advancement of Science.
4.2 Developing the Perpetual Library

4.2.1 Does transcription off of beads require the same concentration of T7 RNA polymerase as traditional transcription?

While several groups have demonstrated the success of on-bead transcription prior to this work, entire libraries of random sequences had not been transcribed off of beads or stored for archival in vitro evolution experiments (Fujita and Silver 1993; Ghosh et al. 2005). Initial systematic questions such as will the T7 RNA polymerase run into the bead and abort transcription? and do on bead transcription reactions require the same time and concentration of T7 RNA polymerase? were examined. To address the first question initial experiments with an 80n and a 40n random library were conducted and full-length transcription products were obtained. To deduce how much T7 was necessary for on-bead transcription the following assumptions were made: an average transcription consists of 250µl of streptavidin beads, which is approximately 1.75E6 beads and 8.2E14 molecules of double stranded DNA. This amounts to 4.7E8 molecules of double stranded material per bead. The same amount of T7 RNA polymerase, 4.7E8 molecules per bead, was used in the initial on-bead library transcription reactions. However, upon further investigation the amount of T7 RNA polymerase was decreased by two logs without a decrease in the amount of transcription product. In fact traditional three hour T7 ~ transcription produced 4.03E-10 moles of RNA transcript while the transcription pictured in Figure 4.3, which was carried out on beads with two logs less T7 RNA polymerase, produced ~2.66E-8 moles of
transcript. Similarly, transcriptions of libraries, with a 40n random region, also produced the same amount or more RNA transcript with two logs less T7 RNA polymerase.

Figure 4.3: Perpetual Library: Efficient and Convenient: The perpetual library technique allows us to drop the T7 RNA polymerase concentration by two logs with no decline in transcription.
4.2.2 Is transcription from beads biased?

Throughout the in-vitro transcription process valuable sequences evolve from a vast library of random sequences. Any system used to optimize the process of in-vitro transcription and preserve each round of transcription must preserve both initial sequence diversity and the functional sequences that the selection converges upon. By preserving the exact sequence environment, the selection can be repeated for verification. An additional utility of preserving each round of a selection is that once a selection has been verified the selection can be repeated with a new selection pressure allowing the process of how changing the selection pressure changes the evolved sequences. Thus, it is essential that when transcription of a random library is carried out in the on-bead format all populations of sequences must be transcribed.

To test the fidelity of the on-bead library transcription system for transcribing all of the sequences in a random library, a 40n random library with the standard 1E14 molecules of random sequences was synthesized and mixed with 200 molecules of a known sequence, Pdase 17 (Pd17). Modified Pdase 17, which is transcribed using 5-(4-pyridylmethyl)-UTP, mediates the formation of hexagonal palladium (Pd) plates upon incubation with tris(dibenzylideneacetone) dipalladium(0) \( \text{[Pd}_2\text{(DBA)}_3\text{]} \) (Gugliotti et al. 2005; 2009). A second 40n random library with 1E14 random sequence molecules was also synthesized and mixed with 20,000 molecules of Pd17. These seeded random libraries were then attached to streptavidin beads and a test selection was carried out on both libraries. The selection pressure consisted of subjecting the RNA transcribed from the beads to a bead-bound single stranded complement to the known sequence
Pd17. Pd17 and all of the members of the 40n random library share the same 5' fixed region but have a different 3' region. Consequently only the Pd17 sequence should have enough sequence complementation to be captured by the bead-bound complement. After several rounds of selection the Pd17 sequence should become enriched. Figure 4.4 further illustrates the steps of this test selection.

Figure 4.4 Illustration of a test selection used to determine if low frequency sequences could be transcribed and enriched from the library on a bead system.
The enrichment of a selection can be monitored by real time PCR (Horisawa et al. 2005). As more of the winning sequences are enriched the initial population of winning single stranded DNA increases and the amplification of the DNA occurs at lower PCR cycle numbers. As indicated by the increase in double stranded PCR product, followed by real time PCR, at cycle eighteen and twelve for rounds one and two of the test transcription the PD17 sequence was enriched using material from on-bead transcription (Figure 4.5). These results indicated that low frequency sequences could be transcribed from the on-bead library.
Figure 4.5 Real Time PCR results from round one and two of a test selection for Pdase 17. The green traces are from the selection that started with a 200 molecules of Pdase 17 and the blue traces are from the selection that started with 20,000 molecules of Pdase 17.
4.3 Results and Conclusion

The promising results of the test selection led to the adoption of the on-bead random library transcription technique for three different selections. Each selection started with a different random library and unique primer sets. In each selection upon round five a shorter band became prominent on the transcription verification gels. All three selections seemed to be converging on shorter sequences than the sequences present in the original starting library. The selections did not involve an inherent mechanism for creating shorter RNA and as shorter sequences for three independent selections with different selection pressures is unlikely, these results implied a systematic error in the selection technique. Two of the selections were started over and again converged on a shorter sequence. Finally one condition of the selections was started over using the on-bead random library and the same library was used for a parallel conventional selection. The shorter sequences emerged in the selection with on-bead random library but did not appear in the conventional selection.

Due to the well-documented reproducibility of RNA amplification with T7 RNA polymerase in microarray experiments, a bias in the transcription fidelity of long versus short sequences in the library seems unlikely (Schneider et al. 2004). Another unlikely possible explanation for the emergence of shorter sequences is that as the selection converges on families of similar sequences the local population of these sequences and the regions of complementary sequences on the beads becomes concentrated enough to cause double stranded DNA templates to become stuck together with transient regions of triplex DNA. Triple helical complexes are thermodynamically stable near
physiological conditions and have half-lives compatible with the length of a transcription reaction making this an attractive explanation (Francois et al. 1989; Maher 1992; Maher et al. 1992). However, experiments by Mahler et al. have shown that triplex DNA does not block elongation, but rather excludes the polymerase from the promoter. Steric hindrance at the promoter by triplex DNA cannot explain the convergence of the selection on the shorter RNA transcripts. Possibly, the condition of having the random sequence library at high density on the surface of the beads is an independent selection pressure that mediates the evolution of sequence dependent termination elements. This possibility could be tested by examining the short sequences of later selection rounds via sequencing and looking for common motifs and comparing those patterns to known termination sequences.

4.4 Materials and Methods

Reagents. All reagents were used without further purification.

PCR amplification of DNA Templates

The 5’-primer for Pd17 (5’-GCT AAT ACG ACT CAC TAT AGG GAG ACA AGA ATA-3’) was purchased from Integrated DNA Technologies, Inc (Coralville, IA). The 3’ biotinylated primer (5’ - BBG CCT GTT GTG AGC CTC CTG TCG AA-3’), the Pd17 sequence (5’-GCT AAT ACG ACT CAC TAT AGG GAG ACA AGA ATA AAC GCT CGG CCC TTT CTA TCC TCA ATG TAC CAA CAA AAA ATG TAT TCC TTC GAC AGG
AGG CTC ACA ACA GGC3’-), the 40N random template (5’- GCT AAT ACG ACT CAC
TAT AGG GAG ACA AGA ACA TAC CGT CAA -40n- TTC GAC AGG AGG CTC
ACA ACA GGC -3’) and the Pd17 complement (5’ –BBGGAATACATTTTTTGTGGA
TACATTGAGGA-3’) were synthesized using phosphoramidites and biotin
phosphoramidite (B in the sequences) from Glen Research (Sterling, Virginia) using
standard phosphoramidite chemistry on an ABI 394 DNA Synthesizer. After synthesis
the primer was deprotected using a 1:1:2 methanol:t-butylamine: Water mixture at 70 °C
for 5 hrs. The synthesized materials were reverse-phase HPLC purified, then gel
purified. Streptavidin used for gel shift analysis was dissolved in phosphate buffered
saline. Pierce UltraLink Immobilized Streptavidin Plus agarose beads (distributed by
Thermo Scientific: Rockford, IL) were washed 3 times with 1 wash buffer (125 mM
NaCl, 40 mM HEPES pH 7.5, 40 mM MgCl$_2$) and resuspended to their original volume
as a 50% slurry immediately before use. All verification gels of DNA were visualized and
quantitated by staining with SYBR Gold nucleic acid gel stain (Invitrogen) and imaged
on a FujiFilm 5100 fluorescent imager.

Two nanomoles of dsDNA template or Pd17 were combined with 1 Taq DNA
Polymerase buffer (New England Biolabs, 10 mM KCl, 10 mM (NH$_4$)$_2$SO$_4$, 20 mM Tris-
HCl, pH 8.8, 2 mM MgSO$_4$, 0.1% Triton X-100), 0.12 mM each of dATP, dCTP, dGTP,
and dTTP, 2 mM MgCl$_2$, 1 µM each of 5’ -primer and 3’ -primer, 0.1 U/µL Taq DNA
Polymerase (New England Biolabs), and 1x SYBR Green (Qiagen, USA). PCR was
performed using the following reaction parameters: 95 °C, 1 minute, 58 °C, 1.5 minutes,
72 °C 10 minutes; hold at 4 °C. DNA was gel purified to remove primers and quantitated using UV-Vis spectroscopy.

Streptavidin Bound Double Stranded DNA Library

An aliquot of Pierce UltraLink Immobilized Streptavidin Plus agarose beads (distributed by Thermo Scientific: Rockford, IL) were washed 4 times with 1 wash buffer (125 mM NaCl, 40 mM HEPES pH 7.5, 40 mM MgCl₂). The pre-washed beads are added in 50 µl aliquots to the purified, biotinylated, double stranded, random DNA library, generated from two-cycles of pcr, described above. After incubating the streptavidin beads with the random library with rotation for 5 minutes the beads are spun down using a bench top centrifuge and the supernatant was examined using UV-Vis spectrophotometry. Aliquots of washed beads were added to the library until the UV-vis absorbance value, at \( \lambda = 260 \text{ nm} \), of the supernatant was stable. Once the UV-Vis absorbance value had stabilized the beads were washed in 1x transcription buffer to remove any unbound material. An analytical 8% PAGE gel of a small aliquot of the beads and the supernatant was run to verify that no double stranded template had been left behind in the supernatant.

RNA Synthesis

The initial random sequence RNA library for the test selection was prepared using 5X T7 RNA polymerase buffer at 1X concentration (Promega: Madison, WI) (4%(w/v) PEG 8000, 40mM Tris-HCl, pH 8.0, 12 mM MgCl₂, 5mM dithiothreitol (DTT), 1mM spermidine HCl, 0.002% Triton X-100), 0.2 mM each of ATP, CTP, GTP, and modified UTP (5-(4-pyridylmethyl)-UTP), 250nM dsDNA template immobilized on
streptavidin beads or in solution for control RNA synthesis, 200 units of T7 RNA Polymerase (Promega), 0.8 U/ul Rnaisin (Promega), incubated at 37 °C for 3 hours to yield 5-(4-pyridylmethyl)-uridine modified RNA transcripts (87-mer): 5’-GGGAGACAAGAATAAACGCTCGG-40 nucleotides-TTCGACAGGAGGCTCACAACAGGC-3’ . The selections that adopted the on bead transcription library were conducted in HEPES transcription buffer which consisted of 40mM HEPES, pH 8.0, 125 mM NaCl, 40mM MgCl₂.
4.5 References


Bibliography:


CDC (2010). Tuberculosis fact sheet, CDC, ed., Division of Tuberculosis Elimination, Atlanta.


