Pulmonary delivery for the treatment of tuberculosis

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SINGAPORE

**PULMONARY DELIVERY FOR THE TREATMENT OF
TUBERCULOSIS**

WILSON POH KWOK CHOON

SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

2019

**PULMONARY DELIVERY FOR THE TREATMENT
OF TUBERCULOSIS**

WILSON POH KWOK CHOON

SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

A thesis submitted to the Nanyang Technological University
in partial fulfilment of the requirement for the degree of
Doctor of Philosophy

2019

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research, is free of plagiarised materials, and has not been submitted for a higher degree to any other University or Institution.

21 August 2019

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.....
Wilson Poh Kwok Choon

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I have reviewed the content and presentation style of this thesis and declare it is free of plagiarism and of sufficient grammatical clarity to be examined. To the best of my knowledge, the research and writing are those of the candidate except as acknowledged in the Author Attribution Statement. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

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Joachim Loo Say Chye

Authorship Attribution Statement

This thesis contains material from the paper published in the following peer-reviewed journal where I was the first author.

Chapter 5 is published as Active pulmonary targeting against tuberculosis (TB) via triple encapsulation of Q203, Bedaquiline, and Superparamagnetic Iron Oxides (SPIOs) in nanoparticle aggregates. *Drug Delivery*, Volume 26 Issue 1 (2019) DOI: 10.1080/10717544.2019.1676841

The contributions of the co-authors are as follows:

- Prof. S.C.J. Loo provided the initial project direction and edited the manuscript drafts.
- Prof. J. Sznitman and his team conducted the *in-silico* and *in-vitro* magnetic targeting experiment.
- Prof. K. Pethe and his team provided the Q203 and Bedaquiline drugs and guided me for the mycobactericidal experiments.
- I prepared the manuscript drafts.
- I co-designed the study with Prof. S.C.J. Loo and performed all the laboratory work at the School of Materials Science and Engineering and the Lee Kong Chian School of Medicine. I also analyzed the data.
- All microscopy, including sample preparation, was conducted by me in the Facility for Analysis, Characterization, Testing and Simulation.

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Abstract

Tuberculosis (TB) has gained attention over the past few decades by becoming one of the top ten leading causes of death worldwide. This infectious disease of the lungs is orally treated with a medicinal armamentarium. However, this route of administration passes through the body's first-pass metabolism which reduces the drugs' bioavailability and taxes the liver and kidneys. Inhalation therapy represents an alternative to the oral route, but low deposition efficiencies of delivery devices such as nebulizers and dry powder inhalers render it challenging as a favourable therapy. It is hypothesized that by encapsulating two potent TB-agents, i.e. Q203 and Bedaquiline, that inhibit the oxidative phosphorylation of the bacteria together with a magnetic targeting component, Superparamagnetic Iron Oxides (SPIOs), into a PDLG carrier using a single emulsion technique, the treatment of TB can be a better therapeutic alternative. This simple fabrication method achieved a homogenous distribution of 500 nm particles with a magnetic saturation of 28 emu/g. Such particles are shown to be magnetically susceptible in an *in-vitro* assessment, viable against A549 epithelial cells, and are able to reduce two log bacteria counts of the BCG organism. Furthermore, through the use of an external magnet, our *in-silico* Computational Fluid Dynamics (CFD) simulations support the notion of yielding close to a 100% deposition in the deep lungs. Our proposed inhalation therapy circumvents challenges related to oral and respiratory treatments and embodies a highly favourable new treatment regime.

Lay Summary

Tuberculosis (TB) is the disease of the lungs which impairs those inflicted with symptoms of severe cough, blood-tinged sputum, fever, and weight lost. It is an epidemic which affects one-third of the global population and causes world-wide mortalities. While TB is treatable with a combination of oral antibiotics, the treatment duration stretches from 6-30 months. This lengthy form of medicinal regime devastates the liver and kidneys. Hence, an alternative delivery system to shorten treatment duration is necessary. The respiratory tract is a fitting avenue to overcome this complication because it directly targets the bacteria in the lungs and bypasses the liver and kidneys. It is hypothesized that the delivery of two potent anti-tubercular agents known as Q203 and Bedaquiline through the lungs can shorten treatment duration. These two anti-tubercular agents have proven their efficacy against the current drugs and when used together have a synergistic effect. The pulmonary delivery of drugs however has its own set of challenges; one of which is the low deposition percentage into the lungs. The current available pulmonary devices are only capable of up to 40% of deposition into the lungs. The TB bacteria are found in the lower airways and in order for the drugs to reach it, the particles that enter the lungs must be of submicron sizes. Hence, for a pulmonary drug delivery system to circumnavigate through these two conundrums, a polymeric carrier of sub-micron size with magnetically susceptible iron oxides loaded are used to increase the deposition percentage and manoeuvre the carrier into the lower airways. The drug carrier shows no toxicity against the lung epithelial cells and is able to completely eradicate an attenuate form of the TB bacteria in an in-vitro environment. The data presented highly favours an inhalation therapy to supersede the current TB treatment.

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Abbreviations

AAD	Adaptive Aerosol Delivery
AMIK	Amikacin
APTT	Activated Partial Thromboplastin Time
ATP	Adenosine Triphosphate
AM	Alveolar Macrophages
BCG	Bacillus Calmette-Guerin
BDQ	Bedaquiline
BH	Breath-Hold (BH)
CFC	Chlorofluorocarbons
CFD	Computational Fluid Dynamics
CFU	Colony Forming Units
COPD	Chronic Obstructive Pulmonary Disease
DCM	Dichloromethane
DLS	Dynamic Light Scattering
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DOTS	Directly Observed Treatment, Short Course
DPIs	Dry Powdered Inhalers
DSPC	Distearoylphosphatidylcholine
ELISA	Enzyme-Linked Immunosorbent Assay
EMB	Ethambutol
ELISPOT	Enzyme-Linked Immunosorbent Spot
ETC	Electron Transport Chain
FDC	Fixed-Dose Combination
FE-SEM	Field Emission Scanning Electron Microscope
FVM	Finite Volume Method
GSK	GlaxoSmithKline
GS-MS	Gas Chromatography Mass Spectrometry

HBEPc	Human Bronchial Epithelial Cells
HIV	Human Immunodeficiency Virus
HPLC	High Performance Liquid Chromatography
HRBCs	Human Red Blood Cells
ICRP	International Commission on Radiological Protection
IFN γ	Interferon Gamma
IL-2	Interleukin 2
IL-7	Interleukin 17
INH	Isoniazid
LEVO	Levofloxacin
LC-MS	Liquid Chromatography Mass Spectroscopy
MBC	Minimum Bactericidal Concentration
MDR-TB	Multi Drug-Resistant Tuberculosis
MIC	Minimal Inhibitory Concentration
MMAD	Mass Median Aerodynamic Diameter
MRI	Magnetic Resonance Imaging
MS	Mass Spectrometry
<i>M.Tb</i>	Mycobacterium Tuberculosis
OA	Oleic Acid
P/S	Penicillin/Streptomycin
PBS	Phosphate Buffered Saline
PDLG	DL-lactide/glycolide copolymer
PLGA	Poly (lactic-co-glycolic acid)
pMDIs	pressurized Metered-Dose Inhalers
PPD	Purified Protein Derivative
PRP	Platelet-Rich Plasma
PTFE	Polytetrafluoroethylene
PYZ+	Pyrazinoic Acid
rBCG	recombinant Bacillus Calmette-Guerin
Re	Reynolds Number
RIF	Rifampicin

RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
SEM	Scanning Electron Microscope
SLF	Simulated Lung Fluid
SPIOs	Superparamagnetic Iron Oxides
TB	Tuberculosis
TEM	Transmission Electron Microscope
TNF _α	Tumour Necrosis Factor Alpha
VNJ	Ventilator-Nebulizer Junction
VSM	Vibrating Sample Magnetometer
VWD	Van Der Waals
WT	Wild-Type
WT-BCG	Wild-Type Bacillus Calmette-Guerin

Chapter 1

Introduction

The chapter begins by introducing the epidemic of Tuberculosis, the current oral therapy regime, and the problems relating to it. An alternative is then proposed to circumvent the problem and is elaborated upon. After which the hypothesis, objectives and scope of this project are delineated. An overview of the thesis is then provided through short summaries of each chapter and ends off by outlining the novelty and findings of the work.

1.1 Background

Tuberculosis (TB) is a contagious disease that has scourged millions of lives around the world and is one of the top ten leading cause of death worldwide [1]. It is caused by a virulent bacterium known as the *Mycobacterium tuberculosis* (*M.tb*) that primarily affects the lungs and is transmitted through the air when a person coughs or sneezes. A person that is diagnosed with an active form of this disease will exhibit symptoms such as coughing, chest pains, fatigue, and fever. While this form of TB can manifest, there exist another that is more inconspicuous in nature.

Known as latent TB, the *M.tb* bacteria are sojourned in a collection of macrophages (known as granuloma) that precludes the bacteria from affliction of any sort. Patients that are infected with latent TB do not exhibit any symptoms, however, in this quiescent state, the *M.tb* can still proliferate [2]. It is postulated that the infection with the *M.tb* hinders the acidification process of the phagosomes, preventing the phagocytosis process from running its course [3, 4]; if the granuloma was to lose its restrains on the bacteria due to a compromise in one's health (Human Immunodeficiency Virus [HIV], old age, etc.), this virulent bacteria will be made active.

In the armamentarium against TB are the four first-line drugs: Rifampicin (RIF), Isoniazid (INH), Pyrazinamide (PYZ), and Ethambutol (EMB) [5]. They are taken in combination for a period of 6-9 months, followed by a post treatment of 1-2 years, depending of the severity of the infection [6]. Though this current oral therapy is convenient, it undergoes the body's first-pass metabolism which significantly lowers the bioavailability of the drug; a higher dosage is required to reach therapeutic levels. Hence, after prolong administration through the gastrointestinal tract, the liver or kidneys will be overworked, resulting in damage [7].

Pulmonary delivery on the other hand circumvents this problem. It is a non-invasive delivery which does not require a higher dosage to reach the desired therapeutic level. On top of that, it addresses the issue on site without needing the drug to be absorbed into the blood stream before taking effect. In this mode of delivery, the particle size places a

crucial role in the deposition location. Particle size bigger than $5\mu\text{m}$ sees an impaction at the oropharyngeal region (**Figure 1.1**), while in the size range of $1\text{--}5\mu\text{m}$ experiences at sedimentation down to the lower airways, which in the case of TB, is the area of objective. This is however, one of many aspects (shape, density, hygroscopicity, respiratory system complex, etc.) that can affect the region in which the particles are deposited.

Figure 1.1 The human respiratory network [8]

One of the major problems of drug delivery is the lack of specificity. When a drug is administered into the body systemically, it affects both the unhealthy cells at pathological site and the healthy cells that it comes in contact with. This general distribution of the drug throughout the entire body can cause side-effects that are deleterious to the patient. For this reason, treatments are moving away from systemic delivery towards local ones like magnetic targeting through the use of Superparamagnetic Iron Oxides (SPIOs).

SPIOs are biocompatible magnetic nanoparticles in the size range of 5-10 nm that can be guided or held at a particular location by a magnetic field. They are inexpensive to produce and are used in numerous *in-vivo* applications such as Magnetic Resonance Imaging (MRI), hyperthermia, and drug delivery. In the body, SPIOs can be navigated by a rare-earth magnet (Nd-Fe-B) to an effective depth of 10-15 cm [9], which is promising because drug administrations can now achieve localized delivery with SPIOs that are incorporated into the drug carriers [10].

The use of the pulmonary route to deliver anti-tubercular agents is not entirely novel. There are many researches out there that focus on the delivery of first-line anti-tubercular drugs loaded into biocompatible polymers [11-14] and the use SPIOs loaded PLGA particles [15-18]. However, the optimization of these carriers to obtain high deposition fractions into the lower airways together with the encapsulation of newer and potentially more potent TB-drugs is still lacking. The existing works on SPIOs loaded PLGA particles do not study geometrical size influence on the deposition fraction and focus rather on the magnetic targeting and its applications. The geometrical size of the particles drastically affects the deposition region, dispersion, and fraction and it is quintessential to optimize this aspect to achieve favourable results. In a recent *in-silico* study [19], it was shown that water droplets, loaded with Superparamagnetic Iron Oxides (SPIOs), in the size range of 0.5-3.0 μm can achieve deposition of up to 100% in the acinar region, under an applied magnetic field. This study demonstrates that size can hugely impact the efficacy of the delivery system and it is beneficial to investigate. However, water droplet carriers are unable to load hydrophobic drugs such as RIF and in the humid environment of the lungs the geometrical dimensions of these carriers will be altered due to its hygroscopicity, upsetting the intended aerodynamic flow. For these reasons, the drug carriers that will be used are hydrophobic in nature and possess a solid structure to maintain the designed deposition mechanism.

In this research, we would like to propose a delivery system that employs the non-invasive pulmonary route of delivery to deliver newer TB-drugs of higher

mycobactericidal activity than first-line anti-tubercular drugs with increased deposition amounts through magnetic targeting.

1.2 Hypothesis

In inhalation therapy, the size of the carrier has an important role in the deposition geography and mechanism. Carriers of 1-5 μm size have been acceptable to be optimal for deep lung delivery and sizes smaller than that, while can enter into the lower airways, sees the particles leaving the lungs during exhalation. Unfortunately, even at the optimal size, the deposition rates into the lower airway are still low [20-22].

The incorporation of SPIOs in 0.5 μm drug carriers can be one solution to improve low deposition rates. At this size range, the carriers can enter into the lower airways. However, because there are no physical forces present to resist the exhalation airflow, the particles ergo gets exhaled out. For this reason, iron oxides are encapsulated into the drug carrier as a means of arresting the particles within the vicinity intended. This will enable particles to reach the lower airways and be kept there until the particles are deposited. In addition, the combination of the 500 nm size carriers and the SPIOs as previously expatiated can achieve high deposition rates of up to 100% in an *in-silico* model.

The endeavor of ending the epidemic of TB has inspired research in the area of drugs development and by this avenue begotten drugs of significant potency. Q203 and BDQ are two of them which are shown to be individually more potent than the first-line drugs [23, 24]. They target different complexes in the oxidative phosphorylation of the bacteria and are theoreticized to have a synergistic effect. They are also hydrophobic in nature which allows them to be able to be encapsulated within a hydrophobic carrier such as the copolymer, DL-lactide/glycolide copolymer (PDLG).

It is therefore hypothesized that the pulmonary delivery of PDLG particles encapsulating Q203, Bedaquiline (BDQ), and SPIOs will allow for an improved pulmonary delivery and a more effective killing of the TB bacteria.

1.3 Objectives and Scope

The research objectives are:

1. Fabrication of solid PDLG particles loaded with Q203, BDQ, and SPIOs possessing specific attributes:
 - a. 500 nm in size for the optimal deep lung delivery
 - b. Magnetic capability which allows the particles to be manoeuvred by a permanent magnet
2. *In-silico* deposition efficiency of the drug carriers into the acinus
3. *In-vitro* magnetic targeting assessment of the drug carriers
4. Cell viability assessment of the drug carrier with A549 lung epithelial cells
5. Mycobactericidal activity of the drug carrier with BCG organisms
6. Comparison of Q203 and BDQ with first-line TB drugs

1.4 Dissertation Overview

The thesis addresses the development of a pulmonary drug delivery system that is capable of penetrating into the deep lung with the use of an external magnet and possesses a mycobactericidal effect on BCG with the two antibiotic agents, Q203 and BDQ.

Chapter 1 provides a rationale for the conceptualization of the work that led to this thesis and outlines the problem statement, objectives and novelty of the work.

Chapter 2 reviews the literature pertaining to pulmonary delivery systems for the treatment of TB. The chapter begins with the background of TB, its impact, and the strategies undertaken to control the epidemic. The chapter then continues to examine the *M.tb* and its attenuate form, i.e. BCG. Next, the anatomy of the lungs and the dynamics of particle deposition within the lungs are discussed. The chapter then advances into the literature concerning pulmonary delivery systems and the challenges accompanying respiratory administration. Subsequent, the components making up the proposed carrier, i.e. Q203, BDQ, and SPIOs will be explained.

Chapter 3 elucidates on the experimental methodologies, working principles, and the characterization involved in the work.

Chapter 4 reports on comparative data obtain on the efficacy of the combination use of Q203 and BDQ compared to dual combination first-line anti-tubercular drugs, and the combination efficacy of Q203 paired with another first-line TB agent.

Chapter 5 reports the results obtained on the inhalable carrier: The characterizations of the carrier using various techniques and the evaluation of its combinational mycobacterial activity on BCG.

Chapter 6 concludes and links the results obtained to the objectives and the hypothesis stated. The chapter also discusses the future experimental work recommendations that can be conducted to further characterize the carrier.

1.5 Findings and Outcomes/Originality

The novelties of this thesis are:

1. An active targeting into the lower airways through 500 nm biodegradable PDLG drug carriers loaded with SPIOs. These particles formulated through a solvent emulsion technique are homogenous and are able to penetrate into the deep lung with a considerable volume and dispersity.
2. The encapsulation of both Q203 and BDQ into a polymeric carrier. The lipophilicities of both Q203 and BDQ enable successful encapsulation into the hydrophobic PDLG carrier. Also, their synergism proved to be effective on the BCG organisms.

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Chapter 2

Literature Review

The chapter summarizes the various literature published in relation to the topic of this thesis, reviewing the relevant contemporary work done, thereby identifying the gaps in developing a pulmonary drug delivery systems for the treatment of TB. The chapter begins with an introduction to TB, its impact on the world, and the strategies that have been put in place to combat the disease. The chapter then progresses on to study the M.tb and the BCG vaccine that is used to protect against it. The chapter then continues by introducing the lungs and expands on the particle deposition dynamics in the different regions. Next, the chapter compares the different routes of administration and contemporary pulmonary delivery systems that are in development. The different determinants affecting deposition are addressed, i.e. the physiological, physicochemical, geometrical, and delivery devices will then be discussed. Finally, the components of the carrier, i.e. the SPIOs and drugs are elucidated. The chapter ends with a short summary on the various research gaps ascertained from literature review, that the thesis addresses.

2.1 Tuberculosis (TB)

TB is one of the top ten leading causes of death worldwide and remains a global health problem. In 2017, the latest estimate of TB mortality is around 1.3 million among HIV-negative people and 300,000 deaths among HIV-positive people. Over 10 million people developed TB in 2017 with the largest portion of people infected living in Africa (72%), followed by India (22%), China (9%), Indonesia (8%), the Philippines (6%), Pakistan (5%), Nigeria (4%), Bangladesh (4%), and South Africa (3%) [1].

Although the epidemic of TB is widespread, the vast majority of TB cases and deaths occur in low-income countries with 150-500 new cases per 100,000 people. While in higher-income countries fewer than 10 new cases per 100,000 people occur. This can be explained by factors such as inaccessible healthcare, higher exposure to unhealthy and crowded working conditions, malnutrition, co-infection with HIV, diabetes, mellitus, smoking, alcohol, drug abuse, etc. These determinants have a direct link in the control of TB and are largely dependent on the macro-level political decisions and nation-wide public health efforts. It is also historically shown that the improvement in social and economic conditions can greatly help the control of TB.

In the beginning of the 21st century, the number of people afflicted with TB disease continues to rise despite it being a treatable illness. To seize and reverse the TB epidemic by 2015, the WHO together with a coalition of partners established the ‘The Global Plan to Stop TB’ to provide an evidence-based approach to reach the target of reducing the prevalence and death due to TB by 50%. This strategy has six major components [2]:

- Pursue high-quality Directly Observed Treatment, Short Course (DOTS) expansion and enhancement;
- Address TB/HIV, Multi Drug-Resistant Tuberculosis (MDR-TB), and the needs of poor and vulnerable populations;
- Contribute to health system strengthening based on primary health care;
- Engage all care providers;
- Empower people with TB, and communities through partnership, and

- Enable and promote research.

The fruits of this implementation are a steady, albeit slow decline of the epidemic. This partnership has now launched a new ‘Global Plan to End TB 2016-2020’ to provide a blue-print to effect the global TB efforts in becoming more ambitious and effective. The milestone of 2020 is to effectuate a 35% reduction in TB deaths and a 20% reduction in the TB incidence rate compared to the 2015 levels.

There are three pillars to the ‘End TB’ Strategy. The first is ‘Integrated, patient-centered TB care and prevention’, the second is ‘Bold policies and supportive systems’ and the third is ‘Intensified research and innovation’. Priorities for TB research and development include:

- A vaccine to lower the risk of infection,
- A vaccine or new drug treatment to cut the risk of TB disease in the 1.7 billion people already latently infected,
- Rapid diagnostics for use at the point of care, and
- Simpler, shorter drug regimens for treating TB disease.

There are currently twenty drugs in Phase I, II, or III trials for the treatment of drug-susceptible TB, MDR-TB, or latent TB infection, and 11 newly developed compounds. These compounds are Contezolid, Delpazolid, GSK-3036656, Macozinone, OPC-167832, Pretomanid, Q203, SQ109, Sutezolid, TBA-7371, and TBI-166. Bedaquiline and Delamanid are two other drugs that have received conditional regulatory approval based in Phase IIb results. A major breakthrough is required so that the rate of TB incidence can fall to an average of 17% per year between 2025 and 2035.

2.2 *Mycobacterium Tuberculosis (M.tb)*

M.tb is a rod shape-like pathogenic species in the family of Mycobacteriaceae that is responsible for the airborne epidemic disease known as TB. Other members of this *M.tb* complex include the *M. Bovis*, *M. Africanum*, *M. caprae*, *M. canettii*, *M. microti*, and

M.pinnipedii. It is transmitted through the aerosol expulsion of a person infected with an active form of the disease and its symptoms include coughing, weight loss, fever, exhaustion, and blood-tinged sputum. While TB primarily affects the lungs, it can also be spread, extrapulmonary, to the kidneys, spine, and brain. Pott's disease is one such example. It arises from a haematogenous transmission from the lungs and can cause bone destruction, damage, or paraplegia.

The cell wall of the *M.tb* is made up of layers of peptidoglycan, arabinogalactan, mycolic acid, and surface glycolipids [3, 4]. The waxy cell surface makes it difficult to perform and distinguish the organism through Gram staining. However, since its cell wall is similar to that of a Gram-negative bacterium, the *M.tb*, in this aspect, can be loosely categorized as a Gram-negative bacterium. The cell wall of a Gram-negative bacterium has an absence of teichoic acid (unlike its counterpart) with a thinner layer of peptidoglycan and other components such as lipopolysaccharides, lipoprotein, and porins. However, in actinomycetes (rod-shaped actinobacteria which are phylum of Gram-positive bacteria), mycolic acids is the only reliable phylogenetic indicator. The cyclopropane mycolic acid synthase present in *M.tb* is ubiquitous in proteobacteria (a major phylum of Gram-negative bacteria), but only present in only a few low G+C Gram-positive bacteria. Hence, strongly supporting that *M.tb* is a Gram-negative bacterium.

2.3 Mycobacterium Tuberculosis Biofilms

Biofilms are communities of microorganisms that embed themselves in an extracellular matrix for the purpose of either reducing or modulating metabolic pathways for becoming more tolerant. The formation of a biofilm is a response to various cues such as cellular recognition of a specific or non-specific attachment site, nutritional signals, or most commonly in drug delivery, the exposure of the planktonic bacteria cells to sub-inhibitory concentration of antibiotics. The latter can be categorized into different levels of resistance from the lower level of Multi-Drug Resistant (MDR), to more unyielding levels of extensively drug resistant (XDR) and even Total Drug Resistant (TDR).

For the treatment of TB, its therapeutic regime entails a lengthy treatment of at least 6 months with an armamentarium of drugs depending of the severity of the condition. The duration is presumably necessitated by biofilm-related tolerance and the ongoing emergence of such strains underscores the limitations of current TB control strategies and brings the TB epidemic to a new level. At a cellular level, the hurdle of antimicrobial access to the bacteria is impaired by the efflux pumps which expel antibiotics out of the cell. The antimicrobial activity is dependent on the metabolic status of the microorganisms, the physicochemical conditions prevailing at the site of infection, and the specific phenotypes frequently found in biofilms, including small colony variants (SCVs) and persister cells [5, 6].

The growth and development of the biofilms are a multistage process which requires dedicated genetic programs expressed in a spatiotemporal order [7-9]. The initial stage of the development involves a substratum attachment of the planktonic cells of which the coverage is determined by the mycobacterial non-ribosomal peptide synthase (Mps)-dependent glycopeptidolipids (GPLs). These cells are then aggregated together and spread itself across the substratum. In the early maturation of the biofilm, the chaperonin GroEL1-dependent free mycolic acids (FM) synthesis is triggered and the Fe-sequestration pathways are induced. Finally, in the maturation stage, the synthesis of the extracellular matrix occurs [8]. The development stages of the biofilm are illustrated in **Figure 2.1**.

Figure 2.1 A model depicting the cellular and molecular events of the development of a biofilm [8]

A study done by Abhishek Trivedi et. al. [10] on the architecture of *in-vitro* thiol reduced stress induced *M.tb.* biofilm found that the extracellular matrix encapsulating the bacteria are composed of proteins, lipids, polysaccharides and extracellular DNA. These components are critical for integrity of the biofilm and the degradation of them can result in the disruption of the film. In addition, the biofilms were found to possess a network of pores and channels which could facilitate the distribution of nutrients throughout the bacterial population in the biofilm.

In highly structured biofilm, the environmental conditions are not consistent throughout the film and as a result, the microbial physiology and metabolism in different parts of the biofilm varies [11]. This spatial organization is shaped by the interactions between different species and/or genotypes and can lead to a spectrum of biofilm organization, ranging from 'intermixed' biofilms, i.e. different microbes live intermingled in the same aggregate to biofilms in which individual aggregates predominantly consist of a single species/genotype [12, 13]. The heterogeneity generates a gradient of metabolites and oxygen within the biofilm, resulting in the physiological divergence with internal gradients of metabolic activity, growth rates, oxygen, and tolerance to antibiotics. Bacteria at the outer layers of the biofilm have a fitness advantage as compared to those in the peripheral parts of the film because oxygen and nutrients are more readily available. This would mean that the subpopulations located internally have low growth rates and low metabolic activity, and makes the bacteria more tolerant to antibiotics because the lack of oxygen in that region prevents endogenous formation of bactericidal reactive oxygen species (ROS), which augment the killing of the bacteria.

In addition to oxygen availability, other strategies such as metabolic adaptations have been discovered that is used by the bacteria to contribute to the tolerance of antibiotics. Using the *Pseudomonas aeruginosa* PA14 transposon mutant library in an *in-vitro* screen for tobramycin resistant mutants, it was observed that a large proportion of the resistant

mutants had the transposon inserted in genes related to metabolism [14]. This affects the ability of the bacteria to maintain a sufficient high Proton Motive Force (PMF) in the electron transport chain (ETC), leading to a reduced of antibiotic uptake and, hence, a reduced susceptibility. This similar response is seen in the exposure of antibiotics to the TB biofilm where the metabolic flux of the TCA cycle is reduced, increasing antibiotic tolerance [15].

2.4 *Bacillus Calmette-Guerin (BCG)*

BCG is a live attenuate form of the *M.bovis* strain which is the only licensed vaccine against pulmonary TB. It was developed by two French scientists, Albert Calmette and Camille Guerin in 1905, through the serial passaging of an isolate from a cow with tubercular mastitis [16]. Globally, it has been extensively administered with an approximately 100 million of newborn babies being vaccinated each year [17]. Its efficacy on TB meningitis and disseminated TB is consistent. However, against pulmonary disease, its success is highly variable. It is reported that 35-80% of recipients of this BCG vaccine are likely to develop TB, and one comprehensive meta-analysis estimated the BCG efficacy to be around 50% [18]. This highly variable protection against the pulmonary disease is a concern because it directly contributes to the global TB mortality and morbidity numbers. Furthermore, it is shown through a randomized controlled trial (RCT) conducted in Brazil that revaccination with BCG during adolescence does not improve the protective efficacy. Thus, new strategies are needed to better existing conventions.

There are two main strategies in development that can be utilized to reestablish TB vaccination. One of the strategies is to introduce recombinant BCG (rBCG) strains that targets specific immune-processing pathways to induce high levels of cellular immunity. Responses from CD4+ and CD8+ T cells, Th-1 cytokines interferon gamma (IFN γ), tumour necrosis factor alpha (TNF α), interleukin 2 and 17 (IL-2 and IL-17) are necessary for protective immunity. Their functions are listed in **Table 2.1**. One example of a recombinant strain is rBCG30 [19], developed at the University of California, which is capable of over-expressing an antigen from *M.tb* that is more protective than the wild-

type strain in a guinea pig model. In a phase I clinical trial, the recombinant strain has demonstrated its compatibility with humans and is immunogenic.

Table 2.1 The functions of the cells involved in immunoresponse

Cells	Functions
CD4 ⁺ T cell	T helper cells that releases T cell cytokines to signal immune cells to destroy infected or damaged cells
CD8 ⁺ T cell	A type of white blood cell that destroys infected or damaged cells
IFN γ , TNF α , IL-2, IL-17	Cell signalling protein (cytokine)

The second strategy in the development of TB vaccine is the bolstering of the vaccine through the introduction of a subunit administered in a later period from the point of BCG inoculation [18]. This later time point could be promptly after BCG vaccination or when the effect of the BCG starts to diminish. AS02, an adjuvant from GlaxoSmithKline (GSK) is one such booster. It is delivered with the recombinant protein, M72, also developed by GSK, with immunostimulants monophosphoryl lipid A and *Quillaja saponaria* fraction 21 as a protein-adjuvant vaccine. A phase I study with this vaccine administered as a three dose regimen in purified protein derivative (PPD)-negative healthy volunteers, showed a Grade 3 adverse event reactogenic profile 75% of them. And in an *in-vitro* characterization using short-term enzyme-linked immunosorbent spot (ELISPOT) assay, the antigen-specific CD4⁺ T cells were present.

2.5 Anatomy, Function and Dynamics of the Lungs

The human lungs are an extremely complex geometrical network of 2^{21} - 2^{23} bifurcating channels with 480-790 million alveoli at the terminal [20]. The respiratory tract can be divided (according to its function) into a conducting zone, i.e. G0 to G16 (trachea, main bronchus, bronchus, bronchioles, and the terminal bronchioles) and a respiratory zone, i.e. G17 to G23 (respiratory bronchioles, alveolar duct, and the alveolus). In the conducting zone, the organs present conduct the air in and out of the lungs and protects it from foreign entities through filtration. Its defense mechanism is an epithelial lining

interspersed with goblet cells that secrete a layer of protective mucus to trap foreign particles accumulated in that region. Particles that are arrested in the mucus are expelled into the stomach through the mucociliary escalator of the cilia. In the respiratory zone, the gas exchange of $O_2 - CO_2$ occurs; 10% of the exchange is accomplished through the respiratory bronchioles and alveolar ducts while the remaining 90% is through the alveoli.

The alveolus (singular for alveoli) has a mean diameter of 200 μm and increases during inhalation. Present within itself are the type I and II pneumocytes and the mobile alveolar macrophages. The type I pneumocytes form the structure of the alveolus and covers an approximately 90-95% of the alveolar surface. They are extremely thin (25 nm) and facilitate the gas exchange of the alveoli and the blood. Type I pneumocytes are unable to replicate. In the event of cell injury, the type II pneumocytes will proliferate and differentiate into type I to compensate. Type II pneumocytes make up a small fraction in the alveolus and secrete pulmonary surfactants to lower surface tension [21].

In the pursuit to accurately predict particle deposition in the human lungs, efforts have been directed onto experimental and computation simulations that mimic the human respiratory system in realistic lung models. Over the years, lung models from Weibel [22], Hosefield [23], and Sznitman [24] has been proposed and used. However, they fall short of being a complete set of realistic pulmonary airway. Simulations of the lungs are often simplified with assumptions of steady laminar, isothermal airflow, rigid airways, spherical non-interacting particles, mono-dispersed, and deposit upon contact. These are inaccurate because for one, the airflow within the lungs is sporadic and undergo different flow regimes, i.e. laminar, transitional, turbulent, secondary, and oscillatory. In addition, because of the limitation of computer geometric resolutions, simulated lung models do not exceed six generations.

In the respiratory tract, the dynamics of airflow differs from region to region. The flow of air is usually laminar (up to $Q = 24$ litres per minute) in the oral cavity and then becomes transitional and turbulent after the constriction formed by the soft palate and throat, and returns to a laminar flow after several generation in the bronchial tree [25]. The onset of

turbulence may take place during acceleration when the Reynolds number (Re) is ≈ 2400 and weakens when it is < 800 . When the airflow reaches the alveolar region, the Re goes down to < 1 , but is complex due to the complicated geometric features and rhythmic expansion and contraction of the alveoli.

2.6 Oral and Pulmonary Delivery

Oral delivery is indubitably the most common route of drug administration. While this is true, it has its own set of advantages and limitations and should not be the only route to be considered when combating a particular disease. The pulmonary route of delivery offers a large surface area of absorption (70 m^2) and has a rapid onset of action upon drug deposition. The lungs' thin alveolar epithelium provides express passage for the drug into the blood stream which bypasses the first-pass metabolism [26]. Because of this, the bioavailability of the drug is largely retained [27]. And in the case of TB, pulmonary delivery targets the infection at the site of interest. A comparison between these two routes of administration is listed in **Table 2.2**.

Table 2.2 Comparison between the different routes of delivery

Oral		Pulmonary
Quick	Application Period	Dependent on delivery device
Easy	Ease of Administration	Difficult
Simple	Method of Fabrication	Complicated
Low	Bioavailability	High
Indirect	Delivery Locality	Direct
Slow	Onset of Drugs	Quick
Taxes lungs and kidneys	Bodily Complications	Benign

2.7 Potential Inhalation Delivery Systems

Countless strategies on therapeutic TB treatments have been proposed over the years to help mitigate the spread of TB. The approach of delivering drugs into the lungs is an attractive avenue for treatment because it is the natural site of infection. The delivery systems proposed follow a template of a vehicle (liposome [28-30], PLGA [31-35],

chitosan [36], etc.) in a breathable size range loaded with anti-tubercular drugs. One of these vehicles used is liposomes. Liposomes are used because its constituents are endogenous to the lungs and are benign upon deposition. A study was done by Oselys R. Justo et al. [37] which uses lipids cholesterol (Chol) and distearoylphosphatidylcholine (DSPC) to encapsulate Isoniazid, Pyrazinamide, Rifampicin, Ethionamide, and Streptomycin. The results show encapsulation efficiencies of 2.5%, 2.2%, and 42.0% for Isoniazid, Pyrazinamide, and Ethionamide, respectively. While Rifampicin and Streptomycin has none of its drug loaded. Other studies such as the one from Anjali Sharma [38] used lection-functionalized PLGA as carriers. The double emulsion technique yielded particles of 350-400 nm with drug encapsulation efficiencies of 54.0%, 64.3%, and 66.8% for Rifampicin, Isoniazid, and Pyrazinamide, respectively. A lean summary of proposed pulmonary delivery systems is compiled in **Table 2.3**.

Table 2.3 A summary of proposed pulmonary drug delivery systems

Pulmonary Drug Delivery System					
Delivery Carrier	Size	Drug(s) Loaded	Morphology	Preparation Technique	Reference
PLGA	1 - 5	Rifampicin	Spherical	Solvent evaporation and spray drying	[39]
	1.5, 3.5, 6.2, 8.9				[40]
	4.2		Spherical and porous	Solvent evaporation and spray drying	[41]
PLA		Rifampicin and Isoniazid		Spray drying	[42]
PLA/PLGA	2.2 - 5.1	Rifampicin	Spherical and shriveled	Spray drying	[43]
Trehalose,	5-10	Isoniazid	Spherical and	Spray drying	[44]

mannose, and lactose			irregular		
Leucine and DPPC	4.14	PA-824	Thin-walled and porous	Spray drying	[45]
	3.95				[46]
Liposomes	1.9	Amikacin		Modified reverse phase evaporation	[47]
	0.24	Rifampicin	A mixture of unilamellar and multilamellar	Chloroform-film	[48]
	3	Isoniazid	Spherical	Spray drying	[49]
	1-3	Pyrazinamide	Spherical and porous	Spray drying	[50]
	3	Levofloxacin	Spherical and porous	Spray drying	[51]
Alginate	0.5	Amikacin and moxifloxacin	Spherical	Solvent evaporation	[52]
Alginate and Chitosan	0.344		Spherical	Ionotropic gelation	[53]
Oleate	8.86	Capreomycin	Spherical	Solvent evaporation and spray drying	[54]
Excipient Free	0.2-5	Rifampicin, Isoniazid, and Pyrazinamide	Spherical	Spray drying	[55]

2.8 Factors Affecting Delivery

2.8.1 Physiological Factors

The lungs have a network of bifurcations that branches from the trachea down to alveolus [56]. As the airways bifurcate, it narrows down in diameter, inspiring an impaction mode of deposition instead of the desired sedimentation and diffusion. For this reason,

aerosolized particles must possess certain characteristics that impede momentum build-up during inhalation. Lung diseases further complicate matters. Respiratory disease can further narrow airways by means of constriction and/or inflammation with mucus; in some cases, the respiratory disease might even change the architecture of the lungs. This change and narrowing of the airways perturb laminar airflow and affect the particles' deposition and distribution patterns.

2.8.2 Physicochemical Interaction with Lung Fluids

Upon successful deposition into the alveolar region, particles will come into contact with the alveolar fluid which consists of 90% lipids with the remainder being made up of surfactant proteins SP-A, -B, -C, and -D. This fluid which lines the inside of the epithelium has an average depth of 100-200 nm. Once in contact with the alveolar fluid, particles that are soluble will start to dissolve in them while the more tenacious ones, i.e. the more insoluble particles get engulfed by the fluid. This makes the particles more predisposed to the neutralization by alveolar macrophages (AM) [57]. The AM are the body's self-defense mechanism against foreign particles. During an encounter with foreign particles, the AM will surround the particles and discharge inflammatory mediators such as cytokines, chemokines, arachidonic acid metabolites, lysozymes, and antimicrobial proteins to phagocytize the particles [58, 59]. Though larger particles can avoid uptake by alveolar macrophage [60], particles in the size range of 1-10 μm are susceptible for phagocytosis. In the case for submicron particles, while they fall out of the aforementioned range, they can be still cleared by the macrophages, but they retain in the lungs for a longer period of time [61].

2.8.3 Particle Size and Deposition Mechanism

As the particles enter into the respiratory tract, it undergoes one, or a combination, of the three deposition mechanism: Inertial impaction, gravitational sedimentation, and Brownian diffusion [62]. Depending on the aerodynamic particle diameter (and not geometrical particle diameter) the mechanism in which the particle experiences would result in different deposition region. The relationship between the aerodynamic diameter (d_{ae}) and the geometrical diameter is as follows:

$$d_{ae} = d_v \sqrt{\frac{\rho}{\rho_0 X}}$$

in which d_v is the volume diameter, ρ is the density of the particle, ρ_0 is the density of control (1g/mL), and X is the dynamic shape factor, which is 1 for spheres. Particles with an aerodynamic diameter of larger than 5 μm are deposited in the upper airways (trachea, bronchi, and bronchioles) by inertial impaction, while in the range of 1-5 μm , experiences a combination of inertial impaction and gravitational sedimentation. As the particle size reduces to a size smaller than 1 μm , it inspires a diffusion deposition mechanism. However, because of the its size, most of them gets exhaled out.

It must be duly noted that though the deposition mechanisms and patterns can be predicted through the aerodynamic size of the particles, it is not the only defining factor; particle characteristics such as shape, density hygroscopicity, electrical charge, lung morphology, and breathing patterns also contribute to the deposition mechanism and patterns [63, 64].

2.9 Drug Delivery Devices

Another important component of drug delivery is the inhalation device. There are three commonly employed devices: Nebulizers, pressurized Metered-Dose Inhalers (pMDIs), and Dry Powdered Inhalers (DPIs). These three platforms have their own unique strengths and limitations.

2.9.1 Nebulizers

The nebulizer is a device that can passively administer aerosolized drugs to patients that do not possess the dexterity of operating an inhalation device such as infants, elderly, and mentally and/or physically challenged people. Nebulizers are generally divided into two categories based on their operating mechanism: Jet-nebulization or ultra-sonication [27].

Jet-nebulizers use pressurized air to generate aerosols by forcing the drug solution through a nozzle as illustrated in **Figure 2.1A**. The high energy airflow breaks the drug solution into tiny droplets and directs them towards the mouthpiece. The baffle that is located downstream regulates these droplets and allows only a certain size range through while directing larger ones down into the drug reservoir. Jet-nebulizers, unfortunately, have a poor delivery efficacy into the lungs with only 12% of the total drug delivered [65]. The remaining 88% of the drugs are lost in the baffles, exhaled or deposited in the oropharynx, or swallowed. Moreover, the pressurized air utilized is incapable in itself to give a full inhalation flow volume. Thus, the patient would require drawing the aerosolized droplets through inhalation. Jet nebulizers are also bulky because they require the use of a compressor to generate pressurized air, making them bulky and limited to home or hospital use.

Ultrasonic nebulizers utilize high frequency acoustic waves to overwhelm the capillary stresses and thus break the drug solution into aerosolized droplets (**Figure 2.1B**). These acoustic waves are the product of a piezoelectric transducer converting alternating electric field into a periodic mechanical vibration. This form of energy dissipation generates a substantial amount of heat which could alter the drug concentration through the evaporation of the aerosolizing liquid and/or possibly cause the denaturing of the drug. This evaporation when compared to jet-nebulizers however is miniscule. In jet-nebulizers, the recycling of the aerosolizing liquid from the baffles into the reservoir has more evaporation taking place. Hence, there is more drug wastage and a lesser consistent drug dose.

Figure 2.2 Illustration of a (A) jet nebulizer and a (B) ultrasonic nebulizer [65]

While nebulizers have their countable shortcomings, their ability to electronically tune the droplet size, nebulization rate, total dose, and spray pattern is their greatest asset. These factors can affect the deposition region and tailor the device towards a specific patient profile. In addition, the development of the intelligent system known as the Adaptive Aerosol Delivery (AAD) helps to reduce drug waste through the synchronization of the pulsed aerosol with the patient's breathing pattern [66]. One such device by Ventavis®, I-neb AAD system, shown an *in-vivo* deposition of ~73% deposition in the entire lungs, with ~26% in the upper airways, ~18% in central lungs, ~29% in the peripheral lung, and only ~0.2% of the emitted dose exhaled out [67].

There have been several studies reported comparing the relative performances between nebulizers. The findings understandably were inconclusive. This is because nebulizer performances are affected by interrelated factors such as respiratory flow rate, measurement method, *in-vitro* aerosol collection method, ambient conditions (e.g. relative humidity), breathing mode (nasal or oral) and pattern (tidal or deep breathing; breath-holding), physiological profile (e.g. lung morphology), etc [68]. Hence, studies

with a systematic parameter study wherein the effects of one parameter on the system's performance are isolated pose a significant challenge to comparison and examination.

2.9.2 Future of Nebulizers

Conventional ultrasonic nebulizers often require a significant amount of power for operation and can sometimes generate a considerable amount of heat that denatures the drug. In order to avoid this issue, nebulizers are operated at a higher frequency to induce cavitation at a lower power than initially required. However, ultrasonic nebulizers are limited by a maximum frequency of 3 MHz due to the resonance modes of the piezoelectric crystal. Moreover, ultrasonic nebulizers have difficulties aerosolizing drug suspensions and viscous solutions. These limitations found a way for a next generation of ultrasonic nebulizers which uses the piezoelectric element to vibrate the mesh instead of the reservoir.

Vibrating mesh nebulizers (**Figure 2.2**) have been reported to provide a more homogenous droplet size without exacting large shear stresses and temperature increases on the drug. The ability to generate narrower droplet size removes the need of baffles which minimizes residual drug loss. Vibrating mesh nebulizers can be battery-operated which makes the overall size comparably smaller and audibly softer to the conventional nebulizer. However, a severe limitation for vibrating mesh nebulizers is the clogging of the meshes that requires careful cleaning in order to clear the block and prevent any bacterial contamination.

Figure 2.3 Schematic of a vibrating mesh nebulizer [65]

2.9.3 Pressurized Metered-Dose Inhalers (pMDIs)

The pMDI is a device that uses a propellant within a pressurized canister to generate a short burst of aerosolized formulation to the lungs [69]. Amongst the three devices, it is the most popular and is commonly used for the treatment of asthma and Chronic Obstructive Pulmonary Disease (COPD). Its reliability and low-cost has won the hearts of end users despite some challenges that it face. The drug delivery using pMDIs is typically restricted to the oropharyngeal region of the lungs due to high velocity that the aerosolized formulation experience after exiting the actuator nozzle. In addition, upon release from the nozzle, aerosolized particles sees a heterogeneous distribution in size, making pMDIs an unfavorable device for the delivery of nanoparticles.

2.9.4 Dry Powered Inhalers (DPIs)

DPIs have received much attention over the years; they are capable of delivering drug powders in the size range of 1-5 μm to the lower airways and are chemically more stable than their counterparts. Some designs of DPI even have a dose counter or indicator that helps patients to keep track of their medication regime [70]. The initial intent of DPIs was to abandon the use of chlorofluorocarbons (CFC) propellant in pMDIs for a more ozone-friendly inhaler. This turns out to be a big success, but there are a couple of disadvantages of DPIs. The largest drawback of DPI is the problem of agglomeration. As the size of the

particle reduces, its surface energy increases due to an increase in the surface area. This makes the particle unstable and in the attempt to regain stability, the particle will agglomerate to reduce surface energy.

Recent developments in DPIs aim to address the problem of agglomeration. One such effort is the use of a turbulent air stream to break up particles using shear force. In this mechanism, particles are fed into a turbulent air stream where the shear force generated upon the agglomerated particles will break them apart [71]. However, it is reported that the efficacy is low. Other mechanisms of breaking up agglomerated particles are jet velocity, shear, sudden acceleration, and expansion of gas within the agglomerates. Aside from agglomeration, DPIs faced an issue with patients' inhalation variability. As DPIs require from the patient a substantial inspiration effort, patients that inhale too forcefully, will have particles depositing at the upper airways, i.e. the oropharyngeal via impaction [26]. Therefore, adequate practice is needed in order to achieve a therapeutic effect.

2.10 Superparamagnetic Iron Oxides (SPIOs)

Superparamagnetism is a form of magnetism which occurs in single-domain magnetic nanoparticles where the thermal energy from the surrounding is sufficient to overcome the materials' magnetic anisotropy, causing randomized flipping of the magnetic dipoles. Magnetite (Fe_3O_4), maghemite ($\gamma\text{-Fe}_2\text{O}_3$), and hematite ($\alpha\text{-Fe}_2\text{O}_3$) are three different types of iron oxides that fall into the category of SPIOs. In this research however, SPIOs will refer to Fe_3O_4 . Fe_3O_4 or $(\text{Fe}^{3+})^{\text{Tet}}(\text{Fe}^{2+}\text{Fe}^{3+})^{\text{Oct}}\text{O}_4$, has a cubic inverse spinel structure with oxide and iron ions forming a cubic close packed array. In one unit cell of Fe_3O_4 , 8 Fe^{3+} ions occupy the tetrahedral sites, while another 8 of both Fe^{2+} and Fe^{3+} ions occupy the octahedral sites. SPIOs are biocompatible, inexpensive to fabricate, has a number of synthesis routes, can be directed by a magnet, and are physically and chemically stable [72]. These attributes are favorable in many biomedical applications which are why SPIOs are employed in areas such as MRI [73-76], hyperthermia, magnetic field assisted radionuclide therapy, and drug delivery.

Fe_3O_4 can be easily synthesized through a co-precipitation method, though there are many other ways in which magnetite can be fabricated (sol-gel, micro-emulsions, and thermal decomposition). In this co-precipitation technique, Fe (II) and Fe (III) salts are mixed together in a stoichiometric ratio of 1:2, and then added into an alkaline environment [77], delineated in the chemical equation below:

This addition consequently produces a black precipitate. It is vital that during the addition of the aqueous salt into the alkaline medium, that the environment is purged from any oxygen as its presence would oxidize the Fe_3O_4 to $\text{Fe}(\text{OH})_3$.

The bubbling N_2 gas to create an oxygen-free environment not only protects the Fe_3O_4 from oxidizing, but reduces the size of the particles [78]. The co-precipitation technique is capable of producing SPIOs in the size range of 20 nm. However, at that geometrical size, the particles agglomerate easily. SPIOs can aggregate via one these three interactions: Hydrophobic/hydrophilic, magnetic dipole-dipole, and Van Der Waals (VWD). At the nanometer range, because of the enormous surface energy that is present in the individual SPIO, the particles will try to bring down their energy by agglomerating with other particles via VWD or hydrophobic-hydrophilic interactions. As they come together, they grow in size where they exhibit strong magnetic dipole-dipole interaction with one another. This interaction is the attraction of an individual clustered-particle to its neighboring particle. As they come closer, the neighboring atom magnetizes the incoming particle, causing mutual magnetization and agglomeration [78]. The agglomeration of SPIOs can damper the efficacy of the drug delivery system; so, in order to prevent agglomeration, SPIOs are coated with surfactants. The most commonly used ones are oleic acid, lauric acid, alkane sulphonic acid, and alkane phosphonic acid.

In drug delivery, SPIOs that are conjugated with a drug or encapsulated within a non-magnetic material such as PLGA can cause a drastic drop in the magnetic saturation [73]. Hence, strategies must be employed to overcome this limitation so that the drop in magnetic saturation will not hinder the effectiveness of delivery.

Recent cytotoxicity studies have shown that at concentrations higher than 100 $\mu\text{g/mL}$, SPIOs can be toxic [79]. The introduction of SPIOs into the body at that concentration, or higher, can generate Reactive Oxygen Species (ROS), free radicals such as superoxide anion and hydroxyl radicals, and non-radical hydrogen peroxide. Particularly for ROS, they enter the interior of the cell and produce oxidative stress through the activation of transcription factors and can react with macromolecules and damage cells by peroxidizing lipids, changing proteins, disrupting DNA and signaling functions, modulating gene transcription, and ultimately causing cell death. SPIOs can also induce toxicity through iron overloading, which is a mechanism where high level of free Fe ions cause imbalanced homeostasis, abnormal cellular responses, and inspire carcinogenesis.

2.11 Synthetic Polymeric Carriers

Over the years, there have been a myriad of synthetic polymers employed in drug delivery. Different polymers classes such as polyesters [80, 81], polyorthoesters [82], and polyanhydrides [83] have been used as matrices for drug delivery applications because of their biodegradable, biocompatible, and non-toxic properties.

Polyester is a category of polymers that contain an ester functional group in the main chain and are considered the most commercially competitive. They have been used for sutures, bone fixture devices, stents, screws and tissue repairs, and a broad range of medical devices and drug delivery applications. The commonly used polyesters are: polylactic acid (PLA), poly(lactic-co-glycolic acid) (PLGA), poly(ϵ -caprolactone) (PCL), poly-3-hydroxybutyrate (or poly- β -hydroxybutyric acid, PHB), poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV), poly(propylene carbonate) (PPC), poly(butylene succinate) (PBS), and poly(propylene fumarate) (PPF) [84].

Among them, PLGA is the most extensively used in drug delivery applications [85]. PLGA is a copolymer of lactic acid and glycolic acid, and is one of the FDA-approved vehicle for therapeutic drugs. Its popularity stems forth from its biodegradable and biocompatible anatomy, its flexibility to encapsulate both hydrophobic and hydrophilic drugs, and also its ability to be tuneable to obtain different release rates which is achieved by varying the molar ratio of lactic and glycolic monomers. Since lactic acid is more hydrophobic than glycolic acid, lactic-rich ratio combinations e.g. PLGA 80:20 or PLGA 75:25 are more hydrophobic and would require a longer time to degrade. The reverse is also true except for PLGA 50:50, which has the fastest degradation rate amongst the other ratios. The degradation of PLGA happens through the hydrolytic cleavage of the ester linkages and occurs throughout the entire material [86]. This is known as bulk degradation.

During bulk degradation, the molecular weight, mechanical strength, and degradation rate of the polymer decrease over time, and goes through two distinct phases: The first phase is the random cleavage of longer polymer chains into shorter ones which initially does not affect the mechanical properties of the polymer as presences of physical cross-linkages such as entanglements and regions of crystallinity persist. This however eventually disappears, resulting in a decrease in the mechanical properties of the polymer. At this phase, there is no mass loss. The second phase is the continual chain scission of the polymer chains into soluble oligomers that are small enough to diffuse out of the network. In this phase, there is a considerable mass loss in the polymer. As the volume of the polymer decreases, the degradation rate too decreases. The representation of these effects is seen in **Figure 2.3(a)** [87].

Figure 2.4 The molecular weight, strength, and mass effects of (a) bulk and (b) surface degradation of polymers.

The majority of polyesters are bulk degraded, whereas polyanhydrides and polyorthoester are surface degraded. In surface degradation, the loss of material is confined to the surface of the polymer only. For surface degraded polymers, their size and mass decreases over time in water, but their molecular weight and mechanical properties remain relatively unchanged as can be seen in **Figure 2.3(b)**. In surface degradation, the rate of mass loss is proportional to the surface area. The rate of hydrolysis of labile bonds in surface degradation is relatively fast in comparison to the diffusion rate of water into the bulk, and the degradation reactions are limited to the surface of the polymer material.

Polyanhydrides have an inherent high reactivity towards water that results in rapid hydrolytic degradation. Polyanhydrides, such as poly(1,3- bis(p-carboxyphe-noxy) propane (PCPP), poly(sebacic acid) (PSA), poly[1,3-bis (p-carboxyphenoxy) propane: sebacic acid] (PCPP:SA), and poly(1-6-bis(p-carboxy phenxoy)hexane) (PCPH), exhibit characteristic of surface degradation with an degradation time from a few days to several years [88]. One of the major shortcomings of polyanhydrides is their limited shelf-life. Handling, storage, and synthesis of polyanhydrides require dexterity and attention because polyanhydrides are susceptible to water and the hydrolysis of its polymer chains can occur from the moisture present in the surroundings [89]. In general, polyanhydrides are unstable at room temperature and requires freezing storage conditions.

Polyorthoesters are hydrophobic, surface eroding polymers with a general structure of $[-R-O-C(R_1, OR_2)-O-R_3-]_n-$. They are used as hydrophobic implant materials for drug depots and are generally more hydrophobic than polyesters or polyanhydrides [90]. Due to their lack of carbonyl functionality, their rate of degradation in aqueous environments is very low. The rate of degradation, pH sensitivity, and glass transition temperatures of polyorthoesters can be controlled by using diols with varying levels of chain flexibility.

2.12 Solvent Evaporation Emulsion Technique

Over the past few decades, there has been a considerable amount of interest in the development of drug delivery systems using micro/nanoparticles. These systems are generally represented in a drug encapsulated polymeric matrix of which a myriad of polymers ranging from natural to synthetic were used. Several microencapsulation techniques such as solvent evaporation, homogenization, ultra-sonication, spray drying have been extensively employed in the pharmaceutical industry for the controlled and/or sustained release of these drug delivery forms [91].

In solvent evaporation, depending on the hydrophilicity and hydrophobicity of the drug and matrix, methods can vary from oil-in-water (o/w), water-in-oil-in-water (w/o/w), solid-in-oil-in-water (s/o/w), oil-in-oil-in-water (o/o/w), and oil-in-oil (o/o). For o/w emulsion, the drug and polymer to be used is dissolved in a suitable water immiscible solvent, typically chloroform or DCM, and dispersed in an aqueous continuous phase, typically PVA solution, to form discrete droplets. Several methods have been utilized to achieve dispersion of the oil phase in the continuous phase. The common methods are: Propeller blade stirring, homogenizing, sonication, and use of a microfluidizer [92]. The microspheres are formed when the organic solvent diffuses into the aqueous phase and is evaporated at the water/air interface. The physiochemical properties of the microspheres, i.e. size, drug encapsulation efficiency, and release profiles can be altered by tuning the processing parameters. These parameters are: Agitation rate, surfactant concentration, rate of solvent removal, solvent type, polymer concentration and molecular weight.

Agitation rate. Agitation rate influences the microsphere's size [93-95]. As the agitation rate increases, the shear forces that disperse the discrete and continuous phases increase, thereby decreasing the particle size. The extent to which the size of the droplets is reduced depends on the viscosities of the dispersed and continuous phases, their volume ratio, the geometry and the number of impeller(s), and the size ratio of the impeller to the mixing vessel. When PLGA is dissolved in a mixture of ACN and DCM at 6.25%, 12.5%, and 25%, the microspheres with mean diameters of 36, 115, and 208 μm were obtained [96].

Surfactant concentration. To prevent coalescence of the droplets after dispersion, a stabilizer or a surfactant such as PVA is usually added to the continuous phase. Increasing of the surfactant ratio decreases the droplet size [97-99]. When increasing amounts of PVA (1-10%) was dispersed with a PLGA/DCM oil phase, the mean diameter of droplets decreases from 8.3 to 3.7 μm [97].

Rate of solvent removal. The rate of solvent removal can be facilitated by the addition of heat. The rapid removal of the solvent through the increase of temperature will create a high concentration gradient of the solvent and the continuous phase, leading to some cases higher encapsulation efficiencies, but wider and larger size distributions. This is reported in the formation of microspheres using pure PLGA and a PLGA-PEG blend encapsulating BSA at different temperatures, i.e. 4-33, 38, and 42°C [100, 101]. When the PLGA microspheres were prepared at 38 and 42°C, the particles obtained were larger and possess a wider size distribution than those prepared at lower temperatures, i.e. 4-33°C. This is because there was insufficient time for mixing to reduce the droplet size and improve the homogeneity. For the encapsulation efficiency, the pure PLGA encapsulating BSA showed no increase in the efficiency (53-63%) when the temperatures increase, but for the PLGA-PEG blend, the BSA displayed a steady improved from 15% at 22°C to 52% at 38°C.

Solvent type. The successful entrapment of the drugs in the polymer matrix depends also on the type of organic solvent used to dissolve the polymer. It was found that for O/W

emulsions, the rate of polymer precipitation from the organic solvent phase was strongly affected by the rate of diffusion of the organic solvent into the aqueous phase. Organic solvents with a lower aqueous solubility have a slower polymer precipitation and thus affect the drug loading adversely.

Polymer concentration. Bigger particles are formed as a result of an increase in polymer concentration [102]. This is because of higher polymer concentrations have higher viscous resistance against the shear forces, which is required to reduce the particle size during the dispersion process. Higher polymer concentration also increases the drug-polymer phase viscosity, which could restrict the movement of the drug into the continuous phase and thus increase the drug encapsulation efficiency.

Polymer molecular weight. A study done on microcapsule containing phenobarbitone was prepared with PLA of different molecular weights, i.e. 5200, 20200, and 13300 to understand its influence on the release profiles [103]. The microspheres produced using W/O emulsion-evaporation method displayed an initial burst release followed by a lag phase. The lag phase was related to polymer weight such that as the polymer molecular weight increases, the lag time of the release also increases. Below in **Table 2.4** is a summary of the process parameters and the influences on the physiochemical properties of the polymer.

Table 2.4 The process parameters of the solvent evaporation emulsion technique and its influences on the particle's physiochemical properties

Process Parameters	Influences	Proportionality
Agitation Rate	Particle Size	Inversely
Surfactant Concentration	Particle Size	Inversely
Rate of Solvent Removal	Particle Size	Proportionate
	Encapsulation Efficiency	Proportionate
Solvent Type	Encapsulation Efficiency	
Polymer Concentration	Particle Size	Proportionate
	Encapsulation Efficiency	Proportionate
Polymer Molecular Weight	Release Profile's Lag Time	Proportionate

2.13 First-Line Anti-Tubercular Drugs

The first-line anti-tubercular drugs used for the treatment of TB are RIF, INH, PYZ, and EMB. They possess the greatest activity against the TB bacteria and are administered either as single-tablet combination or as a combined tablet, known as Fixed-Dose Combination (FDC). In a normal TB regime, the four first-line drugs will be given daily for two months; and in the following four months, rifampicin and isoniazid, three times weekly. Only when the patient has developed a drug resistant towards the first-line drugs will the second line be employed. They are categorized into group A, B, and C, listed in **Table 2.5**. In group A, all three drugs should be included; in group B, one or both drugs should be included; and in group C, drugs are used to complete the regime when drugs in group A and B cannot be used.

Table 2.5 Anti-tubercular drugs used for TB treatment

Group A	Group B	Group C
Levofloxacin or	Cycloserine or	Imipenem-cilastatin or
Moxifloxacin	Terizidone	Meropenem
Bedaquiline	Clofazimine	Amikacin or
		Streptomycin
Linezolid		Ethionamide or
		Prothionamide
		Pyrazinamide
		Ethambutol
		Delamanid
		p-aminosalicylic acid

RIF belongs to the class of Rifamycins, which is a group of antibiotics that are synthesized naturally by the Gram-positive bacteria, *Amiclatopsis Rifamycinica*. It is a bactericidal drug that inhibits the DNA-dependent RNA polymerase, preventing the bacteria from synthesizing messenger RNA and protein. Rifampicin is an inducer of the CYP450 system, which are enzymes that metabolize potentially toxic compounds such as drugs, making difficult for it to have paired with other drugs [104]. RIF also induces the

enzyme, uridine diphosphate-glucuronosyltransferase, which also metabolizes various drugs.

INH is a pro-drug which is activated into an active form by the metabolization of the *M.tb* catalase-peroxidase enzyme, KatG [105]. Once activated, the oxygen-derived free radicals (superoxide, hydrogen peroxide, and peroxynitrite) and organic free radicals will be produced and ipso facto inhibit the formation of mycolic acids which are present in the bacterial cell wall, causing cell death. INH is a hepatotoxic drug and must be administered prudently to women who are pregnant because they are more susceptible to developing hepatitis in the postpartum period. Unlike RIF, INH is an inhibitor of the CYP450 system which can increase the plasma concentrations of certain drugs to a toxic level.

PYZ, similar to INH, is a pro-drug. Its chemical structure comparable to Isoniazid, and has no cross resistance with it. PYZ is converted into its active form through the bacteria enzymes into pyrazinoic acid and causes bacterial death through the accumulation of pyrazinoic acid in the bacterial cytoplasm [106]. As a result, there is a decrease in the intracellular pH that inactivates the enzymes, impairing the mycolic acid biosynthesis. PYZ is also able to eliminate the bacilli that are phagocytosed by macrophages [107].

Ethambutol works by interfering with the cell wall, specifically the biosynthesis of arabinogalactan. It inhibits the arabinosyltransferase enzyme encoded by the *embB* gene, which mediates the polymerization of arabinose into arabinogalactan [108].

Their structure, minimal inhibitory concentration (MIC), and mechanism of action are summarized in **Table 2.6** [109, 110].

Table 2.6 Structure, MIC, and the mechanism of action of the first-line TB drugs

Drugs	Structure	MIC ($\mu\text{g/mL}$)	Mechanism of Action
Rifampicin		0.05 - 0.50	Inhibits of bacterial RNA synthesis by binding to the β -subunit of the bacterial DNA-dependent RNA polymerase.
Isoniazid		0.01 - 0.20	Inhibits the synthesis of mycolic acid which is a vital part of the mycobacterial cell wall through a covalent complex formation of acyl carrier protein (AcpM) and KsaA.
Pyrazinamide		20 -100 at pH 5.5 or 6	Lowers the pH of the immediate residence of the bacteria, preventing the organism from proliferating, by pyrazinamidase itself to active pyrazanoic acid.
Ethambutol		1 - 5	Inhibits the cell wall arabinogalactan biosynthesis

In the conventional prescription of treating patients afflicted with active TB, 9-16 oral tablets of the aforementioned drugs are administered daily for the first two months of the treatment. Subsequently, the intensity of the regime slows down and patients would only require 3-9 tablets daily for 4-6 months. It is paramount in both phases that multi-drug therapy is employed to prevent the mutation of the TB bacteria into a drug-resistant strain [110]. However, this template of treatment is not always followed.

Purchase of the four single drugs are frequent and usually from different manufacturers. In certain cases where delay in shipment occurs, a less-than-ideal treatment curriculum is resulted. Moreover, the drug RIF, known for its versatility in successfully treating other infectious diseases, has been abused in the form of monotherapy to patients who unsuspectedly also has TB. And while the remedy of RIF is able to alleviate the patient from that disease (even for a short period of time), the resistant of the drug by the TB bacteria has already been developing. This primitive way of treating TB raises the risk of drug resistance due to monotherapy and lack of compliance from ingesting huge number of tablets.

Hence, in order to mitigate these risks, the WHO has recommended the use of Fixed-Dose Combination (FDC) tablets as an alternative to separate formulations. The FDC tablet is a combination of the first four-line drugs in different ratios [111], and can vary in terms of its dosage strength and drug combinations (**Table 2.7**) for daily or intermittent use.

Table 2.7 Fixed Dosage Combination (FDC) tablet strengths and formulation for daily or intermittent use

Drugs	Dosage (mg)
Daily	
RIF + INH + PYZ + EMB	150 + 75 + 400 + 275
RIF + INH + PYZ	150 + 75 + 400
RIF + INH	300 + 150
INH + EMB	150 + 400
Intermittent (3 times weekly)	
RIF + INH + PYZ	150 + 150 + 500
RIF + INH	150 + 150

The FDC dosing schedule for children and adults with different weight categories at different phases is shown in **Table 2.8**

Table 2.8 FDC dosing amounts of patients with different bodyweights

Body Weight (kg)	Initial Phase (2 months)		Continuation Phase (4-6 months)	
	RIPE daily	RIP daily	RI daily	RI 3 times/week
Adults				
30 - 37	2	2	2	2
38 - 54	3	3	3	3
55 - 70	4	4	4	4
≥ 71	5	5	5	5
Children				
≤ 7	1	1	1	1
8 - 9	1.5	1.5	1.5	1.5
10 - 14	2	2	2	2
15 - 19	3	3	3	3
20 - 24	4	4	4	4
25 - 29	5	5	5	5

R = Rifampicin, I = Isoniazid, P = Pyrazinamide, E = Ethambutol

The incorporation of the drugs into one tablet helps to alleviate the struggle of ingesting the numerous amounts of single drug formulation down to 3-4 FDC tablets every session. Moreover, since the purchase of individual drugs is made from different manufacturers, FDC tablets could prevent the onset of MDR-TB in the case of a delayed shipment. Though there are no commercial aerosolized FDC in the market, there are a few inhalable FDC formulation that have been proposed; one of which is by John et al. [55]. They have introduced an inhalable dry powder formulation that incorporates three of the first-line drugs, i.e. PYZ, RIF, and INH into a PLGA particle with a MMAD of $3.5 \pm 0.1 \mu\text{m}$. This inhalable dry powder could be an attractive alternative to the standard oral FDC tablet.

2.14 Newer Anti-Tubercular Drugs

In recent years, the development of newer TB drugs has seen the spark of new life. Drugs such as Rifapentine, Moxifloxacin, Clofazimine, and TMC207 (recently renamed as Bedaquiline [BDQ]) are now beyond pre-clinical development and are potential candidates for the treatment of TB [112]. Among them, BDQ reigns supreme; it is the

first-ever FDA-approved TB drug in over 40 years [113], has a low MIC of 0.06 $\mu\text{g/mL}$, a long terminal half-life in humans of more than 24 hours, and has no cross resistance with existing anti-tuberculosis antibiotics. When compared to the first-line drugs in **Table 2.6**, BDQ has comparable inhibitory capabilities with RIF and INH, while having several folds lower drug requirements than PYZ and EMB. BDQ is a class of diarylquinoline drugs that destroys the mycobacteria through the inhibition of its adenosine triphosphate (ATP) synthase, an enzyme which is responsible for the production of ATP. [114]. In humans, within 4 hours after the consumption of 100mg and 400mg of the drug, the C_{max} reached $1.2 \pm 0.39\mu\text{g/mL}$ and $5.5 \pm 2.96\mu\text{g/mL}$, respectively.

Another recent TB candidate is Q203. It belongs to the family of Imidazopyridine Amide (IPA) compound that inhibits the growth of the MTB by targeting the respiratory cytochrome bc_1 complex [115]. The cytochrome bc_1 complex, also known as complex III, is the third complex in the electron transport chain of the bacteria oxidative phosphorylation. It is responsible for the biochemical generation of Adenosine Triphosphate (ATP), which transports the chemical energy within the cells for metabolism. Q203 has an MIC_{50} of 2.7 nM and 0.28 nM in culture broth medium and inside macrophages, respectively. Q203 is easy to synthesize, inexpensive, and scalable, which makes it a compelling candidate to combat TB in low-income countries. Also, when Q203 is compared to INH and moxifloxacin, Q203 is shown to possess better inhibitory capabilities than the two.

Q203 is a bacteriostatic drug. Although it is effective in inhibiting the bacteria growth, it does not inhibit its oxygen respiration. Under aerobic conditions, the Cytochrome $\text{bc}_1\text{-aa}_3$ supercomplex (Cyt- $\text{bc}_1\text{:aa}_3$) and the less efficient oxidase, cytochrome bd oxidase (Cyt-bd) in the third complex of the electron transport chain of the bacteria will be used to pump the protons through the plasma membrane. When Q203 is introduced to the bacteria, the flow of electrons through the Cyt- $\text{bc}_1\text{:aa}_3$ oxidase will be shut down, and the flow of electrons will be then directed through the Cyt-bd oxidase branch, maintaining the bacteria viability. For this reason, Q203 is able to only kill off replicating bacteria and

not non-replicating ones. And is the reason why Q203 is less effective at killing *M.tb* compared to BDQ.

2.15 Summary

The review on existing literatures encapsulating the treatment of TB gives a clear insight on the disease and identifies the challenges and gaps needed to design a pulmonary delivery system favourable for therapy. Currently, the treatment regime for TB is an oral administration of the first-line drugs for 6-9 months. However, because the drugs pass through the first-pass metabolism of the body and reduces the bioavailability of the drugs, patients would then require ingesting large amounts of drugs to compensate. In addition, because the treatment duration is prolonged, the liver and kidneys are taxed. These issues give rise for reasons to pursue an alternative treatment method. The pulmonary delivery of drugs is a promising therapeutic means because it delivers the drugs to the site of infection, possesses a higher bioavailability, and is benign to the kidney and liver. Pulmonary administration does have its own set of limitations and challenges. The physiological and physiocochemical factors are a few hurdles that need to be crossed in order for the pulmonary delivery to be effective. These factors affect the deposition fractions and regions. Current conventional aerosol delivery platforms have low deposition percentages into the lungs which makes this route of administration challenging. The low deposition fractions are due to a myriad of reason ranging from patients' incompetency to the ineffectiveness of the platform itself. Current proposed respiratory technologies marry the use of a vehicle which can be liposomes, PLGA, chitosan, or any analogous delivery carriers with first-line TB antibiotics. These carriers were found fitting because they are benign to the human body and are synthesize to a breathable range. PLGA is one of the most common because on top of being benign to the human lungs, it is FDA approved, and can be easily fabricated into various sizes with the use of an emulsion technique. PLGA can also load both hydrophobic and hydrophilic drugs. However, the use of PLGA alone as an inhalation carrier is not sufficient to delivery high deposition efficiencies. To boost the deposition effectiveness, SPIOs can be utilized as a conjugation to the drug carrier for delivery. Their superparamagnetic characteristic allows them to be directed in the presence of a magnetic field and is useful

when trying to manoeuvre and arrests them into a particular location in the lungs. Combining SPIOs into a pulmonary delivery system with novel but more effective anti-tubercular drugs than current first-line drugs can be an effective treatment of TB. Although, there is a plethora of novel pulmonary systems available for TB treatment, very few systems combine the mycobactericidal activity of its carrier with high deposition fractions. This is why the combination of SPIOs and new TB drugs into a pulmonary platform is a favourable alternative for TB treatment.

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Chapter 3

Experimental Methodology

This chapter outlines the experimental methodologies employed for the study. In each experiment, the operating principles and the procedural method of experimentation are elaborated. The chapter is divided into three parts. The first part of the chapter is concerned with the fabrication of the SPIOs and the drug carrier. The second part of the chapter enumerates the characterization techniques used for analysis of the drug carrier and its components. The last part of the chapter discusses the design of experiment employed for the comparison of Q203 and BDQ against the first-line drugs.

3.1 Fabrication

3.1.1 Fabrication of the Superparamagnetic Iron Oxides (SPIOs)

The synthesis of SPIOs can be accomplished through various means such as co-precipitation, thermal decomposition, sol-gel, micro-emulsion, etc. Co-precipitation is the most common. It involves the mixing of ferric and ferrous ions in a 1:2 molar ratio in a basic solution at room temperature or at elevated temperatures. The resulting chemistry is iron oxides of nano proportions. Without surfactants, the iron oxides will come together forming aggregates that are incompatible with their intended purpose. As such, surfactant such as oleic acid is added to prevent agglomeration.

The SPIOs were produced via a co-precipitation of iron (II) and iron (III) salts in an alkaline environment. The fabrication protocol follows that of Xie et al. [1]. Briefly, 0.30 g of FeCl_2 and 0.82 g of FeCl_3 were dissolved in 20 mL of DI-water that has been deoxygenated, and were heated to 80°C under nitrogen purging. The solution was heated for 30 min before 1 mL of ammonium hydroxide was added, drop-wise, and was heated under the same temperature for another 30 min. 1 mL of oleic acid (OA) was then added to the black precipitate and the temperature was raised to 95°C to be heated for 90 min. The resulting mixture was cooled, centrifuged, and washed thrice before storing in Dichloromethane (DCM).

3.1.2 Fabrication of the Pulmonary Drug Carrier

The pulmonary drug carrier was fabricated using a single emulsion (O/W) technique. Solvent evaporation single emulsion technique is commonly employed for the preparation of polymeric particles and utilizes the immiscibility of oil (O) [discrete] in a water (W) [continuous] to form particles. In certain conditions, a stabilizing agent/surfactant is added to aid the discrete phase in retaining individual particles without agglomerating. Typically, a highly volatile organic solvent, e.g. dichloromethane or chloroform is used to dissolve the polymer to form the oil phase. This is because it has a low boiling point which allows the harmful solvent to evaporate upon stirring. The particles are formed when the organic solvent are eliminated from the initial droplet and

transported into the aqueous phase. The solvent transport through the aqueous phase is diffusion-controlled.

The oil phase (O) consisting of 200 mg of PDLG 5010, 1.0 mg of Q203, 5.1 mg of Bedaquiline, 100 mg of SPIOs, and 10 mL of DCM were homogenized at 19,000 rpm with 20 mL of 6% PVA solution (W). At this O/W ratio, the solvent concentration in the aqueous phase is at its saturation level. This is done in order to form droplets of the same size before the formation of the particles can take place when the solvent evaporates. The resulting emulsion was poured into a beaker containing 250 mL 0.6% PVA solution and then overhead stirred for three hrs at 400 rpm to evaporate off the DCM. Once the DCM has evaporated, the resulting particles were then centrifuged, decanted, and washed thrice before they were freeze-dried.

3.2 Characterization of the Drug Carrier

3.2.1 Size and Morphology of the SPIOs and Drug Carrier

Electron microscopy is a surface-analytical technique used to obtain information of the surface morphology, shape, and size of a specimen. It utilizes a focused beam of electrons that are liberated from a field emission source to interact with the sample to obtain information on the topography. The electrons are accelerated through a vacuum column by an electrical field gradient and are deflected by electronic lenses to produce a narrow scan. A schematic of the Field Emission Scanning Electron Microscope (FE-SEM) is shown in **Figure 3.1**.

Figure 3.1 Schematic diagram of the FE-SEM

In FE-SEM, a very sharp tungsten needle of 100 nm tip radius is used to generate electrons. This is different from the way a SEM generates electron which is by heating the tungsten filament to a temperature of about 2800°C. This “cold” method produces an electron beam 1000 times smaller than the SEM, greatly improving the quality of the image. The electrons liberated by the cathode tip are then accelerated to the anode. The electro-magnetic lenses (condenser lens, scan coils, stigmator coils, and objective lens) focus the beam into a tiny sharp spot.

Depending on the energy of the electron beam, the interaction volume varies producing different signals: Secondary electrons (topographical information), back scattered electrons (information on phase differences), or characteristic x-rays (information on atomic composition). The drug carrier was analyzed using a FE-SEM (JEOL JSM-6340F SEM) at an operating voltage of 5 kV and a probe current of 12 μ A. The sample for analysis was prepared by spreading the freeze-dried drug carriers onto a carbon tape. The sample was then coated with platinum at 20 mA for 60 secs.

The major differences between the TEM and SEM are:

1. Specimen for TEM needs to be very thin
2. Broad static beam instead of a narrow beam is used in TEM
3. Higher accelerating voltage (up to 300 kV in TEM) in order to penetrate the specimen

The SPIOs and SPIOs-loaded drug carrier were observed using a TEM (Carl Zeiss Libra 120 Plus) at an operating voltage of 120 kV. The samples to be analyzed were dispersed in DI-water, sonicated for 10 min, and then pipetted out on a copper grid and left to dry for 24 hrs.

3.2.2 Dynamic Light Scattering (DLS)

Dynamic light scattering (DLS) is an optical technique used to measure the hydrodynamic diameter of particles suspended in a medium. Its operating principle is based on Brownian motion, which is the movement of the particles through the bombardment of solvent molecules. Larger particles inspire slower Brownian motion, while smaller ones stimulate rapid movements. In DLS, the rate of the particles diffusing is measured by the intensity of the fluctuating light scattered. The rate of the fluctuation is dependent of the size of the particles. Smaller particles cause more rapid fluctuation than larger ones. For a system undergoing Brownian motion, the hydrodynamic size of the particle is governed by the Stokes-Einstein equation:

$$d_H = \frac{kT}{3\pi\eta D}$$

where d_H is the hydrodynamic diameter, k is the Boltzmann's constant, T is the absolute temperature, η is the viscosity, and D is the translational diffusion coefficient. The information on the temperature needs to be accurate in order to determine the size of particles because it directly relates to the viscosity of the liquid. In addition, the temperature within the liquid must be stable. Gradient in temperature will cause convectional currents resulting in false measurements.

The size distribution obtained from the measurements plots the relative intensity of light scattered to particle size. There are three different parameters that can be used to view the distribution: Intensity, volume, and number. Number-distributions separate the peaks according to the number of populations. If a sample was to have a 5 nm and a 50 nm distribution, the number-distribution would see a 1:1 peak ratio. If this same sample is converted to a volume-distribution, the plot would be a 1:1000 peak ratio. This is because the volume of the 50 nm population is larger than the 5 nm population. If the sample is converted to an intensity-distribution, a 1:1000000 peak ratio would be observed.

The average particle size and polydispersity was measured using a Malvern Nanosizer. The drug carrier was suspended in DI-water, sonicated for 10 min, and then immediately pipetted out into a cuvette for analysis. The measurements were done in triplicates.

3.2.3 Magnetic Saturation of the SPIOs and the Drug Carrier

The magnetic saturation for both the SPIOs and the drug carrier was determined using a Vibrating Sample Magnetometer (VSM) [VSM Lakeshore 7400] at a maximum magnetic field of 10,000 Gauss (G).

VSMs measure the magnetic properties of a magnetic material by vibrating it across a uniform magnetic field. Its design is based on Faraday's Law of Induction which describes how an electric current produces a magnetic field and, conversely, how a changing magnetic field generates an electric current in a conductor. Before operation, the electromagnets between the sample will be activated to generate a uniform magnetic field, H , and consequently induce a magnetic response (magnetization) from the sample, M . Once the testing commence, the holder arm for the sample will vibrate longitudinally in the z -axis between two electromagnets which generates an electric field in the coil. The pickup coils surrounding the sample holder will then pick up the induced voltage and map out the magnetic profile of the sample. The induced voltage is proportionate to the magnetic moment of the sample.

The samples were prepared by placing 5 mg of the dried SPIOs and drug carrier, separately, into a polytetrafluoroethylene (PTFE) tape. The samples were then placed in the sample holder for analysis.

3.2.4 Drug Encapsulation Efficiency (EE)

The drug encapsulation efficiency was determined using Liquid Chromatography Mass Spectroscopy (LC-MS) (Agilent LC 1260 MS 6120). LC-MS is an analytical technique that combines the separation capabilities of a High Performance Liquid Chromatography (HPLC) and the detection capabilities of a Mass Spectrometry (MS). LC-MS is a powerful instrument that can analyze a myriad of organic compounds which other equipment such as the Gas Chromatography Mass Spectrometry (GC-MS) cannot because it imparts little to no heat to the analyte, making it suitable for a range of specimen from small pharmaceutical compounds to large proteins. It involves the separation of the specimen into individual components and then ionizing them so that they can be detected according to the mass per charge (m/z) ratio. A simple schematic on the LC-MS is shown on **Figure 3.2**.

Figure 3.2 Schematic diagram of the LC-MS.

When the sample is injected into the column, it gets absorbed and interacts with the stationary phase. The analyte is separated into individual compounds depending on their affinity with the packing materials in the column and the mobile phase. These compounds are then pumped into the MS through a nebulizing needle.

Once the effluent is nebulized into liquid droplets, it gets charged from the potential difference present between the needle and the electrode and flows towards the capillary orifice. During that time, a counter-flow of heated nitrogen drying gas is injected into the MS, carrying the uncharged particles away and shrinking the charged droplets. The shrinkage causes the coulombic forces of the charge droplets to exceed the cohesive force, desorbing the droplets into a gas phase. These desorbed ions then pass through the capillary sampling orifice into the low-pressure region of the ion source and into the mass analyzer. The MS process is illustrated in **Figure 3.3**.

Figure 3.3 The MS mechanism of operation

The mobile phase consists of acetonitrile and DI-water (50:50) with 0.1% v/v of formic acid, using Zorbax RX-C8 (150 mm x 4.6 mm) as the stationary phase. The samples were maintained at 25°C and injected into the column with a flow rate of 1 mL/min and analyzed at an m/z of 279 for Q203 and m/z of 555 for BDQ. 20 mg of the drug carrier was dissolved in dimethyl sulfoxide (DMSO) and centrifuged at 13,000 rpm for five min. The supernatant was then collected for analysis. The drug encapsulation efficiency was carried out in triplicates and is expressed as the percentage of actual drug loaded over the initial amount (mg).

$$\text{Encapsulation efficiency (\%)} = \frac{\text{Actual drug loaded (mg)}}{\text{Initial drug loaded (mg)}} \times 100\%$$

3.2.5 Drug Release Studies

10 mg of the drug carrier was placed inside a 2 mL microtube containing 1 mL of Simulated Lung Fluid (SLF) [Table 3.1] and shaken at 37°C. The drug release studies were conducted for 24 hrs in triplicates. At pre-determined time intervals, the release media was pipetted out, transferred to a new microtube, and then frozen in the -20°C refrigerator. The microtube was then freeze-dried before 1 mL of DMSO was added. The samples were analyzed using LC-MS with the same settings used for the encapsulation efficiency studies.

Table 3.1 Constituents of the SLF

Simulated Lung Fluid (SLF)	
Composition	Amounts (g/L)
Magnesium Chloride	0.095
Sodium Chloride	6.019
Potassium Chloride	0.298
Disodium Hydrogen Phosphate (Na ₂ HPO ₄)	0.126
Sodium Sulfate	0.063
Calcium Chloride Dihydrate	0.368
Sodium Acetate	0.574
Sodium Hydrogen Carbonate (NaHCO ₃)	2.604
Sodium Citrate Dihydrate	0.097

3.2.6 Numerical Simulations

Computational Fluid Dynamics (CFD) simulations were performed using a commercial finite volume method (FVM) solver (ANSYS Fluent) to simulate magnetic particle inhalation into an acinar geometry. The geometry mimics the alveolar environment and consists of 372 individual alveoli that densely populate the space surrounding a branching

airway tree, starting with one inlet and bifurcating a mean of 4.38 generations, with up to 8 generations (see Hofemeier et al. for further details [2]). Breathing was modelled through a self-similar motion of the walls of the acinar domain, following a sinusoidal volumetric inflation function with an amplitude of 16.8% off the resting volume (corresponding to functional residual capacity) and a breathing cycle of three secs.

Physics of fluid (i.e. air) motion are described by the transient Navier-Stokes' equations along with the continuity equation, where the inhaled air is assumed to be Newtonian, incompressible and isothermal, with density $\rho = 1.22 \text{ kg/m}^3$ and viscosity $\mu = 1.79 \cdot 10^{-5} \text{ Pa}\cdot\text{s}$. A no-slip boundary condition is implemented on the moving geometry walls, whereas the domain inlet (i.e. outlet during deflation) is defined as a pressure-inlet, which results in air being drawn into the geometry as it inflates, and pushed out as it deflates.

An in-house discrete element method (DEM) solver was used to simulate the motion of the particles [3, 4]. Particles were one-way coupled to the flow and their motion was solved according to Newton's force balance in a Lagrangian framework, where gravitational, drag and Brownian forces were considered. The direction of the gravity was previously shown to have little effect on the particle deposition results [2], and was arbitrarily directed here in the $-y$ direction. Particles with density of $\rho_p = 1490 \text{ kg/m}^3$ and diameter of 0.5 mm are injected into the domain via the inlet after 0.6 secs to account for the lung's dead space (i.e. crossing through the conducting airways) until 1.5 secs (i.e. peak inhalation). Upon contacting a wall, particles are assumed to have deposited. Briefly, following rigorous mesh convergence tests, a total of 4.25×10^6 tetrahedral cells were used to mesh the geometry, whereas a time step of 10^{-3} secs was used for the flow and $2 \cdot 10^{-7}$ secs for the particles, and 10,000 particles were injected to assure accurate deposition statistics.

3.2.7 In-Vitro Magnetic Targeting

A set-up to assess the magnetic targeting of the drug carriers was erected (**Figure 3.4**). The components of this setup consist of a 16.8 mm hollow tube mimicking an adult trachea, a Ventilator-Nebulizer Junction (VNJ), a smart inhaler system (custom-made

nebulizer coupled with an electronic control that releases short pulse of aerosol bolus) , an actuator (LEY25B-300-S16P1, SMC), and a stack of permanent magnets. The smart inhaler and the actuator were connected to the inlet of the hollow tube by means of a VNJ and the outlet of the tube was connected to a chemical hood. A stack of four permanent magnets were placed outside of the tube approximately at the centre (lengthwise) of the tube. The magnetic targeting capability of the drug carrier was assessed by passing an aerosolized brine solution [DI water:NaCl (10:1)] of the drug carriers through the hollow tube and having the magnets along the tube to arrest the particles. A breath-hold (BH) of five secs was calibrated to initiate after the aerosol bolus was directly beneath the magnet. The suspension was aerosolized by a smart inhaler which releases short and controlled pulse of the aerosol. The flow of 1.145 L/min was generated by a linear actuator. The magnets are four disc-shaped (30 mm in diameter, 15 mm in height) rare-earth permanent magnet separated by 5 mm thick plastic plates. The response of the aerosolized suspension in relation to the magnet was also assessed. For this assessment, the magnet's distance in relation to the tube was varied from 0 to 10 mm and finally to 20 mm.

Figure 3.4 Schematic of the in-vitro magnetic targeting setup.

3.2.8 Cell Viability

A549 cells (10,000/100 μ L) were seeded onto 96-well plates and incubated overnight at 37°C under 5% CO₂ atmosphere. The cells were maintained in high-glucose Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS, v/v), and 1% penicillin/streptomycin (P/S, v/v). After incubation, the cells were replenished with 100 μ L of fresh media containing 100–500 μ g/mL of the drug carrier with and without SPIOs, and raw SPIOs corresponding to the amounts loaded into 100–500 μ g/mL of the drug carrier were incubated for 24, 48 and 72 hrs. And at each time point, the media is removed, and 100 μ L of PrestoBlue assay that has been diluted down ten-folds was added. The cells were then incubated for another hour. After that, 50 μ L of the media was pipetted out onto new 96-well plates and analyzed while the remaining media was discarded and replenish with fresh media. The fluorescence readings were done at 570 nm and 600 nm. The statistical evaluation of the cell viability was performed using a one-way ANOVA. The significance between the means of the three groups, i.e. 24, 48, and 72 hours, were evaluated to determine whether there was a difference in the A549 cells' viability. For the comparison of the difference between the free drug and encapsulated Q203 and BDQ, a small-sample hypothesis testing of two means was used. In all cases, a p-value of <0.05 is accepted to reject the null hypothesis.

3.2.9 Assessment of the Number of Viable Bacteria

BCG bacteria at OD 0.005 in the mid-log phase were grown in 7H9 broth with glycerol to a 10⁶ CFU count. MIC₉₉ of the encapsulated Q203 only, BDQ only, and Q203 and BDQ was introduced the BCG bacteria once at the beginning of the experiment. The untreated BCG and the drug carrier with drugs were used as controls. At day 0, 4, 10, and 15, 50 μ L aliquots of the culture was dropped onto agar plates and allowed to incubate at 37°C with 5% CO until bacteria colonies were formed. The MBC was determined by multiplying the visible colonies with the dilution factor.

3.3 Comparison of first-line drugs and Q203 and BDQ

3.3.1 Minimum Inhibitory Concentration

The MIC of RIF, INH, Pyrazinoic Acid (PYZ+), Q203, and BDQ on WT-BCG were determined using a turbidity-based assay. 200 μ L [Optical Density (O.D.) of 0.005] of WT-BCG suspended in 7H9 without glycerol were added to 2 μ L of free drugs, i.e. RIF, INH, PYZ+, Q203, and BDQ with starting concentration of 100 nM, 1 μ M, 10 mM, 100 nM, and 3 μ M, respectively. The subsequent wells have the drug concentration diluted two folds as seen in Table 3.2. The drugs and the bacteria were incubated at 37°C under 5% CO₂ atmosphere for five days. The samples were analyzed using Cytation3 imaging reader at 600 nm in triplicates.

Table 3.2 96-well plate assay of the MIC study

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	RIF	100	50	25	12.5	6.25	3.13	1.56	0.78	0.39	0.20	
C	INH	1000	500	250	125	62.5	31.25	15.63	7.81	3.91	1.20	
D	PYZ+	10	5	205	1.25	0.625	0.313	0.156	0.078	0.039	0.020	
E	Q203	100	50	25	12.5	6.25	3.13	1.56	0.78	0.39	0.20	
F	BDQ	3000	1500	750	375	187.5	93.75	46.88	23.44	11.72	5.86	
G												
H												
I												

3.3.2 Chequerboard Comparison

The combinational efficacy of Q203 and BDQ against the first-line TB drugs was determined using a chequerboard assay. The chequerboard assay is a two-fold serial dilution of two antibiotics at the starting concentration of MIC₅₀ to study the extent of the bacterial growth inhibition of WT-BCG at different concentrations (Table 3.3). The interaction between two antibiotics in combination can be described as synergistic, additive, no effect, or antagonistic using the quotient, x/y (where x is the data obtained when two antibiotics are in combination and y is the data with the lower value of the two agents when tested separately at the same concentration). If,

$x/y < 0.5$ – Synergistic

$0.5 \leq x/y < 0.75$ – Additive

$0.75 \leq x/y < 1$ – Moderately Additive

$1 \leq x/y < 2$ – No effect

$x/y \geq 2$ – Antagonistic

Table 3.3 Example of a chequerboard assay of the combination efficacy of Q203 and BDQ against RIF and INH

	1	2	3	4	5	6	7	8	9	10	11	12					
A	Q203						RIF										
B	MIC ₅₀							MIC ₅₀
C	↓							↓
D
E
F
G		←				MIC ₅₀		←				MIC ₅₀					
H						BDQ						INH					
I																	

It is essential that the starting concentration of the assay is below the 99% inhibition concentration in order to observe the aforementioned effects. Q203 and BDQ will be compared against RIF and INH, RIF and PYZ+, and INH and PYZ+. 2 μ L of the respective antibiotic at different concentrations will added into the well with 200 μ L [Optical Density (O.D.) of 0.005 suspended in 7H9 without glycerol] of WT-BCG. The drugs and the bacteria were incubated at 37°C under 5% CO₂ atmosphere for five days. The samples were analyzed using Cytation³ imaging reader at 600 nm in triplicates.

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Chapter 4

Combination Efficacy of the Anti-Tubercular Agents

This chapter compares the bactericidal activity of first-line anti-tubercular drugs, i.e. RIF, INH, and PYZ+ with Q203 and BDQ. The chapter begins by determining the MIC of all the drugs against WT-BCG. Utilizing the results obtained, the chequerboard method mentioned in chapter 3.3.2 is employed to compare their combination effects. The synergistic, additive, zero, and antagonist effect of the drug combination will be investigated.

4.1 Minimum Inhibitory Concentration

The MIC profiles of the Q203, BDQ, and the first-line drugs are shown in **Figure 4.1**. The starting concentrations of Q203, BDQ, RIF, INH, and PYZ+ are 100 nM, 3 μ M, 100 nM, 1 μ M, and 10 mM, respectively. From **Figure 4.1** it can be seen that amongst the anti-tubercular drugs tested, RIF is the most potent, followed by Q203 and BDQ, and then lastly, INH and PYZ+. In terms of potency, Q203 and BDQ are relatively exceptional, requiring only a small amount of drugs to kill off the BCG bacteria. And although they are not as potent as RIF, Q203 and BDQ have proven themselves to be more effective, individually, than the other gold standards of TB treatment, i.e. INH and PYZ+.

Figure 4.1 MIC profile of the anti-tubercular drugs

The MIC₅₀ values of the anti-tubercular drugs are listed in **Table 4.1**. From the table, it can be seen that amongst the drug tested, PYZ+ requires a considerable amount of drug to inhibit 50% of the BCG organism. Comparatively speaking, PYZ+ would require 1.3 million times more drug than Q203 and 28,500 times more than BDQ. This is indicative that while PYZ+ is used as the first-line anti-tubercular drug, its efficacy is inferior to that of Q203 and BDQ. The potency of Q203 and BDQ stems from the ability to inhibit the

respiratory complexes in the bacteria's oxidative phosphorylation, a different mechanism to that of INH and PYZ+.

Table 4.1 MIC₅₀ values of the anti-tubercular drugs

	Q203	BDQ	RIF	INH	PYZ+
MIC ₅₀ (nM)	1.15 ± 0.09	53.92 ± 4.39	0.033 ± 0.04	197.3 ± 14.06	1,537,000 ± 101432

4.2 Combination Efficacy of Anti-Tubercular Drugs

The comparative data obtained from using the chequerboard assessments on the combinational efficacy of the anti-tubercular drugs on BCG are shown on **Table 4.2**, **Table 4.3**, **Table 4.4**, and **Table 4.5**. These assessments utilize an x/y quotient, where x is the value of the combination efficacy of the drugs on BCG, and y is the lowest value obtained between the two drugs at the same concentration used alone. The combination activity of the drugs are considered synergistic when $x/y < 0.5$, additive when $0.5 \leq x/y < 0.75$, moderately additive when $0.75 \leq x/y < 1$, no effect when $1 \leq x/y < 2$, and antagonist when $x/y \geq 2$. In **Table 4.2**, the combination efficacy of Q203 and BDQ is shown.

Table 4.2 Q203 and BDQ combinational efficacy

Q203	0.66883	0.681818	0.662338	0.62987	0.590909
	0.67857	0.672619	0.690476	0.654762	0.553571	
	0.83152	0.728261	0.728261	0.619565	0.538043	
	0.84080	0.78607	0.696517	0.636816	0.512438	
	0.74661	0.705882	0.687783	0.58371	0.475113	
BDQ						

From **Table 4.2**, it can be seen that majority of the combinations of Q203 and BDQ (21 out of 25) yielded additive results. Three of the combinations had a moderate additive effect, while only one combination produced synergy. At 15.63 nM of BDQ (MIC₅₀) and 0.20 nM of Q203 the combination is most effective in killing off the BCG bacteria. It is noteworthy however that the other concentrations of Q203 in combination with MIC₅₀ of BDQ showed borderline synergy.

In **Table 4.3**, the two first-line drugs, i.e. RIF and PYZ+ are assessed. For the combination efficacy of RIF and PYZ+, only three of the combination yielded additive effects. The other 22 out of 25 combinations are moderate additive. This when compared to the Q203 and BDQ combination is inferior.

Table 4.3 RIF and PYZ+ combinational efficacy

RIF	0.8125	0.8125	0.804688	0.8125	0.828125	Synergistic Additive Moderate Additive No effect Antagonistic
	0.833333	0.833333	0.833333	0.833333	0.886364	
	0.810458	0.816993	0.803922	0.803922	0.830065	
	0.762162	0.751351	0.713514	0.72973	0.735135	
	0.957576	0.945455	0.939394	0.921212	0.951515	
PYZ+						

Next, the combination RIF and INH are compared (**Table 4.4**). In this chequerboard assay, 20 out of 25 of the combinations are moderate additive, while the other five combinations have no effect, when the concentration of RIF is at 0.20 nM with all concentrations of INH. The combination of RIF and INH is comparable to RIF and PYZ+, but again is inferior to the Q203 and BDQ combination.

Table 4.4 RIF and INH combinational efficacy

RIF	0.804688	0.8125	0.796875	0.804688	0.796875	Synergistic Additive Moderate Additive No effect Antagonistic
	0.924242	0.878788	0.886364	0.901515	0.909091	
	0.849673	0.869281	0.888889	0.921569	0.921569	
	0.805405	0.827027	0.843243	0.875676	0.897297	
	1.018182	1.072727	1.084848	1.084848	1.10303	
INH						

Lastly, in **Table 4.5**, the combinational efficacy of PYZ+ and INH are shown. In this combination, 19 out of the 25 concentrations are moderate additive with the remaining six that have no effect. Similar to the other first-line drugs combination, this combination of PYZ+ and INH is inferior to that of Q203 and BDQ.

Table 4.5 PYZ+ and INH combinational efficacy

PYZ+	0.876777	0.919431	0.924171	0.909953	0.92891	Synergistic Additive Moderate Additive No effect Antagonistic
	1.057895	1.015789	0.984211	0.968421	0.989474	
	1.009901	0.935644	0.891089	0.876238	0.891089	
	1.028302	0.882075	0.768868	0.773585	0.811321	
	1.094444	1.05	0.955556	0.972222	0.966667	
INH						

It is conclusive from the chequerboard assay that the combination of Q203 and BDQ is more efficacious than any of the first-line drug dual combinations. In all three first-line drug dual combinations, there was no drug combination concentration that has a synergistic effect; and only 3 out of 25 of the RIF and PYZ+ have an additive effect. On the other hand, Q203 and BDQ have 1 combination concentration that is synergistic, 21 combinations that are additive and 3 that are moderate additive. The comparative data of the Q203 and BDQ against the first-line drugs is summarized in **Table 4.6**.

Table 4.6 Combination efficacy of Q203 and BDQ against the first-line drugs

	x/y				
	Synergistic	Additive	Moderate Additive	No effect	Antagonistic
Q203 + BDQ	1/25	21/25	3/25	0/25	0/25
RIF + PYZ+	0/25	3/25	22/25	0/25	0/25
RIF + INH	0/25	0/25	20/25	5/25	0/25
PYZ+ + INH	0/25	0/25	19/25	6/25	0/25

It is possible that the synergy between the first-line drugs requires three or more antibiotics working together to produce the desired mycobactericidal activity. A study done by Nalin Rastogi [1] compared the *in-vitro* activities of Levofloxacin (LEVO) when used alone and when combined with first- and second-line anti-tubercular drugs against different *M.tb* strains. The comparative study uses the same x/y quotients for assessment where synergism for two, three, and four drug combinations have x/y values smaller than 0.5, 0.33, and 0.25, respectively. One of the strains used to test the activity was H37Rv (a mycobacterium strain resistant to RIF). When the two drug combinations of LEVO and EMB, and LEVO and RIF was tested again this strain, the combinations did not indicate

any enhanced drug action. Only when LEVO was combined with EMB and RIF did a synergy against this strain ($x/y < 0.19$) was witness. The similar observation was made when the two drug combinations of LEVO and EMB, LEVO and RIF, and LEVO and Amikacin (AMIK) produced no bactericidal activity against H37Rv, but when combined together, achieved synergy with an x/y score of 0.14.

While a three drug or four drug combinations with the first-line drug was not conducted, it would be telling of the Q203 and BDQ drug combination that even if the first-line drugs achieved enhanced drug activity at three or four drug combinations, that the two drugs will be enough to produce comparable efficacy than when three or four of the first-line drugs are combined together.

4.3 Combination Efficacy of Q203 with First-Line Drugs

From the MIC profiles in **Figure 4.1**, it can be seen that Q203 stands out from the other anti-tubercular agents by having a superior inhibitory capability on BCG, second only to RIF. In order to further explore the combinational effects of the anti-tubercular drugs, Q203 has been paired with the first-line drugs to determine whether their combination would be comparable or even better than the combination of Q203 and BDQ as can be seen in **Table 4.2**.

In this assessment, the same chequerboard method has been utilized, with the x/y quotient serving as the drug activity marker. The combinations tested will be Q203 and RIF, Q203 and PYZ+, and Q203 and INH. Their comparative data is summarized in **Table 4.7**.

Table 4.7 Combination efficacy of Q203 and the first-line drugs

	x/y				
	Synergistic	Additive	Moderate Additive	No effect	Antagonistic
Q203 + RIF	0/25	0/25	8/25	17/25	0/25
Q203 + PYZ+	0/25	0/25	9/25	16/25	0/25
Q203 + INH	0/25	0/25	8/25	17/25	0/25

Although Q203 and RIF are the two most potent drugs in the assessment, their combination did not have any synergistic or additive effect. The majority of the Q203 and RIF's combination (17 out of 25) yielded no effect, while only 8 combination concentrations have moderate additive effect. Similarly with the other two first-line drugs, Q203 does not yield any synergistic or additive effect with PYZ+ or with INH.

Efficacy studies of analogous comparison have been conducted with new anti-tubercular drugs and the results obtained were different from that of Q203. SQ109 is one such candidate. SQ109 is an ethylenediamine that targets the MmpL3 whose purpose is to transport mycolic acid for the building up of the bacteria's cell wall [2]. The MIC of SQ109 against live *M.tb* is at a range of 0.16-0.64 µg/mL. In a study conducted by Ping Chen et. al [3], a similar checkerboard titration was employed but with SQ109, INH, ETM, PYZ, and streptomycin. The results obtained showed that SQ109 has strong synergistic activity with INH and with RIF, additive efficacy with streptomycin, but have neither synergistic nor additive effects with EMB and with PYZ. There is no definitive explanation for the synergism of SQ109 and RIF. However, it is hypothesized that the synergistic interaction could spring forth from the different drug actions. The targeting of the cell wall from SQ109 could result in an increase of permeability, allowing more of the RIF to enter in the bacteria. The hypothesis was tested with EMB (a cell wall inhibitor like SQ109), but when introduced together with RIF to the bacteria, the combination yielded null efficacy. Another possible reason for the synergism of RIF and SQ109 is that RIF is an inducer of CYP450 (an enzyme that has a vital role in the biosynthesis of compounds such as sterols, steroids, fatty acids, and detoxifies xenobiotics and chemicals) which activates the SQ109 by producing oxidized metabolites of SQ109 within the *M.tb*.

In the case of Q203, neither the increased permeability of INH (targeting the cell wall), nor the activation of the CYP450 from RIF resulted in synergism with Q203. One reason why Q203 is not synergistic with the three first-line drugs is because of the difference in drug actions. It is possible that the drugs that target the oxidative phosphorylation would

need to have accompanying drugs that possess the same drug action in order to produce synergistic or additive effects.

4.4 Discussion

The MIC profiles in **Figure 4.1** illustrate individually the potency of the anti-tubercular agents. From there, it is evident that RIF has the most potent inhibitory capability at a MIC₅₀ of 0.033 nM (35 times more potent than Q203). RIF is a drug which inhibits the bacteria RNA synthesis and is used for many other diseases other than TB such as leprosy and Legionnaires' disease. However, the monotherapy of RIF to a patient with undiscovered TB can be threatening if the TB bacteria within develops resistant to the antibiotic. Hence, monotherapy is never employed for TB treatment. One of the current templates of TB treatment at the initial phase is a combination of RIF, INH, and PYZ+. These drugs are highly efficacious towards *M.tb* and although the use of RIF is an inducer of the CYP450 system, which metabolizes various drugs, INH harmoniously inhibits the CYP450 system, making the use of them together compatible.

It is hypothesized that the combination use of Q203 and BDQ will have a more efficacious mycobacterial activity than the combination first-line drugs, on the basis that individually, Q203 and BDQ are more potent than the first-line drugs and that they shut down the oxidative phosphorylation of the bacteria by targeting the different respiratory complexes within. In order to validate the hypothesis, the dual drug combinations of the TB drugs were compared using the checkerboard method. The concentrations of each drug tested were at sub-MICs and their mycobacterial activity was determined using the x/y quotient. In the combination of Q203 and BDQ, the results in **Table 5.2** show that majority of the combination yield additive results. These additive results are due to the fact that Q203 and BDQ, individually, have potent inhibitory capability as seen in **Figure 5.1**. However, at 15.63 nM of BDQ and 0.20 nM of Q203, there was a synergism observed. At this ratio, the Q203 and BDQ drug worked harmoniously and is the only drug combination concentration among all the combination tested that has an x/y than is lower than 0.5 as can be seen in **Table 5.6**.

For the first-line combination of RIF and PYZ+, RIF and INH, and PYZ+ and INH, above 75% of their combination yielded moderate additive. For the RIF and INH, and PYZ+ and INH combination, 20% of the combination does not have any effect if compared to a single drug use. These results affirm the hypothesis made that Q203 and BDQ combination is more effective against the first-line combination. This combination efficacy could be due to the fact that Q203 and BDQ synergistically targets the different complex of the oxidative phosphorylation of the bacteria. Evidently, this is more effective than if the drug was to be target the cell wall (like INH) or by overloading the bacteria's cytoplasm (PYZ+).

In order to rule out that the synergistic combination of Q203 and BDQ is not due just because of Q203 superior inhibitory capability, Q203 was paired with the first-line drugs in the same checkerboard assessment. In this assay, the combination of Q203 and RIF, Q203 and PYZ+, and Q203 and INH, did not yield favourable results. Around 65% of all three combinations have no effect, and only around 35% of the combination concentrations have a moderate additive. This highly suggests that Q203 is not synergistic with the first-line drugs and only by a combinational targeting of the oxidative phosphorylation similar to that of BDQ would produce favourable results.

References

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Chapter 5

Drug Carrier Fabrication and Characterization

The chapter focuses on the results obtained on the characterization of the drug carrier. First, the result on the fabrication of the SPIOs and the drug carrier will be discussed. This includes the surface characterization and the hysteresis loop of the SPIOs and the drug carrier. Next, the results obtained on the drug encapsulation efficiency and the drug release of both single and combined Q203 and BDQ are examined. The chapter continues, analyzing the in-silico and in-vitro magnetic targeting results, which are works that are conducted from Technion Institute of Technology and ends with evaluation of the toxicity of the drug carrier and its mycobactericidal activity.

5.1 Fabrication of SPIOs and Drug Carrier

The drop-wise addition of ammonium hydroxide into iron (II) and iron (III) salts solution yielded spherical SPIOs with a narrow size distribution of 9 ± 3.92 nm as shown in **Figure 5.1A**. These SPIOs possess superparamagnetic properties with no remanence and coercivity, upon the removal of the magnetic field. The SPIOs possess a high magnetic saturation of 74.8 emu/g, and when encapsulated into PDLG sub-micron particles (the drug carrier), a drop in the overall magnetic saturation to 28.0 emu/g was observed (**Figure 5.1B**), due to the shielding effect of the polymer. This magnetic saturation value is subsequently applied in the CFD simulations to validate the deposition profile of these drug delivery particles in the acinus.

Figure 5.1 Characterization of SPIOs. (A) TEM image of the SPIOs. (B) Hysteresis curve of the raw SPIOs and the drug carrier using VSM.

Next, PDLG drug carriers, encapsulating SPIOs, were synthesized through a single emulsion technique that yielded uniformly-sized, spherical particles with a smooth morphology of around 500 nm, as shown in **Figure 5.2A**. From the TEM image in **Figure 5.2B**, the contrast of the PDLG carrier and the SPIOs can be seen with the bulk of the SPIOs located at the core of the carrier. DLS (**Figure 5.2C**) measurements confirmed a mean hydrodynamic diameter of 476.9 ± 28.7 nm. The DLS also reports a

PolyDispersity Index (PDI) of 0.51, which falls between the desired dispersity range of 0.4-0.7. Solvent emulsion is a suitable technique for the fabrication of narrow size particles. This technique is commonly used for the fabrication of microspheres in other pulmonary systems [1, 2] and is capable of loading not only hydrophobic entities, but also hydrophilic ones through water-in-oil-in-water emulsions.

Figure 5.2 Characterization of the drug carrier using different characterization techniques. (A) FE-SEM image at 8000x magnification. (B) TEM image at 4000x magnification. (C) DLS spectrum of the drug carrier having a mean hydrodynamic diameter of $476.9 \text{ nm} \pm 28.7$ with a PDI of 0.51.

Size has an influence on the particles' deposition fractions, mechanisms, and locations. From the deposition statistics generated by the International Commission on Radiological

Protection (ICRP) [**Supplementary Figure 1**], a statistical model designed according to the semi-empirical equations derived from the deposition data in humans using mono-dispersed particles, it is clearly seen that particles larger than 8 μm are dominated by an impaction mechanism, while particles around 0.5-8 μm inspire a sedimentation mechanism. Also, particles smaller than 0.5 μm are diffusion-controlled. The deposition mechanisms of the particles are governed by the gain of momentum ($p = mv$; where p is the momentum, m is the mass and v is the velocity) of the body during movement. For a given velocity, as the mass of the particle increases, the momentum of the body also increases, altering the deposition mechanism.

Different size particles can also alter the deposition location. Based on this model, for a particle of 500 nm, the particle would only see a deposition fraction of 0.15 in the total lungs and approximate 0.1 in the alveolar (Alv). At this size range, the total deposition is at its lowest, which is the most suitable for pulmonary targeted delivery with iron oxides. The reason is because targeted delivery requires the particle to traverse through the lungs without being deposited prior to the desired location. The external magnet used for this targeted delivery will serve as a beacon for the inhaled particles. And if so desired the particles are to be deposited into the trachea-bronchi, the magnet will be placed near that region, externally, to attract the particles towards that region. In order to achieve higher deposition amounts into trachea-bronchi, the particles must first pass through the headways without being deposited.

A visual comparison between the PDLG drug carrier with and without SPIOs is seen in **Figure 5.3**. The drug carrier without SPIOs (**Figure 5.3A**) has a distinctly clear appearance in the particle whereas the drug carrier with SPIOs (**Figure 5.3B**) can be seen with dark elements which are the iron oxides that were encapsulated within the carrier.

Figure 5.3 TEM images of the drug carrier (A) without SPIOs and (B) with SPIOs

In order to further characterize the drug carriers' size distribution, a NanoSight (NTA) experiment was conducted. In this NTA analysis, the drug carriers were diluted in water to an appropriate concentration and injected into a sample resin on the laser module. The module was then placed under the laser during which the reflection of the laser from the particles was acquired by a gamma camera. The particles were recorded for 60 secs and analyzed using the NTA software at an appropriate camera level, screen gain and threshold. From **Figure 5.4A** the size distributions obtained using the NTA have 3 distinct peaks at 87, 411, and 600 nm. The particles with size distributions at 87 and 411 nm constitute to 10.1% of the concentration while the particles at 600 nm constitute to a 89.1% concentration. The peak located at 87 nm is postulated to be the SPIOs that have been released from the drug carrier during the sonication and vortex procedure conducted prior to the analysis and then aggregated together at the point where the sample was injected into the sample resin. The particles at the 411 nm peak are anomalies that occurring during the fabrication step. The bulk of the particles possess a 600 ± 73 nm particles size range. The particles were again characterized with DLS as seen in **Figure 5.4B** Similarly to the NTA characterization, the particles characterized by DLS have a peak at the 598 ± 38 nm size distribution.

Figure 5.4 The size distribution of the drug carriers using the (A) NTA and (B) DLS.

According to on the ICRP distribution model, the size range between 400 – 600 nm has a total deposition in the lungs of between 14-21% total depositions in the lungs. The purpose for having the size range at 500 nm is to reduce the interaction of the particles with the lungs by having them traverse the respiratory channels without being deposited. The smaller the deposition fraction, the better it is for the particles to possess a targeted delivery when incorporated with SPIOs. At 500 nm, the ICRP shows at 18% total deposition in the lungs. This, from the in-silico at force ratio 10, is able to achieve at 75% deposition into the acinus simulated with a 23% dispersion volume. Having a size distribution deviating from the 500 nm mark, i.e. 400-600 nm, would have little significance on the deposition amounts because the total deposition between the 500 nm particles and the 400 and 600 nm particles differs with only around 4%. The impact on the size distribution can be adjusted in the actual administration by increasing the dosing time to compensate for the lost carriers deposited in other regions of the lungs.

5.2 Drug Encapsulation Efficiency and Release Profiles

The encapsulation efficiencies of Q203 and BDQ were 55% and 57%, respectively, as shown in **Table 5.1**. Both Q203 and BDQ have high log P values which are compatible with the hydrophobic nature of the PDLG polymer, and enable them to dissolve in DCM to form the oil phase (with SPIOs and polymer). Although for high positive log P drugs, the encapsulation within a hydrophobic carrier would usually result in high encapsulation

efficiencies, this occurrence was not witness here. Instead, the incorporation of the SPIOs into the PDLG carrier deterred more of the Q203 or BDQ drug from being loaded, resulting in moderate encapsulation efficiencies. A further reduction in the encapsulation efficiencies for both Q203 and BDQ were observed when both drugs were loaded in combination, likely due to competitive loading of the two drugs within a single carrier.

Table 5.1 Encapsulation efficiency of the Q203 and Bedaquiline drug in PDLG 5010

	Log P	Encapsulation Efficiency (%)
Q203 only	7.62*	54.50 $\pm$ 0.71
BDQ only	7.52	56.78 $\pm$ 2.88
Q203+BDQ		40.57 $\pm$ 1.61 (Q203)
(combination)		49.92 $\pm$ 0.84 (BDQ)

* c log P

These moderately low encapsulation efficiencies of Q203 and BDQ obtained are a result from the tradeoff between the loading of the SPIOs and the drugs. The tradeoff by no means negates the importance of having SPIOs into the carrier. Furthermore, the incorporation of SPIOs would have a higher impact to the pulmonary delivery than the system that does not. In a **Table 5.2**, a hypothetical comparison was being made with a system without SPIOs possessing a 100% encapsulation efficiency of both drugs, and a system with SPIOs possessing a 100% deposition and encapsulation efficiencies seen in **Table 5.1**. It has been reported that conventional pulmonary systems has only 10-40% of deposition rates. So, by taking the average, the pulmonary system without SPIOs would have around 25% of deposition. The loading of drugs into one carrier is taken to be at the MIC₉₉ of Q203 and BDQ i.e. 100 nM and 3000 nM, respectively. It can see that when 100 particles are delivered to the lungs, there are more drugs available in the systems with SPIOs than a system that does not. While there are assumptions made, it can be clearly seen that the advantage of incorporation SPIOs into a pulmonary system outweigh its shortcomings.

Table 5.2 Hypothetical system with SPIOs and without SPIOs at different EE

Hypothetical Pulmonary System				
EE		Deposition Efficiency	Drug available in 100 particles emitted	
With SPIOs	100%	25%	100 nM (Q203)	2500 nM (Q203)
			3000 nM (BDQ)	75000 nM (BDQ)
Without SPIOs	40% (Q203)	100%	40 nM (Q203)	4000 nM (Q203)
	50% (BDQ)		1500 nM (BDQ)	150,000 nM (BDQ)

Figure 5.5 shows the release profiles of encapsulated Q203 only, BDQ only, and the combination of Q203 and BDQ in SLF. For all three delivery systems, the release profiles exhibit similar characteristics. In the first phase (0-8 hrs), the drug(s) was (were) released precipitously, with a slower release in the second phase. The percentage cumulative drug remaining for all three delivery systems when plotted against time corresponds to a first-order release kinetics. This indicates that the release is concentration dependent and diffusion controlled ($R^2 \geq 0.9443$). Release of either drug is also independent of the other as the release kinetics are similar whether encapsulated singly or in combination. After 24 hrs, all drugs were completely released. In order to obtain combination efficacy of the drugs, a simultaneous release of both of them is required. This enables them to kill off both the replicating and non-replicating bacteria through the simultaneous inhibition of the $bc_1:aa_3$ complex and the ATP synthase. In addition, because the macrophages within the lungs take around 24 hrs to clear off foreign particles, the complete release of the drugs from the carrier within that time frame is desired. Other pulmonary systems have displayed similar characteristics. In a formulation developed by T.V.P. Doan et. al [1], 80% of the RIF loaded within 2-8 μ m PLGA microspheres were released in a time period of 12-96 hrs. And in study done by the Novel Drug Delivery Systems Lab [3], 200 nm-sized PLGA particles released 100% of the RIF contents within a five hrs time frame. The release of the drugs into the lungs within 24 hrs would allow the drug delivery system to have lower drug loss through macrophage-activated-phagocytosis after that time period and consequently increase the bioavailability of the drugs.

Figure 5.5 Release profiles of encapsulated Q203 only, BDQ only, and Q203 and BDQ (Q+B) in 1 mL of SLF

5.3 *In-silico* Deposition Efficiency

To validate the deposition efficiency of the pulmonary delivery formulations, CFD simulations were performed with 10,000 carrier particles (500 nm in diameter, 28 emu/g magnetic saturation) in the acinus to analyze the deposition efficiency of these particles with respect to the ratio of the magnetic field applied (F_{magnetic}) and gravitational pull (F_{Gravity}). **Figure 5.6** shows that as the force ratio ($F_{\text{magnetic}}/F_{\text{Gravity}}$) increases, the deposition efficiency of the particles into the acinus increases. When the magnetic field yields a force ratio ≥ 10 , the particles can be deposited in the acinus with little or no loss yielding high deposition efficiency.

Figure 5.6 Deposition efficiency of the SPIOs-encapsulated carriers as a function of the force ratio

However, as the magnetic field increases, the dispersion of the aerosol carriers throughout the acinar region suffers (**Figure 5.7** in which the simulations explore the influence of different force ratios). When the force ratios are at 5 and 10, dispersion of the particles spans across 23% of the acinar volume. However, as the force ratio increases from 10 to 1,000, the dispersion volume (i.e. the volume covered by the particles in relation to the entire acinar volume) decreases from 23% to 2% and then eventually to 0.6%, i.e. one alveolus. Higher force ratios, i.e. 100 and 1,000, are unsuitable as a wider particle-drug distribution within the lungs is always preferred, especially for TB treatment.

Figure 5.7 Simulation of 372 alveoli in the acinus. 10,000 particles of size 500 nm with a magnetic saturation of 28 emu/g were introduced into the acinar model with a breathing cycle and subjected to different force ratios (magnetic force against gravitation force) of 1, 5, 10, 100, and 1000. As the magnetic component in the force ratio increase, the deposition amount also increases but reduces the dispersion.

5.4 *In-vitro* Magnetic Targeting

Aerosolization of the drug carriers suspended in the brine solution is generated via a modified nebulization setup and measured at a Mass Median Aerodynamic Diameter (MMAD) of $0.4506 \pm 0.0302 \mu\text{m}$. After aerosolization, the aerosol bolus produced follows the airflow in and travels along the hollow tube until it reaches beneath the

magnet, during which a BH of five secs was activated (**Figure 5.8A**). It can be seen in **Figure 5.8B** that shortly after the breath-hold, the particles were drawn up towards the magnet. Only a small remainder of the nebulized particles is “exhaled”. A clear elliptical stain is visible when the magnet is removed from its position (**Figure 5.8C**). Since not all the deposited particles are localized in one area, the deposition ratio (i.e. the ratio of the particles deposited near the magnet to the particles deposited elsewhere is infinity) cannot be precisely quantified. The experiment was repeated again, varying the magnet’s distance from the tube to 10 mm and then 20 mm. Similarly, for both these cases, the aerosol bolus was again seen to be arrested by the magnet (**Figure 5.8D and Figure 5.8E**). For most magnets, the strength of the magnetic field is inversely proportionate the cube of the distance (r), i.e. $1/r^3$. This means that as the distance between an object and the magnet increases, the magnetic field strength of the magnet falls off with a cube of the distance. The drug carriers have shown to be able to be arrested by the magnet despite having the magnetic strength rapidly falling off. This would imply that the drug carriers are responsive even at low magnetic field strengths. However, if the distance between the magnet and the drug carriers was to be increased beyond 20 mm, a stronger magnet would be required to obtain the same results.

Figure 5.8 *In-vitro* magnetic targeting. (A) Drug carriers aerosolized with a brine solution of 10:1 DI water:NaCl into a 16.8 mm inner diameter hollow tube. The airflow was set at 1.145 L/min. The magnet was placed 0 mm from the hollow tube. The aerosol bolus was calibrated to stop directly below the magnet (B) The aerosol bolus was attracted towards the magnet. (C) The deposited aerosol bolus onto the inner wall of the hollow tube. The elliptical width was measured at 29 mm. (D) Experiment with magnet placed 10 mm from the hollow tube. (E) Experiment with magnet placed 20 mm from the hollow tube.

5.5 Cell Viability

Cell viability was evaluated using a Prestoblu assay to determine the toxicity of these carriers on the lung epithelium cells (i.e. A549). These are type II pneumocytes that are responsible for secreting lung surfactants in the alveoli, and were used because they are one of the first cells to be exposed to the delivery system. Also, A549 is one of the *in-vitro* cell lines accepted for cytotoxicity assays [4-6] in the lungs. Cell viability was

investigated across different concentrations up to 500 $\mu\text{g/mL}$ (**Figure 5.9**). Although it is unlikely that the cells would be exposed to the particles at such high concentrations, we sought to challenge the cells to mimic a situation where the particles would accumulate in the lungs. The results demonstrate that there is no significant difference in cell viability for the advocated drug carrier with and without SPIOs. This indicates that the carrier is non-cytotoxic and may be suitable as a pulmonary delivery system, which is in agreement with other cytotoxicity studies of PLDG [7-9] and SPIOs [10, 11]. The Q203 and BDQ drugs were omitted from drug carrier for the toxicity studies because the amount present are too low to have an impact on the cells. In 500 $\mu\text{g/mL}$ of the drug carrier, there would only be 0.67 μg of Q203 and 4.25 μg of BDQ. The reported toxicity of Q203 is at 10 mg per kg body weight (for rats) [12] with daily dosing, while the daily recommended dose for BDQ is at 400 mg for the first two weeks of pulmonary MDR TB [13].

Figure 5.9 A549 cell viability measured by Prestoblu assay against (A) drug carrier (DC) without SPIOs at 24, 48, and 72h, (B) drug carrier with SPIOs. The groups are normalized with the control and * $P < 0.05$, indicates a significant difference between the groups

For the cell viability, a one-way ANOVA was used. The one-way ANOVA compares the A549 cell viability at different drug concentrations in the three exposure times, i.e. 24, 48, and 72 hours, and determines whether there is a statistical evidence of the associated populations.

Null hypothesis, H_0 : There is a change in cell viability after 3 days of exposure with the drug carrier without SPIOs

Alternative hypothesis, H_1 : There is no change in the cell viability after 3 days of exposure with the drug carrier without SPIOs

Significance level: $\alpha = 0.05$

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Day 1	9	871.3185	96.81317	2.553485
Day 2	9	889.5533	98.83926	1.184339
Day 3	9	917.5897	101.9544	16.95545

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	120.7245	2	60.36225	8.750996	0.001399	3.402826
Within Groups	165.5462	24	6.897758			
Total	286.2707	26				

Since, the p-value is smaller than 0.05, we can reject the null hypothesis and say that there is evidence for the alternative and that there is no significant difference in the cell viability after 3 days of exposure with the drug carriers without SPIOs.

Null hypothesis, H_0 : There is a change in cell viability after 3 days of exposure with the drug carrier with SPIOs

Alternative hypothesis, H_1 : There is no change in the cell viability after 3 days of exposure with the drug carrier with SPIOs

Significance level: $\alpha = 0.05$

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Day 1	9	882.9999	98.1111	4.090889
Day 2	9	869.7316	96.63684	16.81804
Day 3	9	908.7206	100.969	8.16476

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	87.32402	2	43.66201	4.505311	0.021812	3.402826
Within Groups	232.5895	24	9.69123			
Total	319.9135	26				

Since, the p-value is smaller than 0.05, we can reject the null hypothesis and say that there is evidence for the alternative and that there is no significant difference in the cell viability after 3 days of exposure with the drug carriers with SPIOs.

The graph in **Figure 5.10** shows the cell viability of the raw SPIOs on the A549 cells using Prestoblu assay. The concentrations used for the raw SPIOs correspond to the amounts that are loaded into 100, 150, 200, 250, 300, 350, 400, 450, and 500 $\mu\text{g/mL}$ of the drug carrier. From **Figure 5.10** it is seen that the raw SPIOs are benign to the A549 epithelial cells for all 3 time periods, i.e. 24, 48, and 72 hrs with an approximate of 100% cell viability ($p < 0.05$, $p = 3.9\text{E-}05$ *Please see excel spread for the statistical analysis) normalized with the cells-only-control. This is in line with the cell viability assay done for the drug carriers.

Figure 5.10 A549 cell viability measured by Prestoblu assay on raw SPIOs. The groups are normalized with the control and * indicates a significant difference between the groups

Although, the drug carriers at concentrations 100 – 500 $\mu\text{g/mL}$ have shown to be benign to the epithelial cells, recent cytotoxicity studies have shown that at concentrations larger than 100 $\mu\text{g/mL}$, SPIOs can be toxic [14] (More in Chapter 2.10). Because of this, the concentration of the drug carrier to be administered should be lesser than 400 $\mu\text{g/mL}$. Ideally, 100 $\mu\text{g/mL}$ of the drug carrier would be sufficient to observe a therapeutic condition.

From the *in-vitro* experiment of the magnetic targeting assay, 70 aerosol boluses were produced from 2mL of the drug carrier solution. If all conditions remain similar, 1 mL of 100 $\mu\text{g/mL}$ of the drug carrier can produce 35 aerosol boluses which would take an approximate of 5 min for delivery. This means that 240 nM of Q203 and 1530 nM of BDQ would have been administered into the lower airways. For the mycobactericidal activity, in order to see a killing efficacy on the 10^6 BCG organisms, 100 and 500 nM of Q203 and BDQ, respectively, were used. This would mean that the 35 aerosol boluses administered are sufficient to eradicate the bacteria present. Because the drug carriers to be administered would be acted on by the macrophages upon deposition, assuming that within a 12 hrs time period the macrophages in the body would have removed 50% of the particles deposited from the lungs, the amount to be administered should be twice more than intended to overcome this scenario, which in the case of the 100 $\mu\text{g/mL}$ would have been already met. This summary is seen in **Table 5.3**.

Table 5.3 Hypothetical concentration suitable for the administration of the drug carrier into the lower airways

	Time (min)		Concentration (nM)
Delivery Time	5		
100 $\mu\text{g/mL}$		Q203	240
		BDQ	1530
Mycobactericidal Activity		Q203	100
		BDQ	500

5.6 Bactericidal effect of free drugs and drug delivery system on BCG

Here the efficacy of Q203 only, BDQ only, and the combination of both of them were individually evaluated against wild-type (WT) BCG. To ascertain the activity of the free drug combination and the encapsulated drugs, a bactericidal assessment with these two components at MIC_{50} (3 nM for Q203, 120 nM for BDQ) were evaluated. From **Figure 5.11A**, it can be seen that there is insignificant difference in the efficacy between free drugs (FD) and the encapsulated ones (E), indicating that the delivery system does not interfere with the antimicrobial properties of the drugs. With this, the comparison between single and dual encapsulated drug formulation at MIC_{99} (100nM for Q203,

500nM for BDQ) were evaluated. For this assessment, the dosage of the drugs was increased to observe the bactericidal activity below the inoculum value of the combination. The controls used for this study are the untreated WT-BCG and the drug carrier without drugs (DC). In **Figure 5.11B**, the single encapsulated Q203 is able to deter the growth of BCG consistently throughout the study. However, for the single encapsulated BDQ, the BCG is able to proliferate for up to 10 days (although to a lesser extent than that of the controls) before decreasing slightly at day 15. Individual encapsulated drugs, i.e. Q203 or BDQ, were unable to lower the CFU count. However, when both drugs were combined, a two-fold decrease in the bacteria colony can be observed. For the first 10 days, there was a steady decline in the bacteria colonies. After which from day 10 to 15, the killing plateaus.

Figure 5.11 Minimum Bacterial Concentration (MBC) studies of Wild-Type (WT)-BCG against Q203 and/or BDQ. Time points were set at day 0, 4, 10, and 15. (A) Comparison of the combination free drugs (FD) and encapsulated drugs (E) at MIC₅₀. *P < 0.05, significant difference between means (B) Comparison between encapsulated Q203 only, BDQ only, and a combination Q203 and BDQ at MIC₉₉.

To evaluate if there is a significant difference the bactericidal effect of the free drugs with the encapsulated one, a small-sample difference between two means hypothesis testing is used for the different time points, i.e. day 4, 10, and 15.

Null hypothesis, H_0 : There is no significant difference in the means of the free drugs and encapsulated ones, i.e. $\mu_{\text{free}} - \mu_{\text{encap}} = 0$

Alternative hypothesis, H_1 : There is a significant difference in the means of the free drugs and encapsulated ones, i.e. $\mu_{\text{free}} - \mu_{\text{encap}} \neq 0$

	Day 4	Day 10	Day 15
n_{free}	3		
n_{encap}	3		

μ_{free}	2813333.333	27666667	46066667
$\mu_{\text{encapsulated}}$	3680000	74133333	51466667

S_{free}	213853.5324	6394790	4473626
$S_{\text{encapsulated}}$	628649.3458	15951593	10216327

S_p	=			
$\sqrt{\frac{(n_{\text{free}}-1)s_{\text{free}}^2 + (n_{\text{encapsulated}}-1)s_{\text{encapsulated}}^2}{n_{\text{free}} + n_{\text{encapsulated}} - 2}}$		537649.2041	14457293	9602777
$t = \frac{\mu_{\text{free}} - \mu_{\text{encapsulated}}}{S_p \sqrt{\frac{1}{n_{\text{free}}} + \frac{1}{n_{\text{encapsulated}}}}}$		-1.9742344	-3.93641	-0.68872
Degree of freedom		4		
95%		± 2.776		
		Inconclusive	Reject H_0	Inconclusive

Since for day 10, the t value is more than 2.776, we can reject the H_0 and say that there is a significant difference between the two means.

5.7 Discussion

The one-pot synthesis of the drug carriers using a single emulsion technique produced mono-dispersed particles of 500 nm. The SPIOs endowed the drug carriers with magnetic properties, although a decrease in the magnetic saturation of these carriers was observed

when compared to naked SPIOs. The combination of the SPIOs and the PDLG carrier was assessed for its toxicity and from the Prestoblue assay, the drug carrier was found to possess low toxicity to lung epithelial cells as shown from our *in-vitro* data (**Figure 5.8**).

The rationale for using 500 nm particles as the size range for this pulmonary delivery was based on the deposition statistics generated by the ICRP [**Supplementary Figure 1**]. This statistical model was designed according to the semi-empirical equations derived from the deposition data in humans using mono-dispersed particles [15]. In this model, particles in the size range of 500 nm were shown to have the lowest total deposition in the lungs, attributed to the unresponsive nature to Brownian diffusion or gravity. Such particles introduced into the respiratory tract follow the inhalation airflow in and out of the lungs without being deposited. However, to achieve acinar delivery and avoid unintentional targeting to other organs along the respiratory tract, these carriers were incorporated with magnetically responsive SPIOs, and a large volume of deep lung deposition may thus be observed (**Figure 5.6**). Ostrovski et al. [16] had previously demonstrated *in-silico* that when combined with a magnetic component and an appropriate breath-hold maneuver, the 500 nm water droplets can be leveraged for a targeted delivery to achieve 100% deposition in the acinus, which is similarly observed for our 500 nm PDLG carriers encapsulated with SPIOs. As seen in **Figure 5.6**, when the external field strength increases, the percentage of the deposited particles also increases. At a force ratio ($F_{\text{magnetic}}/F_{\text{Gravity}}$) more than 10, the particles are able to reach 100% deposition, but at the expense of volume dispersion (**Figure 5.7**). An optimal balance between these two components, i.e. deposition and dispersion was found when the force ratio is at 10. At this ratio, 75% of the particles that were inhaled were deposited while achieving a dispersion volume of 23% throughout the acinus. Although, a 100% dispersion is always desired, 23% of dispersity still covers a substantial area in which the bacteria can occupy. This force ratio was therefore favourable for a large deposition, while mitigating drug wastage with a good dispersity.

To validate the above numerical analysis results, these 500 nm drug carriers were next evaluated in an *in-vitro* magnetic targeting set up. The carriers were suspended in a brine

solution and nebulized into an aerosol mist before injecting into a hollow tube. The aerosol bolus that entered the tube was set at a breathable range of MMAD $0.4506 \pm 0.0302 \mu\text{m}$ and was observed to be ‘captured’ beneath the external magnetic source. Within two secs of BH, the majority of the aerosol bolus was pulled towards the magnet, while the remainder followed the airflow out of the tube. The BH simulation was necessary to achieve targeting because it allows time for the particles to get pulled towards the magnet. In an experiment with no BH activated, no deposition of the aerosol bolus onto the walls of the tube was observed. To further validate the effectiveness of this magnetic-targeting ability of the drug carriers, the distances between the magnet and the tube were increased to a distance 10 mm and 20 mm. In both scenarios, the carriers continued to exhibit similar flow and ‘capture’ characteristics of the earlier experiment, confirming the magnetization capability of these active-targeting, sub-micron (500 nm) drug carriers.

Conventionally, TB is treated with Rifampicin, Isoniazid, Pyrazinamide, and Ethambutol. A combination of drugs is used so as to mitigate the evolution of MDR strains through monotherapy. On this basis, any treatment of TB should also be aligned towards multidrug therapy. In order to pair Q203 with another anti-TB agent, the accompanying drug should present no cross resistance with Q203 and also targets the ATP synthase complex in the bacteria oxidative phosphorylation, for synergy. BDQ is therefore one such candidate. From the drug release studies in **Figure 5.5**, it was observed that both drugs were released at the same rate (0.07 hr^{-1}) from the drug carrier. This would allow for a fixed ratio of the two drugs to be administered. The mycobactericidal activity of the combination of Q203 and BDQ on WT-BCG is advocatory of their ability to eradicate the TB bacteria upon deposition. Individually, the drugs were unable to reduce bacteria colonies. This is because while Q203 is able arrest the growth of replicating bacteria by inhibiting the cytochrome- $\text{bc}_1\text{:aa}_3$ pathway in the third complex of the Electron Transport Chain (ETC), the non-replicating bacteria are able to sustain their viability due to an alternate branch (ctyochrome-bd) in the same complex that compensate the flow of electrons [17]. BDQ, which depletes the ATP production in the bacteria, was only able to partially kill off the replicating and non-replicating BCG. It is only when both drugs were

combined that the bacteria log count reduced by two folds. Q203 and BDQ inhibitory capabilities to disrupt the flow of electrons in the ETC are crucial factors to the effectiveness of this delivery system.

A comparison was made with other promising pulmonary platforms to evaluate the efficacy of this delivery system. It is important to note that unless the conditions in which the experiments evaluated were similar, the comparison cannot be done impartially. A study done by Keiji Hirota et al. [18] to examine the bactericidal effects on Rifampicin-loaded PLGA microspheres (RPF-PLGA MS) on BCG-infected alveolar macrophages (NR8383). In that study, the administration of RPF-PLGA MS on the BCG-infected macrophages had a greater mycobactericidal effect than un-encapsulated ones and was able to reduce one and a half log CFU counts of the BCG (initial count of 10^5) with an incubation for four days; and an approximately two log reduction with an incubation of seven days. The mycobactericidal effect was more pronounced in encapsulated Rifampicin because after being engulfed into the macrophages, the drug accumulated within them through the sustained released of microspheres resulted in a larger amount of Rifampicin than un-encapsulated ones. Another study conducted by Sandra Suarez et al. [19] explored the effects of PLGA microspheres containing Rifampicin (R-PLGA MS) on *M.tb*. In this in-vitro study, the growth of 50% of the *M.tb* was inhibited when 2 $\mu\text{g/mL}$ of the R-PLGA MS was introduced while a 100% was observed when higher concentrations (20 and 100 $\mu\text{g/mL}$) were used. In both studies, although their delivery systems were capable of bactericidal activities, there was no evidence to support the capability of a high deposition or targeting into the lungs. The results obtained from our newly developed pulmonary delivery system highlight both the bactericidal synergism between Q203 and BDQ and the communicative ability to achieve high deposition amounts into the lungs, as shown from our in-vitro assays and CFD simulations. The combination of these elements together with the low cytotoxicity of these drug carriers substantiates our claims that this delivery system could be a potential delivery system for pulmonary delivery for the treatment of TB.

The encapsulation of Q203, BDQ and SPIOs into an inhalable PDLG carrier is a promising strategy against active TB. The carrier has demonstrated its ability to be targeted into lungs with high deposition volumes through *in-silico* computations and an *in-vitro* magnetic targeting assessment, have a safe interaction with A549 epithelial cells, and possesses a potent killing efficacy on BCG. This pulmonary delivery system has proven to be comparable in terms of the mycobacterial activity with other existing pulmonary systems, and further addresses the issue of the low deposition efficiencies from aerosolizing platforms which other systems lack. In addition, the method of fabrication is straightforward, using a single emulsion technique to incorporate all the components into the drug carrier.

Conditions of the lungs such as bronchoconstriction, inflammation, and airway narrowing can affect the deposition of the lungs. This is because these conditions decrease the cross-sectional area of the pathways and increases the air velocities and turbulence in regions where airflow is normally laminar.

In the *in-silico* assessment of the PDLG drug carriers, the presence of mucus was not included because the computing capacity of the system has been maximized; to accommodate an additional parameter like the mucus layer at different regions of the lungs would require simplification of other parameters, which is not desired. Although, this is an unavoidable flaw in computational fluid dynamics, the incorporation and understanding of the mucus in the scheme of particle deposition can be developed and discussed. One of the possible solutions for the inclusion the mucus layer into a lung deposition model is to segment the lungs into smaller regions. In this way, the computing capacity of the system will not be taxed and additional parameters can be included. A study conducted by Anita Chvatal et. al. [20] uses this approach to investigate the *in-silico* deposition of meloxicam potassium in the lungs. In this study, a Stochastic Lung Deposition Model (a whole-lung deposition model) computes the fraction of the particles deposited in each anatomical region of the airway system and the exhalation fraction. In addition, different parameters characterizing the particles, i.e. density, shape,

aerodynamic properties and also the breathing characteristics, i.e. inhaled volume, inhalation time, breath-hold time, exhalation, breathing mode, etc. can be included.

Another area that can be explored for the in-silico characterization is the interaction of the particles with the mucus. The mucus is a complex hydrogel biopolymer barrier that is continuously produced, secreted, digested, recycled or discarded. During homeostasis, the mucus in the layer in the nasal cavity has a thickness of 5-15 μm , 10-30 μm in the trachea, and 2-5 μm in the bronchi. An in-silico study conducted by Julian Kirch et. al. [21] investigates the mucus interaction of different size particles (micron, submicron, and nano) with its shape, charge, and surface chemistry. The computational model is a two dimensional model that simulates the mucus and the air above it (**Figure 5.12**).

Figure 5.12 Illustration of the square computation model domain. Left and right boundaries were left periodic during computation

This model is a square computation domain with a side length of 100 μm . In the horizontal direction the domain consisted of 320 cells and in the vertical direction consisted of 100 cells which were not equidistant but were adapted to yield square cells in the area of the mucus. With this model, the mucus layer can be tune in terms of thickness to simulate an inflammation condition and to determine the deposition ability of

the inhaled particles at quicker velocities due to the narrowing of the airway. Other parameters, such as the charge of the particles (negative for the PDLG particles) can be also assessed to see whether they will be repelled by the negatively charged mucus layer.

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Chapter 6

Conclusion and Future Works

This chapter draws together the discussions from the results presented in Chapters 4 and 5, and serves as a means to link the results obtained to the hypothesis and objectives. The first part of the chapter concludes from the results obtained and elucidates how the drug carrier fabricated embodies a favourable pulmonary drug delivery system. The second part of the chapter provides a list of future recommendations to further characterize the drug carrier for safe usage.

6.1 Conclusion

The primary aim of this pulmonary delivery system is to provide an alternative for the treatment of active TB which circumvent the problems related to oral delivery. The hypothesis statement describes that the pulmonary delivery of DL-lactide/glycolide copolymer (PDLG) particles encapsulating Q203, BDQ, and SPIOs will allow for an improved pulmonary delivery and a more effective killing of the TB bacteria. In order to test this hypothesis, six main objectives were outlined:

1. Fabrication of solid PDLG particles loaded with Q203, BDQ, and SPIOs possessing specific attributes:
 - a. 500 nm in size for the optimal deep lung delivery
 - b. Magnetic capability which allows the particles to be manoeuvred by a permanent magnet
2. *In-silico* deposition efficiency of the drug carriers into the acinus
3. *In-vitro* magnetic targeting assessment of the drug carriers
4. Cell viability assessment of the drug carrier with A549 lung epithelial cells
5. Mycobactericidal activity of the drug carrier with BCG organisms
6. Comparison of Q203 and BDQ with first-line TB drugs

PDLG have been selected as the host for the drug and magnetic constituents because it is biocompatible, biodegradable, and FDA-approved. Upon hydrolytic degradation of the ester linkages of the copolymer, lactic acid and glycolic acid are produced; both of which are benign to the body. It is imperative that the drug carrier to be administered is biocompatible because it will ensure that the patient being treated does not incite any immuno-response. PDLG have also selected for the reason of being able to dissolve in an organic solvent together with Q203, BDQ, and SPIOs such that a single emulsion technique can be employed.

The incorporation of SPIOs gives the drug carrier magnetic targeting properties and enables them to be directed and arrested into the lower airways with the aid of an external magnet. This in-tandem with a 500 nm size drug carrier can persuade high deposition

fractions. At this range, the particles are able to follow the inspiration flow into the lower airways without being deposited elsewhere; and with the magnet in place, the particles will be arrested in the locality without following the exhalation flow out of the body. This has been validated through the *in-silico* assessment.

In order to further validate the drug carriers' magnetic susceptibility, a series of *in-vitro* assessments using a hollow tube, a smart system, a nebulizer, and magnets were conducted. In this assessment, the carriers were shown to be able to be 'captured' by the magnet when being aerosolized by the nebulizer. The same flow and 'capture' characteristics were observed when the distance between the hollow tube and the magnet increased, demonstrating that the carriers were effective even at a distance. In all these assessments, only a small fraction of the aerosolized particles were being 'exhaled' out which substantiates the proof-of-concept of high volume magnetic targeting.

The drug carriers have also been assessed on its toxicity on A549 epithelial cells. These cells are adenocarcinomic human alveolar type II pneumocytes which are used frequently in cell viability investigations. The drug carriers with and without SPIOs are introduced with the cells at various concentration and are incubated for 24, 48, and 72 hrs. The prestoblue assay ascertained cell viability for all concentration and for all time points. While *in-vitro* cell viability is an important step towards safe usage, there needs to be a more robust scrutiny in terms of *in-vivo* study and immune-responses assessment.

The mycobactericidal activity of the drug carriers on WT-BCG has shown that the combination of Q203 and BDQ have a synergistic effect. From the agar drop plate method, it can be seen that the individually encapsulated drugs were not able to reduce the initial bacterial count. However, when the drugs are used together, the CFU was reduced to more than 2 log counts. In addition, when compared to the first-line drugs dual combinations, the combination of Q203 and BDQ were able to obtained better mycobacterial activity i.e. smaller x/y values than the first-line drugs. This would support the notion of a shorter treatment of TB by virtue of potent antibiotics.

Thus, with these six objectives met, the hypothesis of having an improved pulmonary delivery performance and a more effective killing of the TB bacteria is verified.

6.2 Future Works

6.2.1 Comparison between Combination Encapsulated First-Line Drugs with Q203 and Bedaquiline

The first-line drugs, i.e. Rifampicin, Isoniazid, Pyrazinamide, and Ethambutol are the forefront of TB medicinal treatment. There are always used in combination to deter the development of the drug-resistant strains. While individually, Q203 and BDQ has proven their efficacy against single first-line drugs, there are currently no studies being done to compare the efficacy of encapsulated combination first-line drugs to encapsulated Q203 and BDQ.

For this experiment, two first-line drugs, i.e. Rifampicin and Pyrazinoic acid (the active derivative of Pyrazinamide) will be compared against Q203 and BDQ. The reason for selecting Rifampicin and Pyrazinoic acid is because they are soluble in an organic solvent which makes the technique of solvent evaporation single emulsion possible. Pyrazinamide is an amphiphilic drug. It is able to dissolve in both water and organic solvent. In its un-protonated form, Pyrazinamide dissolves poorly in organic solvent, which means that in order to encapsulate both Rifampicin and Pyrazinamide into a PLGA carrier, a double emulsion technique would need to be employed instead [1].

This while not impossible, the method of double emulsion has a number of challenges. For one, when the primary emulsion is formed, the water phase (W_1) which contains the drug has a tendency to flow out into the secondary emulsion (W_2). This is because the droplet size of the W_1 is small (<500 nm) and unstable. Hence, in order to lower its energy, the W_1 will flow out to the emulsion during the homogenizing step into W_2 . This makes the encapsulation efficiency extremely low. Also, because the encapsulation efficiency is low it is difficult to pair the ratio of Rifampicin and Pyrazinamide according to the MBC value.

In order to determine ratio needed to be used for both first-line drugs, an MIC with the combinations of drug amounts against BCG will be conducted (**Table 6.1**). From this table, the optimal amount of both Rifampicin and Pyrazinamide can be obtained.

Table 6.1 MIC of the combination of Rifampicin (RIF) and Pyrazinoic Acid (PYZ+) against Q203 and BDQ

	1	2	3	4	5	6	7	8	9	10	11	12				
A	Q203						RIF									
B	MIC ₅₀							MIC ₅₀
C	↓							↓
D
E
F
G		←				MIC ₅₀		←				MIC ₅₀				
H						BDQ						PYZ+				
I																

6.2.2 Bactericidal activity on MTB

The next step in regards to the mycobacterial activity will be the evaluation of the drug carriers MTB. This *in-vitro* study with the TB bacteria will give a better insight to the efficacy of the Q203 and BDQ encapsulated carriers. The setup of the experiment will be similar to that of previously conducted mycobactericidal evaluation with BCG.

Briefly, H37Rv bacteria at OD 0.005 in the mid-log phase were grown in 7H9 broth with glycerol to a 10^6 CFU count. MIC₉₉ of the encapsulated Q203 only, Bedaquiline only, and Q203 and Bedaquiline was introduced the bacteria once at the beginning of the experiment. The untreated H37Rv and the drug carrier without drugs were used as controls. At day 0, 4, 10, and 15, 50 μ L aliquots of the culture was dropped onto agar plates and allowed to incubate at 37°C with 5% CO until bacteria colonies were formed. The MBC was determined by multiplying the visible colonies with the dilution factor.

In addition, upon the successful encapsulation of the first-line drugs into the carrier, a comparison with Q203 and BDQ can be made with the H37Rv bacteria.

6.2.3 In-Vitro Targeting in 3D printed Bronchial Bifurcated Airways

The preliminary study of the *in-vitro* magnetic targeting using a hollow tube has made concrete the concept of using iron oxides for targeting. Although rudimentary, the design of experiment showcases the drug carriers' ability to target into a specific location where a magnetic field is present. In order to move forward, a replica of the human lung can be 3D printed out to future validate the drug carriers' potential. This lung model would be able more closely replicate the actual human lungs and the airflow dynamics within.

To prepare the model, a cast for the airway tree will be 3D printed out. Once completed, the shell will be washed with Isopropyl Alcohol and then coated with a transparent lacquer to create a smooth surface. Next elastosil silicon is poured in. The wet cast will be placed into a vacuum chamber to be de-voided of any air bubbles and allowed to set. The dry cast will be removed, ready for the experiment.

The setup of the in-vitro targeting is seen in **Figure 6.1**. In this experiment, a nebulizer will be used to aerosolize the drug carriers (tagged with fluorescence) that are suspended in a salt solution. The aerosol will be injected into the airway tree and made to travel through the airway channels out of the lung model. Along the airway, magnets will be placed externally to 'catch' the aerosol flowing through. To quantify the deposition of the carriers, the lung model will be cut at each bifurcation and washed with DI-water. The residue is then examined using a fluorescent microscope.

Figure 6.1 Schematic of the in-vitro setup using the airway tree

Because the number of generations of the lungs will affect the complexity of the experiment, the lung model at this stage will only go up to five generations (2^5 channels), working with only one side of the lungs.

6.2.4 Human Bronchial Lung Cytotoxicity

In order to further strengthen the preliminary *in-vitro* cell viability data, an experiment on the cytotoxicity of the drug carriers with Human Bronchial Epithelial Cells (HBEpC) will be conducted. HBEpC comes from the human bronchi and is an excellent model to study its interaction with viral infections [2-5].

In this study, the design of experiment will replicate the previous cell viability test differing in the usage of HBEpC instead of the A549 cell line. Briefly, HBEpC (10,000/100 μ L) will be seeded onto 96-well plates and incubated overnight at 37°C under 5% CO₂ atmosphere. The cells will be maintained in Bronchia/Trachea Epithelial Cell Growth Medium. After incubation, the cells will be replenished with 100 μ L of the fresh media containing 100–500 μ g/mL of the drug carrier with and without SPIOs, and incubated for 24, 48 and 72 hrs. And at each time point, the media is removed, and 100 μ L of PrestoBlue assay that has been diluted down ten-folds was added. The cells were then incubated for another hour. After that, 50 μ L of the media was pipetted out onto new

96-well plates and analyzed while the remaining media was discarded and replenish with fresh media. The fluorescence readings were done at 570 nm and 600 nm.

6.2.5 Platelet Activation Evaluation

Platelets are colourless disc-shaped cells which are involved in blood clotting. During administration, when a foreign object enters into the blood stream, the platelets within the vicinity will interact with it. This is a plausibility that might occur during the administration of the drug carriers. Upon the hydrolysis of the PDLG particles, the components within the carrier, i.e. SPIOs, Q203, and BDQ are released into the surroundings and because the desired location of deposition is at the alveolus, the thin epithelium walls within the vicinity can facilitate the entry of nano-sized SPIOs or the drug carriers of permissible size into the bloody stream. Depending on the interaction, the platelets might be activated causing aggregation and thrombus formation. When this happens, on the activated platelet surface membrane, a 140 kD glycoprotein is expressed. The p-selectin expression can be evaluated using enzyme-linked immunosorbent assay (ELISA) to determine platelet activation.

In this study, similar to one conducted by Zhang et. al [6], human blood will be centrifuged at 1500 rpm for 15 min to obtain Platelet-Rich Plasma (PRP) for p-selectin expressing. The samples (similar to that of the hemolytic assay) will be incubated in PRP for 2 hrs at 37°C and then rinsed with calf serum albumin PBS solution three times. The samples are then injected with 200 µL of rat anti-human CD62P antibody and incubated for 1 hr at 37°C. Then 200 µL of horseradish peroxidase-enzyme goat anti-rat poly clonal antibody will be added. The samples are then washed with PBS solution before 140 µL of TMB solution is injected. After reaction for 10 min, 50 µL of 1 M H₂SO₄ was added to stop the reaction. The samples will then be analyzed using a microplate reader at an absorbance of 450 nm.

6.2.6 Activated Partial Thromboplastin Time (APTT)

Blood coagulation involves a series of proteolytic (the breaking down of proteins into smaller polypeptides or amino acids) reactions. By measuring the APTT, which is the

activity of coagulation factor XII (plasma protein), the effect of the degraded drug carrier on the intrinsic coagulation pathway can be investigated.

In this study, 0.5 mL of platelet-poor plasma, obtained by centrifuging whole blood at 3000 rpm, will be dropped onto the surface of the 10 mg of the drug carrier that has been suspended in SLF for 1, 2, and 3 days and dried off in a 24-well plate [6-8]. The plate will then be incubated at 37°C for 30 min. 100 µL of actin-activated cephaloplastin reagent will then be added into the wells, followed by 0.03M of CaCl₂ solution after 3 min of incubation at 37°C. The clotting time of the plasma solution will be measured by a coagulometer. For a healthy blood plasma, the APTT will be around 31.5 ± 0.3 secs.

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APPENDIX

Supplementary Figure 1 ICRP deposition statistics.