The *in vitro* and *in vivo* pharmacokinetic parameters of polylactic-co-glycolic acid nanoparticles encapsulating anti-tuberculosis drugs

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“It always seems impossible until it’s done.” ~ Nelson Mandela

Dedicated to my love, my husband, my best friend

Eswhin Booysen

Preface

This thesis was written in accordance with the guidelines for postgraduate study as set out by the North-West University, Potchefstroom campus. Unless otherwise referenced, chemical drawings were self-drawn using Symyx Draw 3.2 software. Harvard style referencing was used throughout the thesis. Each Chapter include a list of abbreviations, introduction, body of text and conclusion sections with the references for each Chapter provided at the end of the Chapter. The Introduction and aim of study introduces the reasons for the proposed study and how the study was designed. Chapters 1 and 2 are literature reviews relevant to the focus of this thesis. Permissions for all cited illustrations were obtained from either author or journal as copyright rules indicated. Chapters 3, 4 and 5 consist of experimental chapters demonstrating the results obtained. The work described in chapters 3 and 4 were conducted as part of a PhD studentship by myself at the Council for Scientific and Industrial Research (CSIR) in Pretoria, South Africa. The CSIR and the Department of Science and Technology (DST) provided all funding for the research. I also conducted the work described in chapter 5 at the Department of Microbiology, Pathology and Immunology of the Colorado State University, Fort Collins, Colorado, USA where I was hosted as a visiting scholar in the laboratory of Prof. Anne Lenaerts. A Summary and Future Prospects are included at the end of the thesis to discuss the main findings of the studies presented in the thesis as well as formulated conclusions on what was observed and what can be done to further contribute to the scientific outcomes of this study.

Three Appendices (A, B and C) are included in this thesis. Appendix A includes ethics approval letters for studies conducted at different institutions. Appendix B includes publications and conference proceedings where I made specific contributions as a PhD student. In Appendix B1, a first authorship of a submitted manuscript is included. In this manuscript, some of the work described in Chapter 5 was used to prepare a manuscript for publication. As first author, I conducted the experiments, analysed the data and prepared the manuscript, with co-authors' contribution being in assistance in conducting of experiments and review of the final manuscript. In Appendix B2, a published co-first authorship manuscript is included. In this manuscript, conducting of macrophage uptake and cytokine expression experiments and data analysis was done by me (Chapter 3) and the preparation of the manuscript and additional experiments done by the other co-first author Dr. B. Semete. It

is indicated on the publication that we contributed equally to this work. Appendix B3 and B4 are publications where personal second author contributions are demonstrated in terms of work conducted in Chapter 4. Appendix B5 is a first author contribution poster presentation accepted as part of the Council for Scientific and Industrial Research (CSIR) Conference 2010~ General science, engineering & technology. Appendix C is supporting data referred to but not shown in text.

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Abstract

Tuberculosis (TB) is an infectious, deadly disease, caused by *Mycobacterium tuberculosis (M.tb).* In 2010, there were 8,8 million incident cases of TB globally. South Africa currently has the third highest TB incident cases worldwide. In an attempt to address the challenges facing TB chemotherapy, among which frequent dosing and long duration of therapy resulting in poor patient compliance, a novel poly(DL-lactic-*co*-glycolic) acid (PLGA) nanoparticulate drug delivery system (DDS) encapsulating anti-TB drugs was developed. It is hypothesised that this nanoparticulate DDS will address the challenges mentioned by enabling decreased dosing frequency, shortening duration of therapy and minimising adverse side effects. Therefore, favourable modification of pharmacodynamic (PD) and pharmacokinetic (PK) properties of the conventional anti-TB drugs was demonstrated. Furthermore, the nanoparticles will provide a platform for drug delivery to macrophages that serve as hosts for *M.tb.*

The study design was based on determining specific physicochemical properties of the nanoparticulate DDS to elucidate the hypothesis. Spray-dried PLGA nanoparticles were prepared using the double emulsion solvent evaporation technique. *In vivo* analysis of macrophage uptake and possible immunological response in mice were evaluated. *In vitro* protein-binding assays of PLGA nanoparticles encapsulating anti-TB drugs isoniazid (INH) and rifampicin (RIF) were performed with subsequent *in vivo* tissue distribution assays to support protein-binding data generated. Finally, PK/PD analyses were conducted to evaluate the effect of nanoencapsulation on the anti-TB drugs. These involved *in vitro* assays to determine if sufficient drug was released from the nanoparticles to exhibit minimum inhibitory concentration (MIC) and minimum bactericidal concentrations (MBC). Furthermore, *in vivo* drug distribution and drug release kinetics assays of encapsulated RIF, INH, pyrazinamide (PZA) and ethambutol (ETB) in a mouse model were performed.

The results confirmed that the PLGA nanoparticles (<250 nm, low positive zeta potential) were taken up by macrophages *in vivo* with no significant immunological effect. Furthermore the nanoparticles were present in the brain, heart, kidneys, lungs, liver and spleen for up to 7 days following once-off oral dosing at 13.23± 0.11%, 16.81± 0.11%, 54.89± 0.95%, 15.61± 1.15%, 48.48± 2.28% and 5.73± 0.21%, respectively. This was further confirmed by drug analysis demonstrating the presence of INH, RIF and ETB at different time points up to 7 days

in the lungs, kidneys, liver and spleen. However, PZA was not detected. Nanoencapsulated RIF and INH exhibited MICs and MBCs *in vitro* over 14 days and these drugs were also observed in plasma for up to 7 days post once-off oral dosing. ETB and PZA were observed up to 3 days.

From the results generated, it can be concluded that the nanoparticles were taken up by macrophages without eliciting an immune response. This provides a platform for drug delivery to specific sites. Furthermore, the nanoparticulate DDS exhibited sustained drug release *in vitro* and *in vivo* over a number of days above the MIC for the drugs analysed. Sustained drug distribution was also observed. It can therefore be concluded that the hypothesised reduction in dose frequency and duration of therapy for this DDS is a possibility.

Keywords: tuberculosis; PLGA nanoparticles; drug delivery systems; pharmacodynamics; pharmacokinetics; protein-binding; biodistribution; cytokine expression; drug release

Uittreksel

Tuberkulose (TB) is 'n aansteeklike, dodelike siekte, wat veroorsaak word deur *Mikobakterium tuberkulose (M.tb)*. In 2010 is 8,8 miljoen gevalle van TB wêreldwyd gerapporteer en Suid-Afrika het tans die derde hoogste TB-insidensie ter wêreld. In 'n poging om die uitdagings van TB-chemoterapie, soos gereelde dosering en die lang duur van terapie – wat lei tot swak pasiënt meewerkendheid– aan te spreek, is 'n oorspronklike poli(DL-laktaat-*ko*-glycolitiese) suur (PLGA) nanopartikel afleweringstelsel wat anti-TB-middels enkapsuleer, ontwikkel. Daar word gepostuleer dat hierdie nanopartikel afleweringstelsel 'n platform sal skep vir geteikende aflewering na makrofage, dat dit bio-vereenigbaar sal wees in terme van immunogenisiteit en dat dit gunstige farmakodinamiese (PD) en farmakokinetiese (PK) eienskappe van die konvensionele anti-TB middels sal verseker. Só kan dit die vele uitdagings van TB-behandeling aanspreek, deur ‘n afname in doseringsfrekwensie, ‘n korter duur van terapie en moontlike verlaagde toksisiteit.

Die ontwerp van hierdie studie is gebaseer op die bepaling van die fisies-chemiese eienskappe van die nanopartikel afleweringstelsel, om die hipotetiese bevindinge toe te lig. Spuitgedroogde PLGA nanopartikels is voorberei met die dubbele emulsie oplosmiddel verdampingstegniek. *In vivo* analise van sellulêre opname en moontlike immunologiese reaksie is in muise geëvalueer. *In vitro* proteïenbindingstudies van PLGA nanopartikels, geënkapsuleer met die anti-TB-middels isoniasied (INH) en rifampisien (RIF) is uitgevoer, met die daaropvolgende *in vivo* weefselverspreidingstudies om die proteïenbindingdata wat gegenereer is, te ondersteun. PK/PD analises is gedoen om die studie af te rond. Hierdie analises het bestaan uit *in vitro* studies om te bepaal of genoegsame geneesmiddel vrygestel is van die nanopartikels om minimum inhiberende konsentrasie (MIK) en minimum bakterisidiese konsentrasies (MBK) te bereik. Verder is *in vivo* geneemiddel verspreiding en geneesmiddel vrystellingskinetika-studies met nanopartikels, geënkapsuleer met RIF, INH, pyrazinamied (PZA) en ethambutol (ETB), ook in 'n muismodel uitgevoer.

Die resultate bevestig dat die PLGA nanopartikels (<250 nm, lae positiewe zeta potensiaal) opgeneem is deur makrofage *in vivo*, met geen beduidende immunologiese reaksie nie. Verder was die nanopartikels teenwoordig in die brein (13.23± 0.11%), hart (16.81± 0.11%), longe (15.61± 1.15%), niere (54.89± 0.95%), lewer (48.48± 2.28%) en milt (5.73± 0.21%) vir tot

sewe dae ná eenmalige orale dosering. Dit is verder bevestig deur geneesmiddel-analise, wat die teenwoordigheid van nano-gëenkapsuleerde INH, RIF en ETB op verskillende stadia tot sewe dae in die longe, niere, lewer en milt getoon het. PZA is nie opgemerk nie. Nano-geënkapsuleerde RIF en INH het MIK en MBK *in vitro* oor 14 dae getoon en hierdie geneesmiddels is ook waargeneem in die plasma, vir tot sewe dae ná eenmalige orale dosering. ETB en PZA is waargeneem tot op drie dae.

Uit die resultate gegenereer kan dit afgelei word dat die opname van nanopartikels deur makrofage 'n moontlikheid is, sonder om 'n immuunrespons te ontlok. Verder het die nanopartikel afleweringstelsel genoegsame geneesmiddel *in vitro* en *in vivo* vrygestel oor 'n aantal dae bo die MIK. Volgehoue geneesmiddel verspreiding is ook waargeneem. Dit kan dus afgelei word dat die hipotetiese vermindering in die frekwensie van dosering en die duur van terapie vir hierdie afleweringstelsel wel 'n moontlikheid is.

Sleutelwoorde: tuberkulose, PLGA nanopartikels, geneesmiddel afleweringstelsels; farmakodinamika, farmakokinetika, proteïenbinding; weefselverspreiding, sitokien uitdrukking, geneesmiddel vrystelling

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Introduction and aims of the study

TB is an airborne and deadly infectious disease caused by the bacteria *M.tb* primarily infecting humans. Subsequent to inhalation of the bacteria, infection commences as soon as the bacteria reach the pulmonary alveoli. Here, invasion and replication occurs within the alveolar macrophages (WHO, 2011:3). Systemic absorption of the bacteria may also result in spinal and/or cerebral TB. For the past two decades, the increase in the prevalence of TB has led to much research focus in the medical and biomedical community to address this challenge. It has become the leading killer of young adults on a global scale affecting almost one third of the global population. An estimated 8.8 million incident cases (range, 8.5 - 9.2 million) were reported in 2010, which equates to 128 cases per 100 000 population. The most occurrences of these estimated cases were in Asia (59%) and Africa (26%). Lower occurrences were reported in the Eastern Mediterranean region (9%), the European region (7%) and the region of the Americas (3%) (WHO, 2011:10). Approximately 1.4 million (range, 1.2 – 1.5 million) people died of TB in 2010 (WHO, 2011:17). South Africa, one of the 22 high burden countries, currently has the third highest incidence rate globally (range 0.40 million - 0.59 million) (WHO, 2011:11). Various drug regimens exist today that have shown success in TB treatment (Janin, 2008:2480). However, poor patient compliance due to long duration of therapy and drug side effects in some cases has led to the inefficiency of these treatment regimens resulting in the drug resistant strains, multidrug resistant TB (MDR-TB) and extremely drug resistant TB (XDR-TB) (Rattan *et al*. 1998:195; Raviglione, 2006:1186).

The Directly Observed Treatment, Short Course (DOTS) was an initiative launched to assist with the issue of poor patient compliance and subsequent treatment failure, which is further supported by the Stop TB partnership. The Stop TB partnership strategy is based on six components, namely DOTS expansion and enhancement, address human immunodeficiency virus (HIV) – TB co-infection, contribute to health system strengthening, engage all health care providers, empower communities and people with TB and enable and promote research (Jassal & Bishai 2010:S157).

The research into ways of combating this life threatening disease has also lead to many novel drug discoveries as well as the use of other antimicrobials, antibiotics and antifungals which

are currently undergoing clinical trials (Ahmad *et al*. 2005:20; Ahmad *et al*. 2006:544; Ahmad *et al*. 2008:142; TB Alliance, 2012:1). The various challenges in TB chemotherapy have also provided a platform for on-going development and enhancement of drug delivery systems to reduce dose frequency and potentially increase patient compliance, specifically the applications of nanotechnology employing nanoparticulate drug delivery systems.

DDS can be classified by dividing a broad spectrum of DDS into two groups, namely macromolecular and particulate delivery systems. The latter includes micelles, liposomes and nanoparticulates (nanospheres and nanocapsules). This thesis focuses on nanoparticulate drug delivery systems. The definition of the size range of nanoparticles is a challenging task due to the ubiquitous use of the term nano- and nanotechnology. Oberdörster *et al* (2005:825) described a distinction between nanosized particles which includes all ambient and engineered spherical nanosized particles <100 nm and ultrafine particles (UFPs) which are ambient laboratory-generated nanosized particles that are not produced in an engineered, controlled way, but has the same size range. However, nanoparticles used in pharmaceutical applications have been described as colloidal particulate dispersions or solid particles ranging from 10 – 1000 nm (des Rieux *et al*. 2006:3; Mansour *et al*. 2009:300; Ledet and Mandal 2012:7). Nanoparticles by definition include monolithic nanoparticles (nanospheres) where the drug is adsorbed, dissolved or dispersed throughout the matrix (Gelperina *et al*. 2005:1487; Shegokar *et al*. 2011:102). Nanoparticles usually consist of biodegradable and biocompatible, natural or synthetic polymers (Shegokar *et al*. 2011:102). Nanoparticles as drug delivery systems offer many advantages, as proposed by Couvreur and Vauthier (2006:1417), such as drug protection from degradation, enhanced absorption by diffusion through the epithelium, modify pharmacokinetic and drug tissue distribution profile and/or improve intracellular penetration and distribution. In addition, nanoparticles pose advantages of high stability and high carrier capacity due to its large surface area, their ability to incorporate hydrophilic and hydrophobic drugs, their variable routes of administration and its ability to be designed to enable controlled drug delivery (Gelperina *et al*. 2005:1487).

Nanotechnology-based DDS has already been approved by the Food and Drug Administration (FDA) in the treatment of various ailments and diseases. Among these are Abraxane®, a formulation for the cancer chemotherapy drug paclitaxel, Cimzia®, a formulation of anti-TNF-α antibody to treat Crohn's disease and Pegasys®, a formulation of

interferon-alpha-2a to treat hepatitis C, to name a few (Ledet & Mandal 2012). Nanoparticulate systems encapsulating anti-TB drugs have been previously investigated (Ahmad *et al.* 2008; Du Toit *et al.* 2008; Pandey *et al.* 2005; Sharma *et al.* 2004; Torres-Chavolla & Alocilja 2011; Verma *et al.* 2011). Published nanoparticle formulations for TB chemotherapy are all in development phases and none are currently FDA approved or available on the pharmaceutical market. This study aims to add to the knowledge base of the vast nanoparticulate formulations being investigated for the treatment of TB.

A nanoparticulate system was formulated using the polymer PLGA, using a novel spray-drying technique (Kalombo, 2008). Spray-drying has seen wide applications in the field of drug delivery. However, the application of this technique to produce solid nanoparticles was in fact a drying method of nanocapsules prepared by other techniques. Essentially already prepared nanoparticles were subjected to spray-drying resulting in particles with a very broad size range from nano to micron. Therefore, the need for a spray-drying technique to directly formulate spherical, smooth-surfaced nanoparticles below 250nm with a narrow particle size distribution was identified. This cost effective and fast drying process for application in anti-TB drug encapsulation with the properties mentioned earlier would provide a much needed platform in addressing the problem associated with TB chemotherapy. Since these nanoparticles have not been analysed to determine *in vitro* and *in vivo* capabilities, the aim of this PhD study was to elucidate various PK/PD variables for anti-TB drugs encapsulated in this nanoparticle formulation and in so doing contribute to further development and possible market application.

Aims and objectives of the study

The overall objective of the research was to treat the hypothesis that TB treatment can be improved by modifying the PK and PD properties of ant-TB drugs through nanotechnology drug delivery system. To attain this objective, the specific aims of the study were as follows:

1. To validate a FACS method for PLGA uptake by macrophages;
2. Elucidate the *in vivo* cellular uptake of PLGA nanoparticles post oral and intraperitoneal administration with focus on macrophage uptake;
3. Assess the *in vivo* immunological response post cellular uptake of these particles;
4. To study effect of polymer coating of the particles on *in vitro* protein-binding and *in vivo* biodistribution of the nanoparticles;
5. Develop a method for the determination of INH, RIF, PZA and ETB in plasma and tissue homogenates using liquid chromatography mass spectrometry (LCMS-MS);
6. To investigate the effect of PLGA encapsulating drugs on the *in vitro/in vivo* PK/PD of INH, RIF, PZA and ETB with specific focus on:
    a. *In vitro* efficacy, i.e. is sufficient drug released from the nanoparticles to reach MIC's and facilitate growth inhibition of *M.tb* and MBC's for complete bacterial killing;
    b. Analyse *in vitro* MIC using serum from mice treated with nanoencapsulated drugs to determine whether sufficient drug is bioavailable to exhibit growth inhibition of *M.tb*;
    c. *In vivo* drug release assays over 10 days by analysing drug plasma levels following once-off oral administration in mice. These drug levels should be above the MIC determined in (b) to be a significant finding; and
    d. Harvest organs from the mice in (c) to determine drug distribution of the encapsulated drugs over 10 days by drug analysis of tissue homogenates.

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