Chapter 1: General introduction

1.1 Epidemiology of tuberculosis

Tuberculosis (TB) has returned to become one of the leading causes of preventable deaths in some 200 countries and territories, including South Africa [1]. Paleopathology studies showed that the *M.tb* complex was detected even in Egyptian mummies by making use of modern day DNA and HPLC analysis [2, 3]. The bacillus causing the actual disease, *Mycobacterium tuberculosis* (*M.tb*), was identified and described by the German physician Robert Koch in 1882 [4]. Prior to Robert Koch's work, the disease "consumption" was believed to have various sources depending on the affected community's folklore [5]. Early treatments focused on a healthy diet and the administration of expectorants and purgatives, with the first sanatorium opened in 1854 in Germany where good hygiene and fresh air were thought to stimulate the body's natural immune system. Consequently the success rate was much higher than any previous treatment and the system was adopted by other countries such as Britain. The further critical improvement of public health reduced the number of tuberculosis cases even before the introduction of antibiotics in the mid 20th century [6].

However, an increase in mortality rates from the 1980s changed this perception dramatically. It could be attributed to the breakdown in health services, the spread of HIV/AIDS and the emergence of multidrug-resistant TB (MDR-TB) [5]. Co-infection with the human immunodeficiency virus (HIV), a phenomenon that started in the 1980s and became eminent in the late 1990s, causes a severe burden on the individual's immune system making them susceptible to opportunistic infections, of which TB is the most prominent. The mortality rate for individuals which are co-infected with TB is very high [1]. MDR-TB is a dangerous form of drug-resistant TB, that comes about when the infecting bacillus develops resistance to at least INH and RIF, the two most often used first line anti-TB drugs.

As indicated in the global tuberculosis control report of 2009 from the world health organisation [1], South Africa currently has the highest incidence of TB per 100 000 (358 per 100 000) people in the world. In 2007 an estimated 112 000 people died of

TB in South Africa alone, of which 94 000 (72%) were co-infected with HIV [1]. In 2007, the prevalence of TB per 100,000 people was highest in sub-Saharan Africa, and was also relatively high in Asia [1] as illustrated in figure 1.1.

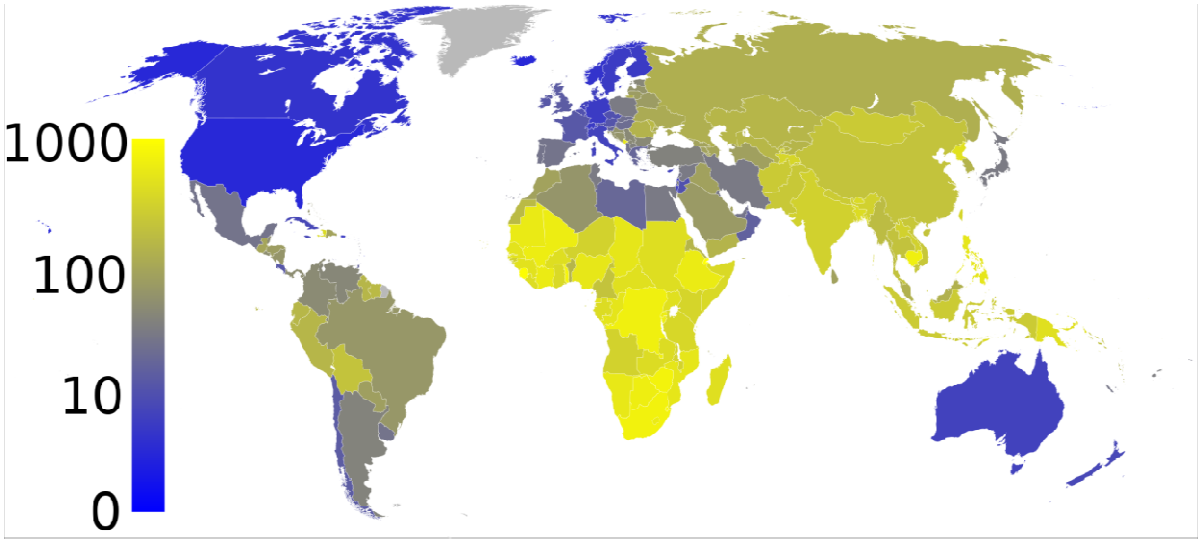

Figure 1.1 *Worldmap indicating TB incidence for 2007 [1].*

1.1.2 MDR-TB in South Africa

The serious threat of tuberculosis, especially MDR and extensively drug resistant (XDR) TB, is a great concern in Southern Africa particularly pertaining to individuals with HIV/AIDS. XDR-TB comes about when resistance to INH and RIF is compounded by an additional resistance to the second line drugs, including any fluoroquinolones and at least one of the three injectables (kanamycin, amikacin or capreomycin) [7].

The concern about XDR-TB was emphasized following a clinical study in 2006 at the Church of Scotland Hospital in KwaZulu-Natal, South Africa. Of the 536 TB patients hospitalized at the time, 221 were found to have MDR-TB, of which 53 were diagnosed with XDR-TB. Of these, 52 died within 25 days. At the time, it was thought that the co-infection of 44 of these patients with HIV was the reason behind their development of XDR-TB [8]. However, recent evidence presented by Dr. Tony Moll at the 2nd TB conference, 1-4 June 2010 in Durban South Africa, indicated that

the XDR-TB primarily originated in the hospital through inadequate infection control. XDR-TB developed in patients that never had TB or HIV infection before, but were hospitalized for other ailments in wards that held one or two undiagnosed XDR-TB patients. In another study done in a HIV co-infected population at a South African gold mine, it was found that existing TB control measures were insufficient to control the spread of drug resistant TB. Furthermore inappropriate therapy as well as a delay in diagnosis contributed to drug resistance and the transmission of the disease [9].

1.2 TB treatment

The era of antibiotics started when streptomycin was discovered in 1946 followed by the successful testing of isoniazid (INH) in 1952, which was shown to be the most important antibiotic in the standard treatment regime against TB. Other drugs were developed in the following years: pyrazinamide in 1954, ethambutol in 1962 and rifampicin (RIF) in 1963 [5].

The treatment of tuberculosis differs from that of other infectious diseases due to the long treatment time needed to cure the patient [10]. A characteristic difficulty of tuberculosis is the persistence of the pathogenic mycobacteria, regardless of prolonged antibiotic treatment. The micro-environment containing dormant bacteria could change over a period of time causing the bacteria to recommence growth, at which stage they are vulnerable to the standard drugs [11, 12]. Because some of the subpopulations of *M.tb* may not be eliminated effectively with standard antibiotics, prolonged periods of the treatments are required [13]. These heterogeneous subpopulations of the bacteria are able to survive within granulomatous lesions surrounded by foamy macrophages in a persistent or latent state, with lacking clinical symptoms [13, 14]. Most bactericidal drugs are only effective against actively growing bacilli and the extended treatment times are needed to then inhibit the regrowth of the bacteria [15]. The length of treatment depends on the presence of non-replicating bacteria and pathogens in a stationary phase present in old lesions of fibrotic tissue [16]. The implementation of a 6 month or longer treatment regime with antibiotics for cases of susceptible TB resulted in a remarkable reduction in the

number of deaths of tuberculosis cases per 100 000 population in the 1960s, such that TB was thought to be a curable disease that was easy to manage.

Antibiotics against TB can be classified into two lines of combination treatment, of which application of the more expensive and less efficient second line is dictated by the development of drug resistance to the first. The decision to commence with a treatment regime for TB is not taken lightly, due to the severe side effects that can occur. For example, first line drugs can cause, drug induced hepatitis, nausea, deafness and progressive loss of vision [10]. The first line of drugs includes INH, RIF, ethambutol, pyrazinamide and streptomycin used for the treatment of drug sensitive TB. There are currently 6 second line drugs used for the treatment of MDR-TB. However theses drugs have more toxic side effects (e.g. cycloserine). Second line drugs are difficult to come by in developing countries (e.g. fluoroquinolones) or are less effective than first line drugs (e.g. *p*-aminosalicylic acid). XDR TB brought the concept of ’third line’ drugs to the fore that may be used in such extreme cases as a last resort. These are drugs that are not listed by the WHO as second line drugs or of which the efficacies are not yet proven [17].

The current standard first line treatment regimen according to the South African tuberculosis control programme of 2004 consists out of an initial (or intensive) phase of 2 months consisting of 4 drugs INH, RIF, pyrazinamide, and ethambutol. Streptomycin is added to the regime when the person is re-treated for TB. A continuation phase of 4 months with INH and RIF follows after the intensive phase to effect sterilization, i.e. the complete elimination of the infecting mycobacteria [10]. Due to the duration of the treatment and the increased probability of non-compliance that this holds for the patient, drug resistance to any one drug can develop.

Most of the standard chemotherapy is not effective for individuals that have MDR-TB and there is practically no cure for XDR-TB. Drug resistant mycobacterial pathogens are increasingly detected in persons who have been previously treated for TB where a possible cause could be the failure to complete lengthy drug regimens and the pathogens becoming resistant to especially the two first line drugs, INH and RIF through mutations [16, 18-20]. The approach to control this disease now is either to discover new chemotherapies effective against *M.tb*, as well as to enhance the potential of existing drugs to treat MDR-TB [21].

Treating TB patients who are co-infected with HIV poses some major challenges. This includes drug-drug interactions between antiretroviral drugs (protease inhibitors and non- nucleoside reverse transcriptase inhibitors) and rifamycins, which could result in subtherapeutic concentrations of anti-retroviral drugs. When overlapping toxicities of the anti-retroviral and anti-tuberculosis drugs increase, discontinuation of the treatment may be required. Another complication is immunopathological reactions and clinical deterioration due to immune reconstitution inflammatory syndrome where a worsening or recurrence of TB occurs when antiretroviral treatment is commenced. It is suggested that antiretroviral therapy should be delayed until the intensive phase of anti-tuberculosis treatment is completed, but delayed antiretroviral therapy on the other hand also increases the risk of morbidity and mortality in patients in the advanced stages of HIV [20, 22].

If a new TB treatment is going to replace the already existing therapy then it should at least shorten the duration of treatment or reduce the number of dosages to be taken. Furthermore the new drug should improve the treatment of MDR-TB or provide effective treatment against latent TB infection [23]. A current approach to address the efficacy of drug treatment lies in the investigation of novel systems for drug delivery. One such approach that was investigated during the course of this thesis investigated the feasibility of using sustained release nanoparticles together with a targeting ligand to target TB infected cells.

1.3 Nanoparticles (NP) as drug delivery vehicles

1.3.1 Background

Improvement of anti-tuberculosis drugs has enjoyed a lot of attention in the past decade [16]. New approaches for TB drug treatment are becoming essential to combat this disease, especially for patients with co-infections and drug resistance. Various therapeutic compounds suffer from limitations which are primarily due to low solubility, short half life, rapid clearance from the biological system and considerable side effects. Some anti-tuberculosis agents such as RIF have several drawbacks with

poor aqueous solubility, low stability and poor bioavailability [16]. A way to address this issue lies in the development of nanotechnology based drug delivery systems.

Nanotechnology has many definitions but can mainly be summarised as "the design, characterization, production, and application of structures, devices, and systems by controlled manipulation of size and shape at the nanometer scale (atomic, molecular, and macromolecular scale) that produces structures, devices, and systems with at least one novel/superior characteristic or property [24].

Under this definition also falls the study of medicine in the nanometer scale. Nanomedicine could be defined as "the use of nanoscale or nanostructured materials in medicine that according to their structure have unique medical effects" [25].

Some of the earliest forms of nanodrug delivery vehicles were lipid vesicles i.e. liposomes described in 1960's by Bangham *et al*. [26]. Since then several organic and inorganic molecules were investigated for their use as vehicles in nanomedicines. The first example of a controlled release polymer was reported in 1976 [27]. Thereafter cell specific targeting of nanomedicines were investigated with vehicles such as liposomes in 1980 [28]. In later studies the use of polyethylene glycol (PEG) to increase blood circulation time for liposomes and polymers followed. The approval of doxorubicin liposomes for the treatment of AIDS associated Kaposi's sarcoma was achieved in 1995 [28]. From there onwards an array of nanomedicine devices were patented and described in literature with the majority aiming at improved drug solubility and addressing poor bioavailability [25]. In a study done by Wagner and colleagues in 2006 they identified 38 nanomedicine products on the market contributed to 75% of the total sales on drug delivery systems. However, none of the current nanomedicines approved for commercial use was for the treatment of TB [25].

Some of the physiochemical properties that make nanomedicine so appealing will be discussed in more detail.

1.3.2 Physicochemical properties

The advantage of nanomedicine lies in the size of the material/particles used, the ability to be able to control the release of the therapeutic agent and being able to cross biological barriers that hindered successful therapies in the past [29]. Further more, NPs can improve drug solubility and facilitate intracellular drug delivery as well as targeting drugs to the site of infection. The small size of the nano molecules can assist in the uptake and biodistribution of the particles in that they could more easily traverse capillaries and therefore penetrate deeper into the target areas, thus increasing accumulation and diffusion in the tissue as was shown by Rijcken *et al.* They also indicated that the size could influence the circulation half life. As the size decreases, the circulation time increases as the smaller particles are less prone to opsonisation, thereby reducing the required frequency of drug intake, increasing the efficacy of the dose and possibly even shortening the duration of treatment [30].

Passive or active targeting can be achieved with nanoparticulate drug delivery systems. Passive targeting is mostly achieved because of the small size and longer circulation time of the particles that facilitate accumulation at the pathological site, possibly due to locally increased capillary permeability [31]. With active targeting, the surface of the nanoparticle is modified with targeting molecules (homing devices) that interact with their ligands in the tissue and may provide a way for the drugs to reach the site of interest with high specificity [31].

The zeta potential or the surface charge of the particle is also a good indicator for the probability of particle uptake. Phagocytosis increases when a particle has a positive charge compared to being neutral or negative [30]. Additionally the stability of the nanoparticles in terms of aggregation in different media can be predicted with the known zeta potential. Neutral nanoparticles tend to aggregate, while charged nanoparticles repel one another [30, 32, 33].

In comparison to more bulky carriers smaller particles have a larger surface area per unit of mass, due to the increase in surface-to-volume ratio. This can influence the release kinetics of the particles. The controlled release of a therapeutic agent from a nanocarrier is a big gain from this technology. Nano encapsulated pharmaceuticals can be obtained by dissolving, entrapping, encapsulating or binding of the drug to the nanoparticle matrix.

The encapsulated or adsorbed drugs can be released from the nanoparticle carriers in a number of different ways. Desorption of the drug from the surface, diffusion through either the nanoparticle matrix or nanoparticle wall, erosion of the nanoparticle matrix and a combined erosion diffusion process [34, 35]. The rate of release of the drugs depends mostly on the degradation, solubility and diffusion of the composition of the matrix material. For example the molecular weight of the polymer determines the degradation rate of the material, wherea higher molecular weight compound degrade slower than a compound with a lower molecular weight. Other advantages of nanoparticles include the chemical control of surface characteristics and the actual polymer composition to form different constituents of the polymer to suit the needs for the different routes of administration [36].

1.3.3 Preparation and modification of polymeric nano carriers

Different nanotechnologies are available that have been applied for medical purposes and drug development. Nanoparticle drug delivery systems can either be formulated with synthetic or natural compounds [35]. Formulation is one of the most important considerations for nanocomposite systems. Nanoparticle composition and morphology of the core and corona determine the type of nanoparticles produced [30]. The nature and density of the nanoparticle coatings, their size and dissolution properties are the primary factors that determine the efficacy of drug delivery.

Polymeric nanoscale vesicles can be chemically modified and manipulated to have different surface ligands and physical properties to contain a number of different therapeutic agents for various diseases, as illustrated in figure 1.2. Typically the materials for forming the nanoparticles have been prepared mainly by dispersion of the preformed polymers or polymerization of the monomers [34].

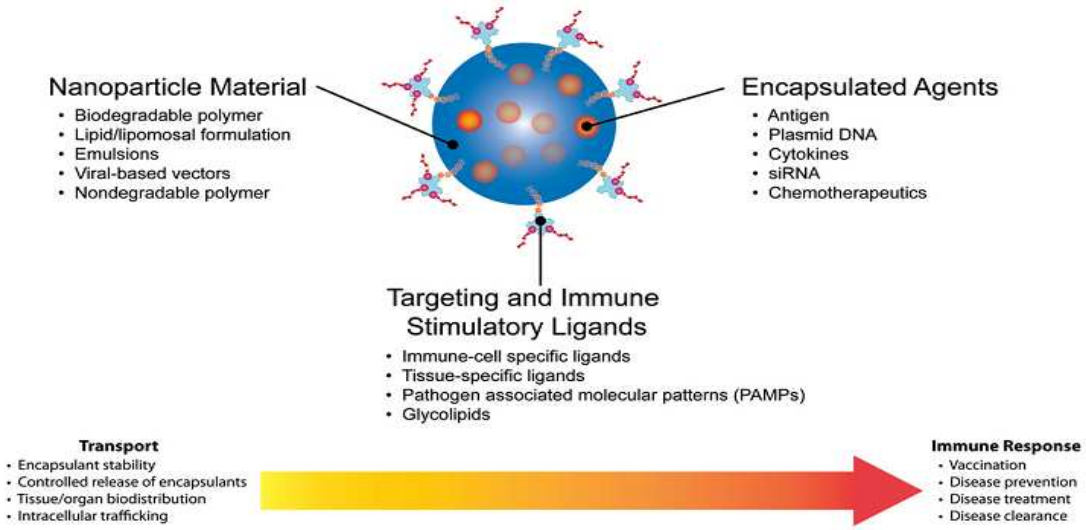

Figure 1.2 *Schematic illustration of different modifications and usages to formulate the nanodrug carrier [37].*

Nanoparticles can also be synthesized from different materials to control the rate of drug release, capacity for drug loading and intracellular trafficking of the particle. The material can be designed to protect and stabilize the particles in adverse physiological and external environments. Physical stability of the nanoparticulate system can be controlled to avoid aggregation and sedimentation over time that could create a potential safety concern [38]. Addition of ligands or modifications to the surface of the nanoparticles have been shown to achieve certain beneficial properties, like increasing retention time in blood by evading opsonisation and targeting to the site of infection to reduce the normal dosage required [35, 37]. Surface antigens such as peptides, nucleic acid aptamers and antibodies have been added to functionalize the surface of the particle and to target certain ligands in the biological environment [30, 39-41].

A major biological barrier in controlled drug delivery is the process of opsonisation, where opsonins such as antibodies or complement molecules bind to a foreign molecule to enhance the process of phagocytosis and therefore clearance of the particle by the immune system. Surface modification of the nanoparticles with ligands aiming at increasing the active life of the material was demonstrated with PEG or PEG containing copolymers. The PEG was grafted or adsorbed to the surface of the

nanoparticle to form a hydrophilic layer that increased the blood circulation time, possibly due to the hindrance of the adsorption of plasma proteins (or opsonins) onto the surface of the nanoparticle [30, 42, 43]. Pluronics, copolymer surfactants made from poly(ethylene oxides) and poly(propylene oxide) mixtures, were also used to increase circulation and were found to be less prone to phagocytosis and better able to accumulate within tumour cells by enhanced permeation and retention (EPR) through passive targeting [44]. Polaxamers, consisting of block copolymers of ethylene oxide (EO) and propylene oxide (PO), have also been used to coat nanoparticle surfaces to prolong circulation time and to reduce the uptake in the liver [31, 43]. Polysorbate 80 (Tween 80) coated nanoparticles are an example of molecules that enable the nanoparticles to cross the blood-brain barrier [45].

1.3.4 Different types of nanodrug delivery systems

A variety of drug delivery systems exist that can be used to improve the efficacy of a drug. Here, the focus will be on the different types of nanodrug carriers that are currently employed for therapeutics (Figure 1.3).

1.3.4.1 Liposomes

Liposomes (lipid vesicles) consist of a phospholipid bilayer with an aqueous core. They can be nano or micro sized. The large variety of phospholipids that can be used, allows for formulation of a range of biochemical and biophysical properties of liposomes [46] to incorporate hydrophobic agents within the membrane layer or entrap hydrophilic molecules in the aqueous core. Liposomes are taken up readily by phagocytic cells of the reticuloendothelial system making them ideal carriers for targeting macrophages, in particular when negatively charged phospholipids are included in the liposome formulation that enhance macrophage binding and uptake. Some limitations that this type of carrier pose is that they tend to be leaky, thus controlled release is not as effective, and also the liposomes is not very stable over time during storage [46, 47].

1.3.4.2 Dendrimers

Dendrimers are well defined hyperbranched macromolecules with a three dimensional structure usually with a size range of 1-100 nm. Dendritic polymers may be symmetrical, monodispersed branched structures or could be irregular, polydispersed assemblies of random hyperbranched polymers, thereby providing a number of options to select for incorporating therapeutic drugs. The end groups of the branched chains can be functionalized in order to bind molecules such as proteins and antibodies, these dendrimers could be challenging to synthesize to obtain the organised architecture required to form the specific dendrimer [48].

1.3.4.3 Nanoemulsions

Nanoemulsions are nanodispersions generated spontaneously and are formed from translucent oil in water dispersions. These emulsions are stable in suspensions due to their small droplet size [38]. Colloidal suspensions of high concentrations of pure drugs together with surfactants are called nanosuspensions. These suspensions are normally used for drugs that have a limited solubility in water and in oils [38]. The technique to form these suspensions is quite cost-effective, because poorly soluble drugs can be handled better, requiring simpler techniques such as high pressure homogenization, in the manufacturing process.

1.3.4.4 Niosomes

Niosomes, another type of nanodispersions are liposome-like vesicles produced from charged phospholipids and non-ionic surfactants. These molecules were developed as alternatives to overcome several limitations of liposomes. Niosomes can be used to host hydrophilic and hydrophobic drugs [16, 49].

1.3.4.5 Solid lipid nanoparticles (SLN)

SLN are formed from nanocrystalline suspensions of lipids prepared in water. It provides better encapsulation efficiency and stability than liposomes [16] and both hydrophilic and hydrophobic drugs can be incorporated [50]. The preparation method uses minimal amounts of organic solvents. In comparison, polymeric nanoparticles require high volumes of organic solvents in the manufacturing process that affect both the price and the quality control of the product [16].

1.3.4.6 Polymeric micelles

Polymeric micelles are formed by the self assembly of amphiphilic polymers in water. The hydrophilic portion of the molecules form the outer most layer of the micelle exposed to the aqueous environments where hydrophilic drugs could be added, whereas the micelles form a hydrophobic core where hydrophobic type drugs could be incorporated in order to facilitate solubility in the aqueous environment [16]. Extended drug release and circulation time are some of the advantages of the polymeric micelles [38].

1.3.4.7 Polymeric nanoparticles

Polymeric nanoparticles are formulated and synthesized according to the requirements needed for the specific type of therapy [35]. Biodegradable polymers are preferred materials as they have the additional advantage of biocompatibility with cells [51]. Upon synthesis of the nanoparticles, the drug molecules can either be entrapped inside or adsorbed onto the surface of the particle. The challenge of these type of particles are the use of solvents during the synthesis process [16, 34, 35].

A number of commonly applied polymers will be described in more detail.

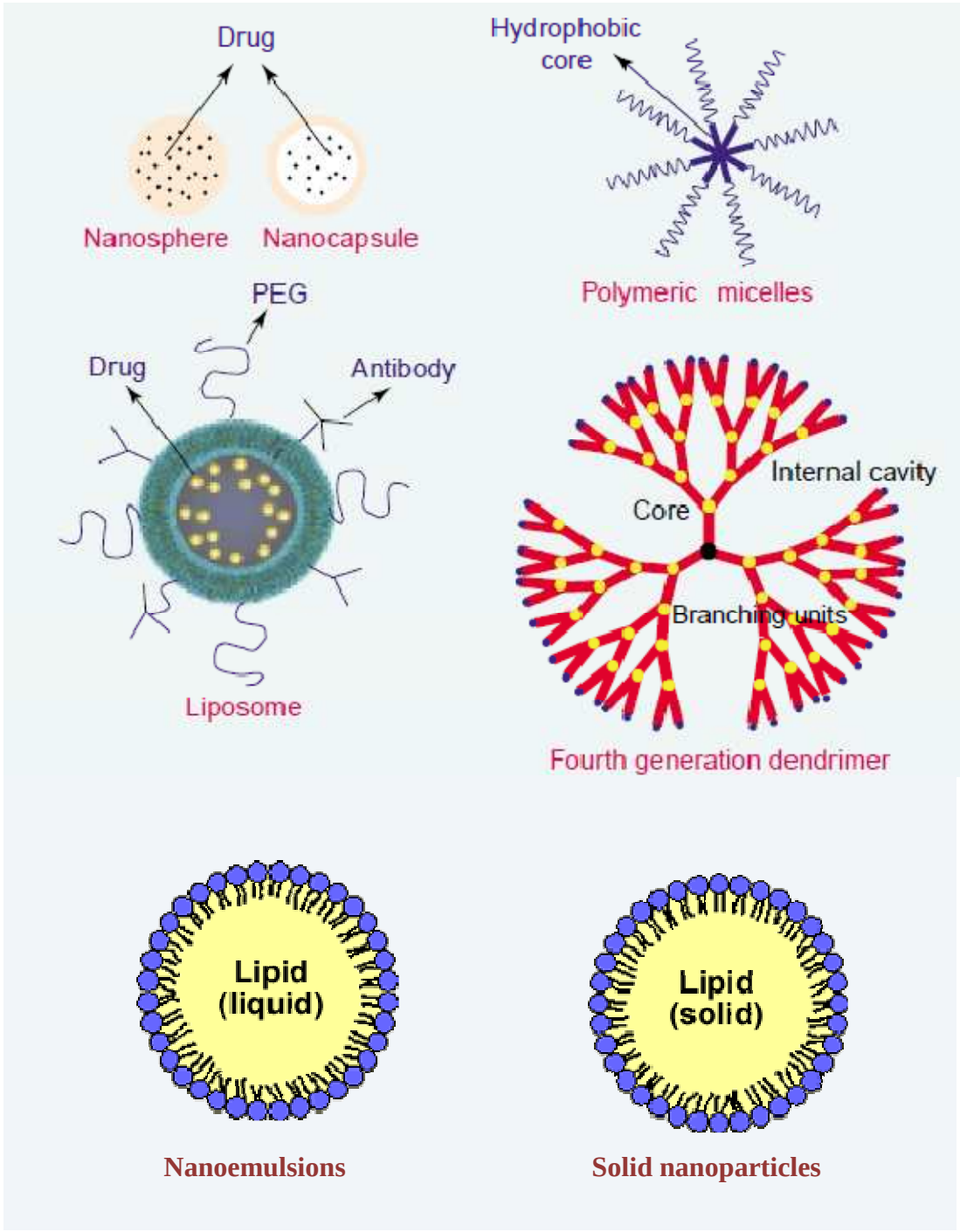

Figure 1.3 *Schematic illustration of a variety of drug delivery systems (adapted from www.scf-online.com/english/25e/images25e/daniels1b25e.gif)[52].*

1.3.4.7.1 Polylactic acid (PLA)

PLA, (Figure 1.4) is a polymer that is often used. It can be prepared with a salting out procedure, thereby avoiding the use of chlorinated solvents. With characteristics of good biocompatibility and biodegradability, PLA is often used to encapsulate proteins with minimal stress [35].

1.3.3.7.2 Poly-ε- caprolactone (PCL)

PCL, (Figure 1.4) is suitable for human use as it is degraded by hydrolysis of the ester linkages under physiological conditions. It is especially suited for long term implantable devices, due to the long degradation time of the polymer [35].

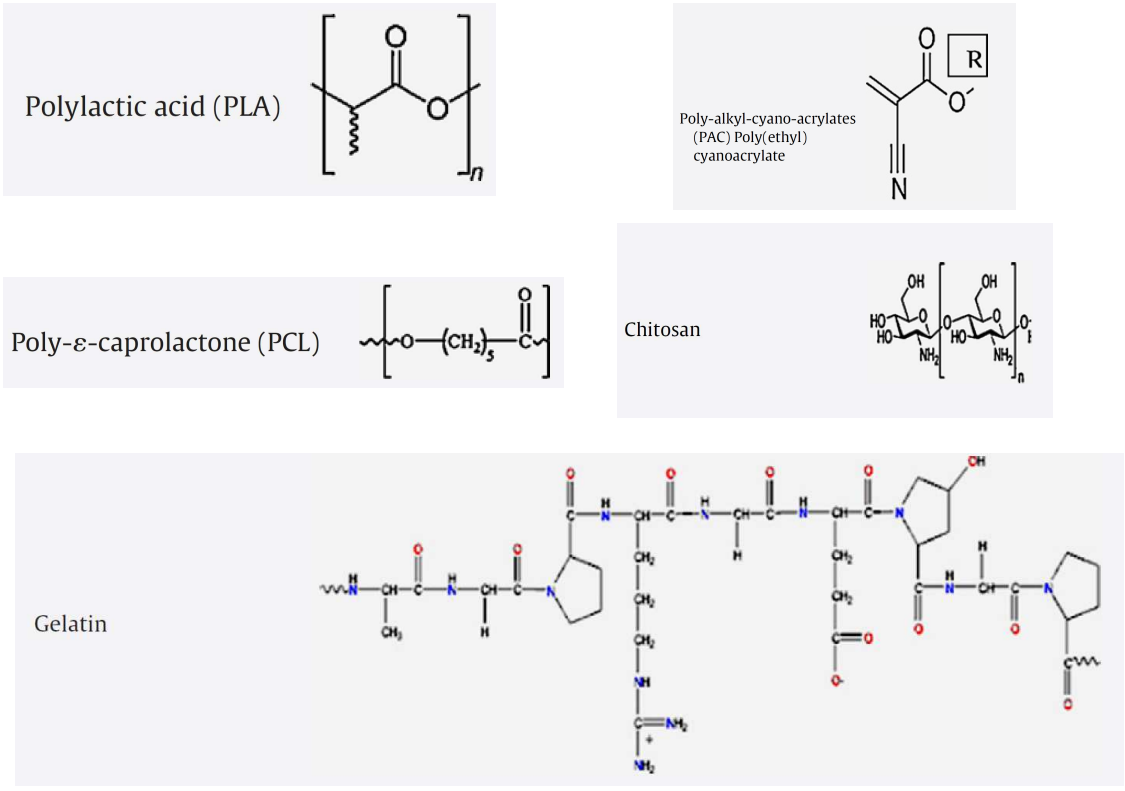

Figure 1.4 *Different polymeric nanoparticles for the use of drug encapsulation [35].*

1.3.4.7.3 Chitosan

Chitosan is a positively charged natural carbohydrate polymer derived from crustacean chitin (Figure 1.4). It is of interest for oral drug delivery, because it was shown that the cationic polymer was able to open tight junctions between epithelial cells making macromolecular uptake possible [53].

1.3.4.7.4 Gelatin

Gelatin is a polyampholyte with anionic, cationic and hydrophilic groups (Figure 1.4) that is extensively used in the food and medical industry. By varying the cross-linking degree of the polymer, the mechanical and thermal properties can be changed. Encapsulated agents are released from these particles via a diffusion controlled mechanism [35]. Gelatin has also been used to coat other polymers such as poly, DL, lactic-co-glycolic acid, (PLGA) to enhance fibronectin recognition and interaction [54].

1.3.4.7.5 Poly-alkyl-cyano-acrylate (PAC)

PAC (Figure 1.4) is degradable by esterases in biological environments, but produces some toxic products that can damage the central nervous system [35]. Compared to polymers such as PLGA and PLA, PAC degradation occurs over a few days rather than a few weeks. The use of different polymers for nanoencapsulation of drugs thus depends on the required rate of drug release.

1.3.4.7.6 Poly, DL, lactic-co-glycolic acid, (PLGA)

PLGA is a biodegradable polymer with very low toxicity. It undergoes hydrolysis in the body to form lactic acid and glycolic acid monomers that are metabolized via the

citric acid cycle as illustrated in figure 1.5 [43, 44, 51, 55]. PLGA has been approved by the United States food and drug administration (USFDA) for human and nanomedicine use [35]. Nanoparticles such as PLGA provide protection to poorly soluble and unstable agents in the body. They are also able to be internalised by cells and escape endosomes due to their small size [29, 34]. Different synthesis techniques have been used to form PLGA nanoparticles of which emulsion diffusion, solvent evaporation and nanoprecipitation are the most popular [35, 56]. PLGA nanoparticles are generally formulated by making use of solvent evaporation or displacement techniques [55].

For these types of techniques, reagents such as polyvinyl alcohol (PVA) have been most commonly used as the emulsifier assisting in the formation of smaller and more uniform particles. Other surfactants such as Pluronics have also been used. PVA enhances particle stability by forming a barrier to the diffusional release of incorporated compounds. Studies done by Sahoo *et al.* showed that different concentrations of PVA influence PLGA nanoparticle characteristics such as size and zeta potential as well as *in vitro* uptake [57] as more time is needed to break the polymer into soluble oligomers by hydrolysis [58]. Drug diffusion and matrix erosion are some of the methods that have been reported to cause the drug to be released from the polymer. Altering of the polymeric characteristics such as surface charge, molecular weight, hydrophobicity and number of monomers lead to varied release kinetics [32, 59, 60]. It has been shown that some nanoparticles (such as PLGA) escape the endo-lysosomes and enters the cytosolic compartment of the macrophage depending on the particle's characteristics [32, 51].

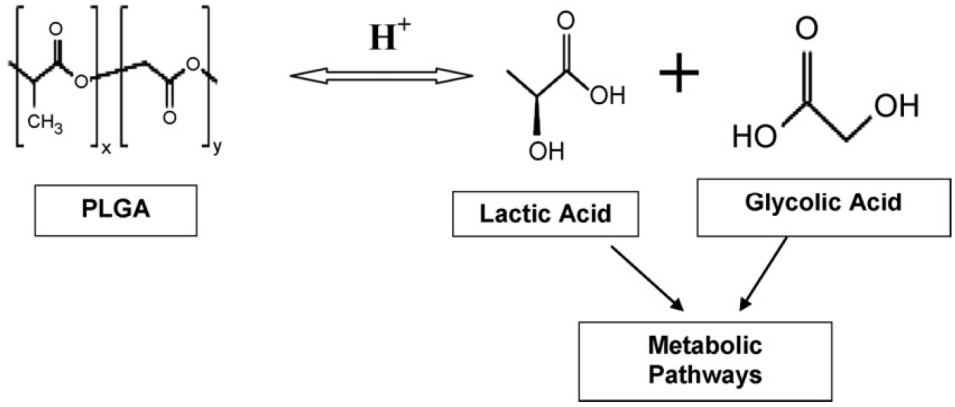

Figure 1.5 *PLGA nanoparticles are hydrolysed in an acidic environment to the monomers lactic and glycolic acid [35].*

1.3.5 Applications of nanoparticles in therapeutic treatment

Therapeutic compounds against several illnesses have been encapsulated to improve their effeciency. An example of PLA nanoparticles used in chemotherapy is the neuroleptic compound savoxepine, where the authors showed that the drug carrier extended the release of the drug for more than a week [42]. It has been shown by Thapa and co-workers that PLA containing alpha-galactosylceramide was selectively taken up by macrophages and dendritic cells [61]. The encapsulation of an anti-tumor agent, ftorafur, with PAC was shown to enhance the effeciency of the drug [62]. Encapsulation also improved the biological activity of anti-cancer agents such as paclitaxel and 9-nitrocamptothecin [56, 63]. Another interesting application for polymers, reported by Di Toro *et al.* was to apply PLGA and PLA biodegradable internal bone fixation devices [64].

A PCL polymer together with a polycationic non biodegradable acrylic polymer was shown to preserve insulin's biological activity in an oral formulation given to diabetic rats [65]. Furthermore, encapsulating insulin in alginate/chitosan nanoparticles showed enhanced intestinal absorption after it was administered orally [66].

Several studies have been conducted on the first line anti-tuberculosis drugs. For example INH, RIF and pyrazinamide were incorporated into SLN's and administered

to TB infected guinea-pigs. The drugs maintained detectable levels for over 10 days in the lung, spleen and liver [50]. Silva and co-workers produced micelle forming carrier-drug conjugates of the anti-tuberculosis agents INH, RIF and pyrazinamide. The results indicated stable micelles *in vitro* with extended release times as well as better anti-mycobacterial activity compared to the original drugs [67].

A number of research groups have turned their focus to making use of nanotechnology platforms for targeted delivery to the lungs, as TB manifests itself mainly in the respiratory system [68]. PLGA containing RIF particles, incorporated into mannitol by a spray drying technique for inhalation therapy, effected improved uptake by alveolar macrophages [69]. RIF was also incorporated into nanoemulsions in another study using excipients such as Sefsol, Tween and saline. The *in vitro* studies showed an initial burst effect with more moderate drug release afterwards [16]. Even niosomes could be used to enhance the effect of RIF, as it was shown to have an increased accumulation in the lymphatic system compared to free drug following intraperitoneal administration [49]. In a study done with RIF loaded mannosylated dendrimers, where the RIF was bound inside the dendritic crevices, the haemolytic effect of RIF was reduced and the drug release was prolonged [70].

Therapeutic compounds against *Mycobacterium avium* (*M. avium)* were also improved by encapsulation. A nanocrystalline suspension of the drug clofazimine was produced and was shown to have reduced toxicity and increased solubility [71]. Liposomal formulations of ciprofloxacin and azithromycin indicated an increase in potency against the bacteria *in vitro* compared to the free drugs [72].

1.3.6 PLGA as preferred nanoencapsulation approach for anti-TB drugs

PLGA is a synthetic copolymer that is biodegradable and biocompatible. The polymer has slow drug release characteristics *in vivo*, is not significantly immunogenic and has the capacity to encapsulate hydrophobic and hydrophilic agents [34, 54, 55]. PLGA particles are easy to prepare with high stability in biological environments [63]. This polymer has lower toxicity compared to other polymers such as PAC, and is more stable than liposomes or SLNs in biological environments [56]. As mentioned

previously, PLGA particles are taken up into macrophages and dendritic cells [61], which comprise the main target cells for delivery of anti-TB drugs [32, 51]. Another advantage of PLGA is that the degradation rate of the particle can be altered by changing the monomer composition and thereby the molecular weight of the polymer [58]. For the purpose of our study, we investigated PLGA as a nanocarrier molecule for the anti-TB agent, INH.

1.4 Targeting of nanoencapsulated anti-TB drugs to the sites of infection

Nanoencapsulation of drugs for delivery allow the opportunity to incorporate targeting strategies to further enhance drug efficiency and limit systemic toxic side-effects to the patient. This demands an intricate knowledge of the cellular and molecular aspects of the disease in order to identify suitable ligands that can be incorporated into the polymeric shell of the nanoparticle. These ligands should bind to disease specific receptors with an affinity high enough to effect a higher local concentration of the encapsulated drug at the site of infection rather than remaining in circulation. Any toxic side-effects that the drug may have will then mainly manifest in the vicinity of the infected tissue, while the mycobactericidal drug dose level is maintained there for longer periods than in the rest of the body.

For infectious diseases a first consideration for drug targeting would be to identify the receptors on the host cell that is used for entry of the pathogen. Nanoencapsulated drugs can then use a ligand that follows the pathogen into the host cell using the same selective mechanism of entry. Another approach would be to identify surrogate markers of infection that characterize the disease at the site where the pathogen is localized in the host tissues. If a non-toxic ligand can be identified that recognizes such surrogate marker receptors, then it can be incorporated into nanoparticle for targeting the drugs.

1.4.1 Macrophage receptor(s) for entry of *M. tuberculosis*

Infection in humans occurs through inhalation of the tuberculosis bacilli, which is taken up by alveolar macrophages in the lungs. When the *M.tb* is inside the phagocyte, the bacteria prevent fusion with acidic lysosomal compartments to circumvent destruction. Granuloma formation then occurs. The granuloma can either contain the infection that could lead to sterilization or localized caseation and necrosis can take place that will lead to the release of infectious bacteria into the airways [73].

Mycobacteria enter macrophages by various receptors such as complement, mannose, Fc and scavenger receptors. Selective receptor blockade studies done by Zimmerli and co-workers indicated that the mycobacteria were able to survive and replicate in human macrophages regardless of the receptors used for binding and entry into the cell. The authors discovered that class A scavenger receptors accounted for the most significant amount of interaction and uptake of *M.tb* into macrophages. Scavenger receptors have the ability to bind and mediate the endocytosis of polyanionic macromolecules and particles such as low density lipoproteins, as well as interact with lipopolysaccharide of gram negative bacteria and lipoteichoic acids of gram positive bacteria [74-76]. Scavenger receptors have been implicated to play a role in cholesterol deposition in atherosclerosis, due to uptake of modified low density lipoproteins, which contain cholesterol, triacylglycerides and phospholipids [77].

1.4.2 Localized tissue markers of TB infection

Receptor mediated drug delivery can be achieved by linking a ligand to the drug-containing nanoparticle to interact with specific receptors, such as scavenger receptors or mannose receptors at the site of infection [78]. For example, mannosylated liposomes were used to target infected alveolar macrophages *in vivo* more efficiently than unmodified liposomes could [79].

Cholesterol may also be considered as a surrogate marker due to its accumulation at the sites of infection [80, 81]. Furthermore, infection of macrophages with mycobacteria results in the accumulation of cholesterol at the entry site. The latter

indicated that cholesterol has a direct role in uptake of mycobacteria by the macrophages as the binding of the mycobacteria to the cell surface required sufficient amounts of cholesterol in the plasma membrane. Entry of *M.tb* could be hindered by prior cholesterol depletion of the macrophage membrane [81-83]. However, these conclusions made by the early studies of Gatfield and Pieters were not as simple as they first thought.

Cholesterol plays an important role in the structural and functional aspects in macrophages [84]. Lipid bodies are observed in macrophages from the very beginning of infection and may play a role in immunomodulation [85] as well as being a nutrient rich reservoir for tubercle bacilli [82].

Peyron *et al.* (2000) argued that the resistance to uptake of tuberculous bacilli in membrane-cholesterol depleted cells is because of perturbed receptor signalling rather than inhibition of binding of the bacilli directly to membrane cholesterol as entry mechanism. Using the human neutrophil model as host and *M. kansasii* as mycobacterial pathogen, they suggested complement receptor type 3 (CR3) as the main entry receptor, which is associated with GPI- anchored proteins localized in cholesterol rich microdomains (lipid rafts) [86, 87]. Without cholesterol in the membrane, the CR3 remains outside the cholesterol-rich lipid raft domains and do not activate the cell to take up the mycobacteria.

Ferrari *et al*. identified a phagosome coat protein (Figure 1.6) termed TACO (tryptophane aspartate containing coat protein), coronin 1 or P57 that was retained at the cytoplasmic face of the phagosomes carrying the viable mycobacteria [88]. This protein is associated with the microtubule network and is present in cells of the lymphoid/myeloid lineage. It is not an integral protein, but is bound to the phagosomal membrane via a steroid moiety, cholesterol. The attraction of TACO protein to the phagosome membranes occurs in a cholesterol dependent way [81]. Viable mycobacteria were shown to maintain the TACO protein on the phagosome thereby hindering lysosome fusion, whereas heat killed bacteria caused a release of the protein resulting in lysosome fusion. In addition, macrophages that did not have TACO (example in Kupffer cells) destroyed mycobacteria after being taken up, suggesting that the protein is involved in the intracellular survival of mycobacteria

[88]. In a review by Pieters and Gatfield they speculated that the coat protein, TACO, could possibly mimic the plasma membrane thereby avoiding lysosomal delivery [89]. It was more recently shown that TACO is responsible for activating the calcium (Ca^{2+}) dependent phosphatase calcineurin, that leads to the inhibition of lysosomal delivery [90].

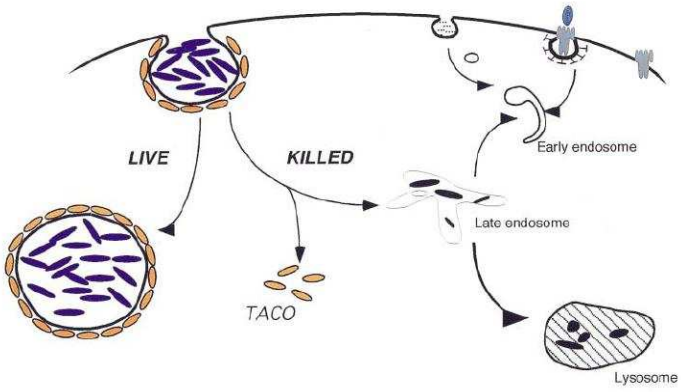

Figure 1.6 *Survival mechanisms of the mycobacteria, in the macrophage host. Phagocytosis of mycobacteria (blue) into the macrophages triggers the recruitment of TACO (yellow) around the emerging phagosome. The TACO coat must be removed in order for the mycobacteria in the phagosome to be delivered to the lysosome where the contents of the phagosome are degraded. Living mycobacteria in contrast to heat killed M.tb are able to retain TACO at the phagosomal membrane, preventing the delivery to the lysosome so that the bacteria can survive within the phagosome [91].*

In addition a human cholesterol specific receptor – C_k like molecule was found that could possibly interact with the cholesterol rich domains on the macrophage surface, forming a synaptic-junction that facilitates signalling events such as the association to the TACO protein [92, 93].

Pathogenic mycobacteria are able to survive within the phagosomes by interfering with the phagosome maturation and therefore do not fuse with lysosomes [94]. A number of different mechanisms have been shown to play a role in the maturation block [87]. Work done by De Chastelier and co-workers derived from morphological observations that a close apposition of the phagosome membrane with the whole mycobacterial surface was necessary for a maturation block [95]. During phagocytic

uptake of mycobacteria plasma membrane cholesterol dependence was observed [81, 86]. De Chastelier and her colleagues then investigated the effect of cholesterol depletion on the close interaction between the internalized bacterium's cell wall and the phagosome membrane. When the cholesterol was depleted, the mycobacterial surface was not closely associated with the phagosome membrane anymore and lysosomal fusion followed, pointing towards an important role for cholesterol in the maturation block. Interestingly, when cholesterol was replenished again, the mycobacteria were able to restore themselves in phagosomes [96].

In conclusion, cholesterol may be considered as a marker of localised TB infection due to its accumulation at the site of mycobacterial entry [81]. It could, however, be quite a challenge to find a non-toxic ligand to cholesterol to facilitate drug delivery. The cholesterol-binding drugs Amphotericin B or cyclodextrins are too toxic to include into the nano-encapsulated anti-TB drug particles.

1.4.3 Mycolic acid ligands for drug targeting

The cell wall envelope of *M.tb*, as depicted in figure 1.7, consists of a cytoplasmic membrane and an outer capsule comprising peptidoglycan and arabinogalactan complexes as well as mycolic acids with intercalated free standing lipids, glycolipids and proteins. This provides an extremely robust and impermeable envelope with high resistance to host-derived and therapeutic anti-bacterial agents [97, 98]. Other predominant components include the lipopolysaccharides, lipoarabinomannan (LAM) and phosphatidylinositides anchored in the plasmamembrane [99].

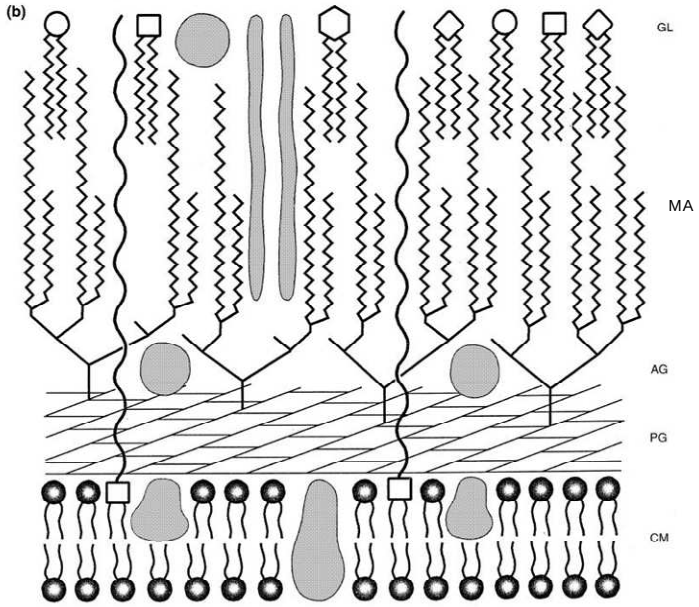

Figure 1.7 *Schematic representation of a mycobacterial cell wall PG: peptidoglycan, CM: cytoplasmic membrane, GL: glycolipids, MA: mycolic acids, AG: arabinogalactan, the shaded areas represent different proteins present in the membranes[100].*

The most abundant high molecular weight lipids present in the envelope are mycolic acids (MA), an extended family of long 2-alkyl 3-hydroxyl fatty acids, typically 70-90 carbon atoms in length. The members of the mycobacterium complex i.e *M.tb*, *Mycobacterium africanum*, *Mycobacterium bovis* and *Mycobacterium microti* are pathogenic and produce α-, keto- and methoxy- MA. Two moieties could be distinguished in the MA structure, the meromycolate moiety and the mycolic motif (Figure 1.8). The mycolic motif is similar for all mycolic acids with a few minor variations in chain length in the α position. The meromycolate moiety defines the subtypes of MA. These sections can be differently substituted with carboxyl, methyl, carbonyl, epoxy groups, double bonds or cyclopropanes in the proximal and distal positions [101]. A variety of MA patterns occur other than the α-, keto- and methoxy-MA found in *M.tb*. Amongst other different mycobacterial species carboxymycolates are found in *M. avium* and epoxy mycolates are produced in *Mycobacterium fortuitim* and *Mycobacterium smegmatis* [99, 101].

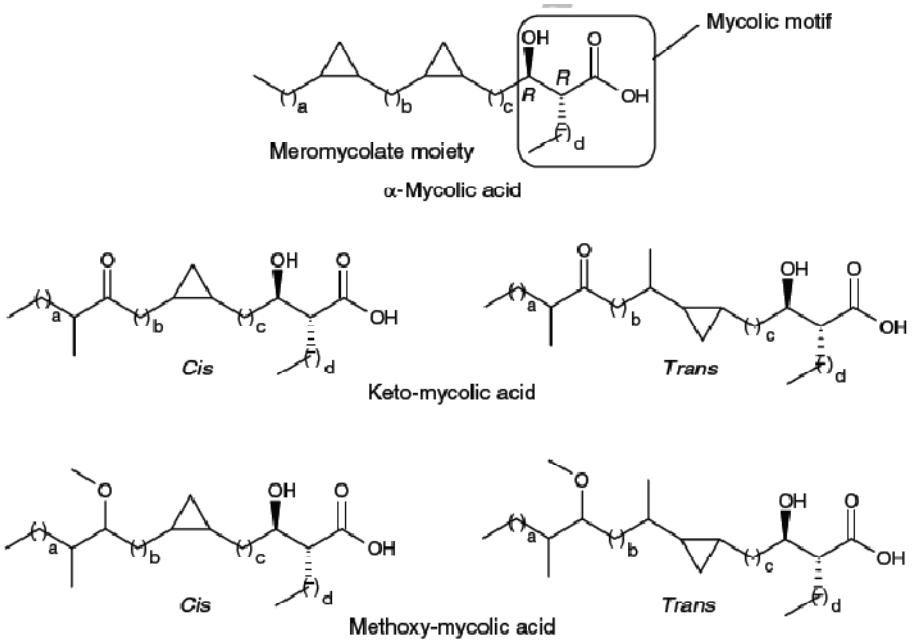

Figure 1.8 *Structures of mycolic acids from M.tb [97].*

MA are predominantly found in the bound form. The MA can either be linked to arabinogalactan polysaccharide via ester linkages, which is linked to peptidoglycan of the cell wall, some MA exist as trehalose monomycolate (TMM) and dimycolates (TDM) or be loosely associated with the insoluble matrix [99]. The folding of the MA molecules differs for each subtype depending on the functional groups present. Langmuir trough studies showed that keto MA tended more towards a W-shaped configuration with exceptional rigidity in monolayers, whereas methoxy- and alpha-MA exhibited a more flexible conformation towards variation of experimental parameters [102, 103]. The precise conformation of the MA within the cell wall structure is complicated to determine as the MA are present in different subclass mixtures with different carbon chain lengths within each group [97].

The biosynthesis of MA is a unique system as it employs the fatty acid synthetases (FAS) type I and II for synthesis. The FAS I and II are also NADH/NADPH dependent for their respective reactions. FAS type II is especially important for drug development and targeting as this synthetase is found in prokaryotic organisms and plants but not in mammalian cells. FAS type I is involved in the *de novo* synthesis of $C_{16\text{-}18}$ and $C_{24\text{-}26}$ fatty acids which is then subsequently used in the FAS type II process for further elongation and insertion of functional groups. The formation of the MA occurs through repetitive cycles of condensation, keto reduction, dehydration and

enoyl reduction in the fatty acid synthesis process, each product of the completed cycle is used as the substrate for the next cycle until the specific chain length is acquired. Several enzymes are involved to catalyse the reactions, β-ketoacyl synthase, β-ketoacyl reductase, β-hydroxyacyl dehydrase and enoyl reductase. For the formation of the functional groups the fatty acids undergo desaturation and cyclopropanation that is all part of the FAS type II pathway [104-106]. Cyclopropanation occurs via the S-adenosylmethionine dependent methyl transferases not found in other bacteria. The next step is the Claisen-type condensation to join the meromycolate to the mycolic motif [99]. In the last step the mycolic acids are esterified with the arabinogalactan complex or with free glycolipids to form trehalose dimycolate (TDM) by the antigen 85 complex [107].

1.4.3.1 MA structure-function relationship

The various subtypes of MA have been shown to date to play a role in the virulence of the pathogen [108, 109]. In a study done by Dubnau and co-workers, they synthesized a mutant strain of *M.tb* that did not produce oxygenated (methoxy- and keto-) MA. This strain was shown to have a decrease in membrane permeability and thus confirmed that the fluidity of the cell wall greatly depends on the type of mycolate produced [101, 110]. The results also showed that the bacteria were attenuated in mice, thus the oxygenated MA playing a role in virulence of the bacteria [108].

An array of mycobacterial cell wall components have been considered as surrogate markers for TB in the past [111, 112]. Antigenic activity of mycolic acids and the glycolipid derivatives such as the lipid extractable trehalose mono- or dimycolates, TMM or TDM respectively (cord factors) have been previously described [97, 109, 113]. Of all the antigens prevalent in the cell wall of the mycobacteria that may be considered for use in TB serodiagnosis, MA provide a special opportunity due to their variability among different species of Mycobacterium and the unique way that they communicate their presence to the immune response of the host [97, 114, 115]. Their ability to elicit CD4, CD8 double negative T cells by means of their presentation on CD1b lipid presentation proteins on dendritic cells [116] may well be the reason that

the antibody titers to mycolic acids in AIDS patients with even very low CD4 T cell counts are maintained, relative to other patients that are not infected with HIV, or have normal CD4 T cell counts [117, 118].

Using mycolic acids as surrogate markers for TB diagnosis was shown to be successful in an ELISA assay [117-119]. Pan *et al*. have shown that the most antigenic part of the cord factor antigen was the mycolic acid [97, 118, 119]. This was also shown in a study done by a group in Japan that the anti-cord factor IgG produced from rabbits, recognized specifically the MA subclasses [112]. Due to the low specificity achieved, this was not the ideal solution

A biosensor approach showed improved accuracy to a level that may be seriously considered for the possibility of commercialization, when using free mycolic acids in liposomes as antigens in a competitive binding assay [117, 120]. This test, subsequently dubbed the MARTI-test (for Mycolic acids Antibody Real-Time Inhibition), can diagnose TB within four hours of sampling by analyzing the serum sample for the presence of anti-mycolic acid antibodies as immune surrogate markers for active TB that is has an accuracy of 84% [120].

The structure of MA showed significant characteristics as was previously investigated by our group. A biosensor study was done which showed that amphotericin B interacted with MA and cholesterol, and that MA associated with cholesterol. This lead to the conclusion that MA exhibit a structural similarity to a sterol such as cholesterol [121].

The contribution that MA makes in the host pathogen interaction has been an area of interest for the past decade. In a study done by Korf *et al*. where they measured the cellular responses in peritoneal and alveolar macrophages when injected with MA, these authors observed the induction of foamy macrophages (intracellular lipid accumulation in macrophages) as well as MA being able to induce an inflammatory response of IL-12 and IFN–γ [14] as was also shown with TDM except TDM also induced TNF-α response whereas MA did not [122, 123]. Therefore, cytokine inducers such as TDM have good potential to be used as adjuvants and immunomodulators [124].

This mycobacterial induced host response for foamy macrophage formation is probably for active lipid import that the bacteria can utilise as metabolic substrates for survival and replication [14]. This was confirmed when it was shown that *M.tb* could utilise cholesterol as an energy and carbon source [125].

Foamy macrophages arise from the onset of infection by the accumulation of lipid bodies as indicated by *M.tb* infected mice and guinea pig studies done by Caceres *et al*. 2009 [85]. Peyron and co-workers illustrated in an *in vitro* human tuberculous granuloma model that virulent mycobacteria (*M.tb, M. avium*) and not the saprophytic type (*M. smegmatis*) induce foamy macrophages. The *M.tb* resides within the foamy macrophages singularly in phagosomes in a non-replicative state. A very interesting observation the group made was that a number of the bacteria were in close apposition to the lipid bodies present in the foamy macrophages. The role that MA plays in the induction of the foamy macrophages was shown to be through the oxygenated mycolic acids, and are thus virulence factors enabling the bacteria to persist in the macrophage over a long time [82].

The MA elicited responses points towards a macrophage steering of T regulatory immune responses. This was illustrated when Korf *et al*. used a mouse model of asthma by treating ovalbumin sensitized mice with MA. The airways of the mice were tolerant to a secondary exposure to the ovalbumin indicating that MA suppressed Th2 reactivity [126]. MA might also serve as a liver X receptor (LXR) ligand, which plays a role in macrophage cholesterol, fatty acid and glucose homeostasis [127]. These functions in effect have an influence on the inflammatory status of the macrophage.

Mycobacteria have been shown to have conserved molecular products (pathogen associated molecular patterns (PAMP) including the glycolipid lipoarabinomannan, lipopeptides and soluble tuberculosis factor recognised by Toll-like receptors (TLR) [128, 129], but MA was excluded being recognised by TLR2 and TLR4 [14]. TDM is an immunogenic lipid associated with pro-inflammatory responses, recognition to TLR2 was shown to be mediated by the macrophage scavenger receptor MARCO and CD14 [130].

1.4.3.2 MA as virulence factors

Infection occurs when inhaled bacilli are phagocytosed by alveolar macrophages. Macrophages that are infected with *M.tb* are the initiators of granulomas, which consist out of lymphocytes, extra cellular matrix components, calcifications and caseous necrotic tissue [73, 82]. Interactions of the bacteria with other leukocytes include dendritic cells, the professional antigen presenting cells. However, experimental work done by Tailleux and colleagues indicated that the bacteria do not grow within dendritocytes, but infected dendritocytes do accumulate in the regional lymph node, adding to the immune response. Within the phagocyte of the macrophage on the other hand the bacteria prevents the fusion with acidic compartments, enabling it to replicate within the macrophages [73, 131].

Pioneering work of Armstrong and D'Arcy Hart showed that *M.tb*-containing phagosomes do not fuse with lysosomes [132]. When mycobacteria enter the macrophages via phagocytosis, they reside and multiply within phagosomes, the process of fusion with lysosomes is inhibited by the mycobacteria to form phagolysosomes [94, 132]. Mycobacterial lipids such as phosphatidylinositol-mannoside (PIM) [133] and TDM [123, 134] have been implicated to slow down the maturation of the phagosomes into phagolysosomes. Mannose-capped lipoarabinomannan (ManLAM) [135] was shown to play a role in the maturation block but more recent findings point to opposite results [136]. Beatty and co-workers found that mycobacterial lipids were released from the mycobacterial phagosome and that these microvesicles entered a secretory pathway (possibly lysosomal like organelles) and then could be phagocytosed by bystander macrophages [137, 138].

A single *M. avium* per phagosome was shown to require a close apposition between the mycobacterial surface and the enclosing phagosome membrane to inhibit phagolysosome formation, when more than one bacteria was present within the phagosome the close apposition was not maintained and the phagosomes fuse with lysosomes [95, 96, 135, 139]. In a study done by De Chastellier in 2009 it was shown that in the event of phagolysosome formation, *M. avium* was able to rescue itself and reside again in immature phagosomes [140]. Similarly Armstrong and D'Arcy Hart

observed that *M.tb* were able to grow even when phagosome lysosome fusion occurred [132].

1.4.3.3 Mycolic acids as surrogate marker antigen in TB

Ojha and colleagues demonstrated in an *in vitro* study that *M.tb* was capable of biofilm formation in the slow turn over, persistent stage of the *M.tb* life cycle. The formation of these biofilms was found to be dependent of iron and zinc metal-ions, as well as gaseous exchange of CO_2. The films were shown to provide resistance to anti-TB drugs, i.e. the bacteria were able to survive in the presence of these drugs *in vitro*. The findings that were most relevant to this were the presence of abundant free MA in these biofilms. The major MAs that were detected with NMR and MS studies turned out to be the methoxy MA and some α mycolates [141]. From observations with *M. smegmatis*, a TDM specific serine esterase is utilized to hydrolyze TDM to form the free MA. It was suggested that *M.tb* could also form free MA from TDM by an esterase. The free MA could then be excreted to form the biofilm matrix [142].

MA incorporated into nanoparticles may interact with the anti-mycolic acid antibodies that should be present in higher concentrations at the infected areas. In this way, targeting may be achieved by an accumulation of the nanoparticles in immune complexes at the site of infection. Alternatively MA could also serve as a ligand for cholesterol-rich areas, due to the cholesteroid nature of MA and the fact that MA is attracted to cholesterol [121].

1.5 Aim of the project

The ultimate aim is to provide a nano-encapsulated anti-TB drug delivery vehicle which will overcome the difficulty of daily supervision of drug-taking by patients and possibly shorten the regime of anti-TB treatment. For this study, MA structure-function properties and the practical feasibility of encapsulating MA into PLGA

nanoparticles as targeting ligand was investigated as well as the product's toxicity to and efficiency of uptake in host macrophages.

In chapter 2 the cholesteroid nature of MA was investigated by testing the tolerance of structural changes of natural mixed MA for recognition by Amphotericin B. In addition, the structure of synthetic mycolic acids in relation to their antigenicity in tuberculosis was determined.

Chapter 3 describes how the MA containing nanoparticles were synthesized and assembled, characterized for their ability to be internalized and processed into macrophage cell lines and what degree they may be toxic to host cells. In addition, the effect that MA may have on the mycobactericidal properties of co-encapsulated isoniazid was explored *in vitro* in *M.tb* infected macrophages.

The potential impact of the results from the experimental chapters on TB treatment in future is discussed in chapter 4.