

The Potential of Nanotechnology for Tuberculosis Treatment

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ABSTRACT

Tuberculosis (**TB**) remains a significant global health threat and is the second leading cause of death from infectious diseases. The inappropriate prescription of anti-tuberculosis drug, the poor compliance due to extended treatment period and the emergence of multi-drug resistance TB result in failure of conventional pharmacotherapy. Besides drug resistant issues, long-term and multiple drugs administration are associated with severe side effects such as organ toxicity. A more effective treatment regimen involving lower dose frequencies, higher residency at infected site and reduced drug-induced toxicities is needed. In this context, nanotechnology-based drug delivery systems offer a promising solution as their nanostructured systems are highly effective in encapsulating considerable amount of TB drugs, highly stable, feasible for various administration routes (i.e. oral, intravenous and inhalation) and could be designed to prevent systemic clearance

and pre-mature degradation. To the end, this approach significantly contributes to higher bioavailability as well as minimizing TB drug resistant issue. The current chapter summarizes the state-of-the-art in the current nanotechnology systems used in the delivery and therapy of anti-TB drugs. These include liposome, solid lipid nanoparticles, synthetic or natural polymeric nanoparticles, micelles, nanosuspension and nanoemulsion.

INTRODUCTION

Tuberculosis (**TB**) is an extremely contagious infectious disease with origin of microbiology and its main etiologic agent is believed to evolve from an early progenitor in East Africa as early as 3 million years ago. The outbreak of TB in Europe and North America during the 1800s resulted in approximately 1,000 deaths out of 100,000 patients. Currently one third of the global population, which is amounting to 2 billion people, is infected with TB. This pervasive and deadly infectious disease has been ranked as the second most fatal disease after HIV/AIDS. Statistics from World Health Organization (**WHO**) reveal that the annual mortality rate is 1.7 million people and at least 10 million people develop this disease each year [1,2]. Pathogenesis of TB is triggered by *Mycobacterium tuberculosis*, which possesses inherent ability to remain dormant for several years or cause active diseases upon infections. The development of active TB differs with factors such as age, gender, geographical locations, immunity level and period of infection. For instance, men are more prone to TB infection compared to women. Apart from gender, young children are also at risk to TB infection, as their lifetime risk of TB was approximately 10% [3]. The incidence of TB also varies in accordance to socioeconomic and geographical locations whereby the majorities of TB cases were in South East Asia (29%), Africa (27%) and Western Pacific (19%) regions. India and China alone contributed to 26% and 12% cases, respectively [2, 4]. Immunocompromised patients (i.e. HIV-positive) are susceptible to the attack of TB infection [3]. Others immune-responsive condition like chronic lung disease, diabetes, malnutrition and alcoholism are also identified as potential factors to trigger TB infection [4].

PATHOGENESIS OF TUBERCULOSIS

M. tuberculosis is an acid-fast, Gram-positive, rod-shaped bacilli which could remain as airborne and stable droplet nuclei for several hours after exhaled into environment [5]. The trademark of *M. tuberculosis* is the existence of two metabolically different growth phases including an active stage (as reflected in progressive, symptomatic disease) and a dormant persistent state [6]. The transmission route of *M. tuberculosis* is through inhalation, in which the bacterium is generated from individuals suffering from pulmonary or laryngeal TB and inhaled by susceptible hosts. Inhaled *M. tuberculosis* aerosols travel through the upper respiratory tract to reach alveoli regions of the lungs and inhabit largely in resident phagocytic cells (i.e. alveolar macrophages) [6]. The phagocytosis of the bacterium by alveolar macrophages occurs via different receptors that available on the macrophage surface such as mannose, Toll-like, Fc, surfactant protein A, CD14 and scavenger receptor [7-9]. Early innate immune response to active

bacilli triggers a pro-inflammatory response leading to recruitment of neighbouring neutrophils, dendritic cells and monocytes in the lungs. This is followed by the formation of cellular matrix of granuloma (containing foamy macrophages, lymphocytes and infected macrophages) [10]. The recruitments of phagocytic cells upon infection of other microorganisms are often sufficient to eliminate invading pathogens. In the case of mycobacterial infection however, the containment of the bacterium provides shielding effects and encourages *M. tuberculosis* to adopt a clinically silent, dormant state for later re-activation when the physiological microenvironment permits. It is established that *M. tuberculosis* has adopted several mechanisms for them to survive within the host. Firstly, *M. tuberculosis* inhibits the maturation of phagosomes, lysosomal fusion and acidification [11]. This inhibitory effect allows the mycobacteria to escape lysosomal killing and degradation [6,12]. In addition, the mycobacteria reduced the action of inducible Nitric Oxide Synthase (**iNOS**) as well as blocked antigen processing of TB. In addition, mycobacteria exploit several virulence mechanisms to their advantage to enhance their spread from cells to cells. The bacterial cells spread to other parts of body via the lymphatic or blood circulation.

CURRENT TREATMENT MODALITY OF TUBERCULOSIS

The recommended WHO guidelines for current treatment of TB involve extended administration of multiple oral drugs regimens over long periods to prevent development of Multi Drug Resistant TB (**MDR-TB**) [1,13]. During the initial intensive phase, combinations of three or more first-line drugs are recommended for at least 2 months to kill rapidly proliferating cells. This is followed by the use of two or three drugs for the next 4 months [14]. The length of treatment is largely due to the presence of a) bacterial cells with low metabolic activity that were not killed during the initial phase treatment and b) stationary phase persistent cells in lesions. Based on the observation of disease progression, the following drug schedule could be chosen in the order of preference (Table 1) [14-16]:

1. First-line drugs such as Isoniazid (**INH**), Rifampicin (**RIF**), Pyrazinamide (**PZA**), Ethambutol (**EMB**) and streptomycin
2. First-line drugs followed by injectable drugs
3. Second-line drugs and injectable drugs such as Ethionamide (ETH), prothionamide, kanamycin, amikacin, terizidone/cycloserine, capreomycin, viomycin and para-aminosalicylic acid.
4. Other drugs including fluoroquinolones (ciprofloxacin, ofloxacin and levofloxacin)

The lack of patient compliance to prescribed regimens and the drug-associated-toxicity result in the prevalence of MDR-TB or extremely drug resistant TB (XDR-TB). MDR-TB strains are resistant to INH and RIF and the treatment usually comprises of the administration of PZA simultaneously with second-line drugs or fluoroquinolones [17].

However the treatment cost is higher and longer treatment time (up to 12 months) is required usually [18]. XDR-TB strains are highly prevalent in HIV-positive individuals. Patients developing XDR-TB fail to respond to INH and RIF along with fluoroquinolone and several second-line anti-tuberculosis drugs (ATD).

Table 1: Classification of ATD, mechanism of action and adverse effects associated with the drugs [19-21].

	Drug	General minimum inhibitory concentration ($\mu\text{g/mL}$)	Mechanism of action	Adverse effect	Solubility mg/mL (in water)	References
First-line drugs	Isoniazid (INH)	0.01-0.2	Bactericidal effect. Inhibits mycolic acids synthesis via formation of complex with NADPH-dependent enoyl acyl carrier protein reductase InhA. Involved in DNA, lipids, carbohydrates and NAD metabolism.	Hepatitis, burning sensation, loss of memory, lung injury, stinging pain in feet, drug interactions. Hepatotoxic.	125-140	[21,22]
	Rifampicin (RIF)	0.05-0.5	Bactericidal effect. Inhibits bacterial RNA synthesis by binding to bacterial DNA-dependent RNA polymerase.	Bleeding, lost appetite, low urine excretion, stomach upset. Hepatotoxic.	2-Jan	[21,23]
	Pyrazinamide (PZA)	20-100 (at pH 5.5-6.0)	Bactericidal effect. Converts to pyrazanoic acid in the presence of pyrazinamidase which acts to lower pH of bacteria. Prevent the process of managing damaged proteins and rescue non-functioning ribosomes in <i>M. tuberculosis</i> . Disturbs membrane potential and energy production needed for survival of bacterium in acidic conditions.	Pains in joints and abdomen. Hepatitis and rashes.	15	[21,24]

Second-line drugs	Ethambutol (EMB)	1–5	Bacteriostatic or bactericidal effect. Inhibits the biosynthesis of arabinoglycan that is an essential component of bacterial cell wall via inhibiting mycobacterial arabinosyl transferases.	Disturbed vision. Hepatotoxicity.	10	[21,25]
	Streptomycin	2–8	Bacteriostatic or bactericidal effect. Acts as an irreversible inhibitor of protein (polypeptide) synthesis by binding to 30S ribosomal subunits, thus resulting in translational disruption.	Hypokalemia, hypomagnesemia, nephrotoxicity, ototoxicity and vestibular toxicity.	> 20	[21, 26]
	Ethionamide (ETH)	0.6-2.5	Bacteriostatic effect. Inhibition of mycolic acid synthesis by binding to the ACP reductase InhA. Inhibition of mycolic acid synthesis by binding to the ACP reductase InhA.	Hepatotoxicity, neurotoxicity, anorexia and gastrointestinal upset.	0.1	[21,28]
	Kanamycin	1–8	Bactericidal effect. Inhibits RNA-dependent protein synthesis and binding to bacterial 30 S ribosomal subunit.	-	-	[21]
	Cycloserin (CS)	5–20	Bacteriostatic effect. Due to the structural analogue to D-alanine, peptidoglycan biosynthesis is interrupted via inhibition of incorporating D-alanine into peptidoglycan pentapeptide by inhibiting alanine racemase.	Peripheral neuropathy, Stevens-Johnson syndrome.	100	[21,28]
	Capreomycin (CAP)	5–20	Bactericidal effect. Inhibits protein synthesis via modification of ribosomal structures at the 16S rRNA.	Nephrotoxicity, hypersensitivity, Hypokalemia, neuromuscular blockade, auditory and vestibular ototoxicity.	Freely soluble	[21,29]

	Para-aminosalicylic acid (PAS)	1–8	Bacteriostatic effect. Prevents iron uptake and folic acid biosynthesis. Prevents iron uptake and folic acid biosynthesis.	Not suitable for individual with renal disease due to build-up of toxic metabolite. Reduces uptake of vitamin B12 and thyroid metabolism. Dermatological effect such as rashes.	1.7	[21,30]
Other drugs	Rifabutin		Inhibits bacterial RNA synthesis via binding to β -subunit of bacterial DNA-dependent RNA polymerase.	Similar adverse effects to RIF.	0.19	[21,31]
	Clarithromycin		Prevents protein synthesis via binding to 50S ribosomal RNA.	Gastrointestinal discomfort (diarrhoea, vomiting, abdominal pain and nausea), headache, and rash. Hepatotoxic. Hypoglycaemia in rare cases.	Insoluble in water	[21,32]
	Clofazimine		Antimycobacterial and immunosuppressive properties. Might be involved in DNA binding.	Gastrointestinal toxicity (abdominal and epigastric pain, diarrhoea, nausea, vomiting, gastrointestinal intolerance (40–50%))	Insoluble in water	[21,33]
	Amoxicillin		Inhibits cell wall biosynthesis.			[21]

APPLICATION OF NANOTECHNOLOGY-BASED DRUG DELIVERY SYSTEMS FOR TB TREATMENT

Side effects from current TB pharmacotherapy are a clinical concern. Oral route administration is the most convenient and least expensive method to deliver ATD. However, slow onset of action, significant first-pass metabolism effect in the liver, instability in acidic condition and high absorption in gastrointestinal tract are some of the limitations faced. More importantly often sub-therapeutic concentrations of ATD at the site of action promote the emergence of MDR strains. From the clinical perspective, even though sufficient ATD levels are delivered to infected sites, they might not penetrate into granulomas where the mycobacteria reside. Nanotechnology-based drug delivery systems offer exciting new opportunities to treat TB including enhanced diagnosis and therapy. These systems are usually comprised of nano-sized carriers, which could be modulated to have different structural or functional properties (Table 2). Some technological advances of these carriers include high stability, high loading capacity, feasibility for various administration routes (i.e. oral, intravenous and inhalation) and relative higher bioavailability. Nanocarriers can also be designed to exhibit selective targeting behaviour. In the case of TB, as macrophages is usually the major infected site, the functionalization of mannose-based ligands on these carriers could result in higher affinities *in vitro/in vivo*. Different ligands such as maleylated bovine serum albumin (**MBSA**), *O*-steroyl amylopectin (**O-SAP**), galactomannan, *p*-aminophenyl-mannopyranoside

(PAM) and *O*-palmitoylpullulan **(OPP)** were utilized for this purpose to improve internalization via mannose or lectin-like receptors on surface of macrophages. In addition, nanocarriers can be manipulated to possess controlled release or stimuli-response release properties. The latter characteristic is particularly relevant for the targeting of intracellular mycobacteria in acidified compartment within macrophages. Moreover, nanotechnology-based carrier system is poised to reduce low bioavailability, high systemic clearance and anatomical barrier issues encountered with conventional drug delivery. Through this, lower adverse effects associated with ATD and doses frequency could be achieved. This chapter discusses the current development of nanotechnology-based delivery vehicles for TB.

Table 2: Advantages and limitations of current nanotechnology-based drug delivery system.

Carrier	Advantages	Limitations	References
Liposome	Promote phagocytic cells uptake and suitable for delivering hydrophobic and hydrophilic drugs. Highly flexible for surface modification (i.e. surface charge regulation and ligand conjugation).	Not stable in room temperature and need to be refrigerated.	[34-36]
Solid lipid nanoparticles	Easy to scale-up, suitable for hydrophobic and hydrophilic drugs Solvent-free	Toxicity might be contributed from lipid matrix.	[37,38]
Polymeric nanoparticles	Excellent to increase drug stability, hydrophobic and hydrophilic drugs can be delivered with different route of administration.	Degradation of polymer could be limited due to lacking of enzymes.	[36,39]
Micelles	Facilitate solubilisation of hydrophobic drugs and prevent chemical and biological degradation. Improve accumulation of ATD in infected cells.	Require stabilizer to prevent micelles aggregation.	[40, 41]
Nanosuspension	Highly suitable for improving drugs with limited water and lipid solubility.	Dimethyl sulfoxide might contribute to toxicity effect.	[42,43]
Nanoemulsion	Improve cellular uptake by phagocytic cells. Thermodynamically stable, remain unchanged up to 3 months in both 4°C and 25 °C. Suitable for filter sterilization.	Initial burst release effect occurs	[44-46]
Dendrimer	Monodisperse, flexible for surface modification and can be delivered by different administration route.	PAMAM dendrimers are toxic to cells	[47-49]

POLYMERIC NANOPARTICLES

Synthetic-Polymer Based Nanoparticles

Among the various polymers used in drug delivery, Poly (Lactic-Co-Glycolic Acid) **[PLGA]** is one the most studied copolymer and has been approved by Food and Drug Administration (**FDA**) for therapeutic applications [34]. PLGA is biocompatible, biodegradable and is broken down to lactic acid and glycolic acid in the body. Excellent stability, versatility to attach homing receptors or targeting moiety on the surface, high loading of various drugs and ease of preparation are some of the advantages of PLGA nanoparticles [34]. In a study by Pandey and co-workers, sustained release PLGA (50:50) nanoparticles encapsulating three first line ATD (RIF, INH, PZA) were produced using modified multiple emulsion and evaporation method [50]. Drug encapsulation efficiencies were 56.9% for RIF, 66.3% for INH and 68.0% for PZA. Following single dose of ATD loaded PLGA nanoparticles administered through oral route, therapeutic drug concentrations (>minimum inhibitory concentration, MIC) in tissues were observed up to day 9. Free drugs were cleared from plasma within 24 h in comparison. Enhanced antibacterial activities were also

evident as five oral doses of the nanoparticle every 10 days eliminated the infection completely while it took 46 equivalent doses of free drugs to achieve the same effect [50]. The same group also demonstrated similar results of this formulation in terms of relative bioavailability and therapeutic potential when administered to *M. tuberculosis* infected guinea pig model [51]. In another study, Johnson and co-workers demonstrated that the non-encapsulated free drugs were more effective to eliminate mycobacterial infection in mediastinal lymph nodes of disseminated TB guinea pig model [52]. The authors suggested that PLGA nanoparticles were unable to penetrate lymphoid tissues as easily as free drugs [52].

In addition, Pandey and co-workers investigated the synergistic combination of EMB loaded PLGA nanoparticles and ATD loaded PLGA nanoparticles (RIF, INH, PZA) against *M. tuberculosis* infected mice [53]. As EMB is prone to degradation in the presence of other ATDs, the authors had to encapsulate EMB separately into PLGA nanoparticles [53]. Hepatotoxicity effect was not evident in mice receiving repeated oral doses of these formulations. The four-drug combination treatment significantly improved drug bioavailability and reduced dose frequency from single dose daily for 28 days to a total of 3 doses administered every ten days [53].

Kumar and co-workers developed negatively charged ETH loaded PLGA nanoparticles with average diameter of 286 ± 26 nm using solvent evaporation method after optimizing various experimental variables [54]. It was demonstrated that the combination of 0.5% polyvinyl alcohol (PVA), sonication time of 3 min and drug:polymer (1:1) produced nanoparticles with optimal size, loading and stability. Despite the moderate encapsulate efficiency (35.2%), the high drug loading at 38.6% implied that minimal amount of carrier would be administered to ensure sufficient drug content. In addition, this combination parameter resulted in stable nanoparticles for as long as 3 months without changes in particle size, surface charge and polydispersity index. ETH loaded PLGA nanoparticles administrated at therapeutic dose (130 mg/kg) were well-tolerated and showed negligible toxicity-associated effects on both body weight gains and clinical signs (i.e. hematology, clinical chemistry and histopathology) [54]. ETH loaded PLGA nanoparticles also exhibited enhanced residency and bioavailability based on pharmacokinetic parameters. The mean residence time (MRT) for ETH loaded nanoparticles and free ETH was 36.11 ± 3.9 h and 1.27 ± 0.2 h, respectively [55].

In a recent study, the mechanistic *in vitro* and *in vivo* drug release behaviours of INH loaded PLGA-PEG-PLGA triblock copolymers were investigated. The drug loaded nanoparticles, as prepared using water-oil-water emulsification method, were spherical and ranged from 150-400 nm in size [56]. The authors demonstrated that these triblock copolymers had higher INH loading and entrapment efficiency compared to di-block PLGA nanoparticles. In addition, oral delivery of the drug loaded tri-block copolymers to Wistar rats resulted in significantly improved bioavailability (28-fold higher) and prolonged circulation in plasma compared to free INH [56]. Long acting PLGA nanoparticles containing rifampin and pentenyl-INH to selectively target macrophage endosomes were prepared [57]. The cellular uptake of rifampin and pentenyl-INH

were 3 fold and 10 fold higher in PLGA nanoparticles compared to free drug, respectively in human monocyte-derived macrophage (**MDM**). Interestingly, PLGA nanoparticles could retain rifampin (0.1 µg/106 cells) and pentenyl-INH (0.2 µg/106 cells) until day 15; however, these drugs in free form were not detectable after 24 h. Regardless of types of drugs loaded nanoparticles used to reduce *M. smegmatis* viability, the antimicrobial activity was only effective on the first day of treatment but started to decrease on day 5. However, when compared to free form drug, the antimicrobial activity from drug loaded PLGA nanoparticles were 3 to 5 fold higher than free form drug. The authors showed that PLGA nanoparticles were protected inside the recycling endosomal compartments and therefore had better antimicrobial activity [57].

Functionalization of nanoparticles to allow stimuli-responsive release of drug cargo in specific microenvironment (i.e. acidic) is sensible especially in the case of targeting endolysosomal compartment in macrophages. INH was covalently attached to mesoporous silica nanoparticles (**MSN**) of different sizes (50 nm and 100 nm) through a pH-sensitive hydrazone bond to form a prodrug nanoparticulate system and coated with PEI-PEG [58]. The nanoformulations were stable at neutral pH and a switch to acidic conditions triggered high drug release. The INH loaded PEI-PEG MSN was also avidly internalized by *M. tuberculosis* infected THP-1 macrophages and killed the intracellular bacteria in a dose- dependent manner. Irrespective of nanoparticle size and route of administration, the authors demonstrated that these nanoformulations were well-tolerated by infected mice. Both subcutaneous and intravenous administrations of INH loaded PEI-PEG MSN were more effective than free drug, which were largely attributable to improved pharmacokinetics [58]. In another work, cyclodextrin-based-pH operated valves which open at acidic conditions were anchored to rifampin loaded MSN (average size of 100 nm) [59]. Similarly, the stimuli- responsive nanocarrier was internalized efficiently by macrophages and trafficked to acidified endosomal compartment of macrophages [59]. Subsequent intracellular high concentration of rifampin resulted in significantly higher killing of *M. tuberculosis* compared to free drugs [59].

The emergence of multi-drug resistant variants of *M. tuberculosis* signals the search for novel classes of drugs and treatment regimens. Mycolic acids are currently investigated as promising mycobacterial ligand anchored to drug loaded nanoparticle carrier to combat tuberculosis [60]. Mycolic acids, comprised of long 2-alkyl 3-hydroxyl fatty acids are dominant lipid found in the outer cell wall envelope of Mycobacterium sp. and are major virulence factors of these pathogenic strains. Lemmer and co-workers reported a simple approach of incorporating mycolic acids to INH loaded PLGA nanoparticles using double emulsion solvent evaporation [60]. Attached mycolic acid was rapidly recognized by cell surface receptors such as scavenger receptors, avidly internalized into mycobacterium- infected macrophages and were processed into phagolysosome within hours. At least 80% of cells displayed one NP where exposed to ligand coated nanoparticles compared to only 53% for unmodified nanoparticles. The authors suggested that lipid-rich mycolic acids served as ligand for cholesterol-rich lipid-raft-like areas on infected cells, thus facilitating their uptake [60].

Another new ATD candidate (TB515) with enhanced *in vitro* efficacy and internalization in macrophage cells has been formulated into surface modified PLGA nanoparticles [61]. Lapazines, an active ingredient of *Tabebuia* sp, has been known to exhibit antibacterial activity against *Mycobacterium* sp. However, inherent low solubility coupled with acute chronic toxicity in Balb/c mice restricted the use of lapazines *in vivo*. In a recent study by Silveira and co-workers, polymeric nanoparticles were developed to encapsulate synthetic lapazine with the aim to preserve its antimycobacterial activity and protect the drugs from physiological conditions [62]. Lapazine loaded PLGA or PCL nanoparticles were relatively monodispersed with mean diameters around 200 nm. The MIC of lapazines against H37RV *M. tuberculosis* and RIF-resistant strains were 3.00 and 1.56 $\mu\text{g/mL}$, respectively [62].

Non-invasive route such as inhalation is becoming a popular choice given the majority of TB manifestations are localized in the respiratory systems. In addition, local delivery of ATD via inhalation to target alveolar macrophage also ensures that systemic toxicities associated with drugs are kept at minimum. Several first-line TB drugs that are conventionally administered systemically are now revamped for targeted delivery to lungs. The lung is a complex structure with narrowing airways and capture particulates through impaction. As a rule, aerosolized particulates should have a mass median aerodynamic diameter (MMAD) $<5\ \mu\text{m}$ to penetrate into the conducting airways and beyond. Single-step synthesis of RIF/PLGA nanoparticles dispersed in mannitol microspheres using four-fluid nozzle spray drier were used to prepare inhalable dry powder formulation [63]. The mean diameter of RIF/PLGA nanoparticles was 213 nm while 50% of spray-dried inhalable nanoparticles-in-microspheres were $2.1\ \mu\text{m}$. Using cascade impactor to evaluate *in vitro* aerosol performance, up to 42% drugs were deposited in stages with cut-off diameter $<5\ \mu\text{m}$. The *in vivo* imaging of rats following intratracheal insufflation suggested that PLGA microspheres were rapidly excreted while PLGA nanoparticle were retained longer in the lungs [63].

Natural Polymer-Based Nanoparticles

Natural polymer-based nanoparticles such as alginate and gelatin have attracted numerous attentions recently as novel drug carriers. Sodium alginate consists of two different components (β -D-mannuronic acid and α -L-guluronic acid) that can exist as homo-polymeric block or hetero-polymeric blocks. Depending on the components' ratio, sodium alginate can be used as thickening agent (high β -D-mannuronic acid) and for gelation purposes (α -L-guluronic) [64]. In addition, physicochemical properties such as porosity, shrinkage, elasticity and swellable behaviour are alterable by modulating the concentrations of β -D-mannuronic acid and α -L-guluronic acid. For instance, alginates with higher α -L-guluronic content are more porous and do not tend to shrink during gelation [64]. On the other hand, alginates containing high β -D-mannuronic acid content adopt gel-like structure with higher elasticity and low porosity. These alterable properties of alginate offer versatility during the design of carrier system depending on the end-point applications [64].

Inhalable alginate nanoparticles as anti-TB carrier was developed using cation-induced gelification of alginate method [65]. Irrespective of the drugs encapsulated (INH, RIF and PZA) the sizes of nanoparticle carrier were approximately 240 nm while drug encapsulation efficiency ranged between 70 to 90%. Up to 80% of the nebulized drugs containing alginate nanoparticles were within inhalable range; with recorded Mass Median Aerodynamic Diameter (**MMAD**) and Geometric Standard Deviation (**GSD**) of $1.1 \pm 0.4 \mu\text{m}$ and $1.7 \pm 0.1 \mu\text{m}$, respectively. Significant increase in relative bioavailability in terms of maximum plasma concentration (C_{max}), time reaching maximum plasma concentration (T_{max}) and AUC was demonstrated for drug loaded alginate nanoparticles compared to free drugs. INH, RIF and PZA were detectable up to 14, 10 and 14 days, respectively when administered in nanoparticulate form. In contrast, aerosolized free drugs were cleared within 24 h of administration. The authors also showed that bioavailability was affected by administration route in which aerosol route could effectively prolonged drug retention and avoided systemic clearance compared to oral route in guinea pig model. In addition, the drug concentrations of the nanoparticle-based formulations in various tissues (lung, liver and spleen) were higher than established MIC of respective drugs against *M. tuberculosis*. From the perspective of therapeutic efficacy, drug loaded alginate nanoparticles administration via inhalation required only three dosing every 15 days while for comparable results, free drugs given orally required a single dose/day for 45 consecutive times [65]. Using the same gelification method, alginate nanoparticles encapsulating four different ATDs (INH, RIF, PZA and EMB) were synthesized [66]. Regardless of the doses administrated into mice, the pharmacokinetic and pharmacodynamics profiles showed that drugs existing as nanoformulations were retained longer in plasma and tissues [66].

As mentioned earlier, high dosing frequency is sometimes associated with ineffective therapy owing to the emergence of bacterial resistance and failure of patients' compliance. Therefore, Ahmad and co-workers attempted to prove that oral administration of alginate nanoparticle containing ATD (i.e. INH, RIF, PZA and EMB) could reduce to dose frequency from 45 to 3 doses over a period of 45 days. No bacilli were detected in lung and spleen of *M. tuberculosis* infected mice after a total of 3 doses drug loaded alginate nanoparticles were administered orally, and this was comparable with conventional dose-schedule (45 doses) [67]. Guar gum- based porous nanoaggregates with excellent flow properties are potential carrier for ATD drugs. Pulmonary administration of ATD (RIF and INH) loaded guar gum nanoparticles produced using solvent-precipitation-spray dried showed prolonged residence at target site as well as multiple-reduction of bacilli replication [68,69].

Azole anti-fungal drugs have demonstrated their potentials as antimycobacterial drug under *in vivo*, *ex vivo*, *in vitro* conditions against murine tuberculosis [70-72]. Specifically, econazole showed comparable chemotherapeutic efficacy to RIF through the reduction of bacterial burden (90%) in lungs and spleen of mycobacterial infection mice [73]. However, the bioavailability of this drug is extremely low through oral route. In a work by Ahmad and co-workers, the combination

of antifungal and ATD was evaluated for their feasibility to control TB infection. Using the optimized gelification method, four different ATDs (RIF, INH, PZA and EMB) and econazole were incorporated into alginate nanocarrier to produce particles with average size of 230 nm. All ATDs were detected for as long as 15 days while the residency time of econazole in lung, liver or spleen was 8 days. In addition, it is obvious that the use econazole loaded alginate nanoparticles resulted in lower dose frequency and potentially reduced toxicity. For instance, the therapeutic efficacy of 8 doses of econazole loaded gelatin nanoparticles was equivalent to 112 dose of free econazole [74].

Gelatin nanoparticles (**GP**) are attractive carrier owing to their biodegradability, biocompatibility, low antigenicity and flexibility for attaching targeting moieties [75]. To date, these nanoparticles have been studied for the delivery of amphotericin B, paclitaxel, doxorubicin and various genes or peptides [76-82]. In a recent study, GP encapsulating RIF (entrapment efficiency: 60%) with uniform shapes were fabricated using two-step desolvation method with acetone acting as desoalting agent [83]. The resulting RIF loaded GPs were spherical in shape and positively charged (15.32 mV), hence the high stability and minimal particles' aggregation in aqueous conditions. In addition, the RIF loaded GPs had a mean diameter of 264 nm and were non-toxic to J774 macrophage cell line (cell viability of 91%) even at high concentration (1 mg/mL). Free RIF on the other hand reduced the cell viability to only 65% at equivalent concentration. The biodistribution study clearly indicated that GP enhanced the bioavailability of RIF in macrophage-rich organs such as liver, spleen and lung. Compared to free drug, the concentration of RIF loaded GPs in lung, liver and spleen were at least 6-fold, 2.5-fold and 3-fold, respectively higher. The *in vivo* antibacterial activity also revealed the potency of RIF loaded GPs to simultaneously killed mycobacteria at fewer dose frequencies [83]. To improve the selectivity towards alveolar macrophages, the same authors introduced a mannose receptor on the surface of GPs loaded with INH [84]. In the presence of mannose, the encapsulated INH reduced from 55.3% to 43.2% but this was compensated by enhanced recognition and engulfment of mannosylated GPs by J774 macrophage cell line. Furthermore, the coating of GPs with mannose enhanced the accumulation of INH to liver, spleen and lungs compared to non-modified GPs [84].

LIPOSOMES

Liposomes are considered the most versatile vesicles for drug delivery and are consisted of phospholipid bilayer surrounding an aqueous core [85]. Liposomes can be classified as large multilamellar liposomes, small unilamellar vesicles and large unilamellar vesicles depending on the number of lipid bilayers. Cholesterol and phospholipid are main components of lipid bilayers and this offer unique properties for liposome in which hydrophobic drugs would locate in the lipid bilayers whereas hydrophilic drugs will be entrapped either in aqueous core or bilayer interface [86]. In another word, liposome has a natural ability to compartmentalize hydrophobic and hydrophilic drugs. Besides being a biocompatible and biodegradable nanocarrier, liposome has demonstrated stronger affinity towards cell surfaces via electrostatic interactions or hydrogen

bonding. Liposomes with variable size have different final fate after administration *in vivo*. Liposomes with size less than 100 nm have longer residency time, slower drug release rate and less rate of opsonisation compared to liposomes with larger sizes (more than 100 nm). Liposomes with size between 50-200 nm are generally well accepted as effective drug delivery.

Both *in vitro* and *in vivo* liposome-mediated or deliveries of antibiotics to mycobacterial-infected cell lines or tissues have been a success to enhance therapeutic effect of drugs [87-92]. During the early 1990s, the antibiotics studied as potential ATD include second-line (reserve) drugs such as aminoglycosides (i.e. amikacin, gentamicin) and fluoroquinolones (i.e. ciprofloxacin, sparfloxacin). In 1990, Klemens and co-workers showed that liposomal gentamicin were more effective to reduce the populations of *M. avium* in mouse model compared to free gentamicin [93]. Intermittent intravenous administration of liposomal gentamicin (20 mg/kg) for 3 weeks caused a 2.5 log reduction in bacterial count in spleen and liver [93]. Nightingale and co-workers previously showed that liposomal gentamicin is a potential anti-TB therapy for associated infections in AIDS patients. Intravenous injection of TLC G-65, a liposome-encapsulated gentamicin was administered twice weekly for 4 weeks to AIDS patients with *M. avium*- *M. intracellulare* Complex (**MAC**). The concentrations of gentamicin per kg per body weight administered varied among patients whereby 4 patients received 1.7 mg/kg, 10 patients received 3.4 mg/kg and 7 patients received 5.1 mg/kg. The bacterial count reduced by 75% in all groups and the patients did not develop drug resistance [94]. In another study, liposomal amikacin resulted in outstanding results as the concentration of amikacin was maintained above MIC for *M. avium* for more than 28 days. In comparison, the concentration of free amikacin was below the detection limit after 9 h of administration. The treatment of *M. avium* infected mice with liposomal amikacin once a week had successfully reduced the bacterial count by more than 2-log and this finding was equivalent to conventional dosing regimens of twice-weekly or even daily injections [95]. Mehta and co-workers have evaluated the efficacy of a series of free and liposomal antibiotics (i.e. cerulenin, sparfloxacin, clofazimine, rifampin, resorcinomycin A, PD117596 and PD117558) against different species of Mycobacterium including clinical isolates from cancer and AIDS patients [96]. Overall, MIC of all Mycobacterium sp. was in the range of 0.5–62 $\mu\text{g/mL}$ and the Minimum Bactericidal Concentration (**MBC**) ranged from 2 to more than 125 $\mu\text{g/mL}$. Among the tested antibiotics, clofazimine either in free or liposomal form demonstrated the highest killing effects including against *M. avium*, *M. tuberculosis* and some drug resistant strains [96]. In addition, liposomal encapsulated antibiotics containing either ciprofloxacin or streptomycin were synthesized using mixtures of phosphatidylglycerol-phosphatidylcholine-cholesterol (molar ratio, 1:9:5) and evaluated against MAC-infected human peripheral blood monocyte/macrophages. Both liposomal streptomycin (10-50 $\mu\text{g/mL}$) and liposomal ciprofloxacin (0.1 $\mu\text{g/mL}$) resulted in 3-fold and 50-fold, respectively higher reduction in bacterial count compared to free solubilized drug [88]. Another fluoroquinolone, sparfloxacin had also been encapsulated into multilamellar liposome consisting of phosphatidylglycerol-phosphatidylcholine-cholesterol (1:1:1 molar ratio)

for treatment of *M. avium* infected murine macrophage-like cell line J774 [87]. No significant differences in antibacterial effect between free and liposomal sparfloxacin formulations were observed which is in contrast with other published data. Therefore the authors suggested that both free and liposomal sparfloxacin were internalized and localized within macrophage cells at similar rate [87]. As most the drugs are non-target specific, developing an efficient way to deliver sufficient dose to treat infections while exerting minimal cytotoxic effect is a challenge. Adams and co-workers has reported high tolerance of clofazimine in *M. tuberculosis* infected mice [97]. In acute murine tuberculosis model, maximum tolerate concentration of clofazimine in free form was approximately 5 mg/kg with little effect on inhibition of *M. tuberculosis* growth. However, higher concentration of clofazimine (at least 10 times higher) did not cause toxicity complications in murine model when delivered using liposomal system. At least more than 3- log reduction of Colony Forming Unit (**CFU**) in spleen and lungs while 2-log in liver was observed. This effect was more pronounced in chronic and established murine tuberculosis model whereby no bacilli were detected after treated with 50 mg/kg of liposomal clofazimine. The bioavailability of high concentration of clofazimine within the macrophages was the main contributing factor to enhanced antibacterial effect compared to free clofazimine (which also caused extensive toxicity to cellular membrane) [97].

First-line TB drugs such as RIF, INH and PZA have also been encapsulated into liposome to prevent degradation and reduce inherent toxicities associated with these drugs. Different lipids used during the preparation of liposome are demonstrated to influence the bioavailability of rifabutin in mice model. Liposomes consisting of high phase transition temperature (T_c) phospholipid such as dipalmitoyl phosphatidylcholine (DPPC, $T_c = 42^\circ\text{C}$), dipalmitoyl phosphatidylglycerol (DPPG, $T_c = 42^\circ\text{C}$) and hydrogenated phosphatidylcholine (HPC, $T_c = 58^\circ\text{C}$) prevented metabolism of rifabutin *in vivo* [98]. Among these phospholipids, combination of DPPC and DPPG was the most superior to prevent degradation of rifabutin and also effectively deliver rifabutin to liver, spleen and lung. In comparison, the recovery of rifabutin from these organs was almost zero after 24 h of administration using free rifabutin. As expected, mice treated with liposomal rifabutin exerted higher antibacterial effect compared to free rifabutin as in reduced bacterial count in spleen (5.53-log vs 5.18-log) and liver (5.79-log vs 5.41-log) [98]. Neutral or charged liposomes with relatively large multilamellar vesicles could be easily prepared by altering DPPC to cholesterol ratio [99]. Using vortex dispersion method to induce swelling at heated condition (52°C), up to 10% of PZA was incorporated to both lipophilic and hydrophilic segments of liposome [99]. PZA liposome exhibited high therapeutic efficacy with significant reduction of bacterial load in mice lung after administration of seven doses (25 mg/kg per dose, twice weekly) via subcutaneous injection. This result is on par with control groups receiving a total of 18 doses with frequency of 6 times per week [99]. Surface modification of PEGylated-liposome with O-streaylamylopectin (O-SAP) produced stealth-like behavior nanocarriers with strong affinity towards lung tissue in mice [100]. To achieve this prolonged circulation effect, Doel and Khuller synthesized sterically

stable multilamellar liposome vesicles containing ATD (INH and RIF) consisting of cholesterol, egg phosphatidylcholine, O-SAP and gangliosides. Biodistribution studies conducted using healthy and *M. tuberculosis*-infected mice via intravenous administration showed that pronounced retention of drugs existing in O-SAP-PEGylated liposomes (31%) in the lungs after 30 min [100]. In comparison, only 5.1% non-coated conventional liposomes were delivered to lungs. Moreover, the pre-treatment of both subject groups with conventional liposomes prior to administration of modified liposome enhanced the uptake in lungs to approximately 40%. This observation could be attributed by the saturation of Reticuloendothelial System (**RES**) with conventional liposome. In addition, *in vitro* cytotoxicity assay with peritoneal macrophages showed reduced toxic effects by liposomal system owing to slow sustained release of drugs [100]. Therapeutic efficacy of free and O-SAP-PEGylated liposomes investigated in *M. tuberculosis* infected mouse for 6 weeks at both therapeutic and sub-therapeutic dose showed that liposomal carrier was extremely effective in inhibit bacterial growth even at concentrations below the recommended therapeutic concentrations. In conjunction with higher antibacterial activity, O-SAP-PEGylated liposomes reduced the hepatotoxicity effect on healthy cells as confirmed by measurements of bilirubin, glutamate pyruvate transaminase and amylopectin [101,102]. In addition, similar results in terms of bioavailability, toxicity and therapeutic activities were also obtained when O-SAP-PEGylated liposomal drugs were administered to guinea pigs infection model [103].

Pulmonary administration of liposome using nebulization is currently the mainstream approach to achieve high local delivery of ATD to lungs. In the meantime, engineering of dry powder liposomal formulations through lyophilization and spray drying are also extensively investigated to warrant particles' stability prior to administration. Vyas and co-workers developed targeting ligand-anchored liposomal formulation containing RIF to pulmonary alveolar macrophages via inhalation [104]. Maleylated bovine serum albumin (**MBSA**) and O-SAP were chosen owing to their specificity towards macrophage scavenger receptors. Pressurized packed systems (chlorofluorocarbon-based) of the RIF liposomal formulations displayed good aerosol performance whereby up to 1.8-fold increase in drug deposition at the base of lung was observed compared to free RIF solution aerosol. In addition, *in vitro* and *in vivo* viabilities of *M. smegmatis* in macrophages after exposure to aerosolized ligand- anchored liposome were in range of 7 to 11%. Meanwhile, the percentage survival of the mycobacteria in the presence of free RIF solution aerosol was approximately 45% [104].

In contrast, passive targeting to alveolar macrophages using RIF loaded liposome was developed using phospholipon 90®, soy lecithin and cholesterol via film hydration method followed by freeze-drying [91]. Lyophilized liposomes adopted multilamellar vesicle morphology with average size of 185 nm and polydispersity index of 0.24. In terms of aerosol performance, the calculated nebulization efficiency and drug content after nebulization were >50% and 65%, respectively. Complete *in vitro* growth inhibition of *M. avium* by liposomal RIF and free RIF occurred at 0.05 µg/mL and 5 µg/mL, respectively. The authors also reported that nebulized

liposomal RIF into rats using nose-only exposure chamber were deposited deeper into lung airways compared to free drug aerosol. However, therapeutic effect of liposomal RIF using infected animal model was not assayed in this work [91]. In another work, DPPC-based liposome encapsulating INH at ratio of 1:1 was developed to produce a dual function drug delivery system that simultaneously acted as exogenous pulmonary surfactant replacement and inhalable drug carrier [105]. The liposomal vesicle had average diameter of 750 nm and encapsulation efficiency of 37%. Combination of INH and DPPC at 1:1 ratio formed monolayers with characteristics of pulmonary surfactant which could contribute to alveolar stabilization owing to anti-atelectatic effect of the surface tension, as suggested by the authors. In addition, respirable DPPC-based liposomal formulations possessed a faster adsorption at the pulmonary air-aqueous interface compared to commercial lung surfactants. As expected, stronger antibacterial activity against *M. tuberculosis* suggested the suitability of this inhalable liposomal formulation to be investigated further as adjuvant local therapy for TB infection [105].

Proliposome are free-flowing dry powders that spontaneously form liposome when in contact with water or biological fluids. They have potential to overcome stability issues associated with dispersions of liposome upon freeze-drying or spray drying. In addition, they present a potential to be delivered locally to the lungs as dry powder form. RIF proliposomes were prepared previously via freeze-drying using mannitol as cryoprotectant [106]. These prepared dry powder formulations were non-toxic to healthy bronchial epithelial cells, small airway epithelial cells and alveolar macrophages while successfully inhibited *M. bovis* growth at low concentration (0.2 μM). In addition, spray drying has been used to develop proliposomes loaded with INH, levofloxacin or PZA [92,107,108]. Proliposomes, consisting of INH, microporous mannitol as well as mixtures of soybean phosphatidylcholine and cholesterol were spray-dried and converted into liposomal dispersion upon reconstitution in water. The mean vesicle sizes ranged from 300 to 1000 nm depending on the ratio of mannitol to INH. INH proliposomes demonstrated good aerosol performance under evaluation using Andersen Cascade Impactor (**ACI**) at flow rate of 60 L/min as up to 35% fine particle fraction was obtained. In addition, these proliposomes did not elicit immunological responses in alveolar macrophages but demonstrated higher antibacterial effect against *M. bovis* [108]. Similar results were also reported for the efficacy and suitability of inhalable levofloxacin proliposomes as potential anti-TB treatment [92].

In a recent study, Patil-Gadhe and Pokharkar employed a systemic approach based on Quality by Design (**QbD**) to produce inhalable proliposome containing rifapentine via fast, single step spray drying process [89]. Using modified Ishikawa diagram, the optimized rifapentine loaded proliposomal formulations (vesicle size: 578 nm; encapsulation efficiency: 72.08%) were prepared in combinations of rifapentine, hydrogenated soya phosphatidylcholine (**HSPC**) and cholesterol at molar ratio of 1:2:1. In addition, stearylamine was included to produce stable vesicles with positive charges (+29.4 mV). *In vitro* lung deposition study demonstrated excellent performance as up to 90% of the proliposomes were within inhalable size ranges [89]. The

rifapentine proliposome was less toxic against lung cell line (A549) compared to free rifapentine, as evidenced by their respective IC_{50} values ($105.28 \mu\text{g/mL}$ vs $72.57 \mu\text{g/mL}$) [90]. In addition, subacute repeated dose toxicity study was conducted using Wistar rats for a period of 28 days. With the exception of control subjects, the subjects were randomized to receive either placebo or different concentrations of rifapentine proliposome (1, 5 or 10 mg/kg) via intratracheal insufflation [90]. Repetitive dosing of inhalable RIF proliposome for 28 days at low (1 mg/kg) and medium (5 mg/kg) concentrations produced negligible cytotoxic and inflammation response in the rats. In addition, the subject groups appeared healthy and did not develop behavioral changes. However, at high dosage (10 mg/kg), the rats could not survive owing to significant pro-inflammatory response as well as liver and kidney failure [90].

MICELLES

Micelles are self-generated nanocarriers which exhibit aggregate-like structures in aqueous conditions when the concentrations of amphiphilic copolymers, polymer-lipid conjugates or surfactant molecules exceed their Critical Micelle Concentration (**CMC**) values. Micelles are consisted of two layers whereby the outer layer is a hydrophilic block, which facilitates the stability of the micelles, and the inner core tends to be more hydrophobic for encapsulation of poorly water-soluble drugs. Numerous polymer-based materials have been widely used for micelles formation such as poly(ethylene oxide)-b-poly(propylene oxide)-poly(ethyleneoxide) [PEO-PPO-PEO] and poly(epsilon-caprolactone)-b-poly(ethylene glycol)-poly(epsilon-caprolactone) [PCL-PEG-PCL] [20,109-111].

Micelle-forming copolymer PEG-poly(aspartic acid) [PEG-PASP] conjugated with INH (drug content approximately 64.87%) was synthesized with sustained release behaviour. The *in vitro* MIC assay using *M. tuberculosis* revealed that this micellar system was 5-folds more effective compared to free INH [112]. Similar approach was applied to incorporate PZA onto PEG-PASP micelles whereby the loading of drug was >80% [113]. The antibacterial activities of PZA loaded PEG-PASP micelles and free PZA were measured using strains Ra and Rv of *M. tuberculosis*. PZA loaded PEG-PASP micelles showed superior activities to inhibit Ra and Rv, corresponding to $0.475 \mu\text{g/mL}$ and $0.950 \mu\text{g/mL}$, respectively. In comparison, free drug exhibited higher MIC values at $6.25 \mu\text{g/mL}$ (Ra) and $12.5 \mu\text{g/mL}$ (Rv) [113]. The authors also attempted to synthesize novel co-formulations containing rifampin, PZA and INH using micelle-drug conjugation technique [114]. Using the condensation techniques in which moieties of the drugs were substituted onto carboxyl groups on the copolymer PEG-PASP derivatives, three micelles systems were successfully synthesized: PZA-PEG-PASP, INH-PEG-PASP and INH-co-rifampin-PEG-PASP. The mean diameters of micelles ranged from 78.2 to 98.9 nm while the amounts of drug substitutions were high (65.0–85.7%). The lyophilized and re-dispersed micelles formulations were stable *in vitro* and exhibited stronger anti-mycobacterial activities (15-fold higher) compared to free drugs irrespective of the *Mycobacterium* sp. strains used [114].

A range of self-assembled PVP-PCL diblock copolymer micelles with sizes varying from 150-205 nm were synthesized to study their potential role as nano-carrier for ATD [115]. First-line ATDs were successfully loaded either as single formulations (RIF or PZA) or dual formulations (RIF and INH) into the core of micelles. Based on the calculation of binding constants, RIF bound stronger to micelle cores compared to PZA and INH. In addition, the increase of hydrophobic chain length enhanced the loading of drugs. For instance, the loading efficacy of RIF changed from 49% to 79% as the portion of hydrophobic PCL segment increased from 50 to 100. In contrast, the release rate of RIF was promoted with higher hydrophilic chains [115].

Flower-like micelles consisting of PCL-PEG-PCL block copolymers were prepared to investigate the encapsulation efficiency of RIF recently [116]. In total, ten derivatives of these micelles were synthesized by Microwave-Assisted Polymer Synthesis (**MAPS**) technique. Physical stabilities of micelles in terms of particle sizes, heterogeneity and aggregation were intimately governed by molecular weight (M_w) and compositions of caprolactone (**CL**) and ethylene oxide (OE). The authors demonstrated that sufficiently long hydrophobic PCL segments were requisite to form stable micellar systems with higher drug encapsulation efficacy. Amphiphilic micelles synthesized with ratios CL/EO ratios >0.3 combined the characteristic of optimal drug encapsulation behaviour and physical stability. However, two main drawbacks were highlighted in this study. Firstly, prolonging the encapsulation period from 10 min to 40 min did not significantly enhance the loading efficiency. Secondly, higher RIF load compromised the physical stability of micelles. As the drug loaded micelles underwent substantial size growth, they had to be freeze-dried immediately upon preparation [116]. Addition of cryoprotectants such as mono- and disaccharides (glucose, sorbitol and sucrose), hydroxypropyl- β -cyclodextrin (HP β CD) and PEG on the physical stability of RIF loaded flower-like micelles were subsequently characterized [117]. The authors found that sugars or polyols alone were ineffective to cryopreserve the size and stability of both loaded and non-loaded drug micelles following freeze-thawing cycles. To maintain particles' stability, dual cryoprotectants with fixed ratios containing PEG3350 in conjunction with either sugar or polyols were required. The low ratio between sugars and polymers used was not enough to cover the matrix around the micelles, thus resulting in aggregation during freezing process. Different from sugar-based compounds,

HP β CD alone was found to be effective in minimizing the micelles grow even at low weight ratios to polymer (1:5) [117]. High encapsulation efficiency of RIF (97.8%), PZA (93.4%) and INH (96.6%) in lecithin-tyloxapol mixed micelles was synthesized by Metha and co-workers [41]. The *in vitro* released profile revealed that the amount of RIF (745 μ g/mL) released in first 5 h was higher than the therapeutic concentration (6 μ g/mL) and followed by sustained release behaviour. The other drugs exhibited burst release and reached the plateau phase before reaching 70 min incubation [41].

SOLID LIPID NANOPARTICLES (SLN)

SLN exist as aqueous nano-crystalline suspensions and are comprised from solid lipids at room temperature [38]. During the preparation of SLN with varying sizes (50-1000 nm), surfactants are often included to stabilize the lipid dispersion [38]. The advantages of SLN include high flexibility, ease of scaling-up and capability to encapsulate hydrophilic, hydrophobic, lipophilic and zwitterionic drugs [38]. In numerous studies, it have been indicated that extremely low concentrations of INH in plasma and tissues are found due to recognition by RES and subsequent extensive hepatic metabolism by liver enzymes [118,119]. As a consequence, repetitive dosing or high INH dosage daily is obligatory to obtain therapeutic effective plasma level. In a recent study, monodispersed INH SLNs with particle sizes of 48.4 nm and high entrapment efficiency (69%) was synthesized [120]. A significant improvement in bioavailability in plasma and brain following single dose oral administration (25 mg/kg) of SLN to rats was observed. Plasma and brain concentrations for INH SLNs were 6-folds and 4-folds, respectively higher compared to free drugs. In addition, prolonged circulation of INH SLN was achieved as evident from increased $t_{1/2}$ from 2.78 h to 13.32 h [120].

Combination therapies involving the administration of multiple ATDs have been used to resolve issues associated with multi-drug resistances. Having said that, poor bioavailability of RIF in combination with INH suggested drug-drug interactions, thus causing the dose of RIF to fall below the required minimum value. Many studies have suggested that in the presence of INH at acidic conditions (i.e. stomach), RIF underwent rapid decomposition [121, 122]. Singh and co-workers suggested that encapsulation of RIF and INH independently into SLN could effectively reduced drug-drug interactions and drug degradation. RIF loaded SLN provided 60% protection against degradation in the presence of free INH. Meanwhile, up to 75% improvement in RIF stability was observed when both drugs were administered in the form of SLN [123]. Rifampin loaded SLN with superior antibacterial activity against *M. fortuitum* was synthesized via ultrasonication aided emulsion approach [124]. The rifampin loaded SLN was spherical with mean diameter of 100 nm and exhibited 8-fold higher antibacterial activity. The MIC values of free rifampin and rifampin loaded SLN corresponded to 22 $\mu\text{g/mL}$ and 2.75 $\mu\text{g/mL}$, respectively [124]. In another approach, emulsion solvent diffusion was employed to incorporate triple ATD into a single SLN [125]. With this technique, the encapsulation efficiency for RIF, INH and PZA was $51 \pm 5\%$, $45 \pm 4\%$ and $41 \pm 4\%$, respectively while residual solvents were not present on the nanoparticles. The higher loading of RIF was probably due to its lipophilic characteristic, which increased the affinity and interaction with lipid components of the nanoparticles. Other physical characterization revealed that the SLN formulations were stable with slow sustained release profile. The *in vivo* pharmacokinetics following the administration of SLN to mice via oral route demonstrated that the drugs remained detectable in plasma up to 8 days and were distributed to lung, liver and spleen. After 10 days, the concentrations of drugs detected in lung, liver and spleen followed the decreasing pattern: PZA (5 mg/kg) > RIF (1 mg/kg) > INH (0.5 mg/kg) [125]. As the orally

administered free drugs were cleared within 48 hours from the tissues, SLN formulation resulted in an increase of 30-folds bioavailability in mice. In addition enhanced therapeutic effect was observed for ATD-SLN formulations compared to free drugs in terms of bacterial load reduction in *M. tuberculosis* H37Rv infected mice. Oral administration of single ATD-SLN dose every 10 days for 5 consecutive times was sufficient to completely eradicate *M. tuberculosis* in lung and spleen. To achieve the similar effect, 46 conventional doses of oral free drugs were needed. Results from serum bilirubin, Alanine Aminotransferase (**ALT**) or Alkaline Phosphatase (**ALP**) indicated that ATD- SLN or empty SLN were well tolerated and did not induce hepatotoxicity effect [125].

Targeting the phagocytosis pathways in macrophages is an alternative method to control internal bacterial replication as mycobacterium resides in host macrophages. As such, Chuan and co-workers demonstrated that submicron RIF loaded SLN (mean diameter of 829 nm) was internalized more effectively by alveolar macrophages compared to free RIF in both *in vitro* and *in vivo* experiments [126]. In another study, inhalable SLN formulations with alterable sizes for delivery of rifabutin into human monocytic cell line (THP-1) have been synthesized through modulation of lipid components. SLN prepared using glyceryl dibehenate had average size of 99 ± 4 nm while larger particles were produced using glyceryl tristearate (210 ± 8 nm). Almost 1-fold higher rifabutin concentration was observed in THP-1 when glyceryl dibehenate-based SLN was used compared to glyceryl tristearate-based SLN. It is suggested that particle size and not the lipid composition which contributed to the differences in the *in vitro* cellular uptake. Both glyceryl-based SLN were also safe for pulmonary drug delivery because did not interfere the plasma membrane of pulmonary cells (A549 and Calu-3) [127].

NANOSUSPENSION

Nanosuspensions are colloidal dispersions of submicron drug particles (100-700 nm) and stabilized by surfactants [43,128]. In nanosuspensions, poorly soluble drugs can be dispersed in aqueous phase and stabilized with addition of small amounts of surfactants such as poly(vinyl alcohol), pluronic or phosphatidyl choline [20,129]. The presence of surfactants or stabilizers inhibits crystal growth and particle aggregation during size reduction [20]. In addition these surfactants also act as cryoprotectant thus allowing the lyophilization of drugs into stable solid particles. These stable colloidal nanosuspensions enhance the possibility of delivering poorly soluble drugs via intravenous administration without blocking blood capillaries. This method also potentially improves drug absorption and bioavailability as smaller sizes confer higher surface area to volume ratio thus resulting in more effective TB therapies. Peters and co-workers have reported a facile approach to prepare clofazimine nanocrystalline suspension using high-pressure homogenizer precipitation. Depending on the preparation conditions, freeze-dried clofazimine nanocrystals ranged between 381 to 932 nm were successfully synthesized and stabilized using trehalose and mannitol. The authors also compared the efficacy of clofazimine nanocrystalline to reduce bacterial load in *M. avium* infected mice following intravenous administration with control liposomal clofazimine. Although the size of liposome was significantly smaller (100 nm)

than that of nanocrystalline (~400 nm), the authors found that clofazimine nanocrystalline was equally as effective as liposomal formulations. The distributions of clofazimine in lungs and spleen for both formulations showed no significant differences while the accumulation of the drugs in nanocrystals form was two-fold higher in liver compared to liposome. Although lung had the lowest clofazimine bioavailability, however the concentration of drugs was always higher than the MIC for *M. avium*. The reduction of bacterial burden data showed that both liposomal and nanocrystalline clofazimine were effective to suppress the replication of pathogens in all organs [130]. The data obtained were in accord with a previous study by Borner et al whereby intravenous injection of either liposomal (mean diameter of 107 nm) or nanocrystalline clofazimine (mean diameter of 420 nm) resulted in similar organ distributions [131]. Another approach employed to prepare nanosuspension could be via semi-continuous Supercritical Anti-Solvent (**SAS**) method coupled with carbon dioxide atomization. For this, Reverchon and co-workers exploited SAS precipitation method to produce RIF nanoparticles with controllable size and distribution, suitable for pulmonary and parenteral drug delivery. It was observed that organic solvents, soluble RIF concentrations and chamber pressure altered the morphologies and size of nanoparticles. For instance, RIF nanoparticles precipitated in Dimethyl Sulfoxide (**DMSO**) at 120 bar were interconnected small aggregates while ethanol resulted in long needle like crystal under same experimental conditions. Single spherical nanoparticles were obtained when operating at lower pressures. Meanwhile, the increment of particle size (400 nm to 1000 nm) was directly correlated to the increase of soluble RIF concentration (10 to 70 mg/mL). The stability assay confirmed that these synthesized RIF nanoparticles were in amorphous form and no degradation occurred during the fabrication process [42].

NANOEMULSIONS

Nanoemulsions are thermodynamically stable oil-in-water emulsions with droplet size typically ranging from 10 to 100 nm [128]. The advantage of this system is that they can be generated spontaneously and could be easily scale-up without requiring high homogenization energy. In addition, the synthesized drug loaded nanoemulsions can be sterilized via filtration. Nanoemulsions are highly suitable for delivery of any drugs (hydrophobic, hydrophilic, amphiphilic and lipophilic) via pulmonary, oral and intravenous routes. For instance, higher drug dosage could be delivered and promoted higher internalization by phagocytic cell in liver via lipoprotein receptors upon oral administration of nanoemulsions [128,132]. To date, the studies describing the use of emulsion to target TB are few.

In a study by Mehta and co-workers, the effects of a binary phase emulsion system containing oleic acid, Tween 80 and ethanol on physicochemical properties such as size, stability and drug loading were evaluated. The microemulsion remained stable after incorporation of anti-TB drug, INH in terms of pH, optical texture and phase separations. Out of the five microemulsion systems investigated, ME-C which contained 68% of surfactant resulted in the optimal loading of INH. In addition, the authors demonstrated that addition of 0.5% INH caused negligible effect

on the droplet size. The reduction of droplet size in the presence of 1.0% INH (260 nm to 210 nm) is probably due to the localization of drug molecules at the interface affecting mobility of surfactant [45]. This system also provided sustained released of INH as approximately 40% of drug was released after 2 h of the study and indicated that the release kinetics followed Non-Fickian behaviour. The same group attempted to prepare triple anti-TB formulations containing INH, RIF and PZA using Tween- based microemulsion systems [46]. In this study, the effect of different surfactants (Tween 20, 40, 60, 80) on phase behaviour was investigated at a constant surfactant/co-surfactant mass ratio ($K_m = 0.55$). It was demonstrated that the incorporation of INH and PZA reduced the droplet size from 260 nm to 210 nm. However, RIF promoted enlargement of droplet size to 320 nm. By using Tween-based microemulsion system, controlled releases of drugs were achieved whereby the release rate of the drugs reached plateau after 30 min. The authors postulated that the gelling of microemulsion system (resulting in higher viscosity) caused low diffusion of drugs from Tween 80. In addition, owing to the differences in hydrophobicities and localization of the drugs in the o/w phase, these drugs showed different release rate pattern. RIF demonstrated the lowest release rate (10%), followed by PZA (35%) and INH (40%). Being highly hydrophobic, RIF molecules were localized in the lipophilic phase of o/w while INH molecules were present in the continuum phase. Therefore the former encountered the highest hindrance in release while the latter demonstrated the highest release rate since the molecules were highly soluble in water [46]. Ahmed and co-workers synthesized six different o/w nanoemulsions entrapping RIF for intravenous administration by modulating the compositions of pharmaceutically acceptable excipients such as Sefsol 218 (RIF soluble agent), Tween® 80 (surfactant) and Tween® 85 (co-surfactant) and saline water as aqueous core [44]. The mean droplet sizes of the formulations ranged from 47 nm to 115 nm. The nanoemulsions were monodisperse irrespective of concentrations of excipients used. Interestingly, the drug entrapment efficiency was close to 100% and the pH of the nanoemulsions was in the range of 6.7-7.3. In addition, the nanoemulsions were stable and did not undergo significant transformations in terms of droplet size, entrapment efficiency and pH for more than 19 months under refrigerated (4 °C) and room temperature (25 °C) [44].

DENDRIMERS

Dendrimers are low molecular weight macromolecules with symmetrical hyperbranched architecture which extend from a central hollow core [49,133]. Depending on the numbers of branch generations, dendrimers showed three-dimensional globular morphologies with steric overcrowding at the periphery. The physical loading of drugs into dendrimers mainly depends on the hydrogen bonding and hydrophobic interactions between core of dendrimers and drugs [49,133]. Complexation and conjugation of drugs onto the dendrimers surface are also involved in drug binding processes. In addition, other functionality and ligands could be incorporated along the branches chain or periphery of dendrimers [128,134].

The first dendrimers synthesized from Polyamidoamines (**PAMAM**) was introduced in early 1980s [135]. It has been reported that cationic PAMAM dendrimers were hemolytic and caused excessive damage to cellular membrane [48,136]. Therefore, Jevprasesphant and co-workers investigated the effect of the incorporating surface modifying agents such as fatty acids or Polyethylene Glycol (**PEG**) on the cytotoxicity and compatibility of PAMAM dendrimers [137]. By introducing six lauroyl acid chains or four PEG chains on the surface of dendrimers, the cytotoxicity against Caco-2 cells were drastically decreased as evident from reduced membrane damages [137]. Optimization of RIF loading into 4th generation PAMAM dendrimer had been conducted by means of molecular dynamic simulations recently [138]. The maximum loading of 20 RIF molecules per PAMAM dendrimer at neutral pH was estimated. The RIF loaded PAMAM dendrimers showed higher stability at neutral pH. At acidic condition, rapid release of RIF was simulated, which in turned suggested beneficial targeting towards acidic cellular structures such as alveolar macrophages. It should be noted that *M. tuberculosis* resides within the acidic domain in macrophages (pH of 4.5) [138].

Recently, Manalan and co-workers have developed RIF-poly(propyleneimine) (**PPI**) dendrimers with the aim to minimize drug metabolism and improve drug solubility [139]. The entrapment efficiency and *in vitro* release of RIF was dependent on the ratio of dendrimer and drug concentration. In terms of hemolytic toxicity, RIF-poly(propyleneimine) dendrimers showed lowered the toxicity effect as compared to void poly(propyleneimine) dendrimers or RIF alone [139].

As mentioned earlier, surface modification with sugar-based molecules such as mannose is favorable to enhance targeting towards macrophages as lectin receptors present on membrane possess high affinities towards mannose. Kumar and co-workers have successfully loaded RIF (drug loading efficiency: 37.34%) into mannosylated 5th generation (5G) Polypropyleneimine (**PPI**) dendrimer with non-covalent interactions between RIF and dendrimer [140]. Owing to the steric hindrance and deprotonation of OH groups on the surface of mannosylated dendrimers, the mannose decorated formulations resulted in 10-fold reduction of RIF's solubility. Despite low solubility, RIF loaded mannosylated dendrimer had extremely low cytotoxic effect towards kidney cells while simultaneously promoted accumulation of RIF in alveolar macrophage compared free solubilised RIF molecules [140].

CONCLUSION

Although tuberculosis is a highly fatal disease with high mortality rate, it has been proven that it can be cured (if not totally) when the management of drug intake is properly controlled. From the drastic emergence of MDR-TB and XDR-TB in infected individuals, the re-evaluation of treatment regimens from a different angle is appealing. To put this into context, TB treatment does not only involve medical doctors and patients' compliance, the understanding of the biological behavior of mycobacterium and other epidemiological or lifestyle factors are needed. For instance,

the profiling of proteins specifically expressed on membranes of infected tissues might shed light to innovative designing of targeted treatment. Besides fluoroquinolone, new additions to ATD regimens have yet been approved although various novel classes of drugs, both natural and synthetic, are investigated. The major disadvantages involved in novel drug development include time, cost and effort as well as the uncertainty of whether these novel drugs could overcome MDR and latent TB issues. Nanotechnology-based drug delivery system seems to be an interesting alternative to overcome issues associated with conventional TB treatment. Using nanotechnology approach, existing ATD drugs could be encapsulated within smart nanocarriers equipped with various functional properties in a system including controlled release, long circulation and specific targeting. Administrations of free ATDs are prone to degradation induced by drug-drug interaction or in physiological microenvironment. It is often observed that higher therapeutic efficacy is found when ATDs are delivered with a suitable nanocarrier system. The enhancement of antimycobacterial activities is not due to the system itself but rather the drug protection conferred by the nanocarrier. Despite the advantages of nanotechnology-based drug delivery systems, numerous issues have to be clarified before large-scale clinical use as TB treatment. There is a valid concern about the toxicity of nanocarriers in human as little is known about the 'life-time' of nanoparticles in human. So far, data extensive *in vitro* and *in vivo* studies vouch for the safety of these nanocarriers especially liposomal-based formulations, as minimal toxicity are present towards organs or tissues.

Among various types of carriers, lipid-based carrier such as liposome and SLN are deemed the most suitable for pulmonary delivery of anti-TB drugs since the lung consists of lipid-rich surfactants. It should be noted that the composition in lipid matrix could result in different level of cytotoxicity towards macrophage cells. In the study conducted by Schöler and co-workers, SLN containing stearic acid and dimethyl-dicotadecyl ammonium at concentration of 0.01% induced significant toxicity towards murine peritoneal macrophage [141]. On the other hand, lipid-based nanoparticles can be exploited for mycobacterial targeting as the bacterial can utilize lipid-rich substrates owing to the wide ranges of lipase secreted. Therefore the coating of a nanocarrier with lipid-rich substrate is therapeutically beneficial as the release of ATDs as a result of lipase-degradation might eradicate the mycobacterium. Lipid-based nanoparticles might also increase internalization into macrophage cells via phagocytosis, passive diffusion and lipid-raft dependent endocytosis. The delivery of antigens and vaccines using nano-carriers has been intensively investigated to induce immunological responses. Synergistic action of ATD and vaccine is fundamentally interesting if the delivered vaccines or antigens could stimulate immune response to recognize TB infections. Therefore, ATD drugs would behave as 'supplementary' to help eradicate the bacteria. In addition discovery of molecules that can switch the metabolically dormant mycobacteria to an active state is useful as most antibiotics act on actively dividing cells.

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