Expert Opinion

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Fighting tuberculosis: old drugs, new formulations

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This review reports the state of the art on innovative drug delivery strategies designed for antitubercular chemotherapeutics. The introduction contains the fundamental biological background concerning tuberculosis and a review of the current antitubercular therapy, and is followed by a critical report of the micrometric and nanometric particulate systems designed and investigated to improve tuberculosis chemotherapy.

Keywords: antitubercular therapy, liposomes, macrophage targeting, microparticles, nanoparticles, pulmonary administration, pulmonary tuberculosis

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1. Introduction

Tuberculosis (TB) is a curable infectious bacterial disease caused by the *Mycobacterium tuberculosis* that, in most cases, affects the lungs. *M. tuberculosis* has been known to be the causative agent of TB since 24 March 1882, when Hermann Heinrich Robert Koch, with extreme meticulousness, communicated to the Berlin Physiological Society the results of his studies on the tubercle bacillus, thereafter named Koch's bacillus [1-3]. For his contribution on the elucidation of the aetiology of TB, Koch was awarded the Nobel Prize in Physiology or Medicine in 1905 [4].

It appears clear that after this breakthrough, the individuation of an effective therapeutic agent against *M. tuberculosis* was just a matter of time. In fact, the following years saw different attempts to treat TB. Koch, first, produced an extract of the virulent tubercle bacilli (called tuberculin) that, if on the one hand failed to treat the disease, in the other hand it turned out to be a very useful diagnostic tool [3]. Unfortunately, the vaccine produced by Albert Calmet and Camille Guérin, using a weakened form of *M. tuberculosis*, also demonstrated a moderate efficacy [2]. The first reliable TB therapy came out only in 1944, when Selman Abraham Waksman and his collaborators isolated and purified the antibiotic streptomycin (S) [5]. The same year, S was found to be active *in vitro* against *M. tuberculosis* and *in vivo* in both animal models and humans [6-8]. 'For his discovery of S, the first antibiotic effective against tuberculosis', Waksman received the Nobel Prize in Physiology or Medicine in 1952 [9].

Now, some questions are mandatory. Why, 127 years after the discovery of the tubercle bacillus, and 64 years after the discovery of S, is an article dealing with strategies to fight TB still needed? A quick answer can be given analysing the global TB burden [10]. In 2006, the World Health Organization (WHO) reported 1.7 million deaths and 9.2 million new cases. More than half of the global cases (55%) were estimated to occur in Asia (South-East Asia and Western Pacific regions), whereas Africa accounts for a further 31%. Above all, India, China, Indonesia, South Africa and Nigeria represent the first five countries in terms of TB incidence [10]. Furthermore, among the 9.2 million new cases of TB in 2006, the WHO estimated that ~ 709,000 (7.7%) were HIV-positive. Sadly, still in 2008, TB is the first

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cause of mortality linked to a unique infectious agent and, more important, is the primary cause of death from a curable infective disease [11].

Why, nowadays, are people dying from a curable disease? First of all we should consider the TB epidemiology. *M. tuberculosis* mainly finds its victims in developing countries with degraded social and health conditions where the access to medicines is limited, if not completely missing. The concomitant presence of conditions compromising the immune system functionality, such as alcoholism and HIV infection, favours the spreading of the infection and makes the treatment more difficult. Finally, the therapeutic regimen and its long duration, especially in the case of resistant TB, further complicate the scenario. It should be easy, now, to understand why many people still die from the *M. tuberculosis* infection. Some can criticise that therapy efficacy is not mentioned at all. This is because TB is, first, a social disease [12]. In fact, the strong decline of this pathology followed the improvement of social and health conditions rather than the advent of antibiotics [2].

The current recommended TB chemotherapy, also known by the acronym DOTS (directly observed treatment, short-course), consists of a 6-month therapy of 4 co-administered drugs [13]. Although this treatment regimen has shown 95% cure rate, in areas with a high incidence of multi-drug resistant TB (MDR-TB) it drops to 50%. In the case of MDR-TB, the WHO recommends the use of DOTS-Plus, which is DOTS plus second-line antitubercular drugs (ATDs). DOTS-Plus therapy has several disadvantages, such as a treatment duration of 24 months, severe toxicity (due to the second-line drugs) and very high costs [14]. Unfortunately, in recent years much concern has arisen from the spreading of a further category of resistant bacteria, defined as extensive drug-resistant (XDR) [15]. XDR-TB is developed when mycobacteria become resistant to isoniazid (H) and rifampicin (R) and to certain second-line drugs, in particular to at least one fluoroquinolone and one among kanamycin (Km), amikacin (Amk) and capreomycin (Cm). XDR-TB is not a new phenomenon as it has been recorded previously in Eastern Europe and Central Asia; however, recent outbreaks in at least 45 countries with a high HIV burden have brought this problem to the renewed attention of practitioners and scientists. All these features contribute to significantly lower patient compliance with an increased risk of failure.

Medical and pharmaceutical research is contributing in many ways to fighting TB. Today, many researchers are working worldwide, for example, to understand the propagation mechanisms, to individuate the genes responsible for the acquired resistance, to design and develop new effective chemotherapeutic agents, and to investigate the possibility of producing a reliable vaccine.

In this scenario, a significant contribution to improving TB therapy might come from pharmaceutical technology. Indeed, innovative drug delivery strategies as well as alternative administration routes may play a fundamental role in improving antitubercular chemotherapy efficacy, enhancing patient compliance.

This paper reports the state of the art on innovative drug delivery strategies designed for antitubercular chemotherapeutics. An introduction containing the fundamental biological background concerning TB and a review of the current antitubercular therapy is provided, followed by a critical report on micrometric and nanometric particulate systems designed and investigated to improve TB chemotherapy.

2. Elements of tuberculosis pathogenesis

TB pathogenesis is a complex subject in and of itself and its review is not the aim of this paper. Exhaustive information on this topic may be found in different monographs of recent compilation [16,17]. As anticipated by the title, the aim of this section is to provide some rudiments of TB pathogenesis, useful for understanding the reliability of different drug delivery approaches proposed in the scientific literature.

In humans, *M. tuberculosis* gains access to the body as well as is transmitted from one subject to another by the respiratory system. An infected individual, by coughing, generates bacilli containing droplet nuclei (diameter ≥ 5 µm) that might be inhaled by others with a high risk of transmission [16]. Even though a single bacillus has the potential to cause the infection, it is generally estimated that a non-immunised person needs to inhale 5 – 200 bacilli to develop the pathology [18]. Once the bacilli (contained in the inhaled droplets) reach the alveoli, they are promptly phagocytised by non-activated alveolar macrophages and either destroyed or multiply. Infected macrophages are then stimulated to produce tumour necrosis factor-α (TNF-α) and other inflammatory chemokines responsible for the recruitment of neutrophils, natural killer T cells, $CD4^+$ and $CD8^+$ T cells that produce their own cytokines and amplify the response [12]. This pro-inflammatory cascade seems to be turned off by the production of interferon-γ (INF-γ) [19]. With the aim of limiting the infection, the cellular immune response evolves with the formation of a tuberculosis granuloma, also called tuberculoma, a core of infected macrophages surrounded by foamy macrophages, mononuclear phagocytes, capillaries and a peripheral layer of lymphocytes. At later stages, the granuloma develops a thick fibrous layer and its centre loses the vascularisation, becoming hypoxic and necrotic. In the progressive tuberculomas, necrosis is followed by caseation (the process by which a tuberculoma is transformed into a structureless mass), rupture of the granuloma wall and bacteria dissemination with successive transmission. The resulting lesion is known as *Ghon focus*. Even though granuloma formation mechanisms have not been completely elucidated, it is clear that its development is mediated by the host immune response against mycobacteria [12]. In fact, recent *in vivo* experiments showed the paramount importance of the bacillus wall lipids in the formation of granulomatous reactions. The sole administration of trehalose dimycolates (one of the components of the bacterial wall), loaded within oil droplets or particles, was sufficient to produce granulomas [20].

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If the primary infection is not completely eradicated it can progress at the infected sites and disseminate (progressive primary TB). The final disease progression, known as disseminated TB or miliary TB, is the invasion of practically all organs by *M. tuberculosis* haematogenous dissemination. The pathogenesis of miliary TB and the pathogenesis of disseminated TB are similar, but they present different histological pictures. In fact, small tubercules (1 – 3 mm) can be observed only in the case of miliary TB [21,22].

The WHO estimates that 1.8 billion people worldwide are infected by *M. tuberculosis* and most of them are clinically latent. The mechanism of this latency is poorly understood and is still a matter of investigation. According to one hypothesis, latency is due to the presence of slow-replicating or non-replicating bacteria, also known as dormants or persisters [23], non-sensitive to ATDs and host immune system response [15,24]. The same bacteria are probably responsible for chronicisation and reactivation. This theory is supported by the observation that the colony-forming units (CFUs) and total bacterial counts remain similar and stable during this phase, indicating a static equilibrium [15,24]. Very recently, data showed a different picture, proposing a revision of some accepted aspects of the persistence models [25]. In fact, it has been demonstrated that *M. tuberculosis*, during chronic TB, is in a replicating (not dormant) state restrained by the host immune system [25]. This will surely relight a debate that has never been closed.

Although brief and non-exhaustive, this introduction clearly shows how far we stand from a complete understanding of *M. tuberculosis* biology and TB pathogenesis.

3. Lights and shadows of the antitubercular chemotherapy

At present, strong efforts are being made to develop DNA vaccines for TB prophylaxis, but the research is still far from a clinical application perspective [26]. Although there is some evidence of improvement in the TB treatment when chemotherapy is combined with new DNA vaccines in animal models [26,27], progress in this field is slow and there is an increasing demand for new drugs and/or delivery strategies able to strike TB infection. In fact, the first appearance of S-resistant strains pushed the establishment of the principle of drug combination in TB therapy. In the 1950s, the combination of H, S and *p*-aminosalicylic acid (PAS) was able to cure patients in ~ 18 – 24 months. Later, the association of R with H allowed reduction of the treatment length to 6 – 9 months [28].

Nowadays, although no new antitubercular drug has been developed recently, other drugs, divided into first- and second-line, are available [28]. First-line drugs (i.e., H, R, ethambutol (E), pyrazinamide (Z), S and rifabutin (Rfb)), generally more effective on non-resistant strains [29], are available in conventional pharmaceutical formulations, such as tablets, capsules and powders for intramuscular (i.m.) injection. Dosages are usually high because of pharmacokinetic issues [30]. First-line treatments imply long periods of intensive care (6 – 9 months) under strict medical control.

As mentioned above, multi-drug treatment is usually recommended to prevent drug resistance and TB recurrences. Current therapy protocols have been well established by the WHO through the previously defined DOTS programs [14]. DOTS consist of an initial intensive administration of H, R, E and Z over 2 months and a continuation phase with R and H for a further 4 months either daily or 3 times a week [14]. So far, these four drugs represent the most effective tool against TB infection as they show a synergic action on most fast, slow replicating and dormant bacteria [14]. Nevertheless, multi-drug treatments suffer from high risk of side effects and low compliance, which can induce high patient drop-out rates in the therapeutic follow-up. This generally produces a decrease in the cure rate and the therapeutic potential of TB treatments, with an increased mortality and acquired drug resistance [14].

So far, in MDR-TB cases, less effective and more toxic anti-TB agents are usually used in combination with first-line drugs [31]. These so-called second-line drugs are divided into four main classes, aminoglycosides, polypeptides, fluoroquinolones, thioamides plus cycloserine (Cs) and PAS, and can be sorted out into old- and new-generation drugs. Old second-line agents are Cm, Km, Amk, ethionamide (Eto), ciprofloxacin (Cpx), ofloxacin (Ofx), Cs, PAS and protionamide (Pto) [29,30]. Beyond possessing features common to the first-line agents, they are characterised by longer administration periods (18 – 24 months), higher toxicity and lower cure rates (~ 60%) [30]. Dosages are always very high (125 – 200 up to 1000 mg/day) and some of them, such as Cm, Km and Amk, are poorly absorbed orally and therefore need to be administered i.m. daily, thereby producing low patient compliance [30]. However, new-generation second-line drugs are also available and can be grouped into rifamycin derivatives (rifalazil, rifapentine, Rfb), fluoroquinolones (moxifloxacin (Mfx), gemiflozacin) and macrolides (clarithromycin (Clr), azithromycin, roxithromycin), and their effectiveness is still under evaluation [30]. The WHO proposed a different classification of ATDs [14]. According to it, drugs have been sorted into five groups: i) first-line oral ATDs (H, R, E, Z, Rfb); ii) injectable ATDs (Km, Amk, Cm, S); iii) fluoroquinolones (levofloxacin (Lfx), Mfx, Ofx); iv) oral bacteriostatic second-line ATDs (Eto, Pto, Cs, terizidone (Trd), PAS); and v) ATDs with unclear efficacy or unclear role in MDR-TB treatment (clofazimine (Cfz), linezolid (Lzd), amoxicillin/clavulanate (Amx/Clv), thioacetazone (Thz), Clr, imipenem (Ipm)).

Strategies and recommendations to design effective MDR-TB treatment regimens have been reviewed recently [14,32]. Generally, as also required by the DOTS-Plus protocol, a five or more drug association is advised. The treatment comprises first-line agents, such as H, R, E, or Z, an injectable drug such as an aminoglycoside or Cm, a lower-generation quinolone, or higher-generation quinolone when resistance is developed,

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and second-line bacteriostatic agents such as Eto and Cs. PAS is frequently used in patients with higher-grade resistance [32]. Even though not recommended by the WHO, further agents, such as Amx/Clv and Cfz, can be used depending on the clinical status, disease burden, degree and pattern of resistance [32].

In recent years, the concern arising from the spread of XDR-TB strongly advised the introduction in therapy of individual drug susceptibility testing over a wide range of agents, including first- and second-line ATDs [15,31]. Unfortunately, *in vitro* susceptibility tests are often poorly reproducible and miscorrelated with *in vivo* data [15,33] owing to lack of significant *in vitro* models and the length and complexity of antimycobacterial and sterilising activity evaluation. This issue is related either to the slow growing of tubercle bacilli or to the long studies (12 – 24 months) required to determine drug sterilising activity. However, attempts to establish new and high-throughput methods to evaluate drug susceptibility are in progress [34]. For these reasons, strict application of DOTS and DOTS-Plus therapies can indeed improve the cure rates and the efficacy of first-line treatments, and only the worldwide rigorous application of the WHO programmes can reduce the current spreading of TB infection [14]. Of course, on the other side, the identification of new drug candidates, drug targets and delivery strategies is as important and essential for the future as a correct therapeutic planning.

4. Drug delivery strategies

The need for new tools to fight TB, and in particular MDR- and XDR-TB, is pushing towards new strategies and drug candidates to improve therapeutic efficiency. The advancement of the research in this field is suffering though from lack of knowledge about the mechanisms of *M. tuberculosis* persistance and thereby of new and effective drug targets. However, attempts to improve TB treatment have been based mainly on new chemotherapy regimens [35-39] and in particular new drug candidates [40], and only marginally on the use of new formulations and alternative routes of administration.

So far, TB therapies have exploited conventional routes of administration, such as oral and i.m., as well as pharmaceutical forms, namely tablets, capsules and injectable solutions. However, pharmacokinetics issues forced the use of high dosages and frequent administrations to maintain the drug therapeutic concentration over time. To try to solve this issue, recent alternatives to conventional oral dosage forms consist of gastroretentive tablets loaded with R and hard gel enteric-coated capsules for H delivery [41]. Such systems have been developed to reduce R degradation, which occurs fast in the stomach in the presence of H in multi-drug regimens. Moreover, R sustained release was achieved with an almost zero-order kinetics. An advancement of the system mentioned may be represented by osmotically regulated multi-drug oral delivery systems [42]. In fact, such devices were derived from the classic osmotic pumps to provide a first-order release kinetics for H and a zero-order release for R in multi-drug oral administration.

In addition to alternative dosage forms, over the past few years different therapeutic regimens have been recommended by regulatory organs and world organisations [14,43]. Particularly applicable to oral treatments, fixed dose combinations have been suggested, as, in this way, either compliance or standardisation of multi-drug therapy can be better achieved. Even though not yet completely disclosed, the use of such strategy may allow some improvements also related to general TB treatment management.

Nevertheless, despite alternative regimens, DOTS programmes and some formulation improvement, as most ATDs are administered orally, it is somehow disappointing to observe that just a few efforts have been made to improve the oral approach to TB therapy. However, some advancement may be provided by new formulations and drug delivery strategies [44,45]. The basic idea consists of reducing side effects and improving drug effectiveness by concentrating the drug at the primary site of infection. As 80% of TB cases affect the lungs, the site of entrance of the bacilli, inhalable ATD pharmaceutical forms would be extremely useful to obtain high drug concentrations in the lungs and to target directly the alveolar macrophages, site of residence of the *M. tuberculosis* (**Figure 1**) [45]. As a consequence, the required dose will be remarkably decreased and systemic side effects significantly reduced. In this sense, microencapsulation, the embedding of the active principle in a wall material to obtain particulate matter in the micrometric range, may decrease drug toxicity further and enhance active accumulation to the target site. Moreover, the prolonged release of drugs would possibly reduce the frequency of administration. These features may be beneficial in order to increase the low compliance of current TB therapies and perhaps to shorten the long treatment duration. Naturally, the recommendations based on the TB treatment guidelines emphasised above remain valid (adequately adapted), even in the case of the use of such delivery strategies [45].

In this regard, an increasing number of studies support the use of particulate systems to treat TB [45]. The advantages of particulate systems versus conventional formulations are clear and have been widely underlined in the literature [46], but there are also some issues to consider. In fact, formulation and large-scale production of these systems are costly, and their use, other than oral, is not convenient for patients, especially in developing countries where economic and social dire straits have a determinant role in the failure of TB therapies [45]. However, the use of particulate drug carriers holds huge potential to improve TB therapy outcomes through the re-evaluation of dismissed drugs.

5. Microparticulate-based delivery systems

In the past two decades, various drug delivery systems have been investigated to deliver ATDs using different administration routes [47-49]. Among the existing drug carriers, in this section studies on microparticulate systems proposed for TB therapy are reviewed according to the proposed route of

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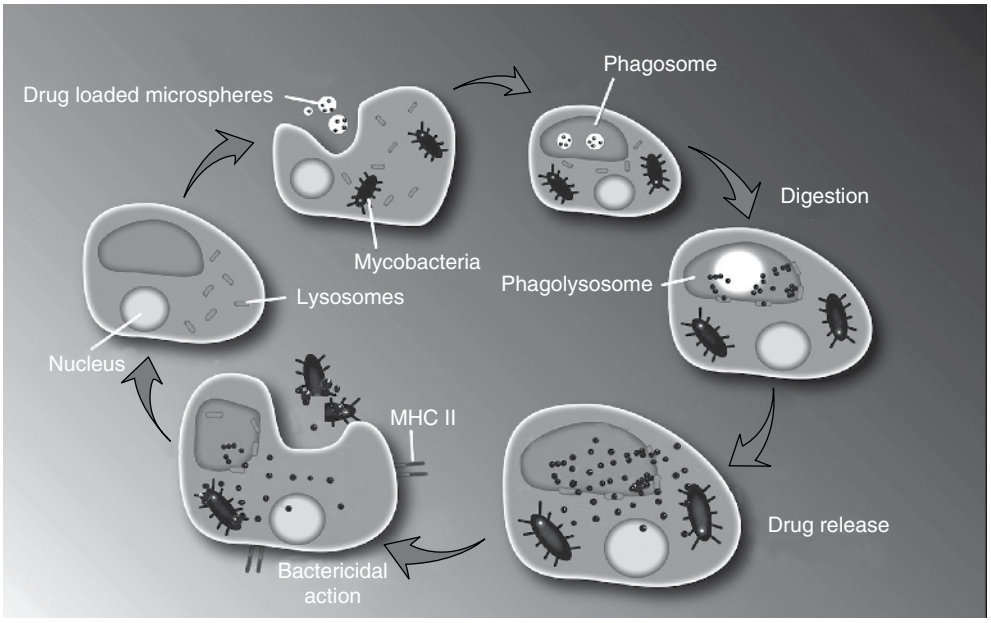

Figure 1. Schematic representation of the fate of insoluble material (e.g., antitubercular drug-loaded microparticles) delivered to infected alveolar macrophages.

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administration. Owing to their biocompatibility, biodegradability and FDA (Food and Drug Administration) status, special attention will be conferred to microparticles (MPs) based on polyesters, such as poly(lactide) (PLA), poly(glycolide) (PGA) and poly(lactide-*co*-glycolide) (PLGA) [50].

5.1 Parenteral route

Different H-loaded MPs have been prepared with PLGA polymers characterised by a degradation rate from 1 to 2 months [51]. In this way, a once-a-month injection would surely improve the treatment compliance with respect to the used daily administration. By these MPs (12 µm), a sustained release up to 7 weeks was obtained both *in vitro* and *in vivo* without experiencing the typical H hepatotoxicity. The same dose of free drug did not last for > 12 h in the plasma [51]. In successive works, two different doses were investigated and the efficacy of a single administration of MPs was compared with the daily administration of free drugs [52,53]. At high dose, the free drug was more effective in reducing the number of bacilli in the lungs, whereas at low dose, a single injection of MPs led to a better lung bacilli clearance. In the spleen and liver, the bacilli clearance obtained with MPs was higher or equivalent to that achieved with the free drugs for both doses. Even if MPs appear to be a valid alternative to avoid hepatotoxicity and to improve the treatment compliance by reducing the administration frequency, some concerns are expressed on the possibility of administering subcutaneously (s.c.) a sufficient amount of particles warranting the dose for a month of treatment [51,52]. In fact, according to the WHO, the recommended oral daily dose (subject of 51 – 70 kg) for H and R is 300 and 600 mg, respectively [14].

MPs have also been investigated for their capability to target macrophages, a reservoir of mycobacteria. *M. tuberculosis* infected macrophages (J774 murine macrophage cell line) have been treated with R-loaded MPs (different batches from 7.5 to 15 µm) in order to evaluate the efficacy of this approach [54]. In this way, R toxicity on both macrophages (J774) and human monocytic cell line MM6 was reduced and an effective targeting was obtained by MPs < 10 µm in size. R-loaded MPs increased the intracellular concentration and thus the CFU clearance compared with the same dose of free R [54]. A successive study investigated the efficacy of small (7.5 – 8.8 µm, administered intraperitoneally (i.p.)) and large (101.1 – 130.2 µm, administered s.c.) R-loaded MPs administered to infected mice [55]. The treatment with large MPs or with the combination of both formulation led to a significant decrease of bacteria counts after 26 days. Also, no bacilli were detected in 2 and 4 of the 10 mice treated with large and the combination of small and large MPs, respectively. MPs were therefore successful in reducing lung CFUs and could efficiently replace the daily oral regimen with the free compound [55]. The small MP formulation was not able to generate a significant CFU reduction. This phenomenon was explained by the formation of aggregates in the peritoneal cavity that reduced the number of particles reaching the circulation. The authors' statement is very doubtful. In fact, it is reported that MPs injected i.p. generally localise over the whole cavity and no large aggregates are observed [56]. Moreover, MPs of 5, 25, 60 and 250 µm in size remained in

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the peritoneal cavity for at least 2 weeks and only nanoparticles (NPs) were cleared from the peritoneum [57]. However, 7.5 – 8.8 μm particles injected i.p. by Quenelle and co-workers could reach the lymphatic system through the lymphatic duct opening present on the subdiaphragm surface as reported by Tsai and co-workers [58], but it is very unlikely that they are able to reach the systemic circulation [55]. The same R-loaded PLGA MPs were administered together with an H oral dosage form in order to reproduce the typical treatment of TB (combination of several drugs) used to minimise MDR [59]. This combination allowed the reduction of H dose (from 1.56 to 0.39 mg/kg), maintaining the same efficacy.

To summarise, owing to their prolonged release property, MPs consent to reduce the dose of the orally administered drug while minimising the side effects and decreasing frequency of administration.

5.2 Pulmonary route

R-loaded PLGA MPs have also been prepared as a respirable formulation and evaluated against TB in infected guinea-pigs [60,61]. A significant reduction of spleen inflammation (expressed indirectly as weight drop) was observed when compared with free R [61]. As R systemic concentration was very low, the inflammation reduction was explained by the lower number of bacteria reaching the spleen. A single and double dose of R-loaded MPs decreased the viable bacteria and inflammation in the lungs, but the same result was obtained in the spleen only when administered before infection [61,62]. Daily doses of free R over 10 or 20 days had a positive effect on pulmonary and splenic inflammation but not on the number of viable bacteria in lungs. Nevertheless, single administration of MPs or free R for 20 days equally decreased the bacteria population in the spleen. In any case, MPs were able to increase the R residence time in the lungs because, after 72 h, a 3.3-fold higher drug amount was found for R-loaded MPs [62].

Particle phagocytosis by alveolar macrophages (NR8383 cell lines) was influenced by particle size and the number of particles added to the cells [63]. Three and 6 μm particles were found to be optimal. However, no uptake was observed for 30% of macrophages at all particle/cell ratios. This ratio was shown to influence the number of MPs internalised per cell. The same number of 1 and 3 μm particles was found in macrophages. Therefore, as larger particles possess a higher drug loading capacity, the use of 3 rather than 1 μm particles is preferable [63]. H direct lung delivery by PLGA MPs was also investigated. This approach allowed minimisation of H first-pass metabolism that generates acetylisoniazid and successively acetylhydrazine, responsible for liver toxicity [64]. Inhalable polymeric MPs have also been prepared by loading a mixture of H and R. To determine phagocytosis efficiency both *in vitro* and *in vivo*, MPs were labelled with a fluorescent probe [65]. After 15 min of incubation, macrophages had already engulfed a large amount of MPs, and drug concentrations in cells were higher compared with drug solution. In spite of a ~ 20 – 30% burst release within 15 min leading to an almost immediate drug serum level, MPs delivered a higher amount of drugs in the alveolar macrophages compared with the control [65]. A combination of Rfb and H into respirable PLA MPs has been used as well [66]. In this case, MP inhalation or intratracheal instillation led to 18-fold higher drug concentration in the cells with respect to that in the plasma. Compared with drug solutions, the MPs were able to increase the intracellular concentrations fourfold [66].

Cm, a cyclic pentapeptide classified in the WHO second group and used for the treatment of MDR-TB [14,67], was formulated as dry powder by spray drying in the presence of L-leucine [68,69]. In a first study, the dry powder was characterised by a drug content of ~ 60% and a mass median aerodynamic diameter (MMAD) of 4.99 μm, whereas in a second one, the final drug contents and MMAD ranged between 90 and 93% and 4.70 and 4.99 μm, respectively. The first study allowed to establish that Cm lung administration led to an increased drug half-life and to an absolute bioavailability of 59%. Therefore, a comparable lung drug concentration can be obtained by inhalation of a reduced dose with respect to the conventional i.m. route, thus lowering the systemic exposure and side effects [68,69]. In addition, the comparison of three different administration routes (i.e., intravenous (i.v.), i.m. and pulmonary) showed that aerosolised Cm was able to lower lung inflammation, bacterial count and granulomas. Even though the higher dose (14.5 mg/kg) of the inhaled dry powder had a positive effect on the spleen histology, only the i.m. administration of Cm (20 mg/kg) reduced the bacterial count in the spleen [68].

Cm was also encapsulated in conventional and large porous PLGA MPs using a simple double-emulsion solvent evaporation method (**Figure 2 A, B**) [48,70]. Formulation parameters were optimised using a computer-generated response surface method in order to obtain the desired MP drug content and particle size. The best MP formulation was characterised by a mean diameter of 12.5 ± 2.50 μm and a drug content of 6 ± 1% (w/w). Large porous MPs combine a MMAD suitable for inhalation and the advantageous rheological features typical of large particles. In fact, small particles (1 – 5 μm), owing to the higher cohesive forces, generally tend to aggregate, losing their optimal aerodynamic behaviour (**Figure 2C**) [71-74]. On the other hand, conventional Cm-loaded MP of ~ 5 μm in size, labelled with fluorescein isothiocianate (FITC), can be efficiently taken up by murine alveolar macrophages, with a complete phagocytosis over < 3 h (**Figure 3**) (unpublished data).

Nevertheless, owing to toxicity and waste disposal concerns, an ideal formulation should possess the highest drug content in order to expose the infected lungs to the minimal amount of excipient (e.g., PLGA). For this reason, to improve Cm content within conventional respirable PLGA MPs, the hydrophobic ion pair (HIP) strategy was used [75]. The successful preparation of a hydrophobic salt of Cm and sodium oleate allowed the encapsulation efficiency to be enhanced up to ~ 90% [76]. By

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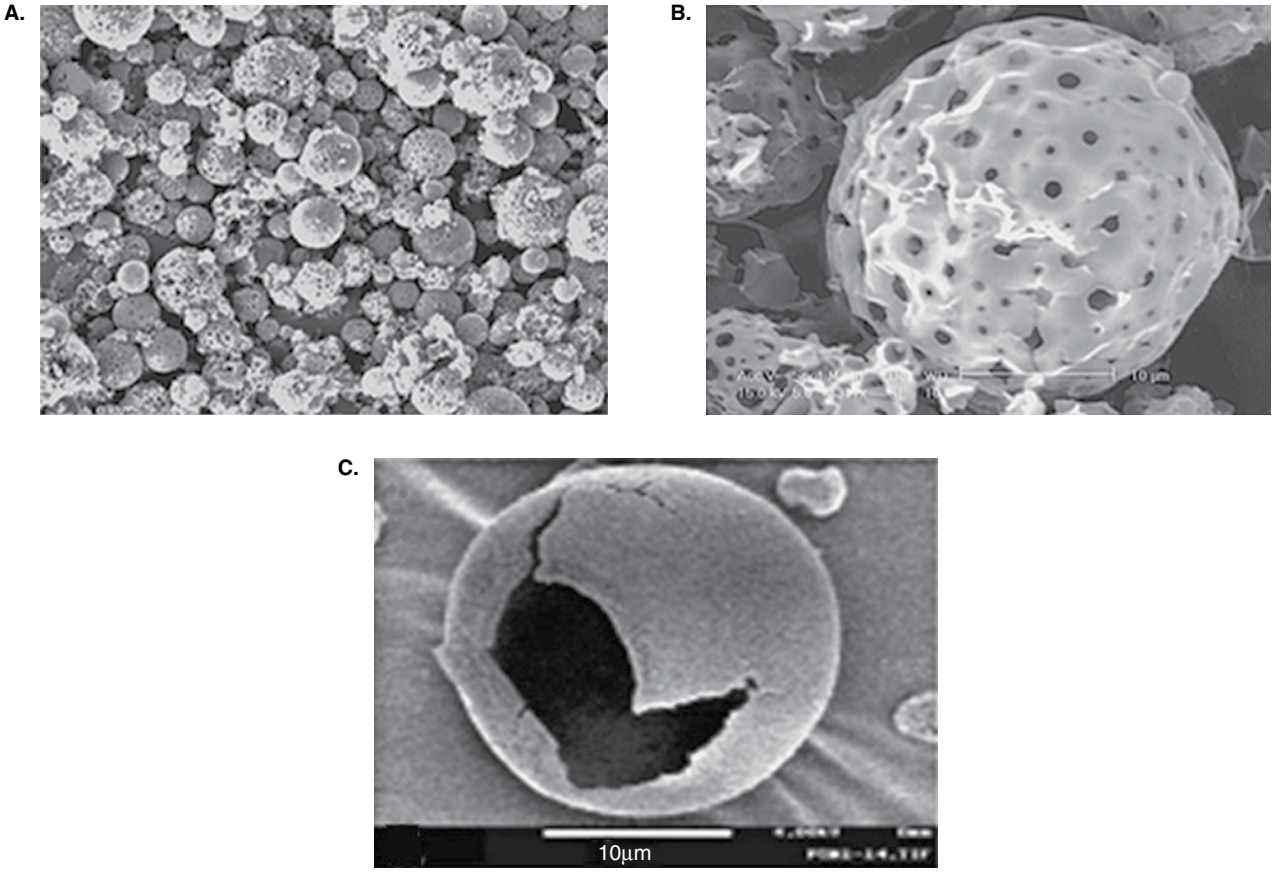

Figure 2. Different kinds of dry powder produced for drug pulmonary administration. A. and **B.** Cm-loaded large porous microparticles at different magnifications [48]. **C.** Large porous carriers of nanoparticles (Trojan particles).
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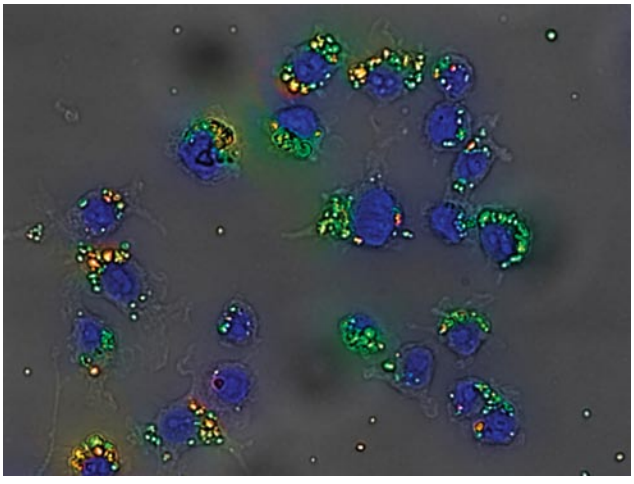

Figure 3. Uptake of conventional Cm-loaded PLGA microparticles for pulmonary delivery labelled with fluorescein isothiocianate on co-incubation with murine alveolar macrophages. In blue are the cell nuclei and in yellow the phagocytosed particles.

combining the HIP strategy and the spray drying technique, it was also possible to obtain MPs of a mean volume diameter of 6.7 µm (**Figure 4A**). In addition, Cm oleate, when properly processed, could be easily shaped into respirable particles (**Figure 4B**) [76]. It can be speculated that both HIP and HIP-loaded PLGA MPs can be easily taken up by alveolar macrophages owing to their hydrophobic character [45].

Investigations on the effect of blank inhalable MPs on the activation of macrophages have also been carried out [77]. An important finding is that infected macrophages are still able to engulf other materials and, successively to MP phagocytosis, a respiration burst indicative of macrophage activation was observed [77]. Therefore, in addition to delivering ATDs, empty MPs appeared to be beneficial and to enhance TB treatment efficacy [45,77].

5.3 Oral route

The oral route is the most used in chemotherapy because it is the easiest method of administration [14]. Even though it is a very attractive route, only a few studies have been done

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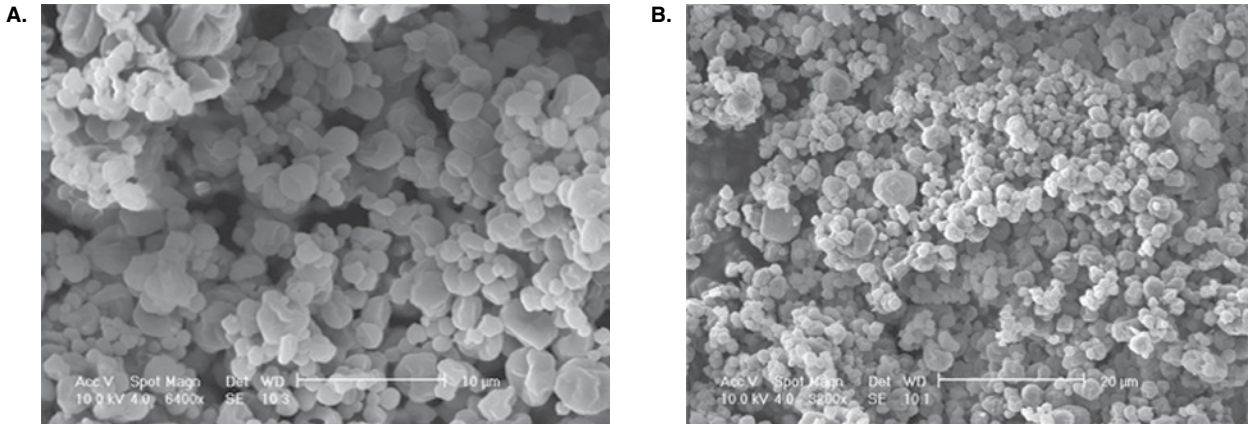

Figure 4. Cm oleate for pulmonary administration. A. Cm oleate-loaded PLGA microparticles. **B.** Respirable Cm oleate dry powder [76].

on ATD encapsulation for oral delivery [78-82]. MPs for oral administration generally obtain a prolonged release of the drug, reducing the administration frequency and, sometimes, even the administered dose [82].

Orally administered R, H and Z, when encapsulated in PLGA MPs, showed a five times higher bioavailability compared with the free drugs [79,80]. This higher bioavailability was explained by the possible uptake of such particles (1.1 – 2.2 µm) by the M cells of the Peyer's patches and an enhanced permanence in the intestinal tract thanks to the PLGA bioadhesive property [79,80]. However, no data on the therapeutic efficacy of this system have been reported [79,80]. Ain et al. encapsulated the three above-cited drugs into alginate MPs of ~ 100 µm in diameter [81]. The results obtained with this system were similar to those observed with PLGA MPs. With alginate, drug bioavailability was further enhanced (nine times) compared with the free drug [81]. Alginate MPs were able to clear bacilli in the same efficient way as the free drugs and a reduced H toxicity was observed when H-loaded alginate particles were administered in combination with R and Z [81]. The higher bioavailability is explained by the mucoadhesive properties of alginate as intestinal uptake is very unlikely to occur because of the MPs' large size. Obviously, the resulting higher bioavailability and prolonged drug release can allow lower frequency of administration. To improve further this system, and in particular to retard particle erosion, alginate/chitosan MPs were prepared as well [82]. In this fashion, drugs were detected up to 7 days after administration and bioavailability was increased 17 – 19 times with respect to free drugs. This formulation reduced both dose and administration frequency, improving therapeutic efficiency [82].

6. Nanoparticulate-based delivery systems

As previously mentioned, site-specific drug delivery may be very valuable to fight TB. The lung is an anatomically unique organ accessible, directly, via the tracheobronchial route, and, indirectly, via the systemic circulation. Direct drug delivery can be obtained by the administration of aerosolised formulation and may lead to the achievement of high local concentrations as well as systemic distribution. Among the existing nanoparticulate systems, liposomes, polymeric and solid lipid NPs have been proposed and investigated to improve mycobacterial infection therapy and are reviewed in this section [83].

6.1 Liposomes

One of the first studies on ATD-loaded liposomes goes back to the early 1980s. Intravenous injection of 40 – 80 nm S-loaded liposomes [84] to infected mice led to a significant decrease of the mycobacterium count in the spleen, but not in the lungs, with respect to the drug solution. Moreover, a prolonged mouse survival and reduced acute drug toxicity were observed with respect to free S. Similar results were obtained with Rfb liposomal formulations, suggesting promising applications for the treatment of extrapulmonary TB [85].

Comparative pharmacokinetic studies showed that 3 h after i.v. administration of ^{3}H-dihydrostreptomycin (DHS)-loaded liposomes, the DHS lung levels were 3 times higher in infected mice with respect to the free drug [86]. The same study performed on healthy mice did not highlight differences. This is consistent with the fact that high concentrations of macrophages and polymorphonuclear leukocytes in infected foci may affect liposome fate [87].

Very interesting were the *in vivo* studies conducted on second-line ATDs, such as Cm, against a mouse model of disseminated Mycobacterium avium complex (MAC) infection [88]. Cm, either in solution or encapsulated into multi lamellar vesicle (MLV) of pure dipalmitoylphosphatidylcholine (DPPC), was administered intravenously (60 or 120 mg/kg) daily for 5 days. The 120 mg/kg dose enhanced Cm accumulation in the spleen, lungs and kidneys and increased the half-life in serum and significantly reduced the number of viable mycobacteria in the liver, spleen and blood compared with controls.

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Starting from these results, the development of a reliable Cm liposomal formulation for pulmonary targeting was carried out [89]. Among the phospholipids studied, distearoylphosphatidylcholine (DSPC) was more suitable if compared with DPPC and hydrogenated phosphatidylcholine (HPC). The vesicles showed a narrow size distribution, from 138 to 166 nm, and a good encapsulation efficiency and morphology. In a following study, the freeze-thawing technique was used and optimised by a response surface methodology [90,91]. In this way, Cm-loaded liposome dry powder, possessing a MMAD suitable for inhalation and an increased peptide content (10 – 13%), was produced by freeze drying (**Figure 5**) [92]. These systems, in particular DSPC liposomes, could represent promising carriers for the pulmonary administration of this peptide.

Cs, a bacteriostatic cyclic amino acid of the WHO IV group, was also successfully encapsulated within MLV using the thin-layer evaporation technique, followed by freezing and thawing cycles [93]. Even though this formulation was aimed at being administered through the nasal route for the treatment of schizophrenia [94], this study may provide interesting information for future development of Cs-loaded liposomes intended for TB treatment.

Very promising results were obtained after the administration in mice of H- and R-loaded liposomes, composed of egg phosphatidylcholine (EPC):cholesterol (CH):cardiolipin (7:2:1 molar ratio). Following a treatment of 30 days, highest survival (~ 85%), lowest CFU value and less inflamed lungs were found in mice [95].

Cfz has been exploited as ATD after encapsulation in MLVs. The liposomal formulation was able to eradicate *M. tuberculosis* from spleen and liver, demonstrating an effective therapeutic response on acute, established and chronic murine models [96].

Even though infected lungs are characterised by a vascular microanatomy that favours extravasation, an extra targeting moiety, such as *O*-stearylamylopectin (O-SAP), on liposome surface should enhance their targeting efficiency [97]. Liposomes containing EPC, CH, dicetylphosphate (DCP), O-SAP and monosialogangliosides (GM_1)/distearoylphosphatidylethanolamine-poly(ethylene glycol) 2000 (DSPE-PEG 2000) were found to accumulate in the lungs rather than in the other reticuloendothelial system (RES) organs, both in normal and infected mice. Pretreating mice with PC:CH (2:1.5) liposomes before the injection of lung-specific stealth liposomes resulted in further enhancement of their uptake in the lung tissue. The efficacy of O-SAP at improving lung targeting was confirmed by the results obtained after injection (twice a week for 6 weeks) of H or R separately encapsulated in lung-specific stealth liposomes [98]. Drug-loaded liposomes were more effective than free drugs against *M. tuberculosis*. Furthermore, liposomal drugs had marginal hepatotoxicities resulting from the levels of total bilirubin and hepatic enzymes in the serum. The elimination of mycobacteria from the liver and spleen was also higher with liposomal drugs than with free drugs. In a successive work, the same formulations administered at one-third of the recommended doses (12 and 10 mg/kg for H and R, respectively) showed a sustained release of these drugs in the plasma (5 days), lungs, liver and spleen (7 days) [99]. At these concentrations, t_{max} and the area under the curve values of liposomal drugs were higher than those observed with free drugs. The 6 once-a-week administrations of liposomal drugs resulted in a significant reduction in mycobacterial load in the lungs, liver and spleen compared with untreated animals. Therefore, this co-administration offers a promising alternative in the chemotherapy of TB treatment.

An active targeting to macrophages can also potentially be achieved by grafting tuftsin onto the liposome surface [100,101]. It was observed that R-loaded tuftsin liposomes (twice a week for 2 weeks) were ~ 2000 times more effective than the free drug in reducing the bacilli count in the infected animal lungs. However, pretreatment with blank tuftsin liposomes did not make the pretreated animals resistant to the *M. tuberculosis* infection or increase the efficacy of the liposomal R.

Liposomes for pulmonary drug delivery in TB treatment have been reported as well [102-104]. Their high value is strongly related to the high biocompatibility of phospholipids, which are natural occurring surfactants in the alveoli, and some products are in the market for paediatric use [105].

A single administration of a nebulised suspension of H- and R-loaded PC and CH MLV to guinea-pigs showed plasma drug levels up to 48 h, whereas the free drugs could not be detected beyond 24 h post-nebulisation [106].

Vyas *et al.* [107] combined aerosolisation and ligand-mediated alveolar macrophage targeting. Specifically, R-loaded maleylated bovine serum albumin (MBSA) or O-SAP tagged liposomes produced barely 7 – 11% survival of *Mycobacterium smegmatis* inside macrophages. On the contrary, viability increased to 45.7 and 31.6% when free drug and conventional liposomes were used, respectively. Therefore, aerosol administration of ligand-tagged liposomes showed preferential accumulation in the lungs with a high detectable drug concentration even after 24 h. These results suggest that these liposomes also possess a high capability in maintaining high drug lung concentrations over time.

The most investigated ATD liposomal formulation is MiKasome® (Gilead, Foster City, CA), an injectable SUV suspension containing Amk, patented and produced by NeXstar, now Gilead [108-111]. Encapsulation in long circulating liposomes prolonged the plasma half-life from 12.6 min to 24.5 h in rats. Of particular interest is the increase of the mean residence time in all tissues (e.g., lungs, 62.9 h; liver, 95 h; spleen, 134 h; kidney, 228 h) that in all cases exceeded the plasma half-life [108]. The same formulation injected intravenously to rhesus monkeys showed significant lower plasma and urinary clearance with respect to Amk solution and Amk was found for over a week after treatment [112]. The modified Amk pharmacokinetic properties, owing to the formulation, were responsible for the enhancement of the activity against murine TB. It was estimated that

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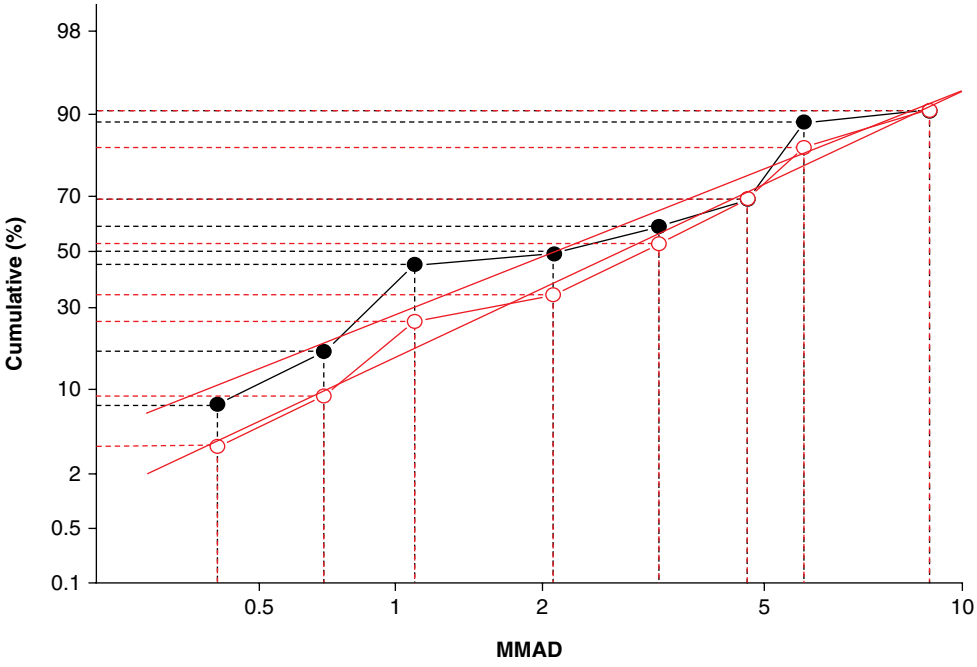

Figure 5. log-prob graph showing duplicate cumulative distribution of respirable Cm-loaded liposomes obtained by *in vitro* respirability studies (MMAD, 2.56 µm) [92].
MMAD: Mass median aerodynamic diameter.

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MiKasome was 2.4 – 5 times more potent than the Amk solution and 3.7 – 6.7 times more than S [113]. Less exciting were the results obtained in humans. In fact, even though high total Amk plasma concentration was detected, there was no evidence of early bactericidal activity. This was probably because of the release of Amk in the macrophages, usually too far from the large extracellular clumps of bacilli in the cavity caseum [110].

6.2 Polymeric nanoparticles

Recently, polymeric NPs have been largely investigated as a promising alternative to liposomes in delivering many classes of drugs, including ATDs [114-116]. The advantages in using NPs instead of liposomes reside in the possibility of obtaining higher drug-loading, higher stability in the bloodstream and more opportunities for surface modifications. On these bases, Anisimova and co-workers developed poly-*n*-butylcyanoacrylate and polyisobutylcyanoacrylate NPs containing R, H and S [115]. In spite of an increased accumulation of the three drugs, only H and S showed enhanced antimicrobial activity, whereas, surprisingly, the encapsulated R showed the same activity of the free drug [115].

PLGA NPs co-encapsulating H, R and Z were also developed for oral and pulmonary delivery [116-118]. Following a single oral administration of these preparations to mice, R and H/Z plasma levels were still detectable after 6 and 9 days, respectively, whereas therapeutic concentrations in the tissues were maintained for an extra 9 – 11 days. Further, upon oral administration (every 10 days) of drug-loaded NPs to infected mice, no tubercle bacilli could be detected in the tissues after 5 doses [116]. Comparable results were obtained after pulmonary administration of drugs co-encapsulated into inhalable PLGA particles (MMAD, 1.88 ± 0.11 µm) [118]. In fact, a single nebulisation in guinea-pigs resulted in sustained therapeutic drug levels in the plasma for 6 – 8 days and in the lungs for 11 days. Moreover, after 5 doses every 10 days, no tubercle bacilli could be detected in the lung homogenates. To achieve the same results, 46 daily doses of orally administered drug were required [118].

Nanoparticle (NP) functionalisation with wheat germ agglutinin (WGA) has also been proposed as a targeting strategy [119]. After oral/aerosol administration, R, H and Z containing WGA-coated NPs guaranteed plasma drug levels up to 6 – 7 days for R and 13 – 14 days for H and Z. In addition, WGA-coated NPs warranted a 15-day residence time in the lungs, liver and spleen. The administration of uncoated PLGA NPs led to detectable plasma levels for 4 – 6 days for R and for 8 – 9 days for Z and H. These studies also revealed that only three doses of oral/nebulised WGA-coated NPs fortnightly were sufficient to yield undetectable CFUs in the lung and spleen homogenates [116,118]. Unfortunately, the authors [116,117] did not report NP stability and *in vitro* drug release studies at different pH conditions (e.g., gastric fluid, intestinal fluid), therefore it is difficult to correlate the results to the postulated prolonged drug release or NP mucosal absorption.

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More recently, R-, H- and Z-loaded PLGA NPs were administrated orally in combination with E-loaded PLGA NPs [120]. Following a single oral dose of ATD-loaded PLGA NPs to infected mice, the supplementation of E resulted in enhanced chemotherapeutic efficacy. In fact, the *Mycobacterium* eradication was achieved after 1 week compared with the 6 weeks needed for PLGA-encapsulated R, H and Z. These results are in contrast to those reported in the literature, which show that negatively charged polystyrene nanospheres, microspheres (from 100 nm to 1 µm) as well as non-ionised polystyrene particles (100 nm, 500 nm, 1 µm, 3 µm) could not be detected in the lungs after oral administration [121,122]. Araujo, *et al*. showed that the NP lung accumulation is generally negligible even though a 50% increase of NP uptake from the gastrointestinal tract may be obtained using peanut oil added with oleic acid [123].

Moreover, the body distribution of ^{14}C-labelled azidothymidine bound to hexylcyanoacrylate NPs was investigated after oral administration. No increase of drug concentration in the lungs compared with the control oral solution was obtained [124]. All these studies demonstrate that NP administered by mouth might not be ideal to reach the lungs and that further mechanistic studies are needed to understand better the results and to prove the real advantages of orally administered NPs compared with other oral drug delivery systems.

A sustained drug release in the plasma up to 32 days was obtained for H-, R- and Z-loaded NPs (s.c. administration), whereas therapeutic drug concentrations were maintained over 36 days in the lungs and spleen of infected mice and no bacterial counts were detectable up to day 21 post-inoculation [125].

The same three drugs were co-encapsulated in alginate/chitosan NPs [126] and their relative bioavailabilities were significantly higher compared with oral free drugs. Following aerosol administration, drug levels above the minimum inhibitory concentration (MIC) were detected in the lungs, liver and spleen up to 15 days, compared with just 1 day for the free drugs. Forty-five daily doses of oral free drugs were needed to obtain the same results. However, in spite of the promising results, a lack of a deep particle characterisation (e.g., morphology, size and size distribution, *in vitro* release) makes the observed results difficult to explain and casts doubt on the speculations drawn by the authors.

6.3 Solid lipid nanoparticles

Solid lipid nanoparticles (SLNs) were proposed at the beginning of the 1990s as an attractive alternative to liposomes and polymeric NPs for drug delivery and targeting [127]. Owing to the generally regarded as safe (GRAS) status of lipids, SLN have been studied and proposed for virtually all administration routes to deliver a large number of actives [128]. ATDs have also been considered to be embedded into SLNs, either for oral or for pulmonary administration.

In this regard, H, R and Z have been co-encapsulated within SLN for oral administration and their efficacy has been evaluated against experimental murine TB [129]. After a single oral administration, SLNs provided therapeutic concentrations of all three drugs in the plasma for 8 days and in the lungs, liver and spleen for 10 days. On the other hand, the free drugs were cleared within 12 h from the bloodstream and 1 – 2 days from the RES organs. Moreover, after 5 oral doses administered every 10 days, no tubercle bacilli could be detected in the lungs and spleen [129]. As stated before in the case of polymeric NPs, again this work suffers from lack of SLN characterisation in terms of size, morphology and drug localisation within the carrier. This may pose serious problems in data interpretation, reducing the value of the results [130-132]. In fact, SLN size has been recognised to influence remarkably the formulation performance after oral administration. SLNs with a size between 50 and 100 nm have been found to afford high drug release rates as well as efficient transport across the gut walls [132,133]. In addition, the plasma drug level profiles obtained [129] contrast with other works [130-133] showing a lower t_{max} compared with the micronised reference material. This corresponds to higher dissolution rate and enhanced drug absorption.

Surprisingly, the same authors obtained almost identical pharmacokinetic profiles after pulmonary administration of lipid MP (1.1 – 2.1 µm) [134] or SLN oral administration [129]. Some concerns are expressed over these results because different kinds of particles as well as different routes of administration are generally not supposed to provide such similar pharmacokinetics behaviour. Moreover, lipid MPs were assessed in healthy and TB-infected animals and, again, it is difficult to accept that both models can provide the same plasma drug profiles.

Therefore, SLN pulmonary delivery remains an open concern as no significant and reliable data support their use for TB treatment. However, SLNs' potential usefulness is unaltered owing to their faster degradation, rapid cellular uptake and low cytotoxicity compared with polymeric particles [128,135,136]. Moreover, SLNs' suitability for pulmonary delivery is strengthened by their good stability on nebulisation [128].

7. Expert opinion

In the last two decades, TB has gone from being a forgotten disease to a modern and recrudescent pathology. Even though TB infection interests mainly South-East Asia, Western Pacific regions and Africa, many worldwide efforts have gathered to fight this worrying disease. Among the diverse scientific expertise combined into attempts to improve TB treatment, the pharmaceutical technology contribution remains limited. This is even worse if considering that TB is a curable infection and most of the negative therapy outcomes are related to extremely low patient compliance, which could be solved by new drug delivery approaches. In fact, as demonstrated extensively in this paper, alternative administration routes, together with micrometric or nanometric particulate carriers, might enhance therapy efficacy and patient compliance.

Achievement of high local drug concentrations, targeting of alveolar macrophages and the possibility of prolonging

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residence time by modifying drug release are the main features supporting the use of the innovative particulate delivery systems reviewed in this paper. Unfortunately, their efficacy has been unambiguously demonstrated in small animal models, such as infected mice, a not completely representative model of the human pathology, and only a few studies have been performed in humans [110,137-139]. For example, MiKasome, which increased Amk activity and residence time in rodents, partially failed in humans, probably because the antibiotic was released in the macrophage too far from the extracellular bacilli in the caseous lesions [108,110,111,113]. From a pharmaceutical technology point of view, the lack of particle comprehensive characterisation in some of the reported papers [116,119,129,134] makes the replication of the experiments extremely difficult, if not impossible, lowering the scientific significance of the results.

Although all the reviewed studies are very promising for improving TB treatment efficacy, some concerns arise on the practical feasibility and on the reliability of the experimental set-ups. For example, long-lasting MP formulations, intended for subcutaneous and/or i.m. administration, have a realistic potential in the case of very potent drugs, such as peptidic hormones. In fact, to be advantageous over conventional formulations, injectable MPs should contain the drug dose needed for weeks or months of treatment in a reasonable amount of powder.

The experimental set-ups encountered when reviewing the available literature are not always optimal for giving reliable information on the ATD delivery system potentiality. In fact, contrary to what has been reported by some authors [55], i.p. administration route is very unlikely to be useful in the treatment of pulmonary TB using microparticulate systems. MPs are generally retained in the peritoneal cavity [57,58] and do not reach the systemic circulation.

From our experience, we strongly support the use of micrometric and nanometric carriers to fight TB and we believe that the inhalation therapy should be privileged. Of course, this approach is still suffering from serious concerns on the possible pulmonary toxicity of the various excipients used. In this regard, a serious shortage of data regarding the biological safety of the used excipients in healthy lungs and, more importantly, in diseased organs, does not afford a conclusive opinion on the realistic application of inhaled MPs against TB. Furthermore, there are almost no studies focused on the effects that inhalation devices can have on particle characteristics and thereby on therapy outcomes. Another point to underline is that, as reported for MiKasome [110,111], even though the drug reaches the infected lungs, it could be unable to attain the bacilli present in the caseous lesions. Therefore, even if inhalation therapy is a very promising strategy, it could suffer from the same drawback.

At this point, it is important to outline that, in order to have the highest possibility of succeeding against TB, industries and academia should invest much more resources in developing pharmaceutical technology strategies to deliver efficiently ATDs. Such consideration does not imply that other fields of TB research are marginal, but emphasises the importance of delivering the right amount of drug for the needed time to the exact target. In fact, one of the main causes of MDR-TB and XDR-TB insurgence is individuated in the misuse of the existing and efficacious ATDs. However, efforts towards the development of a more compliant therapy should be combined with a renewed commitment to the comprehension of *M. tuberculosis* biology and the establishment of new and valuable molecular targets. Following this philosophy, the Stop TB partnership, a network of international organisations, countries, donors from the public and private sectors, government and non-government organisations and individuals, was established in 1998 [140]. Big efforts have been also been made by not-for-profit research organisations, such as MEND-Medicine in Need [141], founded by Harvard University Professor David Edwards, a very active person in transferring new pharmaceutical technologies to the cure of TB [72,74,142,143].

Declaration of interest

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