Synthesis of biocompatible nanoparticle drug complexes for inhibition of mycobacteria

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Received 17 June 2013
Accepted for publication 17 September 2013
Published 28 October 2013
Online at stacks.iop.org/ANSN/4/045015

Abstract

Tuberculosis (TB) is one of the most critical infectious diseases affecting the world today. Current TB treatment involves six months long daily administration of four oral doses of antibiotics. Due to severe side effects and the long treatment, a patient’s adherence is low and this results in relapse of symptoms causing an alarming increase in the prevalence of multi-drug resistant (MDR) TB. Hence, it is imperative to develop a new drug delivery technology wherein these effects can be reduced. Rifampicin (RIF) is one of the widely used anti-tubercular drugs (ATD). The present study discusses the development of biocompatible nanoparticle–RIF complexes with superior inhibitory activity against both *Mycobacterium smegmatis* (*M. smegmatis*) and *Mycobacterium tuberculosis* (*M. tuberculosis*). Iron oxide nanoparticles (NPs) synthesized by gas phase condensation and NP-RIF complexes were tested against *M. smegmatis* SN2 strain as well as *M. tuberculosis* H37Rv laboratory strain. These complexes showed significantly better inhibition of *M. smegmatis* SN2 strain at a much lower effective concentration (27.5 µg ml⁻¹) as compared to neat RIF (125 µg ml⁻¹). Similarly *M. tuberculosis* H37Rv laboratory strain was susceptible to both nanoparticle–RIF complex and neat RIF at a minimum inhibitory concentration of 0.22 and 1 µg ml⁻¹, respectively. Further studies are underway to determine the efficacy of NPs–RIF complexes in clinical isolates of *M. tuberculosis* as well as MDR isolates.

Keywords: tuberculosis, drug delivery, nanoparticle, RIF–NP complexes

Classification numbers: 2.04, 5.09

1. Introduction

Approximately one-third of the World’s population is infected with tuberculosis (TB), of which the highest burden occurs in India [1]. India accounts for more than 20% of the 9.4 million global cases of TB each year which kills approximately 2.5%, 2 million Indian adults annually [2]. The disease mainly affects the lungs but can also develop as extra-pulmonary tuberculosis in the central nervous or circulatory systems or elsewhere in the body. Even though reliable drugs for the treatment of TB are normally accessible, the proliferation of resistant strains and the association of TB and AIDS are the newly emerging threats. An epidemiological model predicted that there would be a total of 225 million new cases and 79 million deaths from TB between 1998 and 2003 [3,4].
TB is an infectious disease caused by bacteria *Mycobacterium tuberculosis* (*M. tuberculosis*). Current TB treatment involves the daily administration of four oral doses of antibiotics for a period of six months or more. Due to high incidence of side effects and the long treatment, a patient’s adherence is low and this results in relapse of symptoms causing an alarming increase in the prevalence of multi-drug resistant (MDR) TB. Additionally, few drug molecules actually reach the lung alveoli, the desired site for drug action [5].

The immediate challenges for the control of tuberculosis include developing curative chemotherapeutic regimens that are shorter or that require patients to take drugs less frequently. Such regimens would greatly facilitate monitoring compliance [6]. From this point of view, there is an urgent need to develop new drugs with novel modes of action, especially early bactericidal action and later sterilizing action when bacteria undergo sporadic metabolic activity and are likely to relapse [7]. Implementation of directly observed therapy short course (DOTS) is also not very successful due to non-compliance from patients to prescribed regimens as these involve continuous, frequent, multiple drug dosing [8].

Mycobacteria are non-motile, acid-fast and Gram positive bacilli from phylum actinobacteria, that do not form spores or capsules. Out of 41 species of mycobacteria, 5 are obligate pathogens, 10 are opportunistic pathogens and the remaining are either animal pathogens or saprophytes. The present study has used *M. tuberculosis*, an obligate human pathogen and *Mycobacterium smegmatis* (*M. smegmatis*), a saprophyte as the type strains. *M. smegmatis* is commonly used in work on the *Mycobacterium* species due to its faster growth and non-pathogenic nature allowing for experimentation under normal Bio Safety Lab-1 (BSL-1) laboratory conditions. This species shows a high degree of genetic homology with *M. tuberculosis* and shares the same unusual cell wall structure of *M. tuberculosis* [4]. *M. tuberculosis* strain H37Rv was used for antibacterial assays. This strain retains full virulence in animal models of TB. It is also susceptible to antitubercular drugs and is amenable to genetic manipulation [4]. Rifampicin (RIF) is one of the first line anti-tubercular drugs with mycobactericidal activity that is crucial for decreasing transmission of infection and preventing disease relapse. The adult dose of Rifampicin is 10 mg kg\(^{-1}\) body wt. (maximum of 600 mg) once a day administrated orally. In normal subjects, the weight serum half-life of RIF is approximately 3 h. However, the adverse reactions to RIF are hepatotoxicity, gastricin tolerance, peripheral neuropathy, hypersensitivity, hematological reactions and neurological effects such as seizures and psychosis, feeling of excessive tiredness or weakness, difficult or painful urination and arthritis. These adverse reactions and continuation of therapy for six months are the major hurdles causing non-adherence to treatment leading to treatment failures and emergence of drug resistant strains of mycobacteria [9]. In the last few years, a great number of new drug delivery technologies have emerged with a view to avoiding or reducing the potential disadvantages involved in traditional chemotherapy. The possibility of designing different drug delivery systems, for a controlled release, improved efficacy and better assimilation in the human body, is an attractive feature of a drug developed from such technologies.

A number of such methods involving drugs in combination with micro- and nanoparticle (NP) systems are being developed which essentially improve drug administration, reduce the frequency of dosing schedule and avoid potential disadvantages of conventional drug therapies. The NP–drug combinations are also stable, biocompatible and biodegradable [8,10–14]. In addition, they deliver both hydrophilic and hydrophobic drugs with higher efficiency across biological barriers, with fewer side effects and via multiple routes, probably due to increased drug uptake through endocytosis of drug carriers [15, 16].

There are reports about successful use of NPs in combination with many antibiotics to control a wide variety of infectious organisms, e.g. amphotericin nanomodification [17] and NPs for its delivery [16], gentamicin NPs against *Brucella* spp. [18], and cytotoxic drug doxorubicin with NPs [19]. However, there are not many reports on successful combination of NPs and antibiotics for control of mycobacterial infections. Successful utilization of polymer NPs for effective control of TB has been demonstrated. Sosnik et al [20] have overviewed the nano based drug delivery systems for encapsulation and release of anti-TB drugs. Pandey et al [21,22] have described NP-encapsulated antitubercular drugs effective against murine TB.

The above-mentioned work has described the use of solid–lipid NPs. It is believed that these NPs are able to target cellular reservoirs of *M. tuberculosis* in alveolar macrophages. Additionally the development of NP-based aerosol vaccine is currently underway, which could serve as new platform for immunization [4]. In this paper we report the formation of a colloidal drug delivery system based on the NPs of iron oxide. RIF–NP complexes were formed and tested to determine *in vitro* inhibitory potential against *M. tuberculosis* (infectious organism for humans) and *M. smegmatis* (non-infectious organism for humans but saprophytic and type organism) along with control samples of plain NPs and United States Pharmacopeial (USP) convention grade RIF.

### 2. Experimental

#### 2.1. Preparation of NPs

NPs of iron oxide required to form the RIF–NP complexes were synthesized by gas phase condensation method using an arc plasma reactor. The details of the synthesis process are described in our earlier publication [23]. The RIF–NP complexes were formed using a solution based technique involving chloroform as a solvent and subsequent vacuum drying under specific conditions [24].

#### 2.2. Morphological characterization and size analysis

The size and morphology of the complexes were characterized using atomic force microscopy (AFM) (Nanoscope III, Digital Instruments). The concentrations and purity of the drug were determined with high-performance liquid chromatography (HPLC) analysis carried out with a Shimadzu LC-2010 system. The x-ray diffraction (XRD) pattern was generated by using XRD technique (Bruker Advance D8). Nickel filtered Cu-Kα radiation (α = 1.54) generated at 40 kV and 40 mA was used for the angle (2θ) range from 20° to 80°.
18–24 h optical density at 600 nm
oleic acid-dextrose-albumin-catalase (ODAC) supplement for
Identity and purity of
was prepared as 1 mg ml$^{-1}$ USP grade RIF was obtained from Lupin Ltd (India). Its stock
2.3. Antibiotics
USP grade RIF was obtained from Lupin Ltd (India). Its stock was prepared as 1 mg ml$^{-1}$ in dimethyl sulfoxide (DMSO).
2.4. Pathogens and growth conditions
Identity and purity of $M$. smegmatis strain SN2 was confirmed with acid fast staining using routine procedures [9]. It was cultured either in minimal medium [25] or in Middlebrook 7H9 broth (Hi Media, India) without oleic acid-dextrose-albumin-catalase (ODAC) supplement for 18–24 h optical density at 600 nm (OD$_{600}$ > 0.5) at 37 $^\circ$C and 180 rpm (Rotary Incubator Shaker, Steel mate, India). $M$. tuberculosis strain H37Rv was cultured in Middlebrook broth in MB/BacT automated culture detection system as per the manufacturer's instructions (BioMerieux, France).
2.5. Antibacterial assay for $M$. smegmatis
The in vitro studies were carried out to compare the anti-tubercular efficacy of NP–RIF complex with the conventional RIF against $M$. smegmatis SN2 strain grown as stated above. Bacterial growth was quantified by recording (OD) at 600 nm on a spectrophotometer (Toshniwal TSUV 75, Pune, India). To these bacterial suspensions, plain RIF, plain NPs and NP–RIF complex were added at serial two-fold dilutions starting from 100 down to 3.90 µg ml$^{-1}$. These cultures were re-incubated under conditions mentioned above and the growth was again quantified as above after 24 h. For all these experiments, plain DMSO and plain uncomplexed NPs of iron oxide were used to check for any harmful effects on the bacterial growth.
2.6. Antibacterial assay for $M$. tuberculosis
The in vitro studies were carried out to compare the anti-tubercular efficacy of NP–RIF complex with the conventional RIF against $M$. tuberculosis H37Rv laboratory strain. The mycobacterial susceptibility testing was carried out as per the standard laboratory operating procedures and manufacturer's (BioMerieux, France) instructions. Briefly, serial two-fold dilutions of conventional (neat) RIF and NP–RIF complex, starting from 4 µg ml$^{-1}$ final concentration were prepared. MB/BacT process bottles were supplemented with reconstitution fluid and then serially diluted neat and NP–RIF complex was added to the respective bottle marked with reconstitution fluid. To each of these process bottles, 0.5 McFarland turbidity standard of $M$. tuberculosis H37Rv culture was added. All bottles were incubated in MB/BacT instrument's incubation module for 45 days or until flagged as positive. If the bottle flagged positive at the same time or before that of drug-free $M$. tuberculosis control, it was considered as resistant to the respective dilution of the added drug. Accordingly the minimum inhibitory concentration (MIC) values for both NP-complexed and neat drugs were noted and compared.
2.7. Statistical analysis
The experiments for both the bacteria were repeatedly carried out and the values in table 1 represent the mean values for observations. The differences between the control (medium with bacteria) and either the neat antibiotic or NP and antibiotic experiments were calculated by $t$-test at 95% confidence level.
3. Results and discussion
The precursor for the synthesis of nano drug particles, in the present study, consisted of NPs of iron oxide ($\text{Fe}_2\text{O}_3$) with sizes ranging between 10 and 30 nm in dimension as seen in atomic force micrograph (figure 1). The particles were spherical in shape and seen to exist mostly in non-agglomerated state. The crystalline purity of the sample was ascertained from the x-ray diffraction (XRD) analysis. The XRD pattern is shown in figure 2. The close correspondence between the line positions on the Bragg angle

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**Table 1. Effect of RIF–NPs on growth of $M$. smegmatis.**

| No. | Experimental | Mean OD600 ± SD (mm) (after 24 h growth) |
|-----|--------------|----------------------------------------|
| 1   | Plain medium 7H9 | Blank                                  |
| 2   | Medium + DMSO  | 0.1325 ± 0.003                         |
| 3   | Medium + uncomplexed NPs | 0.5 ± 0.014                           |
| 4   | Medium + $M$. smegmatis (control) | 0.6 ± 0.0                             |
| 5   | Medium + $M$. smegmatis + 500 µg RIF | 0.245 ± 0.011$^{a,b}$                   |
| 6   | Medium + $M$. smegmatis + 250 µg RIF | 0.205 ± 0.03$^a$                      |
| 7   | Medium + $M$. smegmatis + 125 µg RIF | 0.124 ± 0.005$^{a,b}$                  |
| 8   | Medium + $M$. smegmatis + 500 µg NP + RIF | 0.073 ± 0.003$^b$                   |
| 9   | Medium + $M$. smegmatis + 250 µg NP + RIF | 0.092 ± 0.001$^a$                    |
| 10  | Medium + $M$. smegmatis + 125 µg NP + RIF | 0.0925 ± 0.005$^b$                  |
| 11  | Medium + $M$. smegmatis + 62.5 µg NP + RIF | 0.080 ± 0.01$^a$                    |
| 12  | Medium + $M$. smegmatis + 31.25 µg NP + RIF | 0.097 ± 0.004$^a$                   |
| 13  | Medium + $M$. smegmatis + 15.62 µg NP + RIF | 0.104 ± 0.004$^a$                   |

$^a$ Indicates that results are significantly better than control at 95% level of confidence.

$^b$ Indicates that $M$. smegmatis inhibition by NPs + RIF is significantly better than neat RIF at 95% level of confidence.
Figure 1. Atomic force micrograph of NPs of iron oxide (Fe$_2$O$_3$) showing their diameter ranging between 10 and 30 nm.

Figure 2. XRD spectrum of iron oxide. The close correspondence between the lines positions on the 2θ scale and the standard data file (JCPDS) confirms the perfect crystallinity close to γ-Fe$_2$O$_3$.

2θ scale and the standard data file (JCPDS) confirms the perfect crystallinity close to γ-Fe$_2$O$_3$.

The morphology of the particles, after coating with drug molecules, was studied with the help of AFM. The micrograph for RIF–Fe$_2$O$_3$ NPs complex of Fe$_2$O$_3$ is shown in figure 3. Several RIF–NPs complex are seen in the micrograph of figure 3(a), whereas figure 3(b) is a magnified image of a single RIF–NP complex. It is very interesting to see a structure in which the drug (RIF) forms a cage to hold the NP of Fe$_2$O$_3$. This is how the NPs of drug are formed in the present method. The opening of the cage is expected to have occurred during the process of vacuum drying of the NPs. The average size of the RIF complex NPs is seen to be around 50–60 nm. Content of RIF was estimated in the nano-complex formed, against USP Grade solution of RIF by HPLC. Average area under the curve for the USP standard rifampicin was 2421042 (40 mg in 500 ml) while average area under the curve of RIF–NP complex sample was 2253842 (165 mg in 500 ml). With the mathematical calculations considering the purity of rifampicin standard used i.e. 99.33%, the concentration of rifampicin is 22.4%. Thus, there is only 22.4% rifampicin in the RIF–NPs complex (figure 4). However, peak purity of RIF indicates no contamination or interference on account of complexation. There are no related impurities associated with RIF that are present in any significant amount. This study indicates that pure RIF is maintained through complex product. Moreover the RIF–NPs complex is stable throughout the process. The anti-tubercular efficacy of the RIF–NP complex was tested against *M. smegmatis* SN2 strain as well as against *M. tuberculosis* (H37Rv) laboratory strain. The efficacy was compared with that for the conventional RIF.

The *M. smegmatis* sensitivity results are depicted in table 1. Under normal conditions of growth, *M. smegmatis* exhibited an optical density of 0.6 at 600 nm (OD$_{600}$). When 500 µg neat RIF were added to such a culture, after 24 h, the bacterial growth was inhibited and OD$_{600}$ was 0.245, which translates to an inhibition of 41%. When 500 µg RIF–NPs complex were added to bacterial culture as above, after 24 h, the bacterial growth was further inhibited and OD$_{600}$ was 0.073, which translates to an improvement of 3.4% over inhibition of bacterial growth achieved with neat RIF powder alone. Further improvement of almost 45 and 74% is achieved when we compare the effects of 250 µg and 125 µg RIF–NPs complex versus neat RIF powder at 250 and 125 µg, respectively. All these improvements have statistically also been proven to be significant by t-test at 95% level of confidence (table 1).

It is to be further noted that HPLC analyses have proved that RIF–NPs complexes have only 22% of their weight being represented by the antibiotic (figure 4). Thus 250 µg RIF–NPs complexes have only 55 µg of effective RIF while 125 µg RIF–NPs complexes have only 27.5 µg of effective rifampicin weight. This conclusively proves that RIF–NPs complexes are definitely better means of control of *M. smegmatis* infections as they show better inhibition of bacterial growth at much lower concentrations of active drug on a per weight basis. This result has further significance because it is known that high concentrations of neat rifampicin (even up to 200 µg ml$^{-1}$) are needed to inhibit transcription in *M. smegmatis* [25].
Figure 3. (a) AFM showing RIF–Fe$_2$O$_3$ NPs complexes. Average particle diameter of the complex is 50–60 nm. (b) AFM of a single particle of RIF–Fe$_2$O$_3$ complex.

Our results show that bacterial inhibition is achieved at much lower concentrations with drug–NP complexes. The results of the sensitivity of M. tuberculosis are shown in table 2. Here a difference as compared to table 1 is with respect to RIF concentrations used. Since M. tuberculosis is much more sensitive to this antibiotic than M. smegmatis, lower concentrations of the antibiotic, close to the published values [26] have been used. Preliminary data showed that the H37Rv strain was equally susceptible to both RIF–NPs complex and the conventional RIF at MIC of 0.18 µg ml$^{-1}$ over a period of 30 days.

For slightly higher values of RIF–NPs complex and the conventional RIF, the culture bottles did not show any growth of H37Rv strain even after incubating them for a longer time period of 54 days. Here again it is to be noted that NPs complexes with RIF have only 22% of their weight being represented by the antibiotic. Thus 0.375 µg NP + RIF has effectively only 0.083 µg drug concentrations while 0.1875 µg NP + RIF has effectively only 0.041 µg drug concentrations. Earlier reports in the literature [20,21,27] discuss the use of anti-tubercular drugs encapsulated within various types of NPs. They demonstrated that the NPs provided sustained release of the anti-TB drugs and considerably enhanced their efficacy after oral administration. Also, therapeutic concentrations in the tissues were maintained for 9–11 days. In contrast, free (unbound)
drugs were cleared from the plasma within 12–24 h after administration.

The present work is different from these earlier reports in three aspects. Firstly, we have used iron oxide NPs that are known as biocompatible agents approved by the Food and Drug Administration [28] for human treatment. In addition in this system, the complex of drug (RIF) and NPs is a novel way of treatment as far as TB is concerned. Finally, the most important aspect is that for both the bacteria, use of NP–drug complexes, far less concentration of RIF has proved to be effective in inhibiting their growth which has interesting therapeutic implications.

4. Conclusion

In conclusion, it has been demonstrated that complexes of NPs of Fe₂O₃ and rifampicin can be successfully developed on a laboratory scale which are superior in their inhibitory activity against both M. smegmatis and M. tuberculosis.

Acknowledgments

Lupin India Limited is acknowledged for providing us with rifampicin USP. HB and SVB acknowledge CSIR for RA and ES support. This work was performed under the Department of Biotechnology (DBT), Government of India grant. TB and SS acknowledge DBT for financial support for research. T B also acknowledges DIAT-DRDO nano program for support.

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