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Potential of Improving the Treatment of Tuberculosis Through Nanomedicine

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Current treatment of tuberculosis is inadequate due to lengthy treatment course and drug-related toxicity. To address these setbacks, we developed a nanotechnology drug delivery system that can be administered in a single dose that maintains an active level of drug for at least a week. Polymeric poly(lactic-co-glycolic acid) nanoparticles of 200–300 nm were synthesized, with a drug encapsulation efficiency of 50–65% for isoniazid and rifampicin. The particles were taken up in vitro and in vivo and a slow release profile was observed in mice over 5 days. This study illustrates the feasibility of a sustained release system for tuberculosis treatment.

Keywords Drug release; nanomedicine; PLGA nanoparticles; tuberculosis

Introduction

Tuberculosis (TB) is currently one of the leading causes of death in South African adults. Annually, approximately 250 000 new cases of TB occur and it is ranked 5th amongst the top 22 high burden countries of the world, which collectively account for over 80% of all TB cases [1]. A major reason for the escalation of TB is the evolution of the AIDS epidemic. It is estimated that approximately 70% of adult TB cases aged 15–49 years are HIV-infected and approximately 1.7% of new cases are multi-drug resistant [2]. Although an effective therapeutic regimen is available, patient non-compliance resulting in treatment failure, as well as the emergence of multi-drug resistant (MDR) and extremely drug resistant (XDR) strains of TB still pose a challenge [3]. The first line drugs that are currently administered to treat TB include rifampicin (RIF), isoniazid (INH), pyrazinamide (PYR) and ethambutol (ETB). The current doses administered are high compared to the required minimum inhibitory concentration (MIC) of the drugs [4]. This is because the drugs have poor bioavailability and low permeability, which is a function of elimination or

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clearance of drugs before reaching their target site [4]. Thus the drawbacks of conventional chemotherapy necessitate the development of a carrier system which can release drugs slowly over prolonged time periods and maintain the MIC for a number of days.

The application of nanotechnology in the health care sector, also referred to as nanomedicine, has gained ground over the past 5 years. The field of drug development could potentially benefit greatly from nanomedicine in terms of addressing current shortfalls such as lack of specificity, toxicity of the therapeutic compounds, low solubility leading to lowered bioavailability and thus reduced efficacy [5]. The nanometer size-ranges of delivery systems offer certain distinct advantages for drug delivery. Due to their sub-cellular and sub-micron size, nanoparticles can penetrate deep into tissues through fine capillaries, cross the fenestration present in the epithelial lining and are generally taken up efficiently by the cells. This allows efficient delivery of therapeutic agents to target sites in the body [6].

The small size also facilitates intracellular and para-cellular absorption or uptake of the particles. To date various hydrophilic surfactants such as polyethylene glycol (PEG) have been used to enhance the bioavailability, by increasing the mean residence time of the encapsulated drug through minimizing opsonisation of the particles [7]. Poloxamers, poloxamines and other polymers such as chitosan can also be used for this purpose. Furthermore, the reported ability to functionalize these polymeric nanoparticles with ligands such as antibodies, peptides, carbohydrates and DNA has enabled researchers to target specific diseased cells within the body, thus further increasing the specificity and efficacy of the therapeutic drugs [8,9].

Much of the advances in drug delivery have been primarily on cancer treatment, however various groups are working on anti-TB drug encapsulation. Dutt and Khuller [10] formulated entrapped INH and RIF in PLGA microparticles for sustained drug delivery following subcutaneous administration. In vivo studies revealed sustained release of 6–7 days in mice, whereas a release of only 24 hrs was observed for free drugs [10]. Pandey et al. [11] also encapsulated RIF, INH and PZA in PLGA nanoparticles for oral delivery by the multiple emulsion technique. The nanoparticles were administered at every 10th day and after 5 oral doses of treatment, no tubercule bacilli could be detected in the tissues [10]. This formulation achieved an encapsulation efficiency of 56.9% for RIF, 66.3% for INH and 68% for PZA [11]. The group has conducted various studies in anti-TB drug delivery with very positive data. In addition, Kisich et al. [12] illustrated that encapsulated moxifloxacin accumulated in macrophages approximately three-fold times that of the free drug, and was detected in the cells for at least six times longer than free moxifloxacin at the same extracellular concentration. Inhibition of intracellular *Mycobacterium Tuberculosis (M.tb)* growth with encapsulated moxifloxacin was achieved at the concentration of 0.1 μg/ml, whereas the same effect with free moxifloxacin required a concentration of 1 μg/ml [12].

In this paper we report the encapsulation of anti-TB drugs using a novel spray drying technique as well as a freeze drying technology. The focus is specifically on RIF and INH encapsulation. We will illustrate how we have managed to modify physiochemical properties of the particles and attain sustained drug release over a period of days, both in vitro and in vivo. We further indicate that our particles are taken up by cells and also that the activity of the drugs against *M.tb* is still maintained in the process of encapsulation.

Materials and Methods

Preparation of PLGA Nanoparticles Via Freeze Drying

Poly, DL, Lactic-co-Glycolic Acid (PLGA) 50:50 (Mw: 45000–75000) nanoparticles loaded with anti-tuberculosis drug were prepared using a modified multiple emulsion

solvent evaporation technique [13] followed by freeze drying (FD). A solution of the hydrophilic drug (INH) was dissolved in 2 ml of an aqueous phosphate buffer solution (PBS) pH 7.4 was emulsified for a short period with a solution of 100 mg PLGA dissolved in 8 ml of ethyl acetate (EA), by means of a high speed homogeniser (Silverson L4R) with a speed varying between 3000 and 5000 rpm. The mass ratio of the drug to the polymer was 1:1. This water-in-oil (w/o) emulsion obtained was transferred into a specific volume of an aqueous solution of 1% w/v of the polyvinyl alcohol (PVA) (Mw: 13000–23000 and partially hydrolysed (87–89%)) as a stabiliser. The mixture was further emulsified for 5 min by homogenisation at 5000 or 8000 rpm. When hydrophobic drug (e.g. rifampicin) was used, it was dissolved together with PLGA in the organic solvent and the mass ratio polymer/drug was kept constant to 1:2. The water-in-oil-in water (w/o/w) double emulsion obtained was gently stirred overnight at room temperature to remove the organic solvent. Thereafter, drug-loaded PLGA nanoparticles were harvested by ultracentrifugation (37000 g for 15 min) followed by a series of wash steps with de-ionised water. The recovered pellet was re-dispersed in a small volume of de-ionised water to be lyophilized for 3 days.

Preparation of PLGA Nanoparticles Via Spray Drying

An extensive investigation has been conducted on another method of preparing PLGA nanoparticles via spray drying (SD), where the double emulsion (w/o/w) obtained was directly fed into a bench top Buchi mini-spray dryer (Model B-290) and spray dried at a temperature ranging between 95 and 110°C, with an atomizing pressure varying between 6 and 7 bars. PEG was used in the formulation as an excipient to increase the in vivo residence time of nanoparticles in the blood stream [14]. In order to further increase the residence time and enhance uptake of nanocarriers through the gastro-intestinal tract, nanoparticles were coated with a mucoadhesive and positively charged polysaccharide, i.e. chitosan as recommended in previous reports [15,16]. The summary of formulations investigated is presented in Table 1.

Particle Characterization

Particle Size and Zeta Potential. Particle size and distribution were measured by Dynamic Laser Scattering or Photon Correlation Spectroscopy using Malvern Zetasizer Nano ZS (Malvern Instruments Ltd, UK). For each sample 1–3 mg of nanoparticles were suspended

Table 1. Different formulations investigated

Formulation	1% PVA Vol (ml)	Speed of homogenisation (rpm)[a]	Type of drying	Drug
A	20	3000–5000	FD	INH
A1	20	5000–8000	FD	INH
B	40	3000–5000	FD	INH
B1	40	5000–8000	FD	INH
C	40	3000–5000	FD	RIF
C1	40	5000–8000	FD	RIF
D	40	5000–8000	SD	RIF
F	40	5000–8000	SD	INH

[a] = The first speed for first emulsion and the second refers to the speed of the second emulsion.

in filtered water (0.2 μm filter), then vortexed and/or sonicated for few minutes. Each sample was measured in triplicate. The zeta potential was also determined using the same instrument.

Determination of Encapsulation and Loading Efficiency. To determine drug concentration in the particles, i.e. the encapsulation efficiency (EE), an indirect method was used. For that 10 mg of nanoparticles were suspended in a small volume of de-ionized water (10 ml) and washed by centrifugation to remove the un-encapsulated drug. The supernatant was analysed using a UV-spectrophotometer (UV-Vis, Thermo Spectronic Helios) set at a corresponding wavelength of the specific drug (e.g. 262 and 330 nm for INH and RIF: respectively). Drug concentrations were determined using a standard calibration curve. The amount of drug incorporated into polymeric nanoparticles is then deduced from the difference between the initial amount put in the formulation and the amount found in the supernatant after washing of nanoparticles. Drug encapsulation and loading efficiency were calculated as follows:

$$\mathrm{EE} = [(\mathrm{M_t} - \mathrm{M_u})/\mathrm{M_t}] \times 100 \quad (1)$$

where EE is the encapsulation efficiency in (%), $\mathrm{M_t}$ is the total mass of drug used in the formulation, and $\mathrm{M_u}$ is the mass of un-encapsulated drug obtained from the supernatant.

$$\text{Drug loading (in \%)} = (\mathrm{MD}/\mathrm{MT}) \times 100$$

Where MD is the actual mass of drug encapsulated into nanoparticles and MT is the mass of nanoparticles.

Analysis of Surface Morphology

Surface morphology of anti-TB drugs-loaded PLGA nanoparticles was studied by scanning electron microscopy (LEO 1525 Field Emission SEM).

In Vitro Release Studies

In vitro drug release of INH and RIF was performed in phosphate buffered saline (0.1 M PBS, pH 7.4). In this method, three replicates of each sample containing nanoparticles of a known concentration of RIF and INH were dispersed in 100 ml of PBS in a shaker at 37°C. At specified intervals, 500 μl of sample was sampled and replaced with the same volume of fresh PBS. The collected samples were analysed by HPLC using the method adapted from Mohan et al. 2003 with minor modifications [17]. The second method involved placing the suspended particles in a dialysis membrane with a molecular weight cut off of 10–12 kDa, and immersing the membrane into a PBS solution. For positive controls, 10 mg of each of the drugs was dialysed into 100 ml of PBS. Sample collection and analysis was performed in a similar manner as that of PBS suspensions.

Particle Uptake Studies

In Vitro Particle Uptake. Particle uptake studies were performed in CacCo-2 cells, a colon carcinoma cell line purchased from Highveld Biologicals. The cells were cultured in 25 cm^3 flasks at 37°C in humidified atmosphere (90% humidity), 5% CO_2, DMEM, 1% non-essential amino acid, 1% glutamine, 10% Fetal bovine serum (FBS) and Penicillin, 100

U/ml and streptomycin, 100 μg/ml. Stocks of the cells were prepared in culture medium, containing 20% FBS, and 10% sterile glycerol. The cells were maintained according to routine cell culture procedures. Cells were grown until they reached confluence at 1×10^6 cells per well and treated with 10 μg/ml of Rhodamine 6G labelled PLGA particles. Rhodamine 6G was added to the aqueous phase of the emulsion during the preparation of the particles. In order to visualize the lysosomes, the cells we stained with $1\times$ lysotracker green, following the incubation with the nanoparticles and analysed by confocal microscopy. The incubations were performed over a 3-hr period and the cells were viewed at different time points.

In Vivo Particle Uptake. In vivo studies in Balb/c mice were approved by the Ethics Committee for Research on Animals (ECRA) of the South African Medical Research Council (MRC). The procedures followed in these studies were in accordance with institutional guidelines. Mice weighing between 20–25 g were selected and housed under standard environment conditions at ambient temperature of 25°C and supplied with food and water *ad libitum*. 4 mg in 0.2 ml sterile saline of rhodamine 6G labelled PLGA nanoparticles were administered via the oral route once daily over five days and another group via the intra-peritoneal (ip) route once to unchallenged Balb/C mice. Thioglycolate, which served as a positive control was administered via the oral and intraperitoneal routes respectively for the detection of activated macrophages. Saline was used as a negative control. The cells were harvested from the peritoneal lavage. The particle uptake by macrophages was determined via fluorescence activation cell sorting (FACS). Antibodies specific to macrophage and specifically phagocytotic macrophages, anti-MOMA-2 and anti-CD11c were utilized for the distinction of macrophage cells from the rest of the peritoneal exudate cell (PECS) population.

Determination In Vitro Efficacy

Direct BACTEC 460. BACTEC 12B vials (Becton Dickinson) were primed with 5% CO_2 and 100 μl of the antimicrobial, which contains Polymyxin B, Amphotericin B, Nalidixic acid, Trimethoprim, Azlocillin (PANTA^{TM} Becton Dickinson) in reconstitution fluid. 100 ul of McFarland No.1 stock of the $H_{37}R_V$ (1×10^8/ml) was added to each vial and incubated at 37°C. Control vials were treated with reported MIC concentration of RIF and INH and the samples vials were treated with the same drug concentration however the drugs in this case were encapsulated in PLGA nanoparticles. Three vials were used for each group. Two controls were included, one a 10 X dilution and the second at 100 X dilution of the stock. Readings were taken daily until the bacterial growth index reached a maximum in the control samples.

Determination of In Vivo Release Profile

PLGA-drug loaded nanoparticles were administered via the oral gavage to unchallenged Laca mice on day 1 at a therapeutic dose of RIF 12 mg/kg and INH 10 mg/kg. Mice were divided into two groups. One group received free RIF and INH and another received PLGA-RIF and INH. Mice were bled via the retro-orbital plexus once each day for a period of 6 days. Drug concentrations were determined in plasma by HPLC-MS.

Results and Discussion

Particle Characterization

Particle Size, Size Distribution (SD) and Zeta Potentials (ZP). The size of nanoparticles was optimized against a benchmark of 500 nm as it is reported that smaller particles (average

Table 2. Characteristics of nanoparticles (n = 3)

Formulation	Ave size (nm)	SD	ZP (mV)	EE
A	386 ± 7	0.53 ± 0.11	−18 ± 3	35 ± 4
A1	373 ± 23	0.27 ± 0.07	−11 ± 3	51 ± 3
B	300 ± 9	0.24 ± 0.06	−14 ± 3	53 ± 5
B1	210 ± 13	0.12 ± 0.03	−14 ± 2	60 ± 3
C	328 ± 18	0.3 ± 0.09	−15 ± 2	48 ± 3
C1	280 ± 23	0.21 ± 0.08	−10 ± 4	58 ± 5
D	297 ± 22	0.15 ± 0.06	+16 ± 2	65 ± 2
F	321 ± 33	0.21 ± 0.06	+19 ± 1	70 ± 3

SD = standard deviation; ZP = zeta potential, EE = encapsulation efficiency.

size <500 nm) undergo passive diffusion through cells and different tissues in the body [18]. Various parameters were optimized to obtain an average particle size ranging between 250 and 350 nm and an average polydispersity index of 1.2. The increase of shear rate by increasing the speed of the homogenizer caused a decrease in particle size.

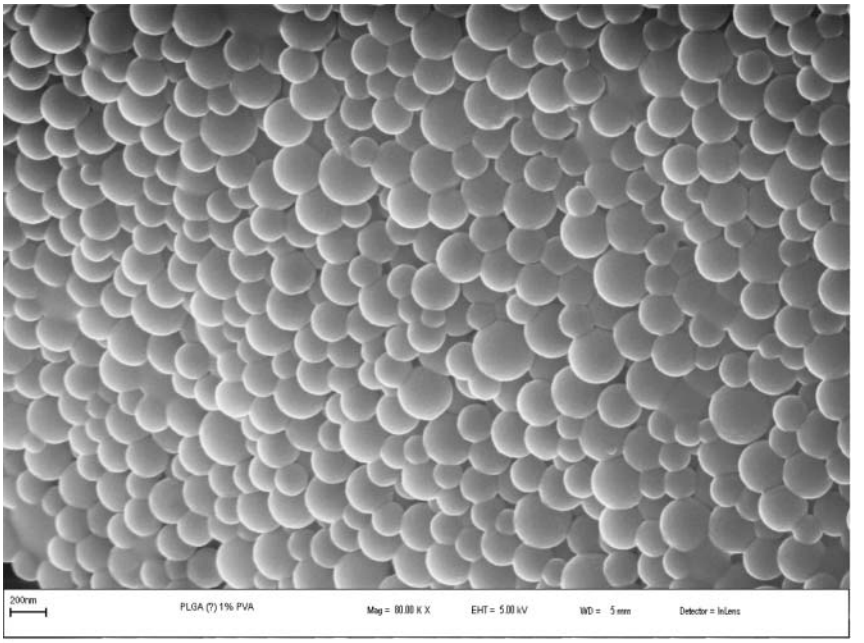
All samples made via freeze drying showed a negative zeta potential. The addition of chitosan to provide positive surface charge resulted in microparticles (data not shown). This problem has been alleviated by spray drying the double emulsion containing chitosan and PEG in the formulation (i.e. D and F in Table 2). All the subsequent assays were thus conducted with the spray dried formulation. Although RIF was loaded in the polymeric matrix of the carrier, there were no significant differences with INH-loaded nanocarriers in terms of all characteristics investigated, i.e. size, size distribution and zeta potential.

Encapsulation Efficiency (EE)

As indicated in Table 2, a considerable fraction of the active compounds (i.e. INH and RIF) were incorporated into the nanoparticles. The encapsulation was improved by increasing the amount of PVA molecules for the same amount of particles. This might be attributed to a better coverage of the stabilizer onto the surface of particles that might limit the escape of drug molecules from the carrier during the preparation process. Although RIF is highly hydrophobic, which should imply high encapsulation efficiency attributed to its low diffusion rate through the aqueous phase, its encapsulation is still relatively low when compared to INH samples. This might be attributed to the fact that it is located in the polymeric shell (as opposed to the inner aqueous core), which might make it susceptible to loss during washing.

Analysis of Surface Morphology

As depicted in Tables 1 and 2, high volume of stabilizer i.e. PVA, led to well distributed and uniform INH-loaded PLGA nanoparticles with an average size around 300 nm, characterized by a very smooth surface as depicted by the SEM image in Fig. 1A, as opposed to particles made with low volume of stabilizer (see Fig. 1B). A dimpling effect was observed with both INH and RIF encapsulation as depicted in Fig. 2A. When lactose (5% w/v) was added in the spray dried formulation, it resulted in smooth spherical particles shown in Fig. 2B.



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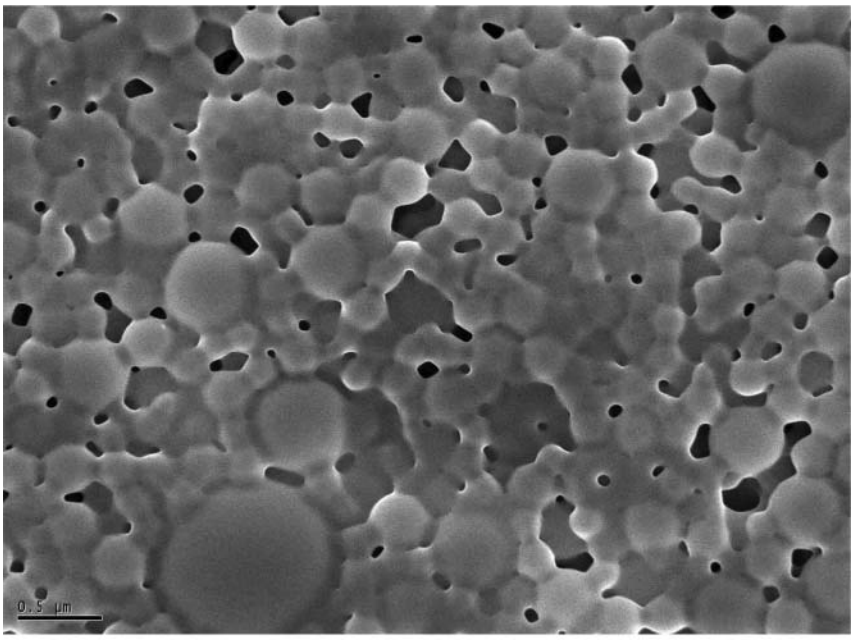
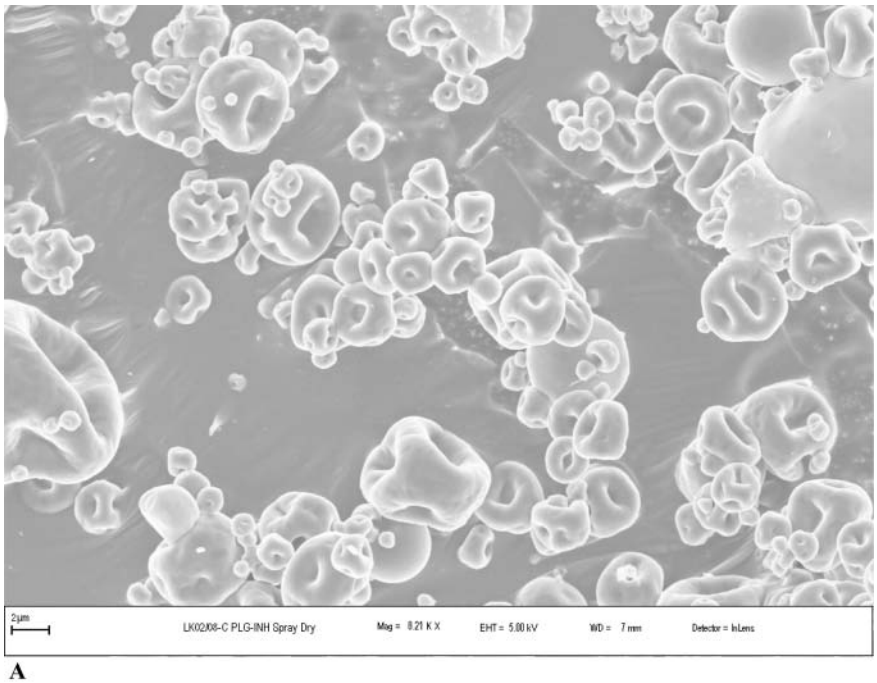


B

Figure 1. A = SEM image of freeze dried formulation B1, (Prepared with 40 ml 1% PVA). B = Freeze dried formulation A1 (Prepared with 20 ml PVA 1% w/w). The bar represents 200 nm.



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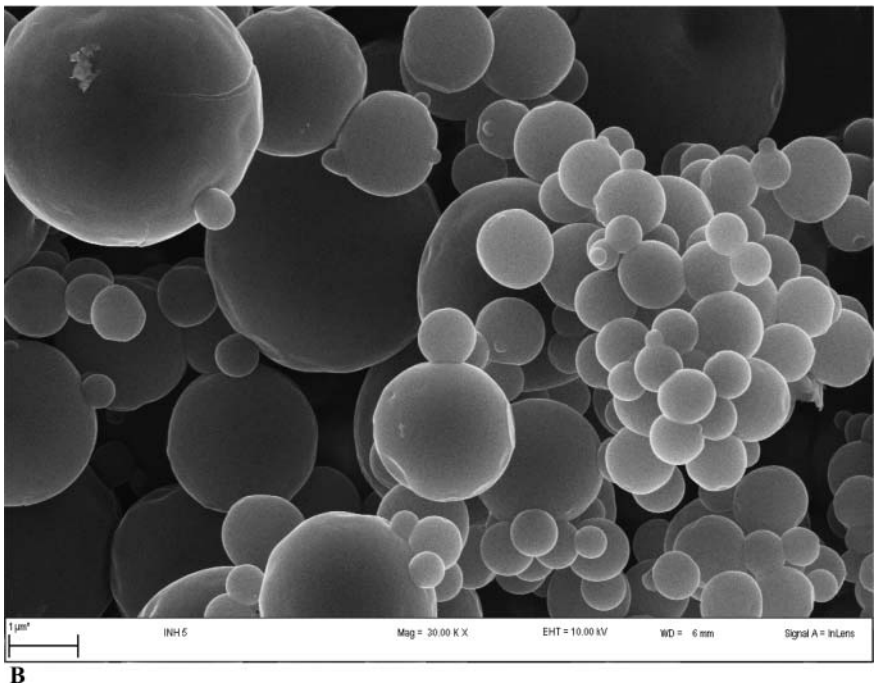


B

Figure 2. A = SEM image of spry dried particles without lactose. B = Spray dried particles with 5% w/v lactose.

It has been reported that small size (less than 500 nm) and a spherical shape give rise to an enhanced efficiency of cell internalization. A spherical particle possesses the right curvature allowing its attachment onto the cell [19]. It is equally admitted that spherical particles offer maximum volume for drug incorporation.

In Vitro Drug Release Profile

Minimal drug release was observed from nanoparticles suspended in PBS or in particles suspended in the dialysis membrane as depicted in Fig. 3. Evidence of initial burst release can be observed in the first 1–2 hours. The release lasted for approximately 24 hours for both drugs as depicted in Fig. 3A, in contrast to free drugs whose release was complete in 6 hours (Fig. 3B). Additionally, there was no significant difference in the amounts of drug released in the freeze dried and spray dried formulations. In the two methods, approximately 15–40% of surface-entrapped drugs were removed during the washing stage when the encapsulation efficiency was determined. Thus, the amount of drugs released from nanoparticles only accounted for less than 2% of encapsulated drugs. We postulate that the rest of the drugs (62–78%) are possibly still trapped in the nanoparticles in these conditions.

Dutt and Khuller [11,20] also had the same observation, where they observed that when pre-washed drug-loaded nanoparticles were suspended in PBS, there was negligible (initial 3–7% followed by $<1\%$) in vitro drug release for 6 weeks [20]. Polymer biodegradation and adequate agitation usually play a crucial role in drug release from nanoparticulate systems [21]. It is also hypothesized that drug release from NPs is through bioerosion of nanoparticles [22]. Since hydrolytic enzymes were not included in PBS, the slow rate of nanoparticle degradation could be attributed to this factor. It is envisaged that faster release rate could be observed in the biological milieu with hydrolytic enzymes present.

Particle Uptake Studies

In Vitro Particle Uptake

To determine particle uptake by Caco-2 cells, cell were treated with 10 μg/ml PLGA at a density of 1×10^5 cells/cm^2. It was evident as indicated in Fig. 4 that the particles of

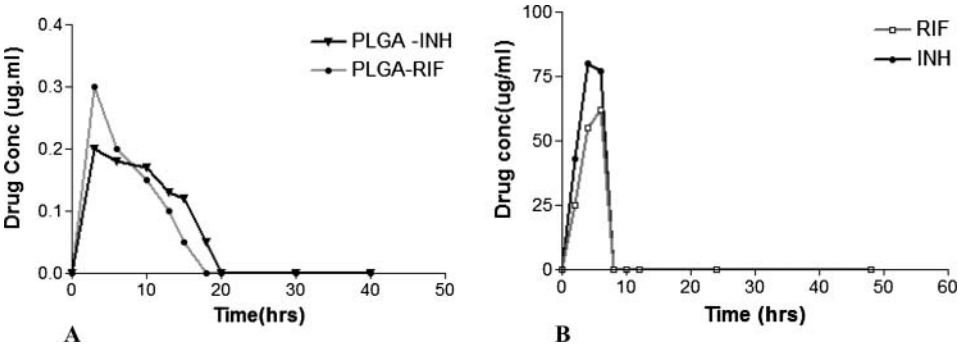


Figure 3. A = *In vitro* release of INH and RIF from PLGA nanoparticles suspended in 0.1 M PBS pH 7.4. B = Positive control of free drugs following dialysis (10 mg/100 ml PBS) with stirring at 37°C.

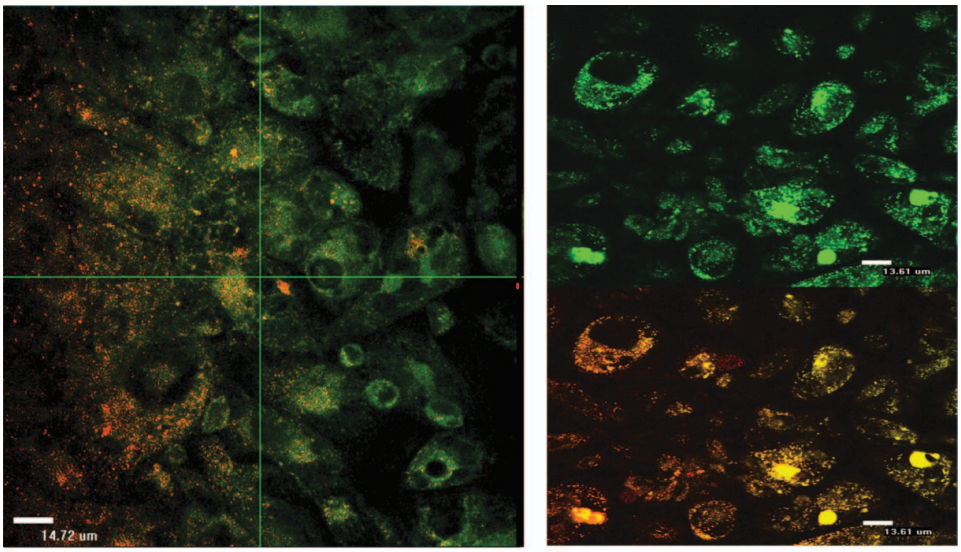


Figure 4. A indicates a Z-stack of 30 min incubation and B depicts a 60 min incubation period.

the specified size range were taken up within 30 min. However, after 60 min, a greater proportion of the particles were taken up. Based on this data, it can be suggested that the Rhodamine loaded nanoparticles co-localized with the lysosomes, as indicated by the orange colour in Fig. 4.

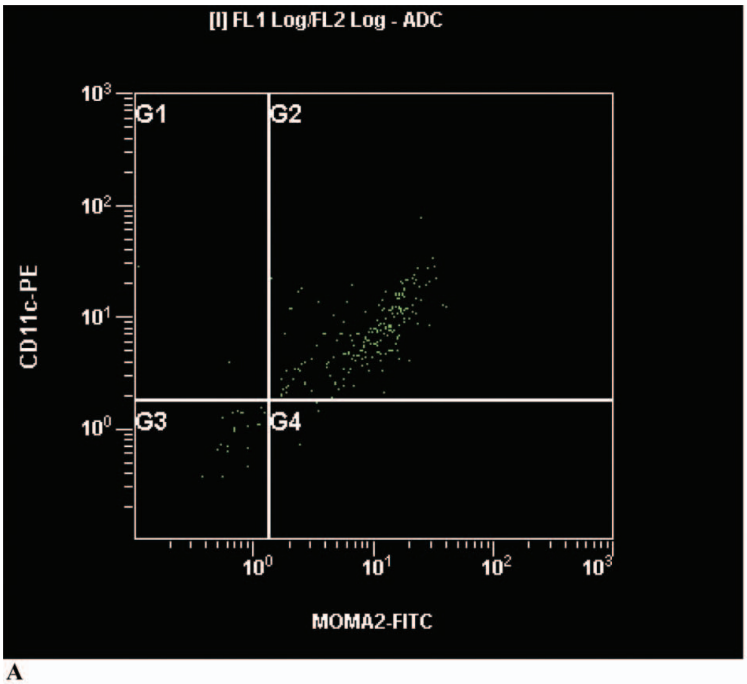
Our results are in agreement with similar reports where it has been illustrated that nanoparticles are taken up by cells. Furthermore it supports the data by Kisich et al. [12], where it was illustrated that free drugs did not accumulate in the cells at the same concentration as encapsulated drugs. Thus, nanoparticulate encapsulation of drugs may enhance drug uptake into the cells. The use of Caco-2 cells in this specific study enabled the in vitro analysis of uptake in a cell model of the intestine. Thus, it can be suggested that the prepared particles should be taken up by cells of the intestine of patients.

In Vivo Particle Uptake

An in vivo study was performed to confirm particle uptake. Initially macrophages were characterized from the subpopulation of PECS as indicated in Fig. 5A and B, using thioglycolate induced macrophage proliferation a positive control and as well as anti-CD11c and MOMA-2 to characterize these cells. In the saline treated group no activated macrophages were detected in the gated channel as depicted in Fig. 5C. In general macrophages are large in size and also granular, thus they are detected in the higher ranges of the forward and side scatters.

The analysis of PECS subsequent to intraperitoneal and oral administration of Rhodamine labelled PLGA nanoparticles indicated that peritoneal macrophages did take up the particle as indicated in Fig. 6A and B respectively.

From this data, it can be suggested that PLGA particles are taken up in vivo by macrophages. This is indicated by a population of macrophages that is positive for the Rhodamine particles. Although uptake of particles by macrophages has previously been



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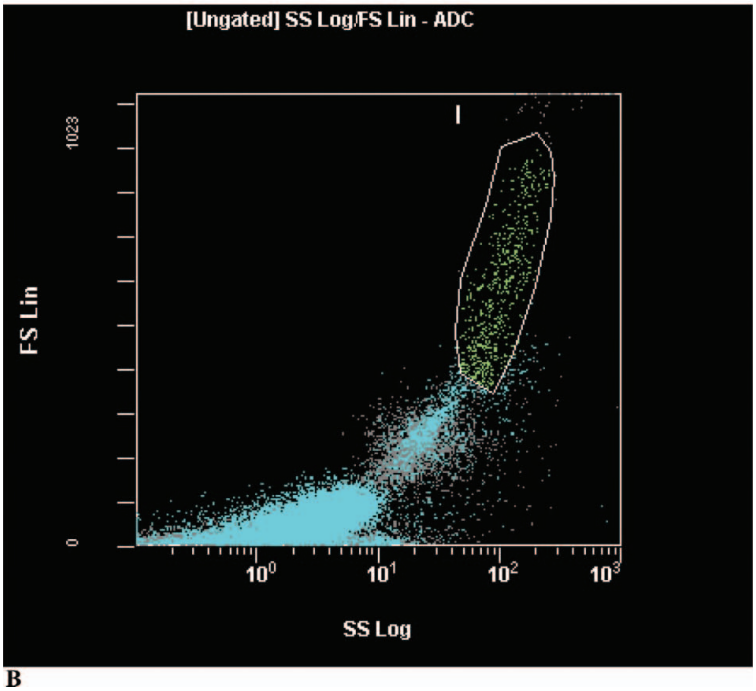

B

Figure 5. A = FACS data of PECS subsequent to thioglycolate administration to mice. B = FACS data of PECS subsequent to saline administration to mice.

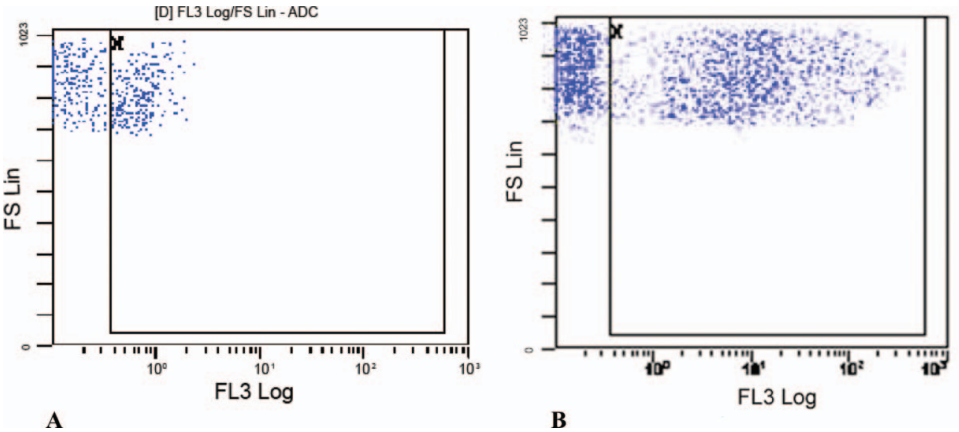

Figure 6. A indicates macrophage cells subsequent to intraperitonel administration and B indicates oral administration of Rhodamine labeled particles.

reported [23], much of the research was conducted in vitro. With this approach, we were able to illustrate that subsequent to intraperitoneal and oral administration, fluorescently labelled PLGA nanoparticles of the reported size range were taken up by the macrophages of the peritoneum. Further assays were conducted to illustrate whether the uptake of the particles elicits immune response or not by analysing the cytokine profile in mice post administration of the particles (data not shown).

In Vitro Efficacy Studies

MIC concentrations of INH and RIF were added into Bactec 460 vials inoculated with $H_{37}R_V$. From the reading taken on the days indicated in Fig. 7, it was evident that the encapsulated drugs still exhibited in vitro efficacy against $H_{37}R_V$, comparable to the free drugs. The Bactec 460 assay is generally conducted to analyse the susceptibility of *M.tb* to

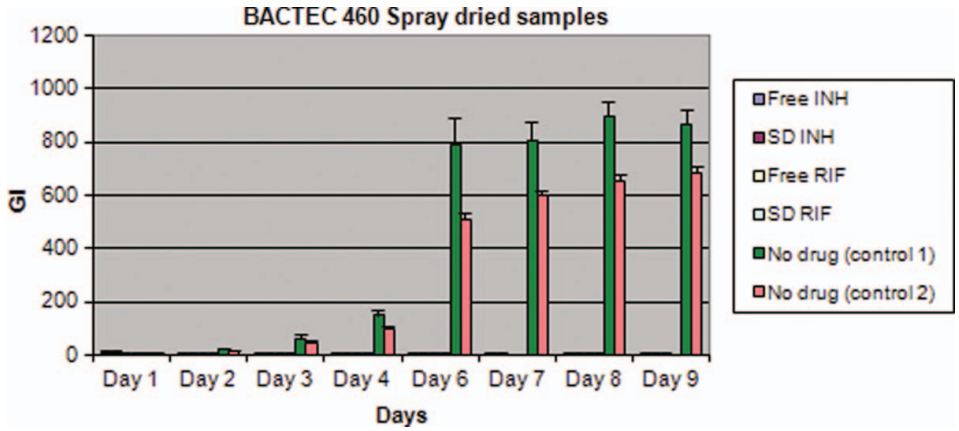

Figure 7. BACTEC 460 data indicating bacterial growth index of $H_{37}R_V$ treated of encapsulated RIF and INH and unencapsulated drugs.

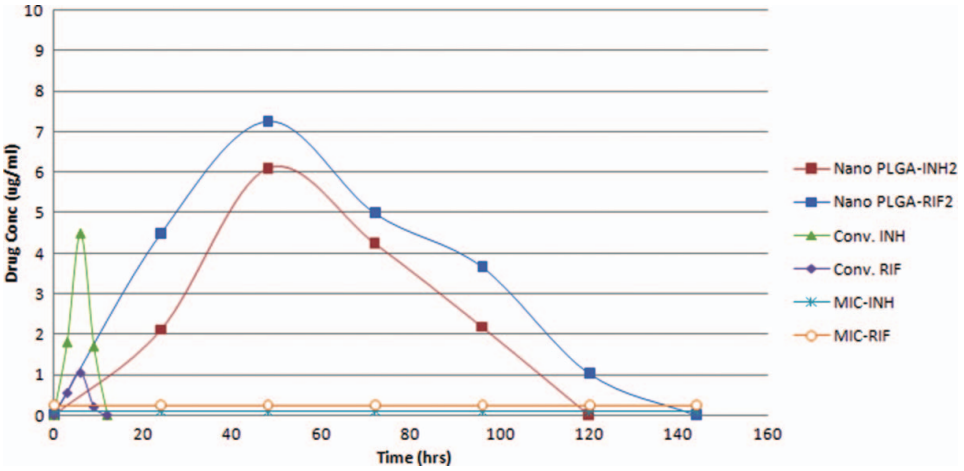

Figure 8. *In vivo* release of free drugs vs spray dried nanoparticles encapsulating RIF and INH and PZA.

the test drugs. Similar to data presented by Kisich et al., 2007, we were able to illustrate in vitro that the method of encapsulation, i.e. multiple emulsion- spray drying technique does not have any effect on the potency of the drugs.

In Vivo Release Profile

When spray dried PLGA RIF and INH particles were orally administered a sustained release profile as depicted in Fig. 8 was observed over a period of at least 5 days when compared to free drugs which reached levels below the MIC within 16 hrs. With encapsulated drugs, drug concentration in plasma above the MIC level of RIF and INH were sustained for 5/6 days.

The difference between our results and those of Pandey et al. [11], is the use of a novel spray drying technology as well as the surface modified of the particles to increase the residence time in the circulation, via the attachment of PEG and chitosan. The scalability of our technology also makes it feasible for easier entry into the market.

Conclusions

This study presents a novel technology that can be applied to encapsulated anti-TB drugs to improve the bioavailability of the drugs. The ability to release the drugs at the MIC concentration over a period of days, presents the promise of reducing the doses currently administered, and also minimizing the dose frequency, from a daily intake of antibiotics to a postulated once a week intake. Furthermore, we have illustrated in vivo that macrophages which are the cells of targeted in TB chemotherapy do take up the particles post oral administration of the nanoparticles.

Nanomedicine presents a science that will greatly advance the field of drug development and drug delivery. With nano-based drug delivery systems, it is envisaged that various drugs that could not enter the market due to poor bioavailability and adverse side effects etc., can be reformulated using nanotechnology for better efficacy and lesser adverse side effects.

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