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Functionalization of PLGA Nanoparticles with 1,3- β -glucan Enhances the Intracellular Pharmacokinetics of Rifampicin in Macrophages

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Abstract

Purpose—*Mycobacterium tuberculosis* which causes tuberculosis, is primarily resident within macrophages. 1,3- β -glucan has been proposed as a ligand to target drug loaded nanoparticles (NPs) to macrophages. In this study we characterized the intracellular pharmacokinetics of the anti-tubercular drug rifampicin delivered by 1,3- β -glucan functionalized PLGA NPs (Glu-PLGA). We hypothesized that Glu-PLGA NPs would be taken up at a faster rate than PLGA NPs, and consequently deliver higher amounts of rifampicin into the macrophages.

Methods—Carbodiimide chemistry was employed to conjugate 1,3- β -glucan and rhodamine to PLGA. Rifampicin loaded PLGA and Glu-PLGA NPs as well as rhodamine functionalized PLGA and Glu-PLGA NPs were synthesized using an emulsion solvent evaporation technique. Intracellular pharmacokinetics of rifampicin and NPs were evaluated in THP-1 derived macrophages. A pharmacokinetic model was developed to describe uptake, and modelling was performed using ADAPT 5 software.

Results—The NPs increased the rate of uptake of rifampicin by a factor of 17 and 62 in case of PLGA and Glu-PLGA, respectively. Expulsion of NPs from the macrophages was also observed, which was 3 fold greater for Glu-PLGA NPs than for PLGA NPs. However, the ratio of uptake to expulsion was similar for both NPs. After 24 h, the amount of rifampicin delivered by the PLGA and Glu-PLGA NPs was similar. The NPs resulted in at least a 10-fold increase in the uptake of rifampicin.

Conclusions—Functionalization of PLGA NPs with 1,3- β -glucan resulted in faster uptake of rifampicin into macrophages. These NPs may be useful to achieve rapid intracellular eradication of *Mycobacterium tuberculosis*.

Keywords

1,3- β -glucan; nanoparticle drug delivery; pharmacokinetic modelling; PLGA nanoparticles; rifampicin intracellular concentrations

INTRODUCTION

Each year tuberculosis (TB) is responsible for close to 1.5 million deaths globally (1). The TB causative organism *Mycobacterium tuberculosis* (*M.tb*) is a predominantly intracellular pathogen and the primary host cell is the macrophage (2). Therefore macrophages are a target for drug delivery and characterizing intracellular drug concentrations within this compartment is important. Generally, anti-tubercular drugs are challenged with respect to entering macrophages. Rifampicin (RIF) is reported to penetrate macrophages poorly (3–6). Low drug concentrations, particularly those falling below the minimum inhibitory concentration can be expected to play a major role in the generation of drug resistant *M.tb* strains, as a result of sub-optimal drug exposure to the microbe. It is reported that high drug concentrations, at early time points, which are sustained over long periods, are necessary for optimal *M.tb* killing (7). Therefore, the effectiveness of drug therapy for *M.tb* eradication can be improved if drug uptake and residence within macrophages is enhanced. TB that is resistant to existing drugs is increasing and given the thin pipeline of new drugs for TB (8), strategies to effectively deliver and use existing drugs are required.

We and others have previously reported that polymeric nanoparticle (NP) drug delivery systems are effective at depositing encapsulated anti-tubercular drugs into macrophages and thereby increasing intracellular drug concentrations (4,5,9). Commonly, NPs have been functionalized with mannose, to achieve specific interactions with the mannose receptor on the macrophage surface, thereby targeting the NP to the macrophage and consequently enhancing uptake of the drug loaded NP (10,11). In previous work, we conjugated the polysaccharide 1,3- β -glucan onto the polymer poly(lactic-co-glycolic acid) (PLGA) and formulated glucan functionalized PLGA NPs. We reported that these NPs were not toxic to macrophages (12). 1,3- β -glucan is of use as a macrophage targeting ligand as it interacts with the Dectin-1 receptor; a receptor predominantly expressed on macrophage surfaces (13). Activation of Dectin-1 is reported to enhance phagocytosis thereby providing an opportunity for enhanced NP uptake and intracellular drug concentrations. Other known Dectin-1 effects include production of ROS/RNS as well as pro-inflammatory cytokines and chemokines, including IL-12 (14,15).

In this work, we describe the intracellular pharmacokinetics of RIF in macrophages delivered by glucan functionalized PLGA NPs. We present a pharmacokinetic model which explains the processes leading to increased intracellular concentrations of RIF by the NPs when exposed to macrophages in culture. We hypothesized that glucan functionalized NPs would be taken up at a faster rate than PLGA NPs, and consequently deliver higher amounts

of rifampicin into the macrophages. We expect our data to support the future investigation of the efficacy of 1,3- β -glucan functionalized NPs towards eradicating *M.tb* within macrophages.

MATERIALS

1-Ethyl-3(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC.HCl), *N*-Hydroxysuccinimide (NHS), *N,N*-Diisopropylethylamine (DIEA), linear chain 1,3- β -glucan, i.e. curdlan from *Alcaligenes faecalis* (MW 189 kDa), rifampicin 97% (RIF), polyvinyl alcohol (PVA): Mw, 13–23 kDa, partially hydrolysed (87–89%), poly(*D,L*-lactide-*co*-glycolide) (50/50) with the terminal carboxylic acid group (PLGA-COOH: MW 24–38 kDa) and non-acid terminated (PLGA, MW 30–60 kDa), phorbol myristate acetate (PMA) and [N-(8-amino-3,6-dioxaoctyl)rhodamine 6G-amide bis(trifluoroacetate)] (Rh) were all purchased from Sigma-Aldrich (St Louis, MO). VivaSpin 20 (polyethersulfone 3000 molecular weight cut-off) were purchased from National Separation (Pty) Ltd (Johannesburg, South Africa). All reagents were analytical grade or above and were used as purchased, unless otherwise stated. Acetonitrile, used as mobile phase in high-performance liquid chromatography (HPLC) and liquid mass spectrometer (LC-MS/MS) was purchased from Merck. Deionised water was obtained from the Barnstead EasyPure (II) UV-ultrapure water system (Thermo Fisher Scientific, USA) and was used throughout the study. THP-1 cells were purchased from Highveld Biologicals (Pty) Ltd (Johannesburg, South Africa). The media and supplements used to maintain cell growth were purchased from LTC Tech (Johannesburg, South Africa).

METHODS

Synthesis of Fluorescing glucan-PLGA Co-Polymer and Fluorescing PLGA

The synthesis of the fluorescing glucan-PLGA-rhodamine (Glu-PLGA-Rh) polymer was synthesized in a two-step synthetic procedure. The first step involved the synthesis of the Glu-PLGA polymer as disclosed previously in Tukulula *et al* (12). The second step was adopted, with modifications, from the synthetic procedure reported by Adamczyk *et al* (16) on the synthesis of Rh conjugates, except that in our case, the aminated Rh, [N-(8-amino-3,6-dioxaoctyl)Rhodamine 6G-amide bis(trifluoroacetate)], was bought commercially. Thus, to prepare the Glu-PLGA-Rh, the Glu-PLGA conjugate (100 mg) was stirred in 10 mL anhydrous DMSO/DMF solvent mixture for 2 h, thereafter EDC.HCl (3.88 mg, 20 μ moles), NHS (1.66 mg, 15 μ moles) and DIEA (3.88 mg, 5.1 μ L) were added at room temperature to activate the excess carboxylic acid functionality of the PLGA moiety of Glu-PLGA. The resultant mixture was allowed to stir for 24 h. After this period, the commercially bought aminated Rh (20 mg, to achieve loading of about 2%) was added to the mixture and the reaction was allowed to stir further for another 48 h at room temperature. The reaction mixture was then dialysed using a dialysis membrane (15 mm $\times$ 23 mm, 12,400 MWCO, 99.99% retention; purchased from Sigma Aldrich) for over 24 h, followed by lyophilization to obtain an amorphous off-white product (120 mg).

The Rh-PLGA conjugate was also synthesized using the same procedure described for Glu-PLGA-Rh above. Briefly, PLGA (200 mg) was dissolved in 5 mL anhydrous DMF followed

by the addition of EDC.*HCl* (3.88 mg, 20 μ moles), *NHS* (1.66 mg, 15 μ moles) and DIEA (3.88 mg, 5.1 μ L). The resultant solution was stirred for 1 h at room temperature to allow for activation of the carboxylic acid functionality of the PLGA. Subsequent to this, the commercially bought aminated Rh (10 mg) was added and the resultant mixture was stirred for a further 48 h at room temperature, followed by dialysis and lyophilization to obtain an amorphous cream-white product (208 mg). All conjugates were analysed by ^1H NMR to determine the % of loading Rh on the polymers.

Synthesis and Characterization of NPs

An emulsion-solvent evaporation technique (12) was used to synthesize RIF loaded PLGA (PLGA(RIF)) and Glu-PLGA NPs (Glu-PLGA(RIF)). To synthesize PLGA(RIF) NPs, a 20 mg/ml PLGA solution in ethyl acetate (containing 2 mg/ml RIF) was added drop-wise to 10 mg/ml PVA solution in de-ionised water under stirring at 1000 rpm. Upon complete addition, the suspension on ice was subjected to high speed homogenization at 8000 rpm for 10 min using a Silverson L4R mixer homogenizer (Silverson, MA). Ethyl acetate was subsequently removed in vacuo at 40°C for 1 h and NPs collected through centrifugation at 10000 $\times g$ for 5 min, followed by washing thrice with de-ionized water, and lyophilization over 72 h in the presence of sucrose as a cryoprotectant. To synthesize C-PLGA(RIF) NPs, the procedure described above was followed, however, a dimethylformamide: chloroform mixture (20:80) was used as solvent. Rh-PLGA and Glu-PLGA-Rh NPs were also synthesized as above, except that the organic phase did not contain RIF.

Suspensions of NPs in de-ionized water (1 mg/ml) were characterized for size, polydispersity index (PDI) and zeta potential using a Malvern Zetasizer Nano ZS (Malvern Instruments Ltd, UK). Measurements were conducted at 25°C in triplicate. The amount of RIF encapsulated in the NPs was determined using a validated HPLC-UV method, following destruction of the NPs through sonication in acetonitrile.

CHARACTERIZATION OF UPTAKE KINETICS

THP-1 Cell Culture

The human monocyte cell line THP-1 was cultured in RPMI 1640 medium supplemented with 10% fetal calf serum and 1% penicillin streptomycin antibiotic mixture in 6-well culture plates at 37°C, 5% CO_2 atmosphere (12). Prior to experiments, cells seeded at a density of 10^6 cells/well were induced to undergo terminal differentiation into macrophages by incubation in the presence of 20 nM of PMA for 24 h. Formation of macrophages was confirmed by microscopy. Fresh complete RPMI was then added to the adherent cells and incubation continued for 24 h.

Pharmacokinetics of RIF in Macrophages

The intracellular and extracellular pharmacokinetics of RIF in THP-1 derived macrophages after delivery by (i) a solution of RIF, (ii) PLGA(RIF) and (iii) Glu-PLGA(RIF) NPs were determined. To determine RIF kinetics, as delivered by a RIF solution, RIF (dissolved in PBS) was added to the cell culture wells to a concentration of 0.1 $\mu\text{g/ml}$ per well. To determine RIF kinetics as delivered by PLGA(RIF) and Glu-PLGA(RIF) NPs, 100 μl of a 1

mg/ml NP suspension in PBS (equivalent to RIF payload of 0.1 µg/ml to the well) were added to designated wells. The culture plates (10^6 cells/ml) were incubated at 37°C, 5% CO₂ atmosphere and at intervals (0.5, 1, 2, 4, 8 and 24 h), the designated plate was removed from incubation and immediately placed on ice. Firstly, the medium surrounding the cells was collected into tubes (for later determination of extracellular RIF concentrations). Cells were then washed thrice with ice cold PBS, and the washings added to the medium collected in the tubes. The 'extracellular' medium was immediately snap frozen and stored at -80°C for later drug quantification using a validated LC-MS/MS assay. After collection of the medium, 1 ml of 0.1% triton X-100 was added to the wells to lyse the cells. The cell lysate was collected into separate tubes, which were also snap frozen and stored at -80°C prior to RIF quantification using LC-MS/MS. Quantification of RIF in the cell lysates provided a determination of the intracellular concentrations of RIF.

Sample Preparation for Analysis

'Released drug' representing drug released from the NPs (or delivered by RIF solution) into medium or cell lysate was measured. 'Total drug', representing drug released from the NPs, as well as that still present within the NPs was also quantified (17). With respect to 'released drug', to 200 µl of medium or cell lysate, 50 µl of ammonium acetate (2.6 mg/ml) was added to precipitate proteins (18). In prior experiments, we determined that this concentration of ammonium acetate could not disrupt the NPs (data not shown). Samples were then centrifuged at 12000 x *g* for 5 min at 4°C, before quantification of RIF using LC-MS/MS. In the case of 'total drug' analysis, to 200 µl of sample, 50 µl of acetonitrile was added to precipitate proteins and concurrently disrupt the NPs (and thereby release any remaining encapsulated drug) (6), before centrifugation at 12000 x *g* for 5 min at 4°C and quantification of RIF using LC-MS/MS. To normalize concentrations of the cell lysates, protein mass was measured in the cell lysates collected. The Bradford assay was employed to determine protein mass, and fluorescence was measured at 620 nm in a microplate reader (Tecan, Männedorf, Switzerland). A standard curve of albumin with a range of 0.15–1.4 mg/ml ($r^2 = 0.99$) was used. The concentrations of RIF in the lysates were expressed as µg per mg of cell protein.

Pharmacokinetics of NPs in Macrophages

The intracellular and extracellular pharmacokinetics of the NPs were determined in the THP-1 derived macrophages. 100 µl of a 1 mg/ml suspension of Rh-PLGA or Glu-PLGA-Rh NPs were added to the cells (10^6 cells/ml) and incubated at 37°C, 5% CO₂ atmosphere and at intervals (0.5, 1, 2, 4, 8 and 24 h), the designated plate was removed from incubation and placed on ice. To determine extracellular NP concentrations, the medium surrounding the cells was collected into tubes. The cells were then washed thrice with 150 µl ice cold PBS, and the washings added to the medium in the tubes, and immediately snap frozen using liquid nitrogen and stored at -80°C for fluorescence measurement. After collection of the medium, 1 ml of cell lysis buffer (0.1% triton X-100) was added to the wells. The lysed cells were collected into separate tubes, which were also snap frozen and stored at -80°C for fluorescence quantification (determination of intracellular NP concentrations). The Bradford assay was employed to measure protein mass as per the RIF quantification procedure above. Rh was quantified in the cell media and lysates by measuring fluorescence in a microplate

reader (excitation 485 nm, emission 535 nm). A standard curve of Rh-PLGA and Glu-PLGA-Rh NPs in cell media and cell lysate over the range 0.001– 15 µg/ml was used for NP quantification.

LC-MS Assay

The LC–MS/MS system consisted of a Shimadzu Prominence Ultra-Fast LC (UFLC) series system (Shimadzu Corporation, Kyoto, Japan) coupled with an ABSciex 3200 QTrap Triple Quadrupole Tandem Mass Spectrometer equipped with Analyst 1.6 software (AB Sciex, Massachusetts, USA). Chromatography was performed using a 9 min gradient elution method with 0.1% formic acid (FA) as mobile phase A and acetonitrile (0.1% FA) as mobile phase B at a flow rate of 0.5 mL/min on a Phenomenex Kinetex 2.6 µ C8 analytical column (50 mm × 4.60 mm). Briefly, the time program for the gradient method used was: 2% B for 1 min; ramped up to 98% B until 2 min; held at 98% B until 7 min; ramped down to 2% B until 8 min; equilibrated at 2% B until 9 min. The mass spectrometer was operated in positive ion mode with the transition of 823.4 m/z – 791.4 m/z for RIF detection. The following ion source parameters were used: curtain gas flow, 35 psi; ion spray voltage, 4500 V; temperature, 500°C; gases 1 and 2, 45 psi; declustering potential, 45 V; entrance potential, 4 V; collision cell entrance potential, 28.87 V; collision energy, 25 V and collision cell exit potential, 6.1 V. The parameters for the quantification method: peak areas integrated; expected retention time of RIF, 3.04 min; 30 s RT window; noise percentage, 50%; peak splitting factor, 2; smoothing width, 5 points; linear fit calibration curve; no weighting of calibration points. The range of the assay was 5 ng/mL - 1 µg/mL ($r^2 = 0.99$).

Pharmacokinetic Data Modelling

The basic model fitted to the experimental data included all the envisaged pathways for the ‘free drug’ and drug encapsulated in the NPs, in the intracellular (lysates) and extracellular compartments (extracellular medium) (Fig. 1). Drug may be released from the NPs in the extracellular environment (kDPE) or carried by the NP into the macrophage (kPI) and released within the cell (kDPI). The NP may be expelled out of the cell (kPE). Apart from passive diffusion of drug out of the NP, the NP may erode/ degrade resulting in drug release (kIPS). From preliminary experimental data the degradation/erosion of the NPs over the 24 period was considered to be negligible (kEPS). Drug released extracellularly may diffuse passively into the macrophage (KDI) or outside of the cell (kDE). Degradation/metabolism of the drug may occur outside the cell (kDStab) or inside the cell (kDM). KDStab was considered negligible over the 24 h period and therefore this pathway was not considered in the model. Linear kinetics were assumed for all the pathways. It was assumed that NP uptake occurred via phagocytosis and was thus dependent on the concentration of NPs in the extracellular compartment and the uptake of drug alone uptake was assumed to occur via passive diffusion (19). Therefore, first-order kinetics were used to describe the uptake kinetics.

To determine the mass of RIF in the extracellular medium at time t , the following equation was used:

$$m_{DE, t} = m_{DE, t-1} + \Delta t \left[k_{DPE} * m_{DPE, t-1} + k_{DE} * m_{DI, t-1} - k_{DStab} * m_{DE, t-1} \right]$$

To determine the mass change of RIF within the macrophages at time t , the following equation was used:

$$\frac{dm_{DI}}{dt} = \left[k_{DPI} * f_{DPI, t-1} * m_{DPI, t-1} + k_{DI} * m_{DI, t-1} - k_{DM} * m_{DI, t-1} - k_{DE} * m_{DI, t-1} \right]$$

To determine the mass change of NPs in the extracellular medium over time, the following equation was used:

$$\frac{dm_{PE}}{dt} = \left[-k_{PI} * m_{PE, t-1} + k_{PE} * m_{PI, t-1} \right]$$

To determine the mass of NPs within the macrophages at time t , the following equation was used:

$$\frac{dm_{PI}}{dt} = \left[k_{PI} * m_{PE, t-1} - k_{PE} * m_{PI, t-1} - k_{PM} * m_{PI, t-1} \right]$$

All the masses in the equations were obtained by multiplying the concentrations by the corresponding volume. The volumes were kept constant as they were fixed (namely the extracellular medium) or estimated according to (19).

The model parameters were adjusted in order to obtain the best fit using ADAPT 5 software (20). In order to obtain a robust estimation of the common rate constants, the model was fitted simultaneously to all the experimental data.

Statistical Analysis

Data were analyzed by the Students' t test using the Statistical Package for Social Science v 22 (IBM, SPSS, Chicago). Statistical differences were considered at $p < 0.05$. All data are presented as mean $\pm$ s.d. ($n = 3$), unless otherwise noted.

RESULTS AND DISCUSSION

Characterization of Polymer Conjugates

The loading amounts of Rh to PLGA and Glu-PLGA was determined by comparing the characteristic aromatic signal intensities of Rh with those of PLGA both in the Rh-PLGA and Rh-Glu-PLGA spectra (Fig. 2). Specifically, comparing the characteristic aromatic Rh signals (21) at approximately 7.01–7.82 ppm with those of PLGA at 5.23 ppm in the Rh-PLGA ^1H NMR spectrum (Fig. 2), indicated a loading of approximately 1.32% (w/w). It is important to note that the aromatic Rh signals in the Rh-PLGA spectrum shifted slightly up

field (7.01–7.82 ppm) compared to those of the neat Rh at 6.78–8.35 ppm. Furthermore, in addition to the up field shifting of aromatic Rh signals, the signal at 6.79 ppm in the neat Rh spectrum, which is due to the free primary NH_2 of Rh, disappeared in the Rh-PLGA spectrum, further confirming the successful formation of an amide bond and hence the grafting of Rh to PLGA. at 3.06 ppm (in Fig. 2), which shifted slight downfield in the Rh-PLGA (to about 3.14 ppm) spectrum, with that PLGA at 3.42 ppm indicated a loading of about 1.32% (w/w).

As in the case of Rh-PLGA conjugate, the aromatic Rh signals at approximately 7.01–7.24 ppm were compared with those of PLGA at approximately 5.23 ppm in Rh-Glu-PLGA spectrum. Based on the intensities of the said peaks, the loading of Rh to Glu-PLGA conjugate was determined to be between 0.6–0.7% (specifically 0.683% w/w). Since there is an extensive overlap of peaks in both the Rh-PLGA and Rh-Glu-PLGA, the major difference between these two compounds is the two signals in the region between 4 and 5 ppm. In the Rh-PLGA spectrum, the distance (~ 0.23 ppm) between the two signals is narrower (4.10 to 4.33 ppm) compared to that in Rh-Glu-PLGA (4.12 to 4.53 ppm) which is wider (~ 0.38 ppm), indicating that these two compounds are not the same. The loading (w/w) of Glu to PLGA was determined in our pervious publication (12). These Rh loading amounts were taken into account in the pharmacokinetic modelling of NP uptake into the macrophages. Figures 3, 4, and 5 are the fully integrated spectra of neat Rh, Rh-PLGA and Rh-Glu-PLGA, respectively.

Characterization of NPs

Conjugation of Rh onto the PLGA and Glu-PLGA copolymer resulted in an increase in the mean size of the NPs (Table I). However, the size distributions of the NPs (Fig. 6) indicated overlap between the PLGA(RIF) and PLGA-Rh and between the Glu-PLGA(RIF) and Glu-PLGA-Rh NPs (Fig. 6). Therefore, we could use the intraand extracellular kinetic data generated using the Rh-conjugated NPs to explain trends observed from the RIF loaded NPs. All NPs were negatively charged in aqueous media. The amount of RIF loaded was 1.0–1.3 μg per mg of freeze dried NPs.

Intracellular Drug and NP Pharmacokinetics

During the experimental period, the total mass (sum of intracellular and extracellular) of RIF and NPs did not show any noticeable decrease over time (24 h). RIF or NPs/RIF loaded NPS were added to the extracellular medium and therefore were the only source of material measured in the intracellular compartment.

Rate constants were estimated as well as their associated uncertainty (95% confidence interval (CI)). The resultant significant parameters are provided in Table II. In order to accommodate the NP drug release ‘burst effect’, we assumed the drug concentration in the NP dosing experiment at $t(0)$ had a value different from zero. In this way we could, without violating the linear pharmacokinetic assumption, address the burst effect which we observed in preliminary *in vitro* drug release experiments (data not shown). We did not detect any difference in the measured intracellular concentrations of ‘free’ and ‘total’ drug therefore, the data reported is that of the ‘free’ drug only.

The rate constants of RIF from solution and NPs, indicated that the NPs increased the uptake of RIF into the cells by a factor of 17 and 62 (kPI/kDI) in the case of PLGA(RIF) and Glu-PLGA(RIF), respectively. For the same amount of NPs, the rate of uptake of Glu-PLGA NPs into the macrophages was more than twice that from the PLGA NPs, as was observed from the rate constants (kPI) which were 0.4198 h^{-1} vs 0.1822 h^{-1} for the Glu-PLGA NPs and PLGA NPs, respectively. The faster uptake of the glucan functionalized NPs could possibly be due to the specific interaction of glucan with the dectin-1 receptor, known to activate macrophages and also enhance their phagocytic activity (14,15). However, it appeared that at the same time, the Glu-PLGA NPs were leaving the cells at a faster rate compared to the PLGA NPs based on the rate constants (kPE) which were 2.093 h^{-1} vs 0.667 h^{-1} for the Glu-PLGA NPs and PLGA NPs, respectively. However, the ratio of kPI to kPE for each NP type was approximately the same ($kPI/kPE = 0.273$ and 0.201 for the PLGA and Glu-PLGA NPs, respectively) and this suggested that at equilibrium the intracellular concentrations of NPs would be similar between the two NP types; as was observed from the experimental data (Figs. 7 and 8). Indeed this typical profile of initial rapid uptake followed by a plateau has been reported in previous studies (17,19). In relation to killing intracellular bacteria, achieving high intracellular concentrations fast, as was observed with the GluPLGA NPs would be desirable.

Measurement of intracellular concentrations of RIF indicated that after 24 h, the amount of RIF delivered by the PLGA(RIF) and Glu-PLGA(RIF) NPs was similar. Approximately 10% of total drug available was delivered into the cells (Fig. 7). However, administration of RIF as a solution resulted in low intracellular concentrations (approximately 1% of total drug available was delivered into the cells). Similar low concentrations delivered by RIF solution have also been reported previously (5). Therefore, the NPs resulted in at least a 10-fold increase in the % uptake of RIF into the macrophages. This serves as direct evidence of the utility of NPs in enhancing intracellular delivery of drugs.

Quantification of NP uptake into the macrophages (Fig. 8) through the direct measurement of rhodamine, indicated that close to 60% of both PLGA-Rh and Glu-PLGA-Rh NPs were present in the intracellular compartment after 24 h. Although the analysis accounted for the % loading of Rh and RIF on the NPs, we found no direct correlation between the % of NPs and % of RIF intracellularly, possibly due to the multiple process which occurred concurrently, i.e. the bi-directional transport of NPs and release of RIF in the extracellular and intracellular compartments. Li *et al.* (17) reported a similar finding regarding the uptake profiles of paclitaxel and the PLGA NP delivery system in the human breast cancer cell line MCF7 cells (17).

CONCLUSION

A simple pharmacokinetic model was described which provided insight into the processes which were key to the intracellular delivery of RIF using NPs. The model suggested that the PLGA and Glu-PLGA NPs serve as ‘Trojan horses’ to deliver RIF into macrophages, consequently increasing the intracellular concentrations several fold in comparison to RIF solution. Functionalization of PLGA NPs with 1,3- β -glucan resulted in faster uptake of RIF and PLGA NPs into THP-1 macrophages, however, expulsion of both types of NPs from the

macrophages was observed, leading to an equilibrium in intracellular concentrations of RIF and the NPs. It remains to be seen whether the faster uptake of RIF and higher intracellular concentrations (in relation to delivery by RIF solution) will lead to improved eradication of *M.tb* resident within macrophages.

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ABBREVIATIONS

DIEA	N,N-Diisopropylethylamine
DMF	Dimethylformamide
EDC	1-Ethyl-3(3-dimethylaminopropyl)carbodiimide hydrochloride
LC-MS	Liquid chromatography mass spectrometry
M.tb	Mycobacterium tuberculosis
NHS	N-Hydroxysuccinimide
NMR	Nuclear magnetic resonance spectroscopy
NP	Nanoparticle
PLGA	Poly(D,L-lactide-co-glycolide)
PMA	Phorbol myristate acetate
RIF	Rifampicin
TB	Tuberculosis

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Fig. 1.

Schematic of pharmacokinetic model to describe the extracellular and intracellular pharmacokinetics of the drug (RIF) delivered by a drug solution and NPs. RIF may be metabolised in the intracellular compartment (and also degrade both in the intra and extra-cell media) and as such its total mass (intracellular + extracellular + nanoparticle) may decrease with time. The same model was adapted to describe the intraand extracellular pharmacokinetics of the NPs, i.e. considering K_{PI} and K_{PE} only.

Fig. 2.
Superimposed ¹H NMR spectra of neat aminated Rh, Rh-PLGA and Rh-Glu-PLGA showing the highlighted aromatic signals of Rh (Blue) used in calculating Rh loading. Also, highlighted in brown is the free amine signal of Rh (neat Rh) that disappears on grafting, consistent with formation of an amide. The distance in the two peaks between 4 and 5 ppm in both Rh-PLGA and Rh-Glu-PLGA is highlighted in red.

Fig. 3.
¹H NMR spectrum of commercially available neat Rh, in DMSO-d₆, prior to grafting in both Rh-PLGA and Glu-Rh-PLGA.

Fig. 4.
¹H NMR spectrum of Rh-PLGA conjugate, in DMSO-d₆, after grafting of Rh to PLGA.

Fig. 5.
¹H NMR spectrum of Rh-Glu-PLGA conjugate, in DMSO-d₆, after grafting Rh to previously synthesised Glu-PLGA conjugate.

Fig. 6. Overlay of representative particle size distributions of the PLGA(RIF), PLGA-Rh, Glu-PLGA(RIF) and Glu-PLGA-Rh NPs. Overlay was observed in the number size distributions indicating that the Rh-conjugated NPs could be used to describe the uptake kinetics of the PLGA(RIF) and Glu-PLGA(RIF) NPs.

Fig. 7. Intracellular fraction (intracellular concentration/total concentration) of RIF in macrophages over a 24 h period following administration of the PLGA(RIF) NPs, Glu-PLGA(RIF) NPs and RIF solution. The error bars represent the pooled standard deviation of the replicate determinations. The PLGA and GLU-PLGA markers time values were offset by ± 0.1 h to improve error bars view clarity.

Fig. 8. Intracellular fraction (intracellular concentration/total concentration) of PLGA-Rh and Glu-PLGA-Rh NPs in macrophages over a 24 h period following administration of NPs into the extracellular compartment. The error bars represent the pooled standard deviation of the replicate determinations regarding each type of NP.

Table I

Mean Size (Z-Average Hydrodynamic Diameter), PDI and Zeta Potential of the NPs. Drug Loading Results are Expressed as μg of RIF per mg of NPs, Excluding Weight of Sucrose Cryoprotectant. The Values are the Mean $\pm$ s.d (n = 3).

Formulation	Mean size (nm)	PDI	Zeta potential (mV)	Drug loading ($\mu\text{g}/\text{mg}$)
PLGA(RIF)	260.5 $\pm$ 12.4 *	0.12 $\pm$ 0.04	-11.6 $\pm$ 0.7	1.2 $\pm$ 0.1
Glu-PLGA(RIF)	283.1 $\pm$ 7.0**	0.19 $\pm$ 0.03	-28.6 $\pm$ 2.0	1.1 $\pm$ 0.1
PLGA-Rh	386.3 $\pm$ 12.5 *	0.27 $\pm$ 0.02	-14.8 $\pm$ 0.4	-
Glu-PLGA-Rh	446.5 $\pm$ 32.7**	0.35 $\pm$ 0.12	-11.4 $\pm$ 0.6	-

* Indicates Statistically Significant Difference in Mean Size between the PLGA(RIF) and PLGA-Rh NPs ($p < 0.05$) and Statistically Significant Difference in Mean Size between the Glu-PLGA(RIF) and the Glu-PLGA-Rh NPs (**)

Table IIModel Parameters (h^{-1}) and Point Estimate (95% CI) after Fitting the Model to the Experimental Data

Parameter	Estimate (CI)		
	RIF	PLGA NPs	Glu-PLGA NPs
Drug entering cell (kDI)	0.01075 (0.008)	0.01075 (0.008)	0.01075 (0.008)
NPs entering the cell (kPI)	–	0.1822 (0.0689)	0.4198 (0.3228)
NPs leaving the cell (kPE)	–	0.6667 (0.3404)	2.093 (1.8241)