NANOPARTICLE FORMULATION OF TWO ANTI-TB DRUGS FOR THE TREATMENT OF MULTIDRUG RESISTANT TUBERCULOSIS

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Abstract - Polymeric nanoparticles have been widely investigated as a controlled release drug delivery platform for the treatment of tuberculosis (TB). These nanoparticles are readily internalised into macrophages and leads to high intracellular drug concentration. In this study two anti-TB drugs, amikacin and moxifloxacin were encapsulated into alginate entrapped PLGA nanoparticles. The alginate entrapped PLGA nanoparticles were within the desirable particle size range of 312-365 nm and the PDI was 0.14-0.31, and therefore were chosen for subsequent studies. Alginate entrapped nanoparticles exhibited an entrapment of moxifloxacin range of10.1-18.7 µg/mg and an entrapment of amikacin range of15-17.4µg/mg. To study the macrophage uptake efficiency, both formulations of the alginate entrapped nanoparticle; non-calcium formulation and calcium wereloaded with coumarin-6 as a marker, seeded toH37RA infected THP-1 derived macrophages and examined by confocal microscopy. The alginate entrapped particles were readily internalised into the macrophages and highly concentrated in the perinuclear region, and the non-calcium alginate entrapped formulation showing the maximum internalisation. Furthermore, the anti-mycobacterial activity of the dually entrapped drug-loaded particles (moxifloxacin and amikacin) was evaluated using M. tuberculosis-infected macrophages, which revealed anenhanced inhibition of viable bacterial count compared to single drug loaded nanoparticle formulations, and tothe untreated group. In conclusion, the amikacin-moxifloxacin alginate entrapped PLGA nanoparticles are promising for further in vivo studies.

I. INTRODUCTION

MDR-TB is an infection caused by M. tuberculosis strains resistance to two of the first-line anti-TB drugs, isoniazid and rifampin. Agents to treat MDR-TB include aminoglycosides (i.e. amikacin, capreomycin, and kanamycin) and fluoroquinolones (i.e. moxifloxacin and levofloxacin) [1]. Amikacin is one of the main injectable aminoglycosides used in the management of MDR-TB. However, on prolonged use the drug often leads to nephrotoxicity, neurotoxicity and irreversible ototoxicity [2].Moxifloxacin is another widely used drug to treat MDR-TB [3]. The drug isa fourth-generation synthetic fluoroquinolone which has favorable pharmacokinetics, that include a large volume of distribution and the ability to permeate the epithelial-lining fluid and macrophages [4]. However, the extensive use of moxifloxacin was reported to lead to the emergence of extensive drug resistant (XDR) M. tuberculosis strains [5]. The combinative administration of these two antibiotics have shown synergy against MDR-TB[6].

The utilization of polymeric drug nanoparticles has been widely investigated for their potential to improve the efficacy and targeting of drugs for various diseases including TB [2, 7-9]. Polymeric nanoparticles offer the advantage of readily internalised into alveolar macrophages by the same defence machinery employed in the engulfment of M. tuberculosis[10-13]. Polymeric nanoparticles also provide a sustained release system and improve the stability of labile drugs from in vivoenzymatic degradation [14, 15]. Anti-TB drug loaded polymeric nanoparticles have been investigated for many routes of administrations including oral, intravenous, and inhalation [16]. In the present study, moxifloxacin and amikacin were encapsulated into nanoparticles targeting for the treatment of MDR. The combinative encapsulation of these two drugsinto polymeric nanoparticles has the potential to decrease the dosing of these drugs, thereby improves patient compliance to treatment, and potentially mitigates the undesirable dose-associated side effects. In this study, the drugs were incorporated into PLGA nanoparticles using water-oil-water (w/o/w) emulsion strategy to produce alginate entrapped PLGA nanoparticles. The particles were characterised in term size, drug loading, and morphology. The macrophage phagocytosis efficiency of the particles was also investigated using confocal microscopy and in vitro intracellular killing of nanoparticles containing single drug or dual encapsulation of amikacin and/or moxifloxacin against M. tuberculosis within the macrophages was investigated and compared to free drug at comparable concentrations.

II. METHODS

Formulation of drug loaded PLGA/alginate nanoparticles

Alginate entrapped PLGA nanoparticleswere fabricated using a modified emulsion evaporation technique. 5 mg amikacin and5 mg moxifloxacin were dissolved in 2 ml of 10 mg/ml alginate solution(waterinternal phase) and heated at 50 ºC for 15 min. The final volume was then adjusted to be 1 ml. Subsequently, 0.3 ml of the above solution was emulsified with 1 ml DCM containing 50 mg PLGA

(oil phase)using a probe sonicator. The resulted w/o emulsion was further emulsified with 5 ml of PVA 2.5% with (calcium formulation), or without 10 mg $CaCl_2$(non-calciumformulation).The emulsion was then evaporated, washed, and lyophilised as mentioned earlier. Single drug-loaded nanoparticles (amikacin or moxifloxacin) were formulated in a similar manner, with only the relevant drug is added to the alginate solution. Nanoparticles were further lyophilised at −30°C for 24 h followed by at 25°C for 48 h in a freeze drier (Christ Alpha 1–4 LOC-1 M, Martin Christ, Osterode am Harz, Germany).

Physical characterisation of the polymeric nanoparticles

The particle size and zeta potential of the nanoparticles were measured using photon correlation spectroscopy Scanning electron microscopy (SEM) was used to examine the morphology of the particles The drug loading of amikacin and moxifloxacinwere determined via indirect loading measurement of the free drug in the supernatant using modified HPLC methods. Drug loading and encapsulation efficiency were calculated as follows.

$$\%\text{Drug loading}\left(\mu\frac{g}{mg}\right) = \frac{\text{Total drug added }(\mu g) - \text{drug quantified in the supernatant }(\mu g)}{\text{Total weight of polymers and drug in the formulation (mg)}} * 100$$

Nanoparticle internalisation by macrophages

THP-1cells were loaded onto LAB- TEK™ chamber slide (Thermo Scientific Nunc™, UK) at $1X10^5$ cells/well in RPMI medium supplemented with 10% FCS and 100 nM PMA and incubated for 48 hours. After incubation, cells were infected with H37Ra for four hoursand then washed thoroughly with Phosphate buffered saline (PBS). The medium was then changed to pre-warmedat 37ºC, serum-free media supplemented with different formulations of coumarin-6 loaded nanoparticles;alginate entrapped-no calcium, alginate entrapped calcium, (10 µg/ml),or PBS solution (Control), and incubated for 48 hours at 37 C and 5% CO2. Immediately after incubation, the medium was changed and cells were washed using ice-cold PBS pH 7.4 (6 x 1min). Cells were fixed using 250 µl ice cold 4% PFA for 20 minutes at room temperature (in darkness). After washing with ice-cold PBS (6 x 1 min), thecells were stained using DAPI at a concentration of 0.5 µg/ml. Slides were then mounted in mounting medium and firmly fixed to glass slide using nail polish. The fluorescence intensity of confocal images was measured using ImageJ 1.46 software.

In vitroviability of M. tuberculosis H37Ra growth within the host

THP-1 cells were plated with 100 nM PMA at $1x10^5$ cells/well in a 96 well plate and incubated for 48 hrs at 37°C, 5% CO_2. The cells were then infected with log phase M. tuberculosis H37Ra at a multiplicity of infection (MOI) of 5. Cells were then incubated at 37°C and 5% CO_2 for 4 h. The plate was then washed with PBS three times to remove free M. tuberculosis cells. Fresh complete medium containing various volumes of drug loaded nanoparticles, free drugs (50 µl, 10 µl, or 1 µl), or PBS (control) was then added and incubated overnight at 37°C and 5 % CO_2. The free drug concentration for both drugs was designed based on the final drug entrapment of the nanoparticles seeded to the cells. After 48 h incubation, the cells were lysed and the viable bacterial number (colony forming units (CFU)/mL) was quantified by plating onto Middlebrook 7H11 agar plates supplemented with 10% (v/v) OADC, and 0.5% (v/v) glycerol[17].

Statistical analysis

Statistical analysis was performed using two-way analysis of variance (ANOVA) to compare the effect of dose and formulation/drug used (Graph-Pad Prism, San Diego, CA, USA). Particle size, Polydispersity, and entrapment efficiency, were compared using student t test for all pairs.Results were presented as the mean ± SD unless stated otherwise. Rejection of the null hypothesis was considered when $p<0.05$.

III. RESULTS AND DISCUSSION

Physical properties of the nanoparticles

Table 1 shows the average particle size, polydispersity index (PDI), drug loading and zeta potential of the alginate entrapped PLGA particles..alginate entrapped PLGA nanoparticles were developed on the basis of entrapping the drugs with alginate in the internal phase. Two formulation strategies were employed; calcium containing formulation, and calcium free formulation. In the calcium containing formulation, $CaCl_2$ was added to the external phase to complex with alginate/ PLGA matrix. In the second formulation, no calcium was added. Results showed comparable drug loading between the formulation; 18.7 ± 7.1 moxifloxacin µg/mg, and 15 ± 3 amikacin for the calcium containing formulation, and 10.3±1.2 µg/mg moxifloxacin and 17.4 ± 1.6 amikacin for the calcium free formulation. However;the particle size and the polydispersity was significantly larger for the calcium containing formulation versus the calcium free formulation.This can be ascribed to the complexation of calcium ions with alginate tend to result in large particle size, as shown in previous studies[18-21]. For that reason, only the calcium free formulation was studied for SEM imaging and the antimycobacterial efficacy.

The size of thecalcium-freeparticleswasvisually concurred with the SEM observation (~300 nm). The nanoparticles were also spherical (Figure 1), which is appropriate for internalisation into alveolar

macrophages.The drug loading of moxifloxacin and amikacin in the calcium free alginate trapped PLGA particles was 10.3µg/mg and 17.4µg/mg, respectively. These loading figures were found also for other hydrophilic drugs [22, 23]. On the basis of these results, the calcium-free alginate entrapped PLGA nanoparticle formulation was chosen for further evaluation.

Formulation	Average size (nm)	PDI	Moxifloxacin drug loading (µg drug per mg powder)	Amikacin drug loading (µg drug per mg powder)	Zeta potential (mV)
Alginate entrapped PLGA nanoparticles					
Alginate entrapped (Moxifloxacin and Amikacin) PLGA (with calcium)	420 ± 38 (*)	0.31 ± 0.04 (*)	18.7 ±7.1	15±3	+5.4±1.4
Alginate entrapped (Moxifloxacin and Amikacin) PLGA (without calcium)	325 ± 12	0.14 ± 0.06	10.3 ± 1.2	17.4 ± 1.6	-5.3 ± 0.9
Alginate entrapped (Moxifloxacin) PLGA (without calcium)	347 ± 21	0.16 ± 0.03	10.1±2.8	----------	-2.8 ± 0.4
Alginate entrapped (Amikacin) PLGA (without calcium)	312 ± 47	0.18 ± 0.05	----------	15.5 ± 3.3	-7.5 ± 1.3

Table 1: Physical Characterization of moxifloxacin loaded, amikacin loaded, and moxifloxacin/amikacin loaded alginate entrapped PLGA nanoparticles, n=3.

Nanoparticle internalisationby macrophages

To visualise the ability of different formulations of nanoparticles (the calcium-free alginate entrapped PLGA nanoparticles, calcium alginate entrapped PLGA nanoparticles to be internalised by the macrophages, the nanoparticles were loaded with coumarin-6, and nanoparticle suspensions (10 µg/ml) were seeded to the THP-1 derived alveolar macrophages and viewed under confocal microscopy. The resultsshowed that the coumarin-6, alginate entrapped non-calcium formulation showed the maximum internalisation. However, calcium alginate PLGA nanoparticles showed significantly less nanoparticles internalisation. In all formulation, nanoparticles weredispersedthroughout the cytosol ofthe macrophages, and more concentrated in the perinuclear region. The cytosol of the macrophages was observed to be predominately-fluorescing light corresponding to the coumarin-6 loaded nanoparticles while thered ofcounterstain DAPI was localised in the nucleus area. Furthermore,the fluorescence intensitywas significantly higher when the concentration of the nanoparticles wasincreased indicating a proportional relationship between nanoparticles concentration and internalisation (Figure 2I, II(A-B)). Thissuggestedan extensive distribution of the nanoparticles throughout the cytosolwith more particles concentrated in regions around nucleus. Comparing the three different nanoparticle formulations revealed the best internalisation for the non-calcium alginate entrapped nanoparticle formulation. This is because non-calcium formulation is negatively charged, and has lessparticle size and polydispersity compared to the calcium containing formulation. Also, the ability of calcium to complex into strong matrix renders the nanoparticles less likely to internalize into THP-1 cells.

In general, the phagocytosis of particles is influenced by the size, hydrophobicity, surface charge and morphology of the particle [24, 25]. The calcium free particles wereless than 1 µm, negatively charged, and spherical therefore efficiently internalised into macrophages. It has been shown that anionic nanoparticles have a higher cellular uptake into phagocytic cells compared to cationic nanoparticles,presumably due to the ingestion of negatively charged bacteria[24]. In one study, anionic polystyrene nanoparticles demonstrated preferential accumulation in the liver, where macrophages of the reticuloendothelial system reside [26]. In another study, 75% of immortal Primary human alveolar type 1(AT1) cells internalized negatively charged particles, while less than 55% of the cells internalized positively charged particles[27]. Moreover; positively charged nanoparticles have demonstrated higher cytotoxicity compared to negatively charged nanoparticles [24].Besides, the Tg of PLGA 50:50 is between 50 and 55°C, suggesting the particles will have an intact morphology at body temperature of 37°C which could further enhance the cellular uptake [28]. In a similar study, rifampicin, isoniazid, and streptomycin encapsulated in poly-n-butylcyanoacrylate and polyisobutylcyanoacrylate particles showed enhanced accumulation of all the three encapsulated drugs in vitro in human monocytes

[29]. This property of the nanocarriers is promising for targeting treatment of latent TB where the M. tuberculosis dormant inside the macrophages [30].

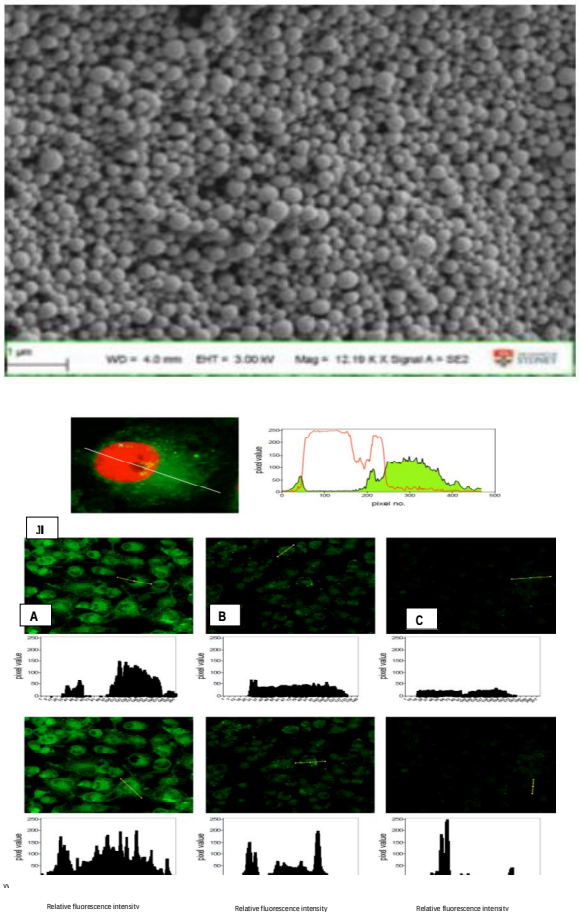


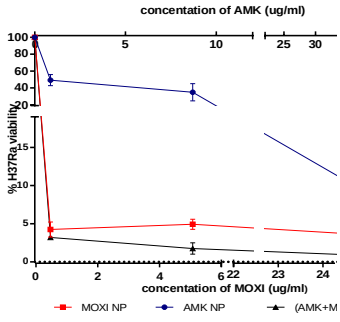
Figure 2(I). The cytoplasmic distribution of nanoparticle of the alginate entrapped nanoparticles into THP-1 cells.(II). Confocal images and ImageJ curves (number pixels vs fluorescence intensity) of coumarin-6 loaded PLGA nanoparticles by THP-1 derived macrophages

The cells were treated for 60 minutes with acridine orange loaded nanoparticles at two different concentrations of: (A) alginate entrapped (noncalcium formulation) loaded with coumarin-6 and (B) alginate entrapped nanoparticles (calcium formulation) loaded with coumarin-6, and The green colour corresponds to the distribution of nanoparticles in the cytosol of THP-1 cells. The red colour corresponds to the DAPI stained nucleus. The ImageJ curves shows that the frequency of pixels with high fluorescence intensity is the maximum in the alginate entrapped calcium free formulation.

In vitro viability of M. tuberculosis H37Ra growth within the host

The efficacy of the drug-loaded nanoparticles was tested against M. tuberculosisH37Ra infected macrophages. Figure 3 shows the viable M. tuberculosis(H37Ra) count after treatment with nanoparticles at various concentrations. Thedual entrapment of amikacin and moxifloxacin into alginate entrapped nanoparticles showed enhanced inhibition of bacterial growth compared to amikacin nanoparticles or moxifloxacin nanoparticles.These phenomena of the drug loaded nanoparticles being less efficacious than the free drug were also reported in the previous study where gentamicin loaded PLGA nanoparticles showed less antimicrobial activity compared to the free gentamicin against Pseudomonas aeroginosa in vitro planktonic and biofilm susceptibility assays. However, when the particles were administered intraperitoneally to the mice that infected with same P. aeruginosa strain, a significant reduction of bacterial count was observed in the blood and bronchoalveolar lavage fluid after 96 hr post-treatment compared to the free drug treatment[9]. Similarly, the nalmefene PLGA microspheres demonstrated a controlled release profile over 4 weeks in PBS (pH 7.4) at 37°C, while the mean residence time was about 16 days when delivered intravenously to mice [31]. Therefore, the alginate PLGA particles are expected to exhibit a faster degradation and improved anti-TB activity in vivo. Nanoparticles are expected to possess better pharmacokinetics in vivo since both amikacin and moxifloxacin have relatively short half-lives [45, 46]. Therefore, the nanoparticles are expected to have higher mean residence time compared to free drugs in vivo. The enhanced activity of the (amikacin + moxifloxacin) loaded nanoparticles was demonstrated by comparing the colony forming units (cfu) and hence the viability of the H37Ra treated with (amikacin + moxifloxacin) loaded nanoparticles with the single drug loaded nanoparticle formulations (amikacin or moxifloxacin). For example, at a concentration of 5.2 µg/ml moxifloxacin and 8.7 µg/ml amikacin, amikacin loaded nanoparticles showed mycobacterial growth of 34.9% and moxifloxacin loaded nanoparticles showed a growth of 3.27% . However; (amikacin or moxifloxacin) loaded nanoparticles showed a growth of 1.75 %(Figure 3A). The free drug treated groups showed a significantly more inhibition effect. For example, a concentration of 6.25 µg/ml of freeamikacin showed 13% H37Ra viability, 6.25 µg/ml of free moxifloxacin showed 0.5% H37Ra viability, and 6.25µg/ml of both amikacin and moxifloxacin showed 0.3% viability (Figure 3B).

A



B

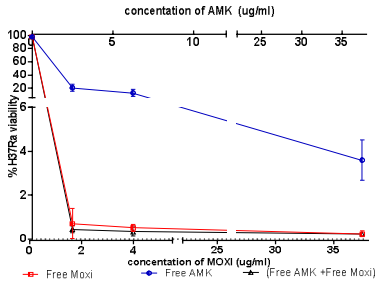


Figure 3: H37Ra viability of A: amikacin and/or moxifloxacin nanoparticles treated, and B: free amikacin and moxifloxacin treated

THP-1 cells were plated with 100 nM PMA at $1x10^5$ cells/well in a 96 well plate and incubated for 48 hrs at 37°C, 5% CO_2. The cells were then infected with log phase M. tuberculosis H37Ra at a multiplicity of infection (MOI) of 5. Cells were then incubated at 37°C and 5% CO_2 for 4 h. The plate was then washed with PBS three times to remove uninfected M. tuberculosis cells. Fresh complete medium containing various concentrations of drug loaded nanoparticles; Amikacin nanoparticles (AMK NP), Moxifloxacin nanoparticles (MOXI NP), (Amikacin and Moxifloxacin) loaded nanoparticles (AMK+MOXI) NP (50 µl, 10 µl, or 1 µl), free drugs (15 µl, 2.5 µl, or 1 µl), or PBS (control) was then added and incubated overnight at 37°C and 5 % CO_2. The free drug concentration for both drugs was based on the final drug entrapment of the nanoparticles seeded to the cells. After 48 h incubation, the cells were lysed and the viable bacterial number (colony forming units (CFU)/mL) was quantified by plating onto Middlebrook 7H11 agar plates supplemented with 10% (v/v) OADC, and 0.5% (v/v) glycerol.

Nanoparticles have been widely investigated for their potential to reduce the duration of anti-TB chemotherapy. Moxifloxacin and amikacin are second-line drugs that have shown synergistically inhibit MDR-TB[6]. Moxifloxacin acts on bacteria by inhibiting the bacterial DNA through binding to DNA gyrase enzyme, while amikacin binds to bacterial 30s ribosomal subunits resulting in misreading of t-RNA[31, 32].As a result,co-administration of these two drugs with different mechanism of action coupled witha polymeric nanoparticulate matrix could enhance clinical outcome. Furthermore, coupling polymeric nanoparticles with inhalation delivery has gained significant interest for treatment of TB [33-35]. In our previous studies, we have successfully formulated anti-TB pure drugs including rifapentine, moxifloxacin and pyrazinamide into inhalable dry powder [36, 37]. Further studies are ongoing to formulate the free alginate entrapped PLGA nanoparticles into inhalable form and testing its efficacy in TB in vivo models.

In conclusion,the ability of the non-calcium alginate entrapped nanoparticles to internalise to a mammalian cells to a higher degree compared to the other nanoparticle formulations resulted in the enhanced activity that was observed on counting agar plates. Based on the above results, it can be inferred that the nanoparticles have a preferential effect compared to the free nanoparticle formulation. Moreover; dually entrapped nanoparticle formulations showed enhanced effect compared to the single entrapped nanoparticle formulations.

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