3 The Influence of Polymers on Alveolar Macrophage Uptake

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3.1 Introduction

It is widely acknowledged that particles having a mass median aerodynamic diameter (MMAD) of <1 μm are predominantly exhaled by an individual exhibiting at normal breathing patterns, and aerosol particles with MMAD values between 5 and 10 μm should be deposited overwhelmingly in the upper airways (or oropharyngeal region), and removed by mucociliary clearance prior to being swallowed [1–3]. However, particles with an MMAD of 1–5 μm should be able to traverse the branching airways of the lung and be deposited in the deep lung following an inhalation manoeuvre. For drugs within this MMAD range, particle dissolution (followed by diffusion through the lung tissue, metabolism) and/or phagocytosis by alveolar macrophages are the mechanisms of clearance in the alveoli that play an important role [1, 2, 4].

Primary particles that range in size from 200–500 nm have a lower probability of being phagocytised by alveolar macrophages compared to particles in the range of 0.5–1 μm [5–7], although it has previously been reported that an adequate amount of phagocytosis may occur with particles in the 100 nm range for imaging and/or diagnostic purposes [8]. Selvam and co-workers have suggested that the use of a hydrophilic swelling polymer within an inhaled formulation might serve to elude macrophage uptake due to an increase in particle diameter that could be well in excess of 1 μm, potentially avoiding uptake and clearance for several days [9].

Meenach and co-workers report that porous microparticles prepared using a biopolymer of acetylated dextran can be loaded with the drug camptothecin, and manufactured to particle sizes between 1 and 4 µm. The authors point out that alveolar macrophage uptake would likely be avoided due to the larger particle size associated with the manufacture of these large porous particles for inhalation [10]. Furthermore, the development of this biopolymer matrix for the manufacture of large porous particles was designed to act as a platform delivery technology, allowing for a variety of pharmaceutically active ingredients that require a sustained residence time in the alveolar space to be administered.

Interestingly, a group has recently tackled the issue of quantification of cellular interactions with particulate drug-delivery systems. Lawlor and co-workers pointed out that most particle–cell interaction studies are conducted using spectrofluorimetry, flow cytometry and fluorescence/confocal microscopy and that these methods are not rapid enough to be considered for high-throughput screening purposes. The researchers presented a new automated high-throughput method to analyse microparticulate drug-delivery systems and their alveolar macrophage uptake [11]. In validation studies related to this new high-throughput analysis, the group manufactured poly(lactic-*co*-glycolic acid) (PLGA) containing particles ranging from 0.8–2.1 µm in size (they indicated that this would be in the optimal range for macrophage uptake), as well as investigating the effect of different adjuvant ingredients coated onto the PLGA microparticles. The results presented demonstrated both the suitability of the method for quantitative evaluation related to alveolar macrophage uptake, and the increased cellular uptake with both gelatin and ovalbumin coatings applied to the PLGA microparticles. Furthermore, they report that gelatin-coated PLGA microparticles were found within human macrophage cells to the same extent as pure gelatin microparticles [11], suggesting material-dependent phagocytosis played a part. In an alternative study conducted by Ruge and co-workers, the advantage of using surfactant protein A to increase alveolar macrophage uptake was described. The authors show that an increase in macrophage uptake of nanoparticles (110–180 nm)

coated with chitosan, poly-maleic-oleic acid or phosphatidylcholine was enhanced in the presence of surfactant protein A and decreased in the presence of serum albumin [12].

3.2 Poly(lactic-*co*-glycolic acid)

The co-polymer PLGA is generally considered to be minimally toxic due to its highly biocompatible biodegradability in the body. During biodegradation physiologically occurring hydrolysis results in the formation of lactic acid and glycolic acid. The monomers are themselves normally present in the body as a result of a variety of metabolic processes. One widely investigated use for PLGA in inhalation is focused on tuberculosis (TB) therapy. *Mycobacterium tuberculosis* (MTB) has been shown to be present disproportionately in alveolar macrophage cells [13]. MTB is a devastating infection of the respiratory tract [14]. The parasitic bacterium is known to infect and display latency in mononuclear phagocytes such as alveolar macrophages [15]. Remarkably, the intracellular pathogen is able to survive by using a number of strategies such as pH neutralisation of the phagosome and interference with cellular autophagy [16]. Following endocytosis of the MTB from the extracellular region into an alveolar macrophage, these evasion strategies result in the development of a latent infection [17]. Due to the intracellular location of MTB at this latent stage of infection, research has been directed toward the delivery of antituberculosis agents directly to alveolar macrophage cells by inhalation, with the goal of direct targeting and a potential reduction of systemic side effects [18]. Lawlor and co-workers report that the use of inhalable microparticles to target MTB could offer a unique opportunity for drug targeting directly to the alveolar macrophages, increasing local drug concentration whilst simultaneously reducing the potential for toxic systemic exposure [11].

Alveolar macrophages were studied in terms of optimal conditions for PLGA microsphere uptake by Hirota and co-workers. Particle size, effect of drug and cellular viability were criteria for investigation [19]. The team was able to demonstrate that particle size indeed

played a critical role in macrophage uptake, as there was a larger extent of proportional uptake of 1 µm or 3 µm particles than 6 or 10 µm microspheres. Additionally, the inclusion of rifampicin into the microspheres at 1–6 µm was shown to have a positive effect on overall uptake. The use of the PLGA with the drug was not observed to affect the rat macrophage viability within the 4 h time frame of exposure, after the commencement of phagocytosis.

Hirota and co-workers studied the extent of alveolar macrophage uptake of microspheres loaded with rifampicin and PLGA with a monomer composition of lactide:glycolide at 75:25 [18]. In a spray-drying process, which combined the rifampicin and PLGA with a molecular weight of 10,000 Da, the researchers were able to manufacture microspheres with a mean diameter of 2 µm that had a drug loading of approximately 5×10^{-7} µg per microsphere [20]. Following the manufacturing process the researchers went on to show that the uptake of rifampicin from a 2.50 µg/ml suspension of the microspheres into cultured alveolar macrophages (rat: NR8383 cell line) was ten times greater than that of a standard solution containing twice the amount of drug (5 µg/ml). Moreover, they were able to demonstrate that the PLGA-based microspheres were able to provide a more potent antibacterial effect on the Bacillus Calmette–Guerin within the macrophage cells than the drug solution following a 7 day incubation period. Furthermore, by comparing the effect of polystyrene latex microspheres of the same diameter on the viability of the macrophage cells, the authors note that the PLGA-containing microspheres had little effect when compared to the polystyrene [18]. In previous reports this group had concluded that phagocytosis of rifampicin-PLGA microspheres was likely to enhance the phagocytic activity of macrophage cells, compared to the relative degree of phagocytosis, which was not significant for control particles of polystyrene latex microspheres [21, 22]. The same group determined that incorporation of a surfactant into the PLGA-loaded rifampicin microspheres had demonstrated a pH-dependent release. Additionally, electrophoretic mobility measurements of PLGA microspheres indicated the surface charge density of PLGA microspheres was changed by surfactant absorption on the surface,

allowing a 'soft surface' to form, ultimately promoting alveolar phagocytosis. The authors indicated that this combination of effects would be ideal to deliver and release the drug directly into the alveolar macrophages where *mycobacterium tuberculosis* bacilli reside, thereby optimising the antibacterial effect through targeted delivery [20]. An additional study by this group has suggested that the rifampicin PLGA microspheres are degraded in the phago-lysosomes allowing the drug to be released into the cytosol whilst maintaining a high potency [23].

Another study had investigated the effects of molecular weight and composition of PLGA on release of rifampicin, in tandem with macrophage uptake [24]. Makino and co-workers manufactured 2 μm diameter rifampicin containing PLGA microspheres using a solvent evaporation method. The researchers found that a low molecular weight of PLGA of 5,000 Da was more efficient at drug loading than a 20,000 Da grade, approximately 91% and 58% respectively. They surmised that this discrepancy in loading efficiency was due to a potential interaction of the rifampicin amino groups with the terminal carboxyl groups of the PLGA back bone. Drug loading in the monomer composition of lactide:glycolide of 75:25 was found to be more efficient than that of a ratio of 50:50 in this study. And whilst these studies demonstrated that drug release was controlled using different molecular weight compositions of PLGA, the authors noted that a highly effective delivery of rifampicin to cultured alveolar macrophages was observed by the use of rifampicin-loaded PLGA microspheres, at apporximately19 times higher than that of a reference solution with the same concentration of drug [24].

Work performed by Diab and co-workers demonstrated internalisation of rifampicin containing microspheres into *ex vivo* rat alveolar macrophages [25]. Previously the same group had reported a solvent evaporation method to produce the microspheres in the size range of 2–8 μm [26]. The solvent evaporation method that was developed used poly(vinyl alcohol) (PVA) in order to stabilise the emulsion-based production process. These PVA-stabilised microspheres were reported to have a drug loading capacity of 4.9–16.5% and a release

rate of 80% from 12 h to 4 days. In their follow-up study, the group replaced the nonbiodegradable PVA with sucrose palmitate to produce the microspheres within the repairable range, which the authors indicated was a biodegradable and biocompatible surfactant [25]. Along with this formulation improvement it was shown that a higher drug loading was possible at approximately 34% w/w. This formulation type was shown to demonstrate seven times greater alveolar macrophage intracellular levels when incubated for 4 h compared with an equivalent amount of free rifampicin solution.

Mannitol combined PLGA-rifampicin nanoparticles, in a single-step spray-drying process, were earlier investigated by Ohashi and co-workers The research team were able to show that rifampicin-PLGA particles of 213 nm could be encapsulated within mannitol, to form a 2–3 μm microsphere, suitable for lung inhalation studies [27]. The researchers were effectively able to demonstrate that the uptake of rifampicin from the mannitol containing microspheres was >9% at 4 h flowing administration to Sprague Dawley rats, and macrophage harvesting in bronco-alveolar lavage (BAL) fluid. The macrophage uptake was shown to be much greater than the uptake exhibited by standard PLGA rifampicin microspheres that did not consist of encapsulated nanoparticles within mannitol. Additionally, a fluorescence study was able to show that the micron-sized PLGA particles were more rapidly removed from the lung than the nanoparticles of the same composition; this was attributed to an effect of mucociliary clearance due to localisation in the more central airways by the larger particles. With the more peripheral localisation, and more overall widespread distribution of the PLGA-rifampicin nanoparticles in the lung, it was hypothesised by the authors that this led to widespread macrophage uptake (as evidenced by the BAL) and no apparent mucociliary clearance. With the lack of mucociliary clearance this would ultimately result in a longer pulmonary retention time. Overall the study demonstrated that particular processing parameters and careful selection of excipients can lead to a complex type of formulation with very desirable macrophage uptake properties and useful therapeutic potential.

With a different use of PLGA researchers in Texas indicated that particle size had an important role in the immune response generated by a hepatitis B vaccine that was for inhalation [28]. In their study Thomas and co-workers prepared hepatitis B surface antigen (HBsAg) containing PLGA microspheres by a double emulsion-solvent-evaporation method. Characterisation of the particles indicated that their mass median aerodynamic diameter ranged from 2–12 µm [28]. For this study rat alveolar macrophages were obtained from male Sprague Dawley rats using a BAL procedure that the authors had previously published [29]. Following the method described in the previous article, the suitably isolated macrophage cells were then exposed to an aliquot of fluorescein isothiocyanate-conjugated bovine serum albumin PLGA microspheres at a concentration of 1 mg/ml. Uptake of the fluorescent microspheres by the alveolar macrophages was viewed by confocal microscopy. The group was able to show that the fluorescent microspheres 4–5 µm in size were preferentially taken up by the macrophages compared to particles that were >10 µm. The macrophage uptake data were in direct agreement with the immunogenicity data presented in this paper, which showed an enhanced immune response with smaller HBsAg-PLGA loaded microspheres of approximately 5 µm, when compared to microspheres that were approximately 12 µm in size.

Phagocytic uptake by alveolar macrophages was also shown to be increased with specific surface modification. Brandhonneur and co-workers showed that by grafting cell-specific ligands on PLGA microparticles, their uptake by a simultaneous occurrence of a linear nonspecific process and a nonlinear specific and saturable process could be modulated [30]. The researchers were able to demonstrate that the highest macrophage uptake was found with mannose and wheat germ agglutinin substituted grafted ligands.

A summary of current research that has utilised PLGA as part of a strategy to target alveolar macrophage uptake is shown in **Table 3.1**.

Table 3.1 Current research incorporating the use of PLGA				
Title	**Authors**	**Journal**	**Notes**	**Reference**
Response of cultured macrophages to *mycobacterium-tuberculosis*, with observations on fusion of lysosomes with phagosomes	J.A. Armstrong and P. Hart	Journal of Experimental Medicine	MTB infection pathology	[13]
The temporal expression profile of *Mycobacterium tuberculosis* infection in mice	A. Talaat, R. Lyons, S. Howard and S. Johnston	Proceedings of the National Academy of Sciences, USA	MTB infection pathology	[14]
Who puts the tubercle in tuberculosis?	D.G. Russell	Nature Reviews Microbiology	Phagocytosis of MTB infection	[15]
Future vaccination strategies against TB: thinking outside the box	S.H.E. Kaufmann	Immunity	Survival strategies of MTB	[16]
Isolation of Mycobacterium TB mutants defective in the arrest of phagosome maturation	K. Pethe, D. Swenson, S. Alonso, J. Anderson, C. Wang and D. Russell	Proceedings of the National Academy of Sciences, USA	MTB latency	[17]
Delivery of rifampicin-PLGA microspheres into alveolar macrophages is promising for treatment of TB	K. Hirota, T. Hasegawa, T. Nakajima, H. Inagawa, C. Kohchi, G-I. Soma, K. Makino and H. Terada	Journal of Controlled Release	Direct targeting of rifampicin to alveolar macrophages	[18]

Optimum conditions for efficient phagocytosis of rifampicin-loaded PLGA microspheres by alveolar macrophages	K. Hirota, T. Hinata, F. Ito, H. Inagawa, C. Kohchi, G-I. Soma, K. Makino and H. Terada	Journal of Controlled Release	Particle size, effect of drug and cellular viability on alveolar macrophage uptake	[19]
Effects of pulmonary surfactant system on rifampicin release from rifampicin-loaded PLGA microspheres	K. Tomoda, S. Kojima, M. Kajimoto, D. Watanabe, T. Nakajima and K. Makino	Colloids and Surfaces B: Biointerfaces	PLGA-rifampicin microsphere manufacture	[20]
Phagocytic activity of alveolar macrophages toward polystyrene latex microspheres and PLGA microspheres loaded with anti-TB agent	T. Hasegawa, K. Hirota, K. Tomoda, F. Ito, H. Inagawa, C. Kohchi, G-I. Soma, K. Makino and H. Terada	Colloids and Surfaces B: Biointerfaces	Phagocytosis of rifampicin-PLGA microspheres. Enhancement of phagocytotic activity	[21, 22]
The behaviour of PLGA microspheres containing rifampicin in alveolar macrophages	T. Onoshita, Y. Shimizu, N. Yamaya, M. Miyazaki, M. Yokoyama, N. Fujiwara, T. Nakajima, K. Makino, H. Terada and M. Haga	Colloids and Surfaces B: Biointerfaces	Degradation of rifampicin-PLGA microspheres in the phago-lysosomes	[23]

Efficient intracellular delivery of rifampicin to alveolar macrophages using rifampicin-loaded PLGA microspheres: effects of molecular weight and composition of PLGA on release of rifampicin	K. Makino, T. Nakajima, M. Shikamura, F. Ito, S. Ando, C. Kochi, H. Inagawa, G. Soma and H. Terada	Colloids and Surfaces B: Biointerfaces	Effects of MW and composition of PLGA on rifampicin release and macrophage uptake	[24]
Formulation and *in vitro* characterisation of inhalable polyvinyl alcohol-free rifampicin-loaded PLGA microspheres prepared with sucrose palmitate as stabiliser: Efficiency for *ex vivo* alveolar macrophage targeting	R. Diab, J. Brillault, A. Bardy, A.V.L. Gontijo and J.C. Olivier	International Journal of Pharmaceutics	Alveolar macrophage uptake of rifampicin containing microspheres	[25]
Formulation *and in vitro* characterisation of inhalable rifampicin-loaded PLGA microspheres for sustained lung delivery	T.V.P. Doan, W. Couet and J.C. Olivier	International Journal of Pharmaceutics	Manufacture of rifampicin containing microspheres	[26]

One-step preparation of rifampicin/PEG nanoparticle-containing mannitol microspheres using a four-fluid nozzle spray drier for inhalation therapy of TB	K. Ohashi, T. Kabasawa, T. Ozeki and H. Okada	Journal of Controlled Release	Manufacture of mannitol combined PLGA-rifampicin nanoparticles by spray drying	[27]
Particle size influences the immune response produced by hepatitis B vaccine formulated in inhalable particles	C. Thomas, V. Gupta and F. Ahsan	Pharmaceutical Research	Size discrimination of fluorescently labelled microspheres for alveolar macrophage uptake	[28]
Specific and non-specific phagocytosis of ligand-grafted PLGA microspheres by macrophages	N. Brandhonneur, F. Chevanne, V. Vie, B. Frisch, R. Primault, M-F. Le Potier and P. Le Corre	European Journal of Pharmaceutical Sciences	Alveolar macrophage. Uptake of PLGA microparticles grafted with cell-specific ligands by alveolar macrophages	[30]

3.3 Poly(ethylene glycol)

In a study conducted by Gursahani and co-workers the extent of uptake of fluorescently labelled poly(ethylene glycol) (PEG) conjugated polymers was described. In this extensive study, which characterised absorption and distribution across the pulmonary epithelia of PEG polymers, the authors found that smaller molecular weight PEG (<2 kDa) may be absorbed across the epithelia. Conversely, larger molecular weight PEG (>5 kDa) were characterised as being responsible for a slow epithelial absorption, followed by an increased macrophage uptake response (due to the extended residence time in the lung) [31]; consequently a biphasic clearance mechanism was proposed following the onset of this increased macrophage activity.

By investigating the PEGylation density on PEGylated nanoparticles prepared using particle replication in nonwetting templates (PRINT) technology Perry and co-workers described its effect on alveolar macrophage uptake [32]. The authors noted that PEG surface coverage increases both protein adsorption and subsequently decreases macrophage association and uptake.

3.4 Mannosylated-gelatin

Tiwari and co-workers investigated the attachment of mannose on the surface of gelatin to promote the targeting of isoniazid (a second-line therapy for TB) to the alveolar macrophages [33]. In a process that utilised an emulsification solvent extraction technique, the researchers reported that an average particle size of approximately 4 µm was achieved, and that the entrapment efficiency of the isoniazid was 56%. An *ex vivo* study, in which alveolar macrophages were obtained by BAL of male albino rats, demonstrated that the mannosylated particles were selectively taken up, and were found to be associated with phago-lysosomal vesicles of the alveolar macrophages. The authors went on to explain that the microspheres were stable in the BAL fluid, and that a therapeutic level of the isoniazid could be maintained for an extended period of time. The maintenance of the therapeutic level was evidenced by a plasma pharmacokinetics,

indicating that less isoniazid was present in the plasma throughout the study (thus more being present in the lung) from the mannosylated gelatin microspheres than for just the standard nonmannosylated gelatin microspheres. In particular the plasma levels during the first 24 h of the study evidenced the differences in systemic absorption.

The phagocytic uptake of rifampicin-loaded mannosylated dendrimers by alveolar macrophages was studied by Kumar and co-workers; they report a clear relationship between drug loading and drug incorporated into alveolar macrophages following incubation with drug loaded, mannosylated dendrimers. When compared to the control of free rifampicin solution, the authors show a vast improvement in drug uptake with the mannosylated dendrimers [34].

3.5 Mannan-Poly(ethylene glycol)-Poly(ethylene) Bioadhesive Poly(lactide-*co*-glycol acid)

A novel synthetic mannan (MN)-PEG-poly(ethylene) (PE) was developed as a surface modification for PLGA, to obtain biodegradable bioadhesive polymeric nanoparticles for use in gene therapy [35]. Liver macrophages (Kupffer cells) were used in order to evaluate gene transfection *in vitro*; the cells were isolated from male Sprague Dawley rats and incubated with the surface modified nanoparticles. Following incubation it was shown that the surface-modified nanoparticles had considerably higher transfection efficiency than naked deoxynucleic acid (DNA), which was used as a negative control. Furthermore the authors were able to demonstrate higher *in vivo* transfection than either naked DNA or PLGA nanoparticles with no surface modification. The authors were able to conclude that mannan containing targeting ligands could significantly improve the transfection efficiency of the biodegradable carriers [35]. These modified vectors could be very useful in targeted gene delivery for a variety of delivery routes including inhalation, especially as PLGA use and modification has been widely investigated.

A summary of current research that has utilised other polymer systems as part of a strategy to target alveolar macrophage uptake is shown in **Table 3.2.**

Table 3.2 Current research incorporating the use of other polymers for alveolar macrophage uptake				
Title	**Authors**	**Journal**	**Notes**	**Reference**
Absorption of PEG polymers: The effect of PEG size on permeability	H. Gursahani, J. Riggs-Sauthier, J. Pfeiffer, D. Lechuga-Ballesteros and C.S. Fishburn	Journal of Pharmaceutical Sciences	The effect of PEG molecular weight on alveolar epithelial and macrophage uptake	[31]
PEGylated print nanoparticles: The impact of PEG density on protein binding, macrophage association, biodistribution, and pharmacokinetics	J.L. Perry, K.G. Reuter, M.P. Kai, K.P. Herlihy, S.W. Jones, J.C. Luft, M. Napier, J.E. Bear and J.M. DeSimone	Nano Letters	PEG surface coating and alveolar macrophage uptake	[32]
Macrophage-specific targeting of isoniazid through mannosylated gelatin microspheres	S. Tiwari, A.P. Chaturvedi, Y.B. Tripathi and B. Mishra	AAPS PharmSci Tech	Mannosylation and its effect on alveolar macrophage uptake	[33]
Intracellular macrophage uptake of rifampicin loaded mannosylated dendrimers	P.V. Kumar, A. Asthana and T. Dutta	Journal of Drug Targeting	Mannosylated dendrimers and their alveolar macrophage uptake	[34]
Novel MN-PEG-PE modified bioadhesive PLGA nanoparticles for targeted gene delivery	G. Wu, F. Zhou, L. Ge, X. Liu and F. Kong	Journal of Nanomaterials	DNA transfection with MN-PEG-PE nanoparticles to Kupffer cells	[35]

3.6 Summary

The strategies for improving cellular uptake into alveolar macrophages may be driven by the desire to target directly the macrophages for an improved therapeutic response, as described in many of the examples in this chapter that focus on TB therapy. Many of the most effective strategies for delivery of the anti-TB agents are modulated by the inclusion of an adjuvant excipient, such as mannitol with a PLGA microsphere. Another strategy is the surface modification of microspheres following their manufacture, with ligands such as mannose. This is an emerging field and the progress is at its early stages of development. However, targeting of macrophages for an improved therapeutic response has been shown to be an exciting prospect for future pulmonary drug-delivery systems.

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