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Research Article

Potential Active Targeting of Gatifloxacin to Macrophages by Means of Surface-Modified PLGA Microparticles Destined to Treat Tuberculosis

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Abstract. Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis* and represents one of the leading causes of mortality worldwide due to multidrug-resistant TB (MDR-TB). In our work, a new formulation of biodegradable PLGA microparticles was developed for pulmonary administration of gatifloxacin, using a surface modifier agent to actively target alveolar macrophages thereby allowing to gain access of the drug to *Mycobacterium tuberculosis*. For this, rapid uptake of the particles by macrophages is beneficial. This process was evaluated with fluorescein-loaded microparticles using PLGA 502 or PLGA 502H as polymers and labrafil as surface modifier. Cell phagocytosis was studied in raw 264.7 mouse macrophage cell line after 3, 5, 24, and 48 h incubation with the microparticles. Labrafil enhanced the uptake rate of PLGA 502H microparticles by macrophages which was directly related to the modification of the polymer matrix. Gatifloxacin-loaded PLGA microparticles using PLGA 502 or PLGA 502H and labrafil were prepared. From our results, only microparticles prepared with PLGA 502H and labrafil exhibited high encapsulation efficiency (89.6 ± 0.2%), rapid phagocytosis by macrophages (3 h), and remained inside the cells for at least 48 h, thereby resulting in a suitable carrier to potentially treat MDR-TB.

KEY WORDS: gatifloxacin; PLGA microparticles; labrafil; tuberculosis; macrophage phagocytosis.

INTRODUCTION

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis* (MTB), and is one of the leading causes of mortality worldwide. In 2017, around 10 million people became ill with TB, and 1.3 million died from the disease (1). First-line antituberculosis drugs exhibit high percentages of adverse effects such as ototoxicity and nephrotoxicity, and the long duration of TB treatments results in low patients' adherence and the development of resistances leading to therapeutic failure (2). Multidrug-resistant TB (MDR-TB) is defined as TB that does not respond to at least isoniazid and rifampicin and represents a major obstacle for effective TB therapy. One of the main reasons for the emergence of TB-resistant strains is the exposure of mycobacteria to sub-therapeutic levels of one or more antibiotics, since current treatments are not able to reach the site of action due to low vascularization of the pulmonary lesions (3). Moreover, bacteria are able to develop "silent" infections inside the cells and to escape from their bactericidal mechanisms due to the fact that these cells are not digested by macrophages. In this case, the digestion of bacteria inside phagosomes is inhibited which requires the fusion of the MTB-containing phagosomes with lysosomes. As a consequence, MTB cells are able to proliferate and accumulate within the macrophages being therefore unable to eradicate the intracellular bacteria. Under these conditions, they may act as reservoirs facilitating the propagation of the infection to other cells and organs. In addition, intracellular location of bacteria protects them from the attack of the immune system and from the effects of antibacterial drugs present in the extracellular fluids (4).

The mononuclear phagocytic system (MPS), especially alveolar macrophages, plays a critical role in this pathology. MTB has the ability to survive and replicate in alveolar macrophages evading host defense mechanisms thereby developing a latent disease. Taking into consideration that phagocytic cells are able to localize and internalize foreign materials, such as for instance polymeric particles, this fact can be used to direct the particles to the interior of macrophages thereby resulting in an interesting approach to

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treat intracellular infections affecting the MPS. Strategies for targeting anti-tuberculosis molecules to macrophages can be divided into two categories: passive targeting and active targeting. Passive targeting involves the optimization of the physicochemical characteristics of microparticles (composition, size, shape, and rigidity) to gain increased uptake by macrophages (5,6). For active targeting, surface modification of the particles with specific molecules is needed to adequately interact with the cell membranes. Several studies have focused on the preparation of biodegradable PLA (polylactic acid) or PLGA (polylactic-co glycolic acid) microparticles for targeting conventional antituberculosis drugs (rifampicin or isoniazid) to alveolar macrophages (7,8). Microparticles prepared with hydrophobic PLGA copolymers and adequate particle sizes are easily captured by macrophages. However, when trying to encapsulate hydrophilic drugs, the use of more hydrophilic PLGA is needed but although it hinders their uptake by the immune cells. To overcome this problem, modification of the superficial characteristics of the microparticles by the incorporation of surface modifying agents is a good strategy.

The use of fluoroquinolones is recommended for MDR-TB treatments. For this, gatifloxacin is an interesting agent which is currently undergoing several phase II and III clinical trials due to its short-term treatment regimens (less than the standard 6-month protocol) (9–12). The clinical results obtained with gatifloxacin suggest that this drug could minimize drug resistance by reducing the duration of TB treatments. To this date, formulations have not been developed for vectorization of gatifloxacin to macrophages. For this, in our work, we have developed the first inhaled gatifloxacin formulation able to access the lung alveolar macrophages achieving therapeutic levels to efficiently reduce MDR-TB. This formulation may be a promising alternative to efficiently treat TB and to reduce systemic toxicity.

The new formulation consists of biodegradable PLGA microparticles for pulmonary administration of gatifloxacin using PLGA 502 and PLGA 502H as polymers. Labrafil, as a surface modifying agent, was used to increase the uptake of microparticles by macrophages thereby gaining better access of gatifloxacin to MTB. The uptake of the new biodegradable gatifloxacin formulation by macrophages as well as the rate of phagocytosis was also studied.

MATERIALS AND METHODS

Materials

Gatifloxacin sesquihydrate (GAT) (1-cyclopropyl-6-fluoro-8-methoxy-7-(3-methylpiperazin-1-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid sesquihydrate) was supplied by Santa Cruz Biotechnology Inc. (Santa Cruz Biotechnology Inc., Heidelberg, Germany); poly (D, L-lactide-co-glycolide) acid (PLGA) with a 50:50 ratio (RG® 502, Mw = 12 kDa and RG® 502H, Mw = 12 kDa) was purchased from Evonik (Evonik, Darmstadt, Germany). Polyvinyl alcohol (PVA) (Mw = 49,000 Da) was obtained from Thermo Fisher Scientific (Thermo Fisher Scientific, Geel, Belgium). Fluorescein sodium salt was supplied by Sigma-Aldrich Química S.A. (Sigma-Aldrich Química, S.A., Madrid, Spain), and Labrafil® M 1944 CS (PEG-5 oleate) was supplied by Gattefossé (Gattefossé, Nanterre, France). Other reagents and solvents used were of analytical grade and provided by Merck KGaA (Merck, Darmstadt, Germany). Distilled and deionized water Milli-Q (Millipore Corporation, Billerica, MA, USA) was used in the preparation of all buffers and solutions.

Preliminary Phagocytosis Study in Primary Cell Cultures

Microparticle Preparation

To carry out preliminary phagocytosis studies, microparticles (MPs) of two different particle sizes (formulations F1 and F2) were prepared using the solvent evaporation-extraction method from an organic/water emulsion (O/W emulsion). For this, 200 mg of PLGA 502 were dissolved in CH_2Cl_2 (1 mL). The aqueous phase of the emulsion was formed by using a solution of 1% PVA (5 mL). The emulsion was obtained using a Polytron homogenizer (PT 10-30, Kinematic, Lucerne, Switzerland) for 2 min at either 2000 rpm or 20,000 rpm when preparing formulations F1 and F2, respectively. Then water (6 mL) was added and the mixture stirred for 2 min at the same rpm previously used. Finally, the immature microspheres were suspended in water (250 mL) and stirred for 3 h (magnetic multi-stirrer AMI4, VELP Scientific, A-Zlab, S.L., Barcelona, Spain) to remove the organic solvent. The high polydispersity obtained in formulation F2 made it necessary to remove larger particles by filtering the suspension of MPs through 5 μm and 0.45 μm filters with this fraction being collected. Finally, the MPs were vacuum-filtered (KNF vacuum pump Neuberger, Inc., Trenton, NY, USA), freeze-dried (Flexi-Dry MP ™, FTS Systems Inc., Stone Ridge, NY, USA) for 24 h, and frozen at −50°C. The lyophilized microspheres were kept in a desiccator until use.

Ex vivo Study of Macrophages Behavior

An *ex vivo* study of phagocytosis was performed in male Swiss mice, weighing between 28 and 32 g. All animal procedures were conducted according to the European Community Council Directive (010/63/UE). To carry out this study, mice were intraperitoneally (ip) injected with 23.45 mg kg^{-1} MPs (formulation F1 or F2) suspended in PBS. In addition, mice were injected with labrafil (0.5 mL) dissolved in PBS (3.2 μg mL^{-1}). Peritoneal wash was performed after 2 h with saline solution containing 10% bovine serum albumin (BSA). MPs along with macrophages were then extracted by aspiration. The aliquots obtained were incubated at 37°C in RPMI 1640 medium with 10% fetal bovine serum (FBS) for 72 h in an atmosphere containing 5% CO_2 and controlled humidity. Then, the primary cultures were observed using an optical microscope (Olympus CKX41 Inverted, The Lab World Group, Woburn, MA, USA). This study was performed in duplicate.

Microparticle Development and Phagocytosis Study in Cell Lines

Optimization of the Procedure to Obtain Microparticles

To choose the method providing the most adequate particle size, blank MPs were obtained using PVA solution as

external phase and PLGA 502 (200 mg) dissolved in CH_2Cl_2 (4 mL) as organic phase. The organic phase was then poured dropwise into the aqueous phase using a 30G needle coupled to a syringe and homogenized for 5 min with a polytron (Polytron 10-35 GT®, Polytron Technologies, Inc., Fisher Scientific, Madrid, Spain). The emulsion formed was maintained under magnetic stirring for 2 h to remove all the organic solvent. The MPs obtained were filtered through 0.45 μm filters (Millipore, Billerica, MA, USA), washed with water, freeze-dried (Flexi-Dry MP ™, FTS Systems Inc., Stone Ridge, NY, USA) for 24 h, and frozen at − 50°C. The lyophilized MPs were kept in a desiccator until use.

The following variables were studied for optimization purposes: PVA volume (20 mL and 40 mL), PVA concentration (0.5% and 1%), and two different stirring rates (8500 rpm and 10,000 rpm). The responses evaluated were particle size, span, and production yield.

The span of a volume-based size distribution is defined as **Span = (D90–D10)/D50.**

This value gives an indication of how far apart are the 10% (D10) and 90% (D90) points, normalized with respect to the midpoint (D50).

When preparing nanosystems, monodisperse populations are obtained with span values < 1. Table I shows the different formulations prepared (MPI-1 to MPI-8) and the results obtained for production yield, particle size, and span. All formulations were prepared in triplicate.

Preparation of Fluorescein-Loaded PLGA Microparticles

The preparation of fluorescein-loaded PLGA MPs was performed by the same method as indicated in the "Optimization of the Procedure to Obtain Microparticles" section using 0.5% PVA (20 mL) as external phase and 10% fluorescein and PLGA (502 or 502H) as organic phase. Stirring rate was set at 8500 rpm. All formulations were prepared in triplicate. Table II shows the formulations prepared (MPF-1 and MPF-2).

Preparation of Labrafil-Modified Fluorescein-Loaded PLGA Microparticles

Labrafil-modified fluorescein-loaded PLGA MPs were prepared by the same procedure as indicated in the "Preparation of Fluorescein-Loaded PLGA Microparticles" section but with the addition of labrafil (14 μL) to the organic phase of the emulsion (Table II, formulations MPF-L1 and MPF-L2). Once filtered and washed with water, MPs were dried for 48 h in vacuum desiccators. All formulations were prepared in triplicate.

Preparation of Gatifloxacin-Loaded PLGA Microparticles

GAT-loaded PLGA MPs were prepared by the same procedure as indicated in the "Optimization of the Procedure to Obtain Microparticles" section but incorporating GAT (10%) and 0.5% PVA (20 mL). To facilitate encapsulation of the active ingredient, the pH of the PVA solution was adjusted to 8.6 with sodium phosphate dihydrate. Table II shows the formulations prepared (MPG-1 and MPG-2). All formulations were prepared in triplicate.

Preparation of Labrafil-Modified GAT-Loaded PLGA Microparticles

Labrafil-modified GAT-loaded PLGA MPs were prepared by the same procedure as indicated in the "Preparation of Gatifloxacin-Loaded PLGA Microparticles" section but with the addition of labrafil (14 μL) to the organic phase of the emulsion and 0.5% PVA (20 mL) (Table II, formulations MPG-L1 and MPG-L2). Once filtered and washed with water, MPs were dried for 48 h in vacuum desiccators. All formulations were prepared in triplicate.

Quantification of Gatifloxacin by HPLC

The apparatus consisted in an HPLC Series 200 Perkin Elmer chomatograph equipped with a 1740 HP computer and a 235C diode array detector (Perkin Elmer, Shelton, CT, USA). A C18 Mediterranea Sea chomatographic column (250 × 4 mm, 5 μm) (Teknokroma, S. Coop., Barcelona, Spain) was used. The mobile phase consisted of acetonitrile:water (80:20, v/v) with 0.3% trimethylamine and the pH adjusted to 3.3 with phosphoric acid. The mobile phase was pumped through the chomatographic column at a flow rate of 1 mL min^{-1}; the injection volume was 20 μL and the detection wavelength was set at 295 nm. All analyses were performed at 25 ± 0.5°C and each determination was carried out in triplicate. Data were collected and processed using the Turbochom navigator version 6.1.1 (Perkin Elmer Shelton, CT, USA). The HPLC method was used to determine the encapsulation efficiency of GAT within the MPs and for the quantification of GAT *in vitro* release studies. The method was validated being linear within the concentration range 2–40 μg mL^{-1}, exact, accurate, and selective with detection and quantification limits of 0.507 μg mL^{-1} and 1.68 μg mL^{-1}, respectively.

Table I. Blank Formulations Prepared. PVA (polyvinyl alcohol).

Formulation	PVA concentration (%)	Volume of PVA solution (mL)	Stirring rate (rpm)	Production yield (%) (mean ± SD)	Particle size (μm ± SD)	Span
MPI-1	0.5	20	8500	57.34 ± 4.78	4.36 ± 1.05	1.05
MPI-2	0.5	20	10,000	46.26 ± 5.91	3.72 ± 1.35	1.61
MPI-3	0.5	40	8500	44.25 ± 6.11	4.58 ± 1.78	1.92
MPI-4	0.5	40	10,000	42.55 ± 8.41	3.90 ± 2.03	2.41
MPI-5	1	20	8500	55.32 ± 3.46	3.55 ± 1.31	1.14
MPI-6	1	20	10,000	44.61 ± 5.62	4.44 ± 2.15	1.65
MPI-7	1	40	8500	45.23 ± 5.45	4.62 ± 2.05	1.47
MPI-8	1	40	10,000	47.78 ± 7.21	4.01 ± 1.97	2.06

Table II. Formulations Prepared. *GAT* Gatifloxacin. PLGA (poly (D,L-lactide-co-glycolide) acid).

Formulation	PLGA	GAT drug/polymer (%)	Labrafil (μL)	Fluorescein (%)
MPF-1	502	-		10
MPF-2	502-H	-		10
MPF-L1	502	-	14	10
MPF-L2	502-H	-	14	10
MPG-1	502	10	-	-
MPG-2	502-H	10	-	-
MPG-L1	502	10	14	-
MPG-L2	502-H	10	14	-

Characterization of Microparticles

Process Yield and Encapsulation Efficiency. Process yield was calculated as the ratio between the total weight of MPs obtained and the total weight of drug/tracer and polymer used. Process yield was determined for all fluorescein and GAT formulations.

Encapsulation efficiency (EE%) of fluorescein within MPs (formulations MPF-1, MPF-2, MPF-L1, and MPF-L2) was determined by UV spectrophotometry (Beckman DU-7, Beckman Coulter, Inc., Brea, CA, USA). Encapsulation efficiency was calculated as the ratio between the amount of drug content in the MPs and the amount of drug used for their preparation.

For this, a weighed amount of MPs (10 mg) was dissolved in 1 mL of CH_2Cl_2. The polymer was then precipitated with water and fluorescein was removed with water (6 mL). Samples were centrifuged at 5000 rpm for 5 min. Finally, all samples were filtered through 0.45 μm filters and analyzed by UV spectrophotometry at 488 nm.

EE (%) of GAT in formulations MPG-1, MPG-2, MPG-L1, and MPG-L2 (Table II) was calculated as the percentage ratio between the amount of drug content in the MPs and the amount of drug used for their preparation.

EE = (GAT amount in MPs/initial GAT amount used in MPs).100

The amount of excipients included in the polymeric matrix was taken into account. The amount of encapsulated GAT was determined by dissolving 10 mg of MPs in 1 mL CH_2Cl_2. Then, PLGA was precipitated by the addition of ethanol (15 mL). The supernatant obtained after centrifugation at 5000 rpm for 5 min (Universal 32, Hettich, Kirchlengern, Germany) was collected. The precipitate was then treated five times with ethanol to achieve complete extraction of GAT. Samples were filtered through 0.45 mm filters and analyzed by the HPLC method previously described.

Morphological Characterization and Size Distribution of Microparticles. The morphology of the MPs was analyzed by scanning electron microscopy (SEM, JEOL JEM 6335F, JEOL Ltd., Zaventem, Belgium). Samples were coated with a thin layer of colloidal gold (15 nm) for 3 min, and applied in a cathodic vacuum evaporator before observation by SEM at 20 KV. The mean diameter and size distribution of the particles were evaluated by laser diffraction using a Microtrac-S3500 particle size analyzer at 25°C (Microtrac S3500, Microtac, Inc., Montgomeryville, PA, USA). For this, the lyophilized samples were suspended in Milli-Q water and sonicated for 30 s before each determination to prevent clumping. Results are described in terms of mean diameter and standard deviation (SD).

In vitro Release Studies. In vitro release of GAT from the MPs was carried out in a Memmert WB22 water bath (Memmert GmbH Co. KG, Schwabach, Germany) heated at 37 ± 0.2°C and with constant shaking (100 rpm). An amount of MPs (10 mg) was suspended in PBS (3 mL) at pH 7.4 under sink conditions. At regular time intervals, 2.5 mL of PBS was withdrawn, centrifuged at 15,000 rpm, filtered through 0.45 nm filters, and replaced with the same volume of fresh medium. Quantification of GAT was performed by HPLC. The *in vitro* release tests were done in triplicate for each formulation (MPG-1, MPG-2, MPG-L1, and MPG-L2).

Aerodynamic Assessment of Fine Particles. This test is used to determine the fine particle characteristics of the aerosol clouds generated by inhaled preparations. For this, we used a glass impinger corresponding to apparatus A of the European Pharmacopoeia (13). The system consists of two impingement chambers; a lower chamber which consists of a conical flask of 250 mL and the upper chamber consisting in a modified round-bottomed flask of 100 mL. Both chambers were adequately connected. Formulation MPG-L2 (Table II) was assayed. To carry out the test, 7 mL and 30 mL of the solvent were respectively introduced into the upper and lower impingement chambers. In both cases, CH_2Cl_2 (1 mL) was used to dissolve the MPs and 6 mL or 29 mL ethanol added to extract GAT from the MPs (final volumes of 7 mL or 30 mL in the upper and lower chambers, respectively). The amount of GAT assayed was 100 mg with the air flow though the apparatus measured and the inlet to the throat was set at 60 ± 5 L min^{-1}. The amount of GAT in each chamber was determined by HPLC. This test was performed in triplicate.

In vitro Evaluation of Microparticle Phagocytosis. In vitro evaluation of phagocytosis was carried out in a raw 264.7 mouse macrophage cell line. This cell line was kindly donated by Prof.

Carolina Hurtado from the Universidad CEU-San Pablo (Spain) and obtained from ATCC (ATCC® TIB-71™, ATCC, Manassas, VA, USA). The cells were cultured under normal conditions (20% O_2, 5% CO_2, 75% N_2) in a humid environment at 37°C using RPMI 1640 medium (Sigma R8758, Sigma Aldrich, Madrid, Spain) supplemented with 10% FBS, L-glutamine (2 mM) and gentamicin (50 μg mL^{-1}). Macrophages were maintained in culture for 48 h, and after reaching 80–100% confluence, cells were centrifuged and the supernatant removed and adjusted to the desired concentration.

Phagocytosis was analyzed by flow cytometry (Flow cytometer, FACS Calibur, Becton Dickinson, San Jose, CA, USA) and microscopy (Olympus CKX41 Inverted, The Lab World Group, Woburn, MA, USA).

For flow cytometry, cells were placed in wells containing 300,000 cells/well. After 72 h, the medium was removed and the different MPs (formulations MPF-1, MPF-2, MPF-L1 and MPF-L2) were suspended in RPMI medium at two different concentrations (0.5 mg mL^{-1} and 0.8 mg mL^{-1}). In addition, two labrafil concentrations (35 μL and 70 μL) were also analyzed. Cells were incubated at three different times: 3, 5, and 24 h. At each time, the culture medium was removed, cells were washed three times with PBS, detached with trypsin (0.5 mL), centrifuged, and resuspended in PBS and propidium iodide (10 μL). Cell viability, granularity, and fluorescence intensity were determined using an FL1 probe (530/15 nm) and 488 nm excitation wavelength. The experiments were performed in triplicate.

Analysis of fluorescent MPs was also performed by confocal microscopy using the same cell line and culture conditions. After reaching 80–100% confluence, cells were centrifuged and the supernatant removed and adjusted to the desired concentration. Cells were then placed in individual plates (35 mm, Glass bottom IBIDI 81151) with 150,000 cells/well for 48 h and incubated with the different fluorescein formulations (MPF-1, MPF-2, MPF-L1, and MPF-L2) at a concentration of 0.8 mg mL^{-1} in RPMI medium. Incubation times were 3, 5, and 24 h. Moreover, GAT formulations MPG-2 and MPG-L2 were incubated at a concentration of 0.8 mg mL^{-1} for 3, 5, 24, and 48 h. At each time, the medium from each well was removed and cells were washed twice with PBS. For nuclear staining, each well was treated with Hoechst 33342 solution for 10 min (blue fluorescence). Hoechst dye is used for nuclear staining of living or fixed cells as it binds nucleic acids of DNA. Then the Hoechst/DNA complex is excited at 350 nm and 461 nm as emission wavelength. The fluorescence of fluorescein MPs (green) was visualized by confocal microscopy (Leica SP5 confocal laser scanning microscope, Leica Microsystems, Wetzlar, Germany). The experiments were performed in triplicate.

Statistical Analysis

Data were expressed as mean ± SD with $n = 3$. The statistical significance of differences between groups was calculated using Student's t test, and p values < 0.05 were considered significant.

RESULTS AND DISCUSSION

The main objective of this work was to develop the first surface-modified GAT-loaded PLGA microparticulate system for pulmonary administration being able to target alveolar macrophages in order to directly act on TB reservoir (MTB). Labrafil was used as a surface modifying agent. Macrophages, as part of the mononuclear phagocyte system, are innate immune cells which respond to different stimulus such as TB. To achieve sustained drug release into alveolar macrophages, particle size is a critical factor when developing formulations intended for pulmonary administration. Moreover, it is known that for passive targeting to macrophages, a combination of physical properties such as shape and particle size also needs to be taken into consideration (5).

In our work, we have performed a preliminary *ex vivo* study in mice macrophages to evaluate the effect of particle size on the behavior of these immune cells. In addition, we have analyzed the response of macrophages to the presence of labrafil, an additive which can be used to modify the superficial characteristics of MPs. Labrafil, an oleoyl polyoxyl-6 glyceride, is a water dispersible surfactant being previously used by our research group due to its ability to both modify the superficial characteristics of MPS and the release of drugs from the particles (14,15).

To carry out this study, mice were given ip 23.45 mg kg^{-1} blank PLGA MPs (formulations F1 or F2) suspended in PBS and with average particle sizes of 40.3 ± 4.7 μm and 1.4 ± 0.7 μm, respectively. A primary culture of macrophages was performed by peritoneal washing and extracting the MPs along with the macrophages. After incubation, the primary cultures were observed under a microscope (Fig. 1). Figures 1b and c correspond to images obtained for formulations F1 and F2, respectively. Cell culture of macrophages obtained after ip administration of MPs with particles sizes around 40 μm (formulation F1) produced a modification in macrophage morphology probably due to the formation of foam cells. This modification occurred only with MPS of particle sizes around 40 μm which enhances the importance of particle size not only regarding phagocytosis. This undesirable effect is strongly associated with possible tissue necrotic damage as a dysregulation in the balance between the influx and efflux of low density lipoprotein (LDL) particles from the serum (16). In respect, several authors have indicated that foamy macrophages play an important role in the maintenance of persistent bacteria and tissue pathologies (17).

On the contrary, MPs with smaller sizes (formulation F2) were not observed in the intercellular space (Fig. 1c) probably due to their internalization by macrophages. Neither have we observed an apparent modification in macrophage morphology (Fig. 1c *versus* a). The shape of macrophages can be modified as their phagocytic activity increases. However, in our case, this did not occur. These results are in accordance with those obtained by Pacheco *et al.* (18) who found that the internalization efficiency of small polystyrene particles (0.5–2 μm) by macrophages occurred without any shape modification.

In addition, we have evaluated the behavior of macrophages in the presence of labrafil as a potential component of our formulation. For this, a dispersion of labrafil (3.2 μg mL^{-1}) in PBS (0.5 mL) was given ip to mice. Figure 1d shows

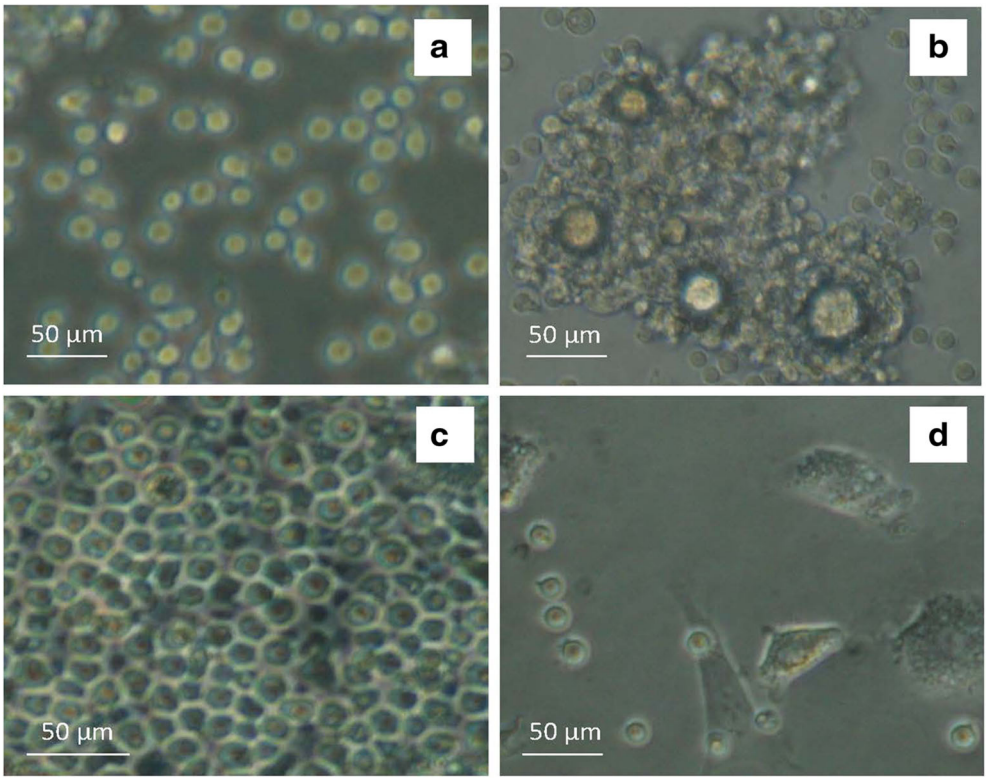

Fig. 1. Microphotographs of control cells (**a**), formulations F1 (**b**), and F2 (**c**) and labrafil (**d**) incubated in murine macrophages for 72 h

a microphotograph of labrafil and murine macrophage cells after incubation in which modification of the cell shape is observed. The results obtained with labrafil did not produce any sign of cytotoxicity at the concentration assayed (Fig. 1d).

In order to continue studying phagocytosis, we have optimized the method for obtaining fluorescein-loaded MPs with particle sizes between 1 and 6 μm since this is a key factor for the recognition of MPs by macrophages (19). In this regard, Hirota *et al.* (20) performed an *in vitro* study with rifampicin-loaded PLGA MPs. The results obtained showed that particle sizes between 1 and 6 μm suffered higher uptake by macrophages than smaller ones. Particles being too large or too small are not recognized by macrophages (4). For this, and in order to establish the most appropriate conditions to obtain MPs intended for pulmonary administration, we have optimized the method used in the elaboration of blank MPS with the following variables being studied: PVA concentration, volume of the external phase, and stirring rate used in preparation of the emulsion. Table III summarizes the results obtained regarding size, span, and production yield.

All MP formulations presented mean particle sizes between 3.55 and 4.62 μm which are adequate for pulmonary administration in which access of the drug at the alveolar level and uptake by macrophages is desired.

The formulations prepared with stirring rate of 10,000 rpm had span values > 1.5 indicating high dispersion in size distribution. When intending for high encapsulation efficiencies of water-soluble drugs, the use of small volumes and low PVA concentrations is preferred. From our results, the following conditions were chosen: stirring rate of 8500 rpm and 20 mL of 0.5% PVA.

In order to evaluate the uptake of PLGA MPs by macrophages, several fluorescein-loaded MP formulations were developed (Table II): formulations MPF-1 and MPF-2 prepared with 10% fluorescein, and formulations MPF-L1 and MPF-L2 prepared with 10% fluorescein and labrafil (14 μL) as surface modifier included to analyze its behavior in the phagocytosis process by macrophages. For these formulations, mean particle sizes (Table III) were around 3–5 μm, being suitable for macrophage phagocytosis (7,19). Mean encapsulation efficiencies were lower than 5% probably due to the hydrophobic characteristics of the polymer which hinders the encapsulation of hydrophilic substances such as sodium fluorescein (water solubility = 600 mg mL^{-1}). This water solubility would be enough to observe fluorescent MPs.

The uptake of MPs by macrophages was studied by flow cytometry in raw 264.7 mouse macrophage cell line regarding intensity of fluorescence and granulometric complexity (internal cellular complexity) at two MP concentrations (0.5 and 0.8 mg mL^{-1}) and after 3, 5, and 24 h incubation. Some authors have indicated that an increase of granularity is directly related with the number of particles phagocytosed by macrophages (21). In addition, in our work, the effect of labrafil on macrophage viability was also studied by flow cytometry. For this, cells were incubated with labrafil (35 and 70 μL). After incubation, no significant differences were found in cell viability. These results are in agreement with previous studies performed by our research team in which high cellular viabilities in primary cell cultures (splenocytes and leukocytes) were obtained when using similar concentrations (14).

Table III. Characterization of the microparticle formulations prepared

Formulation	Particle size (μm) (mean ± SD)	Span	Encapsulation efficiency (%) (mean ± SD)	Drug loading (μg/100 mg MPs) (mean ± SD)	Production yield (%) (mean ± SD)
MPF-1	4.4 ± 2.2	1.26	1.5 ± 0.1	136.2 ± 11.2	42.1 ± 1.3
MPF-2	3.4 ± 1.7	1.28	4.8 ± 0.1	436.7 ± 32.3	55.7 ± 2.9
MPF-L1	3.9 ± 2.0	1.97	1.9 ± 0.8	162.2 ± 14.1	47.3 ± 1.6
MPF-L2	3.5 ± 1.3	1.36	2.4 ± 0.1	204.3 ± 20.8	45.2 ± 3.1
MPG-1	4.9 ± 2.5	1.07	7.3 ± 0.5	663.5 ± 35.4	45.6 ± 2.5
MPG-2	3.9 ± 0.4	1.23	57.9 ± 1.1	5263.2 ± 77.4	63.6 ± 1.5
MPG-L1	4.7 ± 1.1	1.05	34.2 ± 1.1	2886.5 ± 39.3	50.8 ± 4.5
MPG-L2	4.5 ± 0.8	1.30	89.6 ± 1.2	7984.28 ± 53.2	45.7 ± 3.5

Figure 2 shows the results obtained (granularity and intensity of fluorescence) for the different fluorescein formulations assayed (MPF-1, MPF-2, MPF-L1, and MPF-L2). At shorter times (3 and 5 h) and for both concentrations studied (0.5 mg mL^{-1} and 0.8 mg mL^{-1}), the highest levels of granularity ($p < 0.05$) were obtained when macrophages were incubated with formulation MPF-2 which was prepared with PLGA 502H and without labrafil. Figure 3 shows the dot plots (SSC *versus* FSC) and intensity of fluorescence plots obtained for all formulations after 3 h incubation, in which formulation MPF-2 exhibits the highest increase in fluorescence intensity with two maxima being observed. These results are not in concordance with those obtained by Mathaes *et al.* (22) in which particles with lipophilic surfaces were more readily phagocytosed than those with hydrophilic ones. Our results can be attributed to the fact that the technique used (flow cytometry) does not allow us to distinguish between MPs trapped inside the cells and adsorbed on their surfaces. Even though this result is paradoxical, it can be related with the adsorption of the MPs to the cell surfaces and could only be explained once phagocytosis tests were carried out in macrophages in which MPs were not trapped inside macrophages but adhered to the cell surfaces.

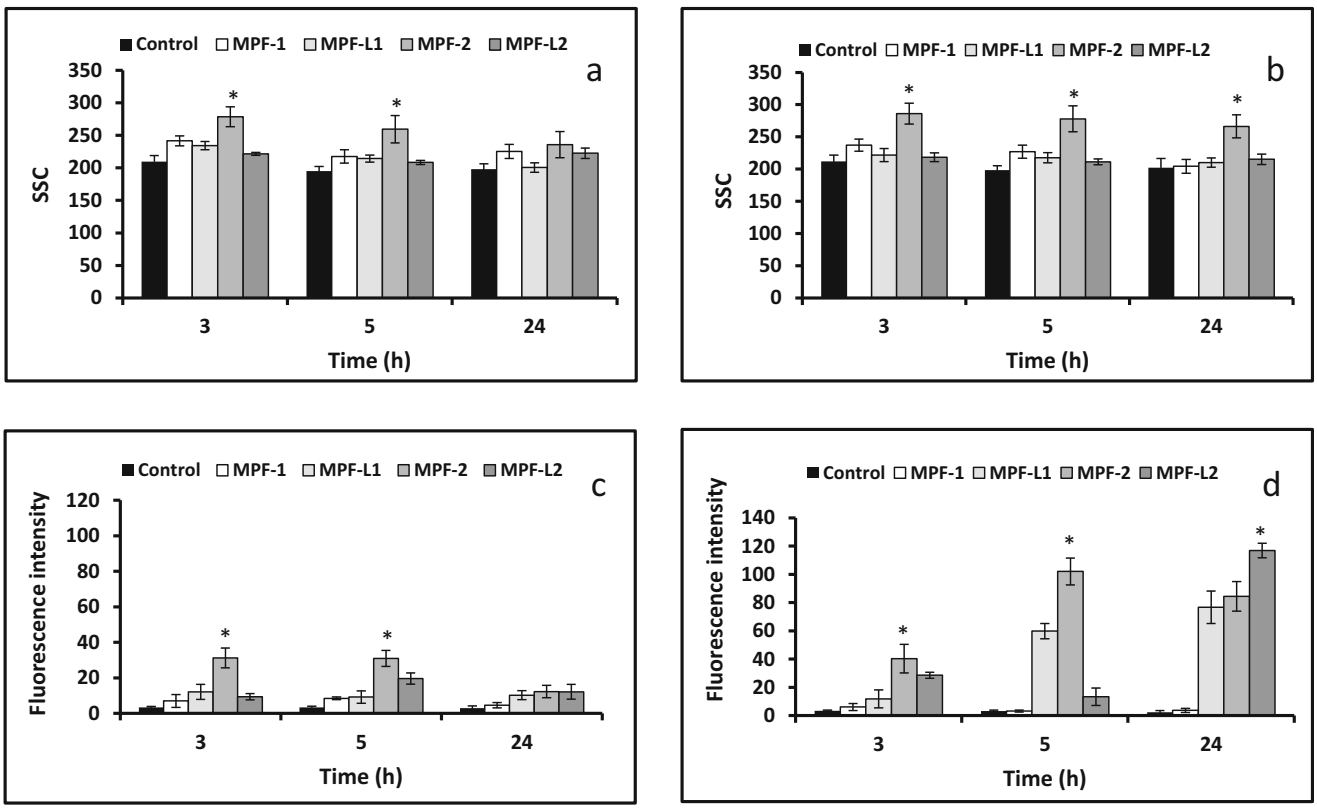

Fig. 2. Granulometric complexity of microparticle formulations (MPs) expressed as SSC (side scatter) parameter ± SD at concentrations of 0.5 mg mL^{-1} (**a**) and 0.8 mg mL^{-1} (**b**). Fluorescence intensity ± SD of MPs at concentrations of 0.5 mg mL^{-1} (**c**) and 0.8 mg mL^{-1} (**d**). MPF-1, fluorescein-loaded PLGA 502 MPs; MPF-2, fluorescein-loaded PLGA 502H MPs; MPF-L1, labrafil-modified fluorescein-loaded PLGA 502 MPs; MPF-L2, labrafil-modified fluorescein-loaded PLGA 502H MPs

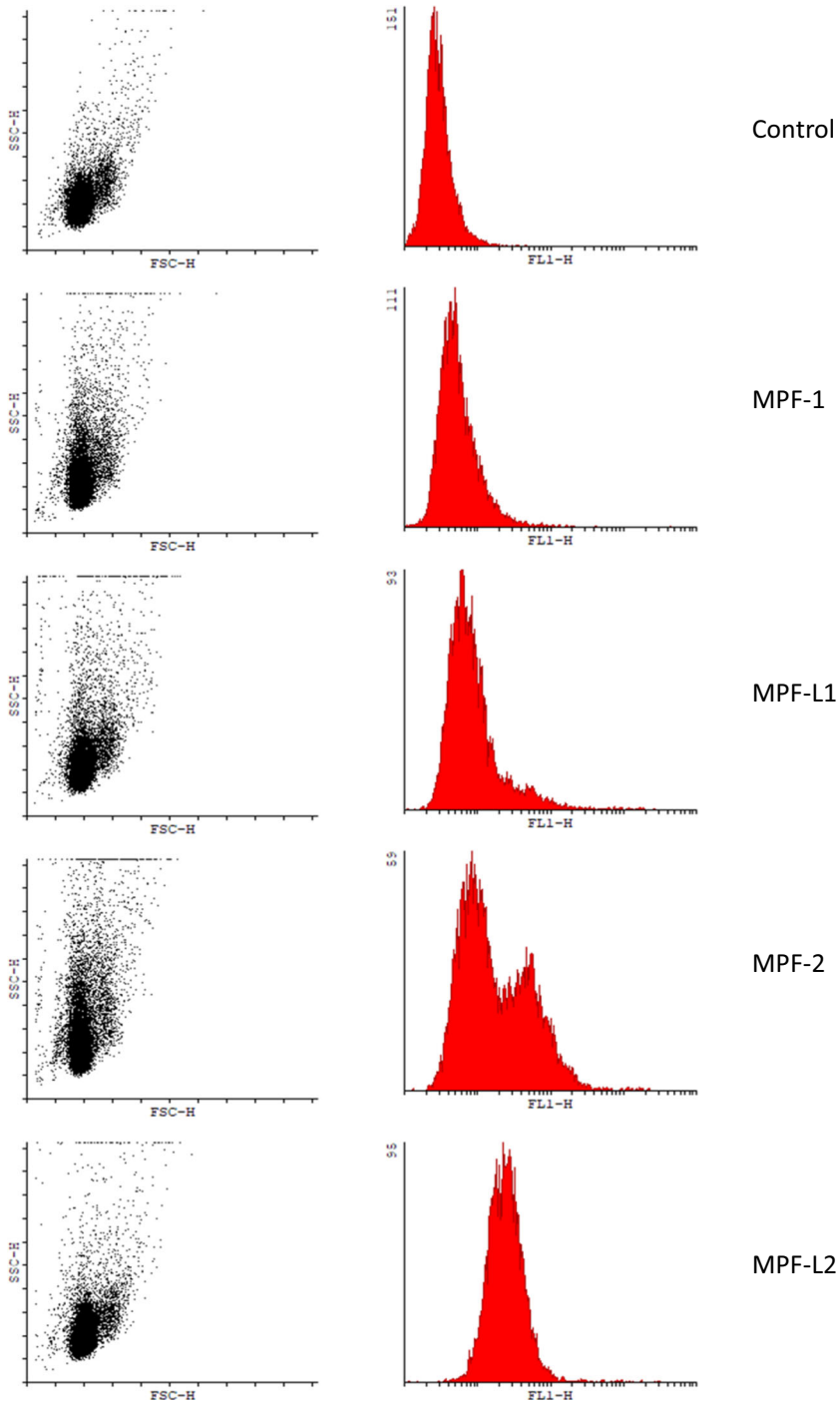

Fig. 3. Dot plots (SSC *versus* FSC) and intensity of fluorescence plots obtained for all formulations after 3 h incubation at a concentration of 0.8 mg mL^{-1}. MPF-1, fluorescein-loaded PLGA 502 MPs; MPF-2, fluorescein-loaded PLGA 502H MPs; MPF-L1, labrafil-modified fluorescein-loaded PLGA 502 MPs; MPF-L2, labrafil-modified fluorescein-loaded PLGA 502H MPs

The effect of labrafil on macrophages was studied at two concentrations (35 and 70 μL). Both concentrations of labrafil did not have any effect on macrophage appearance (Fig. 4a). MPs were then assayed at two concentrations (0.5 and 0.8 mg mL^{-1}) and incubation times of 3, 5, and 24 h.

Microphotographs of MPs obtained by confocal microscopy after 3 and 5 h incubation are shown in Fig. 5. As previously indicated, Hoechst solution produces blue fluorescence when bound to DNA, thereby allowing to differentiate the nuclei of macrophages. Green fluorescence corresponds to fluorescein. Due to the time of exposure and rapid release of fluorescein from the MPs, its fluorescence was not observed in all micrographs. However, it was possible to observe the presence of MPs inside macrophages.

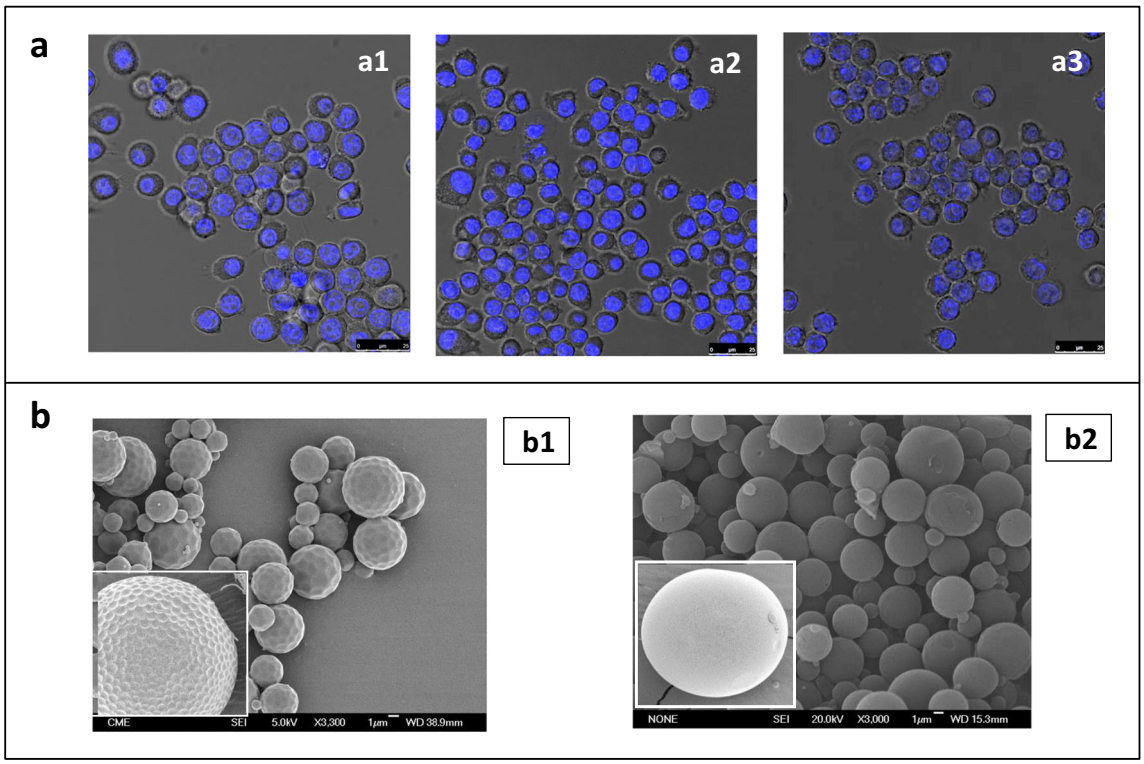

Fig. 4. **a** Confocal images corresponding to control (a1), 35 μL labrafil (a2), and 70 μL labrafil (a3) after 3 h incubation. **b** SEM images corresponding to blank PLGA 502H MPs prepared with (b1) and without labrafil (b2)

Our formulations presented adequate particle sizes for phagocytosis (3–5 μm); however, differences in phagocytosis rate were found. For instance, formulation MPF-1 prepared with PLGA 502 was rapidly phagocytosed (3 h). This result is in accordance with studies performed by other authors (5,23,24). On the contrary, formulation MPF-2 which was prepared with PLGA 502H and without labrafil was not phagocytosed after 5 h probably due to the fact that this formulation has a more hydrophilic surface than MPF-1. Therefore, at shorter times, high amounts of MPs from formulation MPF-2 are adhered to the surface of macrophages. For this, the high values of intensity of fluorescence obtained by flow cytometry for this formulation (Fig. 2) are not related to its uptake by macrophages, but as a result of the adhesion of the particles to the cell surface. Moreover, when labrafil was included as surface modifier (formulation MPF-L2), MPs were readily phagocytosed indicating that the addition of labrafil improved the rate at which MPs were taken up by macrophages due to the modification of PLGA 502H polymer matrix. Figure 3b shows SEM images of formulations MPF-1 and MPF-L1 prepared with PLGA 502 and with (Fig. 4b1) or without labrafil (Fig. 4b2). It can be observed that the presence of labrafil modified the surface of MPs.

It is well known that PEG decreases the uptake of drug carriers by macrophages, while increasing their residence time within the body. However, this behavior only occurs with high-molecular weight PEG (25). Yang *et al.* (26) demonstrated that long *in vivo* circulation times were only achieved when nanosystems were prepared with high-molecular weight PEG. Other authors have also indicated that in order to reduce the recognition of nanoparticles by macrophages, high-molecular weight PEG should be used in the preparation of these systems (27,28). In our case, labrafil, a PEG-derivative surfactant with low-molecular weight (Mw = 300 Da), was used for the preparation of MPs. Labrafil did not protect our particles from phagocytosis. In fact, the incorporation of labrafil improved the rate at which MPs were taken up by macrophages due to the modification of the polymer matrix and thereby representing an adequate strategy for drug targeting (Fig. 5). This process was not observed when MPs were prepared with PLGA 502, with or without the addition of labrafil (formulations MPF-1, MPF-L1) since this is a more lipophilic polymer, and MPs were rapidly phagocytosed by macrophages with independence of the incorporation of labrafil.

Phagocytosis occurred in all fluorescein formulations after 24 h incubation (including formulation MPF-2) as well as survival of macrophages (Fig. 5). Due to the rapid release of the fluorescent tracer, it was not possible to detect any fluorescence derived from the MPs after 24 h incubation, but MPs were observed within live macrophages at this incubation time. When observed by microscopy, the maximum number of MPs captured by macrophages was around 3–4 after incubation with independence of the formulation tested.

Different mechanisms for cellular uptake of MPs have been demonstrated which are largely dependent on particle size. Phagocytosis is the mechanism by which relatively large particles (> 0.5 μm) such as solid particles or dead cells are internalized (29). In our study, the phagocytic process suffered by formulation MPF-L2 is depicted in Fig. 5. Firstly, approximation and adhesion of the MPs to the macrophage surface occurred (Fig. 6a and b), then internalization of the

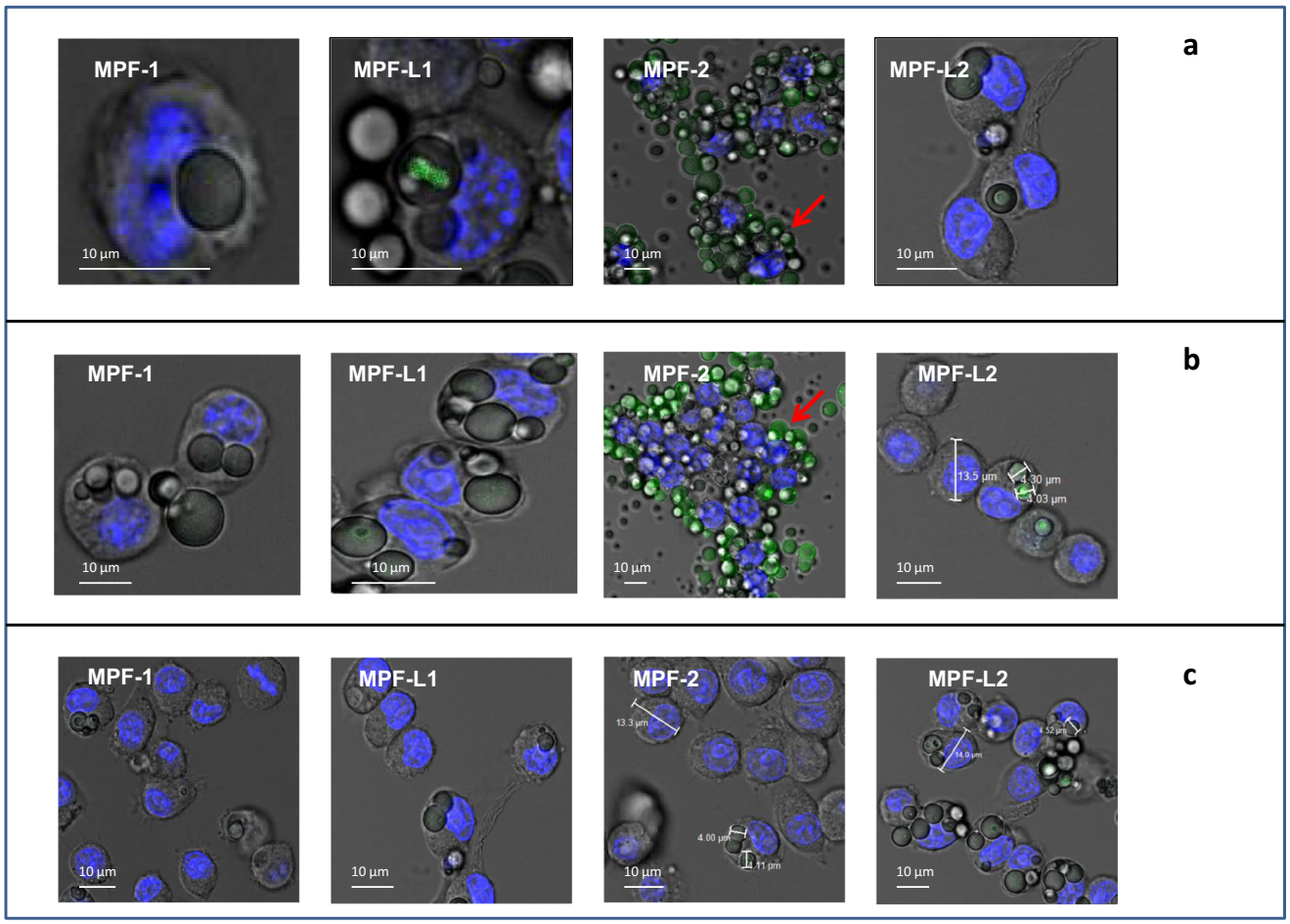

Fig. 5. Confocal images of phagocytosis obtained at 3 h (**a**), 5 h (**b**), and 24 h (**c**). MPF-1, fluorescein-loaded PLGA 502 MPs; MPF-2, fluorescein-loaded PLGA 502H MPs; MPF-L1, labrafil-modified fluorescein-loaded PLGA 502 MPs; MPF-L2, labrafil-modified fluorescein-loaded PLGA 502H MPs

MPs by means of invagination of the macrophage membrane was produced by protrusion of the cell membrane to form a membrane-bound vesicle (Fig. 6c and d), and finally, the possible formation of a phagosome and its subsequent phagocytosis took place while fusing with several compartments of the endocytic system as being transported within the cytosol (Fig. 6e and f). MPs are covered by soluble proteins (opsonins) in a process known as opsonization which makes them more susceptible to macrophage phagocytosis (30). Opsonized MPs will then be bound to receptors located on the membrane of macrophages. Although opsonin absorption to particles occurs preferentially due to hydrophobic interactions, other types of interactions such as electrostatic or hydrogen bonding could occur (27). Without the presence of opsonin proteins absorbed onto the MP surfaces, macrophages are not able to bind or recognize foreign particles. This binding process induces structural changes in the cytoskeleton as pseudopodia formation on the surface of macrophages with the consequent internalization of strange particles due to an endocytosis process (30). For this, bonding of opsonins to the surface of MPs is a critical step.

In our case, modification of the polymer matrix of MPs prepared with PLGA 502H and labrafil probably conducted to a more hydrophobic surface with higher opsonin absorption and faster phagocytosis.

Once the influence of polymer type and labrafil on the uptake of MPS by macrophages is studied, gatifloxacin (GAT)-loaded MPs were developed for the treatment of TB.

It is recommended to treat MDR-TB with the use of fluoroquinolones, with several phase II and III clinical trials being recently conducted on the safety and efficacy of various fluoroquinolones given in short-term treatment regimens (less than the 6-month standard protocol) (9–12). The results obtained with GAT suggest that this agent could minimize drug resistance by reducing the duration of treatment from 6 to 4 months with similar efficacy and safety to the standard treatment guidelines recommended by WHO (11,12).

For this, we have developed an inhalable formulation consisting of biodegradable GAT-loaded PLGA MPs able to achieve active targeting to macrophages thereby directly acting on MTB. An aerosol formulation of slow release MPs (particle size 1–5 µm) offers important advantages since it allows targeting the drug to the macrophages, reduce systemic side-effects, and decrease both dose and frequency of administration. In addition, this route of administration is well tolerated by the patient (19). In our case, the formulation

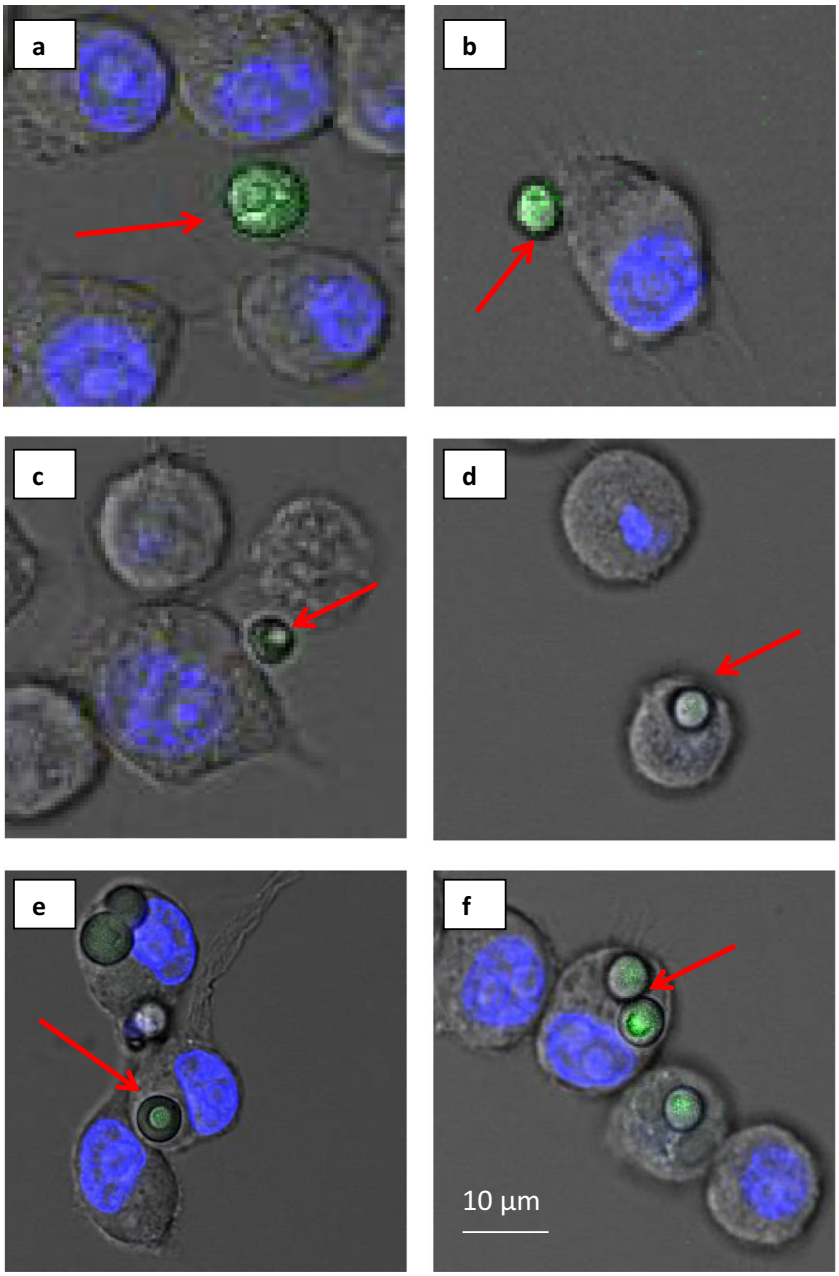

Fig. 6. Mechanism of phagocytosis. Approximation and adhesion of MPs to the macrophage surface (**a** and **b**); internalization of MPs by invagination of the macrophage membrane (**c** and **d**); possible formation of the phagosome and its phagocytosis (**e** and **f**)

developed could be administered by means of a dry-powder inhaler with the MPs included as powder. Therefore, the MP formulations developed in our study were optimized for pulmonary administration of GAT.

GAT-loaded PLGA MPs were prepared using two different PLGA polymers (PLGA 502 and PLGA 502H) with and without the incorporation of labrafil (Table II, formulations MPG-1, MPG-2, MPG-L1, and MPG-L2). Stirring rate was maintained at 8500 rpm since high particle size polydispersity was obtained at 10,000 rpm. Formulations exhibited mean particle sizes < 5 μm (Table III) which are adequate for both the delivery of drugs to the alveolar level and macrophage uptake. As previously indicated, particle size is a key factor for the recognition of foreign particles by macrophages with particles too large or too small not being recognized by them (4,5). In a study performed by Hirota *et al.* (20) using NR8383 cells derived from rat alveolar macrophages, phagocytic uptake of rifampicin-loaded PLGA MPs was enhanced for particle sizes between 1 and 6 μm when compared with smaller particles. On the other hand, it is well known that after pulmonary administration, particles larger than 10 μm are mainly retained in the oropharyngeal region, whereas particles ranging 2–5 μm can access the alveoli and then be captured by alveolar macrophages (31). In our work, all GAT formulations presented adequate size characteristics (small sizes and monodisperse populations) for

this purpose. Regarding particle size, no statistical significant differences were found ($p < 0.05$) when labrafil was used in the preparation of MPs.

Mean values for process yield ranged between 45.6 ± 2.5% and 63.6 ± 1.5% for formulations MPG-1, MPG-2, MPG-L1, and MPG-L2 (Table III). The use of PLGA 502H significantly increased the encapsulation efficacy of GAT ($p < 0.05$) probably due to the presence of more hydrophilic groups which facilitate the encapsulation of hydrophilic drugs (formulation MPG-1 *versus* MPG-2 and MPG-L1 *versus* MPG-L2) (Table III). Therefore, the incorporation of labrafil resulted in increased encapsulation efficiencies (formulation MPG-L1 *versus* MPG-1 and MPG-L2 *versus* MPG-2). The highest value of encapsulation efficiency (89. 6 ± 1.2%) was obtained for formulation MPG-L2 prepared with PLGA 502H and labrafil as surface modifier.

Figure 7 shows the mean *in vitro* release profiles of GAT from all MP formulations (MPG-1, MPG-2, MPG-L1, and MPG-L2). In all cases, biphasic profiles were obtained, with rapid GAT release occurring during the first 3 days followed by slower releases. The incorporation of labrafil did not have any influence on the release profiles of GAT (Fig. 7a). *In vitro* release of GAT was faster from the MPs prepared with PLGA 502H which can be related with their higher encapsulation efficiencies (Fig. 7a). After 2 days, approximately 73–75% GAT was released from formulations prepared with PLGA 502H corresponding these percentages to around 400 μg and 600 μg GAT released from formulations MPG-2 and MPG-L2, respectively (Fig. 7b). MTB has an extremely slow replication cycle; therefore, the rapid GAT release obtained from our formulations during the first 48 h could be adequate to control the multiplication of MTB inside macrophages (32).

On the other hand, when administering a multiparticulate system by inhalation, it is necessary to ensure that the fine particle characteristics of the aerosol clouds generated during inhalation have an aerodynamic size to access the deep lung. For this, the aerodynamic assessment of fine particles from formulations MPG-2 and MPLG-L2 was studied. For both formulations, the percentages of particles that can reach stage 2 (deep lung) were 17.3% and 15.9%, respectively. These values are similar to those reported for powder inhalers (< 20%) (33).

To evaluate the behavior of macrophages in the presence of GAT-loaded PLGA MPs, cells were incubated for 3, 5, 24, and 48 h with formulations MPG-2 and MPG-L2 (prepared

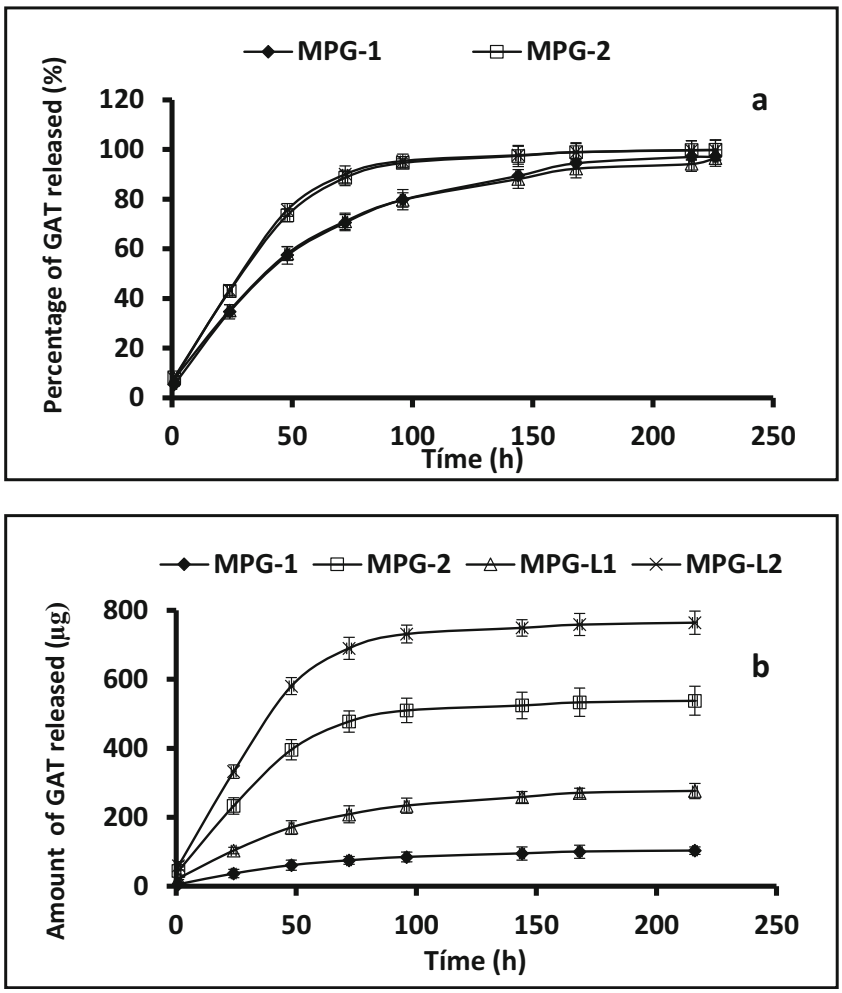

Fig. 7. Mean release profiles of GAT (% ± SD (**a**) and amount ± SD (**b**)) from formulations MPG-2 (GAT-loaded PLGA 502H MPs) and MPG-L2 (labrafil-modified GAT-loaded PLGA 502H MPs). GAT gatifloxacin

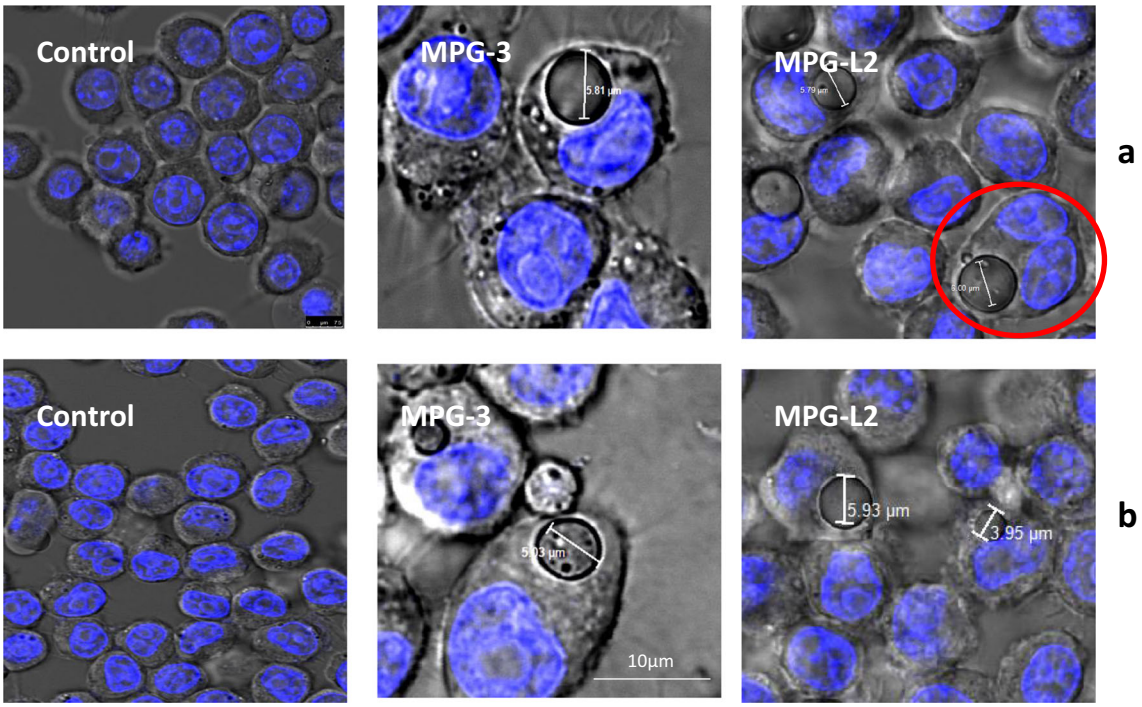

Fig. 8. Phagocytosis of MPs after 24 h (**a**) and 48 h (**b**). MPG-2, GAT-loaded PLGA 502H MPs; MPG-L2, labrafil-modified GAT-loaded PLGA 502H MPs; GAT, gatifloxacin

with PLGA 502H). These formulations exhibited high encapsulation efficiencies, suitable particle sizes, and adequate control of GAT release. Both formulations were phagocytosed after 24 h with formulation MPG-L2 being the only one captured at shorter times (3 and 5 h). It is important to notice that after 48 h, macrophages were still alive, MPs could be observed inside the cells, and cell replication was not hindered by the presence of MPs (Fig. 8).

In this preliminary study, the early release of GAT from formulation MPG-2 and its slow macrophage uptake would result in less access of the active ingredient to its site of action. Therefore, formulation MPG-L2 will be preferred for our purposes due to its high encapsulation efficiency (89.6 ± 0.2%), rapid phagocytosis, and because it remained inside the cells for at least 48 h.

CONCLUSIONS

PLGA 502H is frequently employed for the encapsulation of hydrophilic drugs, but in our work, MPs prepared with this polymer exhibited slow phagocytosis by macrophages (> 5 h). Labrafil can be a good surface modifier for polymers such as PLGA 502H when trying to achieve rapid and active targeting to macrophages.

GAT-loaded MPs prepared with PLGA 502H and labrafil exhibited high encapsulation efficiency, adequate particle size for pulmonary administration, were rapidly phagocytosed by macrophages, and remained in their interior for at least 48 h, thereby resulting in a suitable drug carrier to potentially treat MDR-TB.

These results are promising, but regarding future perspectives, further immunogenicity studies of the developed systems and phagocytosis in MTB infected macrophages should be conducted, as well as the formulations being evaluated in an animal model of TB.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflicts of Interest The authors declare that they have no conflict of interest.

Ethical Approval All animal procedures were conducted according to the European Community Council Directive (010/63/UE).

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