Colloids and Surfaces B: Biointerfaces 136 (2015) 678–686

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

Microspheres prepared with different co-polymers of poly(lactic-glycolic acid) (PLGA) or with chitosan cause distinct effects on macrophages

Claudia da Silva Bitencourt[a,b], Letícia Bueno da Silva[a], Priscilla Aparecida Tartari Pereira[a], Guilherme Martins Gelfuso[c], Lúcia Helena Faccioli[a,b,*]

[a] Departamento de Análises Clínicas, Toxicológicas e Bromatológicas, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo. Ribeirão Preto, SP, 14040-903, Brazil
[b] Centro Universitário das Faculdades Associadas ao Ensino (UNIFAE), São João da Boa Vista, SP, 13870-377, Brazil
[c] Laboratório de Tecnologia de Medicamentos, Alimentos e Cosméticos (LTMAC), Faculdade de Ciências da Saúde, Universidade de Brasília, Brasília, DF, 70910-900, Brazil

ARTICLE INFO

Article history:
Received 18 August 2015
Received in revised form 7 October 2015
Accepted 8 October 2015

Keywords:
Microsphere
Microparticle
PLGA
Chitosan
Macrophage
Phagocytosis

ABSTRACT

Microencapsulation of bioactive molecules for modulating the immune response during infectious or inflammatory events is a promising approach, since microspheres (MS) protect these labile biomolecules against fast degradation, prolong the delivery over longer periods of time and, in many situations, target their delivery to site of action, avoiding toxic side effects. Little is known, however, about the influence of different polymers used to prepare MS on macrophages. This paper aims to address this issue by evaluating *in vitro* cytotoxicity, phagocytosis profile and cytokines release from alveolar macrophages (J-774.1) treated with MS prepared with chitosan, and four different co-polymers of PLGA [poly (lactic-co-glycolic acid)]. The five MS prepared presented similar diameter and zeta potential each other. Chitosan-MS showed to be cytotoxic to J-774.1 cells, in contrast to PLGA-MS, which were all innocuous to this cell linage. PLGA 5000-MS was more efficiently phagocytized by macrophages compared to the other MS tested. PLGA 5000-MS and 5002-MS induced significant production of TNF-α, while 5000-MS, 5004-MS and 7502-MS decreased spontaneous IL-6 release. Nevertheless, only PLGA 5002-MS induced significant NFkB/SEAP activation. These findings together show that MS prepared with distinct PLGA co-polymers are differently recognized by macrophages, depending on proportion of lactic and glycolic acid in polymeric chain, and on molecular weight of the co-polymer used. Selection of the most adequate polymer to prepare a microparticulate drug delivery system to modulate immunologic system may take into account, therefore, which kind of immunomodulatory response is more adequate for the required treatment.

1. Introduction

Microspheres (MS) prepared with biocompatible, biodegradable polymers have been considered as drug delivery systems due to their ability to protect labile molecules and biomolecules against fast degradation [1–3] and to prolong the delivery of these pharmacologically active substances over longer periods of time [4]. In many situations, MS can also target drug delivery to its site of action, avoiding many toxic effects inherent to systemic biodistribution of drugs [1]<

In such context, microencapsulation of bioactive molecules for modulating the immune response during infectious or inflammatory events is a promising approach that has recently been studied [5–11]. It is considered that cells of immune system, especially resident macrophages, recognize MS as foreign and initiate the activation of innate immune response, *i.e.*, the inflammation [12]. Inflammatory process is mediated by cytokines, chemokines and lipid mediators, which are chemoattractant for neutrophils and macrophages and promote cell accumulation into the site of injury [13–15]. It has already known that variables such as shape, size and, mainly, the physico-chemical properties of the biomaterial used to

Abbreviations: MS, microspheres; PLGA, poly(D,L-lactide-co-glycolide); FBS, fetal bovine serum; PBS, phosphate-buffered saline; DMSO, dimethyl sulfoxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; LAL, *Limulus amoebocyte* lysate test; PI, phagocytic index.

* Corresponding author.
E-mail address: faccioli@fcfrp.usp.br (L.H. Faccioli).

http://dx.doi.org/10.1016/j.colsurfb.2015.10.011
0927-7765/

prepare the MS may be responsible for the degree and duration of the inflammatory response [16].

Phagocytosis is one of the main steps for induction of inflammatory mediators release and for triggering of inflammatory response [17]. Thus, phagocytosis, attachment and internalization of IgG-opsonized and non-opsonized polystyrene MS have already been analysed using rat alveolar macrophages and it was verified that the phagocytosis is size-dependent due to the attachment step [18]. However, little is known about the influence of the nature of polymers commonly used to prepare MS on their interaction with macrophages, and on the release induction of inflammation mediators. PLGA (poly [lactic-glycolic acid] co-polymers) are the most used material to produce pharmaceutical MS due to their high biocompatibility and biodegradability [19–20]. Depending on the ratio of lactic to glycolic acid used for the polymerization, different forms of PLGA can be obtained with different molecular weight and physico-chemical properties, as water solubility. For example, PLGA 50:50 co-polymer is comprised by 50% of lactic acid and 50% of glicolic acid in its chain, and is more hydrossoluble than PLGA 75:25 [21]. Chitosan is a natural, hydrophilic polymer obtained from chitin deacetilation, also described as non-toxic and biodegradable, but that presents lower cost and higher mucoadhesive properties if compared to PLGA co-polymers [22]. These features have made of chitosan a good alternative to PLGA as excipient to prepare MS.

The aim of this paper was to study *in vitro* the impact of polymeric MS prepared with different polymers on macrophages, in order to evaluate their potential to be used in the future as appropriate vehicles for delivering active biomolecules *in vivo*. To accomplish this objective, MS were prepared with chitosan, and with four different types of PLGA co-polymers (with different proportions of lactic and glycolic acid, and diverse inherent viscosities). MS were then characterized in relation to their sizes, zeta potentials and morphologic features. Following, macrophage cytotoxicity generated by all samples of MS was assessed, and non-toxic MS were employed in *in vitro* studies of cellular uptake, cytokines induction and NFkB activation.

2. Material and methods

2.1. Material

PLGA co-polymers (PLGA 50:50/i.v. = 0.1 dL/g, PLGA 50:50/i.v. = 0.2 dL/g, PLGA 50:50/i.v. = 0.4 dL/g, and PLGA 75:25 i.v. = 0.2 dL/g) were kindly provided by Purac (Gorinchem, Netherlands). Chitosan of medium molecular weight 190–310 kDa, 75–85% deacetylation, high purity (CAS 9012-76-4, Reference ALDRICH 448877-250G) was purchased from Sigma–Aldrich (Steinheim, Germany). β-glucan was obtained from Calbiochem (Merck Millipore, Darmstadt, Germany). Acetic acid, used in preparation of chitosan MS, and HCl were purchased from Fluka (Steinheim, Germany). Poly(vinyl-alcohol) (PVA, Mowiols 40–88) was purchased from Aldrich Chemicals (Wankee, WI, USA). LPS of *Escherichia coli* (serotype 0127:B8), dimethyl sulfoxide (DMSO), cytochalasin D, sodium dodecyl sulfate (SDS) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Methylene dichloride, acetonitrile (HPLC grade) and β-glucan were purchased from Merck (Dietikon, Switzerland). Panoptic staining was purchased from Laborclin (Paraná, Brazil). For the detection of endotoxin in formulations, a *Limulus amoebocyte lysate* test (LAL test, QCL-1000, Bio Whittaker, CAMBREX Company, East Rutherford, NY, USA) was used. Protein assays were performed using a Coomassie Protein Assay Reagent Kit from Pierce (Rockford, IL, USA). RPMI-1640 medium, fetal bovine serum (FBS) and antibiotics (penicillin and gentamicin) were obtained from Gibco (Grand Island, NY, USA). Plates from Becton Dickinson (BD Falcon, Franklin Lakes, NJ, USA) were used for cell cultures. Commercially enzyme-linked immunosorbent assay (ELISA) antibodies were used to measure cytokines (R&D Systems, Minneapolis, MN, USA). Purified monoclonal IgG antibody to mouse TLR2, LPS from *Rhodobacter sphaeroides*, Normocin™, Zeocin™ and QUANTIBlue™ were purchased from InvitroGen (San Diego, USA). The water used in all preparations was of Milli Q grade (Millipore, Darmstadt, German).

2.2. Preparation and characterization of microspheres

2.2.1. Preparation of PLGA-copolymers' microspheres

Briefly, 120 mg of each polymer (PLGA 50:50/i.v. = 0.1 dL/g; PLGA 50:50/i.v. = 0.2 dL/g; PLGA 50:50/i.v. = 0.4 dL/g; or PLGA 75:25/i.v. = 0.2 dL/g) was added to 10 mL of methylene dichloride and stirred until complete dissolution. This solution was emulsified with 40 mL of a PVA aqueous solution (3%, w/v) and continuously stirred for 4 h using a RW-20Homogenizer (IKA, Labortechnik, Germany) to assure solvent evaporation. All residues of evaporated methylene dichloride were captured by exhaustion system of the laboratory. The dispersion obtained was collected and centrifuged at 23,646 × *g* during 10 min. MS were washed three times with sterile water. After removal of the last supernatant, the MS sediment was freeze-dried, without any lyophilization additives, at −55 °C and 50 mm Hg during 12 h. All preparations were done in sterile conditions, *i.e.*, all flasks and equipment used in their preparation were previously autoclaved and manipulation always occurred under laminar flow, to avoid microbiological contamination of the samples.

2.2.2. Preparation of chitosan microspheres

Chitosan MS was prepared by spray drying as previously described [4]. In sterile conditions, a solution of chitosan at 1.5% (w/v) was obtained by dissolving the polymer under stirring in an aqueous solution containing 1% (w/w) of acetic acid, pH 4. Two hundreds mL of the chitosan solution was spray dried with a 1.0 mm pressurized atomizer at a feed rate of 6 mL/min in a Labmaq model MSD 0.5 spray dryer (Ribeirão Preto, Brazil). The atomizing airflow rate was 6 m^3/min, the inlet temperature was maintained at 135 °C, and the outlet temperature was 75–80 °C. Dried MS were collected in a sterile flask connected to the equipment.

2.2.3. Endotoxin measurements

Each batch of PLGA and chitosan MS were assayed using a *Limulus amebocyte lysate* (LAL) test to determine the possible presence of free LPS in samples. This test was performed according to the manufacturer's instructions and using 1 mg of each MS dispersed in 1 mL of sterile phosphate-buffered saline solution. The maximum level of endotoxin that is considered safe according to the European Pharmacopoeia is 5 EU/kg/h.

2.2.4. Characterization of microspheres

Size distribution of MS was determined using a LS 13 320 Laser Diffraction Particle Size Analyzer (Beckman Coulter, USA). Samples (1 mg) of either PLGA MS or chitosan MS dispersed in 0.4 mL of purified sterile water or ethanol, respectively, were analyzed at 25 °C. Zeta potential of MS was determined using a Zetasizer Nano (Malvern Instruments, England). Each sample was prepared dispersing 1 mg of PLGA or chitosan MS in 0.4 mL of purified water or ethanol/water (40:60 v/v), respectively, all containing 10 mM NaCl, and were analyzed at 25 °C. Morphology of MS samples was assessed by scanning electron microscopy (SEM) using a FEI Inspect S 50 scanning microscope (FEI; Oregon, USA).

2.3. Cytotoxicity assay

J774.1 murine macrophages cell line (obtained from the American Type Culture Collection, ATCC, Rockville, MD, USA) viability after exposure to each kind of PLGA or chitosan MS was evaluated using MTT colorimetric assay [23]. The cells were incubated for 24 h with 1 mL of each MS sample previously dispersed in water at a concentration of 1 mg/mL. Next, the medium were replaced to 5% MTT in RPMI-1640c without red fenol, and incubated for 3 h. Subsequently, 50 μL of 20% SDS in 0.01 M HCl were added to each well and maintained at room temperature until complete solubilization. DMSO was used as positive control of cell death. Absorbance was measured at 570 nm in a spectrophotometer (mQuanti, Bio-Tek Instruments,Inc., Winooski, VT) and was directly proportional to cell viability. MS cytotoxicities were expressed as a percentage of the relative cytotoxicity observed in the unstimulated control cells [24].

2.4. Cell culture and phagocytosis assay

To determine the phagocytic index, J-774.1 cells were cultured (2×10^5 cell/well) in 24-well plates with a 13 mm sterile circular cover slip in each well. After 1 h, the RPMI-c medium was gently removed, and 1 mg of MS dispersed in 1 mL of RPMI-c was added. After 4 h of incubation with the MS, the medium was again removed, and the non-ingested MS were gently washed away using warm, sterile phosphate buffered saline (PBS). The cover slips were then removed, stained with Panoptic solution (similar to Diff-Quick®) and affixed to glass slides. MS uptake was assessed by counting 200 cells, analyzing they ingested or not at least one MS. In the cell that engulfed MS it was counted the total numbers of MS into the cytoplasm. The phagocytic index (PI) was then calculated as following:

$$\mathrm{PI} = \frac{(\#\text{engulfed MS}) \times (\#\text{cells conatining at least one MS})}{\#\text{Counted cells}}$$

In a second set of experiments, cytochalasin D (5 μg), an inhibitor of cytoskeletal, was added 30 min before MS incubation, in order to inhibit the phagocytosis.

In a third set of experiments, J-774.1 cells (2×10^5 cell/well in 24-well plates) were exposed to 1 mg of each MS dispersed in 1 mL of media. After 24 h, the well supernatant was collected and storage at −20 °C to determine cytokines release.

All experiments were performed in quadruplicate, and were conducted in sterile conditions.

2.5. Cytokine concetration

The concentrations of TNF-α, IL-6, IL-1β and IL-10 in culture supernatants were quantified by ELISA using specific antibodies (purified and biotinylated) and cytokine standards, according to the manufacturers' instructions (BD Biosciences). The optical densities were measured at 405 nm and the cytokines concentrations determined using standard curve established with the appropriate recombinant cytokine. The sensitivity of the assays was 10 pg/mL.

2.6. NFkB report assay

RAW-Blue™ cells [RAW264.7 macrophages that stably express a secreted embryonic alkaline phosphatase (SEAP) gene inducible by the NF-κB/AP-1 transcription factors and resistant to Zeocin™, a selectable marker] were kindly donated by Dr Huy Ong (Université du Montréal, Canada). The protocol was adapted from that described before [25]: 2×10^5 cells/well were cultured in 96-well plates in DMEM medium supplemented with Normocin™ (50 μg/mL) at 37 °C in a humidified 5% CO_2 atmosphere. After 18 h, the cells were incubated for 24 h with 1 mg of the differents MS. LPS from *E. coli* (0.5 μg) or β- glucan (0.1 mg) from were used as positive control of TLR4 and TLR2 ligant, respectively, to induce cytokines production. After 24 h of stimulation, the medium was harvested, and 50 μL samples were mixed with QUANTIBlue™, a SEAP detection medium (150 μL), in 96-well plates at room temperature and was left overnight. In another experiment, the cells were pre-incubated during 0.5 h with 100 ng of purified monoclonal IgG antibody to mouse TLR2 (antagonist of TLR2) (anti-mTLR2-IgG—InvivoGen) and/or 10 ng of LPS from Rhodobacter sphaeroides (LPS-RS, an antagonist of TLR4). Then, they were stimulated with 1 mg of MS. After 24 h of stimulation, the medium was harvested, and 50 μL samples were mixed with QUANTIBlue™ (InvivoGen, San Diego, USA) a SEAP detection medium (150 μL), in 96-well plates at 37 °C for 2 h. The optical density was then measured at 650 nm using an ELISA reader.

Table 1
Mean diameter (μm) ± standard deviation (SD) and zeta potential (mV) of the MS prepared with chitosan and different PLGA co-polymers.

MS	Mean diameter ± SD (μm)	Zeta potential (mV)
Chitosan-MS	3.6 (±2.0)	+ 5.4 (±4.0)
5000-MS	4.4 (±2.5)	−8.2 (±39.4)
5002-MS	5.1 (±3.3)	+ 1.7 (±9.1)
5004-MS	4.4 (±2.5)	− 11.9 (±29.0)
7502-MS	4.2 (±2.2)	+ 1.7 (±11.9)

2.7. Statistical analyses

The data were expressed as the mean ± standard error of the mean. Statistical variations between the groups were determined using analysis of variance (ANOVA) and the Newman–Keuls posttest or t test in some set of the experiments. The results were analyzed using GraphPad Prism 5.0 software, and values with $P < 0.05$ were considered statistically significant.

3. Results

MS prepared with chitosan and different PLGA co-polymers presented monomodal population with similar diameter each other, ranging from 3.6 (±2.0) μm to 5.1 (±3.2) μm (Table 1). Zeta potential of all MS were near the neutrality, with quite wide variations in superficial charges (Table 1). Regarding morphology, MS prepared with different PLGA co-polymers (namely: 5000-MS, 5002-MS, 5004-MS and 7502-MS) were nonporous, spherical and non-aggregated. Chitosan-MS also exhibited spherical, non-porous surface, but it was observed a deposition of polymer on their surface, similar with other chitosan MS prepared with the same polymer following the same technique [4].

The endotoxin levels detected in all MS samples were lower than 0.1 EU/kg (data not shown), indicating that prepared MS were proper for therapeutic use, based on the criteria of the European Pharmacopoeia (5 EU/kg/h is the safety level required for endovenous administration).

Cytotoxicity assays, aiming to determine whether MS could be toxic to J-774.1 cells, were then performed. As can be observed in the Fig. 2, different from PLGAs MS, chitosan-MS presented higher cytotoxicity relatively to medium, being comparable to that induced by DMSO, used in this experiment as positive control. By this reason, the use of chitosan-MS to evaluate their phagocytosis was not considered appropriate.

To determine whether different PLGA-MS could be recognized and engulfed by macrophages, J-774.1 cells were incubated with the series of PLGA-MS and the percentage of phagocytosis and PI were determined. As It can be seen in the Fig. 3A, all MS were efficiently internalized by the cells. The PI values of macrophages

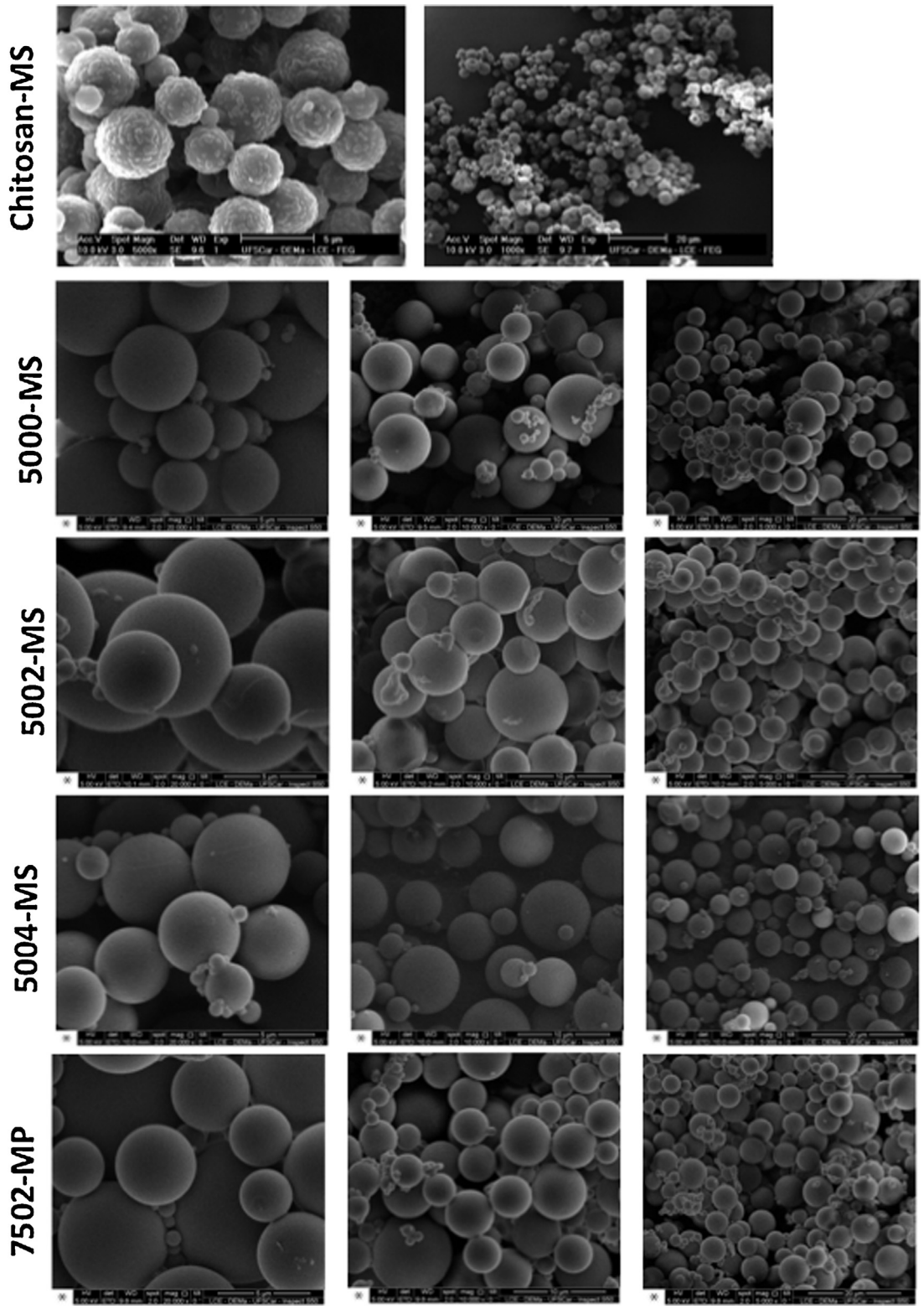

Fig. 1. SEM images of the MS prepared with chitosan and different PLGA co-polymers. Original magnifications of 5×, 10× or 20×.

incubated with 5000-MS and 7502-MS were higher and smaller than the others', respectively. The percentage of phagocytosis, which corresponds to the amount of cells that engulfed at least one MS, was similar for the four MS, being diminished in presence of 7502-MS. Fig. 3C shows macrophages containing the MS in the cytoplasm.

Following, the J-774.1 cells were previously treated with cytochalasin D, a drug that inhibits macrophage fusion and do not significantly decrease macrophage adhesion, spreading, or motility [26]. Fig. 4 shows that for all PLGA-MS, both percentage of phagocytosis and phagocytic index were diminished by the treatment with cytochalasin D, indicating that the entrance mechanism of MS into the cells is typically by phagocytosis.

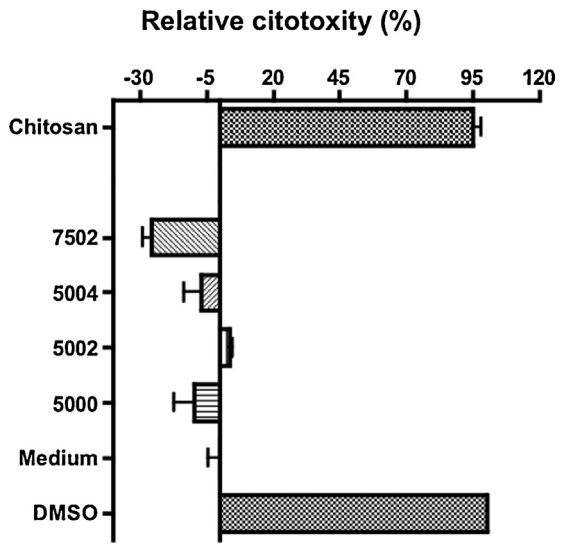

Fig. 2. Effects of MS on J774.1 viability. The J774.1 cells were plated with 1 mL of MS (1 mg/mL) during 24 h in 5% CO_2 at 37 °C, followed by cell viability measurement by MTT assay. Each column represents the mean value ± SEM of four replicates from two independent experiments.

It was also determined the cytokines released in the supernatants of J-774.1 cells after incubation with MS during 24 h. It was not detected either IL-1β or IL-10 in any of the samples. As can be observed in the Fig. 5, only 5000-MS and 5002-MS induced a significant increase of TNF-α compared to the medium (continued line). Interestingly, 5000-MS, 5004-MS and 7502-MS decreased the release ofIL-6 by J-774.1 cells compared to the medium. It is important to point out that all PLGA-MS induced a small release of cytokines, three times smaller than the concentration detected in supernatant of cells incubated with LPS, the positive control.

Finally, it was evaluated NFkB/SEAP activity in RAW-Blue™ cells after exposure to PLGA-MS. Fig. 6 shows that 5002-MS was more efficient in inducing NFkB/SEAP activation, although 5004-MS and 7502-MS also significantly activated this transcription factor. Similarly to the cytokines data, the activation of NFkB/SEAP by the MS was smaller than that observed with LPS, used as positive control. Antagonists of TLR (Toll-like receptors)-2 and -4 were used aiming to verify if the recognition of MS by macrophages was mediated by these receptors, which are present in cells membrane. As it can be observed in Fig. 6, pre-incubation of cells with TLR-2 antagonist did not modify NFkB/SEAP activation induced by the four MS. However, unexpected NFkB/SEAP activity was significantly increased by the pre-incubation of cells with TLR-4 antagonist following by 7502-MS, 5002-MS and 5004-MS. Interestingly, when the cells were pre-treated with TLR-2 and TLR-4 in combination, it was observed distinctive effects, dependent on the nature of the polymer use to prepare the MS.

4. Discussion

Our research group has devoted many effords to employ biological molecules, such as lipid mediatiors (prostaglandins and leukotriens), to modulate inflamatory response during infections [7–11]. Due to the high degree of lability of such biomolecules, microencapsulation have been shown as an effective alternative to delivery these molecules, maintaining their biological effects, and targeting these biomolecules delivery to effector cells, as macrophages, in a prolonged way. It has been observed, however, that different polymers used to prepare therapeutical MS can cause different effects on immune cells, especially macrophages [16].

Macrophages are resident cells that are the first line of defense and recognition of foreign substances and microorganisms [12]. The interation between macrophages and foreign species is mediated by pattern recognition receptors (PRRs) present in cell membrane that sense pathogen-associated molecular patterns (PAMPs) and others molecules such as venom-associated molecules (VAMPs). Among these PRRs, TLR-2 and TLR-4 recognize great range of molecules, such as bacterial and venom derived products, resulting in phagocytosis, cell activation and inflammatory mediators production [25,27].

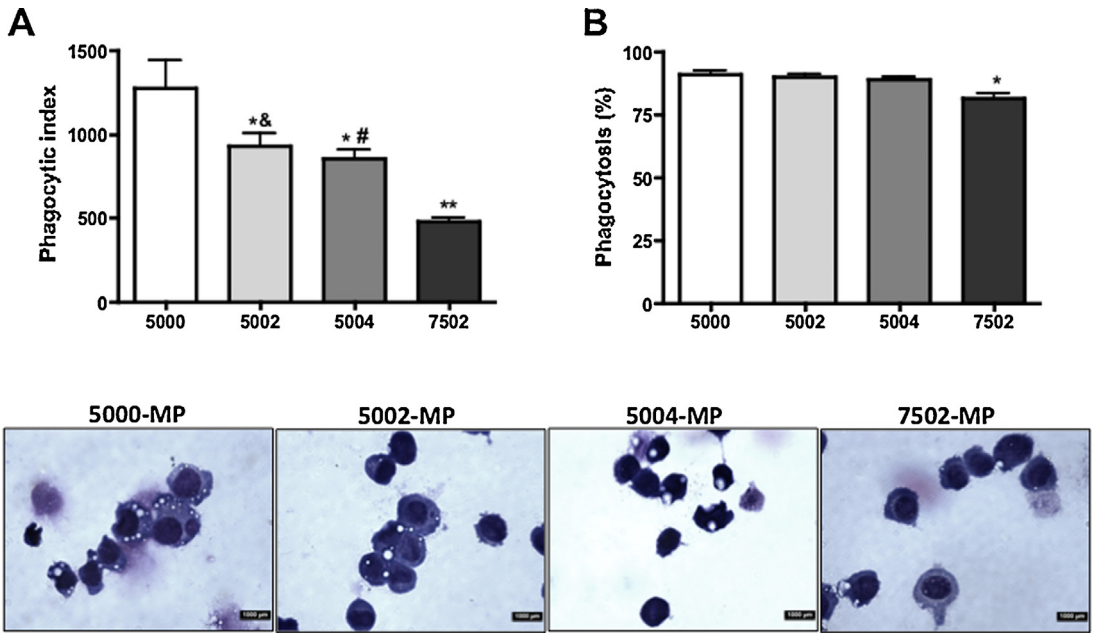

Fig. 3. Phagocytic cells recognize MS. (A) Uptake of the 5000-MS, 5002-MS, 5004-MS and 7502-MS by J-774 cells after 4 h of incubation. The values denote the mean ± SEM from four replicates of two independent experiments. (B) Percentages of J-774 cells that engulfed at least one MS (5000-MS, 5002-MS, 5004-MS or 7502-MS). The values denote the mean ± SEM from four replicates of two independent experiments. (C) Representative optical micrographs of J-774 cells that phagocytized 5000-MS, 5002-MS, 5004-MS or 7502-MS. The cells were stained with Panoptic and imaged using an optical microscope. Magnification: 100×/1.25 oil.

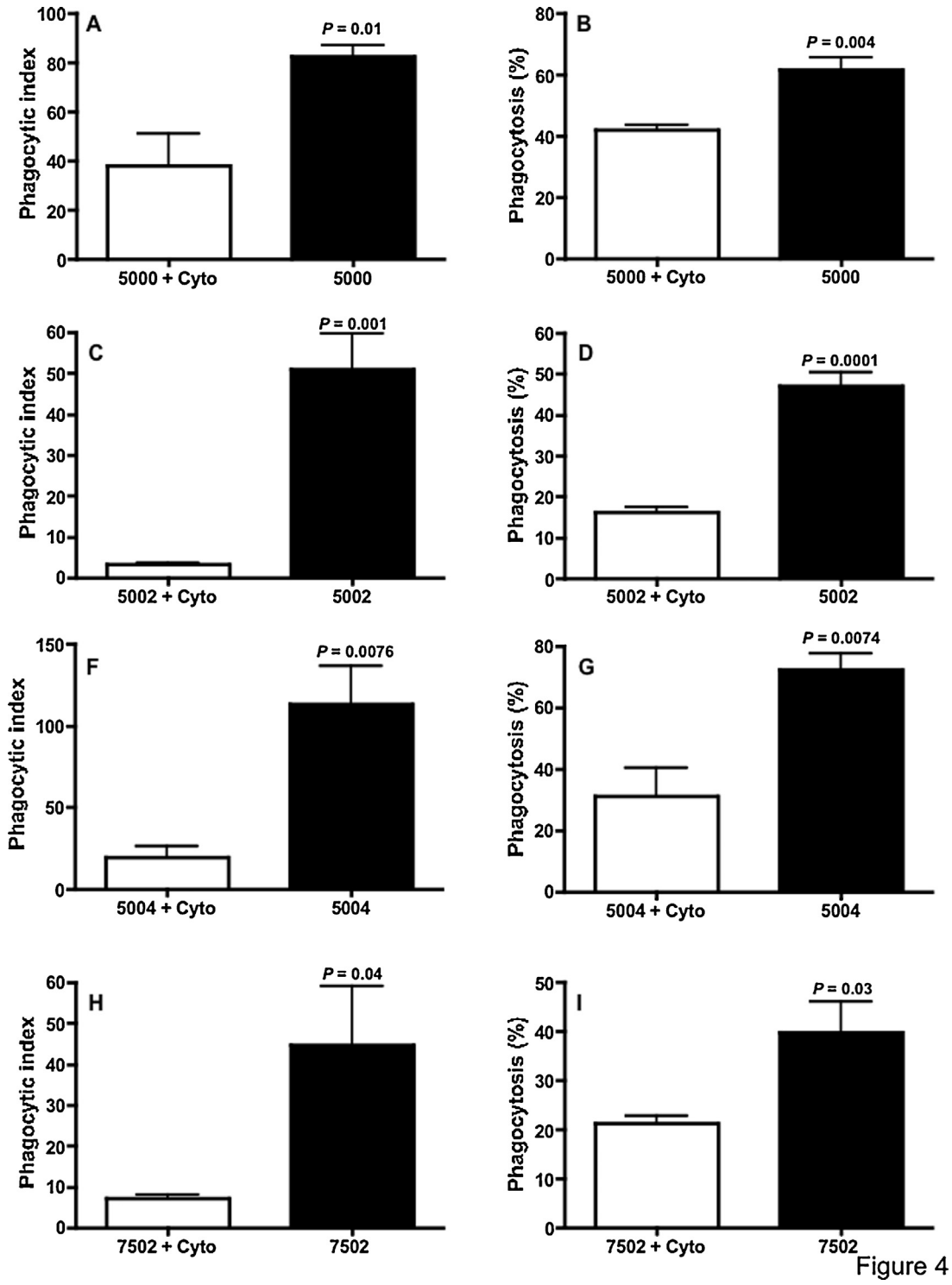

Fig. 4. MS enter the macrophages by phagocytosis. The uptake of (A) 5000-MS, (C) 5002-MS, (F) 5004-MS or (H) 7502-MS by J-774 cells after 4 h of incubation in the presence or in absence of cytochalasin D. The values denote the mean ± SEM from four replicates of two independent experiments. Percentages of J-774 cells that engulfed at least one MS [(B) 5000-MS, (D) 5002-MS, (G) 5004-MS or (I) 7502-MS] in presence or in absence of cytochalasin D. The values denote the mean ± SEM from four replicates of two independent experiments. *P* values are indicated in the figure.

The aim of this paper was to study the consequence of macrophage interaction with MS prepared with chitosan and four distinct PLGA co-polymers, which possess different proportions of glycolic acid/lactic acid in their composition or different molecular weight each other. In this way, it was first determined cell cytotoxicity induced by the each sample of MS, and it was observed that MS prepared with the natural polymer chitosan were quite toxic to the macrophages. On the other hand, the four PLGA MS showed insignificant cytotoxicity to macrophages, comparable to the medium used as negative control (Fig. 2). This finding suggests that chitosan MS should not be used as vehicle to inhalable drugs, since products released with cell destruction caused by the interaction with this kind of MS are described to be potent inducers of inflammatory reactions, as demonstrated before [28,29].

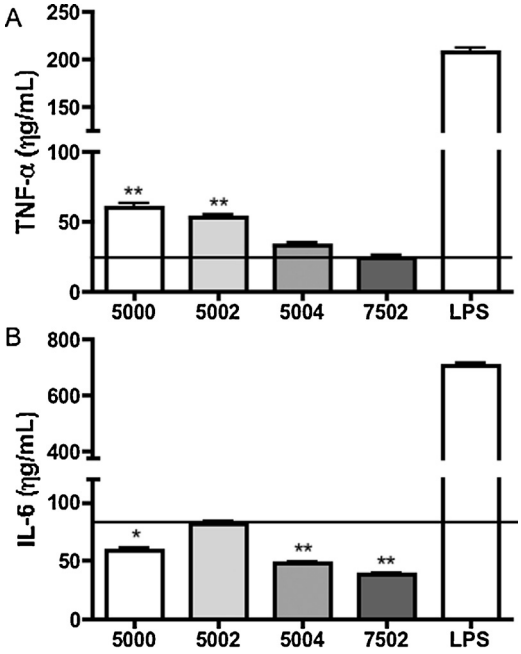

Fig. 5. MS induce cytokines release. (A) TNF-α and (B) IL-6 assayed by ELISA after J-774 incubation with different MS. The black line indicates RPMI-1640 cytokines mean value. A - The values denote the mean ± SEM from one representative result of three independent experiments using ANOVA. $^{**}P < 0.001$ compared to RPMI-1640. B - The values denote the mean ± SEM from one representative result of three independent experiments using ANOVA. $^{**}P < 0.001$, $^{*}P < 0.01$ compared to RPMI-1640.

It was then evaluated the potential of MS prepared with four different PLGA co-polymers to be uptaken by macrophages, and to activate these cells. 5000-MS, 5002-MS and 5004-MS were all prepared with PLGA 50:50 co-polymers, *i.e.*, PLGA co-polymers constituted by the same proportion of lactic acid in comparison to glycolic acid in their composition, but with different molecular weight each other, which result in different inherent viscosity (0.1 dL/g; 0.2 dL/g and 0.4 dL/g, respectively). 7502-MS, in its turn, were prepared with PLGA 75:25 co-polymer, which contain in this molecules 75% of lactic acid and 25% of glycolic acid (inherent viscosity of 0.2 dL/g). The data presented in this paper have shown that these differences in proportion of lactide and glycolide and in molecular weight of PLGA co-polymers in fact plays some influence in the interaction of the resulting MS with the phagocytic cells. 5000-MS were more efficiently internalized by macrophages in comparison with the others, since the phagocytic index of macrophages in its presence was the highest, being 28%, 33% and 62% more internalized than 5002-MS, 5004-MS and 7502-MS, respectively. Kamei and collaborators have found that MS produced with PLGA 75:25 with molecular weight of 10 kDa degraded twice faster than those prepared with the polymer with molecular weight of 20 kDa when in contact with interstitial fluid of the skin, but the degradation rate was the opposite when the MS were placed in contact to the lung [30]. All together, these results can indicate that there is a role on polymer degradation rate, where MS that remain intact for longer periods have more opportunity to be phagocyted by macrophages. Taking into account that the lower the inherent viscosity of the PLGA co-polymer, the more time the PLGA MS remain intact, 5000-MS seems to be more suitable to undergo phagocytosis by alveolar macrophages than the other ones.

It was also established in this paper that all PLGA-MS entered in the cells following the phagocytosis mechanism, since J-774.1 macrophages pre-treated with cytochalasin D, a drug that inhibits macrophages fusion [31], significantly decreased MS engulfment (Fig. 4). A previous published study evaluating chitosan particles also verified that the inhibition of phagocytosis with cytochalasin D suppressed the inflammatory reaction induced by chitosan, and abolished inflammasome activation [32]. The authors, however, did not evaluate cell toxicity induced by chitosan particles in that opportunity. Since it was verified in this paper that chitosan-MS are toxic to alveolar macrophages, it can be suggested that by inhibiting chitosan-MS phagocytosis, cytochalasin D prevents cell lysis and inflammatory reaction, as demonstrated before [32].

Nevertheless, the data presented in this paper have demonstrated that PLGA MS internalization, without inducing cell lysis, provoked NFkB activation and production of inflammatory mediators. It was indeed expected that it could occurs, since inflammation is triggered by phagocytosis, with cells activation after the stimuli be sensed by PPRs during innate immune response [25]. However, diverse intracellular mechanisms seem to activate macrophages, depending on the MS evaluated. 5000-MS, which was more efficiently phagocytized (Fig. 1), also induced high concentrations of TNF-α but inhibits IL-6 production (Fig. 5), with discrete activation of NFkB (Fig. 6). On the other hand, 5002-MS similarly induced TNF-α, did not inhibited IL-6, but was the most efficient MS for NFkB activation. Intriguing, 7502-MS induced NFkB stimulation without promoting cytokines production. These results inferred that the four MS are differently recognized by distinct PRRs (including other PPRs not evaluated in this work). This fact might be related to activation/inhibition of specific signaling pathway, resulting in particular inflammatory mediators production [33]. It is hypothesized that each PLGA MS activates a different intracellular signaling pathway, which can be supported by literature [17,25,34,35]. It is shown that 5002-MS, 5004-MS and 7502-MS activated NFkB compared to the media; however this activation was not so high if compared to that induced by LPS, used as the positive control. These data suggest that after blocking TLR-4, the MS may interact with unoccupied unknown receptors trigging NFkB/SEAP activation, or may decrease production of mediators that inhibits phagocytosis, such as IL-10.

In summary, the results presented in this paper demonstrate that macrophages sense MS by distinct PRRs, resulting in differences in phagocytosis and cell activation, a phenomenon dependent on the nature of PLGA polymers. In other words, proportion of lactic and glycolic acid present in the polymeric chain, and the molecular weight of the polymer chain, play a role on the

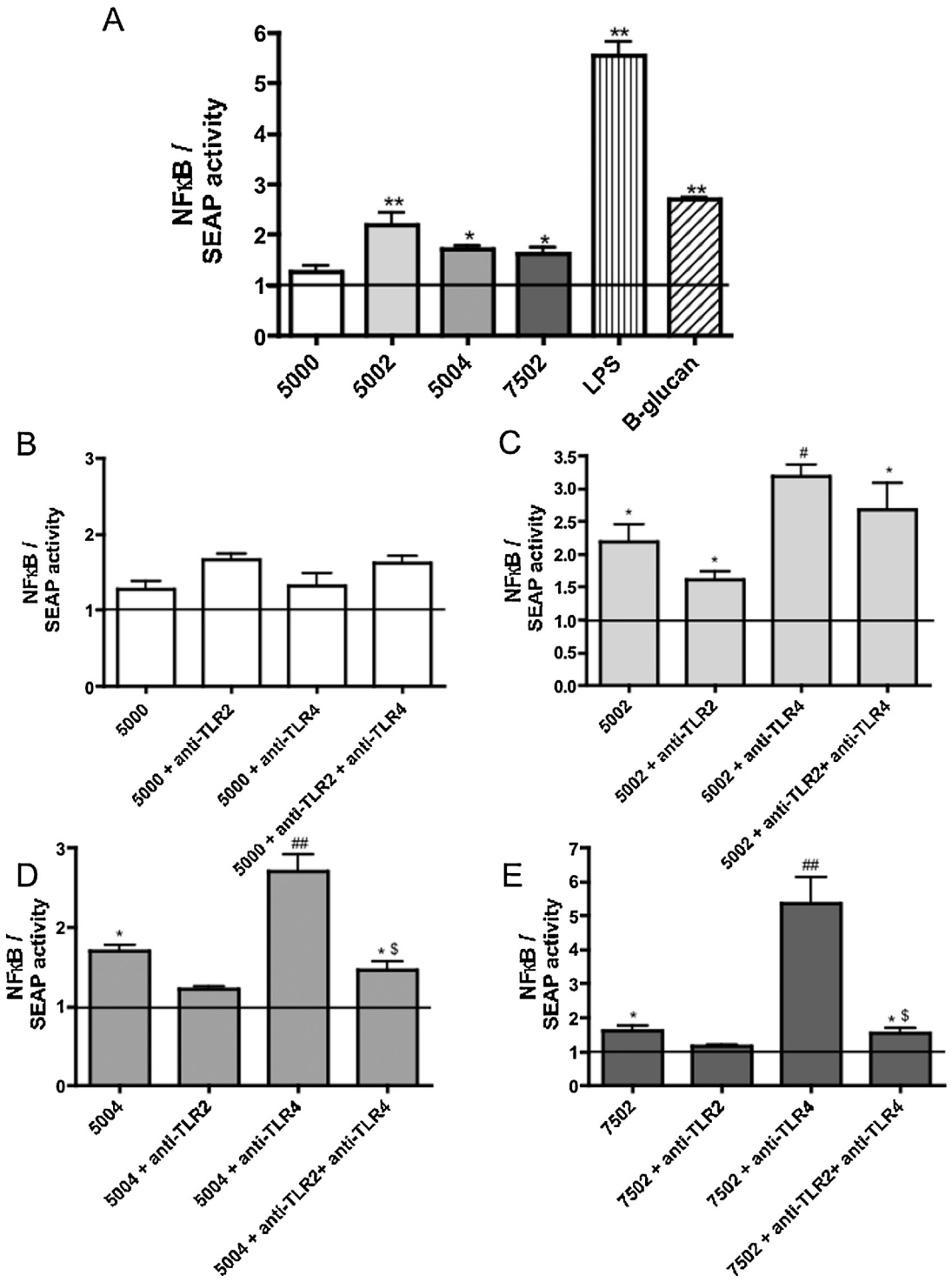

Fig. 6. MS induce NF-κB/AP-1 activation. RAW-Blue™ cells are derived from RAW 264.7 macrophages containing the chromosomal integration of a SEAP reporter construct inducible by NF-κB. The black line indicates DMEM medium mean. The values denote the mean ± SEM from four replicates from two independent experiments. $^{**}P < 0.001$ compared to medium and $^{*}P < 0.01$ compared to medium.

interaction between the MS and cells from innate immunity. Moreover, it was shown that chitosan-MS seems not be an appropriate alternative to be used as delivery system to target macrophages, as it can cause cell death. This hypothesis, however, will be confirmed in future experiments using an *in vivo* model. Finally, these findings have important implications in the selection of the most adequate polymer to prepare microparticulate drug delivery system for using to modulate immunologic system, both in situations where an immunomodulatory response is desirable (*e.g.* enhancing adjuvant in vaccination) and in those where it is not (*e.g.* other pharmaceutical purposes).

Acknowledgments

The authors would like to acknowledge Purac Biomaterials, which kindly donated PLGA polymers used in this work, and J.

O. D. Ciampo for technical assistance. These studies were funded by the São Paulo Research Foundation (FAPESP, Brazil), grants #2007/04741-4, #02/12856-2, #05/54855-0 and #2009/07169-5; and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brazil), grant #150991/2011-8.

References

[1] G.M. Gelfuso, M.A. Barros, M.B. Delgado-Charro, R.H. Guy, R.F. Lopez, Colloids Surf. B Biointerfaces 134 (2015) 408.
[2] M. Parlato, A. Johnson, G.A. Hudalla, W.L. Murphy, Acta Biomater. 9 (2013) 9270.
[3] G. Tiwari, R. Tiwari, B. Sriwastawa, L. Bhati, S. Pandey, P. Pandey, S.K. Bannerjee, Int. J. Pharm. Invest. 2 (2012) 2.
[4] G.M. Gelfuso, T. Gratieri, P.S. Simão, L.A.P. de Freitas, R.F.V. Lopez, J. Microencapsul. 28 (2011) 650.
[5] I.M. Meraz, B. Melendez, J. Gu, S.T. Wong, X. Liu, H.A. Andersson, R.E. Serda, Mol. Pharm. 9 (2012) 2049.
[6] S.M. Moyes, J.F. Morris, K.E. Carr, J. Anat. 217 (2010) 740.
[7] D.F. dos Santos, R. Nicolete, P.R. de Souza, C.S. Bitencourt, R.R. dos Santos Jr., V.L. Bonato, C.L. Silva, L.H. Faccioli, Eur. J. Pharm. Sci. 38 (2009) 548.
[8] D.F. dos Santos, C.S. Bitencourt, G.M. Gelfuso, P.A. Pereira, P.R. de Souza, C.A. Sorgi, R. Nicolete, L.H. Faccioli, Eur. J. Pharm. Sci. 44 (2011) 580.
[9] R. Nicolete, K.M. Lima, J.M. Júnior, P.J. Jose, M.J. Sanz, L.H. Faccioli, Eur. J. Pharm. Biopharm. 70 (2008) 784.
[10] R. Nicolete, A. Secatto, P.A. Pereira, E.G. Soares, L.H. Faccioli, Int. J. Antimicrob. Agents. 34 (2009) 365.
[11] P.A. Pereira, C. da Silva Bitencourt, D.F. Dos Santos, R. Nicolete, G.M. Gelfuso, L.H. Faccioli, Eur. J. Pharm. Sci. 2 (2015) 132.
[12] S. Gordon, Eur. J. Immunol. 37 (2007) S9.
[13] L.H. Faccioli, S. Nourshargh, R. Moqbel, F.M. Williams, R. Sehmi, A.B. Kay, T.J. Williams, Immunology 73 (1991) 222.
[14] R. Medzhitov, Nature 454 (2008) 428.
[15] Y. Tabata, Y. Ikada, Adv. Polymer Sci. 94 (1990) 107.
[16] P. Pacheco, D. White, T. Sulchek, PLoS One 8 (2013) e60989.
[17] T.H. Mogensen, Clin. Microbiol. Rev. 22 (2009) 240.
[18] J.A. Champion, A. Walker, S. Mitragotri, Pharm. Res. 25 (2008) 1815.
[19] K.M. Lima, J.M. Rodrigues Júnior, Braz. J. Med. Biol. Res. 32 (1999) 171.
[20] X.S. Wu, Artif. Cells Blood Substit. Immobil. Biotechnol. 32 (2004) 575.
[21] M.S. Shive, J.M. Anderson, Adv. Drug Deliv. Rev. 28 (1997) 5.
[22] P.P. Shah, R.C. Mashru, A.R. Thakkar, A.C. Badhan, J. Pharm. Pharmacol. 60 (2008) 421.
[23] T. Mosmann, J. Immunol. Methods 65 (1983) 55.
[24] K.F. Zoccal, C.S. Bitencourt, A. Secatto, C.A. Sorgi, K.D. Bordon, S.V. Sampaio, E.C. Arantes, L.H. Faccioli, Toxicon 57 (2011) 1101.
[25] K.F. Zoccal, C.S. Bitencourt, F.W. Paula-Silva, C.A. Sorgi, K. de Castro Figueiredo Bordon, E.C. Arantes, L.H. Faccioli, PLoS One 9 (2014) e88174.
[26] K.M. De Fife, C.R. Jenney, E. Colton, J.M. Anderson, FASEB J. 13 (1999) 823.
[27] H. Kumar, T. Kawai, S. Akira, Int. Rev. Immunol. 30 (2011) 16.
[28] K. Grattendick, R. Stuart, E. Roberts, J. Lincoln, S.S. Lefkowitz, A. Bollen, N. Moguilevsky, H. Friedman, D.L. Lefkowitz, Am. J. Respir. Cell Mol. Biol. 26 (2002) 716.
[29] E.L. Taylor, I.L. Megson, C. Haslett, A.G. Rossi, Cell Death Differ. 10 (2003) 418.
[30] S. Kamei, Y. Inoue, H. Okada, M. Yamada, Y. Ogawa, H. Toguchi, Biomaterials 13 (1992) 953.
[31] J.A. Elliott, W.C. Winn Jr., Infect. Immun. 51 (1986) 31.
[32] C.L. Bueter, C.K. Lee, V.A. Rathinam, G.J. Healy, C.H. Taron, C.A. Specht, S.M. Levitz, J. Biol. Chem. 286 (2011) 35447.
[33] K. Takeda, S. Akira, Semin. Immunol. 16 (2004) 3.
[34] M. Fujihara, M. Muroi, K. Tanamoto, T. Suzuki, H. Azuma, H. Ikeda, Pharmacol. Ther. 100 (2003) 171.
[35] G. Bonizzi, M. Karin, Trends Immunol. 25 (2004) 280.