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Incorporation of PVMMA to PLGA MS enhances lectin grafting and their *in vitro* activity in macrophages

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ABSTRACT

Attachment of lectins, especially those that are recognized and bounded by macrophages and other antigen presenting cells onto biodegradable microspheres surface, may serve as support for a wide variety of applications. The aim of this work was: (1) to prepare microspheres (MS) based on two biodegradable copolymers, poly (lactide-co-glicolide) acid (PLGA) and poly[(methyl vinyl ether)-co-(maleic anhydride)] (PVMMA) with their surface functionalized with Concanavalin A (Con A) and, (2) to evaluate their behaviour in macrophage culture. The incorporation of PVMMA was studied in order to augment the lectin coupling efficiency. MS were obtained by spray-drying and bovine serum albumin (BSA) was loaded as protein model. Particles were characterized for size, morphology, surface charge, entrapment efficiency, FTIR, *in vitro* protein release and conjugation efficiency. Finally, functionalized MS were tested *in vitro* with raw 264.7 murine macrophages (Mϕ) in terms of cytotoxicity, phagocytosis, nitric oxide (NO) production, and oxygen consumption. Conjugated Con A microspheres showed increased grafting efficiency up to four times compared to PLGA alone. The retention of Con A after coupling was confirmed by desorption studies. The attachment of Con A to microspheres induced oxygen consumption, increased phagocytosis efficiency and even NO production by macrophages. The results suggest that Con A and possibly, other lectins, grafted onto PLGA–PVMMA microspheres may serve as potential adjuvants by modulating protein delivery and macrophage activation.

1. Introduction

Efficient efforts have been made so far in order to improve the functionality of microparticulate carriers by modification of their surface with lectins using mainly PLGA nano and microspheres. Covalent coupling of lectins onto carboxylic groups on PLGA microspheres surface has been reported using different methods (Ertl et al., 2000; Montisci et al., 2001; Roth-Walter et al., 2005). However, the main limitation of PLGA microparticles is the low density of reactive groups on their surface. To overcome this disadvantage, we explored a new alternative to improve the covalent immobilization of Con A based on PLGA/PVMMA microspheres that may increase the coupling of lectins to the MS surface by carbodiimide chemistry.

Biodegradable polylactide-co-glycolide (PLGA) microspheres have been widely used for delivering and controlling the release of antigens and proteins encapsulated, coupled or adsorbed to particles (Rafferty et al., 1996; Waeckerle-Men and Groettrup, 2005; Storni et al., 2005). Also, poly[(methyl vinyl ether)-co-(maleic anhydride)] (PVMMA) has been employed as delivery systems in a variety of applications such as oral drug administration or eliciting immune responses (Arbós et al., 2002; Gómez et al., 2006; Salman et al., 2008).

The interest in microparticles as a platform for delivery of antigens has been increasing in the latest years. These particles are recognized by mononuclear phagocytic system cells (mainly, macrophages and dendritic cells) which remove and clear them via unspecific phagocytosis (Juliano, 1988). Size and ζ-potential of particles are critical for efficient phagocytosis. Antigen particulate carriers sized below 10 μm offer several attributes to be used as vaccine delivery systems because microspheres have a similar size to pathogens and thus, they can be efficiently internalized by antigen presenting cells (dentritic cells, macrophages) (Torché et al., 1999; Prior et al., 2002; Luzardo-Álvarez et al., 2005). Also, it is generally recognized that hydrophobic, cationically charged microspheres and the presence of ligands by surface modification can control phagocytosis and could affect the type of response of phagocytic cells (Ahsan et al., 2002; Kempf et al., 2003; Thiele et al., 2003; Wattendorf et al., 2008).

Macrophages (Mϕ) are considered first line host defence against microbiological infections. Their capacity of phagocytosis and antigen processing render the most attractive targets for microspheres

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(Ahsan et al., 2002). Furthermore, Mϕ posses cell surface receptors for a wide variety of molecules, including lectin recognizing receptor (Goldstein, 1976). Concanavalin A (Con A) is a lectin isolated from *Canavalia ensiformis* (Jack bean) well known and well characterized. Con A consists of four identical protomers held together by polar interactions, hydrogen bonds, and electrostatic interactions. At pH above 7, Con A exists as a tetramer with three specific sites: one site for carbohydrate α-d-mannose/glucose, another for divalent cations (Ca^{2+} and Mn^{2+}), which activates the protein for carbohydrate interaction, and yet another hydrophobic site (Edelman et al., 1975; Loris et al., 1998). Carbohydrate binding activity of Con A induces different cellular responses through the specific recognition of glycoproteins (α-D-mannosyl and α-D-glucosyl residue) and glycolipids present on the plasma membrane of different cell types. Also, naturally ingested lectins interact with gut epithelium and degenerate it, enter body tissue and interact with different cells, particularly macrophages, resulting in their activation. Specifically, Con A induces enhanced production of NO and the activation of MAPK, transcription factor, expression of Toll-like receptors and production of proinflammatory cytokines (Edelson and Cohn, 1974a,b; Andrade et al., 1999; Kesherwani and Sodhi, 2007; Sodhi et al., 2007) by means of specific binding to specific receptors or glycosylated binding sites of macrophage membrane (Warton and Papadimitriou, 1984; Peschke et al., 1990; Sodhi and Kesherwani, 2007).

The aim of this work was to develop new biodegradable microspheres based on poly (lactide-co-glicolide) acid (PLGA) and Poly(metyl vinyl ether-co-maleic anhydride) Gantrez® AN (PVMMA) with their surface functionalized with Con A lectin. Physicochemical characteristics of microspheres and the effect of Gantrez to increase the amount of functional groups of the microspheres where lectin can be attached were investigated. Encapsulated protein release and grafted Con A desorption were studied. The capacity of these new delivery systems of enhancing covalent bounding or adsorbed Con A in their surface to modulate *in vitro* activation of macrophages was evaluated by phagocytosis assays, NO release and oxygen consumption.

2. Materials and methods

2.1. Materials

Bovine Serum Albumin (BSA) and Concanavalin A (Con A) and their fluorescein analogues were purchased from Sigma Chemical co. and Fluka Biochemika respectively (Sigma–Aldrich Química, Madrid, Spain). Poly lactic-co-glycolic acid (PLGA), RG503H and RG502H, were both carboxylic end-group uncapped, and they were obtained from Boehringer Ingelheim (Ingelheim Germany). Poly (metyl vinyl ether-co-maleic anhydre) Gantrez® AN119 was kindly gifted by ISP Corporation (Barcelona, Spain). Ethyl formiate and acetone were purchased from Merck, Spain.

2.2. Preparation of PLGA–PVMMA microspheres

PLGA–PVMMA microspheres were prepared by spray-drying using a Mini Büchi 190 B. Four different types of microspheres were prepared. The production of different microspheres was limited by the solubility of polymers on different solvents. For one type of formulation (PLGA RG503H–PVMMA AN119) 2.6% PLGA was dissolved in ethylformiate and a dispersion of 60 mg PVMMA was prepared in acetone and cross-linked with triethanolamine (45 mg/ml). Both polymers were then mixed and spray-dried. For PLGA RG502H–PVMMA AN119 acetone was also used as solvent con PVMMA (90 mg). For the PLGA RG503H–PVMMA AN139 microspheres preparation, a different solvent, acetic acid, was used and triethanolamine was the cross-linker for PVMMA in the same manner. Dispersion of polymers was spray-dried through a 0.7-mm nozzle installed in a Mini Spray-Dryer 190 (Büchi, Flawil, Switzerland). The product was feed at 3 ml/min, inlet and outlet temperatures were at 42 and 72 °C, respectively, and a pressurized air flow of 500 l/h. Finally, microspheres were always collected in cyclone and kept under vacuum at room temperature for 18 h.

2.3. FITC-BSA encapsulation

Bovine Serum albumin was chosen as model protein for entrapment inside microspheres to study protein release stability and microsphere characteristics. FITC-BSA loaded PLGA–PVMMA microspheres were prepared by emulsion W/O. 250 μl of the internal aqueous protein phase of FITC-BSA (2%) was emulsified by ultrasonication Sonopuls, Bandelin Electronic, GmbH, Berlin, Germany) using 2 cycles of 10 pulses in ethyl formiate containing PLGA. Afterwards this emulsion was added into PVMMA solution and was atomized according to the methodology described above.

2.4. Covalent coupling of Con A and FITC-Con A

In order to activate carboxylic groups from PLGA polymer, covalent coupling of protein to microspheres surface was carried out using a variation of the carbodiimide method with few modifications. Three different protocols were tested. 10 mg of PLGA microspheres were suspended in 1 ml MES buffer [2-(N-morpholino)ethanesulfonic acid] 0.1 M pH 3.3 with the following ratios of N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide (EDC) and N-hydroxysuccinimide (NHS) (50:35; 10:7; and 100/70 mM, respectively). The suspension was rotated for 10 min at 20 °C and 500 rpm. After removal of excess reagents by washing three times with MES-buffer, microspheres were incubated for 30 min with 1 ml of Con A or FITC-Con A solution 0.5; 0.7 and 1 mg/ml in phosphate buffered saline (PBS; at pH 7.4). Microspheres were collected by centrifugation 5000 × *g* for 15 min and washed twice with PBS and one last time with water. Microspheres were freeze-dried and stored at 4 °C.

2.5. Microspheres characterization

2.5.1. Microspheres morphology

Surface morphology of the microspheres was analysed by scanning electron microscopy (SEM). After drying, microspheres were mounted onto a double faced adhesive tape, which was fixed on supports. The samples were sputtered with gold using Biorad E5000 sputter coater. Photomicrographs were taken at different magnification 1000×, 3000×, 12,000× with LEO 435 vp (LEO Electron Microscopy Ltd., Cambridge, UK).

2.5.2. Particle size measurement

Microspheres size and size distribution were measured by laser light-scattering method using Mastersizer (Malvern Instruments, UK). Microspheres were dispersed in distilled water and Tween 20 was used as wetting agent to prevent agglomeration. Measurements were taken at room temperature under continuous stirring.

2.5.3. ζ-Potential measurements

Surface charge of microparticles was determinated by zeta potential measurement (Zeta Plus Potential Analyzer; Brookhaven Instruments Corporation, New York, USA) in distilled water and acetic acid (0.05 M). Ten different measurements for each sample were performed.

2.5.4. Determination of carboxylic groups

Acid-base titration method was used to determine the content of acid groups onto microspheres surface. Microspheres (50 mg) were

resuspended in distilled water and mixed with 10 μl of phenolphthalein (10 mg/ml) by stirring. Then, small volumes of NaOH 0.1 M were added carefully to a phenolphthalein end point. The excess of NaOH was back-titrated with HCl 0.1 M. The difference in added volume of HCl was a direct measure for the amount of carboxylic groups.

2.5.5. Encapsulation efficiency

A 5 mg quantity of microspheres was dissolved in 3 ml of ethyl formiate and 1 ml PBS was added. The mixture was stirred vigorously and FITC-BSA was extracted from it to the aqueous phase. BSA content was determined by measuring fluorescence with Fluorostar Optima equipment (BMG Labtech, Biogen, Madrid, Spain) using 485 nm for excitation wavelength and 520 nm for emission wavelength. The encapsulation efficiency of BSA was calculated according to following equation:

$$\text{E.E. (w/w\%)} = \frac{M_{ENC}}{M_0} \times 100$$

where M_{ENC} is the real content of BSA in the MS and M_0 is the theoretical amount of drug in the formulation (theoretical loading).

2.5.6. Coupling efficiency

The amount of conjugated Con A (μg) to microspheres was estimated by quantifying the amount of unbound lectin recovered upon centrifugations and washings of microspheres and substracting it from the initial amount of lectin. Coupling efficiency was expressed as percentage of conjugated lectin compared to the initial amount of Con A. The amount of lectin was determinated by using two methods: Quanti Pro BCA assay (Sigma Aldrich) and also measuring fluorescence intensity with a Fluorostar Optima equipment (BMG Labtech, Biogen, Madrid, Spain) using 485 nm as fluorescence excitation and 520 nm for emission wavelength, respectively.

2.5.7. In vitro release

In vitro BSA release was determinated by incubating microspheres (40 mg microspheres) in 4 ml PBS pH 7.4, 37 °C with rotation (100 rpm; Heidolph Instruments incubator 1000) until 100% of protein release was achieved. The amount of protein released was carried out taking samples at every stipulated times intervals and supernatant and particles were separated by centrifugation. The protein amount was determined by measuring in a spectrophotometer using Quanti Pro BCA protein assay kit (Sigma Aldrich, Madrid, Spain). The experiment was carried out in triplicate.

2.5.8. Infrared studies

IR spectroscopy was carried out to confirm the conjugation of Con A to microspheres. Samples were prepared in KBr discs and transmittance was measured from 400 to 4000 cm^{-1} using Bruker spectrophotometer (IFS-66V, Bruker, Massachusetts, USA).

2.5.9. Lectin desorption studies

40 mg of accurately weighted microspheres were placed in phosphate buffered saline (PBS 10 mM, pH 7.4) and shaken in an incubator at 37 °C. At predetermined intervals, samples were taken and centrifuged (5000 × *g* for 10 min; Hettich centrifuge; Hettich International, Tuttlingen, Germany). The amount of lectin in the supernatant was determined using the BCA assay kit (Sigma–Aldrich Química, Madrid).

2.6. Cell culture studies

2.6.1. Macrophages and cell culturing

RAW 264.7 Mϕ (RAW Mϕ) were purchased from ECACC (UK). Macrophages were cultivated in Dubelcco's Modified Eagle's medium (Sigma Aldrich, Spain) containing 10% of inactivated Fetal bovine Serum (FBS) and 1% of antibiotic/antimycotic mixture consisting of 10,000 Units/ml penicillin, 10,000 units/ml Streptomicin in 0.85% saline (Chemicon International). Macrophages were cultured in a humidified 5% CO_2 atmosphere at 37 °C.

2.6.2. In vitro uptake of microspheres by Raw 264.7 macrophages cells

To investigate whether lectin-coated microspheres would increase particle uptake by macrophages, microspheres were incubated in macrophages culture. With this aim 5×10^5 cells were fed in a 24 well plate in 1 ml DMEM medium for 24 h. Afterwards, 25 μg/ml of microspheres were co-incubated for 1 h. As a reference we used uncoated microspheres and PLGA MS. The extent of phagocytosis was examined by microscopy by counting the amount of cells that had ingested at least one microsphere. Microspheres uptake was determined by quantification of at least 200 cells from each assay. As negative control we have used cells without microspheres and as positive control we used yeast. Cells were fixed in methanol and stained with Giemsa.

In order to determinate if coated microspheres uptake was related with the Con A membrane receptor or binding site, competition assays with manann and mannose were performed in 24 well plates. Microsphere were co-incubated with mannose 10 μg/ml for 15 min and added to the medium for 1 h. Also, in a different assay, microspheres were added to the cells with 8 μg/ml of mannan for 1 h and cells were fixed with methanol and dyed with Giemsa to quantify cells.

2.6.3. NO production

NO production by Mϕ upon exposure to unloaded MS was measured by the Griess method after 48 h. Supernatant from the cell cultures (100 μl) was incubated with an equal volume of Griess reagent mixtures (1% sulfanilamine, 0.1% N-(1-naphtyl)-ethylendiamine dihydrochloride, 2.5% H_3PO_4) at room temperature for 10 min. The absorbance was measured in a microplate reader (Multiskan Ascent; Thermo Fisher Scientific, Madrid, Spain), and concentrations calculated from a sodium nitrite standard curve (1–100 μM). RAW Mϕ were left untreated (negative control), co-incubated with Zymosan (1 μg/ml), Concanavalin A (10 μg/ml) and IFN-γ (10U/ml) (also used as positive controsl), or with MS alone. The inhibitor of NO synthase 2 L-NMMA (N-monomethyl-L-arginine monoacetate) was added at 250 μM.

2.6.4. Respiratory measurements

In order to determinate the effects on respiratory metabolism of Mϕ after uptake of microspheres, we measured the oxygen consumption associated with phagocytic uptake using an Oxymeter (Oxygraph Hansatech Instruments Ltd., Norfolk, England) equipped with a Clark electrode. Exogenous respiration of the cells was determined in 6 well plates. The cell density was adjusted to 5×10^6 cell/well and, after appropriate adherence time, MS were added to each well. Untreated cells were used as reference and Zymosan A incubation with macrophages was used as positive control. Experiments were carried out with all PLGA–PVMMA formulations conjugated with lectin on their surface and without lectin. Respiration rates were expressed as nanomoles of oxygen consumed per minute and per 10^6 cells.

Table 1
Size, ζ-potential and encapsulation efficiency of PLGA–PVMMA microspheres.

	PLGA RG503H	PLGA RG503H–PVMMA AN119	PLGA RG502H–PVMMA AN119	PLGA RG503H–PVMMA AN139
Size (μm)	7.46 ± 0.04	6.31 ± 0.1	5.14 ± 0.037	5.68 ± 0.17
Zeta potential (mV)	−25.38 ± 0.53	−19.22 ± 1.36	−18.44 ± 0.57	−20.01 ± 1.38
Zeta potential with Con A (mV)	−16.38 ± 0.97	−17.49 ± 0.59	−14.82 ± 0.59	−15.45 ± 1.27
E.E. (%)	65.07 ± 0.04	59.93 ± 0,09	57.73 ± 0.05	62.09 ± 0.08

2.6.5. Microphere cytocompatibility

To determinate Mϕ viability upon co-incubation with microspheres an assay based on tetrazolium salt was used (WST-1, Boehringer Mannheim, Germany). Cell viability was tested in 96 well plates where cells were seeded at a density of 2.5×10^4 cell/well and incubated with different amounts of microspheres during 24 h. 10 μl of reagent (a tetrazolium salt, 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) was added to cells and they were incubated in a humidified atmosphere (37 °C, 5%CO_2). Absorbance was measured at a wavelength of 450 nm. Cell viability was expressed as relative optical density measures with test cells relative to untreated control cells.

2.7. Statistics

Comparisons between different conditions were made by two-way analysis of variance (ANOVA) and post hoc Turkey-B test (SPSS, SPSS Inc., Chicago. USA). All data from cell culture experiments were based on at least three individual cell preparations. Statistical significance level was defined as $p < 0.05$ compared with untreated (control cells).

3. Results and discussion

3.1. Characteristics of MS: particle size, ζ-potential and morphology of PLGA–PVMMA MS

In this study, we presented the preparation of PLGA–PVMMA microspheres with enhanced capacity of coupling lectin on their surface as protein carriers for macrophage activation. Size and ζ-potential are important parameters to evaluate the degree of interaction of microspheres with the cell surface and their uptake by phagocytic cells (Piskin et al., 1994; Thiele et al., 2003; Steinkamp et al., 1982). Depending on their size, particles may either be incorporated via phagocytosis if diameter is smaller than 10 μm give rise to a depot at the injection site (Tabata and Ikada, 1990; Ayhan et al., 1995; Foged et al., 2005). Here, three different types of microspheres loaded with a model protein (BSA) were prepared by spray-drying with and without Con A as surface coating using two varieties of PLGA (RG502H and 503H) and two types of PVMMA (AN119 and AN139). Other variations of PLGA–PVMMA polymers different from those here developed were also tried. However, as a result of the type of the equipment and the own physicochemical characteristics of materials, these attempts were unsuccessful.

Table 1 summarizes particle size and zeta potential of PLGA–PVMMA microparticles. As measured by laser light scattering, mean diameters of all batches of microspheres were in a narrow range of 5–7 μm. No significant differences in size were detected amongst formulations. Incorporation of the higher molecular weight PVMMA type did not lead to an increase in the mean diameters of microspheres. Also, BSA encapsulation had no effect on particle size. Independently of BSA encapsulated, the zeta potential was highly negative for non-coated PLGA–PVMMA microspheres as expected. The zeta potential of MS differed when they were coated with lectin, confirming the surface modification of PLGA–PVMMA microspheres (Table 1). Coated MS with Con A in acetic acid showed attenuated values of negative zeta potential compared with non-coated microparticles. No significant differences were found in the values of zeta potential performed in distilled water (about −30 mV). The procedure of Con A binding was carried out at pH 7.4, therefore Con A (pI 5) would show negative under this condition. However, measurements of zeta potential were performed in acetic acid and this may be the reason for the decrease of surface charge of microparticles in this medium.

3.2. Determination of acid groups on microspheres

The aim of these work is the formulation of new protein delivery system with a higher number of functional groups where Con A could be attached. The method used here is a direct measurement of the amount of carboxylic groups generated by inclusion of PVMMA to PLGA microspheres. PVMMA is a copolymer of methyl vinyl ether and maleic anhydride that can easily react with amino groups, which makes easy to bind proteins. However, in aqueous environments, it hydrolyzes rapidly to produce the free acid. Thereby, since PVMMA is dissolved in the protein aqueous phase, it would hydrolyze easily. To take advantage of these new free –COOH groups, we have used the carbodiimide method to conjugate the lectin on microspheres surface.

The results of determining the number of –COOH groups showed a significant augmentation in the acid reactive groups on MS surface for all formulations prepared with PVMMA compared to plain PLGA. The incorporation of PVMMA to PLGA formulations allowed increasing the amount of functional groups in about ten-fold compared to PLGA microspheres without modifying the usual size of microparticles made of PLGA obtained by spray-drying (Table 2). The amount of acid groups in formulation containing PLGA RG502H was found to be higher than the amount of acid groups when RG503H was used. This was due to the fact that the amount of PVMMA AN119 used to prepare PLGA RG502H–PVMMA AN119 microspheres was bigger than the amount of PVMMA AN119 used to prepare all other formulations. In the case of PLGA RG503H–PVMMA AN139 microspheres, the amount of acid groups is also higher due to the fact that we used PVMMA AN139 with a higher molecular weight (M.W.: 1×10^6) and therefore, a bigger amount of functional groups.

3.3. Morphology

Uncoated and coated PLGA–PVMMA microspheres were examined for their surface morphology. SEM micrographs revealed spherical morphology and smooth surfaces without porosity or deformations independently of the presence of Con A (Fig. 1A–C) for all types of microspheres except for PLGA RG502H–PVMMA

Table 2
μmols of acid groups per g of PLGA–PVMMA microspheres.

Microspheres formulation	μmols acid groups/g microspheres
PLGA RG503H	13.3 ± 2.1
PLGA RG503H–PVMMA AN119	82.5 ± 3.5
PLGA RG502H–PVMMA AN119	92.3 ± 3.5
PLGA RG503H–PVMMA AN139	88.4 ± 5.1

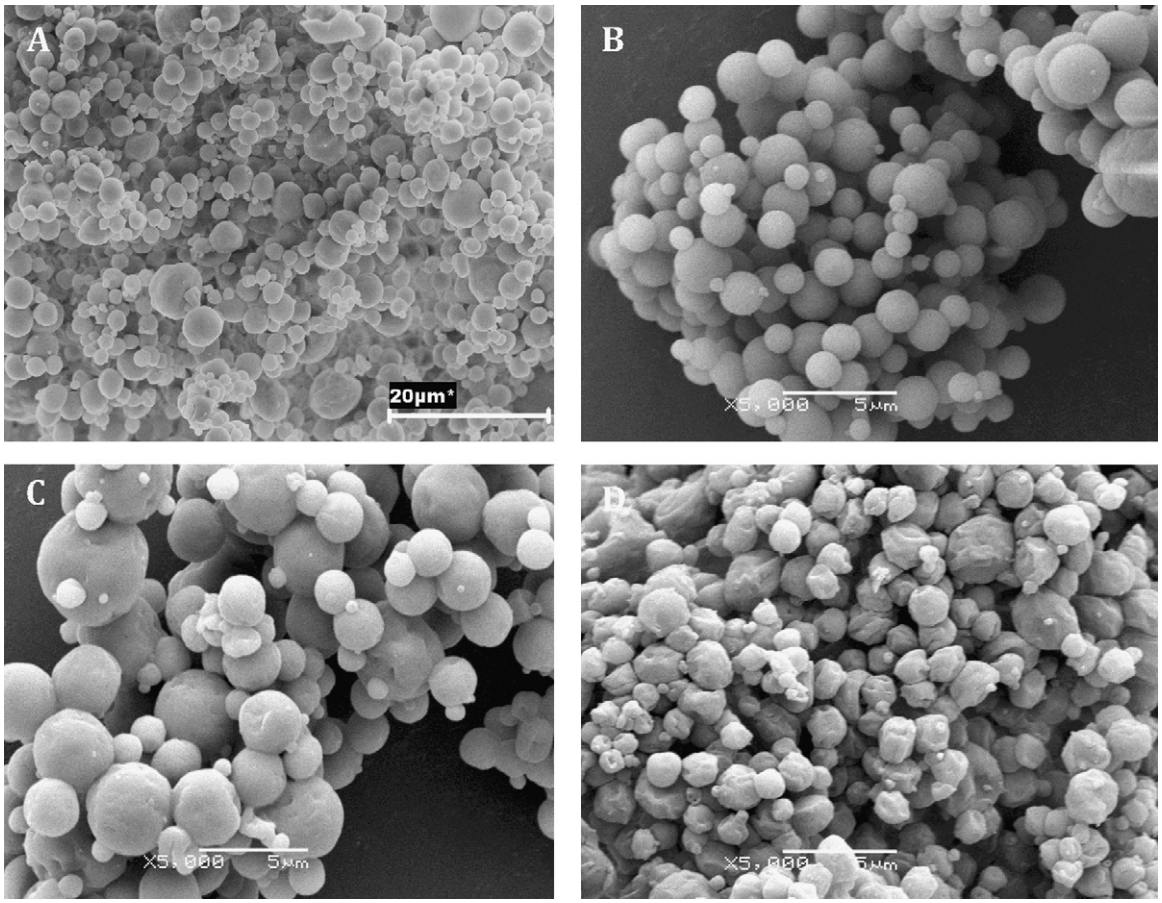

Fig. 1. SEM micrographs of (A) Con A-coated PLGA RG503H–PVMMA AN119 microspheres (3000×), (B) PLGA RG503H–PVMMA AN119 microspheres (5000×), (C) PLGA RG503H–PVMMA AN139 microspheres (5000×), (D) PLGA RG502H–PVMMA AN 119 microspheres (5000×).

AN119 (Fig. 1D). This difference on surface morphology between preparations could be due to the solvent used (acetone) with this type of formulation. During the drying process, the different precipitation ability of the polymer during the acetone removal resulted in the deformation of microspheres.

3.4. Entrapment efficiency (E.E.)

Microencapsulation of BSA as model protein into PLGA–PVMMA microspheres was performed by a previous emulsion before spray-drying. At nominal content of 33 μg BSA/mg MS, entrapment efficiencies of 57% and 65% were achieved (Table 1). The use of the two types of PVMMA did not affect greatly the loading efficiency of MS and they did not differ from usual values of entrapment efficiency for PLGA alone.

3.5. In vitro release

As aforementioned, it would be an interesting feature of MS that these formulations could provide a modulated release of proteins different from those usually obtained for PLGA microparticles by incorporation of PVMMA copolymer. Obtained results from *in vitro* release studies confirmed our expectations. The incorporation of PVMMA exerted a great effect on BSA on the overall release profile. Furthermore, all preparations released 100% of the actual loading in shorter times if compared with what was estimated for pure PLGA formulation (Fig. 2). Microspheres based on PLGA RG503H and PVMMA AN119 in acetic acid released 100% of the protein in five days whereas release from microspheres based on PLGA RG503H and PVMMA AN119 in acetone was completed in six days. Comparing the i*n vitro* release profiles for BSA from PLGA RG503H with PVMMA microspheres reported in bibliography, PLGA has been shown an initial release of a 58% in 24 h (Freitas et al., 2004) whereas microspheres based on only PVMMA AN119 showed a burst effect of 80% in the first 2 h (Arbós et al., 2004). Differences in the release profile may be attributed to the different amount on PLGA component and its more hydrophobic character, which hindered protein diffusion trough polymeric system. Release rate from microspheres

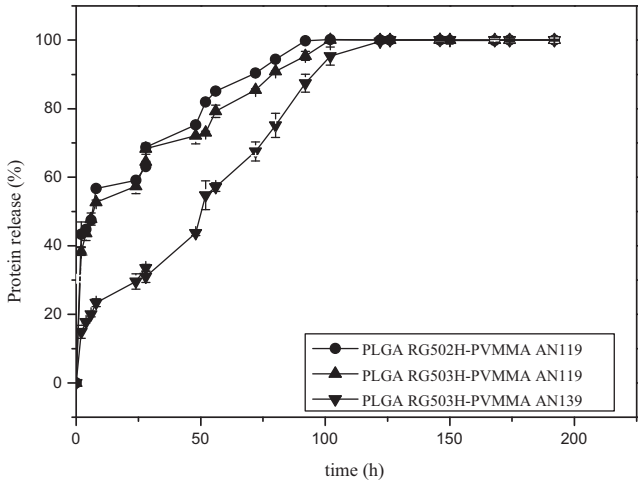

Fig. 2. *In vitro* release profiles of BSA from PLGA–PVMMA microspheres in buffer solution at pH 7.4 (mean ± S.D., $n = 3$).

Table 3
Surface lectin concentration (expressed in μg/mg of PLGA–PVMMA microspheres prepared by spray-drying.

Formulation	Coupling efficiency (μg/mg MS)	Adsorption (μg/mg MS)
PLGA RG503H	6.78 ± 0.43	2.65 ± 0.37
PLGA RG503H–PVMMA AN119	29.26 ± 0.73	23.7 ± 0.52
PLGA RG502H–PVMMA AN119	22.2 ± 0.39	18.1 ± 0.41
PLGA RG 503H–PVMMA AN139	28.3 ± 0.6	20.8 ± 0.9

prepared with PVMMA AN139 was influenced by a higher molecular weight polymer than AN119, resulting in a slower release of protein, suggesting a more gradual disintegration or resistance to swelling of microspheres matrix.

3.6. Determination of the amount of bound Con A to MS surface

Coupling ligands to the surface of PLGA microspheres may be difficult, as the material is chemically low reactive since the surface density of carboxyl groups which may be functionalized is very low. Different approaches for producing coated microspheres formulation based on PLGA have been made and a wide variety of lectins have been covalently coupled to PLGA microspheres by carbodiimide chemistry (Ertl et al., 2000; Montisci et al., 2001; Walter et al., 2004; Roth-Walter et al., 2004, 2005; Keegan et al., 2006). Therefore, it is necessary to increase the amount of reactive sites of microspheres. Here, a new attempt has been made by introducing another polymer (PVMMA) that contributes to increase the carboxylic reactive groups on surface.

The effect of PVMMA on the amount of protein covalently linked on the surface of microspheres was determined by fluorescence and BCA protein assay. Both methods showed comparable results. 100 mM of EDC and 70 mM of NHS in combination with a second step of MS incubation with 1 mg/ml lectin was found to be the best protocol for covalent coupling, and thus, it was used for this work. The increase on –COOH groups density had a great impact in augmenting the amount of lectin bound as shown in Table 3. Non-treated PLGA microspheres were used as control. Furthermore, microspheres were incubated in the presence of Con A to obtain a non-specific adsorption of lectin. The results showed that the total amount in the covalently coupled lectin to PVMMA microspheres was augmented in comparison to PLGA RG503H alone. Differences found between formulations, especially in PLGA RG503H–PVMMA AN119 or AN139 formulations, with coupling efficiency of approximately 80% suggested the increased number of functional groups onto microspheres as the main factor influencing the lectin coupling method. These results indicated that the large increase of concentration of –COOH groups on microsphere surface allowed conjugating larger amounts of Con A compared to PLGA alone.

3.7. Infrared studies

Infrared studies were done in order to determine whether it was established a covalent binding between the protein and –COOH groups of microspheres. IR spectra of empty MS and formulation with Con A were taken and compared. FT-IR spectra showed the existence of bands characteristic of amide linkages formed between the amine group of the lectin and free carbonyls and/or carboxylic groups from polymers. As shown in Fig. 3, the infrared spectrum of microspheres based on PLGA RG503H–PVMMA AN119 showed the existence of a band at 920 cm^{-1} characteristic of the polymer carboxylic groups. Also, it was found an important band at 1762.2 cm^{-1} characteristic of carbonyl groups. Comparing spectra (Fig. 3) IR studies confirmed the conjugation of lectin to PLGA–PVMMA microspheres which were activated through the carbodiimide method previously. The linkage between polymer MS and the lectin was shown because of the emergence of bands at 1650 and 1541 cm^{-1} characteristic of amide I and amide II formed by the covalent binding between the –COOH groups from polymer and NH_2 group of Con A.

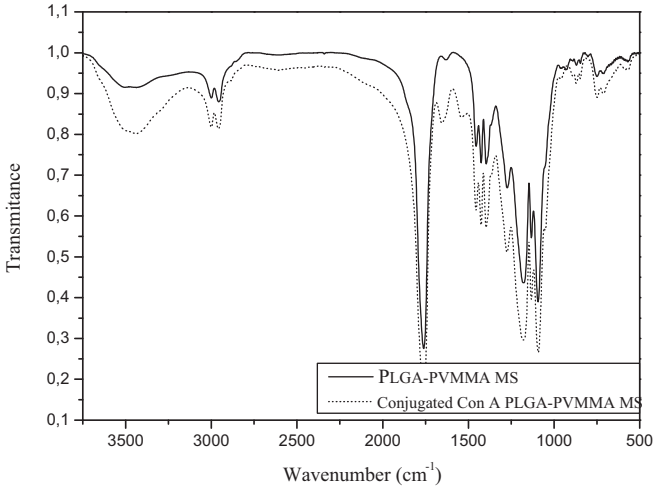

Fig. 3. IR spectra of (a) PLGA RG503H–PVMMA AN119 microspheres. (b) PLGA RG503H–PVMMA AN119 microspheres with lectin in their surface.

3.8. Lectin desorption studies

Fig. 4 shows the *in vitro* total concanavalin A cumulative desorption from PLGA and PLGA–PVMMA microspheres. After a notably burst release of 60% for lectin adsorbed onto microspheres, it was completely desorbed at approximately 4 days (for PLGA alone, 48 h was enough to achieve total release). Covalent coupling of lectin to microspheres had an important effect on desorption since con A was slowly desorbed in a much more controlled manner throughout time.

3.9. Effect on Con A coating on microspheres uptake by macrophages

The role of conjugated Con A on microspheres in the particle uptake by macrophages was evaluated by co-incubation of microspheres after 1 and 24 h. Con A binds avidly saccharide residues (mostly α-D-mannopyranoside) of macrophage plasma membrane and other cells (hepatocytes, T cells (Goldstein, 1976; Barral-Netto and Barral, 1986; Peschke et al., 1990; Irle et al., 1978; Leist and Wendel, 1996) and evokes cell stimulation and phagocytosis events (recognition of the particle to be ingested, adherence to a receptor on the cell surface, the encapsulation and the formation of phagocytic vesicles in the cell cytoplasm) (Edelson and Cohn, 1974a; Maldonado et al., 1994; Loyola et al., 2002). It is known that phagocytosis is initiated by the stimulation of specific receptors on the phagocyte through a ligand on the surface of a particle or invading microorganism. Subsequently, macrophages would potentially increase the rate of uptake of microparticles by interacting with Con A molecules.

Quantification of phagocytic activity was carried out by microscopic examination with previous Giemsa staining. Results are shown in Fig. 5. A significant effect of Con A coating of PLGA–PVMMA microspheres was found on phagocytic activity of macrophages compared to plain MS. Coated microspheres increased phagocytosis compared to those bearing adsorbed Con A due to the existence of major amount of lectin according to the results of coupling efficiency. Since Con A is able to bind mannose

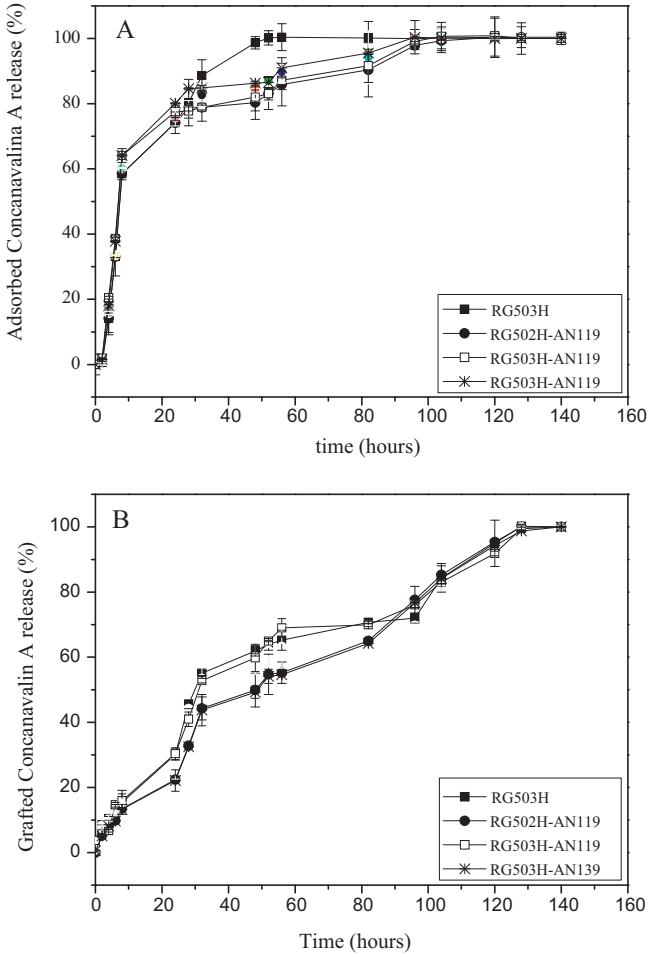

Fig. 4. *In vitro* desorption profile on concanavalin A from PLGA and PLGA–PVMMA microspheres (mean ± S.D.; $n = 3$).

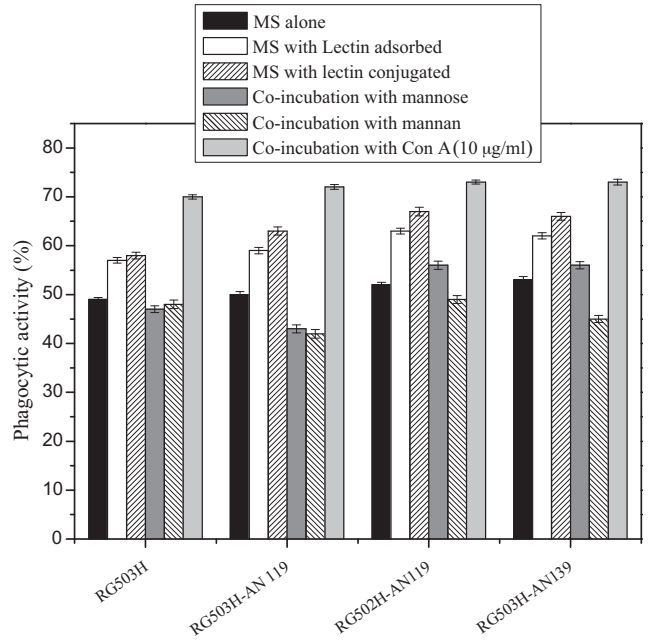

Fig. 5. Uptake of microspheres by macrophages. Microspheres were co-incubated with cells for 1 h, 37 °C in different conditions (in order): MS alone, MS with con A adsorbed on surface, con-A-grafted MS, con-A-grafted MS in presence of mannose, con-A-grafted in presence of mannan and co-incubation with a solution of con A (10 μg/ml).

residues, we evaluated whether the carbohydrate binding capacity of Con A was maintained after lectin attachment to microspheres surface by co-incubating mannose and mannan with macrophages. The presence of mannan in the medium produced a decrease in microspheres uptake. The surface properties of PLGA–PVMMA microspheres had a significant impact on macrophages internalization. We found that presence of Con A on microspheres surface had an important effect as inductor of particle uptake since the presence of Con A inhibitors reduced significantly phagocytosis activity by displacement of MS bearing lectin by the high affinity competitor sugar. The impact on this reduction is especially important in microspheres with PVMMA AN139. The more amount of lectin on surface, the bigger effect of mannose or mannan on particle uptake.

Con A binding to murine cell receptors has been reported to be attenuated *in vitro* in presence of serum even if the serum was proteolytic digested (Leist and Wendel, 1996) what suggests that the effect of attached Con A on particle uptake would be more pronounced in absence of serum. In our case, we also found a great impact of serum. In fact, the results of the extent of phagocytosis studies in the presence of FBS showed similar values of around 50% for all formulations, coated and uncoated microspheres, included plain PLGA. These findings also confirmed the activity of Con A.

3.10. NO production

Nitric oxide (NO) is an important cytotoxic intermediate involved in inflammatory diseases, defence against pathogens and tissue injury which can be synthesized by activated macrophages (Coleman, 2001). It has been reported that stimulation with Con A induces NO release from peritoneal murine macrophages *in vitro* and *ex vivo*, although not with similar intensity values as those obtained with other type of lectins (Andrade et al., 1999; Kesherwani and Sodhi, 2007). Nitrite levels in the supernatants of cell cultures obtained are shown in Fig. 6. As it has been found by other authors, NO production by macrophages was low after microspheres co-incubation in comparison with positive controls (Luzardo-Álvarez et al., 2005). Nitrite levels obtained after 48 h of lectin-grafted microspheres were not significantly different from co-incubation with lectin-adsorbed microspheres, including PLGA MS alone, which get only basal levels. No synergistic effect was found when microspheres were co-incubated with pure concavalin A solution with levels around 14 μM of NO (data not shown). NO production induced by Con A is a time and concentration dependent process. These results indicated that conjugated Con A to microspheres were able to induce NO production by macrophages but more concentrated suspensions were demanded to get augmented levels of nitrite ions released by cells, bearing in mind that the lectin amount determined per well was approximately 0.5 μg/ml for these experiments whereas concanavalin concentration used as positive control reached 10 μg/ml. These results demonstrated that lectin activity was kept unaltered after being grafted onto microspheres. L-NMMA efficiently inhibited NO production by cells stimulated with IFN-γ until values of 4.64 ± 0.007 μM. Plain MS, Con A-adsorbed MS, Con A-grafted MS induced nitrite production in a significantly manner in the presence of IFN-γ and Zymosan A. Furthermore, a synergistic effect was observed when MS were co-incubated in presence of IFN-γ or IFN-γ and Zymosan A, resulting in high a NO production, similar to positive controls or even higher (Fig. 6b). This effect was more pronounced, surprisingly, for naive MS, suggesting a possible inhibition mechanism when con A was associated to MS.

3.11. Oxygen consumption

Upon phagocytosis or stimulation, macrophages display an increase in oxygen consumption. Con A has been reported to

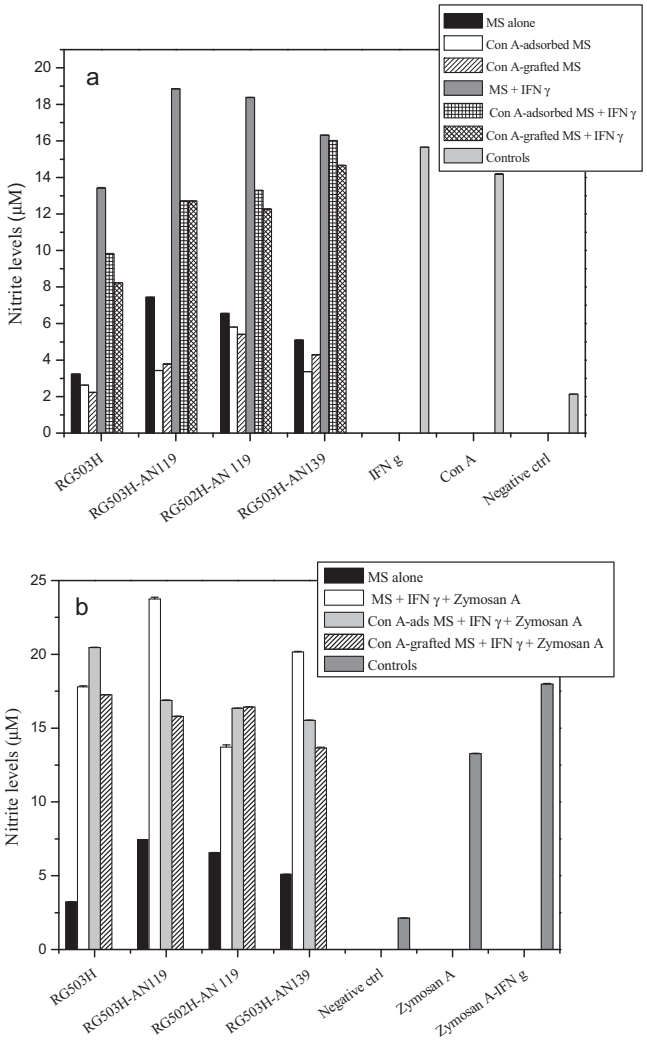

Fig. 6. NO production by macrophages upon incubation with different formulations. (Mean ± D.S.; $n = 3$) and imnunostimulants, Con A solution (10 μg/ml), zymosan A and IFN-γ as positive controls.

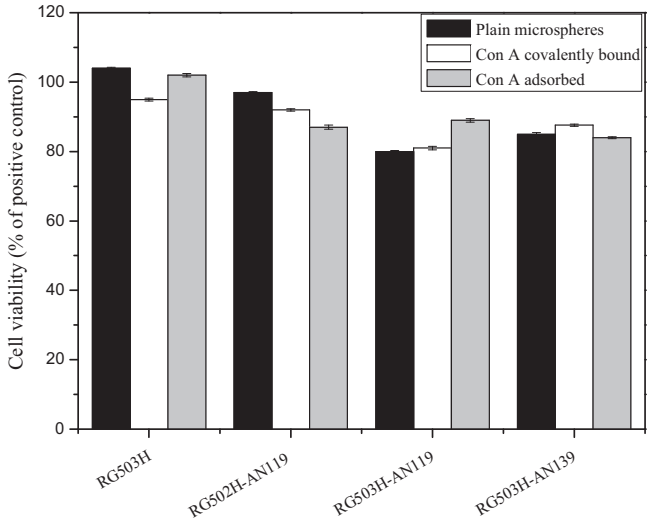

Fig. 7. Cytotoxicity of PLGA–PVMMA microspheres to mice macrophages after 24 h of co-incubation.

interact and activate macrophages and neutrophils inducing different cellular responses and functions (Edelson and Cohn, 1974a,b) as increasing the extent of phagocytosis or binding to murine MHC molecules (Brummer et al., 1983; Maldonado et al., 1994; Moresco et al., 2002; Stoika et al., 2001; Gaziri et al., 1999; Kesherwani and Sodhi, 2007; Sodhi et al., 2007). Upon Con A activation, macrophages will induce NO release, inflammatory cytokines as IL-1β and peroxidase production. Thus, we wanted to obtain further information about *in vitro* cellular effects of MS. In the present study, MS-stimulated oxygen consumption was expressed as percentage of nanomol O_2/min relative to cells stimulated with Zymosan A particles (positive control; 24.73 ± 0.81). The addition of uncoated PLGA–PVMMA MS did not cause any significant change in oxygen consumption by RAW Mϕ (Table 4) when compared to basal values of untreated cells (5.53 ± 0.41). However, the presence of lectin on Con A coupled-MS enhanced the O_2 consumption to 26–27 nmoles/min, except for Con A-PLGA alone, with values nearby reference cells. Slightly lower values of O_2 consumption were obtained when Con A was just adsorbed. These results can be explained as the presence of lectin on their surface significantly impaired the oxygen utilization by phagocytic cells. The attachment of Con A to functionalize particles induced oxygen consumption and therefore it affected the metabolic status of macrophages as concomitant fact that took place during the phagocytosis.

3.12. Viability of macrophages upon microspheres phagocytosis

Macrophage viability was not affected when cells were co-incubated with microspheres. As it can be seen in Fig. 7, microspheres with Con A covalent linked, microspheres with adsorbed Con A and microspheres without Con A have similar viability percentage confirming that our formulations are biocompatible and non cytotoxic. The viability of macrophages after 24 h after being incubated in the presence of Con A (10 μg/ml) was 93.55 ± 0.19 (expressed as percentage of viability respect to the untreated cells). These results were consistent considering that Con A induced time-dependent cytotoxicity *in vitro* cell culture at a concentration about 400 μg/ml, whereas the median lethal concentration was obtained at 30 mg/ml. Therefore, our values of conjugated Con A to microspheres were below of the cytotoxicity threshold concentration.

Table 4
Oxygen consumption (nanomol O_2/min/10^6 cells. Mean ± S.D.; $n = 3$). As reference, cells alone were considered. As positive control, macrophages were incubated with Zymosan A.

Microspheres	O_2 nanomoles/10^6 cells/min (without lectin)	O_2 nanomoles/min (adsorbed lectin)	O_2 nanomoles/min (grafted lectin)
PLGA	5.34 ± 0.30	8.1 ± 0.28	8.04 ± 0.22
PLGA RG502H–PVMMA AN119	5.83 ± 0.03	23.04 ± 2.79	27.76 ± 1.4
PLGA RG503H–PVMMA AN119	5.73 ± 0.19	17.4 ± 0.7	27.47 ± 0.79
PLGA RG503H–PVMMA AN139	6.06 ± 0.21	25.46 ± 1.40	26.71 ± 0.36

4. Conclusions

In this study, PLGA–PVMMA microspheres were prepared successfully by spray-drying as new protein delivery systems with an adequate size and zeta potential to be internalized by macrophages. The presence of PVMMA had a great impact on protein delivery. Also, the addition of PVMMA has shown to augment the functional groups and thereby, the amount of Concanavalin A could be attached on microspheres surface. Con A coupled onto MS stimulated phagocytic activity, oxygen consumption and NO production in macrophage culture. This work provides further argument to investigate Con A and other lectins bounded to microspheres to serve as potential adjuvants by modulating protein delivery and macrophage activation.

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