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Research paper

Co-encapsulation of an antigen and CpG oligonucleotides into PLGA microparticles by TROMS technology

Beatriz San Román [a], Juan M. Irache [a], Sara Gómez [a], Nicolas Tsapis [b,c], Carlos Gamazo [d], María Socorro Espuelas [a,*]

[a] Department of Pharmacy and Pharmaceutical Technology, University of Navarra, Pamplona, Spain
[b] Université Paris-Sud, UMR CNRS 8612, Faculté de Pharmacie, Châtenay-Malabry, France
[c] CNRS, UMR 8612, Faculté de Pharmacie, Châtenay-Malabry, France
[d] Department of Microbiology, University of Navarra, Pamplona, Spain

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Abstract

It seems well established that CpG oligonucleotide Th1-biased adjuvant activity can be improved when closely associated with a variety of antigens in, for example, microparticles. In this context, we prepared 1-μm near non-charged poly(lactic-co-glycolic) acid (PLGA) 502 and PLGA 756 microparticles that loaded with high-efficiency antigen (50% ovalbumin (OVA), approximately) into their matrix and CpG–chitosan complexes (near to 20%) onto their surface maintaining OVA and CpG integrity intact. In the intradermal immunization studies, whereas OVA microencapsulated into PLGA 756 alone induced a strong humoral immune response assisted by a very clear Th1 bias (IgG2a/IgG1 = 0.88) that was decreased by CpG co-delivery (IgG2a/IgG1 = 0.55), the co-encapsulation of CpG with OVA in PLGA 502 particles significantly improved the antibody response and isotype shifting (IgG2a/IgG1 = 0.73) in comparison with mice immunized with OVA-loaded PLGA 502 (IgG2a/IgG1 = 0). This improvement was not correlated with the cellular immune response where the effect of co-encapsulated CpG was rather negative (2030 and 335 pg/mL IFN-γ for OVA PLGA 502 and OVA CpG PLGA 502, respectively). These results underscore the critical role of polymer nature and microparticle characteristics to show the benefits of co-encapsulating CpG motifs in close proximity with an antigen.

Keywords: Microparticles; Antigen; CpG sequences; Th1/Th2 immune response; Poly(lactic-co-glycolic) acid

1. Introduction

Current studies in the field of vaccination are based on subcellular compounds of pathogens [1,2], avoiding the potential pathogenicity of using attenuated organisms. However, the lack of immunogenicity applies for adjuvants and/or immunomodulators. Between the adjuvants described in the literature only aluminium hydroxide (alum) and the emulsion MF59 have been approved for human applications due to their confirmed efficacy and safety [3]. In spite of being the most used adjuvant, alum is a pro-Th2 response enhancer. This outcome is not suitable for some disorders like intracellular infections and allergy [4]. Moreover, the possibility of some undesirable side effects has been reported (including hypersensitivity to aluminium, granuloma and the relationship between the accumulation of aluminium and the Alzheimer disease) [5]. Consequently, the search for novel adjuvants remains a health priority.

Microparticles (MP) have been widely reported in the literature as carriers for antigen delivery [6,7]. Poly(lactic-co-glycolic) acid (PLGA) appears as an attractive candidate for the fabrication of microparticles since its use in resorbable sutures [8] for humans has been approved by the FDA. The protection that microparticles provide to

* Corresponding author. Department of Pharmacy and Pharmaceutical Technology, University of Navarra, Irunlarrea 1, Ap. 177, 31080 Pamplona, Spain. Tel.: +34 948 425600; fax: +34 948 425649.
E-mail address: sespuelas@unav.es (M.S. Espuelas).

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the antigen and the improvement in the uptake by the antigen-presenting cells (APC) result in the enhancement of the antigen-poor immunogenicity [9]. Besides, the nature of degradation of the carrier maintains a sustained release [10] throughout the time which would be in concordance with a few shot protocol of vaccination.

Pathogen-associated molecular patterns (PAMP) have been confirmed as potent immunomodulators due to their interaction with the APC. Their signalization pathway starts in Toll-like receptors (TLR), specific for each PAMP, and ends triggering the immune response. In the case of oligodeoxynucleotides containing immunostimulatory CpG motifs (CpG ODN), the association with their endosomal specific receptor (TLR-9) initiates a complex molecular cascade which encourages the transcription of pro-inflammatory cytokine encoding genes, such as IL-12 and tumor necrosis factor (TNF) [11]. Afterwards, the presence of these molecules in the environment induces the differentiation of naïve Th0 cells into Th1 and cytotoxic T lymphocytes [12]. Thus, the deviation exerted in the immune response could be taken in advantageous to co-administer antigens as well as CpG sequences, leading to a potent antigen-specific Th1 type response and being a promising strategy for a wide range of diseases, such as bacterial infections [13], cancer [14] and allergy [15].

In vivo, both CpG sequences and antigens have been co-administered by admixture in solution or after their covalent association [16]. Also, CpG motifs have been mixed with particulate antigen [17] but all these strategies could result in CpG and/or antigen degradation in the biological environment. Therefore, the association of both antigen and oligonucleotide into the same carrier is supposed to offer several advantages [17]. Despite CpG sequences are under clinical trials, some authors have reported adverse effects such as splenomegaly, lymphadenopathy or the activation of autoimmune responses [18] in animal models after the administration of high doses of CpG motifs (over than 100 μg). In this context, the protection exerted by particulate delivery could reduce the effective dose of CpG. Besides, the co-delivery of both ag and CpG into the same particle guarantees the phagocytosis by the same APC, which results simultaneously in a potent antigen uptake and CpG-induced APC activation [17].

High and efficient loading of CpG sequences into MP is currently impaired by its high hydrophilicity and negative charge. A strategy to solve this obstacle consists in the incorporation of cationic molecules in the MP composition [19]. In our work conditions, preliminary studies have confirmed that the absence of cationic molecules leads to insignificant CpG encapsulation efficiency.

The aim of the present study was to co-encapsulate both ovalbumin (OVA), as an antigen model, and CpG sequences into PLGA 502 and 756 microparticles using a recently described emulsion and solvent evaporation method called Total Recirculation One-Machine System (TROMS). For this purpose, chitosan was used to form a complex with the oligonucleotide, which would decrease its hydrophilicity achieving a high CpG payload. Moreover, the immunostimulatory potency of the CpG-loaded microparticles was analysed *in vitro* after incubation with bone marrow-derived dendritic cells (BMDC) and *in vivo* conditions after immunizing BALB/c mice by intradermal route.

2. Materials and methods

2.1. Materials

The copolymers formed by lactic and glycolic acids, Resomer® RG 502 (M_W 12,000) and Resomer® RG 756 (M_W 98,000), were supplied by Boehringer Ingelheim. Oligonucleotide containing CpG sequences with fully phosphorotioate backbone (#1826, seq. (5′–3′): tccat gacgttcctgacgtt) was obtained from Coley Pharmaceutical Group (USA) and sense oligonucleotide (5′–3′: AACGTCAGCAACGTCATGGA) was purchased from Bonsai Technologies (Spain). Ovalbumin (grade V) and Pluronic® F68 were supplied by Sigma-Aldrich Chemie (Germany) and methylene chloride (reagent grade) was obtained from Scharlau (Spain). Chitosan (M_W 150,000) was purchased from Fluka (Germany) and polyvinyl alcohol (PVA), molecular weight 125,000, was obtained from Polysciences Inc. (USA). Oligreen® ssDNA Quantitation kit and SYBR Green® I nucleic acid gel stain were purchased from Molecular Probes (USA). Microbicinchoninic acid (MicroBCA) protein assay kit was purchased from Pierce (USA). Other chemical compounds were of reagent grade and were obtained from Sigma-Aldrich (USA).

2.2. Microparticle fabrication

Microparticles were fabricated by the solvent evaporation method using TROMS [20,21]. Briefly, the polymer dissolved in methylene chloride (50 mg PLGA 502 or 756, 4% w/v) was injected under a turbulent regime (50 mL/min) through a needle with an inner diameter of 0.17 mm onto a solution of Pluronic® F68 (6% w/v) and chitosan (0.0008–0.0032% w/w chitosan/PLGA) containing ovalbumin (3 mg) and/or CpG sequences (300 nmol), depending on the formulation. Then, this W_1/O emulsion was forced to circulate through the system for 2 min in order to homogenize the droplet size. Afterwards, this emulsion was injected onto the outer water phase, PVA (0.5% w/v), maintaining the pumping flow constant. This turbulent injection resulted in the formation of a double emulsion ($W_1/O/W_2$), which was homogenized for 4 min. Later, the final emulsion was magnetically stirred to allow solvent evaporation and microparticle formation. The microparticles were centrifuged (9300*g*, 12150-H, Sigma 3K30) and washed with distilled water twice. Finally, the microparticles were freeze-dried, lyophilized (Genesis 12EL, Virtis, USA) and stored at 4 °C for their conservation.

Besides, control microparticles were fabricated in the same way as described above without adding ovalbumin or CpG sequences.

2.3. Microparticle characterization

The size of the particles was determined by laser diffractometry using a Mastersizer S laser sizer (Malvern Instruments, UK). The mean size was expressed as the volume mean diameter in micrometers (μm). Microparticle zeta potential was assessed by laser Doppler velocimetry in a Zetasizer Nano ZS (Malvern Instruments, UK). For these analyses, the samples were diluted in distilled water at room temperature and the measurements were performed in triplicate.

The yield of the preparation process was calculated as the difference between the initial amount of polymer used to fabricate the microparticles and the final weight of lyophilized samples, expressed as percentage (%).

The shape and morphology of the microparticles were evaluated by scanning electron microscopy. The samples were coated with a platinum/palladium layer (Cressington Sputter Coater 208 HR, UK) under argon atmosphere. The micrographs were obtained using a scanning electron microscope (LEO 1530, France).

The amount of OVA associated to the microparticles was calculated using the MicroBCA protein assay. Lyophilized microparticles (5 mg) were degraded with 1 mL of NaOH 0.1 N under magnetic stirring at room temperature overnight. The samples were centrifuged (27,100*g*, 10 min) and the resulting pellets were incubated with the MicroBCA reagent for 2 h at 37 °C. Then, the solutions were measured in a spectrophotometer (iEMS Reader MF, Labsystems, Finland) at 562 nm and compared with the absorbance data obtained with OVA in solution. For this purpose, calibration curves (1.5–50 μg/mL) were performed using control OVA dissolved in NaOH 0.1 N. Each sample was assessed in triplicate and the results were expressed as the amount of protein per milligram of microparticle. The encapsulation efficiency was defined as the percentage of OVA-loaded microparticles relating to the initial amount of protein.

To determine the CpG sequences loading, 5 mg of microparticles were shaken with NaOH 0.1 N overnight at room temperature. Oligreen® ssDNA Quantitation reagent was added to the samples and the resulting fluorescence was measured at 522 nm (PerkinElmer LS 50B Luminescence Spectrometer, USA). CpG motifs were dissolved in buffer TE in order to perform a sigmoidal-fitted calibration curve (0.05–1.5 μg/mL). The results were expressed as the amount (in μg) of oligonucleotide per milligram of microparticle. Also, the entrapment efficiency was calculated as the ratio between the loaded and the initial quantity of CpG sequences, expressed in percentage (%).

2.4. In vitro release study

Microparticles containing OVA and/or CpG sequences (5 mg) were dispersed in 1 mL of phosphate buffer saline (PBS 0.15 M, pH 7.4) in eppendorf tubes. Then, the samples were maintained under rotating agitation at 37 °C and at predetermined intervals, the eppendorf tubes were centrifuged at 27,100*g* for 20 min (12150-H, Sigma 3K30). In the supernatants, the amounts of OVA and CpG sequences released from the particles were determined as described above (MicroBCA protein assay and Oligreen® ssDNA Quantitation kit, respectively). Meanwhile the pellets were resuspended in 1 mL of PBS. Empty microparticles were used as controls and subjected to the same procedure. Release data were expressed as the cumulative percentage of OVA and oligonucleotide released in comparison with the initial content of these molecules in the microparticles versus time.

2.5. Structural integrity and immunogenicity of the entrapped OVA

Protein profile of encapsulated OVA and OVA released from the microparticles were determined by SDS–PAGE and its immunogenicity by immunoblotting.

OVA-loaded microparticles (5 mg) were dissolved in methylene chloride. The organic solvent was evaporated with nitrogen and the residue was suspended in electrophoretic sample buffer (Tris–HCl 62.5 mM (pH 6.8), 10% glycerol, 2% SDS, 5% β-mercaptoethanol and 0.05% bromophenol blue). Then, the suspensions were centrifuged (2300*g*, 10 min) to remove polymeric residues. Afterwards, the samples were boiled for 10 min to separate possible ovalbumin degradation fragments.

For SDS–PAGE, samples were analysed by using 15% acrylamide slabs with the discontinuous buffer system of Laemmli [22] and gels stained with Coomasie Brilliant Blue R-250 [23]. Immunoblotting was carried out as described previously [24] with immunoglobulin G against OVA from mouse and with horseradish-conjugated rabbit anti-IgG, and 4-chloro, 1-naphtol as chromogen.

2.6. Integrity of CpG sequences

The integrity of CpG sequences after the preparative process and released from the microparticles was analysed by means of the determination of its melting temperature [25]. On one hand, the oligonucleotide was extracted from the particles using NaOH 0.1 N under shaking overnight at room temperature. On the other hand, microparticles were incubated with PBS under rotating agitation at 37 °C to allow CpG sequences to be released for 35 days. Both solutions were adjusted to a concentration of 4 ng/μL and incubated with their complementary sense oligonucleotide in a ratio 1:1 in the presence of 1 μL of SYBR Green® I nucleic acid gel stain diluted in buffer 100 mM Tris–HCl (pH 8.9), 100 mM NaCl and 14 mM $MgCl_2$. Throughout the incubation period, sense and antisense oligonucleotides formed a double strand, which enclosed the SYBR Green® I. This molecule emitted fluorescence when it is associated to the duplex and its signal is proportional to the hybridization capacity. This parameter was measured in a fluorescence

temperature cycler (Lyghtcycler, Roche Diagnostics GmbH, Germany). Precisely, the duplex was heated at 0.2 °C/s to 95 °C measuring the fluorescence signal during the process and obtaining a melting curve. For improved visualisation of the melting temperature or T_m (temperature at which 50% of the oligonucleotide is forming a double strand) the initial data were derived to obtain melting peaks (fluorescence (F) versus temperature (T) by plotting the negative derivative of fluorescence over temperature versus temperature; ($-dF/dT$) versus T).

2.7. Bone marrow-derived dendritic cell (BMDC) generation and activation

Dendritic cells were generated from C57BL/6 murine bone marrow precursors as previously described by Sarobe et al. [26]. Briefly, femurs and tibia were extracted after cervical dislocation and the bone marrow was flushed out using a 26-gauge needle with RPMI 1640 medium supplemented with 0.1% β-mercaptoethanol 50 mM, 0.5% sodium pyruvate 100 mM, 1 IU/Ml penicillin, 1 μg/mL streptomycin and 10% v/v foetal bovine serum (all from Gibco-BRL, UK). After the lysis of the erythrocytes, the cell suspension was purified and depleted of lymphocytes and granulocytes by incubation with a mixture of antibodies against CD4, CD8, Ly-6G/Gr1 and CD45R and rabbit complement. The resulting solution was grown at 10^6 cells/mL in 6-well plates (2 mL/well) with RPMI 1640 medium containing GM-CSF and IL-4 (25 ng/mL; PrepoTech EC, UK). Two-thirds of the medium was replaced with fresh medium containing GM-CSF and IL-4 at days 2, 4, 5 and 6. The purity of DC on day 7 was found to be greater than 70% (based on the expression of CD11c). Afterwards, BMDC were incubated with CpG microparticles (0.5 and 2 μg CpG/mL) for 24 h and the amount of IL-12 produced was quantified using a commercial ELISA kit (Pharmingen, BD Biosciences, USA). Free CpG (0.5 and 2 μg/mL) and LPS (1 μg/mL) were used as controls.

2.8. Mice immunization

Female BALB/c mice were purchased from Harlan Interfauna Ibérica (Spain), housed in specific pathogen-free conditions and used at 8 weeks of age. The experiments were performed in compliance with the regulations of the Ethical Committee of the University of Navarre in line with the European legislation on animal experiments (86/609/EU).

Groups of 7 mice were immunized twice (days 0 and 14) by the intradermal route with 50 μL of OVA (10 μg) and/or CpG sequences (2 μg) in one of the following combinations: (i) free OVA; (ii) CpG sequences; (iii) free OVA and CpG sequences physically mixed; (iv) OVA-loaded microparticles (OVA PLGA 502 and OVA PLGA 756); (v) CpG-loaded microparticles (CpG PLGA 502 and CpG PLGA 756); (vi) OVA and CpG co-encapsulated into microparticles (OVA CpG PLGA 502 and PLGA 756) and (vii) OVA emulsified with Freund's Adjuvant (CFA).

Blood samples were collected under anaesthesia from the retro-orbital plexus at days 0, 14, 28, 35, 42 and 49 after the first immunization. The samples were centrifuged and the resulting sera were pooled. Finally, the sera were diluted 1:10 in PBS and stored at −80 °C until assayed by ELISA.

2.9. Measurement of anti-OVA antibody levels in serum

An indirect ELISA was performed to determine the level of OVA-specific antibody isotypes in the serum [27]. The experiment was carried out as follows: 96-well microtitre plates (Thermo Labsystems, Finland) were coated overnight with 1 μg per well of ovalbumin in carbonate–bicarbonate buffer (pH 9.6) and maintained at 4 °C. After being washed in buffer (phosphate buffer saline containing 0.05% Tween® 20, PBS-T20), serum samples (100 μl) at different dilutions were added to wells and incubated for 4 h at 37 °C. Then, unbound antibody was washed prior to the addition of 100 μl of goat anti-mouse IgG1 or IgG2a horseradish peroxidase conjugate (Nordic Immunology, Netherlands) diluted 1:1000 in PBS-T20 (37 °C, 1 h). After a final wash step, 100 μl of chromogen and substrate solution (2, 2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) and hydrogen peroxide) were added. The plates were incubated at room temperature for 30 min, and, finally, the absorbance was measured at 405 nm by an iEMS Reader MF (Labsystems, Finland).

2.10. Cytokine assay

Naïve and immunized mice were sacrificed by cervical dislocation at day 13 after immunization and their spleens removed and placed in RPMI 1640 media (Gibco-BRL, UK) under sterile conditions. Each spleen was smashed and cells within experimental groups were pooled in one flask. The cellular suspension was centrifuged at 400g for 10 min, the supernatant discarded and the pellet washed twice with PBS. The splenocytes were suspended in lysis buffer (NH_4Cl 0.15 M, $KHCO_3$ 10 mM, EDTA 0.1 mM) for 2 min to eliminate erythrocytes and refilled with RPMI 1640 to stop the reaction. This suspension was centrifuged (400g, 5 min) and the pellet was resuspended in RPMI 1640 medium supplemented with 0.1% β-mercaptoethanol 50 mM, 0.5% sodium pyruvate 100 mM, 1 IU/Ml penicillin, 1 μg/mL streptomycin and 10% v/v foetal bovine serum (all from Gibco-BRL, UK). The lymphocyte suspension was added to 96-well round bottom microtitre plates (Iwaki, UK) (4×10^5 cells/well) along with test antigen (20, 80 and 160 μg OVA/mL in a final volume of 200 μl per well). Negative (wells without antigen) and positive (wells containing 2 μg/mL concanavalin A, used as mytogen) controls were used. The culture supernatants were collected for cytokine assay at 48 h after the stimulation. Then, the supernatants were kept frozen at −80 °C in a

96-well flat bottom microplate until testing. IFN-γ and IL-4 levels were determined using a commercial ELISA kit (Biosurce International, USA).

3. Results

3.1. Optimization of CpG loading in microparticles

CpG containing PLGA 502 microparticles (without including the model antigen) was fabricated at three different N/P ratios (Table 1). The N/P ratio means the molar relation of amine groups in the cationic molecule, which represent the positive charges, to phosphoric groups in the oligonucleotide, which represent the negative charges. Though the size and the zeta potential were similar in all cases (1–1.4 μm and −5 mV, approximately), the CpG loading tended to decrease to the half (from 11.5 ± 5.1 to 6.4 μg/mg) when the N/P ratio increased. Thus, the following microparticle batches were performed at fixed N/P ratio of 0.26.

3.2. Microparticle characterization

CpG and/or OVA-encapsulated microparticles were prepared using two types of PLGA copolymers, PLGA 502 and PLGA 756. Their differences in molecular weight and hydrophilicity could influence OVA and CpG loading, release and immune response. The results are summarized in Table 2. In all cases the yield of the method of preparation was close to 70%. PLGA 502 microparticles were slightly smaller in comparison with the ones fabricated with PLGA 756 (1.43 ± 0.06 μm for PLGA 502 and 1.94 ± 0.38 μm for PLGA 756). The zeta potential was neutral in all the cases although it moved towards slightly negative when CpG motifs were encapsulated (from 3.1 ± 0.8 to -5.1 ± 0.3 mV, in the case of PLGA 502 microparticles and from 1.2 ± 1.3 to -2.8 ± 2.9 mV, for PLGA 756 microparticles). Irrespective of polymer, OVA loading was very high and significantly increased when the antigen was co-encapsulated with CpG oligonucleotides (42.4 ± 9.7 to 43.1 ± 15.9 versus 60.7 ± 2.9 to 66.4 ± 13.3 for OVA microparticles and OVA CpG microparticles, respectively). Finally, the amount of CpG sequences associated to the particles was close to 12 μg/mg microparticles and was independent of antigen co-encapsulation and polymer nature.

From SEM micrograph studies (Fig. 1), microparticles were found to be rather spherical, homogeneous, without pores and with similar sizes than those obtained by laser diffractometry.

3.3. OVA and CpG sequences release from the microparticles

Fig. 2a shows the release of OVA from the microparticles. As expected, the antigen release was higher for PLGA 502 microparticles than for PLGA 756 microparticles although very slow and continuous for 35 days in any case. On day 35, approximately 40% and 10% of loaded OVA was released from 502 and 756 microparticles, respectively. Further, it seems that CpG motifs affected OVA release because OVA and CpG co-encapsulated formulations displayed a burst effect (around 10% of the protein was released within the first 24 h of incubation) not observed in OVA-loaded microparticles. Regarding the CpG sequences, the oligonucleotide was totally released in the first 24 h in all the formulations, with the exception of CpG-encapsulated PLGA 756 that sustained CpG release until 80% after 7 days of incubation (Fig. 2b). Moreover, the CpG release was not influenced by the OVA co-encapsulation. These results suggest that OVA would be located

Table 1
Influence of chitosan concentration in the physico-chemical characteristics of CpG-loaded microparticles (without OVA)

N/P ratio	Size (μm)	Zeta potential (mV)	CpG content (μg/mg MP)	Encapsulation efficiency (%)
0.26	1.36 ± 0.11	-4.9 ± 2.0	11.5± 5.1	17.5 ± 8.0
1	1.07	-4.99 ± 0.5	5.5	11.0
1.30	1.03	-5.48 ± 0.9	6.4	7.2

The chitosan amount is referred to CpG (N/P ratio). Data are expressed as means ± SD ($n = 1$–3).

Table 2
Physico-chemical characterization of microparticles of PLGA 502 and 756 containing ovalbumin and/or CpG sequences and prepared using chitosan (N/P ratio = 0.260) to form a complex with the oligonucleotide

Polymer	Formulation	Size (μm)	OVA loading (μg/mg MP)	Encapsulation efficiency (%)	CpG content (μg/mg MP)	Encapsulation efficiency (%)
502	OVA	1.47± 0.35	41.4 ± 4.6	42.4 ± 9.7	–	–
	CpG	1.36 ± 0.11	–	–	11.5 ± 5.1	17.4 ± 8.0
	OVA CpG	1.46 ± 0.34	55.1 ± 1.5	60.7 ± 2.9	15.9 ± 4.4	24.1 ± 6.6
756	OVA	2.37 ± 0.54	51.0 ± 15.4	43.1 ± 15.9	–	–
	CpG	1.84 ± 0.68	–	–	10.8 ± 5.8	18.7 ± 6.9
	OVA CpG	1.62 ± 0.47	58.6 ± 6.0	66.4 ± 13.3	14.1 ± 1.7	22.5 ± 4.2

Data are expressed as means ± SD ($n = 4$).

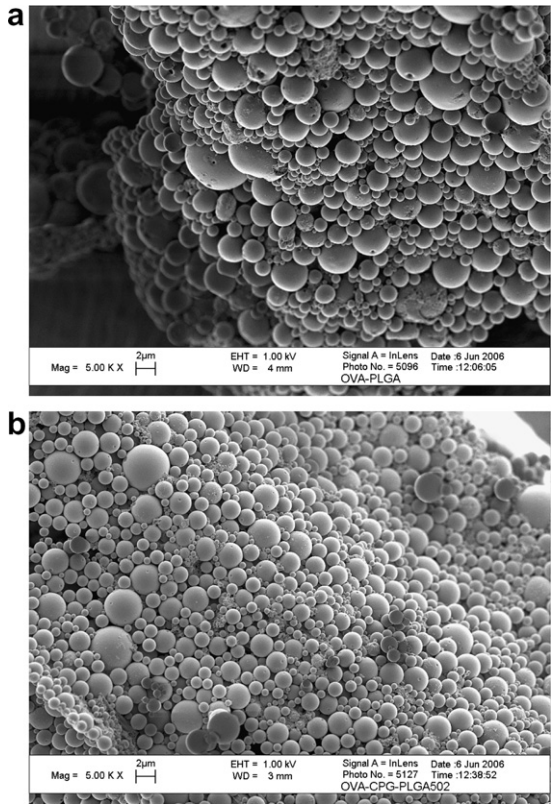

Fig. 1. Scanning electron microscopy of lyophilized OVA (a) and OVA CpG (b) loaded PLGA microparticles prepared by TROMS.

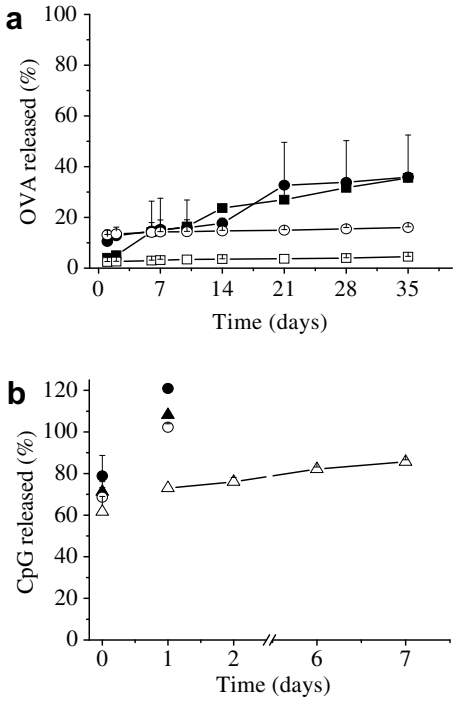

Fig. 2. *In vitro* release of (a) OVA and (b) CpG sequences from OVA PLGA 502 (■), CpG PLGA 502 (▲), OVA CpG PLGA 502 (●), OVA PLGA 756 (□), CpG PLGA 756 (△) and OVA CpG PLGA 756 (○). Microparticles were incubated under rotating agitation in PBS at 37 °C. Data are expressed as the cumulative release (in %) versus time (days) and represent means ± standard deviation of 2–3 experiments.

in the core of the microparticles whereas the CpG motifs would set near the particle surface.

3.4. OVA structural analysis and antigenicity and CpG integrity

The effect of the preparative process on both the structural integrity and antigenicity were studied by SDS–PAGE and immunoblotting, respectively (Fig. 3). All the formulations tested showed a similar band corresponding with the native protein (45 kDa) indicating that the integrity and the antigenicity were not altered following entrapment in any of the microparticle formulations. When studying these parameters in samples from release experiments, the structure and the antigenicity of the protein were unaltered in any of the formulations assessed (Fig. 3, panel B).

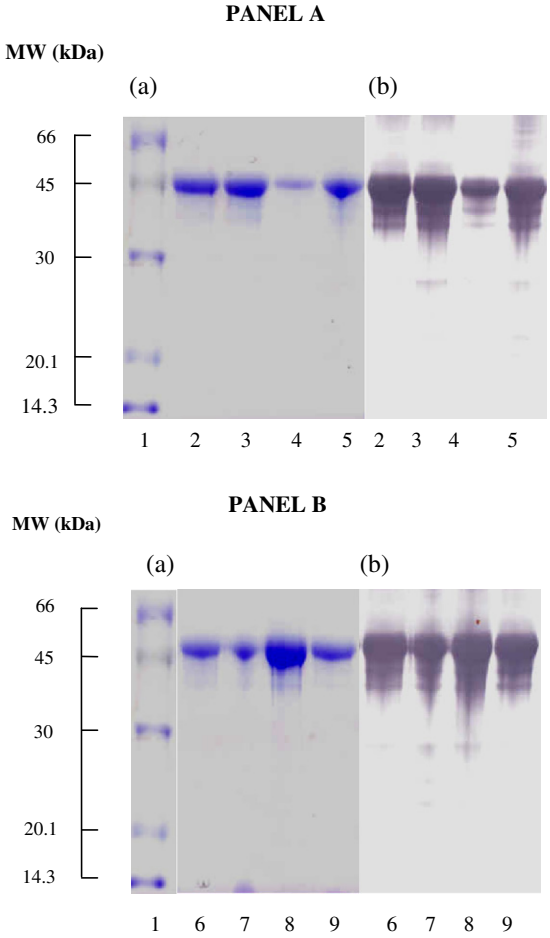

Fig. 3. Study of the integrity of the OVA after encapsulation using TROMS. Panel A shows the results for OVA extracted from lyophilized MP and panel B describes the results for OVA released from MP at day 35 after incubation in PBS. (a) SDS–PAGE stained for proteins (Coomasie Brilliant Blue R-250) and (b) Western blot analysis with an anti-OVA immunoglobulin G and horseradish-conjugated anti-IgG from rabbit. Lane 1: molecular marker; 2 and 6: OVA PLGA 502; 3 and 7: OVA CpG PLGA 502; 4 and 8: OVA PLGA 756; 5 and 9: OVA CpG PLGA 756. Load was the equivalent to 10 µg OVA/well.

The integrity of the CpG sequences was determined by means of the melting temperature, which is related with the capability of the CpG strand to form a duplex. Fig. 4a shows the T_m values for all the microparticles. As it can be observed, encapsulated CpG displayed a similar temperature comparing with free CpG sequences so the method of preparation did not modify the structure of the oligonucleotide. For the CpG sequences released from the microparticles, the T_m values were similar to those obtained for free oligonucleotide (Fig. 4b). Therefore, the integrity of the encapsulated oligonucleotide was maintained throughout the release studies.

3.5. Activation of immature DC by CpG-loaded microparticles

IL-12 production was determined to evaluate if CpG microparticles were able to induce immature DC functional activation (Fig. 5). This cytokine level was assessed after incubation at two different CpG concentrations, 0.5 and 1 μg/mL. The IL-12 produced after incubation with CpG microparticles was slightly higher (14512.5 and 13487.5 pg/mL for CpG PLGA 756 and 502 microparticles, respectively, at 1 μg/mL) than the one produced by

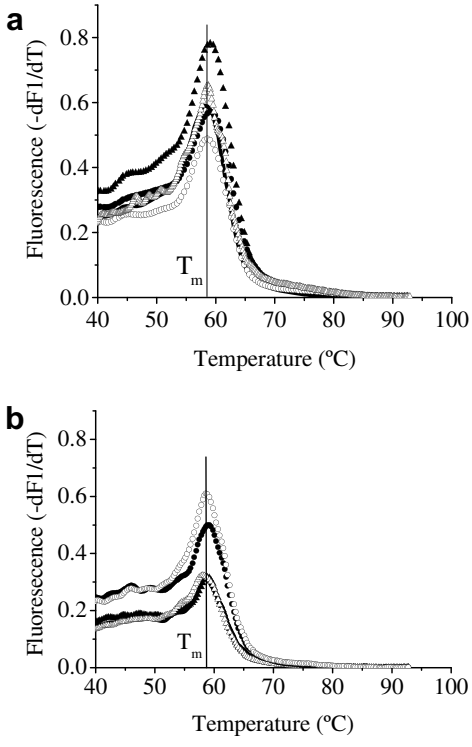

Fig. 4. Fluorescence melting curve analysis of microparticles containing CpG sequences (a) after extracting the oligonucleotide from the particles with NaOH 0.1 N overnight and (b) after the release of the oligonucleotide from the particles in PBS for 3 days. Data are expressed plotting the negative derivative of fluorescence over temperature versus temperature. Free oligonucleotide (—) was subjected to the same experimental conditions and used as control. CpG PLGA 502 (▲), OVA CpG PLGA 502 (●), CpG PLGA 756 (△) and OVA CpG PLGA 756 (○).

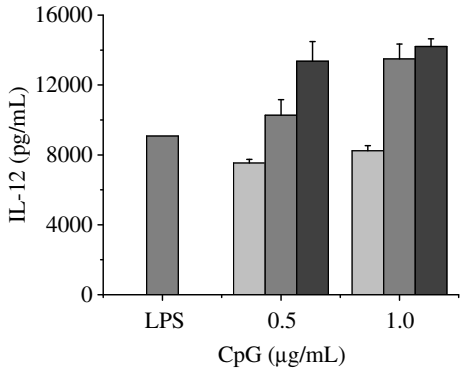

Fig. 5. IL-12 level (pg/mL) produced after the incubation of CpG-loaded PLGA 502 (grey) and 756 (dark grey) microparticles with immature BMDC for 24 h at 37 °C. The experiment was carried out at two different concentrations of CpG (0.5 and 1 μg/mL). Free CpG (light grey) and LPS (first column) were used as control and subjected to the same experimental conditions. Data represent means ± standard deviation of triplicate samples of one experiment representative out of three.

CpG sequences in solution (8241.2 pg/mL at 1 μg/mL) at any CpG concentrations.

3.6. Antibody response

IgG1 and IgG2a antibody levels were studied in BALB/c mice immunized at days 0 and 7 with 10 μg OVA or the equivalent amount of microparticles (Figs. 6 and 7). The administration of OVA-loaded PLGA 756 enhanced the antibody production (15 titres of IgG total (IgG1 + IgG2a) versus 9.5 titres of the mixture of antibodies for OVA in solution) and induced a more balanced immune response than the one elicited by the administration of the antigen in solution (from high titres of IgG1 (IgG2a/IgG1 = 0) toward a balance between IgG2a and IgG1 (IgG2a/IgG1 = 0.88) at the end of the experiment). However, when the protein was encapsulated in PLGA 502 neither the immune profile nor the antibody production was modified (IgG2a/IgG1 = 0.13 and 9 titres of IgG total compared with IgG2a/IgG1 = 0 and 9.5 titres of IgG1 plus IgG2a observed after the administration of OVA in solution).

Consistent with the studies reported by other authors, the co-administration of CpG with OVA significantly increased the production of antibodies (18 titres, approximately) and also IgG2a:IgG1 ratio (IgG2a/IgG1 = 0.54) compared with mice immunized with OVA alone. Despite the antibody production not being enhanced as a consequence of the microencapsulation (approximately, 18 titres of IgG2a and IgG1 for both treatments), the immune profile displayed by OVA CpG PLGA 502 microparticle treated mice was more Th1-biased than the one observed after the administration of the mixture of the antigen and the oligonucleotide in solution (IgG2a/IgG1 = 0.73 for OVA CpG PLGA 502 and IgG2a/IgG1 = 0.54 for the combination of OVA and CpG sequences in solution). Nevertheless, the co-encapsulation of both molecules into PLGA 756 microparticles did not succeed either in the stimulation of

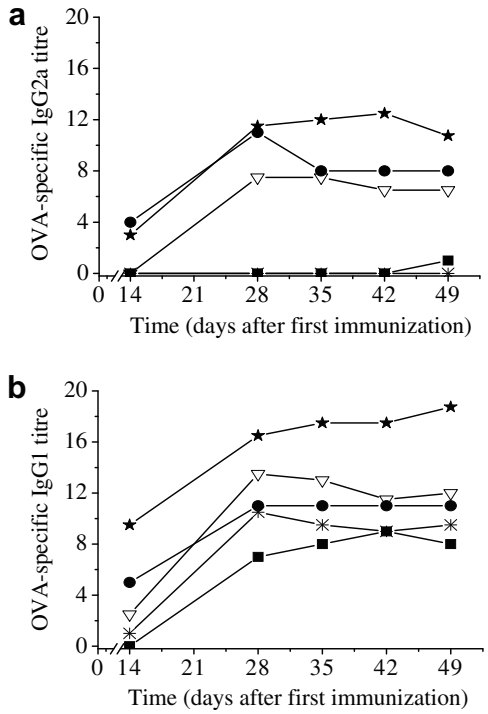

Fig. 6. Serum antibody response to ovalbumin (panel a for IgG2a and panel b for IgG1) measured by indirect ELISA on pooled sera from BALB/c mice intradermically immunized (10 μg OVA) with the following: (i) OVA in PBS (✳), (ii) OVA and CpG sequences physically mixed (▽), (iii) OVA PLGA 502 (■), (iv) OVA CpG PLGA 502 (●) and (v) OVA emulsified with Freund's adjuvant (★). The antibody titre is defined as the reciprocal of a serum dilution whose optical density was equal or above 0.2 than blank samples reading the absorbance at 405 nm, starting from sample dilution 1:40.

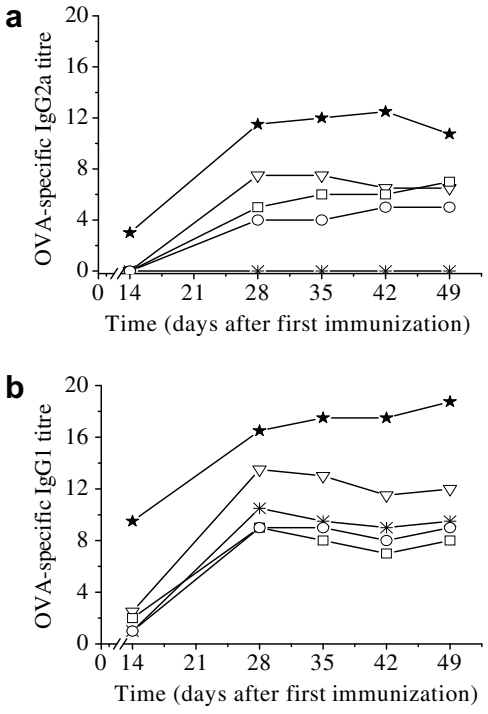

Fig. 7. Serum antibody response to ovalbumin (panel a for IgG2a and panel b for IgG1) measured by indirect ELISA on pooled sera from BALB/c mice intradermically immunized (10 μg OVA) with the following: (i) OVA in PBS (✳), (ii) OVA and CpG sequences physically mixed (▽), (iii) OVA PLGA 756 (□), (iv) OVA CpG PLGA 756 (○) and (v) OVA emulsified with Freund's adjuvant (★). The antibody titre is defined as the reciprocal of a serum dilution whose optical density was equal or above 0.2 than blank samples reading the absorbance at 405 nm, starting from sample dilution 1:40.

the immune system (19 titres of IgG1 and IgG2a) or the deviation of the immunological profile (IgG2a/IgG1 = 0.55) in comparison with the injection of both OVA and CpG sequences in a physical mixture.

3.7. Cellular immune response

IFN-γ (Fig. 8) and IL-4 levels were studied in order to characterize the Th1 and Th2 immune responses, respectively, in BALB/c mice after single-shot intradermal immunization with the previously described formulations. The IL-4 production (the Th2 main cytokine) was almost undetectable (below 5 pg IL-4/mL) for any of the treatments evaluated in this work and under these experimental conditions except for OVA emulsified with CFA, which elicited a poorly higher level (7 pg IL-4/mL). Hence, the single immunization with microparticulated or free OVA did not generate a Th2 cellular response.

Regarding the IFN-γ production, the administration of CpG sequences plus ovalbumin resulted in an increase of the cytokine level compared with the ovalbumin in solution (from 422 to 1094 pg/mL). When the protein was encapsulated in both types of microparticles, the production rose to 2030 pg/mL in the case of PLGA 502 and to 9146 pg/mL in

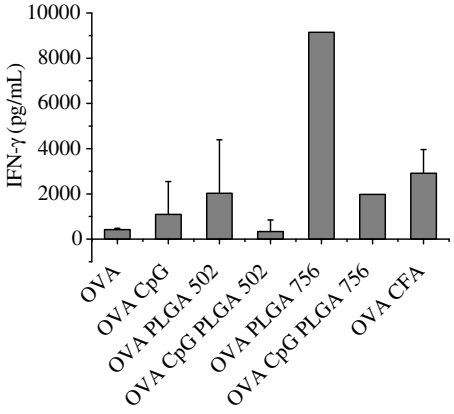

Fig. 8. IFN-γ production by spleen cells obtained from BALB/c mice 13 days after intradermal immunization (10 μg OVA) with one of the following: (i) OVA in solution, (ii) OVA and CpG sequences physically mixed, (iii) OVA PLGA 502, (iv) OVA CpG PLGA 502, (v) OVA PLGA 756, (vi) OVA CpG PLGA 756 and (vii) OVA emulsified with Freund's adjuvant. The resulting splenocyte suspensions were *in vitro* restimulated with 80 μg OVA/mL for 48 h to analyse IFN-γ and IL-4 production (pg/mL). Data represent means ± standard deviation of duplicate samples of one experiment representative out of three.

the case of PLGA 756 microparticles. When the CpG sequences were co-encapsulated into the carriers, OVA

CpG PLGA 502 and 756 produced 335 and 1976 pg/mL IFN-γ, higher level for 756 and lower production for 502 in comparison with the administration of the protein and the oligonucleotide in solution (1094 pg/mL). Hence, the co-encapsulation of CpG sequences with OVA into PLGA microparticles was not able to ameliorate the IFN-γ produced by the formulations containing only the antigen. If compared with the physical combination of the antigen and the oligonucleotide, the polymer induced different results. Whereas OVA CpG PLGA 502 particles decreased the IFN-γ level, OVA CpG PLGA 756-treated mice displayed a more Th1-biased response.

4. Discussion

This work aimed at the preparation of CpG and a model shed antigen (ovalbumin)-loaded microparticles by a new double-emulsion solvent evaporation method called "Total Recirculation One-Machine System" (TROMS). In the recent past, we have shown the performance of this novel technique for loading adenoviruses [28], plasmid DNA [20] and *Brucella* antigens [21] without compromising their integrity. In this study, TROMS is used for achieving non-charged microparticles with high antigen and CpG payloads (Table 2) and avoiding destruction of the protein (Fig. 3) and CpG oligonucleotides (Fig. 4). In the *in vitro* release studies, a rapid disposal of CpG motifs was observed, suggesting their location onto the particle surface (Fig. 2b). On the contrary, the release of the antigen, from the core of the particles, was very slow and sustained over the time (Fig. 2a). The immunogenicity of these formulations was evaluated in BALB/c mice and compared with the co-administration of soluble forms of CpG and antigen.

The improvement of CpG stimulatory potency by liposomes or microparticles-based delivery has been clearly established [29,30]. Nearly all of these reports are based on the absorption of CpG onto the preformed positive particles [31] except for few works which tested the adjuvant effect inside the polymeric matrix with more [17] or less [32] success. In another context, some studies have been applied for the complexation of CpG oligonucleotides or CpG DNA with positive molecules (such as PEI [19] or DOTAP) to modify the intracellular uptake and route with negative or positive effect in their stimulatory activity. In the current study, we investigated the immune response induced by the co-delivery of CpG–chitosan complexes and OVA in non-charged PLGA microspheres. In the first trials, we recorded several cationic molecules and chitosan was almost the only one (i) suitable for adequate loading of CpG into PLGA particles (data not shown) and (ii) that did not have negative effect in CpG stimulatory potency, evaluated as IL-12 production by BMDC *in vitro* (Fig. 5). As compared with the literature [17], it is remarkable that TROMS methodology loaded appreciable amounts of CpG motifs onto around 1 μm PLGA particles that did not exhibit positive zeta potential because of low chitosan percentage (lower than 0.1% w/w) (Table 2). Higher amounts of chitosan that would be necessary for the total complexation of the oligonucleotide at N/P 1 decreased the CpG encapsulation (Table 1). It was probably due to excessive viscosity provided by this polysaccharide, which compromised the stability of the inner aqueous phase.

Also, similarly as previously reported with other antigens [21], TROMS microencapsulated the antigen, OVA, with high efficiency whereas OVA antigenic properties were maintained, also during sustained release periods (Fig. 3). The integrity of CpG molecules was also preserved during the preparative process as long as T_m values remained unchanged (Fig. 4).

There are some factors that can affect the immune response elicited by the administration of microencapsulated antigens. Among them, the polymer type has been shown to be able to modify the antigen intrinsic cytokine profile [33]. In the current study mice were immunized with two different formulations containing PLGA 756 or PLGA 502 as the polymers. Whereas OVA alone or OVA loaded into PLGA 502 microparticles triggered IgG1 antibody response, OVA microencapsulated into PLGA 756 displayed higher antibody titres and increased production of IgG2a antibodies (Figs. 6 and 7), accompanied by strong IFN-γ production. This fact could be explained by the stronger interaction between hydrophobic polymers (such as PLGA 756) and antigen-presenting cells, and, consequently, the promotion of the Th activation [34].

The ability of CpG ODN to trigger the production of T-helper 1 and pro-inflammatory cytokines and IFN-γ dependent IgG2a antibodies has been observed with a variety of co-administered antigens [35]. This adjuvant effect was clearly dose-dependent and mediated by cytokines produced following stimulation of APC, as for example, IL-12. Therefore, the co-association of CpG ODN or CpG DNA with antigens in delivery systems has been previously shown to be so effective to elicit specific T-cell response that CpG simply co-administered with antigen and also that induced by the administration of the antigens microencapsulated alone [17,31,36–38]. On the contrary, the immunopotentiation effect of the particulate delivery on antibody response has not been clearly established [31]. For the first time, our data show the critical influence of PLGA polymer in particulate CpG adjuvanticity.

CpG motifs co-encapsulated into PLGA 502 induced higher antibody titres and increased production of IgG2a antibodies that OVA microencapsulated alone, being more effective that both co-administered freely in solution (Fig. 6). On the contrary, co-delivery of CpG and OVA in PLGA 756 microparticles decreased IgG2a antibody levels (Fig. 7). Either positive or negative influence of CpG particulate delivery in antibody response has been previously reported and it was a function of CpG release rate. So, nanoparticles that loaded CpG inside their matrix could release CpG in a sustained manner that resulted in suboptimal doses for B-cell stimulation [31]. On the

contrary, CpG presented onto the surface of cationic microspheres was able to augment the antibody response and it was suggested due to a certain effect of surface nature in B-cell engagement [36]. Both factors, CpG release (slower from PLGA 756 microparticles) and a certain influence of carrier nature could explain the improved antibody response (stronger and biased towards IgG2a subtype) observed in mice immunized with OVA CpG PLGA 502 in comparison with CpG simply co-administered with the antigen (Fig. 6).

Regarding the results of antigen-specific cytokine production, only the delivery of the antigen (with or without CpG motifs) into PLGA 756 microparticles elicit stronger T-cell response to the immunization with the antigen and CpG in solution (Fig. 8). Irrespective of the polymer, CpG co-encapsulation decreased IFN-production in comparison with the cellular response induced by the antigen microencapsulated alone. The observation contrasts with BMDC stimulation (IL-12 production) induced by both types of microparticles that were similar between them and to that induced by CpG ODN administered in solution (Fig. 5). Our unfavourable results suggest that, in spite of the adjuvanticity at modest doses, a certain antigen:CpG ODN ratio (1:5 or superior) must be preserved (face to 5:1 in the current study) to have clear evidence of the benefits of CpG administration [16]. So far, the absence of correlation with the observed antibody production could be due to the direct effect of CpG oligonucleotide in B cells.

In summary, our results established the critical role of PLGA polymer in the adjuvanticity of microparticles (OVA MP 502 versus OVA MP 756) and encapsulated CpG motifs (OVA MP versus OVA CpG MP). However, we did not find clear benefits of OVA and CpG microparticulate co-delivery (OVA MP or OVA CpG MP versus OVA plus CpG). Some characteristics of the described systems, as antigen:CpG ratio and the form in which CpG motifs are released (free or associated to chitosan) need to be evaluated in detail for better comprehension of their immunological behaviour. Moreover, these and other parameters must be reconsidered in order to justify the superiority of CpG particulate delivery as Th1 adjuvant.

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