



Research paper

Development of poly(anhydride) nanoparticles loaded with peanut proteins: The influence of preparation method on the immunogenic properties

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ABSTRACT

Allergen-specific immunotherapy is based on the administration of allergens with the main disadvantage of inducing an allergic reaction. Within this context, we report the generation of an adjuvant and allergen-delivery system for peanut allergen immunotherapy with reduced IgE induction. Therefore, we prepared and characterized poly(anhydride) nanoparticles loaded with peanut proteins using the solvent displacement method, with some modifications in the manufacturing process. The precipitation of polymer was performed with either a mixture of ethanol and water or water. The resultant nanoparticles were dried by either freeze-drying or spray-drying, respectively. Poly(anhydride) nanoparticles loaded with peanut proteins were successfully developed, achieving both high encapsulation efficiency (70–80%) and manufacturing yield (60–80%). After intradermal immunization of mice (C57Bl/6) with peanut proteins incorporated into poly(anhydride) nanoparticles, a strong mixed T_H1/T_H2 -type immune response was observed. Furthermore, we also provide, to our knowledge for the first time, clear evidence of the influence of formulation design on the immunostimulatory properties of nanoparticles. Taken together, our findings indicate that poly(anhydride) nanoparticles are efficient stimulators of immune responses and promising adjuvants and allergen-delivery systems applied for immunotherapy.

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1. Introduction

Over the past few years, a large increase in the prevalence of allergic diseases has been reported in industrialized countries [1]. Among food allergies, peanut (*Arachis hypogaea*) has attracted great research attention because its prevalence is high, patients do not outgrow the allergy, and it is often severe. Moreover, it has been reported as one of the most common accidental exposures causing anaphylaxis [2,3]. Several studies from North America and the United Kingdom (UK) have reported a significant increase in the prevalence and severity of peanut allergy in recent years [4,5]. For instance, in US, more than 1% of the population (>3 million subjects) currently suffers from this allergy [6].

At present, the only proven therapy for the treatment of peanut allergy is strict avoidance of peanut-containing foods and education of patients to recognize and treat allergic reactions caused

by accidental exposure [7]. Another potential strategy is the use of immunotherapy. However, attempts at standard subcutaneous immunotherapy have been abandoned because of marginal efficacy [8] and adverse reactions including anaphylaxis induced by the vaccine itself [9,10]. In this context, different strategies to develop new immunotherapeutic vaccines are being investigated [11].

In the last decade, nanoparticle-based allergen delivery systems have received much interest as potential adjuvants for allergen immunotherapy [12]. These polymeric nanoparticles may be able to modulate the immune response to the allergens, without triggering adverse reactions [13,14]. Different types of polymers and copolymers have been used to design nanoparticles. Among these, the copolymer of methyl vinyl ether and maleic anhydride (commercialized as Gantrez® AN from ISP Corp.) is a poly(anhydride) that allows an efficient and easy preparation of nanoparticles under mild conditions, using a solvent displacement method [15]. This polymer can easily load soluble proteins and/or bacterial antigens [16–18], including allergens [19–21]. Furthermore, the nanoparticle surface can be “decorated” after incubation with ligands or excipients [22–24], in order to improve their capabilities

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to develop bioadhesive interactions with the mucosa and/or promote their interaction or targeting properties for the mucosa-associated lymphoid system.

Moreover, poly(anhydride) nanoparticles have been recently identified as innate immunity inducers, activating the complement system [25] and Toll-like receptors (TLRs), mainly TLR2 and TLR4 [26]. In addition, previous studies have reported the ability of adjuvants based on poly(anhydride) nanoparticles to induce both antibody and cellular allergen-specific immune responses [19,20]. Thus, Gómez et al. [21] demonstrated that the encapsulation of *Lolium perenne* into poly(anhydride) nanoparticles improved the efficacy of intradermal vaccination against grass pollen allergy. Also, oral immunization with poly(anhydride) nanoparticles loaded with ovalbumin (OVA) protected sensitized mice from death by anaphylactic challenge [20]. All these properties make poly(anhydride) nanoparticles an attractive adjuvant for allergen immunotherapy.

In view of this, we sought to develop an adjuvant and allergen-delivery system for peanut allergen immunotherapy. We therefore prepared and characterized poly(anhydride) nanoparticles loaded with peanut proteins using the solvent displacement method [15], with some modifications in the manufacturing process. The precipitation of polymer was performed with either a mixture of ethanol and water or water. The resultant nanoparticles were dried by either freeze-drying or spray-drying, respectively. The influence of manufacturing process on the immune response was evaluated in C57BL/6 mice.

2. Materials and methods

2.1. Chemicals

Peanut extract (PE) containing peanut proteins were obtained from Diater[®] Laboratories (Madrid, Spain). Poly(methyl vinyl ether-co-maleic anhydride) (PVM/MA), Gantrez[®] AN 119 (MW 200,000), was kindly provided by ISP International Corp. (Barcelona, Spain). 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), PBS containing 0.05% Tween 20 (PBS-T), and protease inhibitor cocktail were purchased from Sigma (Spain). The peroxidase immunoconjugates (GAM/IgG1/PO, GAM/IgG2a/PO, and GAM/IgA/PO) were obtained from Nordic Immunology (The Netherlands). The ELISA IgE Set Mouse was purchased from BD[®] Biosciences (Belgium). Micro-BCA[™] Protein Assay Reagent Kit was from Pierce[®] (Rockford, USA). PVDF (polyvinylidene fluoride) membrane was from Millipore Corporation (Bedford, MA, USA) and 4-chloro-1-naphthol from Merck (Germany). The rainbow colored protein molecular weight marker was from Amersham Pharmacia Biotech (Freiburg, Germany). Viva-Spin[®] 20 300,000 M.W.C.O. was purchased from Vivascience (Sartorius group, Hannover, Germany). All other chemicals used were of reagent grade and obtained from Merck (Spain).

2.2. Preparation of poly(anhydride) nanoparticles

Poly(methyl vinyl ether-co-maleic anhydride) or poly(anhydride) nanoparticles (NPs) were prepared by the solvent displacement method, with some modifications in the manufacturing process. After purification, nanoparticles were dried by freeze-drying [15] or spray-drying [27].

2.2.1. Lyophilized nanoparticles (NP-A)

In order to prepare PE-loaded lyophilized nanoparticles (NP-PE-A), 3 mg of peanut extract was dispersed in 100 µl of deionized water (H₂Odd, pH 3.0) by ultrasonication (Microson[™]) for 1 min (min) and resuspended in 1 mL acetone. The PE dispersion was

added to 4 mL acetone containing 100 mg PVM/MA, and the mixture was stirred for 45 min at room temperature (RT). The polymer was then dissolved by the addition of 20 mL of a mixture of ethanol and H₂Odd (1:1 by volume). The organic solvents were eliminated under reduced pressure (Buchi[®] R-144, Switzerland). Finally, the suspensions of nanoparticles were purified twice by centrifugal filtration (3000g, 20 min) and lyophilized using an aqueous solution containing sucrose (5%, p/v) as cryoprotector (Genesis 12EL, Vir-tis[®], USA). Control lyophilized nanoparticles (NP-A) were prepared in the same way, in the absence of PE.

2.2.2. Spray-dried nanoparticles (NP-B)

Spray-dried PE-loaded nanoparticles (NP-PE-B) were prepared as previously described [27], with some modifications. Briefly, 500 mg PVM/MA were dissolved in 20 mL acetone. In parallel, 3 mg of peanut extract was dispersed in 100 µl of deionized water (H₂Odd, pH 3.0) by ultrasonication (Microson[™]) for 1 min and resuspended in 10 mL acetone. After incubation under magnetic stirring (45 min, RT), the formation of nanoparticles was achieved by addition of 15 mL H₂Odd. After evaporation of acetone, the nanoparticles were purified by centrifugal filtration. Next, a mixture of solution containing 1 g lactose in 15 mL H₂Odd was added under magnetic stirring (5 min, RT). Finally, nanoparticles were dried in a Mini Spray-dryer Büchi B191 (Büchi Labortechnik AG, Switzerland). Control spray-dried nanoparticles (NP-B) were prepared in the same way, in the absence of PE.

2.3. Characterization of nanoparticles

2.3.1. Size and zeta potential

The size and zeta potential of nanoparticles were determined by photon correlation spectroscopy (PCS) and electrophoretic laser Doppler anemometry, respectively, using a Zetamaster analyser system (Malvern[®] Instruments, UK). Samples were diluted with 0.05 mM phosphate-buffered saline (PBS) or KCl 0.1 mM for measurement of size and zeta potential, respectively. All analyses were performed in triplicate, at 25 °C with a scattering angle of 90°. The average particle size is expressed as the volume mean diameter (Vmd) in nanometers (nm), and the average surface charge as mV.

The size and surface charge of the empty nanoparticles obtained by both previously described methods were determined at different intervals of time (0, 1, 1.5, 2, 3, 6.5, and 7 h), in order to evaluate the influence of the manufacturing process on the physicochemical properties of nanoparticles.

2.3.2. Stability study

The stability of the empty nanoparticles prepared by both methods was evaluated by measuring the turbidity of an aqueous suspension of nanoparticle suspension over time. Briefly, 6 mg of either lyophilized or spray-dried nanoparticles were resuspended in 2 mL of purified water and centrifuged (28,000g, 20 min, 4 °C). The precipitate was then resuspended in 4 mL of water and aliquots of 300 µL of each sample were pipetted into microplate wells. The turbidity of the samples was evaluated by measuring their absorbance at 405 nm over time (0–400 min). If necessary, samples were diluted in order to obtain the same initial absorbance in all cases at the beginning of the study. All measurements were performed in triplicate, and the results were expressed as percentage of absorbance reduction (% Reduction) vs. time. The % Reduction was calculated as follows:

$$\% \text{ Reduction} = (A_0 - A_t/A_0) \times 100 \quad (1)$$

where A_0 is the initial absorbance of the sample, and A_t is the absorbance of the sample at each time point.

2.3.3. Morphology

The morphological characteristics of nanoparticles were assessed by scanning electron microscopy (SEM). Prior to analysis, nanoparticles were resuspended in ultrapure water and centrifuged at 27,000g for 20 min at 4 °C. The supernatants (containing the cryoprotector) were discarded, and the pellets were mounted on a glass plate fixed with adhesive tape onto metal stubs and dried under hot flow air. Finally, the particles were coated with gold to a thickness of 16 nm in order to perform the SEM photographs (Ermitech K550 equipment, United Kingdom).

2.3.4. Yield

The amount of polymer transformed into nanoparticles was determined by gravimetry, as previously described [23]. The yield was calculated from the difference between the initial amount of the polymer used to prepare the nanoparticles and the weight of the either lyophilized or spray-dried samples.

2.3.5. Protein loading and encapsulation efficiency

In order to quantify the amount of peanut protein associated with the nanoparticles, 10 mg of either lyophilized or spray-dried nanoparticles was digested in NaOH 0.1 M by shaking at 37 °C the mixture for 1 h. Next, the protein content was measured using the bicinchoninic acid method (MicroBCA), as previously described [28]. Each sample was assayed in triplicate, and results of PE loading were expressed as the amount of protein (in µg) per mg NP. Similarly, the encapsulation efficiency (EE) was calculated as follows:

$$EE (\%) = (Q_{\text{associated}}/Q_{\text{initial}}) \times 100 \quad (2)$$

where Q_{initial} was the initial amount of PE added per mg of polymer that forms the nanoparticles and $Q_{\text{associated}}$ was the amount of loaded PE per mg of nanoparticles, which was calculated by MicroBCA.

2.3.6. In vitro release study of peanut proteins from nanoparticles

PE-loaded nanoparticles (10 mg) were dispersed in Eppendorf tubes by vortexing in 1 mL phosphate buffer saline (PBS, pH 7.4). A release study was conducted at 37 ± 1 °C under rotating agitation. At defined times, the sample tubes were centrifuged at 3000g for 20 min and the protein content was determined in the supernatants by micro-BCA assay.

2.3.7. Structural integrity and antigenicity of the loaded peanut proteins

To evaluate the effects of the manufacturing process on the integrity and antigenicity of peanut proteins, SDS–PAGE and immunoblotting were performed. Briefly, 10 mg of samples was resuspended in 1 mL of ultrapure water and centrifuged (28,000 g, 20 min, 4 °C). Then, the sediment was dissolved in 2 mL of acetone/dimethylformamide (3:1, v/v) and stored for 1 h at –80 °C. Samples were centrifuged at 28,000g (20 min, 4 °C), and the pellet was washed with 1 mL acetone and stored for 30 min at –80 °C. After centrifugation under the same conditions, pellets were resuspended in the electrophoretic sample buffer (Tris–HCl 62.5 mM, pH 6.8; 10% glycerol; 2% sodium dodecyl sulfate, SDS; 5% β-mercaptoethanol and 0.05% bromophenol blue) and boiled for 10 min. SDS–PAGE was performed using discontinuous gels of 15% acrylamide.

For the antigenicity studies, the proteins separated electrophoretically were transferred from the gel to a polyvinylidene fluoride (PVDF) membrane, pore size 0.45 µm, by a semidry blotting system at 0.8 mA/cm² for 60 min (Trans-Blot® SD Transfer Cell, BIO-RAD, Hercules, CA, USA). After the blocking of protein-binding sites with blocking solution (PBS plus 10% fetal bovine serum, 60 min, RT), the membranes were incubated with sera from peanut allergic pa-

tients diluted 1:10, overnight at 4 °C. Peroxidase (PO)-conjugated secondary antibody (Goat anti-human IgE, 1:1000) was then added for 60 min at RT. Finally, membranes were extensively washed and H₂O₂–ABTS solution (Sigma, Missouri, USA) was used as the chromogenic substrate. The apparent molecular masses of peanut proteins were determined by comparison with standard molecular weight markers.

2.4. In vivo studies

2.4.1. Animals

Experiments were performed in compliance with the regulations of the Ethics Committee of the University of Navarra in line with the European legislation on animal experiments (approved protocol number 048/09).

2.4.2. Intradermal immunization

Female C57BL/6 mice (Harlan Interfauna Ibérica, Spain), 8 weeks old (20 ± 1 g), were randomly divided into six groups ($n = 5$). Animals were intradermally immunized with a single dose of free peanut proteins (200 µg) or incorporated into one of the following formulations: (i) lyophilized PE-loaded nanoparticles (NP-PE-A) or (ii) spray-dried PE-loaded nanoparticles (NP-PE-B). Empty nanoparticles (NP-A and NP-B) and free peanut proteins (200 µg of PE) were administered as controls. A group of non-immunized mice (Control, C) were also included in the study. Blood samples from the retroorbital plexus were collected weekly, centrifuged (3000g, 10 min) and the resulting sera were pooled and stored at –80 °C until analysis. All animals were sacrificed five weeks after initial immunization.

2.5. Quantification of anti-PE antibodies by indirect ELISA

The presence of PE-specific antibodies was determined in sera (IgG1, IgG2a, IgA, and IgE) by indirect ELISA. Measurements were taken in triplicate and including negative controls (no mouse serum sample background and no antigen coating control).

2.5.1. Specific IgG1 and IgG2a antibodies

Briefly, microplate wells (Immuno-Maxisorp, Nunc®, Roskilde, Denmark) were coated with 100 µg/mL of peanut proteins diluted in sodium carbonate–bicarbonate buffer (0.05 M, pH 9.6), for 12 h in a moist chamber at 4 °C. The plates were blocked with PBS containing 3% bovine serum albumin (BSA) (Sigma–Aldrich, Barcelona, Spain) for 1 h at room temperature (RT). Wells were incubated with serum samples diluted 1:80 in triplicate (1 h, RT) and subsequently with goat horseradish peroxidase (PO)-conjugated secondary antibody (anti-mouse IgG1 or IgG2a) (Nordic Immunology, Tilburg, The Netherlands) diluted 1:1000 in PBS containing 1% BSA (Sigma–Aldrich, Barcelona, Spain) for 1 h at RT. Finally, the plates were developed using a chromogenic substrate (H₂O₂–ABTS solution, Sigma, Missouri, USA) under shaking. The optical density (OD) was measured at 405 nm (iEMS Reader MF, Labsystems) after 15 min of incubation at RT.

2.5.2. Specific IgE antibodies

Plates were coated with peanut proteins (400 µg/mL) and blocked with PBS plus 10% fetal bovine serum (1 h, RT). After washing, pooled serum samples were added at 1:10 in dilution buffer (0.085% in PBS-T) (2 h, RT). Following incubation, plates were washed and a biotin-labeled anti-mouse IgE antibody was added. After incubation, plates were washed and streptavidin alkaline phosphatase conjugate was added at 1:1000 (in PBS with 10% FBS, pH 7.0). Subsequently, plates were washed and p-nitro-phenyl phosphate (PNPP) substrate was added, according to the manufacturer's instructions (Becton Dickinson, BD®).

2.5.3. IgA antibodies

For the measurement of PE-specific IgA, after coating, plates were blocked with PBS plus 1% non-fatty milk (1 h, RT). Wells were incubated with serum samples diluted 1:20 (4 h, 37 °C) and treated with peroxidase (PO)-conjugated secondary antibody (anti-mouse IgA, Nordic Immunology, Tilburg, The Netherlands), diluted 1:1000 in PBS containing 1% BSA (1 h, RT). The plates were washed, developed, and measured as described above.

2.6. Cytokine production

Five weeks after immunization, mice were sacrificed and spleens were extracted. Spleen cell suspensions were obtained by mechanical disaggregation, and 2.5×10^6 cells were cultured in 96-well flat-bottom plates in RPMI-1640 medium supplemented with 10% fetal bovine serum, 50 μ M 2-mercaptoethanol and 1% penicillin/streptomycin. Cells were incubated at 37 °C in CO₂ and stimulated for 48 h with 150 μ g/mL peanut proteins. Culture supernatants were analyzed for the presence of cytokines (IL-5 and IFN- γ) by a custom-made MILLIPLEX™ MAP (multiple analyte profile) Mouse Cytokine/Chemokine Panel (Millipore Corporation, MA, USA) using a Luminex® 100 IS System. The assay was performed using recombinant cytokines as standard controls, according to the manufacturer's instructions.

2.7. Statistical analysis

Analysis of variance (ANOVA) was employed to analyze the data. When ANOVA indicated a significant difference, the Tukey *post hoc* test was used to assess the difference between groups. Differences were regarded as statistically significant when $P < 0.05$. Data are expressed as the mean $\pm$ SD from independent experiments.

3. Results

Physical–chemical characterization of nanoparticle formulations is presented in Table 1. The mean diameter of poly(anhydride) nanoparticles was, in all cases, below 200 nm; although, for PE-loaded nanoparticles this size was slightly lower than for empty nanoparticles. Interestingly, the polydispersity index (Pdl) was found to be lower than 0.2, indicating homogeneous nanoparticle formulations. This homogeneity was also visually corroborated by SEM (Fig. 1). The microphotographs also demonstrated that the nanoparticle always displayed a spherical shape without any evident differences as a result of the preparation method.

Furthermore, all nanoparticle formulations were found to be negatively charged. However, the zeta potential of empty lyophilized nanoparticles (NP-A) was slightly more negative than for empty spray-dried nanoparticles (NP-B). In addition, the loading and encapsulation efficiency of nanoparticles was similar and inde-

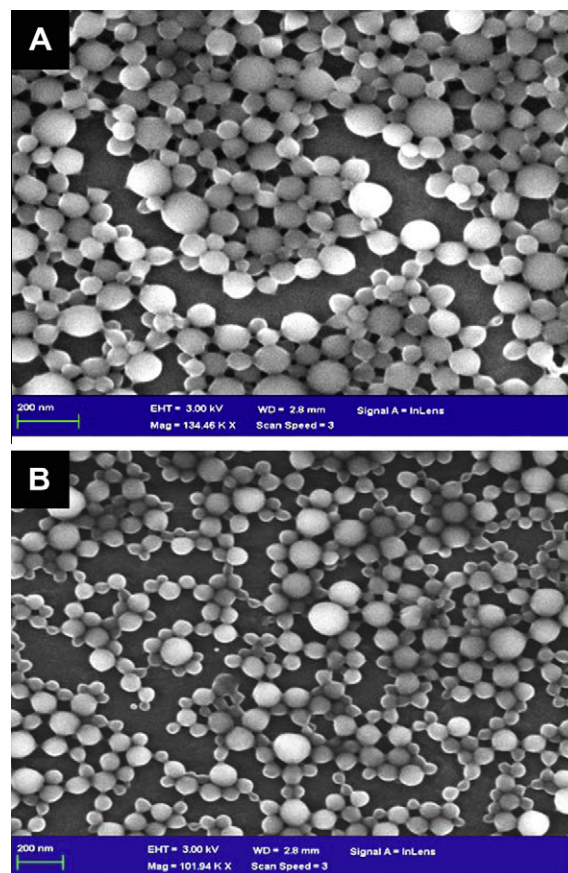


Fig. 1. Scanning electron microscopy of lyophilized (A) and spray-dried (B) empty nanoparticles. Scale bars: 200 nm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

pendent of the method of preparation. In general, the PE loading ranged from 30 to 40 μ g PE/mg nanoparticles, achieving high encapsulation efficiency (70–80%). Finally, the yield of the process was similar for both loaded and empty nanoparticles; although, when nanoparticles were prepared by spray-drying the yield was significantly lower than when freeze-dried. In any case, this difference was directly related to the adherence of the dry powder to some parts of the spray-dry apparatus.

When the effects of the manufacturing processes on the structural integrity of peanut proteins were evaluated (Fig. 2), SDS-PAGE showed similar protein profiles between proteins-extracted from nanoparticles (NP-PE-A and NP-PE-B) and free peanut proteins (PEs). Importantly, immunoblotting demonstrated the same reactivity against sera from peanut allergic patients, confirming that the encapsulation process did not affect the antigenicity of entrapped peanut proteins.

Table 1

Physical–chemical characterization of poly(anhydride) nanoparticles (mean $\pm$ SD, $n = 6$).

Formulation	Size ^a (nm)	Zeta potential (mV)	PE content ^b (μ g/mg NP)	Encapsulation ^c efficiency (%)	Yield ^d (%)
NP-A	196 $\pm$ 1	−54 $\pm$ 3	–	–	79 $\pm$ 3
NP-B	165 $\pm$ 1	−41 $\pm$ 4	–	–	65 $\pm$ 2
NP-PE-A	140 $\pm$ 3	−42 $\pm$ 2	−32 $\pm$ 3	−75 $\pm$ 9	−74 $\pm$ 6
NP-PE-B	145 $\pm$ 4	−46 $\pm$ 1	−38 $\pm$ 5	−79 $\pm$ 8	−60 $\pm$ 5

NP-A, empty lyophilized nanoparticles; NP-B, empty spray-drier nanoparticles; NP-PE-A, lyophilized nanoparticles loaded with peanut proteins; NP-PE-B, spray-dried nanoparticles loaded with peanut proteins.

^a Determination of volume mean diameter by photon correlation spectroscopy.

^b Determination of the protein content by MicroBCA™ protein assay.

^c Determination of the entrapment efficiency, expressed in percentage, by relating the total weight of protein entrapped in the nanoparticles to the initial weight of protein.

^d The percentage yield of the polymer transformed into nanoparticles.

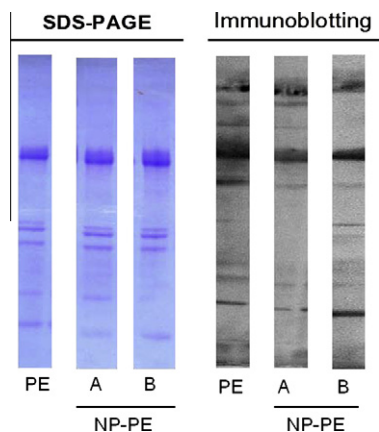


Fig. 2. Structural integrity and antigenicity of peanut proteins. SDS-PAGE (Coomassie brilliant blue staining) and immunoblotting (sera from peanut allergic patients) were performed with free proteins (PE) and proteins entrapped in lyophilized (NP-PE-A) or spray-dried (NP-PE-B) nanoparticles. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

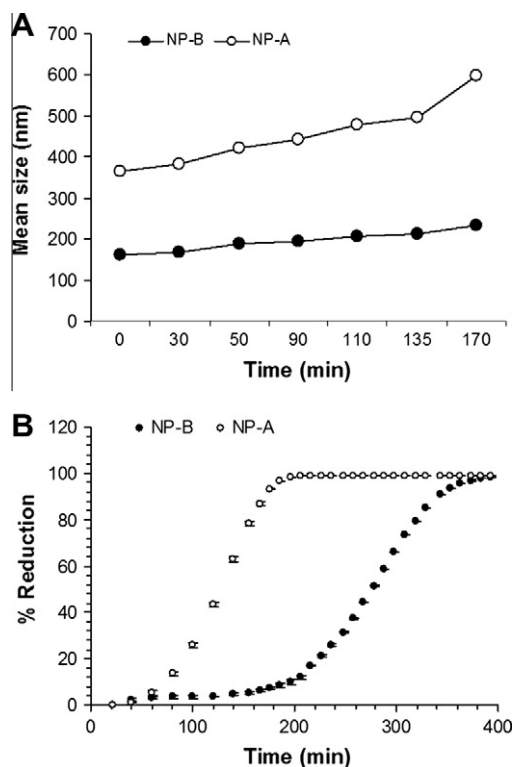


Fig. 3. *In vitro* stability studies. Size evolution (A) and absorbance reduction at 405 nm over time (B) of lyophilized (NP-A) and spray-dried (NP-B) empty nanoparticles (mean $\pm$ SD, $n = 6$).

Fig. 3 summarizes the stability studies of nanoparticles after their dispersion in an aqueous medium. Lyophilized nanoparticles displayed a significantly lower stability in water than spray-dried nanoparticles. This lower stability was characterized by a rapid increase in the mean size (Fig. 3A) and, in parallel, a significant reduction in the turbidity of samples (Fig. 3B). In fact, for lyophilized nanoparticles, 50% of absorbance was achieved after 2 h and the remaining nanoparticles displayed a mean size of 280 nm. For spray-dried nanoparticles, a similar reduction of the turbidity needed 4.5 h.

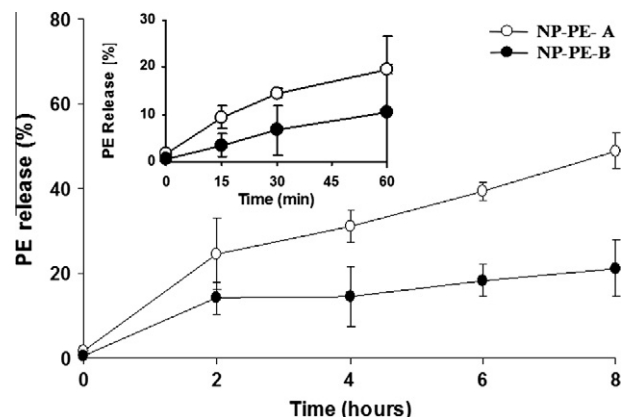


Fig. 4. *In vitro* release of peanut proteins (PEs) from poly(anhydride) nanoparticles. NP-PE-A, lyophilized nanoparticles loaded with peanut proteins; NP-PE-B, spray-dried nanoparticles loaded with peanut proteins. Data express the mean of cumulative amount of PE released vs. time (mean $\pm$ SD, $n = 3$).

Fig. 4 depicts the cumulative percentage of PE released from nanoparticles in PBS (10 mM, pH 7.4) at $37 \pm 1^\circ\text{C}$. The release rate of PE was slower for spray-dried nanoparticles than lyophilized nanoparticles. After 8 h, $\sim 21\%$ of PE was released from spray-dried nanoparticles. For lyophilized nanoparticles, after 8 h, the amount of PE was found to be close to 50% of the loaded allergen.

In order to evaluate the immunomodulatory properties of poly(anhydride) nanoparticles, an immunogenicity study was performed. C57Bl/6 mice were immunized with 200 μg of peanut proteins incorporated into either lyophilized (NP-PE-A) or spray-dried (NP-PE-B) nanoparticles. As controls, peanut proteins (Free PE), empty nanoparticles (NP-A or NP-B), and PBS (non-immunized control group) were used.

Fig. 5 shows the specific antibody response profile elicited in sera after intradermal immunization with the different nanoparticle formulations. All types of nanoparticles induced high levels of both IgG1 and IgG2a ($\text{T}_\text{H}2$ and $\text{T}_\text{H}1$ markers, respectively) in comparison with the controls (empty nanoparticles and free PE) ($p < 0.001$). In addition, both NP-PE-A and NP-PE-B induced higher levels of IgA than the controls. Interestingly, spray-dried nanoparticles induced a higher levels of IgG2a in comparison with lyophilized nanoparticles (days 14th and 28th) ($p < 0.05$), which indicates a strong $\text{T}_\text{H}1$ response. As for allergenicity, animals immunized with spray-dried nanoparticles (NP-PE-B) surprisingly presented lower specific IgE than animals immunized with both lyophilized nanoparticles (NP-PE-A) and free PE. Specific antibodies were undetectable in the groups immunized with empty nanoparticles (NP-A and NP-B) and non-immunized control group.

To further explore the T-cell responses, cytokine levels (IL-5 and IFN- γ) produced after *in vitro* re-stimulation of spleen cells from immunized and non-immunized mice were assessed (Fig. 6). Intradermal immunization with peanut protein-loaded nanoparticles (both NP-PE-A and NP-PE-B) reduced the production of IL-5 (pro- $\text{T}_\text{H}2$ cytokine) and also significantly increased IFN- γ levels (pro- $\text{T}_\text{H}1$) in comparison with free peanut proteins (PEs). In addition, immunization with PE-loaded spray-dried nanoparticles (NP-PE-B) showed a higher $\text{T}_\text{H}1/\text{T}_\text{H}2$ ratio (both IFN- γ /IL-5 and IgG2a/IgG1) than lyophilized nanoparticles (NP-PE-A) ($p < 0.001$), indicating the induction of a strong $\text{T}_\text{H}1$ response (Fig. 7).

4. Discussion

The overall goal in allergen vaccine development is improving both clinical efficacy and safety [29]. Thus, effective and safe vaccines with reduced doses of allergens have been developed using

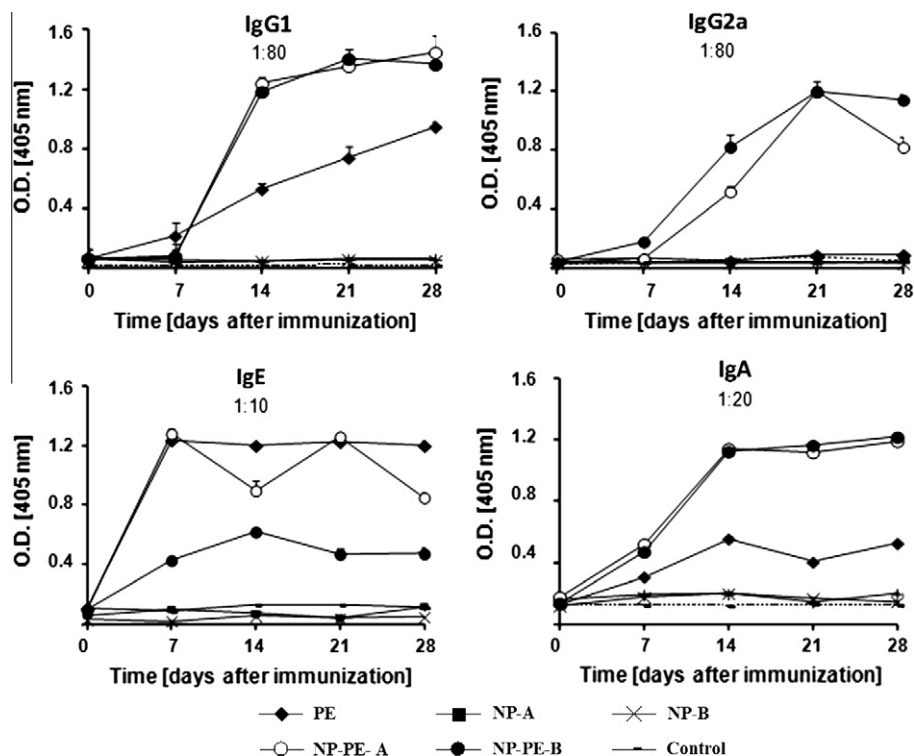


Fig. 5. Specific antibody response (IgG2a, IgG1, IgA, and IgE) induced after intradermal immunization. C57Bl/6 mice were immunized on day 0 with a single dose of peanut proteins (200 μ g) incorporated in one of the following formulations: lyophilized PE-loaded nanoparticles (NP-PE-A) ($\circ$) or spray-dried PE-loaded nanoparticles (NP-PE-B) ($\bullet$). Control mice received one of the following: empty lyophilized NP (NP-A) ($\blacksquare$), empty spray dried NP (NP-B) ($\times$) or free peanut proteins (200 μ g) (PE) ($\blacklozenge$). Data are expressed as the mean $\pm$ SD ($n = 3$).

adjuvants [30]. Nevertheless, traditional adjuvants still have several disadvantages, which limit their use in human vaccines [31,32]. Therefore, several novel adjuvants for allergen immunotherapy are currently being investigated [33]. Among these, nanoparticles-based allergen delivery systems have received much interest as potential adjuvants for allergen immunotherapy [34,35].

Polymeric nanoparticles are controlled delivery systems able to induce protective responses without triggering adverse reactions [13]. These systems act by protecting the encapsulated protein/antigen from enzymatic degradation in the gastrointestinal tract [36]. In addition, nanoparticulate systems have been found to offer a strong immune response and enhanced antigen delivery to gut associated lymphoid tissue (GALT) [37]. On the other hand, after intradermal administration, nanoparticles may remain retained at the injection site, providing local and sustained release of encapsulated antigen/allergen [38,39]. Consequently, nanoparticles could facilitate the antigen/allergen uptake by antigen presented cells (APCs) and the recruitment of competent cells to activate both the innate and adaptive immune system [13].

In the present study, we performed the first study with the same allergen incorporated into different poly(anhydride) nanoparticles (lyophilized and spray-dried), in order to establish a comparative analysis of their immunomodulatory properties. Poly(anhydride) nanoparticles loaded with peanut proteins were successfully developed using the solvent displacement method [15]. The polymer desolvation was performed with a mixture of ethanol and water (lyophilized nanoparticles) or water (spray-dried nanoparticles). Here, high encapsulation efficiency, homogeneous size distribution, and high manufacturing yield were achieved. Importantly, the manufacturing process did not affect the structural integrity and antigenicity of peanut proteins, both key issues on vaccine development.

Moreover, the presented results demonstrate that administration of peanut proteins incorporated in poly(anhydride) nanoparticles significantly enhanced and modulated both the humoral and cellular immune response. In fact, a strong mixed T_H1/T_H2 -type immune response was observed after intradermal immunization with PE-loaded nanoparticles (both lyophilized and spray-dried), with a high level of IgG2a, IgA and IgG1 antibodies. The detection of both T_H1 and T_H2 responses following intradermal immunization using poly(anhydride) nanoparticles is in agreement with previous studies. Gómez et al. [21] demonstrated that poly(anhydride) nanoparticles are efficient carriers for controlled delivery of allergens such as allergenic proteins of *Lolium perenne*. Indeed, the encapsulation of *Lolium perenne* allergens into lyophilized nanoparticles improved the efficacy of intradermal vaccination against grass pollen allergy by induction of significant T_H1 response. In addition, our results are in accordance with previous studies using different formulations of nanoparticles for immunization purposes. For example, Shöll et al. [38] showed that subcutaneous immunization with PLGA nanoparticles loaded with Bet v1 (the major allergen of birch pollen) reduced the predominance of the T_H2 response, increasing the Bet v1 IgG2a levels (T_H1 marker), associated with an increase in IFN- γ and IL-10 levels.

Surprisingly, our results also showed significant differences on the immunomodulatory properties between lyophilized and spray-dried PE-loaded nanoparticles. Concretely, higher IFN- γ production with lower IL-5 secretion and low IgE levels after intradermal immunization with PE-loaded spray-dried as compared with lyophilized nanoparticles. Hence, spray-dried PE-loaded nanoparticles are feasible to efficiently promote T_H1 -type response, which is considered beneficial against allergic inducing processes. It has been reported that allergic diseases are characterized by an imbalance in the responses of activation of T helper (T_H1 and T_H2) and T regulatory (Treg) lymphocytes, with a functional polarization of

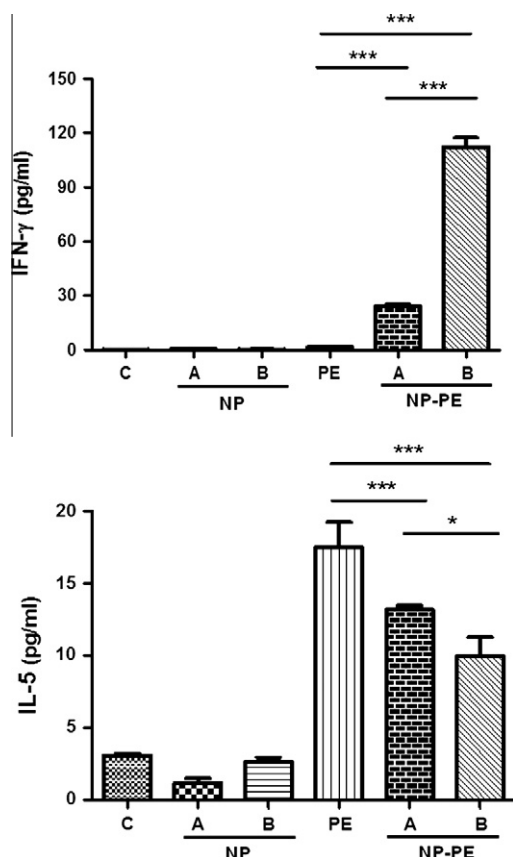


Fig. 6. Cytokine production (IL-5 and IFN- γ) elicited after intradermal immunization. Mice were immunized with lyophilized PE-loaded nanoparticles (NP-PE-A), spray-dried PE-loaded nanoparticles (NP-PE-B), empty lyophilized nanoparticles (NP-A), empty spray-dried nanoparticles (NP-B), or free peanut proteins (PEs). A group of non-immunized mice (C) was used to establish the basal levels of cytokine. Spleen cells were obtained from immunized and non-immunized mice. After *in vitro* re-stimulation with peanut proteins (150 μ g/mL), cytokine levels were measured by ELISA. Data are expressed as the mean $\pm$ SD ($n = 3$). * $p < 0.05$; *** $p < 0.001$.

allergen-specific response of T cells to a T_H2 response. The T_H2 response leads to allergen-specific IgE, proinflammatory cytokines release, and degranulation of basophil and mast cells, hallmarks of allergic diseases [7]. Therefore, one of the main strategies in the development of effective allergen-specific immunotherapy is to redress this imbalance by increasing T_H1 type responses, which counter-balance the T_H2 response [10].

Recent studies have described the role of T_H17 and Treg in the regulation of allergic diseases. It has been demonstrated that these T cells, characterized by the expression of IL-17 [40] or IL-10 [41], regulate pro-inflammatory responses. In fact, the counter-regulatory role of IL-10 has been demonstrated in the induction of tolerance and during specific-immunotherapy, modulating isotype response from an IgE to an IgG4 dominated phenotype [10].

On the other hand, several studies have reported the influence of different factors on the abilities of polymeric particles (nano- and microparticles) to elicit an immune response [42,43]. Among others, particle size [44,45], the nature of the polymer [46], the presence of immunostimulant compounds (i.e. mannose, flagellin) [47], and other physico-chemical factors [48] may affect the capability of particulates to induce immune and protective responses. All of these factors would determine and modulate the interaction of particulates with the APCs, their rate of biodegradability or erosion, and the release profile of the loaded antigen/allergen.

In our case, poly(anhydride) nanoparticles offer different immunomodulatory properties as a function of the preparation method,

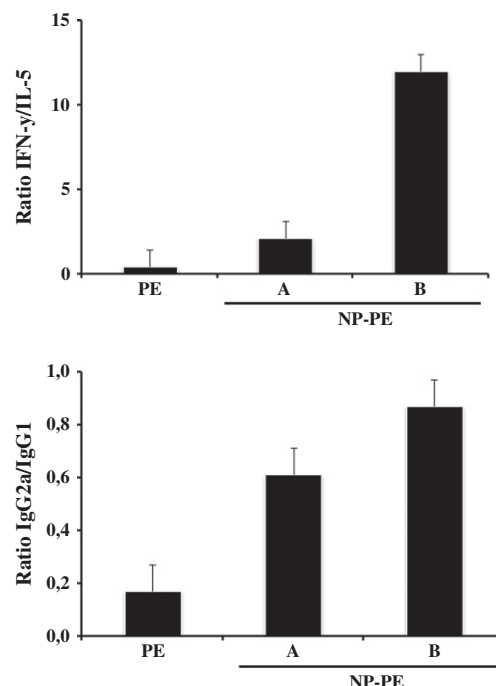


Fig. 7. T_H1/T_H2 ratio elicited after intradermal immunization represented by the ratio IFN- γ /IL-5 (A) and the ratio IgG2a/IgG1 (B). Mice were immunized with lyophilized PE-loaded nanoparticles (NP-PE-A), spray-dried PE-loaded nanoparticles (NP-PE-B), empty lyophilized nanoparticles (NP-A), empty spray-dried nanoparticles (NP-B), or free peanut proteins (PEs). A group of non-immunized mice (C) was used to establish the basal levels of both cytokine and immunoglobulins. For cytokine detection, spleen cells were obtained from immunized and non-immunized mice. After *in vitro* re-stimulation with peanut proteins (150 μ g/mL), cytokine levels were measured by ELISA. The ratio IgG2a/IgG1 represents the levels of specific-antibodies at day 28 post-immunization. Data expressed as the mean $\pm$ SD ($n = 3$). *** $p < 0.001$.

which determines the stability of the resulting nanoparticles in an aqueous environment. Thus, freeze-dried nanoparticles displayed a significantly lower stability than nanoparticles dried by spray-drying (Fig. 2). This lower stability also resulted in a faster release rate of the allergen than for spray-dried nanoparticles (Fig. 4). In any case, this rapid dissolution of lyophilized nanoparticles would decrease the antigen load. In contrast, spray-dried nanoparticles will carry a higher antigen load at time, and hence influencing T_H differentiation. In response to the antigens and the cytokine milieu, naive $CD4^+$ T cells are activated through T-cell and co-stimulatory receptors [49]. These signals induce differentiation into different subsets including T_H1 , T_H2 , T_H17 , and Treg cells [50]. The exact mechanisms regulating these events are not fully defined but both the cytokines and the strength of antigenic stimulus or epitopic density play important roles. Thus, a strong activation tends to a T_H1 differentiation, whereas weak signals favor T_H2 polarization [50,51].

Based on this, a low stability would facilitate a rapid reduction of the antigen/allergen load due to a rapid release from the nanoparticles. This fact would promote the induction of T_H2 responses. On the other hand, for spray-dried poly(anhydride) nanoparticles, a high stability would facilitate the induction of T_H1 responses due to a high antigen/allergen load when interacting with APCs. As a consequence, the preparation method may be a critical factor to take into consideration for adjuvant preparation.

5. Conclusions

In this work, we successfully developed poly(anhydride) nanoparticles loaded with peanut proteins that increased their immu-

nomodulatory activity, inducing a balanced immune response after intradermal immunization. Furthermore, to our knowledge, we provide for the first time clear evidence of the influence of formulation design on the immunostimulatory properties of nanoparticles. Taken together, our findings indicate that spray-dried poly(anhydride) nanoparticles are efficient stimulators of immune responses and promising adjuvants and allergen-delivery systems applied to peanut allergy immunotherapy.

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