Evaluation of nanoparticles as oral vehicles for immunotherapy against experimental peanut allergy

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Abstract

The aim of this work was to evaluate the potential application of an original oral immunotherapy, based on the use of nanoparticles, against an experimentally induced peanut allergy. In this context, a roasted peanut extract, containing the main allergenic proteins, were encapsulated into poly(anhydride) nanoparticles. The resulting peanut-loaded nanoparticles (PE-NP) displayed a mean size of about 150 nm and a significantly lower surface hydrophobicity than empty nanoparticles (NP). This low hydrophobicity correlated well with a higher in vitro diffusion in pig intestinal mucus than NP and an important in vivo capability to reach the intestinal epithelium and Peyer's patches.

The immunotherapeutic capability of PE-NP was evaluated in a model of pre-sensitized CDI mice to peanut. After completing therapy of three doses of peanut extract, either free or encapsulated into nanoparticles, mice underwent an intraperitoneal challenge. Anaphylaxis was evaluated by means of assessment of symptom scores and mouse mast cell protease-1 levels (mMCPT-1). PE-NP treatment was associated with significant lower levels of mMCPT-1, and a significant survival rate after challenge, confirming the protective effect of this formulation against the challenge. In summary, this nanoparticle-based formulation might be a valuable strategy for peanut-specific immunotherapy.

Key words: Peanut allergy; immunotherapy; nanoparticles; oral

1. Introduction

Food allergy has been defined as adverse reactions to food in which immunologic mechanisms have been demonstrated [1]. In general, this type of allergy results when the immune system mistakenly targets a harmless food protein (allergen) as a threat due to the presentation of the allergen to T-cells in presence of Interleukin 4 (IL-4). Then, the naïve T lymphocyte starts its differentiation to a Th2 cell [2]. These Th2 cells produce more IL-4, IL-5 and IL-13 that encourage the production of IgE [2]. Then at the elicitation phase, upon a new exposure to the antigen, allergen becomes attached to the IgE linked to FcεRI on the surface of effector cells (mast cells, eosinophils and basophils), triggering the rapid release of proinflammatory mediators, generally within minutes, including histamine and leukotrienes that cause the symptoms of allergy [3].

Food allergy can result in considerable morbidity, impact negatively on quality of life, and prove costly in terms of medical care. In principle, an allergic reaction to food can affect the skin, the gastrointestinal tract, the respiratory tract, and, in the most serious cases, the cardiovascular system [4]. Allergic reactions can range from mild (hives, eczema, dyspepsia, diarrhoea, angioedema, nasal congestion, etc.) to severe (obstructive swelling of the lips, tongue and/or throat, chest pain, weak pulse, loss of consciousness, etc.) [5]. Severe symptoms alone or in combination with milder symptoms may be signs of a potentially life-threatening condition known as anaphylaxis [5]. Symptoms typically appear within minutes to several hours after eating the culprit food [6]. Fortunately, anaphylactic shock is rarely to happen in response to allergic food compared with more frequent skin rashes or allergic symptoms in the gastrointestinal tract [7]. Although nearly any food is capable of causing an allergic reaction, more than 85% of these disorders are related to the

ingestion of milk, egg, peanut, tree nuts, shellfish, fish, wheat, sesame seed and soy [8]. Allergies to milk, egg, soy and wheat, are usually outgrown within the first ten years of life [9]. In contrast, allergies to peanut, tree nuts, fish, and shellfish tend to be lifelong disorders. Peanut and tree nuts are responsible for the most serious allergic reactions and food-allergy related fatalities [10].

Currently, the only available management in food allergy is the strict avoidance of the culprit food [11]. However this nutritional intervention approach is risky and has to be complemented with training on the recognition of unexpected reactions and their management. For patients at risk of experiencing a severe reaction (anaphylaxis) self-injectable epinephrine is prescribed [12]. In spite of this, severe accidental reactions are common owing to the ubiquitous use of some of these allergens (especially peanut, milk and egg). Nevertheless, in the last years, important efforts to develop effective methods to protect food allergic individuals from severe reactions have been made. Among other important strategies, allergen immunotherapy is being investigated [13,14]. Allergen immunotherapy is based on the continuous administration of small amounts of the allergen in order to provide protection against the allergic symptoms and inflammation associated with the natural exposure to the allergen [15]. If the therapy is effective, ideally, the food can be consumed in normal quantities with no symptoms.

In this context, the use of polymer nanoparticles may be a good strategy to develop effective oral treatments for food allergies. These particulate delivery systems offer interesting abilities as adjuvants, since they boost the delivery of the loaded antigen to the gut lymphoid cells, due to their ability to be captured and internalized by cells of

the GALT [16]. As a consequence, a strongest and more appropriate immune response may be obtained.

Among others, nanoparticles based on poly(DL-lactide-co-glycolide) (PLGA) [17], chitosan [18] or poly(anhydride) [19] have given encouraging results for mucosal allergen delivery. Particularly interesting would be the use of nanoparticles based on the copolymer of methyl vinyl ether and maleic anhydride (Gantrez AN). These poly(anhydride) nanoparticles allow effective immune responses associated with increased Th1 responses [20,21]. This effect would be related with the agonist capability of these nanoparticles of various Toll-like receptors (TLRs) (TLR2, -4, and -5), triggering a Th1-profile cytokine release (gamma interferon, IL-12) [22].

The general aim of this work was to evaluate the rational and potential application of these poly(anhydride) nanoparticles for oral immunotherapy. For this purpose, nanoparticles loaded with a peanut protein extract were evaluated in a peanut sensitized mice model.

2. Material and Methods

2.1. Chemicals

Poly(methyl vinyl ether-co-maleic anhydride) or poly(anhydride) (Gantrez® AN119) was supplied by Ashland, (Ashland, USA). Peanut crude roasted extract was kindly provided by Diater® Laboratories SA (Madrid, Spain). Ethanol and rose Bengal were provided by Panreac (Barcelona, Spain). Acetone was obtained from (VWR-Prolabo). Mannitol, cholera toxin and Tween 20 were from Sigma-Aldrich (Germany). Lumogen® F red 305 was from Kremer (Aichstetten, Germany). Tissue-Tek® OCT compound was obtained from Sakura (Alphen, Netherlands) 4´, 6-diamidino-2-phenylindole (DAPI) was obtained

from Biotium Inc. (Hayward, CA). Micro-BCA™ Protein Assay Reagent Kit was from Pierce® (Rockford, USA). Deionized reagent water (18.2 Mcm resistivity) was prepared by a water purification system (Wasserlab, Pamplona, Spain). Nitrogen gas (ultrapure, > 99) was produced using an Alltech nitrogen generator (Ingeniería Analítica, Barcelona, Spain). ELISA kit (mMCPT-I ELISA Ready-SET-Go! ® was from Affymetrix eBioscience, Inc. (San Diego, CA, USA),

Veratox Peanut Kit was from Neogen (Lansing, MI, USA) and CAPITAN MANI soft peanut butter was from Alimentación Varma, S.L. (Alcobendas, Madrid). Sodium hydroxide (NaOH) was from Scharlau (Spain). All other materials were quality analysis.

2.2. Peanut extract dialysis

Prior the encapsulation of the peanut extract in nanoparticles, crude roasted samples received from the supplier (Diater Laboratories SA) were dialysed in order to remove the existing salts. For this purpose, the peanut extract was dispersed in deionised water and transferred to a dialysis bag (MWCO 3500). The bag was introduced into a vessel with 15 mL water and maintained under agitation for 48 h at 8°C. Finally, the bag contents were transferred to vials and lyophilized in a Genesis 12 EL apparatus (Virtis, USA). For experimental studies, only the dialyzed peanut extract (PE) was used.

2.3. Preparation of peanut extract-loaded nanoparticles (PE-NP)

Poly(anhydride) nanoparticles were prepared by a solvent displacement method [16, 17], with some minor modifications. Briefly, 3 mg of the dialyzed peanut extract (PE) was first dispersed in 100 µL purified water (pH adjusted to 3.0 with HCl 0.1 N) before dispersion in 1 mL acetone. Then, this PE preparation was added to 4 mL acetone

containing 100 mg Gantrez® AN119. Nanoparticles were obtained by the addition, under agitation, of 10 mL of a hydroalcoholic mixture (EtOH 50% by vol.). The organic solvents were eliminated by evaporation under reduced pressure (Büchi R-144, Switzerland) and the nanoparticles were purified by ultracentrifugation (Sigma 3K30 Rot. 12150-H, UK) at 4ºC and 21,000 rpm for 20 minutes. Finally, nanoparticles were resuspended in 3 mL of an aqueous solution of mannitol (2% w/v) and dried by spray-drying in a Büchi Mini Spray Drier B-290 apparatus (Büchi Labortechnik AG, Switzerland). The parameters selected for this purpose were: inlet temperature of 90 ºC, outlet temperature of 60 ºC, spray-flow of 600 mL/h, and aspirator rate at 100% of the maximum capacity.

For the fluorescently labelling of nanoparticles, 4 mg Lumogen® F red 305 was added to the acetone solution containing the poly(anhydride). Then, nanoparticles were prepared, purified and dried as described above.

As control, empty poly(anhydride) nanoparticles were prepared as described above but in the absence of peanut extract. These nanoparticle formulations are identified as NP.

2.4. Physico-chemical characterization of nanoparticles

2.4.1. Size, polydispersity index, zeta potential and yield

The particle size, polydispersity index (PDI) and zeta-potential (ζ) were determined by photon correlation spectroscopy (PCS) and electrophoretic laser Doppler anemometry, respectively, using a Zetasizer analyser system (Brookhaven Instruments Corporation, New York, USA). The diameter and polydispersity index (PDI) of the nanoparticles were determined after dispersion in ultrapure water (1/10) and measured at 25ºC by

dynamic light scattering angle of 90°C. The zeta potential was determined as follows: 200 µL of the samples was diluted in 2 mL of a 0.1 mM KCl solution adjusted to pH 7.4. The yield of the preparative process of nanoparticles was calculated by gravimetry [23].

2.4.2. Peanut extract analysis

The PE loading in the resulting nanoparticles was quantified by microBCA. For quantification, the amount of peanut protein associated with the nanoparticles was measured using the bicinchoninic acid method (MicroBCA), as previously described [20]. Each sample was assayed in triplicate and results of PE loading were expressed as the amount of protein (in µg) per mg NP. The encapsulation efficiency (EE) expressed as a percentage was calculated as the difference between the initial amount of PE added and the amount of protein quantified as entrapped in the resulting nanoparticles.

2.4.3. Surface hydrophobicity evaluation

In order to evaluate the surface hydrophobicity of empty and loaded nanoparticles the Rose Bengal test was performed [24], with some minor modifications. Briefly, 200 µL of nanoparticle suspensions (from 0.03 to 3 mg/mL) were mixed with 400 µL of a Rose Bengal aqueous solution (100 µg/mL). All samples were incubated under constant shaking at 1500 rpm, for 30 min at 25ºC (Labnet VorTemp 56 EVC, Labnet International, Inc.). Afterwards, the samples were centrifuged at 13,500 x g for 30 min (centrifuge MIKRO 220, Hettich, Germany). The amount of Rose Bengal in the supernatants was extrapolated from the absorbance detected at 548 nm by using a microplate reader (BioTek PowerWave XS, USA). Further, the total surface area of each

sample was plotted against the partitioning quotient (PQ) calculated in accordance with the following equation:

$$PQ= (Rose\ Bengal\ Bound/\ Rose\ Bengal\ unbound) \quad \text{[Equation 1]}$$

2.4.4. Stability

The stability of nanoparticles was evaluated by measuring the turbidity changes as a function of time in simulated gastric (SGF) and intestinal fluids (SIF). Analysis was performed for empty and loaded nanoparticles and stability was estimated over 2 hours in the corresponding fluids prepared according European pharmacopeia [25].

Briefly, nanoparticles were first dispersed in purified water (6 mg/mL). Then, each suspension was mixed with a similar volume of either simulated gastric or intestinal fluid (1:1 v/v). The turbidity changes were monitored in a spectrophotometer at 405 nm in continuous kinetic measurements during 2 hours (Labsystems EMS Reader MF). All measurements were performed in triplicate, and the results were expressed as percentage of absorbance reductions vs. time. The percentage of absorbance reduction was calculated as follows (% reduction):

$$\%\ Reduction=(A_0-A_t/A_0) \times 100 \quad \text{[Equation 2]}$$

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

2.5. In vitro release

The release studies were performed under sink conditions by using simulated gastric (SGF) and intestinal fluids (SIF), supplemented with Tween 20 (1% w/v). For these purposes Float-A-Lyzer devices with a MWCO of 300 kDa (Float-A-Lyzer® G2, Spectrum Laboratories) were used. The dialysis bags were filled with 21.6 mg nanoparticles

dispersed in 5 mL SGF and, then, placed into a vessel containing 660 mL SGF. The vessel was maintained under magnetic agitation and, at fixed time intervals, 200 µL samples were withdrawn and replaced with equal volumes of free SGF. After two hours of incubation in the gastric fluid, the device was transferred to a second vessel with 660 mL SIF. Again, at fixed times, 200 µL were withdrawn and replaced with free SIF.

The amount of PE released from nanoparticles was quantified by ELISA with a commercial kit for peanut allergens (Veratox®, Neogen, Scotland, UK).

2.6. In vitro evaluation of nanoparticles diffusion in mucus

The diffusion of nanoparticles through intestinal mucus, as an in vitro measurement of their mucus-permeating properties, was assessed by the Multiple Particle Tracking (MPT) technique [26]. MPT involves video capturing and post-acquisition analysis for the individual movement of hundreds of fluorescently labelled particles within a mucus matrix [27].

In these experiments, pig intestinal mucus was collected from freshly excised intestine. Samples of mucus (0.5 g) were incubated in glass-bottom MatTek imaging dishes at 37 °C. Then, 25 µl of 0.002% fluorescently labelled nanoparticles suspensions were inoculated into each 0.5 g mucus sample. Each sample was incubated for 2 hours prior video microscopy in order to ensure effective particle distribution after inoculation. 2-dimensional videos were captured in Leica DM IRB wide-field epifluorescence microscope (x63 magnification oil immersion lens) using a high speed camera (Allied Vision Technologies, UK) capturing 30 frames/second; 10 seconds videos (i.e. complete video comprised 300 frames). For each 0.5 g mucus sample, minimum of 100 individual

trajectories were tracked and analysed. MPT analysis for each nanoparticles species was carried out in triplicate (i.e. 3 mucus samples each with 100 particles and overall of 300 particles).

Videos were analysed by Fiji ImageJ software to convert the movement of each nanoparticle into individual trajectories which is converted into metric distances in the X-Y directions. The distances moved by each individual particle over time were expressed as particle's squared displacement (SD). The mean square displacement (MSD) of any single particle represents the mean of that particle's squared displacements throughout its entire 30-frame trajectory. MSD was determined as follows:

$$MSD= (X_{\Delta t})^2+(Y_{\Delta t})^2 \qquad \text{[Equation 4]}$$

In any single mucus sample, ensemble MSD <MSD> was determined by calculating the geometric mean for at least 120 individual particles. The Effective Diffusion Coefficient (Deff) for a particular nanoparticle type was then calculated by:

$$Deff= <MSD> /4 \times \Delta t \qquad \text{[Equation 5]}$$

Where 4 is a constant relating to the 2-dimensional mode of video capturing and Δt is the selected time interval.

In order to express the diffusion of nanoparticles after normalising the nanoparticles size, the diffusion of particles was presented as a % ratio [Deff]/[D°] where D° is the absolute diffusion of particles in water depending on their particle sizes and calculated by the Stokes–Einstein equation as follows:

$$D°=\kappa T/6\pi\eta r \qquad \text{[Equation 6]}$$

In which κ is the Boltzmann constant, T is absolute temperature, η is water viscosity and r is the mean radius of nanoparticles.

2.7. Biodistribution of nanoparticles

Experiments were performed in compliance with the regulation of the Ethics Committee of the University of Navarra in line with the European legislation on animal experiments following a protocol previously approved [28]. For this purpose, 10 mg of fluorescently labelled nanoparticles were administered orally by gavage to the animals in order to visualize their presence in close contact with the mucosa. Then, after 2 hours the animals were sacrificed by cervical dislocation and the gut was removed. Different portions of the gut were collected, cleaned with PBS, stored in OCT and frozen at -80 ºC. Each portion was then cut into 5 µm sections on a cryostat and attached to glass slides. Finally, these samples were fixed with formaldehyde and incubated with DAPI for 15 min before the cover assembly. The presence of both fluorescently loaded nanoparticles in the Intestinal mucosa and the cell nuclei dyed with DAPI were visualized in a fluorescence microscope (Axioimager M1, Zeiss) with a coupled camera (Axiocam ICc3, Zeiss) and fluorescent source (HBO 100, Zeiss. The images were captured with the software ZEN (Zeiss).

2.8. Protective studies

Experiments were performed in compliance with the regulation of the Ethics Committee of the University of Navarra in line with the European legislation on animal experiments (approved protocol 006/15).

CDI female mice of about 20 grams were sensitized by orally administration of peanut butter (CAPITAN MANI soft peanut butter; 4.35 mg with an approx. content of 1 mg protein) and 5 µg cholera toxin in a total volume of 200 µL of saline solution on days 1, 7, 15 and 21. Moreover it was applied a tape stripping to obtain a higher sensitization [29]. Mice were shave and barrier-disrupted on back skin. Percutaneous sensitization in the damaged skin was performed by topical application of 100 µg peanut extract in 100 µL saline solution onto the barrier-disrupted skin.

On days 25, 28 and 35 the animals received one oral dose of 1 mg peanut extract either resuspended in purified water or incorporated into nanoparticles. Finally, on days 44 and 45 animals were challenged by an injection of 2 mg PE in 200 µL of saline solution by intraperitoneal route in order to provoke an anaphylactic shock in the sensitized animals.

In order to analyse the intensity of the anaphylaxis shock the following parameters were recorded: body temperature, mobility, bristly hair and cyanosis.

Clinical anaphylactic reactions were scored by two independent observers. Piloerection and cyanosis were scored as follows: (−) absent (normal mouse), (+) weak reaction and/or scratching of the nose and head, (++) moderate, and (+++) strong. In a similar way, the mobility of animals was scored as very low (no reaction after pushing), low (arched back and low movements) or normal.

Furthermore, levels of mouse mast cell protease-1 (mMCPT-1) in serum were measured with an mMCPT-1 enzyme-linked immunosorbent assay kit, following the manufacturer's instructions.

2.9. Statistical analysis

The physico-chemical characteristics of nanoparticles as well as the in vitro studies were compared using the Student´s t test. For in vivo studies, comparisons were performed using the one-way analysis of the variance (ANOVA) and Tukey HSD test. In all cases $p<0.05$ was consider as a statistically significant difference. All calculations were performed using Graphpad Prism v6 (Graphpad Software, San Diego, CA). The Kaplan-Meyer curves were used for analysis of the efficacy experiment using OriginPro 8.5

3. Results

3.1. Preparation and characterization of peanut extract-loaded nanoparticles

Previous to characterization, the commercial peanut extracts were dialyzed in order to remove and eliminate phosphates, calcium salts and other divalent cations that negatively affect to the formation of poly(anhydride) nanoparticles.

Table 1 summarizes the main physico-chemical characteristics of the nanoparticles employed in this study. The encapsulation of PE in nanoparticles decreased significantly the mean size (150 vs. 178) and negative zeta potential (-35 vs. -43 mV) of the resulting nanocarriers. Furthermore, the PE loading was calculated to be close to 14 µg per mg nanoparticles, with an encapsulation efficiency of about 50%.

The surface hydrophobicity of nanoparticles was evaluated by using the Rose Bengal test. The data are plotted as total surface area against the partitioning quotient and the parameter that identifies the hydrophobicity of the NPs is the slope of each line, the steeper the slope the higher the hydrophobicity. As presented in Figure 1, empty

nanoparticles were significantly more hydrophobic than PE-NP ($p<0.001$) and displayed a slope 5-fold higher than for loaded nanoparticles.

The in vitro stability of nanoparticles was evaluated after incubation in simulated gastric (SGF) and simulated intestinal fluids (SIF) in a concentration of 3mg/mL (Figure 2). In case of SGF, NP and PE-NP were stable for at least 2 hours. On the contrary, when nanoparticles were dispersed in SIF, the absorbance of the suspensions decreased rapidly, although, this decrease was more pronounced for NP than for PE-NP.

Figure 3 shows the release profile of PE from nanoparticles as function of time when incubated in SGF (during the first 2 hours), and SIF (from 2 to 24 hours) in sink conditions. In SGF only about 10% of the PE content was released during the first 2 hours of the experiment. In SIF, the release rate of PE from nanoparticles increased during the first 8 hours, when a plateau was reached. At the end of the experiment (24h) only about 40% of the PE content was released from the nanoparticles.

3.2. Evaluation of nanoparticle diffusion in mucus and biodistribution

Table 2 shows the theoretical intrinsic free Brownian motion of nanoparticles in water, the diffusion coefficient in intestinal mucus (Deff) measured by MPT and the ratio as percentage of these two parameters (Deff/D°). This last parameter was employed to compare the diffusion of the nanoparticles in intestinal pig mucus after normalising the effect of particle size. For PE-NP, the diffusion capability in the mucus was found to be 2.25-fold higher than for empty nanoparticles (NP).

Figure 4 shows fluorescence microscopy images of ileum samples from animals treated with fluorescently labelled nanoparticles two hours post-administration. Empty

nanoparticles displayed a restricted localization at the mucus layer and a large amount of the formulation was visualized in the lumen with no interaction with the mucosa. On the contrary, the fluorescence associated to peanut-loaded nanoparticles was mainly observed in areas close to the intestinal villi of mucosa. In addition, PE-NP displayed also an interesting capability to interact with Peyer´s Patches (PP).

3.3. Protective studies

Peanut sensitized animals received an immunotherapeutic schedule on days 4, 7, and 14 after sensitization. Then, on day 20, animals were challenged with the intraperitoneal administration of 2 mg peanut extract. In order to analyse the intensity of the anaphylaxis, several parameters were evaluated. Figure 5 shows the body temperature in the laboratory animals, 10 minutes after challenge. Sensitized- and non -treated animals (Control +) experienced an important decrease of their body temperature. Interestingly, animals treated either with free (PE) or encapsulated (PE-NP) peanut extract displayed a significantly lower temperature variation ($p<0.05$).

Twenty minutes after challenge, mice were visually assessed for symptoms of anaphylaxis and assigned symptoms scores (Table 3). Piloerection and cyanosis seemed to be similar for animals treated with either PE or PE-NP. Moreover, animals from the non-treated group (Control +) were found completely static with a high difficulty to coordinate any simple movement, whereas treated animals were able to coordinate to some extent simple movement or react against pain or pressure in a similar way. Moreover, 20 min post-challenge, the body temperature of sensitized animals maintained a significantly lower body temperature than the basal one. This temperature decrease was higher for non-treated (Control +) and PE-treated animals

than for those animals treated with PE-NP. Non-sensitized animals displayed a normal behaviour with no symptoms of anaphylaxis.

Figure 6 shows the mouse mast cell protease-1 (mMCPT-1) levels on the sera of animals 15 minutes after challenge. Non-sensitized animals displayed lower values of this protease, similar to constitutive expression found in the bibliography [30–32]. Animals treated with PE-NP displayed similar serum levels of mMCPT-1 than non-sensitized animals. In addition, these protease levels were significantly lower than those quantified in animals ascribed to the positive control group or the PE treated group ($p<0.001$).

Figure 7 shows the cumulative survival of animals after the intraperitoneal administration of 2 mg PE. In the control group, all the non-sensitized animals survived to this challenge. On the contrary, one day after challenge, all the animals included in the positive control and PE groups died. For the sensitized animals treated with PE-NP, the survival rate 24 h post-challenge was calculated to be of 40%.

4. Discussion

In previous studies, our research group evaluated the immunogenicity of PE-loaded nanoparticles suggesting their potential for oral immunotherapy purposes due to their pro-Th1 adjuvant properties. Thus, one single dose of PE-loaded nanoparticles induced in mice a balanced Th1 and Th2 antibody response, accompanied by a low specific IgE induction and high serum levels ofIFN-ɣ and IL-10 [33]. In the present work, similar nanoparticles have been prepared in order to gain insight about their behaviour in vivo and their protective capability against a challenge in peanut sensitized animals. In this

particular work a roasted crude extract of peanut was employed after purification by dialysis [34].

The purified extract was then encapsulated into poly(anhydride) nanoparticles (150 nm and -35 mV), with a payload of 14 μg peanut extract per mg nanoparticles (Table 1). Overall, these physico-chemical properties were similar to those described previously by Rebouças and co-workers [20,35]. On the other hand, the encapsulation of the peanut extract produced nanoparticles (PE-NP) with a significantly lower hydrophobicity than empty nanoparticles (Table 1). This finding, as well as the reduction in the negative zeta potential of the loaded nanoparticles (-35 mV for PE-NP vs. -43 mV for NP, table 1) suggested that some of the component of the peanut extract would be localized in the outer layer of the resulting nanocarriers. This decrease of the surface hydrophobicity for PE-loaded nanoparticles correlated well with higher diffusion in intestinal mucus than for empty nanoparticles (Table 2). Among other characteristics, for nanocarriers, a lower surface charge and a lack of hydrophobic regions/domains have been related with a reduced interaction with mucus [36,37]; facilitating the development of nanoparticles with mucus-permeating properties. In our case, and due to the presence of protein components of the peanut extract on the surface of nanoparticles, PE-NP exhibited 2.3-fold higher diffusion through the mucus network in comparison with control NP. This higher diffusivity of PE-loaded nanoparticles in the mucus was also corroborated in the biodistribution study with fluorescently labelled nanoparticles. Thus, NP displayed mucoadhesive properties with a distribution restricted to the mucus layer of the intestinal mucosa (Figures 4C and 4D), whereas PE-NP were capable of reaching the intestinal epithelium and the surface of Peyer`s Patches (Figures 4A and 4B, respectively). Peyer's Patches

are primary inductive sites of mucosal immunity located in mammalian small intestine [38]. In fact, They have been extensively studied for their contribution to mucosal tolerance, although their role is still unclear [39]. In any case, the targeting of Peyer's patches would be desirable for the modulation of tolerance [40].

On the other hand, PE-NP displayed gastro-resistant properties with a relatively rapid degradation under simulated intestinal conditions (figure 2). This behaviour agrees well with the release profile of the peanut extract from nanoparticles. Nevertheless, at the end of the experiment, only 40% of the loaded extract appeared to be released (Figure 3). This apparent anomalous result maybe related with the capability of Gantrez AN of establishing "strong" ionic and covalent interactions with compounds possessing ionisable residues and/or primary amine groups [41,42]. These interactions would induce changes on the conformation of peanut proteins that would reduce the sensitivity of the ELISA analysis. In any case, these possible links between the poly(anhydride) and peanut proteins should be elucidated in a next future study.

The immunotherapeutic capability of PE-NP was evaluated in a model of pre-sensitized mice to peanut. Once the animals were sensitized, the nanoparticles were administered to the mice and afterwards they were intraperitoneally challenged with the peanut extract. Regarding the anaphylactic symptoms, only animals treated with the PE-NP displayed a significantly lower decrease in their basal temperature when compared with animals treated with PE or ascribed to the positive control group (table 3). Another interesting finding was the similar mMCPT-1 (mouse mast cell protease 1) levels in the sera of sensitized animals treated with PE-NP, when compared with normal animals (negative control group) (Figure 6). mMCPT-1 is a chymase expressed by intestinal mucosal mast cells, which are found in the intestinal epithelium [43].

Elevated mMCPT-1 levels are observed during intestinal allergic hypersensitivity reactions. Thus, these sera levels of the protease would be directly related with the severity of the anaphylactic shock [44].

In line with these results, a significant fraction of animals treated with PE-NP survived 24 hours after ip challenge (Figure 7); confirming the protective effect of this formulation against the challenge. In terms of clinical use, a nanoparticle-based immunotherapy with an improved efficacy than the current daily doses of allergens, would facilitate the implementation of safer regimens and its adaptation to individual patients' needs and their progression over time.

In summary, we have corroborated the ability of poly(anhydride) nanoparticles to load and carry a peanut extract, facilitating their biodistribution within the gut, including Peyer`s Patches. In addition, this nanoparticle-based formulation offers a promising protection against the effects induced by an anaphylactic shock in peanut sensitized animals, opening the door to a future clinical employment.

Author contributions

Conceived and designed the experiments: CG, MG, JMI. Performed the experiments: ABC, NMA, JM, MA. Contributed reagents/materials/analysis tools: CG, MG, JMI. Wrote the manuscript: ABC, JMI.

Conflicts of interest

The authors declare that there is no conflict of interest.

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Illustrations

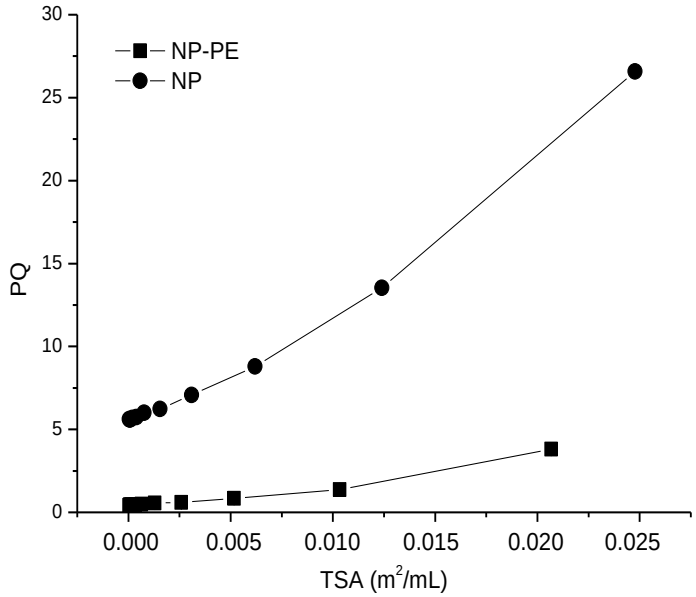

Figure 1. Hydrophobicity of empty and peanut-loaded nanoparticles. Partition Quotient (PQ) versus total surface area (TSA) of nanoparticles. Values expressed as the mean ± SD (n=3).

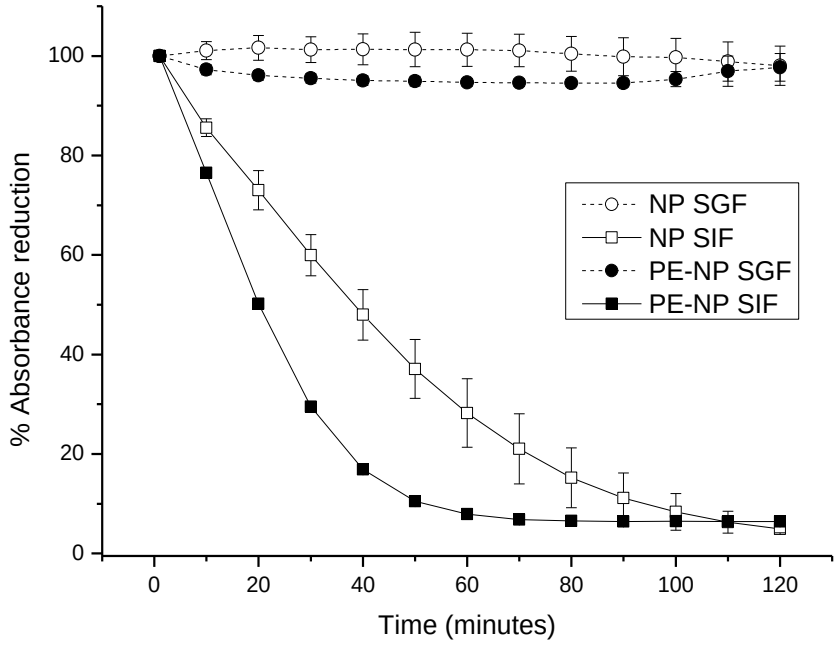

Figure 2. Stability of empty (NP) or peanut loaded nanoparticles (PE-NP) in simulated gastric fluid (SGF, pH 1.2) and simulated intestinal fluid (SIF, pH 6.8). Data expressed as mean ± SD (n=3).

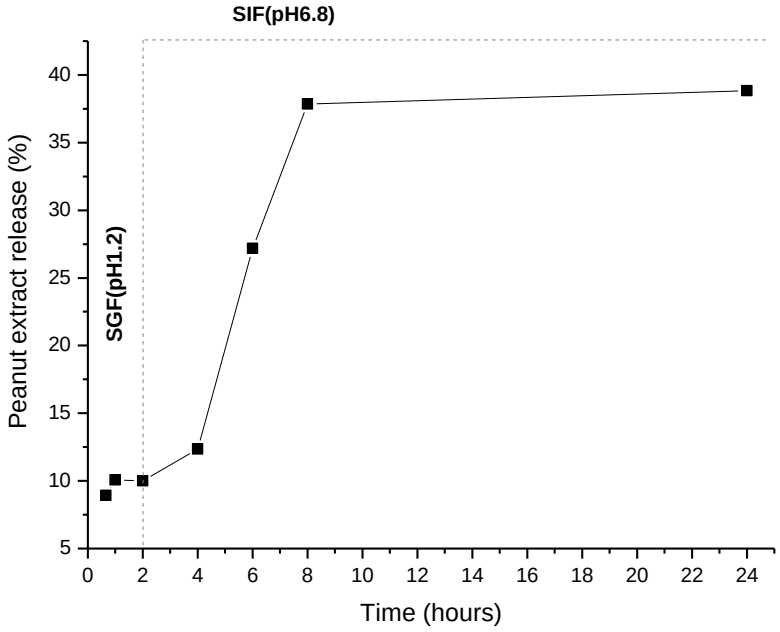

Figure 3. In vitro release of peanut extract from PE-NP. Data expressed as cumulative amount of PE released versus time. Samples were incubated for 2 hours in SGF, and then samples were transferred to SIF till the end of the experiment. Data expressed as mean ± SD (n=3).

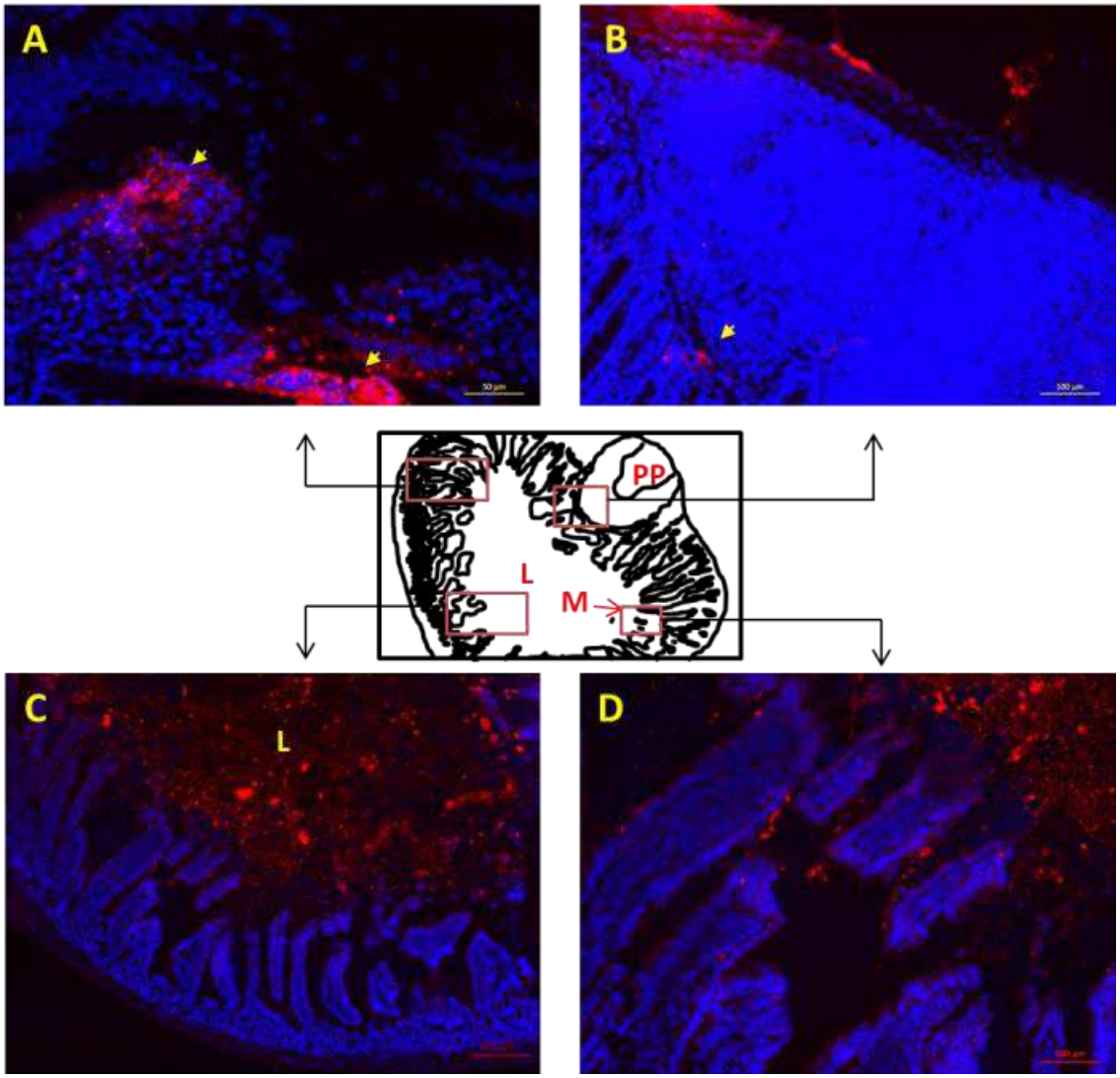

Figure 4. Fluorescent microscopic visualization of Lumogen red-loaded nanoparticles in longitudinal sections of the ileum of rats. Images A and B correspond with peanut extract-loaded nanoparticles (PE-NP), whereas images C and D show the distribution of NP. The draw in the middle indicates the anatomical regions for the mucosal intestinal villi (M), the follicle-associated epithelium of Peyer`s Patches (PP), and Lumen (L). Arrows in figures A and B indicate the intestinal portions with high interaction between nanoparticles and mucosal intestinal villi. DAPI staining of nuclei appears as blue.

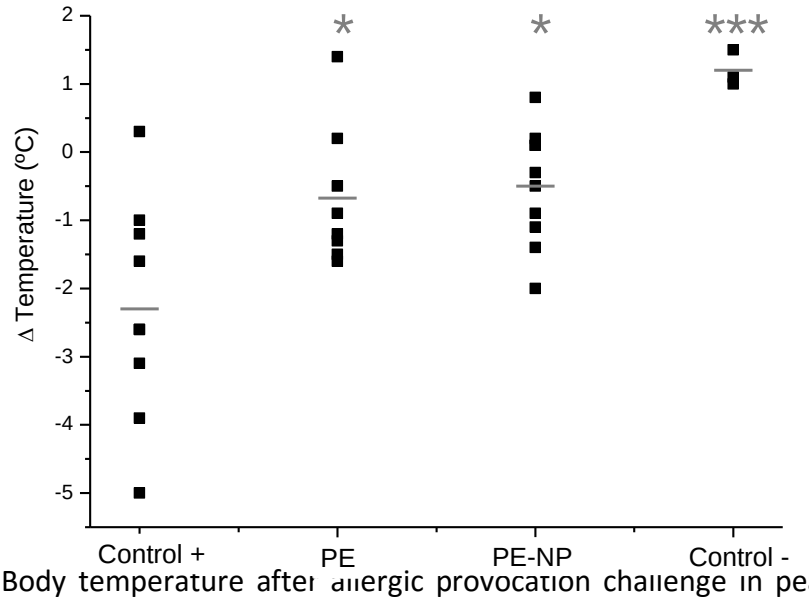

Figure 5. Body temperature after allergic provocation challenge in peanut-sensitized mice. Mice were treated with empty nanoparticles (NP) or peanut-loaded nanoparticles (PE-NP). A group of sensitized untreated mice (control +) and a group of non-sensitized mice (control -) were also included. Data show individual temperature and mean. (* p<0.05 and ***p<0.001 vs. Control +).

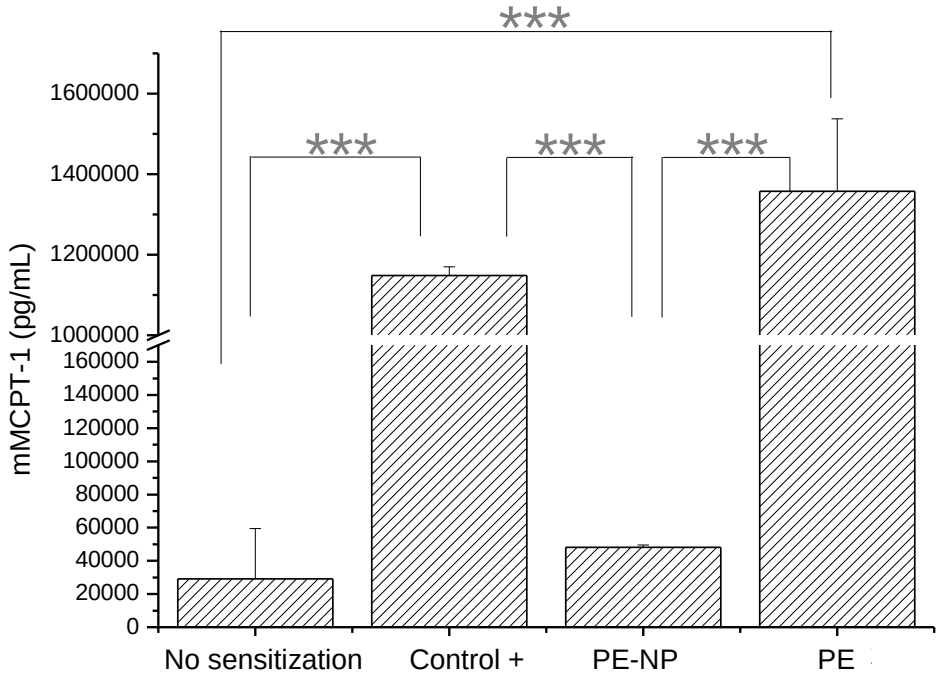

Figure 6. Sera levels of mMCPT-1 after allergic provocation challenge in peanut-sensitized mice. Mice were treated with empty nanoparticles (NP) or peanut-loaded nanoparticles (PE-NP). A group of sensitized untreated mice was also included. A group of sensitized untreated mice (control +) and a group of non-sensitized (control -) were also included. Serum samples were taken 15 minutes after challenge. Data are shown as mean ± SD. ***P<0.001 vs. Control +.

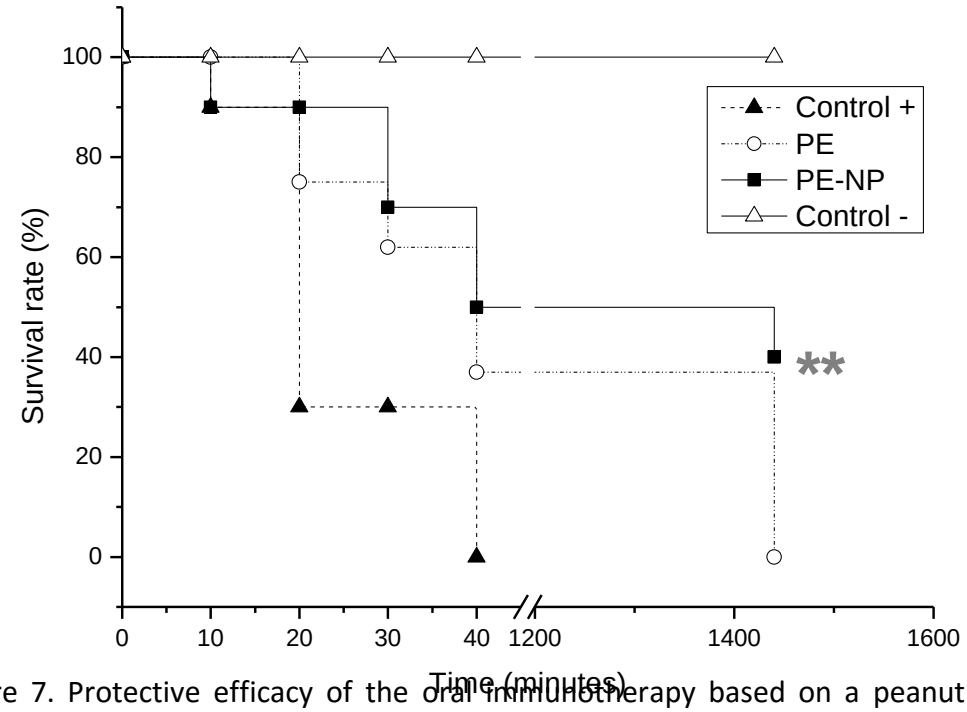

Figure 7. Protective efficacy of the oral immunotherapy based on a peanut extract administered either free (PE) or encapsulated into nanoparticles (PE-NP). Mice were previously sensitized. Sensitized untreated animals (Control +) and non-sensitized mice (Control -) were also included as controls. The figure indicates the percentage of mice that survive the intraperitoneal challenge with a peanut fraction (**p<0.01, Log rank test indicates significant differences between animals treated with PE-NP and non-treated control positive animals).

Tables

Table 1. Physico-chemical characteristics of PE-loaded nanoparticles. PDI: polydispersion index; EE: encapsulation efficiency. The degree of hydrophobicity corresponds with the slope of curves from Figure 1. Data expressed as mean ± SD (n=3).

	Mean size (nm)[a]	**PDI**	**Zeta Pot. (mV)[b]**	**PE loading (µg/mg NP)**	**EE (%)**	**Surface hydrophobicity**
NP	178±2	0.14±0.06	-43±1	-	-	274±2
PE-NP	151±5	0.16±0.01	-35±1	14.0±0.7	50.3±2.6	51±2

[a] $p<0.01$ empty versus loaded NP; [b] $p<0.001$ empty versus loaded NP.

Table 2. Nanoparticle diffusion kinetics. Data expressed as mean ± SD (n=3). D°: diffusion coefficient in water; <Deff>: diffusion coefficient in mucus; ratio %<Deff>/ D°: relative efficiency of particles diffusion; R: ratio of %<Deff>/ D° of the formulations tested.

	D° (water) $cm^2 \times S^{-1} \times 10^{-9}$	**<Deff> (mucus) $cm^2 \times S^{-1} \times 10^{-9}$**	**%<Deff>/ D°**	**R**
NP	25.69	0.00041 (± 0.00032)	0.00160	1
PE-NP	31.44	0.00113 (± 0.00083)	0.00360	2.25

Table 3. Anaphylactic symptoms after allergic provocation challenge in peanut-sensitized mice. Mice were treated with empty nanoparticles (NP) or peanut-loaded nanoparticles (PE-NP). A group of sensitized untreated mice was also included. A group of sensitized untreated mice (control +) and a group of non-sensitized (control -) were also included. Symptoms were scored at 20 minutes post-challenge.

	Δ Temperature (°C)	**Piloerection**	**Mobility**	**Cyanosis**
Control +	-4.5±0.61	+++	Very low	+++
PE	-4.4±0.8	++	Low	-
PE-NP	-2.5±1.8	++	Low	-
No sensitization	0.5±0.8	-	Normal	-

Severity symptoms: (-) absent (+) weak (++) moderate (+++) strong