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Mannosylated Nanoparticles for Oral Immunotherapy in a Murine Model of Peanut Allergy

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ABSTRACT

Peanut allergy is one of the most prevalent and severe of food allergies with no available cure. The aim of this work was to evaluate the potential of an oral immunotherapy based on the use of a roasted peanut extract encapsulated in nanoparticles with immunoadjuvant properties. For this, a polymer conjugate formed by the covalent binding of mannosamine to the copolymer of methyl vinyl ether and maleic anhydride was first synthesized and characterized. Then, the conjugate was used to prepare nanoparticles with an important capability to diffuse through the mucus layer and reach, in a large extent, the intestinal epithelium, including Peyer's patches. Their immunotherapeutic potential was evaluated in a model of sensitized CD1 mice to peanut. After completing therapy, mice underwent an intraperitoneal challenge with peanut extract. Nanoparticle-treatment was associated with both less serious anaphylaxis symptoms and higher survival rates than control, confirming the protective effect of this formulation against the challenge.

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Introduction

Food allergy is a serious and growing problem. For peanut allergic patients, they must maintain a strict allergen-free diet and carry emergency medication (i.e., epinephrine auto-injectors) to treat anaphylactic symptoms following accidental exposure.¹ In spite of improvements in industrial cleaning procedures and food labeling, accidental exposures continue to be the leading cause of anaphylaxis.² Particularly for young patients, the persistent fear to accidental exposures as well as the life-threatening reactions significantly disrupts their social and educational activities.^{3,4} This profoundly impairs health-related quality of life, to an extent greater than that seen in chronic conditions such as diabetes mellitus.⁵

Oral immunotherapy (OIT) has been suggested as a promising therapy for food allergy. OIT is based on the delivery of increasing doses of allergens with the objective of inducing desensitization

and/or tolerance.⁶ The exact mechanism underlying desensitization is not well understood. Nevertheless, from an immunological point of view, OIT would induce allergen-specific IgG4 antibodies, reducing at the same time specific IgE.⁷ IgG4 acts as a blocking antibody for specific IgE, inhibiting IgE-mediated mast cell activation and degranulation.⁸ This activation of mast cells produces the release of vasoactive amines and cytokines, responsible for the symptoms associated to allergic reactions.

Different clinical trials have provided evidences that OIT can effectively desensitize a majority of individuals to a food allergen, although the safety and tolerability of the conventional treatments continue to limit its use.^{9–11} Recently, to improve the efficacy and safety of conventional OIT, the use of polymer nanoparticles as allergen delivery systems has been proposed.¹² In principle, these devices may offer the following advantages: (1) protection of the loaded allergen from its premature degradation by the harsh conditions of the gut,¹³ (2) co-encapsulation of immunomodulators to boost appropriate tolerogenic adaptive immune responses to allergens,^{14,15} and (3) facilitate the presentation of the allergen to the gut-associated lymphoid tissue and promote a protective response.^{16,17}

However, when polymer nanoparticles are orally administered, they are faced to the protective mucus layer that highly hampers

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their arrival to the intestinal epithelium.^{18,19} In fact, the mucus layer acts as a barrier in which nanoparticles remain trapped by mucoadhesive forces and, thus, minimizing their interaction with the immune cells localized in the intestinal epithelium.²⁰ Among others, hydrophobic interactions between the surface of nanoparticles and hydrophobic domains of glycoproteins constituting the mucus play a major role in the mucoadhesion phenomenon.²¹ To minimize this drawback, different approaches have been proposed including the use of “slippery” nanoparticles. These nanoparticles, with mucus-permeating properties, may be obtained by the coating or functionalization of nanoparticles with hydrophilic compounds, such as poly(ethylene glycols)²² or poloxamers.²³ Another interesting approach may be the functionalization of these nanoparticles with hydrophilic compounds with the capability of recognizing and binding to specific receptors localized on the surface of particular intestinal cells. In this context, the use of mannose-modified nanoparticles may be a good strategy to target intestinal dendritic cells and M-cells of Peyer’s patches.^{24,25}

The aim of this work was the preparation and characterization of mannosamine-modified nanoparticles as carriers for a peanut extract (PE) containing the main allergens. In the recent past, the nanoencapsulation of a PE containing the main allergenic proteins (PE) in nanoparticles, from the copolymer of methyl vinyl ether and maleic anhydride (Gantrez® AN), displayed an important capability to induce a balanced Th1 and Th2 antibody response after 1 single dose.²⁶ Specifically, mice treated with these nanoparticles displayed high levels of IFN- γ and IL-10 with lower pro-Th2 cytokines (IL-4, IL-5, and IL-6) and reduced specific IgE levels compared to control.²⁶ In the present work, the capability of nanoparticles based in a polymer conjugate between Gantrez® AN and mannosamine to carry this PE and to offer protection against anaphylaxis in a peanut-sensitized mice model was evaluated. Mannosamine was selected to improve the immunoadjuvant properties of Gantrez® AN²⁷ owing to their capability to specifically bind and activate immune cells (i.e., dendritic cells and macrophages).^{28,29}

Methods

Materials

Poly(methyl vinyl ether-co-maleic anhydride) or poly(-anhydride) (Gantrez® AN 119) was supplied by Ashland, (Ashland, Covington, KY). PE was kindly provided by Diater® Laboratories SA (Madrid, Spain). Ethanol and Rose Bengal were provided by Pan-Reac (Barcelona, Spain). Acetone was obtained from VWR-Prolabo (Llinars del Vallés, Spain). Cholera toxin, D-mannitol, D-mannosamine, and Tween 20 were from Sigma-Aldrich (Hamburg, Germany). Lumogen® F red 305 was from Kremer (Aichstetten, Germany). Tissue-Tek® OCT compound was obtained from Sakura (Alphen, The Netherlands). 4', 6-diamidino-2-phenylindole (DAPI) was obtained from Biotium Inc. (Hayward, CA). Micro-BCA™ Protein Assay Reagent Kit was from Pierce® (Rockford, IL). Veratox Peanut Kit was from Neogen (Lansing, MI), and CAPITAN MANI® soft peanut butter was from Alimentación Varma, S.L. (Alcobendas, Madrid, Spain). Sodium hydroxide (NaOH) was from Scharlau (Barcelona, Spain).

PLGA nanoparticles (PLGA-NP) were kindly supplied by Nanomi B.V. (Oldenzaal, The Netherlands). These nanoparticles displayed a mean size of 161 nm with a polydispersity index of 0.03 and negative zeta potential of -29 mV.

Peanut Extract Dialysis

Prior the encapsulation in nanoparticles, the roasted PE was dispersed in deionized 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, Los Angeles, CA). For experimental studies, only the dialyzed PE was used. The extract was characterized by SDS-PAGE and proteomic analysis to confirm the presence of the main allergenic proteins (Ara h1, Ara h2, Ara h3, Ara h5-8, and oleosins).

Preparation and Characterization of Gantrez® AN-Mannosamine Conjugate

The conjugate was formed by the covalent binding of mannosamine to the anhydride groups of the polymer backbone. For this purpose, 1 g Gantrez® AN [poly(anhydride)] was dissolved in 120 mL acetone. Then, 50 mg mannosamine was added, and the mixture was heated at 50°C, under magnetic agitation at 400 rpm for 3 h. Then, the mixture was filtered through a pleated filter paper, and the organic solvent was eliminated under reduced pressure in a Büchi R-144 apparatus (BÜCHI Labortechnik AG, Flawil, Switzerland) until the conjugate was totally dry. Finally, the resulting powder was stored at room temperature in a hermetically sealed container until use.

For the characterization, Gantrez® AN-mannosamine conjugate (GM) was analyzed by infrared spectroscopy, elemental analysis, ¹H-NMR, and titration. The amount of mannosamine bound to the poly(anhydride) was estimated by the o-phthalaldehyde method.³⁰ More details about the characterization of GM are provided in the “Supplementary Material” section.

Preparation of Nanoparticles

Preparation of Unloaded Nanoparticles

For the preparation of unloaded nanoparticles, 400 mg GM polymer was dissolved in 20 mL acetone to form solution 1. Then 40 mL of a hydroalcoholic mixture (10 mL water and 30 mL ethanol) containing 80 μ L calcium chloride (0.8% w/v) was added to solution 1 where GM-nanoparticles (GM-NPs) were formed under continuous magnetic stirring. The organic solvents were evaporated under reduced pressure (Rotary evaporator, Büchi R-144; BÜCHI Labortechnik AG), and the nanosuspensions were ultracentrifuged (Sigma 3K30 Rot. 12150-H; Newtown, UK) at 4°C and 40,000 $\times$ g for 20 min. Finally, the resulting batch of pellets was dispersed in 40 mL 2% mannitol solution and dried by spray-drying in a Büchi Mini Spray Drier B-290 apparatus (Büchi Labortechnik AG). The parameters for spray drying were as follows: inlet temperature of 90°C, outlet temperature of 60°C, spray-flow of 600 mL/h, and aspirator at 100% of the maximum capacity. These nanoparticles were named GM-NP.

As controls, nanoparticles based on Gantrez® AN (NP) were prepared as described previously.³¹

Preparation of Peanut Extract-Loaded Nanoparticles

PE-loaded nanoparticles were prepared by the dissolution of 400 mg GM in 20 mL acetone. Then, 12 mg of the PE (previously resuspended in 100 μ L of purified water adjusted to a pH 3 with HCl 0.1N) was added to this solution and incubated, under agitation, for 45 min. Nanoparticles were obtained, purified, and dried as described above. The resulting nanoparticles were identified as PE-GM-NP.

Preparation of Fluorescently Labeled Nanoparticles

Nanoparticles were fluorescently labeled by the encapsulation of Lumogen® F Red 305. Briefly, 4 mg Lumogen Red was dissolved in a solution of acetone containing GM or Gantrez® AN polymer prior to the formation of the nanoparticles as described previously. The resulting nanoparticles were purified and dried as described previously.

Physicochemical Characterization of Nanoparticles

For the characterization of nanoparticles, their size and zeta potential were determined by photon correlation spectroscopy and electrophoretic laser Doppler anemometry, respectively, using a Zetaplus apparatus (Brookhaven Inst. Corporation, Holtsville, NY). The yield of the preparative process of nanoparticles was calculated by gravimetry,³¹ whereas the amount of peanut encapsulated into nanoparticles was measured by using the bichinchonic acid method. The surface hydrophobicity of unloaded and loaded nanoparticles was evaluated by the Rose Bengal test as previously described,³² with some minor modifications. Finally, the stability of nanoparticles was evaluated by measuring the turbidity changes as a function of time in buffer solutions with different pH value corresponded to simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) (i.e., pH 1.2 and 6.8, respectively). More details about these studies are provided in “[Supplementary Material](#)”.

In Vitro Release

The release studies were performed under sink conditions by using SGF and SIF, supplemented with Tween 20 (1% w/v). For these purposes, Float-A-Lyzer devices with a MWCO of 300 kDa (Spectrum Labs, Breda, The Netherlands) were used. First, the dialysis bags were washed with ethanol 10% for 10 min and second with water. The bags were filled with 96 mg formulation dispersed in 5 mL SGF and then placed into a vessel containing 660 mL SGF. The vessel was maintained under magnetic agitation, and 200 μ L samples were withdrawn at fixed time intervals and replaced with equal volumes of SGF. After 2 h of incubation in SGF, the bags were transferred to a second vessel with 660 mL SIF. Again, at fixed times, 200 μ L were withdrawn and replaced with free SIF.

The amount of PE released from nanoparticles was quantified with a sandwich ELISA kit for peanut allergy (Veratox[®], Neogen, Scotland, UK).

In Vitro Evaluation of Nanoparticles Diffusion in Mucus

The diffusion of nanoparticles through porcine intestinal mucus barrier was measured by the multiple particle tracking (MPT) technique to assess the permeation of particles through mucus barrier.³³ MPT technique involves using of epifluorescence microscopy to capture videos of particles' movement within the mucus sample followed by tracking of particles trajectories of hundreds of individual particles within the mucus matrix.³⁴ Measuring of particles' displacements enable us to calculate diffusion coefficients of nanoparticles $\langle D_{eff} \rangle$. Diffusion of particles in water (D^0) was calculated by Stoke-Einstein equation. The relative efficiency of nanoparticles diffusion ($\% \langle D_{eff} \rangle / D^0$ ratio) was calculated for comparison between particles restricted diffusion through mucus versus free movement in water. The “[Supplementary Material](#)” section describes in detail these studies.

Gastrointestinal Transit Studies With Radiolabeled Nanoparticles

Prior to *in vivo* studies, nanoparticles were labeled with technetium-99m by reduction with stannous chloride as described previously.³⁵ All procedures were performed following a protocol previously approved by the “Ethical and Biosafety Committee for Research on Animals” at the University of Navarra in line with the European legislation on animal experiments. These studies were carried out in male Wistar rats weighing 250–300 g (Harlan, Barcelona, Spain). Animals were briefly stunned with 2% isoflurane gas (flow of oxygen of 0.2 L/min) for administration of nanoparticles by oral gavage and then quickly awakened. Each animal received a

single dose (1 mL) of nanoparticles (1 mCi; 0.8–1.0 mg with radiolabeled nanoparticles that were completed with up to 10 mg with unlabeled nanoparticles). For analysis, the animals were anesthetized with 2% of isoflurane gas (flow of oxygen of 0.2 L/min) and placed in prone position on the gamma camera (Symbia T2 Truepoint; Siemens Medical Solutions USA, Inc., Malvern, PA). SPECT-CT images were acquired for 25 min, with the following parameters for SPECT: 128 $\times$ 128 matrix, 90 images, 7 images per second, and CT: 110 mAs and 130 kV, 130 images, slice thickness 3 mm. Fused images were processed using the Syngo MI Applications True D software.

Biodistribution of Nanoparticles Within the Gut

These studies were carried out using a protocol described previously²² with minor modifications, after approval by the “Ethical and Biosafety Committee for Research on Animals” at the University of Navarra in line with the European legislation on animal experiments. Briefly, male Wistar rats (average weight 225 g; Harlan, Barcelona, Spain) were placed in metabolic cages and fasted overnight with free access to water. All animals received orally 10 mg of fluorescently labeled nanoparticles dispersed in 1 mL water. Then, after 2 h the animals were sacrificed by cervical dislocation, and the gut was removed. Different portions of the gut were collected, cleaned with PBS, stored in the tissue proceeding medium OCT[™], and frozen at -80°C . Each portion was then cut into 5 μ m 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 stained with DAPI were visualized in a fluorescence microscope (Axioimager M1; Zeiss, Oberkochen, Germany) with a coupled camera (Axiocam ICc3; Zeiss) and fluorescent source (HBO 100; Zeiss). The images were captured with the software ZEN (Zeiss). As control, an aqueous suspension of Lumogen[®] F Red 305 was administered.

Protective Study: Sensitization, Vaccination, and Challenge Studies

Experiments were performed using a protocol previously described^{36,37} and in compliance with the “Ethical and Biosafety Committee for Research on Animals” at the University of Navarra in line with the European legislation on animal experiments. CD-1 female mice of about 20 ± 1 grams were sensitized by oral administration of a mixture between of peanut butter (Capitan Mani[®] soft peanut butter; 4.35 mg with an approx. content of 1 mg protein) and 5 μ g cholera toxin in a total volume of 200 μ L of saline solution on days 1, 7, 15, and 21. Moreover a tape stripping was applied on the back of animals obtain a higher sensitization. For this purpose, mice were shaved and barrier-disrupted on back skin. Percutaneous sensitization in the damaged skin was performed by topical application of 100 μ g PE in 100 μ L saline solution onto the barrier-disrupted skin.

On day 25, the sensitized animals were divided in 3 groups of circa 15 animals each. Each group of animals received 1 of the following treatments: (1) saline (Control +), (2) free PE dispersed in water (PE), and (3) PE-loaded nanoparticles (PE-GM-NP). In addition, a group of nonsensitized animals were also employed as control (Control -). On days 25, 28, and 35, the animals received 1 oral dose of 1 mg PE either resuspended in purified water (PE) or incorporated into nanoparticles (PE-GM-NP). Finally, on days 44 or 45, animals were challenged by an injection of 2 mg PE in 200 μ L of saline solution by intraperitoneal route to provoke an anaphylactic shock in the sensitized animals. [Figure 1S](#) ([Supplementary Material](#)) summarizes the protocol.

Table 1
Physicochemical Characteristics of Empty (GM-NP) and Peanut-Loaded Nanoparticles (PE-GM-NP)

Formulation	Mean Size (nm)	PDI	Zeta Potential (mV)	Yield (%)	PE Loading ($\mu\text{g}/\text{mg}$)	EE (%)
GM-NP	193 $\pm$ 3	0.15 $\pm$ 0.01	-37 $\pm$ 4	87	—	—
PE-GM-NP	274 $\pm$ 2	0.16 $\pm$ 0.01	-33 $\pm$ 4	67	25 $\pm$ 2	81 $\pm$ 6

Data expressed as mean $\pm$ SD ($n = 3$).

EE, encapsulation efficacy; PDI, polydispersity index.

To analyze the intensity of the anaphylaxis shock, the following parameters were recorded: body temperature, mobility, bristly hair, and cyanosis. Clinical anaphylactic reactions were scored by 2 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.

Statistical Analysis

The physicochemical characteristics of nanoparticles as well as the *in vitro* studies were compared using the Student t-test. For *in vivo* studies, comparisons were performed using the one-way ANOVA and Tukey HSD test. In all cases, $p < 0.05$ was considered as a statistically significant difference. All calculations were performed using Graphpad Prism v6 (Graphpad Software Inc., San Diego, CA).

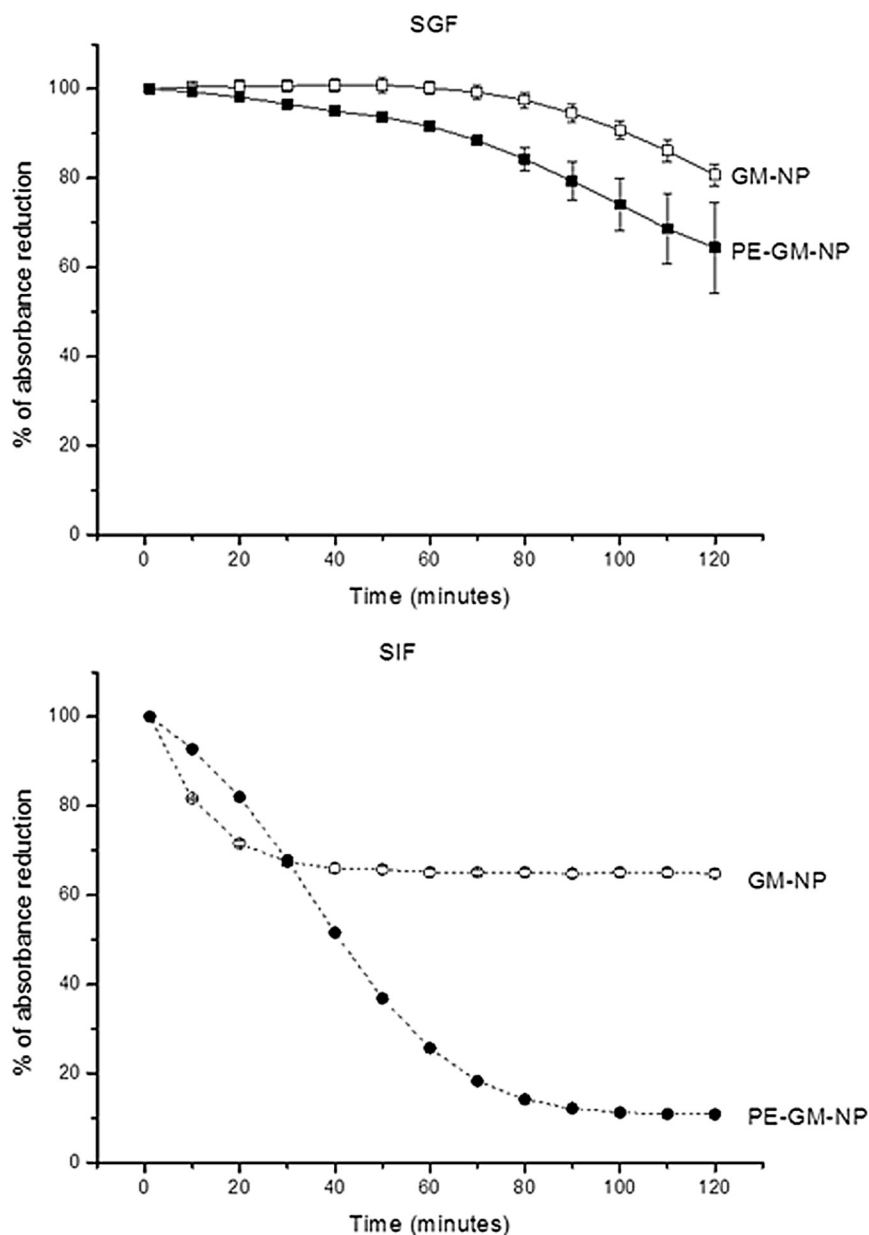


Figure 1. Stability of empty (GM-NP) or peanut-loaded nanoparticles (PE-GM-NPs) in SGF (pH 1.2) or in SIF (pH 6.8). Data expressed as mean $\pm$ SD ($n = 3$).

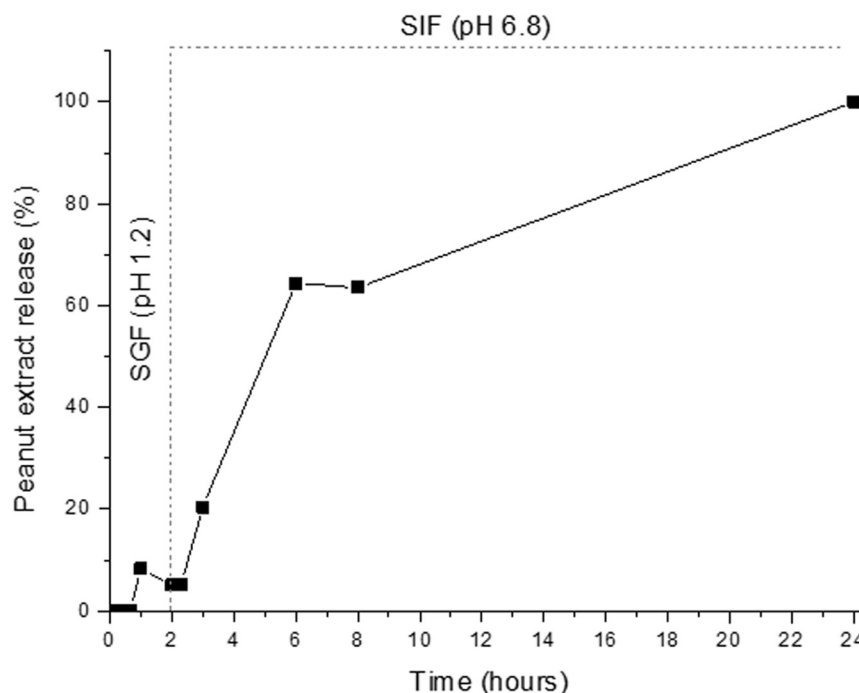


Figure 2. *In vitro* release of peanut extract from PE-GM-NP. Data expressed as cumulative amount of PE released versus time. Data expressed as mean $\pm$ SD ($n = 3$).

Results

Synthesis and Characterization of Gantrez-Mannosamine Conjugate

GM was synthesized by the covalent binding of mannosamine to the anhydride groups of the copolymer of methyl vinyl ether and maleic anhydride (Gantrez® AN). Figure 2S (Supplementary Material) shows a schematic representation of this synthesis.

FTIR analysis, $^1\text{H-NMR}$ analysis (Figs. 3S and 4S; Supplementary Material), and titration (Table 1S; Supplementary Material) were also performed. From these studies, it was calculated that the synthesis produced a polymer conjugate in which about 20% of the carboxylic acid groups from hydrated poly(anhydride) would be used for the covalent binding of mannosamine. In other words, the percentage of substitution means that 20 molecules of the maleic anhydride groups of each 100 residues in Gantrez® AN had reacted with mannosamine to generate amide groups and carboxylic acids. From o-phthalaldehyde analysis, the amount of mannosamine associated with the poly(anhydride) backbone was calculated to be about 21 $\mu\text{g}/\text{mg}$. Finally, with these data, the estimated molecular weight of the conjugate (GM) was 97.54 kDa.

Preparation of Mannosamine-Modified Nanoparticles

Nanoparticles were prepared from GM by a desolvation process using calcium as “bridge” between 2 neighboring carboxylic acid

residues of the polymer backbone (Fig. 2S). Table 1 shows the main physicochemical properties of these nanoparticles. PE-loaded nanoparticles (PE-GM-NP) displayed a slightly higher mean size (270 nm vs. 190 nm) and a similar negative zeta potential (-33 vs. -37 mV) than unloaded ones (GM-NP). Interestingly, the preparative process employed here resulted in homogeneous batches of nanoparticles (polydispersity index lower than 0.2). The PE loading was calculated to be 25 $\mu\text{g}/\text{mg}$ nanoparticles with encapsulation efficiency of 80%. For fluorescently labeled nanoparticles, the amount of Lumogen® F Red 305 incorporated into the nanoparticles was calculated to be similar for all the formulations tested and close to 7.3 $\mu\text{g}/\text{mg}$ (data not shown).

The surface hydrophobicity of nanoparticles was evaluated by using the Rose Bengal test (data not shown). From these studies, the hydrophobicity of GM-NP was 30-times lower than for Gantrez nanoparticles (NP). However, after encapsulation of PE, the resulting nanoparticles (PE-GM-NP) displayed a 6-times higher hydrophobicity than empty ones (GM-NP).

The stability of nanoparticles was investigated by measuring the turbidity changes as a function of time in gastric (SGF) and intestinal (SIF) simulated fluids in a concentration of 3 mg/mL (Fig. 1). Nanoparticles were easily dispersed in both media, and no aggregation phenomenon was observed during the study. When incubated in SGF, both types of nanoparticles (loaded and empty) displayed a similar profile with a quite high stability for at least 1.5 h. In SIF, empty nanoparticles displayed a significantly higher

Table 2
Nanoparticle Diffusion Kinetics in Intestinal Pig Mucus

	D° (Water) $\text{cm}^2 \times \text{S}^{-1} \times 10^{-9}$	$\langle \text{Deff} \rangle$ (Mucus) $\text{cm}^2 \times \text{S}^{-1} \times 10^{-9}$	$\% \langle \text{Deff} \rangle / D^{\circ}$ Ratio	R
PLGA-NP	27.91	0.0001 (± 0.0002)	0.0005	0.03
NP	25.69	0.0004 (± 0.0003)	0.0016	0.084
GM-NP	18.57	0.0035 (± 0.0018)	0.0190	1.00
PE-GM-NP	23.79	0.0531 (± 0.0313)	0.2240	11.79

Data expressed as mean $\pm$ SD ($n = 3$).

$\langle \text{Deff} \rangle$, diffusion coefficient in mucus; $\% \langle \text{Deff} \rangle / D^{\circ}$ ratio, relative efficiency of particles diffusion; R, ratio of $\% \langle \text{Deff} \rangle / D^{\circ}$ of the formulations tested.

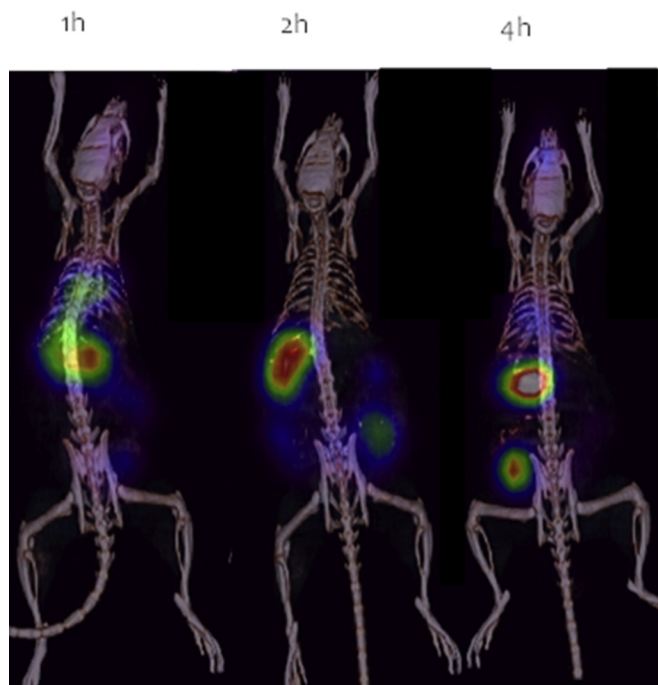


Figure 3. Volume rendered fused SPECT-CT images from representative rats at 1, 2, and 4 h after administration of ^{99m}Tc -labeled GM-NP by oral gavage.

stability than PE-loaded ones. Thus, for GM-NP, the turbidity of samples decreased during the first 30 min to reach a plateau of absorbance. At this moment, the turbidity of samples was about 70% of the initial values. For PE-GM-NP dispersed in SIF, the absorbance of samples decreased rapidly, and after 90 min of incubation, the absorbance was only of about 10% of the initial values.

In Vitro Release Studies

Figure 2 shows the release profile of PE from GM nanoparticles as function of time when incubated in SGF (during the first 2 h) and SIF (from 2 to 24 h). In SGF, only between 5%-8% of the total payload of nanoparticles were released during the first 2 h of the experiment. In SIF, the release rate of PE from nanoparticles increased rapidly and, at the end of the experiment, all the PE content was released, as measured by ELISA.

In Vitro Evaluation of Nanoparticles Diffusion in Mucus

Table 2 shows the diffusion coefficient in water (D°) calculated by the Stoke-Einstein equation, diffusion coefficient in the intestinal mucus $\langle \text{Deff} \rangle$ measured by the MPT technique, and the ratio as percentage of these 2 parameters (Deff/D°) of the tested nanoparticles. This last parameter was employed to compare the diffusion of the nanoparticles in intestinal pig mucus after normalizing the effect of particle size because D° is directly proportional to the particle size. GM-NP displayed a slightly higher diffusion coefficient than NP. For PE-loaded nanoparticles, the diffusion coefficient in the mucus was found to be about 12-fold higher than for unloaded nanoparticles prepared from the Gantrez-mannosamine (GM-NP). In all cases, the relative efficiency of particles diffusion (Deff/D°) of these nanoparticles was higher than the diffusion of PLGA-NP control particles (0.0005 for PLGA-NP vs. 0.0190 and 0.2240 for GM-NP and PE-GM-NP, respectively).

Gastrointestinal Transit Studies With ^{99m}Tc Radiolabeled Nanoparticles

Figure 3 shows the biodistribution (SPECT-CT images) of ^{99m}Tc -GM-NP orally administered to rats. One hour after administration, an important fraction of the given dose of nanoparticles appeared to remain in the stomach of animals. Nanoparticles appeared to slowly enter in the small intestine and move along the gut over time. Likely, at the end of the experiment, the no presence of radioactivity was observed in the liver or the lungs of the animals.

Biodistribution of Nanoparticles Orally Administered

Figure 4 shows fluorescence microscopy images of ileum samples from animals treated with fluorescently labeled nanoparticles 2 h postadministration. Empty nanoparticles (GM-NP) appeared to be mainly localized at the intestinal mucus layer (Figs. 4a and 4b). On the other hand, PE-GM-NP displayed a higher capability for reaching the intestinal epithelium including the surface of Peyer's patches (Figs. 4c and 4d). As a control, an aqueous suspension of Lumogen was administered to rats (data not shown). In this case, Lumogen particles in gut were observed as long aggregates with no capacity to reach intestinal epithelium.

Protective Study

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 PE. To analyze the intensity of the anaphylaxis, several parameters were evaluated. Ten minutes after challenge, sensitized and nontreated animals (Control +) experienced an important decrease of their body temperature. Animals treated either with free (PE) or encapsulated (PE-GM-NP) PE displayed a significantly lower temperature variation ($p < 0.05$).

Thirty minutes after challenge, mice were visually assessed for symptoms of anaphylaxis and assigned symptoms scores (Table 3). Animals from the nontreated group (Control +) displayed a low mobility and strong signs of bristly hair and cyanosis, whereas animals treated with free PE present a slightly better symptomatology, in spite of their apparently lower body temperature than control animals. On the contrary, animals treated with peanut-loaded nanoparticles displayed a better symptomatology in terms of cyanosis and decrease of rectal temperature and even a slightly better ability to maintain some movements than animals from the other groups.

Figure 5 shows the cumulative survival of animals after the intraperitoneal administration of 2 mg PE. In the positive control group, 40 min after challenge, less than 10% of the animals were alive. On the other hand, for sensitized animals treated with PE, the survival rate was close to 30%, whereas for those animals treated with PE-GM-NP, this survival rate was 53%.

Discussion

The success of nanoparticle-based formulations as oral carriers for biologically active compounds (e.g., peptides or proteins) is highly dependent on their capabilities to both protect the cargo against its premature degradation under the harsh conditions of the gut and reach the ideal site for its absorption and/or action. Particularly, nanoparticles for allergy immunotherapy should be able to induce, modulate, and potentiate an adequate immune response to minimize the effect associated with an allergen exposure in susceptible individuals. In this context, recent studies have shown that the encapsulation of a roasted PE in poly(anhydride)

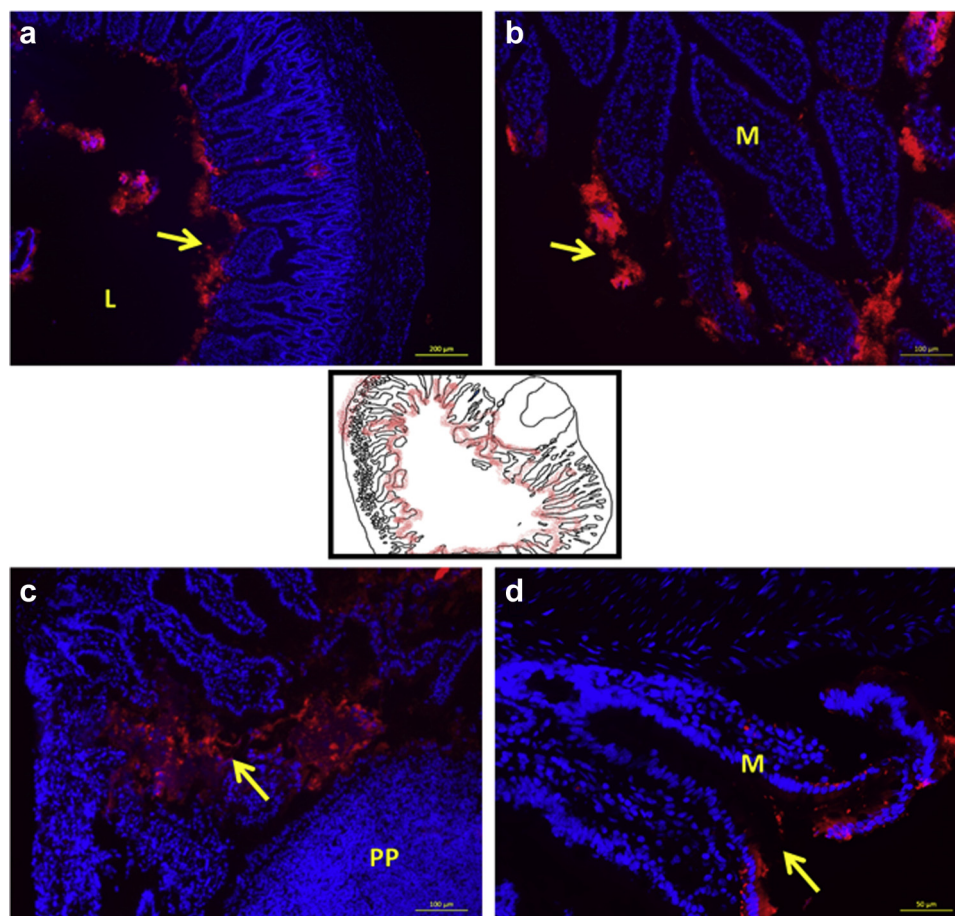


Figure 4. Fluorescent microscopic visualization of Lumogen red-loaded nanoparticles (GM and PE-GM-NP) in longitudinal sections of the ileum of rats. Figures (a and b) correspond to empty formulations (GM-NP) whereas (c and d) refer to peanut-loaded nanoparticles (PE-GM-NP). The draw in the middle indicates the anatomical regions for the mucosal intestinal villi (M), the follicle-associated epithelium of Peyer's patches (PPs), and lumen (L). Arrows in figures indicate the intestinal portions with high interaction between nanoparticles and mucosal intestinal villi. DAPI staining of nuclei appears as blue.

nanoparticles downregulated the allergen-specific Th2 response, potentiating the specific Th1 response²⁶ and, therefore, offering protection in a murine model of peanut allergy.³⁷

To ameliorate these results, one possible strategy may be the use of functionalization of these poly(anhydride) nanoparticles with ligands capable of offering mucus-permeating properties and the ability of specifically binding receptors on the immune cells localized in the gut. In this context, nanoparticles were prepared from a polymer conjugate obtained by the covalent binding of mannosamine to the copolymer of methyl vinyl ether and maleic anhydride (Gantrez[®] AN). The new resulting nanoparticles (GM-NP) displayed a mean size slightly higher than those obtained from Gantrez[®] AN

(193 vs. 178 nm). On the other hand, the zeta potential of both types of nanoparticles were negative and quite similar in an absolute value (−37 vs. −43 mV). On the contrary, GM-NP displayed a 30-fold lower hydrophobic surface and a capability to diffuse in intestinal pig mucus of about 12-times higher than NP (Table 2). All of these evidences suggest that the hydrophilic residues of mannosamine would be mainly exposed on the surface of the nanoparticle. This idea is supported by previous works, where high diffusivity of particles was observed when they were coated with hydrophilic ligands.^{38,39} In line with this, the low stability of mannosylated nanoparticles in SIF (Fig. 1), with an important decrease of their initial size, would facilitate their capability to cross the mucus layer and reach the intestinal epithelium.

Regarding the *in vitro* properties of the peanut-loaded nanoparticles, the release profile of PE from GM-NP was found to be dependent on the pH conditions (Fig. 2). Thus, under simulated gastric conditions (pH 1.2), a small fraction (5%–8%) of the loaded extract was released in 2 h. On the contrary, under simulated intestinal conditions, the peanut content of nanoparticles was completely released in less than 24 h of incubation (Fig. 2). Likely, this behavior is in line with the stability study of these nanoparticles in SGF and SIF (Fig. 1).

Furthermore, the encapsulation of PE increased the hydrophobicity of the resulting nanoparticles, evidencing the presence of components of this extract on the surface. Regarding the diffusion capabilities of nanoparticles in intestinal mucus, it was surprising

Table 3
Anaphylactic Symptoms After Allergic Provocation Challenge in Peanut-Sensitized CD-1 Mice

Treatment	Δ Temperature (°C)	Piloerection	Mobility	Cyanosis
Control +	-4.7 ± 2.6	+++	Very low	+++
PE	-5.2 ± 3.2	++	Very low	++
PE-GM-NP	-3.6 ± 2.7	++	Low	+
Control −	0.5 ± 0.8	−	Normal	−

Severity of the symptoms: (−) absent, (+) weak, (++) moderate, (+++) strong. Animals were treated with a peanut extract administered either free (PE) or encapsulated into nanoparticles (PE-GM-NP). Sensitized untreated animals (Control +) and nonsensitized mice (Control −) were also included.

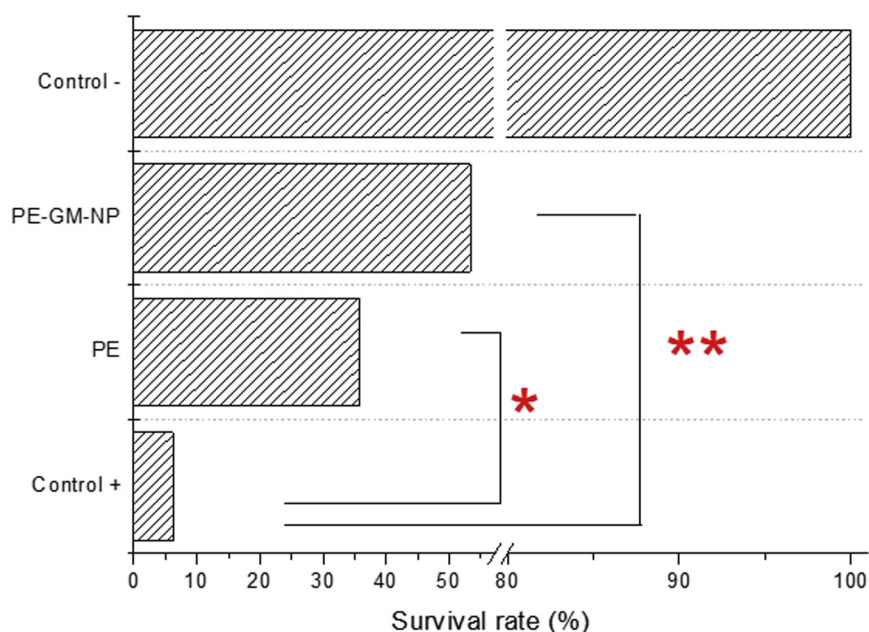


Figure 5. Cumulative survival rate of mice intraperitoneally challenge with peanut. Animals were treated with a peanut extract administered either free (PE) or encapsulated into nanoparticles (PE-GM-NP). Sensitized untreated animals (Control +) and nonsensitized mice (Control -) were also included (** $p < 0.01$, Log rank test indicates significant differences between animals treated with PE-GM-NP and nontreated animals). Log rank test indicate significant differences between free peanut extract and nontreated animals (* $p < 0.05$).

to observe a significantly higher diffusivity for PE-loaded nanoparticles than for empty ones with a high hydrophilic character (Table 2). This result may be explained by a rapid release of PE components located on the surface of the nanoparticles and their disturbing effects on the structural properties of the mucus gel. In fact, peanut proteins possess important emulsifying properties and oil binding capabilities⁴⁰ that may negatively affect the viscoelastic properties of mucus.⁴¹ These effects, as well as their resistance to enzymatic degradation, would facilitate the ability of some peanut proteins (e.g., Ara h2, Ara h6, Ara h7, and oleosins) to cross the mucus barrier and the intestinal epithelium, before exposition to underlying immune cells.⁴²⁻⁴⁴

Likely PE-GM-NPs were capable of reaching both the normal intestinal epithelium and the surface of Peyer's patches (Fig. 4). These findings are in agreement with previous results showing strong interactions of mannosylated nanocarriers within the intestinal epithelium, including Peyer's patches.^{25,45}

Finally, the protective effect of PE-GM-NP therapy was evaluated in a model of presensitized mice to peanut. For this purpose, the animals were intraperitoneally challenged with the PE. Animals treated with the PE-GM-NP displayed both a lower decrease in their basal temperature and less severe symptomatology associated with anaphylactic shock, when compared with animals treated with PE or ascribed to the positive control group (Table 3). In line with these results, animals treated with PE-GM-NP displayed a higher survival rate (53%) than animals treated with PE (35%) or the control group (6%) (Fig. 5). Despite the important differences, no statistical differences from the cumulative survival rate or mice treated with either PE or PE-GM-NP were found. This lack of differences would be related to the limitations of our study, mainly associate with the number of animals involved in the study. Nevertheless, and based on the obtained results, the promising protective effect of PE-GM-NP observed in this study should be confirmed in a larger study involving a higher number of animals.

In conclusion, nanoparticles based in a new GM may be easily prepared by a desolvation procedure followed by a cross-linking process with calcium ions. These nanoparticles may be employed

to load and carry a PE, facilitating their biodistribution within the gut, including Peyer's patches. In addition, this nanoparticle-based formulation offers an important protection against the effects induced by an anaphylactic shock in peanut-sensitized animals. However, further studies are needed to corroborate the promising protection offered by PE-GM-NP in an experimental model of peanut allergy.

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