

# Biodegradable poly (DL-lactide glycolide) microparticles as a vehicle for allergen-specific vaccines: a study performed with Ole e 1, the main allergen of olive pollen

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## Abstract

Ole e 1, the main allergen of the olive pollen, was entrapped in poly (DL-lactide glycolide) microparticles by a solvent evaporation double emulsion technique. The physical properties of the microparticles, such as particle size, surface morphology, allergen entrapment rate and antigen release pattern were investigated. Microparticles with a spherical morphology displayed a size range of less than 2  $\mu\text{m}$  in diameter and antigen loading up to 60–70% (w/w). SDS-PAGE and immunoblotting of the released Ole e 1 confirmed that the molecular integrity and the antigenic properties of Ole e 1 remained unaltered by the encapsulation process or polymer degradation. This finding suggests that microparticles displaying small particle sizes, rapid antigen release and a high allergen/polymer ratio may be a suitable delivery system for antigen in hyposensitization therapy against allergy. © 2002 Elsevier Science B.V. All rights reserved.

**Keywords:** Allergy; Ole e 1; Olive pollen; Microparticle; Poly (DL-lactide glycolide)

## 1. Introduction

Therapy for atopic individuals, at present, mainly comprises specific immunotherapy (SIT) and pharmacotherapy (Frieri, 1999; Barnes, 2000). SIT involves progressive desensitization of the allergic patients by systemic injection of increasing doses of the allergenic extract over a long period of time. Although the effectiveness of SIT in the treatment of allergic disorders has been shown in several clinical studies, the risks of anaphylactic side effects during

hyposensitization represent a major drawback for general application (Nelson et al., 1986). Therefore, the development of more safe and effective immunotherapy is one of the current goals in the field of allergy. In this sense, mucosal delivery of antigens encapsulated into poly (DL-lactide glycolide) (PLG) microparticles would represent an attractive strategy for treating and preventing allergy. PLG microparticles offer the advantage of simultaneously stimulating both mucosal and systemic immunity (Jones et al., 1996; Allaoui-Attarki et al., 1998; Baras et al., 1999; Chattaraj et al., 1999; Johansen et al., 2000; O'Hagan et al., 2000; Pecquet et al., 2000). PLG microparticles are made of biocompatible and biodegradable polymers (Visscher et al., 1987), which have been used for many years in

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humans as resorbable sutures prosthetic devices and drug carriers (Wise et al., 1979; Coombes et al., 1996). These polymers are degraded by nonenzymatic hydrolysis to yield common metabolites, lactic and glycolic acids, which are eliminated from the body through the Krebs cycle. They have the capacity to release an entrapped antigen over extended periods (Redding et al., 1984), and microparticles have been shown to act as adjuvants due to their ability to deliver antigen directly to the antigen-presenting cells (Eldridge et al., 1991). Therefore, the entrapment of allergens into PLG microparticles could represent a safe and promising strategy for hyposensitization therapy.

Olive tree (*Olea europaea*) pollen represents one of the most important causes of pollinosis in Mediterranean countries and some areas of North America where this species is widely cultivated (Bousquet et al., 1985). Ole e 1, a polymorphic glycoprotein of 145 amino acids, is the main allergen of this pollen and it is responsible for the sensitization of more than 70% of the olive pollen allergic individuals. Ole e 1 has been structurally and immunologically characterized (Villalba et al., 1993, 1994). Recently, it has been expressed in the yeast *Pichia pastoris* as a recombinant protein, whose molecular and immunological features were equivalent to that of the natural allergen (Huecas et al., 1999).

The aim of this study was to analyze the viability of PLG microparticles to serve as a vehicle for the administration of the olive allergen Ole e 1. The effect of the encapsulation process on the structural and immunological integrity of Ole e 1 was analyzed.

## 2. Materials and methods

### 2.1. Materials

Poly (DL-lactide glycolide) copolymer, PLG 50:50 (MW 50–75 kDa, Tm 45–50, Sigma-Aldrich, St. Louis, MO, USA), Polyvinyl alcohol (PVA, 87–89% hydrolysed, MW 13–23 kDa, Sigma-Aldrich), Dichloromethane (DCM) (reagent grade, Sigma-Aldrich), Fluorescein isothiocyanate (FITC) (Sigma-Aldrich). All other chemicals were of reagent grade.

Human sera ( $n = 4$ ) from patients allergic to Ole e 1 were selected from a population with a clinical history, a positive skin-prick test and high RAST values (3 to 6) to olive pollen. The presence of IgE antibodies specific to Ole e 1 was determined by ELISA.

### 2.2. Preparation of poly (DL-lactide glycolide) microparticles

Microparticles with entrapped Ole e 1 were prepared by a modification of the solvent evaporation method reported by Jeffery et al. (1993). Briefly, a solution of Ole e 1 in PBS (internal aqueous phase (w), 0.2 ml) was emulsified in a 6% (w/v) solution of the PLG in methylene chloride (oil phase (o), 2 ml), using an Ultraturrax model T8 mixer (IKA Laboratory Technology, Staufen, Germany) for 1 min. The resulting w/o emulsion was then emulsified under the same conditions with a 10% aqueous solution of PVA (external aqueous phase (w), 200 ml) to produce a water-in-oil-in-water emulsion (w/o/w). This w/o/w emulsion was then stirred magnetically for 18 h at room temperature and pressure to allow solvent evaporation and microparticle formation. The microparticles were collected by centrifugation at 10,000 rpm for 10 min, washed three times with ultrapure water and freeze-dried.

### 2.3. Purification of Ole e 1

Ole e 1 was prepared as previously reported (Villalba et al., 1993). The concentration of purified Ole e 1 was determined by amino acid analysis.

### 2.4. Particle size and surface morphology

The size distribution of the microparticle was determined by light scattering with a flow cytometer (EPICS XL, Beckman-Coulter, Hialeah, FL, USA). Estimating the intensity of the scatter signal to be correlated in a linear fashion to the volume of the microparticle size was expressed as the mean volume diameter in micrometer.

The lyophilized microparticles were dispersed in water and air-dried onto metal stubs. The samples were coated with gold–palladium under an argon atmosphere and observed by Scanning Electron Mi-

croscopy (Jeol 6400, Jeol Instrument, Welwyn, Herts, Germany) using an instrument equipped with an image analyzer.

### 2.5. Allergen encapsulation efficiency

The loading efficiency of the antigen in PLG microparticles was determined by extraction of freeze-dried microparticles with SDS/DCM:acetone (Delgado et al., 1999). Briefly, freeze-dried microparticles (5 mg) were suspended in 0.125 ml of 5% (w/v) SDS aqueous solution and shaken for 1 h. A mixture of DCM:acetone (50:50, v/v) was added to dissolve the polymer; the solution was vortexed for a few seconds and shaken for 18 h at room temperature. Following centrifugation, the antigen protein content was determined by micro-bi-cinchoninic acid (BCA) assay (Pierce Chemical, Rockford, IL, USA) following the manufacturer's instructions using known dilutions of Ole e 1 as standard. The percentage (w/w) of Ole e 1 entrapped per dry weight of microparticles was determined. The percentage entrapped efficiency was expressed by the ratio between the experimental entrapped Ole e 1 value and the theoretical value. Each sample was assayed in duplicate.

### 2.6. Ole e 1 distribution within microparticles

Ole e 1 was labelled with FITC in order to analyze the protein distribution within the microparticles. FITC labeling of Ole e 1 was performed as described by Holmes and Fowlkes (1999). Ole e 1 (2 mg/ml) was dissolved in 0.05 M boric acid/0.2 M NaCl, pH 9.2, and incubated with 0.02 ml FITC (5 mg/ml) for 2 h at room temperature. The sample was loaded onto a PD-10 column equilibrated in PBS. Fractions containing the protein were pooled, lyophilized and dissolved in 0.2 ml PBS. Microparticles loaded with FITC-Ole e 1 were examined using a confocal laser scanning microscope (MRC-1024ES Bio-Rad, Hemphstead, UK).

### 2.7. *In vitro* release study

The freeze-dried microparticles (25 mg) were suspended in 1 ml of PBS, pH 7.4 at 37 °C in a waterbath for 42 days under gentle agitation. The

medium also contained 0.02% (w/v) sodium azide as bacteriostatic agent. At predetermined time intervals, the sample was centrifuged at 10,000 rpm for 5 min, and the supernatant was removed and replaced by the same volume of fresh medium. The amount of Ole e 1 contained in this supernatant was measured using the BCA assay. Experiments were performed in duplicate.

### 2.8. Integrity of the microencapsulated Ole e 1

The integrity of Ole e 1 following entrapment in PLG microparticles was investigated using SDS-PAGE, immunoblotting and ELISA inhibition assays.

SDS-PAGE analysis of natural Ole e 1 and Ole e 1 extracted from microparticles were performed as described by Laemmli (1970). Following electrophoresis, gels were stained with Coomassie brilliant blue (0.1%, w/v). For immunoblotting, proteins were transferred onto nitrocellulose membranes (Bio-Rad, Richmond, CA, USA) in glycine/Tris transfer buffer at 1 mA/cm<sup>2</sup> for 1 h. After blocking for 1 h in PBS/0.1% Tween 20 (v/v)/3% skim milk (w/v), the electrophoresed and transferred allergen was incubated for 2 h with a pool of sera (diluted 1/10 in the blocking buffer) from patients allergic to olive pollen (Batanero et al., 1996). After incubating with a mouse anti-human IgE (diluted 1/5000 in PBS/0.5% Tween 20/1% skim milk), blots were incubated with horseradish peroxidase-labelled goat anti-mouse IgG (diluted 1/2500, Pierce) and developed using the ECL immunoblotting reagent and ECL-hyperfilm (Amersham International, Buckinghamshire, UK). Alternatively, a goat anti-rabbit IgG (1/3000, Bio-Rad) or goat anti-mouse IgG (1/2500, Pierce), both horseradish peroxidase-labelled, were used as second antibody for the Ole e 1-specific polyclonal C2 (1/20,000) antiserum and monoclonal 19G6 (1/20,000) antibody binding assays, respectively.

ELISA inhibition experiments were performed as previously described (Batanero et al., 1996). Microtiter 96-well plates (ELISA/RIA plate, Costar, Cambridge, MA, USA) were coated with 100 µl of Ole e 1 (1 µg/ml in PBS) and incubated overnight at 4 °C. After washing four times with PBS/0.5% Tween 20, unoccupied sites on the plates were

blocked with PBS/0.1% Tween 20/3% skim milk for 2 h. Ole e 1-specific rabbit polyclonal antiserum (1/200,000) or monoclonal antibody (1/200,000), previously preabsorbed for 2 h with different concentrations of natural Ole e 1 or released allergen as inhibitors, were added to the wells and incubated for 1 h at room temperature. A goat anti-rabbit IgG (1/3000, Bio-Rad) or goat anti-mouse IgG (1/2500, Pierce), both horseradish peroxidase-labelled, were used as second antibodies as mentioned above. The color reaction was developed with *o*-phenylenediamine (0.63 mg/ml) and 0.04% H<sub>2</sub>O<sub>2</sub>, in 0.1 M sodium citrate buffer, pH 5, containing methanol (4%). Absorbance values were read at 492 nm. All determinations were carried out as duplicate.

### 3. Results

#### 3.1. Microparticle characterization

A representative scanning electron micrograph of PLG microparticles is shown in Fig. 1. The particles were mostly spherical in shape, having smooth surface with no pores. The average size range was between 0.5 and 2  $\mu$ m in diameter, 65% of the particles displaying a mean diameter around 0.5  $\mu$ m. The Ole e 1 loading was 6–7  $\mu$ g of protein per milligram of polymer. The encapsulation efficiency, expressed as the ratio of the experimental and theoretical values, was 60–70%.

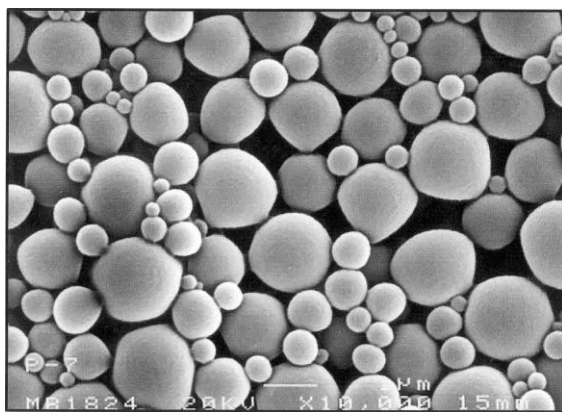


Fig. 1. Scanning electron micrograph of Ole e 1-PLG microparticles (scale bar = 1  $\mu$ m). Magnification, 10,000 $\times$ .

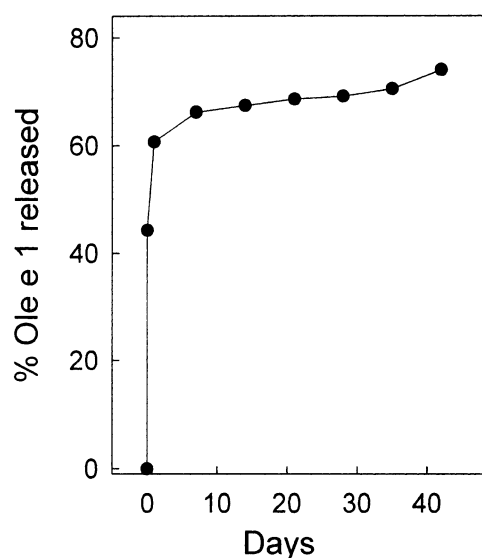


Fig. 2. Analysis of the *in vitro* release of Ole e 1 from PLG microparticles in PBS, pH 7.4, calculated as a percentage of the total entrapped allergen over a period of 42 days.

#### 3.2. *In vitro* release of Ole e 1 from PLG microparticles

To evaluate the suitability of the microparticles to be used as a vehicle for allergen vaccines, the release of Ole e 1 was assayed. After incubation at 37 °C in PBS, the release profile showed a biphasic shape composed of an initial burst and subsequent sustained release phase (Fig. 2). About 45% of the entrapped allergen was released from microparticles within 1 h, following a slow release of the allergen. After 42 days, 74% of Ole e 1 was liberated from PLG microparticles.

The distribution of FITC-labelled Ole e 1 within the microparticles was analysed by confocal laser microscopy. The protein was heterogeneously distributed within the polymer matrix (Fig. 3), mainly close to the surface of the microparticle. The surface localization of the protein may facilitate the initial burst phase that was observed.

#### 3.3. Analysis of the structural integrity of Ole e 1

During Ole e 1 encapsulation, the allergen is exposed to mechanical and chemical treatments which could contribute to a loss of structural and

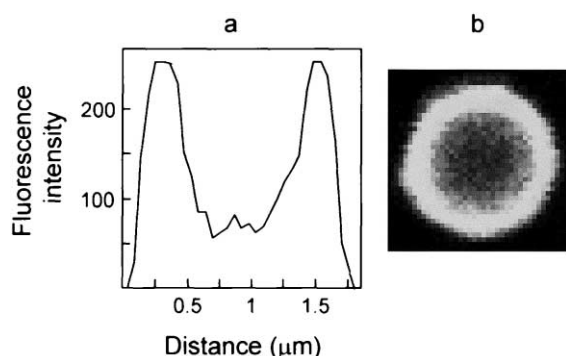


Fig. 3. (a) Profile of the FITC-Ole e 1 distribution within PLG microparticles. (b) Confocal micrograph of microparticles loaded with FITC-labelled Ole e 1.

antigenic integrity. SDS-PAGE patterns of Ole e 1 after release from the PLG microparticles are shown in Fig. 4a, in comparison with preparations of natural Ole e 1. Coomassie blue staining revealed a protein pattern for released Ole e 1 that was identical to that of the natural protein (Villalba et al., 1993). Two main bands of 18.5 and 20 kDa, which probably corresponded, respectively, to non-glycosylated and glycosylated forms, were observed for both prepara-

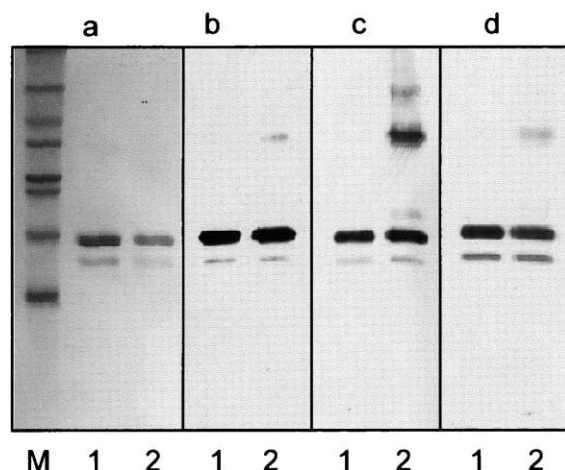


Fig. 4. SDS-PAGE and immunoblot analysis of Ole e 1 released from PLG microparticles. Coomassie blue staining (a) and immunodetection with a monoclonal antibody specific to Ole e 1 (b), a polyclonal antiserum raised against Ole e 1 (c) and a pool of sera from patients allergic to olive pollen (d). (M) Molecular mass markers, (1) natural Ole e 1, (2) Ole e 1 released from PLG microparticles.

tions. There were no additional bands indicating the presence of fragments produced by proteolysis. These data suggest that the structural integrity of this allergen was not affected by the entrapment procedure.

Immunoblot analysis confirmed that the antigenicity of Ole e 1 was not significantly altered following the entrapment in PLG microparticles, since the released allergen was recognized by the monoclonal anti-Ole e 1 antibody 19G6, which only binds to the native form, as well as by a rabbit antiserum raised against the natural Ole e 1 (Fig. 4b and c). Because of the high sensitivity of the polyclonal antiserum, two more bands, corresponding to 40 and 60 kDa—dimeric and trimeric forms of Ole e 1—, were also detected (Fig. 4c). It is known that Ole e 1 shows a strong tendency to aggregate, with the polymeric forms exhibiting higher antigenicity than the monomer. Finally, the released protein retained its ability on immunoblotting to bind IgE in a serum pool derived from olive pollen allergic patients (Fig. 4d). These data demonstrate that the encapsulated Ole e 1 retains the antigenic and allergenic epitopes of the natural allergen.

To test whether the exposure of encapsulated Ole e 1 to the eroding environment of the polymers might affect protein integrity, microparticles containing the allergen were incubated in PBS at 37 °C and

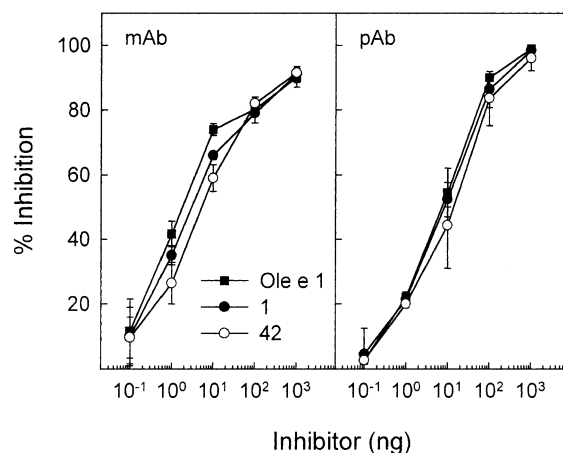


Fig. 5. ELISA inhibition assays of the IgG binding to Ole e 1-coated wells using as inhibitors Ole e 1 released from microparticles on days 1 and 42, as well as natural Ole e 1. The binding to Ole e 1-specific monoclonal antibody (mAb) and to a polyclonal antiserum (pAb) to Ole e 1 were analyzed.

the antigenicity of the released allergen was evaluated at several time points by ELISA inhibition assay (Fig. 5). The Ole e 1 released on day 1 or 42 showed similar inhibitory capacity as non-encapsulated allergen.

#### 4. Discussion

Allergy is an important clinical problem in developed countries. Because of the increasing prevalence of allergy over the last decades together with the lack of effective and safe immunotherapy, the development of new strategies for the prevention and treatment of allergic disorders has become a major goal. Several alternative approaches, including the use of alum as adjuvant, have been investigated to achieve safer and more effective immunotherapy (Bousquet et al., 1998). A promising approach may be the use of PLG microparticles as vaccine delivery systems. PLG is one of the polymeric candidates for the development of microparticles as a vaccine vehicle because it is a biodegradable and biocompatible polymer, which could be used for oral administration of allergens in SIT. PLG microparticles are able to protect a microencapsulated antigen from acid and proteolytic degradation in the gut (O'Hagan et al., 1991) and allow a controlled antigen release and a sustained presentation of the antigen to the immune system (Cohen et al., 1991). Moreover, PLG microparticles are able to induce CD8<sup>+</sup> T cell responses (Partidos et al., 1999), but not alum-based adjuvants. This is particularly advantageous since several studies have shown the importance of CD8<sup>+</sup> T cells in regulating IgE responses (Holmes et al., 1997; Lee et al., 1997). Finally, the stability of microparticles in freeze-dried formulations offers a significant advantage over alum. To date, only allergenic extracts of *Dermatophagoides pteronyssinus* have been successfully encapsulated into PLG microparticles (Sharif et al., 1995). Here, we describe a procedure for efficient encapsulation of the purified allergen Ole e 1, by modifying the method of Jeffery et al. (1993), to yield small size microparticles that retained the antigenicity of the allergen. Microparticles prepared by the solvent evaporation method display mean diameters of less than 2  $\mu\text{m}$ , thus providing optimal uptake into the Peyer's patches.

Although the parameters influencing particle uptake by Peyer's patches overlaying M cells are poorly understood, previous investigations have suggested that uptake is optimal for particles under 2  $\mu\text{m}$  in diameter (Eldridge et al., 1991).

Scanning electron micrographs of PLG microparticles entrapping Ole e 1 revealed particles with a smooth surface and homogeneous size. The antigen entrapment in these microparticles was high, of the order of 60–70%, which is important to supply a small number of doses of the entrapping antigen.

The in vitro release profile of Ole e 1 showed an initial burst of the antigen, followed by a gradual and continuous release of the antigen over the next 42 days. The antigen is released from the microparticles as a result of diffusion through matrix pores, matrix degradation and eventual collapse. This release profile is considered to be desirable for mucosal vaccines due to the limited period of exposure of the microparticle to the mucosal surface and the requirement for a high initial dose for the priming of the immune response, as well as a slow continuous release (booster doses) over extended periods to provide a long-lasting immune response (Aguado and Lambert, 1992).

A major drawback to vaccine development by antigen microencapsulation using the solvent evaporation method is the potential degradation of antigen during the preparation process (Chang and Crupta, 1996). However, neither structural integrity nor the immunogenicity of Ole e 1 was altered by either the microencapsulation process or during the time the microparticles were being disrupted. PLG degradation generates an extreme acid environment (pH 2–3) in which many antigens, such as the tetanus toxin, lose their structural integrity and antigenicity (Park et al., 1995). Previous studies performed by Sharif et al. (1995) with *D. pteronyssinus* allergenic extract entrapped in PLG microparticles indicated that protein integrity was not significantly affected during encapsulation into microparticles, despite the fact that this is a complex mixture of proteins with at least four major allergens. There was a minor reduction in the IgE binding capacity of the whole entrapped allergenic proteins, but this would not affect the ability of the extract to act as a hyposensitizing agent (Sharif et al., 1995). Nevertheless, the IgG binding capacity of Der p 1—one of the major

allergens of this extract—was not affected in comparison to native Der p 1, indicating that the structure of the allergen was maintained during the encapsulation process. It is known that many allergens are remarkably stable to enzymatic and chemical denaturation (Aas, 1978). The results obtained with Ole e 1 encapsulated into PLG microparticles show that the allergen may still be effective in the induction of an immune response and consequently a candidate vehicle for hyposensitization therapy to olive pollen allergy.

Several reports have indicated that IgA deficiency is associated with the development of certain diseases including allergy, upper respiratory tract and oral infections, and autoimmune disorders (Cunningham-Rundles, 1996; Shaffer et al., 1991). As the natural access for many allergens is through the mucosal surfaces of the gastrointestinal and respiratory tracts, an effective protection against allergy should stimulate both mucosal IgA antibodies—the first line of defense against the entrance of allergens—and serum IgG antibodies. The possibility of treating and preventing allergy by mucosal administration of an allergen with a mucosal adjuvant has recently been examined in a mouse model of peanut allergy (Roy et al., 1999). Oral administration of chitosan nanoparticles containing the gene for Ara h 2, the main peanut allergen, resulted in a substantial reduction in the levels of serum IgE, plasma histamine and vascular leakage, and the induction of mucosal IgA antibodies. These data suggest that the induction of specific-IgA antibodies may represent an attractive approach for allergy therapy since IgA could theoretically outcompete IgE for binding to a given allergen. Future studies are required to investigate the in vivo responses following oral immunization with Ole e 1-entrapped in PLG microparticles.

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