



Pharmaceutical Nanotechnology

An allergen-polymeric nanoaggregate as a new tool for allergy vaccination



Mariano Licciardi^{a,1}, Giovanna Montana^{b,1}, Maria Luisa Bondì^c, Angela Bonura^b,
Cinzia Scialabba^a, Mario Melis^b, Calogero Fiorica^a, Gaetano Giammona^a,
Paolo Colombo^{b,*}

^a Dipartimento di Scienze e Tecnologie Biologiche, Chimiche e Farmaceutiche (STEBICEF), Università degli Studi di Palermo, Via Archirafi 32, 90123 Palermo, Italy

^b Istituto di Biomedicina ed Immunologia Molecolare (IBIM), CNR, Via Ugo La Malfa, 153, Palermo, Italy

^c Istituto per lo Studio dei Materiali Nanostrutturati (ISMN), CNR, Via Ugo La Malfa, 153, 90146 Palermo, Italy

ARTICLE INFO

Article history:

Received 6 December 2013

Received in revised form 24 January 2014

Accepted 25 January 2014

Available online 1 February 2014

Keywords:

Allergy

Parietaria pollen

Recombinant allergens

PHEA

Polymeric nanoaggregates

ABSTRACT

A recombinant hybrid composed of the two major allergens of the *Parietaria* pollen Par j 1 and Par j 2 has been generated by DNA recombinant technology (PjED). This hybrid was produced in *E. coli* at high levels of purity. Then, the engineered derivative has been combined with a synthetic polyaminoacidic derivative having a poly(hydroxyethyl)aspartamide (PHEA) backbone and bearing both butyryl groups (C4) and succinyl (S) moieties in the side chain (PHEA-C4-S). The allergen-copolymer nanoaggregate was characterized by means of DLS, zeta potential, electrophoretic mobility and atom force microscopy analysis displaying the formation of a stable complex. Its safety has been proved *in vitro* on a murine cell line, human erythrocytes and basophils. Moreover, the formation of the complex did not alter the ability of the allergens to cross-link surface bound specific IgE demonstrating that the combination of an engineered hybrid with a copolymer did not interfere with its biological activity suggesting its employment as potential vaccine against *Parietaria*-induced allergies.

© 2014 Elsevier B.V. All rights reserved.

1. Introduction

IgE-mediated allergy is one of the most common immunological diseases affecting an increasing percentage of people living in the industrialized countries (Holgate and Broide, 2003).

The symptoms of allergic reactions include several local symptoms such as rhinitis and conjunctivitis, but systemic life-threatening reactions including anaphylactic shock were reported as well. Furthermore, it has been observed that if not properly diagnosed and treated, allergy can progress to a severe and chronic disabling disease such as asthma. The only treatment able to modify the natural outcome of the disease restoring a normal immunity against allergens is specific immunotherapy (SIT) (Bousquet et al., 1998).

So far, immunotherapy is performed by s.c. injection or mucosal administration of a mixture of proteins from natural sources which are not easy to standardize and without taking care of the individual

sensitization profile of the patient (Brunetto et al., 2010; Focke et al., 2008, 2009). In the last years, a few papers demonstrated that clinical immunotherapy trials with recombinant wild-type allergens were safe and effective and can replace allergen extract-based vaccines (Jutel and Cromwell, 2006; Pauli et al., 2008). In addition, it has been reported that allergens can be produced as hybrid molecules incorporating the epitopes of several allergens with the advantage to facilitate vaccine production and to increase immunogenicity (Linhart et al., 2002, 2005).

Parietaria judaica (Pj) pollen allergens are proteins from dicotyledonous weeds of the Urticaceae family. Immunologically, this family represents the most relevant species since its pollen represents one of the main outdoor sources of allergens in the Mediterranean area (D'Amato et al., 2007). In particular, almost 30% of all the allergic subjects in the Southern Mediterranean present a Skin Prick Test (SPT) reactivity toward the Pj pollen and more than 50% of these subjects have experienced bronchial asthma with high levels of bronchial hyper-responsiveness (D'Amato, 2000).

The composition of the allergenic extracts of the Pj pollen has been studied and, by means of molecular cloning, it has been demonstrated that this pollen contains at least 3 major allergens belonging to the Lipid Transfer Proteins (LTP) (Amoresano et al., 2003). In particular, the family of the Par j 1 allergen is

* Corresponding author at: Istituto di Biomedicina ed Immunologia Molecolare, Via Ugo La Malfa, 153, 90146 Palermo, Italy. Tel.: +39 91 6809535; fax: +39 91 6809548.

E-mail address: paolo.colombo@ibim.cnr.it (P. Colombo).

¹ These authors equally contributed to this work.

composed of the Par j 1.0101, a protein of 139 amino acids (Costa et al., 1994), and a shorter variant, Par j 1.0201, composed of 102 amino acids (Duro et al., 1997). The coding regions of the Par j 1.0102 and Par j 1.0201 isoforms show a 95% identity at the amino-acid level within the first 97 amino acids. The two isoforms differs for the presence of a 37 amino acids COOH-terminal tail in the Par j 1.0101 allergen which has been shown to contain a LPS binding region with immunomodulatory activity (Bonura et al., 2013). The Par j 2 allergen is a 102 amino acid long protein with a deduced Mw of 11,344 Da. Sequence similarity, 3D modeling (Colombo et al., 1998) and enzymatic digestion (Amoresano et al., 2003) have shown that major Pj allergens attain a three-dimensional structure consistent with that of the Lipid Transfer Proteins (LTP). Par j 1 and Par j 2 allergens are the species-specific allergens (Stumvoll et al., 2003) and represent the major allergens of this pollen (Costa et al., 1994; Duro et al., 1996).

Following this line of evidence, our research group developed several strategies to design new pharmacological compositions as new tool to cure Pj allergic patients (Bondi et al., 2011; Bonura et al., 2001, 2007; Orlandi et al., 2004). In particular, a hybrid molecule comprising the Par j 1 and Par j 2 allergens was evaluated *in vitro* and *in vivo* showing that heterodimers present improved immunological features (Bonura et al., 2007, 2012).

In this paper, firstly we described the design of a head–tail hybrid molecule expressing two isoforms of the major allergens of the Pj pollen, Par j 1.0201 and Par j 2.0101. This hybrid was expressed in *E. coli* as a His-tagged recombinant protein. Then the engineered derivative has been combined with a properly synthesized biocompatible multifunctional copolymer in order to produce a polymeric nanoaggregate of the allergens. This copolymer is a synthetic polyaminoacidic derivative having a poly(hydroxyethyl)aspartamide (PHEA) backbone and bearing both butyryl groups (C4) and succinyl (S) moieties in the side chain (PHEA-C4-S). Recently, it reported the ability of this copolymer to other complex protein molecules such as insulin and protect this protein against chemical and enzymatic degradation (Licciardi et al., 2013b).

Experimental data reported herein demonstrated that the combination of this copolymer with the engineered hybrid expressing the allergens of the Pj pollen retain its biological activity suggesting its employment as potential vaccine against *Parietaria*-induced allergies.

2. Experimental

2.1. Cloning, expression and purification of a recombinant Par j 2/Par j 1 hybrid

A head-to-tail hybrid expressing the wild type Par j 1 and Par j 2 allergens was generated by PCR (named PjED). Briefly, a DNA fragment containing the full-length coding region of the wild type Par j 2 sequence (EMBL accession number #X95865) was generated by PCR using the Pj2 for (5'-attGGATCCCAAGAAACCTGCGGGACTATG-3') and Pj2rev (5'-cgcGGATCCATAGTAACCTCTGAAAATAGT-3') oligonucleotides. Following the same strategy, a DNA fragment containing the full-length coding region of the Par j 1.0201 allergen (EMBL accession number #X85012) (Duro et al., 1997) was generated using the Parj1for (5'-attGGATCTGAAGAACTTGCGG-3' and the Parj1rev (5'-cgcGGATCCCTAATTTCTTTGTAGTG-3') oligonucleotides. Bold letters indicate the restriction enzyme sites (Bam H1) introduced for cloning and lower case letters the nucleotides inserted to improve restriction enzyme cut efficiency. A head-to-tail dimer (Parj2–Parj1) containing the two cDNAs was prepared cloning the

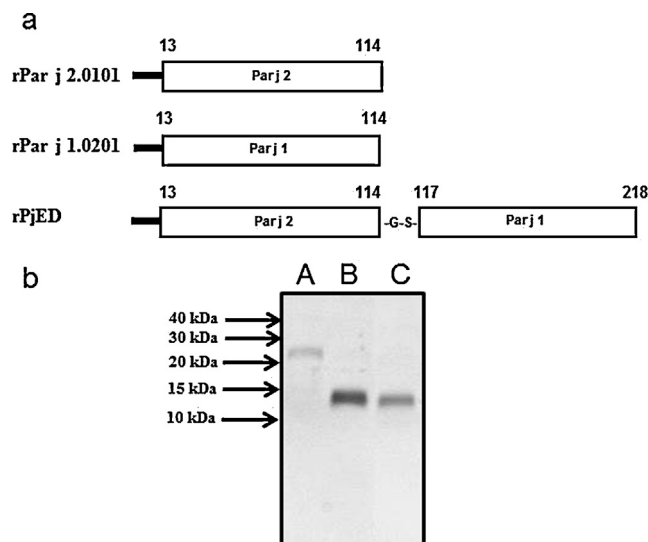


Fig. 1. Structure and Coomassie brilliant blue stained SDS-PAGE of the purified rPjED hybrid. (Panel a) Schematic representation of the Par j 1, Par j 2 and engineered hybrid PjED. Solid bars indicate the amino-terminal tag; open boxes the coding regions of the Par j 2 and Par j 1 allergens. Numbers show the size of the proteins. (Panel b) Coomassie brilliant blue stained SDS-PAGE of the purified recombinant proteins used for the assays. Molecular weights are indicated on the left side. Lane A shows the PjED hybrid, lane B and C the rPar j 1 and rPar j 2 allergens, respectively.

Par j 1 and Par j 2 BamH1 restricted fragments within the BamH1 restriction site of the pQE30 vector (Qiagen, Milan). The correct orientation of the two cDNA was checked by sequencing. Engineered hybrid was transfected for expression into *E. coli* M15 strain (Qiagen, Milan, Italy). The recombinant derivative expresses a fusion protein of 218 AA containing a 12 amino-terminal fusion peptide, a hexa-histidine tag and two additional amino acids (G and S) in position 115 and 116 as a consequence of the cloning procedures (sequence of the Bam H1 cloning site) (see Fig. 1 panel a for details). Engineered hybrid was purified as previously described (Bonura et al., 2007). Briefly, recombinant clones were grown over night at 37 °C in 2YT broth (Bacto-tryptone 16 g/l, Bacto-yeast 10 g/l, NaCl 5 g/l, pH 7.0). A 1:40 dilution was made and the culture was grown for 2 h at 37 °C and, after that, induced with isopropyl- β -thiogalactopyranoside for 2 h at 37 °C. Then cells were harvested, resuspended in a buffer containing 20 mM phosphate buffer pH 7.4, 0.5 M NaCl, 8 M urea and lysed with mild sonication. Cell debris was removed by centrifugation at 10,000 rpm for 30' at 4 °C. The supernatant was filtered using a 8 μ m disk and then loaded on a HisTrap column (GE, Uppsala, Sweden) following the manufacturer's instructions. The recombinant proteins were eluted using a buffer containing 20 mM phosphate buffer pH 7.4, 0.5 M NaCl, 8 M urea and 500 mM imidazole. Then, the fractions containing the recombinant PjED were reload on a HisTrap column for a second run of purification and eluted as above described. The collected fractions were analyzed by 16% SDS-PAGE and Coomassie brilliant blue staining. Fractions containing the recombinant hybrid were dialyzed against a buffer containing 20 mM phosphate buffer pH 7.4, 0.5 M NaCl to allow refolding of the proteins. Buffer exchange was performed using a Sephadex G-25 column (GE, Uppsala, Sweden) in 1 $\times$ PBS. Purity and concentration were determined by Coomassie brilliant blue staining and densitometric analysis. Recombinant proteins for the cell assays were further purified using a Detoxi-gel endotoxin removing gel (Pierce, USA) and tested for the endotoxin content using the Multi-test Limulus Amebocyte Lysate (LAL) pyrogen plus test (Bio-Whittaker, USA).

2.2. General procedure for the synthesis of α,β -poly(N-2-hydroxyethyl)-[(N-4-butylcarbamate)-co-(succinate)]-D,L-aspartamide (PHEA-C4-S) copolymer

α,β -Poly(N-2-hydroxyethyl)-D,L-aspartamide (PHEA) was prepared and characterized according to the previously reported procedure (Mendichi et al., 2003). PHEA-C4-S copolymer was synthesized and purified according to the already reported procedure (Licciardi et al., 2013b). Spectroscopic data (FT-IR and ^1H NMR) and molecular weight of obtained copolymer were in agreement with that already reported (Licciardi et al., 2013b). Briefly, the PHEA backbone has been functionalized step by step with butyryl groups (C4) in order to increase the hydrophobicity of PHEA and with succinyl (S) moieties in order to induce to the copolymer the ability to form ionic or hydrogen bonds. In the first functionalization reaction PHEA backbone has been modified by conjugating at the polymer side chain a certain number of butyryl groups (C4) by the reaction of PHEA with butylamine, using Bis(4-nitrophenyl)carbonate (4-NPBC) as condensing agent and obtaining the copolymer PHEA-C4. After this first functionalization reaction the ring opening reaction of succinic anhydride was used to insert carboxylic acid groups on PHEA-C4 side chain by ester linkage with hydroxyl groups of PHEA. The molar percent of butyl and succinic groups covalently linked to PHEA, calculated as reported by Licciardi and co-workers (Licciardi et al., 2013b) was equal to 15 and 35 mol% respectively, referred to the hydroxyethyl-aspartamide repeating units.

2.3. Preparation of the allergen-copolymer nanoaggregates

Synthesized copolymer (10 mg) was dissolved in sterile/LPS free $1 \times$ PBS at pH 7.4 (1 mL) at room temperature. PjED solution was prepared in the same medium at concentration of 400 ng/ μL ; then aliquots of this allergen solution was mixed with 100 μL of the copolymer solution at 25 °C, in order to obtain different allergen/copolymer weight ratios (1/25, 1/50, 1/100). Mixtures were left to stand for 30 min at 25 °C in a shaker, then used for analysis or stored at –20 °C.

2.4. Characterization of the allergen-copolymer nanoaggregates

2.4.1. Dynamic light scattering (DLS) and zeta potential measurements

DLS and aqueous electrophoresis measurements were performed at 25 °C using a Malvern Zetasizer NanoZS instrument, fitted with a 532 nm laser at a fixed scattering angle of 90°. Freshly prepared allergen, copolymer and allergen/copolymer nanoaggregates dispersions (weight ratios 1/25, 1/50, 1/100), were analyzed at concentrations of 0.1 mg/mL. The intensity-average hydrodynamic diameter and polydispersity index (PDI) were obtained by cumulant analysis of the correlation function. The zeta potential (mV) was calculated from the electrophoretic mobility using the Smoluchowski relationship and assuming that $ka > 1$ (where k and a are the Debye–Hückel parameter and particle radius respectively).

2.4.2. Atomic force microscopy (AFM) analysis

For AFM, a drop (~20 μL) of the allergen/copolymer nanoaggregate dispersion (weight ratio 1/100) in bidistilled water at 0.1 mg/mL concentration was deposited onto freshly cleaved mica and allowed to dry freely in air, then observed with a Multimode V Nanoscope Veeco microscope, driven by a nanoscope controller.

2.5. Electrophoretic analysis of nanoaggregates/peptide complex

In order to characterize the formation of the nanoaggregates/allergen complex a gel retardation experiment was

performed by loading 240 ng of the free hybrid and an equimolar concentration of the PjED/complex in 20 μL of non-denaturing loading buffer (10% glycerol, 62.5 mM Tris–HCl pH 6.8, Bromophenol blue 0.2%). The electrophoresis buffer was 10 mM Tris, pH 7.4, glycine 0.76 M. Electrophoresis of protein and nano/protein complex was carried out in a non-denaturing vertical gel at 10.0 V/cm. Non denaturing gel electrophoresis fractionates roughly spherical complex (particles) both by radius and by average electrical surface charge density. The gel was transferred onto nitrocellulose membranes using a semi-dry trans-blot (Millipore, USA). The complex and the hybrid were detected using a His-tag specific reagent (INDIA™ Hisprobe-HRP, Pierce, USA).

2.6. Cytotoxicity test

Cell viability was measured by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (CellTiter 96 AQueous One Solution Cell Proliferation Assay, Promega, WI, USA) assay according to the manufacturer's instructions. Raw 264.7 cells were previously seeded (1×10^4 cells/well) in RPMI 1640 medium containing 10% fetal calf serum and 0.1% antibiotics (penicillin, streptomycin and gentamicin) and treated in 96-well plates in a final volume of 100 μL plus 10 ng, 100 ng, 1000 ng of polymeric nanoaggregates for 24 h. After treatment of the cells, 20 μL of the MTS solution were added to each well and the incubation was continued for 4, 12 and 24 h at 37 °C, 5% CO_2 . The absorbance was read at 490 nm on a microplate reader (Wallac Victor 2 1420 multilabel counter, PerkinElmer, Waltham, MA). The absorbance of a blank RPMI sample was subtracted from all the absorption measurements corresponding to the different samples. The percentage of the cell viability was calculated as follows: $\% = (\text{ABS}_{\text{sample}} \times 100) / \text{ABS}_{\text{control}}$.

2.7. Hemolysis test

We used *in vitro* hemolysis assay to test hemolytic potential of the polymeric nanoaggregates by hemoglobin release in the plasma. For the hemolysis test, heparinized peripheral blood collected from 3 donors was used. A solution of 6% human erythrocytes was prepared. Erythrocyte concentration was controlled by reading the optical density of an hemolysate of the cell suspension thus made: 0.5 mL blood + 7 mL distilled water should give a reading of 0.7 O.D. at the spectrophotometer at 541 nm. Erythrocytes were incubated in triplicate with an increasing concentration of polymeric nanoaggregates (100 ng, 1 μg , 10 μg , 100 μg), $1 \times$ PBS as negative control and a 0.1% solution of Triton X100 as positive control. Sample reading was carried out at 415 nm.

2.8. CD203c basophil activation assay

Heparinized peripheral blood was obtained from allergic ($n = 3$) and non-allergic ($n = 1$) subjects. Blood aliquots (100 μL) were incubated with serial dilutions of the PjED hybrid (from 1 to 10,000 ng/mL in $1 \times$ PBS) and an equimolar concentration of the PjED-polymeric nanoaggregate for 15 min at 37 °C. $1 \times$ PBS was used as a negative control. Formyl-Methionyl-Leucyl-Phenylalanine (fMLP) was used as a positive control. After incubation, cells were resuspended in 100 μL of FACS buffer (BD Pharmingen™) and incubated with 20 μL /tube of phycoerythrin-labeled anti-CD203c (mAb 97A6, Immunotech, Marseille, France) for 20 min at 4 °C in the dark. Samples were subjected to erythrocyte lyses, washed twice in ice cold FACS Buffer and analyzed by means of flow cytometry on a FACSCalibur flow cytometer (Becton Dickinson). Basophils were detected on the basis of Side-Scatter characteristics

Table 1

DLS and zeta potential data of allergen, PHEA-C4-S copolymer and Par j 1/Par j 2/copolymer nanoaggregates at allergen/copolymer weight ratios (*R*) of 1/25, 1/50 and 1/100.

Sample	<i>D</i> (nm)	PDI	ζ (mV)
Allergen	22.2	0.3	−3.13
Copolymer	9.5	0.35	−12.7
<i>R</i> = 1/25	183	0.35	−3.14
<i>R</i> = 1/50	303	0.36	−6.0
<i>R</i> = 1/100	94	0.29	−10.2

(SSC, y-axis) and expression of CD203c (x-axis). For each sample, 100,000–200,000 cells were analyzed.

3. Results and discussion

3.1. Recombinant expression in *E. coli*

An engineered head to tail hybrid (PjED) containing the full length Par j 2.0101 and Par j 1.0201 open reading frames were expressed in *E. coli* as a fusion protein of 218 amino acids containing an additional 12 amino-terminal peptide, a hexa-histidine tag useful for purification and two additional amino acids (G and S) in position 115 and 116 as a consequence of the cloning procedures (see Fig. 1 panel a for details). This procedure allowed us the production of a single protein containing the whole B and T cell epitopes of the two major allergens of the *Parietaria* pollen. The PjED hybrid was purified by affinity and size exclusion chromatographies and then analyzed on a 16% SDS-PAGE gel (Fig. 1 panel b). Purity and concentration of the hybrid were determined by Coomassie brilliant blue staining and densitometric analysis. In addition, starting from the observation that for the final release of products in the pharmaceutical industries the presence of endogenous endotoxin must be determined, a Limulus Amebocyte Lysate test was performed showing that the recombinant protein contain ≤ 0.003 ng LPS/ μ g of recombinant protein. This procedure allowed to standardize our production in about 1.5 mg/L of highly purified recombinant protein.

3.2. Evaluation of the allergen/copolymer nanoaggregates formation

In order to evaluate the ability of synthesized PHEA-C4-S copolymers of interacting with the allergen and thus generate a potential supramolecular carrier for the allergen, the formation of nanoaggregates between the copolymer and the PjED hybrid (as model allergen protein) has been investigated by means of dynamic light scattering analysis, zeta potential measurements and atomic force microscopy (AFM) observation.

Firstly, the nanoaggregate formation was demonstrated by evaluating the variations of mean diameter (*D*), polydispersity index (PDI) and zeta potential (ζ) values of PjED/copolymer mixtures in function of allergen/copolymer weight ratios (see Table 1). At all the analyzed allergen/copolymer weight ratios, heterogeneous aggregates populations were observed, having mean diameters significantly greater than that of the nude allergen (22.2 nm; Fig. 2 panel a) or the pure copolymer (9.5 nm; Fig. 2 panel b). Nevertheless, it is evident that the aggregates diameter and PDI are the lower at the allergen/copolymer weight ratio of 1/100, because presumably, the interactions among the copolymer and the protein become stronger. This conclusion is supported by the colloidal dimension raised (94 nm; Fig. 2 panel c) and the decrease of PDI values that respect the decrease of the allergen/copolymer weight ratios. Moreover, zeta potential variations were also in agreement with an intensification of the negatively charged copolymer exposition on the nanoaggregate surfaces.

Furthermore, to investigate the shape and surface morphology of the nanoaggregates obtained with the allergen/copolymer nanoaggregate sample at weight ratio 1/100, AFM was used as visualization technique. This technique gave clear 3D morphological images, highlighting a spherical nanoaggregates shape and confirming that only a slight adhesion among nanoaggregates occurs (see Fig. 3 panels a–d). Nanoaggregate diameters observed by AFM were in the range of 50–90 nm, which is slightly smaller than the values determined by DLS in aqueous medium; this phenomenon, otherwise observed, is caused by the shrinkage of nanoaggregates upon drying (Licciardi et al., 2011).

Finally, a non denaturing gel electrophoresis of the free hybrid and nano/protein complexes was carried out. Samples were blotted on membrane and, using a His-tag specific chemiluminescent probe, we were able to detect that the free PjED hybrid migrates according to its molecular weight (Fig. 4 lane F). On the other hand, a large percentage of the nano/protein complexes displayed a gel retardation probably due to the protein/copolymer nanoaggregate formation (Fig. 4 lane C).

3.3. Biological activity

3.3.1. Cytotoxicity and hemolysis assays

To investigate the toxicity of the polymeric nanoaggregate, different amounts of the copolymer were incubated for 4, 12 and 24 h with a RAW 264 cell line. The test was carried out in triplicate and compared to a row of blanks containing exclusively culture medium. After incubation, a spectrophotometer reading was carried out, measuring absorbance at 490 nm. The data reported in Table 2 shows the reading at 24 h. Similar results have been observed at the other two time points. From this analysis, we can demonstrate that increasing concentrations of the nanoaggregate cause no or very low toxic effect on murine cells in culture.

A similar results was obtained in a hemolysis assay using human erythrocytes. TritonX-100-mediated hemolysis of human erythrocytes occurred in a dose response manner in the presence of increasing concentration of the detergent but no almost effect was observed after addition of the polymeric nanoaggregate reaching the final concentration of 100 μ g/ml (Fig. 5 panels a and b, respectively).

3.3.2. CD203c basophil activation assay

The allergenic activity of the PjED/complex and the empty copolymer were studied by basophil activation detecting overexpression of the CD203c marker. As a first analysis, blood from one non allergic subject was incubated with increasing concentration of PHEA-C4-S copolymer showing that it is not able to activate the CD203c antigen demonstrating that this kind of polymeric carrier do not display allergenic activity *per se* (data not shown).

Furthermore, the allergenic activity of the PjED/complex was evaluated by studying the capability of the free PjED hybrid to up-regulate the CD203c surface antigen in comparison to an equimolar amount of the PjED/complex. In all the tested patients, we observed that the PjED/complex was able to activate basophils in a way

Table 2

Cytotoxicity test carried out with RAW 264 cells in culture.

Sample	Mean ($\pm$ SD) of percentage of cell viability
Medium	100%
10 μ g/ml polymeric nanoaggregate	92% ($\pm$ 1)
100 μ g/ml polymeric nanoaggregate	91% ($\pm$ 0.7)
1000 μ g/ml polymeric nanoaggregate	88% ($\pm$ 1)

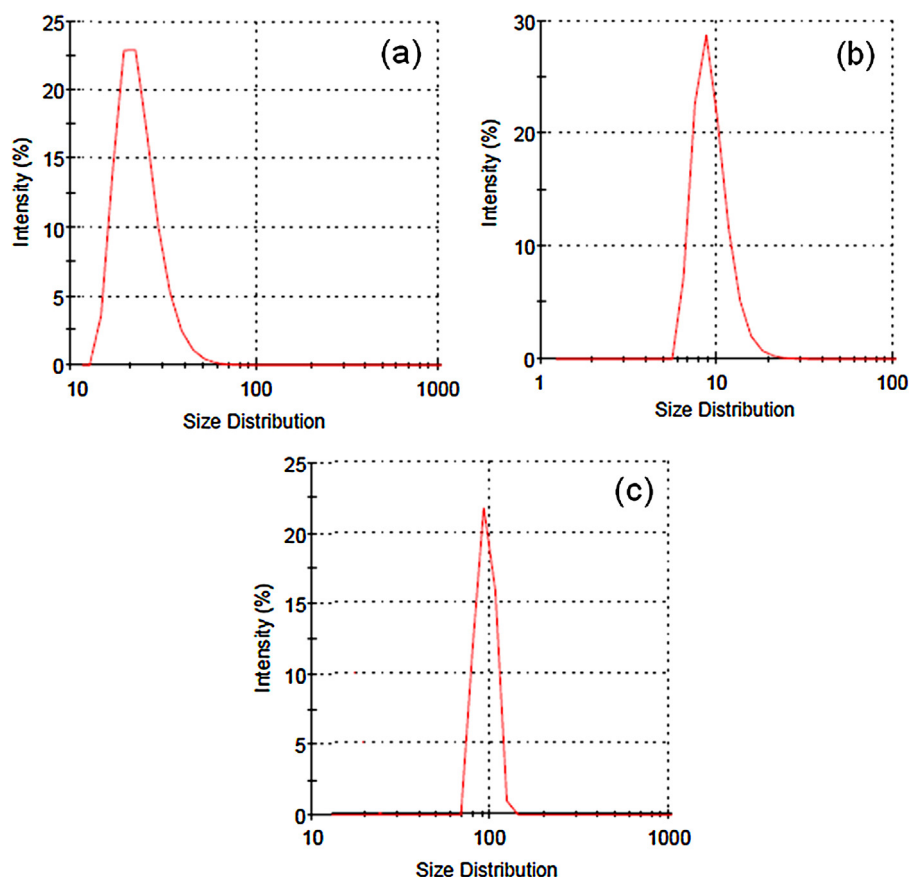


Fig. 2. DLS size distribution histograms of allergen (Panel a), copolymer (Panel b) and allergen/copolymer nanoaggregates at weight ratio of 1/100 (Panel c).

comparable to the nude hybrid. Fig. 6 shows a representative plot of the basophil activation assay from 1 out of 3 *Parietaria* allergic patients. Panels from A to D show the plot of activation of the free PjED hybrid. Panels from E to H display the same assay using the PjED/complex. This analysis demonstrated that the Parj1/Parj2-polymeric nanoaggregate is capable of cross-linking surface bound specific IgE activating CD203c over-expression in *Parietaria* allergic patients in a similar manner than the free derivative.

4. Discussion

In the last decades, the prevalence of allergic disease increased rapidly with up to 25% of population living in the industrialized countries therefore it is of paramount importance the development of new and improved formulations of immune-therapeutics to ameliorate the quality of life of allergic patients. So far, Specific Immunotherapy (SIT) is the only treatment able to prevent the progression of the disease restoring a normal immune response (Bousquet et al., 1998) and reducing the clinical symptoms associated with allergic rhinitis and asthma. In particular, subcutaneous (SCIT) and sublingual immunotherapy (SLIT) with unmodified allergen extracts are the most widely prescribed regimens. Emerging evidence suggests that SIT may be effective in other allergic conditions such as atopic dermatitis, venom sting-induced large local reactions and food allergy. Whereas subcutaneous immunotherapy is of proven value in allergic rhinitis and asthma there is a risk of untoward side effects including rarely anaphylaxis. All preparations that are currently available (standardized extract, allergoids and recombinant allergen) may trigger side effects. For these reasons, in the last few years, new formulations and route of administration

have been exploited demonstrating that such products presented improved efficacy and safety (Dretzke et al., 2013).

Parietaria pollen represents an ideal system to study since it is composed of a few allergens with only two major ones which are recognized by the majority of the Pj allergic population. In this manuscript, we propose the combination of two innovative approaches in *Parietaria* SIT: the use of a hybrid molecule expressing a head–tail dimer of the Par j 1 and Par j 2 allergens which can be produced in a recombinant form in *E. coli* and the loading of such derivative into a nanosized carrier. In fact, the use of nanotechnology platforms although widespread for vaccination purpose, is recently emerging in the field of allergen immunotherapy opening the question whether polymeric nanoparticles can be of interest to develop new therapeutic strategies able to improve both clinical efficacy and safety of allergen vaccines (Gomez et al., 2006, 2007, 2009).

Nanotechnology for biomedical applications offers many advantages, such as improved stability and bioavailability, favorable biodistribution profiles and targeting to specific cell populations. In this field, it was recently explored the ability of some polyaminoacids based graft copolymers to form in water supramolecular nanoaggregates having dimensions below one micron, capable to interact with protein active molecules, and to release the complexed protein *in vivo* in a controlled way (Licciardi et al., 2013b). This approach has been successful proposed to overcome protein instability problems including that related to improve oral protein bioavailability (Licciardi et al., 2013a). With this aim, a synthetic polyaminoacidic copolymers based on α,β -poly(N-hydroxyethyl)-D,L-aspartamide (PHEA), bearing both hydrophobic portions (butyl, C4) and ionizable (COOH, succinic) groups (PHEA-C4-S), have been used to form Par j 1/Par j 2-polymeric nanoaggregate. In these polymer/protein

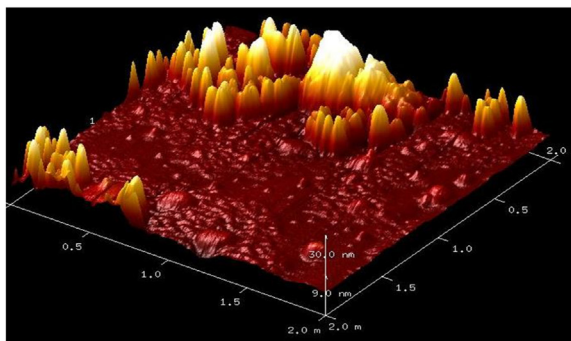
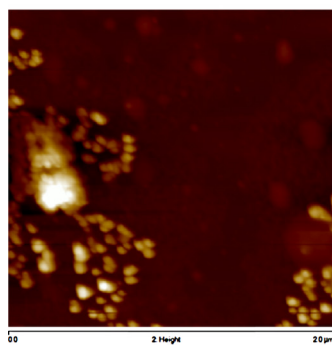
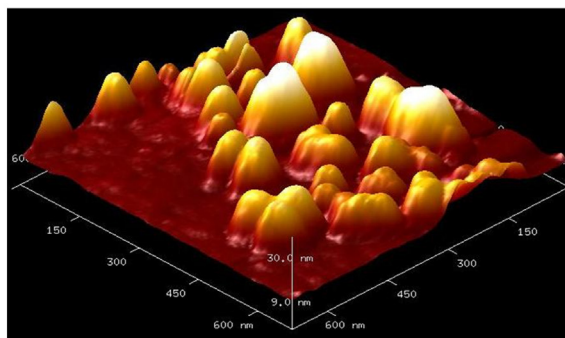
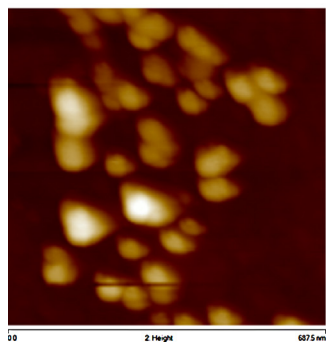
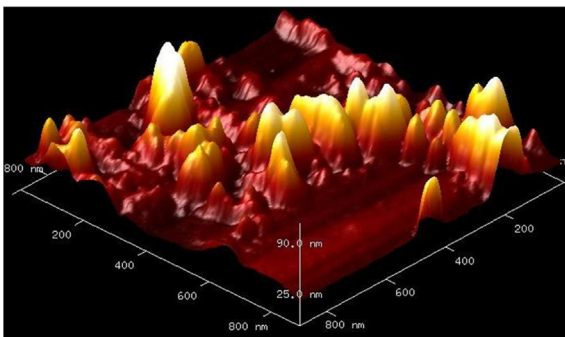
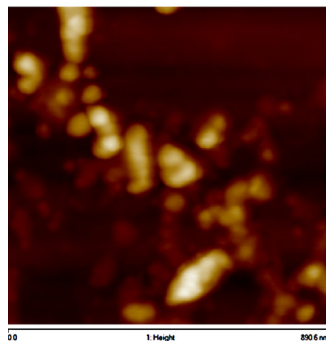
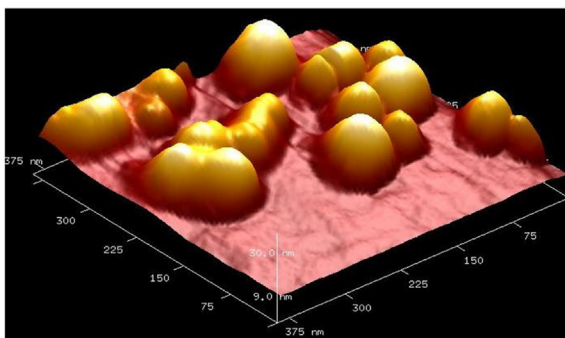
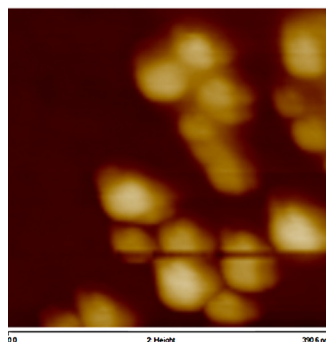
A**B****C****D**

Fig. 3. AFM images of allergen/copolymer nanoaggregates at weight ratio of 1/100 visualized at different scanning planes: 2 μ m (Panel A), 800 nm (Panel B), 600 nm (Panel C) and 375 nm (Panel D).

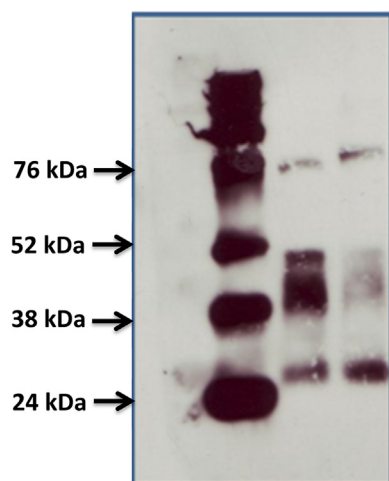


Fig. 4. Influence of nanoaggregate formation on electrophoretic mobility of the PjED hybrid. Lane M: molecular weight marker; Lane C: allergen/copolymer nanoaggregates at weight ratio of 1/100; Lane F: nude PjED hybrid.

nanoaggregates, the main interaction forces involved in the complex formation are hydrophobic interactions and hydrogen bonds. These copolymers, in fact, contain both anionic groups and short hydrophobic chains, such as carboxylic and butyl moieties

respectively, functional groups able of inducing self-assembling properties and interactions with the proteins at the same time (see structure in Fig. 7). An intrinsic advantage of using PHEA as starting polymer, apart from its high biocompatibility, is that this macromolecule has numerous nucleophilic groups (hydroxyl) in the side chain, one for each repeating unit and is soluble in water as well as in organic solvents. These properties make possible its extensive side chain functionalization with different molecules and in consecutive reaction steps. In the case of PHEA-C4-S, the PHEA backbone has been functionalized step by step with butyryl groups (C4) in order to increase the hydrophobicity of PHEA and with succinyl (S) moieties in order to promote the ability of copolymer to form hydrogen bonds. The final copolymer was still hydrosoluble but its multifunctionality make it able to physically interact with protein molecules, increasing the stability and the solubility of the complexed protein, without altering the pharmacological activity.

As a first result, we were able to show that a highly purified engineered recombinant endotoxin-free hybrid containing the two major allergens of the *Parietaria* pollen (PjED derivative) can be produced at higher level of purity and incorporated in a stable allergen/complex as shown using several independent techniques. In particular, DLS, zeta potential and gel retardation assays demonstrated the interaction between the protein and the polymer and, moreover, that this complex became stronger at an allergen/copolymer weight ratio of 1/100. For these reasons, this weight ratio was used for further characterization of the nanoaggregate. In

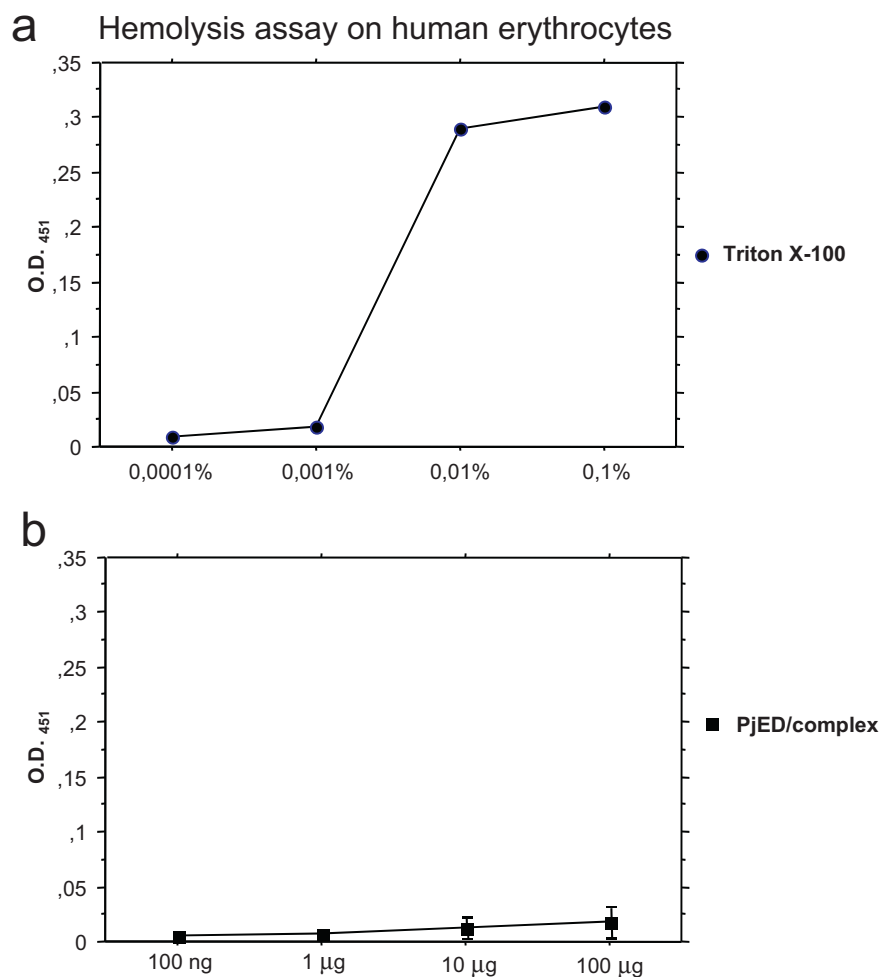


Fig. 5. Hemolysis assay on human erythrocytes. Panel a shows the optical density measured after the incubation of erythrocytes with increasing concentration of the positive control Triton X-100. Panel b displays the same time course with the polymeric nanoaggregate. Numbers on the X-axis indicate the concentration of the reagents used for the assay; on the Y-axis the absorbance detected.

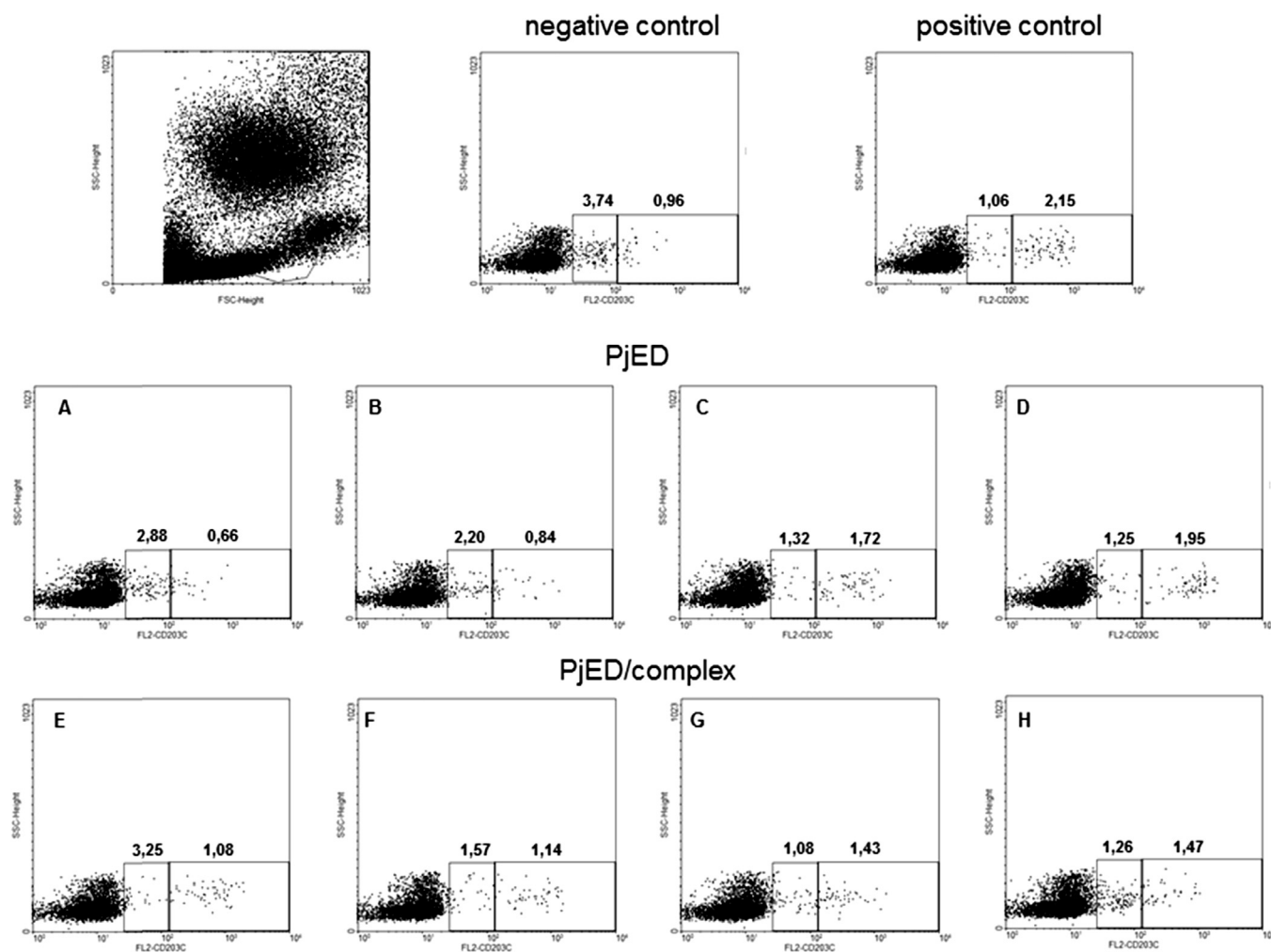


Fig. 6. Basophil CD203c surface expression. Peripheral blood from one *Parietaria* allergic patient was stimulated with serial dilutions of the PjED (Panels A–D) and the PjED/complex (Panels E–H). Negative control shows activation induced by 1 × PBS; positive control shows the percentage of activation using the fMLP peptide as inducer.

addition, AFM was used to study the detailed morphology of the nanoaggregates (Fig. 3 panels a–d) since this technique represents a powerful strategy that allows the observation of delicate and soft samples without the alteration of the sample surface. Nanoaggregate diameters observed by AFM were in the range of 50–90 nm, which is slightly smaller than the values determined by DLS in aqueous medium; this phenomenon, otherwise observed, is caused by the shrinkage of nanoaggregates upon drying (Licciardi et al., 2011).

It is known that properties of nanoparticles such as size, surface charge, hydrophobicity/hydrophilicity can determine its compatibility with the immune system. For these reasons, we looked at the biological activity of the PjED/complex searching for toxic activities *in vitro*. A murine cell line and human erythrocytes were employed to address this question showing that nanoparticles do not have any toxic or lytic effect *per se*. Following this line of evidence, we decided to look at their potential allergenic activity looking at the ability of the complex to activate human basophils from allergic

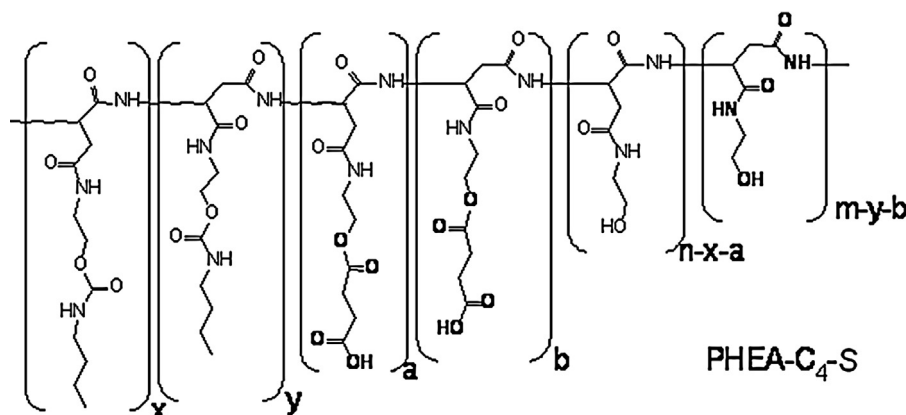


Fig. 7. Chemical structure of PHEA-C₄-S copolymer.

and non-allergic subjects. Basophils are relevant effector cells in allergy since they are responsible for the immediate allergic reaction through the secretion of inflammatory molecules such as histamine, lipid mediators like leukotrienes and several other molecules (Siracusa et al., 2013). The activation of basophils is commonly used in the diagnosis of allergies including drug reactions and it can be investigated *in vitro* by using flow cytometry monitoring the basophil cell surface marker CD203c (McGowan and Saini, 2013). The data reported in this study demonstrated that the nanoparticles are not able to trigger basophil activation even at high concentration using blood from a non-allergic subject (data not shown). On the other hand, when blood from Pj allergic patients was incubated with the nude allergenic hybrid and the PjED/complex, we observed that the formation of the complex did not alter its ability of cross-linking surface bound specific IgE activating CD203c over-expression in *Parietaria* allergic patients.

5. Conclusions

In this study, a head–tail hybrid molecule expressing two isoforms of the major allergens of the Pj pollen, Par j 1.0201 and Par j 2.0101, has been engineered on the attempt to generate a novel formulation for the development of new tool for the vaccination of *Parietaria* allergic population. This hybrid protein was expressed in *E. coli* as a His-tagged recombinant protein and the conditions for its production and purification have been standardized. Then a synthetic polyaminoacidic derivative having a poly(hydroxyethyl)aspartamide (PHEA) backbone and bearing both butyryl groups (C4) and succinyl (S) moieties in the side chain (PHEA-C4-S) has been used as polymeric carrier for this engineered derivative in order to produce a polymeric nanoaggregate of the allergens (PjED/complex). The absence of toxic activity of the polymeric carrier has been demonstrated *in vitro* on a murine cell line and human erythrocytes. Moreover, using blood from a non allergic subject, we observed that the nanoaggregates were not able to trigger basophil activation even at high concentration. Finally, when blood from Pj allergic patients was incubated with the nude hybrid and the PjED/complex, the complex formation did not alter the ability of the allergens to cross-link surface bound specific IgE activating CD203c over-expression in *Parietaria* allergic patients.

Experimental data reported herein demonstrated that the combination of an engineered hybrid expressing the major allergens of the Pj pollen with a copolymer did not interfere with its biological activity suggesting its employment as potential vaccine against *Parietaria*-induced allergies.

References

- Amoresano, A., Pucci, P., Duro, G., Colombo, P., Costa, M.A., Izzo, V., Lamba, D., Geraci, D., 2003. Assignment of disulphide bridges in Par j 2.0101, a major allergen of *Parietaria judaica* pollen. *Biol. Chem.* 384, 1165–1172.
- Bondi, M.L., Montana, G., Craparo, E.F., Di Gesu, R., Giammona, G., Bonura, A., Colombo, P., 2011. Lipid nanoparticles as delivery vehicles for the *Parietaria judaica* major allergen Par j 2. *Int. J. Nanomed.* 6, 2953–2962.
- Bonura, A., Amoroso, S., Locorotondo, G., Di Felice, G., Tinghino, R., Geraci, D., Colombo, P., 2001. Hypoallergenic variants of the *Parietaria judaica* major allergen Par j 1: a member of the non-specific lipid transfer protein plant family. *Int. Arch. Allergy Immunol.* 126, 32–40.
- Bonura, A., Corinti, S., Artale, A., Di Felice, G., Amoroso, S., Melis, M., Geraci, D., Colombo, P., 2007. A hybrid expressing genetically engineered major allergens of the *Parietaria* pollen as a tool for specific allergy vaccination. *Int. Arch. Allergy Immunol.* 142, 274–284.
- Bonura, A., Corinti, S., Schiavi, E., Giacomazza, D., Gianguzza, F., Di Felice, G., Colombo, P., 2013. The major allergen of the *Parietaria* pollen contains an LPS-binding region with immuno-modulatory activity. *Allergy* 68, 297–303.
- Bonura, A., Passantino, R., Costa, M.A., Montana, G., Melis, M., Bondi, M.L., Butteironi, C., Barletta, B., Corinti, S., Di Felice, G., Colombo, P., 2012. Characterization of a Par j 1/Par j 2 mutant hybrid with reduced allergenicity for immunotherapy of *Parietaria* allergy. *Clin. Exp. Allergy* 42, 471–480.
- Bousquet, J., Lockey, R., Malling, H.J., 1998. Allergen immunotherapy: therapeutic vaccines for allergic diseases. A WHO position paper. *J. Allergy Clin. Immunol.* 102, 558–562.
- Brunetto, B., Tinghino, R., Braschi, M.C., Antonicelli, L., Pini, C., Iacovacci, P., 2010. Characterization and comparison of commercially available mite extracts for *in vivo* diagnosis. *Allergy* 65, 184–190.
- Colombo, P., Duro, G., Costa, M.A., Izzo, V., Mirisola, M., Locorotondo, G., Cocchiara, R., Geraci, D., 1998. An update on allergens. *Parietaria* pollen allergens. *Allergy* 53, 917–921.
- Costa, M.A., Colombo, P., Izzo, V., Kennedy, H., Venturella, S., Cocchiara, R., Mistrello, G., Falagiani, P., Geraci, D., 1994. cDNA cloning, expression and primary structure of Par j 1, a major allergen of *Parietaria judaica* pollen. *FEBS Lett.* 341, 182–186.
- D'Amato, G., 2000. Urban air pollution and plant-derived respiratory allergy. *Clin. Exp. Allergy* 30, 628–636.
- D'Amato, G., Cecchi, L., Bonini, S., Nunes, C., Annesi-Maesano, I., Behrendt, H., Liccardi, G., Popov, T., van Cauwenberge, P., 2007. Allergenic pollen and pollen allergy in Europe. *Allergy* 62, 976–990.
- Dretzke, J., Meadows, A., Novielli, N., Huissoon, A., Fry-Smith, A., Meads, C., 2013. Subcutaneous and sublingual immunotherapy for seasonal allergic rhinitis: a systematic review and indirect comparison. *J. Allergy Clin. Immunol.* 131, 1361–1366.
- Duro, G., Colombo, P., Assunta Costa, M., Izzo, V., Porcasi, R., Di Fiore, R., Locorotondo, G., Cocchiara, R., Geraci, D., 1997. Isolation and characterization of two cDNA clones coding for isoforms of the *Parietaria judaica* major allergen Par j 1.0101. *Int. Arch. Allergy Immunol.* 112, 348–355.
- Duro, G., Colombo, P., Costa, M.A., Izzo, V., Porcasi, R., Di Fiore, R., Locorotondo, G., Mirisola, M.G., Cocchiara, R., Geraci, D., 1996. cDNA cloning, sequence analysis and allergological characterization of Par j 2.0101, a new major allergen of the *Parietaria judaica* pollen. *FEBS Lett.* 399, 295–298.
- Focke, M., Marth, K., Flicker, S., Valenta, R., 2008. Heterogeneity of commercial timothy grass pollen extracts. *Clin. Exp. Allergy* 38, 1400–1408.
- Focke, M., Marth, K., Valenta, R., 2009. Molecular composition and biological activity of commercial birch pollen allergen extracts. *Eur. J. Clin. Invest.* 39, 429–436.
- Gomez, S., Gamazo, C., Roman, B.S., Ferrer, M., Sanz, M.L., Irache, J.M., 2007. Gantrez AN nanoparticles as an adjuvant for oral immunotherapy with allergens. *Vaccine* 25, 5263–5271.
- Gomez, S., Gamazo, C., San Roman, B., Grau, A., Espuelas, S., Ferrer, M., Sanz, M.L., Irache, J.M., 2009. A novel nanoparticulate adjuvant for immunotherapy with *Lolium perenne*. *J. Immunol. Methods* 348, 1–8.
- Gomez, S., Gamazo, C., San Roman, B., Vauthier, C., Ferrer, M., Irachel, J.M., 2006. Development of a novel vaccine delivery system based on Gantrez nanoparticles. *J. Nanosci. Nanotechnol.* 6, 3283–3289.
- Holgate, S.T., Broide, D., 2003. New targets for allergic rhinitis—a disease of civilization. *Nat. Rev. Drug Discov.* 2, 902–914.
- Jutel, M., Cromwell, O., 2006. Clinical results from vaccination with recombinant grass pollen allergens. *Clin. Dev. Immunol.* 13, 389–394.
- Licciardi, M., Cavallaro, G., Di Stefano, M., Fiorica, C., Giammona, G., 2011. Polyaspartamide-graft-polymethacrylate nanoparticles for doxorubicin delivery. *Macromol. Biosci.* 11, 445–454.
- Licciardi, M., Pasut, G., Amato, G., Scialabba, C., Mero, A., Montopoli, M., Cavallaro, G., Schiavon, O., Giammona, G., 2013a. PHEA-graft-polymethacrylate supramolecular aggregates for protein oral delivery. *Eur. J. Pharm. Biopharm.: Off. J. Arbeitsgemeinschaft fur Pharmazeutische Verfahrenstechnik e.V.* 84, 21–28.
- Licciardi, M., Pittarresi, G., Cavallaro, G., Giammona, G., 2013b. Nanoaggregates based on new poly-hydroxyethyl-aspartamide copolymers for oral insulin absorption. *Mol. Pharm.* 10, 1644–1654.
- Linhart, B., Hartl, A., Jahn-Schmid, B., Verdino, P., Keller, W., Krauth, M.T., Valent, P., Horak, F., Wiedermann, U., Thalhammer, J., Ebner, C., Kraft, D., Valenta, R., 2005. A hybrid molecule resembling the epitope spectrum of grass pollen for allergy vaccination. *J. Allergy Clin. Immunol.* 115, 1010–1016.
- Linhart, B., Jahn-Schmid, B., Verdino, P., Keller, W., Ebner, C., Kraft, D., Valenta, R., 2002. Combination vaccines for the treatment of grass pollen allergy consisting of genetically engineered hybrid molecules with increased immunogenicity. *FASEB J.* 16, 1301–1303.
- McGowan, E.C., Saini, S., 2013. Update on the performance and application of basophil activation tests. *Curr. Allergy Asthma Rep.* 13, 101–109.
- Mendichi, R., Giacometti Schieroni, A., Cavallaro, G., Licciardi, M., Giammona, G., 2003. Molecular characterization of poly(N-2-hydroxyethyl)-D,L-aspartamide derivatives as potential self-assembling copolymers forming polymeric micelles. *Polymer* 44, 4871–4879.
- Orlandi, A., Grasso, F., Corinti, S., Marinaro, M., Bonura, A., Boirivanti, M., Colombo, P., Di Felice, G., 2004. The recombinant major allergen of *Parietaria judaica* and its hypoallergenic variant: *in vivo* evaluation in a murine model of allergic sensitization. *Clin. Exp. Allergy* 34, 470–477.
- Pauli, G., Larsen, T.H., Rak, S., Horak, F., Pastorello, E., Valenta, R., Purohit, A., Arvidsson, M., Kavina, A., Schroeder, J.W., Mothes, N., Spitzauer, S., Montagut, A., Galvain, S., Melac, M., Andre, C., Poulsen, L.K., Malling, H.J., 2008. Efficacy of recombinant birch pollen vaccine for the treatment of birch-allergic rhinoconjunctivitis. *J. Allergy Clin. Immunol.* 122, 951–960.
- Siracusa, M.C., Kim, B.S., Spergel, J.M., Artis, D., 2013. Basophils and allergic inflammation. *J. Allergy Clin. Immunol.* 132, 789–801, quiz 788.
- Stumvoll, S., Westritschnig, K., Lidholm, J., Spitzauer, S., Colombo, P., Duro, G., Kraft, D., Geraci, D., Valenta, R., 2003. Identification of cross-reactive and genuine *Parietaria judaica* pollen allergens. *J. Allergy Clin. Immunol.* 111, 974–979.