



Encapsulation of hydrophobic allergens into nanoparticles improves the *in vitro* immunological diagnosis of allergic contact dermatitis

Angèle Cortial, MD^{a,b,c,d,e,f}, Aurore Rozières, PhD^{b,c,d,e,f}, Marie Baeck^g,
Laurence De Montjoye^g, Sophie Grande^h, Stéphanie Briançon^a,
Jean-François Nicolas^{b,c,d,e,f,h}, Marc Vocanson, PhD^{b,c,d,e,f,*}

^aUMR CNRS 5007, Laboratoire d'Automatique et de Génie des Procédés, Université Lyon1, Lyon, France

^bCIRI, International Center for Infectiology Research, Université de Lyon, Lyon, France

^cInserm, U1111, Lyon, France

^dEcole Normale Supérieure de Lyon, Lyon, France

^eUniversité Lyon 1, Centre International de Recherche en Infectologie, Lyon, France

^fCNRS, UMR 5308, Lyon, France

^gCliniques Universitaires Saint-Luc, Belgique, Université Catholique de Louvain, Brussels, Belgium

^hAllergology & Clinical Immunology, CH Lyon-Sud, Pierre-Bénite, France

Received 29 September 2014; accepted 6 February 2015

Abstract

The diagnosis of allergic contact dermatitis (ACD) relies on *in vivo* patch testing. *In vitro* immunological assays based on the characterization of circulating allergen-specific memory T cells represent a promising alternative to patch testing. However, their development is hampered by the technical challenge of assessing hydrophobic allergens in serum-based assays. In this study, we show that the encapsulation of fragrance mix 1 (FMI, a mixture of 8 hydrophobic allergens) into poly-2-caprolactone nanoparticle (NP) vectors: (1) dramatically increases the solubilization of allergens in conventional cell culture media and (2) allows for a robust *in vitro* reactivation of allergen-specific T cells in large numbers of fragrance allergic patients. Therefore, the encapsulation of hydrophobic allergens into NP vectors opens new avenues to improve the *in vitro* immunobiological diagnosis of ACD.

© 2015 Published by Elsevier Inc.

Key words: Allergic contact dermatitis; Diagnosis; Immunobiological assays; Fragrances; Poly-ε-caprolactone nanoparticles

Introduction

Allergic contact dermatitis (ACD) is the most prevalent skin inflammatory disease in many European countries.^{1,2} ACD is induced by the repeated contact of individuals with ubiquitous chemical allergens, called haptens. ACD is a delayed-type hypersensitivity reaction and is mediated by the recruitment and activation into the skin of allergen-specific effector T cells (Teff).³

Abbreviations: ACD, Allergic contact dermatitis; EBS, European Baseline Series; FMI, Fragrance mix 1; GC, Gas chromatography; mAb, Monoclonal antibody; NP, Nanoparticle; PBMC, Peripheral blood mononuclear cells; PT, Patch tests; RPMIc, Complete RPMI medium; SI, Stimulation indices; Teff, Effector T cells.

Author Disclosure Statement: The authors declare no competing financial/statement interests.

*Corresponding author at: CIRI-INSERM U1111, Lyon, France.

E-mail address: marc.vocanson@inserm.fr (M. Vocanson).

Currently the diagnosis of ACD relies on clinical investigations by patch testing with suspected haptens. The patch test (PT) method aims at reproducing the ACD lesions in sensitized patients by applying occlusive patches containing the suspected allergens to the patient's healthy skin.⁴

Immunobiological assays based on the detection of allergen-specific Teff cells (and/or Teff cell products) circulating into the blood of allergic patients proved a valuable alternative to PT. Previously, promising results were obtained with hydrophilic compounds, such as metals or dyes.^{5–7} Nevertheless, so far the development of such *in vitro* approaches was hampered by the lack of solubility into conventional cell culture media of the majority of allergens that are responsible for ACD; which is key to restimulate blood leucocytes collected from suspected patients.

In this study, we capitalized on the unique properties of poly-ε-caprolactone nanoparticles (NPs) to improve the solubilization of fragrance mix 1, a mixture of 8 hydrophobic fragrance

Table 1

Clinical characteristics of patients and their response to FMI patch testing.

Patient number	Sex/age	Clinical symptoms	FMI PT result
1	F/59	Generalized eczema	+++
2	M/61	Generalized eczema	+++
3	F/58	Face and generalized eczema	++
4	F/34	Face and neck eczema	++
5	F/50	Face and neck eczema	++
6	F/51	Hand eczema	++
7	F/55	Glossodynia	++
8	F/51	Face and eyelid eczema	++
9	F/51	Eczema	++
10	F/60	Hand and forearm eczema	++
11	M/61	Hand eczema	++
12	M/64	Generalized eczema	++
13	F/42	Generalized eczema	++
14	M/79	Hand eczema	++
15	M/74	Eyelid eczema	+
16	F/49	Hand eczema	+
17	F/52	Face eczema	+
18	F/37	Forehead eczema	+
19	F/57	Eyelid eczema	+
20	F/70	Eczema	+
21	F/61	Angular stomatitis/cheilitis, diffuse eczema	–
22	F/40	Facial erythema, scalp seborrheic dermatitis, friction hives	–
23	F/70	Leg and forearm eczema	–
24	F/32	Eczema	–
25	F/42	Eczema	–
26	F/72	Erysipelas	–
27	M/39	Atopic dermatitis	–
28	F/38	Hand eczema	–
29	M/72	Hand eczema	–
30	F/24	Atopic dermatitis	–
31	F/73	Eczema	–

F, female; M, male; PT, patch test. PT lesions were evaluated according to the ICDRG scoring: negative reaction (–); weak (non-vesicular) positive allergic reaction: erythema, infiltration and possibly papules (+); strong (vesicular) positive allergic reaction: erythema, infiltration, papules and vesicles (++); extreme positive allergic reaction; bullous reaction (+++).

allergens, into cell culture media, and to demonstrate the efficacy of immunobiological assays for the diagnosis of ACD to fragrances.

Methods

See Supplemental Methods for complete information.

Preparation of fragrance mix I-loaded nanoparticles

FMI-loaded NPs were prepared using a nanoprecipitation process.^{8,9} Free, i.e. unloaded NPs were also prepared. NP mean size and size distribution were determined by light scattering measurements. The concentration of each fragrance ingredient loaded into NPs was assessed using gas chromatography (GC).

Solubilization assay into cell culture media

FMI preparations (as allergen-loaded NP suspensions or free allergen solutions) were mixed into complete RPMI medium (RPMIc). Fractions, collected 15 min after introduction and

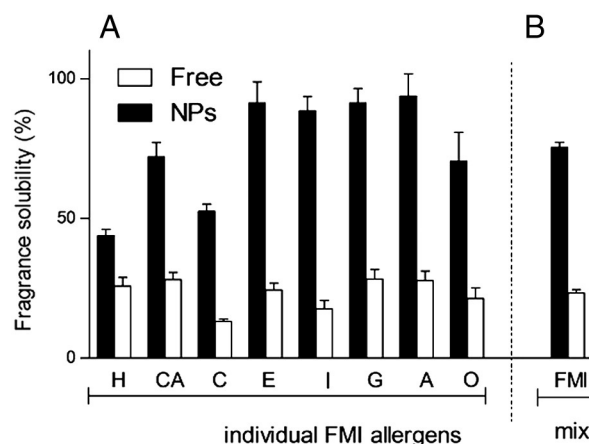


Figure 1. Increased solubilization of allergens and FMI into RPMI when encapsulated into poly-ε-caprolactone nanoparticles (NPs). (A) Percentage of the 8 individual fragrance allergens encapsulated in NPs versus free allergen, solubilized into RPMIc at 37 °C, measured by GC analysis and (B) determination of the solubility of the mix of the 8 allergens (FMI). Results are the mean of 6 measurements of 3 samples. H: hydroxycitronellal, CA: cinnamyl alcohol, C: cinnamal, E: eugenol, I: isoeugenol, G: geraniol, A: (α)-amylcinnamaldehyde, O: oak-moss, FMI: fragrance mix I. See details of solubility calculations in Supplementary Table S3.

mixing, were dissolved in acetone and next analyzed by GC. The percentage of FMI or individual FMI ingredients solubilized into RPMIc was determined as: fragrance concentrations titrated by GC into fractions/fragrance concentrations into FMI preparations (also validated by GC).

Clinical study

A prospective clinical study was conducted to obtain PBMC from FMI-sensitized or non-sensitized subjects. Therein, 31 patients with clinical history of eczema were patch-tested with the European Baseline Series (EBS) (comprising FMI). Blood was collected before PT and PBMC were purified using standard Ficoll density gradient. The characteristics of each individual and PT results are reported in Table 1.

Immunobiological assays for the in vitro diagnosis of ACD

PBMC were cultured with graded amounts of FMI-loaded or free NPs for 5 days. Final concentrations of FMI introduced in each culture varied from 0.01 to 250 µg/mL.

Secondary T cell proliferation and cytokine secretion into culture supernatant were evaluated by radioisotope incorporation and a multiplex ELISA assay respectively.

Results

NP encapsulation of FMI allergens increases their solubility in culture medium

FMI, composed of 8 hydrophobic allergens (cinnamyl alcohol, cinnamal, hydroxycitronellal, amyl cinnamal, geraniol, eugenol, isoeugenol and oak-moss absolute), was encapsulated into poly-ε-caprolactone NP vectors according to a solvent displacement method. This nanoprecipitation process was

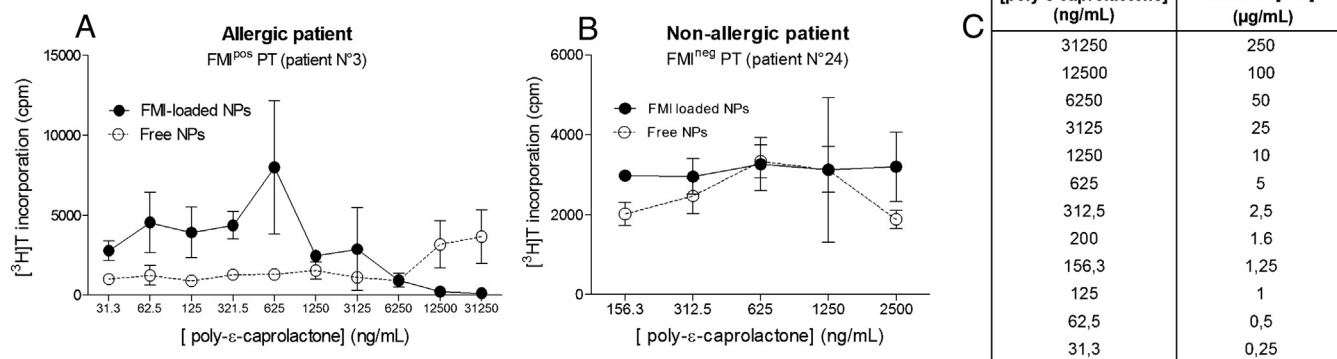


Figure 2. *In vitro* T cell response to FMI. Dose-dependant lympho-proliferation recorded in 2 patients representative of the cohort. PBMC (2×10^5) of 2 patients with FMI^{pos}PT (patient 3) (A) or FMI^{neg}PT (patient 24) (B) were cultured for 5 days with graded doses of FMI-loaded or free NPs (indicated as poly-ε-caprolactone concentrations). Proliferative responses were assessed by [³H]thymidine incorporation for the last 15 hrs of culture. Results represented mean value $\pm$ SD. (C) Correlations between the concentrations of ε-caprolactone polymers in FMI-loaded NP suspensions and the relative FMI concentrations added into cultures.

optimized to obtain NPs with mean size <200 nm and polydispersity index <0.15. The global encapsulation efficiency proved >50%, but varied for each FMI individual ingredient, ranging from 10% (hydroxycitronellal) to >90% (oak-moss) (Cortial *et al*, manuscript submitted).

In contrast to free hydrophobic allergens, which formed a creaming or sedimentation layer when dissolved into conventional cell culture medium, allergen-loaded NPs were homogeneously dispersed due to brownian motion. We then measured the solubilization rate of free- and encapsulated-FMI (or FMI individual ingredients) into RMPic. Solubility percentages at 37 °C, measured by GC, ranged from 40% to >90% when encapsulated into NP vectors, while it proved <30% for all the free compounds (Figure 1).

NP-encapsulated FMI activates allergen-specific T cells *in vitro*

We next assessed the interest of NP encapsulation of FMI for *in vitro* immunological diagnosis of ACD to fragrances. We enrolled a cohort of 31 patients with clinical history of ACD that were patch-tested with FMI and other contact allergens of the EBS. PBMCs were recovered from patients with positive (n = 20) and negative FMI PT (n = 11) and cultured for 5 days with graded doses of FMI-loaded and free NPs. Reactivation of FMI-specific memory T cells was assessed by: (1) proliferation assays using [³H]thymidine incorporation; (2) cytokine secretion assays in the culture supernatant using multiplex ELISA.

FMI-loaded, but not free NPs, induced a significant and dose-dependent T cell proliferation in 10/20 patients with positive FMI PT (FMI^{pos}PT) (Figures 2 and 3, Supplementary Table S1). By contrast, no proliferation was recorded in patients with negative PT (FMI^{neg}PT) (Figures 2 and 3, Supplementary Table S1). We recorded mainly type-1 (IFN-γ, TNF-α) cytokine secretion in FMI^{pos}PT, and to a lesser extent type-2 (IL-5) cytokine secretion. IL-4 or IL-17A secretions were recorded rarely in patients (Figure 3, Supplementary Table S2). Of note, a clear correlation between proliferation and cytokine secretion was observed.

Discussion

In this study, we report that NPs are an innovative solution to improve the solubilization of hydrophobic molecules in culture medium allowing the characterization of circulating allergen-specific T cells in ACD to fragrances. We showed that poly-ε-caprolactone NPs significantly limit molecule partitioning and enhance their solubility into conventional culture media. As a result, we observed that molecule uptake by PBMC subsets (monocytes, B cells and T cells) was significantly augmented when using NP vectors versus free molecules (Supplementary Figures S1 and S2). This explains why memory T cells were efficiently stimulated *in vitro*. In other work, we reported that improved chemical uptake using NP vectors significantly boosted the maturation and stimulatory properties of monocyte-derived dendritic cells that are used for the development of a primary sensitization assay to screen chemical allergens (Achachi *et al*, manuscript submitted).

The results described here address the sensitivity of the immunological assays used for the diagnosis of ACD to fragrances, as half of the patients with positive PT to FMI were negative in the immunological assay. Several reasons can explain the discrepancy between the positive skin PT and the negative immunological assays:

1. Misinterpretation of PT readings; the PT results may be false positives. Indeed, chemicals applied under occlusion for 48 hrs can sometimes cause non-allergic irritant contact dermatitis. Such reactions are often difficult to distinguish from ACD by visual analysis only¹⁰;
2. Insufficient quantities of FMI solubilized into culture and accessible to T cells. FMI is a mixture of 8 compounds and their encapsulation rate into NPs varies depending on their physicochemical properties (Cortial *et al*, manuscript submitted). Hence, depending on the molecules to which patients are sensitized, the amount of the respective chemical solubilized into *in vitro* cultures may be too

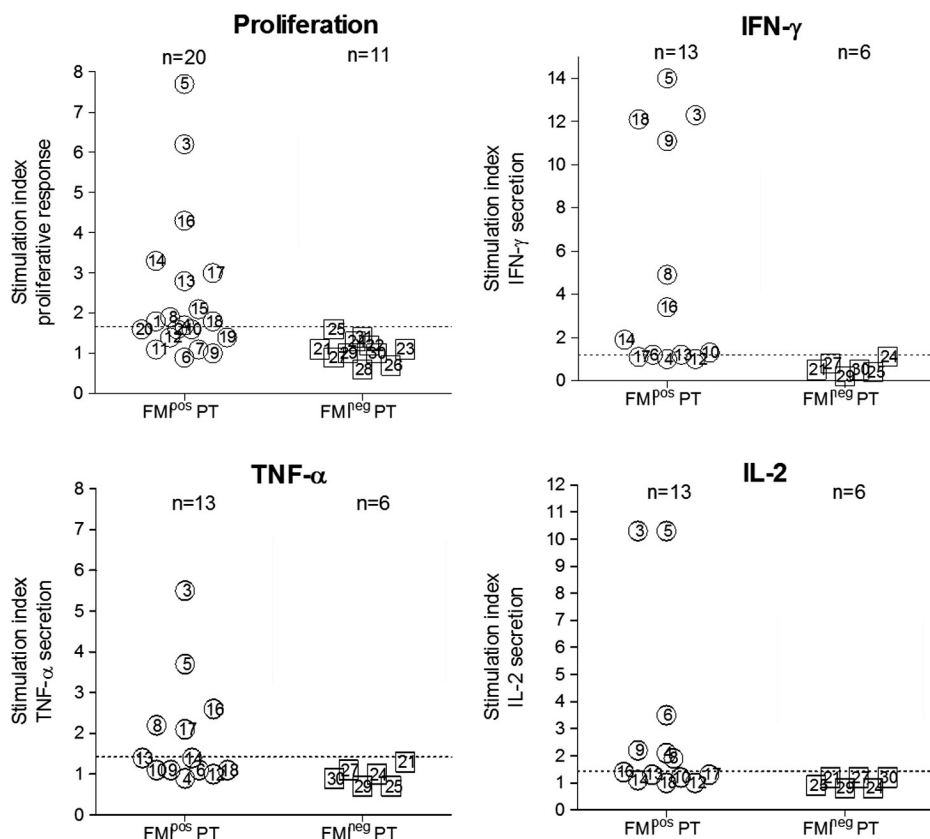


Figure 3. *In vitro* T cell response to FMI. Maximal stimulation indices for proliferation and cytokine secretion readouts. PBMC (2×10^5) of patients with FMI^{pos}PT or FMI^{neg}PT were cultured for 5 days with graded doses of FMI-loaded or free NPs. Stimulation indices (SI) were calculated by dividing values recorded in FMI-loaded NP cultures to values recorded into control free NP cultures, at respective concentrations of poly- ϵ -caprolactone. Results depict maximal SI per individual for both proliferation (20 FMI^{pos}PT and 11 FMI^{neg}PT patients) and cytokine (IFN- γ , TNF- α , IL-2) (13 FMI^{pos}PT and 6 FMI^{neg}PT patients) secretion readouts. Results were considered positive when SI exceeded a threshold value corresponding to “the mean of SI for the FMI^{neg}PT group + 2 $\times$ SD” (dashed line).

low to achieve an effect. Additionally, increasing the dose of FMI also increases toxicity, so the dose necessary to stimulate allergen-specific T cells in the immunological assays may not have been reached.

To overcome this problem, we are currently improving the engineering of poly- ϵ -caprolactone NP vectors to reach an encapsulation rate of >90% for all FMI ingredients. It will be interesting to determine whether these new NP vectors further improve *in vitro* T cell responses to FMI.

In conclusion, the present study suggests that the encapsulation of hydrophobic allergens/mixtures into NP vectors represents a promising approach to improve the immunobiological diagnosis of ACD, offering a valuable and complementary *in vitro* alternative to *in vivo* patch testing.

Q4 Acknowledgments

We thank C. F. Gaudefroy (Unilever R&D, Vlaardingen, Netherlands), N. Gilmour (SEAC, Unilever R&D, Colworth, United Kingdom), M. Elbaz (Chanel, Neuilly-sur-seine, France), G. Ellis (Givaudan, Vernier, Switzerland), B. Smith (Firmenich,

Meyrin, Switzerland) and D. Tennstedt (Cliniques Universitaires Saint-Luc, Belgique) for their support in the realization of this work.

We also thank T. Andrieu and S. Dussurgey (Plateau technique de cytométrie en flux, UMS3444/US8 BioSciences Gerland – Lyon Sud) for their assistance with ImageStream.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.nano.2015.02.001>.

References

- Peiser M, Tralau T, Heidler J, Api AM, Arts JH, Basketter DA, et al. Allergic contact dermatitis: epidemiology, molecular mechanisms, in vitro methods and regulatory aspects. Current knowledge assembled at an international workshop at BfR, Germany. *Cell Mol Life Sci* 2012;69:763–81.
- Diepgen TL. Occupational skin-disease data in Europe. *Int Arch Occup Environ Health* 2003;76:331–8.
- Vocanson M, Hennino A, Rozieres A, Poyet G, Nicolas JF. Effector and regulatory mechanisms in allergic contact dermatitis. *Allergy* 2009;64:1699–714.

- 202 4. Mahler V, Geier J, Schnuch A. Current trends in patch testing - new data
203 from the German Contact Dermatitis Research Group (DKG) and the
204 Information Network of Departments of Dermatology (IVDK). *J Dtsch*
205 *Dermatol Ges* 2014;**12**:583-92.
- 206 5. Summer B, Paul C, Mazoochian F, Rau C, Thomsen M, Banke I, et al.
207 Nickel (Ni) allergic patients with complications to Ni containing joint
208 replacement show preferential IL-17 type reactivity to Ni. *Contact*
209 *Dermatitis* 2010;**63**:15-22.
- 210 6. Kneilling M, Caroli U, Grimm C, Fischer J, Eichner M, Wieder T, et al.
211 Para-phenylenediamine-specific lymphocyte activation test: a sensitive
212 in vitro assay to detect para-phenylenediamine sensitization in patients
213 with severe allergic reactions. *Exp Dermatol* 2010;**19**:435-41.
- 214 7. Coulter EM, Jenkinson C, Farrell J, Lavergne SN, Pease C, White A,
215 et al. Measurement of CD4+ and CD8+ T-lymphocyte cytokine
216 secretion and gene expression changes in p-phenylenediamine
217 allergic patients and tolerant individuals. *J Invest Dermatol*
218 2010;**130**:161-74.
- 219 8. D'Addio SM, Prud'homme RK. Controlling drug nanoparticle formation
220 by rapid precipitation. *Adv Drug Deliv Rev* 2011;**63**:417-26.
- 221 9. Fessi H, Puisieux F, Devissaguet JP, Ammoury N, Benita S.
222 Nanocapsule formation by interfacial polymer deposition following
223 solvent displacement. *Int J Pharm* 1989;**55**:R1-4.
- 224 10. Nosbaum A, Vocanson M, Rozieres A, Hennino A, Nicolas JF. Allergic
225 and irritant contact dermatitis. *Eur J Dermatol* 2009;**19**:325-32.
226



Graphical Abstract

Encapsulation of hydrophobic allergens into nanoparticles improves the *in vitro* immunological diagnosis of allergic contact dermatitis

Angèle Cortial, MD^{a,b,c,d,e,f}, Aurore Rozières, PhD^{b,c,d,e,f}, Marie Baeck^g, Laurence De Montjoye^g,
Sophie Grande^h, Stéphanie Briançon^a, Jean-François Nicolas^{b,c,d,e,f,h}, Marc Vocanson, PhD^{b,c,d,e,f,*}

^aUMR CNRS 5007, Laboratoire d'Automatique et de Génie des Procédés, Université Lyon1, Lyon, France

^bCIRI, International Center for Infectiology Research, Université de Lyon, Lyon, France

^cInserm, U1111, Lyon, France

^dEcole Normale Supérieure de Lyon, Lyon, France

^eUniversité Lyon 1, Centre International de Recherche en Infectiologie, Lyon, France

^fCNRS, UMR 5308, Lyon, France

^gCliniques Universitaires Saint-Luc, Belgique, Université Catholique de Louvain, Brussels, Belgium

^hAllergology & Clinical Immunology, CH Lyon-Sud, Pierre-Bénite, France

FMI-loaded NPs allow for the *in vitro* immunobiological diagnosis of allergic contact dermatitis to fragrances.

Nanomedicine: Nanotechnology, Biology, and Medicine xxx (2015) xxx–xxx

