Lipid nanoparticles for enhancing immune responses to protein antigens

Thomas Courant[*], Hei Lanne Reynaud[**], Antoine Hoang[*], Christian Villiers[**], Frédérique Mittler[*], Isabelle Texier[*], Patrice Marche[**] and Fabrice P. Navarro[*]

[*]CEA, LETI MINATEC, Technologies for Healthcare and Biology Division, 17 rue des martyrs, 38054 Grenoble Cedex 9, France; fabrice.navarro@cea.fr

[**] Institut Albert Bonniot - UJF/INSERM U823, Rond-point de la Chantourne, 38042 Grenoble Cedex 9, France.

ABSTRACT

Nanotechnology presents a great potential for applications in the vaccine field through the controlled manufacturing of synthetic vectors of antigens in the size range of virus. Here, we have developed an original vaccine formulation based-on the grafting of protein antigens onto the surface of lipid nanoemulsions (Lipidots®). Maleimide-functionalized antigen proteins were efficiently grafted on thiol functionalized lipid nanoparticles. These antigens-bearing particles are stable several months at 4°C with marginal protein release over this period. They are very well tolerated *in vitro* and allow the enhancement of the immune responses in mice model using ovalbumin as antigen.

Keywords: biotechnology, lipid nanoparticles, protein delivery, vaccine, immunization.

1 INTRODUCTION

In the near future, novel vaccines will be needed due to the evolution of the society with the increase of elderly and their ageing immune system, the increase of multi-drug resistant strains of pathogens, and also the global warming with the associated spread of tropical diseases in new locations [1]. In parallel, nanotechnology is an emerging field which opens new avenues in medicine for developing innovative diagnosis and/or therapies. In particular, nanoparticles-based vaccine formulations may fight against the spread of infectious diseases where the pathogen is located into the intracellular compartment in host cells or against cancers where a cellular immunity is likely more adapted for reducing tumor growth and metastasis. Moreover, nanoparticles offer the possibilities to combine into the same carrier several kinds of molecules as contrast agents, drugs, proteins, adjuvants or antigens.

Various exemples of protein association with liposomes, solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) can be found in literrature [2, 3]. These carriers have several drawbacks, in particular stability of particles and uncontrolled release of proteins. Furthermore, small sizes (<200 nm) are difficult to reach while keeping a good protein payload. In this context, we have designed a new technology, based on very stable lipid nanoparticles previously described here[4], to vectorize protein antigens with lipid nanoparticles. To overcome release issues, we have decided to covalently graft protein antigens on particle surface. The designed antigen-bearing lipid nanoparticles have been characterized for their physico-chemical properties, their cytotoxicity and their ability to adress antigens to immune system.

2 RESULTS AND DISCUSSION

Lipid nanoemulsions (Lipidots®) are composed of a lipid core, mixture of soybean oil and a wax at different ratios, and a surfactant shell, mixture of phospholipids and PEGylated surfactants (see Fig. 1). Their ingredients are bioabsorbable, since most of them enter natural lipid metabolism, and are biocompatible, as they are already FDA approved for human-use. Thiol-functionalized PEGylated surfactants have been synthetised and incorporated in the formulation to allow the chemical grafting of proteins on the nanoparticle surface via thiol-maleimide chemistry.

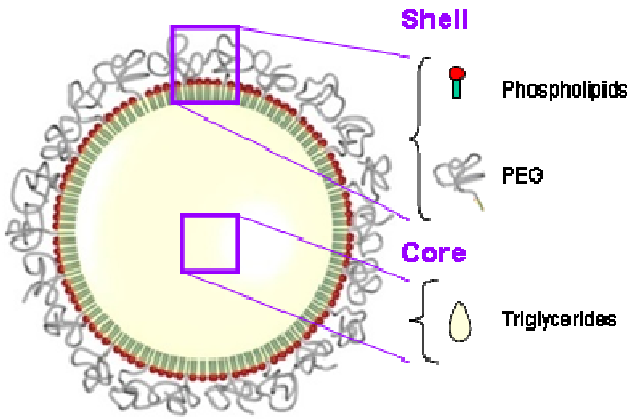

Figure 1: Schematic representation of Lipidots®.

A model protein, namely ovalbumin, was functionalized to introduce maleimide groups and fluorescent dyes in its structure. Optimization allowed the obtention of maleimide mono-functionalized fluorescent ovalbumin. This protein was efficiently grafted on lipid nanoparticles as determined

by fluorescence (coupling yields < 60 %) and unbounded proteins were efficiently removed by gel filtration chromatography (SEC, see Fig. 2).

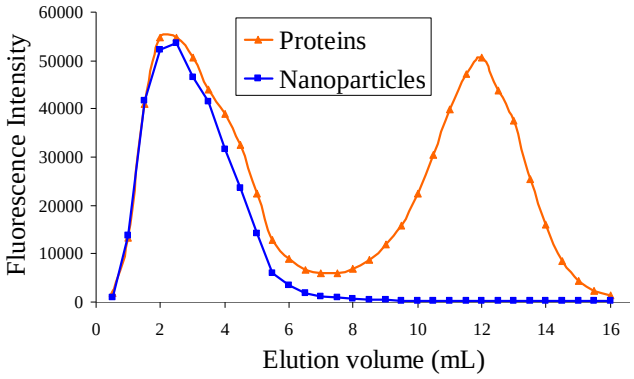

Figure 2: Example of SEC profile obtained for fluorescent-proteins bearing Lipidots.

Absence of unbounded ovalbumin in purified nanoparticle fractions was furthermore analysed by SDS-PAGE with Coomassie blue coloration (see Fig. 3). As control, ungrafted Lipidots® showed no protein signals. Protein smir was observed with Ova-grafted particles but no ovalbumin caracteristic bands were observed, thus confirming the efficiency of grafting and purification.

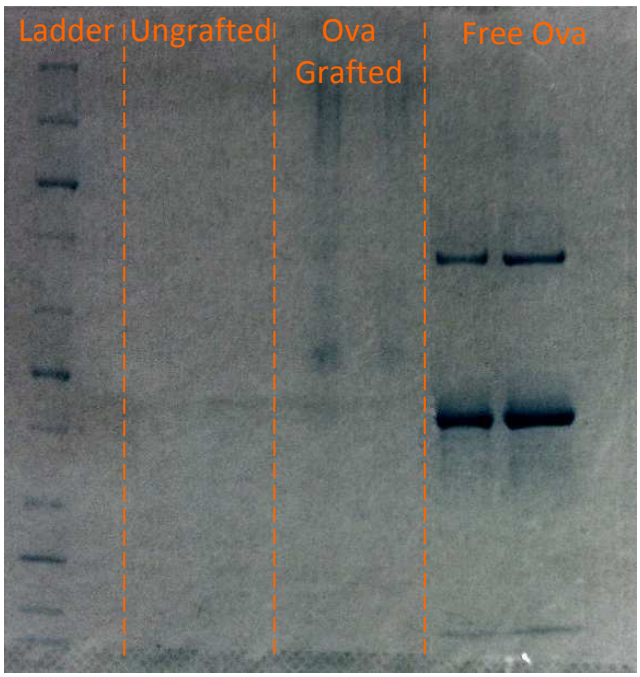

Figure 3: SDS-PAGE analysis.

By varying the amount of thiol bearing PEGylated surfactant incorporated in the formulation, ovalbumin content of the lipid nanoparticles can be tuned from several decades up to 400 proteins per particle. This leads to a maximum ovalbumin loading of 2.9 ± 0.2 % (m/m).

Dynamic Light Scattering (DLS) is used to measure the particle hydrodynamic diameter in PBS or saline dispersions, as well as their time-evolution during storage. The mean diameters of Ova-Lipidots® ranged from 120 nm to 160 nm for particles with highest ovalbumin loading. Protein grafting does not affect the global charge of particles. Ova-Lipidots® keep a slightly negative zeta potential of -8 mV. No significant changes in particle diameter were observed upon 10 weeks of storage at 4°C (Fig. 4). Upon this period of time, no release of ovalbumin was observed by SEC.

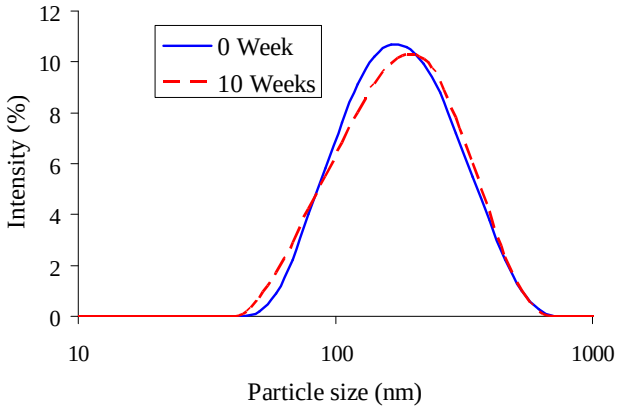

Figure 4: Stability of Ova bearing Lipidots™ at 4°C.

Lipidots® cytotoxicity is assessed by WST-1 assay using NIH 3T3 murine fibroblasts (see Fig 5). Lipidots® without functionalized surfactants and Lipidots® with neutralized surfactants were used as control. Independently of the surface state, lipid nanoparticles display *in vitro* cytotoxicity pattern with an IC_{50} ≈ 1500 µg/mL. It demonstrates that functionalization does not affect the high cellular tolerance of Lipidots®.

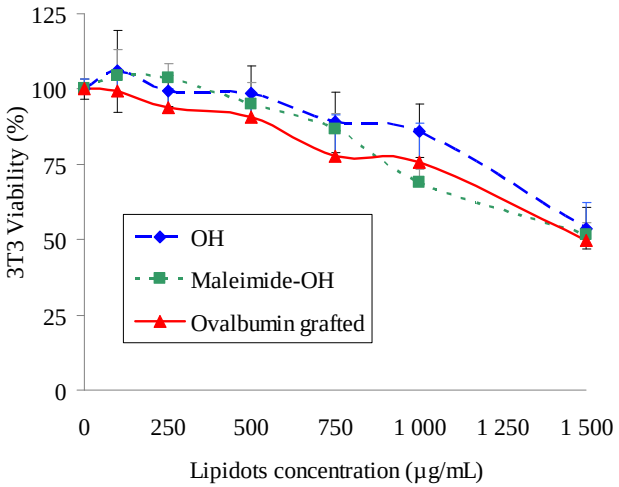

Figure 5: Lipidots® cytotoxicity after 24 hours incubation at 37°C in the presence of 3T3 fibroblasts.

At last, preliminary experiments of *in vivo* immunization in mice were performed using ovalbumin as antigen model. Results seem to indicate an enhancement of immune responses when ovalbumin is associated to lipid nanoparticle formulation compared to free ovalbumin (Figure 6).

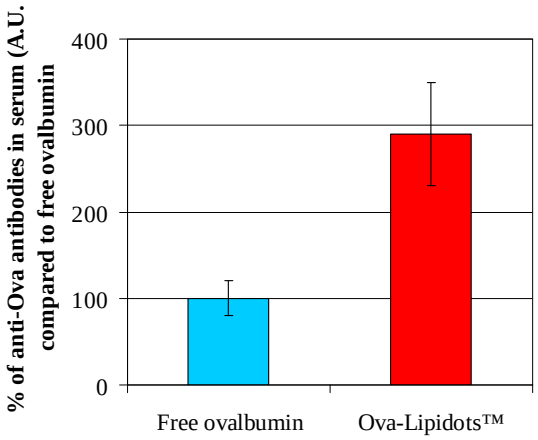

Figure 6: Anti-OVA antibodies titration in mice 28 days after Ova-Lipidots® or free Ova intraperitoneal administration.

3 EXPERIMENTAL SECTION

3.1 Lipidots® synthesis

Fluorescent lipid nanoparticles were prepared and purified as previously described [5]. Typically, a lipid phase containing solid and liquid glycerides with phospholipids and fluorescent dye is emulsified with an aqueous solution of PEG surfactant and thiol-functionalized PEG surfactant in PBS aqueous buffer. Non encapsulated components are separated by overnight dialysis (MWCO: 12 kDa). The amount of each component is adjusted depending on the desired size of nanoemulsion and quantity of thiol functions. The size and size distribution of the prepared nanoparticles were determined by means of dynamic light scattering (DLS) using a Malvern Zetasizer, Nano-ZS (Malvern Instruments, Malvern, UK).

3.2 Proteins coupling

The proteins were functionalized with maleimide groups and fluorophores by the use of Sulfo-SMCC (Sulfosuccinimidyl-4-(Nmaleimidomethyl) cyclohexane-1-carboxylate) and fluoresceine N-hydroxysuccinimide ester. Different ratios of proteins to nanoparticles were used depending of the desired functionalization. Unbounded proteins were separated from functionalized nanoemulsions by size exclusion chromatography on Superdex® 200.

3.3 Cytotoxicity studies

NIH-3T3 murine fibroblast cells (ATCC) are cultured under a humidified (90%) atmosphere of 95% air/ 5 % CO_2 at 37 °C, in Dulbecco's modified Eagle's medium high glucose supplemented with 10% newborn calf serum and 1% penicillin and streptomycin. 5.10^4 cells/ well are incubated for 24 hours at 37 °C, previous to addition of different amounts of nanoparticles. Cytotoxicity is assessed 24 hours after the nanoparticle treatment followed by two washes with cell culture medium, using WST-1 assay.

3.4 *In vivo* immunization

Immunization experiments were performed on BALB/C mice (6/8 weeks old). The mice were divided in 2 groups of 5 animals each. At day 0, groups 1 and 2 were respectively immunized i.p. with 50 µg ovalbumin in PBS and 50 µg ovalbumin equivalent Ova-Lipidots® in PBS. Second injection (booster) was administered at day 21. Animals were sacrificed at day 28. Sera were collected and anti-Ova antibodies were analysed by ELISA.

REFERENCES

[1] R. Rappuoli, C. W. Mandl, S. Black and E. De Gregorio, Nature Reviews Immunology, 11, 865, 2011.

[2] A. J. Almeida and E. Souto, Advanced Drug Delivery Reviews, 59, 478, 2007..

[3] S. Martins, B. Sarmento, D. C. Ferreira and E. B. Souto, International Journal of Nanomedicine, 2(4), 595, 2007.

[4] T. Delmas, H. Piraux, A.C. Couffin, I. Texier, F. Vinet and P. Poulin, Langmuir, 27, 1683, 2011.

[5] M. Goutayer, S. Dufort, V. Josserand, A. Royère, E. Heinrich and F. Vinet, European Journal of Pharmaceutics and Biopharmaceutics, 75, 137, 2010.