Nanostructured Lipid Carriers As Delivery Systems Of Chemically Grafted Protein Antigens

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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 designed a novel vaccine formulation based on lipid nanoemulsions (Lipidots®) carrying protein antigens onto their surface. Ovalbumin, a model antigen, and P24, a protein from Human Immunodeficiency Virus capside, have been chemically modified to be efficiently grafted onto thiol or maleimide-functionalized lipid nanoparticles. The resulted nanoformulations are stable several months at 4°C with no protein release over this period. They are very well tolerated by dendritic cells *in vitro* and allow the enhancement of immune responses. The production of antigen-specific antibodies and activation of cellular immunity have been assessed on mice immunized with ovalbumin and P24-grafted nanoparticles.

Keywords: lipid nanoparticle, protein delivery, immune responses, vaccine, biotechnology

1 INTRODUCTION

Vaccine development is one of the major health advances of the last century. However, new vaccine formulations are still highly anticipated in the near future for facing some health challenges, such as the arrival of new infectious agents or resistant strains, the increase of chronic inflammatory and childhood diseases, the spread of tropical diseases as a result of the global warming and the higher number of elderly whose immune system is getting weaker [1].

Despite the great progress in the field of vaccines, there are still some deadly diseases which are out of control. One of them is caused by Human Immunodeficiency Virus (HIV) which is responsible for more than 30 million deaths until now and keeps infecting new people [2]. The reason why it is so difficult to fight the virus comes from three of its main characteristics. First of all, HIV is constituted of circulating virions which have the ability to infect cells and hide inside them, where they can replicate. Thus, the production of antibodies is likely not enough to overcome the virus because they will only neutralize the circulating virions while the hidden entities inside the cells will remain unaffected. A potent combination of humoral and cell-mediated immunities is indeed mandatory in order to also attack the infected cells and entirely destroy the virus. The second well-known point is that HIV has the ability to irremediably mutate, which makes it extremely complicated to develop efficient defenses. Furthermore, the cells which are preferentially infected by HIV are immune cells, especially the ones supposed to fight against it. One of the most serious options to address all these challenges is to develop a new generation of vaccines able to induce both humoral and cell-mediated immune responses of high quality.

Subunit vaccines, made of synthetic antigens, are generally considered as safer but much less immunogenic, compared to vaccines made of attenuated or killed pathogens. Therefore, they require the co-administration with an adjuvant. The use of Alum, the first approved adjuvant for human vaccines, enhances the humoral immune response but fails to induce potent cell-mediated immunity and its toxicity remains controversial. Novel immunostimulant molecules and adjuvant delivery systems are currently under development, aiming at promoting the immune responses with a better control of their safety [3,4]. For this purpose, we have designed a novel biocompatible lipid-based nanocarrier [5] for the delivery of antigens and the induction of potent immune responses.

2 MATERIALS AND METHODS

2.1 Lipidots® Synthesis

Fluorescent lipid nanoparticles were prepared and purified as previously described [6]. Typically, a lipid phase containing solid and liquid glycerides with phospholipids and a fluorescent dye is emulsified with an aqueous solution of PEG surfactants and thiol or maleimide-functionalized PEG surfactants in PBS buffer. Non encapsulated components are separated by dialysis (MWCO: 12-14 kDa). The amount of each component is adjusted depending on the desired size of nanoemulsion. 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).

2.2 Proteins Coupling

The proteins were functionalized with maleimide or thiol groups and fluorophores by the use of Sulfo-SMCC (Sulfosuccinimidyl-4-(Nmaleimidomethyl) cyclohexane-1-carboxylate) or Traut's reagent (2-iminothiolane) and fluorescein N-hydroxysuccinimide ester. Unbounded proteins were separated from functionalized nanoemulsions by size exclusion chromatography on Superdex® 200.

2.3 Cytotoxicity Studies

JAWSII murine dendritic cells (ATCC) were cultured under a humidified (90%) atmosphere of 95% air/ 5 % CO_2 at 37 °C, in alpha minimum essential medium with ribonucleosides, deoxyribonucleosides, supplemented with 20% fetal bovine serum, 4 mM L-glutamine, 1 mM sodium pyruvate, 1% penicillin and streptomycin and 5 ng/ml murine GM-CSF. 1.10^5 cells/ well were incubated with 1µg/mL lipopolysaccharide for 24 hours at 37 °C, previous to addition of different amounts of grafted and naked nanoparticles. Cytotoxicity was assessed 24 hours after the nanoparticle treatment followed by two washes with PBS and then annexinV-FITC and propidium iodide. The cells were then analyzed by flow cytometry using a BD LSRII cytometer.

2.4 *In vivo* Immunization

Immunization experiments were performed on BALB/c mice (6/8 weeks old). At day 0, mice were immunized i.p. with different nanoparticle formulations with OVA or P24 doses of respectively 10 µg or 5µg. Second injection (booster) was administered at day 21. Animals were sacrificed at day 28. Sera were collected and anti-OVA or anti-P24 antibodies were analyzed by ELISA. Spleens were extracted and splenocytes were exposed to 50µg/mL of OVA or 10µg/mL of P24 for 3 days in RPMI supplemented with 10% fetal bovine serum, 1% penicillin and streptomycin and 1% sodium pyruvate. Supernatants were collected and interferon gamma was dosed by ELISA.

3 RESULTS AND DISCUSSION

Lipid nanoemulsions (Lipidots®) are made of a lipid core, mixture of soybean oil and wax at different ratios, surrounded by a shell of phospholipids and pegylated surfactants (Fig. 1a). All ingredients are bioabsorbable, biocompatible, and FDA approved for human-use. Thiol or maleimide-functionalized PEGylated surfactants have been synthetised and incorporated in the formulation to allow the chemical grafting of different proteins on the nanoparticle surface via thiol-maleimide chemistry (Fig. 1b).

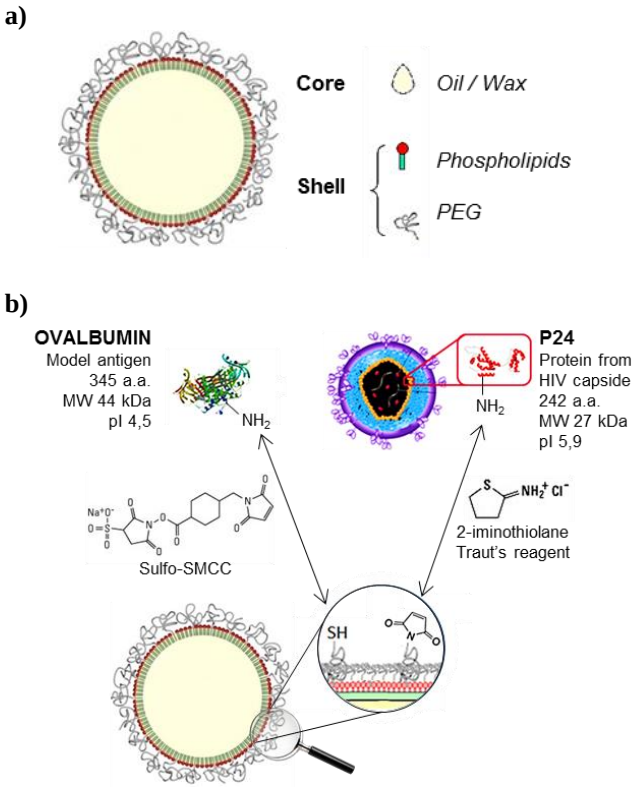
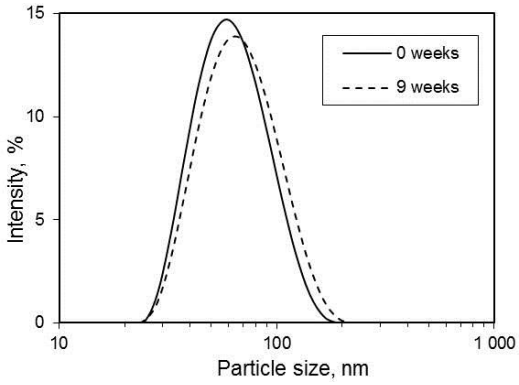


Figure 1: a) Schematic representation of Lipidots® and b) Grafting strategies of OVA and P24 proteins

The protein ovalbumin (OVA) has been chosen as a model antigen for the proof of concept of vectorizing a protein on lipid nanoparticles. Thus, it has been functionalized to introduce maleimide groups and fluorescent dyes in its structure. This protein has been efficiently grafted on Lipidots® with a 60% yield as determined by fluorescence and unbounded proteins were efficiently removed by gel filtration chromatography (GPC). In parallel, a protein from HIV capside named P24, has been treated to be functionalized with thiol groups and fluorescently labeled. This protein has the great advantage to be indifferent to the virus mutations, on the contrary to other antigens from the viral envelope. Here, the P24 antigen has been efficiently grafted onto the lipid nanoparticles, with a yield of 25%.

Dynamic Light Scattering (DLS) was used to measure the particle hydrodynamic diameter in PBS, as well as their time-evolution during storage. The mean diameters of grafted Lipidots® ranged from 80 nm to 90 nm according to the protein loading. No significant change in particle diameter was observed upon 9 weeks of storage at 4°C (Fig. 2a). Upon this period of time, no release of OVA was observed by GPC (Fig. 2b).

a)



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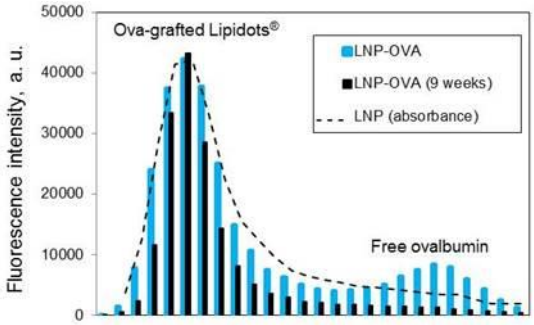


Figure 2: Stability of OVA grafted-Lipidots®
a) suspension by DLS and b) grafting by GPC.

The cytotoxicity of naked and antigen-grafted Lipidots® was assessed by exposition to JAWSII murine dendritic cells. Independently to the load, lipid nanoparticles induced no cell death after a 24-hour exposure at different significant concentrations (Fig. 3). It demonstrates that such engineering of nanoparticles for chemical grafting of proteins does not affect their safety profile.

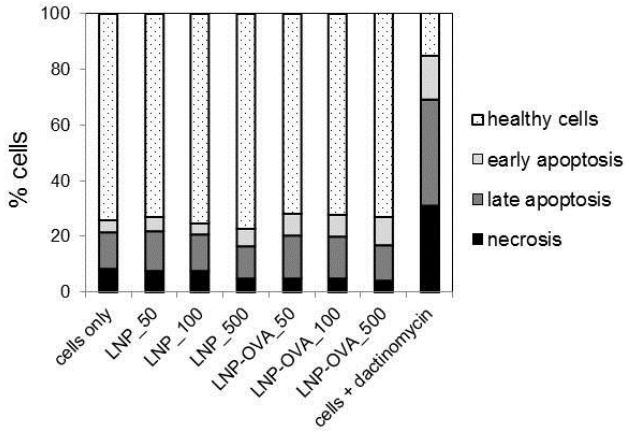
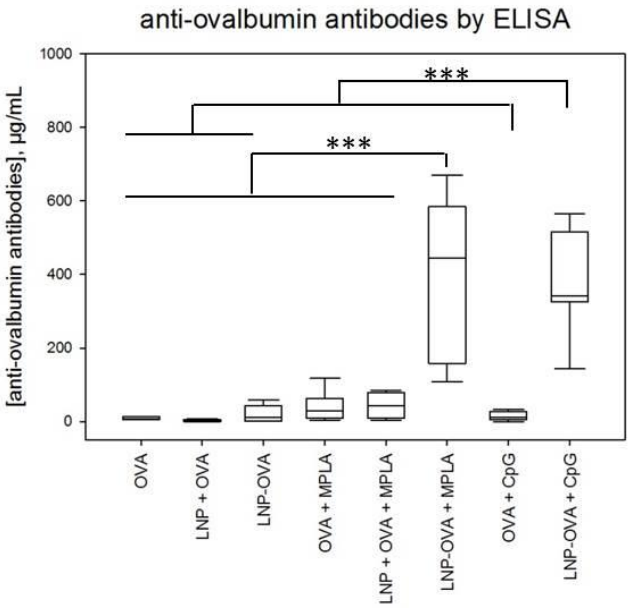


Figure 3: Naked and OVA-grafted Lipidots® cytotoxicity after a 24-hour exposure to JAWSII dendritic cells at different concentrations (50, 100 and 500 µg/mL). After staining with annexinV-FITC and propidium iodide, early, late apoptosis and necrosis were evaluated by flow cytometry.

At last, the ability of OVA and P24-grafted nanoparticles to induce immune responses was evaluated *in vivo* by immunization of mice. The vaccine formulations combined the antigen-grafted nanoparticles with co-administration with MPLA or CpG immunostimulants in order to enhance immune responses. Results are expressed in terms of production of OVA and P24-specific antibodies in the blood and secretion of gamma interferon (IFNγ) by T lymphocytes (Fig. 4).

a)



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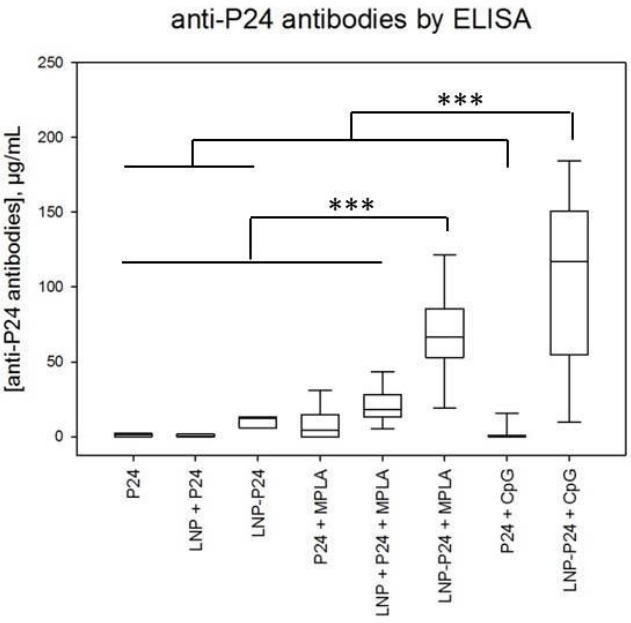

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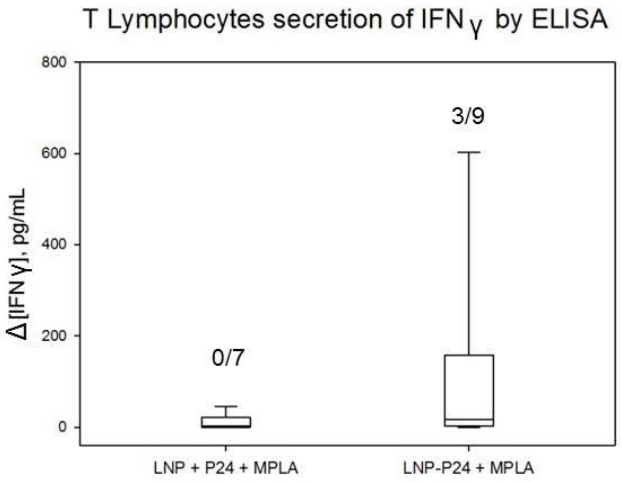

Figure 4: a) OVA and b) P24-specific antibody titers in combination with MPLA or CpG and c) IFNγ secretion by splenic T lymphocytes after re-exposition to P24.

For both OVA and P24 the antibody results show that the antigen vectorized by the lipid nanoparticle in combination with an immunostimulant was much more immunogenic than in a soluble state. It is also demonstrated that the nanoparticle itself had no immunostimulant properties, as it did not enhance the production of antibodies when administrated aside with the antigen. Actually, grafting the antigen onto the nanoparticle improves the immune responses. This can be due to a protection of the antigen from biological degradations and/or to a better uptake by immune cells, especially antigen presenting cells (APCs). To note, 3 mice over 9 having received the P24-lipid nanoparticles displayed also a potent activation of their T lymphocytes in response to exposition to P24. Altogether, these results confirm that Lipidots® act as nanocarriers potentializing the induction of both humoral and cell-mediated immune responses.

CONCLUSION

We have efficiently grafted protein antigens onto lipid nanoparticles. These antigen nanocarriers are highly stable overtime and very well tolerated by cells and rodents. Potent humoral and cell-mediated immune responses have been induced by immunization of mice with these nanoformulations. This study paves the way for the engineering of small synthetic lipid nanoparticles for the delivery of protein antigens in a context of the development of novel vaccine formulations.

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