Nanomedicine: Nanotechnology, Biology, and Medicine
12 (2016) 2439–2448

nanomedicine
Nanotechnology, Biology, and Medicine

nanomedjournal.com

# Effective transcutaneous immunization using a combination of iontophoresis and nanoparticles

Daniela S. Bernardi, PhD[a], Claudia Bitencourt, PhD[a], Denise S.C. da Silveira, PhD[a], Estael L.C.M. da Cruz, MD[a], Marcelo A. Pereira-da-Silva, PhD[b,c], Lúcia Helena Faccioli[a], Renata F.V. Lopez[a,*]

[a]*University of São Paulo, School of Pharmaceutical Sciences of Ribeirão Preto, Ribeirão Preto, SP, Brazil*
[b]*Physics Institute of Sao Carlos-USP, Sao Carlos, SP, Brazil*
[c]*Paulista Central University Center-UNICEP, Sao Carlos, SP, Brazil*

Received 26 November 2015; accepted 3 July 2016

**Abstract**

Needle-free immunization strategies have been sought for years. Transcutaneous immunization using electroporation has been studied, but the high electrical voltage that must be applied may be painful and cause irreversible cell damage. The application of a weak electric field, such as in iontophoresis, has never been attempted. The aim of this work was to verify the potential of employing iontophoresis for transcutaneous immunization using ovalbumin (OVA) as a model antigen. To target the antigen presenting cells that are located in the viable epidermis, a vaccine formulation composed of OVA-loaded liposomes and silver nanoparticles (NPAg) was developed. *In vitro* cathodal iontophoresis of the OVA-liposomes associated with NPAg increased OVA penetration into the viable epidermis by 92-fold in comparison to passive delivery. *In vivo*, transcutaneous immunization with a suitable combination of liposome and iontophoresis induced the production of antibodies, differentiation of immune-competent cells and appeared to present an alternative strategy for needle-free vaccination.


*Key words:* Liposome; Transcutaneous immunization; Iontophoresis; Silver nanoparticles; Ovalbumin

Vaccines are administered, in general, by a parenteral route. The use of needles for this invasive administration is related with a risk of transmission of infectious diseases; additionally, many patients present with needle phobia. Therefore, public health organizations, such as the World Health Organization (WHO), the Global Alliance for Vaccines and Immunization and the Centers for Disease Control and Prevention, encourage the development of new needle-free vaccination strategies.[1] Among the strategies that have been studied, immunization through the skin (*i.e.*, transcutaneous immunization) has been promising.[1]

The skin is an attractive route for needle-free immunization, especially due to the presence of antigen presenting cells (APCs) in the viable epidermis (Langerhans cells) and in the dermis (dendritic cells).[2,3] The APCs capture and process antigens, present them to naive T cells and initiate the cellular immune response.[4–7] To reach the APCs and initiate the immune response, the antigens need to penetrate through the main skin barrier, the stratum corneum (SC), in sufficient concentrations. However, the SC protects the body against the external environment and hampers the permeation of hydrophilic macromolecules, such as peptides and proteins, which are commonly used as antigens in vaccines. To overcome the barrier function of the SC, electroporation has been studied as a transcutaneous immunization strategy.[8]

Electroporation is a physical method that consists of applying short pulses of high electrical voltage (between 50 and 1500 V) to the skin. These pulses promote the formation of transient aqueous pores in the SC lipid bilayer, allowing macromolecules to migrate through them.[9] Although electroporation facilitates

Competing financial interests: The authors declare no competing financial interests.

Financial support: São Paulo Research Foundation (FAPESP grant #2011/13307-1, #2012/05177-3, # 2014/22451-7, # 2014/07125-6) and Brazilian National Council for Scientific and Technological Development (CNPq grant #480962/2013-8).

*Corresponding author at: School of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo, Ribeirão Preto, SP, Brazil.
*E-mail address:* rvianna@fcfrp.usp.br (R.F.V. Lopez).

http://dx.doi.org/10.1016/j.nano.2016.07.001
1549-9634/

antigen penetration through the skin, it can cause the rupture of the SC and lead to irreversible cell damage.[10,11]

Iontophoresis is another physical method that may be used as an alternative to electroporation in transcutaneous immunization. It involves the application of a constant low-density electrical current (maximum of 0.5 mA/cm$^2$) instead of the high voltage pulses that are used in electroporation. Iontophoresis has been widely studied to improve the skin permeation of macromolecules,[12,13] but it has not yet been explored for transcutaneous immunization. During iontophoresis, an electrical current passes through an electrolytic formulation, providing an electrical driving force for the transport of compounds across the SC. Modification of the formulation can modulate drug skin penetration, targeting it to the viable epidermis,[14,15] especially when it is associated with a lipid nanoparticulate delivery system.[16,17]

Liposomes are traditional lipid nanoparticulate delivery systems that promote drug penetration through the skin and can protect substances that degrade easily,[18,19] such as the proteins that are used for immunization. However, liposomal dispersions can also be less conductive than water, decreasing the efficiency of iontophoresis. In this work, we propose to incorporate silver nanoparticles (NPAg) into liposomes to create a vaccine formulation. It was hypothesized that NPAg would facilitate the passage of electric current through a liposomal dispersion, improving iontophoresis efficiency. If Ag could penetrate the skin with the support of iontophoresis, it could also modify the defense mechanisms of the body, such as other metallic materials (aluminum, cadmium, nickel and chrome)[20,21] that stimulate the immune system.

Therefore, the aim of this study was to develop ovalbumin (OVA)-loaded liposomes that included NPAg (OVA-liposome) and to investigate, for the first time, the effect of iontophoresis on the immune response after transcutaneous immunization using OVA-liposomes. OVA was used as a model antigen, and its subcutaneous injection was used as a positive control in *in vivo* experiments.

## Methods

### *Chemicals*

OVA from chicken egg white and albumin from bovine serum were purchased from Sigma Aldrich (St. Louis, MO, USA). Phosphatidylcholine (PC) from soybean (Lipoid S) and 1,2-dioleoylphosphatidylethanolamine (DOPE) (Lipoid PE 18:1/18:1) were obtained from Lipoid GmbH (Ludwigshafen, Rhineland-Palatinate, GER). Chloroform was purchased from J.T. Baker (Center Valley, *CA*, USA). Biotin rat anti-mouse IgG1, biotin rat anti-mouse IgG2a, PE rat anti-mouse CD4, PE rat anti-mouse CD19, FITC hamster anti-mouse CD3e, PerCP rat anti-mouse CD8, APC-CY 7 rat anti-mouse CD45R, APC rat anti-mouse CD44, PE rat IgG2a isotype control, FITC hamster IgG1 isotype control, PerCP rat IgG2a isotype control, APC rat IgG2b isotype control and APC-Cy7 rat IgG2a isotype control were purchased from BD Pharmingen™ (New Jersey, NY, USA), and a TMB substrate reagent set was purchased from BD OptEIA™ (New Jersey, NY, USA). Streptavidin-HRP substrate reagent and stop solution (sulfuric acid, 2 N) were purchased from R&D Systems (Minneapolis, USA). Polysorbate 20 was purchased from Synth (Diadema, Brazil). Deionized water (18.2 MΩ-cm at 25 °C) (Milli-Q, Direct-Q 3 UV, Millipore, Billerica, MA, USA) was used to prepare all of the solutions.

### *Silver nanoparticles*

The NPAg dispersion was prepared *via* the simple reduction of a solution of silver nitrate at 8 mM by the slow addition, under stirring, of sodium borate at 4 mM.[22] The NPAg dispersion was scanned in a spectrophotometer UV/Vis in the range of 200 to 800 nm to verify NPAg formation at 390 nm.[22,23] The mean diameter, polydispersity index (PdI) and zeta-potential of NPAg were measured by dynamic light scattering using a Zetasizer (Nano ZS, Malvern Ltd., United Kingdom).

### *Vaccine formulation*

OVA-loaded liposomes were prepared by a thin-film hydration method using the lipids PC and DOPE in a 3:1 proportion. The lipids were dissolved in 2 mL of chloroform, placed in a round-bottom glass flask and kept in a rotary evaporator (IKA®, Germany) at 30 °C and 100 rpm under vacuum (200 mmHg) for 15 min. The lipid film was hydrated with 50 mL of 0.01 M phosphate buffer, pH 7.4, containing OVA at 5 mg/mL and 5 mL of NPAg (14.5 μM of Ag). After hydration of the film, the suspensions were vortexed for 5 min, stirred for another 20 min over an ice bath in an Ultra Turrax® at 15,000 rpm (IKA®, Germany) and homogenized in a high-pressure homogenizer (EmulsiFlex®, Avestin, Inc., Canada) for 6 cycles. Finally, the dispersion that formed (OVA-liposome) was filtered through a 0.45-μm PVDF membrane.

Liposomes containing OVA without NPAg (OVA-liposome w/o NPAg) and liposomes prepared without OVA and NPAg (Blank liposome) were also obtained.

Liposomes were characterized by mean diameter, PdI, conductivity and zeta potential, using a Malvern Zetasizer ZS 90 (Malvern Instruments Ltd., Worcestershire, England), by atomic force microscopy (AFM), using a Multi Mode Nanoscope V (Bruker, Santa Barbara, *CA*) operating in tapping mode at room temperature and by transmission electron microscopy (TEM), using a JEM-100CXII transmission electron microscope (JEOL, Japan) with an accelerating voltage ranging from 100 kV to 200 kV (Supplementary material, Section S1).

### *OVA encapsulation efficiency (EE)*

Unencapsulated OVA ($Q_{unencapsulated}$) was separated from the OVA-liposomes using an Amicon-Ultra-15 (100 kDa) centrifugal filter at 8000 ×*g* for 40 min at 25 °C. Unencapsulated OVA (45 kDa) was able to cross the membrane. It was quantified in the filtrate by spectrophotometric analysis at 280 nm using a linear calibration curve obtained from OVA ($y = 0.0007\times + 0.0029$, $r^2 = 0.999$) prepared in PBS at pH 7.4 over a concentration range of 100-1500 μg/mL. The *EE* of OVA was calculated using Eq. (1):

$$EE = \frac{Q_{theoretical} - Q_{unencapsulated}}{Q_{theoretical}} \times 100 \quad (1)$$

where $Q_{theoretical}$ is the quantity of OVA that was added to the liposome dispersion.