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Effective transcutaneous immunization using a combination of iontophoresis and nanoparticles

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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.

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

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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

($18.2 \text{ M}\Omega\text{-cm}$ 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 ($14.5 \mu\text{M}$ 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 $0.45\text{-}\mu\text{m}$ 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 ($Q_{\text{unencapsulated}}$) was separated from the OVA-liposomes using an Amicon-Ultra-15 (100 kDa) centrifugal filter at $8000 \times g$ 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 ($y = 0.0007x + 0.0029$, $r^2 = 0.999$) prepared in PBS at pH 7.4 over a concentration range of 100–1500 $\mu\text{g/mL}$. The EE of OVA was calculated using Eq. (1):

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

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

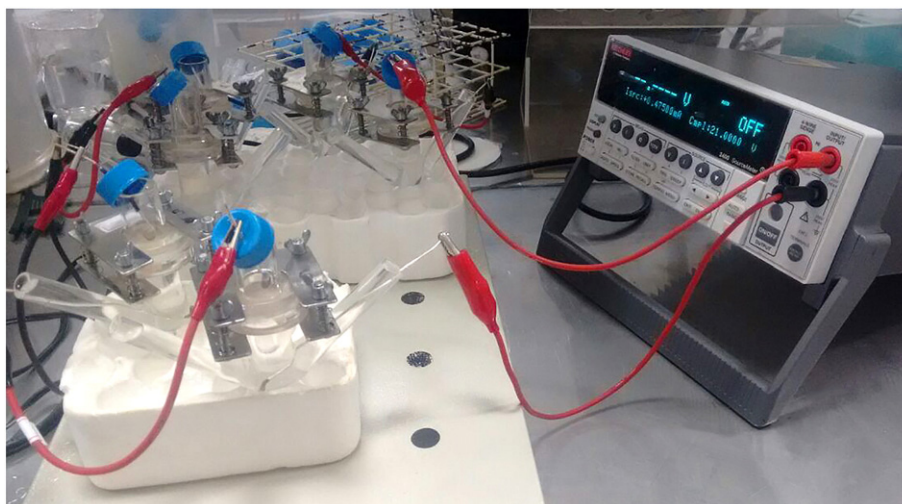


Figure 1. Representation of the setup for the *in vitro* iontophoresis.

In vitro iontophoresis

Skin was collected from pig ears immediately after slaughter (Frigorifico Pontal Ltda, Pontal, Brazil); it was removed from the ear and then dermatomed at 700 μm . The dermatomed skin was stored at -70°C for a maximum of 30 days prior to use. Prior to each experiment, the electrical resistivity of the skin was determined according to Tang et al (2001).²⁴ Any skin with an initial resistivity of $<35\text{ k}\Omega\cdot\text{cm}^2$ was considered damaged and was not used in subsequent experiments.

The experiments were conducted using modified vertical diffusion cells as previously described.²⁵ The dermatomed skin was assembled in the diffusion cells with the SC facing up to the donor compartment. The receptor compartment was filled with 0.01 M phosphate buffer containing NaCl at pH 7.4 and maintained at 37°C . In the donor compartment, 2 mL of the formulation (OVA-PBS, OVA-liposome w/o NPAG or OVA-liposome) was added. Cathodic iontophoresis was performed using a silver chloride electrode placed in contact with the formulation and a silver electrode placed in the receptor compartment. These electrodes were connected to a power source and submitted to a constant electric current of $0.5\text{ mA}/\text{cm}^2$ for 15 min (Figure 1). At the end of the experiment, the skin was removed from the diffusion cell and trapped in a smooth surface with the SC facing up. To quantify OVA in the SC, the skin was tape-stripped 15 times to remove the SC using Scotch Book Tape No. 845 (3M, St Paul, MN). The complete removal of the SC was confirmed after the procedure by glistening of the exposed viable layers of the epidermis.¹⁶ The stripped tapes were added to a sterile polypropylene conical tube containing 10 mM phosphate buffer at pH 7.4 as a solvent extractor of OVA and were left overnight to extract the protein. The remaining skin (viable epidermis) was cut into small pieces, placed into a tube with phosphate buffer at pH 7.4 and left overnight to extract the OVA. Serial dilutions were carried out to obtain appropriate concentrations of OVA for protein quantification by ELISA at 450 nm using a kit which selectively quantifies OVA from chicken egg (Alpha Diagnostic, Texas,

USA). The OVA extracted from the skin was quantified using a linear calibration curve of OVA in the skin ($y = 0.209x + 0.524$, $r^2 = 0.995$) that was prepared in PBS at pH 7.4 over a concentration range of 0.5–4 ng/mL. The recovery of OVA from the skin when using PBS was more than 89%.

Transcutaneous immunization

BALB/c mice at 6–8 weeks old (18–20 g) were used in all of the experiments. The protocol was approved by the Ethics Committee of the University of São Paulo (Protocol no. 10.1.1052.53.9), which is in accordance with the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals.

One day before of the immunization experiment, the hair from the abdominal area was shaved by trichotomy. The resistivity of the skin was measured before and 24 h after the shaving to guarantee that this procedure does not alter the integrity of the skin²⁴ (Supplemented material, section S2).

For the immunization, mice were anesthetized with a solution of xylazine and ketamine at 10 and 75 mg/Kg, respectively. Two milliliters of OVA-liposomes was put into a plastic chamber (1.13 cm^2) that was attached to the shaved abdominal area with silicone grease. Cathodic iontophoresis was applied for 15 min using an AgCl electrode that was soaked in the formulation, and an IOMED patch (Iomed, Inc., Salt Lake City, Utah, USA) was attached around each mouse's tail to serve as a counter electrode. A constant electric current of $0.5\text{ mA}/\text{cm}^2$ was applied using a power supply (digital sourcemeter 2400) manufactured by Keithley (Beaverton, USA). A subcutaneous injection of OVA at 100 $\mu\text{g}/\text{mL}$ was used as a positive control. Passive application (without electric current) was performed for comparison with iontophoretic treatment. Additionally, to evaluate if the application of iontophoresis itself could cause any immune response, cathodal iontophoresis of a PBS solution was performed. Each treatment was performed with 5 animals.

A schematic representation of the groups of animals that were treated can be found in Figure 2. The animals were

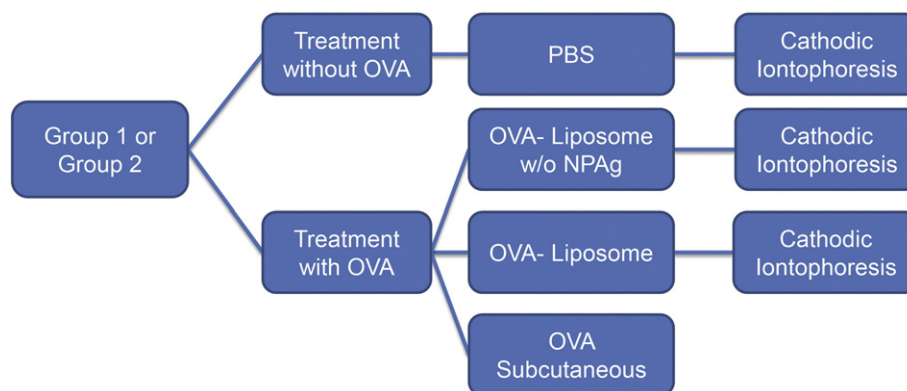


Figure 2. Schematic representation of the group of animals that were administered transcutaneous immunizations.

Table 1

Mean diameter, polydispersity index (Pdl) and zeta potential of dispersions determined by photon correlation spectroscopy.

Formulation	Size (nm)	Pdl	Zeta potential (mV)
NPAg	23 ± 4	0.44 ± 0.14	−8 ± 0
Blank liposome	134 ± 19 ^a	0.30 ± 0.01	−27 ± 7
OVA-liposome	128 ± 2	0.24 ± 0.02	−14 ± 1 ^b
OVA-liposome w/o NPAg	123 ± 8	0.26 ± 0.00	−7 ± 0 ^c

Data represent the mean ± SD of 3 determinations. ^aThe size of blank liposome was statistically higher when compared with the OVA-liposome w/o NPAg (one-way ANOVA, Tukey's multiple comparison test, $P < 0.05$) and similar to the OVA-liposome. ^b, ^cValues of zeta potential of liposomes are statistically different from each other (one-way ANOVA, Tukey's multiple comparison test, $P < 0.05$).

subdivided in two groups. Group 1 was immunized on the first day of the treatment and euthanized on the eighth day, and group 2 was immunized on the first day of treatment, challenged on the twenty-second day of the study and euthanized on the thirty-sixth day.

Humoral immune response was evaluated by the quantification of anti-OVA antibody levels in serum. The levels of anti-OVA immunoglobulin (Ig) G1 and IgG2a were determined by ELISA (Supplementary material, section S3).

To evaluate cellular immune response, immunophenotyping was performed on spleen cells due to the ease of dissecting the spleen and the large number of cells available for analysis. T lymphocyte activation was evaluated using the markers CD3 and CD4 or CD8. B lymphocytes were evaluated using CD19 as a marker. The CD45 marker was used to verify the activation of mature T cells and B cells after treatment. A FACSCanto (TM) cytometer (Becton Dickinson, CA, USA) was used for these analysis (Supplementary material, section S4).

Statistical analysis

The data were expressed as the mean and standard deviation (SD). An analysis of variance (ANOVA) was used for comparisons of more than two samples ($P < 0.05$) using GraphPad Prism 5. Statistical significance of the differences between groups was assessed using Tukey's multiple comparison test and was performed on size and zeta potential, *in vitro*

cathodic iontophoresis, humoral and cellular immune responses. Additionally, Student's *t* test was performed on pH, conductivity and EE analyses, as well as in comparing the groups of animals that were treated with liposomes to the positive and negative control groups.

Results

Dispersion characterizations

Table 1 shows the physicochemical properties of the dispersions that were prepared.

NPAg presented a maximum absorption peak at 390 nm when evaluated by a spectrophotometer, in accordance with the literature.²⁶ Their smaller size of 50 nm and negative zeta potential were similar to other NPAg described in literature.²⁷ The liposomes were 5 to 6-fold larger than NPAg while the addition of OVA to the blank liposome slightly decreased the liposome size ($P < 0.05$, Student's *t* test) and made the zeta potential less negative (from −27 mV to −7 mV) ($P < 0.05$, Student's *t* test). NPAg incorporation into the conventional OVA-loaded liposome dispersion did not significantly change ($P > 0.05$, Student's *t* test) its size (Table 1). However, the zeta potential of the liposome dispersions containing NPAg (OVA-liposome) was more negative ($P < 0.05$, Student's *t* test) than that of the dispersions without NPAg (OVA-liposome w/o NPAg).

The pH and conductivity of OVA-liposome and OVA-liposome w/o NPAg produced similar ($P > 0.05$, Student's *t* test) values of 7.35 ± 0.03 and 7.25 ± 0.04 for pH, 1.37 ± 0.05 mS/cm and 1.42 ± 0.04 mS/cm for conductivity, respectively.

The EE of OVA in OVA-liposomes and in OVA-liposomes w/o NPAg was $78 \pm 3\%$ and $79 \pm 4\%$ respectively. The addition of NPAg to the liposome dispersion did not significantly change ($P > 0.05$, *t* test) the percentage of encapsulated OVA.

Atomic force microscopy images of NPAg, OVA-liposome w/o NPAg and OVA-liposome are shown in Figure 3. An evaluation of NPAg sizes using AFM imaging (Figure 3, A) showed particles with an average size of approximately 20 nm, corroborating the size estimated by photon correlation spectroscopy (PCS) (Table 1). The OVA-liposomes w/o NPAg (Figure 3,

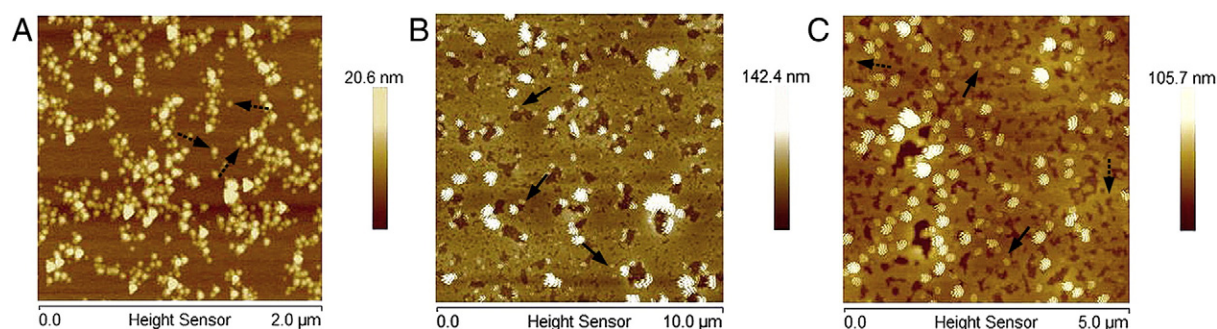


Figure 3. AFM images of (A) NPAG, (B) OVA-liposomes w/o NPAG and (C) OVA-liposomes. The arrows indicate isolated nanoparticles. The brighter the particle, the larger the nanoparticle, while much brighter portions indicate nanoparticles aggregates.

B) were approximately 140 nm, a size slightly higher than that observed by PCS, but the presence of aggregates was clearly observed in Figure 3, B. Aggregates observed could be due to the deposition of the liposomes in the mica substrate which also resulted in the appearance of some flattened nanoparticles.^{33,34} Analyses of the OVA-liposomes (Figure 3, C) presented a mixture of 100 nm and 20 nm nanoparticles due to the presence of liposomes and NPAG.

Figure 4 shows TEM images that were obtained after staining OVA-liposome samples with uranyl acetate, which is an electron-dense material that creates high contrast in liposomal vesicles.²⁸ Larger vesicles of about 100 nm and smaller dots of about 20 nm can be observed in Figure 4, B, suggesting the presence of a mixture of liposomes and NPAG. To prove that the smaller dots observed are NPAG, an image of OVA-liposome prepared without uranyl acetate is shown in the insert in Figure 4, B. With this image, the liposomes cannot be visualized, whereas NPAG can. Figure 4, A shows only vesicles of about 100 nm due to the absence of NPAG in OVA-liposome w/o NPAG formulation.

In vitro iontophoresis

Passive (no-electrical current) penetration of OVA from OVA-PBS into the SC and viable epidermis was $0.03 \pm 0.02 \mu\text{g}/\text{cm}^2$ and $0.47 \pm 0.24 \mu\text{g}/\text{cm}^2$, while OVA encapsulated into the liposomes (OVA-liposome w/o NPAG) resulted in $0.03 \pm 0.01 \mu\text{g}/\text{cm}^2$ and $0.79 \pm 0.35 \mu\text{g}/\text{cm}^2$ of OVA in the SC and viable epidermis, respectively. The addition of NPAG to the liposomes (OVA-liposomes) resulted in $0.06 \pm 0.01 \mu\text{g}/\text{cm}^2$ and $0.05 \pm 0.02 \mu\text{g}/\text{cm}^2$ accumulation of OVA in the SC and viable epidermis, respectively.

Figure 5 shows OVA recovered from the SC and from the viable epidermis after 15 min of cathodic iontophoresis. Iontophoresis of OVA-liposomes increased OVA penetration into both the SC and the viable epidermis. In comparison to passive delivery, iontophoresis applied to OVA-PBS and OVA-liposome w/o NPAG increased OVA penetration by about 3-fold into the viable epidermis, while OVA penetration into viable epidermis increased by about 92-fold when iontophoresis was applied to OVA-liposome (passive penetration: $0.05 \pm 0.02 \mu\text{g}/\text{cm}^2$, iontophoresis: $4.61 \pm 0.12 \mu\text{g}/\text{cm}^2$).

The amount of OVA recovered from the viable epidermis after iontophoresis application to OVA-liposomes was 3-fold and 2-fold higher than the amount recovered after iontophoresis application to OVA-PBS and OVA-liposomes w/o NPAG respectively (Figure 5, B). Iontophoresis of OVA-liposomes w/o NPAG did not increase OVA penetration in relation to OVA-PBS.

Transcutaneous immunization

Humoral immune response

After the first single immunization, the animal groups that were studied showed no statistically significant differences in the levels of anti-OVA IgG1 and IgG2a with respect to the negative control group (naive animals).

Figure 6 shows the anti-OVA IgG1 and IgG2a titers that were produced after the second immunization. After the second immunization, the three groups that were studied showed levels of IgG1 that were significantly superior of those produced by the naïve animals (One-way ANOVA, $P < 0.05$) (Figure 6, A). Iontophoretic treatment with OVA-liposomes – the formulation that contains both liposomes and NPAG – resulted in IgG1 titers similar to those produced following the subcutaneous injection of OVA (positive control) (t test, $P > 0.05$). Although iontophoretic treatment with OVA-liposomes w/o NPAG increased the IgG1 levels in relation to the negative control, the levels were smaller than those produced by the positive control (t test, $P < 0.05$).

Figure 6, B shows the levels of IgG2a expressed after the second immunization. They were larger than the negative control only in the animals that received OVA subcutaneously.

Cellular immune response

Figure 7 shows the percentage of cells expressing activated T CD4 and B CD19 in a region of high fluorescence during flow cytometry. The iontophoretic treatment of OVA-liposome dispersions resulted in a higher percentage of activated T CD4 (Figure 7, A) and B CD19 (Figure 7, B) than the subcutaneous injection of OVA. Interestingly, treatment with iontophoresis alone (single iontophoresis) without OVA also activated the expression of total B lymphocytes (Figure 7, B).

The percentage of cells expressing cytotoxic T CD8 was not significantly different between the naïve animals and the

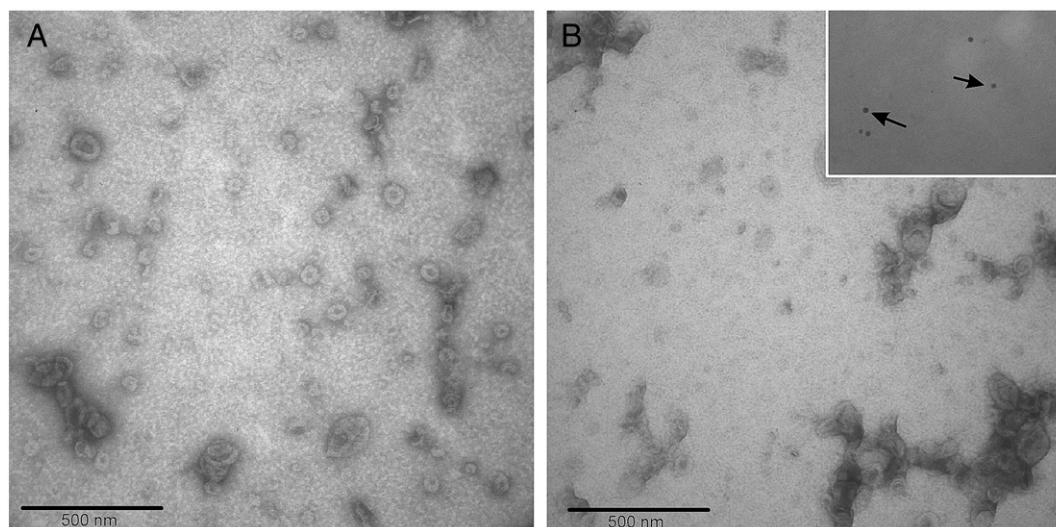


Figure 4. TEM images of (A) OVA-liposomes w/o NPAG and (B) OVA-liposomes. Insert: OVA-liposomes without contrast agent; the arrows indicate NPAG. Scale bar = 500 nm.

animals treated with OVA by a subcutaneous or transdermal route. Therefore, no activation of T CD8 cells was found after the treatments.

Discussion

This study reported a needle-free immunization strategy using a vaccine formulation composed of OVA-loaded liposomes and NPAG and studied the potential of iontophoresis for transcutaneous immunization.

NPAG with size smaller than 25 nm were obtained according to literature. These nanoparticles are stabilized by Ag counter-ions, borate and nitrate present in the dispersion.²⁹ These ions contribute to the negative zeta potential of the NPAG dispersion. The blank liposome presented a size of about 130 nm and a negative zeta potential. This negative zeta potential, despite the zwitterionic characteristic of the lipids used in the liposomes preparation may be explained by the influence of composition and ionic strength of the buffer solution used to hydrate and suspend the liposomes on the surface charge density of the particles.^{30,31} The addition of OVA to these liposomes (OVA-liposome w/o NPAG) slightly decreased the size of blank liposomes and left the zeta potential of the dispersion less negative (Table 1). These changes suggest that encapsulation of the hydrophilic OVA in the liposomes modified the organization of the lipids bilayer, changing the surface of the liposome and consequently, the liposome size and zeta potential of the dispersion. Modifications in liposome size and zeta potential could also be caused by OVA non-encapsulation and adsorption on the liposome surface. However, OVA, which has an isoelectric point of 4.5³² is negatively charged at pH 7.4. It is expected that its adsorption onto liposome surface could leave the liposome more negative and not less negative as suggested by the result. Therefore, it is likely that modifications in the

physicochemical characteristics of the blank liposomes by the addition of OVA could be due to OVA encapsulation.

The addition of NPAG into OVA-liposome w/o NPAG to produce the OVA-liposome did not change the size and the PDI of the drug-liposome dispersion. However, the addition of NPAG increased the zeta potential of the dispersion (from about -7 mV to about -14 mV). This might have occurred because of the negative zeta potential of the NPAG (-8 mV) that seems not to have been encapsulated in the liposomes. Indeed, TEM images (Figure 4, B) also suggest that NPAG are not encapsulated in the liposome vesicle.

The difference of -7 mV in the zeta potential of OVA-liposome (-14 mV) and OVA-liposome w/o NPAG (-7 mV) did not alter the ability of the dispersions to conduct electric current as both OVA-liposome and OVA-liposome w/o NPAG dispersions presented a similar electric conductivity. However, the presence of more ions in OVA-liposome dispersion than in OVA-liposome w/o NPAG dispersion could influence OVA electromigration when iontophoresis is applied. More specifically, the presence of the negative borate and nitrate ions (that were not eliminated from NPAG dispersion) could compete with the negative OVA or with the liposome for the electric current, which could decrease OVA skin penetration. However, because Ag is a metal, its penetration into the SC could facilitate the passage of the electric current across the skin and increase OVA penetration when iontophoresis is applied. Indeed, *in vitro* penetration experiments, discussed further, showed that the OVA-liposome containing NPAG increased OVA penetration when electric current was applied (Figure 5).

The EE of OVA in the liposomes which was higher than 78% did not change significantly after the addition of NPAG. The high EE of OVA in the liposomes is in accordance with other studies in literature.^{35,36} For instance, liposomes composed of phosphatidylcholine and cholesterol (4:1) prepared with the same initial concentration of OVA presented EE of $78 \pm 19\%$.³⁶

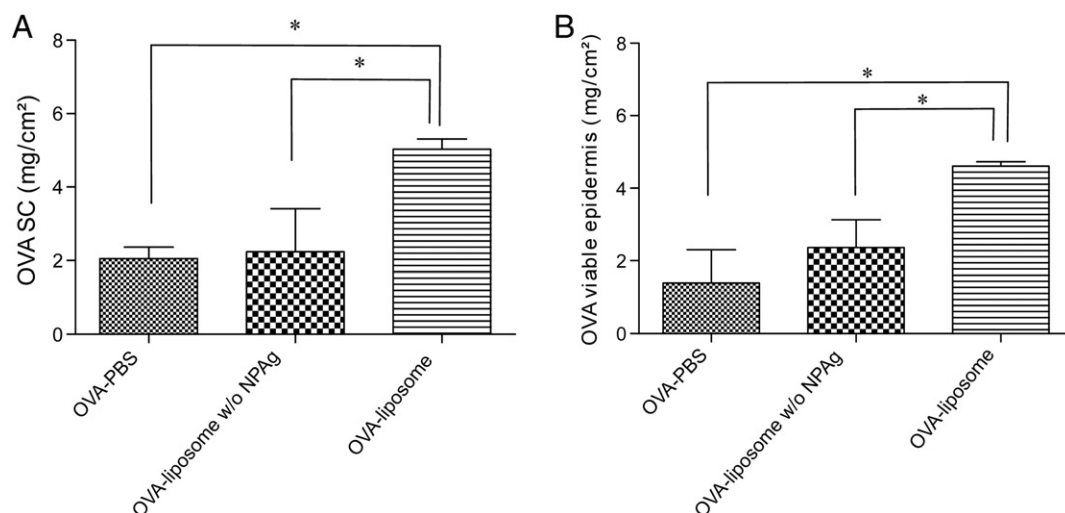


Figure 5. *In vitro* cathodic iontophoresis. OVA recovered from the (A) SC and the (B) viable epidermis of pig ear skin after 15 min of iontophoresis of OVA-PBS, OVA-liposomes w/o NPAG and OVA-liposomes. * $P < 0.05$ ANOVA and Tukey's multiple comparison test.

To evaluate the influence of liposomes and iontophoresis in OVA delivery to different skin layers, *in vitro* skin penetration studies were performed using pig skin. In the absence of iontophoresis (passive experiments), encapsulation of OVA increased OVA penetration to the viable epidermis; however, the addition of NPAG to the liposomes decreased OVA passive penetration. It is possible that NPAG clogged liposome penetration paths into the SC, thus hindering the OVA passive penetration.

Cathodic iontophoresis was performed instead of anodic iontophoresis because of the negative zeta potential of the liposome dispersions (Table 1). Furthermore, OVA presents an isoelectric point of 4.5³² and is also negatively charged when dispersed in PBS (zeta potential of -21 ± 5 mV, determined by PCS). Therefore, the dispersions may benefit from the electro-migration flow that occurs when iontophoresis is applied.

As expected, compared to passive delivery, cathodic iontophoresis increased OVA penetration when applied in all formulations studied (Figure 5), especially in the viable epidermis where APCs are located. Other studies have shown that iontophoresis can influence the permeation of drug-loaded liposome through the skin. However, the results are controversial, as they show that iontophoresis of liposomes can increase the permeation of some drugs such as enoxacin³⁷ and decrease the permeation of others such as insulin³⁸ and enkephalin³⁹ when compared to the permeation of the free drugs. Our results showed that iontophoresis of the liposome without NPAG (OVA-liposome w/o NPAG) did not increase OVA skin penetration when compared to the free drug (OVA-PBS). However, the presence of NPAG in the liposome dispersion (OVA-liposome) contributed to a higher iontophoretic delivery of OVA in the viable epidermis when compared to the formulations without NPAG (OVA-PBS and OVA-liposome w/o NPAG) (Figure 5). It is hypothesized that some Ag penetrate the skin deep when iontophoresis was applied, facilitating the transport of electric current across the skin, improving OVA penetration.

For transcutaneous immunization studies, OVA-liposomes and OVA-liposomes w/o NPAG were selected. Although OVA-liposomes w/o NPAG did not increase OVA *in vitro* skin penetration when compared with OVA-PBS, iontophoresis may push some lipids into the skin.^{15,16} The lipids that enter the skin may act as immunological adjuvants, thereby improving the immune response unleashed by OVA.

After the first single immunization, low titers of anti-OVA IgG1 and IgG2a were observed and expected because immune memory is not yet activated. Due to immune memory, second contact with the antigen leads to a faster and more vigorous response, thus producing high antibody titers.⁴⁰ After the second immunization, all the treatments increased IgG1 titers. However, iontophoresis of liposomes containing NPAG showed again a better response when compared to the OVA-liposomes w/o NPAG (Figure 6, A). The higher IgG1 titers that were induced by the iontophoresis of the liposomes containing NPAG may be related to higher skin penetration of OVA in this formulation or by some additional immune response caused by Ag penetration.

The IgG1 production is related to immune response *via* Th2 while IgG2a production is related to immune response *via* Th1. As shown in Figure 6, B, animals treated with iontophoresis did not express significant levels of IgG2a. Therefore, the iontophoretic administration of OVA generated a greater response *via* the Th2 pathway, as reported for other antigens administered through the skin.^{35,41}

The anti-OVA IgG1 levels that were produced after iontophoresis of the formulations are promising. The combination of OVA-liposomes and OVA-liposomes w/o NPAG with iontophoresis led to favorable transcutaneous immunization after only 15 min of administration. From a practical standpoint, modifications in the formulations and iontophoretic parameters may improve the immune response even more. For instance, changes in iontophoretic polarity from cathodic to anodic iontophoresis may favor OVA skin penetration *via* the contribution of anode-to-cathode electro-osmotic flow, which is typically established across the skin under physiologic

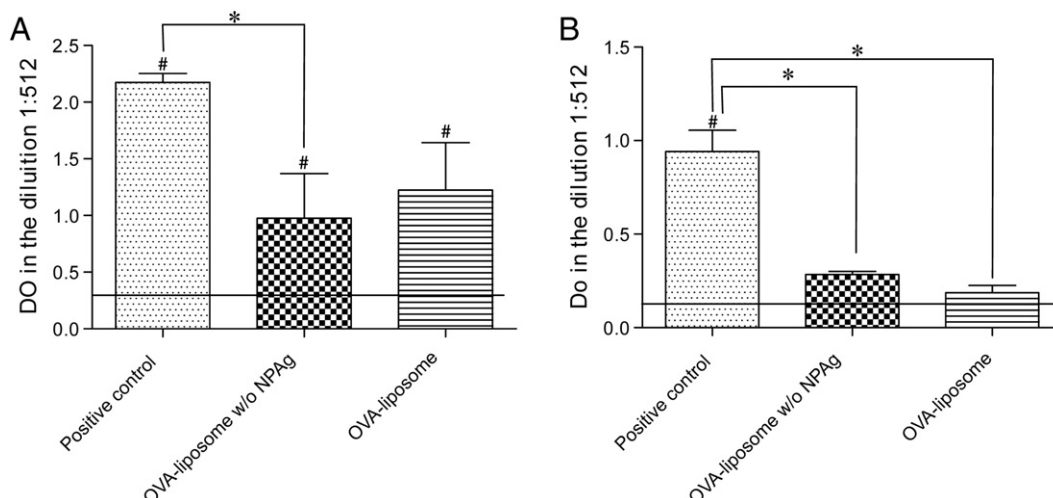


Figure 6. Anti-OVA titers after the second immunization. (A) IgG1, (B) IgG2a. The black line indicates the average of the negative control group. The results are expressed as the average $\pm$ SD, $n = 5$. In the statistical analyses, # shows $P < 0.05$ vs. negative control, * shows $P < 0.05$ vs. positive control. The acronym DO refers to optical density.

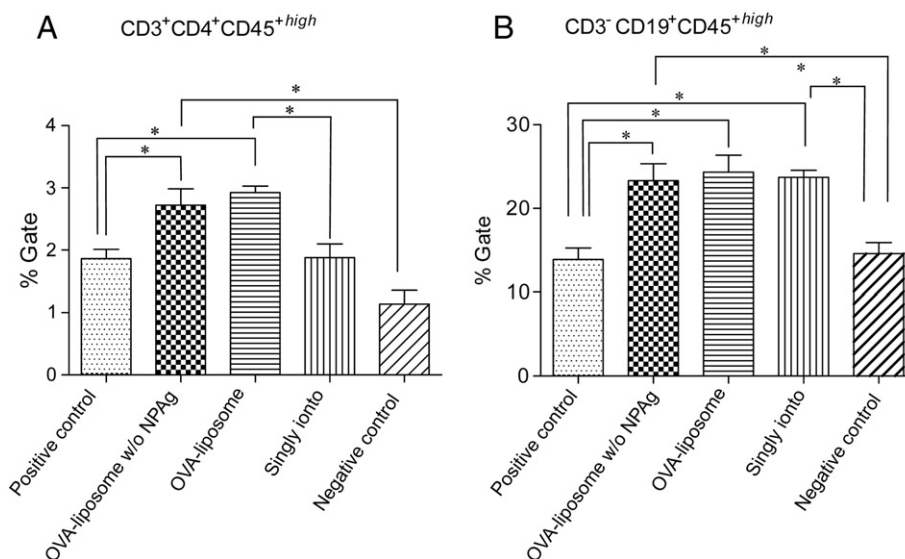


Figure 7. Percentage of total spleen cells that express T cells (A) and B cells (B) activated in the high region. The cell quantity that was used was 2×10^6 in 200 μ L of PBS containing 2% of BSA per animal. The data were analyzed by FACSDiva software. The results were expressed as the average $\pm$ SD, $n = 5$. For the statistical analyses, ANOVA and Tukey's multiple comparison test using the GraphPad Prism 5 were employed, where (*) refers to a significant difference between groups ($P < 0.05$).

conditions.⁴² Additionally, the concentration of OVA in the donor dispersion administered by iontophoresis could be reduced together with a reasonable selection of current density and application time.

Cellular immune response was evaluated in addition to humoral immune response. Interestingly, higher percentage of activated T CD4 and B CD19 was produced after iontophoretic treatment of OVA-liposome than the subcutaneous injection of OVA. Single iontophoresis also activated the expression of the total panel of B lymphocytes. This result suggests that cathodic iontophoresis may be acting as an immunological adjuvant. It is known that cathodic iontophoresis induces intense erythema in the skin.⁴³ This increased activation of local micro circulation

may be one reason for the higher expression of B lymphocytes caused by the cathodal iontophoresis.

The interactions that occur between T and B lymphocytes after vaccine administration are extremely important. T CD4 cells, also called T helper lymphocytes support the differentiation of B cells into antibody producing cells in the plasma and in germinal centers. They also stimulate the production of memory B cells, leading to a more effective immune response.⁴⁴ Figure 7 shows that the cellular immune response obtained after iontophoresis of the liposomes was more efficient than that obtained after subcutaneous injection.

No activation of T CD8 cells was found after the treatments. T CD8 memory cells can kill infected cells and also provide

protection.⁴⁵ Although much is known about the generation of these memory cells following infection by viruses or bacteria, there is far less understanding of how these CD8 memory cells work following immunization.⁴⁴ As demonstrated by Seid Jr. and collaborators,⁴⁶ the addition of immunological adjuvants could contribute to the activation of these cells following topical application.

In conclusion, the combination of iontophoresis and liposomes seems to provide an interesting strategy for needle-free vaccination. This strategy is especially suggested for use in vaccines against diseases that affect the skin, such as melanoma and other skin cancers.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.nano.2016.07.001>.

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Supplementary Material

Effective transcutaneous immunization using a combination of iontophoresis and nanoparticles

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Section S1. Characterization of the vaccine formulation

The mean diameters and PdIs of the OVA-liposomes and the Blank liposomes were determined by photon correlation spectroscopy (PCS) using a Malvern Zetasizer ZS 90 (Malvern Instruments Ltd., Worcestershire, England). For this analysis, the samples were diluted in PBS (1:10), and the measurement was performed at 25 °C with a 50 mV laser at a fixed angle of 90°. The results were recorded using Malvern

Zetasizer Software 2.6, and data processing was performed in the general purpose (normal resolution) mode. All of the samples were analyzed in triplicate.

The zeta potential was determined from the electrophoretic mobility using the same instrument. The samples were diluted (1:10) in PBS, and data analyses were performed using the Helmholtz-Smoluchowski approximation.

OVA-liposomes and Blank liposomes were analyzed using a MultiMode Nanoscope V (Bruker, Santa Barbara, CA) operating in tapping mode at room temperature. Both the height and the phase-imaging data were simultaneously acquired using a commercial silicon cantilever tip (OTESP model, Bruker, Santa Barbara CA) with a stiffness of approximately 40 Nm^{-1} and a resonance frequency of approximately 330 kHz. All of the AFM images were obtained using a scan rate of 0.7 or 1 Hz over a selected area in a dimension of $5 \mu\text{m} \times 5 \mu\text{m}$ or $0.65 \mu\text{m} \times 0.65 \mu\text{m}$ using freshly cleaved mica as a substrate. The force applied to the sample was roughly adjusted by the ratio of the engaged or set-point amplitude to the free-air amplitude. The images were processed and analyzed using NanoScope Analysis software (version 1.40 R1Sr4 Bruker, Santa Barbara, CA). The heights and diameters of the vesicles were measured from the profile sections of AFM line scans. The software Nanoscope was used to measure the average particle size in delimited regions of the micrograph (600x600 nm).

TEM analysis was performed using a JEM-100CXII transmission electron microscope (JEOL, Japan) with an accelerating voltage ranging from 100 kV to 200 kV. The samples were prepared by a negative-staining procedure. Briefly, a carbon-coated formvar grid was floated on top of a drop that contained the sample for 15 min, followed by floating it on top of a 2% osmium tetroxide solution for 10 min. Then, it was floated on top of a 1% uranyl acetate solution for 1 min and, after air drying, it was

observed under the TEM¹. Images of OVA-liposomes prepared without uranyl acetate were also analyzed.

Section S2. Transcutaneous immunization

BALB/c mice at 6–8 weeks old (18–20 g) were used in all of the experiments. The protocol was approved by the Ethics Committee of the University of São Paulo (Protocol nº 10.1.1052.53.9), which is in accordance with the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals.

One day before of the immunization experiment, the hair from the abdominal area was shaved by trichotomy. The resistivity of the skin was measured before and 24 h after the shaving to guarantee that this procedure does not alter the integrity of the skin². To accomplish this, a plastic chamber was attached to the skin and filled with PBS at pH 7.4. An AgCl electrode was inserted into the solution, while a counter-electrode patch (Iomed, Inc., Salt Lake City, Utah, USA) was fixed around the tail of the animal. An alternating current of 100 mV (RMS) potency and 10 Hz frequency was applied, and the electrical current that was generated was measured using a multimeter (Minipa ET 2053). The resistivity ($K\Omega.cm^2$) was calculated using Ohm's law and the area exposed to treatment and was compared with the resistivity presented by the same area of the skin of an unshaven animal. If there was a difference between a shaved and unshaven animal regarding the skin resistivity measurement, those animals were not included in the study.

Section S3. Quantification of anti-OVA antibody levels in serum

After animal euthanasia, blood was collected and maintained at room temperature for 1 h to enable clotting. Then, the blood samples were centrifuged for 10

min at 126 g to obtain serum. The levels of anti-OVA immunoglobulin (Ig) G1 and IgG2a were determined by ELISA. Briefly, ELISA plates (Costar[®], United States) were coated with 100 μ L OVA per well and incubated at room temperature overnight. Then, the plates were blocked with 5% (w/v) BSA in PBS (pH 7.4) for 2 h at 37°C and thoroughly washed, twice with PBST (Polysorbate-20, 0.3% v/v in PBS) and once with PBS, to remove unloaded OVA. Serials dilutions of the serum sample were added to each well, incubated for another 2 h at room temperature and later washed. One-hundred microliters of diluted biotin rat anti-mouse IgG1 or biotin rat anti-mouse IgG2a was added to each well, incubated for 2 h at 37°C and then washed. In the dark, 100 μ L/well of streptavidin diluted at 1:200 in blocking buffer was added, and after 20 min the plate was washed. Next, 100 μ L of substrate reagent (3,3',5,5'-tetramethyl benzidine containing hydrogen peroxide) was added to each well followed by the addition of 50 μ L of 1 M H₂SO₄ to end the reaction. The absorbance was measured at 450 nm using a microplate ELISA reader Multiscan FC (Thermo SCIENTIFIC, USA).

Section S4. Immunophenotyping by flow cytometry

After the euthanasia of the animals, their spleens were removed, placed in a sterile petri dish containing 3 mL of RPMI-I media and maintained on an ice bath. The spleen was macerated, transferred to a sterile polypropylene conical tube and centrifuged for 10 min at 113 g. Then, the cell suspension was added of 10 mL of lysis buffer (0.85% ammonium chloride preheated to 37°C) and incubated for 10 min to induce erythrocytes lysis. Following this, 15 mL of RPMI-I was added, and the system was centrifuged again under the same conditions. The cells were resuspended in 10 mL of RPMI-C media and counted using the trypan blue exclusion method. After adjusting the cells to a concentration of 2×10^6 in 200 μ L of PBS containing 2% of BSA, the

blocking buffer was added, and the cells were incubated for 30 min to 4°C. The cells were then transferred to a FACS tube; conjugated with fluorochromes specific for tracking CD3, CD4, CD8, CD19 and CD45RB; and incubated for 45 min at 4°C. The cells were washed with 2 mL of PBS containing 1% formaldehyde and kept at 4°C until the time of acquisition on a FACSCanto (TM) cytometer (Becton Dickinson, CA, USA). Thirty-thousand events were acquired from each sample. The data were analyzed using FACSDiva software.

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