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Non-invasive delivery of nanoparticles to hair follicles: A perspective for transcutaneous immunization

Ankit Mittal[a,1], Anne S. Raber[a,1], Ulrich F. Schaefer[a], Sebastian Weissmann[c], Thomas Ebensen[c], Kai Schulze[c], Carlos A. Guzmán[c], Claus-Michael Lehr[a,b], Steffi Hansen[a,b,*]

[a] Saarland University, Biopharmaceutics and Pharmaceutical Technology, Saarbruecken, Germany
[b] Helmholtz Institute for Pharmaceutical Research Saarland (HIPS), Helmholtz Centre for Infection Research (HZI), Saarland University, Saarbruecken, Germany
[c] Helmholtz Center for Infection Research Braunschweig, Department of Vaccinology and Applied Microbiology, Braunschweig, Germany

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ABSTRACT

Transfollicular vaccination aims to reach the peri-follicular antigen presenting cells without impairing the stratum corneum (SC) barrier. This would be an optimal vaccination strategy under critical hygienic conditions. Nanoparticles (NPs) are the ideal vehicles for transfollicular delivery of vaccines as they are able to (i) penetrate deeper into the hair follicles than molecules in solution, (ii) can help to stabilize protein based antigen and (iii) improve and modulate the immune response.

This study investigates the potential of transfollicular delivery of polymeric NPs using ovalbumin (OVA) as a model antigen. NPs were prepared by a double emulsion method from pharmaceutically well characterized biocompatible and biodegradable polymers poly(lactide-co-glycolide) (PLGA) or chitosan-coated PLGA (Chit-PLGA) using polyvinyl alcohol as stabilizer. The NP formulations are available as freeze dried product which can be re-constituted with water or cell culture medium before use to yield any desired OVA/NP concentration. OVA was protected from cleavage or aggregation inside the NPs and retained its biological activity to 74% (PLGA) and 64% (Chit-PLGA). Thus, when applying a typical dose of 8.5 $\mu l/cm^2$ NP formulation (50 mg NPs/ml, 54.3 ± 0.047 and 66.5 ± 0.044 μg OVA/mg NPs for PLGA and Chit-PLGA NPs, respectively) an effective dose of 17 $\mu g/cm^2$ (PLGA) or 18 $\mu g/cm^2$ (Chit-PLGA) of active OVA is administered. In a cell culture assay encapsulated OVA stimulated the proliferation of CD4+ (PLGA and Chit-PLGA) and CD8+ T-cells (only Chit-PLGA) to a larger extent than OVA in solution. An adoptive transfer experiment demonstrated that the model antigen OVA can be delivered via the transfollicular route. This preliminary experiment is a proof of concept that by this transfollicular immunization approach it is possible to deliver antigens, thereby stimulating antigen-specific T cells. Both NP formulations improved the delivery efficiency of OVA into the hair follicles on excised pig ears by a factor of 2–3 compared to OVA solution. This delivery efficiency could further be increased by increasing the number of NPs applied per skin area by a factor of ≈2–2.4.

Consequently formulation of OVA into PLGA and Chit-PLGA NPs may offer to reduce the dose which needs to be applied for transfollicular immunization.

1. Introduction

Transcutaneous immunization (TCI) refers to the needle-free application of vaccines across the skin [1]. Major benefits include the possibility of self-administration, improved patient compliance, no requirement of "sharps" waste removal, and reduced storage and transport issues. The skin is a superior organ for eliciting an immune response compared to the muscle due to the abundance of professional antigen presenting cells (APCs) such as dendritic cells (DCs) and Langerhans cells (LCs) in different layers of the skin [2,3]. In addition, TCI can generate both a humoral as well as a cellular response at the site of administration and also at distant mucosal sites [4]. Hence, TCI may allow reducing the antigen dose, as well as eliciting an improved immune response in persons with a disease- or age-compromised immune system [5].

The major challenge for TCI is to enhance the transport of antigens across the stratum corneum (SC) barrier. To this end often barrier disrupting measures are applied, such as chemical permeation enhancers, abrasion, electroporation, micro-needles, PowderJect and gene gun. [6]. In contrast, transfollicular vaccination aims to deliver antigens to the abundant perifollicular APCs

* Corresponding author at: Helmholtz Institute for Pharmaceutical Research Saarland (HIPS), Helmholtz Centre for Infection Research (HZI), Saarland University, Saarbruecken, Germany. Tel.: +49 681 302 2939; fax: +49 681 302 4677.
E-mail address: st.hansen@mx.uni-saarland.de (S. Hansen).
[1] Authors contributed equally to the manuscript.

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[7,8]. Any additional barrier disrupting measures should be expendable as the SC only extents into the upper third of the follicle. Solid NPs are the ideal vehicles for transfollicular delivery as they accumulate in hair follicles and skin folds and penetrate deeper into hair follicles than molecules in solution [9]. Transfollicular delivery has been widely investigated for DNA vaccines, and lately also for non-nucleotide based antigens such as a seasonal flu vaccine as well as nanoparticle (NP) based formulations

Nonetheless, transfollicular vaccination is usually performed after pretreating the skin area by plucking the hairs, waxing, or cyanoacrylate (superglue) stripping. This pretreatment induces the hair cycle into anagen (proliferation) state and markedly increases the transfection efficiency of follicular keratinocytes [10,11]. The pretreatment is therefore helpful for DNA vaccines, however, it may be expendable for protein or peptide based antigens. At the same time the SC is (at least partially) removed so that it is not clear to which extent the antigen penetrates via the hair follicles or across the permeabilized SC. This is especially critical as the reduced barrier increases the risk of pathogen entry which is of particular importance for transfolliclular vaccination in (i) countries with critical hygienic conditions, and (ii) immuno-compromised individuals, such as elderly people with poor wound healing and young children.

This study investigates the potential of transfollicular delivery of ovalbumin (OVA) using polymeric nanoparticles (NPs) without compromising the SC barrier by any pretreatment for the purpose of non-invasive TCI. NPs were prepared by a double emulsion method from poly(lactide-co-glycolide) (PLGA) or chitosan-coated PLGA (Chit-PLGA) and polyvinyl alcohol (PVA) as stabilizer. These are biocompatible and biodegradable polymers which are widely used for biomedical applications. The NPs were characterized physico-chemically in terms of size, surface charge, OVA encapsulation/loading and morphology. The integrity and biological activity of the encapsulated OVA was monitored by SDS page, *in vitro* proliferation of OVA-specific lymphocytes, and ELISA. The follicular delivery efficiency was measured in intact pig skin based on the differential stripping technique and compared to OVA solution [9,12]. An adoptive transfer experiment was performed to verify the usefulness of the non-invasive transfollicular immunization route *in vivo*.

To our knowledge this is the first study to evaluate the potential of transfollicular vaccination using NPs without applying SC barrier reducing measures.

2. Materials and methods

2.1. Material

EndoGrade® ovalbumin (OVA) was obtained from Hyglos GmbH (Bernried, Germany); poly(D,L-lactide-co-glycolide) (PLGA) (Resomer RG 50:50 H; inherent viscosity 0.31 dl/g) was kindly provided by Boehringer Ingelheim (Boehringer Ingelheim GmbH & Co., KG, Ingelheim, Germany); polyvinyl alcohol (PVA) Mowiol® 4–88 was obtained from Kuraray Specialties Europe GmbH (Frankfurt, Germany); ultrapure chitosan chloride salt (Protasan® UP CL113) was purchased from FMC BioPolymer AS (Oslo, Norway); trehalose was obtained from Sigma–Aldrich (St. Louis, MO, USA); fluorescein isothiocyanate conjugated ovalbumin (FITC-OVA) was obtained from Invitrogen (Molecular Probes Inc., Germany). Superglue was kindly provided by Uhu GmbH & Co., KG, (Bühl, Germany). Tesa film was obtained from TESA SE, Hamburg, Germany. The OVA specific ELISA kit was purchased from USCN life science Inc. (Hölzel Diganostik, Germany). All other solvents and chemicals used were of the highest grade commercially available. Deionized water (Milli Q Plus system, Millipore, Bedford, MA, USA) was used throughout the investigation.

2.2. Pig ear skin

Ears from freshly slaughtered pigs were removed before scalding. The ears were washed with water, blotted dry and the hairs were shortened with scissors. The ears were stored at 4–8 °C for a maximum period of 3 days.

2.3. Mice

Female BALB/c (H-2d) or C57BL/6 (H-2b) mice at the age 6–8 weeks were purchased from Harlan (Germany). OVA-TCR transgenic mice OT-I (C57BL/6-Tg(TcraTcrb)1100Mjb/J), OT-II (C57BL/6-Tg(TcraTcrb)425Cbn), and OT-IIxThy1.1 (C57BL/6-Tg(TcraTcrb)425Cbn Thy1^a) were bred at the animal facilities of Helmholtz Centre for Infection Research under specific pathogen-free conditions. The animal experiment permission was given by the local government of Lower Saxony (Germany) with the No. 33.11.42502-04-017/08.

2.4. Nanoparticles preparation and characterization

2.4.1. Nanoparticle preparation

PLGA NPs were prepared by a modified double emulsion method [13]. Briefly, 50 mg of PLGA were dissolved in 2 ml of ethyl acetate at room temperature. Then 400 μl of (1.875% wt/V) OVA solution in water was added to form a primary water-in-oil (w/o) emulsion. After sonicating at 6 W for 15 s, 4 ml of 2% (wt/V) PVA solution was added to the primary emulsion and again sonicated at 12 W for 15 s. Water was added drop-wise to the resulting w/o/w emulsion under constant stirring to allow diffusion and evaporation of the organic solvent. The particles were collected by centrifugation at 15,000 × *g* for 10 min, washed once with distilled water to remove excess surfactant and free drug and freeze dried using trehalose (0.2% wt/V) as cryoprotectant.

Chit-PLGA NPs were prepared in a similar manner as described above with the difference that Protasan® UP CL113 (0.2% wt/V) was added to the PVA solution.

FITC labelled OVA loaded NPs and FITC labelled blank NPs were prepared by replacing OVA or the PLGA polymer with their fluorescent counterparts, respectively.

2.4.2. Nanoparticle characterization

Size and ζ-potential of the NPs were analyzed by photon correlation spectroscopy (PCS) using a Nano-ZS (Malvern Instruments, Malvern, UK). The morphology of the NPs was characterized using scanning electron microscopy (SEM) (JSM 7001F Field Emission SEM (Jeol, Japan)) and atomic force microscopy (AFM) (Multimode V (Veeco, USA)). For SEM, prior to scanning, NPs were sputtered with gold (layer thickness approximately 10 nm). The accelerating voltage was 10 kV with a focal distance of 10 mm. For AFM imaging, NPs were scanned using tapping mode and scan rates of 0.6 Hz. A standard non-contact mode cantilever was used for imaging (OMCL-AC160TS, Olympus, Essex, Great Britain).

The entrapment efficiency (% EE = wt OVA encapsulated/wt OVA added initially) and loading efficiency (% L = wt OVA encapsulated/wt polymer) of OVA in PLGA and Chit-PLGA NPs were determined using a QuantiPro bicinchoninic acid (BCA) assay (Pierce, Rockford, IL, USA) according to the manufacturer's instructions for 96 micro-well plates. Briefly, 5% mg of the lyophilized particles were hydrolyzed in 0.1 N NaOH for 6 h and then neutralized with 0.1 N HCl [13]. Blank NPs were used as a control.