Strategy of topical vaccination with nanoparticles

Sascha Jung
Alexa Patzelt
Charité-Universitätsmedizin Berlin
Department of Dermatology
Center for Experimental and Applied Cutaneous Physiology
Charitéplatz 1
Berlin D-10117
Germany

Nina Otberg
University of British Columbia
Department of Dermatology and Skin Science
835 West 10th Avenue
Vancouver, British Columbia V5Z 4E8
Canada

Gisela Thiede
Wolfram Sterry
Juergen Lademann
Charité-Universitätsmedizin Berlin
Department of Dermatology
Centre of Experimental and Applied Cutaneous Physiology
Charitéplatz 1
Berlin D-10117
Germany

Abstract. Liposomes in the nanosize range have been recognized as a versatile drug delivery system of both hydrophilic and lipophilic molecules. In order to develop a liposome-based topical vaccination strategy, five different types of liposomes were tested as a putative vaccine delivery system on pig ear skin. The investigated liposomes mainly varied in size, lipid composition, and surface charge. Using hydrophilic and hydrophobic fluorescent dyes as model drugs, penetration behavior was studied by means of confocal laser scanning microscopy of intact skin and histological sections, respectively. Follicular penetration of the liposomes was measured in comparison to a standard, nonliposomal formulation at different time points. Dependent on time but independent of their different characters, the liposomes showed a significantly higher penetration depth into the hair follicles compared to the standard formulation. The standard formulation reached a relative penetration depth of 30% of the full hair follicle length after seven days, whereas amphoteric and cationic liposomes had reached ~70%. Penetration depth of negatively charged liposomes did not exceed 50% of the total follicle length. The fluorescence dyes were mainly detected in the hair follicle; only a small amount of dye was found in the upper parts of the epidermis. [DOI: 10.1117/1.3080714]

Keywords: nanoparticles; liposomes; confocal laser scanning microscopy; hair follicle; penetration; topical vaccination; drug delivery system; histological section.

Paper 08247SSR received Jul. 18, 2008; revised manuscript received Nov. 19, 2008; accepted for publication Nov. 26, 2008; published online Mar. 5, 2009.

1 Introduction

Vaccination is one of the most important instruments to fight infectious diseases. Vaccines are mostly applied by intramuscular injection. Injections, however, bare the risk of spreading infectious diseases such as HIV or hepatitis in case of improper usage.[1] According to the World Health Organization (WHO), the number of injections amounts to 12 billion per year. One-third of all injections is applied with improperly sterilized sets of instruments.[2] In Africa, more than 80% of disposable syringes are used more than once.[3,4] This holds a high risk of spreading infectious agents, especially in populations with a high prevalence of infectious diseases.[5]

Oral application of vaccines is a noninvasive alternative strategy for vaccination but reveals insurmountable hurdles. It is for this reason that the oral application against polio was short-lived, because most of the vaccines lost their function after becoming metabolized and worse, many of them functioned as tolerogens.[6] Therefore, the WHO gave precedence to the development of topical vaccination.[7,8]

The skin forms a barrier, protecting our body against environmental hazards. It contains a tight network of immune cells, particularly antigen-presenting Langerhans cells and dendritic cells with the ability to induce a specific immune response. Topical vaccination, in contrast to intramuscular or intradermal application of the vaccine, could therefore be an efficient way to activate effector-T-cells and induce an immune response.[9,10]

Topically applied vaccines have first to pass the skin barrier to reach intraepithelial T-cells and Langerhans cells.[11] Hair follicles were shown to be a promising target for transcutaneous vaccination,[12] as they are rich in immune-competent cells in the infundibular part of the root sheath and around the excretory duct of the sebaceous gland.[11] Hair follicles offer a considerable penetration pathway and potential intracutaneous reservoir for topically applied substances,[13] which makes them an important target for topical vaccination.

For the development of a topical vaccination strategy, a suitable drug delivery system is essential. Nanoparticles appeared to address this issue excellently. Toll et al. investigated the follicular penetration of nanoparticles at different diameters.[14] Particles with a diameter of 600 nm showed deeper penetration into the hair follicles than larger sized particles. Lademann et al., compared the penetration behavior of fluorescent dyes into porcine skin, comparing nanoparticles (320 nm) to a nonparticle formulation.[15] Massage, subsequent to application, resulted in a five-fold increase in follicular penetration depth of the fluorescent dye nanoparticles compared to its nonparticle counterpart. This observation was verified by *in vivo* studies on human skin. Additionally, the *in vivo* studies showed a remarkable follicular reservoir of the dye when applied as nanoparticles. This reservoir was still

Address all correspondence to: Sascha Jung, Charité-Universitätsmedizin Berlin, Department of Dermatology, Center for Experiment and Applied, Cutaneous Physiology, Charitéplatz 1, D-10117 Berlin. Tel.: +49 30 450 518 100; Fax: +49 30 450 518 918; E-mail: saschajung.1@web.de.

1083-3668/2009/14(2)/021001/7/$25.00 © 2009 SPIE

Table 1 Characteristics of the investigated liposome types.

Sample No.	Diameter (nm)	Lipid content of stem solution (mg/mL)	Dye content (mass %)	Liposome type	Charge at pH 7.5	Polydispersity	Zeta-potential at pH 7.5
1	231.4	33.88	0.2	Anionic	Negative	0.05	−27.3 mV
2	448.6	30.14	0.25	Cationic	Positive	0.34	+41.7 mV
3	234.3	92.3	0.38	Anionic	Negative	n.d.	n.d.
4	319.4	26.3	0.16	Amphoteric	Negative	0.25	−13.6 mV
5	253.7	39.1	0.17	Cationic	Positive	0.08	+23.6 mV

detectable 10 days after application.[15] A reasonable explanation for this effect is the gear-pump mechanism, whereby the hair shaft acts like a pump. The particle is pushed deeper into the follicle by the movement of the hair and trapped between the cuticula cells of the hair shaft and the inner root sheath. The mechanism works ideally for particles with a diameter similar to the cuticle cells, which is measured to be approximately 500 nm in human hair.[15–17]

An ideal drug delivery system into hair follicles would therefore be constituted by nanoparticles ranging between 100 and 500 nm[15,17] that can penetrate deeply into the hair follicle and release their cargo, i.e., the vaccine, close to the immune cells. Liposomes as vesicular nanoparticles are an excellent vehicle system for vaccines and therefore a promising drug delivery system for topical vaccination.

The present study addressed the issue of cutaneous and follicular penetration of liposomes. Because of its high resemblance to human skin, regarding structure and distribution of the hair follicles in particular, the experiments were carried out on pig ear skin. Investigated liposomes, which were different in size and other properties such as lipid composition and surface charge, had been prepared from special lipid mixtures, exerting a beneficial effect on their loading rate, membrane stability, cell transfection rate, and membrane surface charge potential. In particular, one of the studied liposome types represents a novel class of liposomes which has the possibility to shift its surface charge depending on the environmental pH. Confocal laser scanning microscopy of intact skin and of histological longitudinal sections was used to determine follicular penetration depth.

2 Materials and Methods

Five different types of liposomes containing a fluorescent dye were provided by Novosom AG (Halle, Germany). Whereas liposome types 1, 2, 4, and 5 enclosed the hydrophilic fluorophore carboxyfluoresceine in their inner hydrophilic compartment, liposome type 3 was prepared with curcumin. Because of its intense hydrophobic character, curcumin was inserted in the membrane of the liposome instead of being enclosed in the inner hydrophilic compartment.

2.1 *Preparation of Liposomes*

Anionic liposomes in sample 1 were prepared from a mixture of dipalmitoylphosphatidylcholine, dipalmitoylphosphatidylglycerole, and cholesterol (50:10:40 mol%). Samples 2 and 5 consisted of cationic liposomes, which were prepared from a mixture of dipalmitoylphosphatidylcholine, 3β-[*N*-(dimethylaminoethane) carbamoyl] cholesterol and cholesterol (50:10:40 mol%), or dimyristoylphosphatidylcholine, *N*-(2,3-dioleoyloxy-1-propyl) trimethylammonium methyl sulfate and cholesterol (50:10:40 mol%), respectively. Amphoteric liposomes in sample 4 were constructed from a lipid mixture of dimyristoylphosphatidylcholine, *N*(α)-L-histidinyl-cholesterolhemisuccinate, and cholesterol (50:10:40 mol%).

In brief, lipid mixtures of the corresponding liposome types were dissolved in chloroform/methanol (3:1). The solvent was evaporated in a rotary evaporator, leading to a thin lipid film. Carboxyfluoresceine (molecular probes) was dissolved at 100 mM in 10 mM *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulpfonic acid] and 150 mM NaCl, and pH was readjusted to 7.5 using sodium hydroxide. Lipid films were hydrated at 60 °C with 2 mL of the carboxyfluoresceine solution with a total lipid concentration of 50 mM in the suspension. The suspensions were frozen and thawed in triplicate and eventually extruded through polycarbonate filters of 400 nm nominal pore width. Nonencapsulated carboxyfluoresceine was removed by gel filtration using PD10 columns (Amersham Pharmacia) in accordance with the instructions of the supplier. Each liposome type was dispersed in a 10 mM *N*-[2-hydroxyethyl] piperazine-*N*′-[2-ethanesulpfonic acid] buffered stock solution (pH 7.5) also containing 150 mM NaCl. Characteristics of the liposome types tested are shown in Table 1. The composition of the carboxyfluoresceine-containing standard formulation that was provided by the Institute of Pharmaceutical Technology and Biopharmacy (Martin-Luther-University, Halle-Wittenberg, Germany) is summarized in Table 2.

2.2 *Preparation of the Model Tissue*

High numbers of biopsies had to be taken. Therefore, pig ear skin served as a model tissue due to its high resemblance to

Table 2 Composition of the standard formulation with carboxyfluoresceine.

Glycerolmonostearate 60	4%
Cetylalcohol	6%
Triglycerides	7.5%
White vaseline	25.5%
Macrogol-20-glycerolmonosterate	7%
Propyleneglycol	10%
Water	39.5%
Carboxyfluoresceine	5%

human skin. The ears were acquired from female pigs (*sus domesticus*) provided by a local butcher. The experiments started less than 5 h after slaughtering. The ears were rinsed carefully with clear water and swabbed with paper towels. Hairs were clipped to a maximal length of 1 cm. Throughout the whole test period of seven days, the ears were stored at 5 °C. The test formulations were applied to the back of the ear; previous studies have shown that this area correlates best to human skin.[18,19] The application areas were free of structural inhomogeneities like scratches, erosions, or scars, as such skin damages could affect the penetration procedure.[20]

2.3 *Preparation of the Test Areas and Application of Samples*

Two test areas (4×6 and 4×2 cm^2) per formulation tested were marked by a barrier (ART-GLASSs by WACOs; peelable dye; Heinrich Wagner, Zurich, Switzerland) on the dorsal side of the pig ears. The barrier was necessary to prevent lateral spreading of the applied formulations. A 0.02 mL sample of the liposome stem solutions, 2 mg of the standard formulation, respectively, was applied per 1 cm^2 of skin. After application, the samples were spread homogeneously with a spatula. Skin biopsies (5×5 mm) were taken at 1 hour, three, five, and seven days after application, each from a different subdivision of the larger test area 1. Another subdivision of this area also served for investigation by noninvasive confocal laser scanning microscopy.[21–24] The smaller test area 2 was used to take cyanoacrylate skin surface biopsies to measure the amount of liposomes in the hair follicle infundibulum. Cyanoacrylate skin surface biopsies were analyzed by laser scanning microscopy. Figure 1(a) illustrates the arrangement of the test areas on the pig ear.

2.4 *Skin Sectioning and Laser Scanning Microscopy of the Histological Longitudinal Sections*

To guarantee an adequate number of hair follicles for the analysis of penetration, five skin biopsies per subdivision were taken as shown in Fig. 1(b). After a seven-day test period, 20 samples had been taken from each test area. For the determination of the penetration depth, cryohistological cross sections were prepared (skin-sectioning device: SLEE-

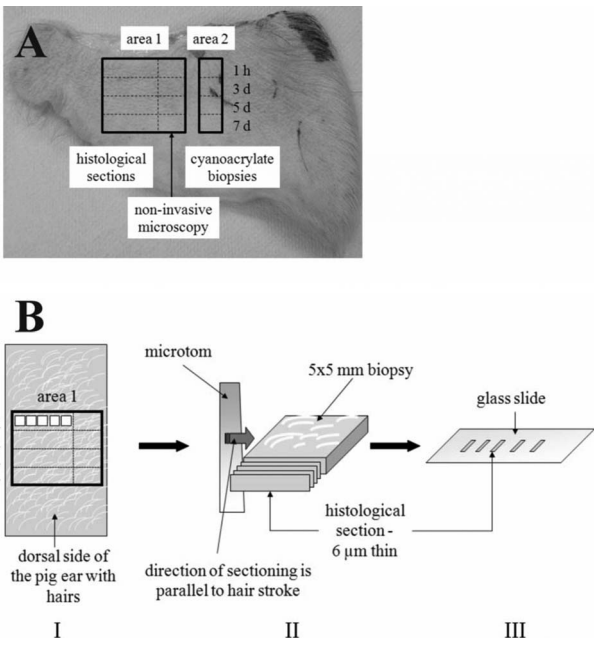

Fig. 1 Experimental procedure. (a) Schematic representation of the arrangement of the application areas on the pig ear. (b) Schematic representation of the preparation of histological sections from biopsies taken 1 h after application. I. Five biopsies were taken from the application area. II. From the biopsies histological sections were prepared. III. The histological sections were collected on a glass slide.

Kryostat MTE, SLEE Technik GmbH, Mainz, Germany; embedding medium: Killiks—cryostat embedding medium, Bio Optica, Milano, Italy; microscope slides, R. Langenbrinck, Emmendingen, Germany). Figure 1(b) shows a scheme of this procedure.

Due to autofluorescence within a range of 520–560 nm exhibited by porcine ear skin, measurements were carried out in the spectral range between 590 and 680 nm using a laser scanning microscope (LSM 410 invert, Zeiss, Germany). Therefore, interference of signals coming from autofluorescence and from fluorophores of the liposomes could be excluded, as verified by skin samples which had been taken from outside the test areas.

The length of every single hair follicle in each skin biopsy and the penetration depth of the applied samples, respectively, were measured by means of a digital image analysis and a special software program (confocal scanning laser microscope, software LSM 410 invert, Zeiss, Jena, Germany). The distinct position of the liposomes and the standard formulation were determined by superimposing the images of fluorescence and light microscopy.

This study was approved by the Ethics Committee of the Charité- Universitätsmedizin, Berlin, Germany.

For statistical analysis, Mann and Whitney's U-test for the comparison of two variables and Kruskal and Wallis's H-test for the comparison of more than two variables and SPSS software (SPSS, Chicago, IL, USA) were utilized.

2.5 *Noninvasive Investigation of Intact Skin and of Cyanoacrylate Biopsies by Confocal Laser Scanning Microscopy*

In addition to the invasive analysis using histological skin sections, noninvasive measurements based on *in vivo* laser

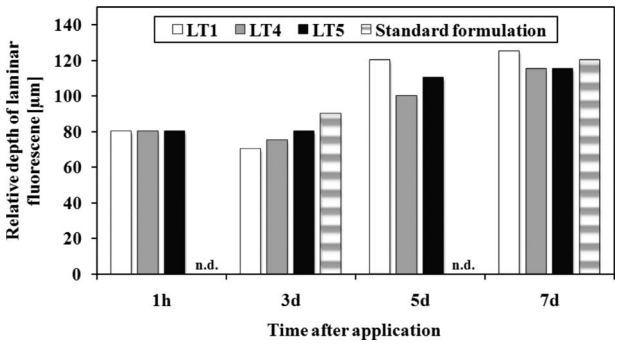

Fig. 2 Cutaneous penetration. Depicted are the relative depths of LT1, LT4, and LT5, and of the standard formulation, respectively, at which a laminar fluorescence signal occurred. Measurements were carried out at 1 h, three, five, and seven days after application. Relative depth values at 1 h and 5 days after application of the standard formulation were not determined.

scanning microscopy were used to investigate the distribution of the dye-containing liposomes in the epidermis and in the hair follicles.

Investigations were carried out with the confocal laser scanning microscopy system "stratum" (OptiScan, Ltd., Melbourne, Australia). An argon laser at 488 nm was used to excite the fluorescence signal. The system and its application have been described in detail by Lademann et al.[25]

Cyanoacrylate biopsies were taken according to the method described by Teichmann et al.[26] In brief, a drop of cyanoacrylate (Uhu GmbH & Co., KG, Brühl, Germany) is placed on the surface of the treated skin and covered with a glass slide under slight pressure. After polymerization (approx. 5 min), the cyanoacrylate is strongly linked with the upper layers of the stratum corneum, the hair shafts, and the casts of follicular infundibula. After removal, the cyanoacrylate surface biopsies were analyzed by LSM (LSM 410 invert, Zeiss, Germany).

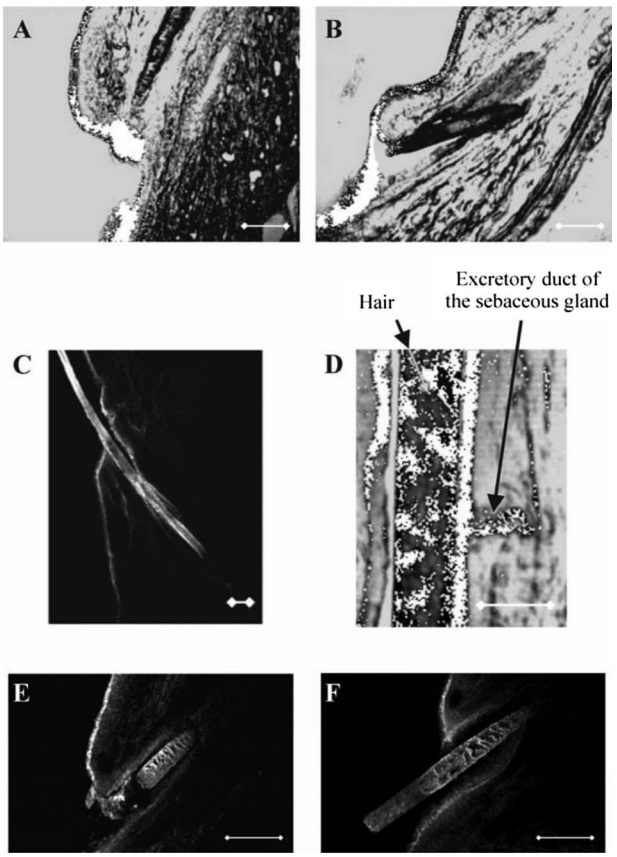

Fig. 3 Fluorescence imaging. (a) (b), Superimposed images depict histological sections of hair follicles taken at day 3 after application of LT3, measured by fluorescence and light microscopy. (c) Fluorescence image of a histological section taken at day 7 after application of LT4. (d) Superimposed image depicts closeup of a hair follicle of a histological section taken three days after application of LT2. (e) (f) Fluorescence images of histological sections taken at day 7 after application of LT4. Bars=100 μm.

3 Results

Noninvasive measurements of the cutaneous penetration process by confocal laser scanning microscopy was carried out for liposome types 1, 4, and 5, and for the standard formulation, respectively, using intact skin. As shown in Fig. 2, 1 h after application of the three liposome samples, the depth of the laminar fluorescence, which marks the plane of highest sample concentration relative to the measurement tip of the microscope, amounted to 80 μm. This depth was used as a reference marked by the dashed line in Fig. 2 to assess whether cutaneous penetration had occurred. Whereas at day 3 all formulations showed similar values of the relative depth of the laminar fluorescence compared to the reference values 1 h after application (dashed line), the liposome samples clearly showed increased depths at day 5 which ranged between 20 and 40 μm. Finally, at day 7 after application, both the liposome samples and the standard formulation showed a similar and significant increase in their relative penetration depth, ranging between 35 and 45 μm compared to the reference value (dashed line).

The present study focused mainly on the investigation of the follicular penetration of the applied liposomes. Twenty-four thousand histological longitudinal sections were prepared. Eight hundred and seventy-one of these sections contained a complete parallel section of a hair follicle, which were analyzed by confocal laser scanning microscopy. Fluorescence was detected in 84% (=735) of the hair follicles. The remaining 136 sections did not show a fluorescence signal in the hair follicle but in the stratum corneum. The access to the follicle depicted in these two figures seems to be impeded by structures at the top of the infundibulum [Figs. 3(a) and 3(b)].

Figure 3(c) shows one of the 871 hair follicles that contained a fluorescence signal. In this follicle the fluorescence signal reached down to the bulbus pili. As already seen in Figs. 3(a) and 3(b), a significant fluorescence signal in the stratum corneum can also be observed. In cases where the section hit the excretory duct of the sebaceous gland, a continuation of the fluorescence signal inside the duct was found [Fig. 3(d)].

Figures 3(e) and 3(f) show fluorescence images of a hair follicle that was sliced in a slantwise manner. Therefore, one can clearly observe a distribution of the fluorescence signal along the edges of the cuticula.

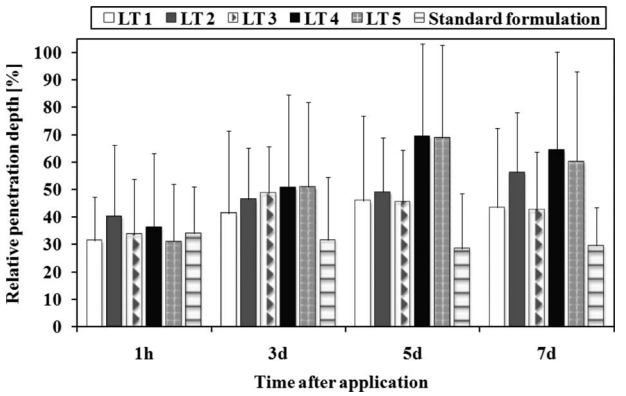

Fig. 4 Survey of the mean relative penetration depth values of the tested formulations.

At least 20 follicles were evaluated for each formulation and at each time point, respectively. The measured absolute penetration depths were normalized by their relation to the full length of the corresponding follicle, resulting in a relative penetration depth value expressed in percent. The relative penetration depth allowed the comparison of the penetration depth within follicles of different lengths.

Figure 4 summarizes the mean values of the relative penetration depth for all tested formulations. One hour after application, all formulations had reached a relative penetration depth ranging between 30 and 40%. Whereas the liposomes showed a significant increase in penetration, the mean relative penetration depth of the standard formulation stagnated during the following six days of monitoring the penetration process.

The highest relative penetration depths with mean values up to 69% were reached by liposome types 4 and 5. The maximum penetration of these two liposome types was reached at day 5. This was also the case for liposome types 1, which reached a penetration depth of 45%. At day 7, the mean relative penetration depth values of the three liposome types decreased in comparison with the previous measurements. In contrast, liposome type 2 showed a continuous increase in penetration, reaching its maximum of 55% at day 7 after application. Liposome type 3 showed a maximum of the relative penetration depth at day 3 after application, at approx. 48%.

The method of cyanoacrylate surface skin biopsies functioned unsatisfactorily. From 21 biopsies only one vellus hair root with a total length of 550 μm was gained (Fig. 5). However, this hair root clearly showed that liposomes were located between the hair root sheath and the hair shaft along the entire follicle, whereas the cutaneous penetration was restricted to the stratum corneum. The deeper epidermis did not show a fluorescence signal.

4 Discussion

Liposomes showed a significantly higher penetration at the follicular path compared to a standard formulation. At the cutaneous path, the penetration of both the liposome samples and the standard formulation were of similar dimensions. Although there is an increase in the cutaneous penetration depth, it was restricted to the stratum corneum, as verified by the histological sections (Fig. 3) and the cyanoacrylate surface

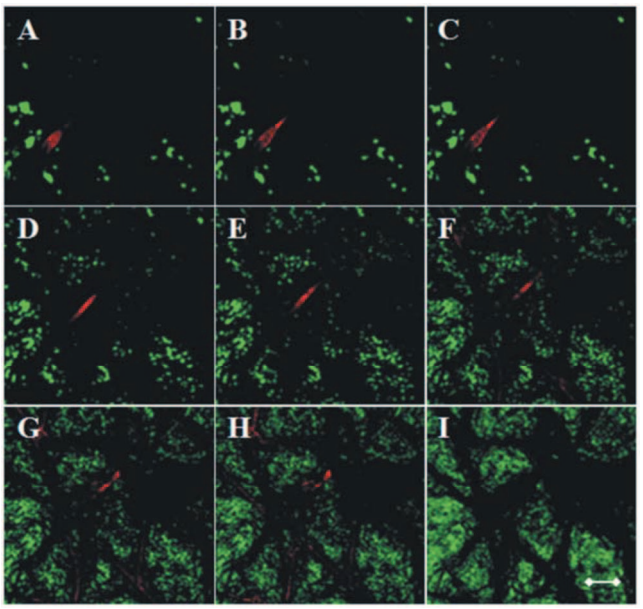

Fig. 5 Fluorescence imaging of a cyanoacrylate surface skin biopsy. Depicted is a series of superimposed images all recorded at a different depth starting at (a) the highest depth or the bulbus pili of the hair, respectively. Total length of the hair root amounted to 550 μm. Sample was taken at day 5 after application of LT1. Bar=200 μm.

skin biopsy (Fig. 5). Therefore, the cutaneous penetration path where topically applied samples must cross the stratum corneum would be less effective regarding the development of topical vaccination strategy, because the applied vaccine would never reach the immune cells, which are located in subepidermal skin layers.

As liposomes penetrated deeply into the hair follicles, this penetration path seems to play a major role in the development of a nanosized liposome-based topical vaccination strategy. Dependent on the liposome type, mean relative penetration depths of 50 to around 70% of the full length of the hair follicle were reached. Therefore, nanosized liposomes would be able to reach the immune-competent cells which are accumulated around the hair follicles. However, comparison of the penetration behavior of the tested liposome types revealed considerable differences regarding the time point at which the maximum of penetration was reached, as well as the dimension of the penetration depth.

The highest penetration depths were reached by the amphoteric liposome type (LT4) and one of the cationic liposome types (LT5). The isoelectric point of the amphoteric liposome type lies at a slightly acidic pH, and because porcine skin shows a pH of 6–7,[27] its surface was presumably neutral or slightly anionic under experimental conditions. The lowest penetration depth maximum was observed for negatively charged liposomes, like liposome type 1. Therefore, it can be concluded that the surface charge plays an important role in the penetration behavior of a given liposome type.

One can further speculate about the effect of surface charge for the penetration of nanoparticles because the skin itself and the hair are negatively charged on their surface. Therefore, the electrostatic interactions with cationic nanoparticles might consequently lead to high local concentrations,

whereas negatively charged particles might be rejected. It remains to be analyzed whether the electrostatic has a beneficial or inhibiting effect. Nevertheless, this perspective might be helpful in the interpretation of the superior penetration of the amphoteric liposomes. The electrostatic effect may have drawn the liposome in penetration direction. Another parameter that may affect the penetration behavior of the investigated liposomes is the phase transition temperature of the lipid types used for synthesis. Liposome types 4 and 5, which reached the highest penetration depths, are based on the lipid dipalmitoylphosphatidylcholine, which has a T_m of 23 °C. This will constitute a more flexible bilayer structure, whereas the liposome types 1 and 2 may be more rigid. Latter ones comprise large amounts of dipalmitoylphosphatidylcholine with a T_m of 41 °C. Higher flexibility may be an advantage within the narrow slot between the hair root sheath and the hair shaft, which depicts the penetration path as verified by the vellus hair gained by cyanoacrylate biopsy (Fig. 5). Penetration of liposomes being of more rigid character may be retarded due to spatial limitations.

No correlation was found between the diameters of the investigated liposome types and their maximum penetration depths. This may be caused by the slight differences in the diameter of the different liposomes. For bigger differences in the diameter of the liposomes, differences in the penetration depths could be expected. Five out of six of the investigated formulations, including the standard formulation, showed a decrease in the penetration depth with time. An explanation for this phenomenon may be a drying process of the skin tissue. As the hair follicles are surrounded by a tight reticulum of connective tissue, the formulation may have been pressed out due to the relaxation of these fibers, which is induced by the decrease in the overall tension of the skin.

All liposomes investigated possess high stability in serum, but nothing is known about their stability in sebum. However, there is evidence to assume that the main portion of the liposomes remained intact during penetration. First of all, the fluorescence pattern in the micrographs is punctuated and uneven (Fig. 3). Secondly, most samples contained carboxyfluorescein, which, in case of release, may not penetrate in sebum. Otherwise, its fluorescence may be quenched because of the low pH of the environment.

The infundibula of human hair follicles ranges between 25 and 33% of the total follicle length.[28] Considering the high penetration depth (up to 69%) in pig ear skin and the smaller absolute length of the human hair follicle, all applied liposome formulations had the potency to act as a follicular drug delivery system in human skin. As penetration reached beyond the infundibulum, this could for several reasons be advantageous for the application of substances that are designed to provoke an immune response. First of all, the immune cells are located in the region ranging from below the infundibulum to the bulbus pili. In can be assumed that the deeper the penetration of vaccine containing liposomes, in particular, beyond the extent of the infundibulum, the higher the immune response. Otherwise, liposomes, which are restricted to the infundibulum, may induce a much weaker immune response. Additionally, these liposomes are exposed to the clearance by the continuous stream of sebum from the outlet of the sebaceous gland toward the follicular orifice. This is no longer of interest once the liposomes penetrated below the confluence of the excretory duct. A further point that must be addressed is the observed attachment of liposomes to the cuticula of the hair shaft [Figs. 3(e) and 3(f)]. On the one hand, this interaction might slow down the penetration process. On the other hand, the interaction may decelerate the clearance of the liposomes by the sebum stream.

Although penetration is a necessary condition for an effective vaccination, this is not sufficient. More important, the vaccine must reach the antigen-presenting cells. Amphoteric liposomes and cationic liposomes can mediate the intracellular delivery of antigens. Further studies will detail the effect on the immune response.

5 Conclusion

In summary, in the present study a methodology was established to assess the strategy of topical vaccination using nanosized liposomes. Interfollicular penetration through the stratum corneum seems to play a minor role for the tested liposomes, whereas follicular penetration of the liposomes seems to be the main route. Liposomes penetrate deeper into hair follicles than a standard formulation. Therefore, they could increase the transfollicular drug uptake. The highest efficacy of follicular penetration is exhibited by liposomes owning the following set of attributes: a positively charged or unloaded surface and a phase transition temperature, which is in the range of the skin temperature or below. Liposomes with a constant high anionic surface charge might be less suitable for follicular drug delivery.

Acknowledgments

The authors thank Novosom AG, in particular Dr. Steffen Panzner, for providing the liposome samples and Professor R. Neubert from the Institute of Pharmaceutical Technology and Biopharmacy, Martin-Luther-University Halle-Wittenberg, Germany, for committing the standard formulation with carboxyfluoresceine. Additionally, we thank the Foundation "Skin Physiology" of the Donor Association for German Science and Humanities for financial support.

References

1. M. J. Choi and H. I. Maibach, "Topical vaccination of DNA antigens: topical delivery of DNA antigens," *Skin Pharmacol. Appl. Skin Physiol.* **16**, 271–282 (2003).
2. M. Naghipour, T. Nakagomi, and O. Nakagomi, "Issues with reducing the rotavirus-associated mortality by vaccination in developing countries," *Vaccine* **26**, 3236–3241 (2008).
3. M. Naghipour, T. Nakagomi, and O. Nakagomi, "Issues with reducing the rotavirus-associated mortality by vaccination in developing countries," *Vaccine* **26**, 3236–3241 (2008).
4. I. Toida and S. Nakata, "Severe adverse reactions after vaccination with Japanese BCG vaccine: a review," *Kekkaku* **82**, 809–824 (2007).
5. M. Ushio, "Amendment of tuberculosis prevention law and prospect of tuberculosis control program," *Kekkaku* **80**, 541–546 (2005).
6. A. A. El-Ghorr, R. M. Williams, C. Heap, and M. Norval, "Transcutaneous immunisation with herpes simplex virus stimulates immunity in mice," *FEMS Immunol. Med. Microbiol.* **29**, 255–261 (2000).
7. M. J. Choi, J. H. Kim, and H. I. Maibach, "Topical DNA vaccination with DNA/lipid based complex," *Curr. Drug Deliv.* **3**, 37–45 (2006).
8. R. E. Thomas, "Preparing patients to travel abroad safely, Part 2: Updating vaccinations," *Can. Fam. Physician* **46**, 646–646 (2000).
9. C. J. Clements, G. Larsen, and L. Jodar, "Technologies that make administration of vaccines safer," *Vaccine* **22**, 2054–2058 (2004).
10. C. M. Huang, "Topical vaccination: The skin as a unique portal to adaptive immune responses," *Springer Semin Immunopathol* **29**, 71–80 (2007).

11. T. Christoph, S. Muller-Rover, H. Audring, D. J. Tobin, B. Hermes, G. Cotsarelis, R. Ruckert, and R. Paus, "The human hair follicle immune system: Cellular composition and immune privilege," *Br. J. Dermatol.* **142**, 862–873 (2000).
12. R. M. Hoffman, "Topical liposome targeting of dyes, melanins, genes, and proteins selectively to hair follicles," *J. Drug Target.* **5**, 67–74 (1998).
13. J. Lademann, N. Otberg, H. Richter, U. Jacobi, H. Schaefer, U. Blume-Peytavi, and W. Sterry, "Follicular penetration. An important pathway for topically applied substances," *Hautarzt* **54**, 321–323 (2003).
14. R. Toll, U. Jacobi, H. Richter, J. Lademann, H. Schaefer, and U. Blume-Peytavi, "Penetration profile of microspheres in follicular targeting of terminal hair follicles," *J. Invest. Dermatol.* **123**, 168–176 (2004).
15. J. Lademann, H. Richter, U. F. Schaefer, U. Blume-Peytavi, A. Teichmann, N. Otberg, and W. Sterry, "Hair follicles-A long-term reservoir for drug delivery," *Skin Pharmacol. Appl. Skin Physiol.* **19**, 232–236 (2006).
16. J. Lademann, H. Schaefer, N. Otberg, A. Teichmann, U. Blume-Peytavi, and W. Sterry, "Penetration of microparticles into human skin," *Hautarzt* **55**, 1117–1119 (2004).
17. J. Lademann, H. Richter, A. Teichmann, N. Otberg, U. Blume-Peytavi, J. Luengo, B. Weiss, U. F. Schaefer, C. M. Lehr, R. Wepf, and W. Sterry, "Nanoparticles—An efficient carrier for drug delivery into the hair follicles," *Eur. J. Pharm. Biopharm.* **66**, 159–164 (2007).
18. W. Meyer and N. H. Zschemisch, "Skin layer thickness at the ear of the domesticated pig, with special reference to the use of the ear integument for human dermatological research," *Berl Munch Tierarztl Wochenschr* **115**, 401–406 (2002).
19. W. Meyer, "Comments on the suitability of swine skin as a biological model for human skin," *Hautarzt* **47**(3), 178–182 (1996).
20. H. Schaefer and J. Lademann, "The role of follicular penetration. A differential view," *Skin Pharmacol. Appl. Skin Physiol.* **14**(Suppl 1), 23–27 (2001).
21. H. Kandárová, H. Richter, M. Liebsch, and J. Lademann, "Stratum corneum architecture of reconstructed human skin models monitored by fluorescent confocal laser scanning microscopy," *Laser Phys. Lett.* **4**(4), 308–311 (2007).
22. J. Lademann, A. Patzelt, M. Darvin, H. Richter, C. Antoniou, W. Sterry, and S. Koch, "Application of optical non-invasive methods in skin physiology," *Laser Phys. Lett.* **5**, 335–346 (2008).
23. J. Lademann, H. Richter, S. Astner, A. Patzelt, F. Knorr, W. Sterry, and C. Antoniou, "Determination of the thickness and structure of the skin barrier by in vivo laser scanning microscopy," *Laser Phys. Lett.* **4**, 311–315 (2007).
24. A. Teichmann, H. Sadeyh Pour Soleh, S. Schanzer, H. Richter, A. Schwarz, and J. Lademann, "Evaluation of the efficacy of skin care products by laser scanning microscopy," *Laser Phys. Lett.* **3**(10), 507–509 (2006).
25. J. Lademann, H. Richter, N. Otberg, F. Lawrenz, U. Blume-Peytavi, and W. Sterry, "Application of a dermatological laser scanning confocal microscope for investigation in skin physiology," *Laser Phys.* **13**, 756–760 (2003).
26. A. Teichmann, U. Jacobi, M. Ossadnik, H. Richter, S. Koch, W. Sterry, and J. Lademann, "Differential stripping: Determination of the amount of topically applied substances penetrated into the hair follicles," *J. Invest. Dermatol.* **125**, 264–269 (2005).
27. W. Meyer and N. H. Zschemisch, "Skin layer thickness at the ear of the domesticated pig, with special reference to the use of the ear integument for human dermatological research," *Laser Phys.* **115**, 401–406 (2002).
28. A. Stevens and A. Lowe, *Histologie*, pp. 356–357, VCH Verlagsgesellschaft mbH, Weinheim (1992).