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Minireview

Nanoparticles for transcutaneous vaccination

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Summary

The living epidermis and dermis are rich in antigen presenting cells (APCs). Their activation can elicit a strong humoral and cellular immune response as well as mucosal immunity. Therefore, the skin is a very attractive site for vaccination, and an intradermal application of antigen may be much more effective than a subcutaneous or intramuscular injection. However, the stratum corneum (SC) is a most effective barrier against the invasion of topically applied vaccines. Products which have reached the stage of clinical testing, avoid this problem by injecting the nano-vaccine intradermally or by employing a barrier disrupting method and applying the vaccine to a relatively large skin area. Needle-free vaccination is desirable from a number of aspects: ease of application, improved patient acceptance and less risk of infection among them. Nanocarriers can be designed in a way that they can overcome the SC. Also incorporation into nanocarriers protects instable antigen from degradation, improves uptake and processing by APCs, and facilitates endosomal escape and nuclear delivery of DNA vaccines. In addition, sustained release systems may build a depot in the tissue gradually releasing antigen which may avoid booster doses. Therefore, nanoformulations of vaccines for transcutaneous immunization are currently a very dynamic field of research. Among the huge variety of nanocarrier systems that are investigated hopes lie on ultra-flexible liposomes, superfine rigid nanoparticles and nanocarriers, which are taken up by hair follicles. The potential and pitfalls associated with these three classes of carriers will be discussed.

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Introduction

Infections are responsible for approximately one-third of all deaths occurring each year in the world (World Health Organization, 2008). Many of these are due to the lack efficient prophylaxis and treatment owing to the unavailability of vaccines and antibiotics or due to the development of drug resistances. In addition, infectious agents are also directly involved in the pathogenesis of many malignant and chronic diseases (Apple *et al.*, 1994; Inman, 2006; Antonelli *et al.*, 2009; Ferreri *et al.*, 2009; Sagaert *et al.*, 2010). Furthermore, infections are often the final cause of death in patients affected by other primary afflictions (e.g. trauma, cardiovascular or respiratory syndromes). Thus, it is of upmost importance to develop strategies for preventing and treating infectious diseases. In this context, vaccines are the most cost-efficient tool to prevent infections. Moreover, their therapeutic application for both infectious and non-infectious diseases, including cancer, chronic auto-immune disorders and neurodegenerative diseases, is attracting general interest (Weiner and Selkoe, 2002; Zur Hausen, 2002; Meyer-Olson and Witte, 2011).

Classical vaccines were based on live attenuated or inactivated pathogens. However, due to their complex and ill-defined nature, such vaccines can vary in quality from batch to batch and can induce adverse events. In recent years we have seen the advent of subunit vaccines. These preparations are based on the use of well-defined subcellular components of the corresponding pathogen, which are in turn critical for the stimulation of a protective response. Subunit vaccines can be prepared with native components derived from the pathogen or obtained by DNA recombinant technologies or *in vitro* synthesis (e.g. recombinant proteins, synthetic peptides, capsular polysaccharides, etc.). Classical vaccines were traditionally very immunogenic, due to the complex nature of these formulations and the presence of pathogen-derived components with built-in adjuvant properties. In contrast, purified components are usually very poor immunogens rendering essential the incorporation of adjuvants in the formulation. Adjuvants do not only allow to improve the

overall strength of the elicited responses but also to reduce the amount of antigen needed and the time required to achieve a threshold of protective immunity. Furthermore, adjuvants can modulate the quality and expand the breadth of the elicited response for example with regard to the balance between T helper cell populations 1 or 2 (Th1, Th2) that is activated. Finally, adjuvants enable the stimulation of long-lasting memory responses, thereby reducing the need for frequent boost vaccinations.

Most of the traditional vaccines have been administered via the parenteral route by subcutaneous (s.c.) or intramuscular (i.m.) injection. However, the use of this route is associated with lack of acceptance by the public and safety issues (e.g. risk of contamination). It also requires skilled health personnel, which in turn represents a logistic constraint. In addition, s.c. or i.m. injections do not deliver the vaccine optimally to antigen presenting cells (APCs), which are the relevant target to prime naïve T cells to initiate an efficient adaptive immune response. In fact, only a limited number of APCs are present in the muscle. In contrast, the skin possesses a very rich immune network, which includes the epidermal keratinocytes (KCs) and Langerhans cells (LCs), dermal fibroblasts (FBs), dendritic cells (DCs) and mast cells (MCs), as well as local draining lymph nodes with T and B lymphocytes (T cells, B cells) and afferent and efferent lymph channels. Targeting the skin immune system is possible either by transcutaneous immunization (TCI), which refers to the needle-free topical application of a vaccine with or without an adjuvant, or by intradermal immunization (IDI), i.e. by direct antigen administration into the dermis (Glenn *et al*., 2000). Both ways of antigen application can be summarized as cutaneous immunization (CI). CI offers several benefits that have recently been reviewed (Karande and Mitragotri, 2010). Specifically, CI can induce strong humoral as well as cellular responses. In fact, the response is usually not restricted to the site of vaccination but can also be observed at distant mucosal sites (Babiuk *et al*., 2000; Martin Mdel *et al*., 2010). Therefore, it is highly attractive to target vaccines to the skin immune system as it can be effective against diseases that manifest within the skin (e.g. smallpox) but also respiratory, enteric (e.g. influenza, bacterial diarrhoea) or sexually transmitted diseases (e.g. HIV, Hepatitis). This can be achieved at much smaller doses than would be needed with deeper s.c. or i.m. injections with obvious benefits as more people can be vaccinated with the same amount of vaccine (Glenn and Kenney, 2006). This is especially attractive in the view of epidemics or pandemics, which threaten the health of many as recently with the outbreaks of swine-flu, avian flu or severe acute respiratory syndrome (SARS).

At the same time, the skin provides a nearly perfect barrier against the invasion of pathogens as well as against topically applied therapeutics and vaccines. The most important barrier is the horny layer or SC due to (i) the close and overlapping alignment of the flat cornified cell layers, which greatly increases intercellular diffusion path length (Talreja *et al*., 2001); (ii) the unusual lipid composition and organization (Wertz and van den Bergh, 1998; Bouwstra *et al*., 2000; Norlen *et al*., 2008; van Smeden *et al*., 2011); and (iii) the poor permeability of the corneocytes, which is not fully understood to date (Hansen *et al*., 2009). Pathways for passive diffusion of small molecules have been identified, among them the intercellular and intracellular pathways, as well the *trans*-appendageal pathway via the hair follicles and pores (Mitragotri, 2003; Hansen *et al*., 2008). Still, healthy skin will be largely impermeable to all but moderately lipophilic (octanol water partition coefficient logP 2–3) and relatively small molecules (< 500 Da) (Bos and Meinardi, 2000). Based on these limitations overcoming the SC barrier is probably the biggest issues in CI. Vaccine antigens are large, complex, and usually very hydrophilic molecules, which do not meet the above criteria for passive diffusion across the SC. Likewise, adjuvants are a very heterogeneous group of compounds (e.g. lipids, short nucleotides, surfactants) and more complex preparations (e.g. emulsions, virosomes, alum gels) that similarly face permeability problems. Further challenges are due to the fact that purified antigens are highly unstable when applied in their native state.

Therefore, innovative delivery strategies are urgently needed, which enable antigen stabilization, facilitate permeation, control release and effectively deliver the antigen to the APCs or achieve a transfection of the target cells. At the same time the delivery system should be biocompatible, biodegradable and producible in high quantities at low cost. Nanotechnology holds great promise for drug delivery, especially in the context of vaccination. Particle mediated delivery systems can efficiently incorporate or bind antigen and adjuvants. Also, some materials used for making nanocarriers have an intrinsic adjuvant effect (Bal *et al*., 2010). Particle born antigen are much easier being taking up by APCs and induce stronger immune responses than soluble antigen due to their resemblance in size to native microorganisms (Clark *et al*., 2001; Friede and Aguado, 2005). It has been demonstrated both *in vitro* and *in vivo* that microparticles with a size below 5 μm are ingested by a wide variety of phagocytic cells (O'HAGAN *et al*., 2004). For submicron particles, there are contradictory reports in the literature with regard to the differential uptake of nanoparticles by APCs, as compared with microparticles, depending on the nature of the particles, the involved antigen, the route of delivery and the study end-point. There is indeed evidence that nanoparticles show increased uptake by APCs, which suggests that they may be better inducers of immune responses than microparticles (Foged *et al*.,

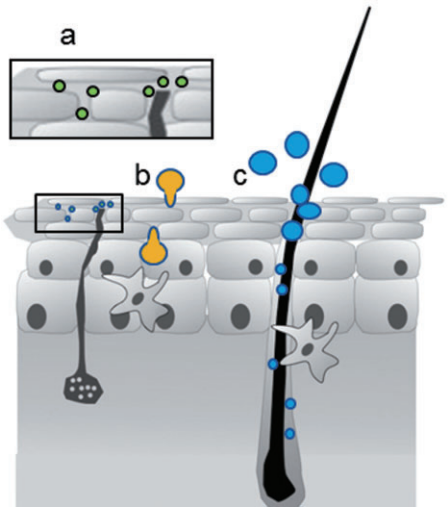

Fig. 1. Three pathways of percutaneous absorption are discussed for nano-sized drug carriers. (a) Due to the small size superfine rigid nanoparticles (< 10 nm) are absorbed via the intercellular lipid channels. (b) Ultraflexible liposomes can squeeze through the intercellular lipid channels despite their nominal diameter being much larger than the size of the lipid channels. (c) Nanoparticles can also enter the hair follicles. This is a size dependent mechanism.

2005). In addition, it was shown that carrier size could play a role in determining the type of response induced. Virus-like particles induced IFN-γ and cell-mediated type 1 responses, whereas larger beads mostly induced type 2 responses (Fifis *et al*., 2004). Delivery of the payload to the APCs or transfection of target cells can be achieved passively, i.e. by taking advantage of the nano-size of the carriers. Delivery may further be improved by attaching active targeting moieties to the carrier surface, such as non-toxic LPS (lipopolysaccharide) derivatives or mannose to target Toll-like receptors or mannose receptors of DCs and macrophages (Cox *et al*., 2006).

This review will focus on opportunities for using nanoparticles for transcutaneous vaccination. The main part of the review will deal with non-barrier compromising methods and outline three possible routes of absorption which are each favoured by different types of nano-delivery devices (Fig. 1). At first rigid nanoparticles are discussed. Below a critical size of less than 10 nm these so-called superfine particles are able to invade via the SC lipid bilayers. Particles larger than that stay on the skin surface in non-barrier compromised skin. For them the *trans*-follicular route is important, which is discussed next. Third, ultradeformable liposomes will be reviewed, which despite their nominal size of normally around 100–200 nm can squeeze through the much narrower SC lipid bilayers due to their flexibility. The potential and draw-backs of using these different types of nano-delivery devices for TCI is discussed. In a second part, shortly, the potential of combining nanoparticle formulations of antigen with barrier disrupting methods will be reviewed. Features, advantages and limitations of all vaccination strategies are summarized in Table 1.

Transcutaneous vaccination using non-Barrier compromising methods

Percutaneous absorption of rigid nanoparticles?

Reports of toxic heavy metal nanoparticles (quantum dots, QDs) and industrial fullerene nanoparticles being

Table 1. Features, advantages and limitations of the discussed strategies for TCI using nanoparticles are summarized.

	Superfine rigid nanoparticles	Trans-follicular route	Ultraflexible liposomes	Nanoparticles and barrier compromising methods
Feature	• Superfine nanoparticles can penetrate the SC and the viable epidermis to some extent	• Nanoparticles bigger than 20 nm penetrate into hair follicles, the invasion depth depends on the nanoparticle size • Inside the hair follicles the nanoparticles do not transfer into the viable epidermis	• Edge activators and/or ethanol fluidize the liposome bilayer, ultraflexible liposomes squeeze through the SC lipid bilayers	• Enhance SC permeability by physical (more effective) or chemical means
Advantages	• Superfine nanoparticles enhance to penetration of co-applied antigens	• Follicles are a reservoir for particles that is slowly cleared • Reduced barrier function in the lower hair follicle due to absence of SC permits the penetration of soluble molecules	• Deep penetration is possible	• No permeability problem • Non-specific immune stimulation
Limitations	• Poor and variable penetration • Poor dosing accuracy • Extremely low carrier capacity	• Dose loss on the skin surface and in wrinkles and skin folds • May require an additional weakening of the follicular epidermal barrier • Carrier capacity	• Carrier capacity	• Barrier disruption enables the invasion of harmful pathogens or chemicals • Expensive equipment • Irritation and sensitization potential of chemical enhancers
Potential for TCI	• x	• xx	• xxx	• xxxx

found in the SC or even in deeper viable skin layers fuelled the discussion on skin-nanotoxicology not only among researchers and regulators but also in the public (Ryman-Rasmussen *et al*., 2006; Xia *et al*., 2010). Through careful control of testing protocols it could, however, be clarified that non-damaged human skin is impermeable to micro- and nanoparticles (Gamer *et al*., 2006; Zhang *et al*., 2008; Monteiro-Riviere and Riviere, 2009; Gratieri *et al*., 2010; Prow *et al*., 2011a,b). The current knowledge on nanoparticle permeation across skin has been summarized by the Scientific Committee on Consumer Products (SCCP) in the following statements (Scientific Committee on Consumer Products, 2008):

i. There is evidence of some skin penetration into viable tissues (mainly into the *stratum spinosum* in the epidermal layer, but eventually also into the dermis) for very small particles (less than 10 nm), such as functionalized fullerenes and quantum dots.
ii. When using accepted skin penetration protocols (intact skin), there is no conclusive evidence for skin penetration into viable tissue for particles of about 20 nm and larger primary particle size as used in sunscreens with physical UV-filters.
iii. The above statements on skin penetration apply to healthy skin (human, porcine). There is an absence of appropriate information for skin with impaired barrier function, e.g. atopic skin or sunburned skin. A few data are available on psoriatic skin.
iv. There is evidence that some mechanical effects (e.g. flexing) on skin may have an effect on nanoparticle penetration.
v. There is no information on the transadnexal penetration for particles under 20 nm. Nanoparticles of 20 nm and above penetrate deeply into hair follicles, but no penetration into viable tissue has been observed.

Statement 3 of the SCCP report points out the lack of data for skin with impaired barrier function. This question is relevant especially for nano-toxicological evaluations of cosmetics, household products, work site safety and of course medical applications. Potentially lesioned skin can show very different uptake of small molecules as well as of nanoparticles. In the meantime some of these knowledge gaps have been amended although the data are still sparse. Mortensen and co-workers looked at the penetration of carboxylated QDs (which, with 20–33 nm, were slightly larger than what is normally considered superfine particles) into UV damaged skin in mice (Mortensen *et al*., 2008). The UV-exposure was comparable to a dose sufficient to cause medium-level sunburn in humans. Low levels of QDs were found in both groups (healthy and UV damaged skin) with higher levels being found in the UV-exposed group (Mortensen *et al*., 2008). There are no data available on the permeability of nanoparticles across sunburnt skin in humans. Prow and colleagues very recently have published some data out of a pilot clinical study on the permeability of ZnO nanoparticles (35 nm) across non-lesional skin versus atopic dermatitis lesions and psoriatic plaques (Prow *et al*., 2011a). By *in vivo* non-invasive multi-photon imaging they could show that ZnO nanoparticles penetrated deeply into lesion furrows and from there spread laterally into the SC. They could not find any particles inside the viable skin layers, neither in lesional nor non-lesional skin. This confirms the results of another *in vivo* study in psoriatic patients where no systemic increase in zinc levels was found after topical application of ZnO nanoparticles (Prow *et al*., 2011a).

In view of these facts the prognosis for using rigid nanoparticles for TCI is disappointing. However, as mentioned also in the SCCP statement the situation may be different for superfine nanoparticles. These are particles such as QDs, metal oxides, silver or gold nanoparticles with sizes usually less than 10 nm. There is one recent report where such superfine Au-NPs (5 nm) were investigated for TCI. Huang *et al*. successfully enhanced the dermal absorption of model proteins (horseradish peroxidase (HRP), β-galactosidase (β-gal) and OVA) in mice by co-administering these proteins with Au-NPs (5 nm). The authors discussed a penetration via the hydrophilic headgroups in the SC lipid bilayers. It should be noted that the skin had previously been hydrated for 10 min by covering it with wet gauze before the application of the formulations, which may have facilitated the uptake. In addition for experiments with OVA the strong mucosal adjuvant cholera toxin (CT) was co-applied with the particles. Cholera toxin is an adenosine diphosphate (ADP)-ribosylating bacterial exotoxin produced by *Vibrio cholera* and is responsible for the life-threatening symptoms of cholera infections. A saline solution of CT applied to shaved mouse back induces high levels of CT-specific IgG antibodies and may work as an adjuvant for co-administered vaccines (Glenn *et al*., 1998). Cholera toxin is also known for its permeabilizing effect. Au-NPs accumulated in high amounts in the SC and in the epidermis. HRP/Au-NPs and β-gal/Au-NPs were detected at similar skin depths. Ova/CT/Au-NPs elicited the production of IgG. However, the immune response was not characterized further (Huang *et al*., 2010). This has so far remained a singular report that superfine nanoparticles may be used for TCI. It remains to be seen whether these results can be repeated in barrier tight animal models or in man or whether further barrier disrupting measures are necessary. What is also not clear at the moment is whether the dose of vaccine that can be transported via this way is sufficient to elicit an effective immune response.

The follicular route

The *trans*-follicular route has long been assumed to be negligible, as follicles only cover about 0.1% of the total skin surface area. However, lately it could be shown that the follicular capacity is very similar to that of the SC lipids, which is commonly considered to be the most important pathway for transport across the horny layer (Schaefer and Lademann, 2001; Otberg *et al*., 2004). Cornified cells are present only in the upper thirds of the hair follicles, whereas the lower part is lined with an epithelium containing tight junctions (TJs) (Furuse *et al*., 2002). Epidermal LCs accumulate around the follicles to build up a second immunologic line of defence in places where the mechanical barrier is weakened. Although LCs represent only about 1% of the total cell population in the skin, they cover a large surface area of approximately 20% through their horizontal orientation and long interdigitating protrusions (Babiuk *et al*., 2000).

Nano- and micron sized particulate carriers that are applied to the skin accumulate in the follicle openings, sebaceous glands or skin folds. We could recently show that a plain aqueous nanoparticle suspension as well as a hydrogel formulation of these particles that was massaged into pig ear skin *in vitro* permeated deeper and to a greater extent into hair follicles than an aqueous solution applied with similar physical force. These nanoparticles were made of the biodegradable and biocompatible polymer polylactic-*co*-glycolic acid (PLGA) using polyvinyl alcohol as a stabilizer. We could further show that such particles may release different encapsulated compounds that then enter the skin (Luengo *et al*., 2006; Stracke *et al*., 2006).

During the last years especially the works of Lademann *et al*. greatly improved our insight in the uptake of particles into follicles. Most of these investigations were done in excised pig skin as this is an appropriate substitute for human skin (Bronaugh *et al*., 1982). The pig ear is especially suitable for studying follicular uptake of particles as the ear cartilage prevents contraction of tensile fibres and closure of follicles, which greatly reduces follicular uptake, when working with excised human skin (Patzelt *et al*., 2008). The invasion depth of particles into the follicle is size dependent. For terminal follicles in pig ear skin a maximum penetration depth was reported for particles with a size of approximately 650 nm (Toll *et al*., 2004; Ossadnik *et al*., 2006; Patzelt *et al*., 2011). It seems likely that the size optimum depends on species, body site and hair type (e.g. vellus and terminal hair follicles). In contrast, the influence of particle surface properties is studied less systematically although current results suggest that this factor may be of less influence than particle size (Patzelt *et al*., 2011). Furthermore, only follicles containing growing hair or showing sebum production took up particles (Lademann *et al*., 2009). Others, 'non-active' follicles, were clogged by cellular debris and needed re-opening by washing or light peeling. This was surprising as the directionality of hair growth and sebum flow are against the invasion of particles. It was hypothesized that this is due to a mechanical process occurring through natural hair movement. which can *in vitro* be mimicked through massaging (Lademann *et al*., 2007). Hair bulbs are also an excellent reservoirs, being only slowly cleared by hair growth and sebum production (Lademann *et al*., 2006).

The invasion of a substance into skin appendages is not yet an absorption process itself. Compounds inside an appendage are still on the outside of the body by definition. Nonetheless, accumulation in such structures may lead to faster and more efficient uptake due to altered barrier morphology in the appendages. In fact, an activation of the LCs via the follicular route is a commonly occurring phenomenon in allergic contact dermatitis in people allergic to pollen antigen. Pollen can be considered as natural micrometer sized carriers of antigen. Antigen release from pollen is triggered by a moist atmosphere with sufficient humidity, such as occurs on the skin by sweating (Jacobi *et al*., 2007). Kubo and co-workers have recently shed some light on how epidermal LCs acquire external antigen, despite the presence of SC and TJs (Kubo *et al*., 2009). According to their findings activated epidermal LCs elongate their dendrites to penetrate KCs to survey the environment located outside of the TJ barrier, just beneath the SC (Kubo *et al*., 2009). From the tip of these dendrites antigens are taken up into the LCs by endocytosis probably via Langerin-positive Birbeck granules. At the same time a dynamic reorganization of TJs between KC-KC and KC-LC contacts ensures that the TJ barrier remains intact to protect the body interior (Kubo *et al*., 2009).

There is evidence in literature that the *trans*-follicular pathway can be used for TCI. Fan *et al*. reported that topical application of naked plasmid expression vectors for lacZ and the hepatitis B surface antigen (HBsAg) to intact mouse skin (V57/BL6 mice, black-haired) induced antigen-specific immune responses that displayed Th-2 features (Fan *et al*., 1999). HBsAg-specific antibody and cellular responses were induced to the same order of magnitude as those produced by i.m. injection of the commercially available recombinant HBsAg polypeptide vaccine (Fan *et al*., 1999). In contrast no immune response could be elicited in nude mice, which lead the researcher to the conclusion that the presence of normal hair follicles was a prerequisite for eliciting a response (Fan *et al*., 1999). It seemed that successful transfection depends on the presence of hair follicles entering the anagen (growth) state which could be induced for example by hair plucking (Shaker *et al*., 2007; Yu *et al*.,

2011). Supposedly, this was due to the fact that hair follicles only proliferate during the anagen state and that proliferating cells express DNA more efficiently compared with quiescent cells. This is difficult to evaluate as plucking may damage the follicular epithelial barrier as well as elicit an unspecific inflammation. At least the physical damage could also enhance the delivery of the plasmid DNA across the follicular epithelium while local inflammation has been shown not to facilitate an immune response to a topically applied DNA vaccine (Yu *et al*., 2011).

Despite its high immunologic potential the *trans*-follicular pathway has not been widely studied as a route of application of nanoparticle for TCI (apart from those using an additional barrier compromising technique such as cyanoacrylate stripping; Vogt *et al*., 2006). Mattheolabakis *et al*. reported the successful *trans*-follicular delivery of ovalbumin (OVA) with poly (lactic acid) particles when applied to shaved mouse skin (Mattheolabakis *et al*., 2010). The particles had been produced by a double emulsion method using sodium cholate as a stabilizer and probe sonication to improve emulsification. The particles had a size of around 150 nm, a negative zeta potential and a relatively low encapsulation efficiency for OVA of around 20%. *In vitro* after a moderate burst of 10% within the first hour hardly any additional OVA was released within the first day. The immunological results of the nanoparticle formulation was rather moderate or even inferior when compared with an OVA solution (antibody and cytokine response; IgG, IFN-γ, IL-2, IL-4, IL-10) but could be stimulated by the additional administration of CT (Mattheolabakis *et al*., 2010).

Apart from overcoming the skin barrier one of the big challenges for *trans*-follicular vaccination is the loss of dose on the skin surface and within wrinkles, which does not invade the hair follicles where it cannot contribute to APC activation. Consequently, *trans*-follicular immunization requires much higher amounts of vaccine to be applied than which is finally going to reach the target site.

Percutaneous absorption of ultraflexible liposomes

Liposomes are non-immunogenic vesicles made of 1,2 *sn*-glycerophospholipids, which may contain cholesterol for bilayer stabilization (Lilia Romero and Morilla, 2011). Variations from this classic composition have been synthesized and decorated with distinctive names, such as niosomes (containing non-ionic surfactants; Rentel *et al*., 1999), ethosomes (containing ethanol; Touitou *et al*., 2000), bilosomes (containing bile salts; Senior, 2001), archaesomes (containing archael ether lipids from archae bacteria; Sprott *et al*., 1997), and virosomes (containing virus derived molecules; Hunziker *et al*., 2002). They can incorporate molecules either within the hydrophilic core, within the lipophilic or hydrophilic areas of the bilayer walls, or attached to the surface. By adding surfactants as edge activators (e.g. bile salts) or ethanol (ethosomes) to the formulation highly deformable or ultraflexible liposomes are received (Cevc and Blume, 1992; Touitou *et al*., 2000; Geusens *et al*., 2010). Both ways the membrane fluidity is increased, which allows the carrier to squeeze through pores that are smaller than the nominal carrier diameter when under pressure (Cevc *et al*., 2002). According to Cevc flexible liposomes are pulled across the SC via the inter-corneocyte lipid channels by the *trans*-epithelial hydration gradient (Cevc and Gebauer, 2003). This mechanism is supposed to depend on non-occlusive conditions. During the passage the lipid bilayers of the vesicles remain intact so that the cargo is protected from potential degradation or undesired distribution (Cevc *et al*., 2002). Also this mechanism brings them rapidly towards the deeper layers of the SC, close to the epidermis, so that they possibly get accessible to LCs sampling the environment (Honeywell-Nguyen *et al*., 2002; Honeywell-Nguyen *et al*., 2004; Kubo *et al*., 2009). In addition when using ethosomes ethanol may work as a fluidizing agent on the intercellular lipid bilayers of the SC, which may further enhance their transport across the SC (Goldstein, 1984). In contrast, when conventional liposomes are applied to the skin they spread on the skin surface, dehydrate and coalesce without entering the lipid bilayers (Verma *et al*., 2003).

Flexible liposomes have been used to improve skin penetration of a wide range of active ingredients and have demonstrated their superiority compared with conventional liposomes (Cevc, 2003; Honeywell-Nguyen and Bouwstra, 2003; Cevc *et al*., 2008). The first mention of ultraflexible liposomes (soy bean phosphatidylcholine (Soy-PC), sodium cholate, sodium dodecyl sulfate) being used for TCI goes back to Paul *et al*. reporting the induction of serum IgG and IgA in mice upon topical application of 'transferosomes' containing gap junction proteins (Gap-transferosomes) with or without the addition of monophosphoryl lipid A (Paul *et al*., 1998). Gap-transferosomes which were applied onto intact skin achieved comparable IgG and IgA responses as if applied by s.c. injection. Responses to topically applied Gap-transferosomes were strongly superior to topically applied Gap-mixed micelles and Gap-conventional liposomes while responses were comparable to s.c. applied Gap-mixed micelles and Gap-conventional liposomes (Paul *et al*., 1998). Gupta *et al*. compared ultraflexible liposomes (Soy-PC, sodium deoxycholate) with niosomes (Soy-PC, Span-85 and cholesterol) and conventional liposomes (Soy-PC, cholesterol) containing tetanus toxoid (Gupta *et al*., 2005). In an *in vitro* diffusion cell experiment using nude rat skin tetanus toxoid was found in the receiver compartment with all formulations after a short incubation time. This once more shows the excessive permeability of nude

rodent skin which is no suitable indicator for the permeability in man (OECD, 2004a,b). However, the *in vivo* immunization study in these animals showed that the ultraflexible carriers were most efficient in inducing serum IgG (Gupta *et al*., 2005). Interestingly, Mishra *et al*. reported two studies using identical protocols one using ultraflexible liposomes composed of Soy-PC and Span-80 the other using ethosomes both for the transcutaneous delivery of HBsAg. These two studies allow some insight into the comparative efficiency of ultraflexible liposomes made with surfactants and ethosomes by relating both results to the same positive control (i.m. injection). Both topical formulations induced serum IgG and systemic and mucosal IgA response in BALB/c mice. In both cases serum IgG was comparable to i.m. injection, serum and salivary IgA levels were even superior (Mishra *et al*., 2006; 2008).

Wang *et al*. applied positively charged ultraflexible liposomes (Soy-PC, octadecylamine) containing hepatitis B surface antigen plasmid DNA (HBsAg-DNA) on shaved mouse back (BALB/c). The IgG response towards the ultraflexible liposomes was superior to non-flexible liposomes and comparable to i.m. injection of naked DNA or HBsAg. Cytokine response (IFN-γ, IL-4) was highest with the ultraflexible liposomes and naked DNA i.m (Wang *et al*., 2007). Xu *et al*. also reported the application of ultraflexible liposomes (Soy-PC, cholate) for transcutaneous DNA vaccination using a plasmid encoding for the F-gene of respiratory syncytial virus (RSV-F DNA). Ultraflexible liposome encapsulated RSV-F DNA; both RSV-specific mucosal antibody response and IFN-γ-producing cells were detected. Intramuscular vaccination of naked RSV-F DNA only induced a significant anti-RSV IgG antibody response but no remarkable sIgA antibody and virus-specific cellular activity. Lungs from mice receiving topical vaccination had fewer histopathologic anomalies after RSV challenge than did mice receiving i.m. vaccination or controls (Xu *et al*., 2008). By combining a cationic lipid (DOTAP, dioleoyl trimethylammonium propane), sodium cholate, ethanol and cholesterol ('SECosomes', surfactant-ethanol-cholesterol-omes), Geusens and co-workers created the first cationic deformable liposomes, which contain both surfactants and ethanol (Geusens *et al*., 2010). These carriers achieved high transfection efficiencies of siRNA *in vitro*. Additionally, upon *in vivo* application of encapsulated siRNA to intact human skin changes in the keratinocyte cell state were demonstrated by FLIM-measurements (fluorescence lifetime imaging) (Geusens *et al*., 2010). This may be a very interesting system for DNA vaccination in the future.

An excellent review on the use of liposomes and similar vesicles for mucosal and transcutaneous vaccination purposes has recently been published and the reader is referred to there for further detail (Lilia Romero and Morilla, 2011).

Transcutaneous vaccination using nanoparticles aided by barrier compromising methods

Barrier compromising methods rely on enhancing the permeability of the very effective SC barrier by chemical or physical means to facilitate access of the antigen to the APCs. Chemical permeation enhancers are a very heterogeneous group of substances with widely varying physico-chemical properties as well as mechanisms of action. Examples are surfactants, terpenes, laurocapram, dimethylsulfoxides, fatty acids, alcohols, water and urea (Kaushik *et al*., 2008). An ideal permeation enhancer should be pharmacologically inert, chemically stable, potent in low concentrations, non-toxic, non-sensitizing and non-irritant. It should also act rapidly and the barrier function should recover quickly. The main principles permeation enhancers may use for improving percutaneous penetration of other compounds are: (i) perturbation, fluidization or disruption of SC lipids; (ii) extraction of membrane components; (iii) increase of the compound solubility within the SC membrane; (iv) improvement of skin hydration; and (v) interaction with keratin. Especially the more potent enhancers use several of the above mechanisms. Typical enhancement ratios for a small molecule such as hydrocortisone are in the range of 2.8–28 in human skin *in vitro* (Ibrahim and Li, 2009). For nanoparticles there are only few reports. Experiments with nude mice indicated that skin permeation of zinc oxide nanoparticles (10 nm) could be enhanced by the addition of oleic acid or ethanol which fluidize the intercellular SC lipid bilayers and extract lipids (Mortensen *et al*., 2008; Kuo *et al*., 2009). It should be kept in mind that especially rodent skin acts differently towards chemical penetration enhancers when compared with human skin (Bond and Barry, 1988; Simon and Maibach, 1998). In human and pig skin the absorption of superfine Au-NPs and QDs required the combined application of ultrasound and sodium lauryl sulfate (SDS) (Seto *et al*., 2010; Lopez *et al*., 2011). Penetration was seen mostly in the hair follicles as well as seen in localized spots in the dermis which they reach supposedly via imperfections in the intercellular lipid layers (Lopez *et al*., 2011). Such imperfections are sufficiently rare to explain the spot-like distribution of the particles (Paliwal *et al*., 2006; Lopez *et al*., 2011). The use of SDS *in vivo* is problematic due to its strong sensitization potential (Kaushik *et al*., 2008).

In contrast, a lot of work has been done to support that physical barrier disruption methods may be used for TCI. A historical example where this concept has successfully been employed is the immunization against smallpox that finally led to the eradication of the disease. An effective

and strong immune response was induced scratching or printing the vaccine into the skin, called scarification (Henderson, 1999). According to the clinical trial database of the US National Institute of Health (2011), skin permeabilizing techniques that are currently evaluated in clinical trials include jet injection and gene gun devices, microneedle patches, exfoliation, electroporation and thermal ablation techniques. This concerns vaccines against seasonal influenza, different kinds of cancer (e.g. melanoma, prostate cancer, leukaemia, and multiple myeloma), human papilloma virus, hepatitis A and C, HIV, and malaria.

Apart from facilitating the invasion of the vaccine the barrier disruption also leads to a non-specific immunostimulation, which improves the antigen-specific immune responses. This includes the secretion of pro-inflammatory cytokines such as TNF-α, IL-1α, IL-1β, and GM-CSF by KCs. TNF-α and IL-1β trigger the upregulation of the expression of a6 integrins, intercellular adhesion molecule 1 (ICAM.1), CD86 and MHC class II molecules, which then promote the dissociation of LCs from KCs and their subsequent maturation and migration (Streilein *et al*., 1982; Proksch *et al*., 1996; Kupper and Fuhlbrigge, 2004; Karande and Mitragotri, 2010). The time unto re-population of the epidermis with LCs is 24 h, which implies that the epidermal pool of LCs can quickly be replenished when needed (Streilein *et al*., 1982). One of the sources of LCs is the hair follicles (Gilliam *et al*., 1998).

Several such physical permeability enhancing methods have been combined with innovative nano- (or micro-) formulations of vaccines in order to combine the advantages of the particulate formulation with those of the barrier disruption. To this end different delivery vectors, such as liposomes, virosomes, transferosomes, nano- or micro-beads and viral vectors, have already been tested mainly in animal models (Babiuk *et al*., 2000). A system that has made it into clinical testing is the HIV-1/AIDS DermaVir patch. It contains a plasmid DNA vaccine (pDNA) encoding for all major HIV-1 antigen and the formation of virus like particles. This was built on a clinical observation from a German HIV infected individual whose immune system learned to control virus replication by producing antiviral T cells (Lisziewicz *et al*., 1999). The pDNA is formulated as pathogen-like mannosylated polyethyleneimine nanoparticles (80–400 nm). The pDNA is adsorbed to the positively charged polymer by ionic interactions and forms a nanoplex, which protects the antigen from degradation during application, absorption and endosomal passage. Mannosylation further supports antigen presentation by targeting the pDNA to LCs and induces a Th-1-type cellular response as shown in mice and macaques (Lisziewicz *et al*., 2005; Cristillo *et al*., 2007). In order to facilitate access of the nanoplex bound antigen to the cutaneous APCs an exfoliation step is performed before the vaccine is applied to break down the skin barrier. The clinical trial database of the US National Institute of Health lists several studies using the DermaVir patch evaluating safety, tolerability and efficacy, which have been completed or are ongoing (US National Institute of Health, 2011). Lately, the results of one study have been published. This study looked at 12 individuals on combined antiretroviral therapy (cART), who were immunized with the vaccine with or without the addition of hydroxyurea or received a placebo patch (Gudmundsdotter *et al*., 2011). Those patients receiving the vaccine patch developed higher and broader levels of CD8+ T cells compared with placebo although it had no effect on CD4+ T-cell count. The vaccination procedure was generally well tolerated with local irritation described at the site of patch application (redness, itching, swelling, pain). Systemic effects were usually mild with headache, fatigue, and muscle pain being reported. The DermaVir vaccine still requires a very large application area for the application of (4 skin sites, each 80 cm^2, two sites each on the thighs and the upper back). One total immunization schedule consisted of six individual vaccinations. One important goal in immunotherapy in patients with HIV-1 is to strengthen the patients' immune system and to reduce viral load to enable intermission of combined antiretroviral therapy for longer periods (Gudmundsdotter *et al*., 2011). In the view of these therapeutic improvements HIV-1 patients will put up with the bothersome vaccination schedule. Skin pre-treatment, large application sites and repeated dosing are however important obstacles which might prevent the DermaVir patch from being widely acceptable by the public for prophylactic vaccinations.

Despite the great potential of barrier disrupting techniques they also raise concerns as the vital skin barrier is weakened more or less temporarily which may open the door for the invasion of pathogens. Also, some of the techniques mentioned require expensive equipment. Both points will have to be considered especially for mass vaccination campaigns in developing countries with poor hygiene standards and low funds.

Concluding remarks

There is no doubt that nanotechnology holds great promise for vaccination and especially TCI. Key issues that will have to be solved in the future are how nano delivery devices can transport a sufficient dose of the vaccine across the SC and how this can be achieved without opening the skin barrier for the invasion of pathogens or harmful material. Currently, ultra-flexible liposomes are the best investigated carriers in this aspect. However, also superfine rigid nanoparticles and the hair

follicular route have shown some potential to be used successfully for TCI.

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