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Review article

Follicular transport route – Research progress and future perspectives

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ABSTRACT

The important role of hair follicles as penetration pathways and reservoir structures for topically applied compounds has been validated in numerous animal models as well as in humans. Follicular penetration rates are modulated by regional variations in size and proportions and the functional status. Advances have especially been made in the targeting of hair follicle-associated cell populations including antigen-presenting cells and stem cells. Improved investigative methods based on differential stripping, spectrophotometry and confocal laser scanning microscopy have led to the determination of the penetration profiles and kinetics for a multiplicity of drugs and drug delivery systems. The observation that particulate delivery systems aggregate and remain in hair follicle openings and their penetration along the follicular duct occurs in a size-dependent manner, which has led to advanced concepts of targeted drug delivery of bioactive compounds in the field of solid particles, as well as semi-solid particles, such as liposomes. This review summarizes the recent progress in this field, and underlines the necessity for pilot studies in human volunteers to further the development of clinical applications for follicular targeting.

1. Introduction

Penetration of topically applied compounds may occur via the stratum corneum [1–4] as well as via skin appendages, i.e., sweat glands and hair follicles. Initially, skin appendages were not considered to be significant transdermal penetration routes, as evidence suggested that they accounted for only approximately 0.1% of the skin surface area [5]. These calculations, however, did not take into the account that the hair follicles represent invaginations, which extend deep into the dermis with a significant increase in the actual surface area available for penetration. With a rich perifollicular vascularisation and changes in the differentiation pattern along the follicular duct, the follicle possesses distinct characteristics which favour penetration, and multiple studies suggest that the follicular penetration route may be especially relevant for hydrophilic and high molecular weight molecules, as well as by particle-based drug delivery systems [3,6–13].

Earliest reports on the participation of hair follicles in percutaneous absorption were based primarily on qualitative, histological studies of dye and stain localization [14–19]. Later studies led to increasingly quantitative data, characterizing follicular transport as a highly complex phenomenon [20]. Complementary to these findings, Weigmann et al. [21] reported that substances are mainly located in the uppermost cell layers of the stratum corneum, where they are continually depleted due to the physiological process of desquamation. These findings suggest that the stratum corneum only provides a short-term reservoir function. The hair follicles in contrast represent efficient long-term reservoirs (up to 10 days) for topically applied substances, as their depletion occurs only through the slow processes of sebum production and hair growth [11,22,23].

The term pilosebaceous unit describes the integrated structure of the hair follicle, the adjoining arrector pili muscle and the associated sebaceous glands [24]. It is a complex and dynamic three-dimensional structure [25], consisting of more than 20 different cell populations [26], which regulate various biochemical, immunological and metabolic activities. At its most basic, divisions can be made between the permanent superficial structure and the transient cycling component of the hair follicle, which includes the hair bulb [27]. Therefore, in addition to representing important reservoir structures and penetration pathways, the hair follicles also contain multiple target structures for innovative therapeutic approaches, which will be discussed in this review. These include specific cell populations in and around the hair follicles, such as immune cells, stem cells and melanocytes, sebaceous glands and perifollicular blood vessels [28–31].

Abbreviations: CSSS, cyanoacrylate skin surface stripping; APCs, antigen-presenting cells.

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Moreover, various factors are able to influence follicular penetration. While regional variations of follicular density and morphology and the hair cycle activity were found to control follicular penetration, also penetration enhancement strategies were developed to increase follicular penetration.

2. Investigation techniques of follicular penetration

The development of novel techniques to determine penetration profiles and kinetics *in vitro* and *in vivo* has tremendously helped to establish reliable quantitative model systems. While the first studies in this field were based on the microscopic evaluation of tissue sections, tape stripping and the addition of cyanoacrylate skin surface stripping (CSSS) have led to the so-called differential tape stripping [32]. The combination of these techniques provides precise information on the penetration behaviour *in vivo*. On the contrary, diffusion cell systems allow quantifying penetration processes *in vitro* under static and flow-through conditions [33,34]. Comparison of different body regions, animal models, artificial introduction of hair follicles in skin equivalents, as well as blockage of hair follicles by nail polish [24,35–37] compared to untreated skin, was used to determine the contribution of the follicular route to overall penetration. Barry [38] recently introduced a technique based on a sandwich made from human stratum corneum and epidermis, which consists of its own stratum corneum and nucleated epidermis. The top stratum corneum blocks shunt penetration pathways such as hair follicle openings. Meidan et al. [39] demonstrated in Wistar rats and guinea pigs that mild heating or low intensity ultrasound application leads to a discharge of sebum, which blocks the follicular pathway for the penetration of hydrophilic molecules.

The number of non-invasive techniques applicable *in vivo* on human volunteers, however, is still limited. Complete differential tape stripping with the removal of the entire stratum corneum and subsequent CSSS procedure is limited to *in vivo* measurements. Additionally, CSSS alone is well tolerated when used *in vivo,* and is a valuable tool to enhance the penetration [36]. The use of advanced optical systems such as autoradiography [40], microimaging [41], as well as *in vivo* confocal laser scanning microscopy and *in vivo* Raman spectroscopy, recently established in our group, represent promising new approaches, which, in future, may allow to determine the kinetics of fluorescent dye penetration in the epidermis and into hair follicle openings and alterations of such penetrations in response to stimuli or stress factors (Fig. 1) [42,43].

3. Target structures within the hair follicles

3.1. The hair follicle infundibulum serves as a reservoir and provides an interface for interactions with hair follicle-associated cell populations

Due to its unique anatomy, the hair follicle infundibulum is the key compartment for penetration processes. It is a reservoir structure as well as an interface for intensive interactions with topically applied compounds, potentially hazardous compounds and allergens [12,44].

While an intact and relatively impermeable horny layer similar to that of the interfollicular epidermis covers the acroinfundibulum, this barrier is interrupted in the lower follicular infundibu-

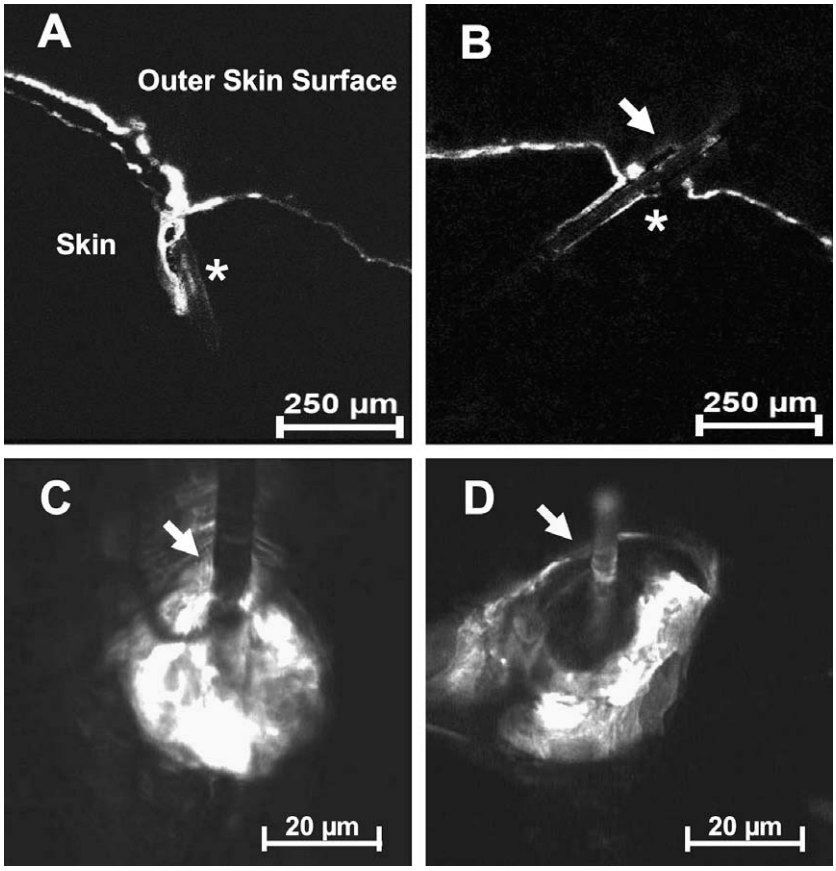

Fig. 1. Confocal laser scanning microscopy. Confocal laser scanning microscopy is a valuable method to visualize the penetration of fluorescent model compounds and particles. Analysis of tissue sections yields high-resolution optical images. (A and B) Sodium fluorescein solution (white signal) spreading on the skin surface and penetrating into hair follicle openings (*) (5 µm longitudinal cryosections of porcine skin, arrow: hair fibre preserved in hair follicle opening). The same technique has been used by our group to visualize follicular penetration in human volunteers *in vivo* (C and D) (optical cross-sections of vellus hair follicle openings in human volunteers, arrow: hair fibre) [30,43].

lum. The corneocytes in this area appear smaller and crumbly, suggesting that the skin barrier is incomplete and permeable in this region [45,46] providing the opportunity for intense interactions between topically applied compounds and the hair follicle epithelium (Fig. 2A). The glassy membrane surrounding the entire follicle and the keratinous layers of the inner and outer root sheaths, in contrast, may physically restrict the passage of molecules into the deeper regions of the hair follicles [19,47]. A perifollicular network of capillaries associated with the upper dermal vasculature supplies the upper region of the hair follicles with blood. The blood vessels of the deep dermis and the subcutaneous tissue nourish the lower region of the hair follicles. A dense network of capillaries also surrounds the sweat glands. Once topically applied substances reach the cutaneous vasculature, they generally permeate into the central circulatory systems. The resorption into the systemic compartments results in rapid dilution [5] and dispersion of the applied substances within the entire circulatory system [19], which is highly significant for the efficacy of systemic drugs. This was demonstrated in a recent study, in which hair follicles were selectively blocked to investigate their influence on the penetration of caffeine into the skin and subsequently into the blood vessels. Blocking of the follicles resulted in a later detection as well as a decreased amount of caffeine in the blood [48]. Multiple cell populations, especially the cells associated with the immune system, such as antigen-presenting cells, mast cells and others, are located in and around the infundibular epithelium, where they are readily accessible and represent important target structures [30]. Over the past years, cell-specific targeting strategies are gaining more and more importance in the design of transcutaneous drug delivery systems. Transcutaneous administration of immunomodulators and vaccines, for example, may allow targeting of skin antigen-presenting cells in their natural environment leading to innovative approaches in immunization strategies. In a recent study on healthy human volunteers, we used CSSS, a technique, which increases the number of hair follicles open for penetrations and the penetration rate of topically applied compounds via hair follicles, and subsequently applied an anti-influenza vaccine. This novel transcutaneous vaccination strategy proved to be safe and efficient in inducing anti-influenza immune responses [49].

3.2. Sebaceous glands – modifier of penetration environment and target structure

Each hair follicle is associated with one or more flask-like sebaceous glands. Ducts join these multilobular holocrine glands to the upper part of the follicular canal from which they release sebum, creating an environment enriched in neutral, non-polar lipids [50]. Size and secretion activity is strongly related to the hair follicle type and body region, as well as to the age and hormone status of the individual [51]. The glands are implicated in the aetiology of acne and androgenetic alopecia [52], as well as in other sebaceous gland dysfunctions [51]. They thus represent an obvious therapeutic site for follicular targeting. Hueber et al. suggested that the sebaceous glands specifically promoted the penetration of hydrocortisone and testosterone into the skin [52]. Considerable effort has been directed towards maximizing the accumulation of various molecules into these glands, including adapalene [51], an erythromycin–zinc complex [53], isotretinoin [54], as well as anti-androgen RU58841 [55,56]. Evidence suggests that topically applied compounds entrapped in liposomes accumulated not only in the hair follicle, but also in the sebaceous glands [55]. Although the process of sebum synthesis and secretion on the skin surface is slow [57], the upward flowing sebum may pose a physical as well as a chemical barrier for drug penetration [19,47]. Efficient drug delivery and the pharmacological effect of the applied compound presumably depend on the interactions between the drug and sebum as well as upon the physicochemical properties of the vehicle.

3.3. Targeting of hair follicle stem cells offers unique therapeutic options for genetic hair and skin therapy and regenerative medicine

The bulge region in the outer root sheath near the insertion of the M. arrector pili is known to be a reservoir for keratinocyte stem cells in humans and rodents [58–64]. According to our own measurements, it is located at 1191 ± 23 μm in terminal hair follicles of the scalp and 362 ± 88 μm in retroauricular vellus hair follicles [65]. This region is responsible for the ability of hair follicles to reconstitute themselves during hair regeneration and hair cycling [66].

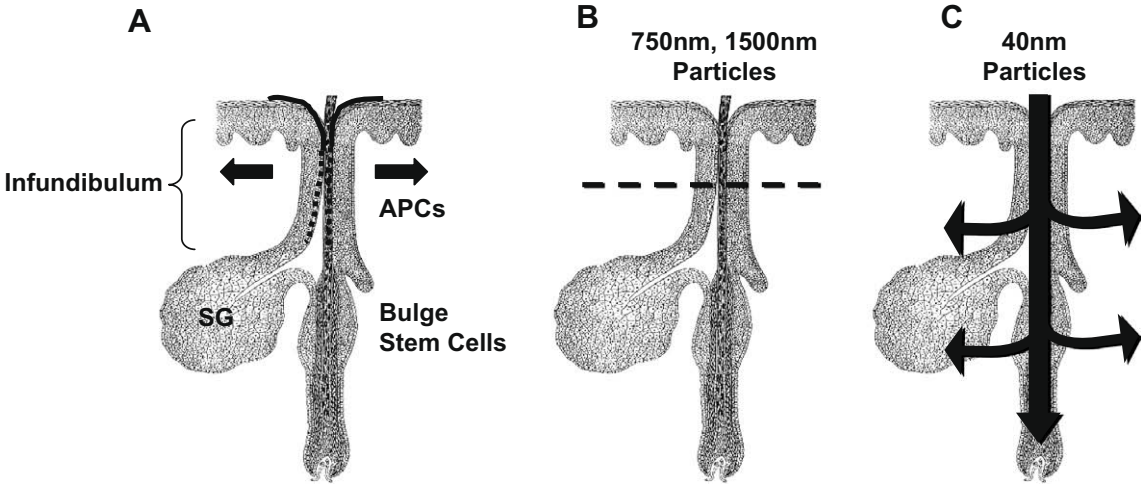

Fig. 2. Targeting of hair follicle-associated cell populations. Its unique anatomy and strategic position within the skin makes the hair follicle infundibulum an ideal long-term reservoir and site of penetration. Cell populations in and around the hair follicle epithelium such as antigen-presenting cells (APCs), are easily accessible via the porous barrier in the lower parts of the infundibulum (A). The sebaceous gland (SG) as well as the bulge region as the site of epithelial stem cells and multiple other precursor cell populations are further important target structures [30]. The follicular route is the preferred penetration pathway for micro- and nanoparticles. Penetration depths and capacity to penetrate the epithelium depend on the particle size, suggesting that larger particles may be used to deposit high concentrations of compounds in the follicular duct from where they can be released (B), while small particles in the size range of 40 nm, especially in barrier-disrupted skin, may be used to directly deliver particle-bound bioactive compounds to specific cell populations (C) [13,31].

Recent advances have made it possible to isolate living bulge cells, to delineate their molecular signature and to study their biological behaviour *in vivo*, as well as *in vitro* [64,67–69]. Results show that bulge stem cells are endowed with a high proliferative capacity and multipotency [70]. Cultured and individually cloned bulge cells from adult mice were shown to form hair follicles in skin reconstitution assays [67,71], which demonstrated that multipotent adult stem cells isolated from the mammalian dermis can proliferate and differentiate into neurons, glia, smooth muscle cells and adipocytes in culture. Hoffman [72] also showed that hair follicle stem cells differentiated into neurons, glia, keratinocytes, smooth muscle cells and melanocytes *in vitro. In vivo* studies indicated that hair follicle stem cells can differentiate into blood vessels and neural tissue after transplantation into the subcutis of nude mice. Furthermore, it was found that hair follicle stem cells implanted into the gap region of a severed or sciatic tibial nerve greatly enhanced the rate of nerve regeneration and the restoration of nerve function. The follicle cells predominantly transdifferentiated into Schwann cells, which support neuron regrowth.

In the light of these results, bulge cells have attracted attention as a stem cell source for cutaneous regenerative medicine. One goal is to treat alopecia with new hair follicles bioengineered from the bulge cells of patients [73]. Gene delivery to specific bulge stem cells may possibly facilitate long-term gene correction of congenital hair diseases [74]. In addition, as the bulge cells are able to repopulate the interfollicular epidermis, the targeting of bulge cells by gene transfer is considered to be a promising approach for the treatment of genetic skin disorders [74,75]. Genetic therapies may as well be utilized to correct carcinogenic stem cell mutations, which tend to accumulate in these slow-cycling cells over time [76].

The identification of melanocyte stem cells in the follicular bulge area [77], immature Langerhans cells [78] and the stem cell potential of follicular dermal sheath cells further extends the range of putative applications of cell-specific drug delivery approaches [79].

3.4. Hair follicle papilla and hair matrix are important in the regulations of hair growth

The hair follicle papilla and the hair matrix, located at the base of the hair follicle, are the regions of key cellular interactions, which are important in the regulations of hair growth and undergo continuous remodelling during the hair cycle.

4. Influences on follicular penetration

4.1. Hair cycle activity influences follicular penetration

Multiple earlier studies, as well as the recent studies underline that the penetration properties vary not only with the hair follicle morphology but also with the functional status of the hair follicle. Each hair follicle undergoes continuous cycling, which includes the complete remodelling of its non-permanent portion, extending from the lower end of the bulge region down to the bulb. Hair growth is traditionally divided into the growth phase (anagen), involution (catagen) and the resting phase (telogen). The duration of the different phases depends on the type and localization of the hair follicle. Under physiological conditions, 85% of the scalp hair is in anagen phase and approximately 15% is in the telogen phase. The anagen phase of the scalp hair follicles typically persists for 2–6 years [80–82]. During each cycle, the hair follicle undergoes substantial changes in the immune and gene expression status, as well as in its vascular supply [83], all of which must be considered for the design of drug delivery systems [7]. Lademann et al. found that it has to be distinguished between active and inactive hair follicles concerning the penetration of topically applied substances into the infundibulum. These hair follicles receptive to penetration showed sebum flow and/or hair growth, and were thus active, while inactive follicles showed neither. The follicular orifices of inactive follicles were found to be covered with a mixture of dry sebum, desquamated corneocytes and other cell detritus [84], which could easily be removed by gentle peeling [32]. Based on these observations, CSSS was developed as a pre-treatment technique of the skin to enhance the follicular penetration rates. CSSS has been shown to open hair follicles for the penetration by removing cellular debris from the hair follicle openings [7]. According to our own data, one CSSS procedure, when applied on excised human skin, removes approximately 33% of the stratum corneum, which corresponds to a mild barrier disruption, *in vivo*, without further damaging the viable epidermis. The procedure is well tolerated and provided the base for a novel transcutaneous vaccination protocol developed in our laboratory [49].

4.2. Regional variations need careful consideration prior to the design of drug delivery system

Density and proportions of the pilosebaceous units vary greatly according to the body regions [36], leading to divergent follicular drug permeation rates. In humans, the palms of the hands, the soles of the feet, and the lips are devoid of hair follicles [24]. Hair follicle formation is completed during the early fetal period. After birth, the absolute number of hair follicles remains the same. As the body proportions change, however, the hair follicles move apart according to the growth of the body and the skin leading to the significant regional differences in hair follicle densities. Due to the reduced growth of the head in comparison with the extremities, hair follicles are therefore much more numerous on the scalp and in the face than on the arms and legs [84–86]. On the scalp and the face, approximately 500–1000 pilosebaceous units per cm^2 can be found. The combined areas of the follicular openings can constitute as much as 10% of the total surface area of the scalp and face. In other parts of the body, such as the calf region, the follicular orifices represent only approximately 0.1% of the total skin area. Otberg et al. analyzed cyanoacrylate biopsies of hair follicle openings in human volunteers to analyze the volume and surface area and the reservoir capacity in different body regions [36]. The largest surface was found on the forehead with approximately 13.7% of the skin surface, and the smallest on the forearm with 0.95%.

Hair follicle size and proportions differ significantly among the body regions. While lanugo hair is produced in utero and generally shed before or shortly after birth, vellus hair follicles of different size and density cover most of the skin surface area in adults. Vellus hair fibres are usually thin with a diameter <30 μm and a length <2 mm. Terminal hair characterized by hair fibres >60 μm diameter and >2 mm length is mainly found on the scalp, as well as in hormone-dependent body regions such as the beard, the axilla and the pubic region [24,27]. Overall size and position of key target structures differ significantly among the different hair follicle types (Fig. 3). In our own studies on scalp terminal hair follicles and vellus hair follicles of the retroauricular regions, however, we found only minor intra- and inter-individual variations, suggesting that specific hair follicle types within one skin area are mostly homogeneous [65]. These findings are of special relevance, because they illustrate that the concept of targeted drug delivery to the different hair follicles types appears to be a feasible approach also in a higher numbers of patients.

4.3. Enhancement of follicular penetration – rationale for particle-based drug delivery

Various efforts have been made in the past in order to develop strategies, which enhance follicular penetration rates, in order to

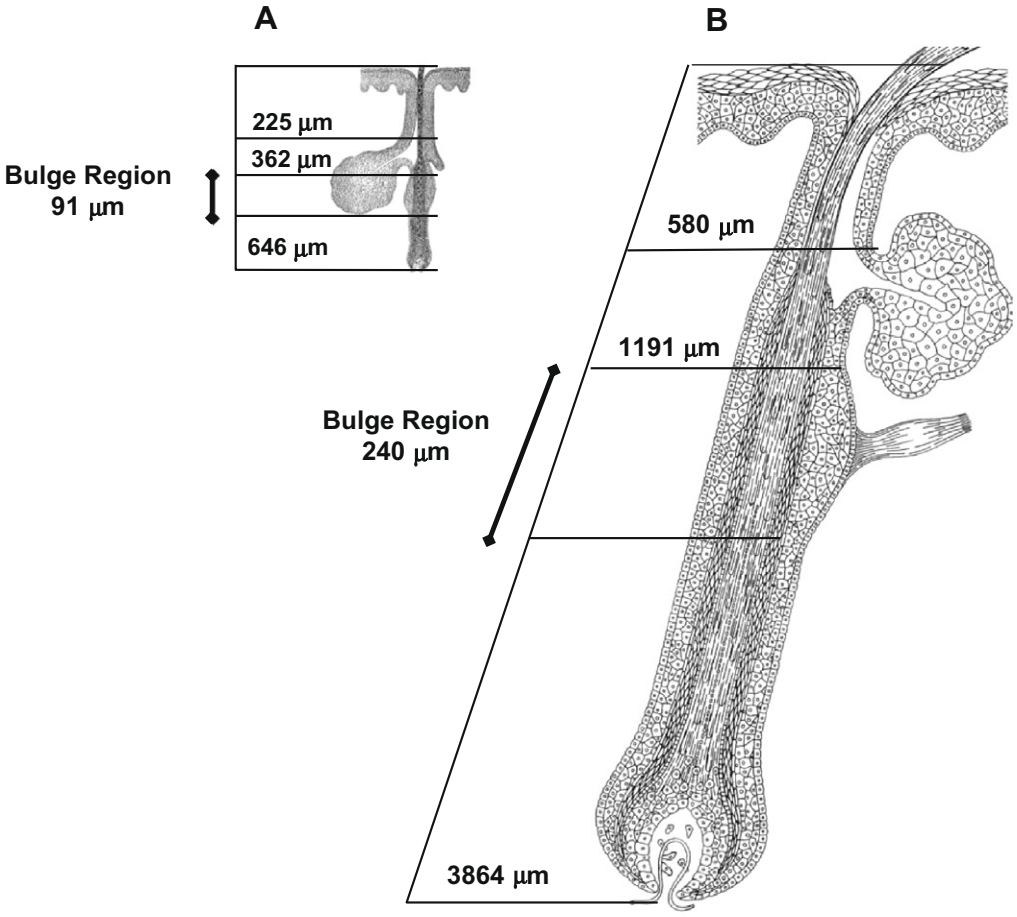

Fig. 3. Regional variations of hair follicles require careful planning of targeting strategies. Overall size and position of key target structures differ significantly among the different hair follicle tapes. Significant deeper penetration is necessary to target the bulge region of terminal hair follicles of the scalp (B) compared to retroauricular vellus hair follicles (A). In our own studies, however, we found only minor intra- and inter-individual variations, suggesting that specific hair follicle types within one skin area are mostly homogeneous [65]. The data illustrate that the concept of targeted drug delivery to the different hair follicle types is a feasible approach, also in a higher number of patients.

reach the maximum concentrations of topically applied compounds in and around the hair follicle.

Among the different compounds and systems studied, particle-based systems show a clear tendency to aggregate and remain in hair follicle openings. Since early observations by Rolland et al., who detected solid PLGA-fluorescence microspheres dispersed in an aqueous gel with the pilosebaceous structures of hairless rats and human skin after 35 min of passive permeations, while penetration via the stratum corneum was not observed, particulate systems have been implemented in various topical applications to enhance the percutaneous transport of drugs into and across the skin barrier [51]. Over the past years, the penetration profile of solid micro- and nanoparticles has been a major field of interest for our group. Schaefer et al. [87] already demonstrated that polymeric microparticles with a diameter ranging from 3 µm to 10 µm selectively penetrated into the follicular ducts, whereas particles larger than 10 µm remained on the skin surface. Rolland et al. found that while microspheres sized 1 µm loaded with adapalene were randomly distributed on the stratum corneum and in the hair follicles, particles sized 5 µm were exclusively located in the pilosebaceous units [51]. Microparticles sized 20 µm did not permeate the skin and remained on the stratum corneum. The follicular aggregation of particles was observed for multiple particle types with different physicochemical compositions. Lademann et al. [88] confirmed the follicular aggregation of coated titanium dioxide microparticles commonly used as UV-filter substances in commercial sunscreen products into skin using tape stripping and skin biopsies in combination with spectroscopic measurements. In a large series of investigations, Toll et al. documented the penetration depths of topically applied polystyrene particles sized 0.75 µm to 6 µm in the follicular duct of human terminal hair follicles of the scalp [13]. Different pre-treatment techniques of the skin samples, including heating, massage and stripping, were also evaluated in this study and revealed that especially CSSS increased the follicular penetration of topically applied particles.

While the retention of the solid particles in the follicular duct has been well documented, there are only few reports on the penetration of solid particles into the viable tissue. Shim et al. [89] reported that minoxidil-loaded nanoparticles penetrated into the hair follicles of hairy guinea pig skin, whereby particles sized 40 nm penetrated more efficiently than particles sized 130 nm. Alvarez-Román et al. used confocal laser scanning microscopy to visualize the distribution of fluorescent polystyrene nanoparticles sized 20 and 200 nm across porcine skin [90]. At present, however, there is little evidence that nanoparticles at a size exceeding 100 nm penetrate into intact skin [1]. E.g., Gamer et al. [91] found that neither titanium dioxide <160 nm nor 80 nm zink oxide penetrated porcine stratum corneum. Although Baroli et al. [92] reported penetration of metallic nanoparticles as small as 5.9 nm across the stratum corneum and via hair follicles using a diffusion cell, the penetration of small particles is probably also limited to barrier-disrupted or otherwise damaged skin. In a recent study,

we identified fluorescent 40 nm polystyrene nanoparticles in the perifollicular tissue of human skin explants pre-treated with cyanoacrylate follicular stripping and confirmed uptake by Langerhans cells and concluded that cell-specific targeting with nanoparticle-bound compounds, e.g., antigens and vaccines, may be a promising new approach in transcutaneous vaccination strategies [31] (Fig. 2B and C). Similarly, flexing of porcine skin significantly increased the penetration of 3.5 nm fullerene amino acid-derivatized peptide nanoparticles [93].

With regard to unintended exposure to nanoparticles in the environment or cosmetic products, however, such results underline the importance of further risk assessment, especially in the light of the increasing number of individuals suffering from skin disorders and diseases, such as eczema, which cause significant barrier disruption and damage.

In contrast to most dermatotherapeutic systems based on the solid particles, which are still experimental, multiple semi-solid preparations, such as liposomes are commonly implemented in clinical practice and represent valuable tools for future targeting approaches.

In accordance with the work on solid particles, follicular aggregation and penetration along the hair follicle duct were also confirmed for the liposomal preparations [94,95]. Du Plessis et al. evaluated the effect of the particle size of liposomes carrying cyclosporin-A on the deposition of drugs into the skin strata of hairless mice, hamster and porcine skin [96]. They found that the intermediate particle size of 300 nm resulted both in the highest reservoir in the deeper skin with the exception of the porcine ear and in the highest drug concentration [97].

In the recent studies, the focus of subsequent studies shifted to the direct delivery of biological compounds as well as vectors for DNA expression. For example, topically applied melanin entrapped in phosphatidylcholine liposomes induced hair shaft pigmentation in white-haired mice [98]. Several researchers have shown that the envelopment of a vaccine in liposomes elicited a clearly increased humoral or cellular immune response, compared to the non-enveloped vaccine [99–102]. Balsari et al. reported that liposomes can deliver monoclonal antibodies into the hair follicles of rats for protection against doxorubicin-induced alopecia [103]. Liposomes loaded with DNA have been used to target high molecular weight DNA to hair follicles in histocultured skin, as a model for gene therapy of hair growth processes. The successful targeting of liposomes loaded with lacZ reporter gene or the repair gene T4 endonuclease V demonstrates the potential of particulate preparation for drug delivery in the context of gentherapeutic approaches [104,105].

In summary, encapsulations using nano- and microparticulate systems, which are an increasingly implemented strategy in drug targeting and delivery, may provide a base for a new generation of transcutaneous, more precisely, "transfollicular" delivery of bioactive compounds [106]. Compared to conventional preparations, such systems may enable sustained release, resulting in an extended activity or enhanced uptake [107,108], and the possible reduction of adverse effects [109]. Furthermore, encapsulated substances are shielded from degradation in the particles [107,110]. Functional coatings can also facilitate the targeted accumulation and release of drugs at their therapeutic sites [111,112]. Several studies have investigated the targeting of specific anatomical sites such as the eye [113], nose [114,115], brain [116,117] and intestine with particles [118,119].

5. Conclusions

The important role of hair follicles as penetration pathways and reservoir structures for topically applied compounds has been validated in numerous animal models as well as in human skin. This body of experimental work provides the base for the design of advanced drug delivery systems, which, in future applications, may allow specific targeting of bioactive molecules to hair follicle-associated cell populations. Targeting of sebaceous glands was the first concept, which was evaluated in clinical applications. Recently, very promising first attempts to translate the experimental knowledge on cell-specific targeting into clinical applications have been undertaken in the fields of immune cell targeting and transcutaneous vaccination as well as stem cell targeting and gene therapy. Among the newly emerging concepts, drug delivery systems based on nano- and microparticles, which efficiently penetrate via the follicular route, are highly promising approaches.

The finding that small nanoparticles are capable of penetrating barrier-disrupted otherwise damaged skin, however, requires the careful investigations of both the therapeutic potential and the possible hazards related to nanoparticle exposure. Additional preclinical data and well-designed pilot studies are necessary for the further development of follicular targeting techniques in order to bring this interesting and promising new concept to clinical applications.

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