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Nanocarriers for drug delivery into and through the skin - Do existing technologies match clinical challenges?

Annika Vogt[1, #], Christian Wischke[2,#], Axel T. Neffe[2], Nan Ma[2], Ulrike Alexiev[3], Andreas Lendlein[2, *]

[1] Clinical Research Center for Hair and Skin Science, Department of Dermatology and Allergy, Charité—Universitätsmedizin Berlin, Berlin, Germany

[2] Institute of Biomaterial Science and Berlin-Brandenburg Centre for Regenerative Therapies, Helmholtz-Zentrum Geesthacht, Kantstr. 55, 14513 Teltow, Germany

[3] Institute for Experimental Physics, Freie Universität Berlin, Arnimallee 14, 14195 Berlin, Germany

[#] Authors contributed equally.

* Corresponding author: andreas.lendlein@hzg.de, Phone: 0049 (0) 3328 352 450

Abstract

The topical application of drug-loaded particles has been explored extensively aiming at a dermal, follicular or transdermal drug delivery. This review summarizes the present state of the field of polymeric nanocarriers for skin application, also covering methodologies to clinically characterize their interaction and penetration in skin in vivo. Furthermore, with a focus on a clinical perspective, a number of questions are addressed: How well are existing nanoparticle systems penetrating the skin? Which functions of new carrier concepts may meet the clinical requirements? To which extend will instrumental imaging techniques provide information on the biological functions of nanocarriers? Which issues have to be addressed for translating experimental concepts into a future clinical application?

Keywords: Nanocarrier, Polymer nanoparticle, Skin delivery, Follicular delivery, Topical application

1. Introduction

The opportunity of delivering bioactive molecules by penetration into or permeation through the skin has tremendous implications for a local therapy of skin diseases as well as vaccination or systemic delivery of drugs with poor peroral bioavailability. According to their natural function, however, the skin is a very efficient barrier, at least in a healthy state. This outlines the clinical challenges, which are associated with topical administration: How to realize a transport of pharmaceutics to their respective target site, ideally allowing for a long lasting pharmacological effect?

More specifically, one challenge is to find the right balance between enhancing the penetration of active compounds through the skin barrier and at the same time ensuring sufficient retention to maintain therapeutic drug concentrations within the skin. Increasing the selectivity by delivering bioactives specifically to lesional skin is another important task to address. Last but not least, encapsulation may allow to effectively deliver molecule classes, which are sensitive to degradation and not yet available for topical therapies such as peptides, proteins or nucleic acids.

A variety of concepts has been proposed to overcome skin barrier functions and improve drug delivery compared to conventional creams or ointments particularly for substances, which are hardly penetrating the skin because of their size or relative hydrophilicity. For enhanced drug penetration, substances, which chemically disturb the skin structure [1], have been more critically discussed compared to occlusion-mediated skin hydration and diffusion enhancement. Low-frequency ultrasound can be used to temporarily disturb the skin barrier by means of shock waves and acoustically-induced microjets resulting from cavitation [2]. Electroporation by high voltage pulses temporarily creates aqueous pores in cell membranes for substance diffusion [3]. Ablation by laser techniques allows destroying skin layers in a controlled fashion, thereby enhancing drug penetration depth through artificial vertical channels [4]. Microneedle arrays mechanically damage the skin, forming diffusion paths for subsequently applied formulations. If coated with drug, used as hollow needles for microinjection, or being made from an immediately dissolving material, microneedles can directly deposit drug substances into the skin [5-7].

Nano-sized carrier systems represent alternative approaches, but could also be powerful additions to the above mentioned skin delivery technologies to facilitate drug delivery by topical application. Ideally, they should allow the transport of incorporated or coupled substances into the skin without the need to previously damage the natural barrier function. Nanocarriers may facilitate drug delivery to structural features of the skin like hair follicles, interact with skin lipids to mediate transportation and/or allow creating depots of drug in the skin for a sustained or stimuli-induced release [8-10].

Considering possible routes of nanoparticle transport into healthy and damaged skin, this review reports on the functions of various polymer-based nanocarriers for skin application, present techniques to study nanoparticle skin penetration and cellular interaction, and finally discusses clinical achievements and perspectives of nanocarriers for dermal applications.

2. Skin anatomy and skin diseases

Healthy skin is very efficient in fulfilling its task as a biobarrier. It is organized as a multilayer structure, which can roughly be categorized into hypodermis, dermis and the non-vascularized epidermis, the latter being covered by the stratum corneum (Fig. 1) [11]. This uppermost anatomic compartment of the skin consists of terminally differentiated corneocytes embedded in a complex lipid matrix and is a major physicochemical and anatomic barrier. Corneocytes and matrix are not only stacked in a “brick-and-mortal” pattern [12], but are interconnected by corneodesmosomes and specialized anchoring structures, which contribute to the mechanical stability. Advances in high-resolution microscopy techniques including electron microscopy [13] and Raman spectromicroscopy revealed a highly organized matrix system containing hydrophilic and hydrophobic compartments as a result of a sophisticated lipid organization [14]. Although hardly detectable under normal conditions or on tissue sections, the presence of aqueous regions has been postulated based on sonophoresis experiments [15] and studies on the penetration of deformable carrier systems across intact skin [16]. The stratum corneum essentially contributes to the fact, that penetration of most molecules across intact skin barrier is limited. Despite a more refined view on its ultrastructure with more differentiated

insights on penetration pathways of large molecules [17], the original "500 Dalton rule" of dermatopharmacy as maximum molecular weight (MW) for skin penetration is still relevant [18].

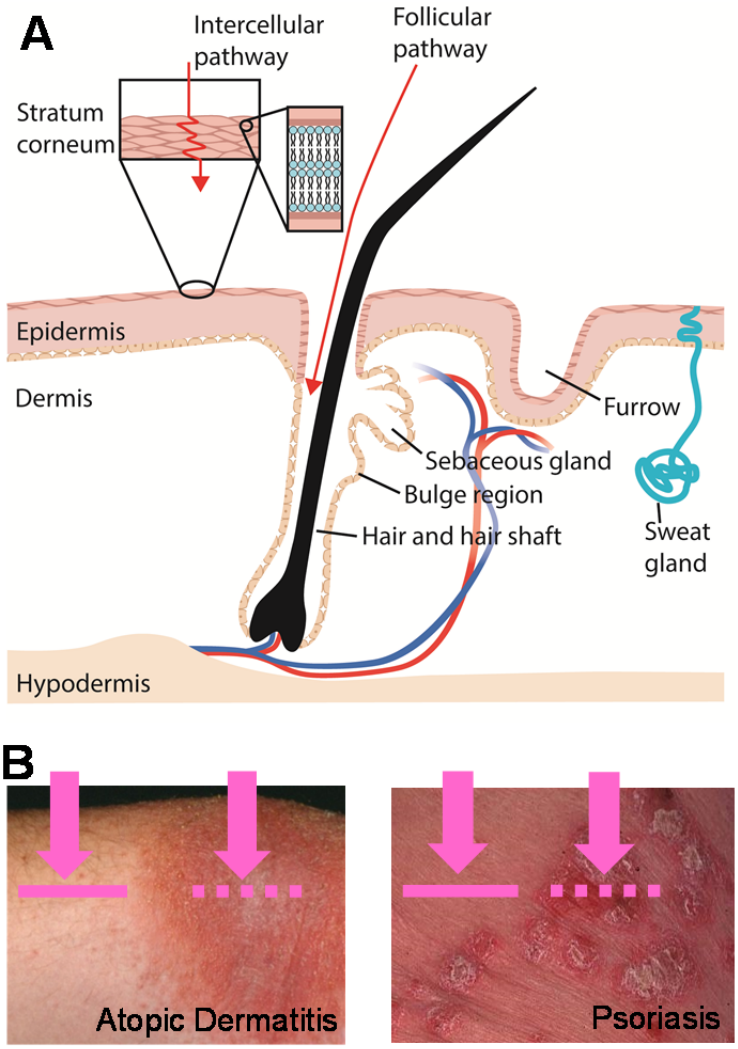

Fig. 1: Schematic representation of skin anatomy and main entrance routes for nanoparticles (A) as well as clinical images of intact and diseased skin (B). As illustrated, inflammatory processes differentially affect the skin barrier. Acute atopic dermatitis is characterized by confluent papulovesicles, psoriasis patients exhibit hyperproliferative plaques with characteristic scales.

In contrast, the hair follicles represent important reservoir structures and shunt penetration pathways. In fact, the pilosebaceous units are key anatomic compartments for particle-based drug delivery

systems. Hair follicle types and dimensions vary significantly among the different body regions [19]. While scalp terminal hair follicles and dilated acne pores can conveniently be reached with particles in the submicron size range [20], penetration in body vellus hair follicles is limited to smaller particles sizes [21].

Furthermore, sebum and sweat flow contribute to the complex microenvironment on the skin surface. As a result, topical formulations have to be adjusted to the intended body region for treatment, e.g., scalp hair treatment versus acne therapy or barrier restoration of dry skin of lower legs. All in all, the impact of the skin surface microenvironment and, similarly, of the skin microbiome on penetration processes and carrier-skin barrier-interactions is only poorly understood.

Once a carrier passes the stratum corneum, tight junctions between stratum granulosum cells act as paracellular diffusion barrier as demonstrated for soluble proteins, including immunoglobulins and bacterial toxins [22]. Only limited information is available on the interactions of nanocarriers with this important skin barrier element. Yet, the observation that activation of epidermal Langerhans cells can result in increased scanning activities and active modulation of tight junctions to take up large molecules [23], illustrates the dynamics in the interplay between barrier, microbiome, topical actives and biological processes in the skin. Those insights also provide the rationale for the exploration of transcutaneous immune cell targeting strategies [24].

In case of diseased skin, the skin barrier structure and function can change in many different ways. In chronic lesional skin affected by inflammatory disorders like atopic dermatitis or psoriasis, the differentiation process of the keratinocytes, the biosynthesis of the stratum corneum [25], its lipid composition and organization are altered [26]. Inflammatory infiltrates, the release of mediators as well as altered microbial colonization all contribute to significant changes in the microenvironment of lesional skin, which are associated with shifts in conditions like pH or transepidermal water loss. Also, the function of tight junctions is affected by the disease process [27]. Although both skin diseases can be easily distinguished clinically and exhibit distinct histological changes, impaired barrier function and increased percutaneous absorption rates have been demonstrated for both diseases [28,29]. However, an increased penetration into skin did not apply to all molecules studied [30]. In fact, the

penetration rates in diseased skin obtained in clinical studies on humans show rather moderate increases [31].

With a better understanding of penetration pathways across diseased skin, polymer-based nanocarrier systems specifically designed to take advantage of the skin barrier alterations in lesional skin could help addressing some of the clinical challenges in dermatotherapy. E.g., atopic dermatitis and psoriasis respond well to treatment with topical corticosteroids, but disadvantages result from the chronic course of the diseases with recurrent episodes and long-term corticosteroid treatments, which are associated with the risk of dermal atrophy and vasculopathy [32]. With respect to such low molecular weight drug molecules, patients would greatly benefit from delivery systems, which improve the selectivity of the therapy, i.e., by targeted delivery of the corticosteroids to the infiltrates or by a prolonged release from reservoirs, which maintain high concentrations at the site of action, hereby allowing for reduced applications frequencies. In contrast, as a result of its high molecular weight, the calcineurin-inhibitor cyclosporin A (MW = 1203 $g \cdot mol^{-1}$) does not reach sufficient penetration rates for topical applications [33]. Topically applied tacrolimus (MW = 804 $g \cdot mol^{-1}$) is effective for the treatment of acute dermatitis, but only to very limited extent for psoriasis [33], although both diseases are associated with compromised skin barrier function compared to healthy skin respond to systemic treatment with calcineurin-inhibitors. For such molecules, effective transportation across the skin barrier is the challenge.

Pathologic processes along hair follicles, as another example, are especially hard to reach and treat by topical therapies, partly because of insufficient penetration, but also as a result of an effective drainage by the highly developed perifollicular vascularization. The topical treatment of inflammatory hair diseases like alopecia areata or cicatricial alopecia subtypes with chronic progressive destruction of the stem cell niche largely relies on corticosteroid application, despite the fact that other immunosuppressive agents, including cyclosporine A and tacrolimus, were shown to be effective when used systemically [34]. This challenge can most effectively be addressed by encapsulation of the bioactives in nanocarriers, which enable sustained release, because hair follicles are a preferred penetration pathway for nanoparticles, as outlined in the following. Possible applications for particle-based drug delivery into hair follicles include the treatment of inflammatory hair diseases, but also hair

growth modulation, targeting of stem cell niches to modify hair growth, improvement of wound healing or gene therapy [35].

The differentiation between lesional and non-lesional skin is especially relevant for the topical treatment of epidermal malignancies. Several approaches for topical treatment of early-stage non-melanoma skin cancer like actinic keratoses or superficial basal cell carcinomas using chemotherapeutic agents or immunomodulators are available, but are restricted to very superficial lesions [36]. Reaching deeper infiltrates or tumor nests, as in the case of basal cell carcinoma, remains a challenge [37]. Here, the use of carrier-based systems, which accumulate actives in tumors while minimizing exposure of non-lesional skin would be a great benefit. This also refers to selective delivery of photosensitizers for improved photodynamic therapy, a field increasingly explored for nanocarrier-based delivery [38,39,40] and option for treatment of malignant melanoma [41].

Thus, anatomic particularities and the specific changes, which are observed in diseased skin, also offer great potential for the design of selective therapies (Fig. 1).

3. Routes, targets and risks of drug delivery by polymer-based nanocarriers

Nanoparticles and carrier-based drug delivery systems can be designed to interact differently with skin barrier components, which opens a multitude of highly interesting clinical applications. More specifically, by use of particulate carriers, distinct transport mechanisms may be facilitated that do not apply, e.g., for dissolved bioactive molecules. Also depots of drug may be created in the skin for sustained release, i.e. the free drug may access deeper skin layers after release from a particle that resides in a specific skin compartment. Accordingly, applications include (i) penetration improvement for established molecules, large molecules with limited penetration, or molecules hard to formulate, (ii) delivery of novel drug classes, or, (iii) targeted delivery to specific cell populations (Fig. 2) [35]. While use of endogenous triggers for stimuli-controlled drug release have widely been explored by other disciplines including oncology and radiology, the field is currently emerging in skin research addressing skin pH [42], temperature and micromilieu of particularities in skin diseases to enable selective entry of actives [43]. Furthermore, the easy access to the skin surface opens a wide field for use of external triggers including UV light and heat. Combinations of topical drug application and UV

light or laser energy are routinely been used in clinical practice for treatment of inflammatory skin diseases or superficial skin tumors, respectively.

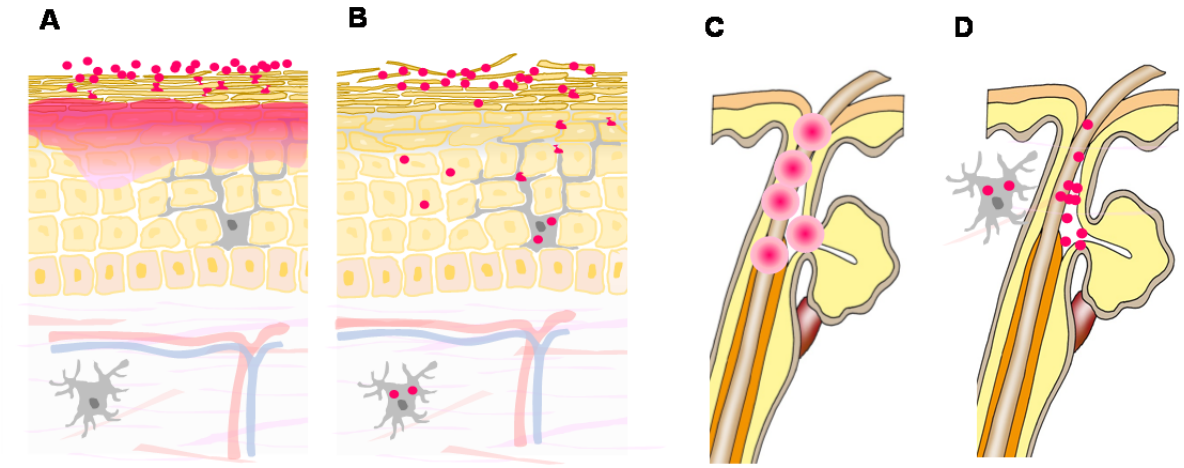

Fig. 2: (Nano-)particles can be designed to interact differently with skin barrier components. (A) While micelles, liposomes and lipid particles act via penetration enhancement and usually disintegrate during the penetration process, polymer particles may penetrate as intact particles and, by variation of composition, may allow for prolonged release kinetics. (B) Nanoparticles may reach viable cells especially in barrier-disrupted skin, which builds the rationale for transcutaneous targeting of cell populations such as skin antigen-presenting cells or stem cells. (C) The preferred penetration into hair follicle openings has been well established for particles size up to several micrometers. While such larger particles can help maximize the concentration of active compounds in the hair follicle, (D) nanoparticles <200 nm do not only penetrate deeply into hair follicles but also have the potential to penetrate the barrier and be internalized, e.g. by activated antigen-presenting cells.

First evidence for using carrier-based systems to bring actives not only across the skin but to cell populations creates highly interesting opportunities for immune cell and stem cell targeting approaches with clinical implications in vaccination [44], wound healing, tissue regeneration and gene therapy [45].

The polymer-based nanocarriers predominantly explored for skin delivery may roughly be classified based on the nature of their matrix material and polymer architecture, which will be discussed below in more detail. Inorganic, metal or lipid-based nanoparticles as well as nanocrystals will not be focused here, but may be briefly touched in the context of selected fundamental studies. In principle, for penetration of nanocarriers into the skin, the intercellular and follicular route, at least in healthy skin, may be most relevant. Particles deposited in furrows atop the stratum corneum may continuously release their payload, which may become effective only if these molecules show suitable features for skin penetration such as moderate lipophilicity and low MW.

Parameters affecting the penetration of intact nanocarriers include physical properties such as size or shape as recently summarized [11], but also the deformability of vesicles has been proposed as a concept to enhance skin penetration [46]. Importantly, it should be noted that skin penetration of blank carriers may not necessarily be predictive for drug loaded systems, at least in cases where drug loading would alter relevant particle properties such as size (e.g. by aggregation or due to necessary modifications in the particle preparation process) or surface properties (e.g. altered hydrophilicity/hydrophobicity due to drug molecules associated to the particle surface). Chemical cues, which origin from the carrier material such as specific moieties interacting with skin components, or external triggers (e.g. ultrasound, microneedles) may support skin penetration [47].

The relevance of size for nanoparticle diffusion, despite obviously existing as explored in numerous model studies [11], has not been conclusively led to a general rule as comparable to the “500 Da rule” for dissolved molecules. This may be, because in addition to size, other physicochemical properties of nanoparticles such as their chemical composition or their capacity for deformation or dynamic reorganisation as in case of some lipid-based carriers [48] could additionally affect the penetration behavior.

However, since the first clinical studies using microparticles to deposit acne therapeutic in face hair follicles [49], the hair follicle has emerged as highly interesting target for carrier-based applications despite relatively low numbers of follicles on a given skin area [11]. Penetration depth can be varied by particle size and the release of encapsulated substances can be directed to the targets of interest, e.g.

the hair follicle infundibulum surrounded by a rich network of antigen-presenting cells, the sebaceous gland and the stem cell niche being rich in epithelial and mesenchymal stem cells [50]. The transport into the hair follicle was shown to be enhanced by massage, which was postulated as a mechanically stimulated ratchet mechanism mediated by the anatomy of hair follicle and hair [51].

The shape of particles has repeatedly been mentioned as possibly relevant parameter for skin penetration, but only rarely studied. In some model studies with human and mouse skin, small gold nanorods showed enhanced skin penetration compared to spheres, but a deposition only in the top skin layers [52], while others suggested penetration in human skin only in case of pricking to destroy the barrier provided by the stratum corneum [53]. Small silver nanorods, compared to spheres and triangle shaped particles, showed highest blood concentration after dermal application in hairless mice with skin occlusion were assigned to a proposed intercellular permeation pathway [54].

Besides the appreciated therapeutic effect of the released bioactive, a potentially undesired systemic bioavailability or stronger site effects by penetration enhancement need to be included in the clinical evaluation of nanocarriers (e.g., skin irritancy testing; see Section 5.2). Possible irritative effects may also result from to the presence of the carrier themselves, e.g. when the evaporation of water from applied aqueous suspensions or hydrogels leads to accumulation of high local concentrations of carrier material in furrows and hair follicles. Accordingly, the fate of the carrier system requires an explicit attention. This includes both acute effects as well as a potential long-term toxicity, which may go beyond the dermal application site due to nanoparticle permeation and biodistribution. A wide range of studies confirmed that the vast majority of particles topically applied on the skin surface do not penetrate into deeper stratum corneum layers and that the likelihood of reaching viable epidermis is very low. E.g., skin penetration across human skin as well as long-term exposure studies in animals suggested no or minimal penetration for titanium dioxide and zink oxide particles frequently used in sunscreen formulations [55,56]. Similar results were obtained with polymeric particles [57], but some investigations also point towards increased penetration in disrupted skin [58,59]. Thus, degradable nanocarriers such as from hydrolytically cleavable polymers producing biocompatible degradation products may be advantageous from the perspective of long-term toxicity, especially when designed

for applications on lesional skin in patient populations prone to adverse side effects, e.g., in atopic patients.

4. Variety of polymer-based nanocarriers

Three important classes of polymer-based nanocarriers discussed here are based on i) hydrophobic polymers, which form stable particulate carriers in an aqueous environment, ii) large molecules with dendritic structures, and iii) hydrophilic nanogels, i.e. polymer networks.

Hydrophobic polymers are attractive carrier matrices for hydrophobic, small molecule drugs, because of a probable 'affinity' of matrix material and payload, e.g. allowing to molecularly distribute relevant quantities in an amorphous polymer phase. Particle formation and drug loading generally occurs in one step and is often based on using water and an organic solvent. E.g. in emulsions, nanoprecipitation, or solvent-displacement methods, in which the polymer and the drug co-localize leading to reasonable encapsulation efficiencies and drug loadings. One versatile and representative class of (co)polymers with easily tunable properties are polymethacrylates, which are formed through free radical polymerization, and whose hydrophobicity and potential charge can be determined by the use of different readily available monomers. In a recent example, nanoparticles with a size of 40-150 nm were obtained by a solvent-displacement method [60]. Three types of such nanocarriers were investigated, neutral carriers based on methacrylate copolymers, positively charged carriers, which contained trimethylammonium ethylmethacrylate chloride (TMAEMAC), and negatively charged carriers, to which sodium lauryl sulfate was added. The copolymer composition ruled the glass transition of the matrix, and therefore its deformability. The carriers were loaded with coenzyme Q_{10} or Nile Red dye. In an in vitro skin permeation assay (abdominal guinea pig skin), particle transport into the stratum corneum and drug release resulted in a deeper penetration of the drug than in case of a drug formulated in capric/caprylic triglyceride. Beneficial for the penetration were positive charges on the particles as well as deformability of the particles, reached through particles with a T_g < body temperature. However, such carriers are mainly useful as model particles, as the long-term fate of non-degradable particles in the skin is unclear and may potentially be inducing critical cellular responses.

Therefore, materials suitable for hydrolytic and/or enzymatic degradation, which are then fragmented to nontoxic products, add an additional functionality to nanocarriers for skin delivery. For this purpose, matrices based on hydroxyalkanoates such as poly(lactic acid) (PLA), poly(lactide-*co*-glycolide) (PLGA), or poly(*ε*-caprolactone) (PCL) are the common choice. For PCL particles in a size range of 500-900 nm prepared by vibrational spray-drying [61], it could be shown that the particles promote the penetration of dexamethasone into the dermis, as shown on pig skin using a HPLC method for drug quantification. Though not investigated in this study, it was speculated that the enhanced drug penetration may be related to the uptake of the particles into the hair follicle, acting as reservoir. Nanoparticles of ~170 nm diameter prepared from PLGA, poloxamer and oil in a nanoprecipitation process promoted the penetration of loaded dye into the epidermis but not into the dermis of presumably pretreated skin [62]. As introduced in Section 2, particles >100 nm are mostly considered for follicular transport, such as reported for PLA particles of ~150 nm diameter prepared from a nanoprecipitation process with narrow size distribution and high yields [63]. Fig. 3 shows the penetration of such particles loaded with 0.05 wt.% Nile Red into the hair follicle of porcine skin. The in vitro release data of FITC and Nile Red from those particles are quite typical in that the release is often limited to the first 12-24 h, as the low diffusion lengths and low loading leads to fast depletion from the particle matrix. Follicular transport can be enhanced by suitable surface modification as shown for phospholipid coated compared to uncoated or chitosan coated PLGA particles of 160-170 nm prepared by an emulsion technique [64].

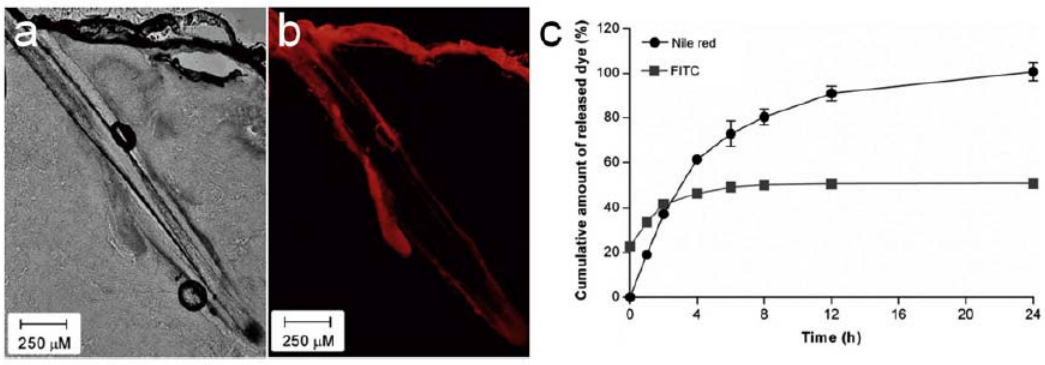

Fig. 3: Bright field (a) and fluorescence (b) image of a hair follicle after 24 h of incubation with Nile Red loaded poly(D,L-lactide) nanoparticles. c) In vitro release profile of Nile Red and fluorescein 5(6)-isothiocyanate from poly(D,L-lactide) nanoparticles. (Reproduced from [63] with permission, © 2014 Society of Cosmetic Scientists and the Société Française de Cosmétologie.)

In addition to hydroxyalkanoates, also other degradable matrices such as polyalkylcyanoacrylates (PACAs) have been suggested as nanocarriers for skin applications. Poly(*n*-butylcyanoacrylate) carriers of 190 nm diameter could e.g. be loaded with >1wt.-% of indomethacin [65]. After topical application on rat skin, the determination of plasma levels showed transdermal drug uptake. However, the uptake mechanism remains to be clarified.

Carrier systems with different phases have been shown to influence carrier penetration as well as drug release and uptake by different mechanisms. For PLGA particles, it could e.g. be shown that incorporation into hydrogels increases drug uptake, probably by utilizing the occlusion effect to enhance drug delivery into deeper layers of dermatomed human skin [66]. Combination of hydrophobic and hydrophilic segments in block copolymers also enhanced the penetration of loaded drugs, which was e.g. shown for PACA-block copolymers on rat skin [67] and PCL-*b*-PEG copolymers, especially in combination with β-cyclodextrin on porcine ear skin [68]. Here, as discussed above, the carrier itself is only transported into the SC, and the drug is locally released and penetrates into deeper layers. While the investigation of further polymer matrices is ongoing, it should be noted that such hydrophobic carriers need to be stabilized by surfactants [69], whose influence on skin penetration has not been fully addressed nor clarified. In addition, the formation of a protein corona in in vivo experiments will have a strong influence on the biodistribution, cellular recognition, and release profiles of nanocarriers [70-71].

As alternative to top-down techniques with processing of preformed hydrophobic polymers to small particles, macromolecules as carriers with distinct molecular structures such as dendrimers or dendritic (hyperbranched) architectures can be obtained by a bottom-up synthesis, where the particle size can be tailored by the synthesis conditions. By choosing appropriate end groups exposed at the surface, such

carriers might not require the use of classical surfactants for stabilization. Typical investigations of the structure-property-relationships of such carriers have been performed for polyamidoamines (PAMAM), where the particle sizes were varied between 10 and 18 nm by using dendrimers of different generations and -NH_2, -COOH, and –OH moieties were investigated as surface functional groups [72]. Here, an enhanced porcine skin penetration could be shown for positively charged compared to negatively charged or neutral nanocarriers. Furthermore, the porcine skin penetration was inversely correlated to MW and a transport could be detected up to the viable epidermis. Fig. 4 shows that predominantly an intercellular pathway is followed through the SC. Iontophoresis increased the efficacy of penetration. While a large variety of (model)drugs including furfural, tamsulosin, 5-fluoruracil or riboflavin can be incorporated and transported by PAMAM dendrimers [73], the toxicity of PAMAM dendrimers remains problematic and the use of degradable and less toxic carriers is investigated.

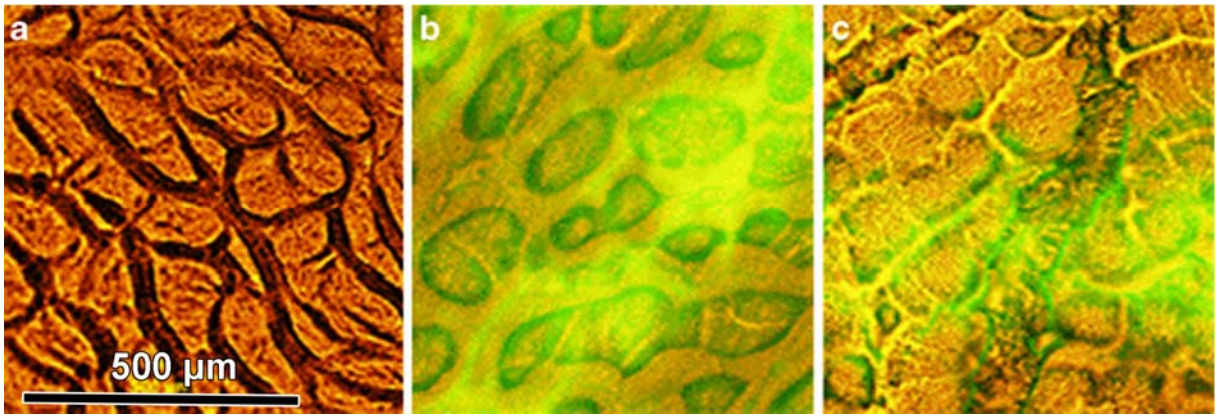

Fig. 4: Fluorescence microscopy images of tape-stripped porcine skin: (a) blank, (b) after treatment with free fluorescein isothiocyanate (FITC) and (c) after treatment with FITC-labeled polyamidoamine dendrimers of generation 4 with terminal NH_2-groups (PAMAM-G4-NH_2) for 2 h. In the case of free FITC, the fluorescence was diffuse in the SC, while in the case of the FITC-labeled PAMAM-G4-NH_2 dendrimer, fluorescence was detected primarily in the intercellular regions. This can be interpreted as a specific uptake route for FITC when bound to the carrier. (Reprinted from [72], © Springer Science + Business Media, LLC 2011. With permission of Springer.)

One exemplary class of hyperbranched carriers that incorporate different compartments are oligoglycerol-based core-multishell (CMS) carriers, which may consist of a hydrophobic first shell, e.g. based on fatty acids, and a hydrophilic outer shell such as based on PEG [74]. Such CMS can carry hydrophilic as well as hydrophobic drugs distributed in the respective compartments, while the payload per individual particle will be restricted by the compartment volume available in such small particles. Again, by employing moieties or segments exposed at the surface that well interact with the dispersion medium like the hydrophilic PEG shell with water, good colloidal stability can be achieved.

While hydrophobic carriers are often suitable for typical small molecule drugs [75], such carriers may have shortcomings in regard to encapsulating and releasing the generally more hydrophilic biologicals (e.g. proteins, DNA) [76], so that alternative types of carriers have attracted attention. In this context, nanogels, i.e. polymer networks of defined dimensions are of interest. Nanogels are based on hydrophilic components, which swell in aqueous environment. They have not been used extensively, yet, for topical administration. Networks based on *N*-isopropylacrylamide (NIPAM) and dendritic polyglycerols, which are accessible through radical copolymerization, can be loaded with proteins such as bovine serum albumin, L-asparaginase II, or transglutaminase-1 up to 70 wt.% loading capacity [77]. At temperatures >35 °C, the thermoresponsivity of NIPAM-based segments resulted in collapse of the gels, which was correlated with a protein release after administration of the particles to porcine skin or human reconstructed skin. These temperatures are typically reached within the deeper layers of the stratum corneum, which may be a hint towards an access of the nanogel to the stratum corneum, while the released proteins could also be detected in the epidermis.

Taking up one observation from methacrylate and PLGA carriers, in organogels, the release of drugs is inversely correlated with the rigidity of the gel [78], and can be related to the easily determinable complex modulus of the gel. In this case, the skin penetration is not depending on the carrier transport, but rather by the reduced diffusion of the load in the organogel.

Overall, while fundamental studies using polymeric nanocarriers for skin delivery have been showing promising results, these carrier systems have not entered, yet, clinical trials or wide-spread application.

5. Analysis of nanocarrier penetration and skin interaction

5.1 Label-based and label-free instrumental methods

Understanding of drug penetration into skin and enhanced penetration into skin facilitated by nanocarriers have been addressed so far by a variety of microscopic, spectroscopic and structural methods. The focus of the studies ranges from localization and penetration/permeation depth measurements, investigation of penetration pathways and diffusivities in the skin or skin constituents, to cellular uptake and intracellular interactions as well as skin condition. Most of these methods can also be applied for the characterization of the nanoparticles in terms of size, shape, morphology, surface properties, softness, or electrostatics. The choice of the technique also depends on the skin or the skin sample to be investigated, i.e. human skin in vivo, ex vivo skin explants and reconstructed skin models including skin sections, stratum corneum sheets as obtained by tape stripping (for a review see [79]), or isolated cells (for cellular uptake techniques see review by [80]).

For characterization of nanoparticle properties and penetration in skin, either label-based or label-free instrumental methods may be used. Depending on the nanoparticle or drug (e.g. solid nanoparticles such as Ag NP, polymer-based nanocarrier, lipid nanoparticles) label-free methods include TEM/SEM, Cryo EM, soft X-ray spectromicroscopy, Raman microscopy, surface enhanced Raman scattering (SERS), and light and fluorescence microscopies as well as FTIR and infrared microscopy. Label-based techniques include fluorescence spectroscopy and microscopy (intensity-based, spectrally-resolved, time-resolved, raster scanning based correlation techniques), single molecule fluorescence microscopy, and EPR techniques. These techniques vary in sensitivity, specificity (information content), spatial resolution, and technical requirements. In this paragraph an overview of the different techniques and their applications will be given.

5.1.1 TEM/SEM, Cryo-EM, AFM, Neutron scattering, X-ray diffraction. Transmission electron microscopy (TEM), cryo-electron microscopy (CryoEM), scanning electron microscopy (SEM) as well as atomic force microscopy (AFM) are routinely used to characterize the shape and surface morphology of nanoparticles [81,82,83] and can be applied to collect information on nanoparticle localization in skin tissue.

For penetration studies, TEM is sensitive to electron-dense areas in the sample and provides valuable images of skin sections particularly for metallic or silica nanoparticles ([81] human skin; [84] murine skin) with ultrastructural spatial resolution down to 0.1 nm. Recently, a correlative microscopy approach combining TEM, light and fluorescence microscopy has been developed [85]. SEM, using a raster scan of an electron beam over a surface, and in combination with an X-ray microanalyzer (SEM-EDX) that is sensitive in the excited volume element, allows for a simultaneous visualization and elemental analysis of the specimen [81]. AFM provides information on height differences in the nm range. Using this technique, clusters of disperse nanoparticles (particle dimension: 37.5 nm width at half height) in the collagen network of the dermis were visualized in human skin cryosections [86].

Since lipids are a major constituent of the stratum corneum, knowledge of their molecular arrangement provides valuable information about the physical structure of the skin barrier in health and disease states of the skin. This information, in the nanometer range, can be obtained by neutron scattering of deuterated samples [87-88] or by X-ray diffraction [89]. Nanoparticles, drugs or penetration enhancer exert different effects on stratum corneum lipid microstructure as shown by wide and small angle X-ray diffraction (WAXS, SAXS) [90].

5.1.2 Soft X-ray Spectromicroscopy. X-ray microscopy is a powerful label-free technique that relies on the resonant excitation of the target samples (e.g. polymers or drugs) in the soft and hard X-ray regime [91,92,93]. The high spatial resolution of a few nanometers is combined with chemical selectivity by tuning the photon energies in the soft X-ray regime to an energy, e.g. required for the excitation of a 1 s electron (K-edge) of carbon or oxygen. These experiments are usually performed at a synchrotron. Fig. 5 shows as an example the penetration profile of the drug dexamethasone in human skin ex vivo as measured by scanning X-ray microscopy of skin sections [93]. By tuning the soft X-rays to a photon energy, at which the absorption of skin is efficiently suppressed, the absorption of the drug (here at the oxygen K-edge at 530.6 eV) can be selectively measured and visualized in the image. Concentrations of dexamethasone as low as ~1-2 $\mu g/cm^2$ were detected in human skin [93].

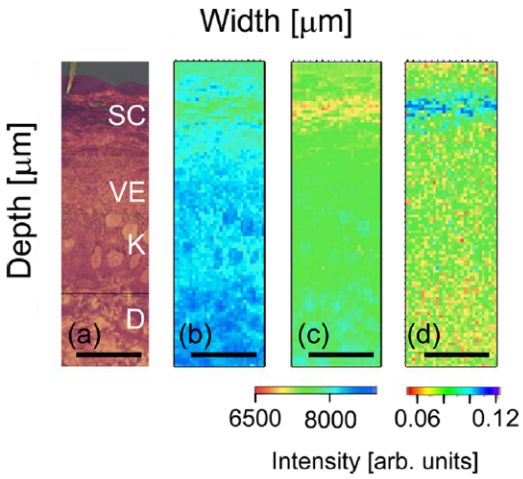

Fig. 5: Penetration of dexamethasone into human skin analyzed by Soft X‑ray spectromicroscopy (SC, stratum corneum; VE, viable epidermis; K, nuclei of keratinocytes; D, dermis): (a) optical micrograph of a vertical skin section of 30 µm × 100 µm; (b) absorption of the same skin section at 528.0 eV (pre-edge regime); (c) absorption of the same skin section at 530.6 eV (O 1 s → π* resonance); (d) optical density of taken up dexamethasone as a function of depth. The scale bar corresponds to 20 µm. (Reprinted with permission from [93]. Copyright 2015 American Chemical Society.)

5.1.3 Vibrational spectroscopy and microscopy including infrared (IR), Fourier transformed infrared (FTIR), Raman, surface enhanced Raman scattering (SERS), coherent anti-stokes Raman scattering (CARS). The absorption in the infrared spectral region provides information on the vibrational states of molecular bonds. Accordingly, information on molecular composition and structural organization, e.g. of lipid lamellar phases, becomes available. Investigations of nanocarrier penetration and drug release were performed *in vivo* by using the tape-stripping technique to collect different layers of stratum corneum. In combination with Fourier-transformed infrared spectroscopy (FTIR) [94] and FTIR photoacoustic spectroscopy [95], quantitative distribution profiles in human stratum corneum were obtained. Furthermore, FTIR measurements on isolated stratum corneum sheets can provide information on skin hydration, protein and lipid composition as well as secondary protein structure [96]. The lateral organization and conformational ordering of the lipids in the sample can be observed

via the methylene scissoring and stretching modes [97]. Due to the strong IR absorption of water, however, in vivo IR spectroscopy experiments are sensitive only to the superficial layer of the skin.

Raman spectroscopy gives information on Raman-active bonds that change their polarizability upon excitation with a certain frequency of light and therefore provides selectivity to specific bonds. Since the intensity of the Raman peak is directly proportional to the concentration of the respective molecule, non-invasive detection and quantification of Raman-active compounds in skin is feasible in vivo [98,99]. This label-free technique also provides images, i.e. direct visualization (resolution down to ~1 μm), together with chemical analysis when using Raman microspectroscopy [100] and can be used for in vivo applications on human skin [101] [102]. Water profiles, stratum corneum thickness and penetration profiles of hydrophilic substances with a depth resolution of 5 μm were reported for in vivo confocal Raman measurements [101]. The penetration profiles of Raman-active nanoparticles can be obtained from the overlapping Raman spectra of the nanoparticles and the skin compounds, such as cholesterol, ceramide, keratin, urea, water, and other skin compounds, by deconvolution with the known model spectra [100]. SERS and CARS are further advanced label-free imaging techniques based on molecular vibrational spectroscopy with resolutions down to the submicrometer region, which have been used for the detection of nanoparticles and drugs in the skin ([100] porcine skin, [103] murine skin).

5.1.4 Light microscopy and diffuse reflectance spectroscopy. Light microscopy is a standard technique, e.g. to evaluate skin sections. Diffuse reflectance spectroscopy (DRS) relies on the UV-VIS absorption of drugs or nanoparticles and can be applied for in vivo skin detection. Human skin penetration kinetics of drugs that absorb in this wavelength region have been measured in vivo [104]. This technique does not provide spatial information.

5.1.5 Fluorescence spectroscopy and microscopy. Similar to DRS, fluorescence spectroscopy can be applied to follow skin penetration in vivo [105]. Fluorophores endogenous to the skin (e.g. porphyrins, NAD/NADH, collagen, elastin), drugs that fluoresce or exogenous fluorophores e.g. coupled to the nanocarrier can be excited (normally in the UV to near IR spectral range). Besides using fluorescence intensity or emission wavelength as a readout, fluorescence lifetime and time-resolved anisotropy can

be employed. Fluorescence lifetime is highly sensitive to the environment and therefore allow, among others, sensing of polarity, pH, calcium concentration or biomolecular interaction. The analysis of time-resolved anisotropy provides insights in the molecular dynamics of the nanocarrier [106,107].

To obtain spatial information, fluorescence microscopy is an essential tool. Conventional intensity-based fluorescence microscopy was used to analyze the penetration of fluorescently tagged nanocarriers into human skin [108]. Confocal laser scanning microscopy provides higher sensitivity and spatial resolution, when assessing nanocarrier penetration into skin [109,57]. Similar to conventional fluorescence spectroscopy, the fluorescence lifetime information can also be exploited by using Fluorescence lifetime imaging microscopy (FLIM). Most FLIM setups combine confocal laser scanning microscopy with time-correlated photon counting, thus enabling the detection of the excited state fluorescence decay traces (fluorescence lifetime curves) for each pixel. Fig. 6 shows FLIM images of skin sections either using the endogeneous skin fluorescence lifetimes for false color coding the pixels (Fig. 6 A,C) [110] or the lifetime of a fluorescently tagged dendritic nanocarrier topically applied to the skin (Fig. 6 D,F). Here, the lifetime information allows distinguishing between the localization of the nanocarrier in the human stratum corneum and in the viable epidermis [108]. Additionally, by the lifetime information, signals from autofluorescence and from the nanocarriers can be separated. This provides background free penetration profiles of the nanocarrier (Figure 6 G) [110] as well as the concentration of nanocarriers in subcellular compartments [111], allowing for a quantitative assessment. Anyhow, skin autofluorescence in combination with FLIM is very useful for determination of the cellular metabolism by means of NAD(P)H autofluorescence [112].

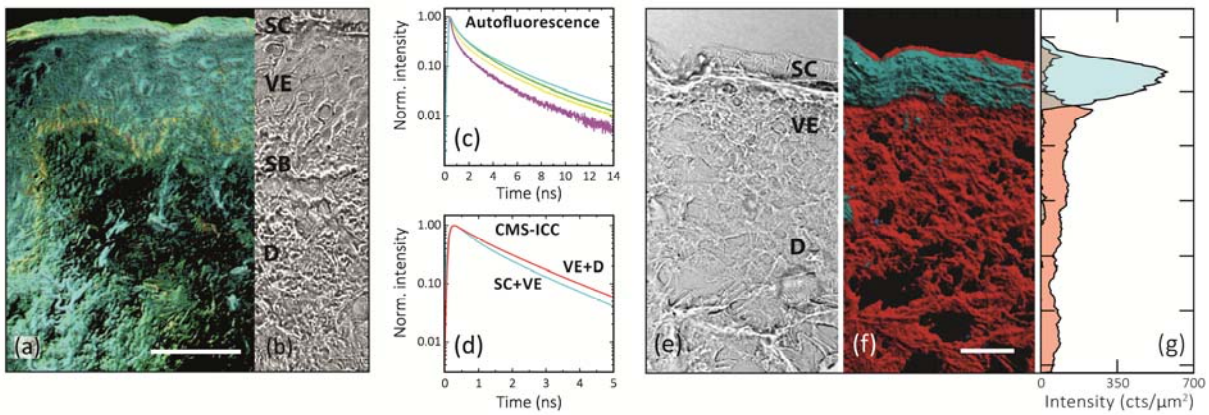

Fig. 6: Label-free and label-based FLIM (a) Autofluorescence based FLIM image of human skin section. (b) Bright-field image of skin adjacent to the FLIM image. (c) Fluorescence lifetime traces corresponding to (a). (e) Bright-field image and (f) corresponding FLIM data for nanoparticle penetration into human skin. The corresponding fluorescence lifetime traces are shown (d). (g) Background-free nanoparticle penetration profile. Scale bars correspond to 50 μm (a, b, e, f); (d, e, f) adapted and modified from [108], Copyright 2014, with permission from Elsevier. SC: stratum corneum, VE: viable epidermis, SB: stratum basale, D: dermis, CMS-ICC: dye loaded core multishell nanocarrier.

Recently multiphoton FLIM is being used for non-invasive imaging of human skin physiology and percutaneous penetration [113] or stratum corneum pH gradients [114]. Similarly, spectrally resolved multiphoton microscopy was applied to image skin tissue in vivo and ex vivo [115]. Local diffusion properties of nanocarriers and drugs in skin ex vivo were shown to be accessible by cross-correlation Raster image correlation spectroscopy (CC-RICS) [48]. In particular multiphoton microscopy using femtosecond lasers offers several advantages over other microscopy techniques when applied to skin such as inherent axional sectioning, penetration depth and many two-photon contrast mechanisms, including second harmonic generation [116]. This has led to several technical developments for medical/clinical optical diagnostics [117,118,119].

5.1.6 Single Molecule Microscopy. Studying fluorescent molecules in turbid tissue, such as skin, at the single molecule level is challenging. Single fluorescently labeled nanoparticles were visualized in the skin using multiphoton microscopy [120]. Recently, a single particle tracking based approach was developed using ex vivo skin combined with tape stripping to visualize and determine the diffusion constants and spatial confinements of diffusion (i.e. penetration pathways) of a model drug in human stratum corneum with nanometer resolution at a depth of about 2-4 μm as shown in Fig. 7 [121]. This technique is also applicable for in vivo topical application of nanocarriers and drugs.

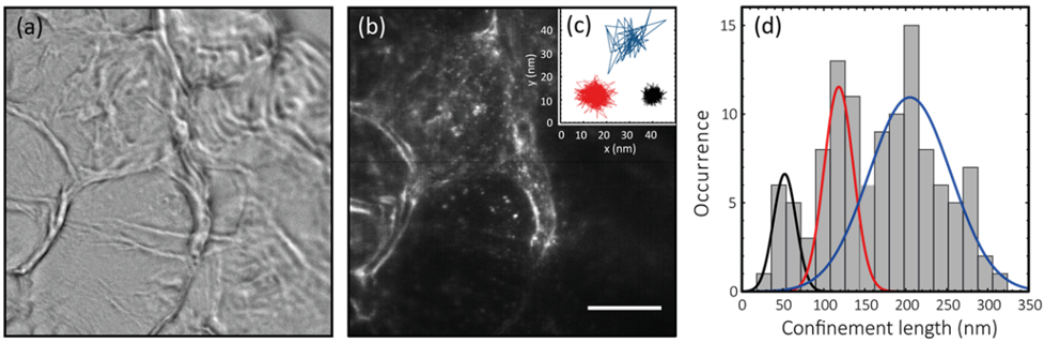

Fig. 7: Penetration of a large amphiphilic molecule in the stratum corneum of human skin by single molecule microscopy. (a) Bright-field image of a stratum corneum tape strip, (b) corresponding TIRFM image of penetrated the large amphiphilic molecule ATTO-Oxa12 (bright spots). The scale bar corresponds to 20 µm (a, b). (c) Three ATTO-Oxa12 single molecule traces in stratum corneum sheets, each representative for one of the three subpopulations shown in (d), the histogram of confinement lengths. (Modified and adapted from [121], with kind permission.)

5.1.7 Electron paramagnetic resonance (EPR) spectroscopy. EPR spectroscopy measures paramagnetic molecules with unpaired electrons. EPR usually involves the labeling with a paramagnetic probe (typically a nitroxide free radical). This approach was used to investigate the penetration of hyperbranched nanotransporters or nano-structured lipid carriers in porcine skin ex vivo and in vivo using spin labeled molecules as cargo [122] as illustrated in Fig. 8. These measurements, however, are not spatially resolved, i.e. provide no visualization. The penetration is followed via the change of the EPR signal within the skin due to the sensitivity of the paramagnetic probe to the immediate environment. Free radicals are also naturally occurring in tissues as they are essential for various metabolic processes. These reactive oxygen species (ROS), containing one or two unpaired electrons, may be overproduced under stress situations (e.g. nanoparticle uptake) and therefore constitute a measure of cytotoxicity as well as antioxidant status using EPR [123].

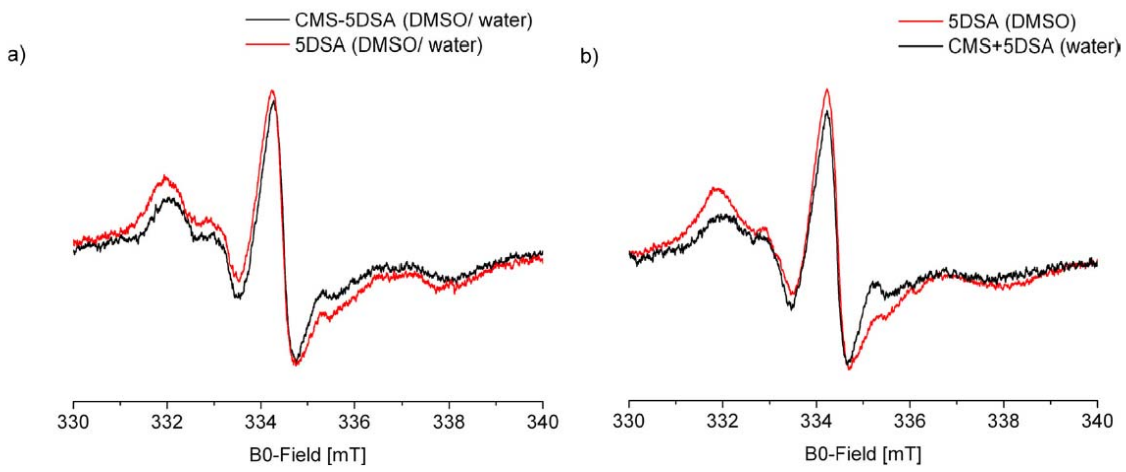

Fig. 8: Ex vivo skin EPR spectroscopy of a lipophilic drug loaded in core multishell (CMS) nanocarrier. (a, b) X-band EPR spectra of spin-labeled 5-doxyl stearic acid (5DSA), after application of different formulations and penetration into porcine skin (ex vivo). Ex vivo studies on porcine skin demonstrated nearly the same properties for both stearic acids loaded to CMS and in solution, independent of the solvent used. (Reproduced from [122], Copyright 2016, with permission from Elsevier.)

5.2 Clinical evaluation

Preclinical models including excised human skin, reconstructed skin and animal models all help elucidate different aspects of drug delivery into the skin. However, the complexity of skin barrier function and drug penetration in diseased skin underlines the importance of proof-of-concept study in humans. Non-invasive imaging technologies such as high frequence ultrasonography, optical coherence tomography, laser scanning microscopy as well as *in vivo* multiphoton microscopy, and most recently also approaches that make use of photoacoustic microscopy are applicable on human volunteers and have opened a refined view on *in vivo* skin barrier anatomy [124]. The inclusion of spectroscopic information such as from *in vivo* Raman spectromicroscopy allows to extract chemical information, which can be used to draw conclusions on water content, lipid organization, and other skin barrier components in stratum corneum material [101] and directly in human volunteers [125].

Topically applied compounds [126] or biological molecules can be localized within the skin, e.g., carotenoids as indicator of the antioxidative potential [127]. The effectiveness of therapies can be followed non-invasively over time as illustrated for psoriasis plaques [128] or tumor bearing skin [129].

Compared to those advanced instrumental analysis methods, classic skin barrier parameters such as transepidermal water loss (TEWL), stratum corneum hydration, skin surface pH, skin surface lipid content and sebum production are easy to conduct using portable probes and are widely applied screening tools to characterize skin barrier function in clinical trials [130]. A larger number of data sets and, in the case of TEWL, also meta-analyses are available, which can be used as guidance for the design of carrier systems [131]. E.g., substantial TEWL increases in humans have been clearly associated with disturbed skin barrier function and stratum corneum. However, TEWL is a sensitive parameter, easily affected by measurement conditions, not specific and subject to high biological variability [132]. The degree and nature of barrier disruption can be further characterized by quantification of biological markers, e.g. inflammatory mediators, collected non-invasively from the skin surface of human volunteers using adhesive tapes [133].

Finally, functional analyses can help characterizing both, the general vulnerability of skin or the efficacy of topicals. Sodium lauryl sulfate (SLS) as inducer of experimental irritant contact dermatitis [134] can be used to monitor pro- or anti-inflammatory effects of topicals in vivo. For instance, in a 5-day repetitive irritation test with topical application of 0.5% SLS twice daily, the capability of topical tacrolimus to enhance the irritative effects of SLS has been demonstrated [135]. In other settings, the responsiveness to SLS application was used to study ethnical differences in skin barrier function [136], the effects of preventive barrier crème [137], or of long-term use of moisturizers [138]. The application of negative pressure can be facilitated to induce suction blisters suitable to determine blistering time as read-out for barrier vulnerability, or to collect blister fluid and blister roofs to study inflammatory mediators and immune cells [134, 139]. Specific characteristics of drugs can also be used to monitor penetration and biological activity, e.g. the blanching effect of corticosteroids by use of skin color reflectance or vascularization measurements [140].

6. Clinical perspectives of nanocarriers for dermal application

With the expansion of knowledge and technical capabilities in the field of nanotechnology, we are now entering an era, where medical indication, way of application and choice of carrier architecture have to be specified very clearly. As outlined previously, applications include penetration improvement for established molecules, delivery of novel drug classes, or, targeted delivery to specific cell populations [35].

In experimental studies, modern carrier technologies allowed for introduction of special physicochemical features such as formability, biodegradability, stability in skin environment including temperature, pH-sensitivity or enzyme-mediated degradation of specific cleavage sites. The use of external triggers such as for phototherapy or photodynamic therapy has a long tradition in dermatology, but also endogenous triggers offer many interesting applications, e.g. stimuli-responsive polymers for anti-microbial therapy [141], wound dressings containing enzyme-responsive nanocomposites, which release their content only in the presence of pathogens [142], light-activated hypoxia-responsive conjugated polymer-based nanocarrier to treat malignancies [143] or acid-responsive polymeric nanoparticles [10], to name only few examples. Complex delivery systems may combine different elements, e.g. contain targeting units and additional functionalities, e.g., anti-oxidative properties.

Incorporation of peptides, proteins or nucleic acids is successfully being addressed as these compounds are increasingly being explored for clinical applications. While biologics have revolutionized the systemic treatment of severe psoriasis and are further explored for atopic dermatitis, urticaria and many less frequent diseases, topical delivery has not yet been achieved. With regard to the numerous genetic disorders associated with severe skin symptoms, topical enzyme-replacement strategies could have incredible impact on the life quality of patients suffering from diseases like ichthyoses [144], DNA repair deficiencies [145], and others. Genetic diseases are also primary indications for gene therapeutic approaches. Furthermore, modification of gene expression with RNA derivatives also holds great promise for wider use including chronic inflammatory skin diseases as illustrated by studies on liposomal formulations [146] or fusogenic nucleic acid lipid particles [147]

for topical treatments of psoriasis. However, the translation from highly promising experimental approaches to clinical applications is a challenge of its own.

The use of polymer microparticles for selective delivery of retinoids to dilated facial hair follicles of acne patients was among the first applications of particle-based drug delivery into the skin, which was brought into clinical trials [49]. Clearly, also liposomes and lipid particles have found broad entry into dermatological formulations [148]. Since then, the scientific basis for the use of many different carrier types for a wide range of indications related to drug delivery into and through the skin has been well established. In contrast to polymer therapeutics like drug-polymer conjugates already being in clinical use for parenteral applications, skin delivery may benefit from nanoparticle functions that can be implemented by transferring concepts realized in bulk material to nanocarriers [149]. However, the field still largely remains in preclinical investigations. This emphasises the need of determined efforts towards the production of medical grade material and translational work to demonstrate surplus value of carrier systems for clinical applications.

Another important aspect from the clinical perspective is the predictive power of in vitro/ex vivo data towards clinical efficacy of new carrier systems. While being valuable for an early testing phase, standard models for the quantification such as Franz diffusion cell assays face limitations as they are frequently conducted on undisrupted skin using infinite doses of drug. Likewise, stratum corneum barrier, epidermal thickness and hair follicle density are such important determinants for penetration, that studies in animal models are of limited predictive value. In order to identify the value of a carrier system for a dermatological condition, suitable preclinical models are needed, which give information on skin penetration, tissue concentration of the drugs and their biological efficacy.

Based on this, a number of key issues may need to be addressed when aiming to implement new nanocarriers as therapeutics in clinical routine:

1. The current research mostly focuses on the highly fascinating state of exploration of the experimental potential of new carriers. One challenge in the future will be to select those systems and indications where nanotechnology truly brings a convincing surplus value for patients – which could be a significantly easier application (reduced frequencies), reduced systemic

absorption or more selective application on diseased skin areas, or even delivery to specific cell populations.

2. The complexity of the skin barrier limits the predictability of routine preclinical assessment tools. Traditional Franz diffusion cells and flux rates alone will not allow deciphering the future therapeutic value of newly designed systems. While high-end research technologies are available for selected questions, robust and straight-forward preclinical screening models, which enable quantification of drug penetration, ideally in line with biological read-outs of efficacy, are greatly needed.
3. The technical possibilities arising from new carriers need to be weighed against health economic aspects and patient interests. After all, a new carrier system will have to compete with existing therapies and show significant improvements to justify potentially higher treatment costs. E.g., higher penetration by a factor of two may not necessarily result in significantly better biological responses, which could motivate further development towards a product.
4. The capacity of loading bioactive molecules by physical entrapment into the particle matrix naturally decreases exponentially with the particle size, i.e. the available volume in the particle core. The same applies to the capacity of the matrix material to act as a diffusion barrier of physically entrapped drugs. Therefore, even if downscaling particles towards (very) low sizes may possibly enable intercellular penetration at least in damaged skin, some desired features for dermal delivery may be negatively affected, namely transport of relevant quantities of a bioactive molecule and its sustained release from a depot build inside a specific skin layer.
5. The requirement of a “nano-“formulation has to be individually evaluated for each specific application. Depot formation in hair follicle openings and treatment of scalp hair diseases with large terminal hair follicles can easily and probably even more effectively be achieved by larger, i.e. submicron- and microparticles, which have an extremely low likelihood of penetrating into the viable tissue. Yet, nanocarrier capable of penetrating into the tissue may enable delivery of drugs, which need to enter cells such as nucleic acids.
6. A bottle neck often neglected is the translational gap between academic research exploring new carrier functions and industrial development, which typically becomes interested to take over

product candidates only after some early proof of clinical efficiency. Accordingly, translational efforts are needed, which demand the cost- and labor intensive production of nanocarriers under conditions that fulfill the regulations of Good Manufacturing Practice.

7. Conclusions

In the light of the wide field of newly emerging nanocarrier technologies, close interactions between medicine, pharmaceutic technology and chemistry are needed to dissect the true potential and surplus value of a specific carrier system for a specific indication. Such interdisciplinary partnerships should allow to better understand the role of the nanocarriers' physicochemical properties defined by material chemistry and processing to specific formulations as well as skin (patho-)physiology on drug delivery into and across the skin. The combination of a rational design of nanocarriers with a detailed analysis of benefits that may be achieved clinically by such systems should be the basis to bring the most promising approaches in clinical trials and potentially lead to true innovations.

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