Aus dem

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Improvement of Dermal and Transdermal Therapies by Nanocarrier-Based Drug Delivery

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Aller Eifer, etwas zu erreichen, nutzt freilich gar nicht, wenn du das Mittel nicht kennst, das dich zum erstrebten Ziele trägt und leitet.

Marcus Tullius Cicero (106-43 v. Chr.),
römischer Redner und Staatsmann

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Abbreviations

Antigen presenting cell, APC
Cell penetrating peptide, CPP
Cluster of differentiation, CD
Cyanoacrylate Skin Surface Stripping, CSSS
Dendritic Cell, DC
Deoxyribonucleic acid, DNA
Enzyme-linked Immunosorbent Assay, ELISA
Human, adult, low calcium, high temperature, HaCaT cells
Human Immunodeficiency Virus-1, HIV-1
Human Leukocyte Antigen, HLA
Immunoglobulin, Ig
Interferon gamma, IFNγ
Interleukin, IL
Infrared, IR
Janus kinase, JAK
Signal Transducer and Activator of Transcription, STAT
Langerhans cell, LC
Lipopolysaccharide, LPS
Nitric oxide, NO
Poly(amidoamine), PAMAM
Poly(ε-caprolactone), PCL
Poly-ethylene glycol, PEG
Poly(glycolic acid), PGA
Poly-lactic-co-glycolic acid, PLGA
Poly-lactic acid, PLA
Poly(propylenimine), PPI
Regulatory T cell, Treg cell
Small interfering ribonucleic acid, siRNA
Stratum corneum, SC
Stratum granulosum, SG
T helper cell, Th cell
Toll-like receptors, TLR
Transforming growth factor beta, TGF-β
Tumor necrosis factor alpha, TNFα
Ultraviolet, UV
Virus-like particle, VLP

1. Introduction[1]

1.1 How Can Nanocarriers Improve Classic Topical Treatments

Skin is the largest and most accessible organ of the human body and the topical delivery as well as targeting of molecules to the affected site should be uncomplicated and effective. However, due to poor penetration or low retention of adequate amounts of drug at the target site, a number of skin diseases can be treated topically only when symptoms are mild and low drug concentrations are sufficient to get a satisfying therapeutic outcome. Thus, despite the fact that topical treatments are associated with less side effects and are well tolerated by patients, most of severe skin conditions are treated by systemic therapies combined to a topical basic treatment that will be continued as maintenance therapy once the systemic treatment is no longer needed. For example, the European guidelines recommend the systemic administration of methotrexate or cyclosporin A as induction treatment for severe forms of psoriasis vulgaris (Nast, Gisondi et al. 2015). Similarly, systemic immunosuppressive treatments with oral glucocorticoids or cyclosporin A are used in the case of atopic dermatitis refractory to topical treatments (Ring, Alomar et al. 2012). Hence, better drug delivery strategies are needed in order to enable the topical treatment of severe skin inflammatory diseases. According to current guidelines for treatment of moderate acne vulgaris (that normally resolves after teen-age) several topical treatments are available. However, patients with severe acne non-responding to topical treatments have to be treated with oral drugs such as antibiotics and retinoids or, as an alternative for females, oral contraceptive pills containing antiandrogens (Nast, Dréno et al. 2016). Most of these therapies are associated with considerable side effects, e.g. teratogenicity as in the case of isotretinoin (Williams, Dellavalle et al. 2012). Improved topical drug delivery would be also useful for the treatment of actinic keratosis. This condition is characterized by skin lesions that can evolve to non-melanoma skin cancer and are therefore surgically removed to eliminate those damaged cells with high potential to transform into malignant lesions. Topical pharmaceutical treatment of small lesions reduces side effects such as scarring, post-operative infections and pain (Ceilley and Jorizzo 2013). A more efficient topical delivery of anti-cancer drugs, such as 5-fluorouracil, diclofenac and imiquimod, would increase drug local concentration and efficacy. In the case of antimicrobial therapies, next to reduction of systemic side effects, topical treatments may be also advantageous as compared to systemic treatments because oral antibiotics used at low concentrations and for long periods may enhance the risk for selection of antibiotic-resistant strains (Eady, Cove et al. 2003).

Thus, one of the rationales for developing new topical administration strategies is to enhance the efficacy of commercially available classic drugs reducing systemic toxicity as well as cutaneous off-target effects.

Most of the drugs used in dermatology face three main problems depending on their chemical structure: i) low penetration across the skin barrier; ii) rapid clearance through blood and lymphatic drainage; iii) rapid metabolism or degradation in the skin. In the past years, a number of studies has shown that the use of delivery systems is a promising strategy to overcome these problems and significantly improve existing topical therapies. Such new topical therapeutic approaches can strongly profit from nanotechnology. A number of nano-architectures have been developed in the past few years in order to enhance the

[1] Part of Introduction and Discussion was included in: Rancan F. (2016): Biodegradable, Biocompatible, and Bioconjugate Materials as Delivery Agents in Dermatology, in: M.R. Hamblin, P. Avci, T. W. Prow, Nanoscience in Dermatology, Elsevier Academic Press, S. 73-87.

transport of different drug moieties across body barriers, protect them from degradation, and control their delivery over time. The use of such nanocarriers for topical therapeutic treatments can revolutionize a number of currently available dermatotherapies.

1.2 Rationales for the Use of Nanocarriers to Deliver New Generation Drugs

Advances in genetics and molecular biology have increased our knowledge on the pathological mechanisms underlying several skin diseases. For example, the genetic background of atopic dermatitis has been widely explored, allowing for the identification of new therapeutic targets. The improved understanding of the skin immune system and the identification of new cellular and molecular actors as well as regulatory feedback mechanisms, has opened the possibility of designing active molecules for the specific treatment of inflammatory and allergic conditions as well as for vaccination purposes (Fig. 1). The translation of this knowledge into new therapeutics has been fostered by the advances in genetic engineering and biotechnology allowing for the production of biological active agents (biologics or biopharmaceuticals) with potent and more specific activity. Biopharmaceuticals such as antibodies, interleukins, interleukin-receptor antagonists, protease and kinase inhibitors, antimicrobial peptides, nucleic acids, as well as antigens and adjuvants for vaccination purposes are widely explored and some of them have already reached the clinical stage. For example, antibodies targeting inflammatory mediators such as TNFα, IL-12 and IL-23, IL-17 receptor (infliximab, ustekinumab, and brodalumab, respectively) are efficiently used for the treatment of severe psoriasis with reduced systemic side effects in comparison to classic anti-inflammatory drugs such as calcineurin inhibitors (Prieto-Pérez, Cabaleiro et al. 2013). The antibody towards the IL-4 receptor, dupilumab, has been recently introduced in the clinic for the therapy of atopic dermatitis with promising results (Beck, Thaçi et al. 2014). Antimicrobial peptides are promising biologicals for the treatment of infected acute and chronic wounds (Mangoni, McDermott et al. 2016). Such actives are preferred to antibiotics, especially when treating immune suppressed patients, due to reduced risks for sensitization and selection of antimicrobial-resistances (Lipsky, Holroyd et al. 2008).

These new classes of pharmaceutics, however, are not available for topical application yet and are administered orally or by intravenous and subcutaneous injection. This leads to a systemic distribution of the drug and, consequently, to systemic suppression of the specific arms of the immune system with related side effects such as increased risk of infections and even cancer (Rongioletti, Burlando et al. 2014). A further drawback of biopharmaceuticals is their rapid degradation by enzymes. For examples, drugs like siRNA, should be administered locally and in a protective pharmaceutical form otherwise it would be mostly degraded before reaching its target. Nanocarriers have been explored since decades as delivery systems for proteins, peptides and nucleic acids to improve their delivery profile and reduce unwanted side effects. The possibility to deliver such macromolecules by topical means reaching therapeutic concentrations in the target skin region would drastically improve the benefit-risk ratio of these drugs. In addition, nanocarrier could help to target drugs to specific skin cell populations (Schlapbach and Navarini 2016), like pathological cell swarms, found in inflamed skin, where dendritic cells (DCs) were observed in close contact to skin resident memory T cells (Natsuaki, Egawa et al. 2014).

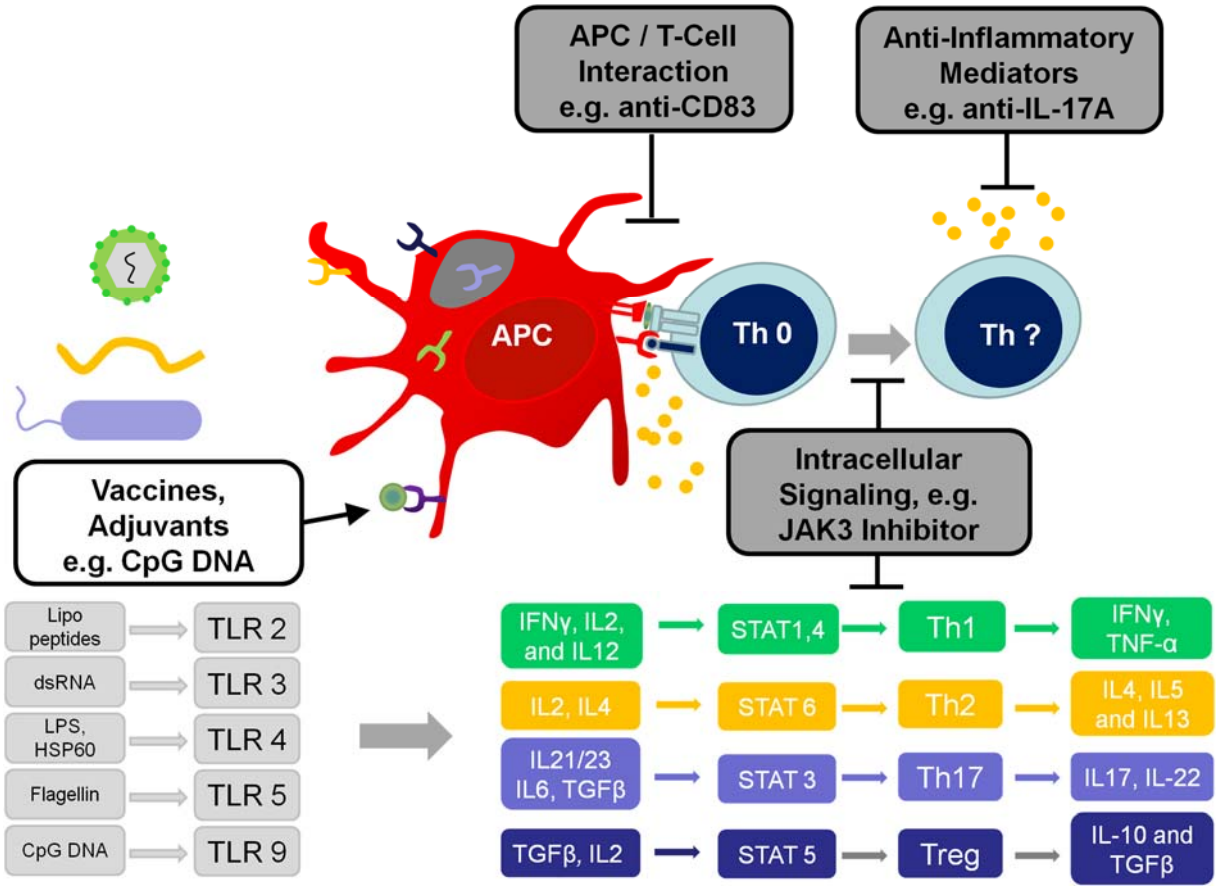

Figure 1. Schematic and simplified representation of the mechanisms and mediators involved in the different arms of skin immune response and examples of new generation drugs and their mechanisms of action. Microbial components induce the activation of APCs through the binding to Toll-like receptors (TLR), which are under investigation as possible targets for vaccine adjuvants. Antigen presenting cells (APCs) capture pathogenic organisms, process them, and present antigens to naive T helper (Th0) and memory T-cells. Depending on the encountered microorganism and on the cytokine environment, different intracellular signaling pathways (e.g. Janus kinase/ Signal Transducer and Activator of Transcription, JAK/STAT) are induced leading to the differentiation of T-cells into different subsets (Th1, Th2, Th17, Treg, etc.) which will mount a pathogen specific immune response. New generation anti-inflammatory drugs and biopharmaceuticals act at different but specific levels, like APC/T-cells interaction, intracellular signaling, or cytokines, suppressing only certain arms of the immune system. Figure modified from (Tamiya, Kashiwagi et al. 2011, Pennock, White et al. 2013, Schlapbach and Navarini 2016).

An increasing number of new carriers has been designed to penetrate the skin barrier and to protect biological drugs from undesired degradation. Furthermore, many other properties of nanocarriers make them attractive tools to improve the performance of new generation drugs. Considering the needs of topical dermatotherapies, nanocarrier peculiar interactions with skin confer them the following properties and advantages:

(i) They accumulate within the SC, in skin furrows as well as in hair follicle canals where they form depots for a sustained drug delivery of encapsulated drugs;

(ii) They may possess intrinsic skin permeabilizing properties or can be supplemented with penetration enhancers.

(iii) Once translocated to the viable skin layers, they allow for cellular up-take of several drug moieties by specific endocytotic pathways influencing drug intracellular concentration;

(iv) A more selective drug delivery can be achieved by means of nanocarriers functionalized with targeting units specific for skin components;

(vi) Two of more active agents can be carried simultaneously, e.g. for vaccination purposes, nanocarriers can be designed to carry simultaneously antigens and adjuvants for specific recognition as well as activation of APCs;

(vii) They can be stimuli-responsive in order to sense the environment or respond to external stimuli realizing a more controlled and selective release of the cargo.

1.3 How Nanocarriers Can Improve Transdermal Drug Delivery

The transcutaneous route of systemic drug delivery can be a very attractive route especially for biopharmaceuticals. Compared to intravenous and oral delivery, it allows very specific pharmacokinetics and biodistribution (Choi, Lee et al. 2012). Whereas intravenous injection results in high bioavailability but also rapid renal clearance, the transcutaneous route allows for lower but prolonged blood levels. In addition, the intravenous administration has low patient compliance and requires trained personnel. Oral delivery is a challenging issue for biopharmaceuticals due to the degrading gastro-intestinal environment and to the first pass effect that further reduces the bioavailability of the adsorbed drug. Subcutaneous injection is currently the preferred delivery route, but it requires needles, is painful and certainly less comfortable than the transdermal delivery *via* patches. Although in the last years transdermal delivery has gained more and more interest for the delivery of biopharmaceuticals and vaccines, commercialized products are still limited. Hormone and painkiller patches (e.g. estradiol and fentanyl) are the most used preparations. Recently, patches for the administration of rivastigmine have been developed to treat mild forms of Alzheimer disease (Cummings, Froelich et al. 2012). The reason for the low number of transdermal applications is that only small and amphiphilic molecules can cross skin barrier and reach adequate blood levels, whereas only limited amounts of high molecular weight pharmaceuticals can cross the skin barrier. Several studies has been done to test the transdermal delivery capacity of nanoparticles using different types of materials and different drug classes. Beside progesterone, lidocaine and anti-inflammatory drugs like corticosteroid, nimesulide (Lenz, Guterres et al. 2012) and flufenamic acid (Prow, Grice et al. 2011), insulin was also loaded on nanocarriers for transdermal drug delivery. King and co-workers have shown that vesicles loaded with insulin and applied by means of transdermal patches delivered insulin to the lymphatic system and resulted in prolonged controlled glucose levels (King, Michel et al. 2003). Transdermal drug delivery to the lymphatic system by means of nanoparticles has also been used for delivery of anticancer drugs to target tumor metastasis (Kong, Hou et al. 2015).

Because the amounts of macromolecules that can cross the intact skin barrier is rather low, recent research has focused on delivery systems or methods that can transiently modify skin barrier permeability. Physical methods like abrasion, cyanoacrylate skin surface stripping (CSSS), electroporation, iontophoresis, sonophoresis, laserporation and others have been developed (Alexander, Dwivedi et al. 2012). In addition, nanotechnology has focused on sophisticated delivery systems such as microneedles, nanoparticles decorated with cell penetrating peptides (CPPs) or other penetration enhancers (Azarbayjani, Khu et al. 2011,

Mitragotri, Burke et al. 2014). These works show that the combination of nanocarriers to safe methods for skin permeabilization is a promising strategy for an efficient and convenient transdermal administration of biopharmaceuticals.

1.4 Barriers and Targets for Nanocarrier-Based Dermal Drug Delivery

Skin is a complex organ with several functions. It is the boundary between body and the external environment, and, thus, it acts as a barrier hindering at the same time the loss of water and the penetration of potentially dangerous exogenous materials. Stratum corneum (SC), viable epidermis, and dermis are not only a sophisticated physical barrier but also an immunological first line defense which efficiently blocks the penetration of microorganisms. These important features are disturbed in certain diseases, like atopic dermatitis, and needs to be restored. On the other side, skin barrier represents an obstacle for the successful local treatment of skin diseases or for the transcutaneous administration of biopharmaceuticals. Depending on the disease to be treated and on their targets, drugs need to be delivered to different skin layers and reach different cell subsets or intracellular compartments. In the following, the different skin layers will be described with special attention to their role as a barrier to nanocarrier-based drug delivery or as targets for dermatotherapies.

The SC is the outermost layer of the epidermis and the main physical barrier regulating the permeation of both hydrophobic and hydrophilic substances. It has a thickness of 10-20 μm (approx. 40 μm in palms and foot soles) and consists of several layers of cornified cells, the corneocytes, tightly bound to each other and embedded in an extracellular matrix based on highly ordered lipid layers (Hirao 2010). Corneocytes are tightly connected to each other by means of intercellular protein structures called corneodesmosomes as well as hook-like structures on the edges of the corneocytes (Richter 2004) (Wepf, Richter et al. 2006). Corneodesmosomes support both its stability and barrier function. The lipid extracellular matrix is made of ordered lipid lamellae that surround the corneocytes. Ceramides, free fatty acids, and cholesterol are the major components of SC lipids and form predominantly a crystalline phase, with liquid phases occurring to a smaller extent (Bouwstra, Honeywell-Nguyen et al. 2003). However, it has to be considered that in several skin conditions the physiological barrier function is altered (Harding 2004). For example, in the case of atopic dermatitis, filaggrin defects, increased serine protease activity, and decrease of total lipids levels result in a dysfunction of the SC with consequent skin dehydration and infections by bacteria of the physiological skin flora such as Staphylococcus aureus (Levin, Friedlander et al. 2013). In psoriasis, the hyperproliferation of keratinocytes results in a thicker SC where cells are not completely cornified and retain their nucleus (parakeratosis). The terminal differentiation of cells is incomplete with lower levels of mature keratin types (K 1, 2, and 10). Also expression of barrier proteins like filaggrin and loricrin is reduced, resulting in impaired skin barrier (Kim, Howell et al. 2011). Recently, genetic variations effecting genes responsible of skin barrier function have been reported to have similar weight for the predisposition and pathogenesis of the disease than those genes involved in skin immune functions (Bergboer, Zeeuwen et al. 2012). Thus, in such diseases the SC does not represent a barrier to overcome but is rather a target for those formulations aiming at restoring skin barrier function. In fact, for several inflammatory skin disorders, formulations improving SC acidity, hydration, and lipid content may possibly prevent the symptoms or their exacerbation (Man, Man et al. 2015).

The viable epidermis is made mostly by keratinocytes (approximately 90% of all cells). They are organized in a squamous epithelium where cells are ordered in differentiating layers called stratum basale, stratum spinosum, and stratum granulosum. In the last differentiation

steps, the keratinocytes lose their nucleus, become korneocytes, and form the SC (Fuchs 1990). In the second layer of the stratum granulosum (SG2), a further physical barrier, blocking the penetration of exogenous substances, is given by tight junctions. These intercellular structures are made of proteins such as claudins, occludin, and junctional adhesion molecules that connect the apical part of granulocytes and control the penetration of macromolecules as well as ions and water (Kirschner, Rosenthal et al. 2013). Beside keratinocytes, Langerhans cells (LCs, 2-3 % of all epidermis cells) represent the first line of skin immunological barrier. Like DCs, they can sample self and non-self antigens and present them to naive and memory T- and B-cells. Thus, LCs represent important actors in skin homeostasis as well as immune defense and are important targets for both vaccination and anti-inflammatory strategies (Pasparakis, Haase et al. 2014). In several inflammatory skin conditions, a special sub-set of DCs can be found in the epidermis, called inflammatory dendritic epidermal cells (Segura, Touzot et al. 2013). In addition to LCs, resident T-cells in the epidermis are involved in immune responses as well as wound healing (Toulon, Breton et al. 2009). Different types of cancer cells can develop from melanocytes (melanome), basal cells (basal cell cancer), and keratinocytes (squamous-cell cancer, actinic keratosis) and are ,therefore, possible targets for dermatotherapies.

The dermis consists of connective tissue and is made predominantly of collagen and elastin fibers organized in bundles and embedded in an extracellular matrix rich in hyaluronic acid and other glycoproteins. The dermis contains cells such as fibroblasts, which are responsible for collagen and elastin production, fibrocytes (i.e. mesenchimal stem cells involved in wound healing), and a variety of immune cells, like mast cells, macrophages, dendritic and T-cells that reside in the dermis or circulate between the dermis and the lymphatic and blood compartments. During immune responses, allergic reactions, and chronic inflammation, dermis cells can cross talk and activate or suppress each other in a complex interplay regulated by released immune-modulating molecules and their receptors. Depending on the type of inflammatory process, different sub-populations of DCs can be detected (Pasparakis, Haase et al. 2014). Being reach in blood vessels, the dermis represents the target skin layer for transdermal drug delivery systems, whereas the different immune cell populations can be possible targets for a number of dermatological treatments such as local anti-inflammatory therapies.

The lowest skin layer, the subcutis, is made of loose connective tissue and fatty tissue (adipocytes grouped in lobules) with blood and lymphatic vessels. This region is often used to create drug depots because of the lower blood flow with respect to muscles. This allows for a slow and sustained delivery of the drug to the blood and lymphatic compartments. The subcutaneous injection is currently the administration route of neutralizing antibodies for the treatment of severe inflammatory skin conditions and is believed to be the most effective route for the delivery of new biological drugs (Rothstein and Gottlieb 2015). Subcutaneous injection of nanoparticles was shown to improve the targeting of drugs to the lymphatic system so that, in the last years, this mode of administration has also been explored for the visualization and treatment of lymphatic metastasis (Singh, Swami et al. 2014).

The pilosebaceous unit is a special structure of the integument with several functions comprising that of protecting (hair shaft), acting as endocrine and exocrine system (sebaceous gland), and contributing to body temperature homeostasis (arrector pili muscle) (Chen and Zouboulis 2009). It is a sort of invagination of the skin, where microorganisms or applied substances can accumulate. The SC is thinner in the upper part of the hair follicle and, thus, this is the site where the entry of applied substances as well as the interactions between skin cells and exogenous material are favored. Interestingly, in this region the

network of Langerhans and DCs is particularly dense and represents an additional immunological barrier in contact with microorganisms but also with delivered vaccine components. An important effect of particle-based delivery is their preferential uptake by cell specialized in endocytosis, like DCs and macrophages. For this reasons, the transfollicular route was tested as preferential pathway to target antigens to APCs for vaccination purposes (Mahe, Vogt et al. 2009) (Rancan, Amselgruber et al. 2014).
The hair follicle infundibulum acts as reservoir where material like nanoparticles can accumulate and persist for several days before skin turnover and sebum clear them (Lademann, Richter et al. 2006). This property can be exploited to create depots of drug-loaded particles in order to deliver their cargo in a controlled and sustained manner. The pilosebaceous unit is the target for treatments of hair growth disorders and sebaceous gland dysfunctions. Because particles of different sizes penetrate to different depth within the hair follicle canal (Vogt, Combadiere et al. 2006), it was also proposed to use nanocarriers with different sizes to target different cell populations such as stem cells, sebocytes, or keratinocytes located at different depths of the hair follicle.

1.5 Nanocarriers

The term "nanocarriers" is used in nanomedicine for nanoparticles that have been designed to carry and deliver therapeutic or imaging agents. They are supramolecular architectures loaded with a cargo that has to be transported to a specific target. The cargo can then be released constantly over time or upon an endogenous or exogenous stimulus. Nanoparticles have been defined as particles with at least one dimension in the size range of 1-100 nm, but recently it has been proposed to consider as nanoparticles those ones whose properties change when size is getting in the nanometer range. Nevertheless, in nanomedicine nanocarriers comprehend also particles with submicron sizes due to their special interactions with biological barriers and immune cells specialized in endocytosis (Nicolas, Mura et al. 2013). The increasing use of nanocarriers in medicine is due to the several special properties of these delivery systems. They can change or influence drug bioavailability, distribution, degradation, and excretion. In addition, further special characteristics such as long circulating ability, targeting properties, as well as stimuli-responsiveness, can be added to enhance selectivity.
One of the most important aspects of nanocarrier-based drug delivery is biocompatibility. Considering the unique interactions between nanocarriers and biological materials (Nel, Mädler et al. 2009), their compatibility with the host organism is of major importance to ensure the safety of nanotechnology-based therapies. Depending on the toxicity of the constituting material, and the ways it is metabolized or excreted after administration, nanocarriers are classified as biodegradable and/or biocompatible. From the ecological point of view, the term biodegradable stands for environmental friendly degradation of a material as a result of biological processes. In medicine, a material can be considered biodegradable when it decomposes to products that have low toxicity and can be metabolized and/or excreted. On the other hand, a matter can be considered biocompatible when, even if not biodegradable, it exerts no local or systemic side effects. Biocompatible materials are utilized since decades, e.g. orthopedic implants, fillers. However, others than medical implants, penetrated nanocarriers cannot be removed. Thus, even if they have no acute toxicity they have to be eliminated by biliary or renal excretion to be considered as safe. In addition, even if nanocarriers are made of non-toxic material, impurities may cause toxicity, e.g. reagents and solvents used in the synthesis or biological contaminants in the case of biological nanocarriers. Therefore, each promising nanocarrier intended for human use needs to be

subjected to comprehensive toxicology as well as pharmacokinetics studies. Different *in vitro* and *in vivo* tests have to be run, depending on: administration route, doses and duration of the therapy as well as target organ and possible non-specific toxicity. Carcinogenic risks have to be identified as well as possible metabolites or degradation products, which might exert toxic effects. In addition, the potential induction of an immune reaction has to be evaluated. Nanocarriers are often taken-up by macrophages and other immune cells with consequent release of cytokines and induction of inflammatory or allergic reactions. Thus, systemic and local increase of markers for irritation and inflammation as well as inflammatory cell infiltrates at the site of application should be monitored.
Because biodegradability and biocompatibility are strictly dependent on the constituting materials, the most relevant drug delivery systems can be classified according to their composition as well as their compatibility and degradability in human body (Fig. 2).

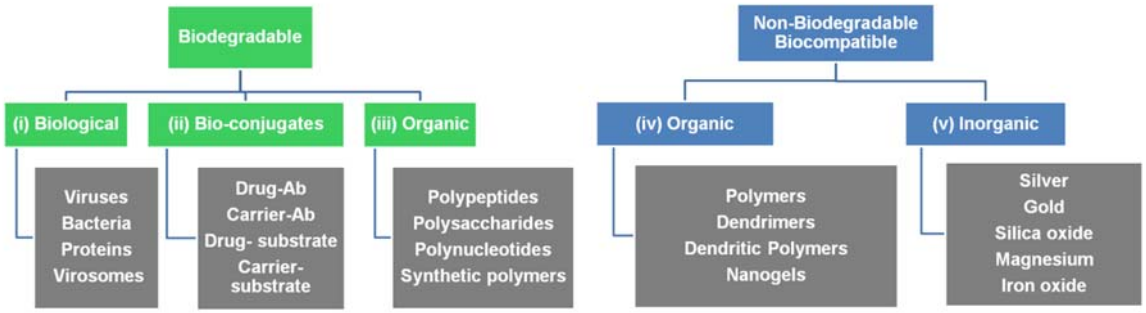

Figure 2. General classification of nanocarriers considering their composition, biodegradability, and biocompatibility. Not all types of nanocarriers fit in this classification, e.g. nanocarriers made of biodegradable subunits coupled to non-biodegradable biocompatible moieties. Ab= antibody

When considering the constituting material, classification can been done into inorganic nanocarriers, made of metals or metal oxides, and organic nanocarriers, made of molecules derived from living organisms and containing predominantly the elements carbon, hydrogen, as well as oxygen and nitrogen. Two types of organic nanocarriers can be distinguished: synthetic or biological ones. Synthetic nanocarriers are made of polymers, branched polymers, and dendrimers, which organize in supramolecular structures like micelles, nanocapsules, spherical or elongated nanoparticles. Biological nanocarriers are complex particles derived from or resembling living organisms or part thereof. They are made of lipids, proteins and nucleic acids. Viruses, bacteria, proteins, virosomes, and virus-like particles belong to this category. Bioconjugates can also be considered as nanocarriers. These macromolecules are drugs covalently conjugated to biological molecules that serve as targeting units, e.g. drug-antibody or drug-substrate conjugates.
Most bioconjugates and biological nanocarriers are biodegradable. On the other side, among organic nanocarriers it can be distinguished between biodegradable and non-biodegradable ones. Polymers for medical use are considered biodegradable when, once administered, they hydrolyze to products that are non-toxic and small enough to be excreted. Depending on the type of synthesis, polymers with different backbones can be synthesized. Radical polymerization leads to carbon-carbon bonds, which are not hydrolysable and therefore non-biodegradable, whereas condensation, ring opening polymerization, or metal catalyst reactions result in hydrolysable esters, amide, urethane, and carbonate bonds (Fig. 3). The introduction of hydrolysable bonds in the backbone of a non-biodegradable polymer makes it

degradable to small products, which can be excreted. The degradation rate is different depending on the type of chemical bond, with esters having the fastest reaction rates. In addition, it is possible to take advantage of specific enzymatic activity found in the target cells or tissues. For example, following this concept, a biodegradable cationic polymer, cleavable by HIV-1 protease, was used to achieve the selective transcription of a therapeutic gene exclusively in HIV-infected cells (Asai, Kuramoto et al. 2010). Another example is given by DNA-polymer complexes that have been engineered with the feature to release the DNA cargo specifically in target inflamed tissue with pronounced activity of the Iκ-B kinase beta enzyme (Asai, Tsuchiya et al. 2009).
Synthetic polymers have been largely used to prepare biodegradable material for implants, joints and stents and in their particulate form, they are also investigated as drug delivery systems. The most studied biodegradable synthetic polymers are poly(lactic-co-glycolic acid) (PLGA), poly-lactic acid (PLA), and poly(ε-caprolactone) (PCL). Whereas the degradation kinetics of PLA polymer is slow and requires several months, poly(glycolic acid) (PGA) hydrolyses more rapidly. PLGA, the copolymer of PLA and PGA, has therefore been prepared, adjusting the ratio of the two monomers in order to control the degradation rate depending on the desired degradation kinetics. In fact, beside the safety issue, the degradation process plays a central role with respect to the release of loaded drugs. Materials with selected degradation rate can be used to get fast or slow drug release, depending on the therapeutic application.

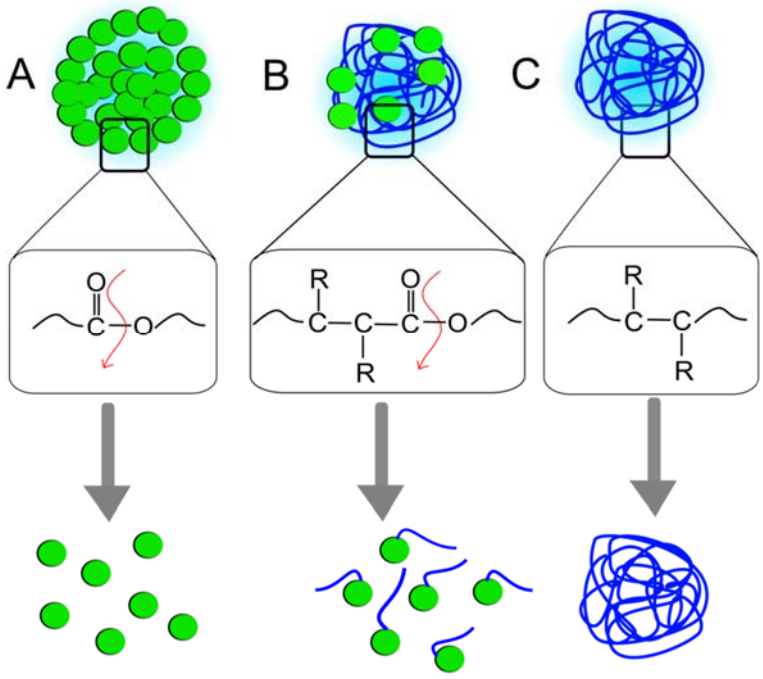

Figure 3. Schematic representation of three nanocarriers made of a synthetic biodegradable polymer, where all monomers are linked by hydrolysable bonds (A), a polymer made biodegradable by the introduction of hydrolysable bonds (B), and a non-biodegradable polymer, where all monomers are linked by non-hydrolysable bonds (C).

1.6 Skin Penetration of Topically Applied Nanocarriers

The penetration of a drug into the skin depends on several parameters like drug physicochemical characteristics (e.g. partition coefficient, molecular weight), skin status (e.g.

metabolism, hydration, pathology) and application conditions (e.g. temperature, occlusion) (Bucks and Maibach 1999). In 2000, Bos and Meinardi (Bos and Meinardi 2000) formulated the "500 Dalton rule" for the skin penetration of chemical compounds and drugs. The authors considered the available data on skin penetration of allergens as well as topically applied drugs and concluded that molecules with molecular weight higher than 500 Dalton cannot penetrate across the SC, at least not in clinically relevant concentrations.

The SC is considered to be the main physical barriers to the penetration of exogenous materials. Three main routes across the SC have been described: i) the intracellular route through the hydrophilic and protein-rich environment of the corneocytes, ii) the intercellular routes through the extracellular lipid matrix, and III) the transfollicular route. The intercellular route is considered as the most relevant penetration pathway for almost dermatological relevant drugs, whereas the hair follicles represent the skin structures where nanoparticle accumulate and deliver drugs to the skin in a sustained manner. In fact, the hair follicle infundibulum has a large surface area that extends in depth down to the epidermal-dermal boundary with the consequence that released drugs have an easier access to both epidermis and upper dermis. Several studies have been conducted on skin penetration and release properties of different types of nanocarriers using model drugs or macromolecules and showing the potential of these delivery agents in the field of dermatotherapy. A few examples follow. Choi et al., delivered enzymes to human skin via chitosan- functionalized Pluronic-based nanocarriers and showed that the activity of the delivered enzyme persisted after skin penetration (Choi, Lee et al. 2012). Lipid-based nanoparticles have been widely investigated for dermal delivery due to their affinity for the hydrophobic components of skin barrier and their ability to enhance skin barrier permeability (Prausnitz and Langer 2008). PLA particles were shown to accumulate in hair follicles (and were used to deliver drugs and peptides for transcutaneous vaccination (Rancan, Amselgruber et al. 2014) Fernandes, Silva et al. 2015). Core-multi-shell nanocarriers based on biocompatible polymers were effective in enhancing the skin penetration of biological active peptides (Do, Weindl et al. 2014).

Over the last decades, the increasing number of products containing nanoparticles has arisen questions about the safety of these new type of materials. According to the 500 Dalton rule, nanoparticles should not cross skin barrier, and, in fact, almost all studies confirmed the absence of nanoparticle skin penetration. Nevertheless, in exceptional cases translocation to the living skin layers could be detected, depending on the skin condition, the mode of application, the addition of skin permeabilizing substances, and the type of nanocarrier. Interestingly, the used detection method makes often the difference, especially when very low amounts of nanoscopic material need to be detected. However, the quantities of nanoparticles that were detected in the lower skin layers were always small, also in those cases where skin barrier was damaged by UV radiation or stripping (Vogt, Combadiere et al. 2006, Mortensen, Oberdörster et al. 2008). Thus, it seems that methods enhancing skin barrier are necessary when it is intended that a consistent amount of nanocarrier overcome the skin barrier. On the base of these considerations, a broad spectrum of physical techniques such as laser microporation, sonophoresis, iontophoresis, microneedles, as well as permeabilizing agents have been developed (Choi, Lee et al. 2012, Zhang, Zhai et al. 2015). Most of these methods aim at inducing a temporary disruption of the SC microstructure in order to facilitate the permeation or translocation of applied material in a minimally invasive way. Several chemical penetration enhancers have been investigated and water is one of the most studied one. In fact, it has been shown that SC hydration can enhance the skin penetration of several drugs. In normal conditions, the SC contains about 20% of water but under occlusion this percent increases leading to a localized perturbation of

the SC structure. Water pools are formed in the corneocytes as well as in the extracellular matrix within the corneocytes (Bouwstra, Honeywell-Nguyen et al. 2003). Nanocarriers like polyglycerol-based nanogels can carry large amounts of water and enhance SC hydration once applied topically. This mechanism has been proposed to explain the good penetration of nanogels in the deepest layers of the SC and their ability to enhance the delivery of loaded cargos (Giulbudagian, Rancan et al. 2016). Other chemical penetration enhancers are sulfoxides, azones, pyrrolidones, alcohols and alkanols, glycols, surfactants, and terpenes (Williams and Barry 2012). Ethanol, dimethyl sulfoxide, propylene glycol, and surfactants like sodium lauryl sulfate are mostly used in dosage forms to improve skin penetration but they have the drawback that they can cause local skin irritation. Biological agents such as CPPs have also been used to enhance the skin penetration of drugs and nanoparticles (Rothbard, Garlington et al. 2000) (Patlolla, Desai et al. 2010).

1.7 Fate of Nanocarriers Applied to Skin

Different routes of nanocarrier administration to skin can be chosen, depending on the therapeutic application. Nanocarriers can be applied on skin surface, with or without skin penetration enhancement, they can be delivered directly to the viable epidermis by means of microneedles and other physical methods, and they can be injected in the dermis or in the subcutis (Fig. 4).

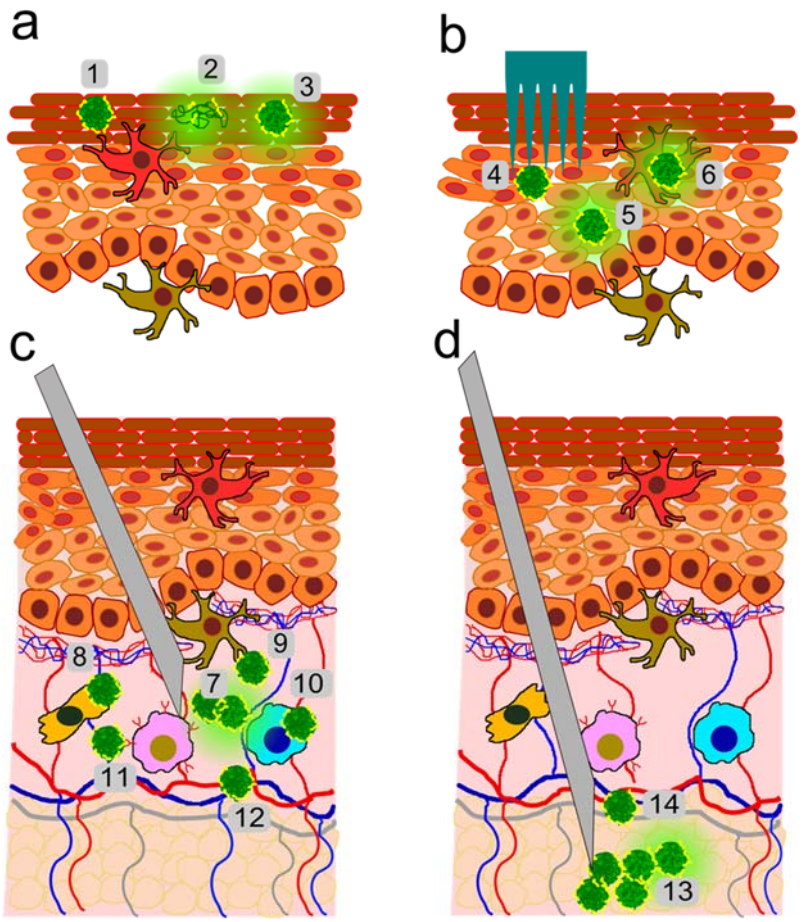

Figure 4. Schematic representation of nanocarriers' possible interactions with skin. Nanocarriers can be applied to different skin layers: a) on skin surface; b) to the epidermis, e.g. by means of microneedles; c) in the dermis or d) in the subcutaneous tissue by means of injection. Independently

to the administration route, nanocarriers are likely to adsorb proteins on their surface (yellow). Topically applied nanocarriers can accumulate in the SC (1), destabilize and lose their particulate state (2), or undergo degradation (3). Nanocarriers that reach the epidermis can localize in the intercellular space or be taken-up by keratinocytes. Non-biodegradable nanocarriers (4) will be eliminated during the epidermal turnover. Biodegradable nanocarriers (5) can hydrolyze spontaneously or by extracellular or lysosomal enzymatic digestion. They can also be recognized by LCs (6) and be degraded or transported to the lymph nodes. Intradermally injected nanocarriers can accumulate and/or degrade in the intercellular space (7). They can interact or be taken-up by cells like fibroblasts (8), dermal DCs (9), macrophages (10), and mast cells, or translocate to the lymphatic and blood vessels reaching lymph nodes and the systemic compartment (12). Subcutaneous injection results in accumulation of nanocarriers in the fat tissue, where they can persist until their degradation (13) or translocate to the lymphatic and blood systems (14).

Once nanocarriers are applied to the skin, different interactions and reactions can take place, which will determine nanocarriers' distribution, biodegradation, drug release kinetics as well as toxicity or biocompatibility. The biocompatibility of a material is highly dependent on the site of application. Thus, when developing nanocarriers for dermal and transdermal delivery, it is important to understand how they interact with skin, including possible effects that the delivery method may have on skin physiology. Possible degradation and clearance processes, eventual reactions of skin immune system as well as toxicity towards skin cells have to be taken into consideration. Biocompatibility is not an absolute but rather a relative attribute, which depends on the specific properties of the nanomaterial, possible impurities, dose and duration of the treatment as well as risk/benefit considerations (Naahidi, Jafari et al. 2013).

Topically applied nanocarriers may accumulate in the SC and degrade or disassemble there while interacting with the components of the horny layer. Extracellular proteins may adsorb on the surface of applied nanocarriers, including products of skin bacterial flora (Wigginton, Titta et al. 2010). The protein corona will influence all further interactions with skin, e.g., the cellular uptake by immune cells may take place through pattern recognition receptors (Fadeel 2012). In the case nanocarriers can cross the skin barrier, due to penetration enhancement or skin barrier impairment, they will interact with epidermal and dermal cells. Non-biodegradable nanocarriers taken-up by keratinocytes will be possibly eliminated together with terminally differentiated corneocytes during the physiological epidermal turnover. On the other hand, keratinocytes are also immune active cells and can release cytokines when they are damaged or exposed to dangerous exogenous materials (Partidos and Muller 2005). If methods such as surfactants, tape stripping or microneedles are used to disrupt skin barrier, cytokines will be released (Glenn, Kenney et al. 2003). These will attract immune cells like LCs, dermal DCs, and macrophages, induce their activation, and promote the uptake of penetrated material (Vogt, Hadam et al. 2015). Intradermally injected nanocarriers may also interact with fibroblasts and immune cells. For example, mast cells have been shown to react to nanoparticles reaching the dermis (Aldossari, Shannahan et al. 2015). Nanocarriers penetrated or administered to the dermis or the subcutis can enter the blood system or be transported to the lymph nodes by lymphatic drainage and/or by DCs (Mahe, Vogt et al. 2009). Once in circulation, nanocarriers can redistribute to other organs, accumulate in the reticular connective tissue, or be excreted by renal clearance.

The degradation of a nanocarrier, once it has penetrated the skin, can take place by spontaneous hydrolysis or by means of skin enzymatic activity and depends on the type of constituting material, skin temperature, pH and oxidative status. Skin is rich in enzymes and biological nanocarriers can be degraded by proteases, lipases, hyaluronidase etc. In the SC, proteases like kallikreins are found (Rawlings and Voegeli 2013) which may degrade

topically applied nanocarriers. After internalization by endocytosis, nanocarriers encounter degrading enzymes like chatepsins in lysosomes and several other types of proteases in the granulosomes of mast cells and neutrophils (Hellman and Thorpe 2014). once in these acidic organelles, nanocarriers can be accumulated, degraded, excreted or released in the cytoplasm due to endosome disruption (endosomal escape) (Nel, Mädler et al. 2009). After endosomal escape or exocytosis, nanocarriers might have a different protein corona acquiring new properties or toxicity (Lundqvist, Stigler et al. 2011, Sakhtianchi, Minchin et al. 2013).

Immunogenicity is one of the main concerns related to nanocarriers, especially those based on biological material, e.g. cross-linked proteins. Depending on the type of cells coming in contact with the nanocarrier and the involved mediators, different reactions can be triggered that range from immune responses to allergic reactions towards nanocarrier constituents behaving as antigen or haptens (Fadeel 2012). Different uptake pathways exist involving different receptors on APCs which might recognize molecules adsorbed or linked to the surface of nanocarriers (Dobrovolskaia and McNeil 2007). Some of these pathways induce pro-inflammatory responses whereas other pathways do not. Coating nanocarriers with hydrophilic negatively charged polymers, such as poly-ethylene glycol (PEG), has been shown to reduce nanocarrier recognition and internalization by APCs. Thus, depending on the nanocarrier components, surface functionalization (e.g. polysaccharides, antibody, PEG) or on the type of adsorbed proteins (e.g. complement) the immune system can be activated or avoided. Once internalized, biological nanoparticles or material loaded on nanocarriers can be processed and presented as antigen to T-cells and B-cells. Depending on the antigen concentration and on the presence of additional stimuli such as cytokines and co-stimulatory molecules, tolerance or an immune response specific for the nanocarrier or the associated antigens can be generated (Irvine, Swartz et al. 2013). Immune responses are of convenience for vaccination purposes, whereas they lead to undesired reactions when nanocarriers are employed for other therapeutic purposes. Nanoparticles can also act as adjuvant enhancing an immune responses or allergic reactions. It has been shown that negatively charged silica nanoparticles could act as adjuvant in allergy processes, whereas positively charged silica nanoparticles were found to be safe and did not exacerbate induced allergic contact dermatitis in mice (Ostrowski, Nordmeyer et al. 2014). Another point to be considered is that most of nanocarriers investigated for dermatological therapies will be applied on diseased skin . In case of atopic dermatitis, psoriasis, skin cancer, or infections, skin immune system is often activated and nanocarriers might amplify or suppress ongoing immune reactions. For example, it has been found that zinc oxide nanoparticles can induce the release of Ig E, when applied on a mouse model for atopic dermatitis (Landriscina, Rosen et al. 2015). Application of delivery agents that exert toxicity towards immune cells or interfere with the immunogenic processes can also have immunosuppressive or anti-inflammatory activity. For example, PAMAM-based dendrimers were shown to inhibit cytokine secretion, whereas polymerized lipid nanoparticles and butyrate-conjugated solid lipid nanoparticles have been shown to inhibit leukocyte adhesion to activated endothelial cells reducing cell infiltration in inflamed tissue (Dobrovolskaia and McNeil 2007). Silver nanoparticles have been shown to exert anti-inflammatory activity, e.g. in a model of contact dermatitis (Nadworny, Wang et al. 2008). The mechanism of action is not fully understood but it seems to be related to increased apoptosis and reduction of pro-inflammatory cytokines.

Independently on the constituting material, nanocarrier physicochemical properties can influence their interaction with skin and determine their toxicity or biocompatibility. Size

influences the penetration profile of nanoparticles in both healthy and diseased skin. If injected, nanocarrier depots are formed preferentially by large particles, whereas smaller particles are more easily transported to the lymph nodes (Mahe, Vogt et al. 2009). Most types of skin cells can internalize particles with size below 200 nm, whereas particles with size higher that 200 nm are more suited for the targeting of phagocytic cells (Rancan, Gao et al. 2012). Consequently, size can also influence the immunomodulating properties of nanocarriers. Nanoparticle shape can influence skin penetration, and cell internalization. It has been shown that phagocytic cells can internalize spherical particles more readily than elongated ones, especially when the aspect ratio increases (Liu, Tan et al. 2012). Surface charge influences particle colloidal stability, penetration across skin barrier and cellular uptake. Positively charged nanocarriers are internalized by epidermis cells better than negatively charged ones (Rancan, Gao et al. 2012). Whereas negatively charged dendrimers were found to cross the skin barrier, dendrimers with positive surface charge were shown to accumulate in different skin layers depending on their size (Yang, Sunoqrot et al. 2012). Surface functionalization can also determine biocompatibility. Quantum dots with neutral, cationic or anionic coatings were found to have different cytotoxicity towards human keratinocytes, with particles coated by carboxylic acid being the most toxic ones (Ryman-Rasmussen, Riviere et al. 2007). Beside reducing the absorption of proteins on particle surface, functional groups like PEG confer colloidal stability upon nanocarrier topical application. For example, PEG was used to stabilize liposomes loaded with calcipotriol (Knudsen, Rønholt et al. 2012). Finally, softness of particles is an important parameter for the penetration in the SC. Elastic vesicles or particles are claimed to squeeze between the lamellar lipid layers as shown for transferosomes by Cevc and Blume (Cevc and Blume 1992). Softness was also an important parameter for the penetration on thermoresponsive nanogels in the SC (Rancan, Asadian-Birjand et al. 2016).

1.8 High Resolution Microscopy and Spectromicroscopy

The introduction of nanotechnology in several fields of biology and medicine has boosted the fields of high resolution imaging methods for the analysis of nanomaterials and their interaction with biological matters. Several microscopic and spectromicroscopic methods are available or are being developed to overcome the limited resolution of conventional light microscopy and/or to detect molecules in a label-free manner. Each microscopic method has advantages and disadvantages and its application in experimental dermatology requires specialized knowledge about output, specificity, and sensitivity of the method as well as about the advantages and disadvantages of the often complex sample preparation procedures.

Commonly, imaging of skin morphology and biomolecular features has been done by optical and fluorescence microscopy of stained or immune-labeled skin sections. However, the high intensity of skin auto-fluorescence is often a drawback along with the low resolution. Confocal laser scanning microscopy (CLSM) has improved the contrast and resolution of conventional fluorescence microscopy, thanks to the introduction of a pinhole that enables the collection of light originating in the focal plane and eliminate out-of-focus fluorescence. Laser or diodes are employed as excitation sources whereas oscillating mirrors allow scanning the beam across the sample. CLSM is one of the most used imaging methods in several science research fields, included experimental dermatology. CLSM was used to detect the penetration of fluorescently labeled nanoparticles in the SC, the penetration of released fluorescent dyes as well as the interaction of nanocarriers with skin immune cells on

immune-stained skin sections. Fluorescence life-time imaging microscopy (FLIM) uses a pulse laser to excite fluorescent molecules and measure their fluorescence life-time in a spatial resolved fashion. The possibility to distinguish between the lifetime of investigated molecules and that of skin components allows subtracting skin background signals. Being fluorescence life time sensitive to the environment, the method enables also to register different decays depending on the pH or hydrophilicity of the analyzed skin region, e.g. it is possible to register if a fluorophore is encapsulated in a nanocarrier or has been delivered once applied topically on skin. Conventional fluorescence microscopy is not adequate to image small structures like nanocarriers because of the Abbe diffraction limit, which set the size limit of optical microscopes to approximately the half of the excitation wavelength, e.g. 244 nm in the case of an Argon laser with emission at 488 nm. Super resolution microscopy is a group of techniques based on light microscopy that were further developed to overcome the Abbe diffraction limit. One of the most famous techniques is stimulated emission depletion (STED) microscopy that uses two pulse lasers, one to stimulate a fluorophore and one to deplete fluorescence out of the focal point, thus generating images with low background fluorescence and a resolution of about 80 nm. Using this technique to image cryosections of human skin, it was demonstrated that intact liposomes could not penetrate the SC (Dreier, Sørensen et al. 2016). Single-molecule fluorescence techniques have also been developed to enhance optical microscope resolution. One example of such methods is total internal reflection fluorescence (TIRF) microscopy, which takes advantage of an evanescent wave formed at the glass/specimen interface to induce fluorescence emission in a small region at a surface of the sample. This method is generally used to study events taking place on the plasma membrane, but it was successfully applies to measure the diffusion constants and spatial confinements of a model dye on the skin surface with the aim to elucidate the penetration pathway of single molecules across the SC (Volz, Boreham et al. 2015).

The first high-resolution images were generated in the 30s by means of electron microscopy. Depending on the instrument, resolution down to 40-0.1 nm can be achieved. Transmission electron microscopy (TEM) allows the imaging of specimens which absorbs electrons like tissue treated with contrast agents or metal and metal oxide nanoparticles. For example the uptake of silver nanoparticles by HaCaT keratinocytes (Ahlberg, Meinke et al. 2014) as well as the penetration of iron and silver nanoparticles in human skin could be visualized by this method (Baroli, Ennas et al. 2007, Larese, D'Agostin et al. 2009). When the sample is embedded in resins such as LR-White, it is also possible to visualize molecules of interest by immuno-labelling with antibodies conjugated to gold nanoparticles. Particles based on organic materials need to be stained (e.g. with uranyl acetate) to be visualized by this technique and are often not well distinguishable from the tissue or cells components. Thus, TEM analyses of nanoparticle interactions with skin components are limited to electron-dense materials. Another limitation of TEM is that samples needs to undergo extensive processing including fixation, contrastation, dehydration, and embedding in polymer-based resins to get solid blocks that can be cut into 70-80 nm thick sections and are stable under vacuum. These procedures can cause artifacts, e.g. dislocation of nanocarriers or drugs to be detected. TEM was developed to visualize biological samples and the fixation procedures are specific for components like proteins with amino groups that can be cross-linked with glutaraldehyde or lipids with carbon double bonds, which can react with osmium tetroxide. Thus, skin components or exogenous materials devoid of these groups will be lost during the preparation steps. Scanning electron microscopy (SEM) is a sample-scanning method that allows the imaging of surfaces by collecting electrons backscattered by the sample. For this

imaging technique, the sample needs also to be fixed and dehydrated because the measurement takes place in vacuum. For an optimal imaging, the specimen needs to be sputter-coated with an electrically conducting metal in order to enhance the number of scattered electrons. This method allows the analysis of tissue blocks whose surface and longitudinal faces can be imaged to obtain high-resolution images and investigate the interaction of nanoscaled structures like bacteria, viruses, or nanoparticles with the components of skin barrier and tissue.

Cryo-SEM does not require sample fixation because the specimen is frozen and imaged at temperatures around -130 °C. Plunge freezing in liquid propane, ethane or liquid nitrogen at low pressure (−130 °C, ~10−3 Pa) is necessary to avoid the formation of water crystals that would damage the sample. High pressure freezing can help improving the quality of frozen samples, however tissue samples must be previously sliced because the preservation of morphology is guaranteed only for 1-3 mm thin samples. Nevertheless, lipid organization is little influenced by the plunge freezing procedure and, thus, cryo-SEM of plunge-frozen skin sections could be used to image the SC and nanocarrier within it. Interestingly, some of the nanocarriers seemed to be wrapped-up by fibrous material probably skin proteins (Rancan, Asadian-Birjand et al. 2016). SEM can be coupled with energy-dispersive X-ray spectroscopy (EDX) which allows the elemental analysis of the scanned area. It relays on the element specific x-ray radiation emitted when the sample adsorbs incident electrons. This technique was successfully used to detect the penetration of gold nanoparticles with different shapes into human skin (Graf, Nordmeyer et al. 2015).

Atomic force microscopy (AFM) is another high-resolution scanning probe technique which can sense the roughness of a sample by interaction of a cantilever with the surface of the specimen. The displacement of the cantilever is registered by changes in reflection of a laser beam pointed on the cantilever tip. The sample is scanned with nanometer precision thanks to piezoelectric elements. AFM has been shown to be useful for the imaging of skin structures and measurements of their mechanical properties with nanoscale resolution (Chang, Liu et al. 2017). This method can also be coupled to spectroscopic techniques like infrared or Raman spectroscopy, whereby the sample can be analyzed also chemically. Another implementation of AFM is the peak-force tapping which is based on force-distance curves generated when the cantilever is moved and taps on the sample. This method give information of the deformability of biological samples like cells (Haase and Pelling 2015) and was used to measure the deformability on thermoresponsive hygroscopic nanocarriers (nanogels) at different temperatures (Rancan, Asadian-Birjand et al. 2016).

X-ray microscopy uses electromagnetic radiation in the soft X-ray range to produce contrast images with a resolution between that of conventional optical microscopes and that of electron microscopes. The used energies are between the absorption edges of carbon (at 284 eV, 4.36 nm) and that of oxygen (534 eV, 2.34 nm), where biologic materials have high absorption and generate images with sufficient contrast. A full-field transmission x-ray microscope based on a laboratory x-ray source (a laser-produced nitrogen plasma) has been used to image skin and skin cells and has been developed for 3D imaging. Samples are measured under vacuum and thus need to be dehydrated and embedded as for TEM, with the only difference that sample thickness can reach up to 10 µm (Dehlinger, Blechschmidt et al. 2015). Scanning transmission x-ray microscopes (STXM) are associated with synchrotron radiation sources. The X-ray radiation is focused to a point by a zone plate, whereby the specimen is scanned by moving the sample holder. Not only dehydrated embedded samples in vacuum, but also wet samples under helium atmosphere can be measured, e.g., fixed cells in between two silicon nitride windows. Using STXM both the uptake of silver

nanoparticles by HaCaT skin cells and the penetration of gold nanoparticles in skin with intact and damaged barrier could be investigated (Graf, Nordmeyer et al. 2015, Ahlberg 2016). Another further development of X-ray based microscopes is x-ray spectromicroscopy, i.e. the possibility to record absorption spectra in a spatial resolved mode. This allows to record images and at the same time to associated each pixel to an X-ray absorption spectrum, which in turn gives information on the chemical composition of the specimen. Using STXM spectromicroscopy, it was possible to image skin sections and detect in a label-free manner the penetration of topically applied dexamethasone formulations (Yamamoto, Flesch et al. 2015), and even detect the effect to skin barrier disruption on the penetration rate of this drug (Yamamoto, Klossek et al. 2016). Because the samples need to be embedded in resins as described for TEM, it is important that the compound under investigation can be fixed to the sample components like proteins and lipids to avoid that it is washed out during the several sample preparation steps. Another major problem by these measurements is that the drug needs to absorb at a specific wavelength in the soft x-ray region where the skin components do not absorb. In case of spectral overlap, the gain in differential absorption at selected wavelength can be used to improve the signal to noise ratio. This method was used to measure the signal of polymer-based nanocarriers and analyze their penetration pathway within the lipid lamellae of the SC (Yamamoto, Klossek et al. 2016). 3D images can be collected by cryo-soft X-ray tomography (cryo-SXT). The tomographic data are used for the reconstruction of images from planes within the samples with resolution of approximately 60 nm. The limiting factor of this method in the plunge freezing procedure, which is optimal for samples with a thickness up to 5-8 µm. In addition, the optimal thickness of the amorphous ice covering the biological sample should be of some nanometers in order to avoid too higher absorption but at the same time to protect the sample from the x-ray radiation. This method is ideal for the investigation of plunge frozen cells in a close-to-native state, whereas the preparation of properly plunge-frozen skin sample is more complex. Similar to electron microscopes, X-ray microscopes are suitable for the label-free detection of metal and metal oxide nanoparticles. For example, cryo-SXT was used to identify the interaction of superparamagnetic iron oxide nanoparticles with a human breast cancer cell line (Chiappi, Conesa et al. 2016).

Raman spectromicroscopy is another valuable label-free method for the analysis of biological specimens. It detects scattered light that originates from sample molecules when they are excited, e.g. by a laser source, to different vibrational and rotational levels. Raman spectra give, similar to x-ray and infrared spectra, information on the specific chemical composition of the sample. Thus, Raman spectromicroscopy can be used to identify the position of a test substance within a specimen. For example, confocal Raman spectromicroscopy has been used to analyze the effect of different penetration enhancers on flufenamic acid skin penetration (Pyatski, Zhang et al. 2016). The technique can be used on full-thickness skin or skin sections without the need for pre-treatment like fixation or labeling. There are different variants of the method like stimulated Raman-spectroscopy (SRS) and coherent anti-stokes Raman scattering (CARS). Recently, SRS was combined to spontaneous Raman scattering to reduce the cross-sensitivities between the lipid and protein signals in order to improve the analysis of lipid distribution within the skin layers (Klossek, Thierbach et al. 2016). SRS was also used to monitor the perturbation of lipid as well as protein supramolecular organization in SC upon topical application of nanogels and hydration of the horny layer (Giulbudagian, Rancan et al. 2016).

2. Original Works

2.1 Skin Penetration and Cellular Uptake of Amorphous Silica Nanoparticles with Variable Size, Surface Functionalization, and Colloidal Stability

Rancan F, Gao Q, Graf C, Troppens S, Hadam S, Hackbarth S, Kembuan C, Blume-Peytavi U, Rühl E, Lademann J, Vogt A, ACS Nano, 2012, 6, 6829-6842.

http://dx.doi.org/10.1021/nn301622h

In this study, the effects of particle size and surface charge on cellular uptake and skin penetration were investigated. First, the uptake of solid silica nanoparticles by different types of skin cells was tested *in vitro* to find out which size and surface charge allow for a preferential uptake of nanoparticles by epidermis cells. The internalization capacity of a keratinocyte cell line (human, adult, low calcium, high temperature, HaCaT cells) was compared to that of freshly isolated human keratinocytes and LCs. The last were separated from epidermal cells suspensions by means of magnetic cell sorting. Whereas HaCaT cells were found to internalize all particles with sizes between 40 and 300 nm, different uptake capacity was observed for primary cells shortly after isolation. Whereas primary keratinocytes were able to uptake only nanoparticles with diameter of 42 ± 3 and 75 ± 6 nm, LCs could internalize also bigger particles with size of 190 ± 9 nm. These results confirmed that LCs can uptake particles by phagocytosis and are more prone to internalize particulate material than primary keratinocytes. In other words, particles with size about 200 nm can be used to target drugs or antigens specifically to skin DCs. The positive surface charge favored the uptake of particles. However, aggregation occurred and this, in turn, resulted in a reduction of particles available for cellular uptake. A further issue of this study was the eventual penetration of such solid silica nanoparticles across disrupted skin barrier and their internalization by skin cells. The results show that after mild disruption skin barrier is still a strong limiting factor to the penetration of solid nanoparticles. Only particles with size of 42 ± 3 nm were detected in epidermis cells, with minimal selectivity for the uptake by LCs.

2.2 Investigation of Polylactic Acid (PLA) Nanoparticles as Drug Delivery Systems for Local Dermatotherapy

Rancan F[1], Papakostas D[1], Hadam S, Hackbarth S, Delair T, Primard C, Verrier B, Sterry W, Blume-Peytavi U, Vogt A, Pharmaceutical Research, 2009, 26, 2027-2036.

https://doi.org/10.1007/s11095-009-9919-x

One of the most interesting behavior of topically applied nanoparticles is their preferential accumulation in skin appendages, especially the hair follicles. This characteristic can be exploited to deliver drugs to the sebaceous gland, which is one of the target of acne vulgaris therapies. In this study, the hair follicle penetration of biodegradable PLA particles was investigated along with the release of encapsulated fluorescent dyes simulating drugs with different lipophilicity. The particles, with diameter sizes of 228 and 365 nm, were found to accumulate in 50 % of investigated hair follicles. The distribution of the released fluorochromes was followed over time. Interestingly, the lipophilic dye nile red was found to accumulate in the sebaceous gland, where the fluorescent signal was detected up to 24 hours after topical application. *In vitro* experiments showed that, whereas no dye release occurred in PLA particles aqueous suspension, dye release occurred when particles were put in contact with a lipophilic solvent. This work underlines that PLA nanoparticles allow for the targeting of drugs to the pilosebaceous unit and especially for the sustained drug delivery to the sebaceous gland.

[1] these authors contributed equally

2.3 Particle-based transcutaneous administration of HIV-1 p24 protein to human skin explants and targeting of epidermal antigen presenting cells

Rancan F, Amselgruber S, Hadam S, Munier S, Pavot V, Verrier B, Hackbarth S, Combadiere B, Blume-Peytavi U, Vogt A, Journal of Controlled Release, 2014, 176, 115-122.

https://doi.org/10.1016/j.jconrel.2013.12.022

Transcutaneous vaccination is one of the most promising field of application for nanocarrier-based transdermal drug delivery. This vaccination route allows to target antigens to potent APCs and even to target different DCs sub-populations, e.g. LCs, in order to control the type of induced immune response. In this study, PS and PLA particles were used to transport an antigen, the HIV-1 p24 peptide, to the skin layers and target it to LCs. In order to improve particle penetration in the hair follicle canals and to activate epidermal LCs, a mild skin barrier disruption was induced by removing the first layers of the SC and the material filling the hair follicle infundibulum by means of CSSS. Particle penetration in the hair follicles and delivery of the HIV-1 p24 peptide to epidermal and dermal skin layers was imaged by immunohistochemistry. Antigen uptake was detected also in DCs isolated from skin, especially from the dermis. In addition, activation and maturation of LCs was detected after CSSS and particle-mediated antigen delivery. These results show that nanocarriers allow for transcutaneous antigen delivery via the transfollicular route. In addition, it was shown that a mild skin barrier disruption, not only facilitate the translocation of particles and antigen to the viable skin, but also activate skin immune system in an adjuvant-like manner. Hence, antigen delivery to CSSS-treated skin by means of biodegradable nanoparticles resulted to be a promising strategy for the development of new vaccination approaches.

2.4 Effects of Thermoresponsivity and Softness on Skin Penetration and Cellular Uptake of Polyglycerol-Based Nanogels

Rancan F, Asadian-Birjand M, Dogan S, Graf C, Cuellar L, Lommatzsch S, Blume-Peytavi U, Calderón M, Vogt A, Journal of Controlled Release, 2016, 228, 159-169.

http://dx.doi.org/10.1016/j.jconrel.2016.02.047

A sophisticated strategy of targeting drugs to diseased tissue is to use smart nanocarriers able to sense the environment in the target region or to release drug on demand, e.g. upon an external trigger. Inflamed tissue is characterized by hyper permeability of the blood vessels and localized high temperature. This can be exploited to deliver drugs more selectively. Thermoresponsive nanogels can change their conformation in dependence to the temperature thereby expelling water and incorporated molecules. This property makes them ideal carrier systems for drug delivery to inflamed tissue or for triggered drug delivery by use of a warming external source, e.g. infrared (IR) radiation. The nanogels investigated in this study are nanoparticles made of hygroscopic brunched polymers, like dendritic polyglycerol, cross-linked with linear thermoresponsive polymers. Such nanocarriers are soft and water soluble but become stiff und water insoluble above a specific temperature, called transition temperature. In this study it was verified whether these properties can influence the way nanogels interact with skin barrier and skin cells. PeakForce Quantitative Nanomechanics was used to confirm the changes of nanogels softness at temperatures above and below the transition temperature. The skin experiments showed that soft nanogels penetrated the SC more efficiently than stiff ones. Combining CLSM, cryoSEM and FACS analyses, it could be shown that small amounts of nanogels were able to cross to the viable epidermis and be taken-up by skin cells.

2.5 Drug Delivery across Intact and Disrupted Skin Barrier: Identification of Cell Populations Interacting with Penetrated Thermoresponsive Nanogels

Rancan F, Giulbudagian M, Jurisch J, Blume-Peytavi U, Calderón M, Vogt A, European Journal of Pharmaceutics and Biopharmaceutics, 2016, 116, 4-11.

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In this study, the ability of thermoresponsive nanogels to release their cargo on demand was demonstrated by inducing a temperature increase on skin surface using an IR lamp. The investigations were done on skin with both intact and disrupted barrier to simulate the altered barrier function typically found in skin inflammatory conditions. The delivery of fluorescein and the penetration of nanogels in the skin layers increased in barrier-disrupted skin and upon application of the external trigger. In a previous study, using TEM and stimulated Raman spectromicroscopy, we could show that, thermoresponsive nanogels above the transition temperature can induce the swelling of the SC, thereby disrupting the organization of both lipids in the extracellular matrix and keratin in the korneocytes. In addition, several experiments had evidenced the propensity of nanogels to cross the SC barrier and to be taken-up by skin cells. These findings suggested to use of nanogels to overcome the skin barrier and target drug to specific skin cells. At this purpose, different cell populations of both epidermis and dermis were analyzed in order to find out which cell type primarily interact with penetrated nanocarriers. FACS analyses showed that, especially in the dermis of skin with disrupted barrier, antigen presenting cells (HLA-DR+ and CD206+ cells) were associated with nanogels. These cell populations play a central role in inflammatory skin conditions, allergies, autoimmune diseases as well as adaptive immune responses. Hence, the possibility to target drugs to modulate the activity of specific DCs would open new perspectives for the treatment of several skin diseases using soft thermoresponsive nanocarriers.

3. Discussion

The effective and selective delivery of a drug to its target is a crucial factor that can determine the success or failure of a therapy. Nanoparticles have been recognized since years as potential delivery systems (Foldvari and Rafiee 2015). By transporting and releasing defined amounts of drugs in specific body or cellular compartments, they completely change the pharmacokinetics and bioavailability of drug molecules. Modifications of nanocarrier surface, size, form, softness, and hydrophobicity give the possibility to control the way they interact with body barriers, different types of cells, proteins and so on. Changing nanocarrier parameters enables to control the mechanisms of cellular uptake and subcellular distribution as well as the kinetics of drug release. The coupling of nanocarriers to targeting units favors the accumulation of nanocarriers in the target tissue and a more selective delivery of the actives to the target cells. The use of nanocarriers for drug delivery to and across the skin holds the promise to improve the topical treatment of several skin conditions and opens the way to new strategies for the transdermal delivery route. In order to develop nanocarriers for dermatotherapies, it is important to develop materials with different penetration and delivery properties in dependence on the disease to be treated. The main aim of the studies presented in this thesis was to improve the understanding as to how the physicochemical properties of nanocarriers may influence their penetration across the skin barrier, as well as their interactions with epithelial and immune active cells. A deeper knowledge of the principles regulating skin penetration, cellular uptake and skin immune responses will foster the development of innovative and tailor-made nanocarriers adapted for the disease to be treated, the drug to be transported, and the needs of the single patient.

3.1 Tuning Particle Size and Surface Charge Allows to Target Specific Skin Cell Populations

It is known that, when injected intravenously, nanoparticles are taken-up by the cells of the mononuclear phagocyte system. This is due to the formation of a protein corona on particle surface, which makes them identifiable by macrophages (Song, S Petschauer et al. 2014). Thus, if particle surface is not modified, most of nanoparticles will be rapidly removed from the systemic circulation and will accumulate in the connective tissue of organs like liver, spleen and kidneys. In addition, when nanocarriers are applied locally and can cross the skin barrier, they will adsorb components of the extracellular space and will then interact with endocytic cells in the epidermis or dermis. Particle size has been shown to influence their uptake by cells, whereby the smaller the particle, the highest the cellular uptake is. On the other hand, particles in the size range of 200-1000 nm are taken-up preferentially by phagocyting immune active cells. In the study reported in chapter 2.1, particles with different sizes were tested, in order to determine the optimal particle size resulting in high uptake by LCs but low internalization by keratinocytes. The results of the study show that 200 nm is the optimal size in order to target nanocarriers preferentially to skin immune cells. Nevertheless, the same study shows that, when the skin barrier is intact or only slightly disrupted, solid particles with such a size can hardly cross the SC. Thus, besides developing nanocarriers with better penetration ability, methods to overcome the skin barrier need to be further developed. Microneedles, electroporation and laserporation are only a few examples of new strategies under investigation in order to increase skin permeability (Zhang, Zhai et al. 2015).
Another crucial point for drug delivery is the amount of drug that can be delivered to a single cell. Decorating nanocarrier surface with specific moieties enhancing cellular uptake is the

most used strategy to improve cellular uptake. Usually, positively charged nanocarriers are internalized by cells better than negatively charged ones. For these reasons, in the same study, the uptake of positively versus negatively charged nanoparticles was tested on freshly isolated skin cells and skin explants. The results confirmed that a positive surface charge increases particle uptake by skin epithelial cells as well as DCs. Different other strategies have been used to increase the amount of cellular uptake, e.g. ligands recognizing specific receptors or agents changing cell membrane permeability. Decorating nanocarriers with albumin, cholesterol or folic acid supports caveole-madiated endocytosis that, other than clathrin-mediated endocytosis, is mostly not associated with the lysosomal pathway (Elsabahy and Wooley 2012).

3.2 Nanoparticle Selective Accumulation in Hair Follicles Improves the Targeting of Drugs to Hairs and Sebaceous Glands

Skin has a very inhomogeneous surface, especially when observing at a nanometer scale. Several structures like shedding corneocytes, microorganisms, hair shafts as well as invaginations such as furrows, sweat glands and hair follicle openings are structures that can influence the distribution on topically applied nanocarriers (Fig. 5). Most of nanoparticle in aqueous dispersions distribute with a typical pattern that has been observed by many groups using different types of nanomaterials, i.e. accumulation into furrows and hair follicle canals. In addition, it has been shown that hair follicles are long-term reservoirs for particulate material. In an *in vivo* study on volunteers, it was found that a hydrogel formulation of biodegradable commercially available nanoparticles (size of 320 nm) accumulated in the hair follicle infundibulum and could persist there for at least 10 days (Lademann, Richter et al. 2006).

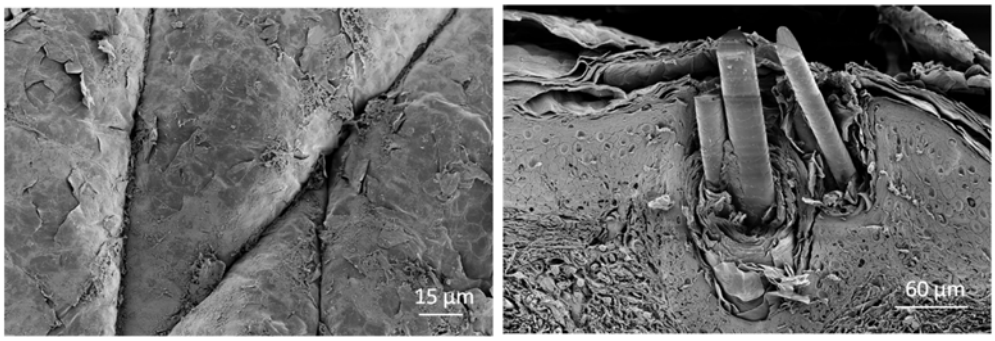

Figure 5. Scanning electron microscope images of human skin. Skin surface view from the top (left) looks as a rough surface interrupted by furrows. The skin section (right) shows the opening of a vellus hair follicle with a diameter of around 50 µm, a sort of skin invagination were nanocarriers can accumulate. Images were taken in collaboration with Dr. Schaudinn, Electron Microscopy Unit, Robert Koch Institute.

Similarly, Prow et al. showed that silver nanoparticles (10-60 nm) can accumulate in skin furrows and that, in tape stripped skin, they persisted there for 10 days (Prow, Grice et al. 2011). Such a distribution behavior has been exploited to create high local drug concentrations or nanocarrier depots for sustained drug delivery to the skin as well as to the perifollicular tissue. The pilosebaceous unit is a complex structure containing several regions of interest. Beside the bulb and hair papilla, which are important target areas for hair growth

therapies, the infundibulum and the sebaceous gland are the targets of topical and oral acne vulgaris treatments with estrogens, antiandrogens, and antimicrobial agents. Thus, nanocarriers accumulating in the infundibulum and releasing drugs to the surrounding environment and the sebaceous gland are promising tools for innovative treatments of acne and hair disorders.

In the study presented in chapter 2.2, biodegradable PLA particles loaded with different fluorescent dyes were investigated. The study showed that PLA particles are suitable carrier systems for the targeting of hydrophobic drugs to hair follicles and the sebaceous glands. The use of nanocarriers improved the penetration of the dye and achieved a sustained delivery to the target skin region for up to 24 hours. The fact that PLA particles are biodegradable and that large amounts of particles with size above 200 nm were never found to cross the intact skin barrier makes these nanocarriers safe and promising candidates for the local treatment of acne. Interestingly, PLA particles can incorporate hydrophobic molecules and make them “soluble” in aqueous vehicles such as hydrogels, which have higher patient compliance than unpleasant greasy formulations.

3.3 Nanocarrier-Based Transcutaneous Delivery Favors the Targeting of Antigens to Dermal Dendritic Cells

Many studies have shown that monocytes, macrophages, and DCs can take-up nanoparticles (Duan and Li 2013). After mild skin barrier disruption and opening of the hair follicles orifices using CSSS, Vogt el al. have shown that 40 nm particles could penetrate deep in the hair follicle canals, translocated to the viable epidermis, and were taken-up by LCs (Vogt, Combadiere et al. 2006). In addition, as shown in section 2.1, LCs could selectively take-up 200 nm silica particles, whereas freshly isolated keratinocytes internalized only particles of smaller size (Rancan, Gao et al. 2012). These findings suggested the possibility to use nanoparticles in the size range around 200 nm to target vaccine components to skin APCs. Skin pre-treatment with CSSS had several functions. On the one side, it resulted in partial removal of the superficial SC along with the content of the upper part of the hair follicle canals, thereby favoring the penetration of nanocarriers in SC and hair follicle infundibulum. On the other side, CSSS caused the release of inflammatory mediators involved in the activation of APCs and the initiation of the immune response. Especially, the released IL-1α was show to activate LCs (Vogt, Hadam et al. 2015) and induce clustering of DCs with effector T-cells (Natsuaki, Egawa et al. 2014).

The results of the study in section 2.3 confirmed our hypothesis that CSSS in combination with antigen delivery *via* nanoparticles is a successful strategy to target peptides to skin APCs. The HIV-1 p24 antigen could be delivered not only in the skin but also to Langerhans and dermal DCs as revealed by ELISA and intracellular immunostaining of cells isolated from treated skin. In addition, DCs from samples treated with CSSS and antigen-loaded nanocarriers showed enhanced expression of activation markers like HLA-DR, CD80, and CD86 as compared with cells treated with CSSS only. These results encourage further investigations exploring the use of nanocarriers for the simultaneous delivery of both antigen and new classes of adjuvants such as TLR ligands (Climent, Pavot et al. 2014). All together, these data suggest also the possibility to use nanocarriers as transporter for the delivery of other macromolecules like proteins and nucleic acids.

3.4 Soft Thermoresponsive Nanogels Cross Skin Barrier, Target Skin Immune Cells and Enable a Triggered Drug Release

Nanocarriers like PLA particles were effectively used to deliver model molecules like fluorochromes and peptides to desired skin regions (hair follicles and sebaceous glands) or cell populations. However, drawbacks such as instability, aggregation, and fast release of adsorbed compounds upon application on skin surface were evidenced in the ex-vivo skin studies. In follow-up investigations, glycerol-based dendropolymer networks, called nanogels, were tested as nanocarriers for dermal delivery. Nanogels are more stable than PLA particles because the different units are cross-linked to form a single macromolecule. In addition, these soft nanocarriers release the loaded cargo when temperature increase over a certain value, transition temperature, thanks to thermoresponsive units that cause a conformational change of the entire moiety. The transition temperature can be tuned by changing the type and concentration of cross-linkers. Thus, nanogels represent attractive alternative carrier systems, whose supramolecular structure and physicochemical properties promise higher stability, improved SC penetration, and controlled drug release.

By comparison of two nanogels with different transition temperatures, it could be shown by means of PeakForce Quantitative Nanomechanics that nanogels are soft and deformable but became stiff when they undergo a temperature driven conformational change. Softness correlated with higher penetration within the SC, whereas cellular uptake was not influenced by this parameter (Rancan, Asadian-Birjand et al. 2016). Thus, in order to improve the penetration of nanogels within the SC, these nanocarriers should be soft at the temperature of skin surface (~32 °C) and change their conformation releasing the loaded cargo at higher temperatures like that of deeper skin layers (~36.5 °C) or inflamed tissue (~38 °C). Alternatively, nanogels with transition temperature above 32 °C can be applied topically while the release of the loaded drug is triggered by means of an external thermal source.

Another important finding was nanogel ability to hydrate the SC by delivering water after topical application, thereby favoring the transport of loaded molecules across the skin barrier (Giulbudagian, Rancan et al. 2016). The induced swelling of the SC suggest the possibility to use nanogels as penetration enhancers to make skin more permeable to bulky moieties such as proteins or nucleic acids. Remarkable improvements of dermal delivery have been achieved by coupling drugs to nanocarriers or by using penetration enhancers. While in the past, the transdermal delivery of biopharmaceuticals was considered as inapplicable, several studies, among them those presented in this thesis, have shown that nanocarriers are attractive and feasible means to deliver drugs in both local as well as systemic treatments.

The results of these studies have also shown that, especially when skin barrier is damaged, nanogels can get in contact with skin viable layers. Uptake by epidermal as well by dermal cells was observed for different types of nanogels. The specific analysis of different cell populations and their interaction with penetrated nanogels revealed that, while in the epidermis nanogels were predominantly taken-up by keratinocytes and not by LCs ($CD1a^+$ cells), in the dermis also DCs ($CD206^+$ and $HLA\text{-}DR^+$ cells) had taken-up the penetrated nanocarriers. These results suggest that these nanocarriers could be developed for the specific targeting of drugs to DCs, which are involved in skin inflammatory and autoimmune diseases.

3.5 Perspectives and Challenges for Nanocarriers-Based Dermal and Transdermal Drug Delivery

Several skin diseases can be treated only symptomatically. The therapies are often not satisfactory when considering that these diseases are not life threatening but can be managed only with drugs displaying moderate to severe side effects, which have to be applied during prolonged time or even the entire life. On this regard, biopharmaceuticals that can modulate cell functions or cell signaling inhibitors designed to block specific arms of the immune system represent important steps towards more selective therapies. In the following years, thanks to the growing knowledge on the molecular processes behind cancer, autoimmune and inflammatory diseases, new drugs with high therapeutic potential are going to be developed. Nevertheless, in order to reduce side effects as well as the costs of the therapy, these new drugs need to be delivered efficiently and specifically to the target body region. Thus, delivery systems designed to tailor the biodistribution of a drug represent essential tools for the development of next generation therapies. The amounts of drug delivered by topical application will never be comparable to that delivered by intravenous injection. Expensive drugs are therefore administered preferentially by intravenous, oral, or subcutaneous route. Nevertheless, the choice of dermal or transdermal delivery can be of advantage in a number of cases: (i) when high local concentrations in the skin can be reached together with a reduction of systemic side effects, (ii) when drugs have severe adverse effects towards blood cells or low stability after injection, (iii) when the drug is very sensitive to first pass metabolism, and (iv) when sustained drug delivery to skin adjacent tissue is desired (Cleland, Daugherty et al. 2001). Combination approaches using systemic induction treatment followed by maintenance local treatments could be of benefit for moderate to severe diseases, especially for elderly or multi-morbid patients, who would greatly benefit from localized treatments.
In the following sections, representative examples of nanocarrier-based strategies currently investigated to improve dermal and transdermal drug delivery are presented.

Inflammatory Skin Diseases
Several studies have shown that nanocarriers can effectively reduce the rapid diffusion of small hydrophilic drugs like glucocorticoids to the dermis (and from there to the systemic compartment) favouring a prolonged release and local retention of the drug in the inflamed skin layers (Hussain, Katas et al. 2013) (Jaques, Rezer et al. 2012). On the other side, nanocarriers enhancing drug solubility hold the potential to improve the topical administration of hydrophobic high molecular weight drugs, like the calcineurin inhibitors, not only by favouring drug permeation across the skin barrier but also by protecting them from enzymatic degradation and targeting them to specific immune active cells. Using a human skin organ-culture model, the penetration and biological effects of PCL nanoparticle-mediated delivery of cyclosporin A were investigated. It was shown that the particles induced no toxic effects and significantly reduced the release of inflammatory mediators (Frušić-Zlotkin, Soroka et al. 2012). Similarly, micelles of the biocompatible methoxypoly(ethylene glycol)-dihexyl substituted polylactide (MPEG-dihex-PLA) diblock copolymer were used to deliver tacrolimus after topical application showing their superiority with respect to a commercial preparation (Protopic®) (Lapteva, Mondon et al. 2014).
Biologicals like antibodies against inflammatory mediators (TNFα, IL-23, IL-17 and its receptor) are currently administered subcutaneously in order to avoid degradation in the gastrointestinal tract. The delivery of such biologics by means of nanocarriers can improve their stability but also help reducing possible hypersensitivity reactions. Even if the topical

delivery of such macromolecules is challenging, their penetration can be accomplished by means of physical and chemical skin permeabilizing methods. For example, laser-assisted topical administration of the TNF-α inhibitor etanercept (Enbrel®) is currently being investigated in the clinical trial on patients with mild to moderate plaque-type psoriasis (https://clinicaltrials.gov/ct2/show /NCT02999776). Another interesting application is the topical administration of filaggrin as a strategy to restore skin barrier function in atopic dermatitis. The protein was conjugated to a CPP in order to increase skin penetration and cellular uptake. Once applied on filaggrin-deficient mice, the recombinant protein was reprocessed with consequent restauration of the normal skin phenotype (Stout, McFarland et al. 2014). Because of their ability to protect the encapsulated cargo, nanocarriers have also the potential to optimize the topical delivery of nucleic acids such as siRNA for the regulation of cytokines' expression and control of inflammatory skin diseases (Bak and Mikkelsen 2010).

Skin Cancer

Nanocarriers has been extensively investigated for the imaging and treatment of melanoma. PLA nanospheres with and without PEG coating were used to deliver an antitumor drug, camptothecin, to mice that had been inoculated with B16-F10 melanoma cells and had metastatic spreads in the lungs (Loch-Neckel, Nemen et al. 2007). Core-shell silica nanoparticles loaded with imaging agents and coated with methoxy-terminated PEG chains were designed to visualize a M21 melanoma in a xenograft mouse model (Benezra, Penate-Medina et al. 2011). Advanced multifunctional nanocarriers have been also developed aiming at improving the selectivity of drug delivery to melanoma cells using targeting ligands. Liposomes functionalized with anisamide, a ligand that is overexpressed in many cancer cells, could be successfully targeted to B16F10 murine melanoma cells (Chen, Bathula et al. 2010).

Actinic keratosis are skin lesions that can evolve to non-melanoma skin cancer and, therefore, they are normally operatively or pharmaceutically removed in order to eliminate cells with high potential to become cancerous. It was shown that hyaluronic acid favors the localization of diclofenac in the epidermis limiting its percutaneous diffusion and, thus, increasing its activity (Brown, Ingham et al. 1995). A 3% diclofenac in 2.5% hyaluronic acid gel, Solaraze® is now available for the topical treatment of actinic keratosis (Brown and Jones 2005). The mechanism for the enhanced penetration and retention in the skin is not well understood. However, nanocarriers based on hyaluronic acid hold the potential to improve the topical treatment of diseases that demand a sustained drug concentration in the epidermis.

Photodynamic therapy (PDT) is currently used in dermatology for the treatment of actinic keratosis and acne. PDT is based on chemical compounds (photosensitizers), which can produce single oxygen and other radical species upon irradiation with light. The penetration of the photosensitizer in the skin layer to be treated is essential for the outcome of the treatment. Small hydrophilic or highly hydrophobic photosensitizers can take advantage of the transport capacity of nanocarriers. For example, invasomes containing different penetration enhancers like ethanol, cineole, citral, and d-limonene, were shown to be useful for the delivery of temoporfin to both epidermis and dermis (Dragicevic-Curic, Scheglmann et al. 2009).

Delivery of Nucleic Acids

The genetic basis of several skin conditions is increasingly being elucidated and it is to expect that the number of DNA and RNA-based therapeutic options will grow in the

upcoming years. Delivery of DNA and siRNA to skin is however a challenge. Nucleic acids have to cross not only the skin barrier but also the plasma membrane and, in the case of genes, even the nuclear membrane. Furthermore, nucleic acids need to be protected from nucleases present in the skin. The use of nanocarriers in combination with methods enhancing skin permeability represents a promising strategy. Chitosan nanoparticles conjugated to PGA were shown to enhance skin penetration and delivery of DNA to skin (Liu, Jiao et al. 2008). Cationized gelatine microspheres could deliver siRNA to hair follicles in a mice model of alopecia areata (Nakamura, Jo et al. 2008). Liposomes loaded with siRNA have been used in combination with low-frequency ultrasound to increase skin permeability and treat early or invasive cutaneous melanoma (Tran, Gowda et al. 2008). Depots of DNA or siRNA in the dermis may allow for a prolonged and sustained delivery of the therapeutic nucleic acid. Tristearin solid lipid nanoparticles were used to deliver siRNA to skin. After intradermal injection in mice footpads, prolonged siRNA release over a period of 10–13 days could be measured (Lobovkina, Jacobson et al. 2011). The delivery of siRNA in wounds of type-2 diabetic mice by means of spherical gold-nanoparticles conjugated to nucleic acids has also been reported (Randeria, Seeger et al. 2015).

Wound Disinfection and Healing

Nanocarriers have been largely investigated in the field of wound healing and wound microbial infections. In chronic wounds, re-epithelialization is impaired due to the chronic inflammatory status and poor blood perfusion. The administration of growth factors by means of carrier systems can promote or accelerate wound healing. Nanofibers were used as scaffold for the growing of new tissue and delivery of growth hormones (Xie, Paras et al. 2013). Fusion proteins between elastine and growth factors that self-assembled to form nanoparticles were also fabricated (Koria, Yagi et al. 2011). Foam formulations or wound dressings containing silver nanoparticles are one of the few examples of nanoparticles already available for clinical use. Despite of their toxicity and their not fully understood mechanism of action, different types of silver nanoparticles are still under investigation (Chernousova and Epple 2013).

NO-releasing matrices and nanocarriers represent an attractive alternative to silver, thanks to their antimicrobial and vasodilatation properties (Eroy-Reveles and Mascharak 2009, Weller 2009, Lu, Slomberg et al. 2014, Quinn, Whittaker et al. 2015). The design of nanocarriers with defined release kinetics is of critical importance to develop an effective and safe wound healing strategy (Carpenter and Schoenfisch 2012). Nanocarriers made of tetramethyl-ortho-silicate, chitosan, and polyethylene glycol able to release NO over a prolonged time were shown to effectively treat wound infection and favor wound healing (Martinez, Han et al. 2009). NO-releasing poly(propylene imine) dendrimers were shown to have low toxicity towards fibroblasts by maintaining antibacterial properties (Sun, Slomberg et al. 2012). Size and surface hydrophobicity seem to have an impact on antimicrobial activity especially against biofilm building strains as shown for NO-releasing PAMAM (Lu, Slomberg et al. 2013).

Transcutaneous Vaccination

There are several rationales for the investigation of transcutaneous antigen delivery as vaccination strategy. The amount of antigen needed to elicit an immune response upon transcutaneous vaccination was shown to be about 5 times lower than that needed for intramuscular vaccination (Kenney, Frech et al. 2004). Transcutaneous antigen delivery has been shown to induce potent cellular immune responses (Hammond, Walwender et al. 2001, Vogt, Mahé et al. 2008). Depending on the targeted skin layer, different arms of the immune

response could be activated (Liard, Munier et al. 2011). Skin and mucosal vaccination protocols result in the production of tissue immunoglobulin A (IgA) that is particularly important for the local prevention of bacterial and viral infections. On the other side, it has been extensively demonstrated that nanocarriers can be used to deliver antigens to skin APCs via the transfollicular route (Vogt, Combadiere et al. 2006) (Mittal, Raber et al. 2013). In particular, upon mild skin barrier disruption activation of skin immune system occurs as well as nanoparticles translocation to viable skin where they can be taken-up by perifollicular dendritic cells (Vogt, Combadiere et al. 2006). In line with these findings, PLA particles loaded with the HIV-1 p24 peptide enabled the delivery of the antigen to activated LCs after topical application on barrier-disrupted excised human skin (Rancan, Amselgruber et al. 2014). One of the issues of transcutaneous nanoparticle-mediated antigen delivery is the stability of the carrier system. In fact, delivering antigens by means of nanocarriers allows to transport and protect the antigen but also to generate adjuvant-like effects. Ideally, to act as adjuvant and deliver a sufficient amount of antigen to the immune cells, antigen-loaded nanocarriers should translocate across the skin barrier and be taken-up by APCs as intact particles. Thus, stability and degradation kinetics play an important role with regard to both antigen delivery and biocompatibility. Interestingly, a superior immune response was elicited when ovalbumin-loaded particles made of trimethyl chitosan and hyaluronic acid were stabilized by cross-linking (Verheul, Slütter et al. 2011). Stable calcium phosphate nanoparticles coated with sugar and albumin induced higher antibody titers than albumin-only samples when applied intradermally or topically on mice pre-treated with tape stripping (Sahdev, Podaralla et al. 2013). Biological nanocarriers such as VLPs have also been utilizes for transcutaneous vaccination strategies. A VLP-based influenza vaccine was administered *via* intradermal injection or microneedles, showing host responses including activation and migration of DCs (Pearton, Pirri et al. 2013). Because of the above-mentioned advantages, antigen delivery through the skin via the intradermal or subcutaneous routes is also pursued by most of the currently investigated nanoparticle-based antitumor vaccination strategies (Amoozgar and Goldberg 2014).

Transdermal Drug Delivery

Drugs that need only low blood concentrations to exert a therapeutic activity, such as opiates and hormones, are already delivered transdermally by means of patches. Nanocarriers could improve the transdermal delivery of drugs such as non-steroidal anti-inflammatory drugs, insulin, and calcitonin. Thus, such delivery agents can provide the platform for a sustained release of drugs and biopharmaceuticals like proteins, antibodies, and nucleic acids. Starch nanoparticles modified in order to make the polymer more hydrophobic, improved the transdermal delivery of flufenamic acid (Santander-Ortega, Stauner et al. 2010). Using male Wistar rats, it was demonstrated that PCL-based nanoparticles increased the anti-inflammatory activity of nimesulide in the models of chronic arthritis and granuloma formation (Lenz, Guterres et al. 2012). The superiority of nanocarrier formulations could also be verified in a clinical study comparing the efficacy of hydrogels with estradiol-loaded particles to estradiol gels, (Nicklas, Schatton et al. 2009). Recently, transdermal lymphatic delivery of doxorubicin was conceived to target tumor metastases in lymph nodes (Kong, Hou et al. 2015). Similarly, the transdermal lymphatic pathway was used to deliver proteins like insulin by means of topically applied lipid-based biphasic vesicles (Biphasix) (King, Michel et al. 2003).

3.6 Conclusions

A number of reviews have been published on nanoparticle-based carrier systems and their applications in dermatology (Papakostas, Rancan et al. 2011, Prow, Grice et al. 2011, DeLouise 2012, Gupta, Agrawal et al. 2012, Badri, Eddabra et al. 2014, Rancan, Blume-Peytavi et al. 2014, Foldvari and Rafiee 2015, Vogt, Wischke et al. 2016). Results from several dermatological and pharmacological research fields show that nanocarriers have the potential to improve the outcome of many existing treatments and may be helpful for the development of new treatment strategies. The number of nanoscaled delivery systems is continuously growing, whereby properties like loading capacity, targeting ability, deformability, and stimuli responsiveness are being continuously improved. Especially interesting is the potential of nanocarriers to deliver bioactive molecules to phagocyting cells like neutrophils, macrophages and DCs. This characteristic could be used not only to deliver antigen and adjuvants for vaccination purposes but also to target drugs to these cells in order to modulate or interfere with their activity. DCs are the bridge between innate and adaptive immunity and a selective control of their interaction with different T and B cell subsets is supposed to modulate allergic, immune, autoimmune as well as inflammatory reactions (Ganguly, Haak et al. 2013, Mekori, Hershko et al. 2016).

In order to realize and even accelerate the clinical translation of these new therapeutic options, an important challenge for nanotechnology is the improvement of nanocarrier skin penetrating and targeting properties. In addition, nanocarrier biodegradability and biocompatibility are mandatory. Systematic investigations directed at developing biodegradable or biocompatible nanocarriers will help to develop safe drug delivery systems for dermatological treatments (Rivera-Gil, Jimenez De Aberasturi et al. 2012). Importantly, a deeper understanding of nanocarrier effects on both the physical and the immunological skin barriers, along with further insides on nanocarrier possible mechanisms of toxicity are necessary. For example, the effects of nanocarriers on LCs ability to internalize and present antigens has not been investigated yet (DeLouise 2012). Similarly, nanocarriers' influence on mast cell functions is still an open question. In addition, systematic studies on the potential cancerogenicity of these new delivery agents in healthy as well as diseased skin are necessary. Finally, along with the further improvement of nanocarriers' drug delivery efficiency, nanodermatology would strongly profit from increased knowledge on the parameters and mechanisms influencing nanocarriers' penetration, uptake by skin cells, as well as re-distribution and degradation, especially taking into consideration differences between healthy and diseased skin. A bright spectrum of potential interactions exists between skin and the different types of nanocarriers leading to different drug release kinetics, nanocarrier uptake, biodegradation, clearance, and toxicity. Only systematic studies investigating how nanocarrier characteristics influence their mode of interaction with skin will allow exploiting the potential of nanotechnology applied to dermatology.

4. Summary

Skin is, from the pharmaceutical and therapeutic point of view, a very accessible organ. However, delivery of drugs to specific target regions of the skin and maintenance of a constant drug concentration is still a challenge for current topical treatments. Newly developed dermatological therapies are based on expensive and, in certain cases unstable molecules, that need an efficient delivery strategy especially when topical delivery is pursued. Approaches based on delivery systems may help overcoming such challenges. Nanocarriers are nanoscaled vehicles that can transport defined amounts of drugs. Depending on the type of nanocarrier, different drug release profiles can be achieved as well as different interactions with body barriers and cell populations. These characteristics make them attractive for several type of therapies.

Skin represents a very tight barrier to the penetration of exogenous material. In fact, skin barrier is not only a physical hurdle made of several lipid layers, protein-rich corneocytes, and tight junctions, but it is also an immunological barrier made by a dense network of immune cells, e.g. dendritic cells, macrophages, and mast cells. It has been shown that these barriers mostly block nanocarriers. Nevertheless, hair follicles represent special skin structures, where nanoparticles can accumulate, build depots for drug delivery and, in small amount, even translocate to the viable skin and interact with cells of the perifollicular epithelia. In addition, different more or less invasive methods (e.g., mild barrier disruption, microneedles, or intradermal injection) can be chosen in order to target drugs to the desired skin layer. Importantly, depending on the skin layer, the nanocarrier will come in contact with different skin tissue components and cells resulting in different drug release mechanisms. Thus, the proper nanocarrier has to be chosen considering its physicochemical characteristics, the type of interaction with skin, and the purpose of the therapy. For example, nanocarriers that are supposed to overcome skin barrier and reach the systemic compartment need to be biodegradable or at least biocompatible and small enough to be excreted.

In the last years, the number of nanocarrier types and variations thereof has tremendously increased. Several of studies investigated the penetration of different nanocarriers and released cargos upon topical administration on skin and many applications have been proposed for the use of nanocarriers in dermatology as well as for transdermal drug delivery. Nevertheless, systematic studies directed to correlate nanocarrier physicochemical characteristics to their skin penetration, drug delivery properties as well as biocompatibility in skin are still missing. Such investigations are needed to fully exploit the advantages of nanocarrier-based drug delivery, foster the further development of carrier systems with improved characteristics and specificities for dermal and transdermal therapies.

In the studies presented in this dissertation nanocarrier physicochemical characteristics were modulated in order to test the effects of size, surface functionalization, type of cargo, softness, and thermoresponsivity on skin and hair follicle penetration as well as nanocarrier interactions with different cell populations. One of the main goals was to enhance the selectivity of the therapy by targeting cargos to specific skin areas or immune active cells. The collected data show that it is possible to adjust nanocarrier characteristics to obtain specific targeting properties. Nanocarrier size could be tuned in order to preferentially target skin resident DCs. Nanocarriers localizing preferentially in the hair follicle canals could be used to deliver the HIV-1 p24 peptide to skin DCs and fluorochromes (mimicking drugs with

different hydrophilicity) to the sebaceous gland and the perifollicular tissue. Soft thermoresponsive nanogels could easily penetrate the SC, enhance skin hydration and permeability to released cargos in a temperature dependent manner, and interact with dermal DCs in skin viable layers. The herein presented biocompatible nanocarriers represent an attractive approach to improve the selectivity of dermatological and transcutaneous therapies. Interestingly, all investigated nanocarriers were found to be associated with significant percentages of antigen presenting cells depending on the degree of skin barrier disruption. DCs are key cell populations involved in adaptive immune responses as well as in inflammatory, allergic or autoimmune processes. Thus, a nanocarrier-based approach that targets drugs of these cell populations and modulate their activity could improve the therapy of several skin conditions.

Zusammenfassung

Aus pharmazeutischer und therapeutischer Sicht ist die Haut als Zielorgan sehr gut geeignet. Dennoch ist das Einbringen von Wirkstoffen und die Erhaltung einer konstanten Wirkstoffkonzentration in den angezielten Regionen der Haut gegenwärtig eine Herausforderung. Neu entwickelte dermatologische Therapien basieren auf teuren und teilweise unstabilen Verbindungen, die einer effizienten Einbringung bedürfen - insbesondere wenn eine topische Behandlung angestrebt wird. Trägersystem-basierte Ansätze könnten solche Herausforderungen überwinden. Nanocarrier sind nanoskalige Vehikel, die definierte Mengen an Wirkstoffen transportieren. In Abhängigkeit vom Typ der Nanocarrier, können unterschiedliche Wirkstofffreisetzungsprofile, sowie Wechselwirkungen mit der Körperbarriere und den Zellpopulationen erreicht werden. Diese Eigenschaften machen Nanocarrier attraktiv für viele verschiedene Therapiearten.

Die Haut stellt eine äußerst dichte Barriere gegen das Eindringen exogenen Materials dar. In der Tat ist die Hautbarriere nicht nur ein physisches Hindernis, das aus mehreren Lipidschichten, proteinhaltigen Korneozyten, und Tight-Junctions im Stratum Granulosum besteht, sondern auch eine immunologische Barriere, die aus einem dichten Netzwerk von Immunzellen wie dendritischen Zellen, Makrophagen und Mastzellen gebildet wird. Es hat sich gezeigt, dass Nanocarrier von diesen Barrieren größtenteils abgewehrt werden. Dennoch stellen Haarfollikel besondere Strukturen der Haut dar, in denen Nanopartikeln akkumuliert und Depots für die Wirkstofffreisetzung gebildet werden. Geringe Mengen an Nanocarrier können sich sogar in die lebenden Hautschichten verlagern, wo sie mit Zellen des perifollikuläre Gewebes interagieren. Darüber hinaus können unterschiedliche mehr oder weniger invasive Methoden (z.B. milde Schädigung der Hautbarriere, Mikronadeln oder intradermaler Injektion) gewählt werden, um Wirkstoffe in die gewünschte Hautschicht gezielt einzubringen. Bemerkenswert ist auch, dass -in Abhängigkeit zur Umgebung, in die die Nanocarrier eingebracht werden- unterschiedliche Wechselwirkungen mit Gewebebestandteile und Zellen stattfinden können, so dass sich unterschiedliche Mechanismen der Wirkstofffreisetzung daraus ergeben. Daher sollte man nur nach Abwägung der physikalisch-chemischen Eigenschaften, der Interaktion mit der Haut und dem Therapieziel, den geeigneten Nanocarrier auswählen. So sollten zum Beispiel Nanocarrier, die entwickelt wurden, um die Hautbarriere zu überwinden und das systemische Kompartiment zu erreichen, bioabbaubar oder zumindest biokompatibel sein.

In den letzten Jahren haben die Anzahl der Nanocarrier-Typen und deren Variationen enorm zugenommen. Eine Reihe von Studien untersuchte das Schicksal verschiedener Nanocarrier und freigesetzter Cargos hinsichtlich der topischen Verabreichung auf der Haut. Dabei wurden viele Anwendungen für Nanocarrier in der Dermatologie, sowie für die transdermale Verabreichung vorgeschlagen. Nichtsdestotrotz fehlen noch systematische Untersuchungen, die darauf abzielen, die physikalisch-chemischen Eigenschaften der Nanocarrier mit ihrer Hautpenetration, ihren Wirkstoffabgabeeigenschaften sowie ihrer Biokompatibilität im Hautgewebe zu korrelieren. Solche Untersuchungen sind erforderlich, um die Vorteile der nanocarrier-basierten Wirkstoffabgabe voll auszuschöpfen, die Weiterentwicklung von Trägersystemen mit verbesserten Eigenschaften zu fördern, und Nanocarrier spezifisch für dermale und transdermale Therapien entwickeln zu können.

In den Arbeiten, die in dieser Dissertation vorgestellt sind, wurden die physikalisch-chemischen Eigenschaften der Nanocarrier variiert, um die Auswirkungen von Parametern wie Größe, Oberflächen-Funktionalisierung und -Ladung, sowie Verformbarkeit und Temperatur-Ansprechvermögen, auf Haut- und Haarfollikelpenetration sowie zelluläre Aufnahme zu testen. Der Hauptzweck war eine Erhöhung der Selektivität der Therapie durch die gezielte Abgabe von Cargos in spezifische Hautbereiche oder immunaktive Zellen. Die erzeugten Ergebnisse zeigen, dass es möglich ist, die Eigenschaften des Nanocarriers zu bestimmen, um spezifische Targeting Effekte zu erhalten. Die Größe des Nanocarriers konnte so bestimmt werden, um vorzugsweise hautresidente dendritische Zellen zu erreichen. Nanocarrier, die sich vorzugsweise in den Haarfollikelkanälen lokalisierten, konnten verwendet werden, um das HIV-1 p24-Peptid zu dendritischen Zellen, sowie Farbstoffe, die Arzneimittel mit unterschiedlicher Hydrophilie simulierten, selektiv in Talgdrüsen und perifollikuläres Gewebe zu liefern. Verformbare und thermoempfindliche Nanogele konnten leicht ins SC penetrieren, in Abhängigkeit zur Temperatur die Haut-Hydratation und -Durchlässigkeit erhöhen, und in den lebenden Hautschichten mit dermalen dendritichen Zellen wechselwirken.

Die in dieser Arbeit beschriebenen biokompatiblen Nanocarrier stellen einen attraktiven Ansatz zur Verbesserung der Selektivität dermatologischer und transkutaner Therapien dar. Interessanterweise, wurden alle untersuchten Nanocarrier - in Abhängigkeit von dem Grad der Hautbarrierestörung - von einen signifikanten Prozentsatz von Antigen-präsentierenden Zellen aufgenommen. Dendritische Zellen sind in adaptive Immunantworten, sowie entzündliche, allergische oder autoimmun Prozesse involviert. Das selektive Targeting dieser Zellen durch Nanocarriers und die Modulation deren Aktivität, könnten die Therapie vieler immunologischer Hauterkrankungen erheblich verbessern.

5. Literature

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Erklärung

§ 4 Abs. 3 (k) der HabOMed der Charité

Hiermit erkläre ich, dass

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Ich erkläre ferner, dass mir die Satzung der Charité – Universitätsmedizin Berlin zur Sicherung Guter Wissenschaftlicher Praxis bekannt ist und ich mich zur Einhaltung dieser Satzung verpflichte.

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