Dermal drug delivery with drug nanocrystals: hair follicle targeting, passive penetration and the novel method for their determination

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Dermal drug delivery with drug nanocrystals: hair follicle targeting, passive penetration and the novel method for their determination

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has been written entirely by myself and is a record of work performed by myself. The research was carried out at the Philipps-Universität Marburg, institute of pharmaceutical technology and biopharmacy at the campus Robert-Koch-Straße, 4 under the supervision of Prof. Dr. Cornelia M. Keck. All citations that have been taken over completely or partly are marked as such.

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Die vorliegende Arbeit entstand auf Anregung sowie unter der Leitung von

Prof. Dr. Cornelia M. Keck

am Institut für Pharmazeutische Technologie und Biopharmazie

der Philipps-Universität Marburg

Für meine Mama und meinen Papa

Für meine Coronafamilie

„Ich weiß nicht, ob es recht war“, antwortete Jonathan.
„Aber es gibt Dinge, die man tun muss,
sonst ist man kein Mensch, sondern nur ein Häuflein Dreck.“

Astrid Lindgren „Brüder Löwenherz“

Abstract

In this thesis, drug nanocrystals (NC) as a formulation approach for poorly soluble active compounds (AC) for the follicular and passive dermal penetration were systematically investigated. This thesis aimed to establish the optimum method for the determination of the follicular and passive dermal penetration efficacy of NC formulations and using this method to investigate the influences of essential formulation aspects on the penetration efficacy of NC formulations into the hair follicles and into the skin.

The previously established "universal" method for characterization of the follicular and the passive dermal penetration efficacy of dermal formulations - differential skin stripping - was found to be insufficient to characterize the follicular and dermal uptake of dermal NC formulations.

Thus, a novel "two-in-one" method for sound characterization of follicular and passive dermal penetration of NC formulations was established. The novel method is based on the visualization of the penetration of the AC into the hair follicles and into the skin using fluorescence microscopy. The quantification of the follicular penetration is carried out by measuring the penetration depth of NC in µm directly from fluorescence images, whereas for quantification of the passive dermal penetration, a method for digital image analysis with the software ImageJ was established in this thesis. The novel method represents the unique "two-in-one" technique allowing for investigation of the follicular and the passive dermal penetration of one formulation using only one method, one skin penetration model and within the skin penetration model only one skin area. The novel method provides a detailed time and space resolved determination of the penetration fate of a dermal formulation. With this, it represents a sound, reliable and universal tool for smart characterization of the follicular and passive dermal penetration of dermal formulations that overcomes all substantial disadvantages of conventional methods and provides optimized solutions. Thus, from now on, a new chapter in penetration experiments with dermal formulations begins.

Using the novel method, the influences of essential formulation aspects on the follicular and passive dermal penetration of NC formulations were detailed and mechanistically investigated. Based on the outcome of these investigations, from now on, by tailored optimization of formulation components, tailor-made NC formulations for effective hair follicle targeting, effective passive dermal penetration and targeted dermal drug delivery can be "designed". This approach – tailored optimization of NC formulations by formulation components – should now be realized in the development of highly effective dermal products with the drug NC, that would for sure enter the market in the next years.

Abstrakt

In dieser Arbeit wurden Wirkstoff-Nanokristalle (NC) als Formulierungsansatz für schwerlösliche Wirkstoffe (WS) für die follikuläre und passive dermale Penetration systematisch untersucht. Das Ziel dieser Arbeit war es, die optimale Methode zur Bestimmung der follikulären und passiven dermalen Penetrationseffizienz von NC-Formulierungen zu etablieren und mit dieser Methode die Einflüsse wesentlicher Formulierungsaspekte auf die Penetrationswirksamkeit von NC-Formulierungen in die Haarfollikel und in die Haut zu untersuchen.

Die bisher etablierte "universelle" Methode zur Charakterisierung der follikulären und der passiven dermalen Penetration von dermalen Formulierungen – die differenzielle Abrissmethode – erwies sich als unzureichend sowie ungeeignet für die Charakterisierung der follikulären und der passiven dermalen Penetration von dermalen NC-Formulierungen.

Deswegen wurde eine neuartige „Zwei-in-Eins“-Methode zur fundierten Charakterisierung der follikulären und passiven dermalen Penetration von NC-Formulierungen etabliert. Die neue Methode beruht auf Visualisierung der Wirkstoffpenetration in die Haarfollikel sowie in die Haut mit Fluoreszenzmikroskopie. Die Quantifizierung der follikulären Penetration erfolgt durch Messung der Eindringtiefe der NC in µm, während für die Quantifizierung der passiven dermalen Penetration eine Methode zur digitalen Bildanalyse mit der Software ImageJ etabliert wurde. Die neuartige Methode stellt eine einzigartige "Zwei-in-Eins"-Technik dar, die es erlaubt, die follikuläre und die passive dermale Penetration einer Formulierung mit nur einer Methode, einem Hautpenetrationsmodell und innerhalb des Hautpenetrationsmodells mit nur einem Hautareal zu untersuchen. Die neuartige Methode ermöglicht eine detaillierte zeit- und ortsaufgelöste Bestimmung des Penetrationsverhaltens einer dermalen Formulierung. Somit stellt sie ein fundiertes, zuverlässiges und universelles Tool zur gründlichen Charakterisierung der follikulären und passiven dermalen Penetration von dermalen Formulierungen dar, das alle wesentlichen Nachteile herkömmlicher Methoden überwindet. Damit wird von nun an ein neues Kapitel im Bereich der Penetrationsstudien mit dermalen Formulierungen geschrieben.

Mit der neuartigen Methode wurden die Einflüsse wesentlicher Formulierungsaspekte auf die follikuläre und passive dermale Penetration von NC-Formulierungen detailliert und mechanistisch untersucht. Basierend auf den Ergebnissen dieser Untersuchungen können von nun an, durch gezielte Optimierung der Formulierungskomponenten, maßgeschneiderte NC-Formulierungen für ein effektives Haarfollikel-Targeting, eine effektive passive dermale Penetration und eine gezielte dermale Wirkstoffabgabe konzipiert werden. Dieser Ansatz soll nun in der Entwicklung von hochwirksamen dermalen Produkten mit NC umgesetzt werden.

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Chapter 1

Introduction

Chapter 1

1.1. Introduction

1.1.1. Double challenge: local treatment of dermatologic conditions meets poorly soluble active compounds (AC)

Despite the high frequency of different skin and hair follicle diseases, they have so far not been regarded as a significant health problem. The reason for this may be the assumption that skin and hair follicle disorders represent a benign, not life-threatening minor nuisance. However, skin and follicular diseases are more common than we think. For example, data from the United Kingdom suggests that each year 54% of the population are affected by skin diseases, and 23 - 33% have a skin disease at any given time that would benefit from medical care [1]. Thus, there seems to be a high demand for more consideration to be given to skin and hair follicle diseases. The impact of dermatological disorders in society is also steadily increasing. The accessibility and visibility of skin and hair make each dermatological condition – independent of its severity – highly visible, leading to a strong influence on the patients' appearance, well-being and life quality. Therefore, there is a great need for suitable treatment options. Dermatological conditions can be treated locally and systemically. Topical application of active compounds (AC) directly to the skin builds a mainstay of the skin and hair follicle disorders treatment and provides a series of benefits compared to systemic therapy. Firstly, the dermal application allows for direct delivery of the AC to the right target – to the skin and hair follicles – in the right concentration and at the right time point. On the contrary, systemically applied AC needs to be metabolized to reach the skin and the hair follicles leading to a higher required dose and a high risk of systemic side effects. Secondly, local dermal therapy represents a very simple and clear application form for the patient leading to high therapy adherence and high compliance. Thus – if possible – local therapy should be preferred. However, at present, not every skin and hair follicle disease can be treated locally. The reason for this is a lack of suitable topical therapeutical options, because not every potent drug substance can be sufficiently applied, i.e., be taken up by the skin and hair follicles. It means, not every AC possesses sufficient dermal bioavailability. Poor dermal bioavailability is often a consequence of poor aqueous solubility. Currently, the poor aqueous solubility of AC represents one of the major challenges in dermal – but of course, not only dermal – drug delivery because this problem associates with a high number of drug substances. According to the current statistic, 40% of the AC in the development stage and about 60% of NCE's (new chemical entities) are poorly soluble [2,3]. AC with poor solubility and consequently poor dermal bioavailability cannot be delivered to the skin and hair follicles by conventional classical formulation strategies. To enable the dermal application of poorly soluble AC, special drug delivery systems and formulation strategies are required. To

understand the prerequisites for these “special formulation strategies” and to conceptualize the optimum formulation strategy for poorly soluble AC for dermal application, a deep and mechanistic understanding of the targets of dermally applied AC and of the penetration mechanisms of the AC into these targets is essential.

1.1.2. Dermal application of AC: major targets

Two main targets of dermally applied AC are the skin and the hair follicles. Typically, a dermally applied AC should be delivered into the skin, through the skin, and/or into the hair follicles. The state of the current scientific opinion considers the hair follicles not just as a “byway” of the skin but as a separate target with essential importance. The reasons for this are outlined further on. Therefore, the targets the skin and the hair follicles and the penetration into and through the skin (via the intercellular, transcellular, or corneodesmosomal pathway) and into the hair follicles (via the follicular pathway) is presented further on separately.

1.1.2.1. The skin

The skin is the largest organ of the body – on average, accounting for 10% of the body mass and covering nearly 2 m^2 of the body surface area [4]. The skin can be broadly divided into three different layers: subcutis, dermis and epidermis (fig. 1A – schematic view). The epidermis is the outermost layer of the skin and can be further subdivided into the viable epidermis and the stratum corneum (SC) (fig. 2 – sectional view). The viable epidermis comprises of, from the inside to the outside, the stratum basale (basal cell layer), followed by stratum spinosum (prickle cell layer) and stratum granulosum (granular layer) (fig. 1B). The horny layer – the stratum corneum (SC) – builds the outermost layer of the skin. According to the current scientific opinion, the SC makes the most significant contribution to the major function of the skin – namely, its protective function – and represents a “defensive wall” against external influences. This contribution is achieved by the specific morphological structure and unique physicochemical composition of the SC [5–8]. The SC consists of 2 compartments (fig. 1C) [9]. The first compartment consists of dead skin cells, the so-called corneocytes. Typically, the SC comprises of 10 - 15 layers of corneocytes and is 10 - 20 µm thick [4]. Corneocytes are nonliving cells derived from terminally differentiated keratinocytes that have originated from the deeper layers of the epidermis. Corneocytes have a cornified envelope (CE) in place of a plasma membrane. The CE contains of proteins, which are surrounded by a lipidic coat (corneocyte lipid envelope, CLE) (fig. 1C) [5,10]. They lack nuclei and cytoplasmic organelles but are filled with keratin filaments. The corneocytes are connected to each other by corneodesmosomes (fig. 1C) [11,12]. It is assumed that each corneocyte is connected to other corneocytes by approximately 400 to 600 corneodesmosomes, which explains the enormous strength and resistance of the SC [9,13–16]. The second compartment of the SC is a very specific mixture of different lipidic structures, which are deposited between the corneocytes as an

intercellular lipid bilayer [6,9,17–25]. With this, corneocytes are interspersed in a lipid-enriched extracellular matrix. This structure, consisting of corneocytes and intercellular lipid bilayer, is termed “brick-and-mortar” structure of the SC, where the corneocytes are likened to bricks and the extracellular matrix analogous to the mortar in a brick wall [26]. Due to the outlined “wall” structure, the SC provides a strong protective barrier and is highly selective to what it lets in, and what out. This barrier helps, on the one hand, to protect the body from mechanical, thermal and physical injury and hazardous substances, but, on the other hand, it limits the dermal penetration of topically applied AC. With this, dermal drug delivery represents a highly challenging issue. Since the dermal application of AC aims to deliver the AC into the skin or through the skin – because the target site of most dermally applied AC is localized in viable epidermal and dermal layers – the AC needs to overcome the “defensive wall” of the SC to reach its target site. The understanding of the structure of the SC in combination with the mechanisms of the dermal penetration allows to “crack” this wall.

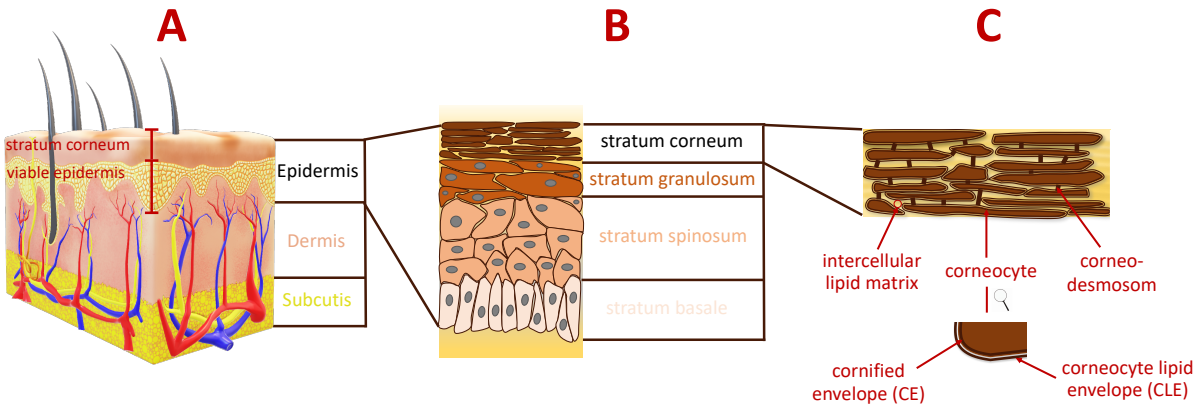


Fig. 1: Skin structure: schematic view. A: Skin. B: Epidermis. C: Stratum corneum (SC), modified after [9].

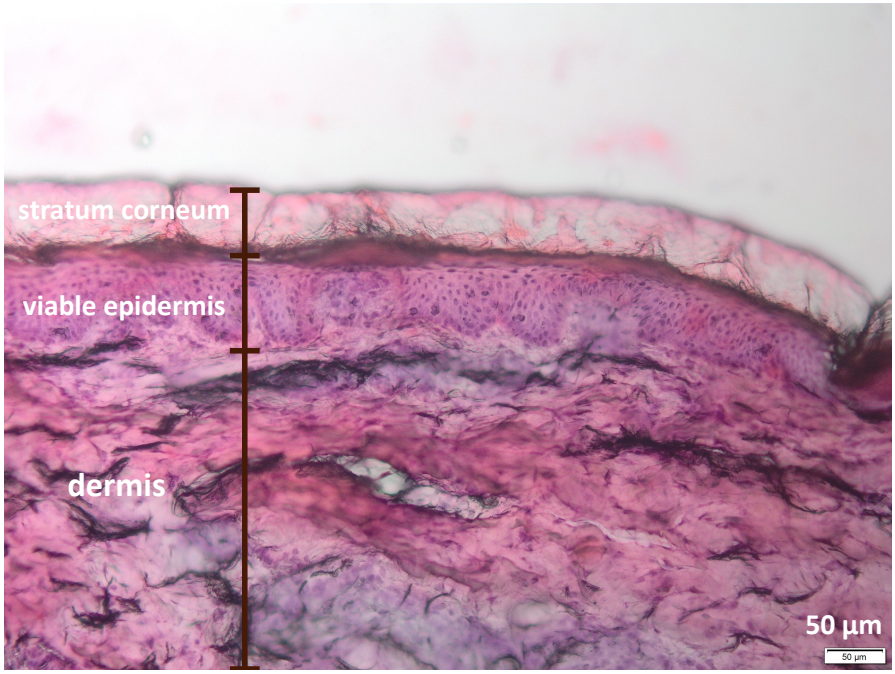


Fig. 2: Skin structure: sectional view (vertical cross-section of the porcine ears skin, stained with haematoxylin/eosin).

1.1.2.2. Passive dermal penetration of AC into or through the skin – mechanisms & challenges of poorly soluble AC

The dermally applied AC can penetrate into or through the SC either via the intercellular, or the transcellular pathway or via the corneodesmosomes (fig. 3) [26].

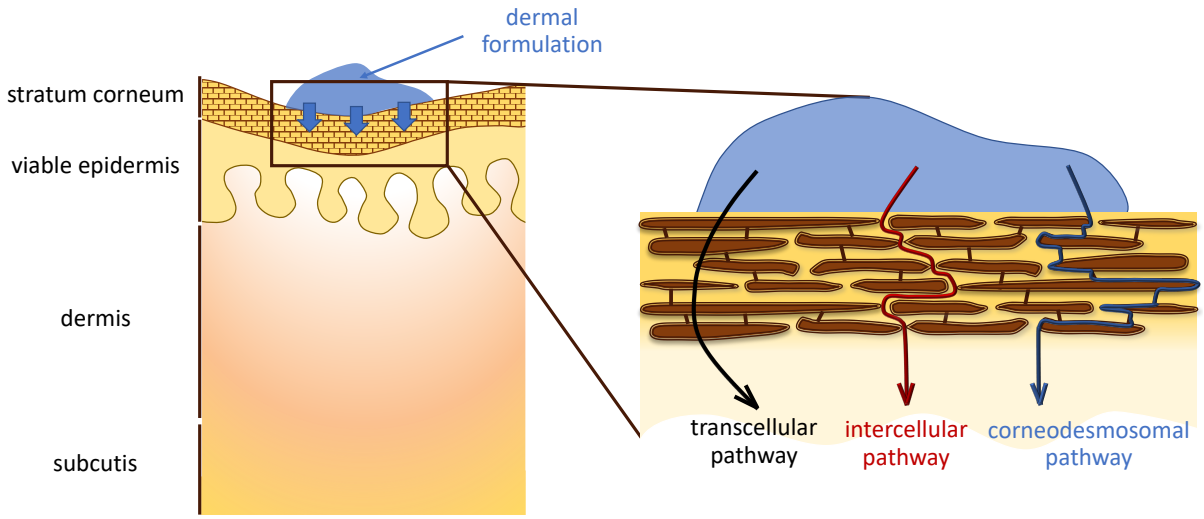


Fig. 3: Penetration pathways of dermally applied AC into or through the SC.

The transcellular pathway – the way through the corneocytes – requires that the AC penetrates the alternating layers of cells and extracellular matrix. This involves a sequence of partitioning and diffusion into alternating hydrophilic and lipophilic domains. With this, the value of this pathway is controversially discussed in the current literature [26]. The way through the corneodesmosomal structures, which molecularly connect individual corneocytes to each other, is often termed in the literature the hydrophilic route because this way is assumed to be the predominant pathway for hydrophilic AC [26]. The corneodesmosomal pathway is also controversially discussed in the literature, and most researchers assume that only small hydrophilic molecules can enter this pathway [26]. The intercellular pathway via the lipid layers surrounding the corneocytes is often termed the lipophilic route and represents, according to the current scientific opinion, the dominant pathway for dermally applied AC to penetrate into or through the SC [26–28]. The main prerequisite for the AC to enter all three outlined possible pathways is that the AC is dissolved. The penetration obeys the physical principle of the passive diffusion – in this thesis, termed passive dermal penetration – and can be described by Fick's first law of diffusion (formula 1):

$$\frac{dQ}{dt} = \frac{D \cdot V_k \cdot A}{d} \cdot c_v$$

Formula 1: Fick's 1st law of diffusion.

In this formula, *dQ/dt* is the amount of AC penetrating out of the formulation – the so-called flux – *D* is the diffusion coefficient in the SC, V_k is the distribution coefficient between the SC and the formulation, *A* is the area of skin on which the formulation is applied, *d* is the thickness of the SC and c_v is the concentration of dissolved AC in the formulation, respectively. Based on this law, $c_{v,}$ which is the amount of dissolved AC in the formulation, is the key parameter to achieve sufficient flux values. Thus, representing the driving force for passive diffusion. It means, the higher is the amount of dissolved AC in the formulation, the higher is the concentration gradient between the dermal formulation and the skin. The higher is the concentration gradient, the more effective is the passive diffusion. With this, the penetration efficacy of the AC from a dermal formulation depends primarily on the solubility – and the dissolution velocity – of the AC in the formulation because these two values are vital in achieving a sufficient amount of dissolved AC in the formulation. Here, the challenges of poorly soluble AC occur because they possess both limited solubility and limited dissolution velocity. Thus, values representing rate-limiting parameters for effective dermal penetration of AC via passive diffusion. Therefore, in the case of poorly water-soluble AC, such a formulation strategy is required to allow for effective dermal uptake via passive diffusion, which increases the solubility and the dissolution velocity of poorly soluble AC along with their dermal bioavailability.

1.1.2.3. The hair follicles

The hair follicles are appendages of the skin. They represent complex and many-faceting three-dimensional structures. The prime function of the hair follicle is to regulate hair growth, whereas the hair itself plays a significant role in protecting the skin from, for example, external forces, light, heat and other external influences. Anatomically, the triad of the hair follicle, sebaceous gland and arrector pili muscle makes up the pilosebaceous unit [29]. As a hair follicle, the layer of tissue that encloses the hair is termed [30]. The hair follicle begins at the surface of the epidermis and extends into the deeper layers of the skin. For follicles that produce terminal hair, the hair follicle extends into the dermis and sometimes subcutis [31,32]. Follicles producing vellus hair extend only to the upper dermis [31,32]. The hair follicle can be subdivided, from the outside to the inside, into three parts (fig. 4) [29–32]. The part of the hair follicle localized between the skin surface and the sebaceous gland opening is termed infundibulum (fig. 4, 5). The infundibulum represents a funnel-shaped structure filled with sebum. In human terminal hair follicles, the infundibulum has an average length of 0.6 - 0.8 mm, accounting for 15 - 20% of the total hair follicle length [33]. The infundibulum plays a vital role in hair follicle biology and pathology because the infundibular epithelium is an important source of the production of chemokines and various antimicrobial peptides [34]. Further, the infundibulum possesses a rich residential microflora [34]. Its interaction with the skin immune system and role for the skin and hair follicle physiology is a topic of current research.

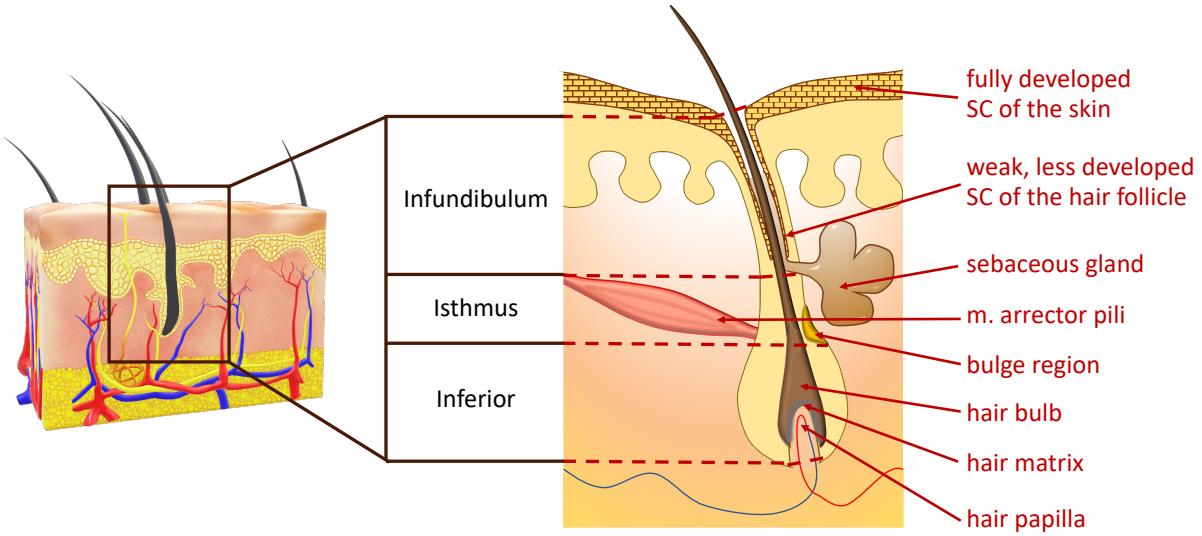


Fig. 4: Hair follicle structure: schematic view.

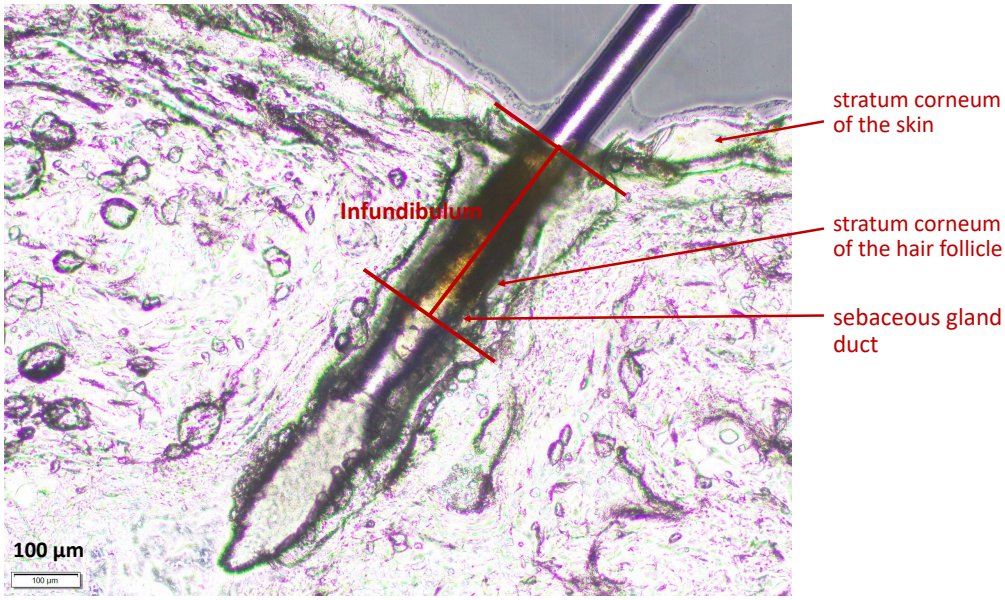


Fig. 5: Hair follicle structure: sectional view (vertical cross-section of the porcine ears hair follicle).

Morphologically – since the infundibulum builds an "invagination" of the epidermis – the infundibulum of the hair follicle possesses a comparable structure to the epidermis of the skin, i.e., comprises of the viable epidermis "covered" with SC. However, the SC of the hair follicle possesses a substantial specificity when compared to the SC of the skin. Its keratinization level decreases from upper infundibulum (skin surface, proximal) to lower infundibulum (deeper hair follicle, distal) [29,30]. The region of the lower infundibulum possesses the lowest keratinization level, also termed trichilemmal keratinization [30,35]. It means that only a few differentiated corneocytes are present within the SC of this follicular region. Thus, the SC of the lower infundibulum of the hair follicle is "weak", i.e., less developed when compared to the SC of the skin. This morphological specificity makes the SC of the

lower infundibulum of the hair follicle highly permeable for dermally applied AC. The part between the sebaceous gland duct opening and the bulge region, marked by the insertion of the arrector pili muscle, is termed isthmus (fig. 4). The central region of interest within the isthmus is the bulge region because it contains several epidermal stem cells. The part of the hair follicle localized between the bulge region and the base of the hair follicle is termed inferior (fig. 4) and can be subdivided into the bulb and the suprabulbar region. The bulb contains the follicular matrix surrounding the sides and top of the hair papilla (fig. 4). The hair papilla contains interactions with the matrix, which has the highest mitotic rate of any organ [32]. The matrix is the part of the hair follicle where matrix keratinocytes proliferate to form the hair shaft of growing hair.

1.1.2.4. Hair follicle targeting: value & features

Surprisingly, the hair follicles were long time assumed to be just an insignificant "byway" of the penetration into or through the skin, were not considered to have an important influence on the penetration process – mainly because they account only for approximately 1% of the skin surface area [29] – and stayed more or less "forgotten" in the past. Things changed in 1999, when the research group of professor Lademann from Charité Berlin showed for the first time that titanium dioxide particles penetrated into the hair follicle orifices [36]. In the last twenty years, much research was done in the field of follicular penetration. Now it is well proved that hair follicles represent not just a "byway", but a highly important and "multitasking" target for dermal drug delivery (fig. 6A, B). "Multitasking" means that the targeting of the hair follicles with AC allows addressing various particular issues (fig. 6A, B).

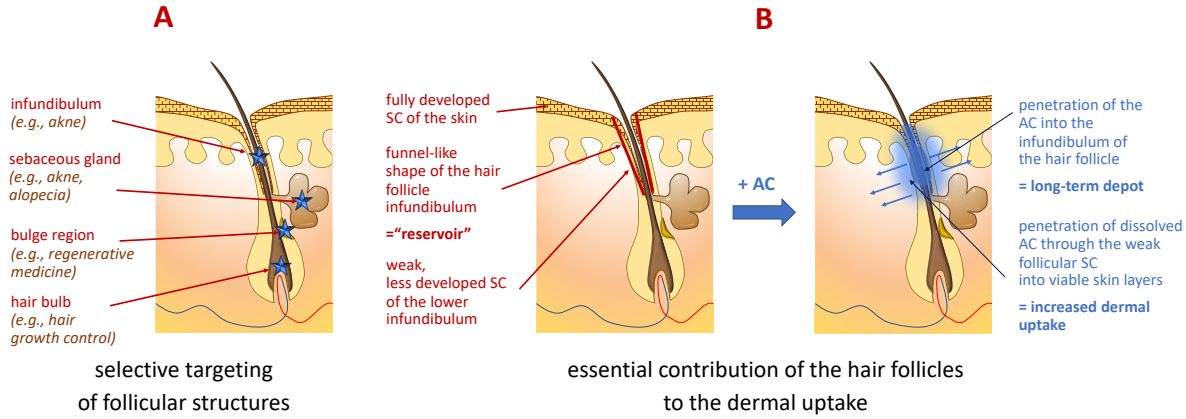


Fig. 6: Hair follicle, a "multitasking" target: A: essential targets within the hair follicle; B: increased dermal uptake via hair follicle targeting. Killing two birds with one shot –1) formation of the AC depot within the hair follicle infundibulum + 2) penetration of dissolved AC from the follicular depot into viable skin layers through the weak infundibular SC = improved dermal uptake.

On the one hand, the hair follicle as such represents a target for selective, local treatment of different hair follicle and hair follicle-associated conditions (fig. 6A). Within the hair follicle, different regions of interest can be defined (fig. 6A) [37]. The bulb region – the region of the hair matrix cells – is a

promising target in regard to hair growth control. The bulge region, which is built up of epithelial stem cells with high proliferative capacity, is a target for regenerative medicine. The sebaceous gland is associated with many disorders – also called hair follicle-associated disorders – for example, different forms of alopecia or acne. Also, the infundibulum of the hair follicle represents the essential target site within the hair follicle, for example, in the treatment of acne or in terms of topical vaccination [37]. On the other hand, the hair follicles make a unique and significant contribution to the dermal uptake of dermally applied AC (fig. 6B). Therefore, the word "multitasking" was chosen to describe the hair follicle as a target for dermally applied AC. This contribution to the dermal uptake is achieved mainly due to the specificities of the hair follicle infundibulum, namely its funnel-like shape and weak SC in its lower part (fig. 6B, left). Due to its funnel-like shape, the hair follicle infundibulum possesses considerable reservoir capacity for the dermally applied AC. Recently published studies demonstrated that the AC penetrated into the infundibulum of the hair follicle can be stored there for up to 10 days [38]. With this, forming a follicular depot. Since the lower infundibulum possesses less developed and with this, high permeable weak SC, the AC stored within the follicular depot can be step-by-step released from the carrier within the depot and penetrate through the weak follicular SC into the viable skin layers bypassing the "defensive wall" of the high developed SC of the skin. With this, the targeting of the hair follicle infundibulum with AC allows to kill two birds with one shot: the reservoir capacity allows to store the AC within the hair follicle and to form a follicular depot, and the weak SC allows the AC released from the follicular depot to penetrate through the weak follicular SC into the viable skin layers (fig 6B, right). Thus, allowing for a long-acting, continuous dermal uptake of the AC from the follicular reservoir and contributing significantly to improved dermal uptake of the dermally applied AC. The outlined "multitasking" character makes the hair follicle to an essential and highly important target for dermally applied AC.

1.1.2.5. Follicular penetration of AC – mechanisms & challenges of poorly soluble AC

Whereas the passive dermal penetration of dissolved AC into or through the skin is based primarily on passive diffusion, the penetration of AC into the hair follicles is based on other mechanisms. Previously published studies postulated for the follicular penetration the so-called ratchet mechanism [39]. Here, the hair shaft, due to its specific zig-zag structure, together with the hair follicle, build the ratchet-like complex, which by each its motion brings the AC deeper into the hair follicle (fig. 7).

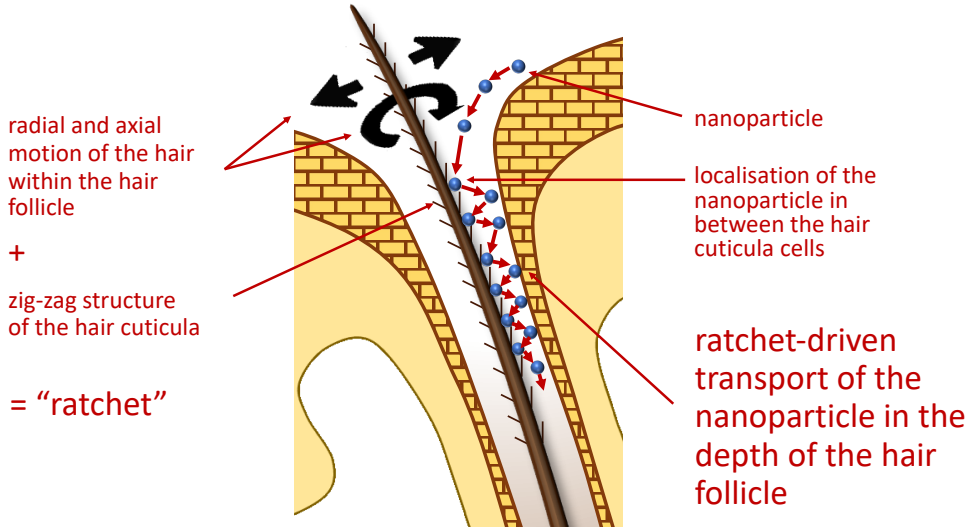


Fig. 7: Principle of the ratchet mechanism of the follicular penetration of nanoparticles.

Based on the ratchet mechanism, previous studies defined two substantial formulation properties required for effective penetration of the AC into the hair follicle. Firstly, it was shown that the AC in particulate form penetrates into the hair follicle more effectively than dissolved AC [40]. Secondly, the particle size was determined to be the major factor influencing the penetration depth of particles into the hair follicles – and with this, the efficacy of follicular penetration [37]. Previously published studies demonstrated that particles with a size in the range of 400 - 700 nm penetrate into the hair follicle deeper than smaller or larger ones [37]. Thus, for effective penetration of poorly soluble AC into the hair follicle, such a formulation approach is required that i) allows for an increase of solubility and thus, the dermal bioavailability of the AC and ii) allows for the formulation of the AC as a nanoparticle with tailored size.

1.1.3. Concept of the optimum formulation strategy for the most effective dermal application of poorly soluble AC

Since both the skin and the hair follicles represent essentially important targets for dermal drug delivery, the optimum formulation for poorly soluble AC for dermal application should be able to capture both targets and to transport the AC both into or through the skin and into the hair follicles. Such a formulation approach with the “dual action” would represent the most powerful, potent and universal optimal solution for effective dermal drug delivery. With this, such a formulation approach for poorly soluble AC should meet the requirements for the optimum formulation for both passive dermal and follicular penetration. It means it should i) allow for an increase of solubility and thus, the dermal bioavailability of the AC (an essential requirement for effective passive dermal penetration) and ii) allow for the formulation of the AC as a nanoparticle with tailored size (an essential requirement for effective hair follicle targeting). Does such a formulation already exist?

1.1.4. Overview of available formulation strategies for poorly soluble AC

In recent years, much research has been done to overcome poor aqueous solubility and to improve the bioavailability of poorly water-soluble AC. At present, several formulation strategies are already established. Here, two basic strategic formulation approaches can be distinguished [41–43]. The first approach to improve the solubility of AC is based on the chemical, molecular optimization of the AC. It can be achieved, for example, by salt formation, by adding water-soluble substituents to the molecule, or by the formation of prodrugs [44,45]. However, the applicability of these formulation strategies to the dermal application of poorly soluble AC provides important limitations. The derivatization to salts or prodrugs leads to new chemical compounds with possibly changed or modified properties and/or biological activity. With this, no one can guarantee that the new compound would lead to the same effect when compared to the unmodified compound. Furthermore, this approach is only “valid” for passive dermal penetration of AC and not for the hair follicle targeting because follicular penetration requires the AC in particulate form. The second approach to improve the solubility and bioavailability of poorly soluble AC is the use of innovative micro- and nanocarriers. Examples are micro- and nanoemulsions, micelles, cyclodextrins, micro- and nanoparticles (for example, liposomes, matrix nanoparticles (polymeric matrix/lipidic matrix), or nanocrystals) [46–56]. Within all outlined innovative carriers, nanoparticles represent the group of the highest interest because the conceptualized optimum formulation approach for poorly soluble AC should possess the “dual action” and should meet the requirements for both effective passive dermal penetration (an increase of solubility and thus the dermal bioavailability of the AC) and effective hair follicle targeting (allow for a formulation of the AC as a nanoparticle with tailored size). Among all outlined nanoparticles, drug nanocrystals (NC) represent the most powerful and advantageous formulation strategy for especially dermal application of poorly soluble AC.

1.1.5. Drug nanocrystals (NC)

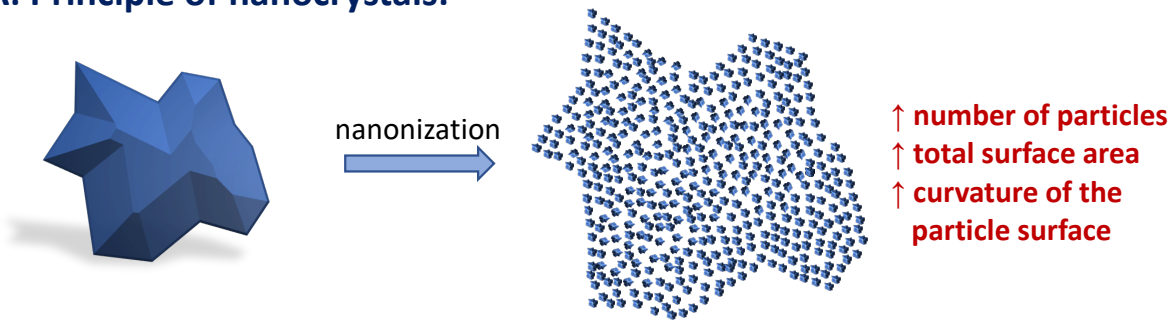
By definition, NC consist of 100% of AC without any matrix material and are typically stabilized by a surrounding surfactant or polymer layer [48]. Compared to other nanoparticles – for example, matrix-nanoparticles consisting of a polymeric matrix (polymeric nanoparticles) or a lipidic matrix (liposomes, lipid nanoparticles) that would theoretically also meet the requirements for the optimum formulation for both effective passive dermal penetration and effective hair follicle targeting and possess the required “dual action” – NC provide a series of advantages, rendering them to the formulation of choice for dermal application of poorly soluble AC. Firstly, in comparison to other nanoparticles, which consist of a matrix material loaded with AC (for example, adsorbed on the surface or incorporated inside the matrix), NC are composed of 100% AC without any matrix material. This advantage plays a substantial role especially for the dermal application. The maximal possible loading capacity makes NC

very efficient in transporting the AC to the target site because much lower numbers of particles and much lower "volume" of the formulation are needed to reach the required therapeutical concentration when compared to drug-loaded nanoparticles, which typically do not exceed a loading capacity of more than 10% [48,53]. With this, other nanoparticles are associated either with high application frequency and/or with high application dose. Thus, limiting substantially their dermal application. In contrast to other nanoparticles that are often characterized by specific requirements to the formulated drug substance, NC represent a universal formulation strategy that applies to any AC with poor aqueous solubility. Further, NC can be simply produced by well-established techniques without the demand for expensive equipment. Thus, making NC to the formulation of choice within all outlined other nanoparticles.

1.1.5.1. Special features of NC

NC are obtained by milling larger-sized bulk material to sizes below 1 µm (fig. 8A). The nanonization leads to a change in the physicochemical properties of the material. The transfer from bulk to nano leads to an increase in the total number of particles, the total surface area and the curvature of the particle surface (fig. 8A). The increased curvature leads to an increased dissolution pressure, which results in an increased kinetic saturation solubility (fig. 8B). The basis for this is the Kelvin equation, describing the relation of the vapor pressure of a liquid to the effect of the surface curvature. The increased surface area improves the dissolution velocity of the AC (fig. 8B). The physical explanation for this is the Noyes-Whitney equation. According to the Noyes-Whitney equation, the nanonization increases the surface area A and the saturation solubility c_s, thus increasing the dissolution velocity dc/dt of the AC. Since the nanonization increases the number of particles and the total surface area, the mass: surface ratio decreases, which in turn leads to an increased adhesiveness on surfaces (fig. 8B). To sum up, the resulting special features of NC are increased kinetic saturation solubility, increased dissolution velocity and increased adhesiveness on surfaces compared to larger-sized bulk material (fig. 8B). The outlined special features of NC make them highly effective for dermal application and allow for a dermal uptake of the poorly soluble AC via both possible ways – penetration of dissolved AC from NC into the skin via passive diffusion, i.e., passive dermal penetration, and penetration of NC as particles into the hair follicles, i.e., hair follicle targeting.

A: Principle of nanocrystals:



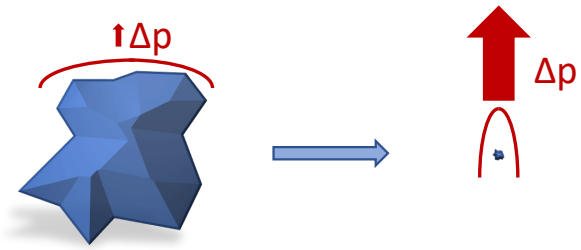
B: Special features of nanocrystals:

1. Increased saturation solubility:

physical principle: Kelvin equation

$$\Delta p = \frac{2\gamma}{R}$$

Δp: dissolution pressure
γ: surface tension (curvature)
R: radius of the curvature



bulk material
low curvature
low dissolution pressure
low saturation solubility

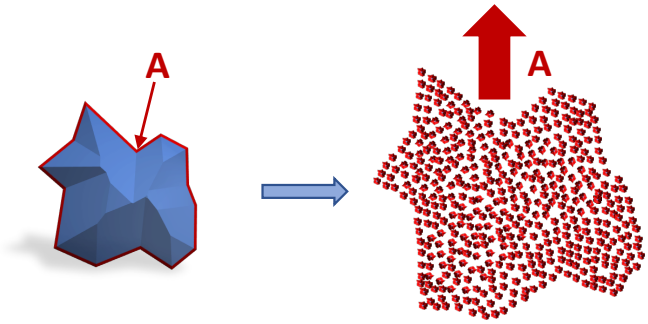
NC
high curvature
high dissolution pressure
high saturation solubility

2. Increased dissolution velocity:

physical principle: Noyes-Whitney equation

$$\frac{dc}{dt} = \frac{D \cdot A \cdot (c_s - c_x)}{h}$$

dc/dt: dissolution velocity
A: total surface area
D: diffusion coefficient
$c_s - c_x$: concentration gradient
h: thickness of the diffusional layer

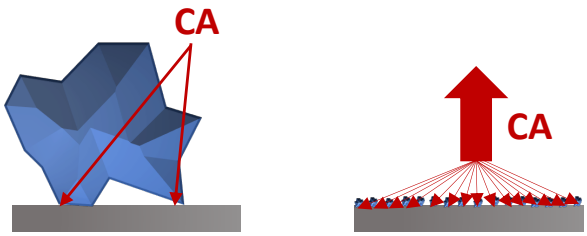


bulk material
low total surface area
low dissolution velocity

NC
high total surface area
high dissolution velocity

3. Increased adhesiveness:

principle:
↑ number of particles
↑ total surface area
↓ mass : surface ratio
↑ **contact area (CA)**
↑ **adhesiveness**



bulk material
low contact area
low adhesiveness

NC
high contact area
high adhesiveness

Fig. 8: Principle and special features of NC (modified after [48]).

1.1.5.1.1. NC for dermal application: improved passive dermal penetration

The improvement of the passive dermal penetration of poorly soluble AC by NC is realized by the following effects (fig. 9, 10):

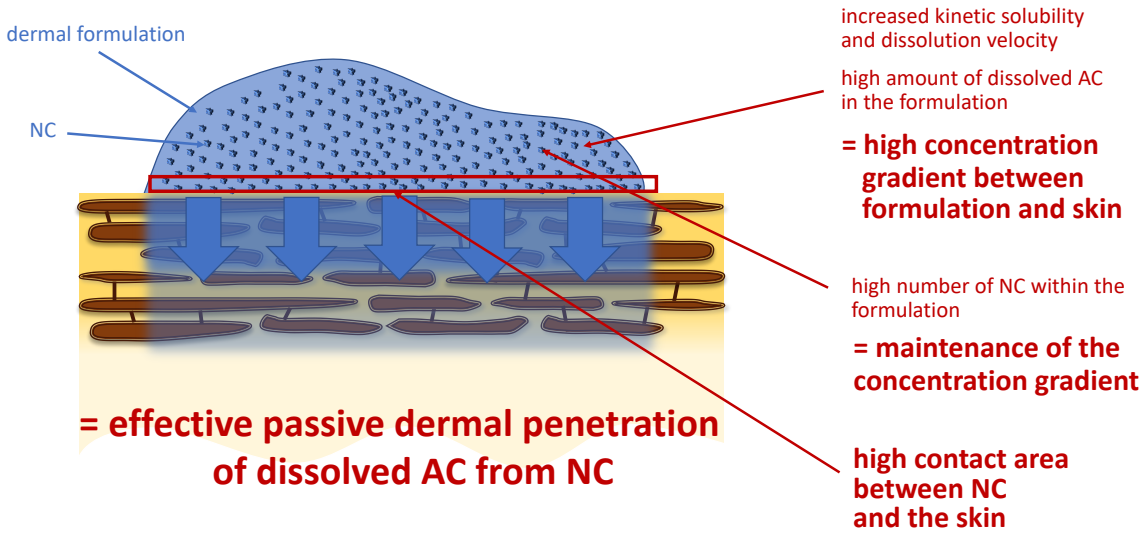


Fig. 9: Principle of NC for effective passive dermal penetration of poorly soluble AC.

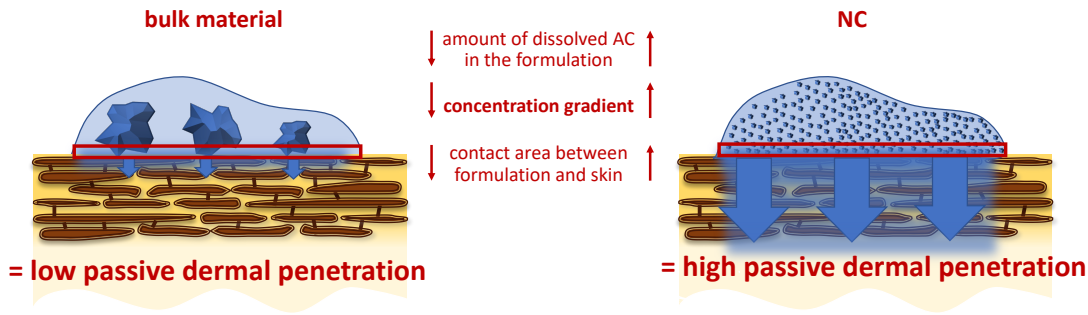


Fig. 10: Comparison of the efficacy of the passive dermal penetration of the AC from bulk material and NC.

1. Increased concentration gradient between formulation and skin:

The physical principle of the passive dermal penetration of dermally applied AC is passive diffusion. The driving force for the passive diffusion is the concentration gradient between the dermal formulation and skin. The higher is the concentration gradient, the more effective is the passive dermal penetration. In the case of NC for dermal application, the increase in the dissolution velocity and kinetic saturation solubility through nanonization generates a higher concentration of the dissolved AC in the formulation when compared to the bulk material. Thus, a higher concentration gradient between the formulation and skin is realized, resulting in effective passive dermal penetration.

2. Depot effect:

When the AC from NC dissolves and penetrates into or through the skin, it is immediately replaced by the next dissolved AC molecule from the NC "depot" within the dermal formulation. With this, the increased concentration gradient achieved due to the increased dissolution velocity and kinetic solubility of NC is not only realized but also maintained.

3. Increased adhesiveness:

The increased adhesiveness of the NC prolongs their retention time on the skin surface. Since each NC is covered by the layer of dissolved AC – the so-called "diffusional corona" – the longer the NC retain on the skin surface, the more effective is the passive dermal penetration.

All outlined advantages allow expecting the effective passive dermal penetration by formulating poorly soluble AC as NC.

1.1.5.1.2. NC for dermal application: improved hair follicle targeting

As outlined above, the hair follicle represents a "multitasking" target. It means that on the one hand, the hair follicle as such represents a target for selective treatment of the follicular and follicle associated disorders, and on the other hand, the targeting of the hair follicle contributes significantly to the improved dermal uptake (depot effect + penetration of the AC from the depot through the weak follicular SC into the viable skin layers). NC represent the formulation of choice for effective hair follicle targeting because NC can capture both cases (fig. 11).

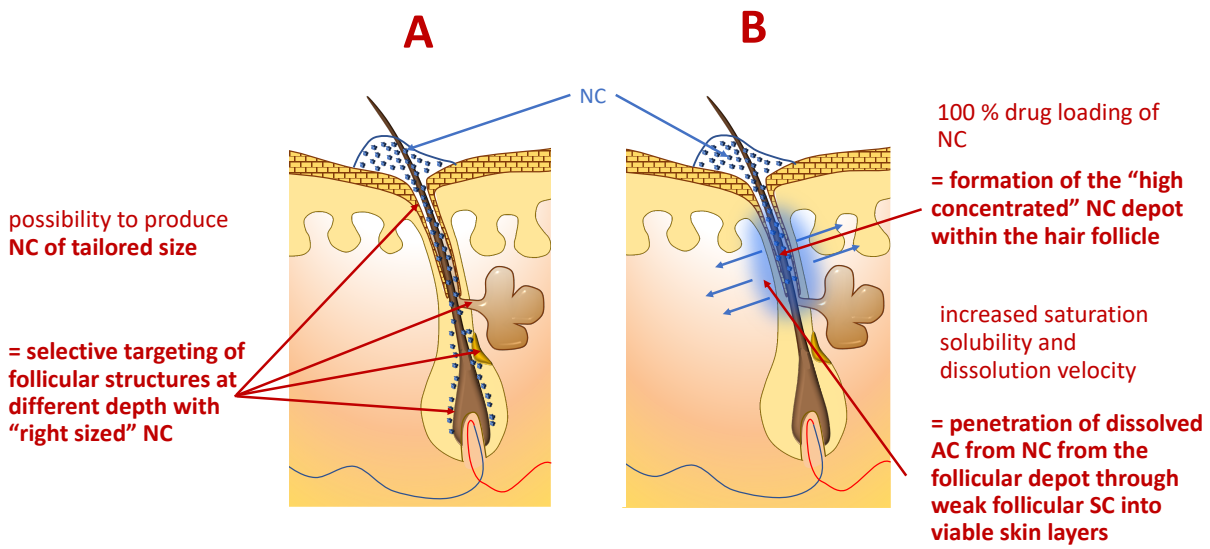


Fig. 11: Principle of NC for effective hair follicle targeting. A: selective targeting of follicular structures. B: contribution to the increased dermal uptake.

Considering the hair follicle as a target – "the first case" – so, within the hair follicle, the target sites responsible for different follicular disorders are localized on different depths (fig. 11A). Thus, for selective treatment of different follicular structures, the AC should be transported to the different depths within the hair follicle. According to the current state of knowledge and studies with other nanoparticles, the penetration depth of the nanoparticles into the hair follicle is size-dependent [37]. Thus, to target different structures within the hair follicle, particles with tailored size should be produced. At present, different production techniques for NC are established enabling the production of NC of tailored size [57]. With this, in theory, the production of NC of tailored size would allow for selective treatment of different targets within the hair follicle (fig. 11A). Considering the "second case" – significant contribution of the hair follicles to the improved dermal uptake of dermally applied AC – NC also represent the formulation of choice (fig. 11B). Their 100% drug loading allows the formation of the most effective and "high concentrated" follicular depot when compared to other existing nanoparticles. Since the NC principle increases the kinetic solubility and the dissolution velocity of the AC from NC, the AC from the NC depot within the hair follicle can step-by-step dissolve from the NC surface and penetrate through the weak SC into the viable skin layers via passive diffusion. Thus, allowing for a long-term NC depot within the hair follicles and a long-acting penetration of the AC into viable skin layers (fig. 11B).

1.1.5.2. NC for dermal application: current state

Surprisingly, even though NC – due to all their outlined advantages – represent the formulation of choice for dermal application of poorly soluble AC for effective passive dermal penetration and effective hair follicle targeting, at present, no systematic data are available that prove or even disprove it! Here, two essential issues are "pending". Firstly, and mainly, a proof-of-concept study showing that NC can effectively penetrate into the hair follicles is not yet available. At present, it is believed that NC due to all outlined properties represent the formulation of choice for effective hair follicle targeting, but no one confirmed it by systematic studies until now. With this, the state of the art of NC for the hair follicle targeting is best described as - NC represent in theory the effective nanocarrier for the hair follicle targeting. Secondly, the most substantial benefit and power of NC for dermal application is their "dual action", i.e., the ability to transport the AC both into the skin and into the hair follicles. And also this ability was not yet proved by systematic studies. It means, the proof-of-concept study confirming that NC formulations can effectively transport the AC both into the skin via passive dermal penetration and into the hair follicles as particles is also not yet available. With this, the major beauty of NC for dermal application is still theoretical, and the state of the art of dermal NC before this thesis is best described by the following sentence – NC represents in theory a highly effective formulation approach for dermal application of poorly soluble AC for passive dermal penetration and for hair follicle

targeting. Thus, the theory must be urgently moved to practice, and the outlined pending issues must be urgently addressed scientifically!

Further, until now, no one did the next step and questioned – if NC are so effective for passive dermal penetration and for the hair follicle targeting, what does it mean for their application use? How should dermal NC be formulated to achieve effective dermal and follicular uptake, and which formulation aspects need to be considered? How do the relevant formulation aspects need to be optimized to achieve the most effective penetration of NC formulations into the skin and/or into the hair follicles? To answer these questions, the influences of essential formulation aspects on the passive dermal and follicular penetration of the NC formulations need to be systematically investigated, and it was not yet done. At present, dermally applied NC are formulated by transferring theoretical statements and textbook knowledge to dermal NC without any systematic studies behind it – and with this, blind. The prominent example of such "theory-driven" formulation approaches is the size of NC. It is believed that small-sized NC are most effective for passive dermal penetration of dissolved AC from NC because the improvement of passive dermal penetration is mainly achieved due to the increased concentration gradient, which is achieved by the increase in kinetic solubility. As the increase in solubility is size-dependent, it is advisable to use small-sized NC for effective passive dermal penetration. This theory is, of course, plausible, but without systematic studies, it is just a theory. With this, the status quo at present is that no one knows whether the size influences the passive dermal penetration efficacy of the AC from NC. The same "game" takes place for NC for effective hair follicle targeting. It is believed that "bigger-sized" NC (with the size in the range of 400 - 700 nm) are most effective for the hair follicle targeting because studies with other nanoparticles revealed that particles in the outlined size range penetrate into the hair follicles deeper than smaller or larger ones. However, also this statement represents without systematic studies only a theoretical assumption. The size-dependent "rule" was previously postulated for nanocarriers with a spherical shape [37]. NC are obtained by milling larger-sized bulk material. Thus, NC are not spherical and differ considerably in their form and shape from the other already investigated nanoparticles. With this, they might be considered to interact differently with the hair follicle structures. Therefore, the size-dependent principle of the follicular penetration of NC cannot be "blind" transferred to NC without systematic studies. Another prominent example of transferring the textbook knowledge to NC without systematic studies is the choice of the vehicle for NC. At present, the researches tend to use hydrogels as vehicles for the NC because, according to the conventional classical formulation strategies, it is meanwhile textbook knowledge that AC suspended in the vehicle (principle of the suspension ointment or, in general, topical suspensions) demonstrates more pronounced penetration when compared to dissolved AC (principle of the solution ointment). As NC are especially poorly soluble in water, hydrogels, which contain only aqueous ingredients, are believed to be the best choice for the incorporation of NC. The keywords in the last sentence are "are

believed" because a systematic study investigating the influence of the vehicle type on the passive dermal and follicular penetration of the AC from NC does not yet exist. With this, at present, any choice of the vehicle – and other formulation components, for example, excipients – for NC is blind. Thus, also this issue – the influence of essential formulation aspects on the passive dermal and follicular penetration of NC formulations – must be addressed scientifically because without this knowledge any development of dermal NC formulations leads to a dead-end.

To address the outlined issues scientifically, sound systematic penetration studies with NC formulations should be performed. Sound penetration studies require a suitable method that can sufficiently characterize the penetration of NC formulations into the skin and into the hair follicles. Until now, dermal NC formulations were tested using previously established "common" universal methods. And until now, no one questioned – are these methods sufficient to characterize the passive dermal and the follicular penetration of the AC from NC? Which method is most suitable for penetration studies with dermal NC formulations? Does such a method already exist or should it be first established? Which skin model should be used for this method?

Based on the outlined issues and questions, the aims of this thesis were defined.

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Chapter 2

Aims of the Thesis

Chapter 2

2.1. Aims of the Thesis

As outlined in the introduction section, at present, it is believed that NC represent a highly effective nanocarrier for dermal application of poorly soluble AC for hair follicle targeting and for passive dermal penetration. However, no systematic studies were yet performed to prove or even disprove it. Further, no one did the next step and questioned – if NC are so effective for dermal drug delivery, what does it mean for their application use? How should NC for effective hair follicle targeting and/or passive dermal penetration be formulated, which formulation aspects need to be considered, and how they should be optimized to achieve the most effective penetration into the hair follicles and/or into or through the skin? To answer these questions, first, the influence of essential formulation aspects on the follicular and passive dermal penetration of dermal NC formulations should be systematically investigated, and it was not yet done. Without this knowledge, any development of dermal NC formulations is blind and leads to a dead-end. Thus, this issue must be urgently addressed scientifically.

To address this issue, sound penetration studies with NC formulations should be performed. A substantial prerequisite for sound penetration studies is a suitable method that can investigate detailed and mechanistically the follicular and passive dermal penetration efficacy of NC formulations. Until now, NC formulations were tested using available previously established “universal” methods. However, until now, no one questioned – are available methods sufficient to characterize the penetration efficacy of dermal NC formulations? Which method is most suitable to characterize NC formulations? Does such a method already exist, or does it need to be first established?

Based on the outlined questions, the **two aims** of this thesis were defined:

First aim: To establish the optimum method for characterization of the follicular and passive dermal penetration of dermal NC formulations.

For this, determine first whether available “universal” methods are sufficient to characterize the follicular and the passive dermal penetration efficacy of NC formulations. If not, optimize them or develop and establish a new method.

Second aim: Using the optimum method to investigate the influences of essential formulation aspects on the follicular and passive dermal penetration efficacy of dermal NC formulations and to optimize them to achieve the most effective follicular and/or dermal uptake.

Here, the prime focus should be set on the hair follicle targeting with NC formulations, because at present, it is believed, that NC due to their special features outlined in the introduction section represent the nanocarrier of choice for effective hair follicle targeting, but no one proved (or disproved) it yet by systematic studies. Therefore, the order used in this thesis should be "follicular and passive dermal penetration".

The outlined aims should be realized in three steps:

First step: Prove

The first step should investigate whether already available "universal" methods are sufficient to characterize the follicular and the passive dermal penetration efficacy of NC formulations. To investigate whether the method is suitable, it needs to be applied.

Thus, the **first step aimed to**:

- Investigate the influences of theoretically relevant essential formulation aspects on the follicular and passive dermal penetration efficacy of NC formulations using the most suitable available "common" previously established method.
- Analyze thoroughly the outcome of the penetration studies. Is the outcome sufficient? Could all influences of the formulation aspects on the follicular and passive dermal penetration of NC formulations be studied detailed and mechanistically? Were NC formulations soundly characterized regarding follicular and passive dermal penetration efficacy, were all necessary penetration parameters captured?
- If not – analyze the disadvantages and "leaks" of the method. What exactly was not possible with the "common" method? How should the method be optimized?

Second step: Optimize

The second step should come up with the optimum method for the characterization of follicular and passive dermal penetration of NC formulations. For this, such a method should be conceptualized and established.

Thus, the **second step aimed to:**

- Based on the outcome of the first step and on the elaborative analysis of available methods, conceptualize the method that i) would build the "perfect match" with NC formulations and characterize sufficiently the follicular and the passive dermal penetration of NC formulations ii) would overcome substantial disadvantages of available methods regarding the outcome, performance and handling and thus, build "the optimum method".
- Establish the conceptualized method. For this, either optimize the already existing method or develop a new method.

Third step: Realize

The third step should realize detailed and mechanistic studies of the follicular and passive dermal penetration of NC formulations with the optimum method and come up with systematic elaborative investigations of the influences of essential formulation aspects on the follicular and passive dermal uptake of NC formulations.

Thus, the **third step aimed to**:

- Investigate the influences of theoretically relevant essential formulation aspects on the follicular and the passive dermal penetration efficacy of NC formulations using the optimum method. For this, modify essential formulation aspects of NC formulations and investigate detailed and mechanistically their influences on the follicular and dermal uptake of NC formulations.
- What do these influences mean for the application use of NC for dermal application? Based on these investigations, identify how the formulation aspects should be optimized to achieve the most effective follicular and/or passive dermal penetration with dermal NC formulations.

To sum up:

Prove the applicability of the universal method -> **optimize** the method -> **realize** sound penetration studies with NC formulations **->** come up with the optimum method for the penetration studies with dermal NC formulations and with optimum formulation aspects for tailor-made NC formulations for the most effective penetration into the hair follicles and/or into the skin.

Chapter 3

Results

Chapter 3

3.1. Guide on Research

The work was performed in three steps:

First step: Prove

The most substantial benefit of NC for dermal application is their "dual action". It means the ability of NC as particles to penetrate into the hair follicles (termed in this thesis follicular penetration or hair follicle targeting) and the ability of dissolved AC from NC to penetrate into/through the skin via passive diffusion (termed in this thesis passive dermal penetration). Thus, the method for sufficient penetration experiments must determine the penetration via both pathways.

From all available methods, differential skin stripping was identified to be most suitable for the aims of this thesis, primarily because with this method, both follicular and passive dermal penetration can be determined. Thus, in the **first step**, the influences of essential theoretically relevant formulation aspects on the follicular and passive dermal penetration of dermal NC formulations were tested using differential skin stripping. Penetration experiments were performed using porcine ears skin as a skin penetration model.

As essential theoretically relevant formulation aspects, the size of NC, different excipients and different vehicles were investigated. Hesperetin and rutin were used as model AC. The influence of NC size was tested on the example of previously established hesperetin NC with different sizes (160 nm, 350 nm, 450 nm, 700 nm, PCS data). To investigate the influence of excipients on the penetration efficacy, prior to NC application, the skin was pre-treated with different hydrophilic (urea, glycerol, ethanol, PEG300) and lipophilic (olive oil) excipients. Subsequently, hesperetin NC were applied. To investigate the influence of the vehicle type, rutin NC were incorporated into a hydrogel, a cream and an oleogel. Penetration experiments were conducted with differential skin stripping and a porcine ears skin model. Follicular penetration was determined with cyanoacrylate skin surface stripping (CSSS) and passive dermal penetration was determined with classical tape stripping. The amount of the AC that penetrated into the hair follicles and into the skin was quantified using HPLC.

The results of the investigations of the passive dermal penetration are presented in the manuscript "Nanocrystals for improved dermal drug delivery" in section 3.2. Results of the investigations of follicular penetration are not presented in the published manuscript. The reasons for this are discussed in the Summary and Discussion section. The outcome of the studies of passive dermal penetration, detected advantages and limitations of differential skin stripping and the suitability of this method for

penetration studies with dermal NC formulations are discussed in the Summary and Discussion section as well.

Second step: Optimize

Differential skin stripping failed as a stand-alone method for sufficient characterization of the follicular and the passive dermal penetration of dermal NC formulations. Thus, in the **second step**, the novel method for sound penetration experiments with dermal NC formulations was established. The novel method was thoroughly conceptualized based on the outcome of the first step and the evaluation of the advantages and limitations of available methods for investigations of the follicular and passive dermal penetration of dermal formulations. The novel method is based on the visualization of the follicular and passive dermal penetration using fluorescence microscopy. The prerequisite for the use of this method is that the AC is fluorescent. The novel method represents the unique "two-in-one" technique. It means that only one skin penetration model (porcine ears skin) is used, and within this model, only one skin area needs to be treated with the investigated formulation. On this treated skin area, both follicular and passive dermal penetration of the formulation are determined. For this method, the investigated formulation is applied onto the skin of intact porcine ears, after the defined penetration time the skin biopsy is punched, embedded and frozen. The frozen skin biopsies are cut with the cryomicrotome into hair follicle sections and skin sections according to the embedding and cutting technique established in this thesis. After cutting, the skin and hair follicle sections are visualized using fluorescence microscopy. After visualization, the follicular and passive dermal penetration are quantified. The follicular penetration is quantified by measuring the penetration depth of NC into the hair follicle in µm directly from original fluorescence images according to the method previously established by the research group of professor Lademann from Charité Berlin. For the quantification of the passive dermal penetration from fluorescence images, the method of digital image analysis with the software ImageJ was established in this thesis. To establish this method, a study with standard formulations with predictable penetration efficacies was conducted. As model AC, curcumin was used due to its pronounced autofluorescence. As standard formulations, solutions of curcumin in ethanol with different curcumin concentrations (0.025%, 0.05%, 0.1%, 0.15%, 0.2%, 0.25% (m/v)) were used due to their predictable penetration properties. Based on Fick's 1st law of diffusion, the concentration of dissolved AC in the formulation is the driving force of the passive diffusion. Thus, it was predicted that the increase of the curcumin concentration in the solution would lead to an increase in its penetration efficacy and vice versa. Thus, enabling the so-called "dilution series on the skin" providing a good example of formulations with "poor" penetration and "good" penetration. For penetration experiments, curcumin solutions were applied onto the skin of intact porcine ears. After the defined penetration time, the skin biopsies were punched, embedded and frozen. Subsequently, skin sections were prepared using the cryomicrotome. The passive dermal penetration of curcumin

was visualized using fluorescence microscopy. To quantify the passive dermal penetration, a sound method for digital image processing using ImageJ software was developed. The development of this method based on the penetration experiments with curcumin standard formulations is presented in the manuscript “Dermal penetration analysis of curcumin in an ex vivo porcine ear model using epifluorescence microscopy and digital image processing” in section 3.3.

The advantages and limitations of previously established conventional methods for determination of follicular and passive dermal penetration efficacy, the advantages and limitations of available skin models, the concept of the novel method and its advantages compared to differential skin stripping used in the first step and other conventional methods are discussed in the Summary and Discussion section.

Third step: Realize

With the establishment of the novel optimum “two-in-one” method, the essential prerequisite for sound penetration studies with dermal NC was fulfilled. Thus, in the **third step**, the influences of the essential formulation aspects on the efficacy of the follicular and passive dermal penetration were investigated using the novel method. As essential formulation aspects, the type of the vehicle and the excipients were investigated because they build the “core” of dermal formulations. The influences of vehicle type and excipients on the follicular and passive dermal penetration efficacy were already investigated in the first step using differential skin stripping. However, the outcome of the penetration studies was insufficient. Thus, these issues were addressed in the third step using the novel method.

The prime focus was set on the hair follicle targeting with NC formulations because, at present, it is believed that NC represent the nanocarrier of choice for effective hair follicle targeting with poorly soluble AC, but no one proved (or even disproved) it yet by systematic studies. The investigations focused primarily on the application use of NC. It means the prime issue was to investigate how essential formulation aspects – vehicle type and excipients – influence the follicular and passive dermal penetration efficacy of NC formulations. Based on this, it was investigated how the formulation aspects should be optimized to achieve the most effective follicular and/or passive dermal uptake.

To investigate the influence of the vehicle type on the follicular and passive dermal penetration of NC formulations, curcumin NC were used as model NC formulation due to the pronounced autofluorescence of curcumin. Curcumin NC of comparable sizes were incorporated into vehicles with varied properties. All formulations contained 1% (w/w) curcumin. As vehicles, an aqueous nanosuspension and 14 semisolid gels were evaluated. The gels varied in their medium, gelling agent and concentration of the gelling agent. The properties of the vehicles were modified in such a way that the influence of the viscosity, polarity and lipophilicity of the vehicle on the follicular and passive dermal penetration was systematically investigated. Penetration experiments were conducted using

the novel method established in the second step of this thesis and the porcine ears skin as a skin penetration model. Results of these investigations are presented in the manuscript “Hair follicle targeting with curcumin nanocrystals: influence of the formulation properties on the penetration efficacy” in section 3.4.

The influence of the excipients on the follicular and passive dermal penetration of NC formulations was also investigated using curcumin NC as a model formulation. To investigate the influence of excipients, curcumin nanosuspension was produced and aliquoted. The aliquots were modified by the addition of different excipients. As excipients, glycerol, propylene glycol, urea, ethanol and olive oil were investigated. Penetration experiments were conducted using the novel method established in the second step of this thesis and the porcine ears skin model as a skin penetration model. Results of these investigations are presented in the manuscript “Hair follicle targeting and dermal drug delivery with curcumin drug nanocrystals—essential influence of excipients” in section 3.5.

The influences of the vehicle type and excipients on the follicular and passive dermal penetration of NC formulations, the investigated mechanisms behind it, and the identified essential key formulation properties for tailor-made NC formulations for effective follicular penetration, passive dermal penetration and targeted dermal drug delivery are discussed in the Summary and Discussion section.

3.2.

Nanocrystals for improved dermal drug delivery

Nanocrystals for improved dermal drug delivery

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Abstract

Nanocrystals are composed of 100% active and possess an increased solubility and dissolution velocity when compared to larger sized materials. Nanocrystals can be used to improve the bioavailability of poorly soluble actives not only for oral, but also for topical application. In this study nanocrystals of different sizes were produced and the influence of size on dermal penetration was investigated. The influence of different excipients and vehicles on the penetration efficacy upon dermal application was also investigated. Results confirm that dermal penetration of poorly soluble actives increases with decreasing size of the nanocrystals. Unexpectedly, it was observed that many classical penetration enhancers failed to promote the penetration of actives from nanocrystals. Also, hydrogels were found to be non-suitable vehicles for the formulation of nanocrystals. As most suitable vehicles for nanocrystals oleogels and creams were identified.

1. Introduction

Nanocrystals are an established formulation principle and have been invented in the early 1990ties to overcome poor solubility and associated poor bioavailability of poorly-water-soluble actives, i.e. slightly soluble drugs according to Ph. Eur. and the class II drugs according to the biopharmaceutical classification system (BCS) [1–5]. The principle of nanocrystals is simple but smart. Nanocrystals are composed of 100 % active and are typically obtained by milling large sized bulk material to sizes to below 1µm. Due to the decrease in size physico-chemical properties are modified, which lead to an increase in kinetic solubility. The phenomenon can be explained by the increased curvature of the nanocrystals, which leads to an increased dissolution pressure and thus to an increase in solubility [6, 7]. The increased solubility and the increased surface area improve the dissolution velocity (Noyes-Whitney equation) of poorly soluble actives and the higher solubility results in an increased concentration gradient, which will promote passive diffusion through biological membranes. In addition, nanosized material possesses an increased adhesiveness to membranes, because the surface to volume ratio increases with decreasing size (gecko effect) [7]. In case of drug delivery this is advantageous, because a longer retention time at the place of absorption will further promote the penetration of actives (fig. 1). The beneficial properties are used in various pharmaceutical drug products for oral application examples are Rapamune® (sirolimus, Pfizer), Emend® (aprepitant, MSD) or Tricor® (fenofibrate, AbbVie) [1, 5, 8].

The benefits of nanocrystals can also be used for dermal application, because also dermal penetration is mostly driven by passive diffusion [7, 9, 10]. However, until now, no pharmaceutical product for dermal application entered the market. Nevertheless, many cosmetic products exploiting the nanocrystal principle are already available on the market. Examples are Juvedical (rutin, Juvena), Edelweiss (rutin, Audorasan) or Platinum rare (hesperidin, la prairie). Despite many articles explaining the principle of nanocrystals for both oral and dermal use [5–7, 11, 12], relatively little research is published in the field of nanocrystals for dermal application. Especially a proof-of-concept study investigating the influence of nanocrystal size on the dermal penetration efficacy of poorly soluble actives is not yet available. Another important issue, that is not yet addressed scientifically, is a study investigating the influence of different excipients and vehicles on the penetration efficacy of poorly soluble actives from nanocrystals.

Today, in most cases, nanocrystals are formulated in hydrogels, because it is believed that classical text book knowledge can be directly transferred to nanocrystals. Classical formulations are formulated by dispersing drug actives in semisolid formulations, because suspended particles of actives are generally known to allow for a more pronounced and deeper penetration of actives than formulations where actives were dissolved in the vehicle [14, 15]. As nanocrystals are especially poorly soluble in water,

hydrogels, which contain only aqueous ingredients, are believed to be the best choice for the incorporation of nanocrystals. In some cases, penetration enhancers are used to further promote the penetration of the actives.

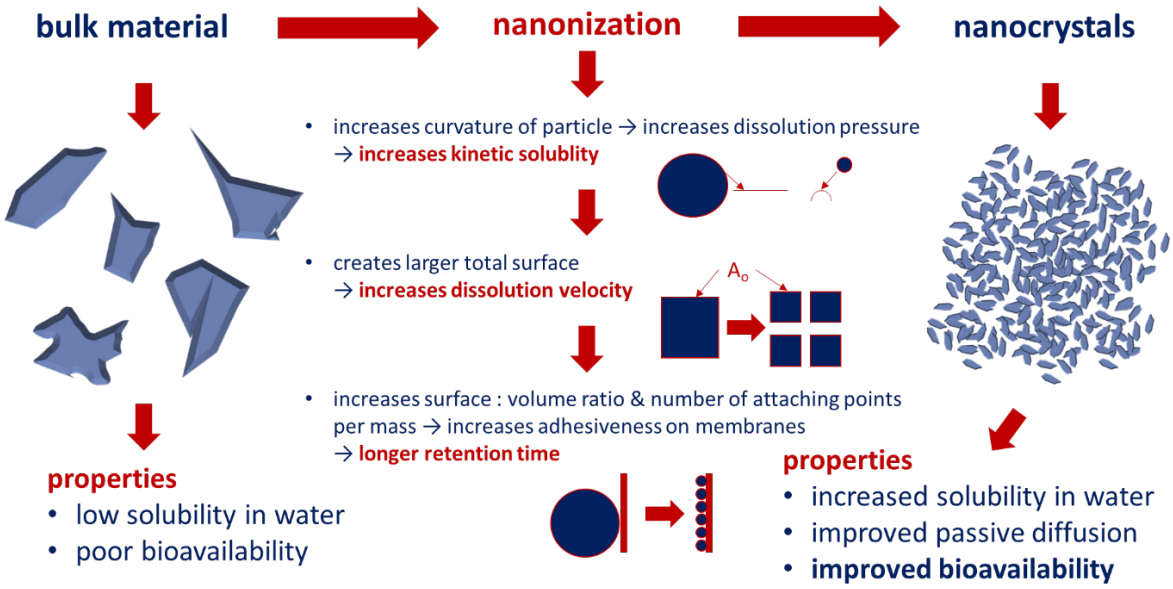

Figure 1: Scheme of improved properties of nanocrystals [5, 6, 13].

A recently performed study by Staufenbiel et al. proved the classical textbook knowledge for the active azithromycin [16]. A formulation containing 10% active dissolved in ethanol, was less effective in penetration when compared to a formulation where 5% active were suspended in a hydrogel. The addition of propylene glycol as penetration enhancer could further improve the penetration of the active and the formulation of azithromycin as nanocrystals resulted in the highest and deepest penetration of the active. Unexpectedly, it was found that propylene glycol, when used as penetration enhancer for the nanocrystals, led to a strong decrease in the penetration of the active. The results were explained by the fact that propylene glycol can also act as a solvent. The small size of the molecule might enable a fast diffusion of propylene glycol into the skin, whereas the larger molecules of the active cannot penetrate quickly. As the solvent diffuses into the skin, high amounts of dissolved active are “left behind” and will precipitate on the skin by forming large crystals with low solubility, thus hampering the penetration of the active.

In fact, even though nanocrystals are now used for more than one decade for improved dermal penetration, a proof of concept study showing the effect on size of the nanocrystals on the penetration efficacy of poorly soluble actives is still not available. An addition, little is known about the influence of type of excipient or vehicle in the dermal penetration efficacy of poorly soluble actives from nanocrystals. Therefore, the aim of this study was to investigate these effects in more detail.

2. Materials and Methods

2.1. Materials

Hesperetin and rutin (Exquim S. A., Spain and Denk Ingredients GmbH, Munic, Germany) are natural flavonoids with high antioxidant capacity, therefore they are considered as promising compounds for both cosmetical as well as pharmaceutical industries. Hesperetin has a water solubility of 273 mg/l, and rutin´s water solubility is 125 mg/l [17, 18]. Hence, they are considered slightly water-soluble materials according to the Ph. Eur. [4]. Additionally, both hesperetin (logP = 2.6) and rutin (logP = 0.15) are considered lipophilic compounds with logP values > 0 [17, 18]. Due to their slightly water solubility, lipophilicity and antioxidant capacity, they were used as model drugs in this study. Plantacare 2000UP (decyl glycoside) was a kind gift from BASF (Ludwigshafen, Germany) and was used as skin-friendly, non-ionic stabilizer for the nanocrystals. As excipients and vehicles urea (Caesar & Loretz GmbH, Hilden, Germany), polyethylene glycol 300 (Caesar & Loretz GmbH, Hilden, Germany), glycerol (Sigma Aldrich, Hamburg, Germany), ethanol (Ethanol ROTISOLV® HPLC Gradient Grade, Carl Roth GmbH, Karlsruhe, Germany), olive oil (REVIDERM AG, Sauerlach, Germany), dimethyl sulfoxide (DMSO, VWR International GmbH, Darmstadt, Germany), Basiscreme DAC (Caesar & Loretz GmbH, Hilden, Germany), petroleum jelly (Caesar & Loretz GmbH, Hilden, Germany) and polyacrylate (Carbopol®980, Caesar & Loretz GmbH, Hilden, Germany) were used. All other analytical chemicals were of analytical grade and were used as received. Purified water was obtained from a PURELAB Flex 2 (ELGA LabWater & Veolia, Germany).

2.2. Methods

2.2.1. Production of nanocrystals

Hesperetin nanocrystals of different sizes were obtained by using small scale bead milling and high pressure homogenization, respectively. Small scale bead milling was performed to produce small sized nanocrystals with a size of about 200 nm. Beads with a size of 1 mm and three magnetic stirrers with a size of 6 x 10 mm were placed in a round vial with a size of 12 x 35 mm. The vial was sealed and placed on a magnetic stirrer. Stirring was performed for 8h with a stirring speed of about 1200 rpm. Larger sized crystals were obtained by high pressure homogenization by using a LAB 40 (APV Gaulin, Unna, Germany). For particles possessing a size of about 400 nm, pre-milling was performed by applying 3 cycles of 200, 300, 500, 750 and 1000 bar respectively, followed by 20 subsequent homogenization cycles at 1500 bar. Larger sized nanocrystals were obtained by reducing the number of homogenization cycles and the pressure applied. Rutin nanocrystals were produced as described before [19, 20]. Briefly, nanocrystals were obtained by applying the ArtCrystal®-technology which is a

combination of high speed stirring with extremely high rotational speed and subsequent high pressure homogenization with low pressure and low number of homogenization cycles. All nanocrystal suspensions contained 5% (w/w) active and 1% (w/w) surfactant.

2.2.2. Characterization of nanocrystals

Nanocrystals were characterized regarding size by using a combination of dynamic and static light scattering techniques and light microscopy. Dynamic light scattering (DLS), also known as photon correlation spectroscopy (PCS), was used to assess the mean particle size and the size distribution (z-average and polydispersity index), whereas laser diffraction was used to detect possible larger particles within the small sized main fraction. Light microscopy was used to further prove the data obtained by laser diffraction. PCS data were analyzed by using the general-purpose mode and a Zetasizer Nano ZS (Malvern Instruments, Germany). Laser diffraction data were analyzed by using Mie-theory with 1.57 for rutin or 1.59 for hesperetin and 0.01 (both) as real and imaginary refractive indices with a Mastersizer 3000 (Malvern Instruments, Germany). Light Microscopy was performed with an Olympus BX53 light microscope (Olympus Cooperation, Tokyo, Japan), which was equipped with an Olympus SC50 CMOS color camera (Olympus soft imaging solutions GmbH, Muenster, Germany).

2.2.3. Determination of dermal penetration

2.2.3.1. Influence of size of nanocrystals on dermal penetration

In the first part of the study the influence of the size of nanocrystals on the dermal penetration was assessed. Differently sized nanocrystals were applied to skin and tape stripping was performed after 30 min of incubation time at 32°C.

2.2.3.2. Tape stripping procedure

Fresh pig ears, which were obtained from a local slaughter house, were used for this study. Prior to the experiment's ears were cleaned with purified water and then patted dry with a soft paper tissue. An investigation area of 1.5 cm × 1.5 cm was defined and only intact skin with no wounds, scratches or other visible skin changes was used. Formulations (50µL, 5% liquid nanosuspension) were applied by pipette tips and saturated gloved finger. After an incubation time of 30 minutes at 32°C, formulations were wiped out using a soft tissue for 1 minute. Subsequently, up to 30 adhesive tapes a soft tissue for 1min. Subsequently, up to 30 adhesive tapes (Tesafilm® crystal clear, tesa SE, Beiersdorf AG, Norderstedt, Germany) were used to remove the stratum corneum layer by layer. If not mentioned otherwise, each experiment was performed in triplicate.

Following tape stripping, an indirect determination of the amount of adhering corneocytes representing the thickness of removed horny layer, was performed by using a SquameScan850

(Heiland electronic GmbH, Wetzlar, Germany) at a wavelength of 850nm. The removed hesperetin was quantitatively extracted from the tapes by using 3.0mL of ethanol while shaking with an Edmund BühlerSwip KS-10 (Edmund Bühler GmbH, Bodelshausen, Germany) for 2h at 125rpm, 25°C. The concentration of hesperetin on each strip was subsequently analyzed through high-performance liquid chromatography (HPLC).

2.2.3.3. Screening for suitable excipients

In the second step of the study a screening for suitable excipients and vehicles in which the nanocrystals could be formulated was performed. Firstly, the influence of different excipients, i.e. urea, glycerol, propylene glycol, ethanol and DMSO on the penetration efficacy of hesperetin from nanocrystals was investigated. For this – prior to the application of the nanocrystals – the excipients (50µl) were applied to the skin and allowed to penetrate the skin for 5 min. After this time non-penetrated liquids were carefully removed with a tissue from the skin. Subsequently the nanocrystal formulation was applied to the skin and the procedure was continued as described above. Excipients were used in different concentrations and experiments were performed once.

2.2.3.4. Screening for suitable vehicles

Based on the results obtained, in the next step a more detailed screening was performed by using olive oil and urea as excipients, respectively. Experiments were performed in triplicate all other procedures were according to the procedures described above. Transepidermal water loss (TEWL) and skin hydration were determined by using a Multi Probe Adapter System equipped with a Corneometer® CM 825 and a Tewameter® TM 300, respectively (Courage + Khazaka electronic GmbH, Germany). The last part aimed at identifying suitable vehicles for the formulation of dermal products containing nanocrystals. Rutin nanocrystals were incorporated into a hydrogel (consisting of 1% (w/w) polyacrylate, sodium hydroxide (q.s.) and purified water), an oleogel (petroleum jelly) and a cream (Basiscreme DAC), respectively. Each formulation contained 2% (w/w) nanosuspension, i.e. 0.1% active. Nanocrystals were admixed to the vehicles by using an Unguator system (Wepa, Germany).

2.2.3.5 HPLC analyses

The concentration of hesperetin on each strip was subsequently analysed through high-performance liquid chromatography (HPLC) by using an Agilent Infinity Lab LC Series 1260 Infinity Quaternary System (Agilent Technology, Germany), equipped with a pump: G7111A 1260 Quat Pump VL; sampler: G7129A 1260, column: Agilent Poroshell 120 EC-C18, 4.6×50nm, 2.7µm and UV detector: diode array detector (G7117C 1260 DAD HS, wavelength 288 nm). Analysis was performed under isocratic elution by using a mixture consisting of methanol : water : acetic acid = 50 : 48 : 2 (V/V/V) as mobile phase as with a

flow rate of 0.450 ml/min and a temperature of 45°C, the injection volume was 5µL. The retention time was about 2.8 min.

3. Results and Discussion

3.1. Production and characterization of nanocrystals

Rutin nanocrystals possessed a size of 410 nm and a narrow size distribution (PdI 0.219). LD results confirmed the small size and proved the absence of larger particles, i.e. 95% of the particles possessed a size below 1.25 µm. The absence of larger particles was also confirmed by light microscopy (fig. 2). Hesperetin nanocrystals were obtained in four different sizes with sizes of about 700, 450, 350 and 160 nm, respectively. The size distribution was relatively broad for the largest particles, which was due to the application of reduced numbers and homogenization cycles, but still sufficiently narrow for use in the further experiments (fig. 3).

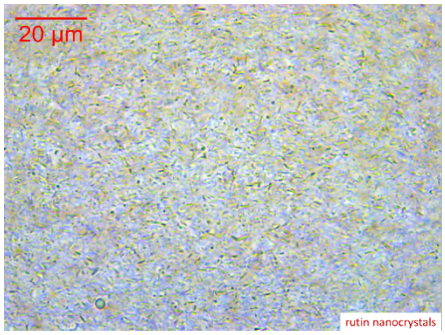

Figure 2: Microscopic image of rutin nanosuspension (magnification 1000x).

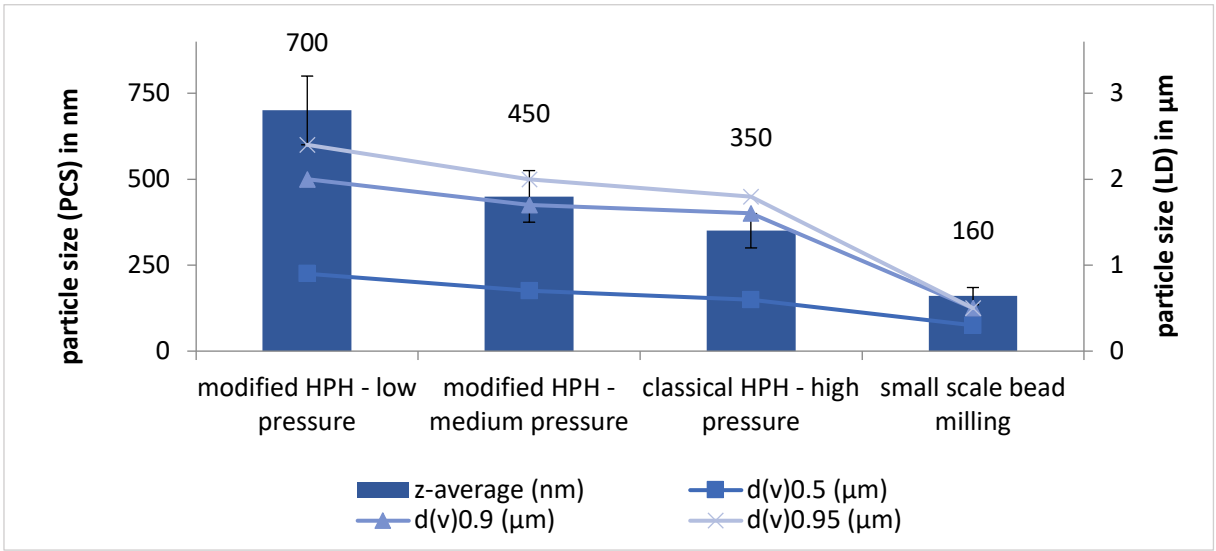

Figure 3: Particle size analysis of differently sized hesperetin nanocrystals (PCS and LD data).

3.2. Determination of dermal penetration

3.2.1. Influence of size of nanocrystals on dermal penetration

Until now it was not possible to demonstrate the size effect of nanocrystals on the dermal penetration efficacy of poorly soluble actives. The reason was a lack of production techniques enabling the production of differently sized nanocrystals. By using different modifications of established methods for the production of nanocrystals, it was now possible to produce particles with sizes of about 700, 450, 350 and 160 nm. Thus, enabling to investigate the influence of size of nanocrystals on dermal penetration. The penetration results nicely demonstrate the postulated effect and prove that smaller sizes improve the passive penetration of poorly soluble actives and promote the penetration into deeper skin layers (fig. 4).

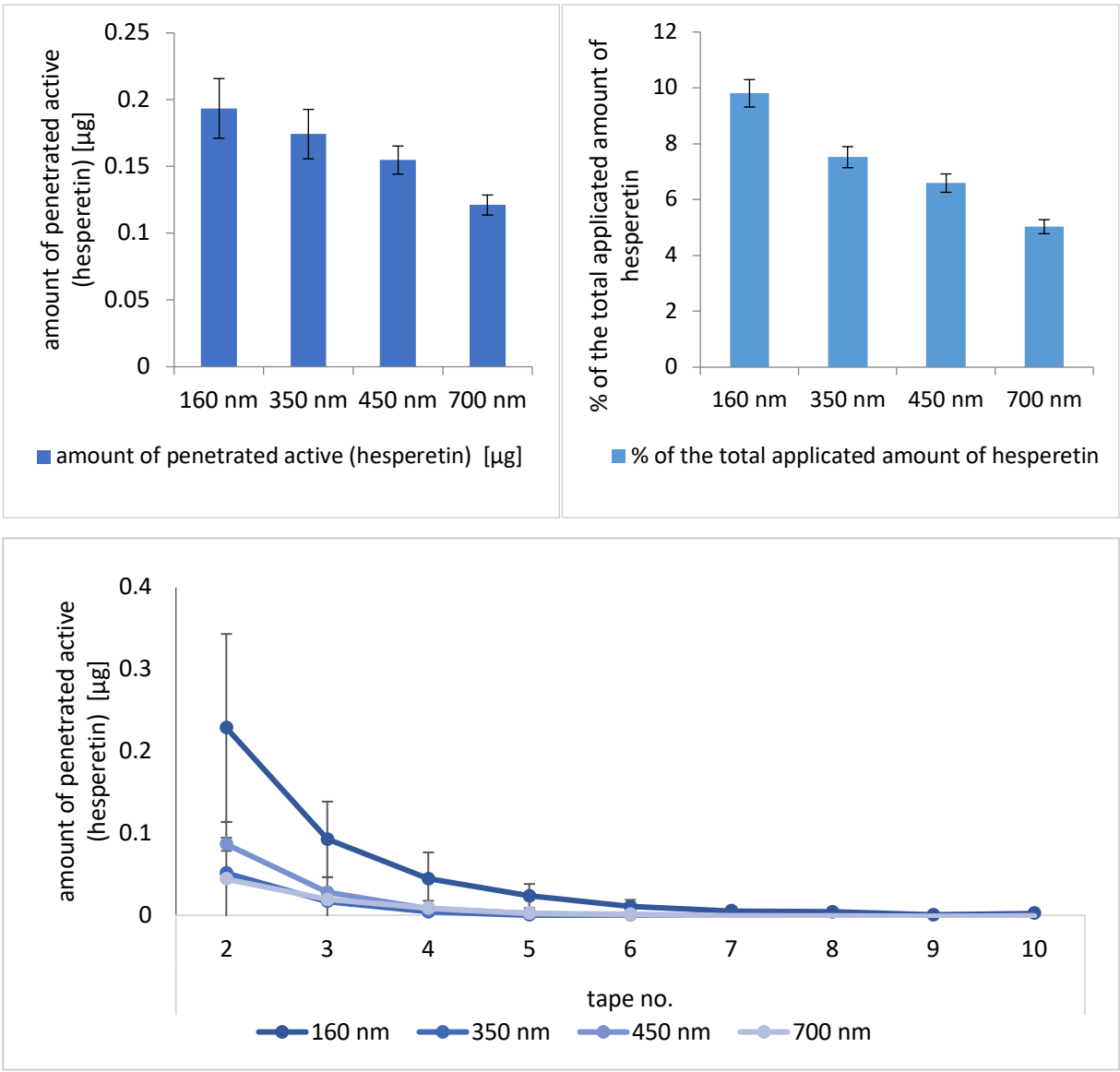

Figure 4: Dermal penetration of hesperetin from nanocrystals with different sizes. Upper: total amount penetrated. Lower: penetration profiles.

3.2.2. Screening for suitable excipients

In the first part of the study different excipients, that are frequently used in cosmetically and/or pharmaceutical applications, were used to investigate the influence on the dermal penetration efficacy of hesperetin from nanocrystals. It was found that all excipients, when applied to the skin prior to the application of the nanocrystals, hampered the penetration of the active (fig. 5). These results were unexpected, because in general, all these excipients are frequently applied as penetration enhancers.

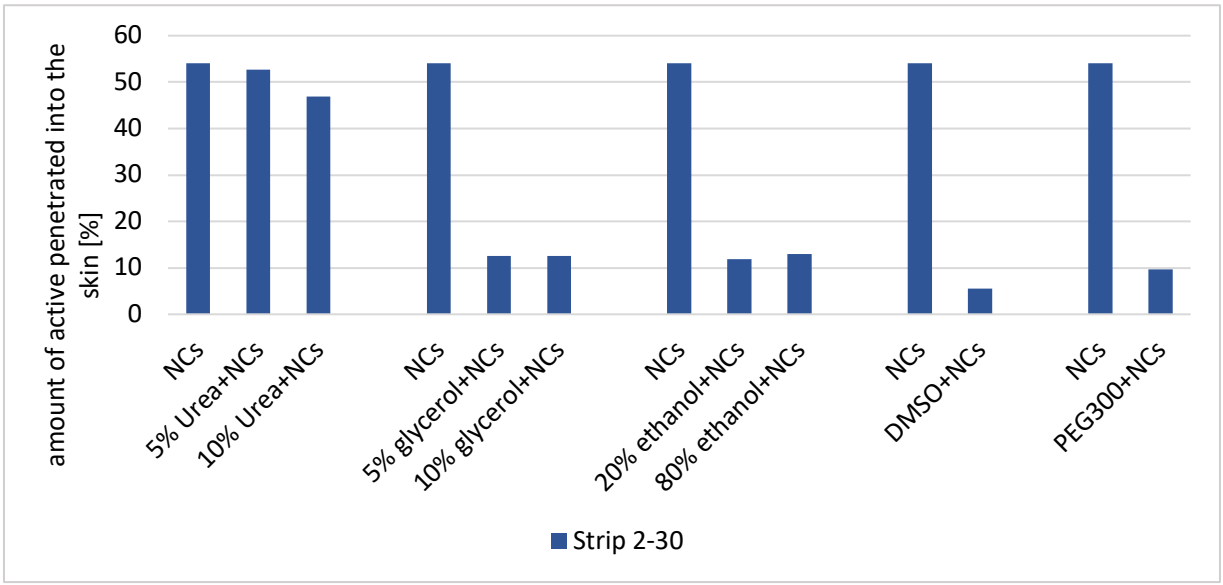

Figure 5: Influence of different excipients on the penetration efficacy of hesperetin from nanocrystals (NCs) with a size of about 400 nm.

A possible explanation for these results could be the hydrophobic nature of the compound hesperetin. Neubert et al. suggest that actives may penetrate the stratum corneum (SC) not only via the classical intercellular or inter cellular pathway (fig. 6A) but may also use a so called corneo-desmosomal (hydrophilic) pathway (fig. 6B). The assumption is based on new findings regarding the molecular structure of the SC. In vitro studies using skin lipid mixtures, e.g. ceramides, cholesterol, cholesterol sulphate und palmitic acid, and the use of neutron scattering proved the existence of lipid bilayers in the stratum corneum [21–26]. This means skin lipids, that are composed of amphiphilic molecules, arrange in symmetric structures, where lipophilic and hydrophilic heads are grouped together (fig. 6B). This results in the formation of hydrophilic and lipophilic regions respectively. Consequently, it is hypothesized that hydrophilic compounds are more prone to penetrate through the hydrophilic regions, whereas lipophilic active will more likely penetrate via the apolar regions (fig. 6B).

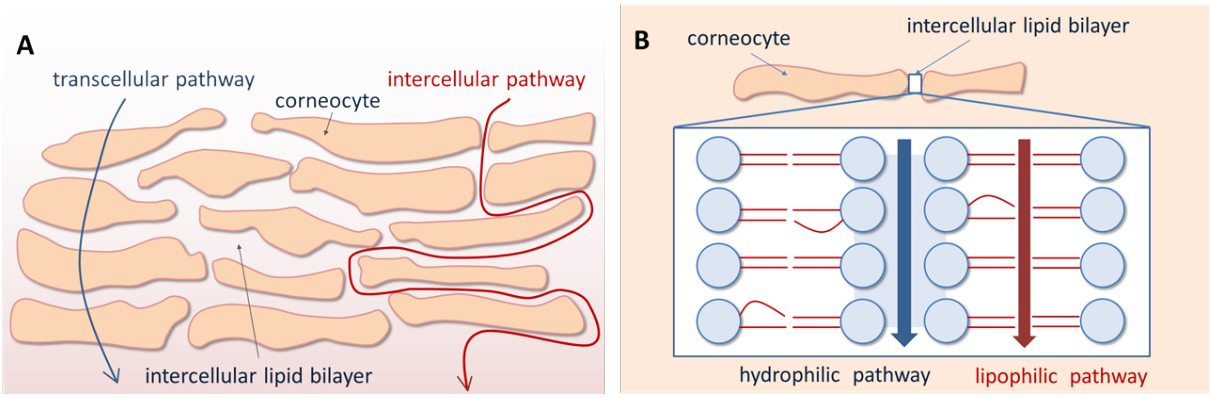

Figure 6: Scheme of dermal penetration of actives (modified after [26]).

Based on these findings it was further suggested that different excipients will promote the penetration of actives in a different way. Hydrophilic excipients, i.e. urea, will promote the penetration via the hydrophilic pathway, whereas lipophilic excipients are rather believed to promote the hydrophobic pathway. This means, in a very simple way, one could imagine the SC as sponge-like skeleton. If the "sponge" is soaked with hydrophilic compounds, or compounds that increase the water content in the skin, an increased penetration of hydrophilic actives is expected, whereas the penetration of lipophilic actives will be reduced and vice versa (fig. 7).

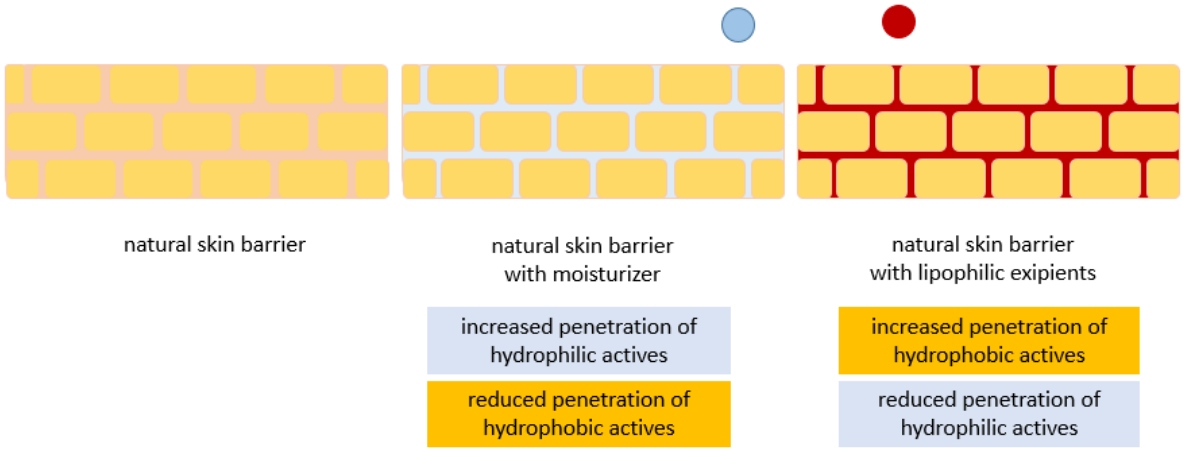

Figure 7: Scheme of dermal penetration – influence of vehicle and excipients on penetration efficacy of hydrophilic and hydrophobic actives.

To investigate if this theory might be applicable for the penetration of poorly soluble actives from nanocrystals, a hydrophilic and a lipophilic excipient was applied to the skin prior to the application of the nanocrystals. In addition to the penetration efficacy, transepidermal water loss (TEWL) and skin hydration were analysed. Results confirm an increase in skin hydration and TEWL when urea is used as excipient, whereas both skin parameters are reduced if olive oil is used as excipient (fig. 8). Also, the results obtained from the tape stripping experiments were in line with this theory, e.g. urea hampered the penetration of hesperetin from nanocrystals, whereas olive oil increased it, even though to a limited extend (fig. 9).

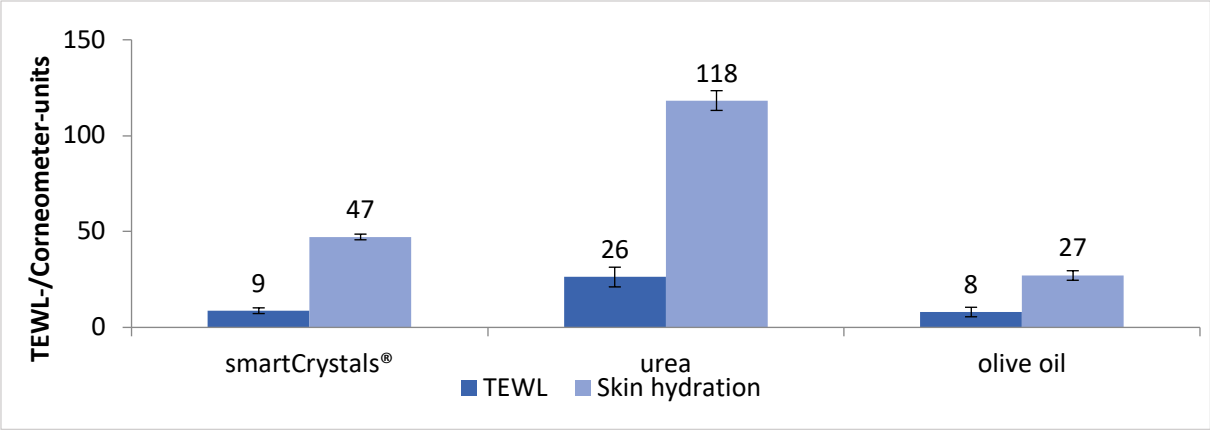

Figure 8: Influence of urea and oil on TEWL and skin hydration.

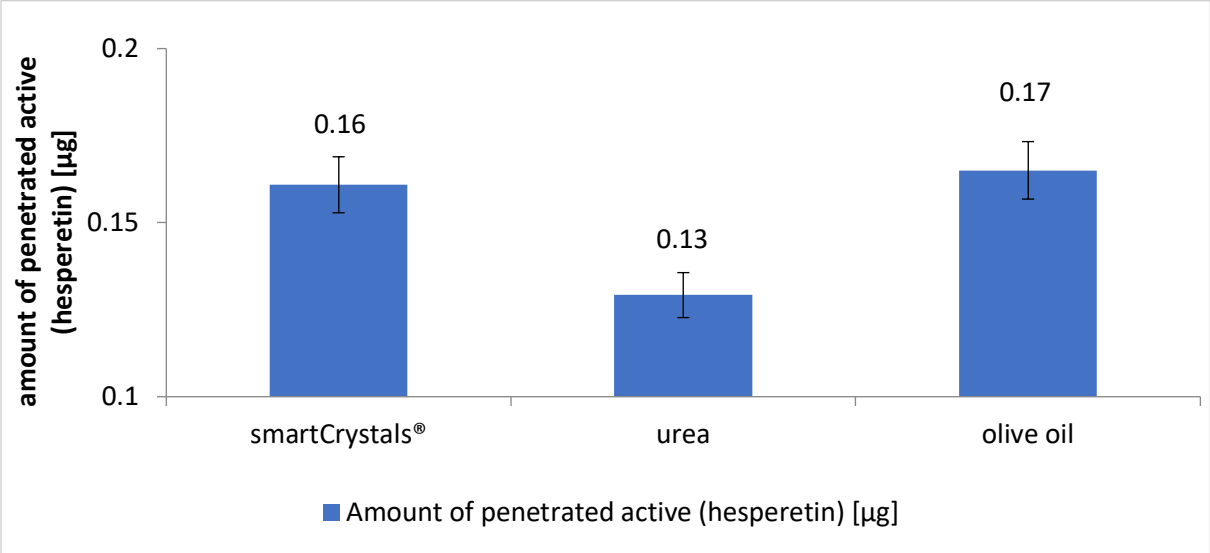

Figure 9: Influence of urea and oil on the penetration efficacy of hesperetin from nanocrystals with a size of about 400 nm.

Based on these findings it was now interesting to extend these studies to real dermal formulations. Therefore, in the last part of this study, nanocrystals were incorporated into a hydrogel, an oleogel and a cream, containing both hydrophilic and lipophilic excipients. Results confirm that incorporation of nanocrystals into the hydrogel results in a very limited penetration of the active into the skin. Incorporation into an oleogel resulted in a 400-fold higher penetration and incorporation into the cream increased the penetration even more than 2500-fold (fig. 10). However, not only the total amount, but also the depth of penetration was influenced by the type of vehicle. When the hydrogel was used as vehicle, no penetration into deeper layers of the SC was detected. Petroleum jelly increased the penetration and penetration depth and cream as vehicle was able to reach even deeper layers of the SC (fig. 11).

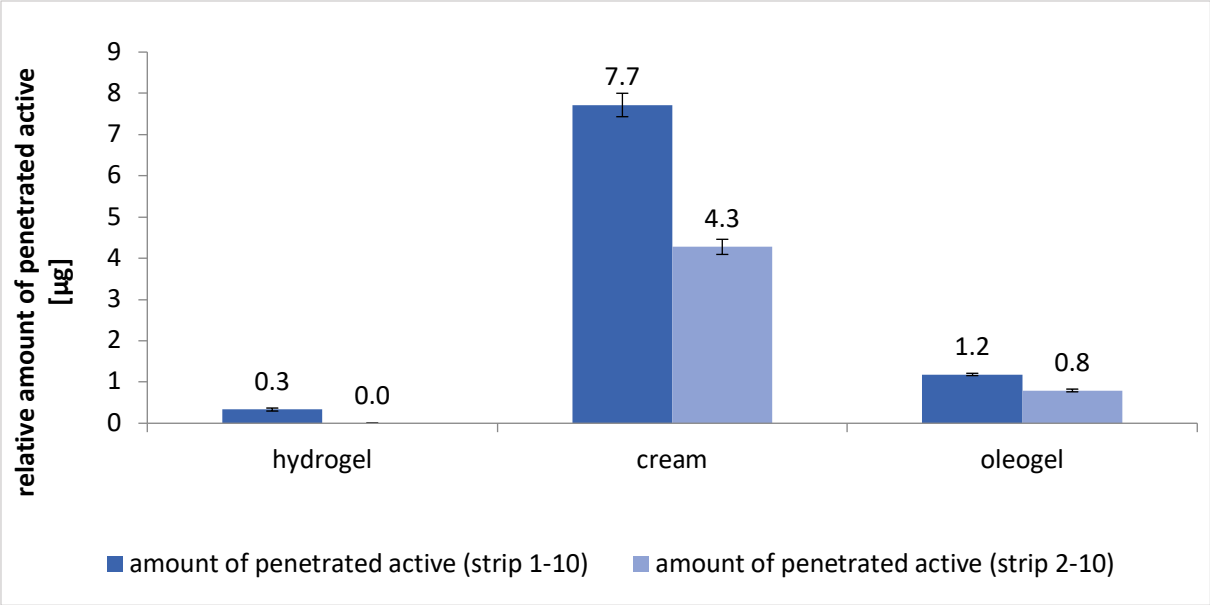

Figure 10: Influence of vehicle on the penetration efficacy of rutin from nanocrystals with a size of about 400 nm – total amounts penetrated.

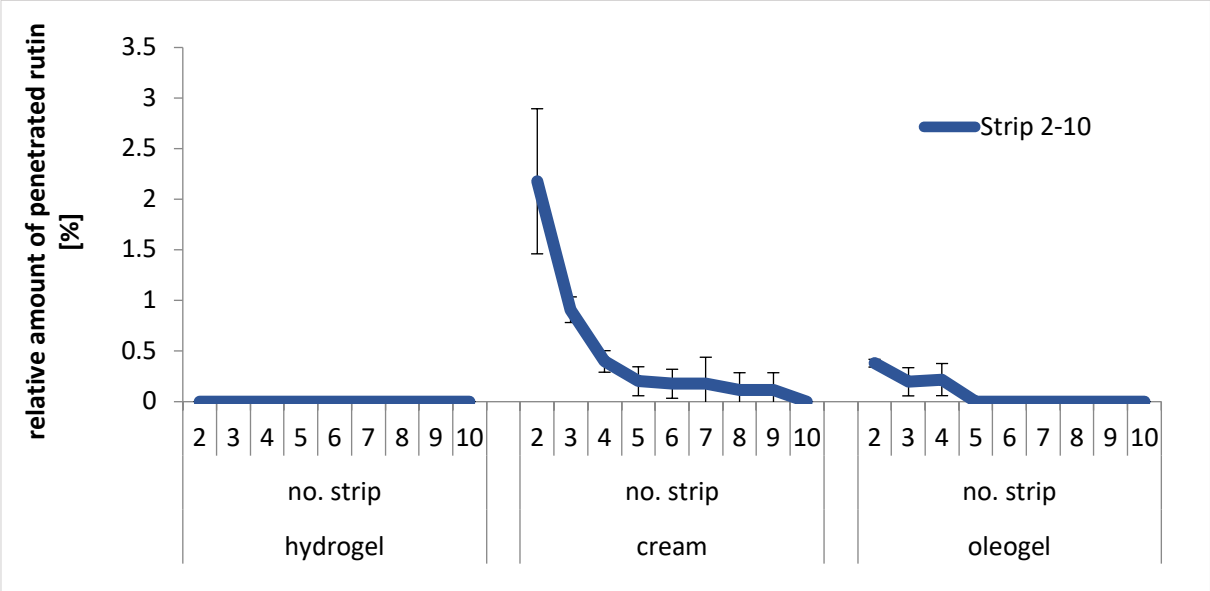

Figure 11: Influence of vehicle on the penetration efficacy of rutin from nanocrystals with a size of about 400 nm – penetration profiles.

Results prove how greatly vehicles contribute to the penetration of dermally applied actives. Data indicate that hydrogels are not suitable for the formulation of nanocrystals for improved passive diffusion of poorly soluble actives, whereas oleogels seem to be a better choice. The cream used in this study was superior and resulted in an excellent penetration of the active. This was again not expected initially, because it contains both, water (40%) and propylene glycol (10%) as penetration enhancer, thus limited penetration would be expected. On the other hand 50% of the ingredients of the cream are of lipophilic nature and especially Miglyol (7.5%) is described by Neubert et al. to promote dermal penetration of lipophilic actives by improving the penetration via the apolar pathway

[21, 26, 27]. Thus, it can be concluded that the effect of different ingredients might lead to interactions that might not be predictable with today's knowledge.

Also, other factors might influence the penetration efficacy of the active from different formulations. Based on Fick's law, which is generally used to describe the dermal penetration of actives from dermal formulations, the concentration gradient, i.e. the amount of dissolved active in the vehicle, is a major driving parameter for the dermal penetration of actives. As described above, nanocrystals possess an increased solubility when compared to larger sized bulk material and thus promote passive diffusion of poorly soluble actives. However, when considering the data obtained in this study, and by assuming that the type of excipient used is not the only driving parameter for improved dermal penetration, it might be worse to analyse the influence of the vehicle on the concentration gradient. A recently performed study by Vidlářová et al. revealed that low viscosity of a vehicle improves the penetration of actives from nanocrystals and that the saturation solubility is not the only determining factor for the penetration. They proposed a new mechanism of penetration of actives from nanocrystals and suggest that the local concentration of dissolved active on the site of application and not the concentration in the whole formulation is the determining parameter for optimal penetration of actives from nanocrystals [28]. This means dermal penetration would be further favoured if the amount of nanocrystals which are in direct contact with skin is high, as this would lead to many high local concentrations of active on the skin (fig. 12).

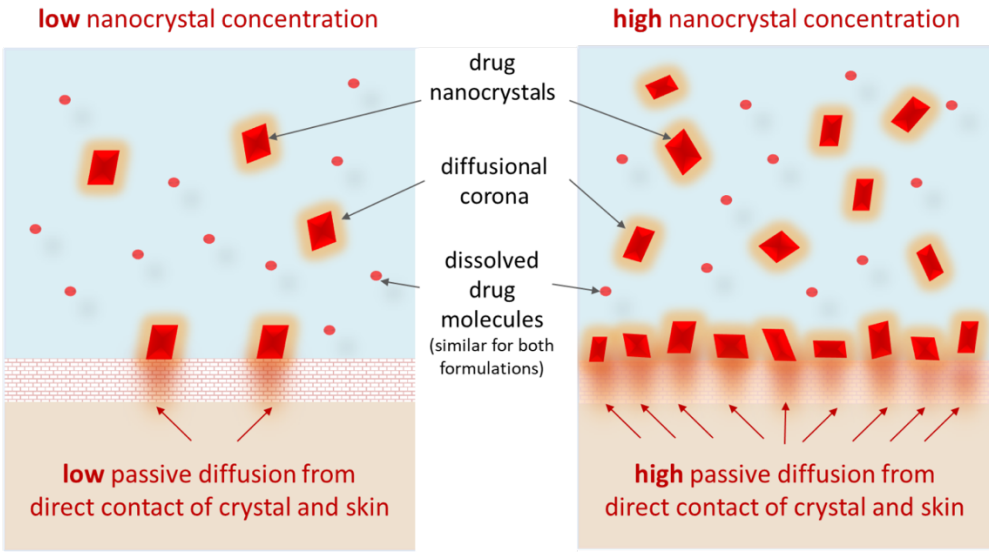

Figure 12: Influence of number of nanocrystals on penetration efficacy of poorly soluble actives from nanocrystals (modified after [28]).

To investigate if this effect might also apply for the formulations used in this study, microscopic images of the formulations were taken (fig. 13). The nanocrystals are homogeneously distributed within the hydrogel. In the cream the nanocrystals are also homogeneously distributed in the water phase. However, the number of nanocrystals/ml in the water phase is about two times higher, because the formulation consists of 50% aqueous phase and 50% lipophilic phase. The elevated levels of

nanocrystals might therefore further contribute to the improved penetration from the cream. In the petroleum jelly the nanocrystals are dispersed as droplets within the lipophilic outer phase. Within the droplets the concentration of the nanocrystals is high and corresponds to the concentration of the original nanosuspensions, i.e. 0.1% active. However, the droplets are dispersed as inner phase within the vehicle, hence, upon dermal application of this formulation, only limited amounts of water droplets containing nanocrystals might get in direct contact with the skin, thus explaining – at least in parts – the lower penetration efficacy of the active from this formulation.

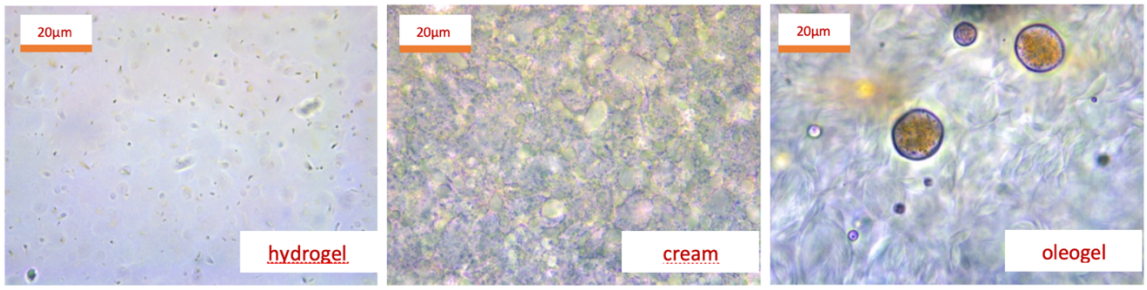

Figure 13: Microscopic images of dermal formulations containing 0.1% rutin nanocrystals. Left – hydrogel, middle – cream, right – oleogel.

4. Conclusions

Nanocrystals of different sizes were produced in this study and tested in regard to dermal penetration efficacy. Results prove that nanocrystals can be used to enhance the dermal penetration of poorly soluble actives in a size dependent manner, i.e. small sized nanocrystals improved the penetration of poorly soluble actives and promoted a deeper penetration of the active into deeper skin layers.

Data obtained in this study also prove the tremendous influence of vehicle and excipients on the penetration efficacy of actives from nanocrystals. Hydrophilic excipients and vehicles, e.g. urea, glycerol, ethanol, propylene glycol or a hydrogel decreased the penetration of poorly soluble actives, whereas oil, cream and oleogel increased it. The reason might be the improvement of the hydrophilic pathway with hydrophilic excipients and vehicles, which in turn hampers the penetration of poorly soluble, hydrophobic actives. Based on the results it suggested to avoid excipients and vehicles which promote the hydrophilic pathway and to use excipients which improve the apolar pathway. Hydrogels, which were often used as vehicle to formulate nanocrystals in the past, were identified as non-suitable vehicles for the formulation of nanocrystals. Improved dermal penetration can be obtained, if high numbers of nanocrystals can come into direct contact with the skin after dermal application. This is achieved if highly concentrated nanocrystals are used or – more cost efficiently – if creams with high lipid content and low water content (in which the nanocrystals are dispersed) are used as vehicles.

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3.3.

Dermal penetration analysis of curcumin in an ex-vivo porcine ear model using epifluorescence microscopy and digital image processing

Dermal penetration analysis of curcumin in an ex-vivo porcine ear model using epifluorescence microscopy and digital image processing

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Abstract

Curcumin is a promising drug candidate, but its use for dermal application is limited due to its poor aqueous solubility. Thus, formulations that increase the solubility of curcumin are needed to fully exploit the therapeutic potential of curcumin. Various previous studies address this issue, but a comparison of the efficacy between these formulations remains difficult. The reason for this is a missing standard formulation as benchmark control and an easy to use skin penetration model that allows for a fast discrimination between different formulations. Thus, the aims of this study were the development of a curcumin standard formulation and a screening tool that allows for a fast discrimination between of the dermal penetration efficacies of curcumin from different formulations. Ethanolic curcumin solutions were selected as simple and easy to produce standard formulations and the ex-vivo porcine ear model, coupled with epifluorescence microscopy and subsequent digital image analysis was utilized to determine the dermal penetration efficacy of curcumin from the different formulations. Results show that the utilized skin penetration model is a suitable and versatile tool that enables not only a fast determination of the dermal penetration efficacy of curcumin from different formulations but also a detailed und mechanistic information on the fate of chemical compounds after dermal penetration. Ethanolic solutions containing 0.25% curcumin were found to be the most suitable standard formulation. Results of the study provide a new, effective screening tool for the development of dermal formulations for improved dermal delivery of curcumin.

1. Introduction

Curcumin is a poorly water-soluble compound that is well-known for its diverse pharmacological properties. Examples include antioxidative, antibacterial, antifungal, antitumor or anti-inflammatory activities [1, 2]. Besides systemic administration, also the dermal application of curcumin can be very useful [3]. For example, it can be used for the treatment of acne, alopecia, atopic dermatitis, facial photoaging, oral lichen planus, pruritus, psoriasis, radiodermatitis or vitiligo [4]. Recent studies could further demonstrate the positive effects of curcumin on the wound healing process, the management of wound restoration and scare treatment [5–7].

Unfortunately, the use of curcumin is limited due to its poor absorption, i.e. poor bioavailability, which is caused by its low solubility in water [8]. Consequently, innovative formulations that can overcome its poor aqueous solubility are needed to exploit the full potential of curcumin. In this sense, many scientific studies addressed this issue and many of those could already prove the beneficial effects of innovative drug delivery principles on the dermal penetration efficacy of curcumin [9–29]. However, a direct comparison of the results from the different studies and a reliable decision which formulations are most promising in comparison to the others is not possible. Two main reasons are responsible for this. The first reason is a missing internal standard formulation for curcumin and the second reason is that the dermal penetration efficacy is often determined with different models, methods and/or measurement setups.

Conducting skin penetration experiments with the currently available methods (e.g. the tests based on the OECD guidelines or the classical tape stripping procedure) is tedious work, requires good skills and highly sensitive analytical equipment to obtain reliable and reproducible data [30–33]. A comparison between data remains often complicated, because many variables – for example differences in the type of skin, skin thickness, skin integrity, sample preparation, skin preparation, type and amount of acceptor medium and of course the penetration time – can lead to great variations in the results obtained [33–39]. In addition, results obtained from these studies, for example from the classical tape stripping procedure, cannot always provide the full picture of the fade of the chemical substance after dermal application. This means, at present a skin penetration model that allows for a simple but meaningful comparison of the dermal penetration efficacy of curcumin from different formulations is not yet available.

Therefore, to allow for a better comparison of the penetration efficacy between different curcumin formulations, the aim of this study was to develop a skin penetration model that would enable a fast discrimination between “good” and “bad” penetrating curcumin formulations. Still, enabling a detailed information on drug penetration and fate of curcumin after dermal application.

1.1. Theoretical aspects for the development of the skin penetration model

A simple skin model should be easy to use and should not require extensive practical experience to perform such studies. In addition, the model should mimic physiological skin conditions and thus, any manipulation of the skin prior to the experiments should be avoided. Sample preparation and data analysis should be fast and – if possible – should not require any high-price analytical equipment.

Today, in many cases, dermal penetration is determined in-vivo or in-vitro [30–32, 40]. Following the 3R strategy, in-vivo methods should be avoided whenever possible and thus, are not appropriate to be used for early formulation development and optimization. In-vitro models, e.g. Franz-diffusion cells, are not easy to handle, and experiments require good skills and are tedious work (c.v. section above). Hence, the new model should overcome these disadvantages.

A possible model for this is the ex-vivo pig ear model. It uses fresh pig ears on which the formulations are directly applied. Hence, the skin is not manipulated prior to the experiment, i.e. cut, squeezed, or stretched, but remains in its original state throughout the whole experiment. The ex-vivo pig ear model is typically used for the classical tape stripping procedure [31, 41–44], but can also be used to evaluate the penetration depth into the stratum corneum by epifluorescence microscopy if the drug substance is fluorescent [26]. For this, punch biopsies of the treated skin areas are taken, cut into thin slices, and analysed via confocal laser scanning microscopy or simply by fluorescence microscopy (Fig. 1).

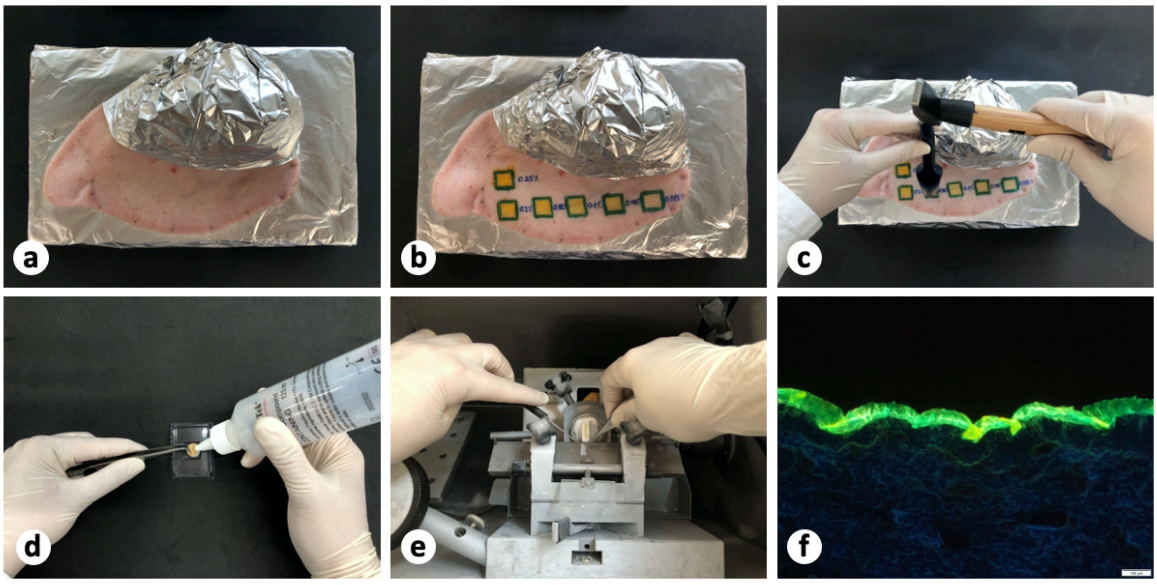


Figure 1: Scheme of ex-vivo skin permeation model. A: fresh pig ear with test formulations, B: skin biopsy, C: embedding of the skin biopsy, D: skin sectioning with cryomicrotome, E: image of skin section visualizing the fate of an auto fluorescent chemical compound (model drug used: curcumin) after dermal application.

Curcumin possesses a strong autofluorescence and thus can be simply visualized by means of epifluorescence microscopy (Fig. 1, E). However, any quantification of the penetration efficacy cannot be done by simple visual inspection of the images obtained. Hence, the present method would not

allow for an objective comparison of the dermal penetration efficacy of curcumin from different formulations.

Of course, the problem that simple visual inspection of images cannot be used for a quantitative analysis of the data is not novel and also very prominent in other disciplines. Hence, the problem is well understood and solutions to overcome this issue are already exploited in various other disciplines. One method to analyse images and to transfer the data from visual inspection into objective measurable results, is the use of image analysis with ImageJ software [45, 46]. ImageJ software enables processing of images and image analysis for various disciplines, e.g. in biology, medicine or biochemistry [47–56]. The extensive use of the ImageJ tool for image analysis in other disciplines and the broad spectrum of analysis tools being available in ImageJ software encouraged us to investigate if the software tool can also be used to evaluate the dermal penetration efficacy of curcumin.

1.2. Theoretical aspects for a curcumin standard formulation

A skin model investigating the penetration efficacy of curcumin should include the use of an internal standard. The internal curcumin standard should be easy to prepare and should give reproducible dermal penetration results. Ideally, a positive and a negative control standard should be available, i.e. curcumin standards mimicking “good” and “bad” dermal penetration, respectively. Hence, curcumin formulations leading to “good” and “bad” dermal penetration need to be identified for this.

Dermal penetration can be described by using Fick’s first law of diffusion (formula 1). In this formula dQ/dt, which is also referred to the flux, is the amount of a chemical substance penetrating out of the vehicle over time. D is the diffusion coefficient of the chemical molecule in the stratum corneum, V_k the partition coefficient, A the area of the skin on which the formulation was applied, d the thickness of the stratum corneum and c_v the concentration of dissolved chemical substance in the vehicle, respectively.

$$\frac{dQ}{dt} = \frac{D \cdot V_k \cdot A}{d} \cdot c_v \qquad \textit{(formula 1: Fick's 1st law of diffusion)}$$

Based on this law the concentration gradient, i.e. c_v, which is the amount of dissolved active in the formulation, can be defined to be one of the most driving parameters to yield sufficient flux values, i.e. good penetration into the skin. As curcumin is poorly soluble in water, but soluble in ethanol, good penetration of curcumin can be expected when using ethanolic solutions of curcumin for dermal drug delivery of curcumin. Ethanolic solutions are simple to prepare and thus represent a good formulation approach that could be used as internal standard.

Based on Fick’s law it can be expected that the amount of penetrated curcumin increases with an increasing concentration of dissolved curcumin. Hence, good penetration can be expected especially

from ethanolic solutions that contain high amounts of dissolved curcumin, whereas lower concentrations are expected to lead to poor penetration (total amount penetrated and depth of penetration).

A very recent study investigated the dermal curcumin delivery in vitro for different nanoemulsions and used an ethanolic curcumin solution with 0.5% (w/w) curcumin as reference, which was shown to enable good transdermal penetration of curcumin already after 3h of penetration [57]. Other studies that systematically investigate the penetration efficacy of curcumin from ethanolic solutions and the effect of the concentration of dissolved curcumin are not available. Consequently, this study also aimed at investigating the dermal penetration efficacy and the influence of the concentration of dissolved curcumin from ethanolic solutions to allow for the selection of appropriate curcumin standards that mimic “bad” and “good” penetrating formulations, respectively.

2. Materials and Methods

2.1. Materials

Curcumin was obtained from Receptura Apotheke (Cornelius-Apothekenbetriebs-OHG, Germany). Ethanol 99.8% v/v (HPLC grade) was obtained from Fischer Scientific GmbH (Germany).

2.2. Methods

2.2.1. Preparation of the ethanolic curcumin solutions

The solubility of curcumin is 10 mg/ml, corresponding to about 1% (m/v). Higher amounts of curcumin cannot be dissolved in ethanol and some studies even report lower solubility values [58], which was also confirmed by our data (Fig. 2). In this study solutions containing either 0.025, 0.05, 0.1, 0.15, 0.2 or 0.25% curcumin (m/v) were prepared. Higher concentrations were not prepared because ethanol is highly volatile and large amounts of the solvent can quickly evaporate upon dermal application. The reduced volume of solvent can lead to an increased concentration of curcumin and might create an oversaturated system, which could then easily lead to re-crystallization of curcumin and the formation of large, undissolved curcumin crystals on the skin. This would lead to a reduced amount of dissolved curcumin molecules and thus – based on Fick’s law (c.f. formula 1) – to a reduced flux of curcumin into the skin. To minimize this effect, curcumin concentrations below the saturation solubility of curcumin in ethanol were selected. The different ethanolic curcumin solutions were prepared by producing a stock solution containing 2.5 mg/ml curcumin. The stock solution was diluted accordingly to obtain the required test solutions. Solutions were freshly prepared and immediately used for the skin penetration experiments.

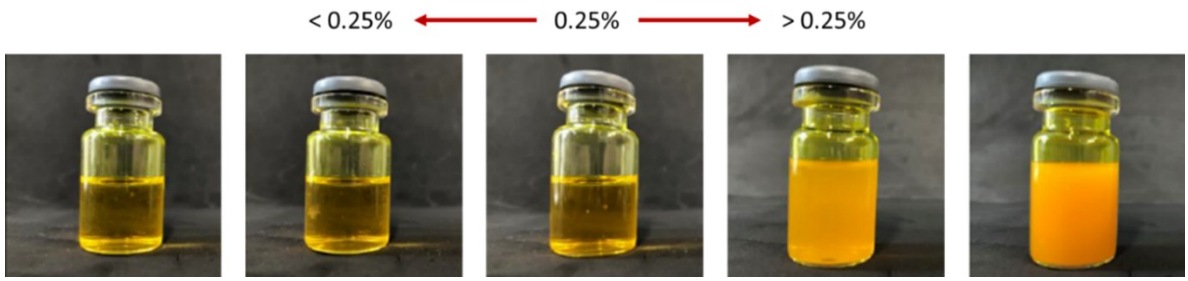


Figure 2. Ethanolic solutions of curcumin with different concentrations of curcumin. The solubility of 1% curcumin in ethanol – often cited in the literature – could not be obtained in this study. Reason for this might be the use of curcumin with a purity of only 80.87 % in this study, which decreased the solubility to < 0.5%.

2.2.2. Dermal penetration of curcumin

Dermal penetration of curcumin was assessed on the ex-vivo pig ear model [59]. Briefly, fresh porcine ears were obtained from a local slaughterhouse and cleaned with lukewarm water (23-25°C). The washed ears were dried with a soft tissue and the formulations were applied without massage on the dorsal site of the ear. The application area was 1.5 x 1.5 cm^2, the amount applicated was 25 µl and the penetration time was 4 h. During the penetration, ears were kept at a temperature of 32 °C to simulate external skin conditions. After the penetration time, non-penetrated formulation was carefully removed from the ear with a soft tissue. Afterwards, skin punch biopsies (Ø 15 mm) were taken, embedded with liquid embedding compound (Tissue-Tek® O.C.T.™, Sakura Finetek Europe B.V., Netherlands) and frozen until further use. All formulations were tested on three different ears and three different, independent skin areas, respectively.

2.2.3. Visualization of dermal penetration

Dermal penetration of curcumin was visualized via epifluorescence microscopy. For this, 20 µm thick sections of the frozen skin biopsies were prepared using a cryomicrotome (Frigocut-2700, Reichert-Jung, Germany). The slices were subsequently analysed using the inverted epifluorescence microscope Olympus CKX53 (Olympus Deutschland GmbH, Germany) equipped with an Olympus DP22 color camera (Olympus Deutschland GmbH, Germany). The samples were analyzed using the DAPI HC filter block system (excitation filter: 340 – 390 nm (LP), dichroic mirror: 410 nm, emission filter: 420 nm (LP)). All images were taken in the dark room under identical light conditions. The intensity of the fluorescence light source (130 W U-HGLGPS illumination system, Olympus Deutschland GmbH, Germany) was chosen to be 50% for all samples analyzed. The fluorescence images were taken at 200fold magnification with the software Olympus cellSens Entry (Olympus Deutschland GmbH, Germany). The exposure time was 50 ms.

2.2.4. Image analysis for semi-quantitative evaluation of penetration efficacy

Images obtained from epifluorescence microscopy were analysed with ImageJ software [45, 46]. The software was used to analyse the penetration depth of curcumin and to evaluate the total amount of curcumin that was able to penetrate into the skin.

2.2.4.1. Autoscaling

Prior to analysis all images were scaled by using the scale function of the software. This was done to synchronize the scale bar from microscopy to a specific number of pixels. The scale was set to 2.84 pixels/µm for the images with 200fold magnification.

2.2.4.2. Determination of penetration depth

The penetration depth was determined manually by analysing the maximal penetration depth of curcumin from each image. For this a line, corresponding to the maximum penetration depth of the respective biopsy or skin section, was manually placed in each image and the length of the line was then automatically analysed by the software.

2.2.4.3. Determination of the total amount of penetrated curcumin

The fluorescence intensity, this means the detected light intensity, is a measure for the total amount of curcumin penetrated into the skin. High intensities represent high amounts of penetrated curcumin and vice versa. In ImageJ analysis the light intensity is expressed in grey values (GV), i.e. high GV represent a high light intensity and low values correspond to low light intensity. The grey scale in ImageJ software varies from 0 – 255. Higher or lower values cannot be obtained. This means, when analysing an image with the ImageJ software, a GV ranging from 0 – 255 is assigned to each pixel within the selected area. From all the G-values that were analysed within an image or image section, the mean grey value (MGV) can be obtained. Hence, the MGV is the GV-average from all pixels in an image or image section, giving an estimate of the total intensity of detected light within the image or image section. Consequently, in our study, the MG-values were used as a measure to estimate the total amount of penetrated curcumin into the skin.

2.2.5. Statistical analysis

From each formulation 3 samples (biopsies) were obtained and from each of these biopsies 60 skin sections were prepared. Thus, covering all treated skin areas. By analysis of the prepared sections via the epifluorescence microscopy, about 30 representative pictures were taken for each biopsy, leading to at least 90 images for each formulation that were subsequently processed via digital image analysis. Descriptive statistics and the comparison of the mean values was performed with JASP software (version 0.13.1) [60]. Normal distribution was determined with the Shapiro-Wilk test and variance

homogeneity was tested with the Levene's test. The mean values of normally distributed data were compared by a one-way ANOVA and Welch-adoption was applied in case of variance heterogeneity. Tukey and Games-Howell post-hoc tests were performed to compare the mean values between each other. The non-parametric data were subjected to the Kruskal-Wallis analysis of variance with Dunn`s post-hoc tests [61]. Bonferroni-Holm adjustment was applied to avoid alpha error accumulation. P-values < 0.05 were considered statistically significant.

3. Results and Discussion

3.1. Determination of dermal penetration efficacy of curcumin from ethanolic solutions

3.1.1. Analysis of original images

Images obtained from the epifluorescence microscopy indicated – as expected – an increasing fluorescence intensity with an increasing concentration of curcumin (Fig. 3). However, by analysing the data with the ImageJ software, the trend could not be verified (Fig. 4). Instead, no correlation between the concentration of curcumin applied on the skin and the resulting fluorescence intensity (expressed as MGV) was observed. Hence, image analysis of the original images could not be used to evaluate the differences in the dermal penetration efficacy of curcumin form the different ethanolic solutions.

The reason for this was attributed to the relatively high autofluorescence of the untreated skin, which was not only high, but also highly variable (Fig. 5). Hence, in order to allow for a semiquantitative analysis of the amount of penetrated curcumin, it was necessary to define a threshold method that could be used prior to image analysis to reduce the autofluorescence of the skin to a minimum.

Thresholding is an algorithm that can be used to split the original image into two classes of pixels. In this study, class I pixel would be the pixels being related to the penetrated curcumin (foreground) and class II pixel would be the pixels being related to the autofluorescence of the skin (background). After image splitting only class I pixels would be kept for further analysis. Because only these are the pixels of interest, as they represent the amount of penetrated active. In contrast, the background pixels, i.e. the pixels being caused by the autofluorescence from the skin, will be discarded.

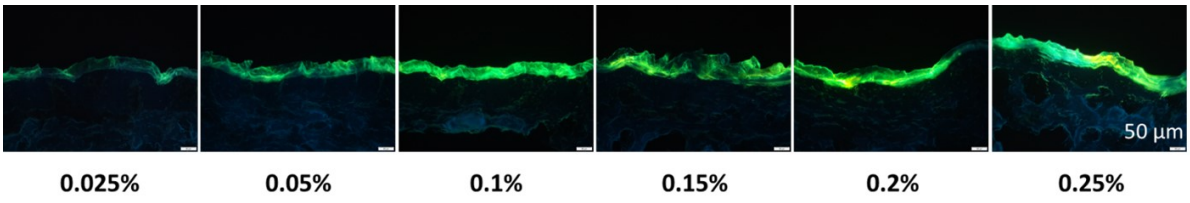


Figure 3: Visualisation of dermal penetration efficacy of curcumin form ethanolic solutions with different concentrations of dissolved curcumin (0.025-0.25%m/v) by epifluorescence microscopy (magnification 200x).

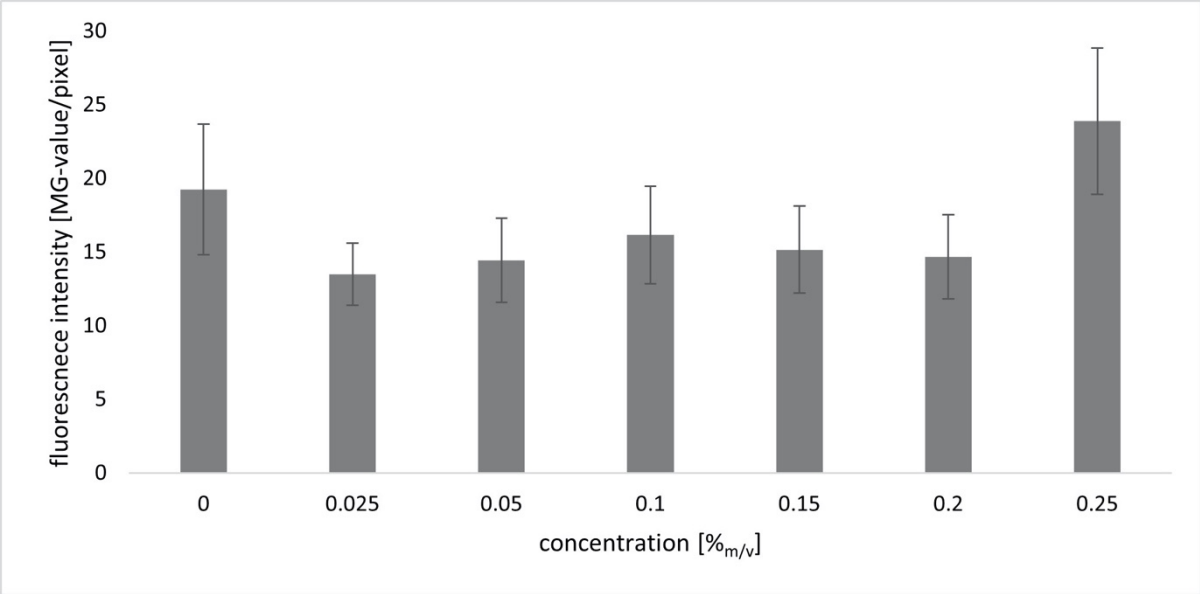


Figure 4: Influence of concentration on penetration efficacy of curcumin into the skin – determination of fluorescence intensity (mean grey values/pixel) form original fluorescence images.

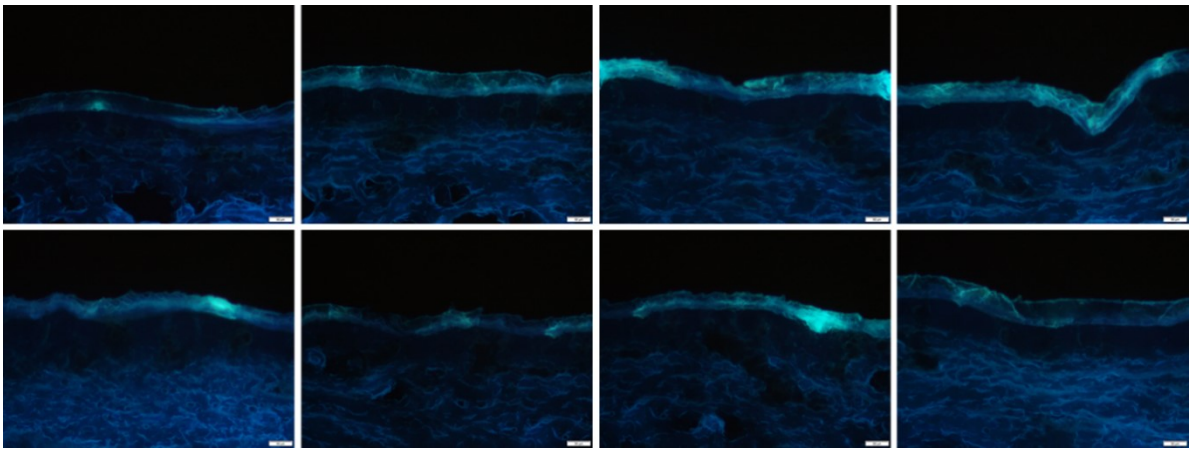


Figure 5: Images of non-treated skin obtained from epifluorescence microscopy (magnification 200x).

3.1.1.1. Determination of threshold algorithm

In a first attempt, the images of the skin were analysed for their respective red, green, and blue image components. Hence, based on the RGB colour model, images were spilt into separate images, each only containing one of the three RGB colours, i.e. the red, green, or blue component, respectively (Fig. 6). The colour-split algorithm revealed that the autofluorescence of the skin is mainly attributed to green and blue components and only very small amounts are contributed form the red channel (Fig. 6, left). In contrast, curcumin was found to contribute from all RGB-channels (Fig. 6, right). The pixels related to curcumin in the red channel were small for small concentrations, probably because only very small amounts of curcumin were able to penetrate the skin. Hence, bright spots, representing penetrated curcumin, were almost not visible in the images shown in Fig. 6. However, they became more visible by digital zooming of the images and by converting the black and white images into white and black images, respectively (Fig. 7). However, digital zooming and black/white inversion also

revealed that a simple colour split could not yet fully eliminate the autofluorescence of the skin. Hence, also in the red channel some minor signals were visible for the untreated skin (Fig. 7, left).

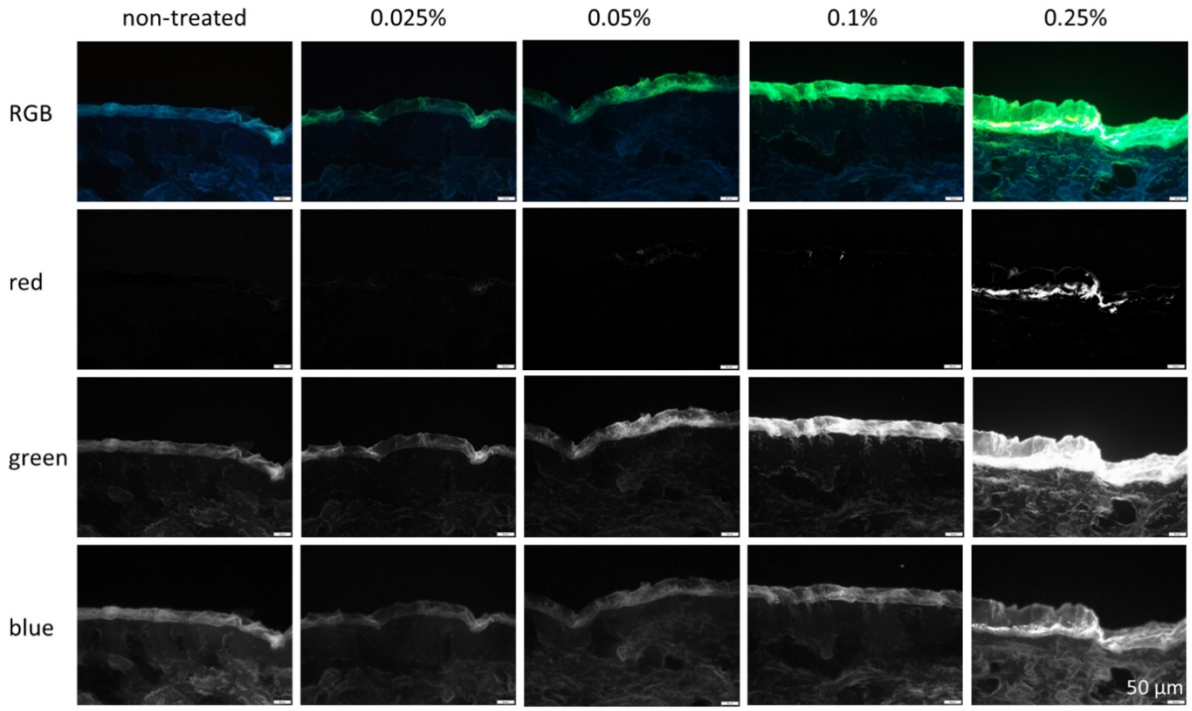


Figure 6: Images obtained from the color-split of the original RGB-images.

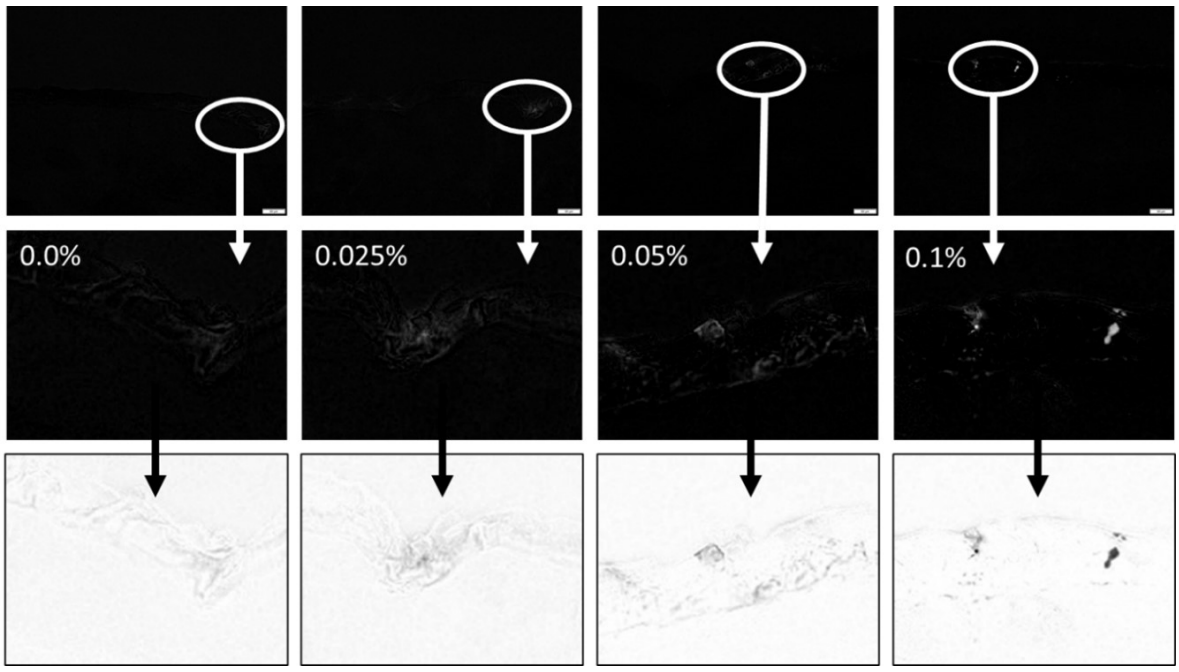


Figure 7: Red-component images obtained from the original images after color-splitting. Upper: original image; Middle: after digital zooming (white circles represent the enlarged area); Lower: inverted black/white images to visualize the detected light intensities and the differences between the different images.

Therefore, to further quench the autofluorescence of the skin, the amount of light intensity of skin in the red component was determined and compared to the intensity derived from curcumin. For this, GV-histograms of the red channel split images were created (Fig. 8). The histograms revealed that all

images possessed MG-values < 10. However, the width of the histograms was different. Curcumin treated biopsies revealed broad histograms and led to maximum grey values between 100 and 255. In contrast, most non-treated skin biopsies did not exceed maximum grey values > 33 (Fig. 8). Only some untreated skin biopsies yielded larger values, which were caused by some bright spots on the top of the stratum corneum. These bright spots on top of the skin can be attributed to dirt, sebum or bacteria, which can also possess high autofluorescence [62]. Remaining dirt, sebum and bacteria can be discarded by an optimized cleaning procedure and thus were not considered for the setting of the threshold for clean skin.

Based on the analysis of the histograms, the red component (red-MGV) contributing to the autofluorescence of clean skin was defined to include all red-MGV ≤ 33. Accordingly, all red-MGV ≤ 33 were defined to belong to class II pixels and only red-MGV > 33 were used as class I pixels for further analysis. Any pixels from the green and blue RGB-components were also not included. Subsequently, the defined threshold-algorithm (R>33, G=0, B=0) was programmed as ImageJ-macro (c.f. suppl. material-S1) and was subsequently applied for all images analysed in this study (for images obtained – c.f. suppl. material-S2).

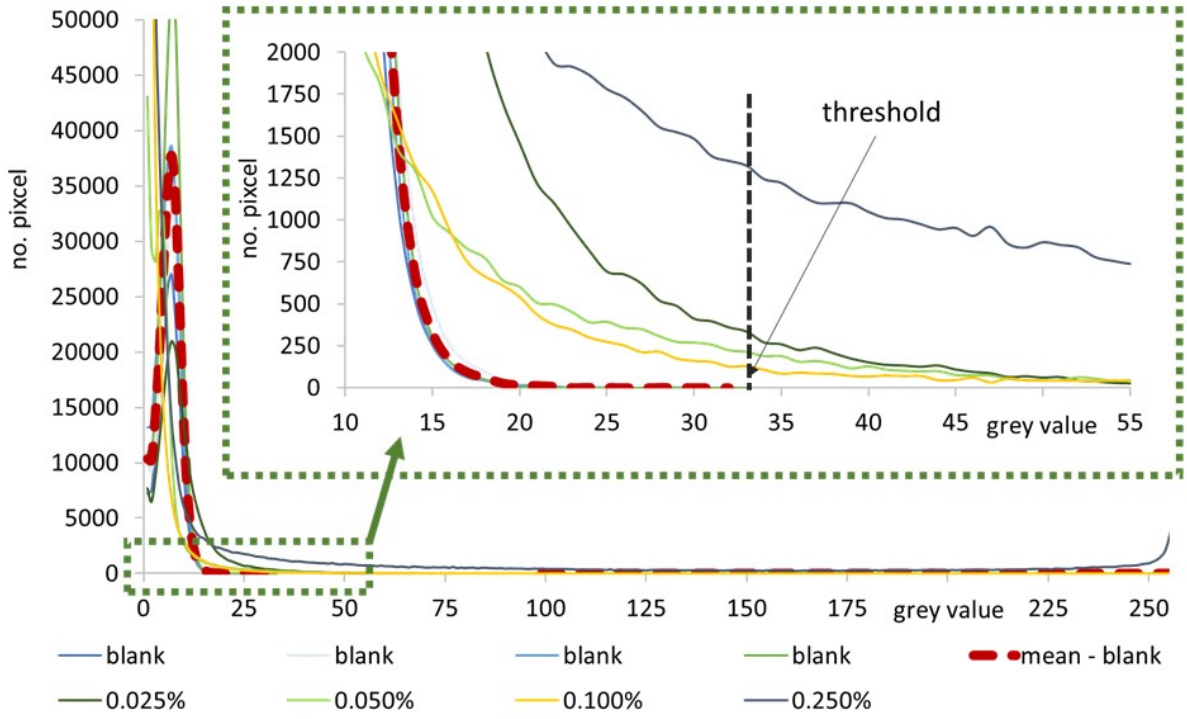


Figure 8: Histograms of grey-values from the red-component images obtained from biopsies of untreated (blank) skin and from biopsies treated with different concentrations of curcumin. Clean skin possesses maximum grey values of ≤33, meaning that greater values that are obtained in image analysis can be attributed to curcumin. Based on the results the threshold of the red-MGV was set to 33.

3.2. Analysis of thresholded images

3.2.1. Visual inspection

The trend of increasing fluorescence intensity with increasing concentration of curcumin was even more pronounced after the images were thresholded to minimize the influence of the autofluorescence for the skin (Fig. 9 – lower). Moreover, despite the increasing fluorescence intensity with increasing concentration, it could now be observed, that the samples that were treated with lower concentrations of curcumin showed high variations in regard to drug distribution within the skin (Fig. 9, 10). Hence, skin biopsies treated with low concentrations of curcumin possessed many blank skin areas, i.e. areas without any curcumin. The variability seemed to be concentration dependent and seemed to decrease with increasing drug concentration. Hence, with increasing curcumin concentrations, the number of blank skin areas decreased. The penetration depth was also affected by the concentration of dissolved curcumin and increased – as expected by Fick's law – with increasing amounts of dissolved curcumin. Lower concentrations led to a penetration of curcumin into the SC, concentrations ≥ 0.15% led to a penetration of the curcumin into the lower epidermis and only the formulation containing 0.25% curcumin enabled some transdermal penetration of the curcumin (Fig. 9, 10).

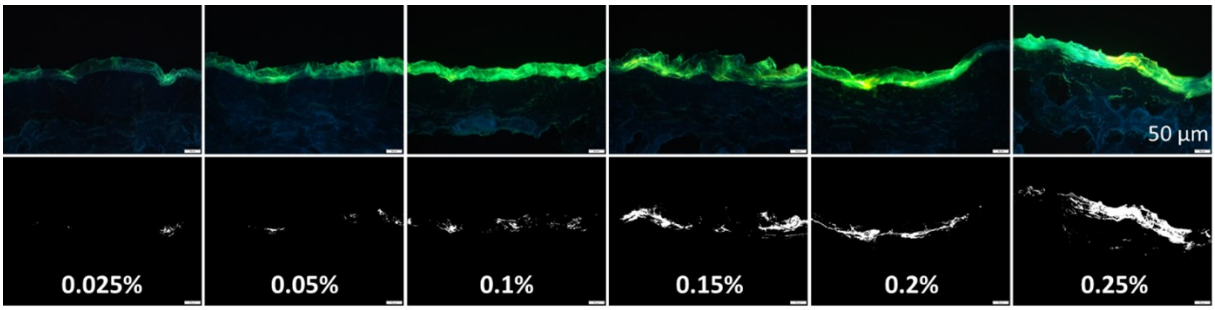


Figure 9: Visualisation of dermal penetration efficacy of curcumin form ethanolic solutions with different concentrations of dissolved curcumin (0.025-0.25%m/v) by epifluorescence microscopy (magnification 200x). Upper: original images, Lower: thresholded images (R>33, G=0, B=0).

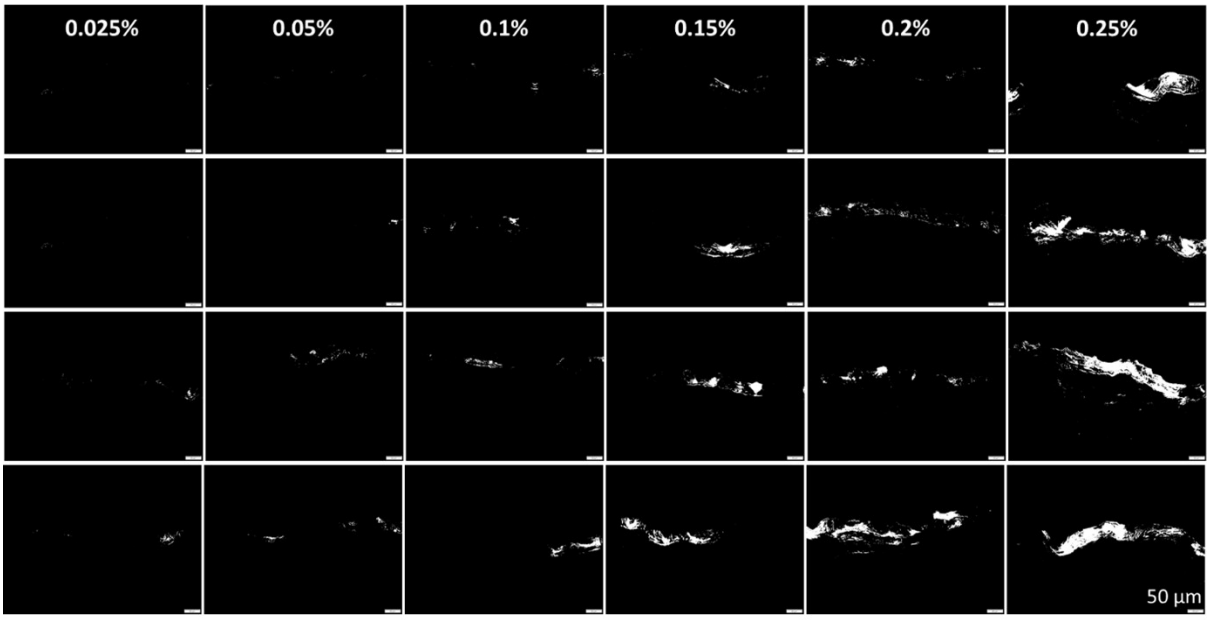


Figure 10: Representative images (magnification 200x) of the thresholded images (R>33, G=0, B=0) obtained from skin biopsies treated with different ethanolic curcumin solutions (0.025-0.25%m/v). Samples were found to possess strong inter- and intra-individual variations regarding drug distribution within the skin. To visualize this, for each curcumin concentration four images were selected. Two samples represent the average of observed penetration profiles (middle), one represents the poorest penetration profiles (upper) and one represents penetration profiles with the best penetration for each concentration tested.

3.2.2. Image analysis

Image analysis was used to transfer the results obtained from visual inspection of the images into objective measures. For this, the MG-values of the thresholded images were used as a measure for the total amount of penetrated curcumin. In addition, the penetration depth was evaluated. Results obtained could prove the observations from visual inspection, i.e. an increasing amount of penetrated curcumin and an increase in penetration depth with increasing concentration of dissolved curcumin in the ethanolic solutions (Fig. 11, 12). Both trends followed a linear progression with Pearson coefficients of about 0.9 (± 0.1). Thus, confirming the validity of Fick's law to predict the dermal penetration efficacy of chemicals. However, no statistical differences in MG-values and penetration depth were found between the biopsies treated with 0.025% and 0.05% curcumin and between the biopsies treated with 0.15% and 0.2% curcumin. Also, the penetration depth of the sample treated with 0.1% curcumin was not significantly different to the samples treated with 0.15% or 0.2% curcumin, respectively.

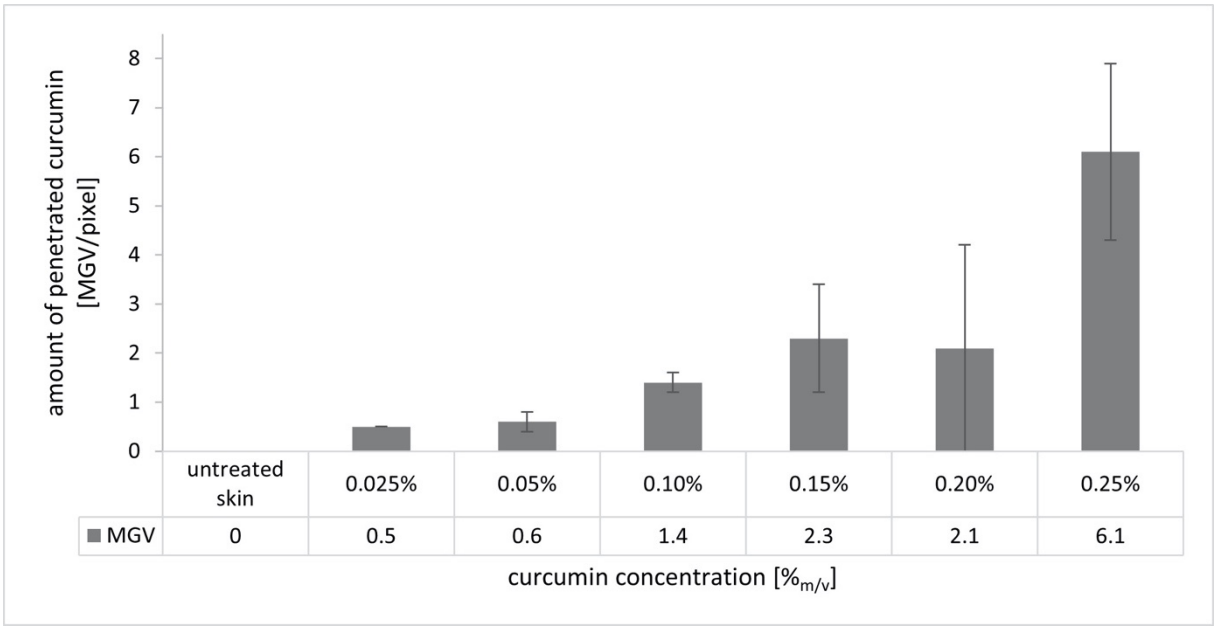


Figure 11: Influence of concentration of dissolved curcumin on dermal penetration efficacy of curcumin from ethanolic solutions – analysis of mean grey value (MGV) as surrogate for the total amount of penetrated curcumin.

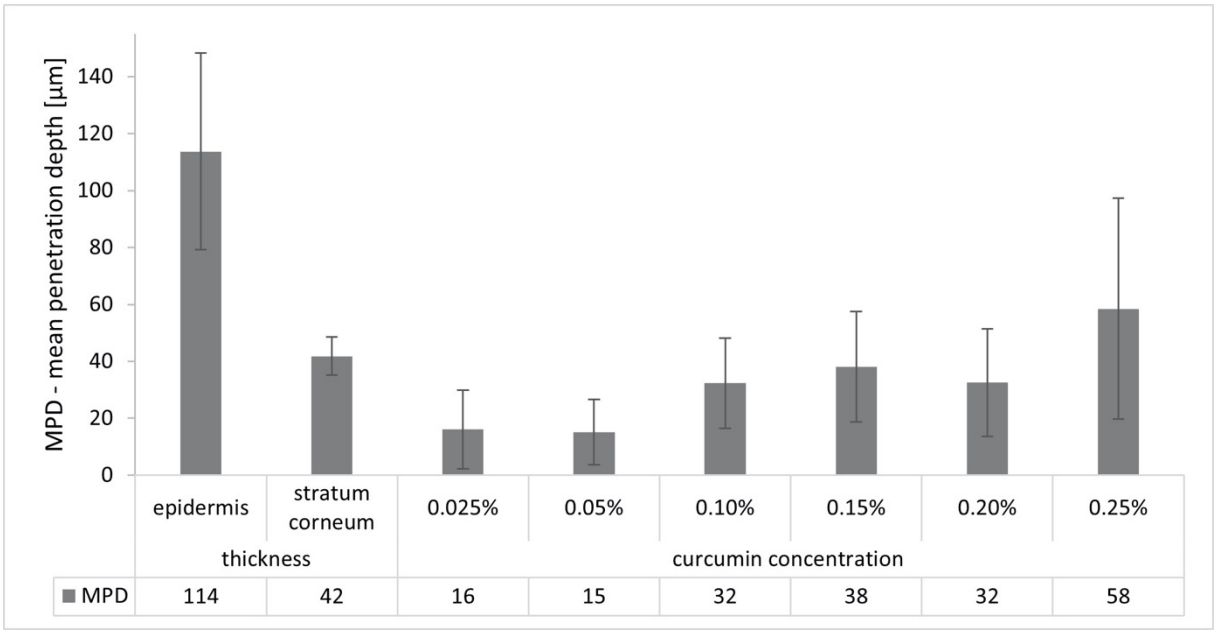


Figure 12: Influence of concentration of dissolved curcumin on dermal penetration efficacy of curcumin from ethanolic solutions – analysis of mean penetration depth (MPD). In addition to the penetration depth of curcumin the thickness of the stratum corneum and the epidermis was analysed. In this way the localization of curcumin in the skin can be estimated.

Based on the results obtained, the different curcumin formulations tested can be divided into three groups. Group 1 (curcumin concentrations 0.025% and 0.5%) led to poor penetration of curcumin. This was reflected by low MG-values and low penetration depths which only corresponded to about 33% of the thickness of the stratum corneum. Group 2 (curcumin concentrations from 0.1-0.2%) led to about 3fold higher MG-values and hence to good penetration of curcumin. In contrast to group 1, group 2 formulations were able to penetrate the whole stratum corneum (c.f. Fig. 12). Group 3 (represented by the formulation containing 0.25% curcumin) led to about 10fold higher MG-values

when compared to group 1 and to about 3fold higher MG-values, when compared to the group 2 formulations. The penetration depth was about 4times higher than for the group 1 formulations and about 2fold higher than for the group 2 formulations. The mean penetration depth (MPD) for the group 3 formulation was 58 µm and with this significantly greater than the thickness of the stratum corneum, which was determined to possess a thickness of about 42 µm (Fig. 12). This means the group 3 formulation was able to penetrate through the stratum corneum and could reach the deeper layers of the epidermis. However, the MPD of the group 3 formulation was still lower than the thickness of the whole epidermis (approx. 114 µm), indicating that also group 3 formulations were not able to enable transdermal penetration of curcumin into deeper layers of the viable dermis (c.f. Fig. 12). Nonetheless, in comparison to group 1 and 2, group 3 formulations were defined to lead to a very good dermal penetration of curcumin.

3.2.3. Determination of variability and inhomogeneity in dermal penetration

The MPD-value obtained from image analysis for the group 3 formulation indicated that transdermal penetration could not be achieved. However, this finding was opposite to the findings from the visual inspection of the images (c.f. section 3.2.1). A possible reason for these mismatching results might be the fact that for visual inspection representative images were selected, that means images that represented not only the average penetration profiles but also to the poorest and the best penetration profiles for each formulation, respectively. In contrast, image analysis only represents the average of all images analysed. Hence, the high variations that were observed from visual inspection, were not represented by the data obtained from image analysis by a sole analysis of the mean grey values. However, inhomogeneity and variations in skin penetration are of high importance, because only if a homogeneous penetration into the skin is obtained, a reliable and reproducible drug penetration and/or permeation can be assured. Therefore, it was interesting to define a method that would allow to estimate the different degrees of variation and inhomogeneity in skin penetration from the different samples.

As a first approach, histograms, that means diagrams plotting the frequency of the measured MGVs and penetration depths from each respective sample, were created for each concentration of curcumin (Fig. 13, 14). Different histograms were obtained for the different biopsies treated with different amounts of curcumin. Histograms for group 1 formulations were narrow, the peak maxima were high and represented small MGV and penetration depths, respectively.

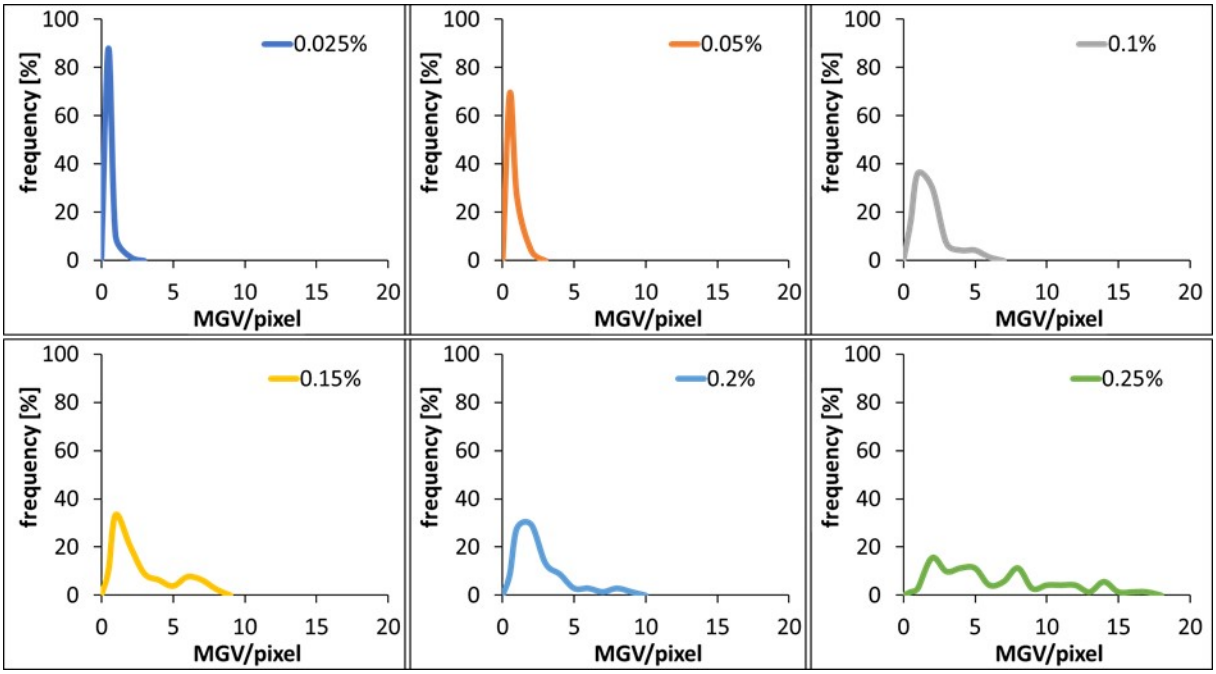


Figure 13: Histograms of MG-values representing the variations of the total amount of curcumin that was able to penetrate the skin. The histograms indicate that an increase in curcumin concentration leads to a broadening of the distribution curves and a constant shift of the mean grey value.

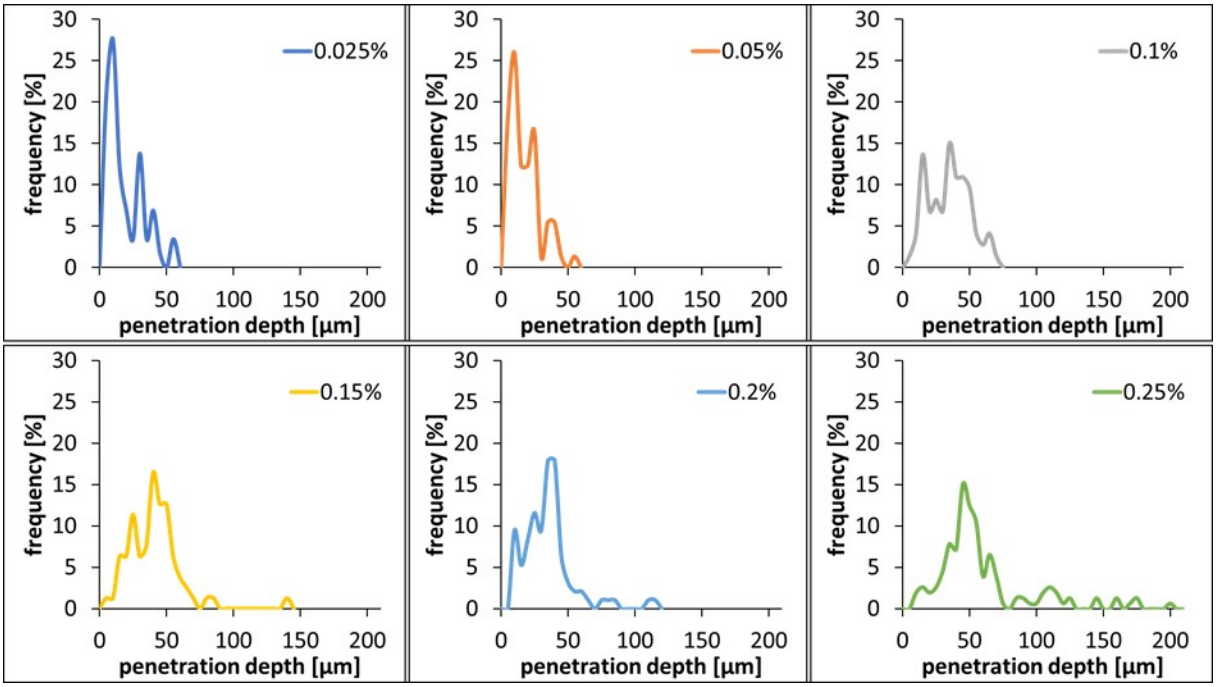


Figure 14: Histograms of mean penetration depth, representing the variations in penetration depth. The histograms indicate that an increase in curcumin concentration leads to a broadening of the distribution curves and a constant shift of the penetration depth.

Group 2 formulations showed broader distributions, lower peak maxima and represented higher MGV and penetration depths, respectively. The broadening of the histogram, the decrease in peak maximum and the increase in MGV and penetration depth was even more pronounced for the group 3 formulation.

The histograms could confirm the variations in skin penetration that were already seen from visual inspection. However, they were also able to provide a much deeper and more mechanistic understanding of the influence of the curcumin concentration on the dermal penetration efficacy of curcumin. For example, it can be seen from the histograms, that low concentrations of curcumin lead to many skin biopsies without any curcumin (blank areas). Treating the skin with increasing concentrations of curcumin, gradually reduces the number (frequency) of these blank areas and increases the number of biopsies with curcumin. Hence, skin penetration becomes more homogeneous and thus more reproducible with higher amounts of dissolved molecules within the vehicle. Moreover, the constant broadening of the histograms, the decrease in peak maximum and the constant shift of the peak to higher values, nicely visualizes that an increasing concentration of curcumin is not causing an abrupt increase in the penetration depth and in the total amount of penetrated active. Instead, it shows that there are not only two but three different parameters that can influence the dermal penetration efficacy of chemical substances. One parameter is of course the total amount of penetrated active and the second parameter is of course the respective penetration depth yielded.

However, the histograms revealed a third aspect, and this is that also the number of skin areas that take up curcumin and the number of biopsies or skin areas without uptake contribute to the all over penetration efficacy of a chemical substance. Hence, it can be assumed that formulations that will lead to similar results regarding the total amount of penetrated active, might possess differently distributed penetration patterns, i.e. histograms. One example could be a formulation that leads to homogeneous but poor penetration (Fig. 15, left) and another that leads to very inhomogeneous penetration into the skin but creates “bright spots” on the skin that possess very high skin penetration (Fig. 15, right).

As a reliable and reproducible drug penetration and/or permeation is the base for the development of functionable, effective and safe dermal drug products, a measure to express the variability in skin penetration might be useful. Therefore, to yield an estimate of the variability of the skin penetration from the different curcumin concentrations, data from the histograms were clustered and divided into four groups. Group 1 represented MG-values ≤ 0.61, representing the MGV obtained for the group 1 formulations, that represent poor penetration. Accordingly, group 2 and 3 values were set to be 2 and 6.1, representing good and very good penetration, respectively (c.f. Fig. 11). The fourth group represented all blank skin areas, that means biopsies with MG-values corresponding to blank, non-treated skin (Fig. 16). In a similar manner, clustered diagrams were also established for the penetration depth (Fig. 17).

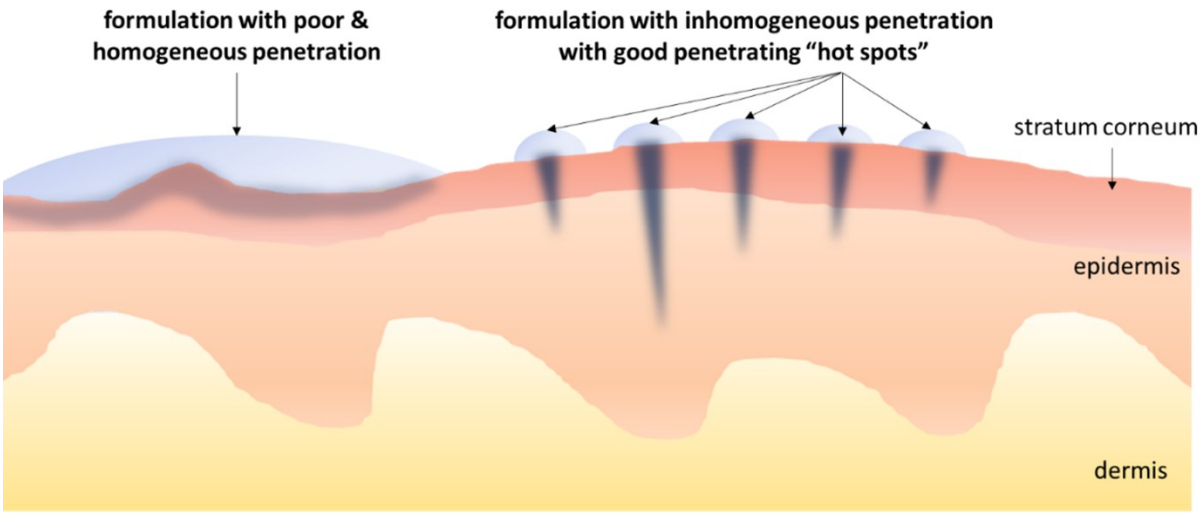
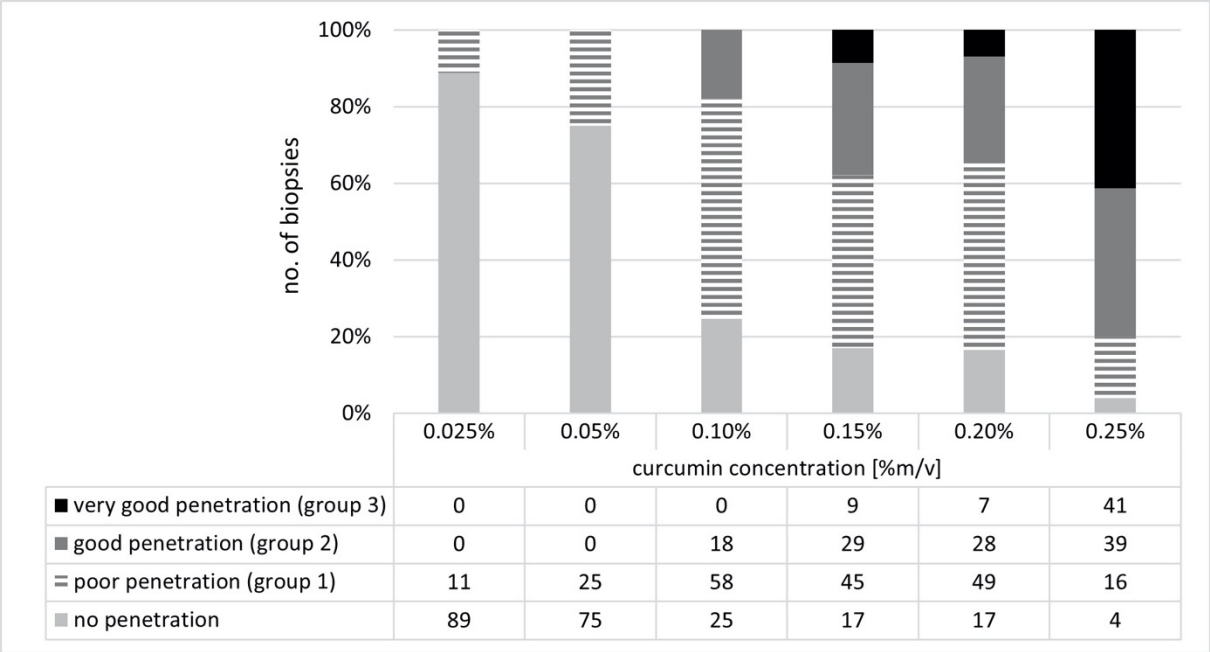


Figure 15: Scheme of possible penetration profiles of a chemical compound from different dermal formulations leading to similar amounts of total amounts of penetrated active. Left: poor and homogeneous penetration, right: inhomogeneous penetration with "hot spots" that cause high concentrations on some areas of the skin and leave other skin areas non-treated.

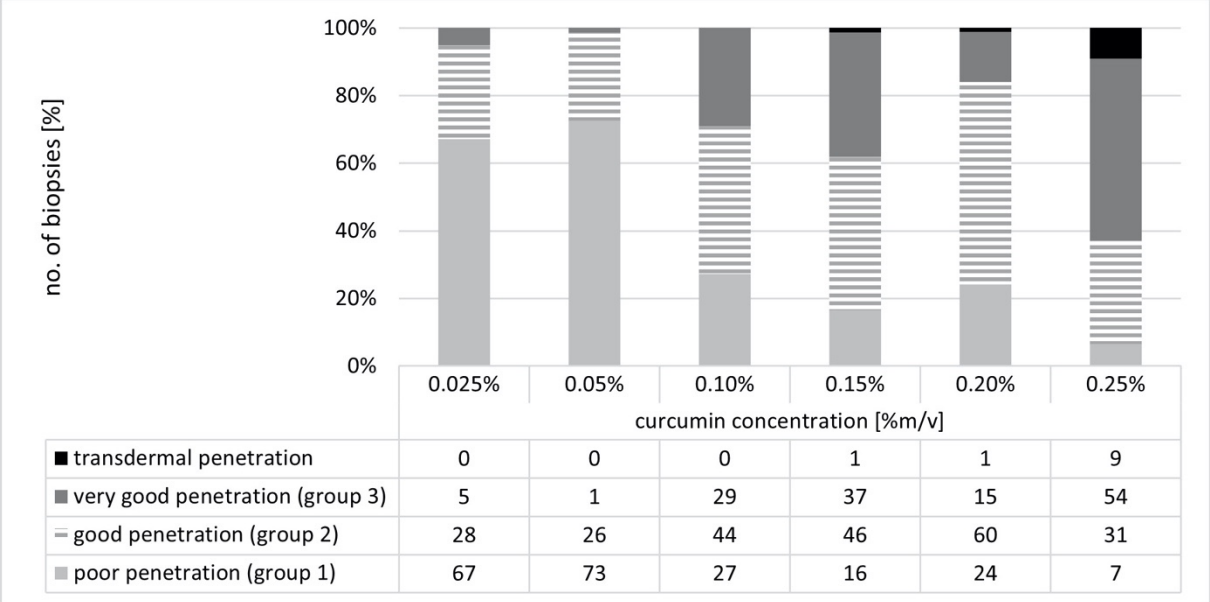
Results show that almost 90% of the skin biopsies treated with 0.025% curcumin did not show any uptake of curcumin. The number of blank skin areas ("dark spots") decreased with increasing concentrations of curcumin and was still 4% for the skin treated with 0.25% curcumin. Group 1 biopsies, representing poor penetration of curcumin, were not only present in the skin biopsies treated with 0.025% and 0.05% curcumin but were also found in biopsies treated with higher amounts of curcumin. The number of biopsies with poor penetration was about 50% for the skin samples treated with 0.1 – 0.2% curcumin solutions and was 16% for the skin treated with 0.25% curcumin. Good and very good penetration was not found for biopsies treated with low concentrations of curcumin and higher concentrations resulted in a constant increase in the number of biopsies possessing good or very good penetration. For example, a good penetration of curcumin was found for only 18% of the skin biopsies when the skin was treated with a 0.1% curcumin solution. Treating the skin with 0.15% or 0.2% curcumin doubled the number of biopsies with good and very good penetration and a treatment with a 0.25% curcumin solution increased the number of biopsies with good and very good penetration to even 80% (Fig. 16).

Similar trends could also be observed for the penetration depth (Fig. 17). Moreover, by defining an additional class, representing all biopsies with a penetration depth > 114 µm (value corresponds to the average of the thickness of the epidermis, c.f. Fig. 12), it was possible to estimate the number of biopsies possessing transdermal penetration of curcumin. No transdermal penetration was observed for the skin treated with 0.025%, 0.05% and 0.1% curcumin. Biopsies treated with 0.15% or 0.2% curcumin revealed transdermal penetration in 1% of the cases and almost 10% of all biopsies treated with 0.25% were found to show transdermal penetration of the curcumin (Fig. 17).



	0.025%	0.05%	0.10%	0.15%	0.20%	0.25%
very good penetration (group 3)	0	0	0	9	7	41
good penetration (group 2)	0	0	18	29	28	39
poor penetration (group 1)	11	25	58	45	49	16
no penetration	89	75	25	17	17	4

Figure 16: Stacked column charts for MG-values to visualize the no. of biopsies belonging to group 1, 2 or 3, i.e. poor, good or very good penetration, respectively.



	0.025%	0.05%	0.10%	0.15%	0.20%	0.25%
transdermal penetration	0	0	0	1	1	9
very good penetration (group 3)	5	1	29	37	15	54
good penetration (group 2)	28	26	44	46	60	31
poor penetration (group 1)	67	73	27	16	24	7

Figure 17: Stacked column charts for mean penetration depth to visualize the no. of biopsies belonging to group 1, 2 or 3, i.e. poor, good or very good penetration, respectively.

The data obtained from the clustered and stacked column charts are well in agreement with the visual inspection from the images, which already revealed a transdermal penetration of curcumin for some of the biopsies treated with 0.25% curcumin. However, by analysing the clustered histograms, it became obvious that already lower curcumin concentrations enabled transdermal delivery of curcumin. In addition, with the use of the histograms, it was possible to characterize the penetration profiles and the differences in the penetration profiles between the different formulations accurately,

not only in regard to penetration depth and the total amount of penetrated active, but also in regard to penetration variability.

Based on the results from this study, it can be concluded that classification of the penetration parameters and the assessment of the penetration variability are sensitive measures to evaluate the homogeneity of drug penetration. In this study it enabled a more detailed and mechanistic understanding of the penetration process of curcumin from ethanolic solutions. Of course, the variability in dermal penetration is not analysed so far, but might be a useful parameter for future studies, that can be used to quickly discriminate between “good” and “bad” samples, that means between samples that lead to homogeneous or inhomogeneous dermal penetration, respectively.

Besides histograms that represent the variations in penetration efficacy between the different replicates (biopsies), i.e. the interindividual variations, it would also be interesting to have an estimate of the variations within a single biopsy, i.e. the intraindividual variations. This value could be of interest, because it would exclude all interindividual variations, for example biological variations of the skin from different ears, and thus, could directly measure if a formulation leads to homogeneous or inhomogeneous penetration of the active.

Therefore, to allow for an estimation of the intraindividual variation in dermal penetration, the coefficient of variation (CV) was calculated from the standard deviations of the MG-values and the respective MG-values from each image (Fig. 18). The coefficient of variation is also referred to be the relative standard deviation (RSD) and is a standardized measure of a dispersion of a frequency distribution. The CV is expressed in %, is independent on units and thus is optimal to estimate the frequency distribution, i.e. variation, of MG-values. Hence, high CV-values would indicate large variations in the penetration profiles, whereas low CV-values would represent lower dispersity and hence, a more homogenous penetration of the drug substance into the skin.

Analysis of the biopsies treated with different concentrations of dissolved curcumin led to statistically different CV-values between the three different sample groups and no statistical differences were found between the samples within the groups. Besides, data confirm the results obtained from visual inspection and reveal that the inhomogeneity in penetration is highest for the samples treated with low curcumin concentrations. With increasing curcumin concentrations, the inhomogeneity decreases and is lowest (about 66%, less when compared to the 10fold less concentrated samples) for the biopsies treated with 0.25%.

For the formulations tested in this study, image analysis revealed significant differences in the penetration efficacy for the different concentrations of curcumin and enabled to discriminate quickly between good and bad penetrating samples. Hence, data from this study provide first evidence that image analysis with ImageJ is a suitable and versatile tool that helps to evaluate the dermal penetration

efficacy of curcumin form different formulations objectively. In addition, it also revealed that dermal penetration efficacy is not only attributed to the penetration depth and the total amount of penetrated chemical compound, but also influenced by the penetration homogeneity. In this study three groups of formulations were identified. Group 1 led to poor and inhomogeneous penetration, group 2 led to good penetration but still very inhomogeneous penetration, which was characterized by alternating "bright spots" and "dark spots", i.e. blank skin areas without penetrated active. Due to the high variability in penetration efficacy, both groups are expected to cause poor reproducibility with repeated experiments. Hence, these formulations cannot be recommended to be used as internal standards for future experiments if reproducible MG-values or penetration depths are needed. The group 3 formulation resulted in good dermal penetration and a more homogeneous penetration and thus – from this set of data – is the most suitable formulation that can be used as an internal standard for future experiments.

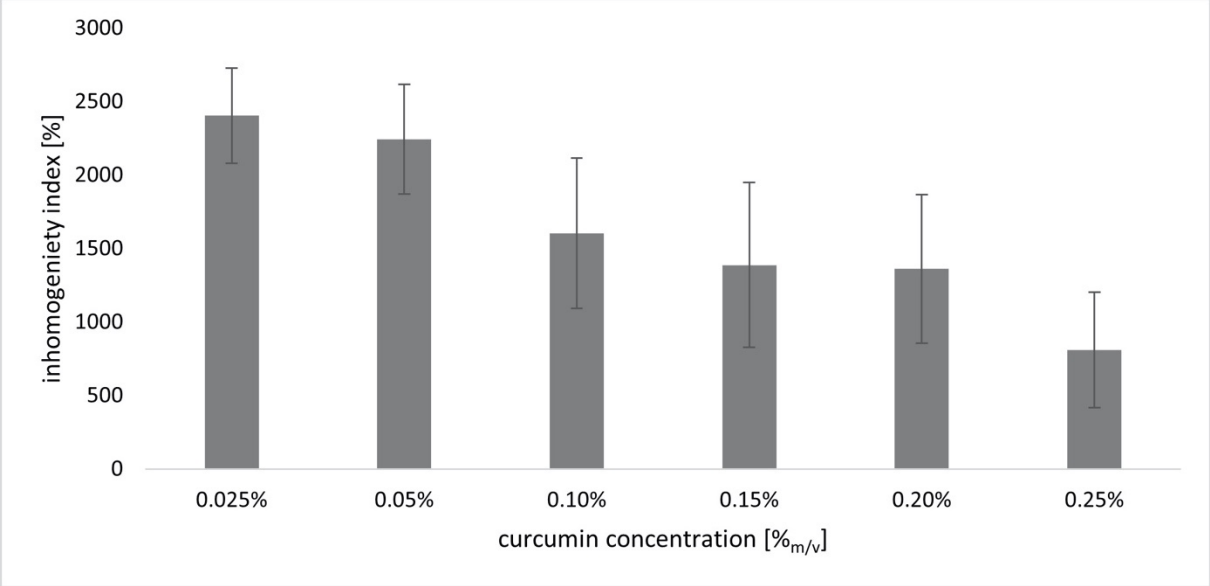


Figure 18: Influence of concentration of curcumin on the penetration homogeniety. The inhomogeniety is expressed as inhomogeniety index (IHI) and corresponds to the coefficient of variation (cv) of the MGV and their respective standard deviations from each image. High IHI-values correspond to a high inhomogeniety and vice versa.

4. Discussion

Based on the results it is concluded that image analysis with the use of ImageJ software can give valuable information about the dermal penetration behaviour of curcumin from differently concentrated ethanolic solutions. The total amount of penetrated curcumin could be evaluated by determining the MGV and the penetration depth was measured directly. In addition to conventional skin penetration models, image analysis enabled also to estimate the homogeneity of the dermal penetration. Thus, giving a much broader and more mechanistic image on the fate of a chemical substance after dermal application. The inhomogeneity of the penetration was assessed with histograms and by calculating the relative standard deviation of the MG-values from the different

biopsies. The latter value is a simple to assess parameter and an objective number at the same time. Thus, it might also be useful to exploit this value further, for example as the “inhomogeneity index (IHI)” to express the inhomogeneity of penetration from different formulations in future studies.

In this study the ex-vivo pig ear model was used to investigate the dermal penetration efficacy of curcumin from ethanolic solutions. The penetration efficacy was determined from skin biopsies by visual inspection and by using ImageJ software for further image analysis. Whereas visual inspection of the images obtained from epifluorescence microscopy could only give a rough hint on the influence of the concentration of dissolved active on the penetration efficacy into the skin, image analysis revealed clear and statistically significant results. Thus, allowing for a number-based discrimination between “good” and “bad” penetrating curcumin formulations. Based on the data obtained it is concluded that image analysis with ImageJ software can be used as a sensitive tool to compare the dermal penetration efficacy of curcumin from different formulations.

The ex-vivo pig ear skin penetration model with subsequent image analysis of the skin biopsies is simple to perform and has several advantages when compared to other skin penetration models. For example, it does not require any manipulation of the skin prior to the measurements and thus mimics physiological skin conditions, which for example cannot not be maintained if Franz-diffusion cells are used for the determination of the dermal penetration efficacy. In contrast to other models, sample preparation is fast and no expensive analytical equipment, e.g. HPLC or LC/MS instruments, are required. The method, however, does not allow for an exact quantification of the amount of penetrated curcumin, but allows for a semi-quantitative analysis. For example, by comparing the results obtained to an internal standard.

Ethanolic curcumin solutions with different concentrations were used in this study and the influence of the concentration of dissolved active on dermal penetration was evaluated. Data proved the validity of Fick’s law and demonstrated – as expected – that both, the total amount of penetrated active and the penetration depth, increase with increasing concentrations of dissolved curcumin. However, data also showed that dermal penetration efficacy can also be affected by penetration inhomogeneity. Thus, a parameter addressing this, e.g. the inhomogeneity index (IHI), should be included in future studies to investigate and express the fate of chemicals after dermal application in more detail.

In this study high penetration inhomogeneity was found for ethanolic solutions containing only low concentrations of curcumin. As high penetration inhomogeneity can be associated with low reproducibility of the data, these formulations cannot be recommended to be used as an internal standard in future studies. Higher concentrations showed less variations and thus are more suitable as internal standard. Based on the IHI-value, the most suitable standard seems to be the formulation containing 0.25% curcumin. The formulation shows low inhomogeneity in dermal penetration and

enables an efficient penetration of curcumin into the epidermis and even some transdermal penetration. Thus, allowing a good and reproducible trackability of curcumin after dermal application.

In this study, the ex-vivo pig ear skin penetration model with subsequent image analysis of the skin biopsies was demonstrated to be a highly suitable tool for the evaluation of the dermal penetration efficacy of curcumin from different formulations. Data suggest that the method might also be suitable as a fast but efficient screening tool for other dermal formulations. A prerequisite for the exploitation of the method is the use of (model) compounds that possess sufficient autofluorescence which can be traced in the skin by means of epifluorescence microscopy. Another possibility would be the fluorescence labelling of the compounds of interest. Future studies should investigate this in more detail.

5. Conclusions

From the results obtained in this study it is concluded that the skin penetration model used, and the curcumin standard developed in this study are suitable to evaluate and compare the fate and the penetration efficacy of curcumin from different formulations. The ex-vivo skin penetration model and the subsequent image analysis of images obtained from epifluorescence microscopy allow for a simple comparison between “good” and “bad” penetrating curcumin formulations and provide detailed information about penetration depth, the total amount of penetrated substance and the homogeneity in penetration. With this, the model provides a detailed picture – not only on drug penetration – but also on the fate of curcumin after dermal application and thus, can be the base for an efficient development and comparison of innovative curcumin formulations for dermal application in the future. Future studies should also investigate if the model can also be used for the inspection of the dermal penetration efficacy of other fluorescent chemical substances.

6. Statement of Ethics

The study complies with internationally-accepted standards for research practice and reporting.

7. Conflict of Interest Statement

Authors declare no conflict of interest.

8. Funding Sources

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9. Author Contributions

OP and SRP collected data. OP, SRP and CMK analyzed data. OP and CMK wrote the manuscript. All authors reviewed and approved the manuscript.

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S. Supplementary material to manuscript: Dermal penetration analysis of curcumin in an ex-vivo porcine ear model using epifluorescence microscopy and digital image processing

S1: Threshold algorithm to quench the autofluorescence of the skin to a minimum. Programmed macro for auto-thresholding the image of curcumin skin biopsies obtained from epifluorescence microscopy and analyzed using the DAPI HC filter block system (excitation filter: 340 – 390 nm (LP), dichroic mirror: 410 nm, emission filter: 420 nm (LP)). **M1:** Macro for setting of the RGB-threshold, **M2:** Macro for white to black inversion of images.

S1:

M1:

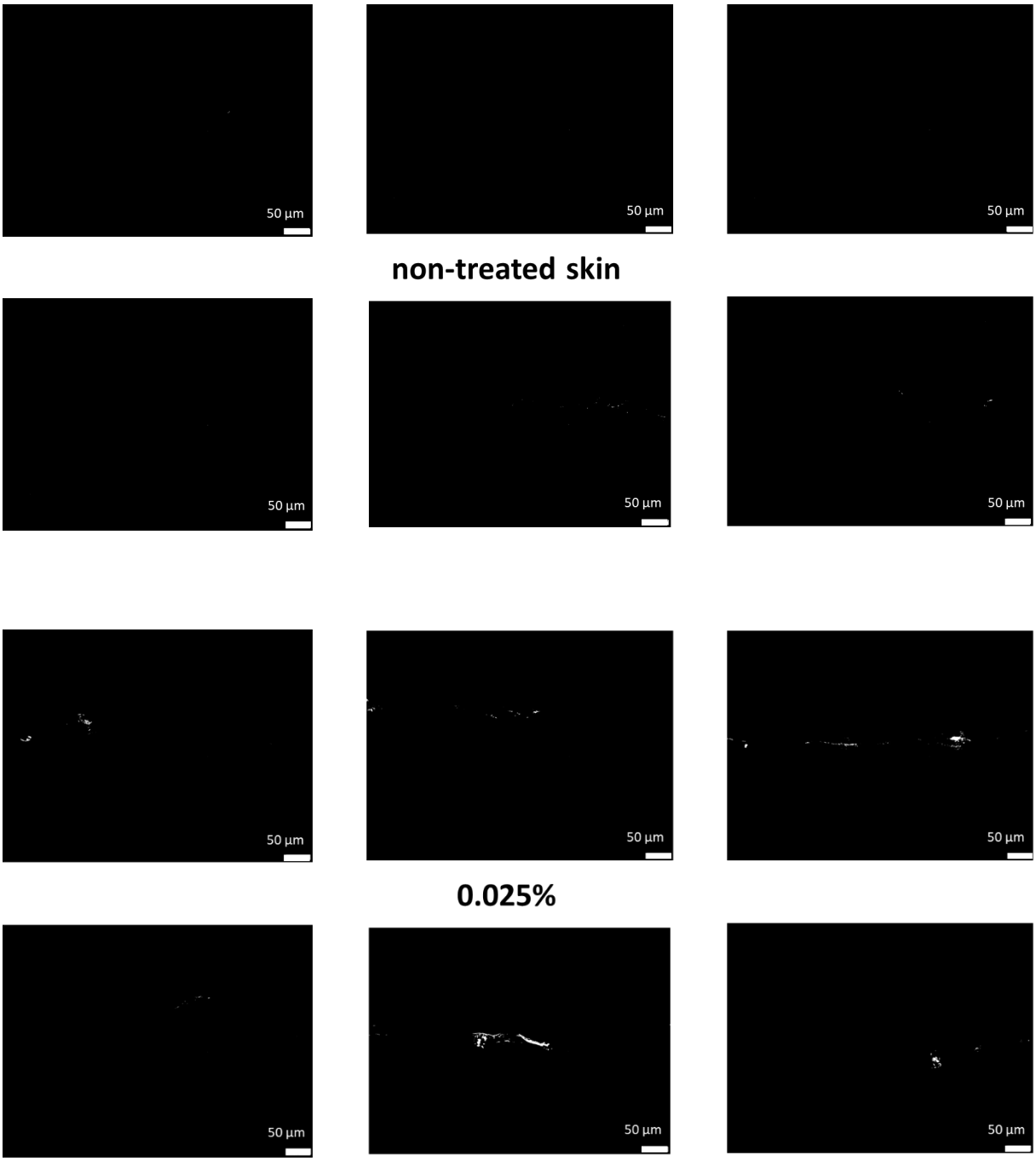
```
/ Color Thresholder 1.52a
// Autogenerated macro, single images only!
min=newArray(3);
max=newArray(3);
filter=newArray(3);
a=getTitle();
run("RGB Stack");
run("Convert Stack to Images");
selectWindow("Red");
rename("0");
selectWindow("Green");
rename("1");
selectWindow("Blue");
rename("2");
min[0]=33;
max[0]=255;
filter[0]="pass";
min[1]=0;
max[1]=0;
filter[1]="stop";
min[2]=0;
max[2]=0;
filter[2]="stop";
```

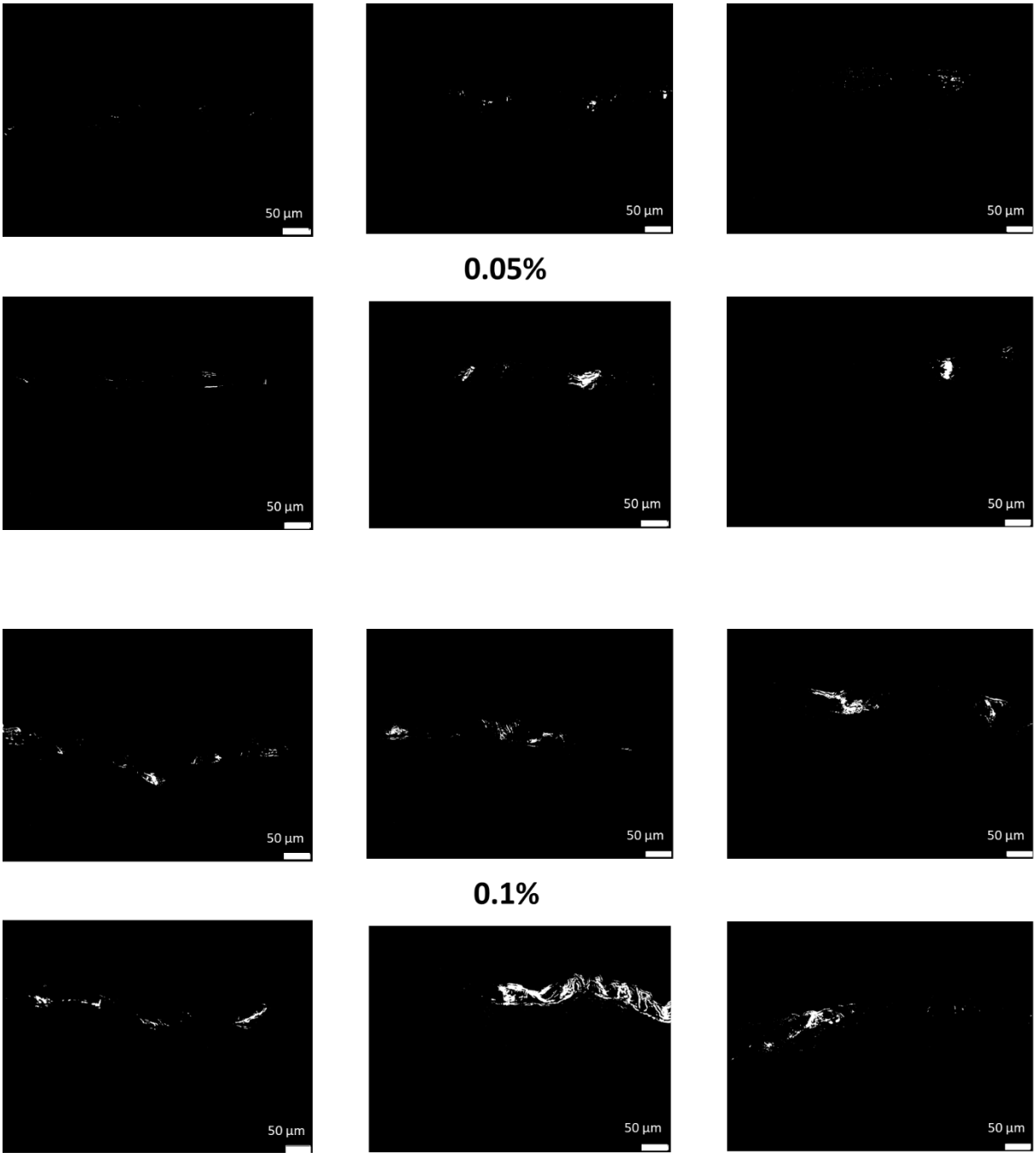
```
for (i=0;i<3;i++){
  selectWindow(""+i);
  setThreshold(min[i], max[i]);
  run("Convert to Mask");
  if (filter[i]=="stop")  run("Invert");
}
imageCalculator("AND create", "0","1");
imageCalculator("AND create", "Result of 0","2");
for (i=0;i<3;i++){
  selectWindow(""+i);
  close();
}
selectWindow("Result of 0");
close();
selectWindow("Result of Result of 0");
rename(a);
// Colour Thresholding----------
```

M2:

```
run("Invert");
```

S2: Representative images for blank, untreated skin and skin biopsies treated with ethanolic solutions containing different concentrations of curcumin after auto threshold.




50 µm
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0.05%
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50 µm
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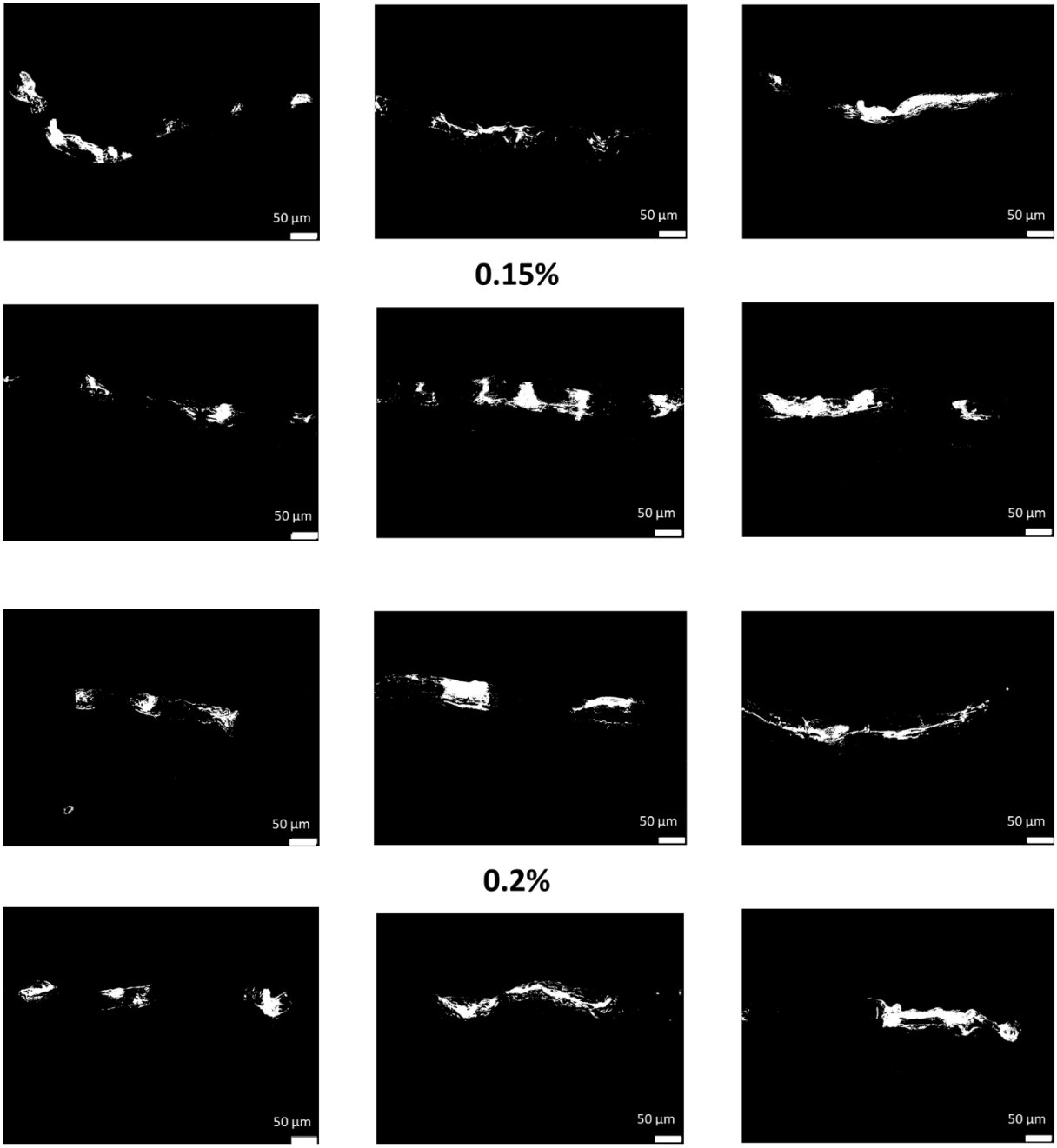
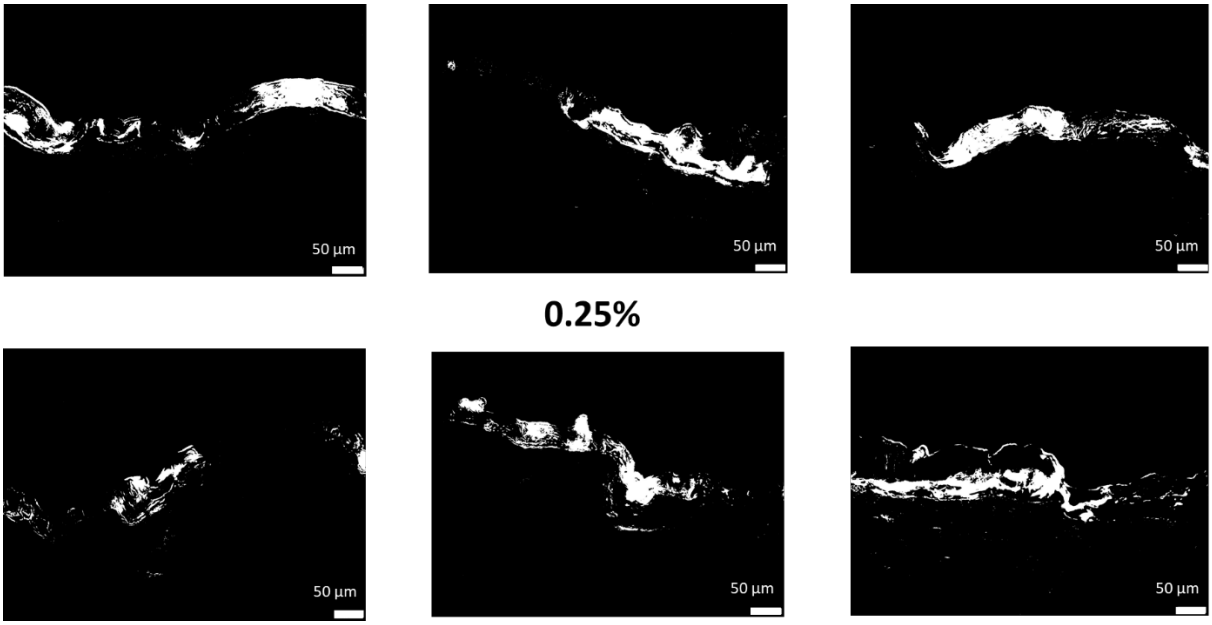
0.1%
50 µm
50 µm
50 µm


50 µm
0.15%
0.2%


50 µm
50 µm
50 µm
0.25%
50 µm
50 µm
50 µm

3.4.

Hair follicle targeting with curcumin nanocrystals: influence of the formulation properties on the penetration efficacy

Hair follicle targeting with curcumin nanocrystals: influence of the formulation properties on the penetration efficacy

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Abstract

Nanocrystals are a universal formulation approach for improved drug delivery of poorly water-soluble drug substances. Besides oral application, also topical application of the nanocrystals is feasible, because the increased kinetic solubility of the nanocrystals results in an increased concentration gradient, thus fostering passive dermal penetration. Also, nanocrystals are also promising for targeting drug substances into the hair follicle. After penetration into the hair follicle, the nanocrystals could form a depot from which the active is released into the hair follicle. Thus, leading to a long-lasting and very efficient dermal drug delivery. The efficacy of nanocrystals to penetrate the hair follicles and the influence of the vehicle in which the nanocrystals are suspended was not yet investigated. Therefore, in this study curcumin nanocrystals with a size of about 300 nm were produced and incorporated into gels with different properties. The efficacy to penetrate the hair follicles, as well as the passive dermal penetration, was assessed on the ex-vivo pig ear model. Nanocrystals were efficiently taken up by the hair follicles and reached the lower part of the infundibulum. This region is optimal for efficient drug delivery because the barrier of the lower infundibulum is not fully developed and thus more permeable, which results in a less hindered passive diffusion of drug substances. The penetration efficacy of the nanocrystals into the hair follicles was not affected by the different types of vehicles, which represented either oleogels or hydrogels that varied in viscosity as well as in the type and the concentration of the gelling agent. All gels possessed a shear-thinning flow behaviour and it is hypothesized that all gels fluidized during the skin massage, whereby leading to similarly low viscosities than the aqueous nanosuspension and thus to similar penetration results. The passive dermal penetration of curcumin was different for the different gels and the main driving parameter leading to good passive diffusion was caused by good skin hydrating properties of the vehicle. The best passive penetration was achieved from hydrogels that contained a humectant. However, the addition of the

humectant reduced the efficacy of the nanocrystals to penetrate the hair follicle. Data so far, therefore, suggest that hair follicle targeting with nanocrystals that are suspended in water or simple, shear-thinning gels is highly effective. However, the addition of other excipients, e.g. humectants, to these vehicles might cause changes in the penetration profiles. More research in this regard is needed to understand these observations in more detail.

1. Introduction

Selective targeting of the hair follicles represents a very promising tool in the topical therapy of hair follicle-related disorders. These include infectious diseases, various forms of hair loss, autoimmune diseases, androgen-associated diseases, or disorders of the hair follicle-related structures, e.g. sebaceous glands and acne vulgaris [1–4]. Selective and targeted follicular delivery of drug substances enables the local treatment of such diseases, reduces systemic side-effects, and thus opens new important therapeutic strategies. However, a suitable drug delivery system that enables efficient hair follicle targeting is the main prerequisite for this. Penetration of actives into the hair follicles from different formulation principles has been investigated previously and it has been reported, that drugs in particulate form penetrate the hair follicles in a more pronounced manner than dissolved drugs molecules [5]. Consequently, different drug carriers, for example, PLGA nanoparticles, liposomes, and microspheres were utilized in subsequent studies and could be identified to represent efficient carriers for intrafollicular drug delivery [5–7]. However, until now only a few studies have investigated hair follicle targeting with nanocrystals [8–11]. A detailed study regarding the penetration depth or exact localization of nanocrystals within the follicles and/or information on the influence of the vehicle in which the nanocrystals are formulated is not yet available. This information is of high importance, because within the hair follicle, key target sides at different depths could be identified, whose selective treatment enables the therapy of different follicular diseases [1]. The present study was therefore conducted to address this issue in more detail.

Compared to other nanoparticles, nanocrystals provide a series of advantages, making them a highly attractive formulation principle for drugs for the hair follicle targeting. In comparison to other nanoparticles, which consist of a matrix material loaded with drugs, nanocrystals are composed of 100% drug without any matrix material [12,13]. The high loading makes them very efficient in transporting the drug to the target side because much lower numbers of particles are needed to reach the required therapeutically concentration when compared to drug-loaded nanocarriers, which typically do not exceed loading capacities > 10%. Nanocrystals can be simply produced by well-established techniques without the demand for expensive equipment. Furthermore, nanocrystals possess an increased surface area and increased apparent solubility when compared to larger-sized material and can therefore enhance the dissolution rate and bioavailability of poorly water-soluble drug substances [14,15]. In fact, due to their special properties nanocrystals represent a very promising drug delivery system not only for oral and topical application but also for hair follicle targeting.

In this study, the follicular penetration of nanocrystals and the influence of vehicles were examined by using curcumin as a model drug. Curcumin is a natural compound extracted from the rhizome of the turmeric plant (*curcuma longa*). It has been used in Indian Ayurvedic medicine for at least 4,000 years

for the treatment of various inflammatory conditions and other diseases [16,17]. Due to its anti-inflammatory, antimicrobial, antioxidant and antineoplastic properties, curcumin offers a variety of potential applications and is widely used for several therapeutic approaches. For example, biliary disorders, rheumatism, hepatic disorders and wounds [18]. Some studies also revealed positive effects of curcumin in the treatment of several skin diseases such as acne, atopic dermatitis, psoriasis and androgenetic alopecia [19]. Hence, targeting curcumin to the hair follicles could have a high clinical impact on the treatment of these diseases. Due to its high lipophilicity curcumin possesses a low solubility and consequently a low bioavailability (BSC class IV). Formulating curcumin as nanocrystals is therefore also a suitable strategy to overcome this issue [20].

Nanocrystals are typically produced via wet milling. If the liquid dispersion medium is not removed after the milling process, the nanocrystals remain suspended in the liquid. Thus, yielding nanosuspensions, respectively. A liquid formulation is not very convenient for the patient, especially if it is aimed to apply the formulation on the scalp. A semi-solid formulation is more convenient to apply and would therefore lead to better compliance and acceptance by the patients. To date, a bright range of semi-solid vehicles (e.g. gels, creams, ointments) with different properties is available and previously published studies could already show that the type of vehicle that is used for the formulation of the nanocrystals can have a tremendous influence on the dermal penetration efficacy of the active compound [21]. Moreover, it was also shown that the vehicle can also affect the effectiveness of the penetration of particles into the hair follicles [22–24]. However, the previous studies investigated only selected formulation parameters and revealed some controversial outcomes [22–24]. Hence, conclusive data in this regard and a systematic study that investigates formulation parameters that can theoretically influence the efficacy of hair follicle targeting of nanocrystals is not yet available.

Therefore, this study aimed to investigate systematically the influence of the formulation and its properties on the penetration efficacy of curcumin nanocrystals into hair follicles and to identify suitable vehicles for effective hair follicle targeting of nanocrystals. The major formulation parameters that were considered to possibly affect the penetration efficacy of the nanocrystals were the viscosity of the vehicle as well as the lipophilicity and polarity (charge) of the vehicle, respectively. It might be assumed, that changes in viscosity and/or the polarity of the formulation could influence the release of nanocrystals from the vehicle which could then lead to an altered penetration efficacy of nanocrystals into the follicles. Other formulation properties, e.g. skin hydration or occlusive properties, might also affect the conditions of the hair follicles and could, for example, lead to swelling or shrinking of the hair follicle infundibulum, which in turn could then affect the permeation ability for the particles. To investigate the influences of these vehicle properties on the hair follicle targeting with nanocrystals, pharmaceutical gels with different properties (different in viscosity, polarity and lipophilicity) were chosen as semi-solid model formulations in this study. Gels can simply be manufactured and modified

to obtain tailored properties and represent a convenient formulation principle for topical application on the skin and the scalp. Thus, with the knowledge gained from this study, it should be possible to formulate gels with tailor-made properties for improved delivery of actives from nanocrystals via the follicular route.

The study was performed in two steps. In the first part, 14 different curcumin nanocrystal-loaded gels were produced and characterized regarding particle size and viscosity. In the second part, the ability of the nanocrystals to penetrate hair follicles from the different gels was determined ex-vivo on fresh pig ears. Additionally, selected formulations were also examined regarding their penetration enhancing effects on the passive dermal and transdermal penetration of curcumin from the nanocrystals.

2. Materials and methods

2.1. Materials

Curcumin was obtained from Receptura Apotheke (Cornelius-Apothekenbetriebs-OHG, Frankfurt am Main, Germany). TPGS (d-α-tocopherol polyethylene glycol 1000 succinate) was used as a stabilizer for the nanocrystals was and purchased from Gustav Parmentier GmbH (Frankfurt am Main, Germany). Purified water was obtained from a PURELAB Flex 2 (ELGA LabWater, High Wycombe, England) and was used as a liquid phase for the nanocrystals and the hydrogels. As non-polar gelling agents for the production of the hydrogels hydroxyethyl cellulose (HEC) (Natrosol® 250 G Pharm., Caesar & Loretz GmbH, Hilden, Germany) and xanthan gum (Special Ingredients Ltd, Chesterfield, UK) were used. Polyacrylic acid (PAA) (Carbomer 50.000/Carbopol® 980, Caesar & Loretz GmbH, Hilden, Germany) and polyacrylate cross polymer-6 (Sepimax Zen™, Seppic, Paris, France) were used as gelling agents for the production of the polar hydrogels. Lipophilic gels, i.e. oleogels, were produced from olive oil (Gustav Heess GmbH, Leonberg, Germany) as liquid phase and colloidal silicon dioxide (Aerosil® 200, Evonik Industries AG, Essen, Germany) as a gelling agent, respectively. All chemicals were used as received.

2.2. Methods

2.2.1. Production and characterization of curcumin nanocrystals

Nanocrystals were produced by bead milling by using a state of the art milling equipment for the production of nanocrystals in industrial scale (NanoWitt-LAB 100, Frewitt fabrique de machines S.A, Switzerland) by using a previously established production protocol [25]. Briefly, nano-milling was carried out in continuous mode configuration of the mill with a suspension circulation rate of 500 mL/min for 45 minutes. The tip speed was set to 4.7 m/s and the bead/suspension-ratio was 60/40 (v/v). Yttrium stabilized zirconium oxide beads (SiLibeads®, Sigmund Lindner GmbH, Switzerland) with

a size of 0.3-0.4 mm were used for the milling and the temperature of the suspension was controlled throughout the milling process to not to exceed temperatures > 20 °C. The so obtained nanocrystals contained 5.0 % (w/w) curcumin, 1.0 % (w/w) TPGS and water to up to 100 % (w/w). Nanocrystals were characterized regarding their size and size distribution by using three independent techniques, i.e. dynamic light scattering (DLS), static light scattering (SLS) and light microscopy. Size analysis was performed directly after production and before the preparation of the nanocrystal-loaded gels. The Zetasizer Nano ZS (Malvern Panalytical Ltd, Malvern, UK) was used for the DLS measurements, as well as for the analysis of the zeta potentials. SLS measurements were carried out with a Mastersizer 3000 (Malvern Panalytical Ltd, Malvern, UK). SLS data analysis was done with Mie-theory with optical parameters set to 1.87 for the real refractive index and 0.1/0.01 for the blue light (470 nm) and the red light (633 nm) imaginary refractive indices, respectively. Light microscopy was performed with an Olympus BX53 light microscope (Olympus Cooperation, Tokyo, Japan), which was equipped with an Olympus SC50 CMOS color camera (Olympus soft imaging solutions GmbH, Münster, Germany).

The original curcumin nanosuspension was used as a stock formulation and was incorporated into the different gel bases, leading to gels that contained 1% (w/w) curcumin (c.f. Table 1). As a control, a nanosuspension, that contained equal amounts of curcumin nanocrystals than the gels, was prepared by diluting 1.0 g of the original nanosuspension with 4.0 g purified water. The diluted nanosuspension contained 1 % (w/w) curcumin nanocrystals and was also characterized regarding size by using of DLS, SLS and light microscopy. To avoid changes in size, e.g. due to Ostwald ripening, the diluted nanosuspension was applied onto the skin immediately after preparation.

2.2.2. Preparation of gels

In total, 14 different gels were produced (Table 1). All gels contained 1% (w/w) curcumin nanocrystals but varied in the type and the amount of gelling agent used. Depending on the type of gelling agent used, slightly different production methods were required to obtain the gels. The methods applied are described in more detail below.

Table 1: Overview of the compositions of the gels formulated and tested in this study.

Gelling agent		Composition of the liquid phase	concentration of the gelling agent in the gel (w/w)	curcumin concentration in the gel (w/w)
polar hydrogels	PAA	water	0.08%	1%
		water	0.2%	1%
		water	0.4%	1%
		water	0.8%	1%
		water	2%	1%
	SepimaxZen®	water	2%	1%
non-polar hydrogels	HEC	water	1.6%	1%
		water	3.6%	1%
		water	4.4%	1%
		water	4.8%	1%
		water + glycerol	4.8%	1%
	Xanthan gum	water	2%	1%
		water	5%	1%
		water + glycerol	5%	1%
oleogels	Aerosil®	olive oil	4%	1%
		olive oil	5.6%	1%

2.2.2.1. Preparation of PAA gels

Plain carbomer gels were prepared by dispersing the required amount of PAA in purified water under stirring with a pestle in a mortar. For gel formation, the pH of the hydrogels was subsequently adjusted to 7 with a 5% (w/v) NaOH solution until a clear homogeneous gel was formed. Subsequently, to obtain the nanocrystal-loaded gels, 2.0 g of the original curcumin nanosuspension was added to 8.0 g of the freshly prepared gel bases. The nanocrystal-loaded gels that contained 0.08% (w/w), 0.2% (w/w), 0.4% (w/w) or 0.8% (w/w) PAA were mixed by stirring with a pestle in the mortar. The 2% (w/w) carbomer gel with nanocrystals was prepared by using the automatic mixing system TopiTec® (WEPA Apothekenbedarf GmbH & Co KG, Hillscheid, Germany). The mixing program adopted for gel preparations (5 min stirring at 500 rpm) was used for the preparation of this gel.

2.2.2.2. Preparation of HEC gels

Plain HEC gels were prepared by using the dispersion method. The required amount of HEC was sieved into purified water in a mortar, dispersed homogeneously by stirring with a pestle for a short time and allowed to swell for 2 hours until thick gels without air bubbles were formed. Subsequently, to obtain the nanocrystal-loaded gels, 2.0 g of the original curcumin nanosuspension was added to 8.0 g of the freshly prepared gel bases by stirring with a pestle in a mortar. The preparation of the glycerol-

containing HEC gel was performed as described above and instead of pure water a mixture of glycerol and water, i.e. 5% (w/w) glycerol in water, was used as aqueous phase (Table 1).

2.2.2.3. Preparation of xanthan gum gels

For xanthan gum gels, the thickener was homogeneously dispersed in the purified water by stirring with a pestle in the mortar. The thick gels were built immediately without additional swelling time. Nanocrystal-loaded gels were prepared by adding 2.0 g of the original nanosuspension to 8.0 g of the freshly prepared plain gels. The nanocrystal-loaded gel containing 2% (w/w) xanthan gum was prepared in the mortar by stirring with a pestle, whereas the 5% (w/w) xanthan gum gel, due to its higher viscosity, was mixed with the original curcumin nanosuspension by using the automatic mixing system TopiTec® (5 min stirring at 500 rpm). The glycerol-containing xanthan gum gel was prepared as described above but by replacing the water by a mixture of water that contained 5% (w/w) glycerol (Table 1).

2.2.2.4. Preparation of SepimaxZen® gel

The plain SepimaxZen® gel was prepared by dispersing the gelling agent in purified water. The gel was built immediately without additional swelling time. Nanocrystal-loaded gels were obtained by adding 2.0 g of the original nanosuspension to 8.0 g of the plain gel by stirring with a pestle in the mortar.

2.2.2.5. Preparation of oleogels

For oleogels, the required amount of Aerosil® was rubbed with a small portion of olive oil in the mortar. Subsequently, the remaining olive oil was added by gentle stirring with a pestle. The gel was allowed to swell for 2 h. The nanocrystal-loaded oleogels were then obtained by admixing the original nanosuspension to the gel base by using the TopiTec® system (5 min mixing at 500 rpm).

2.2.3. Characterization of nanocrystal-loaded gels

The nanocrystal-loaded gels were characterized regarding their microstructure and regarding particle size and size distribution of the nanocrystals within the gels. In addition, the rheological properties of the gels were assessed.

2.2.3.1. Microstructure

Light microscopy (Olympus BX53 light microscope equipped with an Olympus SC50 CMOS color camera, Olympus Life Science Solutions, Hamburg, Germany) was used to study the microstructure of the gels and to detect potential agglomerates of the nanocrystals within the gels. Microscopic observation was not only done for the original samples but was also used to investigate the influence of the skin massage on the microstructure of the gels and the resulting distribution of the nanocrystals within the gels. For this, the formulations were applied with a pipette tip onto a microscopic slide and

“massaged in” for 3 min to simulate the skin application (c.f. section 2.2.4). After the massage, a microscopic examination was performed and images were taken with the cellSens Entry software (Olympus Life Science Solutions, Hamburg, Germany).

2.2.3.2. Determination of particle size of nanocrystals within the gels

The Feret’s diameter of the nanocrystals within the gels was determined from the microscopic images by subsequent digital image analysis with ImageJ software [26,27]. For this, for each formulation 3 representative, independent images were selected and evaluated. The images (1000fold magnification) were color-adjusted, and threshold analysis was performed to select and mark the individual particles and possible agglomerates within the gels. Subsequently, the Feret’s diameter of each marked particle or agglomerate within the image was assessed by the software. Obtained results were visualized as size distribution curves and JASP software [28] was used to calculate the number based median particle size diameters d(n)0.10, d(n)0.50, d(n)0.90, d(n)0.95 and d(n)0.99, respectively. A d(n)0.50 for example represents the size were 50% of all particles are smaller or equal to the given number. Consequently, when looking not only for the d(n)0.50 but also for smaller and larger median values, valuable information about the particle size and the particle size distribution of the particles within the gels can be obtained.

2.2.3.3. Rheological studies

The rheological behaviour of the nanocrystal-loaded gels was determined using a Haake RheoStress 1 rheometer (Thermo Fisher Scientific Inc., Waltham, Massachusetts, US) with the cone-plate configuration, equipped with a 20mm/1° cone. A gap of 0.052 mm was maintained between the cone and the plate throughout the whole study. To allow for an estimation of the thixotropic properties of the gels, the viscosity measurements were carried out as a three-step-loop experiment. First, the shear rate was ramped up from 0 to 1000 s^{-1} within 60 s. Afterwards, the steady shear rate of 1000 s^{-1} was held for 30 s, followed by a ramp down element back to 0 s^{-1} within 60 s. The flow curves obtained were analyzed by the software RheoWin DataManager (Thermo Fisher Scientific Inc., Waltham, Massachusetts, US). All measurements were performed at 20°C and each measurement was performed in triplicate.

2.2.4. Determination of follicular and dermal penetration

The diluted nanosuspension and the nanocrystal-loaded gels were investigated regarding their ability to target the nanocrystals into hair follicles. In addition, the dermal penetration of curcumin through the skin was assessed. The experiments were carried out ex-vivo on the porcine ear skin model [29]. For the experiments, fresh porcine ears were obtained from the local slaughterhouse and used within a few hours after butchering. The preparation procedure of the ears included washing with lukewarm

water (approx. 23 – 25°C) and subsequent drying. Drying was performed by careful dabbing of the skin – area by area – with soft lint-free paper towels without rubbing and any mechanical forces. The penetration experiments were performed on the ventral ear side due to the higher density of the hair follicles in comparison to the dorsal side. After fixing the ears on flat polystyrene plates that were covered with aluminum foil, the skin barrier integrity was ensured by TEWL measurements (Tewameter® TM 300, Courage + Khazaka electronic Gmbh, Cologne, Germany). Intact skin areas of 2x2 cm2 without visible wounds and scratches were selected, marked and the hair within these test areas was cut short with scissors to a length of about 1-3 mm. On each test area, 250 mg formulation (equivalent to 625 µg curcumin/cm^2 skin) were applied and massaged in for 3 min with a saturated, gloved finger. After a penetration time of 6h at 32°C, the formulations were carefully washed off from the ears and punch biopsies (15 mm drive punch) were taken from each test area. The skin biopsies were immediately embedded (Tissue-Tek® O.C.T.™, Sakura Finetek Europe B.V., Alphen aan den Rijn, Netherlands), frozen and stored at -20°C until further use. Experiments were performed in triplicate and each formulation was tested on three different ears.

2.2.4.1. Determination of follicular and dermal penetration via epifluorescence microscopy

Before microscopic analysis, 40 µm thick skin sections were prepared with a cryomicrotome (Frigocut 2700, Reichert-Junk, Nußloch, Germany). The cutting was carried out from right to left in order to avoid contamination with the curcumin from the surface. The skin sections were analyzed by epifluorescence microscopy (Olympus CKX53 equipped with an Olympus DP22 color camera, Olympus Deutschland GmbH, Hamburg, Germany). To quench the autofluorescence of the skin and the hair follicles to a minimum, the intensity of the fluorescence light source (130 W U-HGLGPS illumination system, Olympus Deutschland GmbH, Hamburg, Germany) was set to 50% and 25% for the skin and the hair follicle skin sections, respectively. The exposure time was kept constant and was set to 50 ms for all images taken and the filter selected for analysis of the skin sections was the DAPI HC filter block system (excitation filter: 340 – 390 nm (LP), dichroic mirror: 410 nm, emission filter: 420 nm (LP)).

2.2.4.2. Digital image analysis

Digital analysis of the images was performed to obtain more detailed information. The penetration depth of nanocrystals into the hair follicles was measured via the software cellSens Entry and could be estimated quantitatively in µm. For each formulation, 5-10 follicles per porcine ear and 3 ears for each formulation in total were analyzed. The passive dermal penetration efficacy of curcumin from the different formulations was determined by semi-quantitative image analysis according to a previously established protocol [30]. For this, all images were subjected to an automated threshold protocol to eliminate the autofluorescence of the skin. The remaining light intensity within the images corresponded to the penetrated amount of curcumin and was used to determine the mean grey values

(MGV) of the fluorescence images, the mean penetration depth (MPD) and the inhomogeneity index (IHI) as respective measures for the total amount of penetrated curcumin, the penetration depth into or through the skin, and the degree of penetration homogeneity. Digital analysis of the images was performed via ImageJ software [31–33] and for each formulation, 24 images (8 images from each ear) were analyzed.

2.2.5. Statistical analysis

Descriptive statistics were calculated by using Microsoft Excel® and are reported as mean ± standard deviation (SD). Further statistical analysis was performed by using Minitab 19 (Minitab Inc, State College, Pennsylvania, US) and JASP software (Version 0.13.1) [28]. Normal distribution and variance homogeneity of the data were tested with the Shapiro-Wilk and the Levene's test, respectively. For the normally distributed data, the mean values were compared by a one-way ANOVA, that was Welch-adopted in case of variance heterogeneity. Tukey post-hoc and Games-Howell post-hoc tests were performed to compare the mean values between each other. For the non-parametric data sets a Kruskal-Wallis analysis of variance was performed. Additionally, some selected data were directly compared to each other. For this, the Student's t-test for independent samples was applied for the normally distributed data and the Mann-Whitney-U-test was used for the data that were found to possess a non-normal distribution. P-values < 0.05 were considered statistically significant.

3. Results and Discussion

3.1. Characterization of curcumin nanocrystals

The original curcumin nanosuspension possessed a size of about 290 nm (DLS analysis, z-average) and a relatively narrow size distribution, indicated by a polydispersity index of 0.264. SLS measurements confirmed the absence of larger particles and agglomerates (d(v)0.9 < 2 µm), which was also confirmed by light microscopy. The original nanosuspensions possessed a slightly negative zeta potential of about – 16 mV. The diluted nanosuspension, containing equal amounts of curcumin than the gels, possessed a zeta potential of – 11 mV. The z-average was 267 nm, the polydispersity index was 0.307 and the d(v) 0.9 was < 1.5 µm. Data indicate a slight decrease in size that can be explained by partial dissolution of some of the nanocrystals that occurred upon the addition of more water. Nonetheless, the particle size and the size distribution were not significantly changed when compared to the original suspension. Hence, the diluted nanosuspension showed a comparable size and size distribution to the original nanosuspension and thus was regarded to be suitable for the intended use in the skin experiments.

3.2. Characterization of nanocrystal-loaded gels

Curcumin nanocrystals were successfully incorporated into all gels and macroscopic evaluation of the gels obtained revealed no visible inhomogeneity. Microscopic analysis and subsequent size analysis of the nanocrystals within the gels, however, revealed some slight differences in the distribution of nanocrystals within the different vehicles (Fig. 1). Homogeneously distributed nanocrystals without agglomerates were found in the gels being composed of 0.4% PAA, 0.8% PAA, 2% PAA, 2% SepimaxZen® or xanthan gum, respectively (Fig. 1). A slight agglomeration of the nanocrystals but without the presence of larger agglomerates were found in the gels that contained 0.08% PAA, 0.2% PAA or HEC as gel building agent and the addition of the aqueous nanosuspension to the oleogels resulted in the formation of suspo-emulsions, where water droplets that contained the curcumin nanocrystals were dispersed in the outer oleogel phase (Fig. 1 lower, right). Results obtained from size analysis could confirm these results (Fig. 2). Due to the numeric size distribution and the relatively low number of larger-sized particles and/or agglomerates of the nanocrystals that were observed in some of the formulations, the larger sized particles had only a small impact on the all-oversize results obtained. Thus, leading to relatively small and similar d(v)0.9, d(v)0.95 and d(v)0.99 values for the different formulations, respectively. However, the slightly increased size, due to the formation of some agglomerates in the gels containing either low concentrations of PAA, HEC or aerosol as a gelling agent, were reliably detected by this method (Fig. 2).

As larger sized particles or agglomerated particles can be expected to behave differently on the skin and/or to differently impair the dermal penetration efficacy and/or the ability of the particles to penetrate the hair follicles, it was interesting to inspect if the particle size and the size distribution of the gels would change during the application on the skin. To investigate this in more detail, all formulations which showed a tendency of agglomeration were subjected to further investigations. Further analysis was done by simulating the skin application protocol, which includes a 3 min mechanical massage, on the microscopic slide. After the simulated massage, the formulations were again subjected to microscopic analysis. However, without a coverslip, to avoid any artefacts due to the technical preparation of the samples. Results revealed that a 3 min massage was able to destroy all agglomerates within the gels, leading to homogeneously distributed nanocrystals within all formulations (Fig. 3, lower). From the results obtained it was concluded that all formulations would possess comparable particle sizes and size distributions after topical application with a subsequent mechanical massage. Thus, all nanocrystals-loaded gels were found to be well suited to be used for the intended skin experiments.

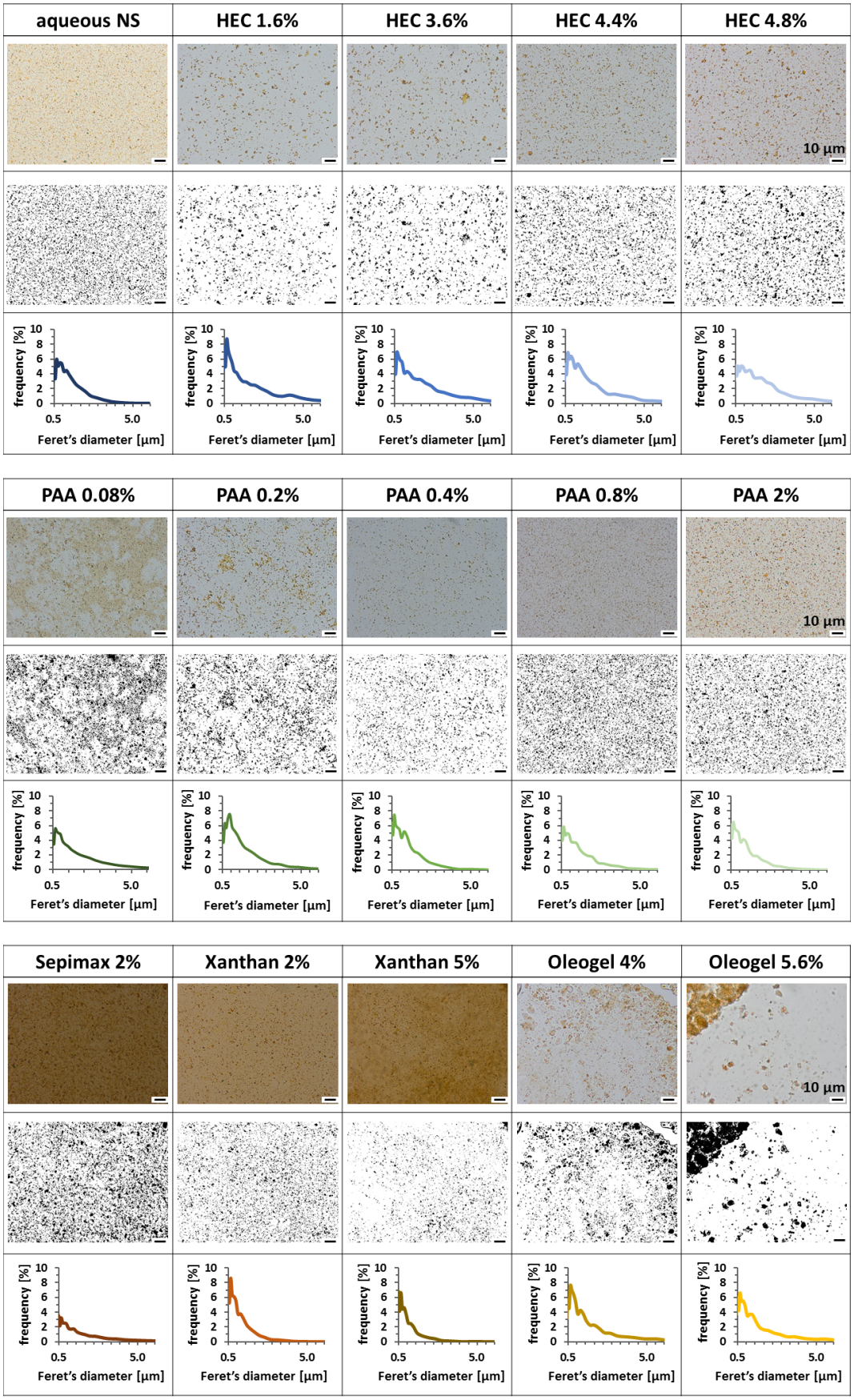


Figure 1: Microstructure of the curcumin nanosuspension and the nanocrystal-loaded gels determined by light microscopy (upper rows), microscopic images obtained after color-adjustment and auto-threshold for size analysis (middle rows) and number-based size distribution curves obtained from image analysis (lower rows).

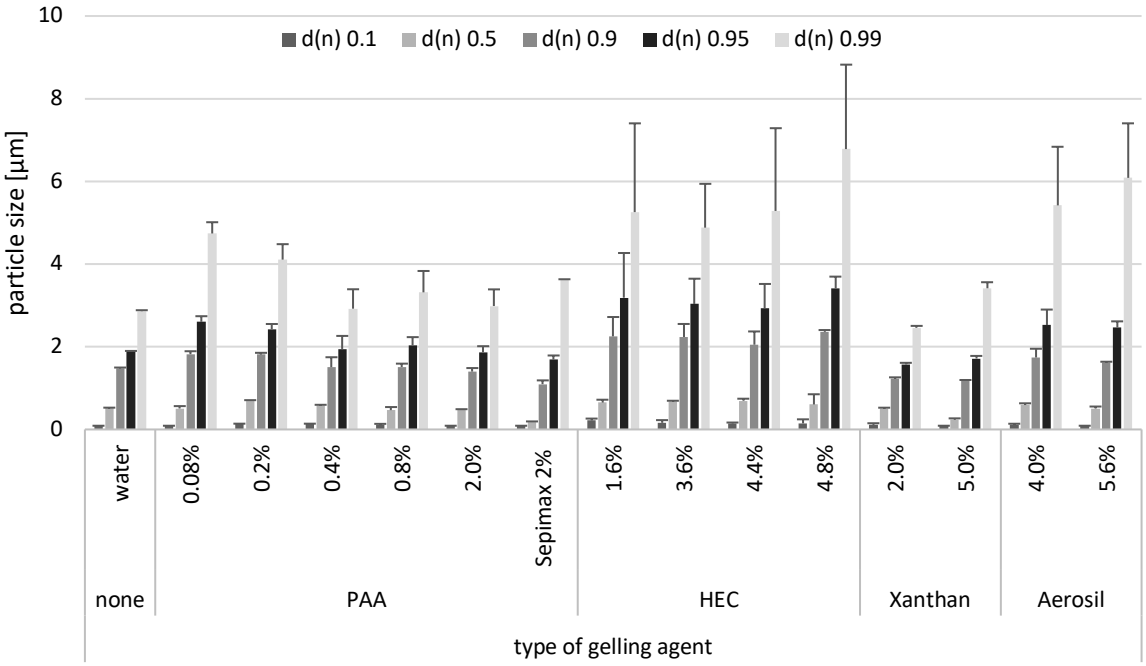


Figure 2: Numeric particle sizes of the nanocrystals based on the determination of Feret`s diameter from microscopic images for the aqueous nanosuspension and the different nanocrystal-loaded gels.

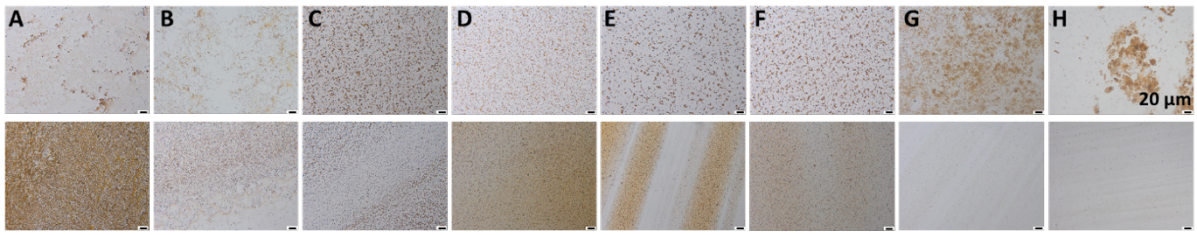


Figure 3: Microstructure of the curcumin nanosuspension and the nanocrystal-loaded gels. Upper: appearance before simulated skin massage. Lower: appearance after simulated skin massage. Magnification: 400fold. A: PAA 0.08%. B: PAA 0.2%. C: HEC 1.6%. D: HEC 3.6%. E: HEC 4.4%. F: HEC 4.8%. G: Oleogel 4%. H: Oleogel 5.6%.

3.2.1. Rheological properties of the gels

The viscosity of the gels was different (Fig. 4). As expected, higher concentrations of a gelling agent resulted in higher viscosities and similar concentrations of different types of gelling agents also resulted in different viscosities. Based on the results obtained, it was possible to divide the gels into four groups. In group 1 the gels possessed very low viscosities ($\eta < 0.5$ Pa•s), group 2 gels possessed gels with low viscosity ($\eta < 2$ Pa•s), group 3 gels possessed a medium viscosity between 3-4 Pa•s and group 4 gels possessed high viscosities in the range between 5-7 Pa•s (Fig. 4). All different gelling agents used were present in group 2 and 3, respectively. Thus, enabling to study the influence of the viscosity and the different types of gels (different in polarity and lipophilicity) on hair follicle targeting efficacy at the same time. The flow curves of the gels revealed a shear-thinning and thixotropic behaviour for all gels (Fig. 5). Only the gel that contained 1.6% HEC was only shear-thinning but not thixotropic (Fig. 5-2B).

The shear-thinning and thixotropic flow behaviour of the gels mean that the semi-solid gels will fluidize during the massage on the skin, leading to a good distribution of the formulation on the skin. After removing the shear forces, i.e. at end of the massage, the gels will recover in their structure and return completely or at least partly to their original viscosity (except the 1.6% HEC gel). The re-gelling of the formulation on the skin will lead to a film formation of the formulations on top of the skin and will prevent the formulations from flowing down from the skin surface. Thus, based on the data, it can be concluded that the nanocrystal-loaded gels (except the 1.6% HEC gel) possess an optimal flow behaviour for topical application on skin and the scalp.

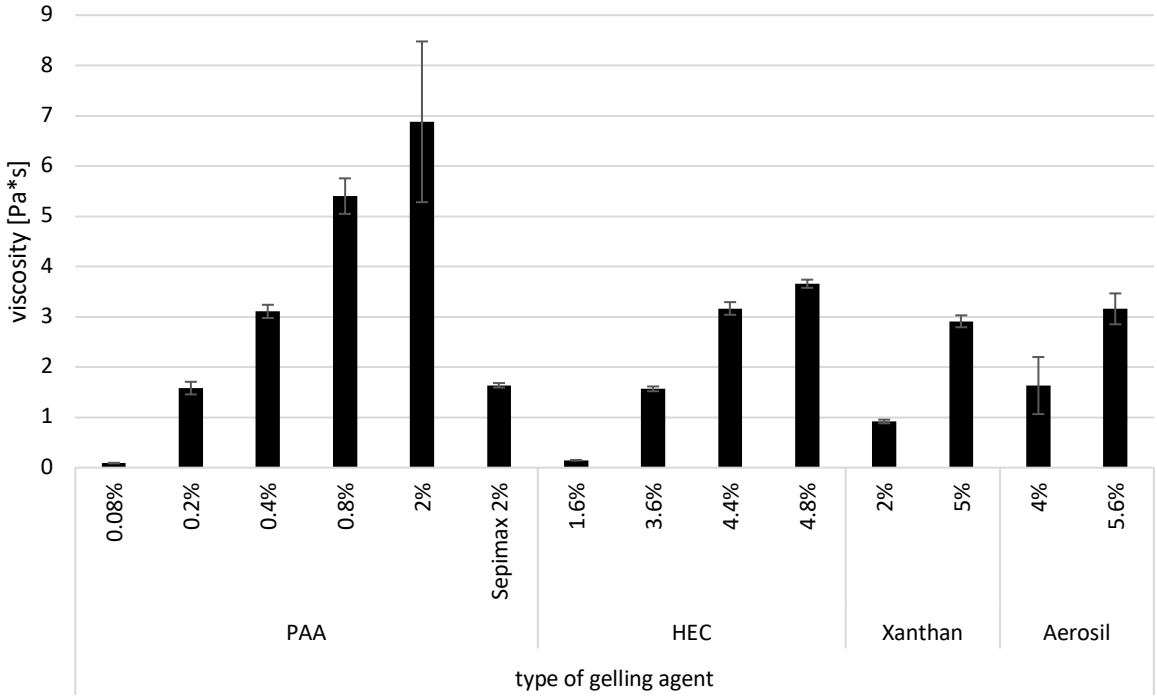


Figure 4: Viscosities of the different nanocrystal-loaded gels at a shear rate of 1/100s.

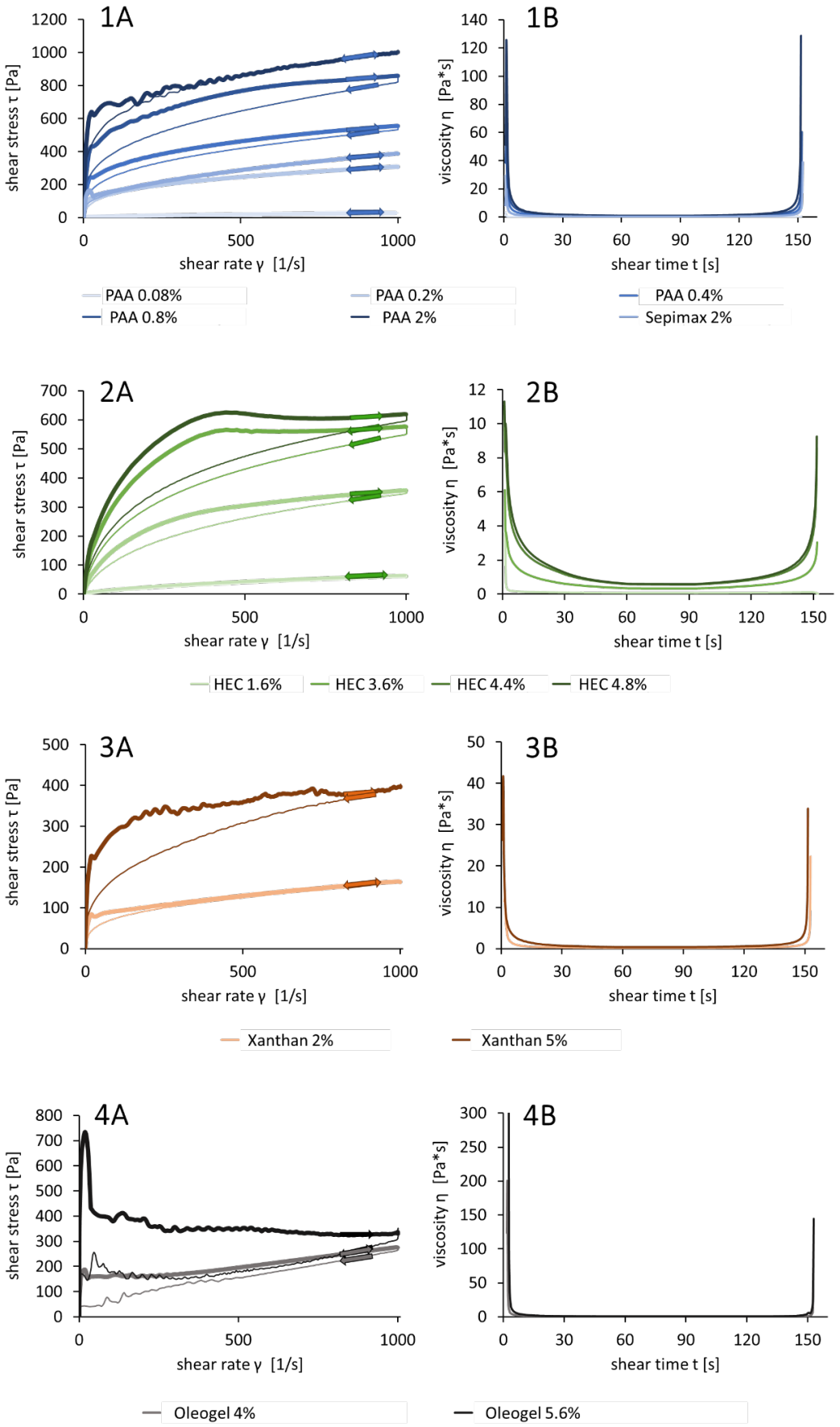


Figure 5: Rheograms of the different nanocrystal-loaded gels. 1: carbomer gels, SepimaxZen® gel. 2: HEC gels. 3: xanthan gum gels. 4: olive oil-Aerosil® gels; A: flow curves (thick: ascending curve, thin: descending curve). B: time-dependent changes in viscosity.

3.3. Role of the vehicle on hair follicle targeting of nanocrystals

In this study, gels with different properties, that were systematically varied in polarity, lipophilicity, and viscosity, were loaded with similar amounts of nanocrystals and tested regarding their ability to target nanocrystals into the hair follicles. All nanocrystal loaded gels, independent on type and concentration of the gelling agent used, led to an efficient uptake of the nanocrystals into the hair follicles (Fig. 6, supplementary material section S1). Nanocrystals could be detected in every single investigated hair follicle. Meaning, that all gels used in this study represent suitable vehicles for efficient delivery of curcumin nanocrystals into hair follicles.

A more detailed investigation of the influence of the varied formulation parameters on the follicular penetration of nanocrystals from the different formulations was enabled by digital image analysis and by determining the relative mean penetration depth (rMPD) of the nanocrystals into the hair follicles (Fig. 7). The mean penetration depth of the nanocrystals into the hair follicles was 271 µm ± 33 µm. This corresponds to an rMPD of about 25% when compared to the whole length of an ordinary hair follicle length. The hair follicle region being present at a depth of about 300 µm represents the lower part of the infundibulum of a hair follicle (Fig. 8A). This region is characterized by a weak, i.e. less developed, stratum corneum and thus is more permeable for drug substances.

The ability to target nanocrystals to this region thus opens a promising perspective for highly efficient drug delivery. After the transport of the nanocrystals into the lower infundibulum of the hair follicle, it can be expected that the nanocrystals will remain there for several days or even weeks [5–7]. This enables the nanocrystals to act as a long-term drug reservoir from which the drug can be released continuously. Due to the weak stratum corneum barrier in the lower infundibulum region (Fig. 8A), a very efficient drug uptake, i.e. passive diffusion of the dissolved drug substance into deeper skin layers, can be reached. Thus, leading to a constant flux of the drug substance into the viable dermis (Fig. 8B). As the nanocrystals are composed of 100% active, relatively high amounts of the drug substance can be transported into the hair follicle, when compared to other nanocarriers, that are typically composed of a matrix in which the drug substance is embedded. Hence, results obtained in this study could confirm the theory and showed that the formulation of drug substances into nanocrystals offers an efficient approach to target drug substances into the hair follicle.

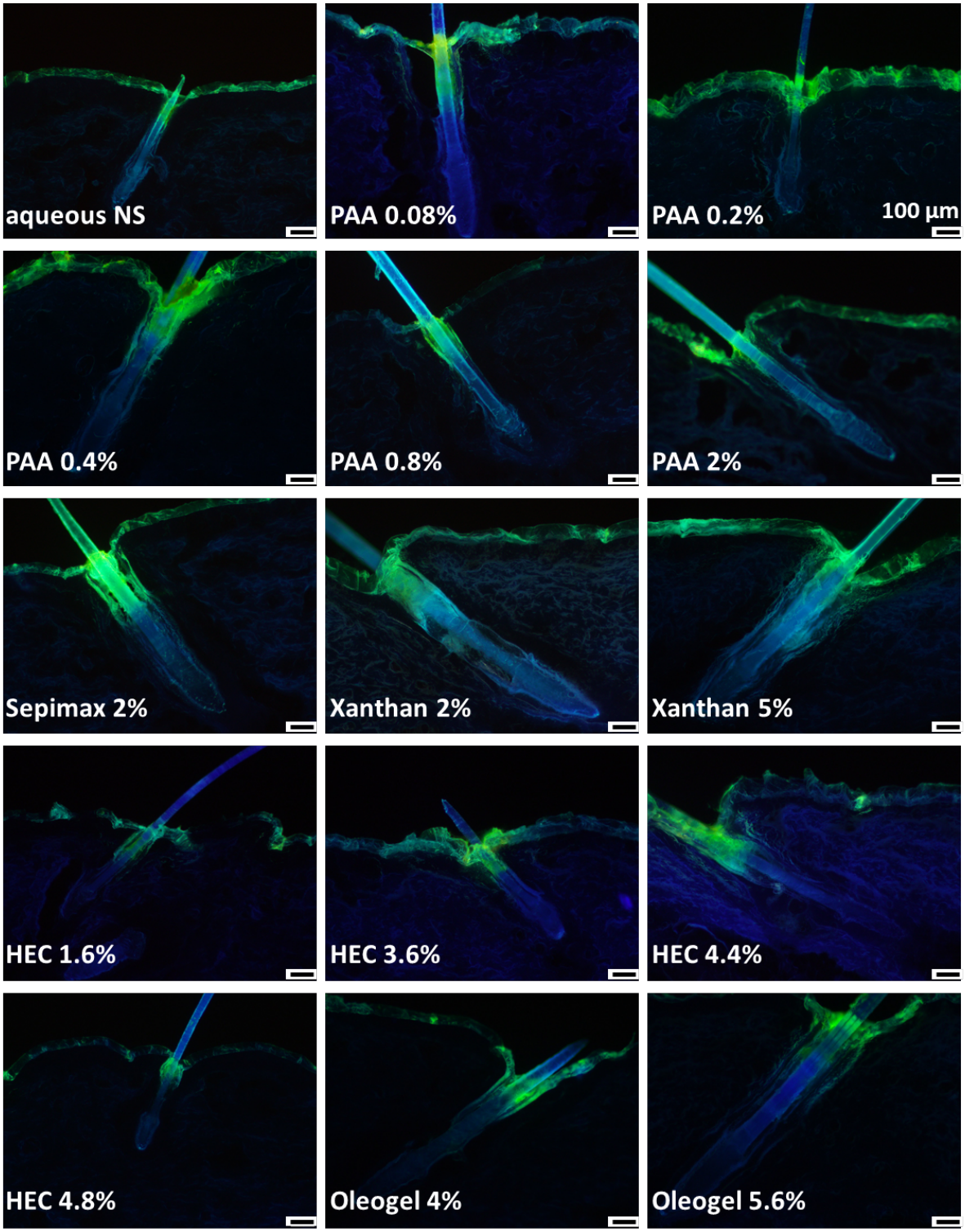


Figure 6: Penetration of curcumin nanocrystals into the hair follicles from an aqueous suspension and gels with different properties. Magnification: 100fold. For additional and more detailed images, please refer to supplementary material section S1-S16.

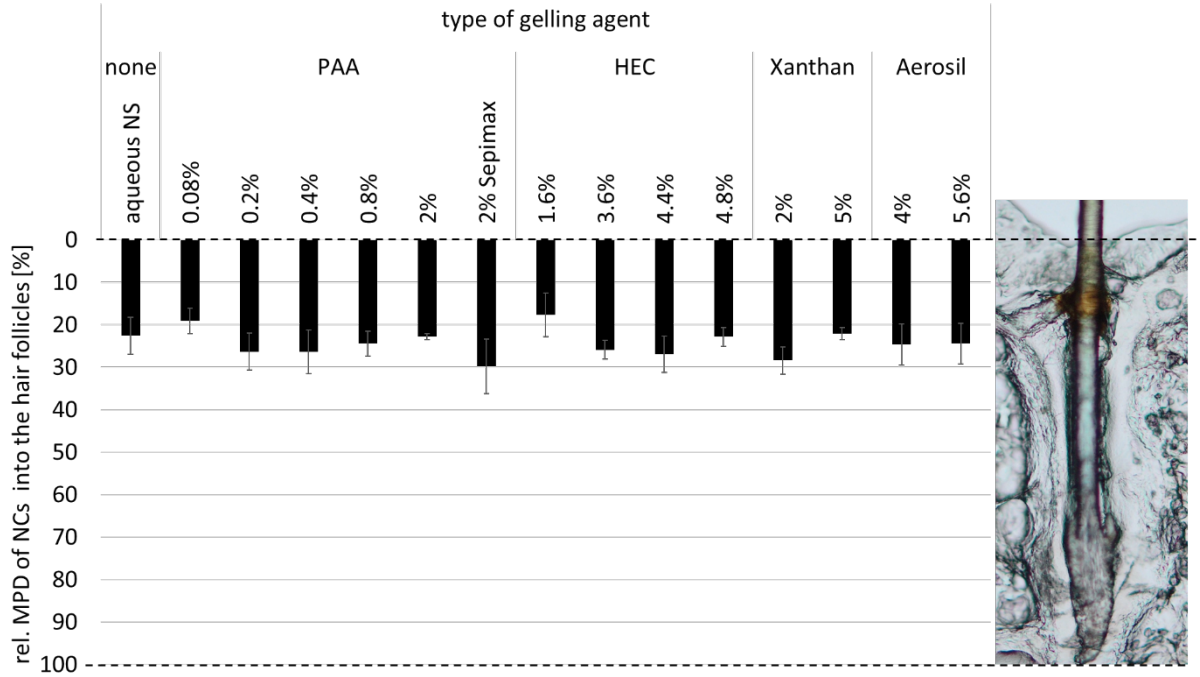


Figure 7: Relative penetration depths of curcumin nanocrystals into the hair follicles from an aqueous suspension and gels with different properties.

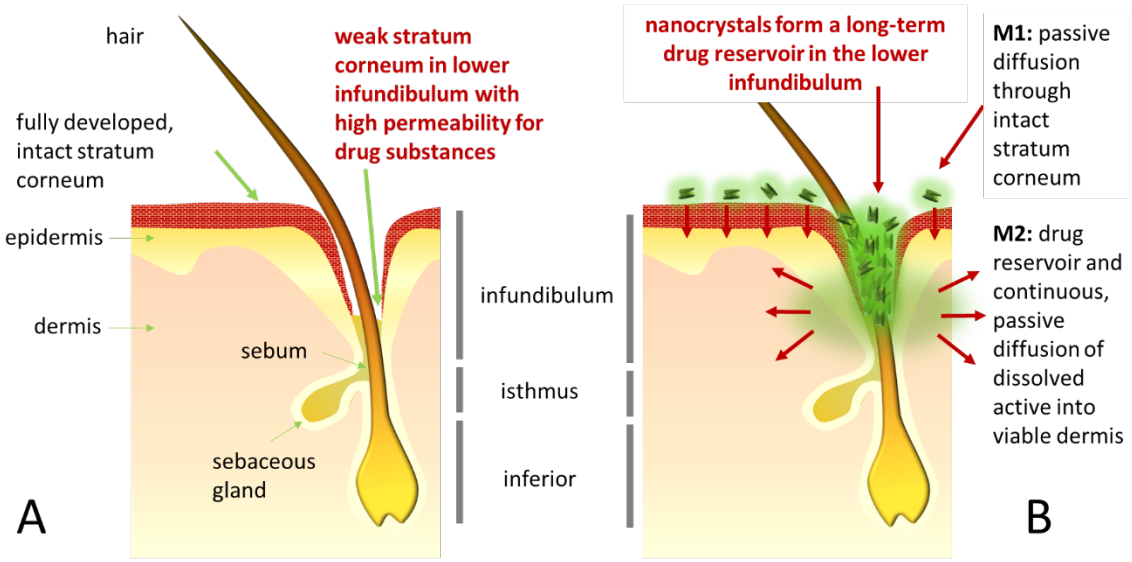


Figure 8: Scheme of the anatomy of hair follicle (A) and suggested mechanisms of dermal drug delivery with nanocrystals (B). Nanocrystals adhere to the surface of the skin and release the drug substance. Passive diffusion takes place due to a high local concentration gradient (M1). In addition, nanocrystals locate in the infundibulum of the hair follicles and form a depot that can last for up to several days. The drug substance is continuously released from the nanocrystals and can diffuse through the weak stratum corneum being present in the lower part of the infundibulum. This results in long-term drug diffusion, directly into the viable dermis (M2).

Interestingly, no statistical differences in the rMPD were found for the nanocrystals from the different gels. Hence, the hair follicle targeting with nanocrystals was found to be not influenced by the type and the concentration of the gelling agent in which the nanocrystals were dispersed. Also, the

lipophilicity and the polarity of the liquid gel seem to have no significant influence on the penetration depth of the nanocrystals (Fig. 7).

The penetration of particles into the hair follicles from different formulations has been partly investigated previously. However, controversial data were reported. For example, Suwannateep et al. investigated the follicular penetration of encapsulated curcumin, formulated as an aqueous suspension, o/w lotion and w/o lotion, respectively [23]. According to the published data, curcumin spheres penetrated significantly deeper into the follicles from lotions than from the aqueous suspension. The results were explained by an agglomeration tendency of the hydrophobic particles in the aqueous medium. Furthermore, the w/o lotion, i.e. the more lipophilic vehicle, enhanced the penetration of the particles in a more pronounced manner. Also, a study by Patzelt et al. revealed comparable results [22]. They investigated the follicular penetration of particles from liquid suspensions and semi-solid gels and found a significantly deeper penetration with the gel preparations compared to the liquid suspensions. Data were also explained by an aggregation tendency of the particles in the liquid suspension. In contrast, results obtained by investigations of Główka et al. revealed an opposite outcome [24]. The authors compared the follicular penetration efficacy of nanoparticles from an aqueous suspension and an organogel and observed a slightly deeper penetration of the particles from the liquid suspension. The observation was explained by the higher viscosity of the gel. This assumption appears reasonable because also other studies already demonstrated a decreased passive diffusion of actives from vehicles with higher viscosity [34–36]. However, due to the different penetration mechanisms, data obtained from passive diffusion of molecules and hair follicle targeting of nanosized particles might not allow for direct comparison and a cross-link between the data. In fact, to date, no conclusive data about the influence of the type of vehicle on the hair follicle targeting efficacy of nanocarriers were available and no data are available for nanocrystals formulated in different types of vehicles. Also the results from this study appear not conclusive at the first glance, because it was initially assumed that the formulation may affect the conditions of the hair follicles leading to a modified permeability for the particles.

Due to the fact, that the hair follicle infundibulum is at least partly filled with lipophilic sebum build of neutral and non-polar lipids, it could be presumed that vehicles with varied lipophilicity and/or polarity may, in theory, cause different interactions with the sebum components [37,38]. That could influence the sebum fluidity, leading to altered hair follicle permeability of the nanocrystals. In addition, according to our initial theory, different viscosities of the vehicles may lead to different fixations of the nanocrystals within the vehicles that would theoretically also influence the extent of the hair follicle penetration. However, none of these assumptions have been confirmed by the results obtained in this study.

One possible reason for this outcome might be the shear thinning flow behaviour of the gels used in this study, which leads to fluidization of the gels during massage (Fig. 5). This means that during the massage all gels will possess relatively low viscosities. The low viscosity might result in almost similar diffusion coefficients for the nanocrystals from all formulations. Thus, enabling a similarly good penetration of the nanocrystals into the hair follicles from all formulations. If this theory is valid, this would mean that most of the diffusion of the nanocrystals into the hair follicles would already take place during the short massage and that only small amounts of nanocrystals (if any) will penetrate later on. This would mean that long penetration times are not required to transport nanocrystals into the hair follicles. Instead, a short massage would be sufficient. Such a simple and time-saving application procedure would be very desirable, especially if it is aimed to transport active substances into the hair follicles of the scalp, where long penetration times and the necessity to apply leave-on products on the scalp hair, might strongly impair the patients or costumers' sense of well-being. Of course, data obtained in this study are too preliminary to draw a conclusion for this. However, the theory seems plausible and – if true – it will be very relevant for clinical (or cosmeceutical) applications. It should therefore be kept in mind for further – more detailed – investigations in this regard.

A further reason that might explain the results of the present study is the fact all formulations contained similar particle sizes. Previous studies postulate that the particle size is a main factor influencing the penetration depth of particles into the follicles and conclude that particles of identical size will reach identical depths within the hair follicles [39]. The explanation for this is the penetration mechanism of nanoparticles into the hair follicles that can be explained by the so-called ratchet mechanism. This means the moving hair shaft, due to its structure, acts as a ratchet, which by its motion brings the particles deeply into the hair follicles [40]. The motion of the hair shaft can be achieved by the application of the nanocarrier-loaded formulations and subsequent mechanical massage [5]. However, so far, the ratchet effect was only postulated for nanocarriers which possess a spherical shape. Nanocrystals are obtained by milling larger sized bulk material to sizes to below 1 µm. Due to this, nanocrystals are not spherical and thus differ considerably in their form and shape from the other already investigated nanoparticles and may therefore be considered to interact differently with the hair follicle structures.

However, based on the data obtained in this study, it can be hypothesized that the ratchet mechanism is also applicable for the hair follicle targeting with nanocrystals. All nanocrystal-loaded gels, as well as the diluted nanosuspension, were similarly applied onto the skin by mechanical massage. All formulations contained identical amounts of curcumin nanocrystals of identical size and only the properties of the vehicles were varied. Due to the fact, that the variations of the formulation properties were considered to disappear during massage (viscosity of the gels) or to have no influence (lipophilicity and polarity of the gel phase) on the penetration depth of the nanocrystals into the

follicles, it can be assumed, that the curcumin nanocrystals penetrate the hair follicles following the mechanical ratchet-mechanism described previously for other nanoparticles [39,40]. As the ratchet effect is described to be a size-dependent effect, the identical size of the nanocrystals resulted in identical penetration depths within the hair follicle, independent of the vehicle used. The optimal size of nanoparticles for targeting the hair follicle is assumed to be in the range between 500-700 nm [39,40]. With a size of about 300 nm the nanocrystals used in this study where distinctly smaller and thus it can be speculated that larger sized nanocrystals, i.e. in the range between 500-700 nm, might reach even deeper regions in the hair follicle. Thus, enabling an even more effective drug targeting effect into the hair follicle. However, more detailed investigations are needed to prove this assumption in more detail.

Data from this study provide a clear trend, showing that the polarity and the lipophilicity of the gel phase have no influence on the penetration of the nanocrystals into the hair follicle. Also, the viscosity of the gel will not significantly influence the penetration efficacy of the nanocrystals if the gel possesses shear thinning properties. The results suggest good flexibility for the development of nanocrystal-based formulations for drug targeting into hair follicles. This is because vehicles do not need to full-fill special requirements for efficient nanocrystal targeting into the hair follicles. This means the properties of the vehicles can be adjusted to other requirements, e.g. type of skin, ease of application, physical and chemical stability of the nanocrystals, etc.

3.4. Role of the vehicle on passive dermal penetration of curcumin from nanocrystals

In addition to the hair follicle targeting, it was also interesting to examine the influence of the type of vehicle on the passive dermal diffusion of curcumin through the skin (M1, Fig. 8B). For this, skin sections treated with different gels, covering a broad range of the varied vehicle properties, were selected and inspected regarding their passive dermal penetration of curcumin (Fig. 9).

In contrast to the results obtained from the hair follicle targeting study, where the type of vehicle was found to have no influence on the penetration of nanocrystals into the hair follicles, pronounced differences in the passive dermal penetration efficacy of curcumin were found from the visual inspection of the skin biopsies for the different vehicles. Poor passive diffusion was obtained from the aqueous nanosuspension and the hydrogels that contained low amounts of PAA or HEC as a gelling agent (Fig. 9, upper). Also, the incorporation of the nanocrystals into oleogels did not result in a pronounced passive dermal penetration of curcumin (Fig. 9, middle). However, the incorporation of the nanocrystals into PAA gels with higher viscosity and the use of xanthan gum as gelling agent resulted in a pronounced increase in the passive diffusion of curcumin (Fig. 9, lower).

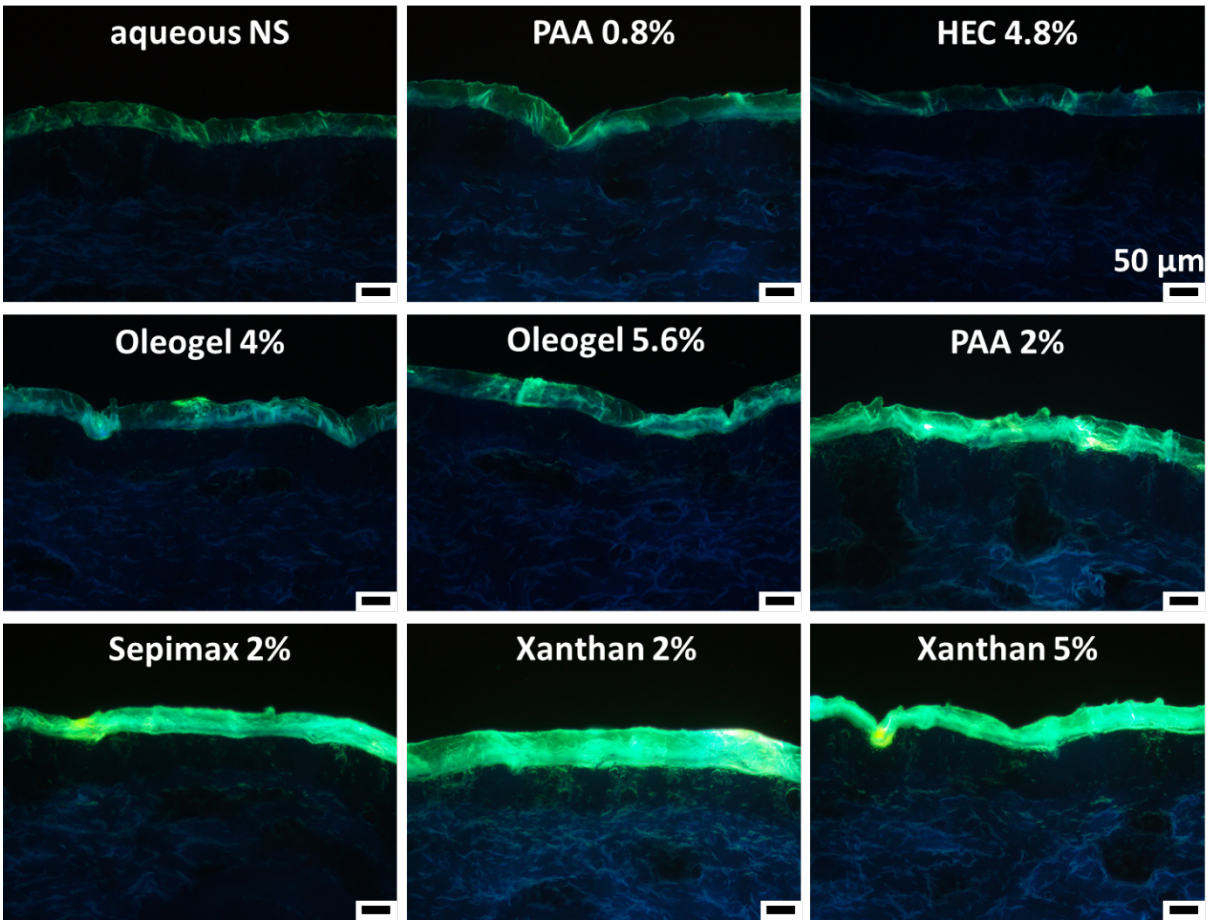


Figure 9: Passive dermal penetration of curcumin from an aqueous nanosuspension and curcumin nanocrystals suspended in different types of gels. Magnification: 200fold.

Even though there was no clear trend that could suggest a specific property of the vehicle being responsible for the different penetration results obtained, it was possible to split the vehicles into two groups. The first group included the aqueous nanosuspension as well as gels is based on 0.8% carbomer, 4.8% HEC and both oleogels. These formulations could be characterized by a poor and inhomogeneous penetration of curcumin, which was limited to the upper layers of the skin, i.e. the stratum corneum (Fig. 9 A-E). Formulations belonging to the second group, i.e. 2% carbomer gel, 2% SepimaxZen® gel and both xanthan gum gels, demonstrated a more pronounced and more homogeneous penetration of curcumin, which was not only limited to the stratum corneum but was found to reach also deeper layers of the skin (Fig. 9 F-I).

Digital analysis with ImageJ software, followed by statistical data evaluation, was employed to transfer the observations from visual inspection into objective numbers. Results obtained confirmed the results of the visual inspection and revealed two types of vehicles with different penetration profiles (Fig. 10).

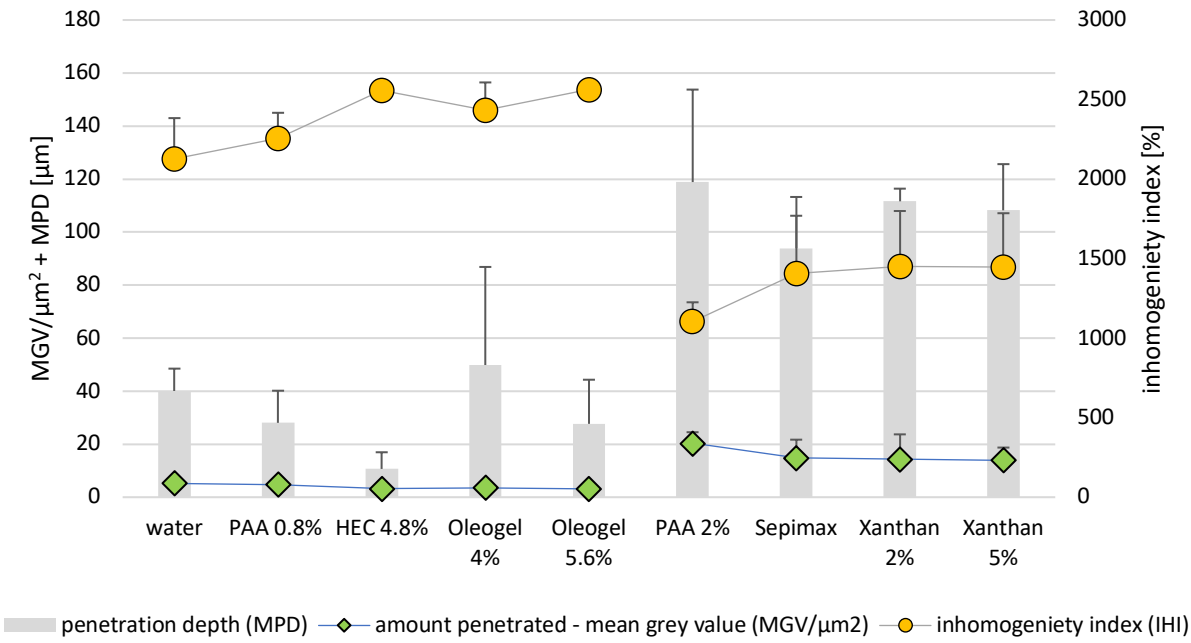


Figure 10: Influence of the type of vehicle on the dermal, passive penetration efficacy (penetration depth, the total amount of penetrated curcumin and penetration inhomogeneity) of curcumin from nanocrystals. Data were assessed via digital processing of the images obtained from epifluorescence microscopy.

The aqueous nanosuspension, the 0.8% carbomer gel, the 4.8% HEC gel, and both oleogels, i.e. the group 1 formulations, showed low amounts of penetrated curcumin with penetration-values MGV/µm^2 ≤ 5 (Fig. 10, green diamonds – left) and could not reach deeper skin layers (Fig. 10, grey columns – left). In contrast, about 4-fold higher penetration values were found for the group 2 gels (Fig. 10, green diamonds – right). The average penetration depth of curcumin from nanocrystals incorporated in group 2 formulations was about 108 µm, indicating that curcumin penetrated not only into the stratum corneum but could also reach deeper skin layers (Fig. 10, grey columns – right). In addition, a more homogeneous and consistent skin penetration of curcumin was obtained from group 2 formulations (Fig. 10, orange circles).

A less efficient penetration of the lipophilic curcumin from the oleogels can be expected and explained when considering the 1. Fick's law of diffusion, where one driving parameter is the partition coefficient of the drug substance between vehicle and skin. If the lipophilic curcumin is added to a lipophilic vehicle, the partition coefficient is lower than by adding the curcumin to a hydrophilic vehicle, respectively. In addition, a small effect on the viscosity could also be seen for these gels. A slightly lower, significant penetration was found for the oleogel with higher viscosity. Thus, confirming the validity of Fick's first law of diffusion for nanocrystal-loaded gels, as well as the results from previous studies were increased viscosities were also shown to hamper the flux of drug molecules into the skin [34–36]. However, the poor penetration of curcumin from the HEC gel and the 0.8% PAA gel could not be related to this. Also, differences in viscosity, particle size or polarity of the gels could be excluded to cause the differences between these hydrogels. Hence, other reasons and a probably more complex

interplay between the different penetration influencing parameters need to be considered to be responsible for the different penetration profiles obtained from these gels.

To understand the cause of the pronounced differences in penetration efficacy between group 1 and 2 gels (c.f. Fig. 9 and 10), data were re-evaluated. The re-evaluation of the data resulted in two observations. First, it was observed that during the penetration experiment, that was carried out for 6h, the hydrogels belonging to group 1 dried out, whereas the hydrogels belonging to group 2 remained in gel state on top of the skin (Fig. 11). In addition, a slightly thicker, i.e. more hydrated, stratum corneum could be estimated for all group 2 gels, whereas the thickness of the SC seemed to remain unchanged for the group 1 hydrogels when compared to untreated skin (Fig. 9).

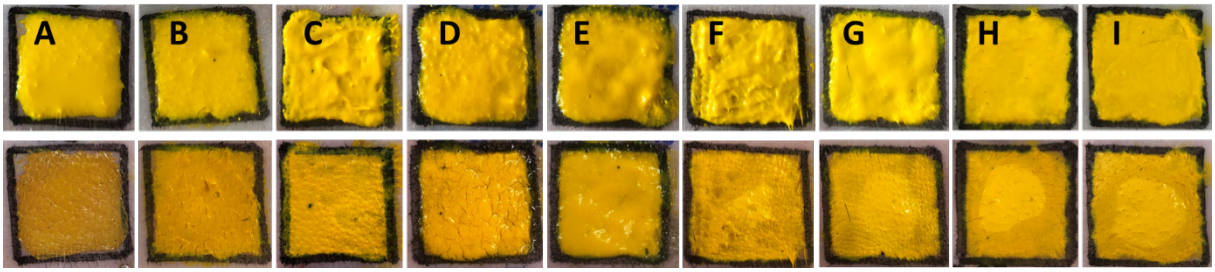
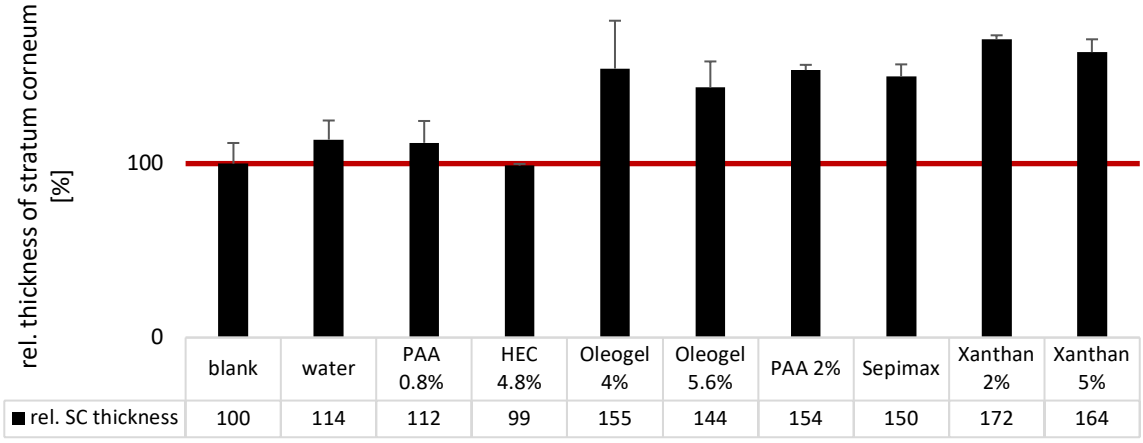


Figure 11: Macroscopic observation of the different curcumin formulations on the pig ear. Upper: appearance directly after topical application. Lower: appearance 6h after topical application. A: aqueous nanosuspension, B: PAA 0.8%, C: HEC 4.8%, D: Oleogel 4%, E: Oleogel 5.6%, F: PAA 2%, G: Sepimax 2%, H: Xanthan 2%, I: Xanthan 5%.

The visual observation of a swollen stratum corneum could be confirmed by measuring the thickness of the stratum corneum by employing ImageJ software, respectively (Fig. 12). Data showed significant differences in the stratum corneum thickness between the group 1 hydrogels and the group 2 gels, whereas no differences were found between the SC thickness of untreated skin, skin treated with the aqueous nanosuspension and the group 1 hydrogels (Fig. 12 left). Also, no differences in the SC thickness were found between the oleogels and the group 2 gels.

Data prove that dermal application of the oleogels and the group 2 gels leads to a swelling of the SC by about 60%. The oleogels, due to their lipophilic nature, can be regarded to possess occlusive properties. Hence, they form an oily film on top of the skin, thus preventing the evaporation of water from the skin, which then results in the hydration of the SC. In contrast, the hydrogels of group 2 can be considered to hydrate the skin directly, i.e. water from the vehicle penetrates into the stratum corneum and causes the SC to swell. The swelling and the increased hydration of the SC increase the flux for the drug substance [41] and thus caused an increased penetration of curcumin from these vehicles. In contrast, only limited penetration was observed for the formulations that were not able to hydrate the stratum corneum efficiently.



	blank	water	PAA 0.8%	HEC 4.8%	Oleogel 4%	Oleogel 5.6%	PAA 2%	Sepimax	Xanthan 2%	Xanthan 5%
■ rel. SC thickness	100	114	112	99	155	144	154	150	172	164

Figure 12: Relative thickness of the stratum corneum, measured 6h after dermal application of the different gels.

Another reason for the poor penetration of curcumin from the group 1 gels can be related to the fact, that drying of the vehicle will also reduce the solvent being available to keep the drug substance in solution. Nanocrystals possess an increased apparent solubility when compared to larger size bulk material and thus create a higher concentration gradient between vehicle and skin than larger sized crystals. However, if the solvent, i.e. the water in the vehicle, evaporates dissolved drug might re-crystallize, precipitate on the skin and thus form large crystals on the skin with reduced solubility. This leads to a reduced concentration gradient of the dissolved active between vehicles and skin and thus, represents another reason that could explain the poor penetration of curcumin from the group 1 hydrogels. Based on these considerations it was speculated that the addition of humectants might help to protect the formulations from drying out on the skin, thereby, increasing the penetration efficacy of the curcumin. In contrast, it was assumed that the addition of humectants to formulations that already possess excellent skin hydration properties, i.e. group 2 gels, will not cause pronounced changes in the dermal penetration efficacy of curcumin.

To prove this theory, HEC 4.8% gel and xanthan gum 5% gel were selected to represent gels from group 1 and 2, respectively. Curcumin-nanocrystal loaded gels were now produced with and without humectant (5% (w/w) glycerol 5%) and were subsequently subjected to skin penetration studies (Fig. 13). Macroscopic observation of the gels after 6h penetration confirmed that the addition of humectant prevented a drying-off of the formulations and inspection of the images obtained from the skin section could indeed prove the expected penetration enhancement for the group 1 gel after addition of the humectant (Fig. 13).

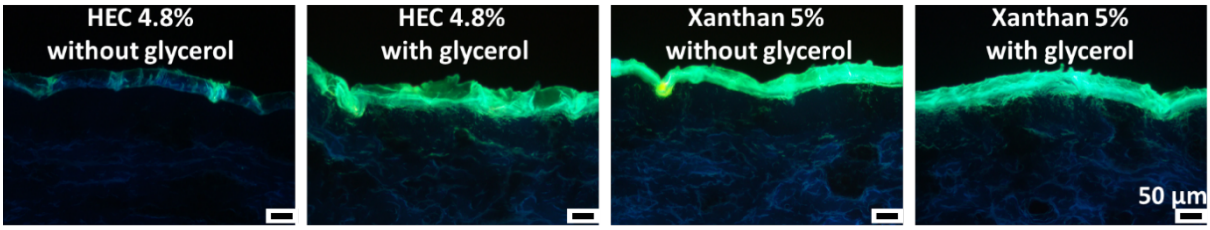


Figure 13: Images obtained from epifluorescence microscopy to determine the influence of humectant on the dermal, passive penetration efficacy of curcumin from nanocrystals. Magnification: 200fold.

Digital image analysis revealed an increase in stratum corneum thickness by about 70% for the group 1 gel and no changes in SC thickness for the group 2 gel (Table 2). The total amount of penetrated curcumin was about 5fold increased for the group 1 gel, whereas the factor was only 1.3 and not significantly different to the group 2 gel without humectant. The penetration depth for the group 1 gel was increased from about 10 µm to about 130 µm, whereas the penetration depth of the group 2 gel increased from about 110 µm to 125 µm. After the addition of the humectant to the group 1 gel, the penetration of curcumin became very consistent and less inhomogeneous, i.e. without skin blank spots and thus was now similar to the penetration homogeneity of the group 2 gels (Table 2, Fig. 13).

Table 2: Comparison of skin penetration data for the different selected gels with and without humectant.

skin and penetration properties	**group 1 gel**		**group 2 gel**	
	HEC 4.8 %	**HEC 4.8 % + glycerol**	**Xanthan gum 5%**	**Xanthan gum 5% + glycerol**
drying on skin	yes	no	no	no
thickness of stratum corneum [µm]	99 ± 9	166 ± 17	164 ± 13	158 ± 13
penetrated curcumin (MGV/µm²)	3.2 ± 0.2	18.4 ± 3.3	14.0 ± 4.8	18.0 ± 4.6
penetration depth [µm]	11 ± 6	129 ± 25	108 ± 17	125 ± 24
inhomogeneity [%]	2556 ± 46	1320 ± 164	1446 ± 340	1231± 150
penetration depth into hair follicle [µm]	275 ± 216	247 ± 89	286 ± 104	198 ± 72

The results could confirm the theory and show that the hydration of the stratum corneum is a key playing parameter for efficient drug delivery into the skin. Skin hydration can be achieved by direct hydration from the formulations and/or by the addition of a humectant. Results obtained from this part of the study suggest that dermal drug delivery of curcumin nanocrystals is most efficient if the nanocrystals are incorporated into a hydrogel with strong skin hydrating properties. Oleogels or hydrogels that dry out quickly after dermal application should not be used. It is likely that the findings from this study can also be transferred to other drug nanocrystal formulations. However, if this assumption is true will need further investigations in this regard. The first evidence for the transferability of the findings from this study to other types of nanocrystals is already given by the data obtained from a previous study by Pelikh et al., where the authors could already show that the type of vehicle in which the nanocrystals are suspended can have a great impact on the penetration efficacy of the drug substance [21].

However, another observation that requires more detailed investigations in future, is the fact that the addition of glycerol caused a significant decrease in the efficacy of the nanocrystals to penetrate the hair follicle (Table 2). Reasons for the decreased follicular targeting efficacy of nanocrystals after the addition of humectants to vehicles might be a swelling of the hair shaft and/or changes in the structure of the hair cuticula. These changes in the hair properties might cause a modified rachet-effect, which could then result in a modified transport of the nanoparticles into the hair follicle. However, data obtained in this study are too preliminary to draw conclusions in this regard. More detailed and systematic studies are suggested to investigate this observation in more detail.

4. Conclusions

The study investigated the influence of vehicle properties, i.e. viscosity, lipophilicity and polarity, on the penetration efficacy of curcumin nanocrystals into hair follicles. Unexpectedly, it was found that none of these properties affected the penetration of the nanocrystals into the hair follicles. Hence, no special requirements or criteria are needed for the formulation of drug nanocrystals for hair follicle targeting. Thus, enabling the development of nanocrystal-loaded vehicles that can be individually adapted to the needs of the patient or customer without affecting the penetration efficiency of drug nanocrystals into the hair follicles.

Dermal drug delivery with nanocrystals can also be used to improve the passive diffusion of poorly soluble compounds through the skin and thus it was also interesting to investigate the effect of the different vehicles on the passive dermal penetration of curcumin. Results indicate that hydrogels are more suitable for the formulation of nanocrystals than oleogels. However, only if they possess good skin hydrating properties. Formulations that are not able to hydrate the skin were found to result in poor penetration. The addition of humectants increased the skin hydration and thus the passive dermal penetration of curcumin through the skin. However, the addition of humectants was also found to decrease the penetration efficacy of the nanocrystals into the hair follicles.

Thus, data of the study show that the properties of the vehicles in which the nanocrystals are incorporated can have a tremendous influence on the dermal penetration efficacy and the ability to target the hair follicle. The influence of excipients, e.g. humectants or other ingredients, needs further and detailed investigations.

5. Acknowledgements

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6. References

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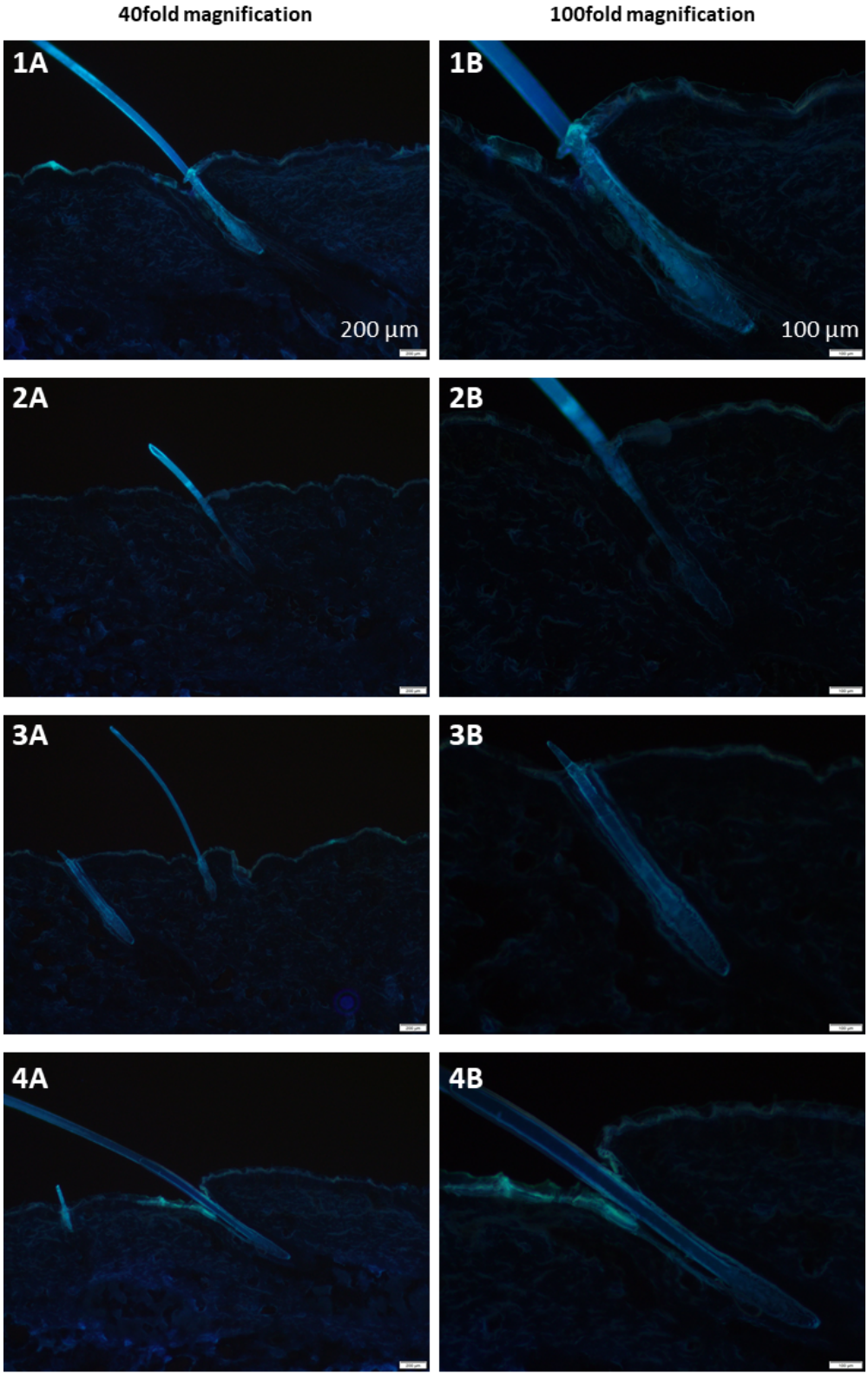
S. Supplementary material to manuscript: Hair follicle targeting with curcumin nanocrystals: influence of the formulation properties on the penetration efficacy

S1: Untreated hair follicles. A: 40fold magnification. B: 100fold magnification.



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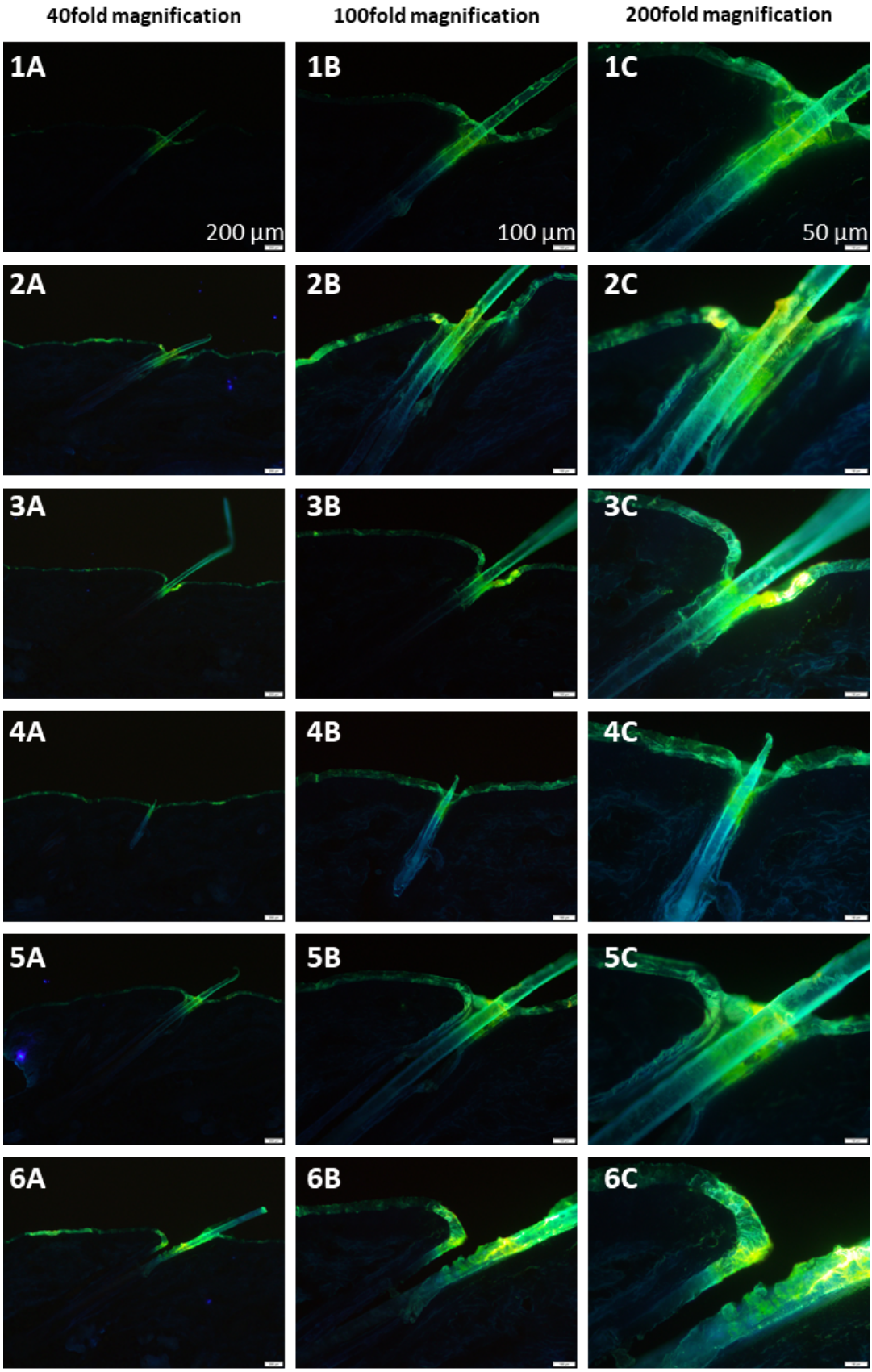

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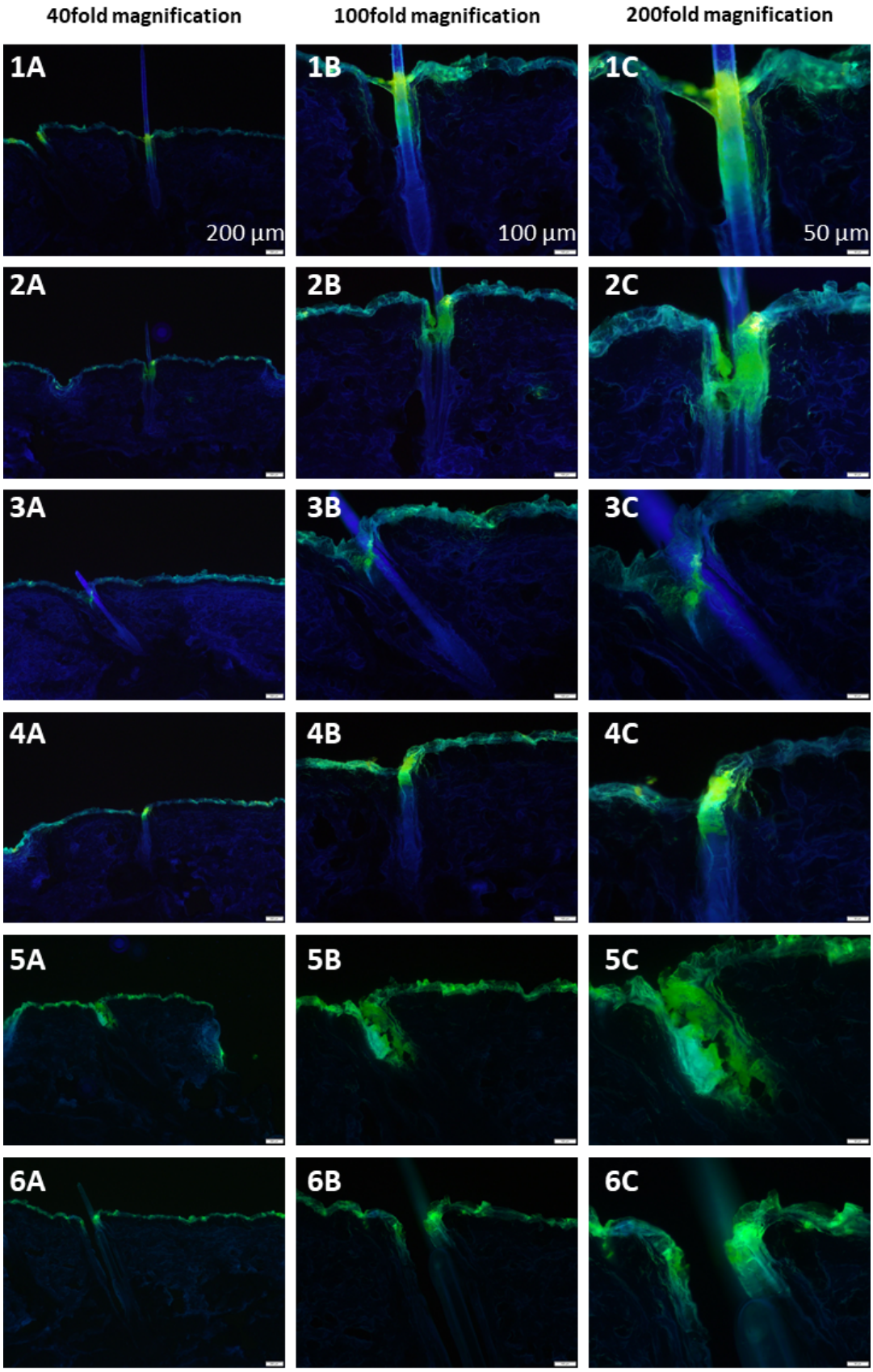
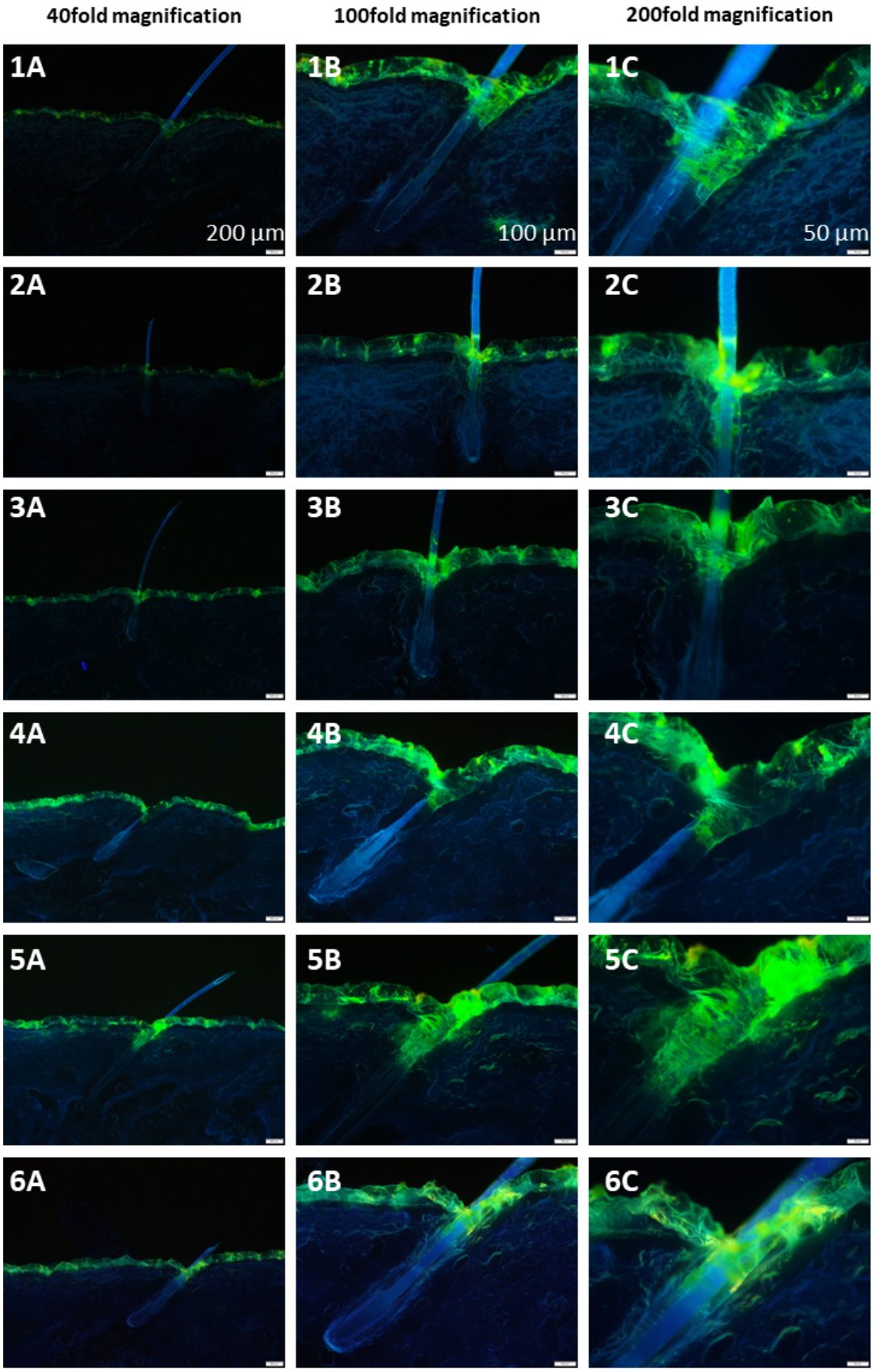
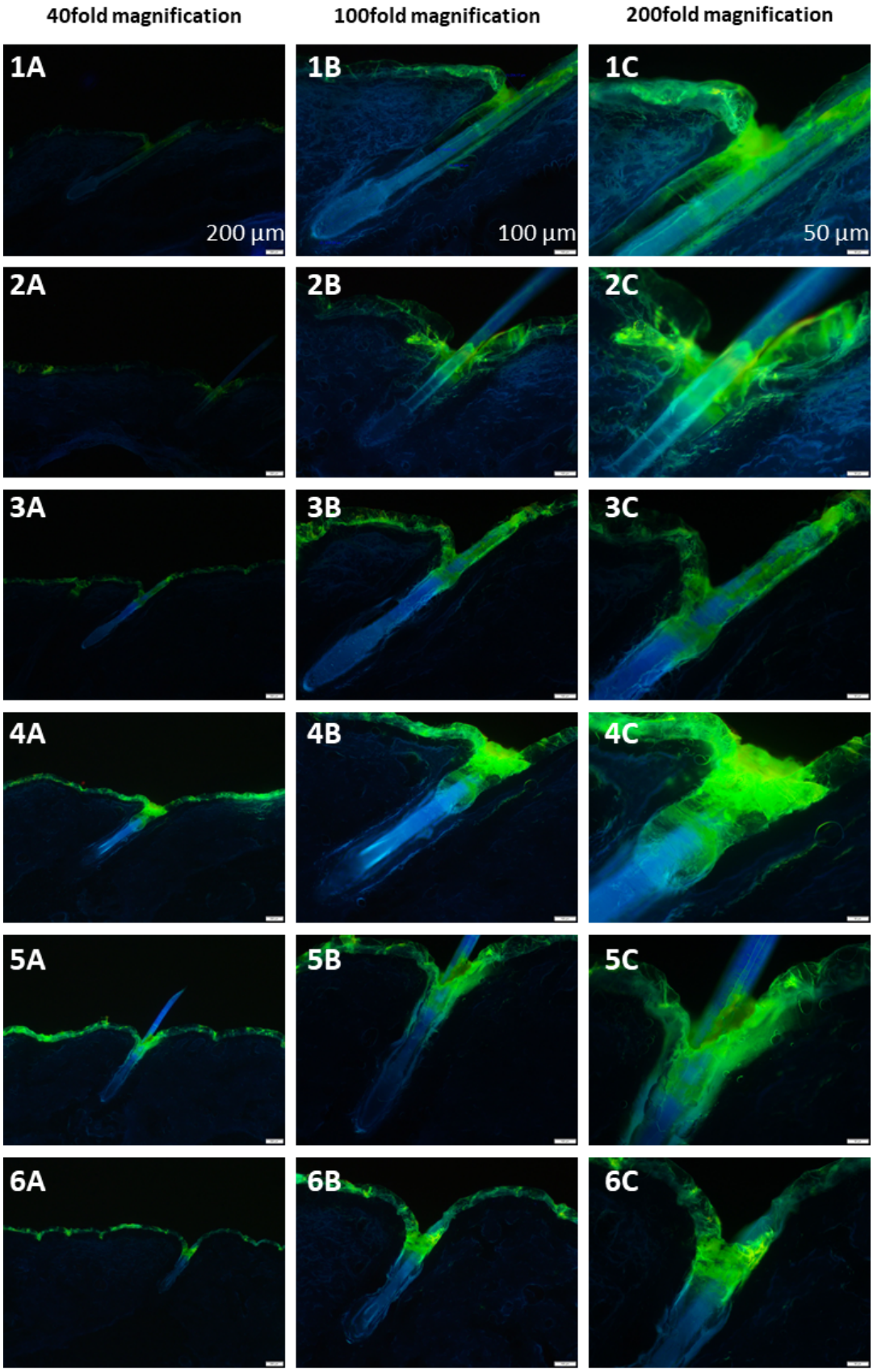


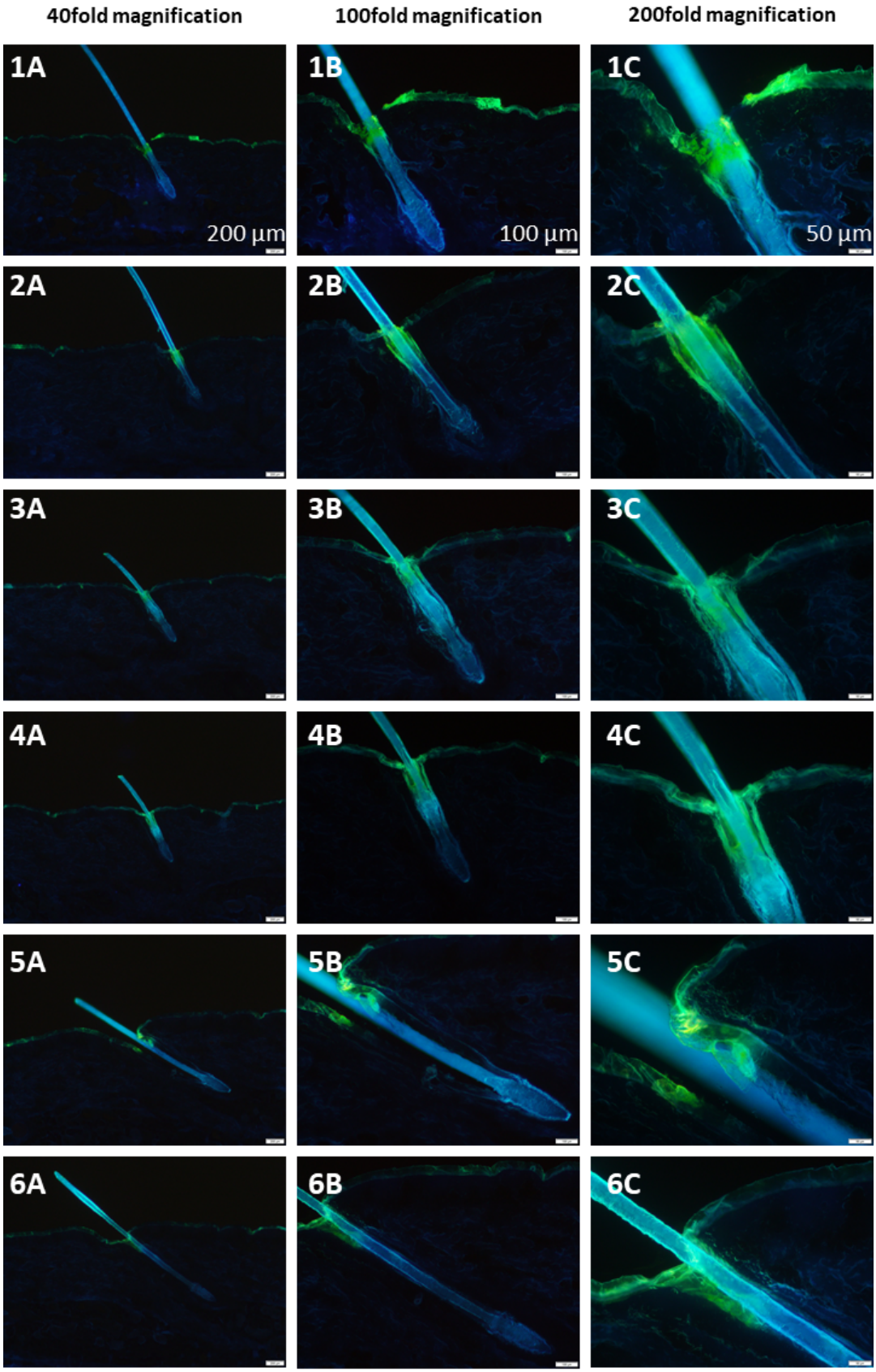
S4: Hair follicles treated with 0.2% carbomer gel. A: 40fold magnification. B: 100fold magnification. C: 200fold magnification.



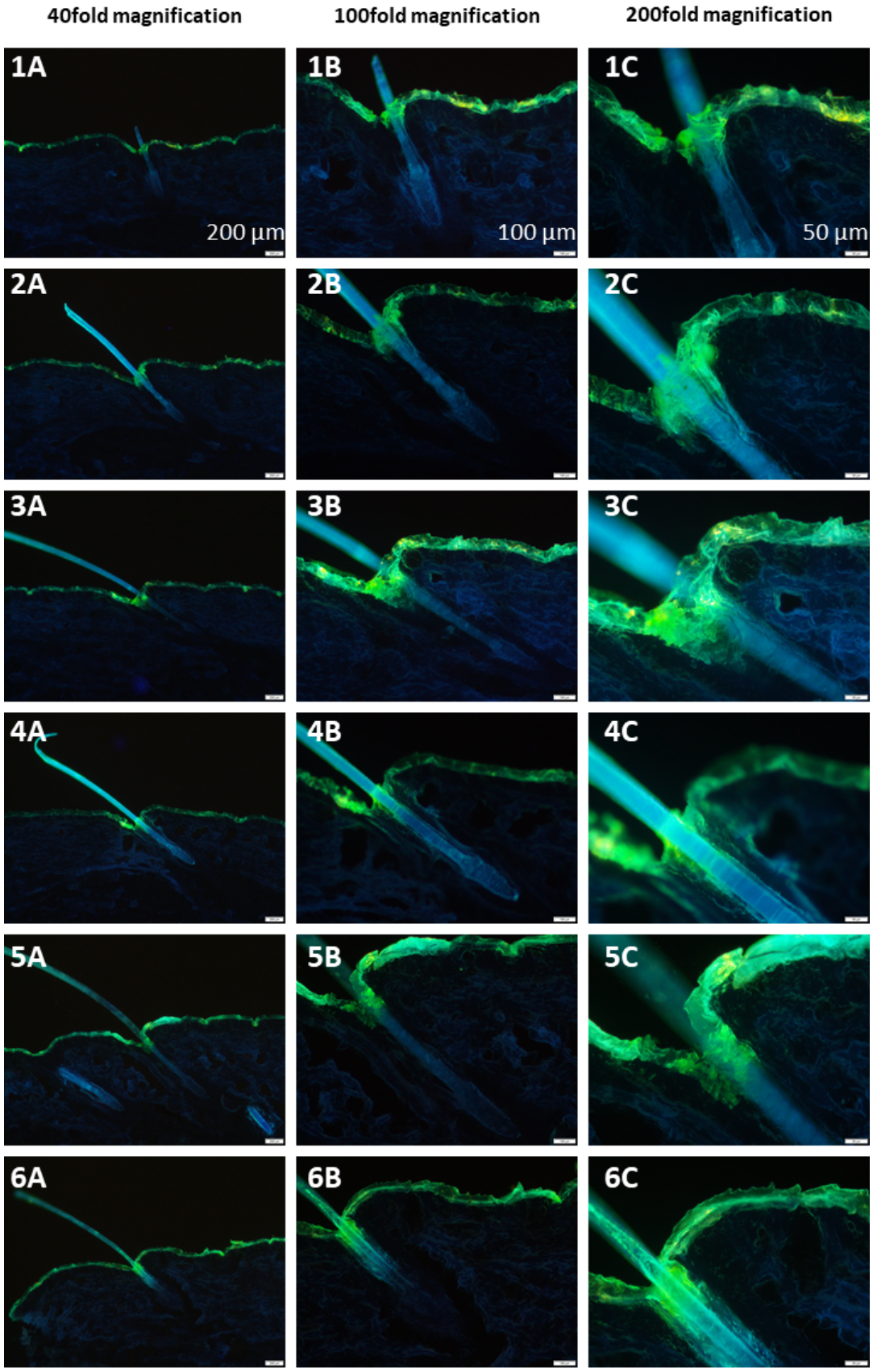
S5: Hair follicles treated with 0.4% carbomer gel. A: 40fold magnification. B: 100fold magnification. C: 200fold magnification.



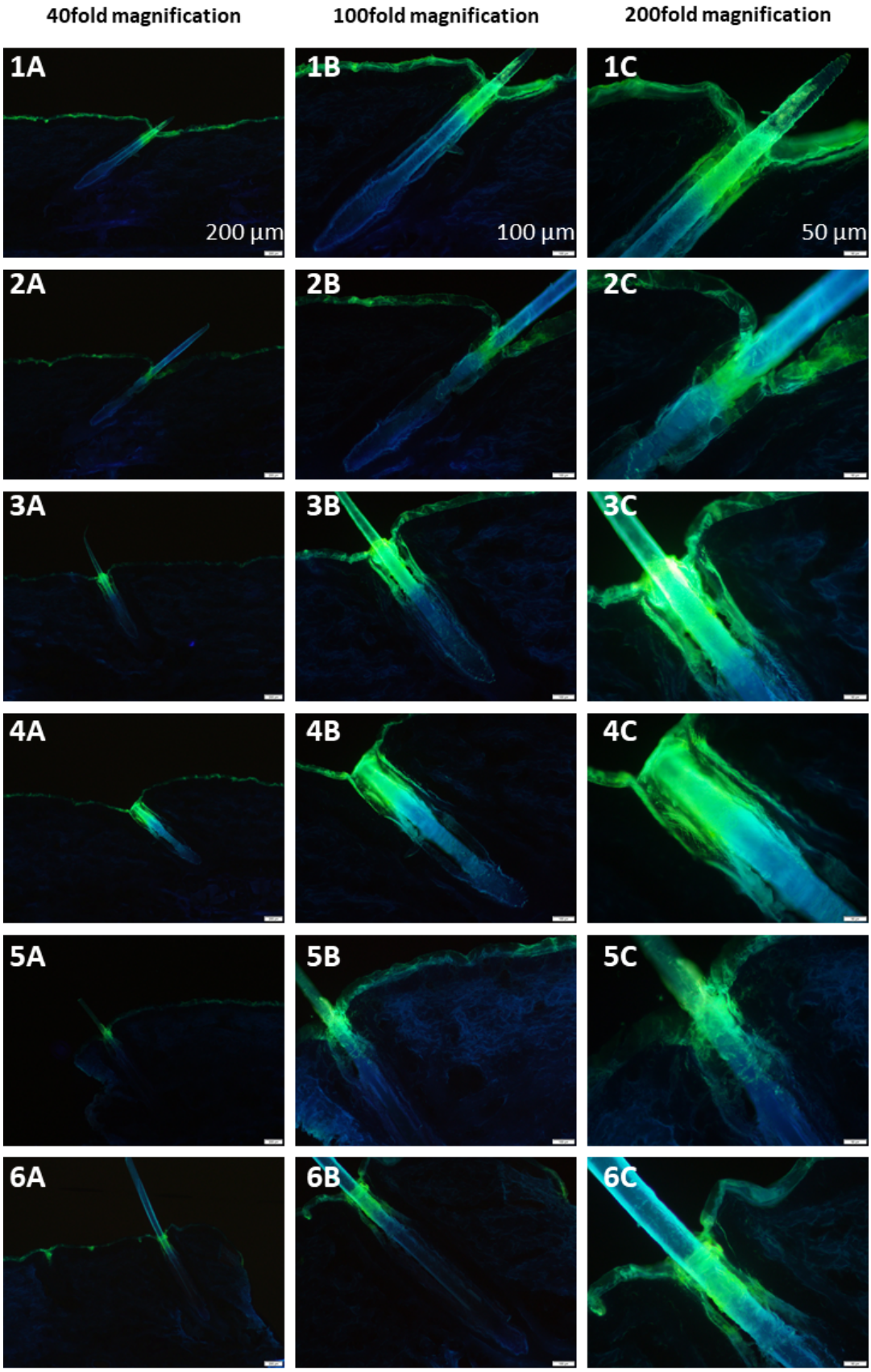
S6: Hair follicles treated with 0.8% carbomer gel. A: 40fold magnification. B: 100fold magnification. C: 200fold magnification.



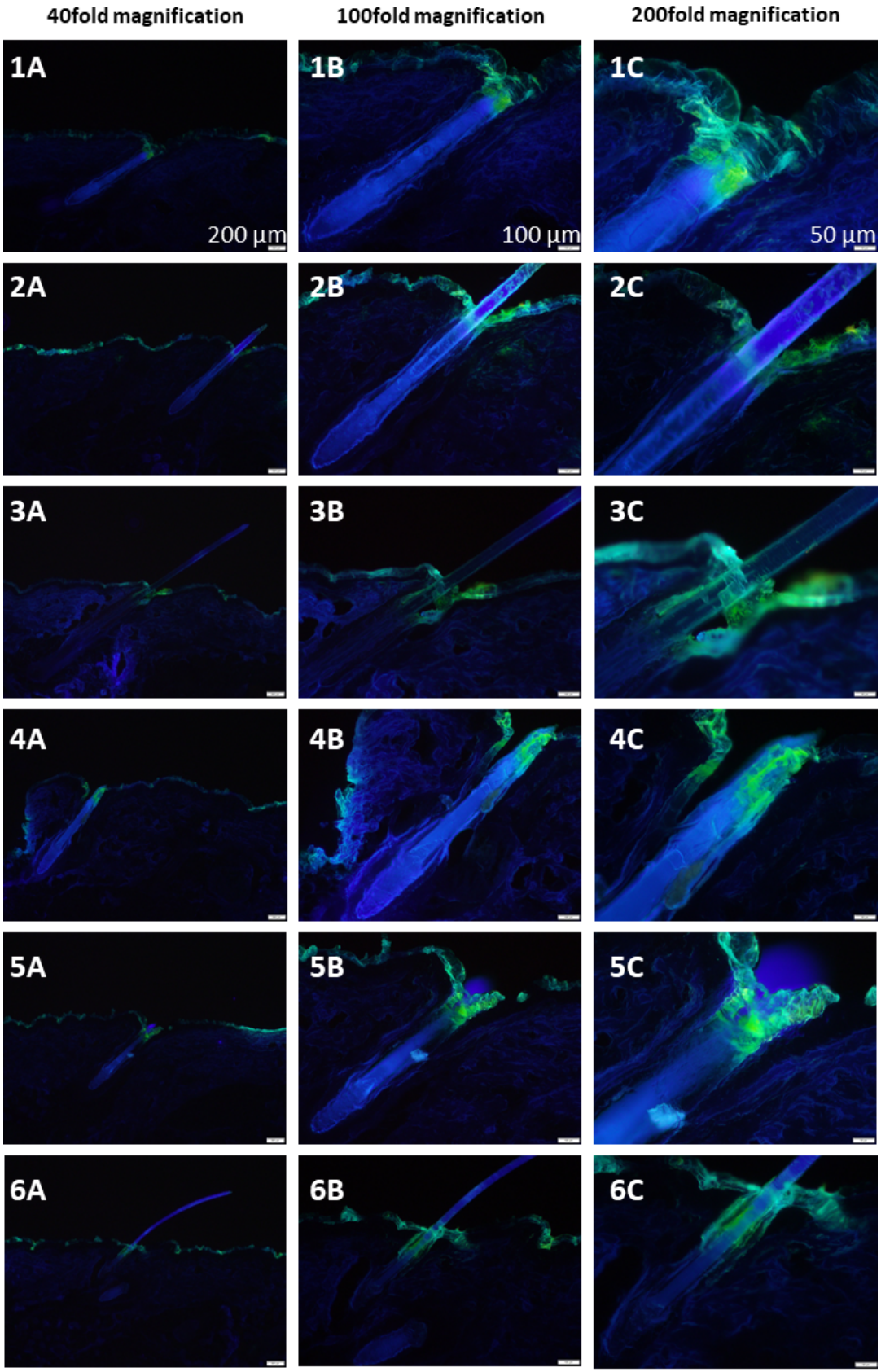
S7: Hair follicles treated with 2% carbomer gel. A: 40fold magnification. B: 100fold magnification. C: 200fold magnification.



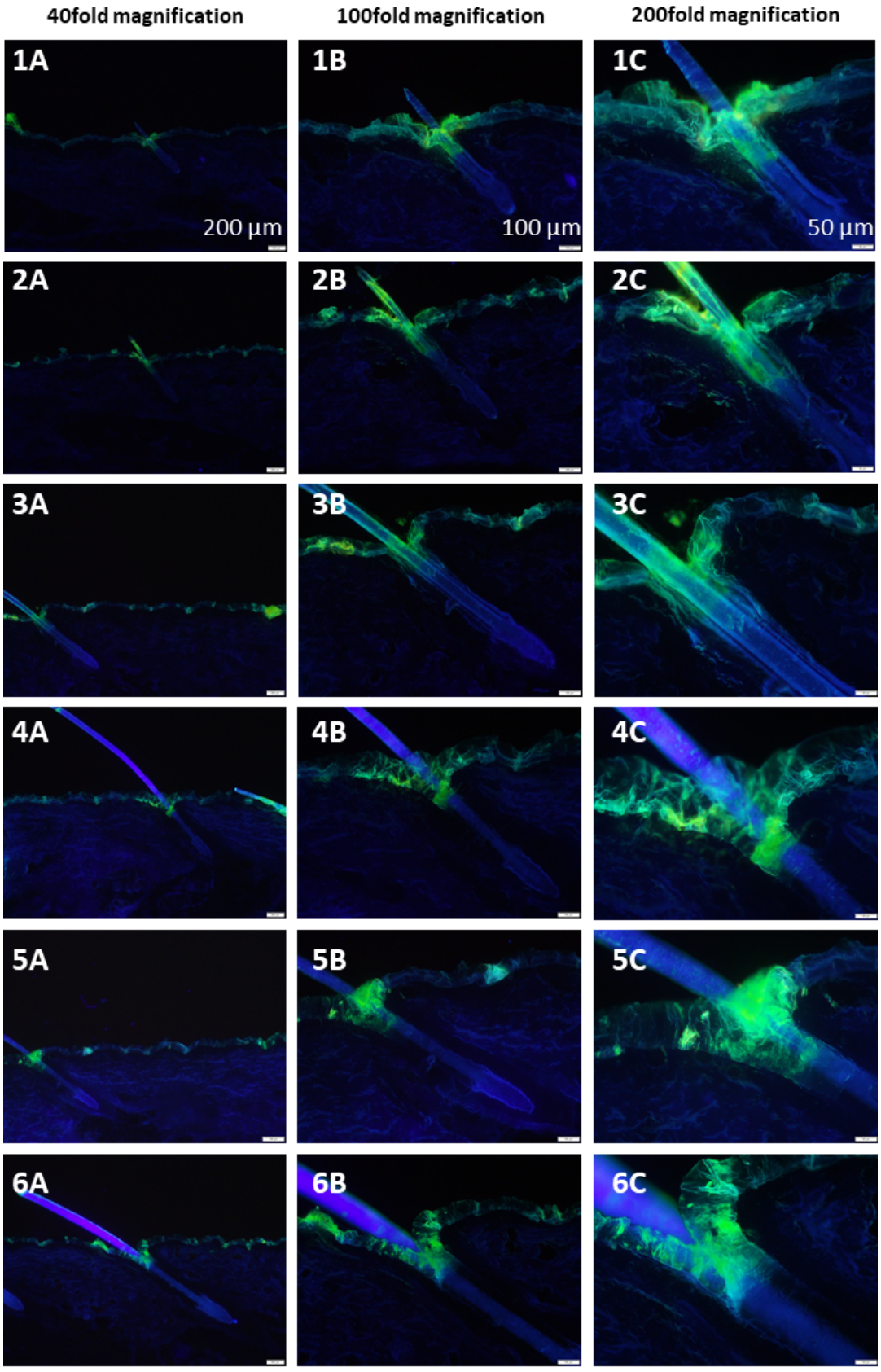
S8: Hair follicles treated with 2% SepimaxZen® gel. A: 40fold magnification. B: 100fold magnification. C: 200fold magnification.



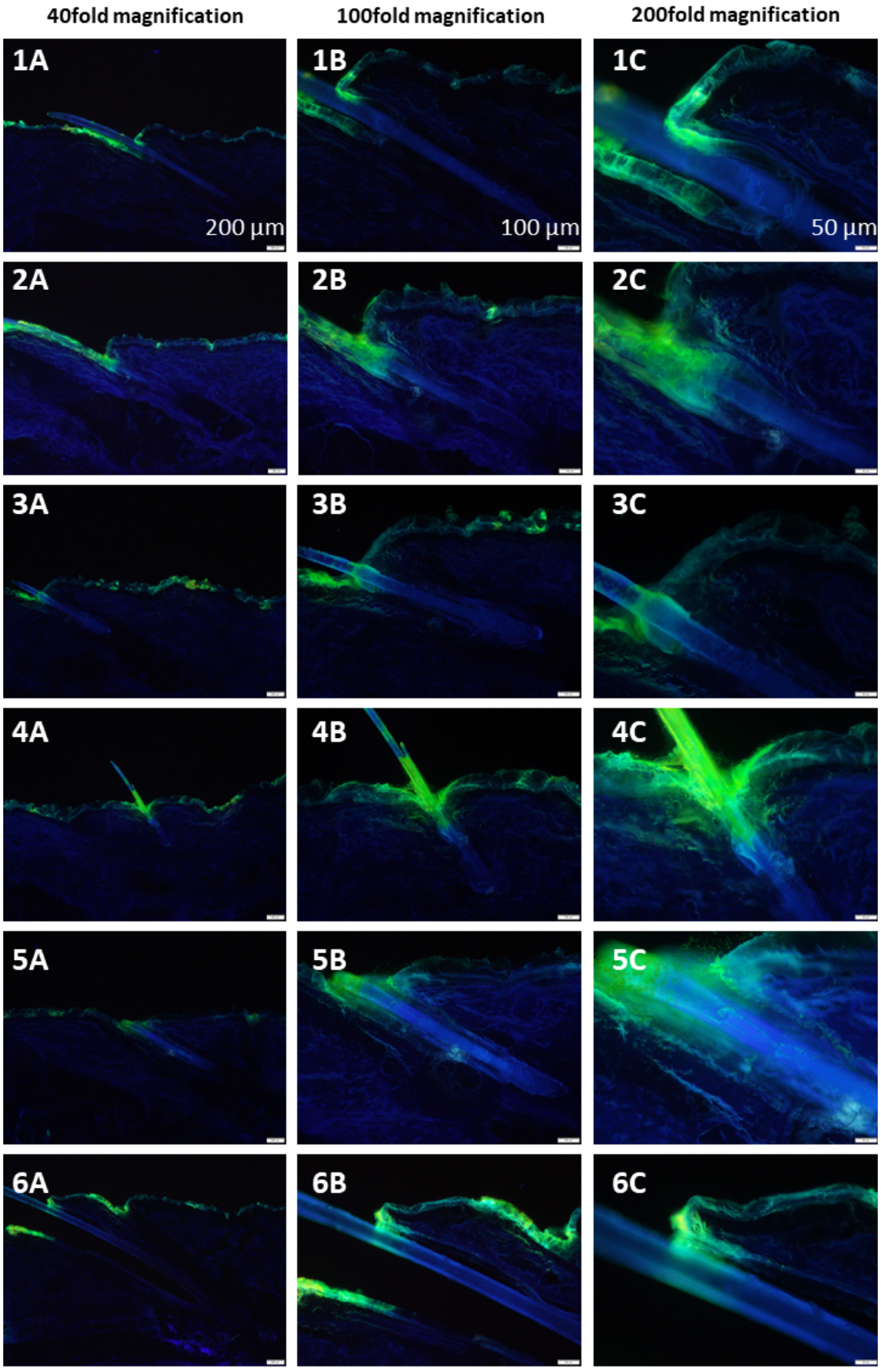
S9: Hair follicles treated with 1.6% HEC gel. A: 40fold magnification. B: 100fold magnification. C: 200fold magnification.



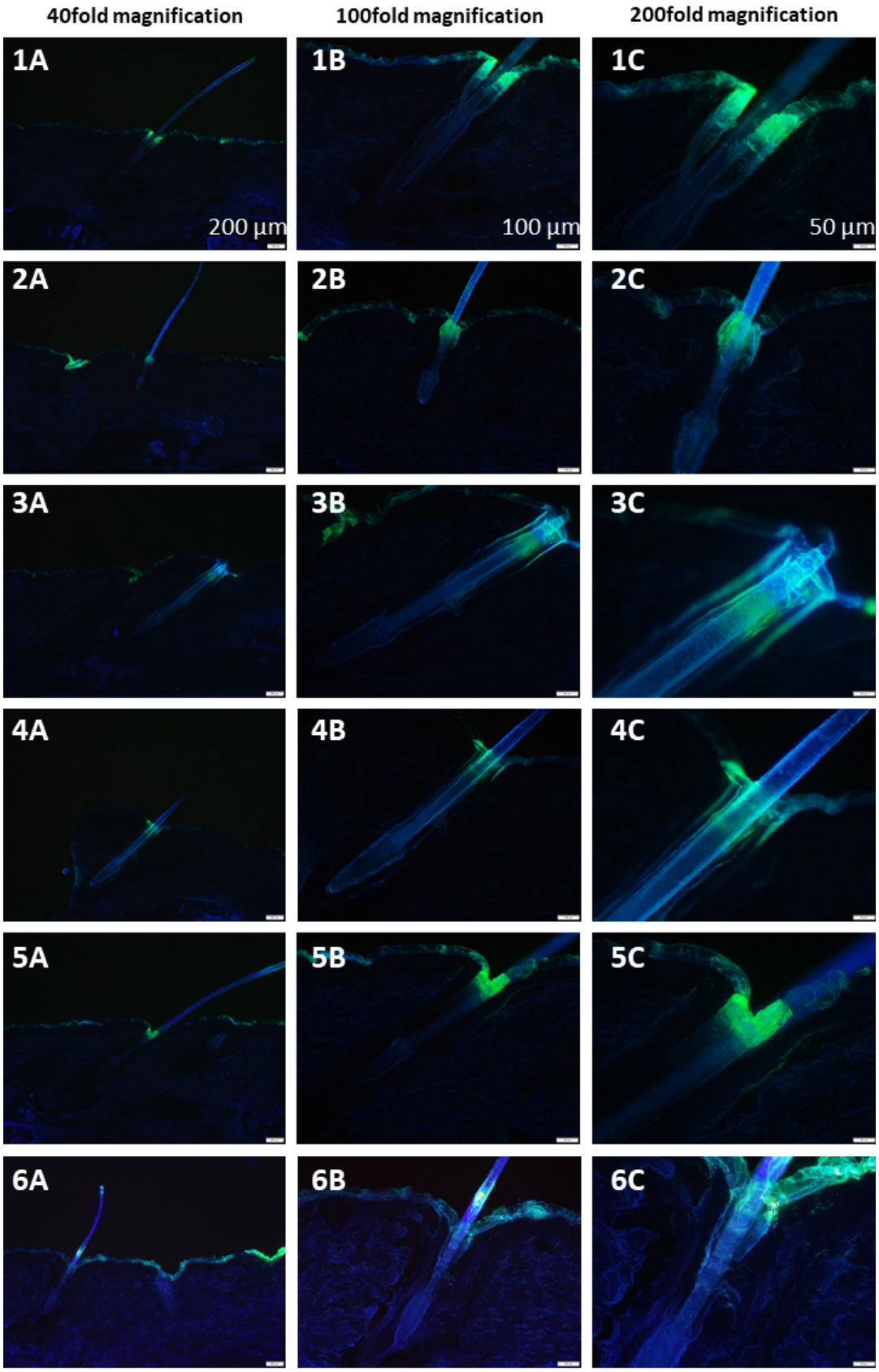
S10: Hair follicles treated with 3.6% HEC gel. A: 40fold magnification. B: 100fold magnification. C: 200fold magnification.



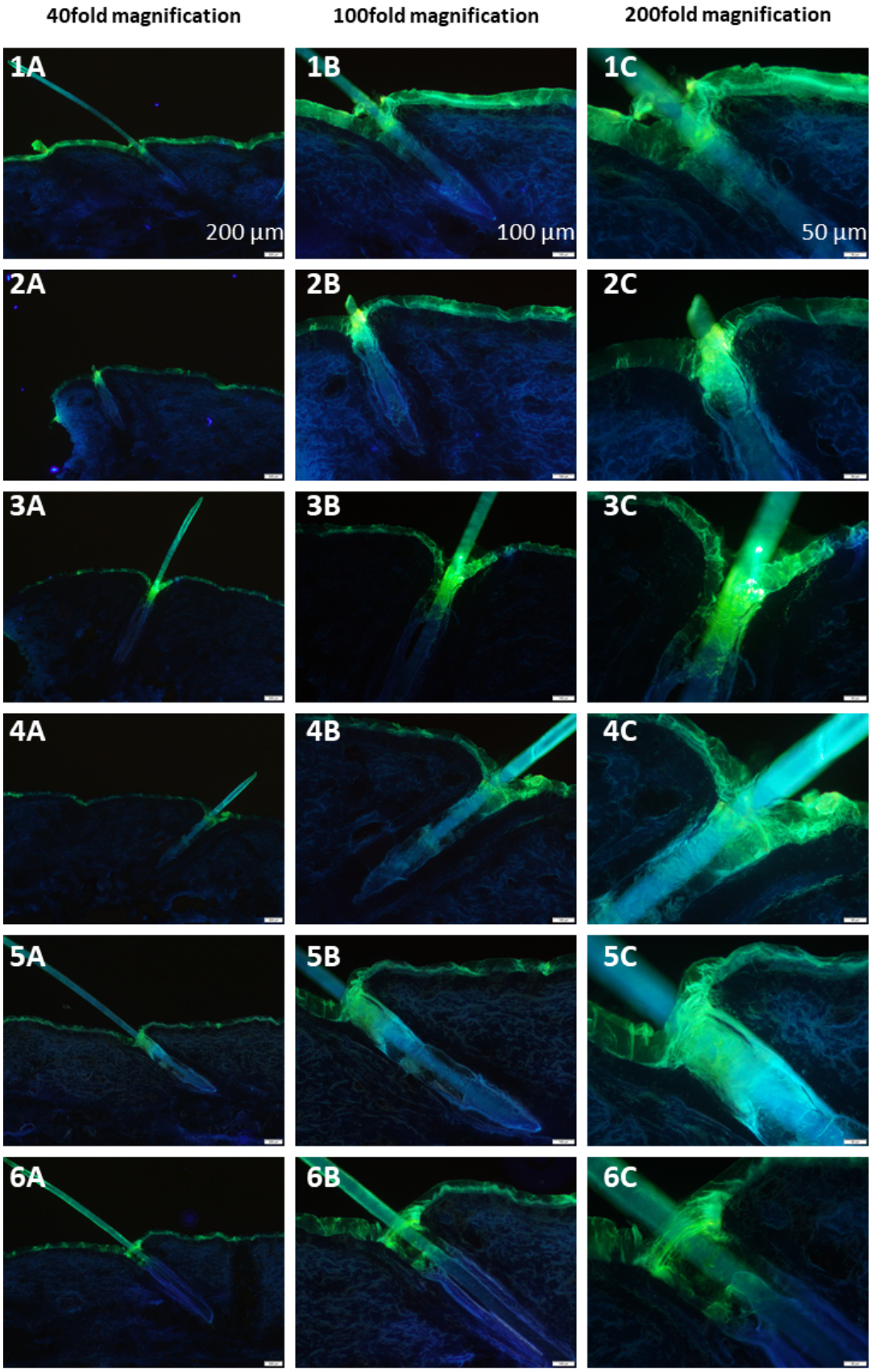
S11: Hair follicles treated with 4.4% HEC gel. A: 40fold magnification. B: 100fold magnification. C: 200fold magnification.



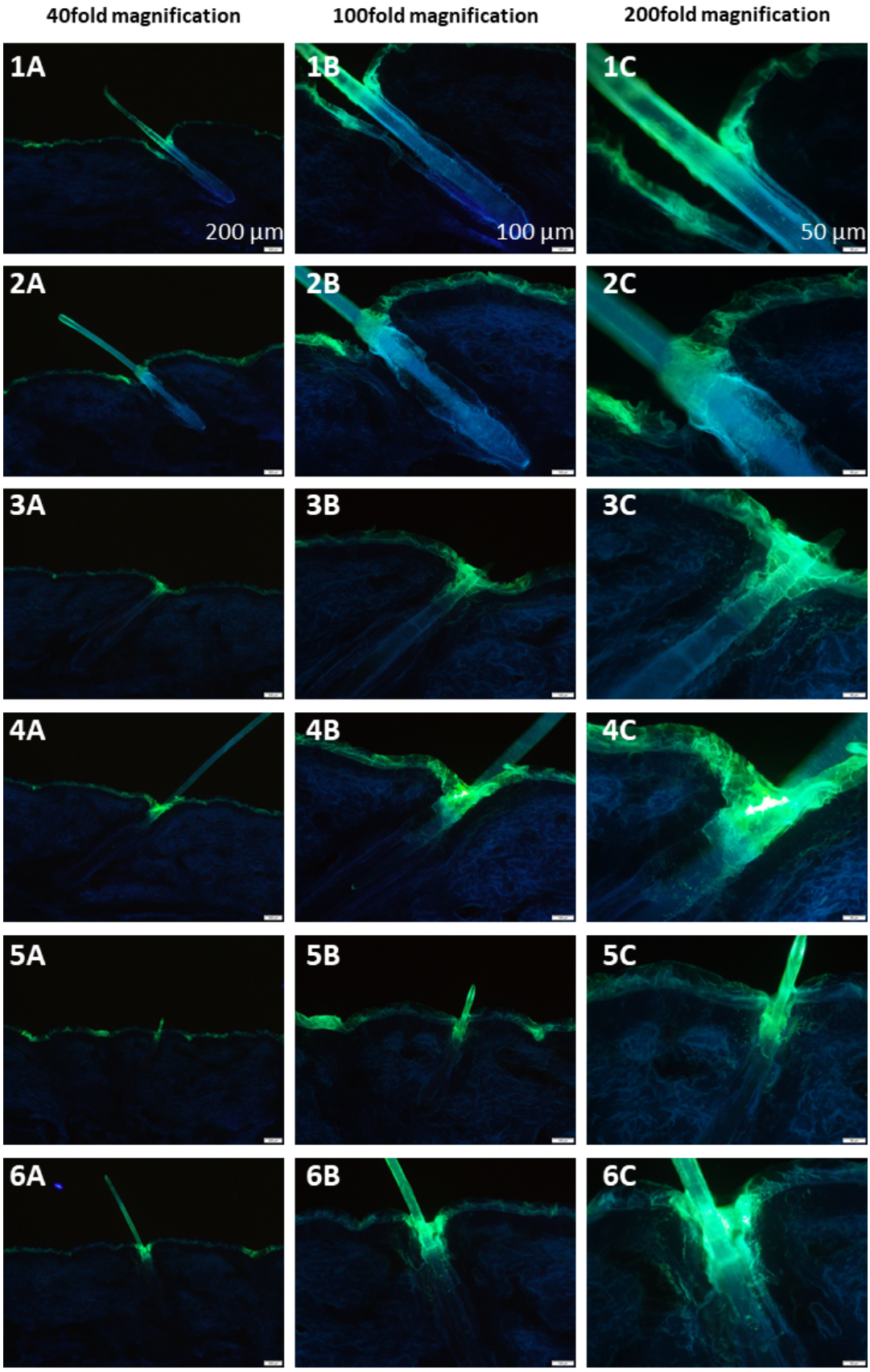
S12: Hair follicles treated with 4.8% HEC gel. A: 40fold magnification. B: 100fold magnification. C: 200fold magnification.



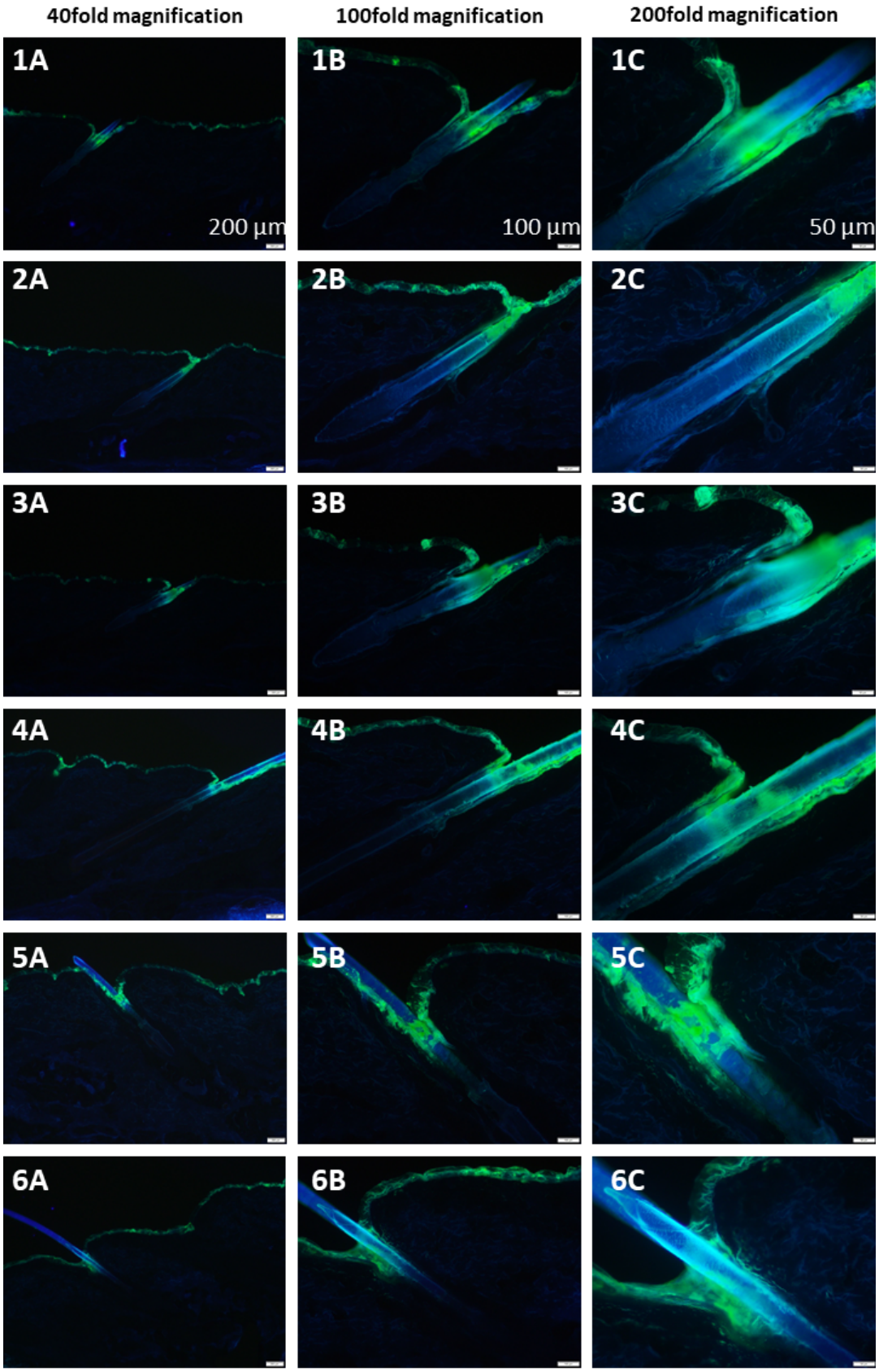
S13: Hair follicles treated with 2% xanthan gum gel. A: 40fold magnification. B: 100fold magnification. C: 200fold magnification.



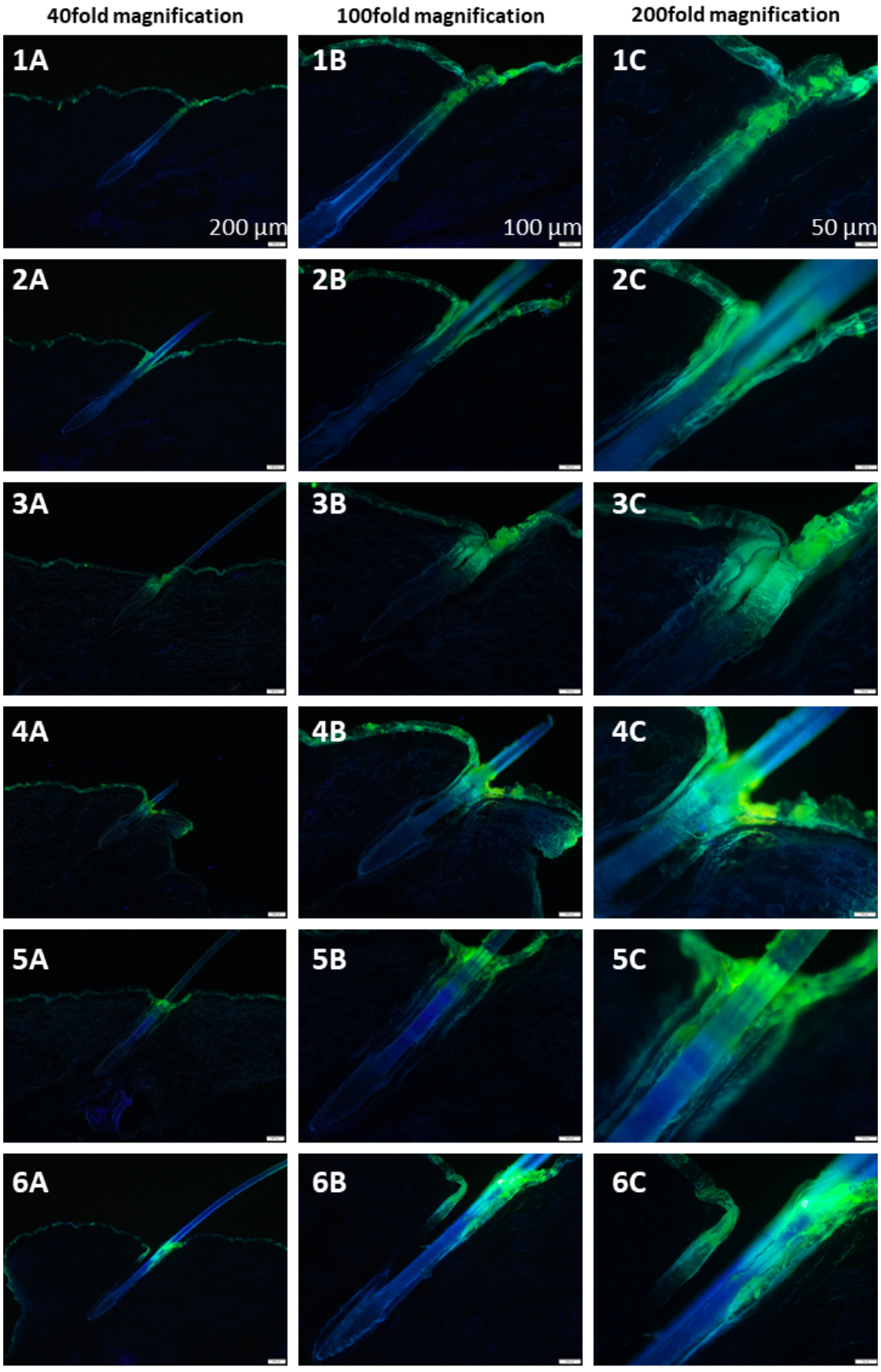
S14: Hair follicles treated with 5% xanthan gum gel. A: 40fold magnification. B: 100fold magnification. C: 200fold magnification.



S15: Hair follicles treated with olive oil-Aerosil® gel, 4% Aerosil®. A: 40fold magnification. B: 100fold magnification. C: 200fold magnification.



S16: Hair follicles treated with olive oil-Aerosil® gel, 5.6% Aerosil®. A: 40fold magnification. B: 100fold magnification. C: 200fold magnification.



3.5.

Hair follicle targeting and dermal drug delivery with curcumin drug nanocrystals: essential influence of excipients

Hair follicle targeting and dermal drug delivery with curcumin drug nanocrystals: essential influence of excipients

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(adapted from Nanomaterials 2020 Nov;10(11):2323)

Abstract

Many active pharmaceutical ingredients (API) possess poor aqueous solubility and thus lead to poor bioavailability upon oral administration and topical application. Nanocrystals have a well-established, universal formulation approach to overcome poor solubility. Various nanocrystal-based products have entered the market for oral application. However, their use in dermal formulations is relatively novel. Previous studies confirmed that nanocrystals are a superior formulation principle to improve the dermal penetration of poorly soluble API. Other studies showed that nanocrystals can also be used to target the hair follicles where they create a drug depot, enabling long acting drug therapy with only one application. Very recent studies show that also the vehicle in which the nanocrystals are incorporated can have a tremendous influence on the pathway of the API and the nanocrystals. In order to elucidate the influence of the excipient in more detail, a systematic study was conducted to investigate the influence of excipients on the penetration efficacy of the formulated API and the pathway of nanocrystals upon dermal application. Results showed that already small quantities of excipients can strongly affect the passive dermal penetration of curcumin and the hair follicle targeting of curcumin nanocrystals. The addition of 2% ethanol promoted hair follicle targeting of nanocrystals and hampered passive diffusion into the stratum corneum of the API, whereas the addition of glycerol hampered hair follicle targeting and promoted passive diffusion. Propylene glycol was found to promote both pathways. In fact, the study proved that formulating nanocrystals to improve the bioefficacy of poorly soluble API upon dermal application is highly effective. However, this is only true, if the correct excipient is selected for the formulation of the vehicle. The study also showed that excipients can be used to allow for a targeted dermal drug delivery, which enables to control if API should be delivered via passive diffusion and/or as drug reservoir by depositing API in the hair follicles.

1. Introduction

Besides oral drug delivery, topical drug delivery is a major and constantly growing market in the pharmaceutical field. The compound annual growth rate (CARG) is >5% and the global market is projected to reach 123.2 billion U$ by 2024 [1]. The high prevalence of skin diseases or injuries, as well as the increasing demand of topically applied drugs for transdermal drug delivery, require the development of innovative and effective formulations that can overcome current challenges in this field. Challenges that need to be addressed are for example a sufficient penetration of the active compound into or through the skin without skin irritation, e.g., due to the use of penetration enhancers. Ideally, the formulation should not only deliver the active compound but should also help to maintain or restore the skin barrier function. The therapy concept that combines efficient drug delivery and skin protecting or restoring properties at the same time, is known as advanced corneotherapy and can help to improve the treatment efficacy and compliance of the patients [2]. In addition, there is an increasing demand to formulate topical drug products as a depot, i.e., formulations that are applied once and then release the active compound over an extended period of time. Most prominent examples for extended dermal drug delivery are transdermal patches that can be worn for several days or microneedle patches where biodegradable drug-loaded microneedles are deposited into the skin where they can act as a long-term reservoir for drugs [3–5].

Another method to form drug depots for long-term drug release is the delivery of active compounds via the hair follicles. Hair follicle targeting gained much attention in the last years and is now known to be most efficient if the drug is encapsulated or linked to a nanocarrier that preferentially possesses a size of about 400–700 nm [6–10]. The improved penetration of active compounds in particulate form is explained by the ratchet effect. This means that the cuticular hair structure acts as a ratchet and transports the particles deeper into the hair follicle [11]. Once the drug-loaded carrier has reached the hair follicle, it will remain for up to several days, forming a long-lasting drug reservoir from which drug is constantly released. Due to the special anatomy of the hair follicle stratum corneum, which is less developed and thus more permeable for chemical compounds compared to the stratum corneum of the skin, the drugs can diffuse transfollicularly and directly into the viable layers of the skin. The possibility to combine long-lasting drug depots and efficient drug delivery into the viable dermis at the same time and without the need of barrier disruption or the use of skin irritant excipients, makes drug delivery via the follicular route highly attractive.

However, even though the superiority of nanocarriers for hair follicle targeting is now well-known, an efficient application of nanocarriers as drug product will always require the formulation of such carriers in appropriate vehicles. Vehicles can be hydrophilic, lipophilic and can vary in viscosity, polarity and many other parameters. When looking at the passive dermal penetration of chemical compounds, it is

well-known that different vehicles with different properties can strongly affect the penetration properties of active compounds into the skin. Consequently, it can be assumed that vehicles with different properties might also affect the penetration of drug nanocarriers into hair follicles. A recent study already addressed this issue and investigated the influence of the type of vehicle on the penetration efficacy of curcumin nanocrystals into the hair follicles. In this study nanocrystals were incorporated into different types of gels that varied in polarity, lipophilicity and viscosity [12]. Results demonstrated that the penetration efficacy of the curcumin nanocrystals into the hair follicles was not affected by variations in viscosity, polarity or lipophilicity of the vehicle. In contrast, it was found that the variation in vehicle properties led to tremendous differences in the passive dermal penetration of curcumin from the drug nanocrystals. The most influencing parameter was the ability of the vehicle to hydrate the stratum corneum. Hence, vehicles with good skin-hydrating properties led to the best dermal penetration of the curcumin. To substantiate this finding, a humectant was added to a formulation with poor hydrating properties and to a formulation with good skin hydrating properties, respectively. Results could nicely demonstrate the increase in skin hydration upon the addition of the humectant to the formulation with former poor skin hydration. And, as expected, the increase in skin hydration resulted in a pronounced increase in passive dermal diffusion of curcumin. In contrast the addition of the humectant to the formulation that already showed good skin hydration could not further increase the skin hydration. Consequently, the passive dermal diffusion of curcumin through the skin was not much increased.

Besides, it was also observed, that the addition of the humectant reduced the follicular uptake of the curcumin nanocrystals by about 20%. Based on obtained results, it was hypothesized that a swelling of the hair shaft and/or changes in the structure of the hair cuticula might be responsible for this. The changes might cause a reduced ratchet effect, which could than result in a less efficient transport of the nanoparticles into the hair follicle. Therefore, the aim of this study was to investigate and to understand this observation in more detail. For this, different excipients with and without known skin-hydrating properties were selected. Subsequently, curcumin nanocrystals were produced after a previously established protocol [12,13], and the different excipients were added in different concentrations to the nanosuspension. The obtained nanocrystal formulations with and without excipients were first characterized regarding size and size distribution and were then investigated via the ex-vivo porcine ear model regarding passive dermal diffusion of curcumin, their influence on the biophysical properties of the skin (i.e., skin hydration, barrier function and skin roughness) and their ability to transport curcumin nanocrystals into the hair follicles (i.e., hair follicle targeting).

The present study is aimed to identify excipients that are able to promote hair follicle targeting and/or passive diffusion of curcumin through the skin. Based on these findings it is then possible to optimally formulate drug nanocrystals for a targeted dermal drug delivery. Hence, with purposeful selection of

excipients, one can choose if the active compound should be delivered via passive diffusion and/or as long-lasting drug reservoir by depositing drug nanocrystals within the hair follicles.

2. Materials and Methods

2.1. Materials

Curcumin was used as the model drug in this study. It is a poorly water-soluble active substance (BCS class IV) that can be detected in the skin and in the hair follicles due to its pronounced autofluorescence, which enables the visualization of penetrated active from the different formulations. It was obtained from Receptura Apotheke-Cornelius Apothekenbetriebs OHG (Frankfurt am Main, Germany). TPGS (d-α-tocopherol polyethylene glycol 1000 succinate) was used as stabilizer for the nanocrystals and was purchased from Gustav Parmentier GmbH (Frankfurt am Main, Germany). Purified water was obtained from a PURELAB Flex 2 (ELGA LabWater, High Wycombe, UK) and was used as a liquid phase for the nanocrystals. The excipients used were glycerol (extra pure, Acros Organics, Geel, Belgium), urea (Caesar & Loretz GmbH, Hilden, Germany), propylene glycol (Caesar & Loretz GmbH, Hilden, Germany), ethanol (HPLC grade, Fischer Scientific GmbH, Schwerte, Germany), and olive oil (Gustav Heess GmbH, Leonberg, Germany). All chemicals were used as received.

2.2. Methods

2.2.1. Production and Characterization of Curcumin Nanocrystals with Different Excipients

Nanocrystals were produced by using the small-scale bead milling approach [14] and by using a previously established protocol [12,13]. The nano-milling was carried out in an 8 mL brown glass bottle filled with Yttrium stabilized zirconium oxide beads (0.3–0.4 mm, SiLibeads®, Sigmund Lindner GmbH, Warmensteinach, Germany) and equipped with a magnetic stirrer (Ø: 8 mm, 13 mm, Rotilabo®, Carl Roth GmbH + Co. KG, Karlsruhe, Germany). The bead/suspension-ratio was 60/40 (*v*/*v*) and the coarse bulk suspensions contained 5.0% (*w*/*w*) curcumin, 1.0% (*w*/*w*) TPGS and water to up to 100% (*w*/*w*). The closed bottle was fixed horizontally to a magnetic stirrer (MIXdrive 15, 2mag AG, Munich, Germany) with adhesive tapes and the milling process was performed at 1000 rpm for 24 h. The so obtained nanocrystals were allocated and different excipients with different concentrations were added to these aliquots (Table 1). Subsequently, the formulations were characterized regarding their size and size distribution by using three independent techniques, i.e., dynamic light scattering (DLS, Zetasizer Nano ZS, Malvern Panalytical Ltd., Malvern, UK), laser diffraction (LD, Mastersizer 3000, Malvern Panalytical Ltd., Malvern, UK) and light microscopy (Olympus BX53 equipped with an Olympus SC50 CMOS color camera, Olympus soft imaging solutions GmbH, Warmensteinach, Germany). DLS data were analyzed with the general purpose mode and LD data analysis was done with Mie-theory

with optical parameters set to 1.87 for the real refractive index and 0.1/0.01 for the blue light (470 nm) and the red light (633 nm) imaginary refractive indices, respectively.

Table 1. Overview of formulations produced in this study.

Formulation Code	Type of Excipient	Concentration of Excipient (w/w)
NS	-	-
Gly 2%	glycerol	2%
Gly 5%	glycerol	5%
Urea 5%	urea	5%
Urea 10%	urea	10%
PG 5%	propylene glycol	5%
EtOH 2%	ethanol	2%
oil 2%	olive oil	2%

2.2.2. Ex-Vivo Model for Passive Dermal and Follicular Penetration

The influence of excipients on the passive dermal penetration of curcumin and the influence on the follicular penetration of the curcumin nanocrystals was tested by utilizing the ex-vivo porcine ear skin model [15]. For this, fresh porcine ears were obtained from a local slaughterhouse and used within a few hours after butchering. Prior to the application of the nanosuspensions on the ventral side of the ear, ears were washed with lukewarm water (approx. 23–25 °C), dried with paper towels and fixed to flat polystyrene plates that were covered with aluminum foil. Intact skin areas of 2 × 2 cm^2 without wounds and scratches were selected and marked. The hair within these areas was cut to a length of about 1–3 mm. On each test area, 50 µL formulation were applied and massaged in for 3 min with a saturated, gloved finger [12]. Formulations were allowed to penetrate into the skin and the hair follicles for 6 h in an oven with a temperature set to 32 °C. After incubation, the formulations were carefully washed off and punch biopsies (15 mm drive punch) were taken from each test area. The skin biopsies were immediately embedded (Tissue-Tek® O.C.T.™, Sakura Finetek Europe B.V., Alphen aan den Rijn, The Netherlands), frozen and stored at −20 °C until further use. Experiments were performed in triplicate and each formulation was tested on three different ears.

2.2.3. Determination of Follicular and Dermal Penetration via EpifluorescencecMicroscopy

Curcumin possesses a strong autofluorescence. Therefore, epifluorescence microscopy can be used to detect curcumin and curcumin nanocrystals that penetrated either into the skin and/or into the hair follicles [12]. Prior to microscopic analysis, 40 µm of thick skin sections were prepared with a cryomicrotome (Frigocut 2700, Reichert-Junk, Nußloch, Germany). The cutting was carried out from right to left in order to avoid contamination with the curcumin from the skin surface. The skin cuts

were then subjected to inverted epifluorescence microscopy (Olympus CKX53 equipped with an Olympus DP22 color camera, Olympus Deutschland GmbH, Hamburg, Germany). The intensity of the fluorescence light source (130 W U-HGLGPS illumination system, Olympus Deutschland GmbH, Hamburg, Germany) was kept constant and was set to 50% and 25% for the skin and the hair follicle sections, respectively. Also, the exposure time was kept constant and was set to 50 ms for all images taken. The fluorescence filter used was the DAPI HC filter block system (excitation filter: 340–390 nm, dichroic mirror: 410 nm, emission filter: 420 nm (LP)).

2.2.4. Digital Image Analysis

Digital image analysis was used to measure the mean penetration depth (MPD) of curcumin into and through the skin as well as the penetration depth of the curcumin nanocrystals into the hair follicles. In addition, it was used to quantify the mean grey value (MGV) of each image as a surrogate for the total amount of penetrated curcumin into the skin [16]. In addition, changes in stratum corneum thickness caused due to the treatment of the skin with the different formulations were also analyzed. The passive dermal penetration efficacy of curcumin from the different formulations, i.e., MPD and MGV, were determined according to a previously established protocol [16] with ImageJ software [17–19]. For this, all images were subjected to an automated threshold protocol in order to eliminate the autofluorescence of the skin. Consequently, the remaining fluorescence intensity (MGV) within the images corresponded to the penetrated amount of curcumin and was used as semi-quantitative surrogate for the total amount of penetrated curcumin. The MPD corresponds to the mean penetration depth that was determined by measuring the maximal penetration depth of curcumin from each image. The mean thickness of the stratum corneum was assessed similarly. For the measurements, 36 images (12 images from each ear, 3 ears in total) were analyzed for each formulation. The penetration depth of the nanocrystals into the hair follicles was determined on 5–10 follicles per porcine ear and on 3 different ears for each formulation. The measurements were performed with the cellSens Entry software package (Olympus Cooperation, Tokyo, Japan).

2.2.5. Determination of Biophysical Skin Properties

The biophysical properties of the skin after the application of the different formulations were determined in order to investigate the effect of the different excipients on the barrier function, skin hydration and skin friction. The transepidermal water loss (TEWL) is a measure for the amount of water that passively evaporates through the skin. It is therefore frequently used to characterize skin barrier function [20] and was measured with a Tewameter (Tewameter® TM300, Courage & Khazaka electronic GmbH, Cologne, Germany). The skin hydration was assessed by measuring the capacitance of the skin surface (Corneometer® CM825, Courage & Khazaka electronic GmbH, Cologne, Germany), where a high capacitance represents a high hydration level of the skin and vice versa. The skin friction is the force

needed to rotate a small disc on the skin surface and was measured by using a frictiometer (Frictiometer® FR700, Courage & Khazaka electronic GmbH, Cologne, Germany). The influence of the excipients on TEWL, skin hydration and skin friction was assessed on fresh pig ears that were conditioned and treated as described above. The biophysical skin properties were assessed on each test area prior to application of the formulations and after a penetration time of 6 h. Untreated skin areas that possessed TEWL values >13 indicated an impaired barrier function and thus were excluded from the study. Each formulation was tested in triplicate on three different ears.

2.2.6. Statistical Analysis

Descriptive statistics and the comparisons of the mean values were analyzed with JASP software (version 0.13.1) [21] and Minitab 19 (Minitab Inc., State College, Pennsylvania, USA), respectively. Parametric data were subjected to a one-way ANOVA, that was Welch-adopted in case of variance heterogeneity. Tukey post-hoc and Games–Howell post-hoc tests were performed to compare the mean values. For the nonparametric data, Kruskal–Wallis analyses of variance with Dunn`s post-hoc tests were performed [22]. In case of multiple comparisons, the Bonferroni–Holm adjustment was applied to avoid alpha error accumulation. *p*-values < 0.05 were considered to be statistically significant.

3. Results and Discussion

3.1. Production and Characterization of Curcumin Nanocrystals with Different Excipients

The coarse bulk material contained curcumin crystals in the upper micrometer range (Table 2). The small-scale bead milled curcumin nanosuspension (NS d1) possessed a size of about 250 nm and a polydispersity index (PDI) of 0.25 (Table 2). Thus, possessing similar properties than the previously produced curcumin nanocrystals [12,13]. Light microscopy and laser diffraction revealed the presence of some larger curcumin microcrystals (approx. 4–10 µm), which were not destroyed during the milling process. The larger crystals caused some physical instability, because the broad size distribution of the nanosuspension initiated Ostwald ripening during storage for 14 days (NS d14) at room temperature. During this time, the z-average and the PDI almost doubled, leading to sizes of about 500 nm and to a broad size distribution (PDI > 0.4). Particle growth due to Ostwald ripening was also confirmed by laser diffraction. However, LD and light microscopy could also confirm that, despite a moderate increase in the mean size, no larger aggregates were formed during storage (Table 2, Figure 1).

Table 2. Particle sizes and size distributions (DLS data and LD data) for the curcumin bulk material, the nanosuspension without excipients and for the nanosuspensions after the addition of different excipients.

Formulation	DLS data		LD data			
	z-average [nm]	PDI	d(v) 0.5 [µm]	d(v) 0.9 [µm]	d(v) 0.95 [µm]	d(v) 0.99 [µm]
bulk material	n.a.		22.6 ± 0.2	50.3 ± 0.5	60.1 ± 0.9	77.3 ± 1.7
NS d1	253 ± 6	0.25 ± 0.05	0.7 ± 0.0	4.2 ± 0.1	5.5 ± 0.1	8.2 ± 0.2
NS d14	483 ± 75	0.48 ± 0.05	1.1 ± 0.0	4.8 ± 0.1	6.5 ± 0.1	10.6 ± 0.2
Gly 2%	325 ± 10	0.25 ± 0.03	0.7 ± 0.0	4.3 ± 0.1	5.6 ± 0.1	8.4 ± 0.1
Gly 5%	278 ± 7	0.30 ± 0.03	0.8 ± 0.0	4.4 ± 0.1	5.7 ± 0.1	8.5 ± 0.1
Urea 5%	270 ± 7	0.24 ± 0.05	0.5 ± 0.0	4.0 ± 0.0	5.2 ± 0.0	7.7 ± 0.1
Urea 10%	277 ± 7	0.29 ± 0.02	0.4 ± 0.0	3.8 ± 0.0	5.0 ± 0.0	7.5 ± 0.1
PG 5%	389 ± 47	0.39 ± 0.07	1.1 ± 0.0	4.6 ± 0.1	6.0 ± 0.2	8.9 ± 0.3
EtOH 2%	389 ± 37	0.45 ± 0.09	1.1 ± 0.0	4.7 ± 0.1	6.1 ± 0.1	9.4 ± 0.2
oil 2%	459 ± 169	0.54 ± 0.25	1.4 ± 0.1	8.5 ± 0.9	13.0 ± 1.6	27.0 ± 3.6

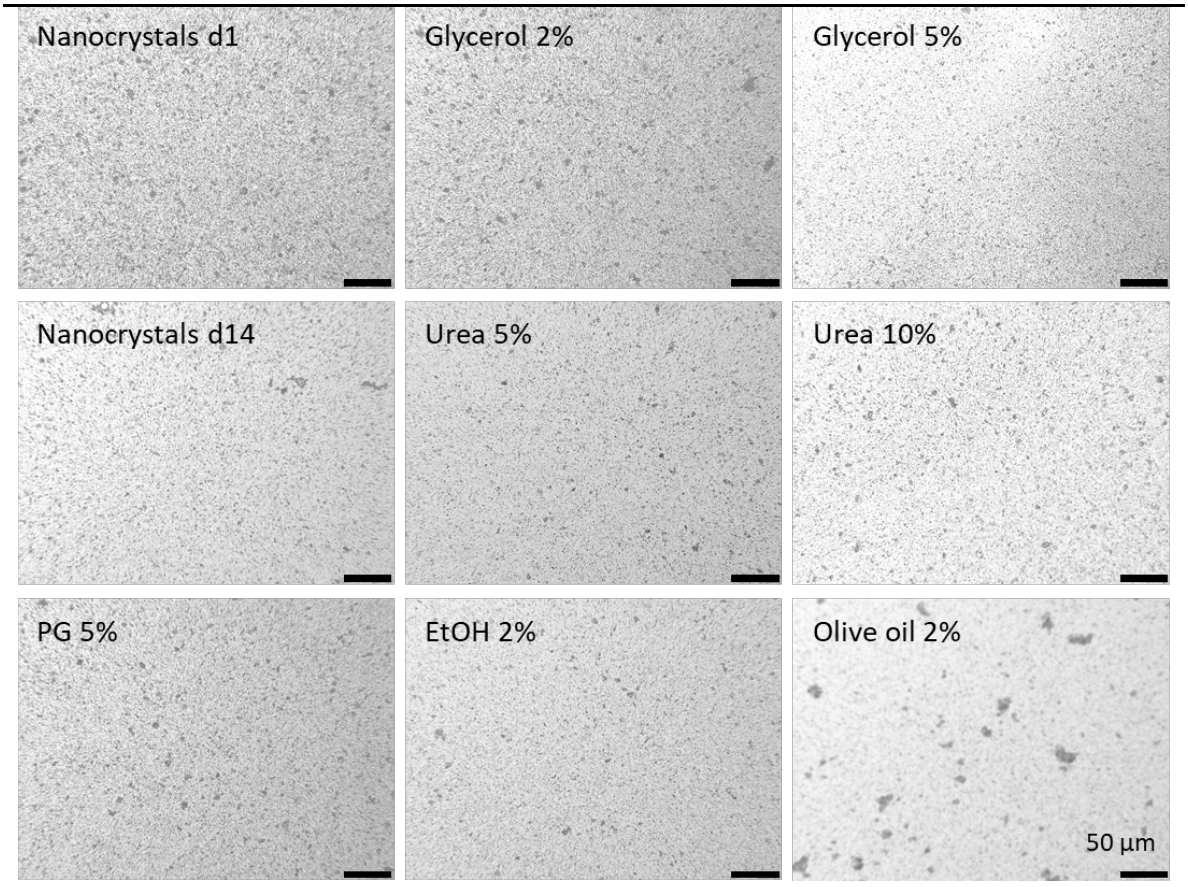


Figure 1. Microscopic images of the curcumin nanosuspension at the day of production, after 14 days of storage and after the addition of different excipients. Magnification: 400-fold.

The addition of excipients caused small changes in the size and size distribution (Table 2, Figure 1). Reasons for these changes are the increased solubility of curcumin in these excipients. The addition of excipients to the original nanosuspension leads to a partial dissolution of the nanocrystals, which can result in either larger or lower particle sizes (c.f. Table 2, LD data), when compared to the nanosuspension without excipients [23,24]. Additives that cause only a minor increase in solubility will

decrease the particle size, because only small amounts of curcumin are dissolved from the surface of each particle. In contrast, if the excipients are good solubility enhancers, they cause a pronounced dissolution of curcumin. As small particles possess a higher kinetic solubility and increased dissolution velocity compared to larger sized crystals, the small crystals will dissolve first and will leave the larger particles behind. This will then lead to an all-over increased particle size of the whole formulation.

The solubility of curcumin is very good in ethanol and propylene glycol [25] and the largest particles were thus found for the formulations to which either ethanol or propylene glycol were added (DLS data, Table 2, Figure 1). The addition of a small quantity of olive oil resulted in the formation of some oil droplets within the aqueous phase of the nanosuspension and some of the curcumin nanocrystals even adhered to the surface of these droplets. Consequently, from all the formulations produced, the addition of olive oil resulted in the formulation with the largest measured particle size and the broadest size distribution, respectively (LD data, d(v) 0.9–0.99 values, Table 2, Figure 1).

In a previous study by Pelikh et al., the particle size of nanocrystals was shown to influence the passive diffusion of hesperetin from drug nanocrystals [26]. However, a tremendous increase in passive diffusion was only achieved with particle sizes <200 nm. Larger sizes did not show pronounced differences. In addition, it was found that the effect decreased with increasing penetration depth into the skin and was already cancelled out after 10 tape strips, which corresponds to less than half of the thickness of the stratum corneum. The present study aims at investigating not only the penetration of curcumin into the upper layers of the stratum corneum but also into the deeper layers of the skin. All nanocrystals used in this study possessed a size well above 200 nm and therefore the differences in size—caused by a partial dissolution of the nanocrystals upon the addition of the solvents—were considered to affect the passive dermal penetration only to a minor extend.

3.2. Determination of Passive Dermal Penetration

3.2.1. Microscopic Imaging by Inverted Epifluorescence Microscopy

The passive diffusion of curcumin was found to be strongly influenced by the addition of the different excipients to the original curcumin nanosuspension (Figure 2). Especially the addition of 5% glycerol or 5% propylene glycol seemed to increase the penetration efficacy of curcumin. The addition of these excipients increased the amount of curcumin that penetrated into the stratum corneum and enabled a transdermal penetration of the curcumin into the viable dermis. A penetration enhancing effect, even though it was not as pronounced as for the above-mentioned excipients, was also detected for glycerol 2% and urea 5%. No differences compared to the nanosuspension without additives were found for the formulations contained olive oil or 10% urea. For ethanol even a slightly decreased penetration was observed. In addition to affecting the penetration of curcumin, the additives were

also found to affect the thickness of the stratum corneum (SC). When compared to untreated skin, the SC seemed to be thicker after the application of the nanosuspensions. The effect seemed most pronounced for the nanosuspensions that contained 5% glycerol or 5% propylene glycol. In contrast, the addition of ethanol seemed to prevent the swelling of the SC. The increase or decrease in SC thickness correlates with the amount of penetrated curcumin. Hence, data indicate, that excipients that increase the SC thickness can improve the penetration efficacy of the hydrophobic curcumin, whereas excipients that cannot increase the SC thickness hamper the penetration.

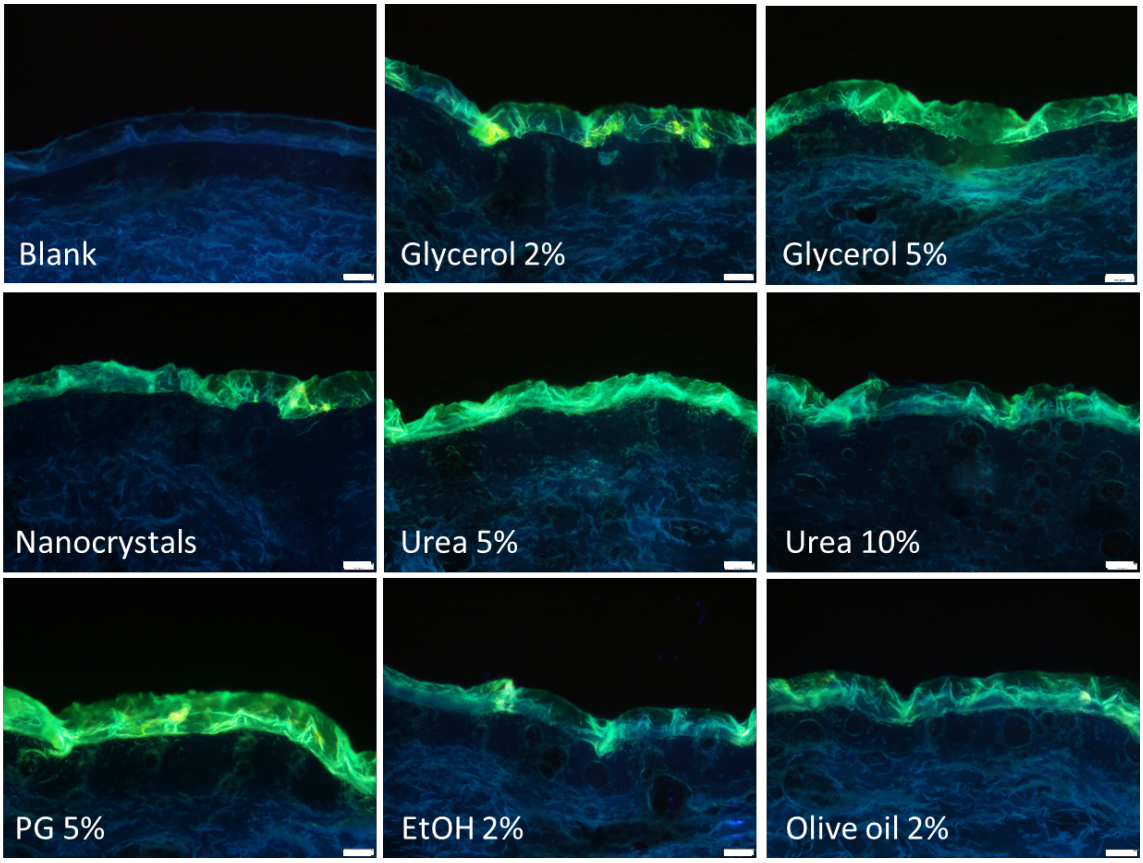


Figure 2. Influence of excipients on passive dermal penetration of curcumin from drug nanocrystals. Representative images from vertical skin sections. Non-treated (Blank), treated with aqueous nanosuspension without further excipients and treated with curcumin nanosuspensions that contained different excipients, i.e., glycerol 2% and 5%, urea 5% and 10%, propylene glycol 5%, ethanol 2% or olive oil 2%. For more detailed inspection of the data, please refer to the Supplementary Material Section S1–S9.

3.2.2. Digital Image Analysis

Data obtained from the visual inspection of the images enabled a first impression of the influence of the different excipients on the dermal penetration efficacy of curcumin from nanocrystals. However, a clear picture can only be obtained by subjecting the images to a digital image analysis which enables the transfer of the observations into exact numbers. Digital processing enables to exactly measure the penetration depth of curcumin and to estimate the total amount of dermally penetrated curcumin [16].

Data obtained for the penetration depth of curcumin from the different formulations are shown in Figure 3. In comparison to the nanosuspension without additives, the penetration depth of curcumin was significantly increased when glycerol, urea or propylene glycol were added as excipients. Olive oil had no influence on the penetration depth of curcumin and ethanol decreased the penetration depth. The results are in line with the results obtained from the visual inspection and can be explained by the well-described penetration enhancing properties of glycerol, urea and propylene glycol [27–34]. Also, olive oil—due to its occlusive properties—could have been expected to yield an improved penetration depth compared to the original nanosuspension. However, the low amount that was added to the formulations might not be sufficient to yield an occlusive effect.

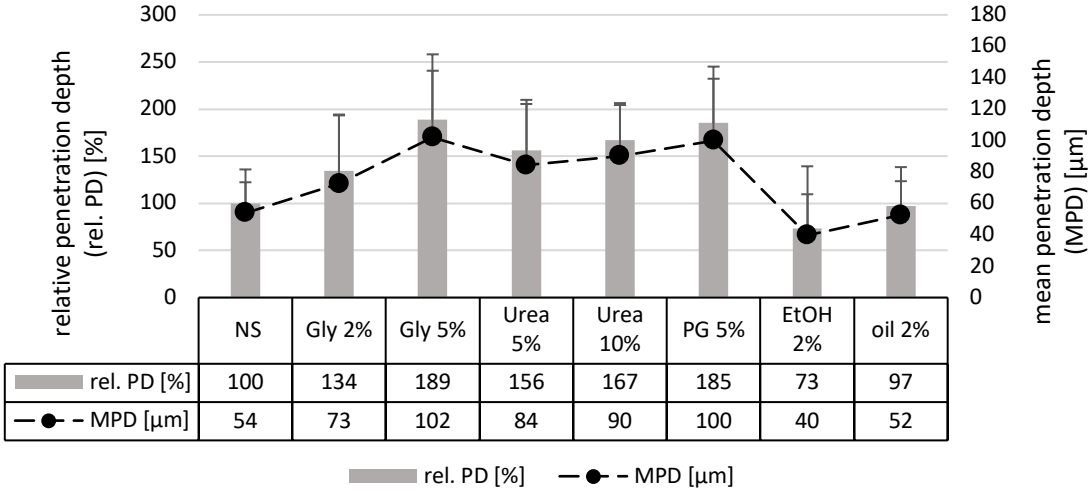
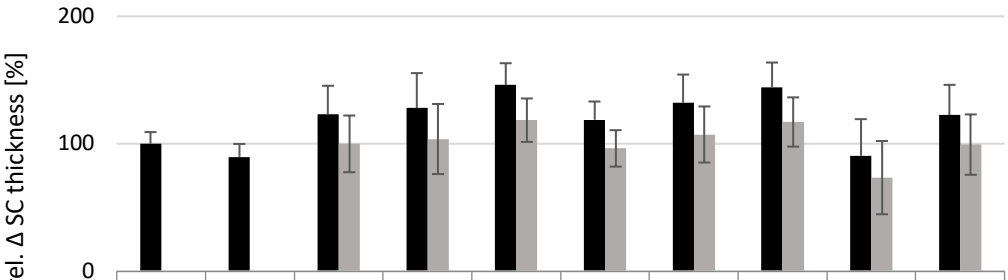


Figure 3. Influence of excipients on the penetration depth of curcumin determined by digital image analysis. MPD = mean penetration depth [µm], rel. PD = relative penetration depth [%] in comparison to the penetration depth of curcumin from the nanosuspension without additives.

The decreased penetration efficacy of ethanol was not expected, because also ethanol is known to possess penetration enhancing properties [30,35–37]. However, it is also known to possess dehydrating properties [38]. The nanosuspension contained water and surfactant. Both compounds can increase the penetration on their own [39,40]. Therefore, it can be assumed that the skin-hydrating properties, and with this the penetration enhancing properties of water and surfactant in the nanosuspension without additives, were stronger than the penetration enhancing effect of ethanol. The addition of ethanol to this formulation might have caused a “dry-out” effect, which then reduced the swelling of the SC and with this the passive diffusion of the curcumin. The reduced thickness of the SC due to the addition of ethanol was already observed from the visual inspection of the microscopic images and could be further proven be measuring the SC thickness with ImageJ software (Figure 4).



	Blank 0h	Blank 6h	NS	Gly 2%	Gly 5%	Urea 5%	Urea 10%	PG 5%	EtOH 2%	oil 2%
■ to blank 0h	100	90	123	128	146	119	132	144	91	123
■ to NS			100	104	119	96	107	117	74	99

Figure 4. Influence of excipients on the stratum corneum thickness determined by digital image analysis. Black columns: relative thickness of SC when compared to non-treated, fresh skin. Grey columns: relative thickness of SC when compared to the SC of the skin treated with aqueous curcumin nanosuspension.

Measuring the thickness of the SC of the untreated skin prior to the application and after 6 h of incubation revealed a decrease in the stratum corneum thickness by about 10%. This is considered to be due to a drying-out effect of the skin during the incubation time. The treatment with the nanosuspension without additives increased the stratum corneum thickness and prevented the drying of the skin during the incubation. A similar strong hydrating effect was found for the formulations containing 2% glycerol, urea or olive oil. Data confirm the observations from the microscopic images and confirm that these excipients do not alter the skin hydration when compared to the nanosuspension without excipients. In contrast, ethanol was proven to dehydrate the skin, whereas glycerol 5% and propylene glycol could further increase the skin hydration.

The determination of the penetration depth and the SC thickness gave a clear image of the influence of the different excipients on the penetration depth of curcumin and their skin hydrating properties. However, to evaluate the penetration efficacy holistically, it is also important to assess the total amount of penetrated active. In this study the amount of penetrated curcumin was assessed by analyzing the fluorescence intensity of each image after the autofluorescence of the skin was fully extracted from the original image by means of an automated threshold algorithm. Hence, the detected fluorescence intensity corresponded to the amount of penetrated curcumin [16]. Results obtained from this analysis (Figure 5) substantiate the observations from the visual inspection of the microscopic images and from the analysis of the penetration depth (Figures 2 and 3). However, they are not fully in line with the results obtained from the analysis of the penetration depth, because the addition of urea was now found to be less effective than the addition of glycerol.

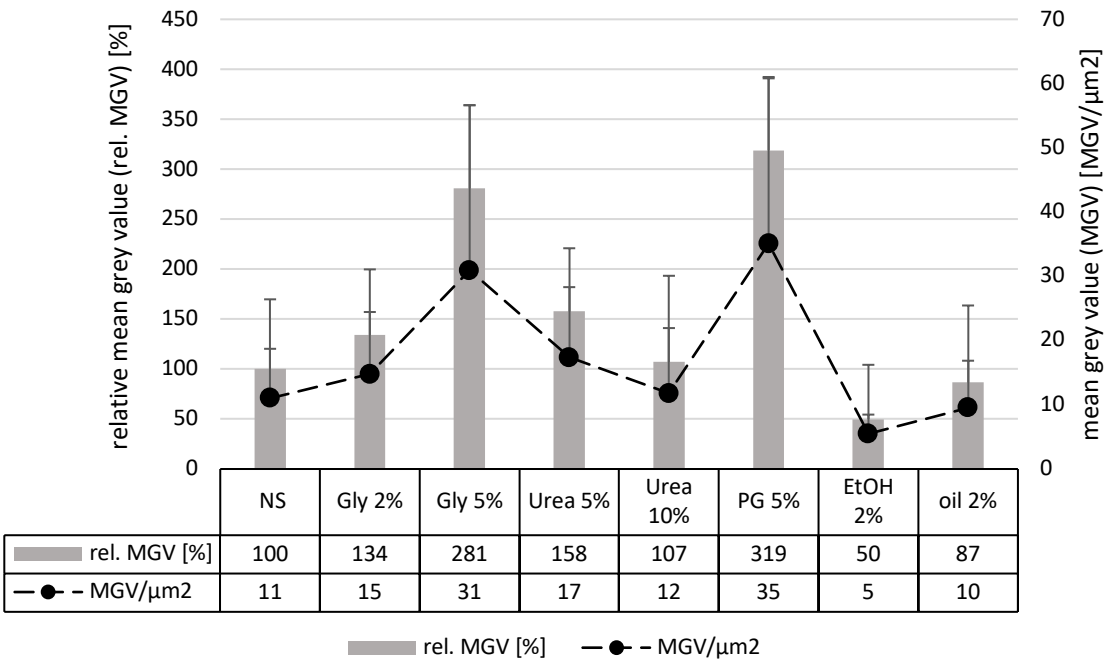


Figure 5. Influence of excipients on the penetration efficacy of curcumin determined by digital image analysis. MGV/µm2 = mean grey value per µm2 skin—representing the total amount of penetrated curcumin. rel. MGV [%] = relative amount of penetrated curcumin in comparison to the MGV from the nanosuspension without additives.

The differences between glycerol and urea are plausible. Water is essential to hydrate the SC and only hydrated SC will allow for a good dermal penetration of actives. The pure application of water would shortly moisture the skin and thus allow for good penetration. Over time, the water evaporates, and the hydration of the SC will decrease. With this, the penetration efficacy decreases. Glycerol acts as humectant and prevents water loss of the stratum corneum [41–43]. The penetration is therefore increased compared to the nanosuspension without additives. The penetration enhancement is concentration dependent, because the increase of the concentration of glycerol, i.e., from 2% to 5%, increased the skin moisturizing properties [44].

In contrast to glycerol, which will mainly localize in the intercellular space of the SC, urea is considered to penetrate into the corneocytes which causes the corneocytes to swell [45–47]. Consequently, with increasing concentrations of urea, the corneocytes increase in size leading to a decrease of the intercellular space. With this, the penetration efficacy of the lipophilic curcumin, which is considered to penetrate the skin via the extracellular pathway, is decreased (Figure 6).

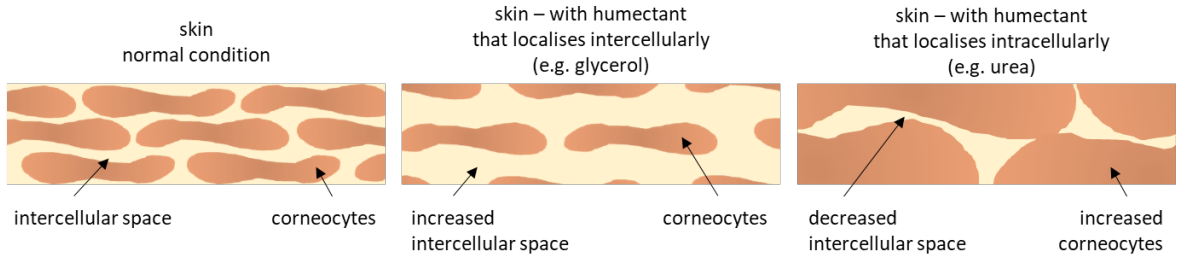


Figure 6. Principle of penetration enhancing properties of different humectants.

Data show that excipients, even though if they are used in very low concentrations, can distinctly influence the passive dermal penetration of curcumin from nanocrystals. As a major penetration enhancing effect, a long-time hydration effect of the stratum corneum was identified, which could be achieved by adding hygroscopic excipients to the formulations. Therefore, additives that dehydrate the stratum corneum should be avoided if effective passive dermal penetration is desired.

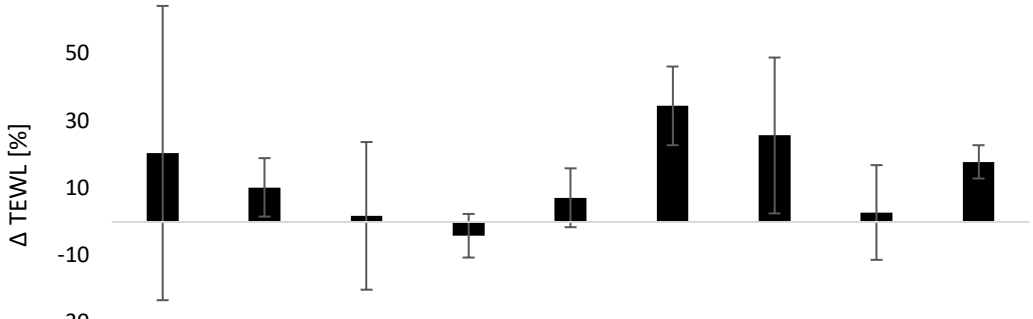
3.3. Influence on Biophysical Skin Parameters

In the next part of the study, the influence of the different excipients on the major biophysical skin parameters (TEWL, skin hydration and skin friction) was determined.

3.3.1. TEWL

The TEWL is a measure for the skin barrier function. High values indicate a disrupted barrier and thus an impaired barrier function. The TEWL of the non-treated skin increased during 6 h of incubation time, indicating a slight breakdown of the SC barrier during this time. The application of the nanosuspensions decreased the TEWL, which can be explained by the formation of a film on top of the skin, which reduces water evaporation from the skin (Figure 7). The reduction in TEWL seemed to be more pronounced for the formulations that contained glycerol, urea in a concentration of 5% or 2% ethanol. This can be explained by the hygroscopic properties of glycerol and urea, which further reduce the water evaporation from the skin and from the formulation on top of the skin. The decreased TEWL of the skin treated with ethanol might be explained by the dehydrating properties of ethanol. This means, it can be assumed that ethanol and water will evaporate quickly after topical application. Thus, leading to a dry skin and to the formation of a dry formulation on top of the skin. The addition of olive oil did not alter the TEWL. However, data in this part were not significant and thus further experiments are necessary to solidify these observations.

A slight but nonsignificant increase in TEWL was seen for the skin treated with the propylene glycol containing nanosuspension. This indicates an SC impairment, which is—even though not significant in this study—well-described in the literature [27] and thus further supported by this data. The TEWL of the skin treated with the nanosuspension that contained 10% urea was increased by about 35%. The effect was significant. Hence, the barrier function-improving properties of urea which are well described in the literature [48] could not be observed in this study. A possible reason is that the barrier-protecting properties of urea are caused by the urea-induced upregulation of involucrin, filaggrin and transglutaminase-1 (TG-1) expression [48], which of course could not take place in the short-term ex-vivo model used in this study. Therefore, the barrier disrupting effect observed in this study might be explained by the keratinolytic properties of urea.



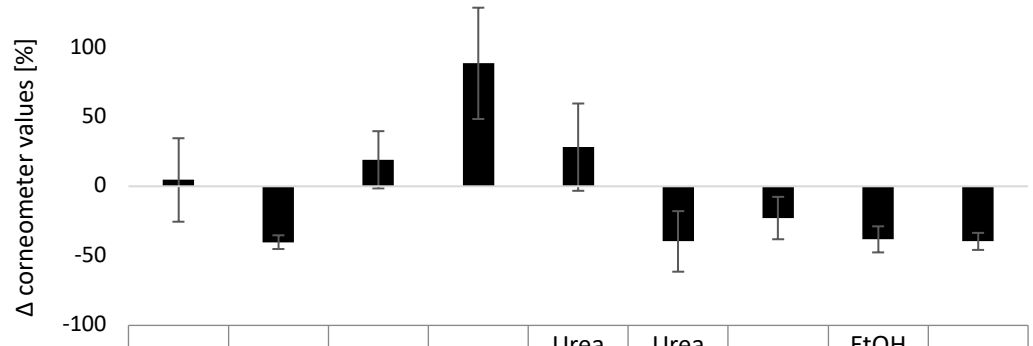
	Blank	NS	Gly 2%	Gly 5%	Urea 5%	Urea 10%	PG 5%	EtOH 2%	oil 2%
■ Δ TEWL [%]	20	10	2	-4	7	35	26	3	18

Figure 7. Influence of curcumin nanosuspension without (NS) and with different excipients on the skin barrier function (TEWL measurements are expressed as Δ TEWL [%], representing the relative changes in TEWL 6 h after the treatment, compared to the TEWL values prior to the skin treatments).

Data obtained show that the curcumin nanosuspension without excipients possesses no barrier-disruptive properties. Also, the addition of 2% or 5% glycerol, 2% olive oil, 2% ethanol or low amounts of urea (5%) did not alter the skin barrier function. Some impairment was found when 5% propylene glycol or 10% urea were added

3.3.2. Skin Hydration

Skin hydration was measured with a corneometer which determines the capacitance of a surface. As water has a high capacitance, high corneometer values correspond to a high skin hydration. After 6 h of incubation no significant changes in skin hydration were observed for the untreated control skin area (blank) and the areas treated with formulations that contained 2% glycerol, 5% urea or propylene glycol (Figure 8). All other changes were significant. The nanosuspension without additives and the formulations that contained 10% urea, ethanol, or olive oil were found to decrease the corneometer values by about 40%.



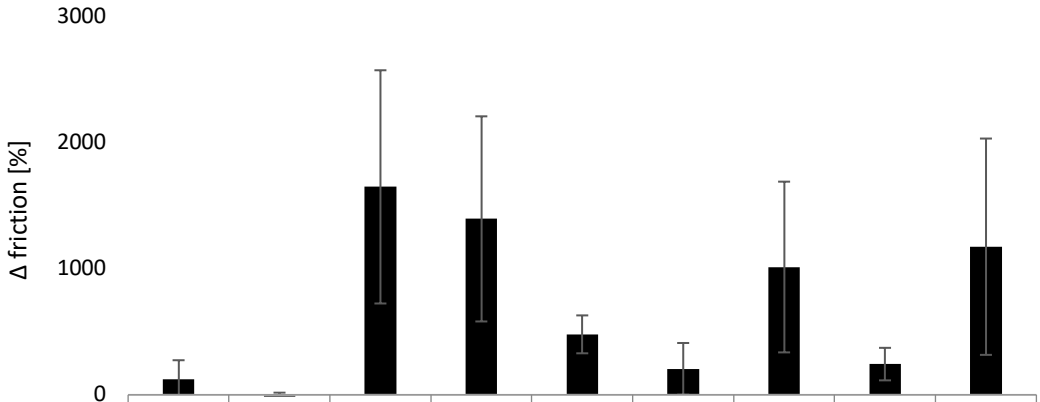
	Blank	NS	Gly 2%	Gly 5%	Urea 5%	Urea 10%	PG 5%	EtOH 2%	oil 2%
■ Δ hydration [%]	5	-40	19	88	28	-40	-23	-38	-40

Figure 8. Influence of curcumin nanosuspension without (NS) and with different excipients on the skin hydration (Corneometer measurements. The values obtained after 6 h of incubation are expressed as relative changes compared to the untreated skin prior to the application).

The observed decrease in capacitance is not corresponding to a real decrease in skin hydration but indicates a film formation of the formulations on top of the skin. As the formulations dry out, a lower capacitance is measured after 6 h of incubation, because the formed film is an insulator that decreases the capacitance reading. The effect has been previously observed and thus data of this study are in agreement with previous findings [49]. In contrast, the addition of 5% glycerol increased the measured skin hydration significantly. The skin-hydrating effect can be explained by the hygroscopic properties of glycerol which prevents the drying of the formulations and thus results in a higher water content in the formulation film that was formed on top of the skin. A similar but not significant trend was seen for the formulations containing 2% glycerol or 5% urea. This is reasonable because lower concentrations will possess less hygroscopic properties.

3.3.3. Skin Friction

Treating the skin for 6 h with the different formulations did not significantly alter the skin friction (resistance against a rotating probe) for the nontreated control, the nanosuspension without excipients and the nanosuspensions with 10% urea or 2% ethanol. The other formulations tremendously increased the skin friction (Figure 9). Data also show that the addition of excipients led to a higher skin friction than the use of the nanosuspension without excipients. The differences are related to the different properties and interactions between formulation, skin and the rotating disc of the frictiometer. The most pronounced increase in skin friction was found after adding 2% or 5% glycerol, 5% urea or olive oil to the nanosuspension. The increased skin friction is plausible because only glycerol and 5% urea were shown to increase the skin hydration. Thus, the increased skin hydration and the swollen SC can be considered to make the skin “stickier”. Thus, leading to the higher skin friction. Likewise, propylene glycol possesses hygroscopic properties [50] and was shown to increase the thickness of the stratum corneum (c.f. Figure 2 and Figure 4). Hence, it can be considered, that propylene glycol increases the skin friction by a similar mechanism like glycerol. In contrast, olive oil can be considered to form a small, oily film on the skin. The oily film—due to the hydrophobic nature of the oil—causes a sticky and slightly greasy skin feeling, which causes an increase in the measured skin friction values.



	Blank	NS	Gly 2%	Gly 5%	Urea 5%	Urea 10%	PG 5%	EtOH 2%	oil 2%
Δ friction [%]	125	-14	1648	1395	479	206	1013	244	1174

Figure 9. Influence of curcumin nanosuspension without (NS) and with different excipients on the skin friction (The values obtained after 6 h of incubation are expressed as relative changes compared to the untreated skin prior to the application).

The measurement of the biophysical skin parameters is not yet a standard procedure when testing dermal formulations in-vitro or ex-vivo. Therefore, only little scientific data are available in this regard. On the contrary, many in-vivo studies use the analysis of the biophysical skin properties as a basic tool to investigate the influence of dermal formulations on the skin conditions for different health conditions. Overcoming the missing link between in-vitro, ex-vivo and in-vivo data is thus needed to improve the power for the development and formulation of novel and efficient dermal drug products. Hence, the long-term aim of such testing is to establish a valid in-vitro / in-vivo correlation, which would then allow to forecast the in-vivo performance of topical formulations in regard to their skin care properties. If the strategy is successful, the use of skin probes in in-vitro and ex-vivo experiments can help to reduce the number of in-vivo experiments, which would not only help to reduce costs, but would also allow to reduce the number of animal experiments. With this, the proposed strategy is a straightforward approach in regard to the 3R strategy [51] and is also helpful for the development of science-based (European) cosmetic products, where animal testing is prohibited [52].

This strategy followed in this study include the ex-vivo testing of the three major biophysical skin properties (TEWL, skin hydration and skin friction) prior to and after application of the different formulations. Results show relatively large standard variations between the different skin areas tested. Hence, future tests should increase the number of test areas and studies to optimize and standardize the test procedure on the ex-vivo pig ear model, which in turn should improve the statistical strengths of such data.

The data obtained in this study already provide evidence that the determination of the biophysical skin parameters, along with the assessment of the penetration efficacy, is helpful for the selection of suitable excipients. For example, the TEWL data enabled to pinpoint formulations that tend to impair the stratum corneum, whereas corneometer values were not affected by these compounds but clearly

differentiated between film forming formulations and formulations with hygroscopic—thus skin-hydrating—properties.

Whereas data that are obtained from dermal penetration testing can select the most suitable excipients for optimal passive dermal penetration, the additional testing of the biophysical skin properties can filter excipients that provide optimal skin care or skin-protective properties. In this study, the addition of 5% propylene glycol or the addition of 5% glycerol to a curcumin nanosuspension was found to be most suitable for improved passive diffusion of curcumin. However, assessing the biophysical properties that are caused on the skin due to the addition of the excipients to the nanosuspension, clearly showed that glycerol possesses skin-caring properties, whereas propylene glycol showed a trend towards barrier-impairing properties. Based on the data, glycerol could be selected to be the most suitable excipient to foster passive dermal penetration of curcumin from drug nanocrystals and to provide skin-caring properties at the same time.

3.4. Determination of Hair Follicle Targeting

The efficacy to target nanoparticles into the hair follicle is considered to be size-dependent [10]. As the different formulations were found to possess slightly different particle sizes that were caused due to partial dissolution of the curcumin nanocrystals upon the addition of the excipients (c.f. 3.1.), it was important to estimate the impact of these differences on the penetration efficacy of the nanocrystals into the hair follicles. This was done by determining the penetration depth of the curcumin nanocrystals without excipients directly after production (NS) and after 14 days of storage at room temperature (NS d14). During this time, the particle size increased from about 700 nm to > 1 µm (LD data, d(v) 0.5). The DLS data (c.f. Table 2) indicated the existence of an additional small sized particle population that possessed a size of about 250 nm in case of the freshly prepared nanosuspension and a size of about 500 nm after 14 days storage. With this, from all formulations produced in this study, the fresh nanosuspension possessed the smallest sizes, the NS d14 the largest particle sizes and all other formulations particle sizes within this range (c.f. Table 2). Therefore, it was assumed that differences in the penetration efficacy due to differences in sizes would be most pronounced between these two formulations without excipients and less pronounced for all other formulations that contained excipients.

The mean penetration depth of the freshly prepared nanocrystals (NS) was 293 ± 103 µm and decreased by about 20% to 233 ± 63 µm for the aged nanocrystals (NS d14) with larger particle size. The difference became significant by direct comparison of the two means with the student`s *t* -test ($p = 0.02$) but was not significant with the post-hoc tests used after one-way ANOVA. Based on the data, it was concluded that the differences in size might contribute to the penetration depth of the nanocrystals to a minor extend. Further, it could be expected that the larger sized formulations would

lead to a slightly decreased penetration depth. Hence, if the size would be the most influencing parameter for the penetration of nanocrystals into the hair follicles, there should be a trend showing that the formulations possessing particle sizes close to the aged nanosuspension, i.e., the formulations that contained propylene glycol, ethanol or olive oil (c.f. Table 2), cause a reduced penetration depth in comparison to the freshly prepared nanosuspension with smaller particle size. Accordingly, the formulations that possess similarly small particle sizes than the freshly prepared nanosuspension, i.e., the formulations containing glycerol or urea (c.f. Table 2), should lead to penetration profiles being similar or close to the penetration profile of the freshly prepared nanosuspension without excipients. The results obtained disproved the expected decrease in penetration depth for the larger sized formulations and the expected similar penetration profiles for the formulations with similar particle sizes (Figure 10 and Figure 11).

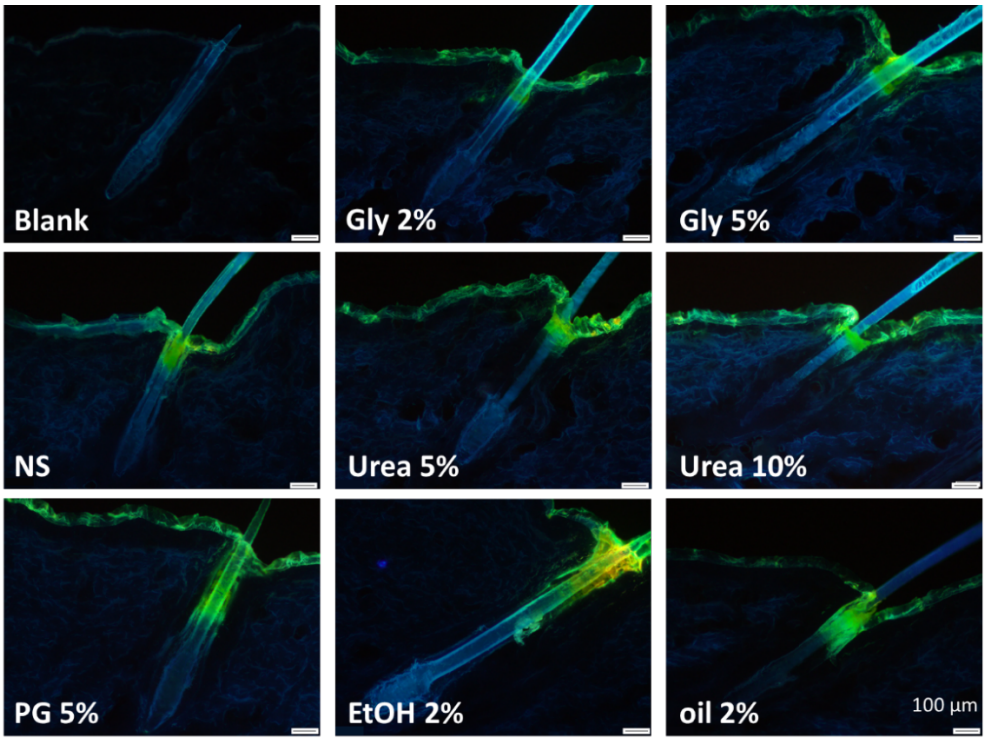


Figure 10. Images obtained from the skin sections (magnification 100-fold) to visualize the influence of excipients on the penetration depth of curcumin nanocrystals into hair follicles. Upper: original images. Lower: images after digital processing (transfer into black-white images and contrast enhancement) to improve the visibility of the penetrated curcumin nanocrystals. Penetrated curcumin nanocrystals appear black (indicated with black arrows). Images obtained from the skin sections (magnification 100-fold). For more detailed inspection of the data, please refer to the Supplementary Material Section S10–S18.

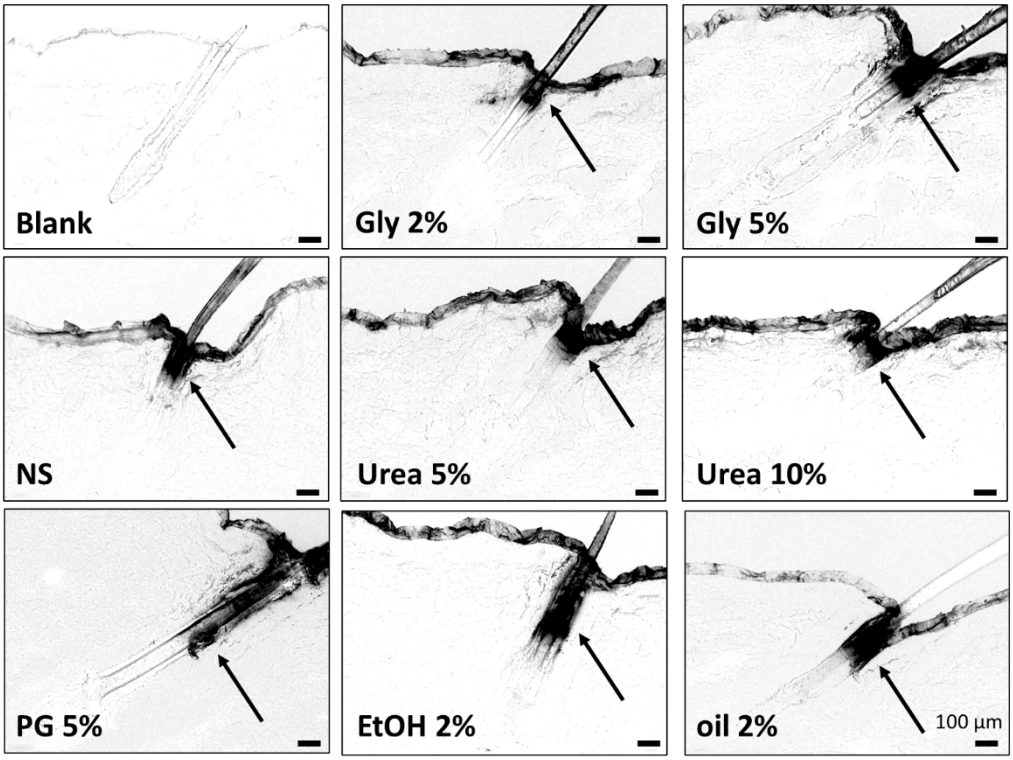


Figure 10. Images obtained from the skin sections (magnification 100-fold) to visualize the influence of excipients on the penetration depth of curcumin nanocrystals into hair follicles. Upper: original images. Lower: images after digital processing (transfer into black-white images and contrast enhancement) to improve the visibility of the penetrated curcumin nanocrystals. Penetrated curcumin nanocrystals appear black (indicated with black arrows). Images obtained from the skin sections (magnification 100-fold). For more detailed inspection of the data, please refer to the Supplementary Material Section S10–S18.

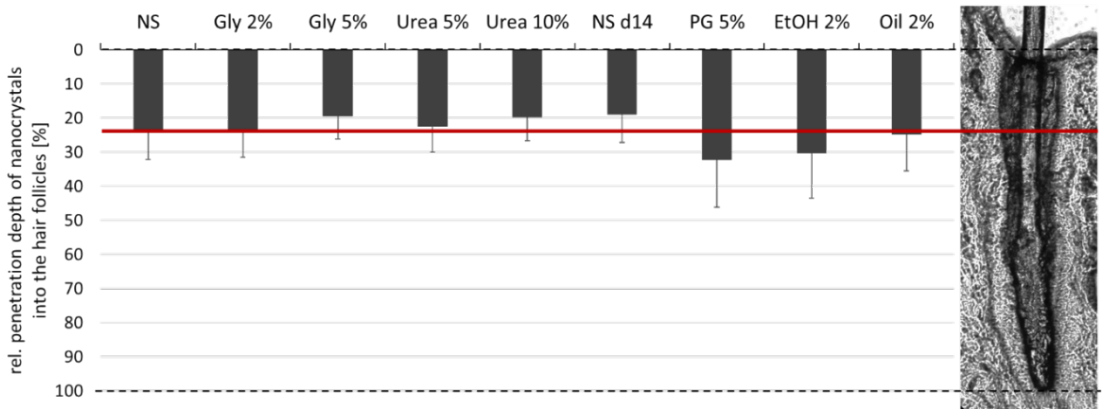


Figure 11. Relative penetration depth [%] of curcumin nanocrystals into the hair follicles from formulations containing different types and amounts of excipients. The relative penetration depth was calculated from the determined mean penetration depth obtained from each formulation and was related to the mean lengths of the hair follicles (porcine ear—ventral side) that was determined to be 1222 µm.

Instead, a significant increase in the penetration depth was found for the formulations that contained propylene glycol and ethanol. The increase in penetration depth was about 30–35% compared to the freshly prepared nanosuspension and about 60–70% compared to the aged nanosuspension without excipients. Likewise, the addition of olive oil led to a slight increase in the penetration depth of the nanocrystals. The difference was not significant when compared to the freshly prepared nanosuspension and became significant by a direct comparison with the penetration depth of the aged nanosuspension (Mann–Whitney-test, $p < 0.05$). The addition of 5% glycerol or 10% urea decreased

the penetration depth of the nanocrystals by about 15–20%. The effects were concentration dependent and became significant by a direct comparison with the freshly prepared nanosuspension (unpaired *t*-test (one-tailed), $p < 0.05$).

One-tailed tests compare mean values of two independent groups in only one direction and thus are considered to provide more power to detect whether a mean of a population is larger or smaller than the mean of another population [53]. Based on the observations in our previous study, where the addition of 5% glycerol was found to decrease the penetration depth of the curcumin nanocrystals [12], we hypothesized that also in this study the addition of glycerol would lead to a decrease in penetration depth. As we assumed that the effect occurs due to a swelling of the hair shaft and/or changes in the structure of the hair cuticula and is caused by the hygroscopic properties of the glycerol, we also hypothesized that urea and propylene glycol—which are also known to possess hygroscopic properties – will cause similar effects. Based on the significant results from the *t*-tests, the hypothesis was confirmed for glycerol and urea but was disproved for propylene glycol. The results therefore indicated that the size of the nanoparticles and the hygroscopic properties of the excipients might not be the sole parameters that affect and modulate the penetration efficacy of nanocrystals into hair follicles.

The main driving parameter for an efficient uptake of nanoparticles into the hair follicle is considered to be the ratchet effect [10,11,54]. Therefore, it was assumed that the different excipients might differently influence the hair and/or hair follicle structure which could then explain the different effects observed. In order to prove this assumption, hairs that were incubated for 6 h with the different formulations were collected and subjected to microscopic analysis (Figure 12). All hairs had adsorbed nanocrystals on their surface that were dried during the 6 h of incubation. The hair cuticula was clearly visible for the hairs treated with the nanocrystals without any additional excipients. However, nanocrystals were not detected within the overlapping cells of the hair cuticula when the hairs were treated with the pure nanosuspension and with the nanosuspensions that contained either glycerol or urea (Figure 12, upper row). In contrast, nanocrystals seemed to accumulate preferentially in between the cuticular structures, when the hairs were treated with propylene glycol or olive oil (Figure 12, lower row). The treatment of hairs with the nanosuspension that contained ethanol caused the formation of a crust. Due to this it was not possible to determine exactly if nanocrystals were also located in between the cuticular structures. However, the small sections that were not covered with the crust suggested this (Figure 12, lower row—middle). The observations were in line with the results obtained from the hair follicle penetration experiments and could link increased hair follicle penetration to an increased localization of the nanocrystals in between the overlapping cells of the hair cuticula, which might have caused an improved ratchet-like transport of the particles into the hair follicles.

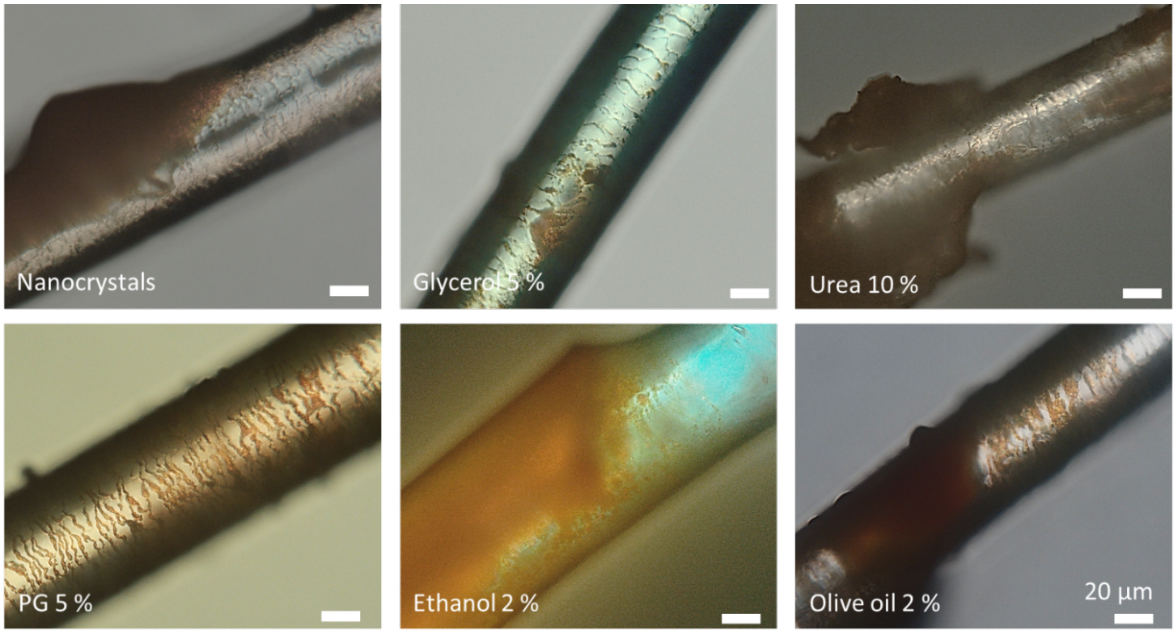


Figure 12. Microscopic images of hairs removed from porcine ears treated with nanosuspensions without and with excipients after 6 h of incubation (digital zoom of images taken at 400-fold magnification).

In the next step, the hairs were rehydrated with their original dispersion medium and microscopic images were taken (Figure 13). The results showed not only differences in the adhesiveness of the nanocrystals but also differences in the structure of the hair cuticula. Almost no changes in the adherence of the nanocrystals were found for the hairs treated with the nanosuspension without excipients. The hairs treated with glycerol and urea had a smooth surface and only a few nanocrystals were found to be attached to the surface of the hair. In contrast, the re-hydrated hairs treated with propylene glycol, ethanol and olive oil showed a rougher surface of the hair cuticula and more nanocrystals were adhered to the surface of the hairs. The localization of the particles in between the cuticular structures was clearly visible and was most pronounced for the hairs treated with propylene glycol and olive oil. The results therefore further substantiate the theory that excipients can influence the penetration of nanocrystals into hair follicles due to a modification of the adhesiveness of the particles and/or due to changes in the hair cuticula structure.

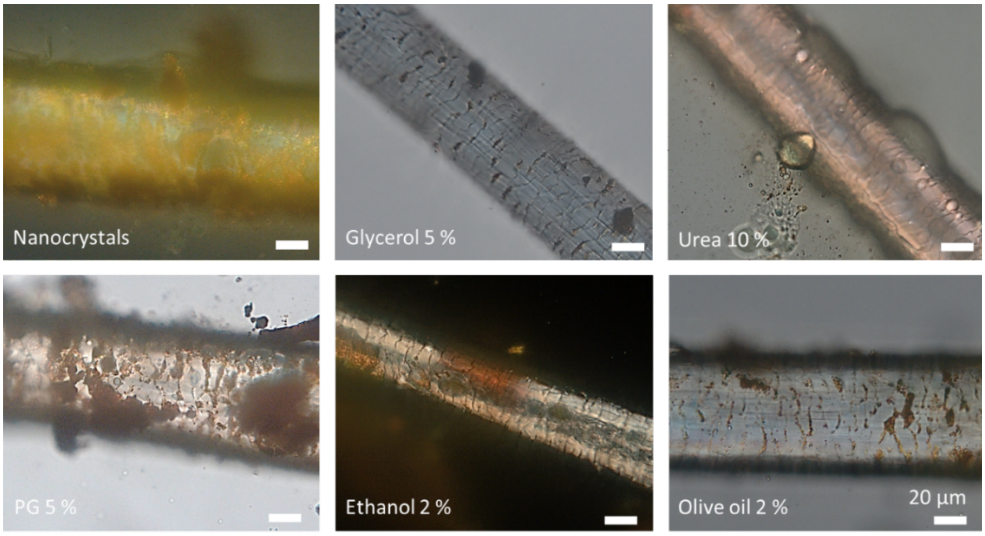


Figure 13. Microscopic images of hairs removed from porcine ears treated with nanosuspensions without and with excipients after addition of dispersion medium (digital zoom of images taken at 400-fold magnification).

The modification of the adhesiveness of the particles and/or changes in the hair cuticula structure due to the addition of excipients were further tested by simulating the application of the nanocrystals to hairs that were freshly obtained from the ventral side of different pig ears. The dry hairs were placed on a microscopic slide and 20 µL of nanosuspension with or without excipients were added. After about 5 min of incubation the hair was transferred to a clean microscopic slide and original dispersion medium was added to the hair (Figure 14 and Figure 15). The results demonstrated again clear differences in the adhesiveness of the nanocrystals between the differently treated hairs. However, the previously observed differences in the hair cuticula structure were not observed and also the adhesiveness was slightly different compared to the results obtained after 6 h of incubation. The least adhesiveness was found again for the hairs treated with glycerol. Urea led to a strong adherence of the nanocrystals, whereas ethanol and olive oil led to a formation of a crust. The crust was washed off upon redispersion of the hair in case of ethanol (Figure 14, black arrow) but remained on the hair surface in case of the hair treated with olive oil. Digital zooming of the images taken indicates that the nanocrystals are homogeneously distributed around the hair (Figure 15). Nonetheless, a detailed inspection shows that the particles start to localize in between the overlapping structures of the hair cuticula (Figure 15, areas marked with arrows). The results therefore suggest that changes in the hair cuticula structure occur not instantly and that the adhesiveness of the particles might also change over time. However, based on the assumption from our last study where we concluded that most of the nanocrystals are transported into the hair follicles within the first minutes after the application [12], the effects that were observed from the last experiment should be considered to be the main effects that cause the differences in the penetration depth of the nanocrystals.

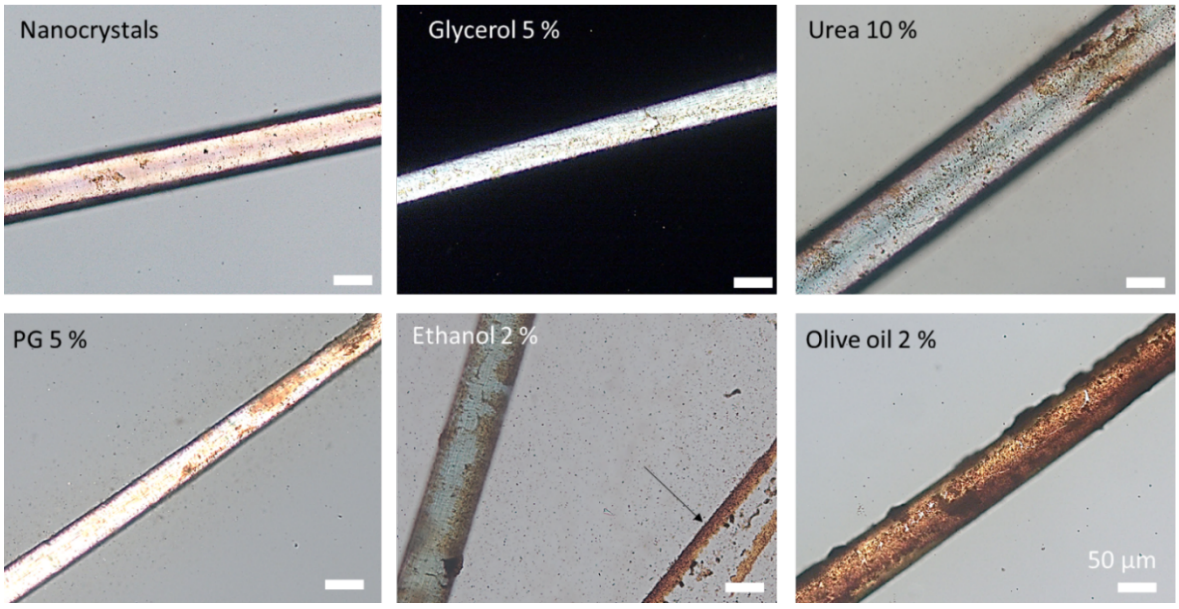


Figure 14. Microscopic images of porcine hairs incubated with nanosuspensions without and with excipients for about 5 min and subsequent redispersion in the respective dispersion medium (digital zoom of images taken at 200-fold magnification).

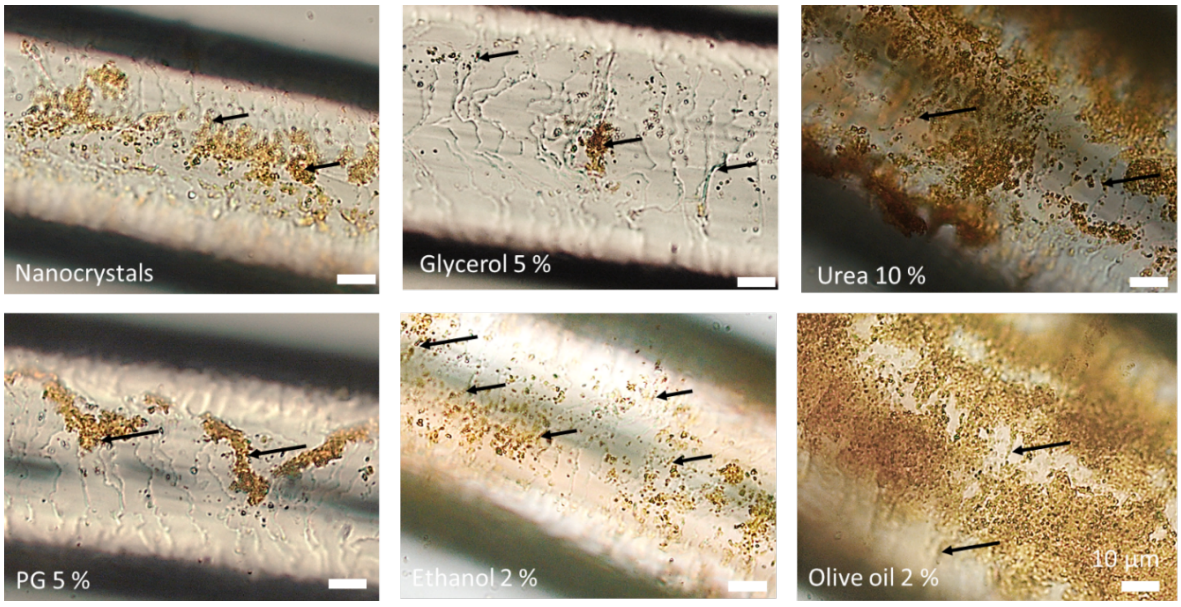


Figure 15. Microscopic images of porcine hairs incubated with nanosuspensions without and with excipients for about 5 min and subsequent re-dispersion in the respective dispersion medium (digital zoom of images taken at 1000-fold magnification).

All the results obtained in the last part of this study lead to a conclusion that the addition of excipients can alter distinctly the penetration efficacy of nanocrystals into hair follicles. The concrete mechanisms that caused the increase or decrease in penetration depth when compared to the nanocrystals without excipients could not be finally ascertained from the data obtained in this study. Nonetheless, the data suggest that the mechanism by which particles are transported into hair follicle is not only influenced by the particle size but also by some additional parameters.

One parameter that was identified in this study is the adhesiveness of the nanocrystals to the hairs. Particles that stick too tightly to the hair cannot move and—consequently—will remain on top of the surface of the hair and cannot penetrate into the hair follicle. Particles that possess extremely poor

adhesiveness to the hair or are even repelled from the hairs, cannot enter the ratchet-mechanism, and thus will also not penetrate into the hair follicle. This means particles that penetrate well into the hair follicle need to adhere to the hair loosely and should preferentially locate in between the gaps of the overlapping cells of the hair cuticula to enter the ratchet mechanism. It can be speculated that excipients that modify the structure of the hair cuticula might increase the ratchet effect if they increase the roughness of the hair cuticula, whereas excipients that reduce the roughness of the hair surface will decrease the ratchet effect. Other mechanisms that promote or impede the adhesiveness of particles to hairs might be related to the charge of the hair and/or the particles and to other attractive or repulsive forces that need to be elucidated in future studies.

A second parameter that might affect the penetration efficacy of nanocrystals into the hair follicles is the condition of the hair follicle, which acts as the “pawl” and is thus indispensable as “countermovement” for a functioning ratchet mechanism and an efficient transport of the particles into the hair follicles. Consequently, it can be hypothesized that excipients that modify the conditions of the hair follicle, might also modify the “pawl” and with this the penetration efficacy of particles into the hair follicles. Possible changes that can occur involve changes in the structure of the hair follicle stratum corneum, which could become softer or more rigid and could swell or shrink. This could further modify the grip and/or the distance between the ratchet and the pawl leading to changes in the penetration efficacy.

A third condition to be considered to change upon the addition of excipients is the fluidity of the sebum. The upper part of the hair follicle, i.e., the infundibulum, is located between the duct of the sebaceous gland and the stratum corneum surface [53–55] and is filled with lipophilic viscous sebum that consists of neutral and nonpolar lipids [55–57]. A high viscosity of the sebum can be considered to counteract the movement of the ratchet-pawl mechanism. Hence, it can be assumed that a decreased sebum viscosity can accelerate the ratchet-pawl mechanism and with this, the penetration efficacy of particles into the hair follicles. On the contrary, compounds or conditions that increase the viscosity should then decrease the penetration efficacy.

Based on these considerations, the penetration of particles into hair follicles can be assumed to be a three-step process. In the first step, the particles need to adhere loosely to the surface of the hair. From there—in the second step—they need to locate in between the overlapping cells of the hair cuticula to enter the ratchet-mechanism. Finally, in the third step, the ratchet together with the pawl will then transport the particles into the hair follicle. The particle size can be assumed to mainly influence the efficacy of the particles to locate in between the overlapping cells of the hair cuticula [10]. Based on the findings of this study, it was shown that excipients can “overwrite” the size effect of the particles. In addition, they can be considered to enable a modification of all three steps of the

hair follicle penetration process. This means that not only the production of tailor-made particles but mainly a purposeful selection of the excipients can help to improve or to impede the penetration of particles into hair follicles. Both aspects might be interesting in formulation development of topical products. Impeded penetration of particles might be interesting to avoid excessive uptake of unwanted particles, for example sun blockers, e.g., titanium dioxide particles [58]. Improved uptake is highly interesting for drug delivery.

The excipients used in this study were found to lead to different effects and can be considered to influence the penetration process of the particles at different stages. Glycerol reduced the adhesiveness of the particles and thus was found to reduce the penetration efficacy of the nanocrystals. If the effect was due to a decrease in the roughness of the hair cuticula and if the effect was further boosted by a change in the hair follicle surface could not be demonstrated in this study. Urea was also found to decrease the penetration depth of the nanocrystals. However, the mechanism that hampered the penetration is probably not like that of glycerol. Urea has keratinolytic properties [59–62]. After a short incubation time this caused a strong adhesion of the particles to the surface of the hairs. Over time, keratolysis resulted in a smoother hair structure (c.f. Figure 13) and low adhesiveness of particles to the hair surface. Both effects can be regarded to contribute to the observed decrease in the hair follicle penetration efficacy. Propylene glycol, ethanol and olive oil increased the penetration efficacy of the nanocrystals. As all three excipients can act as solvents for polar, lipophilic compounds [63–65] these excipients can also be considered to interact with the sebum and to decrease its viscosity [66,67], which then leads to an improved ratchet-effect and a resulting increase in the penetration efficacy. The more pronounced increase in penetration depth for ethanol and propylene glycol might be explained by a slight increase in hair roughness (c.f. Figure 13), which increased the space between the overlapping cells of the hair cuticula and thus allowed more particles to locate in between these cells which than allowed for an increased penetration efficacy of the particles into the hair follicles.

The data obtained for the interpretation of the different penetration mechanisms are yet too preliminary to draw a final conclusion and more research is needed in this regard. However, findings so far suggest that the addition of 2% ethanol or 5% propylene glycol to a nanosuspension are useful to enhance the uptake of nanocrystals into hair follicles. Depending on the desired route of administration, ethanol should be selected for effective hair follicle targeting without intense passive dermal penetration and propylene glycol should be used for formulations that should allow for hair follicle targeting with intense passive dermal penetration at the same time.

4. Conclusions

This study investigated the influence of different excipients on the penetration efficacy of curcumin nanocrystals into the hair follicles and on the passive dermal penetration of dissolved curcumin from nanocrystals. The results revealed that the excipients influenced the penetration pathway tremendously. The addition of glycerol to nanocrystals impaired the hair follicle penetration and improved the passive dermal penetration. Ethanol significantly enhanced the follicular penetration efficacy and reduced the passive penetration of the active at the same time. Propylene glycol improved both penetration routes. Thus, the addition of different components to the nanosuspension could define the penetration pathway of nanocrystals. The different effects of the various excipients on the passive dermal penetration of curcumin are related to their different mechanisms of actions and are in line with the current scientific opinion about the different penetration enhancing properties of the different excipients, whereas the effects of excipients on the efficacy of the hair follicle targeting seemed to overwrite the previously published size effect of particles.

The effects observed can be explained by a three-step mechanism that transports nanocrystals into the hair follicle. Each step of this mechanism can be altered or modified upon the addition of excipients, thus leading to increased or decreased penetration of particles into the hair follicles (Table 3).

Table 3. Overview of influence of excipients on passive dermal penetration and hair follicle targeting.

Type and concentration of excipient	Effect on passive dermal diffusion	Effect on hair follicle targeting
Glycerol 2%	↑	↔
Glycerol 5% *	↑↑↑↑	↓
Urea 5%	↑↑	↔
Urea 10%	↑	↓
Propylene glycol 5% **	↑↑↑↑	↑↑↑
EtOH 2% ***	↓	↑↑↑
Olive oil 2%	↔	↑

*Suggested for drug delivery via passive dermal penetration without depot effect. ** Suggested drug delivery via passive dermal penetration with simultaneous depot effect. ***Suggested drug delivery via hair follicles without passive dermal penetration.

The findings of the present study suggest that fixed combinations of nanocrystals and selected excipients can be used for targeted dermal drug delivery. This means one can choose if the active compound should be delivered via passive diffusion and/or as drug reservoir by depositing active

compounds such as drug nanocrystals into the hair follicles. With this, the findings provide a new perspective for the formulation of highly effective topical products.

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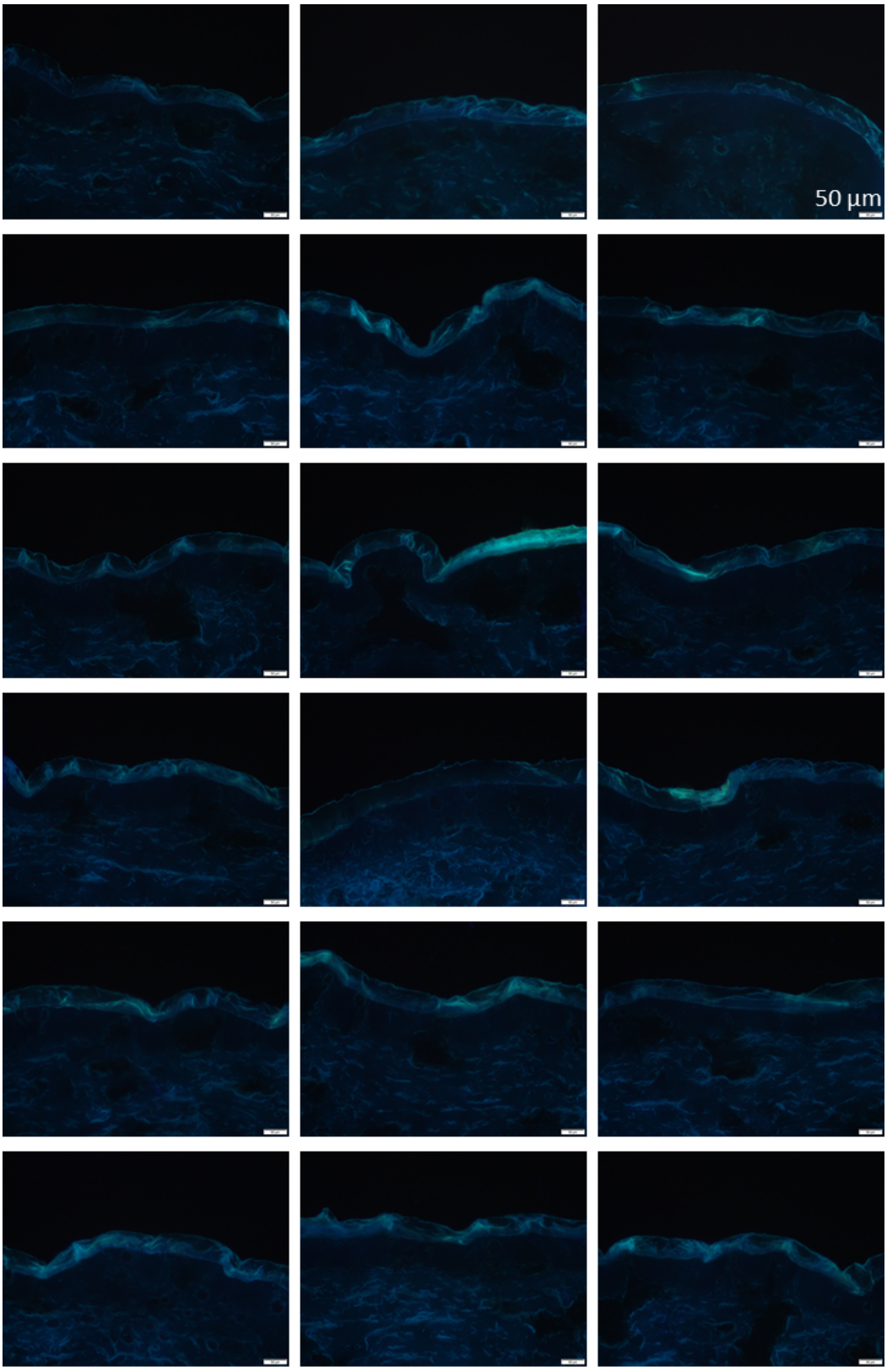
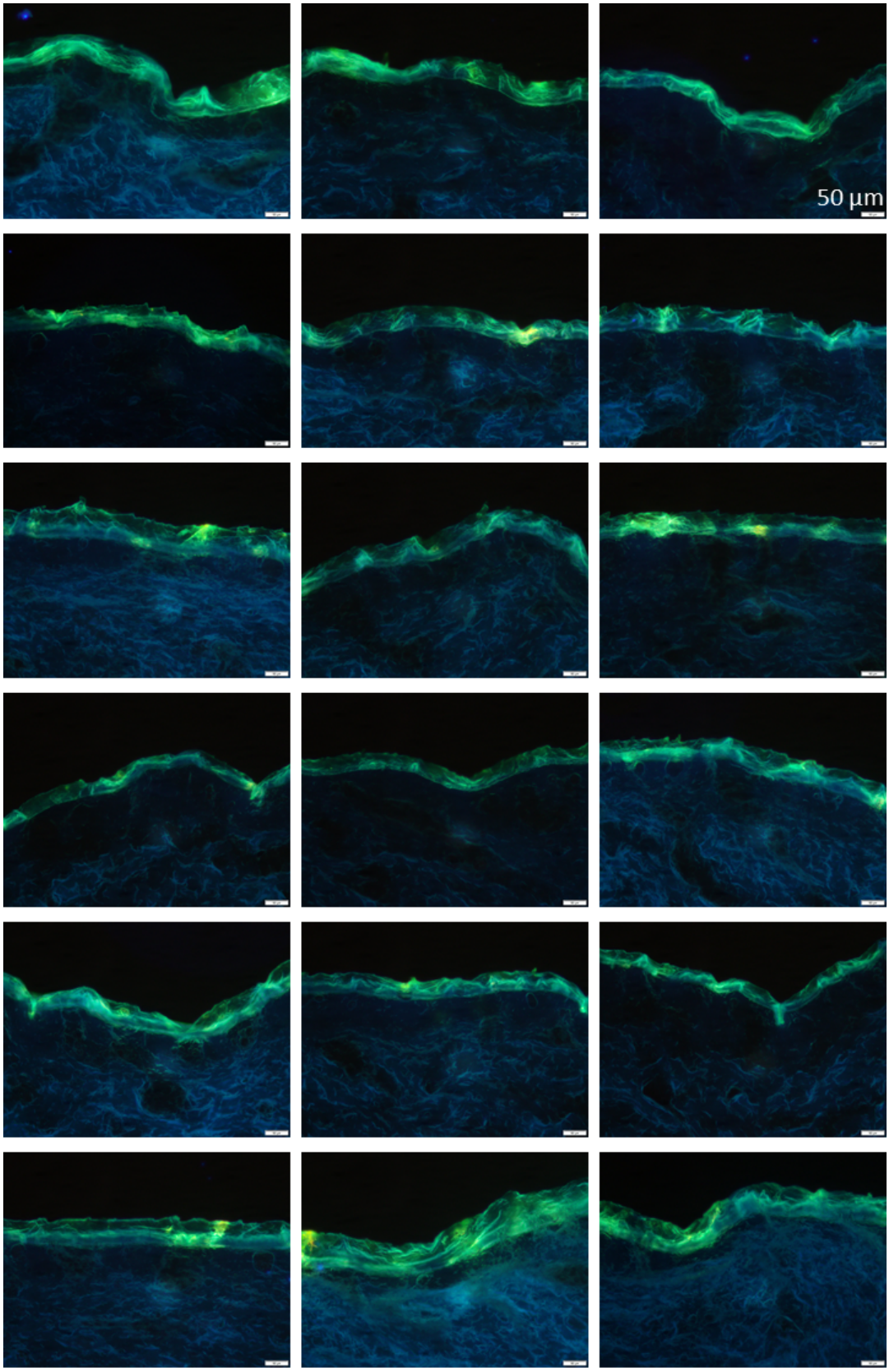
S. Supplementary material to manuscript: Hair follicle targeting and dermal drug delivery with curcumin drug nanocrystals: essential influence of excipients

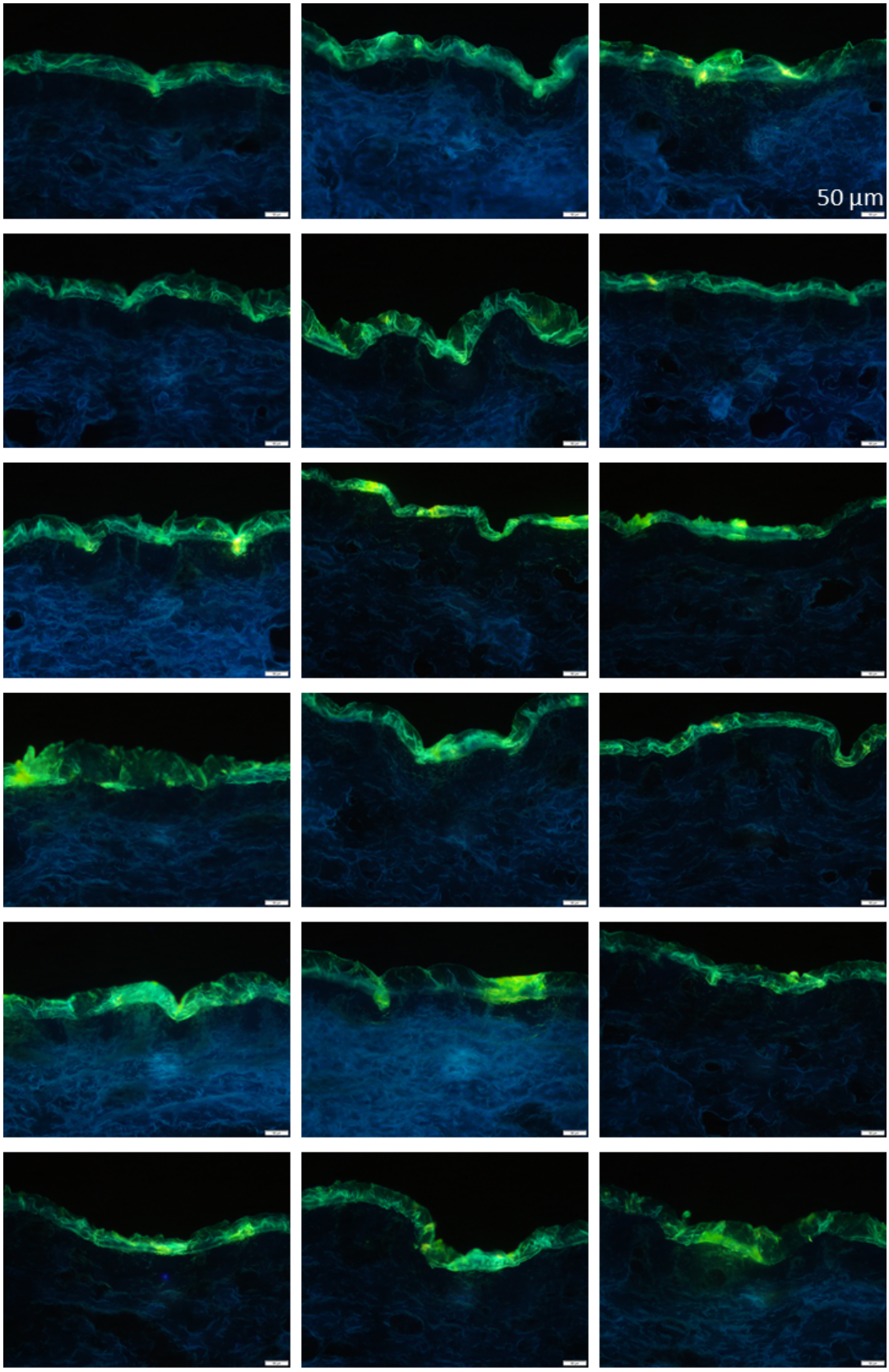
S1: Skin sections of untreated skin. Magnification: 200fold.



S2: Skin sections of skin treated with nanosuspension without additives. Magnification: 200fold.



S3: Skin sections of skin treated with nanosuspension contained 2% glycerol. Magnification: 200fold.



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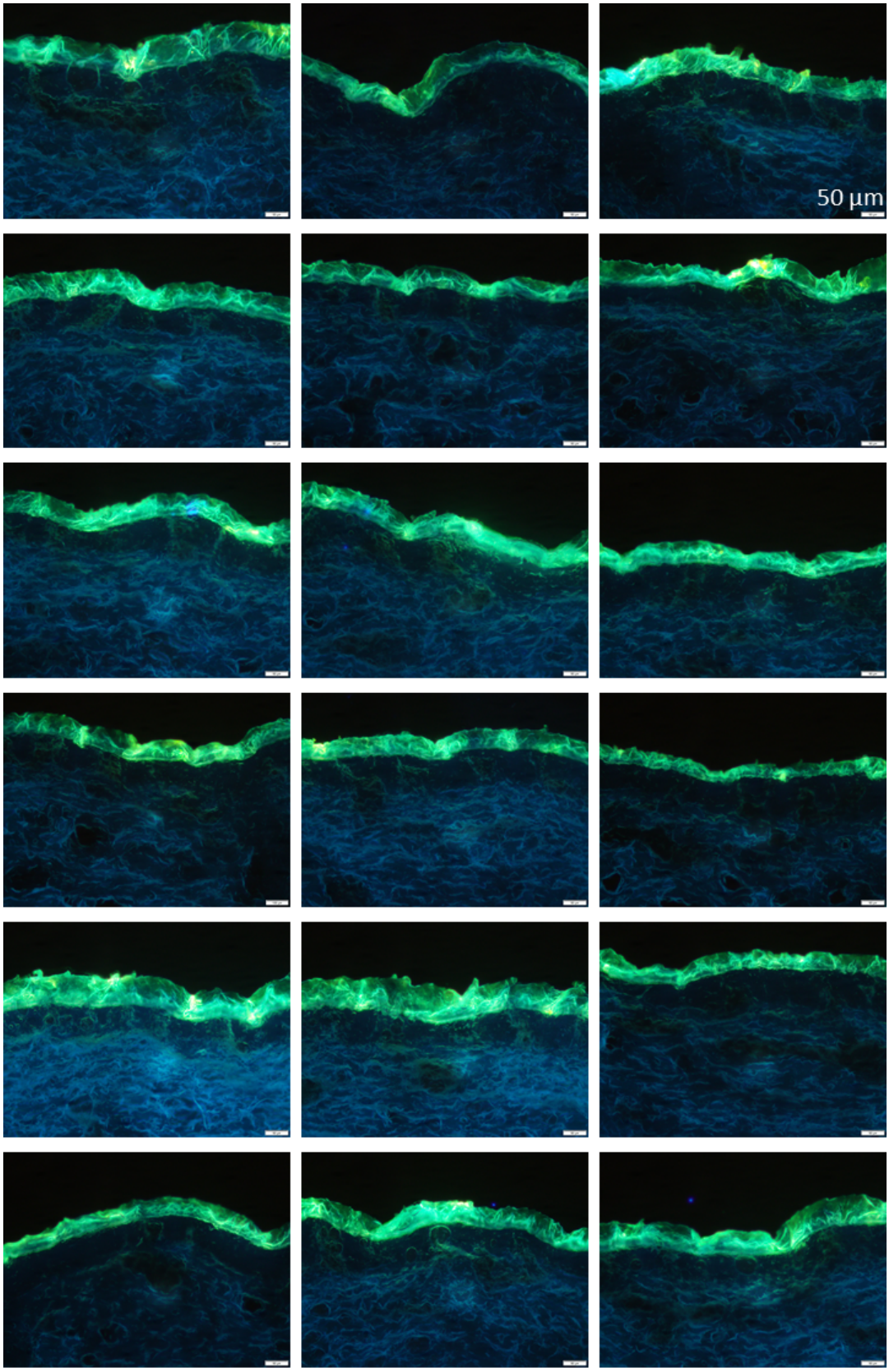

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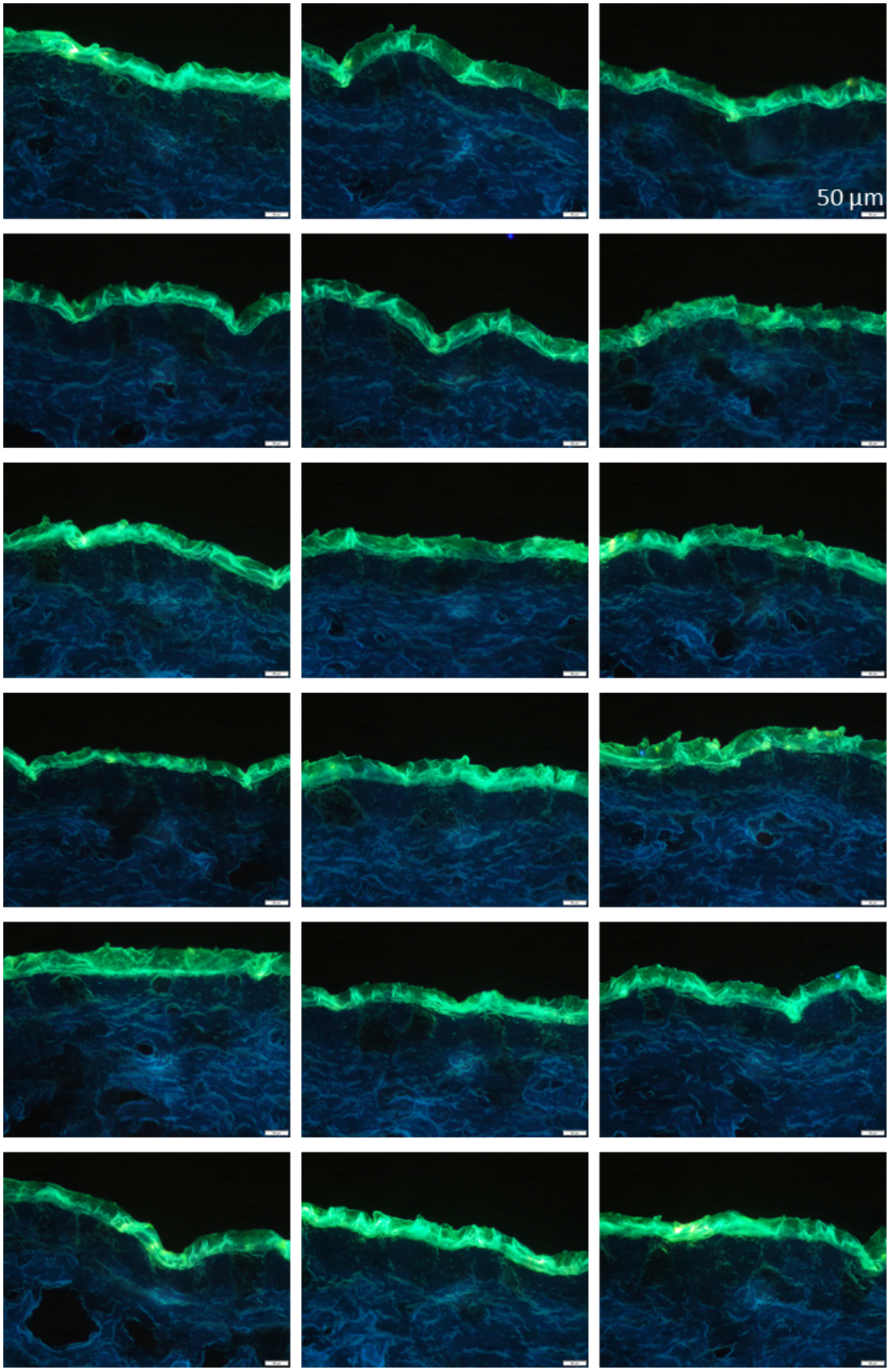
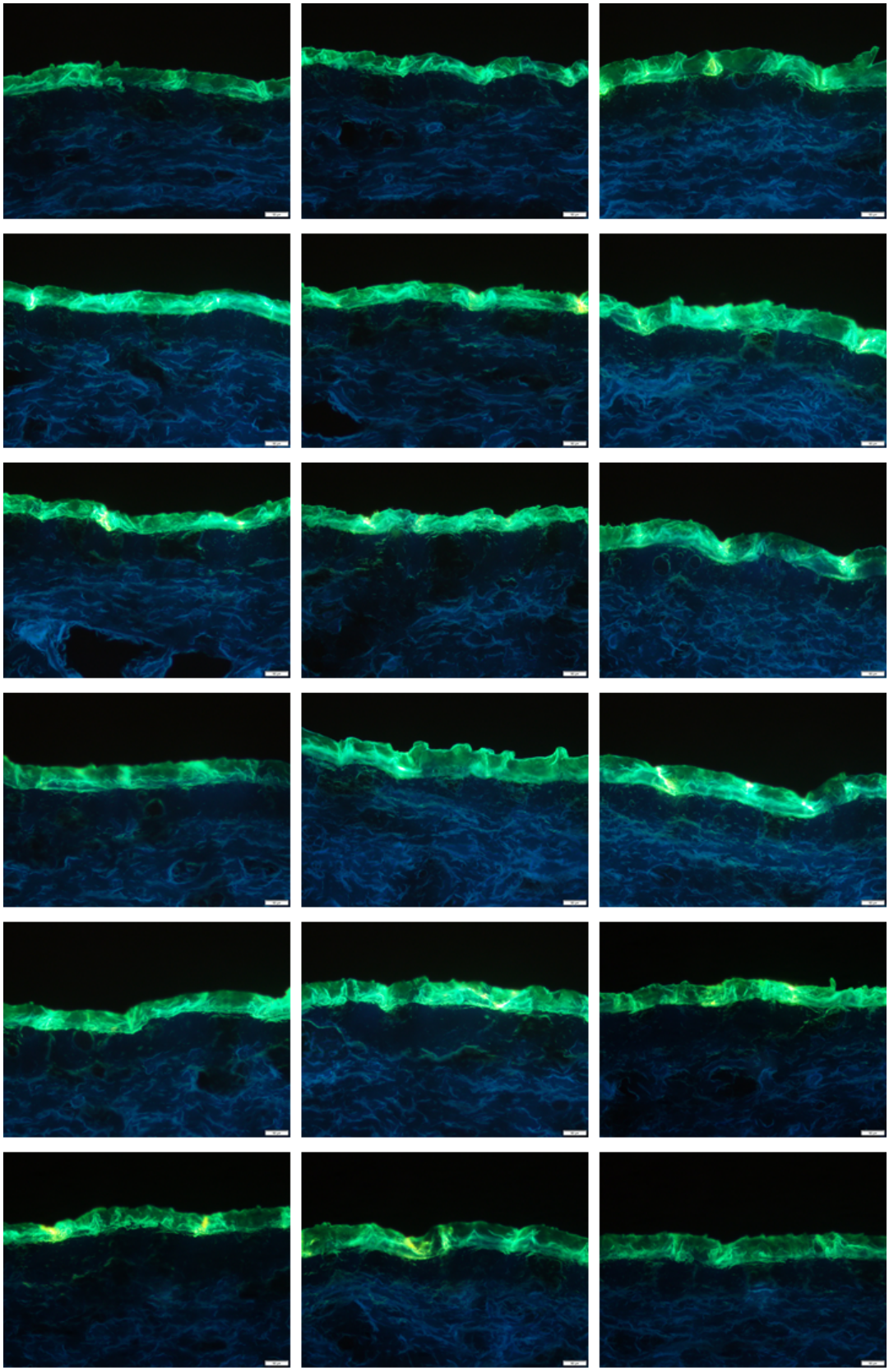
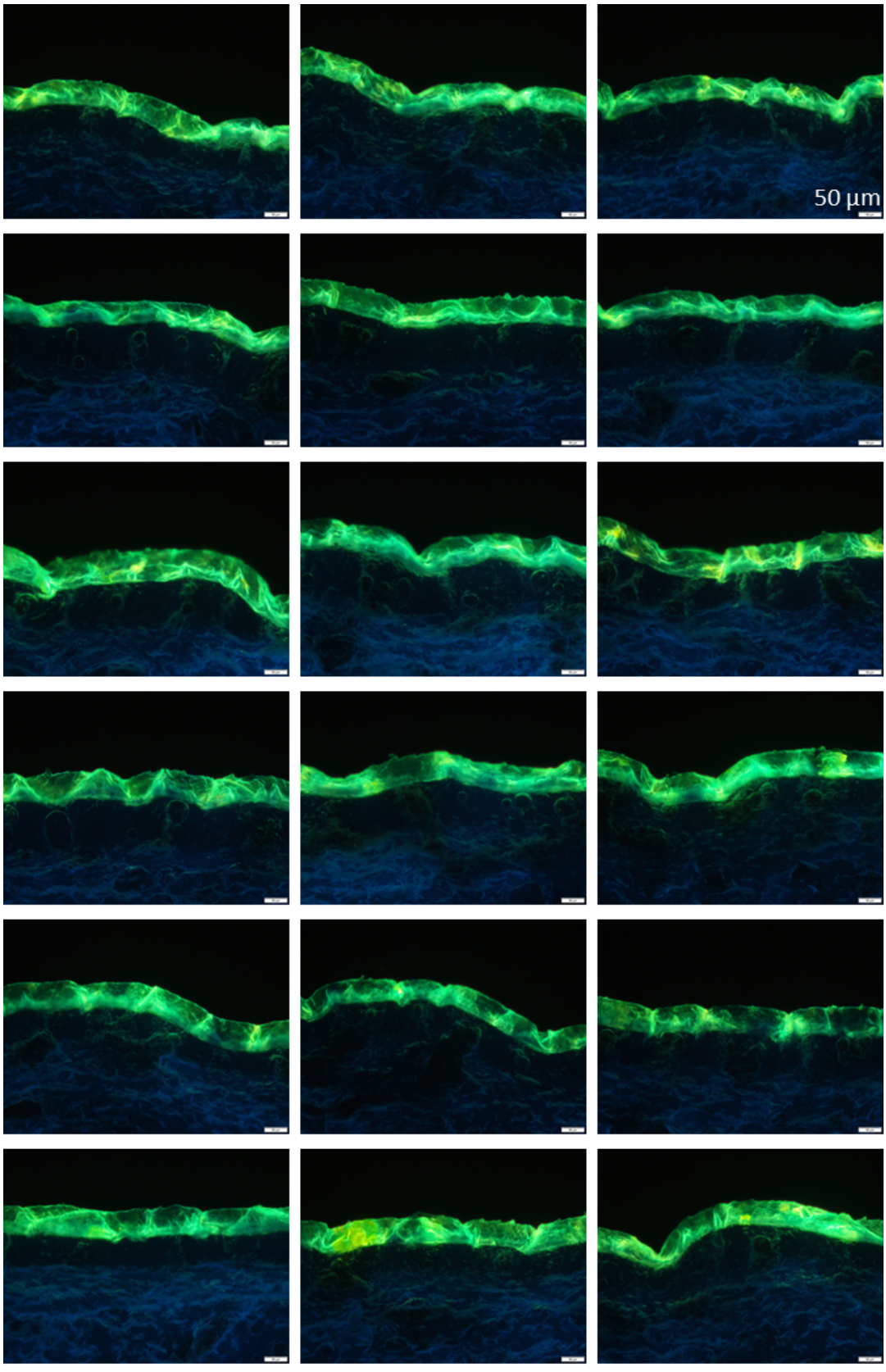


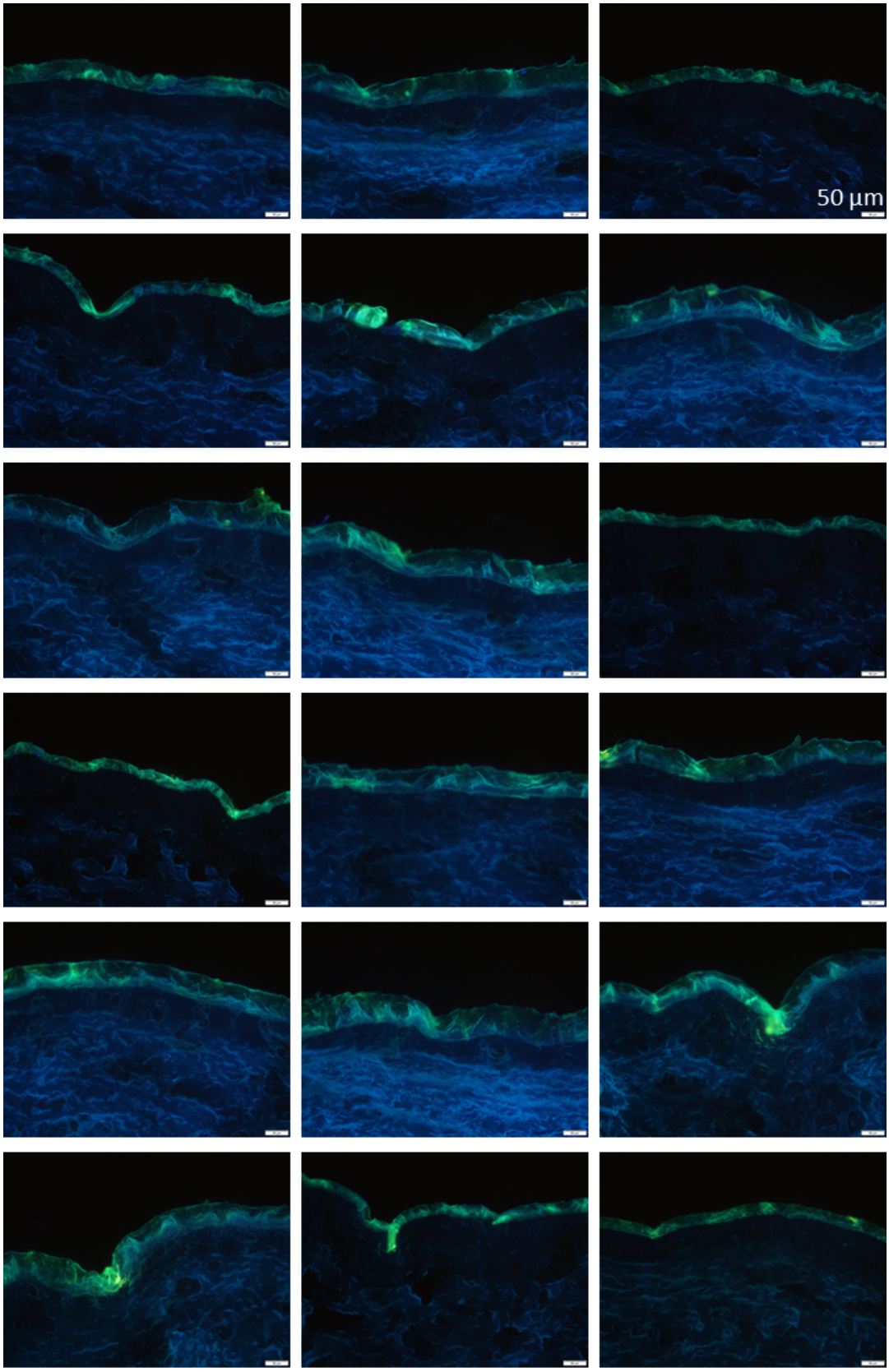
S6: Skin sections of skin treated with nanosuspension contained 10% urea. Magnification: 200fold.



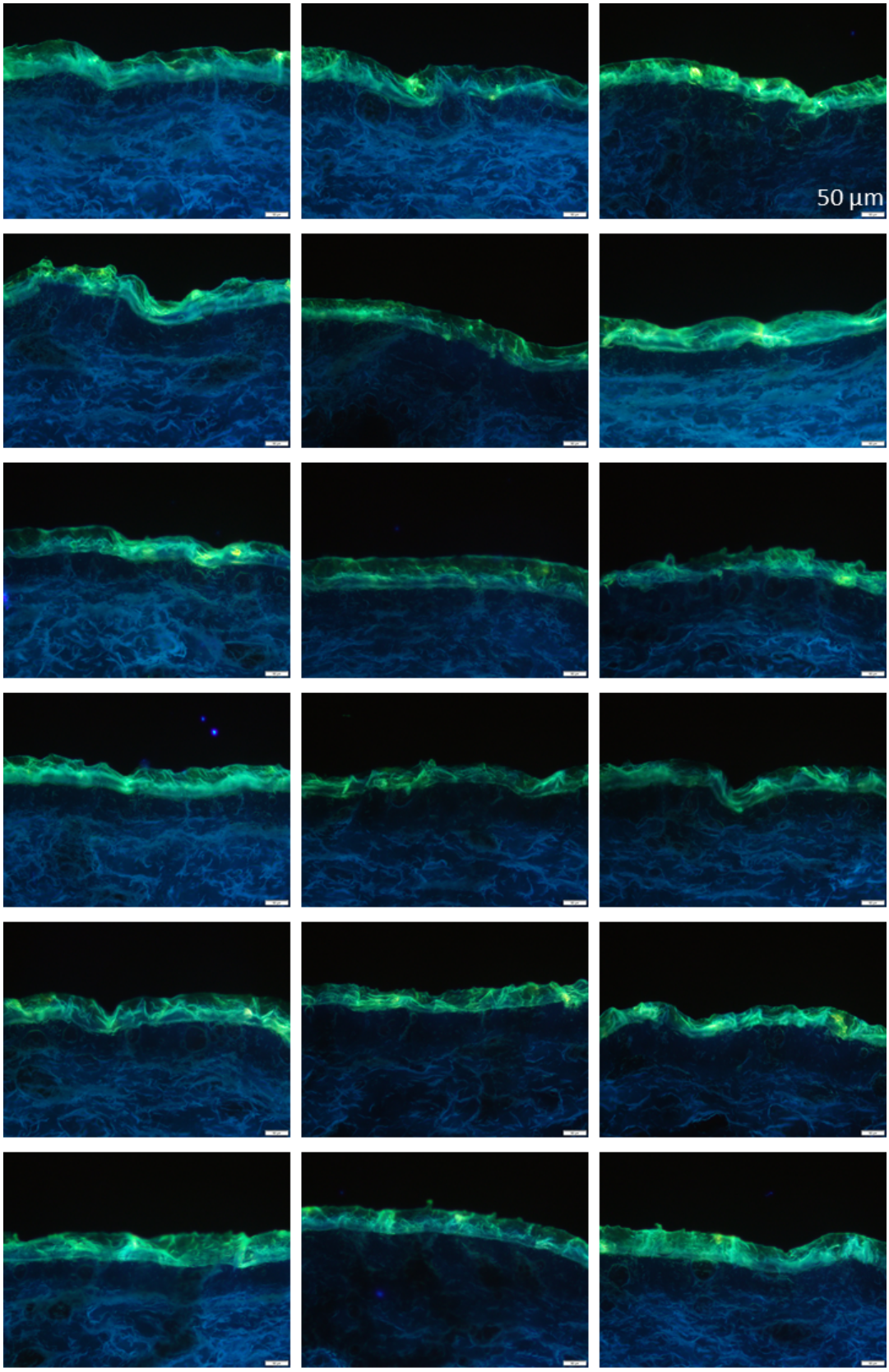
S7: Skin sections of skin treated with nanosuspension contained 5% propylene glycol. Magnification: 200fold.



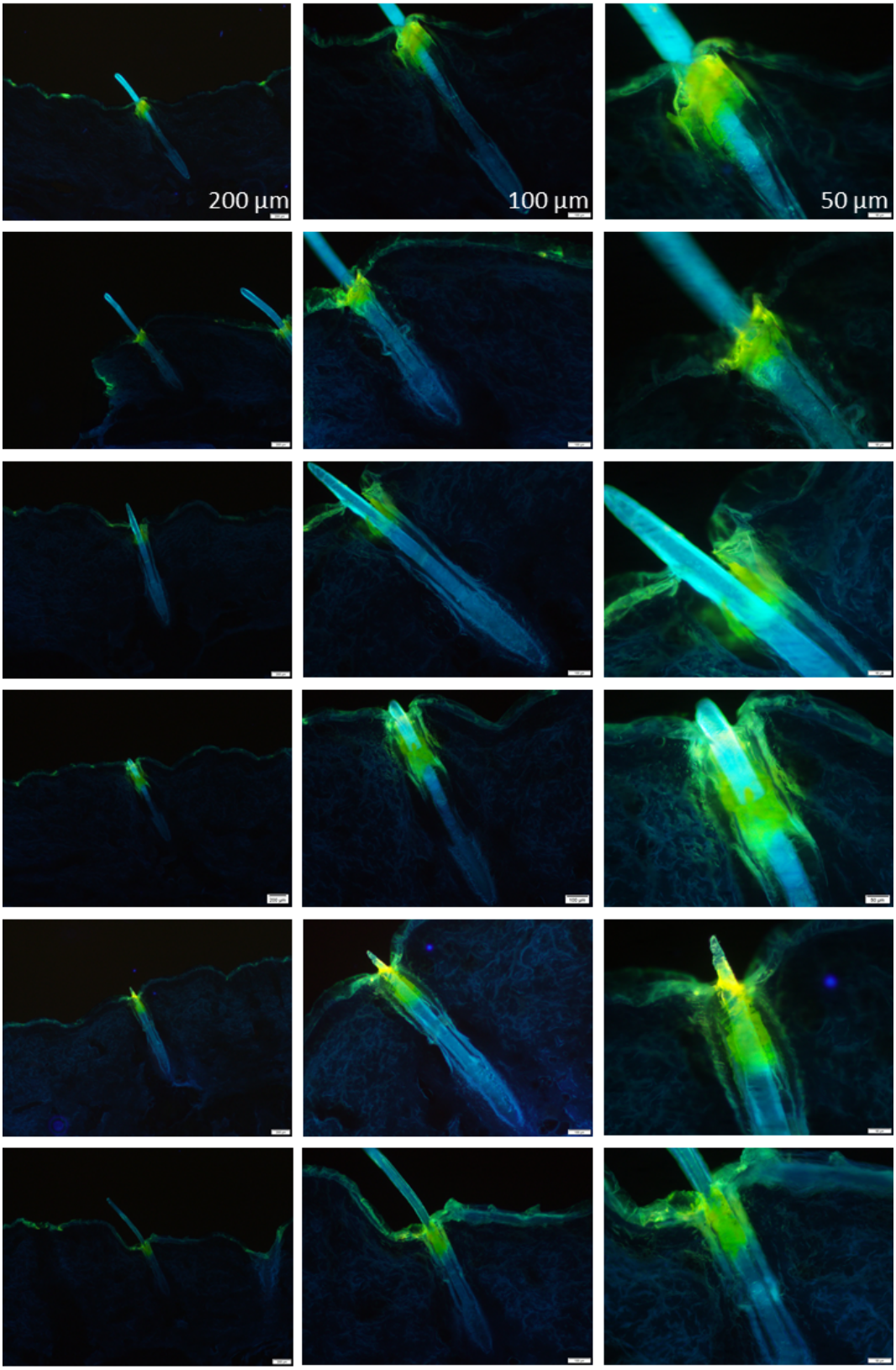
S8: Skin sections of skin treated with nanosuspension contained 2% ethanol. Magnification: 200fold.



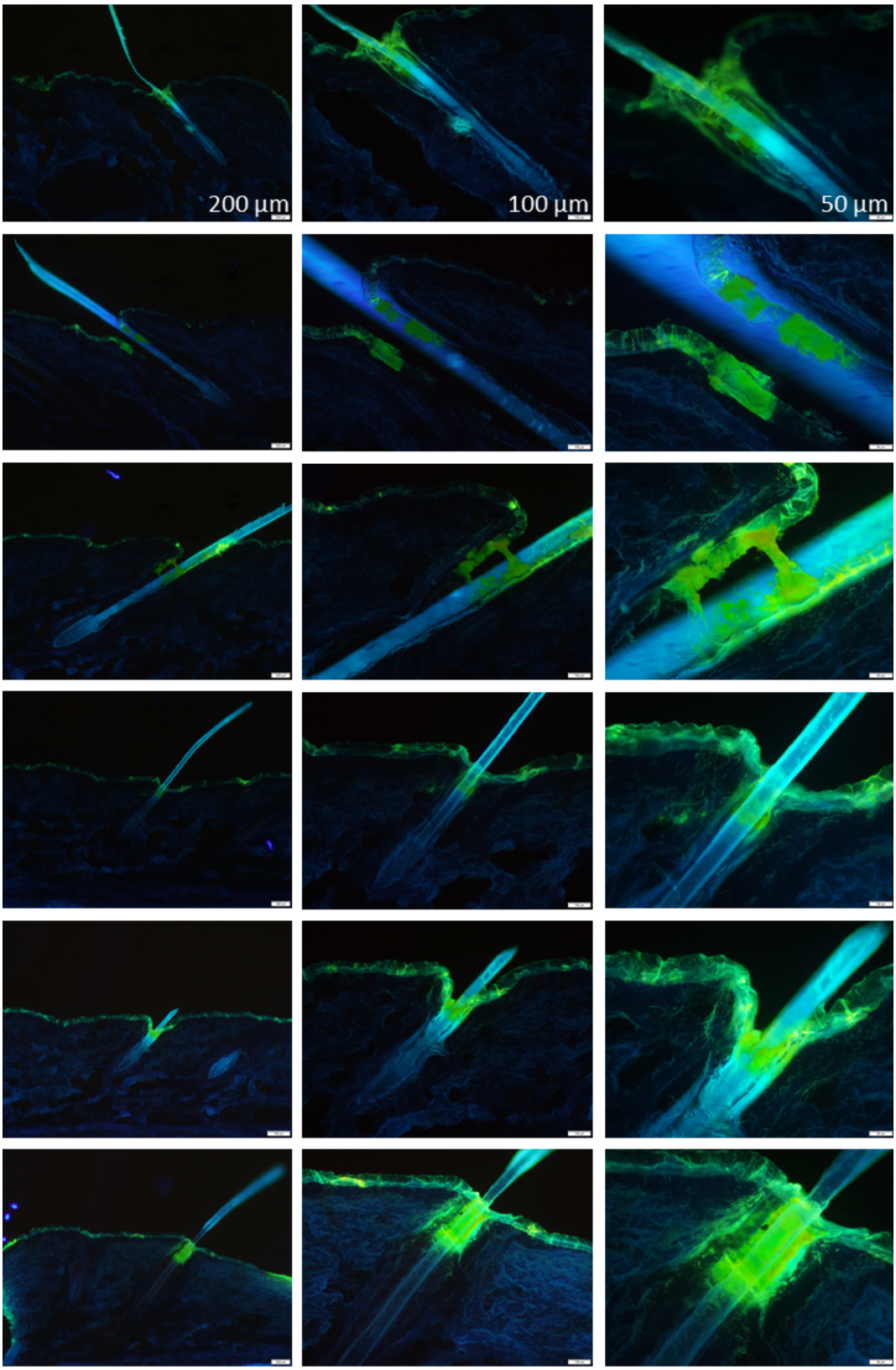
S9: Skin sections of skin treated with nanosuspension contained 2% olive oil. Magnification: 200fold.



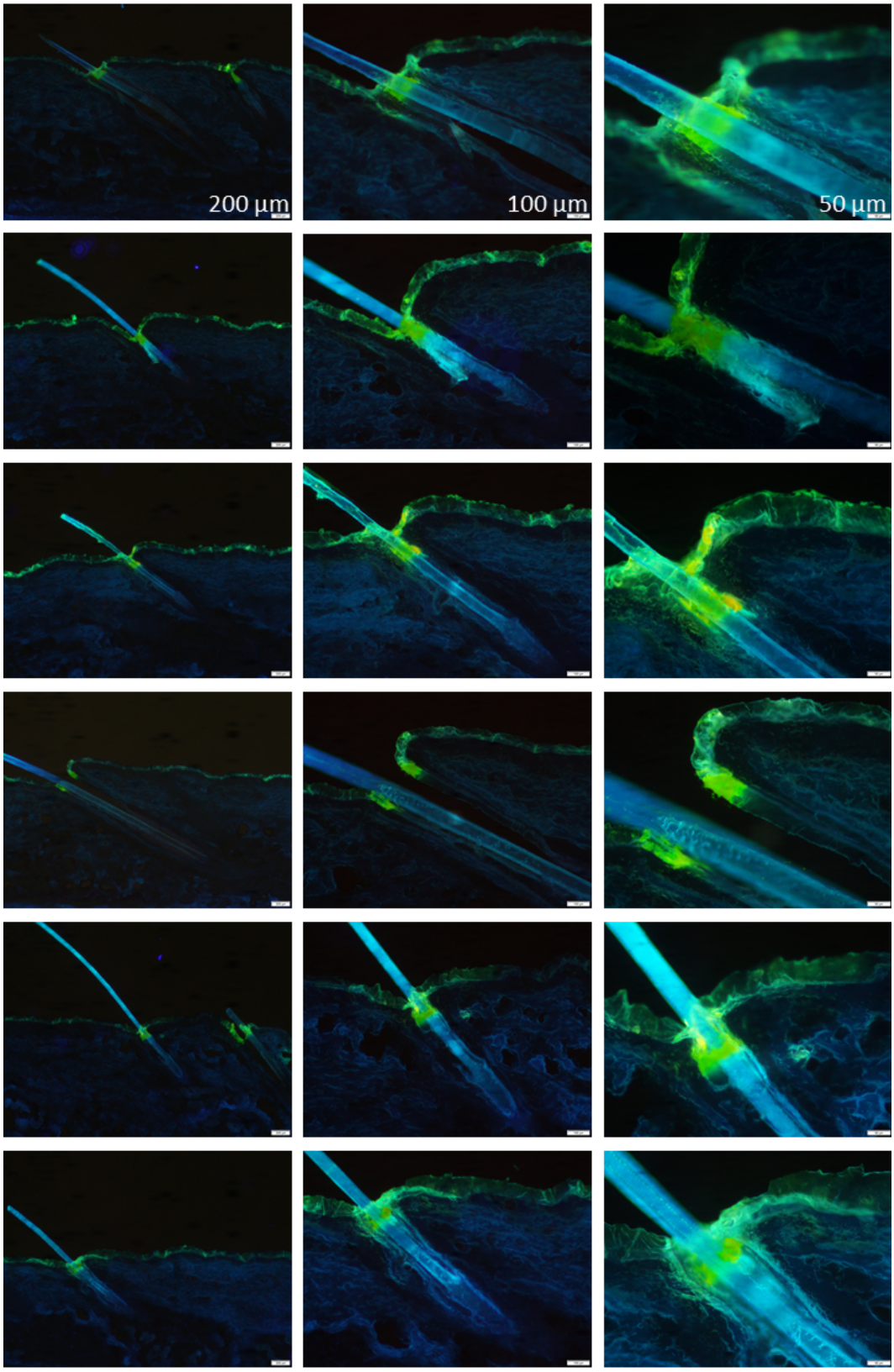
S10: Hair follicles treated with nanosuspension without additives. Left column: images taken at 40fold magnification. Middle column: images taken at 100fold magnification. Right column: images taken at 200fold magnification.



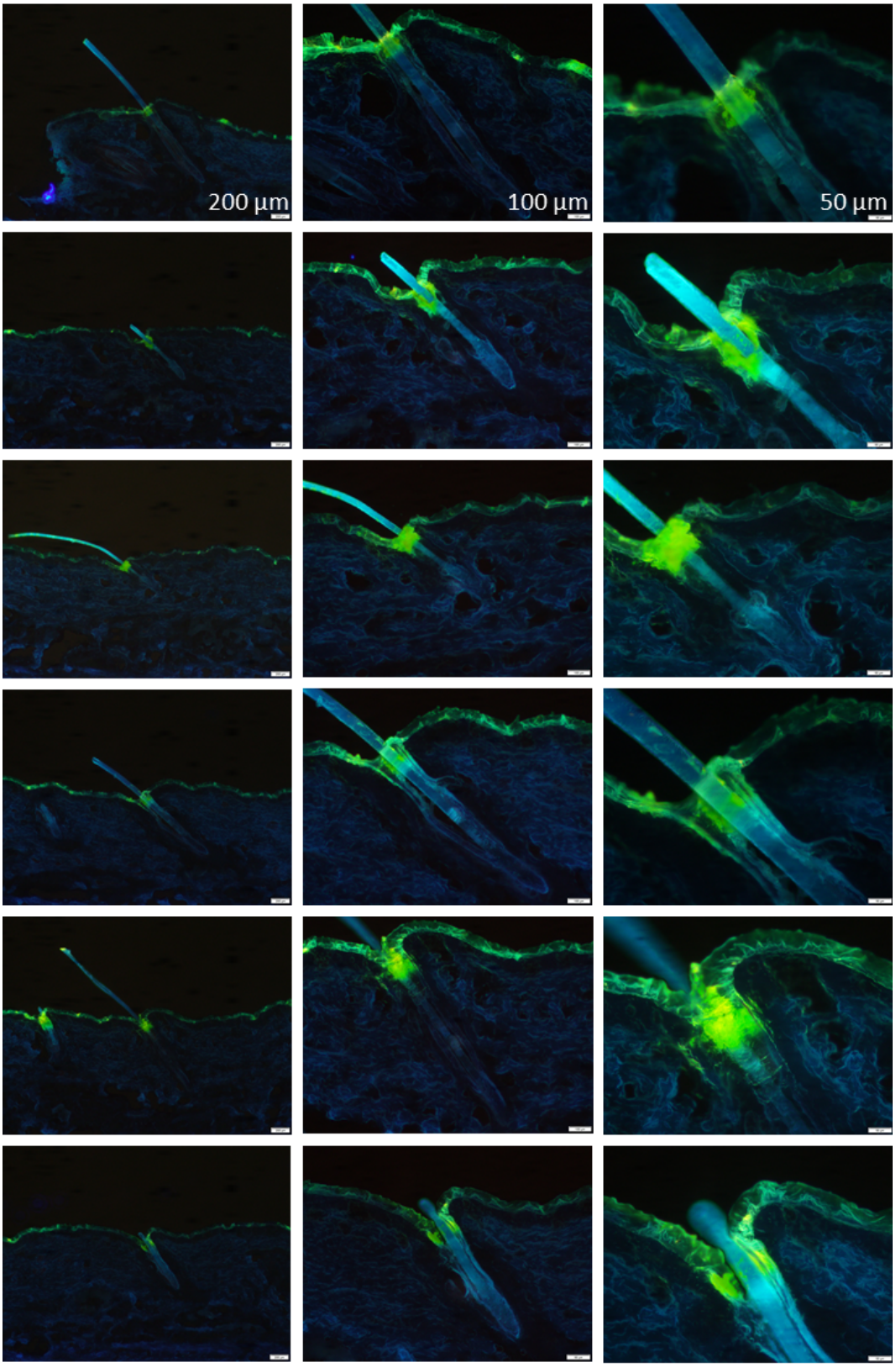
S11: Hair follicles treated with nanosuspension with 2% glycerol. Left column: images taken at 40fold magnification. Middle column: images taken at 100fold magnification. Right column: images taken at 200fold magnification.



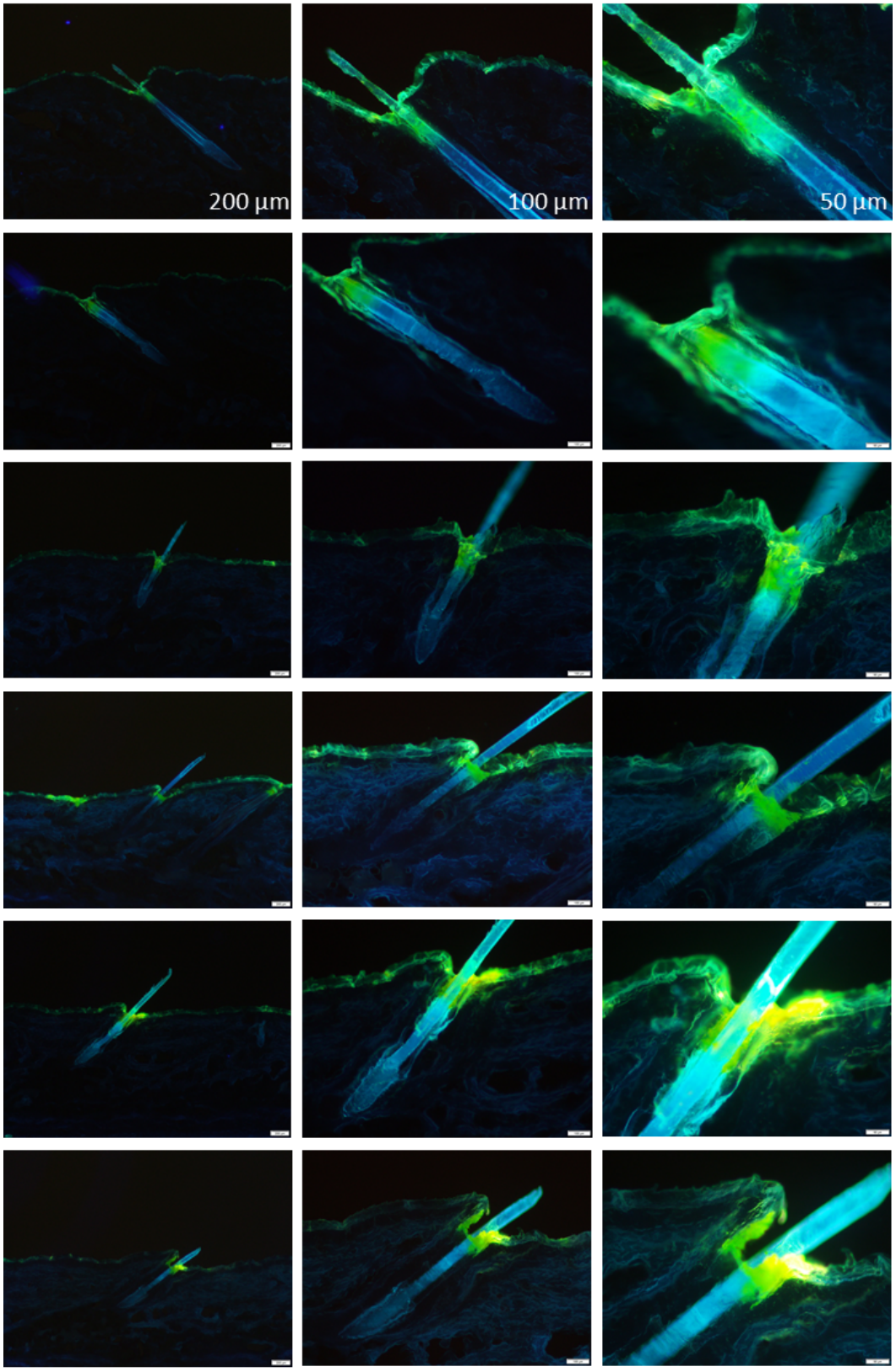
S12: Hair follicles treated with nanosuspension with 5% glycerol. Left column: images taken at 40fold magnification. Middle column: images taken at 100fold magnification. Right column: images taken at 200fold magnification.



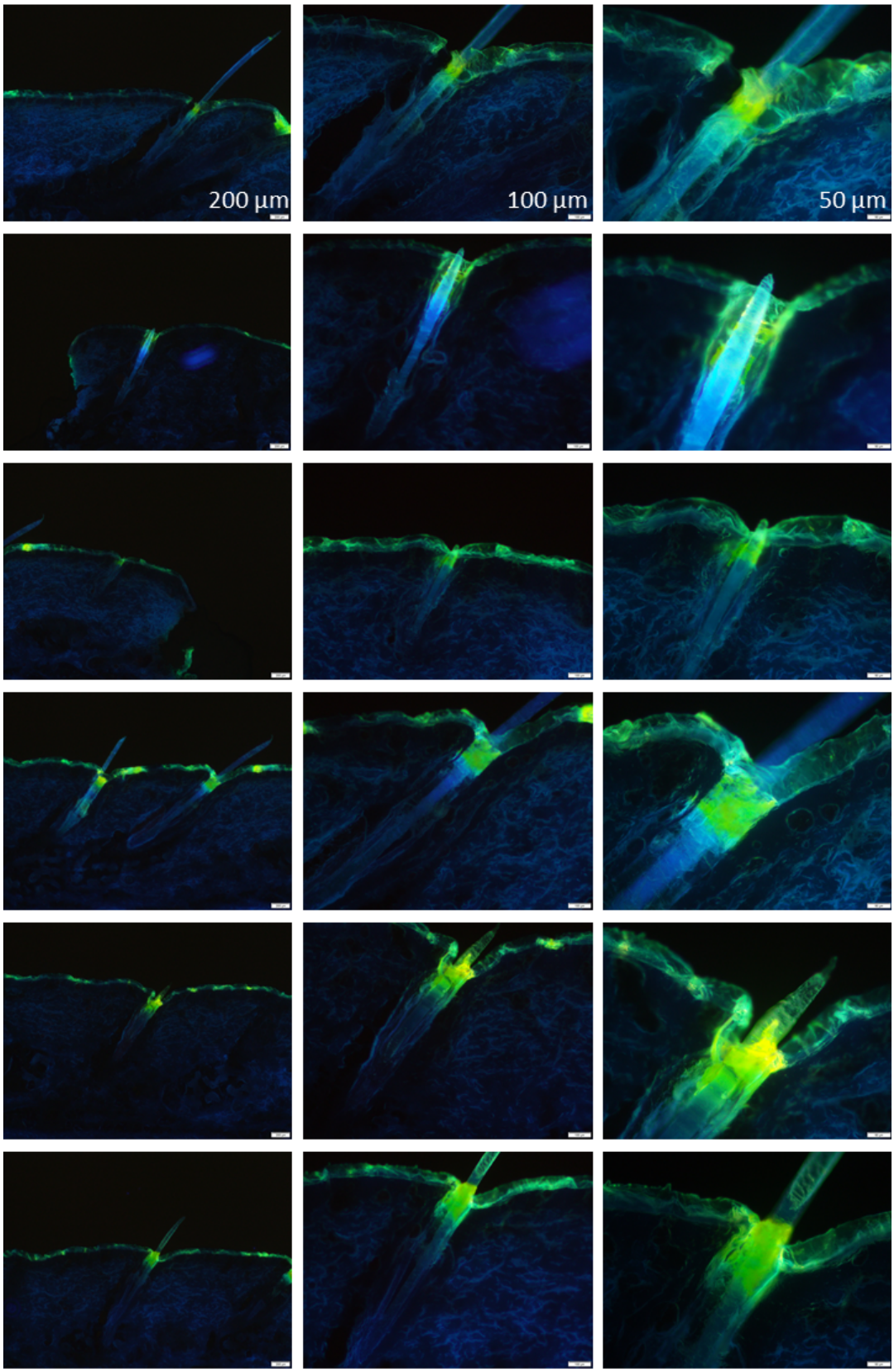
S13: Hair follicles treated with nanosuspension with 5% urea. Left column: images taken at 40fold magnification. Middle column: images taken at 100fold magnification. Right column: images taken at 200fold magnification.



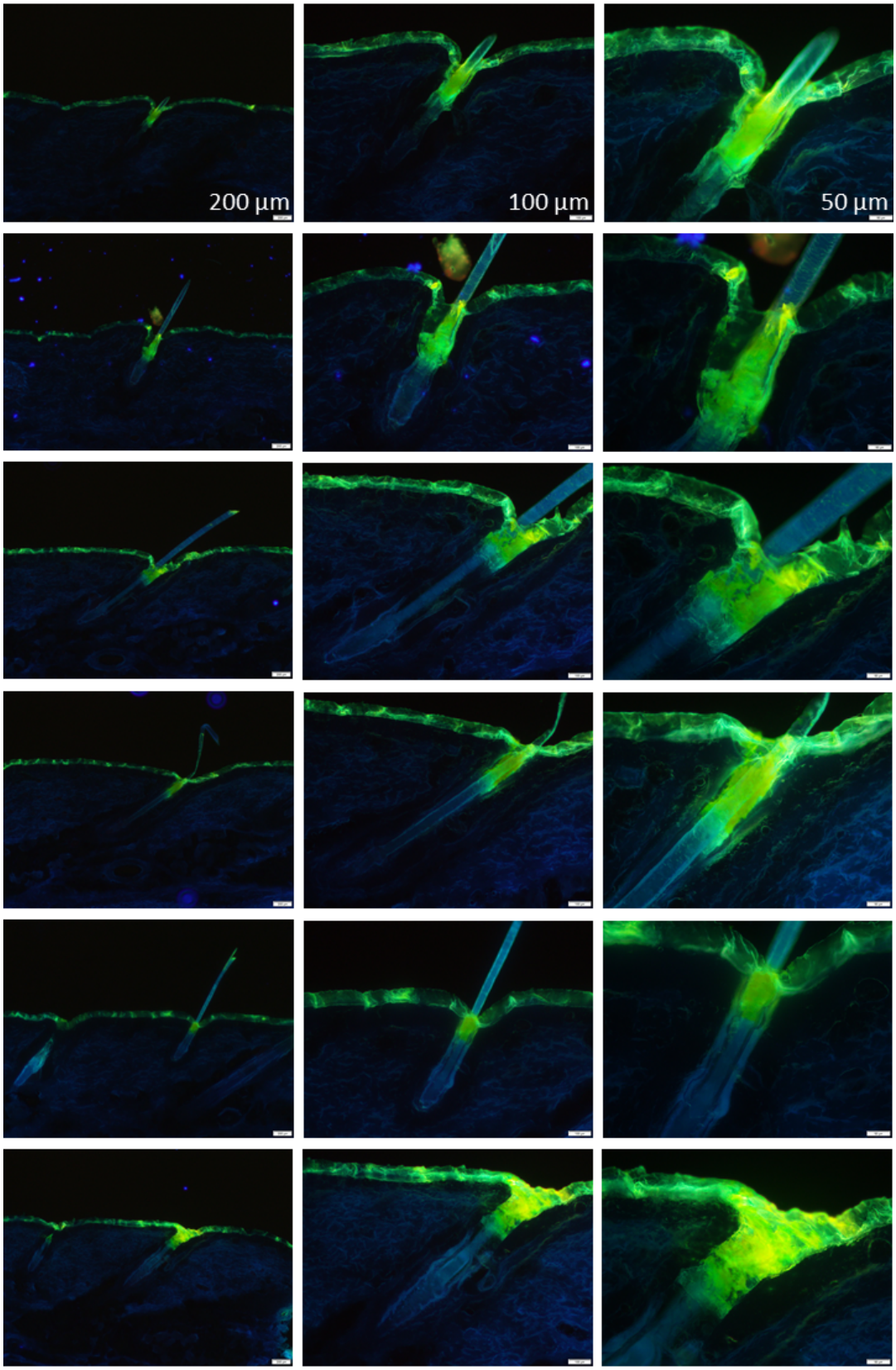
S14: Hair follicles treated with nanosuspension with 10% urea. Left column: images taken at 40fold magnification. Middle column: images taken at 100fold magnification. Right column: images taken at 200fold magnification.



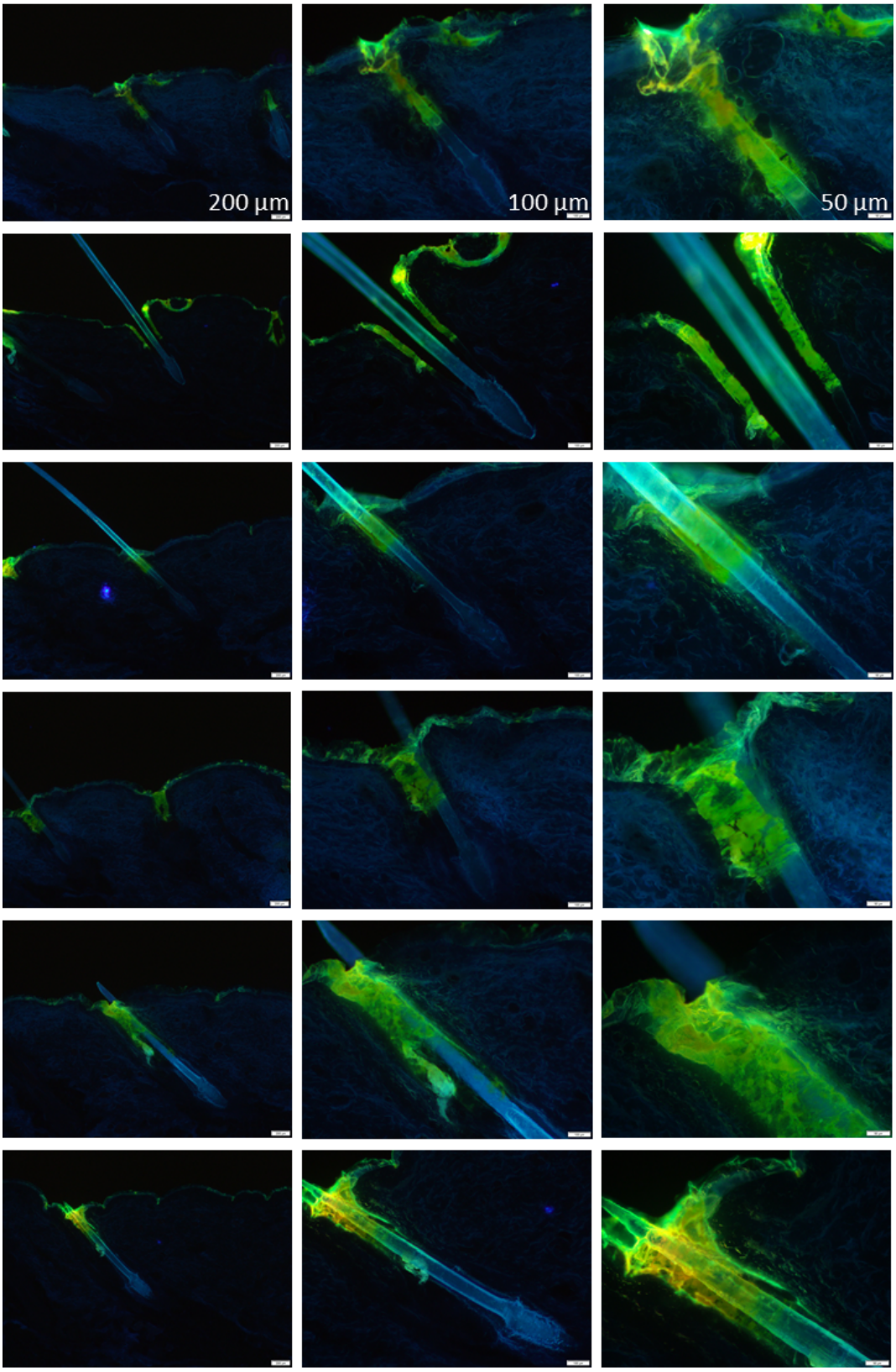
S15: Hair follicles treated with nanosuspension without additives after 14 days storage. Left column: images taken at 40fold magnification. Middle column: images taken at 100fold magnification. Right column: images taken at 200fold magnification.



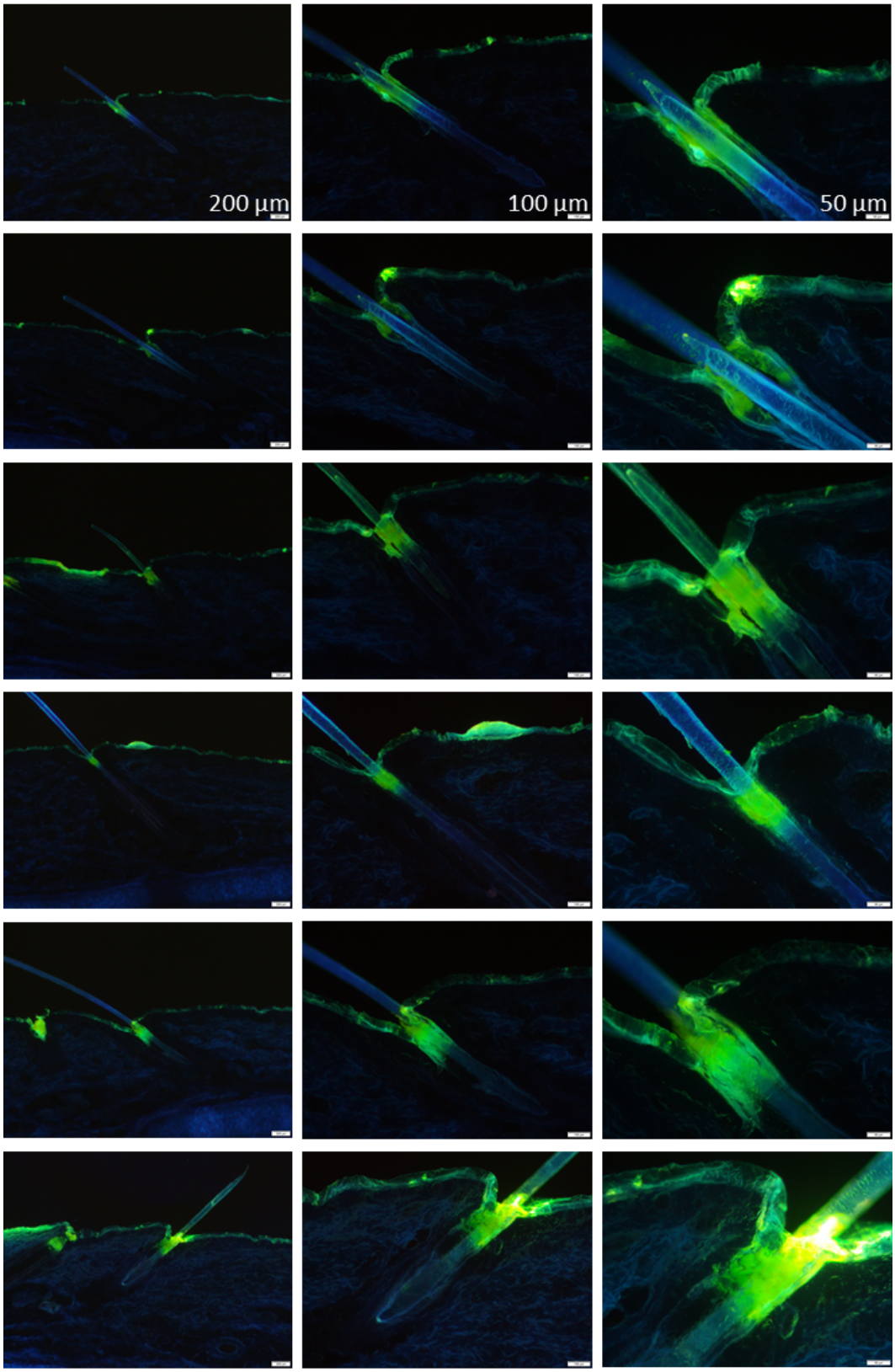
S16: Hair follicles treated with nanosuspension with 5% propylene glycol. Left column: images taken at 40fold magnification. Middle column: images taken at 100fold magnification. Right column: images taken at 200fold magnification.



S17: Hair follicles treated with nanosuspension with 2% ethanol. Left column: images taken at 40fold magnification. Middle column: images taken at 100fold magnification. Right column: images taken at 200fold magnification.



S18: Hair follicles treated with nanosuspension with 2% olive oil. Left column: images taken at 40fold magnification. Middle column: images taken at 100fold magnification. Right column: images taken at 200fold magnification.



Chapter 4

Summary and Discussion

Chapter 4

4.1. Summary and Discussion

The aims of this thesis were realized in three steps:

First step: Prove

The **first step** aimed to determine whether available universal methods are sufficient to characterize the follicular and the passive dermal penetration efficacy of NC formulations. The most substantial benefit of NC for dermal application is their proposed “dual action”, i.e., the ability of NC as particles to penetrate into the hair follicles and the ability of dissolved AC from NC to penetrate into or through the skin via passive diffusion. Thus, the central prerequisite for the method for penetration studies with NC formulations is to investigate the penetration both into the hair follicles and into the skin. Further, to allow for a clear comparability and combinability of the penetration of the NC formulations into the hair follicles and into/through the skin, the experiments should be performed on one skin model and one skin area.

Only the method of differential skin stripping fits the outlined criteria [1]. Thus, experiments of the first step were performed using the method of differential skin stripping and the porcine ear model as a skin penetration model. The term differential skin stripping describes the combination of two methods – cyanoacrylate skin surface stripping (CSSS) and classical tape stripping. Hence, differential skin stripping is carried out in two steps. In the first step, the upper part of the stratum corneum (SC) is removed by classical tape stripping to determine the passive dermal penetration [2–9]. Afterwards, the cyanoacrylate skin surface biopsies are taken to determine the penetration into the hair follicles [1]. The amount of the AC penetrated into the hair follicles and into the skin can be analyzed quantitatively using classical analytical methods.

Investigations of the hair follicle targeting were performed using the method of CSSS. This method was introduced by Teichmann et al. in 2005 and is based on the removal of the hair follicles from the treated skin area using an adhesive tape and cyanoacrylate-containing superglue [1]. For this, onto the treated and tape stripped skin area a drop of cyanoacrylate is placed and covered with a piece of an adhesive tape. After polymerization, the tape is ripped off. Consequently, the hair follicles removed from the treated skin area are fixed on this tape (fig. 1). To determine the amount of the AC penetrated into the hair follicles, the follicular content needs to be extracted with solvents and subsequently analyzed. In this thesis, HPLC analysis was performed to quantify the follicular penetration.

The achieved results are not presented in the published manuscript and in this thesis because the method of CSSS failed to investigate the follicular penetration efficacy of NC formulations. Failed means that the follicular penetration of NC could not be accurately quantified and with this, determined. The amount of the AC penetrated into the hair follicles was above the limit of detection of the HPLC but out of the lower range of the calibration curve. Thus, the NC penetrated into the hair follicles, but no proper quantification of the penetrated amount was possible. Diverse possible reasons could explain the unsuccessful quantification. These reasons could concern each step of the CSSS method, i.e., the removal of the hair follicles, subsequent extraction, detection. The experiments were performed on the dorsal ear site, i.e., the site with "less" hair. The area of the treated skin in this thesis was 2.25 cm^2. From this skin area, approximately 20-30 follicles were removed by CSSS. The volume of each hair follicle behaves in the picoliter range. Due to the small hair follicle volume and low number of removed follicles the amount of NC that is available for the detection is also very little. Thus, it might be assumed that for the detection of such a low amount analytical methods with broader analytical range and higher sensitivity are required. The next possible explanation could concern the extraction procedure. The extraction aims to bring the AC from NC from the hair follicle into the extraction solution to allow for a subsequent quantification. Thus, during the extraction the solvent should reach the NC within the hair follicle and dissolve the AC from NC. Due to the principle of the CSSS, the follicles are strongly connected to the tape by the polymerized cyanoacrylate layer (fig. 1).

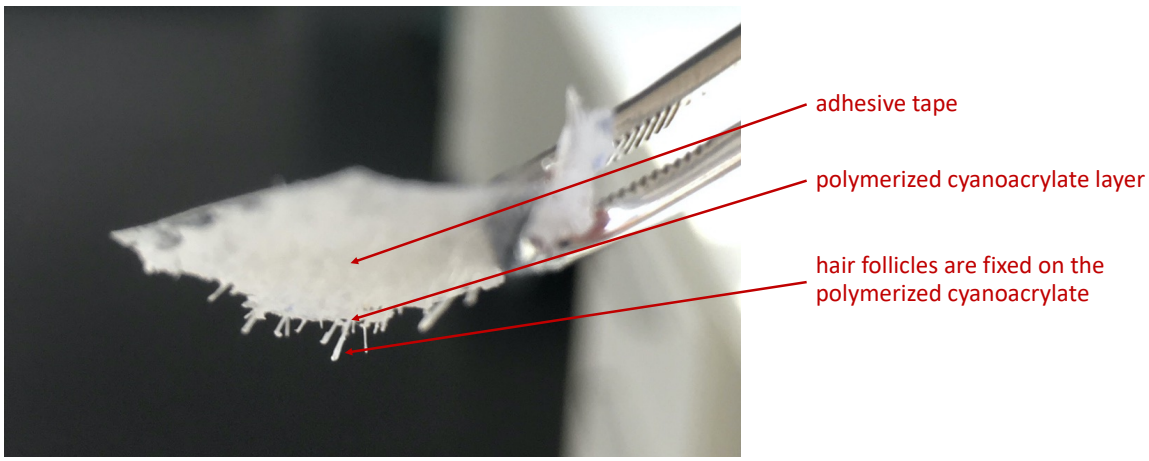


Fig. 1: Principle of the cyanoacrylate skin surface biopsy. The removed hair follicles are strongly connected to the tape by the polymerized cyanoacrylate layer.

The principle of the CSSS foresees that the cyanoacrylate remains not only on the skin surface but penetrates also to a certain extent into the infundibulum of the hair follicles [1]. Thus, the cyanoacrylate might "bind" the NC penetrated into the hair follicle. With this, to extract the follicular content, the solvent should both reach the NC within the hair follicle and extract the AC from NC fixed in the polymerized cyanoacrylate. With this, making the extraction procedure highly challenging because several possible "error sources" can be suspected. Such error sources might be, on the one

hand, that the solvent could not sufficiently reach the follicular content. For this, it needs either to separate the follicle from the tape and on that way open the follicle, or it should penetrate through the hair follicle, dissolve there the NC and the dissolved AC from NC should penetrate back from the hair follicle into the extraction solution, or the solvent needs to destroy the hair follicle and the follicular cells and release the follicular content on that way. Each outlined way can “fail”. On the other hand, the error source might be that the solvent could not sufficiently extract the NC fixed within the polymerized cyanoacrylate. In the original manuscript, which presented the method for the first time, the experiments were performed with a dissolved fluorescent dye (sodium fluorescein) [1]. For the extraction performed during the experiments of this thesis, the same solvent (ethanol) but a longer extraction time were chosen when compared to the original manuscript. Based on the outcome of this thesis, it might be assumed that for NC an utterly different extraction procedure needs to be established. All outlined reasons allow to state, that the method of CSSS is not suitable for the characterization of the follicular penetration of NC formulations. With this, since the prime focus of this thesis is set on the determination of the hair follicle targeting with NC formulations, the aims of this thesis cannot be realized using the method of CSSS.

The passive dermal penetration of NC formulations was determined via classical tape stripping. This technique was firstly introduced in 1939, when Wolf et al. removed successive layers of the SC by repeated stripping with adhesive cellophane tapes [10]. On that way morphologic details of human SC cells were studied [10]. In the following years, tape stripping was established as a method for the determination of the penetration efficacy of dermal formulations [2,3,11–16]. Due to its simplicity and fastness, it is often used by many researchers worldwide for many different kinds of dermal formulations. Also, several previous penetration studies with NC formulations were performed using tape stripping [17–20]. The principle of tape stripping is based on the removal of the SC layer by layer from the treated skin area using adhesive films. The removed tapes contain corneocytes and the penetrated formulation. Previous studies showed that approximately 60-100 tapes are needed to remove the SC completely [21]. However, it is not necessary because it is believed that when an AC penetrates the SC, then it penetrates also into viable skin layers [22]. Therefore, in practice, approximately 20-30 tapes are removed from the treated skin area. In theory, with classical tape stripping, the penetration of a dermal formulation can be characterized by two parameters. First, the total amount of penetrated AC into the removed SC (by summarizing the amounts of penetrated AC on all removed tapes), and second, the penetration depth of the AC into the SC (by plotting the amount of penetrated AC as a penetration profile, i.e., the amount of the penetrated AC in correspondence to the associated tape number/SC thickness). Thus, in theory, classical tape stripping represents a sufficient method to characterize the penetration efficacy of dermal formulations.

In practice – in this thesis – investigations of the passive dermal penetration of NC formulations performed by classical tape stripping – generally – worked out. The procedure of tape stripping and the performance of the penetration studies were feasible, the amount of the penetrated AC was determined, and the penetration profiles were displayed. Based on these data, the influence of theoretically relevant formulation aspects – NC size, excipients, vehicles – on the passive dermal penetration efficacy of dermal NC formulations was analyzed.

Investigations of the influence of NC size on the passive dermal penetration confirmed the existing theories. Results of tape stripping showed that – as initially expected – the decrease in size led to a slight increase in the amount of the AC that penetrated from NC into the skin. Therefore, the theory-driven approach indicating that the penetration efficacy increases with the smaller size of NC was proved. However, the change of NC size improved the penetration only to a limited extent. The differences between the total amounts of the AC penetrated into the skin from differently sized NC were not significant. Furthermore, the evaluation of the penetration profiles showed that the effect of the size was already cancelled out after the 10^{th} tape, which corresponds to less than half of the removed thickness of the SC. Thus, the size did not take a significant influence also on the penetration depth of the AC from NC. Therefore, it was concluded that even though the trend was detected that small-sized NC slightly improved the penetration efficacy, the effect of size does not play a crucial role for the passive dermal penetration of the AC from NC. Since NC of each investigated size delivered the AC successfully into the skin, it can be stated that no specific size of NC is required to achieve effective dermal drug delivery.

In contrast, excipients and vehicles showed a strong influence on the penetration of the AC from dermally applied NC. Studies with different excipients showed that although all investigated excipients are well-known penetration enhancers, the penetration of the AC from NC was both improved or even impaired depending on the obtained excipient. The excipients modulated the penetration based on their physicochemical properties. Lipophilic excipients increased the amount of the AC penetrated from NC into the skin compared to NC without excipients. In contrast, hydrophilic excipients did not increase the penetration of the AC from NC but even hindered it. It means that lower amount of the AC penetrated into the skin from NC using hydrophilic excipients compared to blank NC. Based on the obtained results it is essential to consider three important points regarding dermally applied NC: i) the physicochemical properties of the AC (in this step, lipophilic hesperetin was used as model AC), ii) its possible penetration pathway into/through the SC (hydrophilic pathway through the corneodesmosomes/lipophilic pathway through the lipid surroundings of the corneocytes), and iii) the mechanism of action of the excipients (improvement of the hydrophilic or lipophilic route). In this way, the right match of the AC and the excipient can be found, and effective dermal penetration is achieved.

In this thesis, to increase the penetration of lipophilic hesperetin from NC, olive oil was identified as a suitable penetration enhancer.

Also, the experiments with vehicles demonstrated differences in the amounts of penetrated AC from NC. Results proved that the textbook knowledge indicating that the vehicle influences the penetration efficacy of the AC from dermal formulations is also valid for dermal NC. Interestingly, the most "popular" vehicle for NC in the past due to the old galenic principle of the suspension ointment ("topical suspensions") – the hydrogel – turned out to be unsuitable for NC because no penetration of the AC into the skin was detected. The oleogel showed moderate penetration of rutin from NC, while the best penetration was achieved from a cream. Different theories were discussed to explain achieved results. One of the most plausible theories was the theory of the contact area of NC and the skin (that was previously suggested and discussed by Vidlářová et al.) [18]. This theory postulates that for passive dermal penetration, the contact area of NC and the skin plays a substantial role because every single NC is covered by a so-called diffusional corona of dissolved AC. Thus, the more NC are in direct contact with the skin, the more dissolved AC is in direct contact with the skin, and the more effective is the passive diffusion. Transferring this theory to the current study, it might be assumed that within the cream – because NC were distributed primarily within the water phase and the cream consists of 50% lipophilic and 50% hydrophilic phase – the number of NC/mL in the water phase is about two times higher compared to the hydrogel and the oleogel. Thus, it might contribute to the improved penetration of the AC from NC incorporated in the cream formulation.

In conclusion, classical tape stripping generally worked out, and the passive dermal penetration of investigated formulations was characterized. However, it was very difficult to work with the outcome of the penetration studies and to explain achieved results because all information was only related to the penetration of the AC into the upper layers of the SC. Thus, it cannot be concluded that penetration studies were successful – they worked out, but not more because the information behind was less. If one questions the outcome of the penetration studies critically, the only fact that was determined with tape stripping is that the investigated formulation aspects modulated the amount of the AC penetrated from NC formulations into the upper layers of the SC. It could not be determined whether the formulation aspects influenced only the penetration into the upper layers of the SC or into the entire SC because the amount of the penetrated AC could not be accurately quantified already after the 30th tape. It does not mean that the AC did not penetrate into deeper SC layers, it just means that the AC could not be detected there by available methods. Consequently, it could not be determined whether the formulation aspects influence only the penetration into the SC or also through the SC into the viable skin layers. Thus, indicating the most substantial limitation of the tape stripping. This method captures only the upper part of the SC and blends out the real targets of the NC formulations – and also other dermal formulations – namely the entire SC and the viable skin layers. Thus, making such

penetration studies meaningless. To sum up, after the tape stripping experiments, we still don't know whether the AC from NC formulations penetrates only into the upper layers of the SC, into the entire SC or also into the viable skin layers, to what extent and how the formulation components influence the penetration behaviour.

Another parameter that was not determined via tape stripping – despite initial expectations – was the penetration depth. It was initially expected that the penetration depth of the AC can be determined by plotting the data as a penetration profile. In practice, this approach did not work out properly. Thus, a meaningful comparison between the penetration depths of different formulations could not be performed. The first reason for this is the fact that the effect of each investigated formulation aspect was cancelled out already after the 10^{th} tape. The 10^{th} tape represents less than the half of the removed SC. Considering the information that for the removal of the whole SC approximately 60-100 tapes are needed, the penetration profile allowed only for a comparison of the penetration depths of the NC formulations into the upper layers of the SC. The second reason is that with tape stripping, the amount of the penetrated AC corresponds to the number of the tape and not to the nominal thickness of the SC in µm. Thus, only a "generic" penetration depth can be estimated. Some research groups calculated the total thickness of the SC in µm by completely removing the SC and estimating the total absorption of the removed SC by IR densitometry or UV/VIS [21,23–26]. For this, the researchers removed the SC layer by layer with adhesive tapes, measured the absorption of the SC proteins on each removed tape via IR densitometry or UV/VIS, detected the point where the measured absorption reached the zero point (i.e., when no SC proteins could be measured anymore), calculated the total absorption of the removed SC and corresponded it to the theoretical thickness of the SC in µm. This approach was also addressed in our research group using IR densitometry [27]. However, this approach was found to be insufficient [27]. It was found that the device doesn't measure specifically SC proteins but also compounds that absorb light in the wavelength of the device (850 nm) [27]. Since the viable skin layers also contain compounds that show measurable light absorption at 850 nm, with IR densitometry, no clear endpoint indicating that the SC was removed completely could be determined [27]. Thus, no accurate calculation of the SC thickness via IR densitometry was possible. Therefore, in this thesis, the amount of penetrated AC corresponded to the tape number. However, if one questions this approach critically, one can find several "leaks". For example, one cannot ensure that the tapes removed from the skin treated with different formulations contain a comparable amount of corneocytes. Previous studies postulated that the properties of the investigated formulations influence the amount of SC removed with each tape strip [28–31]. It was shown, that different number of corneocytes sticks on each tape when the skin is treated by the very oily formulation compared to a formulation that is not oily [28]. With this, the same tape numbers correspond to different nominal thicknesses within the SC, and the data about different formulations are not directly comparable [28]. The same problem may

occur by the total number of removed tapes. In practice, researchers working with tape stripping remove 20-30 tapes from the treated skin area. However, no one can ensure that with 20-30 tapes, the same amount of SC is removed from the areas treated with different formulations. If not, the researchers compare incomparable data – and it can, in the case of tape stripping, unfortunately not be avoided because this method is very susceptible to “little” particularities that influence the outcome of penetration studies tremendously. Also, the handling of the tape stripping is very susceptible. Several previously postulated studies defined critical parameters that must be kept constant during the experiments because they take a considerable influence on the outcome of the penetration studies. Such parameters are, for example, application pressure, removal velocity, type of the adhesive tape, the extraction procedure etc. [28,32]. Further, the importance of the constant working protocol was also already reported [5,31]. In the experiments of this thesis, all mentioned parameters were kept constant. However, it was observed that even though the handling was constant, there will always things exist that cannot be kept constant. For example, the porcine ears skin – it was observed, that the tapes stick and behave differently on different skin. The number of hairs was also observed to influence the stickiness of the tapes. Since the CSSS was performed after classical tape stripping to investigate the efficacy of the hair follicle targeting, the skin areas were chosen that contained a sufficient number of hairs. On the other hand, it was observed that the hairs influence the stickiness of the tapes, and fewer amounts of corneocytes were removed when the treated area contained more hair. All outlined particularities indicate that tape stripping is highly susceptible to artefacts or misinterpretation of results, which is a clear “no-go” for the method for dermal penetration experiments.

Furthermore, not only the outcome and the handling but also the performance of tape stripping possesses limitations. It is often said that tape stripping is a fast method, but in practice, it is not the case. As such, the stripping procedure takes approximately 1 h to strip one skin area (one formulation, 30 tapes). In total, to perform all experiments in triplicate for each formulation approximately 3 h are required. Subsequently, the tapes content should be extracted. In dependence of used protocol, it takes further 2 h. Then, the samples for HPLC should be prepared (2 h), and the HPLC analysis should be performed (12 h). In total, approximately 24 h are needed for one sample. Moreover, the analytical equipment needs for quantification of the AC amounts on the tapes – in our case HPLC – is expensive, requires experienced user and validated methods. Thus, indicating that tape stripping is not as simple and fast as it might be assumed.

All outlined limitations lead to a clear conclusion that classical tape stripping is not suitable to characterize the extent of the passive dermal penetration of NC formulations. Viewed abstractly, the tape stripping only provides information on whether the AC "rang" into the skin and whether it opened “the door” into the skin. It cannot be determined which pathway the AC went after this. Tape stripping

could be useful when, for example, the reservoir capacity of the SC needs to be investigated. Then, the penetration of the AC into the SC is precisely the correct and suitable parameter to describe it. But this aim represents a special case. All other penetration studies aiming to investigate the penetration fate of a dermal formulation into its real targets must use other methods. Further, the outcome of tape stripping provides only the amount of the AC penetrated into the upper layers of the SC and the generic penetration depth. It was in this step only enough to identify that investigated formulation aspects influence the penetration into the SC, but not enough to investigate these influences. The difference between "identify" and "investigate" is that to identify something, no mechanistic understanding of the process is needed. Thus, achieved outcome is enough. But to investigate something, in our case the influences of the formulation aspects on the penetration efficacy, a detailed, sound and mechanistic understanding of the penetration process is essential. Precisely this mechanistic understanding of the penetration process of dermal formulations was not gained using tape stripping. However, it is essential for the aims of this thesis. Only if one has the mechanistic understanding of how the formulation aspects influence the penetration process of NC formulations and why, one can tailored optimize these aspects to make the penetration even more effective. To sum up, for sufficient characterization of passive dermal penetration of NC formulations – and also other dermal formulations – the method must be improved, or another method should be developed.

It is astonishing that most researchers who use this method daily tend just to overlook the outlined limitations. Based on the analysis of the research published in the field of dermal formulations, tape stripping still belongs to the often-used methods for penetration studies. However, a trend can be followed that more and more research groups tend to step back from tape stripping and tend to use alternative methods for the characterization of dermal penetration.

To sum up, with differential skin stripping, no sufficient characterization of dermal NC formulations was possible. The method of CSSS failed to determine the follicular penetration because no accurate quantification of the amount of the NC penetrated into the hair follicles was achieved. Classical tape stripping failed to determine the passive dermal penetration properly because i) only the penetration into the upper layers of the SC was determined and the penetration into the real targets of the dermal formulations – the whole SC and the viable skin layers – cannot be determined ii) no mechanistic and detailed understanding of the penetration process can be achieved iii) the handling and the performance represent tedious work for too less outcome. Due to the outlined reasons, the differential skin stripping is not suitable as a stand-alone method for characterization of dermal NC formulations. To perform sound penetration studies with NC formulations, a new method needs to be established.

Second step: Optimize

In the **second step**, a new method was established. First, the method was thoroughly conceptualized. The advantages of differential skin stripping should be kept, whereas disadvantages should be overcome with optimized solutions. The most substantial advantage of differential skin stripping is its ability to determine the penetration into the hair follicles and into the skin by using one skin model and one skin area. Since it is the most convenient way to determine the follicular and the dermal uptake of one formulation, the new method should keep this advantage. The most significant disadvantage of differential skin stripping regarding follicular penetration was the challenging quantification of the penetrated AC. Thus, the new method should provide more convenient determination and quantification of the follicular penetration. The major disadvantage of the differential skin stripping regarding passive dermal penetration was the determination of the penetration only into the upper layers of SC. Thus, the new method should determine the entire penetration fate of the AC from the dermal formulation, i.e., the penetration into the whole SC and into the viable skin layers, and provide a detailed time and space resolved characterization of the fate of the dermal formulation. Further, the new method should provide reliable discrimination between the follicular and dermal uptake. The quantification of both penetration pathways should be sufficient, reliable and sound. Also, the new method should be fast and "handy". It means that the sample preparation, experiment conduction and data analysis should be fast and should not require any high-priced analytical equipment. The performance of the new method should be simple and implementable in the daily lab work.

The conceptualized method requires a suitable skin model. The main prerequisite for such a skin model is to mimic as good as possible physiological skin conditions. Mainly the skin tension must be maintained during the entire experiment. Especially for the determination of follicular penetration, the skin tension is crucial. With this, any contraction of the skin and any manipulation of the skin prior to the experiments must be avoided. The arrangement of the skin model should be feasible, and the handling of the skin should be simple. Thus, complex preparation steps prior to the experiments should be avoided.

At present, a bright spectrum of skin models with different properties is available. In general, penetration studies can be performed using the in-vivo or the ex-vivo/in-vitro skin penetration models [33]. In-vivo skin penetration models include humans or animals. Human in-vivo experiments require considerable effort regarding ethical, practical and financial reasons. With this, they are unsuitable for the formulation development and optimization phase. Based on the ethical 3R principle (Replace, Reduce, Refine), in-vivo experiments on animals should be kept to a necessary minimum. Therefore, they also don't represent the first choice for early phase penetration studies. An alternative is offered

by biological ex-vivo/in-vitro skin models for the investigation of dermal penetration [34–36]. In-vitro skin models – e.g., various cell culture models – represents surrogates of human skin. These models are very elaborative to prepare and to handle and are far away from the real "natural" skin. Ex-vivo skin is represented either by excised human skin, which arises as a "by-product" during human surgeries and can be used for scientific purposes, or animal skin. The availability of human skin can often be problematic and can be associated with many complications, for example, availability, deliverability, storage, shelf life, integrity, reproducibility. Therefore, ex-vivo skin from slaughtered animals offers an optimal solution. The most used skin from slaughtered animals is porcine ear skin. The similarities of the properties of porcine ear skin to the properties of human skin have already been described in several studies [37–43]. Porcine ear skin has a comparable structure and a comparable arrangement of corneocytes and lipids when compared to human skin [40]. Thus, porcine ear skin is often used as a surrogate for human skin. The skin of porcine ears can either be used for the ex-vivo/in-vitro test models – for example, Franz diffusion cells – or the experiments can be performed using the so-called porcine ear model [6,44–46]. The porcine ear model, which was used in this work, offers a number of advantages when compared to other conventional skin models and test systems. The porcine ear model is based on the fact that the experiments are conducted on the intact porcine ear. It means the skin does not need any additional complex preparation steps – the ear just needs to be washed and dabbed dry with soft tissues. With this, the porcine ear model represents the most "natural" and "real" skin model from all existing. With the porcine ear model, the skin is also not manipulated prior to testing, i.e., not stretched or squeezed. On the contrary, for example, for Franz diffusion cells, the skin needs a complex preparation including the cutting step with a dermatome to a defined thickness. When the skin was stretched, or the hole was created during the preparation, another piece of skin must be used. When no more skin is available, the experiment needs to be re-scheduled. Using the porcine ear model, outlined problems cannot occur by definition. Since the whole intact ear is used for penetration experiments, the skin stays fixed on the cartilage during the entire experiment. Thus, allowing for the maintenance of the natural skin tension during the whole experiment and mimicking physiological skin conditions. The maintenance of the tension is important for both skin and hair follicle penetration, but for a skin model for sufficient determination of the hair follicle targeting, it is a must. That is one of the reasons why excised skin cannot be appropriately used for the determination of the hair follicle targeting. Previous studies investigating the follicular reservoir within excised human skin versus in-vivo human skin showed that within the same human donor, the in-vivo follicular reservoir was 90% larger than in excised skin [47]. Thus, indicating a strong contraction of the excided skin. Comparable studies were not conducted for the porcine ear skin, but it might be assumed that follicular reservoir would also be affected when the skin is removed from cartilage. Furthermore, the porcine ears are comparatively simple and quickly to arrange. Thus, the porcine ear

model was selected for the penetration studies in this thesis and for the establishment of the new method.

Before establishing a completely new method, first, an extensive evaluation of the advantages and limitations of the available methods was performed:

Considering follicular penetration, there are only a few methods available that are established for the determination of follicular penetration efficacy. It might represent the reason why hair follicles were long time not believed to be an important penetration pathway – because for a long time, no sufficient method existed to determine especially the follicular penetration. Things changed in 1999 when Lademann et al. investigated the penetration of coated titanium dioxide (TiO_2) microparticles into the SC of living human skin by tape stripping in combination with spectroscopic measurements [48]. The observation that small white spots of the sunscreen material could be recognized visually in several orifices of the follicles after removing the tape strips from the treated skin surface was the first hint that follicles must be considered when analyzing the penetration of TiO_2. The determination of the follicular penetration was conducted firstly by visual inspection of the hair follicle orifices and secondly by staining the tape strips with osmium tetroxide (OsO_4). Since then – over the past 20 years – several further methods were developed. The method of cyanoacrylate skin surface stripping (CSSS) was presented by Teichmann et al. in 2005 and was already discussed in the first step of this thesis [1]. It is based on the removal of the hair follicles with adhesive tape and superglue. The follicular depot can be analyzed either qualitatively by simple visual inspection or by fluorescence microscopy if the penetrated AC is fluorescent, or quantitatively by extracting the follicular content with solvents. With this method, one of the important milestones of the follicular research was reached. Namely, the finding was done indicating that hair follicles represent a long-term reservoir where the particles can be stored as a depot for up to 10 days [49]. The next developed method by Teichmann et al. in 2006 was the method of selective follicular closure [50]. This method enables the determination of the extent of the transfollicular penetration. For this method, the hair follicle orifices are plugged by placing dots of the closure material – for example, nail varnish – on top of each follicular opening. The investigated formulation is applied onto the skin area with blocked follicles and with opened follicles as a control. Comparing the penetration into these two treated areas, the extent of the transfollicular penetration can be determined. This method allowed to reach the next significant milestone of the follicular research. Using selective follicular closure, it was shown that percutaneous absorption of caffeine and minoxidil is significantly lower and more delayed on the skin area with closed hair follicles compared to the skin area with open hair follicles [51,52]. Thus, providing fundamental evidence for the importance of the follicular penetration pathway. In 2006/2007, the next method was introduced – the cross-sections of the hair follicles with subsequent analysis of the follicles using fluorescence microscopy [53]. The prerequisite for using this method is that the AC is fluorescent, or special

fluorescent dyes should be used. The penetration depth can be determined quantitatively in µm directly from the fluorescence images. With this method, the finding was done that AC in particulate form penetrated deeper into the hair follicles than dissolved AC [53]. In recent years, also microdialysis – and the combination of the follicular closure method and microdialysis – was established to determine the extent of the transfollicular penetration [54,55]. Further, also Raman microscopy and multiphoton tomography were established to determine follicular penetration [22]. However, these methods are not suitable for all substances, require complex equipment and extensive data analysis and are therefore only rarely used.

For the aims of this thesis, only the method of CSSS and the cross-sections of the hair follicles are suitable because with other outlined methods (selective follicular closure, microdialysis or combination of both methods) the extent of the transfollicular penetration is determined. Hence, mechanistic studies investigating, for example, the influence of excipients on the penetration into the hair follicles are not possible with these methods. The method of CSSS was already tested in the first step and failed to determine the follicular penetration efficacy of dermal NC formulations. Thus, only the cross-sections of the hair follicles represent the optimal solution. But the main disadvantage of this method is its ability to determine only the follicular penetration and not the passive dermal. Thus, the existing methods for determination of the passive dermal penetration need to be evaluated.

For determination of the passive dermal penetration, various ex-vivo or in-vitro methods are available at present. Most of the ex-vivo penetration experiments are carried out using classical tape stripping, Franz diffusion cells, microdialysis, electron paramagnetic resonance (EPR) spectroscopy, or methods of vibrational spectroscopy – confocal Raman microspectroscopy (CRM) or attenuated total reflection-Fourier-transform infrared (ATR-FTIR) spectroscopy [6–9,56–69]. Classical tape stripping was already presented and discussed in the first step. The principle of the Franz diffusion cell is based on the penetration of the dermal formulation from the donor compartment through the membrane into the receptor compartment [56]. With this, the experimental setup comprises of the donor chamber and the receptor chamber between which the membrane is fixed. As a membrane, either excided human or animal skin, or different synthetic membranes can be used (for example, porous synthetic membranes such as cellulose acetate membrane, or different artificial skin membranes such as Strat-M® membrane for transdermal diffusion testing) [70–72]. The Franz diffusion cell enables the determination of the amount of AC penetrated into the receptor compartment from the donor compartment over defined time, and of the penetration kinetics. The principle of microdialysis is based on the determination of the dermally penetrated AC in the extracellular space in living tissue [73]. The fundamental technical principle is that small catheters/probes equipped with a semipermeable hollow fiber are inserted into the dermis parallel to the skin surface. The probe is perfused with a perfusate. The exchange of diffusible molecules happens across the semipermeable probe membrane from the

probe to the tissue and vice versa, according to the principle of diffusion. The amount of the penetrated AC in the perfusate is measured by classical analytical methods. With this, microdialysis allows for the determination of the amount of AC penetrated into the skin from the dermal formulation. EPR spectroscopy is based on the interaction of electromagnetic radiation with magnetic moments [74]. This method allows for the detection and quantification of paramagnetic molecules and is utilized for dermal penetration experiments. For this method, paramagnetic material needs to be added to the dermal formulation. It means that either the AC needs to be spin-labeled, or special spin probes (for example, nitroxides) should be used. The method of ERP spectroscopy allows to investigate the interaction of the AC with the carrier – and with this, the drug release – and the dermal penetration of topical formulations [74]. The investigation of the drug release is possible by studying the localization of the AC within the carrier system and the microenvironment of the AC within the carrier (e.g., microviscosity, micropolarity). The determination of dermal penetration efficacy is possible because the EPR spectroscopy allows to quantify the amount of AC in the skin after its topical application. Regarding methods of vibrational spectroscopy, CRM and ATR-FTIR have been optimized and utilized for skin analysis [58,63,75–80]. These methods allow both to investigate the penetration of dermal formulations into the skin and to study the skin properties [75,79]. The principle of CRM is based on measuring the vibration frequencies of chemical bonds and parts of molecules in the skin by recording of Raman spectra [75]. The interpretation of Raman spectra enables the determination of the AC distribution within the skin by plotting depth-concentration profiles of penetrated AC. Additionally, CRM gives information about skin lipids and proteins. With this, it allows to study the skin structure (for example, the recording of the CRM spectra of the SC lipids allows to study the lipid ordering) and the interaction of the penetrated AC with skin components. The prime focus of the ATR-FTIR spectroscopy covers mainly the investigation of the molecular effects of different formulations or excipients on the conformational order of SC lipids and proteins [78,79]. Due to a probing depth of about 1 µm, a majority of these studies only provide information about the skin surface [78]. With ATR-FTIR also the quantification of the penetrated AC and the determination of its penetration depth is possible. For this, for example, a combination of the tape stripping and ATR-FTIR was established, allowing for the detection of the penetrated AC in different layers of the SC [63,76,78]. To sum up, all outlined methods represent sufficient and established tools for the characterization of the passive dermal penetration. Nevertheless, they possess several limitations. First, the outlined methods – and many other existing methods – are associated with tedious work. For example, complex skin preparation (e.g., Franz diffusion cells: complex skin preparation is required before an experiment can even start), complex sample preparation including specific labeling (EPR spectroscopy), complex performance that requires high-tech and high-priced equipment (EPR spectroscopy, CRM, ATR-FTIR), extensive handling of the methods that requires a lot of time and highly experienced users (EPR

spectroscopy, CRM, ATR-FTIR), and extensive analysis of the test results (e.g., CRM and ATR-FTIR: complex interpretation of the skin spectra, tape stripping: due to the very small amount of AC on the tapes, often HPLC-MS is required). Second, a comparison between data or between different formulations is often complicated because a large number of variables – such as differences in skin type, skin thickness, sample preparation, skin preparation – can lead to artefacts that can strongly influence the results. Thus, it can falsify the comparison between formulations and cause an incorrect interpretation of the results [81–86]. Third, the existing methods provide often not the full and detailed picture of the penetration fate of dermally applied AC, since the characterization is performed either by a limited number of characteristic parameters (e.g., Franz diffusion cell: only the amount of penetrated AC and the penetration kinetics is measured and the information of the penetration depth of the AC is missing) or only the penetration of the AC into the SC can be studied (tape stripping, ATR-FTIR). However, the most meaningful limitation of all the outlined methods is that they can only be used to determine the passive dermal penetration or cannot distinguish between dermal and follicular penetration. With this, reliable discrimination between the hair follicle uptake and the passive dermal penetration is missing.

Since NC represent a formulation with the proposed “dual action”, the optimum method for penetration studies with dermal NC formulations must determine both the follicular and the dermal uptake. Also, the method must provide reliable discrimination between the follicular and passive dermal penetration. Further, to achieve a direct combinability and comparison of the penetration efficacy of the NC formulations into the hair follicles and into the skin, the experiments should be performed using one skin model and one skin area. At present, only differential skin stripping can determine the follicular penetration and the passive dermal penetration by using one skin model and one skin area. Therefore, this method was tested in the first step of this thesis but was not successful. Another two-in-one method is not yet available. Another possibility to determine both the follicular and the passive dermal penetration of a formulation simultaneously is to combine two methods. For example, to determine the follicular penetration using the method of cross-sections and separately determine the efficacy of the passive dermal penetration using, for example, a Franz diffusion cell. However, this approach represents the ultima ratio because these two experiments cannot be performed on one piece of skin and on one treated skin area since each method has different requirements for the skin model. Further, conditions for both experiments – due to two different skin models – would be different because the skin would be treated differently, and it would complicate the combinability of the results. Thus, at present, an optimal solution to determine both follicular and passive dermal penetration of a dermal formulation does not yet exist.

This thesis presents the optimal solution. In this thesis, a powerful two-in-one method was established. Thus, representing a dimensional step forward into the determination of the penetration efficacy of

dermal formulations. The method established in this thesis is based on the visualization of the follicular and passive dermal penetration utilizing fluorescence microscopy. The prerequisite for using this method is that the AC possesses a sufficient autofluorescence, or special fluorescent dyes should be used. The handling of the method is very simple. The investigated formulation is applied onto the skin of intact porcine ears, after a defined penetration time, the treated skin area is punched using a drive punch, embedded in a special medium and frozen. Subsequently, thin cross-sections of the skin biopsy are prepared using a cryomicrotome and analyzed using fluorescence microscopy. Thus, the established method does not require any high-priced and high-tech equipment since only two devices are needed – a cryomicrotome and a fluorescence microscope. The novelty of the established method is its unique two-in-one concept. It means the ability to determine the penetration into the hair follicles and the passive dermal penetration into/through the skin simultaneously by applying the formulation only once onto one skin area within one skin model. Also, the method can reliably distinguish between follicular and dermal uptake. How does it work? On the porcine ear, the skin area with a sufficient amount of hair follicles is treated by the formulation, then the biopsy is punched, embedded and cut into thin cross-sections. Here is the crux – in this thesis, such an embedding and cut technique of the skin biopsies was established, that one treated skin area and with this, one biopsy provides both proper hair follicle sections (proper means that the hair follicles are cut vertical, straight and the full hair follicle is “caught”) and proper skin sections. With this, to determine the follicular and passive dermal penetration efficacy of the dermal formulation, only one skin area within one skin model needs to be treated and only one skin biopsy needs to be cut. It helps to minimize the work by improved outcome. The outlined two-in-one concept provides the unique beauty, that the hair follicles and the skin are treated by completely equal conditions, i.e., the same skin conditions, same application technique of the formulation, same conditions of incubation during the penetration time etc. In this way generated data about follicular and passive dermal penetration of the dermal formulation are directly combinable and comparable because all conditions are – by definition – equal. The reliable discrimination between follicular and dermal uptake is achieved because the hair follicle sections and the skin sections are visualized using fluorescence microscopy. With this, one sees the hair follicles and the skin with own eyes and can clearly separate the follicular and the passive dermal penetration.

The individual components of the established method are not novel. The porcine ear skin model was established at the beginning of the 2000s and is since then relatively often used for dermal penetration experiments [44]. The use of fluorescence microscopy for the determination of follicular penetration was established by the research group of professor Lademann in 2006/2007 [53]. Also, several studies already utilized fluorescence microscopy to study the passive dermal penetration. But in most cases, fluorescence microscopy is used simply as a tool to track the formulation within the skin [87–92]. Thus, allowing just for the absolute statement – the formulation was detected in the skin/the formulation

penetrated the skin – yes or no. However, until now, no one combined all these individual components to one powerful system, that can determine follicular and passive dermal penetration using only one skin model and one – two-in-one – method. Precisely this combination determines the beauty and the novelty of the method established in this thesis.

The established method enables not only the visualization but also the quantification of the penetration efficacy. The follicular penetration of NC was quantified according to the method established by the research group of professor Lademann, i.e., measured in µm directly from fluorescence images [53,93]. But the method to quantify the extent of the passive dermal penetration using fluorescence microscopy did not yet exist before this thesis.

This thesis established the novel method for the determination of the extent of the passive dermal penetration using fluorescence microscopy and digital image analysis using ImageJ software. The method of digital image analysis is based on the processing of the fluorescence images so that the amount of the AC penetrated into the skin, the penetration depth and the homogeneity of the penetration can be determined using just two tools – fluorescence microscopy and the software ImageJ. The established method considers all specificities of the skin samples and is purposefully adapted to determine the penetration efficacy of dermal formulations. For example, a very meaningful specificity of the skin biopsies is their highly variable autofluorescence. It complicates the image analysis because it is difficult to distinguish between the fluorescence of the AC within the skin and the fluorescence of the skin itself. To overcome it a suitable threshold method for image processing was established in this thesis. The established threshold method is based on dividing the pixels of the fluorescence images of the skin samples into two classes. Class I pixels represent the AC penetrated into the skin and are evaluated, and class II pixels represent skin autofluorescence and are eliminated. After thresholding, only AC pixels are left on the images. The established threshold method builds the prerequisite for sound and accurate subsequent image analysis. Via image analysis, the amount of penetrated AC, its penetration depth and the homogeneity of the penetration are measured. The amount of penetrated AC is measured as the mean grey value (MGV) of the thresholded images. The penetration depth of the AC is directly measured in µm. Since the skin is visualized and one sees the penetrated AC with own eyes and measures its penetration depth with own hands, it can be clearly distinguished in which skin layer the AC is penetrated – only into the upper layers of the SC, into the entire SC, into the epidermis or even into the deeper skin layers. The next established parameter is the so-called inhomogeneity index. As the name says, it measures the extent of the penetration homogeneity. It is important because the visual inspection of the fluorescent images revealed that some images do not possess consistent homogeneous penetration of the AC into the entire SC, but only some “hot spots” of penetrated AC. Since the homogeneous penetration into the entire treated

skin area should be strived by every dermal formulation, the measure of penetration homogeneity helps to characterize the penetration behaviour of a dermal formulation even more preciously.

To minimize the work and increase the power (amount of done work per unit time), and further to automatize the image analysis, a macro was written. At present, the digital image analysis is done automatically (except for the measurements of the penetration depth). It helps to eliminate all possible influencing factors that could occur by manual image analysis and to keep every single processing parameter constant for each evaluated image. Thus, digital image analysis can now be done easy and quick.

As good as the automatic image analysis is, the “simple” visual inspection of fluorescence images takes always place by every penetration experiment prior to digital image analysis. The beauty of seeing the skin and the penetration provides a series of advantages. For example, the measurement of the thickness of the SC allows to determine how the formulation influences the skin conditions, i.e., hydration state of SC. With this, the hydration – or even dehydration – ability of the formulation can be estimated. Further, the visualization of the skin helped to optimize the ears preparation procedure. In the past, the porcine ears were shaved with an automatic razor to remove the hair. The visualization of the skin showed that the shaving influence tremendously the appearance and conditions of the SC. On shaved ears, it possesses a “zig-zag” structure on its surface, whether the not-shaved SC looks smooth and homogeneous. Since shaving influences the SC structure, the outcome of the penetration studies may also be influenced. Thus, the visualization of the skin helped not only to optimize penetration experiments but also to optimize the ears preparation procedure.

To sum up, the method established in this thesis represents a dimensional step forward in determining the penetration efficacy of dermal formulations. Looking back to differential skin stripping (and also to further conventional methods), the new method overcomes all detected disadvantages and comes up with an optimized solution. The most significant benefit of the two-in-one method established in this thesis when compared to conventional test methods is its ability to determine both follicular and passive dermal penetration of the AC from the investigated dermal formulation using only one method and one skin model. Compared to differential skin stripping – that can also determine the follicular and passive dermal penetration of one formulation on one skin area – the new established method brings significantly more outcome by significantly less effort regarding time, handling and equipment. With differential skin stripping, the follicular penetration was characterized only by the amount of the AC penetrated into the follicles. This approach did not work out in the first step of this thesis. The new method allows for the visualization of the hair follicles and for the measurement of the penetration depth of the AC directly in µm. With this, the quantification of the penetration efficacy is much simpler because only fluorescence images are required. Thus, no expensive and complex analytical equipment

is necessary. With differential skin stripping, the passive dermal penetration was characterized only by the amount of the AC penetrated into the upper layers of the SC and by the generic penetration depth. The new established method enables the determination of the amount of AC penetrated into the whole SC and viable skin layers, the measurement of the penetration depth in µm, the discrimination in which skin layer the AC penetrated in, and the measurement of the penetration homogeneity. With this, allowing for a detailed time and space resolved determination of the full fate of the dermally applied formulation. Further, the new method allows to determine the thickness of the SC. Thus, predicting the influence of the dermal formulation on the skin conditions, i.e., on its hydration state. The handling of the new method is much simpler compared to differential skin stripping because fewer working steps are needed. With tape stripping, for one dermal formulation approximately 24 h were needed. The new method allows for testing for up to 3 different formulations (n=3, skin and hair follicle sections) in 8 h. With this, the power is increased to almost 10 times. Thus, the new method allows to minimize the work by the improved outcome – and with this, to be "faster, higher and stronger".

Taking together all outlined advantages, the established method enables a detailed sound characterization of the follicular and passive dermal penetration of dermal formulations. With this, the main prerequisite for sound penetration studies with NC formulations was fulfilled and the first aim of this thesis was achieved. Thus, using the established method sufficient characterization of NC formulations is possible.

Third step: Realize

The second aim of this thesis was to investigate the influences of essential formulation aspects on the follicular and passive dermal penetration of the NC formulations using the optimum method and to tailored optimize them to achieve effective penetration. The investigations of the first step already hinted that essential core formulation components – vehicles and excipients – influence the passive dermal penetration of the AC from NC. However, due to limitations of differential skin stripping, the detected influences could not be investigated mechanistically and detailed. Moreover, the investigations of the follicular penetration were not even possible. The new method established in the second step overcomes all substantial and most limiting disadvantages of differential skin stripping. Thus, allowing for sound penetration studies with NC formulations, which were performed in the **third step** of this thesis.

NC for effective hair follicle targeting

The prime focus of the penetration studies was set on the hair follicle targeting with NC. The reason for this is - in theory, due to their special features, NC represent the formulation of choice for poorly soluble AC for effective hair follicle targeting, but no one proved it yet, i.e., no systematic studies with NC for hair follicle targeting were yet performed. This thesis focused primarily on the application use

of NC for effective hair follicle targeting. It means, the aims were i) to prove (or even disprove) that NC represent effective nanocarrier for the hair follicle targeting and ii) to investigate how formulation aspects (vehicles, excipients) influence the penetration efficacy of NC into the hair follicles and to tailored optimize them to achieve effective follicular penetration.

At the beginning of this work, the theoretical background concerning NC for hair follicle targeting was poor because no systematic studies with NC for hair follicle targeting were yet performed. Considering hair follicle targeting in general, solid theoretical background existed. Previous studies demonstrated that particles penetrate into the hair follicles deeper and more effective than dissolved AC [53]. The superiority of particles versus non-particle formulations was demonstrated not only for penetration efficacy but also for storage behaviour. The dye in non-particle form was detected within the hair follicles for only up to 4 days, whereas the dye in particle form could still be detected in the hair follicles 10 days after application [53]. Further, it was demonstrated that particle size is the key factor for the regulation of follicular penetration depth. Studies with nanoparticles revealed that particles with sizes in the range of 400 – 700 nm penetrate into the hair follicles deeper than larger or smaller ones [94]. The size-depended principle was proved for different kinds of nanocarriers, i.e., PLGA nanoparticles, silica particles, liposomes, microspheres [93–95]. This finding was explained by the penetration mechanism of particles into the hair follicle, which can be described by the so-called ratchet mechanism [96]. Here, the hair follicle together with the hair shaft – due to its characteristic zig-zag structure – build a ratchet-like complex, that by its motion brings particles deeper into the hair follicle. The most effective function of outlined ratchet mechanism – and with this, the deepest follicular penetration – is then achieved when the size of particles corresponds approximately to the distance between the follicle wall and the hair shaft and to the thickness of the hair cuticula cells (i.e., about 400 – 700 nm) [96,97]. For the function of the ratchet mechanism, the motion of the hair within the hair follicle is an essential prerequisite [53]. In ex-vivo experiments, this motion can be achieved by the application of the formulations with a massage. The state of the current scientific opinion indicates that the mechanism of follicular penetration is primarily of mechanical nature. Thus, it is believed that physical parameters influence follicular penetration in a more pronounced manner compared to chemical parameters [22]. However, the influence of the chemical parameters – such as excipients or vehicles – on the hair follicle targeting was not yet systematically investigated with none of the nanocarriers. There exist some studies that investigated this issue in a “spot-on” manner, but the outcome of these studies is very heterogeneous. For example, some studies reported deeper follicular penetration of particles from semi-solid vehicles compared to liquid vehicles, whereas others observed opposite effects [98–100]. The influence of further parameters of the vehicles on the follicular penetration efficacy, such as lipophilicity, was also evaluated very controversial [98–100]. However, for the application use and further development of formulations with NC for effective hair follicle

targeting, the knowledge of whether and how the formulation aspects influence the follicular penetration and how they should be optimized to achieve effective follicular penetration is substantial. Thus, this issue was systematically and detailed addressed in this thesis.

Firstly, and mainly, this thesis confirmed by systematic studies that NC represent a highly effective nanocarrier for hair follicle targeting. Each in this thesis investigated formulation transported NC successfully into the hair follicles. NC were detected in 100% of investigated hair follicles treated with each NC formulation independent of the formulation properties. Thus, the findings of this thesis combined with the special features of NC outlined in the introduction section allow for a clear conclusion that NC represent – not only in theory but also in practice – the nanocarrier of choice for effective hair follicle targeting.

The follicular region that was effectively targeted by each formulation investigated in this thesis (semi-solid gels with varied properties loaded with NC, aqueous nanosuspensions modified with different excipients) is the hair follicle infundibulum. The infundibulum represents a very important target within the hair follicle due to its special properties. Firstly, the infundibulum can act as a kind of reservoir for the particles penetrated into the hair follicles. Recently published studies demonstrated that particles can be stored as a depot within the infundibulum for up to 10 days [49,53]. Secondly, the hair follicle infundibulum possesses a special morphological structure. It is characterized by a weak, i.e., less developed SC in its lower part [101]. Therefore, targeting the hair follicle infundibulum with NC addresses two important issues. Firstly, it enables the formation of the interfollicular NC depot as a long-term reservoir, from which the AC from NC can be step-by-step released. Secondly, the released AC from NC can penetrate through the weak SC of the infundibulum into deeper layers of the skin. Thus, enabling a passive penetration into viable dermis bypassing the “real” SC barrier of the skin. NC consist of 100% AC and thus, possess a maximal possible AC loading. Thus, comparatively high AC concentrations within the hair follicle can be achieved by hair follicle targeting with NC in comparison to other nanoparticles, which usually have a loading capacity of less than 10% [102–106]. Thus, confirming the superiority of NC for hair follicle targeting compared to other nanocarriers.

To allow for a sufficient application use of NC for effective hair follicle targeting, suitable formulation components are required. To find the formulation components enabling the most effective penetration, the influence of the formulation aspects on follicular penetration must be investigated. This thesis addressed this issue and investigated thoroughly and systematically the influence of essential core formulation aspects – vehicle type and excipients – on the efficacy of the hair follicle targeting with NC. The outcome of these investigations provides a statement on how the essential formulation components influence the follicular penetration of NC. Thus, allowing for a “design” of tailor-made NC formulations for the most effective hair follicle targeting.

The type of vehicle showed no significant influence on the efficacy of the hair follicle targeting with NC. To study the influence of the vehicle type, NC of comparable size were incorporated in gels with varied properties (viscosity, lipophilicity, polarity). The follicular penetration of NC from different vehicles was systematically investigated. Results revealed that independent of the vehicle properties NC reached within the hair follicle comparable depths. This outcome was explained by the rheological properties of obtained gels in combination with previously postulated mechanical ratchet-mechanism of the follicular penetration of particles. All obtained gels possess thixotropic flow behaviour, meaning, low viscosities at high share rates. All gels were applied onto the skin by a 3 min mechanical finger massage – that is in accordance with the original manuscript essential to start the ratchet mechanism [53]. When a thixotropic gel is applied onto the skin by mechanical massage, its viscosity decreases and the gel fluidizes. Thus, indicating that all obtained gels possessed comparable low viscosities during the application by massage. The liquification of the gels during application might result in similar diffusion coefficients for the NC from all formulations. Thus, enabling a similarly good and comparable penetration of NC into the hair follicles from all vehicles independent of its other properties (in this thesis, varied initial viscosity/lipophilicity/polarity). A further reason that might explain that the vehicle properties did not influence the penetration efficacy of NC into the hair follicles is the fact that all formulations contained similar NC sizes. Since the variations of the formulation properties were considered to disappear during massage (viscosity of the gels) or to have no influence (lipophilicity and polarity of the gel phase) on the follicular penetration depth of NC and all formulations contained identical amounts of NC of identical size, it can be assumed, that NC penetrate into the hair follicles following the size-depended mechanical ratchet-mechanism described previously for other nanoparticles. Thus, the identical size of NC in combination with the massage application and thixotropic flow behaviour of the semi-solid gels resulted in identical penetration depths of NC within the hair follicle, independent of the vehicle used.

Obtained findings are of substantial importance for application use. When the semi-solid formulation possesses thixotropic flow behaviour and is applied by mechanical massage, other properties of the vehicles that showed no influence on the hair follicle targeting with NC can be adjusted to other requirements. For example, type of skin (dry skin/oily skin), patients' or customers' wishes or needs, ease of application and physical and chemical stability of the NC. Thus, representing good freedom for further formulation development of tailor-made NC formulations for effective hair follicle targeting.

To modulate the follicular penetration, meaning to improve or even prevent it, the NC formulations should be modified by the addition of excipients. This thesis demonstrated that excipients influence the penetration efficacy of NC into the hair follicle tremendously. Thus, utilizing the penetration enhancer principle for hair follicle targeting and representing a novel approach of the regulation of follicular penetration efficacy. Moreover, excipients modified the penetration efficacy of NC into the

hair follicles beyond the particle size. Thus, they were able to overwrite the previously postulated size rule. Until now, because no systematic studies addressed this issue elaboratively, it was believed that follicular penetration can be mainly affected by physical parameters and that chemical parameters play only a minor role. Thus, the outcome of this thesis indicating the essentiality of chemical parameters represents a highly significant milestone in the research field of hair follicle targeting.

To investigate the influence of excipients on the efficacy of the hair follicle targeting, the nanosuspensions were modified by the addition of different excipients. The follicular penetration efficacy of NC from modified nanosuspensions was studied. Results revealed significant differences in penetration depths of differently modified NC in dependence of obtained excipient. Some excipients (ethanol, propylene glycol and olive oil) improved the follicular penetration efficacy compared to NC without the addition of excipients, whereas other excipients (glycerol and urea) impaired the penetration efficacy significantly. Since the detected excipients-effect has not been described previously and could not be explained by the available knowledge, it was investigated in detail in this thesis. For this, the effects of the excipients on the hair structure were examined by microscopic analysis. It was demonstrated that the excipients modulated three parameters. First, the structure of the hair cuticula and thus, the hair roughness, second, the adhesiveness of NC and, finally, the adhesion pattern of NC to the hair. The outlined effects seemed to affect the follicular penetration efficacy beyond the particle size. Thus, this thesis did not only demonstrate that excipients influence the follicular penetration efficacy of NC, but also investigated the mechanisms behind it.

With this new knowledge, the entire mechanism of follicular penetration can now be viewed from a new angle. The findings of this thesis demonstrated that in addition to the size-driven ratchet effect, also other effects – that stayed unnoticed until now – seem to be involved in the process of follicular penetration. By including the identified effects of the excipients in the overall picture of the follicular penetration of particles, it can now be proposed that the mechanism of follicular penetration possesses, in addition to the ratchet effect, two further steps. Thus, representing a complex process that takes place in three successive steps (a three-step mechanism):

1) in the first step, the particles need to adhere loosely to the surface of the hair;

2) in the second step, they need to locate in between the overlapping cells of the hair cuticula to enter the ratchet mechanism;

3) in the third and final step, the ratchet mechanism starts, i.e., the directed transport of particles into the hair follicles, driven by the radial and axial movement of the hair.

The particle size may primarily influence the second step, i.e., the efficacy of the particles to locate in between the overlapping cells of the hair cuticula. This is also supported by the previously postulated

correspondence of the sizes (particle size/thickness of the cuticula cells – 400 - 700 nm) [94,97]. On the contrary, excipients modulate all three steps of the hair follicle penetration process.

Based on the proposed mechanism, tailored optimization of NC formulations by excipients is now possible. To increase the follicular penetration of NC formulations, for example, excipients which increase the hair roughness should be chosen (in this thesis, ethanol and propylene glycol). Thus, improving the second step of the three-step mechanism and increasing the follicular penetration efficacy. To even prevent the follicular uptake, such excipients are required, which, for example, increase the adhesiveness of NC to the hair shaft. Thus, impairing the first step of the three-step-mechanism (in this thesis, urea). If NC stick too much to the hair shaft, they cannot effectively enter the ratchet mechanism. Thus, the penetration efficacy decreases. The prevention of follicular penetration can be utilized, for example, in formulations that are specifically designed to prevent the penetration of unwanted particles into the hair follicles, such as titanium dioxide particles in sunscreens or pollen allergens [107,108].

The outcome of this thesis opens several new perspectives. For example, regarding the development of tailor-made NC formulations and formulations with other nanocarriers for effective hair follicle targeting, their application use, and the testing of the follicular penetration efficacy of dermal formulations. Firstly, and mainly, from now on, tailor-made NC formulations with desired properties can be designed by purposeful optimization of the existing formulations by addition of excipients. The approach to regulate the follicular penetration efficacy by formulation components is very convenient and elegant. To achieve a formulation with desired penetration efficacy, it is necessary to optimize and modify an already existing formulation instead of the developing of a new one with the required properties, for example, particle size. This approach saves both time and money. Further, the understanding of the three-step mechanism allows to “play” with gained knowledge and to try new approaches that can be derived from the proposed mechanism. For example, combinations of excipients with different effects on each step could be investigated. Considering, for example, an excipient that increases the hair roughness effectively (2. step) combined with an excipient that reduces the sebum fluidity (3. step) - would it be a “superpower” formulation leading to the most effective penetration of NC or also other nanocarriers into the hair follicle? Or, in contrast, would the combination of excipients that impair each step of the possible three steps prevent the follicular penetration completely and represent “the formulation” for the prevention of follicular penetration of unwanted particles? Do different excipients affect each step to a different extent? Which excipient is most powerful for each step? Since the effect of excipients is hair structures-related and not formulation-related, it should theoretically be applicable to every nanocarrier. Thus, representing the universal approach for regulation of the hair follicle penetration efficacy. Now, this issue should be proved by systematic studies. All outlined approaches represent the path in the future of hair follicle

targeting. Secondly, the outcome of this thesis indicates that more attention should be paid to the composition of the formulations for effective hair follicle targeting with particles, and to the combination of the products for effective hair follicle targeting with common products. For example, a lot of common formulations for hair care or scalp care that at present exist at the market contain glycerol and/or urea. The outcome of this thesis showed that these components prevent follicular penetration of particles. Thus, on the one hand, the use of common products containing these components should be avoided when effective hair follicle targeting with particles is required. Or, on the other hand, such products can be utilized as a prevention of the follicular penetration of unwanted particles. This approach was already addressed by Meinke et al. [108]. However, the prevention of follicular penetration by common dermal products was explained by their occlusive properties and not by the effects of the formulation components on the efficacy of the hair follicle targeting because this knowledge was not yet available [108]. Thirdly, the outcome of this thesis indicates that the testing of formulations for hair follicle targeting should include not only the actual investigation of the follicular penetration efficacy but also consider the effects of the formulations on the follicular structures and on the adhesiveness of particles to the hair shaft. In that way, the complete picture of the effects of the formulation on the hair follicle targeting can be created, and the sound and mechanistic understanding of the follicular penetration of dermal formulations can be gained.

NC for effective passive dermal penetration

This thesis provides a significant contribution to a deep, sound and mechanistic understanding of the influences of the formulation aspects on the efficacy of the passive dermal penetration of the AC from NC. Based on this understanding, key parameters of the vehicles and excipients were identified, whose tailored optimization enables effective passive dermal penetration with NC formulations. The best way to present this contribution is to compare the outcome of the first step performed with the conventional method (classical tape stripping) with the outcome of the third step performed with the novel method established in this thesis.

Concerning the vehicles, with classical tape stripping, only the amount of the AC penetrated into the upper layers of the SC and the generic penetration depth were estimated. Based on these data, it was shown that the vehicle type influences the amount of AC penetrated from NC into the upper layers of the SC. Since the mechanisms of the detected influences could not be studied, results could not be explained by the state of knowledge existing at the time point when the experiments of the first step were performed. Therefore, in the third step, the influences of the vehicle type on the penetration efficacy of dermal NC formulations were performed with the novel method established in this thesis. The influence of vehicle type was studied on the example of different gels with varied properties. In contrast to tape stripping, the novel method allows a detailed time and space resolved determination

of the fate of the dermally applied formulations. The novel method enabled the visualization of the skin and the penetration of the AC from NC formulations. It allowed to see the entire SC and the viable skin layers, to see the penetrated AC and to determine in which skin layer the AC penetrated in (only parts of SC/entire SC/entire SC and viable skin layers). The visual inspection of the fluorescence images revealed that each vehicle transported the AC from NC either into the entire SC thickness or into the entire SC and even deeper into the viable skin layers. Thus, confirming that the outcome of the tape stripping studies determining only the penetration into the upper part of the SC is meaningless. Further, simple visual inspection enabled the evaluation of the penetration homogeneity. Some vehicles showed only some "hot spots" of penetrated AC and, consequently, a very inhomogeneous inconsistent penetration, whereas other vehicles demonstrated a consistent homogeneous penetration of the AC into the entire treated skin area. Since tape stripping can estimate only the amount of penetrated AC, this method cannot capture the differences in the penetration homogeneity detected with the novel method. The extension of the visual inspection by digital image analysis allowed for the measurement of the amount of penetrated AC in MGV/px, its penetration depth in µm and the penetration homogeneity in %. Thus, vehicles with good penetration of the AC from incorporated NC and vehicles with poor penetration were discriminated. Vehicles with poor penetration showed low amounts of penetrated AC and a very inhomogeneous penetration limited only to the SC. In contrast, other vehicles demonstrated good penetration – it means a consistent homogeneous penetration of higher amounts of AC not only into the SC but also into the viable skin layers. The visualization of the skin allowed to see and to detect that the penetration efficacy of the AC from NC from different formulations correlates directly with the thickness of the SC, i.e., with its hydration state. Formulations with good penetration showed thicker, i.e., more hydrated SC, whereas formulations with poor penetration showed no differences in the SC thickness compared to untreated skin. The measurement of the SC thickness in µm confirmed the results of the visual inspection. Thus, the conclusion was drawn that the ability of the vehicle to hydrate the SC represents the key vehicle property allowing for effective passive dermal penetration of the AC from NC. Thus, with the novel method, the influences of the vehicle type on the entire penetration fate of the AC from NC were detailed and soundly investigated, the mechanism of these influences were explained and the key vehicle property allowing for effective passive dermal penetration with NC formulations was identified. Thus, compared to tape stripping and to the outcome of the first step, the novel method established in this thesis represents a dimensional step forward to sound and meaningful penetration studies with dermal NC formulations.

The determined correlation between the ability of the vehicle to hydrate the SC and the penetration efficacy of the AC from NC could only be detected using the novel method established in this thesis. The visualization of the skin enabled the visualization of the changes in the SC structure. It was possible

initially by simple visual inspection of the fluorescence images and subsequently by measuring the thickness of the SC after the treatment of the skin with different formulations. Thus, enabling the determination of the hydration state of the SC and the influence of the formulation on the SC hydration. The detection of this effect is not possible using either conventional models for determination of the dermal penetration (classical tape stripping, Franz diffusion cells, microdialysis etc.) or the conventional test systems and techniques for determination of the biophysical skin parameters such as skin capacitance or TEWL. For example, when a gel – or a dermally applied formulation in general – dries out on top of the skin, the determination of the biophysical skin parameters does not enable the accurate measurement of the skin hydration and thus, the accurate determination of the influence of the formulation on the skin properties. If one measures the biophysical skin parameters over the dried gel without washing it off, only the film properties and not the skin properties can be measured. If the gel is washed off with water, the subsequent measurement of skin capacitance and TEWL is not meaningful because one cannot ensure where the moisture comes from – from the washing or from the formulation itself. Thus, the novel method provides meaningful additional information about the skin, which contributes significantly to the detailed and mechanistic understanding of the penetration of dermally applied formulations and the mechanisms behind it. The determination of these "add-on's" is not possible with conventional test models, which are usually specialized in the determination of only one penetration parameter.

From the perspective of the knowledge gained in the third step, the results of the first step can be better understood. The first step showed, that across initial expectations and the principle of the topical suspensions, the hydrogel demonstrated the weakest penetration of the AC from NC, whereas from the cream and the oleogel higher amounts of the AC penetrated into the skin. Now, based on the outcome of the third step it might be assumed, that the hydrogel dried out on top of the skin. Thus, it might not hydrate the SC during the entire application time leading to a poor penetration of the AC from NC incorporated into the gel.

The influence of excipients on the penetration efficacy of dermal NC formulations was also investigated with the novel method. Results showed a good correlation to the vehicles' investigations. The most effective passive dermal penetration was achieved using excipients with pronounced skin moisturizing properties. Thus, the ability to hydrate the skin was confirmed to be the key formulation property for effective penetration also on the example of excipients. The novel method allowed to study the influences of the excipients on the entire penetration fate of the AC from a dermal formulation what was not possible in the first step with tape stripping. It was demonstrated that excipients influence all aspects of the penetration efficacy, i.e., the amount of penetrated AC, its penetration depth and penetration homogeneity. Based on these findings, tailored optimization of the NC with excipients to achieve the desired target is now possible. To target only the SC, the NC without any excipient

represent the most suitable formulation. To transport the AC into the viable skin layers, NC should be modified with excipients with good skin moisturizing properties. This thesis demonstrated that simple modification of NC with glycerol, propylene glycol and urea improved all three “components” of the penetration efficacy - compared to blank NC, higher amounts of the AC were transported not only into the SC but also into the deeper layers of the skin, and the penetration homogeneity was improved. To target only the upper layers of the SC, excipients with skin dehydrating properties are required. In this thesis, the addition of ethanol to NC reduced the amount of penetrated AC, its penetration depth and impaired the penetration homogeneity. Thus, the knowledge gained with the novel method enables a purposeful modification of NC with excipients. From now on, tailor-made dermal NC formulations with the required extent of the passive penetration can be designed. As already outlined in the hair follicle targeting section, this approach is elegant and convenient because it is much simpler to optimize an existing NC formulation instead of developing a new one.

The sensitivity of the novel method enabled the detection of the smallest differences between the influences of different excipients on the penetration efficacy of the AC from NC. With the new method, it was possible to detect and investigate differences between excipients that improved the penetration efficacy of dermal NC formulations. Consequently, the most potent excipient could be identified. Interestingly, glycerol and propylene glycol showed a more effective penetration-enhancing effect compared to urea and were consequently more powerful. All three excipients increased the penetration depth of the AC compared to blank NC, but in the case of urea, the increase of the amount of penetrated AC was less pronounced. Since all three excipients hydrated the SC to a comparable extent, this finding demonstrated that not only the ability of the formulation to hydrate the skin contributes to the penetration efficacy, but also the hydration mechanism plays a substantial role. Glycerol and propylene glycol are assumed to penetrate into the intercellular space between the corneocytes, whereas urea is considered to penetrate into the corneocytes [109–111]. The penetration of urea into the corneocytes leads to an increase in their size and, in turn, to a decrease in the volume of the intercellular space. Thus, leading to a reduction of the penetration space for the AC and therefore to a less pronounced penetration enhancement effect compared to humectants with other hydration mechanisms.

In conclusion, the outcome of the third step demonstrated the essential importance of a suitable method for penetration studies. The method and the formulation should build the perfect match. Only when this perfect match is found, the aims of the penetration studies can be achieved. This thesis demonstrated two possible cases – no match (classical tape stripping + NC) and perfect match (novel method + NC). With tape stripping, as the tapes only “touched” the upper layers of the skin, so the penetration efficacy of different NC formulations was only “touched on”. With the novel method, the detailed time and space resolved determination of the penetration fate of dermal NC formulations was

possible. With this, the aims of the thesis were achieved. The power and the meaning of the novel method established in this thesis are best described by the slogan of one of the microscope manufacturers – capture it all. Since we "got eyes" and made the skin and the penetration efficacy not only visible with the fluorescence microscopy but also measurable with digital image analysis, from now on, by performance of the penetration studies, everything is captured. The novel method is now used daily in the research group. It builds the irreplaceable tool not only for sound penetration studies – not only with NC, but also with other dermal formulations because the method is not specified for only NC formulations and is therefore universally applicable – but also for investigations of other skin-related issues.

NC for targeted dermal drug delivery

This thesis demonstrated that NC formulations can effectively target both the hair follicles and the skin. The influence of essential formulation aspects on the follicular and passive dermal penetration was investigated and key vehicle and excipient properties required for effective penetration were determined. However, investigations of excipients' influence showed that both the follicular and the passive dermal penetration efficacy can not only be improved using excipients but also impaired. At the first glance, the impairment of the penetration is nonsense and cannot be utilized for practical reasons. But in the case of NC formulations, this finding opens a perspective for a completely new approach. If one combines the knowledge about follicular and passive dermal penetration of NC formulations and considers the influence of excipients on both penetration pathways in complex, from now on, by the purposeful combination of NC and excipients, NC formulations with effective and selective penetration can be designed. Selective penetration means that the AC can be delivered only into the hair follicles, both into the hair follicles and into/through the skin, or only into/through the skin. With this, from now on, targeted dermal drug delivery with tailor-made NC formulations is possible. Based on the outcome of this thesis, such targeted dermal drug delivery is achieved by a tailored combination of NC and selected excipients:

When selective penetration into the hair follicles is desired, excipients are required that improve the follicular penetration and impair the passive dermal penetration. In this thesis, ethanol showed these effects. Selective penetration into the hair follicles is desirable, for example, for AC for targeted treatment of follicular diseases with many side effects – for example, finasteride or dutasteride. When both effective follicular and passive dermal penetration are desired, those excipients should be used which improve both pathways. This thesis detected this effect for propylene glycol. Penetration via both pathways should be strived, for example, for the treatment of dermal diseases when a formulation with both fast-acting (passive dermal penetration) and long-acting (deposition of the AC within the follicular depot and subsequent release into the viable skin layers) drug delivery properties

is required. When only passive dermal penetration is desired or when the follicular penetration route needs to be specifically blocked, excipients that improve the passive dermal penetration and impair the follicular penetration represent the best choice. In this thesis, glycerol showed these effects. Such excipients can be added to the formulations which are specifically designed to prevent the penetration of unwanted particles into the hair follicles, such as titanium dioxide particles in sunscreens or pollen allergens. This combined outcome proved the substantial importance of the two-in-one (follicular + dermal uptake) characterization of dermal formulations. Such two-in-one characterization is at present – worldwide – possible only with the novel method established in this thesis. With this, confirming the power and the meaning of the novel method and indicating that with this method, a new chapter in the penetration studies with dermal formulations is now started.

To **sum up,** in the third step, the second aim of this thesis was achieved. Before this thesis, it was believed that NC represent in theory an effective nanocarrier for dermal application of poorly soluble AC for hair follicle targeting and for passive dermal penetration. The findings of this thesis allow for a clear conclusion that NC represent in practice a highly effective nanocarrier for dermal drug delivery via hair follicle targeting and via passive dermal penetration. Before this thesis, dermal NC were formulated blind by transferring the theoretical assumptions and textbook knowledge to dermal NC, because no one investigated systematically how formulation aspects influence the penetration efficacy and what does it mean for the application use. This thesis bridged this gap and investigated systematically and elaborative the influences of essential core formulation aspects – vehicles and excipients – on the follicular and passive dermal penetration of NC formulations. The outcome of this thesis allows to skip the blind formulation of dermal NC and enables from now on to design effective tailor-made NC formulations for targeted dermal drug delivery by the purposeful selection of formulation components. With this, a conclusion can be drawn that NC formulations are not only a highly effective formulation principle, but represent the formulation of choice for effective dermal drug delivery with poorly soluble AC. From now on, based on the outcome of this thesis, the sound development of tailor-made dermal products with NC for targeted dermal drug delivery can be performed. These products would for sure enter the market in the next years.

4.2. References

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Chapter 5

Conclusion

Chapter 5

5.1. Conclusion

In this thesis, drug nanocrystals (NC) as a formulation principle for dermal drug delivery of poorly soluble active compounds (AC) were systematically investigated. This thesis aimed to establish the optimum method for the characterization of the follicular and the passive dermal penetration of dermal NC formulations and using this method to investigate the influences of essential formulation aspects on the penetration efficacy of dermal NC formulations into the hair follicles and into the skin. The prime focus was set on the investigations of the hair follicle targeting because, at present, it is believed that NC due to their special properties represent the formulation of choice for effective hair follicle targeting, but no systematic studies are available that proved (or even disproved) it. The outlined aims were realized in three steps:

First step: Prove

Before this thesis, the penetration efficacy of NC formulations was characterized using “universal” previously established methods. However, until now, no one questioned whether the “common” methods are sufficient especially for the characterization of dermal NC formulations. The **first step** of this thesis addressed this issue and investigated the follicular and passive dermal penetration of dermal NC formulations using the previously established method. From all available methods, differential skin stripping was identified as most suitable for the aims of this thesis. Thus, experiments of the first step were performed using differential skin stripping, i.e., the combination of cyanoacrylate skin surface stripping (CSSS) and classical tape stripping. Differential skin stripping failed to characterize both the follicular and the dermal uptake of NC formulations. With CSSS, follicular penetration could not be characterized at all because no proper quantification of the follicular uptake was possible using available analytical methods. With classical tape stripping, passive dermal penetration was characterized insufficient because only the penetration of the AC from NC into the upper layers of the stratum corneum (SC) was studied. Thus, the penetration into the actual targets of the NC formulations (and 99.9% of all dermal formulations worldwide), i.e., entire SC and viable skin layers, was ignored, making such penetration studies meaningless. It was concluded that classical tape stripping might be useful only for specific purposes, for example, to determine explicitly the reservoir capacity of the SC, and not as a stand-alone method for the characterization of the passive dermal penetration of dermal NC formulations. This thesis detected several further limitations of classical tape stripping regarding the outcome, the handling and the performance. With this, it was shown that each researcher

performing penetration experiments with dermal formulations should re-evaluate and critically question the value of classical tape stripping for penetration experiments.

The outcome of differential skin stripping led to the conclusion that this method did not represent the optimum method for the characterization of the follicular and passive dermal penetration of dermal NC formulations. Thus, another method needs to be established.

Second step: Optimize

In the **second step**, a new method was established. The novel method is based on the visualization of the follicular and passive dermal penetration using fluorescence microscopy. The prerequisite for the use of the novel method is that the AC is fluorescent, or special fluorescent dyes should be used. To perform the experiments with the novel method, the formulation should be applied onto the porcine ears skin, after the defined penetration time, the skin biopsies are punched, embedded and frozen. Subsequently, the hair follicle sections and skin sections are cut with the cryomicrotome and visualized with fluorescence microscopy. The follicular penetration efficacy is subsequently quantified by measuring the penetration depth of the AC directly from the fluorescence images. The passive dermal penetration efficacy is quantified using the method of digital image analysis with the software ImageJ established in this thesis. The novel method was conceptualized based on the elaborative analysis of the available methods for the determination of the follicular and the passive dermal penetration and on the outcome of the first step. Thus, it keeps the essential advantages of available methods and overcomes the disadvantages by optimized solutions. The most substantial benefit of the novel method is its “two-in-one” concept. It means that the dermal formulation should be applied only once on one skin area within one skin penetration model (porcine ear skin), and with only one method, both follicular and passive dermal penetration can be determined. This two-in-one concept is realized by the special embedding and cutting technique of the skin biopsies established in this thesis. This technique allows to prepare only one skin biopsy and to get proper hair follicle sections and skin sections. Thus, determining follicular and dermal uptake. With this, the novel method allows a direct combinability and comparability of the data about the follicular and passive dermal penetration of the dermal formulation because all conditions – skin area, treatment of the skin area, application of the formulation, incubation conditions – are equal for both the hair follicles and the skin. Thus, building the key benefit of the novel method. The visualization of the hair follicles and the skin with fluorescence microscopy allows a reliable discrimination between dermal and follicular uptake because one sees the hair follicles and the skin with own eyes. Thus, building the next unique and substantial advantage of the novel method. The skeptics might say that differential skin stripping can also determine both follicular and passive dermal penetration of the dermal formulation using one skin model and one skin area and discriminate between both penetration pathways. In general, yes, but i) this method was tested in the first step of this thesis, did not properly work out, and several

limitations were detected, and ii) the novel method allows for significantly more outcome with much less effort. Much less effort means that to perform penetration experiments with the novel method, only porcine ears, a freezer, a cryomicrotome and a fluorescence microscope are needed. It is often believed that the more complex and sophisticated the method and the more expensive and "fancy" the equipment is, the better is the outcome. The novel method established in this thesis destroyed this stereotype and showed clearly - no. Significantly more outcome means that since we "got eyes", the penetration fate of the dermally applied formulations can now be viewed from the new angle. The beauty of seeing the hair follicles, and with this, the follicular penetration allows to measure in µm how deep the formulation penetrated into the hair follicles. Thus, enabling mechanistic experiments to investigate the influence of formulation aspects – or many other factors – on the follicular penetration efficacy of dermal formulations. The beauty of seeing the skin, and with this, the passive dermal penetration coupled with digital image analysis with the ImageJ software established in this thesis represents the most precise characterization of the passive dermal penetration of a dermal formulation that can even be imagined and that exists at present. From now on, with only one tool, it can be determined how much AC is penetrated into the skin, how deep – either only into the upper layers of the SC, into the entire SC, or into the entire SC and viable skin layers – and how homogeneous the penetration is. Thus, the novel method provides a detailed time and space resolved characterization of the penetration fate of the dermal formulation. Further, the novel method provides meaningful "additional" information. For example, it allows to measure the thickness of the SC and to estimate the influence of a dermal formulation on its hydration state. Before this thesis, the determination of all the outlined parameters was only possible using the combination of different methods (or was not possible at all) and often also different skin models and different equipment. From now on, it is possible with only one tool, one skin model and one "equipment set". With this, the power and the value of the novel method can be best described with the slogan of one of the microscopes manufacturers – capture it all. With the novel method, each relevant penetration parameter is captured. Since the novel method is not formulation-related, it can be used for any type of dermal formulations. Such a method has, at present, no equivalent worldwide. Thus, the method established in this thesis opens a new chapter of sound, simple and smart penetration experiments with dermal formulations.

Third step: Realize

With the establishment of the novel method, the first aim of this thesis was achieved, and the prerequisite for sound penetration studies with NC formulations was fulfilled. Thus, in the **third step,** mechanistic penetration studies with NC formulations were performed using the novel method.

Before this thesis, it was believed that NC represent in theory a highly effective nanocarrier for dermal application of poorly soluble AC for hair follicle targeting and for passive dermal penetration, but this assumption was not yet proved. This thesis addressed this issue and showed that each NC formulation investigated in this thesis successfully delivered the AC into the hair follicles and into the skin. Thus, based on this thesis, from now on, it should be said – systematic studies proved that drug NC represent in practice a highly effective nanocarrier for dermal application of poorly soluble AC for both follicular and passive dermal uptake.

Before this thesis, no studies were available investigating the application use of NC. It means, until now, no one investigated how NC for dermal application should be formulated to achieve the most effective penetration into the hair follicles and into the skin and which formulation components need to be considered. With this, before this thesis, NC for dermal application were formulated blind because the influence of the formulation components on the penetration efficacy of dermal NC formulation was not yet investigated. This thesis bridged this gap and investigated systematically the influence of core formulation aspects – vehicle type and excipients – on the follicular and passive dermal penetration of NC formulations, and fixed the essential "key points" relevant for the application use.

Concerning **follicular penetration**, the influence of the formulation aspects on the hair follicle targeting was not yet investigated systematically for any type of nanocarriers. Previous studies focused primarily on particles as such and not on the application use of the particles. This thesis addressed this issue and investigated systematically the influence of vehicle type and excipients on the follicular uptake of NC formulations. Results showed that the penetration efficacy of the NC into the hair follicles was not affected by the different types of vehicles. As vehicles, different gels that varied in their viscosity, lipophilicity and polarity were evaluated. The results were explained by the thixotropic flow behaviour of obtained gels in combination with its application by mechanical massage. It was assumed that due to their thixotropic flow behaviour all gels liquified during the massage application. Thus, leading to comparable low viscosities at high shear rates and comparable diffusion coefficients for NC from all formulations. Thus, explaining similar follicular penetration of NC from all gels independent of other varied vehicle properties (lipophilicity, polarity). This finding means for the application use that for effective hair follicle targeting with NC, the key vehicle property is its thixotropic flow behaviour in combination with massage application. Thus, other properties of the vehicles that showed no influence on the hair follicle targeting with NC can be adjusted to the patients' needs – for example, skin type – and other requirements – for example, formulation stability. Excipients influenced the follicular penetration of NC tremendously. They improved or even impaired it across the previously postulated size-dependent rule and were able to overwrite the previously postulated size effect. The effects of excipients were explained by their influence on the hair structure and on the adhesion and adhesion

pattern of NC to the hair. Before this thesis, the state of the scientific opinion indicated that particles penetrate into the hair follicles according to the size-dependent ratchet mechanism. This thesis showed that the mechanism of the follicular penetration includes not only the size-dependent ratchet-driven transport but also two additional steps. Thereby, a three-step mechanism of follicular penetration was elaborated and established in this thesis. The elaborated three-step mechanism offers attractive and elegant opportunities for the application use of NC for hair follicle targeting. From now on, tailor-made NC formulations that improve or impair the follicular penetration can be "designed" by purposeful optimization of existing formulations by excipients. This approach – to optimize the existing formulation by excipients rather than develop a new one – saves both time and money. Further, understanding of the three-step mechanism allows "playing" with gained knowledge and trying new approaches that can be derived from the elaborated mechanism. Would the combination of excipients that improve all three steps represent the "superpower" formulation for the most effective hair follicle targeting? Would the combination of excipients that impair all three steps prevent the follicular penetration completely and be "the formulation of choice" for the prevention of follicular penetration? Which excipient has the most pronounced effect on each step? All outlined approaches represent the path in the future of the hair follicle targeting not only with NC but also with other nanocarriers.

Concerning **passive dermal penetration**, its efficacy was affected both by the properties of the vehicle and different excipients. The most effective passive dermal penetration of the AC from NC was achieved using vehicles and excipients with good skin hydrating properties. It means for the application use that the ability of the formulation to hydrate the SC was identified as a key formulation parameter influencing the passive dermal penetration of NC formulations. This ability, and therefore the efficacy of the passive dermal penetration, can be modulated using excipients. When the formulation cannot sufficiently hydrate the SC, excipients with skin hydrating properties should be used to improve the hydrating ability and thus the penetration efficacy. In contrast, when the passive dermal penetration should be hampered, excipients with dehydrating properties should be preferred. With this, the outcome of this thesis allows the design of tailor-made NC formulations with desired "extent" of the passive dermal penetration. All these findings were only possible with the novel method established in this thesis. With classical tape stripping in the first step, the passive dermal penetration was characterized only by the amount of the AC penetrated into the upper layers of SC and the generic penetration depth. Herewith, the outcome was strictly speaking meaningless. With the novel method, the passive dermal penetration was characterized by the amount of penetrated AC, by its penetration depth – allowing for a statement in which skin layer (upper layers of SC, entire SC, entire SC and viable skin layers) the AC penetrated in – and by the penetration homogeneity. With this, each penetration aspect was captured, and mechanistic elaborative penetration experiments were possible. The

visualization of the skin and the measurement of the SC thickness allowed the determination of the correlation between the penetration efficacy and the hydration state of the SC. Thus, allowing to explain achieved results mechanistically. With this, the substantial importance of a suitable method for penetration experiments for every dermal formulation was confirmed – before you start your penetration experiments, prove first whether your method is sufficient!

The two-in-one concept of the novel method allowed us to combine the results of the follicular and passive dermal penetration. It proves detecting that by a tailored combination of NC and excipients **targeted dermal drug delivery** with NC is possible. It means that by the purposeful choice of excipients with required properties, one can choose if the AC from NC should be delivered via a drug reservoir by deposition of NC into the hair follicles and/or via passive diffusion. Thus, opening up the novel approach of targeted dermal drug delivery with tailor-made NC formulations. This approach should now be implemented in the formulation development of highly effective dermal products with NC. These products would for sure enter the market in the next years.

5.2. Overall Conclusion

In this thesis, drug nanocrystals (NC) as a formulation approach for poorly soluble active compounds (AC) for the follicular and passive dermal penetration were systematically investigated. This thesis aimed to establish the optimum method for the determination of the follicular and passive dermal penetration efficacy of NC formulations and using this method to investigate the influences of essential formulation aspects on the penetration efficacy of NC formulations into the hair follicles and into the skin. The outlined aims were realized in three steps.

The **first step** investigated whether the previously established common "universal" methods are sufficient to characterize the penetration efficacy of dermal NC formulations. From all available methods, differential stripping was identified as most suitable for the aims of this thesis. Thus, in the first step, the influences of core formulation aspects on the follicular and passive dermal uptake of dermal NC formulations were investigated using differential skin stripping. This method was found to be insufficient to characterize the follicular and dermal penetration of dermal NC formulations. Several meaningful disadvantages were detected that limited the outcome, the handling and the performance substantially. Thus, it was concluded that for the sound characterization of the follicular and dermal uptake of dermal NC formulations, another method needs to be established.

Thus, in the **second step**, a novel "two-in-one" method was established. The novel method is based on the visualization of the penetration into the hair follicles and into the skin using fluorescence microscopy and subsequent quantification of the penetration efficacy. To perform the experiments with the novel method, the dermal formulations are applied onto the porcine ears skin, after the defined penetration time, skin biopsies are punched, embedded and frozen. Subsequently, the biopsies are cut with the cryomicrotome to hair follicle sections and skin sections. The hair follicles and the skin are then visualized using fluorescence microscopy. The quantification of the follicular penetration is carried out by measuring the penetration depth of NC in µm directly from fluorescence images, whereas for quantification of the passive dermal penetration, a method for digital image analysis with the software ImageJ was established in this thesis. The novel method represents the unique "two-in-one" technique allowing for investigation of the follicular and the passive dermal penetration of one formulation using only one skin penetration model and within the skin penetration model only one skin area. Consequently, all conditions are keeping equal for both targets enabling a direct combinability and comparability of the penetration efficacy of one dermal formulation into the hair follicles and into the skin. Thus, allowing for the "two-in-one" characterization. The novel method provides a detailed time and space resolved determination of the penetration fate of a dermal formulation. With this, it represents a sound, reliable and universal tool for smart characterization of the follicular and passive dermal penetration of dermal formulations that overcomes all significant

disadvantages of conventional methods and provides optimized solutions. Thus, from now on, a new chapter in penetration experiments with dermal formulations is opened.

Using the novel method, in the **third step**, the penetration of dermal NC formulations into the hair follicles and the skin was studied, and the influences of essential formulation aspects on the follicular and passive dermal uptake of NC formulations were detailed and mechanistically investigated.

This thesis demonstrated by systematic studies that NC represent not only in theory but also in practice a highly effective nanocarrier for hair follicle targeting and for passive dermal penetration. Each in this thesis investigated NC formulation delivered the AC successfully into the hair follicles and into the skin.

The formulation aspects influenced the penetration efficacy tremendously. Concerning follicular penetration, the efficacy of the hair follicle targeting with NC was not affected by the type and properties of the vehicle, which in this thesis represented either aqueous nanosuspension, or oleogels, or hydrogels that varied in their viscosity and in the type and concentration of the gelling agent. In contrast, the follicular uptake was strongly influenced by excipients. It has been demonstrated that excipients can both improve and impair the follicular penetration efficacy of NC formulations. These effects were explained by a three-step mechanism of follicular penetration that was investigated and established in this thesis.

Concerning passive dermal penetration of NC formulations, its efficacy was affected both by the properties of the vehicles and excipients. The most effective passive dermal penetration of the AC from NCs was achieved using vehicles and excipients with good skin hydrating properties, whereas excipients that dehydrate the skin hampered the penetration efficacy. Thus, the ability of the formulation to hydrate the SC was identified as a key formulation parameter influencing the passive dermal penetration of NC formulations.

The two-in-one characterization of dermal NC formulations with the novel method enabled the combination of the results of the follicular and passive dermal penetration efficacy. It allowed to detect that tailored combinations of NC and excipients can be used for targeted dermal drug delivery. It means that by a purposeful choice of excipients, one can choose if the AC from NC should be delivered as a reservoir by deposition of NC within the hair follicles, and/or via passive diffusion. Thus, from now on, by tailored optimization of formulation components, tailor-made NC formulations for effective hair follicle targeting, effective passive dermal penetration and targeted dermal drug delivery can be designed. This approach – tailored optimization of NC formulations with formulation components – should now be realized in the development of highly effective dermal products with the drug NC, that would for sure enter the market in the next years.

Chapter 6

Zusammenfassung

Chapter 6

6.1. Zusammenfassung

In dieser Arbeit wurden Wirkstoff-Nanokristalle (NC) als Formulierungsprinzip für die dermale Applikation schwerlöslicher Wirkstoffe (WS) systematisch untersucht. Das Ziel dieser Arbeit war es, die optimale Methode zur Charakterisierung der follikulären und der passiven dermalen Penetration von dermalen NC-Formulierungen zu etablieren und mit dieser Methode die Einflüsse wesentlicher Formulierungsaspekte auf die Effektivität der Penetration von dermalen NC-Formulierungen in die Haarfollikel und in die Haut zu untersuchen. Das Hauptaugenmerk wurde auf die Untersuchungen des Haarfollikel-Targetings mit NC-Formulierungen gelegt, da derzeit angenommen wird, dass NC aufgrund ihrer besonderen Eigenschaften die Formulierung der Wahl für effektives Haarfollikel-Targeting darstellen, jedoch liegen derzeit keine systematischen Studien vor, die dies belegen (oder auch widerlegen). Die gesetzten Ziele wurden in drei Schritten realisiert:

Erster Schritt: Überprüfe

Vor dem Anfertigen dieser Arbeit wurde die Penetration der NC-Formulierungen in die Haarfollikel und in die Haut mit bereits etablierten "universellen" Methoden charakterisiert. Bisher wurde jedoch nicht hinterfragt, inwiefern diese „gängigen“ Methoden speziell für NC-Formulierungen geeignet sind und welche Methode die optimale Methode darstellt. Der **erste Schritt** dieser Arbeit widmete sich dieser Frage und untersuchte die follikuläre und passive dermale Penetration von dermalen NC-Formulierungen mit der bereits etablierten Methode des differenziellen Abrissverfahrens, d.h. der Kombination von Cyanacrylatabrissmethode und klassischer Abrissmethode. Die Ergebnisse zeigten, dass differentielle Abrissmethode für die Charakterisierung der follikulären sowie dermalen Aufnahme der dermalen NC-Formulierungen nicht geeignet ist. Die follikuläre Penetration konnte gar nicht charakterisiert werden, weil die Quantifizierung der follikulären Aufnahme mit den verfügbaren analytischen Methoden nicht möglich war. Die passive dermale Penetration wurde nur unzureichend charakterisiert, weil nur die Penetration des WS von NC in die oberen Schichten des Stratum corneums (SC) untersucht werden konnte. Somit wurde die Penetration in die eigentlichen Targets der NC-Formulierungen (und 99,9 % aller dermalen Formulierungen weltweit) – das ganze SC und die lebenden Hautschichten – ignoriert, was solche Penetrationsstudien redundant macht. Dies führte zu der eindeutigen Schlussfolgerung, dass die klassische Abrissmethode nur für bestimmte Zwecke verwendet werden kann, zum Beispiel zur Bestimmung der Reservoirkapazität des SC, und nicht als eigenständige Methode zur Charakterisierung der passiven dermalen Penetration von dermalen NC-Formulierungen. In dieser Arbeit wurden einige weitere Einschränkungen des klassischen

Abrissmethode hinsichtlich der Ergebnisse, der Handhabung sowie der Durchführung festgestellt. Diese Einschränkungen zeigen, dass jeder Forscher, der Penetrationsuntersuchungen mit dermalen Formulierungen durchführt, die Bedeutung sowie den Wert der differentiellen Abrissmethode für Penetrationsuntersuchungen re-evaluieren und kritisch hinterfragen muss.

Die Ergebnisse der mit der differentiellen Abrissmethode durchgeführten Penetrationsuntersuchungen führten zu der Schlussfolgerung, dass diese Methode nicht die optimale Methode für die Charakterisierung der follikulären und passiven dermalen Penetration von dermalen NC-Formulierungen darstellt. Daher musste eine andere Methode etabliert werden.

Zweiter Schritt: Optimiere

Im **zweiten Schritt** wurde eine neue Methode etabliert. Die Voraussetzung für die Anwendung der neuen Methode ist, dass der WS fluoreszierend ist, alternativ können spezielle Fluoreszenzfarbstoffe verwendet werden. Um die Experimente mit der neuen Methode durchzuführen, wird die Formulierung auf die Haut von Schweineohren aufgetragen, nach der definierten Penetrationszeit werden die Hautbiopsien gestanzt, eingebettet und eingefroren. Anschließend werden die Haarfollikelschnitte und Hautschnitte mit dem Kryomikrotom geschnitten und mit der Fluoreszenzmikroskopie visualisiert. Die Quantifizierung der follikulären Penetration erfolgt durch die Messung der Eindringtiefe des WS in µm direkt von den Fluoreszenzbildern. Die Quantifizierung der passiven dermalen Penetration erfolgt mit der digitalen Bildanalyse mit der Software ImageJ, die in dieser Arbeit ausgearbeitet sowie etabliert wurde. Die neuartige Methode wurde auf der Basis der ausführlichen Analyse der verfügbaren Methoden zur Bestimmung der follikulären und der passiven dermalen Penetration sowie auf der Basis der Ergebnisse des ersten Schritts sorgfältig konzipiert. Somit behält die neuartige Methode die wichtigsten Vorteile der verfügbaren Methoden bei, verbessert sie und überwindet die Nachteile. Der bedeutendste Vorteil der neuen Methode ist ihr "Zwei-in-Eins"-Konzept. Das bedeutet, dass die dermale Formulierung nur einmal auf ein Hautareal innerhalb eines Hautpenetrationsmodells (Schweineohrhaut) aufgetragen werden muss, und mit nur einer Methode sowohl die follikuläre als auch die passive dermale Penetration bestimmt werden kann. Dieses Zwei-in-Eins-Konzept wird durch die in dieser Arbeit etablierte spezielle Einbettungs- und Schneidetechnik der Hautbiopsien realisiert. Diese Technik erlaubt es, mit nur einer Hautbiopsie ordentliche Haarfollikelschnitte und Hautschnitte zu erhalten und damit sowohl die follikuläre als auch die dermale Aufnahme zu bestimmen. Damit ermöglicht die neuartige Methode eine direkte Kombinierbarkeit und Vergleichbarkeit der Daten über die follikuläre und passive dermale Penetration der dermalen Formulierung, da alle Bedingungen für beide Ziele - Hautfläche, Behandlung der Hautfläche, Applikation der Formulierung, Inkubationsbedingungen - gleich sind. Daraus ergibt sich der entscheidende Vorteil der neuartigen Methode. Die Visualisierung der Haarfollikel und der Haut mit der Fluoreszenzmikroskopie ermöglicht eine zuverlässige Unterscheidung zwischen dermaler und

follikulärer Aufnahme – weil man die Haarfollikel und die Haut mit eigenen Augen sieht – und bildet den nächsten einzigartigen und bedeutenden Vorteil der neuartigen Methode. Die Skeptiker mögen sagen, dass man mit der differentiellen Abrissmethode ebenfalls mit einem Hautmodell und einem Hautareal sowohl die follikuläre als auch die passive dermale Penetration der dermalen Formulierung bestimmen und zwischen beiden Penetrationswegen unterscheiden kann. Generell, ja – aber – i) diese Methode wurde im ersten Schritt dieser Arbeit getestet und wurde als unzureichend bewertet, weil mehrere Einschränkungen festgestellt wurden und ii) die neuartige Methode ermöglicht deutlich mehr Ergebnisse mit deutlich weniger Aufwand. Deutlich weniger Aufwand bedeutet – um Penetrationsexperimente durchzuführen, werden nur Schweineohren, ein Gefrierschrank, ein Kryomikrotom und ein Fluoreszenzmikroskop benötigt. Es existiert derzeit eine verbreitete Meinung, dass das Ergebnis eines Experimentes umso besser ist, je komplexer und anspruchsvoller die Methode und je teurer, "cooler" und "schicker" die Ausrüstung ist. Die in dieser Arbeit etablierte neuartige Methode zeigt eindeutig – nein. Deutlich mehr Ergebnis bedeutet – seitdem wir "Augen bekommen" haben, kann die Penetration der dermal applizierten Formulierungen nun aus dem neuen Blickwinkel betrachtet werden. Die Möglichkeit, die Haarfollikel und damit die follikuläre Penetration zu sehen, erlaubt es, eindeutig zu bestimmen und in µm zu messen, wie tief die Formulierung in die Haarfollikel eingedrungen ist. Somit können mechanistische Experimente durchgeführt werden, um den Einfluss von Formulierungsaspekten – oder vielen anderen Faktoren – auf die follikuläre Penetrationseffizienz von dermalen Formulierungen zu untersuchen. Die Möglichkeit, die Haut und damit die passive dermale Penetration zu sehen – gekoppelt mit der digitalen Bildanalyse mit der in dieser Arbeit etablierten Software ImageJ – stellt die effektivste und vollständigste Charakterisierung der passiven dermalen Penetration einer dermalen Formulierung dar, die man sich überhaupt vorstellen kann und die derzeit weltweit existiert. Ab sofort, kann mit nur einem Tool bestimmt werden, wie viel WS in die Haut penetriert ist, wie tief – entweder nur in die oberen Schichten des SC, in den gesamten SC oder in den gesamten SC und die lebenden Hautschichten – und wie homogen die Penetration ist. Damit liefert die neuartige Methode eine detaillierte zeit- und ortsaufgelöste Charakterisierung des Penetrationsverhaltens einer dermalen Formulierung. Darüber hinaus liefert die neuartige Methode aussagekräftige "zusätzliche" Informationen, z. B. erlaubt die Messung der Dicke des SC und damit die Abschätzung des Einflusses einer dermalen Formulierung auf dessen Hydratationszustand. Bevor diese Arbeit geschrieben wurde, war die Bestimmung aller beschriebenen Parameter nur durch die Kombination unterschiedlicher Methoden (oder eben gar nicht) und oft auch verschiedener Hautmodelle und verschiedener Geräte möglich. Jetzt ist sie mit nur einem Tool, einem Hautmodell sowie einem "Gerätesatz" möglich. Damit lässt sich die Leistung und der Wert der in dieser Arbeit etablierten neuartigen Methode am besten mit dem Slogan eines der Mikroskopen-Herstellers beschreiben – capture it all (engl. “erfasse alles”). Mit der neuartigen Methode wird jeder relevante

Penetrationsparameter erfasst. Da die neuartige Methode nicht formulierungsspezifisch ist, kann sie für jede Art von dermalen Formulierungen verwendet werden. Eine solche Methode hat zurzeit weltweit kein Analogon. Die in dieser Arbeit etablierte Methode ermöglicht es somit, ein neues Kapitel fundierter, einfacher sowie smarter Penetrationsuntersuchungen mit dermalen Formulierungen aufzuschlagen.

Dritter Schritt: Realisiere

Mit der Etablierung der neuartigen Methode wurde das erste Ziel dieser Arbeit erreicht und die Voraussetzung für fundierte Penetrationsuntersuchungen mit NC-Formulierungen erfüllt. So wurden im dritten Schritt mechanistische Penetrationsstudien mit NC-Formulierungen mit der neuartigen Methode durchgeführt. Der Einfluss wesentlicher Formulierungsaspekte auf die follikuläre und passive dermale Penetration von dermalen NC-Formulierungen wurde untersucht und die wesentlichen, für den Anwendungseinsatz relevanten Kernpunkte wurden festgelegt.

Vor dem Anfertigen dieser Arbeit wurde angenommen, dass NC in der Theorie einen hocheffektiven Nanocarrier für die dermale Applikation schwerlöslicher WS für das Haarfollikel-Targeting und für die passive dermale Penetration darstellt. Allerdings wurde diese Annahme noch nicht durch systematische Studien bewiesen. Die vorliegende Arbeit zeigt, dass jede in dieser Arbeit untersuchte NC-Formulierung den WS erfolgreich in die Haarfollikel und in die Haut transportiert. Somit kann auf der Grundlage dieser Arbeit festgehalten werden, dass systematische Studien bewiesen haben, dass NC in der Praxis einen hocheffektiven Nanocarrier für die dermale Applikation schwerlöslicher WS sowohl für die follikuläre als auch für die passive dermale Aufnahme darstellen.

Vor dieser Arbeit gab es keine Studien, die die Anwendungsnutzung von dermalen NC-Formulierungen untersuchten. Das bedeutet, dass bisher noch nicht untersucht wurde, wie NC für die dermale Anwendung formuliert werden sollten, um eine möglichst effektive Penetration in die Haarfollikel und in die Haut zu erzielen, und welche Formulierungsaspekte dabei zu berücksichtigen sind. Somit wurden vor dieser Arbeit NC für die dermale Anwendung blind formuliert, weil der Einfluss der Formulierungsaspekte auf die Penetrationswirksamkeit der dermalen NC-Formulierungen noch nicht untersucht wurde. Die vorliegende Arbeit schloss diese Lücke und untersuchte systematisch den Einfluss zentraler Formulierungsaspekte – der Vehikel und der Excipients – auf die follikuläre und passive dermale Penetration von NC-Formulierungen und legte die wesentlichen, für den Anwendungszweck relevanten Kernpunkte fest.

Bezüglich der **follikulären Penetration** wurde der Einfluss der Formulierungsaspekte auf die Effektivität der follikulären Penetration noch nicht systematisch untersucht und zwar für keine Art von Nanocarrier. Die bisher durchgeführten Untersuchungen konzentrierten sich in erster Linie auf die Partikel als solche und nicht auf den Anwendungszweck dieser Partikel.

Die vorliegende Arbeit nahm sich dieses Themas an und untersuchte systematisch den Einfluss des Vehikeltyps sowie Excipients auf die follikuläre Aufnahme von NC-Formulierungen. Ergebnisse zeigten, dass die Penetrationseffektivität der NC in die Haarfollikel durch die unterschiedlichen Vehikeltypen nicht beeinflusst wurde. Als Vehikel wurden verschiedene Gele untersucht, die sich in ihrer Viskosität, Lipophilie sowie Polarität unterschieden. Die Ergebnisse wurden durch das thixotrope Fließverhalten der untersuchten Gele in Kombination mit der Applikation durch mechanische Massage erklärt. Es wurde angenommen, dass sich alle Gele aufgrund ihres thixotropen Fließverhaltens während der Massageapplikation verflüssigten. Dies führte zu vergleichbar niedrigen Viskositäten bei hohen Scherraten und vergleichbaren Diffusionskoeffizienten für NC aus allen Formulierungen. Dies erklärt die ähnliche follikuläre Penetration von NC aus allen Gelen unabhängig von anderen variierten Vehikeleigenschaften (Lipophilie, Polarität). Diese Erkenntnis bedeutet für die Anwendung, dass für ein effektives Haarfollikel-Targeting mit NC die "Schlüssel"-Vehikel-Eigenschaft ein thixotropes Fließverhalten in Kombination mit der Massage-Applikation ist. Somit können andere Eigenschaften der Vehikel, die keinen Einfluss auf das Haarfollikel-Targeting mit NC zeigten, auf die Bedürfnisse der Patienten – zum Beispiel, der Hauttyp – sowie andere Anforderungen – zum Beispiel, Stabilität der Formulierung – abgestimmt werden. Die Excipients beeinflussten die follikuläre Penetration von NC wesentlich – sie verbesserten oder sogar verschlechterten die Penetrationseffizienz sogar über die zuvor postulierte Größen-Regel hinaus und waren in der Lage, den Größeneffekt zu überschreiben. Die Effekte der Excipients wurden durch ihren Einfluss auf die Haarstruktur und damit auf die Adhäsion und das Adhäsionsmuster von NC an den Haaren erklärt. Der aktuelle Stand der Wissenschaft vor dieser Arbeit legte nahe, dass Partikel nach dem größenabhängigen Ratschen-Mechanismus in die Haarfollikel eindringen. In dieser Arbeit wurde gezeigt, dass der Mechanismus der Follikelpenetration neben dem größenabhängigen ratschen-getriebenen Transport noch zwei weitere Schritte beinhaltet. Somit wurde in dieser Arbeit ein dreistufiger Mechanismus der Follikelpenetration erarbeitet und etabliert. Der etablierte Drei-Schritt-Mechanismus bietet attraktive und elegante Möglichkeiten für den Einsatz von NC für das Haarfollikel-Targeting – und Partikeln im Allgemeinen – denn von jetzt an können maßgeschneiderte NC-Formulierungen mit gewünschten Eigenschaften (Verbesserung/Verhinderung der Follikelpenetration) durch gezielte Optimierung bestehender Formulierungen durch Excipients konzipiert werden. Damit kann eine bestehende Formulierung optimiert werden und nicht eine komplett neue Formulierung entwickelt werden, und dieser Ansatz spart Zeit und Geld. Das Verständnis des Drei-Stufen-Mechanismus erlaubt es, mit den gewonnenen Erkenntnissen zu „spielen“ und neue Ansätze auszuprobieren, die sich aus dem etablierten Mechanismus ableiten lassen. Wäre die Kombination von Excipients, die alle drei Schritte verbessern, die Superpower-Formulierung für das effektivste Haarfollikel-Targeting? Würde die Kombination von Excipients, die alle drei Schritte verschlechtern, die Follikelpenetration komplett verhindern und damit

die Formulierung der Wahl für die Verhinderung der Follikelpenetration darstellen? Welcher Excipient hat den stärksten Effekt auf die einzelnen Schritte? Alle skizzierten Ansätze stellen den Weg in die Zukunft des Haarfollikel-Targetings mit NC – und mit Partikeln im Allgemeinen – dar.

Bezüglich der **passiven dermalen Penetration** wurde die Penetrationseffizienz sowohl durch die Eigenschaften des Vehikels als auch durch verschiedene Excipients beeinflusst. Die effektivste passive dermale Penetration des WS von NC wurde durch die Verwendung von Vehikeln und Excipients mit guten hautfeuchtenden Eigenschaften erreicht. Das bedeutet für die Anwendung, dass die Fähigkeit der Formulierung, das SC zu hydratisieren, als wesentlicher Formulierungsparameter identifiziert wurde, der die passive dermale Penetration von NC-Formulierungen beeinflusst. Diese Fähigkeit, und damit die Wirksamkeit der passiven dermalen Penetration, kann durch Excipients moduliert werden. Wenn die Formulierung das SC nicht ausreichend hydratisieren kann, sollten Excipients mit hauthydratisierenden Eigenschaften verwendet werden, um die Hydratisierung der Haut und damit die Penetrationswirksamkeit zu verbessern. Im Gegensatz dazu sollten, wenn die passive dermale Penetration verhindert werden soll, Excipients mit dehydratisierenden Eigenschaften verwendet werden. Damit erlauben die Ergebnisse dieser Arbeit, maßgeschneiderte NC-Formulierungen mit gewünschtem Ausmaß der passiven dermalen Penetration zu konzipieren. All diese Erkenntnisse sind nur mit der in dieser Arbeit etablierten neuartigen Methode möglich gewesen. Mit der klassischen Abrissmethode im ersten Schritt dieser Arbeit wurde die passive dermale Penetration nur durch die Menge des in die oberen Schichten des SC penetrierten WS und die generische Eindringtiefe charakterisiert. Damit war das Ergebnis nicht aussagekräftig und streng genommen bedeutungslos. Mit der neuartigen Methode wurde die passive dermale Penetration durch die Menge des penetrierten WS, durch seine Eindringtiefe – und damit war eine eindeutige Aussage möglich, in welche Hautschicht (obere Schichten des SC, gesamtes SC, gesamtes SC und lebensfähige Hautschichten) der WS penetriert ist – und durch die Penetrationshomogenität charakterisiert. Damit wurde jeder Penetrationsaspekt erfasst und mechanistische, fundierte sowie gründliche Penetrationsuntersuchungen waren möglich. Die Visualisierung der Haut und die Messung der SC-Dicke erlaubten es, die Korrelation zwischen der Penetrationseffektivität und dem Hydratationszustand das SC zu bestimmen und damit die erzielten Penetrationsergebnisse mechanistisch zu erklären. Damit bestätigt sich die essentielle Bedeutung einer geeigneten Methode für Penetrationsuntersuchungen für jede dermale Formulierung – bevor man die Penetrationsuntersuchungen startet, muss unbedingt geprüft werden, ob die Methode geeignet ist!

Das Zwei-in Eins-Konzept der neuartigen Methode erlaubte es uns, die Ergebnisse der follikulären und passiven dermalen Penetration zu kombinieren. Solch eine komplette Charakterisierung erlaubte es zu erkennen, dass durch die maßgeschneiderte Kombination von NC und Excipients eine gezielte dermale Wirkstoffabgabe mit NC möglich ist. Dies bedeutet, dass durch die gezielte Auswahl von Excipients mit

gewünschten Eigenschaften bestimmt werden kann, ob der WS von NC als Wirkstoffreservoir durch Deponierung von NC in den Haarfollikeln und/oder durch passive Diffusion abgegeben werden soll. Damit eröffnet sich der neuartige Ansatz der gezielten dermalen Wirkstoffabgabe mit maßgeschneiderten NC-Formulierungen. Dieser Ansatz soll nun in der Formulierungsentwicklung von hochwirksamen dermalen Produkten mit NC umgesetzt werden. Diese Produkte würden mit Sicherheit in den nächsten Jahren auf den Markt kommen.

6.2. Allgemeine Zusammenfassung

In dieser Arbeit wurden Wirkstoff-Nanokristalle (NC) als Formulierungsansatz für schwerlösliche Wirkstoffe (WS) für die follikuläre und passive dermale Penetration systematisch untersucht. Das Ziel dieser Arbeit war es, die optimale Methode zur Bestimmung der follikulären und passiven dermalen Penetrationseffizienz von NC-Formulierungen zu etablieren und mit dieser Methode die Einflüsse wesentlicher Formulierungsaspekte auf die Penetrationswirksamkeit von NC-Formulierungen in die Haarfollikel und in die Haut zu untersuchen. Diese Ziele wurden in drei Schritten realisiert.

Der **erste Schritt** untersuchte, inwiefern die bisher etablierten "universellen" Methoden ausreichen, um die Penetrationseffektivität von dermalen NC-Formulierungen zu charakterisieren. Für die Ziele dieser Arbeit wurde von allen verfügbaren Methoden die differentielle Abrissmethode als am besten geeignet identifiziert. Somit wurden im ersten Schritt die Einflüsse wesentlicher Formulierungsaspekte auf die follikuläre und passive dermale Penetration von dermalen NC-Formulierungen mittels differentieller Abrissmethode untersucht. Diese Methode erwies sich als unzureichend, um die follikuläre und dermale Penetration von dermalen NC-Formulierungen zu charakterisieren. Es wurden mehrere bedeutende Nachteile detektiert, die das Ergebnis, die Handhabung sowie die Durchführung erheblich einschränkten. Dies führte zu der Schlussfolgerung, dass für eine fundierte Charakterisierung der follikulären und dermalen Aufnahme von dermalen NC-Formulierungen eine andere Methode entwickelt werden muss.

Deswegen wurde im **zweiten Schritt** eine neuartige „Zwei-in-Eins“-Methode zur fundierten Charakterisierung der follikulären und passiven dermalen Penetration von NC-Formulierungen etabliert. Die neue Methode beruht auf der Visualisierung der Wirkstoffpenetration in die Haarfollikel sowie in die Haut mit Fluoreszenzmikroskopie und anschließender Quantifizierung der Penetrationseffizienz. Um die Experimente mit der neuen Methode durchzuführen, werden die dermalen Formulierungen erst auf die Haut von Schweineohren aufgetragen. Nach der definierten Penetrationszeit werden Hautbiopsien gestanzt, eingebettet und eingefroren. Anschließend werden die Biopsien mit dem Kryomikrotom in Haarfollikel- und Hautschnitte geschnitten. Die Haarfollikel und die Haut werden mittels Fluoreszenzmikroskopie visualisiert. Die Quantifizierung der follikulären Penetration erfolgt durch Messung der Eindringtiefe von NC in µm direkt von Fluoreszenzbildern, während für die Quantifizierung der passiven dermalen Penetration in dieser Arbeit eine Methode zur digitalen Bildanalyse mit der Software ImageJ etabliert wurde. Die neuartige Methode stellt eine einzigartige "Zwei-in-Eins"-Technik dar, die es erlaubt, die follikuläre und die passive dermale Penetration einer Formulierung mit nur einer Methode, einem Hautpenetrationsmodell und nur einem Hautareal zu untersuchen. Folglich werden alle Bedingungen für beide Targets gleich gehalten, was eine direkte Kombinierbarkeit und Vergleichbarkeit der Penetration in die Haarfollikel und in die Haut

ermöglicht. So wird eine vollständige komplette "Zwei-in-Eins"-Charakterisierung einer dermalen Formulierung erzielt. Die neue Methode ermöglicht eine detaillierte zeit- sowie ortsaufgelöste Bestimmung des Penetrationsverhaltens einer dermalen Formulierung. Damit stellt sie ein solides, zuverlässiges und universelles Tool zur gründlichen Charakterisierung der follikulären und passiven dermalen Penetration von dermalen Formulierungen dar, das die wesentlichen Nachteile herkömmlicher Methoden überwindet. Damit wird von nun an ein neues Kapitel bei Penetrationsuntersuchungen mit dermalen Formulierungen aufgeschlagen.

Mit Hilfe der neuartigen Methode wurde im **dritten Schritt** die Penetration von dermalen NC-Formulierungen in die Haarfollikel und die Haut getestet. Die Einflüsse wesentlicher Formulierungsaspekte auf die follikuläre und passive dermale Aufnahme von NC-Formulierungen wurden detailliert und mechanistisch untersucht. In dieser Arbeit wurde durch systematische Studien bewiesen, dass NC nicht nur in der Theorie, sondern auch in der Praxis einen hochwirksamen Nanocarrier für das Haarfollikel-Targeting sowie für die passive dermale Penetration darstellen. Jede in dieser Arbeit untersuchte NC-Formulierung transportierte den WS erfolgreich in die Haarfollikel und in die Haut.

Die Formulierungsaspekte beeinflussten die Penetrationseffizienz erheblich. Bezüglich der follikulären Penetration, so wurde die Wirksamkeit des Haarfollikel-Targetings mit NC nicht von der Art und den Eigenschaften des Vehikels beeinflusst. Im Gegensatz dazu wurde die follikuläre Aufnahme wesentlich durch Excipients beeinflusst. Es wurde gezeigt, dass Excipients die Effektivität der follikulären Penetration von NC-Formulierungen sowohl verbessern als auch verschlechtern können. Diese Effekte wurden durch einen dreistufigen Mechanismus der follikulären Penetration erklärt, der in dieser Arbeit ausgearbeitet sowie etabliert wurde.

Die passive dermale Penetration von NC-Formulierungen wurde sowohl durch die Eigenschaften der Vehikel als auch durch die Excipients beeinflusst. Die effektivste passive dermale Penetration des WS von NC wurde mit Vehikeln und Excipients mit ausgeprägter feuchtigkeitsspendender Wirkung erzielt, während Excipients, die die Haut austrocknen, die Penetrationseffektivität hinderten. Somit wurde die Fähigkeit der Formulierung, den SC zu hydratisieren, als ein Schlüsselparameter der Formulierung identifiziert, der die passive dermale Penetration von NC-Formulierungen beeinflusst.

Die vollständige Zwei-in-Eins-Charakterisierung der dermalen NC-Formulierungen mit der neuen Methode ermöglichte die Kombination der Ergebnisse der follikulären und passiven dermalen Penetration. Somit können von nun an, durch gezielte Optimierung der Formulierungskomponenten, maßgeschneiderte NC-Formulierungen für ein effektives Haarfollikel-Targeting, eine effektive passive dermale Penetration und eine gezielte dermale Wirkstoffabgabe konzipiert werden. Dieser Ansatz soll nun in der Entwicklung von hochwirksamen dermalen Produkten mit NC umgesetzt werden.

Chapter 7

Outlook

Chapter 7

7.1. Outlook

The outcome of this thesis offers several new issues to struggle with and several new roads to drive forward:

Novel method for sound penetration studies

This thesis established the method for digital image analysis with the software ImageJ for sound characterization of the passive dermal penetration efficacy of dermal formulations using fluorescence images. The efficacy of the hair follicle targeting was determined in this thesis by measuring the penetration depth of NC into the hair follicles in µm directly from the fluorescence images without additional digital image processing. The penetration depth of NC into the hair follicles already represents a sufficient characterization of the follicular penetration of the dermal formulation. However, the characterization of the follicular penetration by more than one characteristic parameter – for example, to determine not only the penetration depth but also the amount of penetrated AC – would characterize the follicular penetration even more precise and provide more detailed information about the ability of different formulations to deliver the AC into the hair follicles. Such a method that can determine both – follicular penetration depth and the amount of penetrated AC – does not yet exist. The method of digital image analysis for the hair follicle images that would be able to determine both the follicular penetration depth and the amount of penetrated AC would represent the perfect optimal solution. However, establishing a method of digital image analysis for hair follicles is not as easy as it sounds. The hair follicles exhibit an extremely pronounced autofluorescence, with both intraindividual and interindividual variations [1]. This autofluorescence is caused by the presence of different sebum components and bacteria within the hair follicle and, additionally, by the refraction of the light by the hair itself [1]. Thus, special threshold algorithms are required to separate the autofluorescence of the hair follicle from the fluorescence of the AC within the hair follicle. Threshold methods developed in this thesis for the elimination of the autofluorescence of the skin are not feasible for the hair follicles. Thus, the establishment of the method of digital image analysis of hair follicle images with the software ImageJ represents a highly challenging task. But if it works and the method would be established that can determine both the follicular penetration depth and the amount of the penetrated AC – then the outcome would outweigh the effort.

NC for effective hair follicle targeting

This thesis proved by systematic studies that NC represent a highly effective nanocarrier for effective hair follicle targeting. Each investigated formulation successfully delivered the NC into the infundibulum of the hair follicles. Thus, NC built within the infundibulum the intrafollicular depot. Now it would be interesting to investigate the NC depot build within the hair follicles. How long do NC stay and remain within the hair follicle? How do transfollicular penetration of NC and/or dissolved AC from NC behave? Is it possible to trigger/accelerate/control the transfollicular penetration?

Comparing all formulations investigated in this thesis, the hair follicle region targeted by all investigated formulations was the infundibulum. The deepest penetration of NC into the hair follicles was approximately 35% of the ordinary hair follicle lengths. It corresponds still to the region of the lower infundibulum. However, it was initially expected that NC can also reach deeper regions of the hair follicles, for example, the hair bulb. Now the question arises – why did NC not penetrate deeper than the region of the lower infundibulum? This is a struggling question that cannot be answered by the currently available state of knowledge. However, several theories can be suggested. One theory is that the penetration of NC deeper than the lower infundibulum is generally anatomically impossible. It might be assumed that the hair follicle provides a kind of narrowing after the lower infundibulum, and this narrowing might prevent the penetration of NC into deeper follicular regions. Another theory is that the hair shaft within the hair follicle localized deeper than the lower infundibulum does not provide the zig-zag structure required for the ratchet mechanism. With this, the ratchet mechanism cannot occur at all or occurs to a lower extent. Thus, the penetration into deeper follicular regions is impaired. However, outlined theories represent only theoretical assumptions. Thus, the issue should be addressed scientifically – is it possible to transport NC or particles in general deeper into the hair follicles than it was achieved in this thesis?

The next important outlook was already outlined in the Summary and Discussion section. The path of the regulation of the follicular penetration by excipients and the three-step mechanism established in this thesis should be followed. Different approaches derived from this mechanism should be tested. Firstly, it should be proved by systematic studies whether the “excipients effect” is also applicable to other nanocarriers. Secondly, different combinations of the excipients should be tested. Would the combination of excipients that improve all three steps and build the most effective formulation for follicular penetration? Would the combination of excipients that impair all three steps prevent follicular penetration completely? Thirdly, the differences between different excipients should be investigated – which excipient is most effective for each step?

NC for effective passive dermal penetration

This thesis investigated the influence of the vehicle type and excipients on the passive dermal penetration of the AC from NC. The novel method allowed to perform detailed and mechanistic penetration experiments. With this, all initial questions were answered. Thus, this path should also be followed in the future. In the future, also the influence of other formulation components – for example, stabilizers for the NC, preservatives for the formulation, fragrances etc. – should be studied with the novel method.

7.2. References

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Danksagung

Danksagung

„Alle haben Superhelden verdient. So ist das einfach.
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Curriculum vitae

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Personal details

Name	Olga Pelikh
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Place of Birth	Saint Petersburg, Russian Federation
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Education and Training

Feb 2018 – present	PhD Dept. of Pharmaceutics and Biopharmaceutics, Philipps-Universität Marburg
Dec 2017	Registration as Pharmacist
Mai 2017 – Nov 2017	Second Part of the Registration Year for Pharmacists, Dept. of Pharmaceutics and Biopharmaceutics, Philipps-Universität Marburg
Nov 2016 – Apr 2017	First Part of the Registration Year for Pharmacists, Lahn-Apotheke Marburg
Okt 2016	Second Final State Exam in Pharmacy
Okt 2012 – Okt 2016	Study of Pharmacy, Philipps-Universität Marburg

Schooling

Sep 2000 – Jun 2010	Intermediate Education School №111, Saint Petersburg, Russian Federation

„Krümel Löwenherz“, sagte Jonathan, „hast du Angst?“

„Nein ... doch, ich habe Angst! Aber ich tue es trotzdem, Jonathan, ich tue es jetzt ...

jetzt ...

und dann werde ich nie wieder Angst haben. Nie wieder Angst ha ...

Oh, Nangilima! Ja, Jonathan, ich sehe das Licht! Ich sehe das Licht!”

Astrid Lindgren „Brüder Löwenherz“