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Influence of molecular properties and delivery system design on the transfollicular transport across the skin

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Citation

Grams, Y. Y. (2005, January 12). *Influence of molecular properties and delivery system design on the transfollicular transport across the skin*. Retrieved from <https://hdl.handle.net/1887/598>

Version: Corrected Publisher's Version

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Note: To cite this publication please use the final published version (if applicable).

VIII

Summary and future perspectives

INTRODUCTION

Local drug delivery in the skin is important for the efficacy of a drug or a nutrient. Optimisation of the physical and chemical properties of the penetrant itself and the vehicle can improve the efficacy of the delivery to a certain skin region. Target regions of interest are for example sweat glands, pilosebaceous units, the basal lamina of the epidermis and the Langerhans cells within the epidermis. The pilosebaceous unit consists of a sebaceous gland and a hair follicle with the duct of the sebaceous gland joining the follicular duct in the upper part of the dermis. In terminal hair, the bulb of the hair follicle is located in the subcutaneous fat. The hair follicle moves upwards from the bulb and emerges from the skin at the surface. The follicle itself consists of various layers (Figure 1) which form, protect and guide the hair shaft. From the dermis towards the hair shaft, the layers of the hair follicle are outer root sheath and inner root sheath. Within the inner root sheath the Henle's layer, Huxley's layer and the cuticle can be distinguished. The cuticle of the inner root sheath is in direct contact with the cuticle of the hair shaft.

When examining delivery to and along the hair follicle, changes in accumulation in the various structures of the hair follicle have to be investigated. Due to the deep location of the hair follicle, the method of analysis is crucial. Currently several techniques are used, which provide either qualitative or quantitative information about the accumulation of active agents in the various skin regions. Some of these techniques include fixation procedures or other post-experimental treatments of the skin. This procedure has the potential to delocalise the model substance thereby bearing the danger of introducing artefacts. Additionally various *in vitro* studies, which focussed on (trans-) follicular delivery, have been performed using animal models such as mice and hamster. While fundamental questions regarding (trans-) follicular delivery can be addressed, extrapolation to the human subject is often very questionable.

The overall aim of this thesis was to determine the influence of permeant lipophilicity and vehicle composition on the accumulation and transport pathway of the permeant in the various regions of the hair follicle. For these studies fresh human scalp skin was used. In order to be able to compare the accumulation and transport in the various parts of the hair follicle a new method had to be developed. This method had to give access to changes in accumulation in deep layers of the hair follicle, while minimising the danger of artefact formation. Even more effort is required for obtaining information about the actual diffusion processes of model penetrants. In this case it is inevitable to visualise on-line the diffusion of the penetrant in fresh unfixed skin. Not only the diffusion process in superficial layers of the skin, but also the permeation processes in time and depth in deeper regions of the hair follicle have to be visualised to get access to

the permeation pathways. These permeation pathways are important to understand the targeting to the hair follicle and in particular the hair bulb.

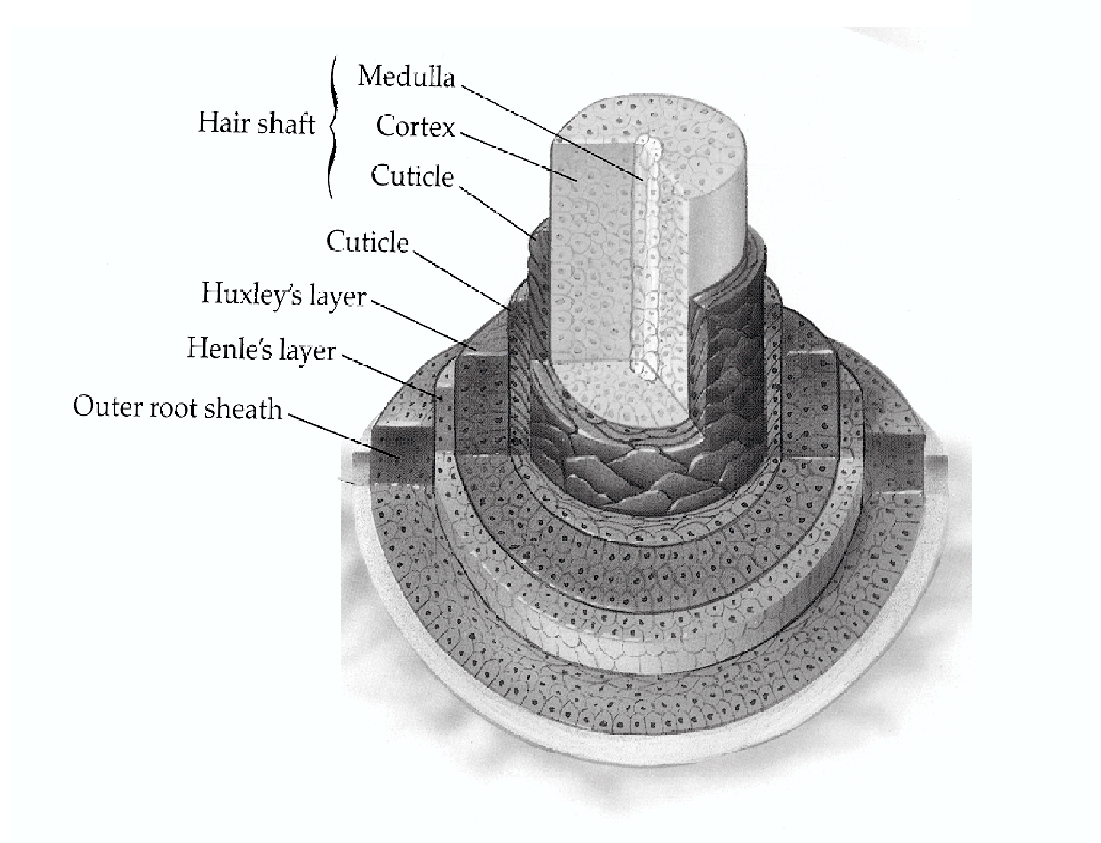


Figure 1. Hair follicle with the outer root sheath, inner root sheath (Henle's layer, Huxley's layer and cuticle of the inner root sheath) and the hair shaft (cuticle of the shaft, cortex and medulla).

Static diffusion analysis

In chapter II a new method is presented, which allows a semi-quantitative comparison of fluorophore distribution in the substructures of the hair follicle and the non-follicular regions of unfixed skin. This method is based on the fluorophore intensity measured with confocal laser scanning microscopy in images parallel and perpendicular to the skin surface.

A lipophilic dye (Bodipy[®] 564/570 C₅) was applied on fresh human scalp skin in citric acid buffer pH 5.0 containing 30 % (v/v) ethanol. After 18 hours of diffusion, the skin was removed from the flow-through diffusion cell and processed for subsequent visualisation in the confocal laser scanning microscope. The skin was visualised parallel to the skin surface at the dermal side. This image provides information about the fluorescent distribution in the hair

follicles and the dermis. In addition the cross section perpendicular to the skin surface provides information about the fluorescent distribution in the epidermis, dermis and stratum corneum. By combining the information of these images, the relative intensity values of the various regions in the skin including the hair follicle can be calculated. If model geometry based on the properties of the skin is assumed, the relative contribution in intensity of each skin region to the total intensity can be calculated. This contribution in intensity for each skin region is referred to as the relative accumulation factor of that skin region.

Subsequently (see chapter III) the influence of permeant lipophilicity on the permeation and distribution in human scalp skin was investigated. The fluorophore was applied in a buffer solution containing 30 % (v/v) ethanol. For these studies the newly developed method described in chapter II was used. The dyes that were selected are in sequence of increasing lipophilicity Oregon Green[®] 488, Bodipy[®] FL C₅ and Bodipy[®] 564/570 C₅. Additionally the presence of 30 % ethanol on diffusion and distribution of the dye with the lowest lipophilicity (Oregon Green[®] 488) was studied. Diffusion studies with fresh human scalp skin of 1100 µm thickness were performed for 18 or 72 hours using flow-through diffusion cells. Subsequently the skin was processed immediately to visualise the intensity distribution within the skin and the hair follicle using confocal laser scanning microscopy. The relative fluorophore distribution in the skin was calculated as described in chapter II. Ethanol (30 % (v/v) in CAB) increases the penetration rate of Oregon Green[®] 488 across the skin and promoted the transport of Oregon Green[®] 488 into and along the hair follicle slightly. Furthermore, an increase in permeant lipophilicity resulted in an increase in penetration rate across human scalp skin. The relative distribution in the skin is also affected by the lipophilicity of the permeating dye. A high lipophilicity of the label promotes the deposition of the label in the hair follicle as demonstrated by the relative accumulation values. Therefore we conclude that delivery to the hair follicle can be improved by the use of a lipophilic substance.

In chapter III, the combined effect of vehicle composition and dye lipophilicity on diffusion and distribution in human scalp skin is described. Formulations were prepared containing surfactants frequently used in shampoo formulations with and without propylene glycol. These surfactant formulations containing one of the dyes were diluted 1:1 with citric acid buffer pH 5.0. Similarly as in the previous study, permeation was measured over a period of 18 hours, which was immediately followed by visualisation using confocal laser scanning microscopy. As in the previous study, the relative accumulation of the dyes in the various parts of the skin and the hair follicle was determined. In the presence of surfactants with or without propylene glycol, medium and highly lipophilic dyes accumulated more in the follicular regions than the dye with the lowest lipophilicity. Additionally after 18 hours of permeation the amount of dye in the surfactants/propylene glycol formulation, that penetrated through the skin was

significantly higher for the most lipophilic dye, Bodipy[®] 564/570 C₅. This phenomenon was not observed for the most hydrophilic dye, Oregon Green[®] 488. These studies demonstrated that the highest accumulation in the hair follicles is observed with lipophilic dyes in surfactant formulation with propylene glycol.

With the newly developed method important information about the localisation of a dye in various skin regions can be generated. Unfortunately, no information can be gained about the actual route of permeation. Therefore an entirely new method was developed with the aim to visualisation the transport *on-line* in superficial as well as in deep layers of the skin, including the subcutaneous fat and the hair follicles.

REAL-TIME DIFFUSION

The aim of the studies described in chapters V to VII is establishing a technique that enables to examine *on-line* the penetration of a fluorophore from the skin surface, across the viable epidermis into the dermis. Importantly, the aim is also to visualise the permeation along the hair follicles in the various depths of the skin even as far as the hair bulb being located in the subcutaneous fat.

The first goal was to develop a method to study *on-line* permeation in the stratum corneum, viable epidermis and superficial layers of the dermis. In order to accomplish this, the skin has to be sliced perpendicular to the skin surface. This was achieved with a specially designed cutting device previously used to create the manual cross sections described in chapters II, III and IV). This cutting device was modified such that immediately after cutting, a donor and acceptor compartment is created (Figure 2). The acceptor and donor compartment were sealed with dental clay and a cover slip. The same cover slip was also covering the skin cross section. This enables to visualise the skin cross section with the confocal scanning laser microscope. Immediately after assembling the on-line diffusion cell, the donor and acceptor phase were injected through the dental clay. The donor phase consisted of the medium lipophilic dye (Bodipy[®] FL C₅) in citric acid buffer pH 5.0. Directly after application of the dye, images were obtained every 10 minutes for 8.5 hours using a high magnification of the microscope. The images revealed the stratum corneum, the epidermis and the dermis. After image acquisition, the change in fluorescence intensity was quantified and evaluated as function of time and location in the skin. Focussing on the epidermis and the dermis, detailed information regarding the change in fluorescence intensity in time and depth (pixel resolution) was obtained. In the stratum corneum, the fluorescence gradient was steep in the superficial layers and became gradually less steep in the deeper layers, demonstrating that the stratum corneum is not a homogeneous layer for diffusion. The fluorescent gradient was less steep in viable epidermis and dermis. At the junction of stratum

corneum/viable epidermis, a sharp increase in fluorescence is observed while at the epidermal/dermal junction, a sudden drop in fluorescence was detected. This indicated that the dye was more easily dissolved in the epidermis than in the stratum corneum and dermis. From this study we can conclude that with the newly developed technique, depth and time resolved visualisation of dye penetration into unfixed skin is obtained.

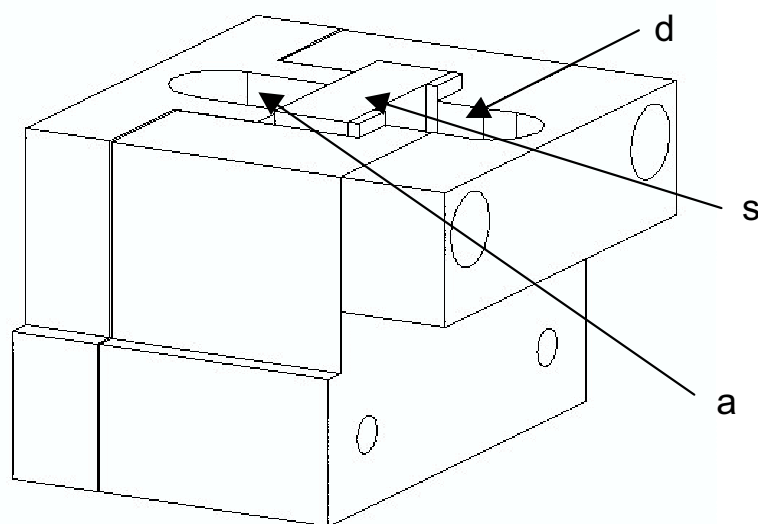


Figure 2. On-line visualisation device containing cross sectioned skin (s), donor compartment (d) and acceptor compartment (a). The stratum corneum of the cross section is facing the donor compartment. The cutting plane of the cross section is sealed with a cover slip and dental clay (not depicted) enabling on-line visualisation of the diffusion process by confocal laser scanning microscopy.

Chapter VI describes the penetration of Bodipy[®] FL C₅ into the hair follicle visualised by CLSM *on-line*. The combined cutting device/on-line diffusion cell was used with a lower magnification as described in chapter V. In this study the focus was to visualise larger structural units as the hair follicle. Images were obtained at a 30-minute interval for 16 hours. The donor phase was identical to the one used in the previous study (Bodipy[®] FL C₅ in citric acid buffer pH 5.0). During image evaluation, the relative quantification method developed for the static visualisation (chapter II) was adapted to include additional regions like the gap of the hair follicle. Additionally accumulation could be calculated at different depths of the same skin cross section (chapter VI). In the initial period the penetration of the medium lipophilic dye, Bodipy[®] FL C₅, occurred mainly via the gap and cuticle. After this period penetration via the epidermis became more important. Label in the cuticle originated mainly from the gap and permeated in

the cuticular region deeper into the skin. Dye in the outer root sheath originated either from the gap or from the epidermis.

However the question remained how the diffusion from the hair follicle close to the surface proceeded into deeper skin regions and whether label would reach the hair bulb. In order to study the diffusion *on-line* in deep skin regions, additional diffusion studies using fresh human scalp skin were performed as described in chapter VI with identical donor phases, image acquisition and magnification. However this time, on-line image series were obtained at increasing depth, namely at the surface and typically at 800 μm , 2100 μm and 4000 μm in depth (chapter VII). Due to the dilemma that a high magnification in CLSM cannot provide an image, which includes the stratum corneum at 0 μm and the hair bulb at > 2000 μm depth, different donors had to be used for each selected depth in the skin. Close to the skin surface, the gap and the cuticle of the hair follicle was stained at a very early stage of the permeation process. Label, which reached deeper layers in the cuticular region, was permeating from the cuticle into the surrounding areas, namely the inner and outer root sheath. At depth of up to 1000 μm , diffusion via the follicular route (cuticle, outer root sheath) was dominating in the initial time period, while the surrounding dermis was stained afterwards. At greater depths, the diffusion via the dermis gained more importance. This was indicated by the earlier staining of the subcutaneous fat as compared to any part of the hair follicle. Although the hair bulb was visualised, no label diffusion into the bulb was detected.

The results of these studies demonstrate that the on-line visualisation technique is a very powerful tool to visualise diffusion processes into deeper regions of non-fixed fresh skin. It has also the potential to study *in vitro* transport processes on cellular level including gene transport studies. In the upper regions of scalp skin, the follicular route is of great importance in the initial diffusion period. Deeper in the skin, diffusion via the dermis gains importance as well. Results indicate that targeting a drug substance comparable to our lipophilic model penetrant to the hair follicle in the upper regions of the dermis appears possible, especially in the cuticle and outer root sheath. Depths where the bulge region (highly proliferative cells) is present might be reached. However penetration of the drug substance (especially when lipophilic) via the stratum corneum into the dermis and subsequently the systemic circulation cannot be excluded. Targeting the hair bulb solely via topical application appears to be difficult and only feasible for therapy if highly potent molecules are used.

FUTURE PERSPECTIVES

Improvement of on-line visualisation technique

In chapter V, VI and VII, on-line images are presented reflecting the diffusion process up to 16 hours. It has been observed, that after a certain period of diffusion, the fluorescence intensity in the donor compartment decreases. As mentioned in those chapters, photobleaching is not expected to be the main reason for this decrease. Since the donor phase of the on-line diffusion cell was not stirred and the donor compartment had a small volume, depletion of the donor phase is expected to be the main reason for this decrease. In future experiments a method should be developed, circumventing donor depletion. Pilot studies already indicated that an on-line diffusion cell could be designed with a flow-through donor phase. This ensures a constant donor concentration and therefore a constant rate of partitioning of the permeant into the skin. The acceptor phase can also be adjusted to generate a flow-through system. This provides a continuous removal of the dye from the dermis and the acceptor compartment resulting in a very low concentration in the acceptor phase. In this way a true steady state flux can be achieved, which will provide more detailed information on the diffusion process.

In the presented work, on-line diffusion was investigated with one lipophilic label. These on-line studies should also be extended to include labels of different physicochemical characteristics. In that way it can be investigated whether the route of transport depends on the chosen permeant. Simultaneous application of two labels followed by on-line visualisation of the diffusion process allows a direct comparison of the permeants diffusion processes. This would result in more accurate data as the comparison of the labels can be made within the same piece of skin and in the same visual plane. Inter donor variation can also be excluded. This is an important advantage especially in scalp skin that shows large inter donor variation. However care has to be taken during the selection of the permeants. The optical properties (excitation and emission wavelength and the fluorescent intensities) and the applied concentration of the dyes are crucial for the investigation.

In visualisation of fluorescent substances, no absolute mathematical correlation can be made between the intensity and the concentration of a fluorophore. Therefore mathematical approaches have been developed in this thesis to obtain information of the distribution of a dye. For these calculations, assumptions had to be made. It was assumed that the fluorescence is independent of the different skin tissues and each skin region was approached by geometric forms. The values obtained using this combined experimental/mathematical approach has to be confirmed by an additional method. Alternative approaches circumventing fluorescent labels could be raman

microscopy, autoradiography and magnetic resonance imaging. The optimal approach would be a model penetrant which can be detected by confocal laser scanning microscopy and one of the above mentioned methods.

The diffusion studies described in this thesis were performed using fresh human scalp skin without any long-term storage and conservation of the skin. Therefore these experiments can be considered to be as close to the human situation as one can achieve with *in vitro* experiments. In most diffusion studies a skin thickness of approximately 200 μm is used. This thickness is selected as this contains mainly the epidermis. In diffusion studies the flow through acceptor phase can therefore simulate the blood flow. However when the hair follicle is the main target, full thickness skin with subcutaneous fat is required to access the deeper layers of the hair follicle and the bulb. A limitation of the *in vitro* studies is that the hair follicle is not highly vascularised as is in the *in vivo* situation. This might influence the transport from the hair follicle into the circulation and the redistribution of the dye from the epidermis/dermis via the blood vessels into the hair follicle. Therefore we suggest that fresh human scalp skin is the best model for investigation of follicular delivery *in vitro*, however results have to be judged critically when extrapolating to the *in vivo* situation. For *in vivo* follicular delivery, magnetic resonance imaging or raman microscopy might be the methods of choice in the near future.

Follicular drug delivery

When addressing delivery of compounds to the hair follicle two approaches have to be considered. The first approach is that the basic ingredients of the formulation have been selected and the transported active ingredient can be adapted. This first approach has been investigated in this thesis. A clear accumulation of substances has been observed in the upper parts of the hair follicle depending on the nature of the penetrant and the formulation. In these upper regions, the cuticle of the inner root sheath and of the hair shaft, the outer root sheath, the duct of the sebaceous gland and the bulge area are candidates for target regions. However, only little staining was observed in the follicle deeper in the skin. No labelling was detected in the hair bulb when using skin including the subcutaneous fat. The *in vivo* situation might influence the accumulation due to higher amount of applied drug, presence of blood vessels and massaging during the application of the formulation. However, since the experiments were carried out over a long time period, the chance of delivering high amount of drug to the hair bulb is estimated to be rather low using this first approach. Therefore, molecules suitable for follicular targeting into deep skin areas have to be highly potent since only a low amount of penetrant reaches the hair bulb. Next to hormones, DNA molecules might be interesting candidates for follicular targeting. Since only few molecules have to reach the site of action it can be a promising penetrant to treat DNA related disorders. However size might

be a critical factor when delivering large molecules such as DNA. The developed on-line visualisation technique can provide a possible tool for future investigations on a cellular level.

The second approach is that a molecule can only be modified slightly while the vehicle can be selected without limitation. Vehicles which are suitable to deliver molecules to the hair follicle such as particulate molecules have to be tested for their efficacy and customer acceptance. Next to their use in pharmaceutical products, they might form an improvement in the hair care industry. They have the potential to accumulate in the follicular opening and slowly release an active ingredient. In case this approach does not result in a therapeutic level of the active at the target site, a combination of topical application with systemic delivery should be considered. This combination might be a good alternative to topical application only since the hair follicle is highly vascularised and therefore susceptible to both routes of delivery. For future approached, it would be very interesting to investigate formulations containing small particles with our newly developed method. The main question would then be if effective transport to the hair root is possible when using particulate formulations in order to avoid systemic applications.

