Stratum corneum hydration : mode of action of moisturizers on a molecular level

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Chapter 2

Methods used in this Thesis

1. Tape-stripping

Tape-stripping is a well known method in which the SC is removed layer by layer by successively applying adhesive tape, after which it is removed with an adhering layer of SC. (Lademann, et al., 2005; Loffler, et al., 2004; Zhai, et al., 2007). Ideally, each tape-strip removes one cell layer of SC. However, the heterogeneous distribution of corneodesmosomes in the SC results in the first tape-strips removing relatively more corneocytes than the following strips. Tape-stripping is used in order to mimic the loss of barrier function in experiments when assessing topical toxicity (Zhai et al., 1998), to study barrier recovery after treatment or insults to the SC (Kucharekova et al., 2002), or to generally study the skin barrier of healthy or diseased skin (Gfesser, et al., 1996; Goon, et al., 2004; Jiang, et al., 1998; Tanaka, et al., 1997; Vandekerkhof, et al., 1995). However, it is also used to determine the diffusion of a topically applied compound into the SC in depth. In this case, tape-stripping is combined with analytical techniques (Herkenne et al., 2006; Honeywell-Nguyen et al., 2004; Kalia et al., 1996; Weigmann et al., 1999).

The reduction in skin barrier function is most often determined by transepidermal water loss measurements (TEWL, see *2 Transepidermal Water Loss Measurements*), which provides information as to the amount of barrier removed by measuring the water flux from inside the body to the environment. However, because tape-stripping as well as TEWL measurements are prone to large inter and intra individual variations (Chilcott and Farrar, 2000; Wesley and Maibach, 2003; Loffler et al., 2004) this may lead to inconsistent results. Therefore, much work has focussed on the quantification of SC removed by tape-stripping by either visual inspection (Lindemann et al., 2003b), weighing (Herkenne et al., 2006; Kalia et al., 1996), UV/VIS absorption (Lindemann et al., 2003a) or protein analysis (Chao and Nylander-French, 2004; Dreher et al., 2005). Additionally, methods to determine the total SC thickness have been devised (Herkenne,

et al., 2006; Kalia, et al., 1996). By combining tape-stripping with SC quantification and a detection method to determine the amount of a penetrant in the tape-stripped SC, it is possible to determine penetration profiles of topically applied substances (Bunge et al., 2006; Kalia et al., 1996).

In this thesis, tape-stripping is combined with freeze fracture microscopy to visualize the interaction of lipophilic moisturizers with SC intercellular lipid at different depths (*Chapter 6*) and with ATR-FTIR (see *Infrared Techniques*) to determine the penetration depth of the moisturizers (*Chapter 8*).

2. Transepidermal Water Loss Measurements

Transepidermal water loss (TEWL) can be defined as the amount of water per skin surface area and per time unit that passes from inside the body through the epidermis to the surrounding atmosphere via diffusion and subsequent evaporation. When the skin barrier is damaged, the amount of water that evaporates increases. TEWL can therefore be used to determine the barrier function of the skin (Fluhr et al., 2006). It is however not always a good indicator to determine how easily a compound will penetrate the skin (Levin and Maibach, 2005).

TEWL is used in the dermatological field to determine whether the skin barrier is damaged by disease (Shahidul, 1969; Werner, 1985), but it can also be useful to determine whether applied substances or skin treatment have an effect on the barrier function. This can for instance be an unwanted effect of soap and chemicals (Ahaghotu et al., 2005; Pedersen et al., 2005; De Jongh et al., 2006) or pharmaceutical excipients (Tanojo, 1997; Levang, 1999; Fang et al., 2003). Additionally, it can be used to monitor the repair of barrier function after such an insult (Coderch et al., 2002; Loden, 2003; Loden, 2005).

Although TEWL measurements are easy to perform, interpretation of the collected data is not always straightforward for at least two reasons. Especially when applying formulations, the formulation itself might change the TEWL measurement due to water evaporating from the applied formulation. Therefore, even though moisturization can increase the barrier function of the skin due to its promoting effect on SC maturation, TEWL may appear as increased after moisturizer treatment due to water evaporation from the formulation. Another problem in interpreting the TEWL data is that a reduction in TEWL cannot be interpreted simply as a repair of the skin barrier function: an additional layer of lipophilic substance on the skin barrier will also reduce the TEWL values.

Two sets of guidelines for the use of TEWL measurements in dermatological and cosmetic

research have been published (Loden, 2003; Pinnagoda et al., 1990; Rogiers, 2001).

In this thesis, TEWL measurements are used to monitor the barrier function after tape-stripping (*chapters 6 and 8*). As both tape-stripping and TEWL measurements are difficult to interpret, additional methods that provide more details are required to study the change in skin barrier function or the interaction of moisturizers or other substances with the skin barrier. Some of these methods will be discussed below.

3. Visualisation techniques

Cryogenic Scanning Electron Microscopy (cryo-SEM)

Cryogenic scanning electron microscopy (cryo-SEM) is a version of SEM in which cryo-fixed samples are kept below -100°C during visualization (Fatouros, et al., 2006; Nijsse and van Aelst, 1999; Richter, et al., 2004). Because of these low temperatures, samples usually do not require any chemical preservative methods. Once frozen, the sample is fractured or sliced and free water is sublimated under vacuum from the obtained fresh surface. The surface is then covered with e.g. gold-palladium after which the sample is immediately transferred into the electron microscope at liquid nitrogen temperature. Particularly when slicing the sample, cryo-SEM can be used to visualize the water distribution in the sample, as sublimation of free water results in small holes that increase the contrast in the image.

In this thesis, cryo-SEM is used to visualize the distribution of water in the SC (Bouwstra et al., 2003; Richter et al., 2004) in order to determine the effect of moisturizer on water levels throughout the SC (*Chapter 4*).

Freeze Fracture Transmission Electron Microscopy (FFEM)

Freeze fracture electron microscopy (FFEM) is a combination of transmission electron microscopy (TEM) and freeze fracturing. In TEM, a micrograph image is generated by transmitting a beam of electrons through a specimen treated with various methods to enhance the visualization of structural details. The resolution of TEM is sufficiently high to visualize not only structures, but also processes in the epidermis. Using different techniques, epidermal granules (Ishida-Yamamoto et al., 2004), Langerhans cells (Demarchez et al., 1992) and the lipids in SC and epidermis (Norlen et al., 2003), amongst others, have been shown.

In FFEM, samples are frozen and subsequently longitudinally fractured approximately parallel to the original skin surface under high vacuum (Holman et al., 1990). Next, platinum is evaporated under high voltage at an angle of 45°, which creates a shadowing pattern of the

fracture plane. This enables visualization of the fracture by TEM. A backing of carbon is applied to create a replica from which the original sample can be removed chemically. In this manner the lipid organisation and the formation of water domains in hydrated SC (vanHal et al., 1996) and in *vernix caseosa* (Rissmann et al., 2006) were determined. Furthermore, this method can provide information on the interaction between the formulation and the skin, such as topically applied vesicles (van den Bergh et al., 1999). Since the fracture will always run along the plane of least resistance, FFEM micrographs of stripped SC most often show the lipid coated surfaces of corneocytes or the lipid lamellae themselves.

In this thesis, FFEM is combined with tape-stripping (see *1 Tape-stripping*), making an examination of the lipid changes in SC in depth possible (Honeywell-Nguyen et al., 2003b) (*Chapter* 6).

4. Small Angle X-ray Diffraction (SAXD)

In X-ray diffraction, X-rays are scattered by a sample and the intensity of the scattered X-rays is measured as a function of its scattering angle. When interested in the larger structural units in the sample, such as a lamellar phase, the sample is measured at very small scattering angle. In that case the technique is referred to as small angle X-ray diffraction (SAXD). The X-ray pattern of a lamellar phase is characterized by a series of sequential maxima, which are positioned at equal interpeak distances at increasing scattering angle. The sequential peaks are referred to as the 1st order (positioned at distance Q1), the 2nd order (Q2), the 3rd order (Q3), etc, in which Q is directly related to the scattering angle. The repeat distance (d) of a lamellar phase can be directly calculated from the peak positions $d = 2\pi/Q1 = 4\pi/Q2 = 6\pi/Q3$, etc.

In skin research SAXD was used to study the lamellar organization of the lipids in the intercellular matrix of SC of humans (Bouwstra, et al., 2000; Bouwstra, et al., 1991a) and other mammals (Bouwstra et al., 1995; Hatta et al., 2006). Furthermore, SAXD measurements using lipid mixtures of ceramides (CER), cholesterol (CHOL) and free fatty acids (FFA) have revealed the role of the various lipid classes in the lamellar phases. (Bouwstra et al., 1996b; Bouwstra et al., 2002b; Bouwstra et al., 1998; de Jager et al., 2005). Additionally, it has been used to study effects of topically applied substances (Bouwstra et al., 1991; Cornwell et al., 1996; Brinkmann and Muller-Goymann, 2005) or physical SC perturbation methods (Jadoul et al., 1996).

In this thesis, SAXD is used to study the effects of hydrophilic and lipophilic moisturizers on the lamellar organization isolated SC and their interaction with porcine CER:CHOL:FFA samples (*Chapters 6 and 7*).

5. Infrared Techniques

Fourier Transform Infrared Spectroscopy (FTIR)

Fourier transform infrared spectroscopy (FTIR) records the amount of IR radiation absorbed by atom bond vibrations in a specimen (Naik, 1997) that is placed between two crystal windows. Detailed insights into the organization of the SC can be gained through the study of the vibrations of amide, amine and carboxylic groups and the frequencies of the methylene stretching, scissoring and rocking vibrations. FTIR is used to study the lateral lipid organization of the intercellular lipid matrix in SC, which is essential for the barrier function of SC, as more densely organized membranes are less permeable to substances. The stretching vibrations are used to determine whether lipids are in an ordered (hexagonal or orthorhombic lateral packing) or disordered packing (liquid phase), while the scissoring and rocking vibration provide detailed information on the presence of orthorhombic phases. By performing measurements at different temperatures, also the thermotropic behaviour of the lipids can be determined.

Substitution of the hydrogen atoms of e.g. lipids with deuterium atoms, i.e. using perdeuterated materials, causes a shift in absorption wavelength. This makes it possible to study the mixing properties of hydrogenated and perdeuterated compounds in the same mixture, by comparing the order-disorder transitions indicated by both the hydrogenated and perdeuterated methylene stretching bands.

Using FTIR, detailed knowledge on the lipid organization has been gathered, e.g. on the importance of FFA for the formation of the orthorhombic lateral packing (Mendelsohn et al., 2000) and the heterogeneity of the lipid components (Chen, et al., 2007; Gooris and Bouwstra, 2007). Naturally, it is also possible to use FTIR to study the effects of topically applied substances or physical penetration enhancement (Mimeault and Bonenfant, 2002; Sznitowska et al., 2003; Wang et al., 2004). Finally, FTIR is a method that can be used for the quantification of lipids (Tsai et al., 2004) and of course also of other substances.

In this thesis, FTIR is used to determine the effect of glycerol on the lateral organization of intercellular lipids in isolated SC and of equimolar CER:CHOL:FFA samples (*Chapter 4)* and of lipophilic moisturizers on the lateral organization of equimolar CER:CHOL:FFA samples (*Chapter 7*).

Attenuated Total Reflectance FTIR (ATR-FTIR)

Attenuated total reflectance FTIR (ATR-FTIR) is a modified version of FTIR, in which IR radiation is not transmitted *through* the sample but reflected *by* the sample. Consequently, a specimen is not placed between two IR windows, but onto an IR crystal. This makes it possible to perform measurements on SC *in vivo*, as the skin can be placed on the ATR crystal. The IR radiation beam has only a limited penetration depth into SC however, approx. 1 m in case of a zinc-selenide crystal. To detect substances *in* the SC, it is therefore necessary to remove SC layers, for instance by tape-stripping. In this case, it is also possible to generate a penetration profile of an applied substance in SC in depth (Coderch et al., 1999; Curdy et al., 2004; Honeywell-Nguyen et al., 2004). ATR-FTIR has been used to determine effects of topically applied substances or physical penetration enhancement on the lipid organization in the SC (Ayala-Bravo, et al., 2003; Coderch, et al., 1999; Jadoul, et al., 1996; Pouliot, et al., 1999; Tanojo, et al., 1997).

In this thesis, ATR-FTIR is combined with tape-stripping to determine the penetration profile of hydrophilic and lipophilic moisturizers in SC in depth (*Chapter 8*). Additionally, the water profile in the SC is determined.

6. References

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