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## **Development of a vernix caseosa substitute : a novel strategy to improve skin barrier function and repair**

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Summary and perspectives

## Summary

### Background

Vernix caseosa (VC) is the creamy white skin-surface biofilm that covers the skin of the fetus and the newborn. VC consists of dead cells (corneocytes) that are embedded in lipids. Structurally, this is very similar to stratum corneum (SC), the uppermost layer of the skin. VC is a highly hydrated protein-lipid rich material composed of approximately 80% water, 10% proteins and 10% lipids [1, 2]. The corneocytes represent the main water reservoir of VC and act thus as hydrophilic sponges. They are mostly polygonal and flat in shape with a diameter of approximately 30  $\mu\text{m}$  [3]. VC lipids consist of a large variety of different lipid classes, including squalene, sterol esters/wax esters (SE/WE), triglycerides (TG) and dihydroxy wax esters (DIOL). All SC barrier lipids, i.e. cholesterol (CHOL), free fatty acids (FFA), and ceramides (CER), are present but in lower levels than in SC [1].

VC is suggested to feature multiple biological functions such as waterproofing and facilitation of the skin formation *in utero* [2, 4]. During delivery it acts as a lubricant, while exhibiting anti-oxidant, skin cleansing and antibacterial properties postnatally [5-7]. The latter might be due to I) mechanical obstruction of bacterial passage [7] and II) the presence of antimicrobial peptides, proteins and branched fatty acids [8, 9]. Moreover, VC was shown to be of importance for the adaptation process after birth as the skin hydration and the acid mantle development were facilitated [2, 10]. These multiple biological functions and unique properties of natural VC suggest that it has great beneficial potential for underdeveloped skin of preterm infants, barrier-deficient or diseased skin. However, its clinical application is restricted due to the limited availability and the risk of transmission of diseases [11]. Hence, the generation of a synthetic VC equivalent with similar structure and properties could lead to a new generation of biofilms.

The aim of this thesis was the rational design of synthetic biofilms which mimic VC. In order to do so, the main objectives of the thesis are defined as follows:

- I) Thorough characterization of VC with focus on (lipid) composition, ultrastructure and physicochemical properties which will be used for the development of synthetic biofilms in a later stage of the project.
- II) Design of lipid mixtures that mimic composition and organization of VC lipids.
- III) Generation of synthetic corneocytes which closely resemble the hydrophilic natural counterpart. This research was conducted by M.H.M. Oudshoorn (Utrecht University) and resulted in the dissertation entitled "Composite of microgels and lipids as biofilm to restore skin barrier function" [12].

- IV) Preparation of synthetic biofilms which are composed of the synthetic corneocytes and the selected lipid mixtures followed by their characterization regarding homogeneity, stability, (ultra-) structure, rheological and water-handling properties in comparison to natural VC
- V) Evaluation of the synthetic biofilms *in vivo* to prove their efficacy and potential application

Objectives IV and V were carried out in collaboration with M.H.M. Oudshoorn.

*Part I – Detailed characterization of vernix caseosa*

Although VC characteristics have been frequently reported in literature, we realized that several features remained unaddressed: I) No integral overview of the free VC lipids was available. II) The presence and composition of the lipids bound to the cornified envelope of the corneocytes had not yet been reported. III) The fatty acid chain distribution of composed lipids such as SE/WE and TG was only determined up to a chain length of 20 carbon atoms.

In **Chapter 2**, we obtained a thorough and detailed analysis of the free and bound VC lipids as well as a complete picture of the fatty acid distribution within the different lipid classes. The free lipids of VC show a wide distribution in polarity: mainly nonpolar lipids such as SE/WE and TG with a chain length up to 32 carbon atoms predominate in VC. Although the lipid composition of VC is different from that of the SC, some similarities could be observed, e.g. the CER profile. It was demonstrated for the first time, that bound lipids, covalently linked to the cornified envelope of corneocytes, are present. Similarly to SC, these bound lipids consist of fatty acids,  $\omega$ -hydroxyacids and  $\omega$ -hydroxyceramides CER(OS) and CER(OH) [13]. Ultrastructural studies were conducted using freeze-fracture electron microscopy (FFEM) as well as cryo-scanning electron microscopy (cryo-SEM). The latter revealed the presence of highly hydrated corneocytes embedded in lipids. Additionally to the study of Pickens *et al.* [2], the internal structure of the corneocytes was clearly visualized. A sparse network of keratin filaments was shown to constitute a scaffold and creates a body to store water within the corneocytes. A low but discernable degree of orientation of the corneocytes was apparent while the intercellular lipids occasionally accumulated as lipid pools. FFEM showed smooth surfaces of corneocytes and a heterogeneous appearance of intercellular lipids. In contrast to SC, our results demonstrate a lower degree of ordering of VC lipids which was confirmed by small-angle X-ray diffraction (SAXD).

Further characterization of VC was performed focusing on the structural and physicochemical properties of VC upon variation of environmental parameters, such as temperature and humidity (**Chapter 3**). This is of great interest in order to better understand VC's role at birth. Interestingly, a difference was observed in VC's water release and uptake properties: dehydration and rehydration processes take place 2-4 times faster at 37°C than at room temperature (RT). The dehydration was irreversible as the rehydration was only possible to a final

weight of 55% (37°C) and 46% (RT) of the pre-desiccation weight. Differential scanning calorimetry (DSC) showed two different overlapping phase transitions with onset temperatures at ~20°C and ~26°C, respectively. Investigation of the lipid organization by Fourier transform infrared spectroscopy (FTIR) and SAXD revealed a more disordered state of lipids at 37°C than at RT, which can explain the faster dehydration and rehydration process at 37°C as well as the changes in thermotropic rheological behaviour. In conclusion, the results of **Chapter 3** clearly illustrate I) the irreversibility of water holding properties of the VC and the subsequent ultrastructural changes and II) the reversibility of the lipid organization after birth. This demonstrates the existence of temperature and hydration-dependent changes in properties of VC within the physiological temperature range and suggests that VC adjusts to the drastic change from the intrauterine to the postnatal environment.

As the main classes of SC barrier lipids are also present in VC, the question rises whether VC lipids are also able to form a comparable lipid organization observed in SC. In SC the lipids are organized in two lamellar phases with a repeat distance of approximately 6 and 13 nm. The 13 nm phase is very characteristic and is referred to as the long periodicity phase (LPP). In order to investigate the formation of this phase, the lipid organization of VC lipid mixtures prepared by various methods was studied by SAXD (**Chapter 4**). To mimic the physiological situation as closely as possible, no equilibration step at elevated temperatures was included. When applying VC lipid extracts by spraying or by spreading after freeze-drying, a long range ordering was observed with a spacing of 4.7 nm. Only when the solution of the lipid mixture was dried under nitrogen at 37°C and subsequently spread onto a support, the LPP was formed. The VC lipids exhibited a repeat distance of 13 nm, similarly to human, pig and mice skin [14-16]. As the equilibration temperature is 37°C, the *in vitro* results of **Chapter 4** demonstrate that the LPP can be formed at body temperature.

#### *Part II – Mimicking vernix caseosa (lipids)*

In order to mimic the properties and structure of VC, synthetic biofilms with a similar structure were prepared. The natural corneocytes were replaced by synthetic corneocytes which were made of hyperbranched polyglycerol derivatized with glycidyl methacrylate (HyPG-MA). This polymer was cross linked using photo-lithography and particles were prepared with similar shape and size (i.e. hexagonal, Ø 30 µm) to natural corneocytes. The design and preparation of these synthetic corneocytes was conducted by M.H.M. Oudshoorn and is presented in her thesis [12]. Besides the corneocytes, also the VC lipid mixture needed to be imitated as closely as possible. The optimization of synthetic lipid mixtures regarding thermotropic behaviour, lipid composition and organization mimicking that of the natural VC lipids is presented in

**Chapter 5.** Anhydrous lanolin, i.e. wool wax from sheep, was used as primary component since I) it consists of lipid classes (i.e. SE/WE and DIOL) which are the most abundant lipids of VC and II) it contains a high level of branched fatty acids. Nonpolar lipid fractions, mainly consisting of SE/WE and DIOL, were isolated from lanolin. Three isolated fractions with different composition were mixed with squalene, barrier lipids and TG. Then, the lipid organization and thermotropic behaviour of extracted VC lipids, isolated lipids (i.e. fractions from lanolin) and lipid mixtures (i.e. isolated fractions from lanolin + barrier lipids + squalene + TG) were examined. Biophysical evaluation revealed that TG play an important role in the (lateral) lipid organization and the thermotropic behaviour of the (semi-) synthetic lipid mixtures (SSLM). Excellent resemblance of VC lipids was obtained when including unsaturated TG in the SSLM. Based on the biophysical properties the most appropriate SSLM was selected and used for the preparation of the synthetic biofilms.

The preparation and characterization of synthetic biofilms are presented in **Chapter 6.** The hydrated particles were embedded in the optimized SSLM using a micromixer. For optimization of these formulations, the water content of the particles (i.e. 50% and 80% (w/w)), an additional lipid coating of the particles (pre-coated) and different particle/lipid ratios were varied. Characterization with confocal laser scanning microscopy (CLSM) showed a homogeneous distribution of the labelled particles in the lipid matrix. Regarding structural appearance, particle density and distribution, the formulations with a high particle/lipid ratio (5:1) resembled native VC most closely. Concerning water handling-properties, the formulation with the pre-coated particles (80% water content) and a particle/lipid ratio of 2:1 exhibited a sustained water release for 140 h mimicking the water release of VC most closely. The formulations were stable for at least one month at 4°C and exhibited – similar to VC – two overlapping phase transitions at physiological temperature range as investigated by DSC. Rheological properties showed a comparable quotient of viscosity and elasticity between the formulations and native VC. An excellent resemblance was achieved in composition, structure and properties between natural and synthetic biofilm(s).

*Part III – In vivo studies to test biological efficacy of natural and synthetic biofilm(s)*

The excellent *in vitro* resemblance of synthetic biofilms to native VC gave also rise for a testing in an *in vivo* setting. However, a suitable model to study the effects of topical applications was of crucial importance. In **Chapter 7** and **8** we focused on the generation of a reliable model for skin barrier disruption and repair to study the effects of topical applications. Different levels of barrier disruption in hairless mouse skin, accomplished by sequential tape stripping, were evaluated (**Chapter 7**). The status of the barrier was monitored by measuring the transepidermal water loss (TEWL). The skin barrier disruption models moderate, severe #1 and severe #2 showed complete recovery of the

TEWL within 72 h. However examination of light microscopic images of skin cross-sections clearly revealed that not all corneocytes were removed after tape stripping. Therefore, two additional models, severe #3 and severe #4 with a gradual increase in the number of tape strippings were evaluated. Model #3 still showed complete recovery within 72 h. With model #4 a crust was formed, resulting in only 50% recovery in 72 h. For both treatments no difference was discernable with histology as all corneocytes were removed while the remaining epidermis was intact. With model #4 almost complete recovery (~90%) was obtained within 8 days. The effect of VC application on the recovery of disrupted skin was evaluated with model #3 and #4. Model #3 showed that application of VC predominantly influenced initial recovery and is therefore merely appropriate to study the effect of formulations in the initial stage of recovery. Topical application of VC on model #4 considerably increased initial and long-term recovery. Moreover, VC application promoted rapid formation of SC and prevented epidermal thickening and crust formation. These observations show the ability of VC to enhance barrier recovery, but also suggest the potential use of this treatment clinically. As model #4 is an excellent model to study long-term recovery, this model was used to evaluate the repair properties of the synthetic biofilms (Chapter 9).

In **Chapter 8** the acetone-induced barrier disruption was investigated and compared to the tape stripping method. For both treatments the TEWL directly after disruption and the subsequent barrier recovery profile were similar. Histological assessment showed significant lower number of corneocyte layers in acetone-treated and tape stripped skin compared to untreated skin, while there was no statistical difference between the two treatments. Lipid analysis revealed that predominantly nonpolar lipids were extracted by acetone. Importantly, the ratio of the barrier lipids remained similar between control and acetone-treated skin. This reflects the undisrupted lipid organization, as determined by SAXD measurements: the LPP was still present after acetone perturbation. These results contradict earlier studies reporting no mechanical SC removal, a substantial extraction of lipids and disruption in lipid organization. In contrast, we observed clearly the removal of corneocytes, the presence of a lamellar lipid organization and only little extraction of lipids by acetone. Our results clearly demonstrate that compared to tape stripping, acetone treatment is a less suitable method for studying the long-term effect of topical applications.

**Chapter 9** describes the effects of application of synthetic biofilms in the tape stripping model. Therefore, various biofilms were applied topically on disrupted mouse skin to determine which formulation could improve barrier recovery, as was observed previously for the natural biofilm VC (Chapter 7). We evaluated biofilms with differences in I) water content of the particles (i.e. 50% and 80% (w/w)), II) absence or presence of a lipid coating of the particles and III) varying particle/lipid ratios (Chapter 6). It was observed that application of all tested formulations improved the skin barrier recovery and reduced crust formation

and epidermal hyperproliferation. However, the biofilm composed of uncoated particles with 50% (w/w) initial water content and particle/lipid ratio of 2:1 mimicked the effects of native VC most closely. This indicates that the balanced ratio of hydrated particles and lipids improve barrier recovery while the lipids play a more prominent role in barrier recovery than the synthetic corneocytes. Altogether, these observations suggest the potential use of the optimized biofilm for clinical treatment.

## Perspectives

The synthetic biofilm formulations resemble excellently the structure, composition and properties of natural VC (Chapter 6). Thereby, the synthetic corneocytes were coated with lipids to control the release of water and to achieve a similar long-term skin hydration as observed for native VC ([10] and Chapter 3). The coating would also enable a sustained release of therapeutic drugs, as one of the future aims is to use the biofilms as drug delivery matrix. Furthermore, several other applications are of prospect e.g. treatment of barrier-deficient preterm infants or therapy of dry skin. Based on the first *in vivo* results, the different applications for the biofilms are elucidated below.

### *Applications of the biofilms*

#### *a. Dry skin conditions*

The symptoms of dry (xerotic) skin, i.e. itching, scaling and redness, are observed in several skin disorders such as psoriasis and atopic dermatitis [17]. As a consequence in dry skin the activity of several enzymes in SC, which are dependent on the water level in their microenvironment, is impaired. This concerns the activity of I) enzymes involved the desquamation process, namely proteases such as SC chymotryptic-like enzyme, kallikrein 5 and 7 and cathepsin D and E [18, 19], II) trans-glutaminases (e.g. transglutaminase 1) involved in the maturation process of the cornified envelope [18, 19] and III) enzymes involved in the hydrolysis of filaggrin, which is involved in the formation of the natural moisturizing factor (NMF). As the NMF plays a crucial role in retaining the water level in the skin [20], the lower level of NMF results in a further decrease in SC hydration and in this way a vicious circle has been created. In addition to that, the lipid composition, especially the CER levels, are changed in xerotic SC and is suggested to cause a perturbed lamellar organisation and consequently in an impaired barrier [18, 21]. Altogether the improper water levels and the changed lipid composition and organisation within the SC cause altered barrier properties of the skin [18].

For effective treatment of dry skin, the water level of SC should be increased to an optimal level. This may help to restore NMF production, corneocyte



maturation and the desquamation process. The application of emollients, i.e. water-containing formulations which smoothen and hydrate the skin, is recommended as therapy [17, 22]. However, alternatively the application of the synthetic biofilm with the water-containing corneocytes may also restore the water levels in dry skin. Compared to the existing emollient treatment, the biofilm presumably offers an advantageous therapy because: 1) The presence of barrier lipids is known to be of importance for skin barrier recovery (in mice) [23, 24]. As the barrier lipid composition in the biofilm mimics that in SC, the barrier lipids may also enrich the SC lipids and compensate for the reduced levels of skin lipids in the diseased state. Supplying barrier lipids may also facilitate skin maturation as the lipids play also a role in epidermal differentiation through signalling [17]. The presence of corneocytes in the biofilm might increase the skin barrier as the permeation pathway should be along the corneocytes. A high amount of water is present in the biofilm, which is released in a controlled manner without changing the structure of the biofilm. This water release may help to restore the SC moisture level for a proper function of the skin barrier. These properties might not only be beneficial for the aforementioned skin disorders but are also of promise for the underdeveloped skin of preterm infants, as described below.

*b. Barrier cream (for preterm infants)*

Another promising application for the biofilms is the treatment of preterm infants. At birth the skin of (very low birth weight) preterm infants is characterized by a substantially reduced skin barrier function and the lack of VC [25, 26]. By improving the barrier, the excessive dehydration and fluid loss of the infant could be prevented. Topical treatment with ointments has been shown to reduce TEWL and moreover, improved skin condition in very low birth weight infants [27]. However, others reported no significant improvement upon topical application of emollients [27, 28] while the practice of ointment application holds a higher risk of secondary infection from the nursing personal.

In addition to the treatment of preterm infants, the generated biofilms can also find applications as barrier cream, i.e. skin protectant, in adult population. Regular exposure to detergents (e.g. hair-dressers) causes skin irritation which can ultimately lead to dermatitis [29]. The treatment with the newly developed biofilms might be of benefit as they presumably offer possibilities to improve the hydration level in the SC in a controlled way and to protect the skin.

*c. Drug delivery matrix*

The biofilms can also be used as a delivery matrix enabling the incorporation of other substances such as anti-infectives or growth factors. The latter can be used to stimulate or facilitate processes e.g. wound healing. Pilot studies of our carrier system have shown that degradable particles on HyPG basis loaded with

lysozyme as a model protein are characterized by a prolonged release [12]. This underlines its possible application as a drug delivery matrix.

The synthetic corneocytes have been enveloped by an additional lipid coating. The binding between the synthetic corneocytes and the lipid coating is based on electrostatic interactions. This coating resulted in a reduction of the water release rate (Chapter 6). However, by changing the composition of the lipid coating, the release may be modulated. It would also be of interest to chemically link the lipid layer with the particle, mimicking more closely the bound lipids of the corneocytes.

*d. The use of the corneocytes and lipid mixtures separately*

Besides the versatile applications of the entire biofilms, also the components alone might have interesting fields of utilization. The hydrogels, serving as synthetic corneocytes in the biofilms, are used increasingly for various applications, because of their tailorable properties and uniform particle distribution. The morphology of the gels (i.e. size and shape) can be adjusted in accordance to their aimed application and function. Currently, a SC substitute is being developed in our laboratories [30]. The purpose of such a substitute is mainly to mimic the permeability barrier of the skin. This is achieved by spraying a lipid mixture (similar to SC lipids) on a support. The incorporation of synthetic corneocytes into the synthetic lipid membrane would enhance the structural resemblance with SC. Also the penetration pathway of drugs across such a membrane will more closely resemble that in SC.

Not only the corneocytes but also the lipid mixtures can be used individually. The outstanding performance of the optimized (semi-) synthetic lipid mixture in the barrier recovery model (Chapter 9) indicates the great potential of this water-free formulation for our current model. In future investigations, also variation of the composition of the lipid mixture, e.g. increased barrier lipid content, should be tested.

*Improvement of the biofilms*

Of course, the final aim is to test those biofilms in a clinical setting. However, prior to clinical application some general issues concerning upscaling, stability and safety should be addressed. For an upscaling of the production of the synthetic biofilms, the basic components, i.e. the lipid mixture and the synthetic corneocytes, need to be generated in larger quantities. For this purpose, the support and collaboration from companies is required. Of interest is also the physical, chemical and microbiological stability of the biofilms. Investigations towards structural changes of the biofilm upon storage under more stressful conditions e.g. higher temperature (40°C) or heating-cooling cycles will be necessary. To comply with microbiological requirements of the pharmacopoeia

[31], the biofilms need to be prepared aseptically and the addition of preservatives is likely. Chemical stability can be improved by adding antioxidants, e.g.  $\alpha$ -tocopherol, which is also present in native VC [6]. Besides the stability, the biofilms should also be tested for safety. The application on severely disrupted mouse skin showed neither irritation, abnormal animal behaviour nor any other side effects (Chapter 9). In the future, the biocompatibility and possible irritation and toxicity of the biofilms and its degradation products should be studied in human models. This can be performed in the first place on human skin equivalents, i.e. *in vitro* cell culture models resembling the composition, structure and function of the human skin [32]. Besides irritation and toxicity, also cellular functions such as tissue repair after inducing a wound could be tested in those models.

In conclusion, this thesis described the successful generation of novel, synthetic biofilms which closely mimic the complex structure and different properties of VC. The *in vivo* studies clearly demonstrated beneficial effects on barrier recovery in a mouse model, as similarly observed for VC. In future, these biofilms must show efficacy tested in diseased, dry or healthy human skin.

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