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

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Effect of synthetic vernix biofilms on barrier recovery of damaged mouse skin

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Abstract
The aim of this work was to investigate whether topical application of synthetic biofilms supports and accelerates the recovery of the murine skin barrier, disrupted by sequential tape stripping. Therefore, various biofilms were applied topically on disrupted mouse skin to determine which formulation could improve barrier function, as was observed previously for the natural biofilm vernix caseosa. The biofilms mimic closely the physicochemical properties of vernix caseosa that consists of corneocytes (dead cells) surrounded by a lipid matrix. Various formulations were prepared using different particle/lipid ratios, particles with different initial water-content and uncoated or lipid-coated particles. It was observed that application of all tested formulations improved the skin barrier recovery and reduced crust formation and epidermal hyperproliferation. However, only one of the biofilms (i.e. B1; composed of uncoated particles with 50% (w/w) initial water content and particle/lipid ratio of 2:1) mimicked the effects of native vernix caseosa most closely. This indicates the importance of the presence of individual components, i.e. barrier lipids and water, as well as the ratio of these components. Consequently, these observations suggest the potential use of this biofilm treatment clinically.

Introduction
Vernix caseosa (VC) is a lipid-rich, natural biofilm which normally covers the skin of the developing fetus during the final stage of the gestational period [1, 2]. However, in premature infants this protective surface film is absent [3, 4]. Macroscopically, VC is a thick, viscous, white cream that consists of hydrated dead cells (corneocytes) dispersed in a lipid matrix. Lipids covalently bound to the cornified envelope of the cells form the interface between the corneocytes and the lipid matrix. The structure of VC is very similar to that of stratum corneum (SC), the outermost layer of the skin, although VC lacks intercorneocyte desmosomal connections and the lipids are in a less ordered state [1, 5]. Consequently, VC exhibits a viscous fluid character and might therefore be perceived as mobile SC [1, 2, 5, 6].
A variety of biological properties has been assigned to VC. In utero it is suggested to act as a waterproof film promoting the formation of the horny layer of the fetus [1, 6]. During delivery it acts as a lubricant and postnatally it exhibits, anti-infective [7], anti-oxidant [8], skin hydrating [9] and skin cleansing properties [4]. Moreover, VC shows a temperature-dependent dehydration behaviour, enabling the hydration of the newborn's skin in a sustained manner [9, 10]. Because of these excellent properties, VC holds promise as a clinical effective therapeutic agent promoting the repair of the skin barrier of preterm

infants [3, 4] or enhancing wound healing in adult skin [6]. Previously, it was already shown that topical application of native VC on disrupted mouse skin considerably increased the skin barrier recovery, promoted a rapid formation of SC and prevented epidermal hyperproliferation [11]. Application of VC in clinics, however, is restricted by the limited availability of VC and the risk of disease transmission. Therefore, the generation of a synthetic VC equivalent could lead to new biofilms, mimicking closely the unique composition and properties of natural VC. Recently, we presented the development of synthetic VC resembling closely the physicochemical properties of natural VC [12]. These biofilms were composed of particles embedded in a lipid matrix. The particles, structured hydrogel microparticles based on hyperbranched polyglycerol, were prepared by photolithography [13] and used as synthetic corneocytes (referred to as particles) in our biofilms. The lipid matrix consisted of a lanolin-derived synthetic lipid mixture and showed similar composition and organization as natural VC [14]. Various formulations were prepared using different particle/lipid ratios, particles with different initial water-content and uncoated or lipid-coated particles to obtain a biofilm mimicking natural VC [12]. In order to test these new formulations, the use of an animal model is essential to investigate efficacy and safety. An excellent mouse model for severe skin barrier disruption and repair was developed previously for this purpose [11]. This model showed a slow recovery (i.e. 8 days) and is appropriate to evaluate the effect of formulations on recovery.

The present study aims to investigate whether topical application of our synthetic biofilms supports and accelerates the recovery of the murine skin barrier, disrupted by sequential tape stripping. Various biofilms were applied topically on the disrupted mouse skin to determine which formulation could improve and accelerate, such as VC, the skin barrier repair. Changes in transepidermal water loss (TEWL) were used to monitor barrier recovery. In addition, biopsies were harvested to evaluate the recovery of the SC by histology. Results were compared to the natural biofilm VC, applied on disrupted mouse skin, which showed previously the ability to enhance skin barrier recovery [11], and to commonly used oil-based ointments Vaseline (petrolatum; Vas) and Eucerin cum aqua (Euc).

Materials & Methods

Materials

Black D-squame (rectangles from 70 mm x 25 mm) was obtained from CuDerm (Dallas, USA). Vaseline (petrolatum) was purchased from Elida Fabergé (London, UK) and Eucerin cum aqua (unguentum alcoholum lanae aquosum; consisting of

petrolatum, wool wax alcohols, cetylstearyl alcohol and water) was supplied by Pharminnova B.V. (Warregem, Belgium). Tissue-Tek® O.C.T.™ compound was obtained from Sakura Finetek Europe B.V. (Zoeterwoude, The Netherlands). Safranin O was purchased from Sigma (Schnellendorf, Germany).

Collecting vernix caseosa and extracting its lipids

VC was scraped off gently immediately after vaginal delivery or caesarean section of healthy full-term neonates. The samples were transferred into sterile plastic tubes and stored at 4°C until use. The collection of VC was approved by the ethical committee of the Leiden University Medical Center and informed consent was given by the parents.

Preparation of biofilms

Synthetic biofilms, mimicking closely the unique composition and properties of natural VC, were prepared as described previously [12]. In brief, fully hydrated synthetic corneocytes (structured HyPG-MA hydrogel microparticles [13]) were mixed with lipids (mimicking closely lipid composition and organization of natural VC [14]) using an automatic ointment-mixer Topitec® (WEPA, Germany) modified for small-scale purposes. Various formulations were prepared using different particle/lipid ratios (i.e. 2:1 and 5:1) and particles with different initial water-content (i.e. 50% (w/w) and 80% (w/w)). Additionally, uncoated or lipid-coated particles were used to obtain biofilms. The composition of the various formulations is given in table 1. The particles and lipids were mixed for 5 min with a rotation speed of 400 rpm to obtain a homogeneous biofilm formulation.

Table 1. Composition of the various biofilms applied on the disrupted skin. The biofilms (B) were prepared from lipid coated (c) or uncoated particles with the water content being 50% (B1, B3) or 80% (w/w) (B2, B4) and a particle/lipid ratio of 2:1 (B1, B2) or 5:1 (w/w) (B3, B4).

Entry	Sample	Composition			
		Particle/lipid ratio	Initial water content particles (% w/w)	Lipid coated particles	Total water content biofilm (% w/w) §
B1	2:1_50	2:1	50	No	60
B1c	2:1_50_coated	2:1	50	Yes	51
B2	2:1_80	2:1	80	No	56
B2c	2:1_80_coated	2:1	80	Yes	57
B3	5:1_50	5:1	50	No	ND
B4	5:1_80	5:1	80	No	ND

ND= not determined; § Experimentally determined as reported in [12]

Skin barrier disruption

Male hairless mice (SKH-hr1), 7-9 weeks old and 28 g ± 2 g in weight, were purchased from Charles River Laboratories (St Aubin Les Elbeuf, France). All animal experiments were conducted in conformity with the Public Health Service Policy on use of laboratory animals and had been approved by the Research Ethical Committee of Leiden University (UDEC, nr. 07002). The mice were maintained in the animal care facility of the Gorlaeus Laboratories, Leiden University, with temperature- and humidity-controlled rooms, and fed standard laboratory chow and tap water *ad libitum*.

The animals were anaesthetized using a mixture of Ketamine (150 mg/kg body weight; Nimatek®, Euovet Animal Health B.V., Bladel, The Netherlands) and Xylazine (10 mg/kg body weight; Rompun®, Bayer B.V., Mijdrecht, The Netherlands) by intraperitoneal injection (i.p.). During anaesthesia, the mice were kept on a warm mattress with their face down and their eyes wetted with Visgel® (Eurovet, Bladel, The Netherlands). The mice were grouped randomly (six per group), with each group receiving a different treatment. The skin of the mice was washed carefully with deionised water prior to marking two areas (~1 cm^2, both left and right) on the upper flank of the back of the mice, near the head. An impaired skin barrier was induced by sequential tape stripping by a single individual. Tape strips (black D-squame) of ~1 cm^2 were cut and applied on the marked areas. The strips were compressed for 5 seconds before being removed in alternated stripping direction. A severe barrier disruption (i.e. model severe #4 as described in our previous paper) [11], defined as TEWL of 79 ± 6 $g/m^2/h$ (12 tape strips), was induced. After treatment, the mice were housed individually to avoid fight-induced skin injury. No scratching of the treated area or any abnormal behaviour was observed during the studies.

Topical applications

Immediately after disruption of the skin barrier, one test area per mouse was treated with natural VC, Vaseline, Eucerin cum aqua or with one of the biofilm formulations (Table 2; 5 mg/cm^2). Additionally, various lipid mixtures were evaluated: synthetic lipid mixtures (synthetic counterpart of VC lipids; L1), a similar synthetic mixture without the barrier lipids - ceramides, free fatty acids and cholesterol - (L2) and isolated VC lipids [14]. A single individual applied the samples onto the treatment area with a spatula. The bilateral untreated site served as control.

Table 2. List of the lipid mixtures, natural biofilm and commercially available formulations applied on the disrupted skin.

Entry	Sample
VC lipids	Natural, isolated VC lipids
L1	Synthetic lipid mixture
L2	Synthetic mixture without barrier lipids
VC	Vernix caseosa
Vas	Vaseline
Euc	Eucerin cum aqua

Biophysical evaluation of the skin

a. Macroscopic observations

Digital photographs were taken at predetermined time points using a canon ixus 40 (Canon Inc., Japan). The photographs of at least three mice were blinded and then independently scored by three independent investigators. The redness as well as the formation of a crust was classified into four different levels: obvious (++), intermediate (+), slight (+/-) or absent (-). The mean of the data was used for interpretation.

b. Transepidermal water loss

The level of barrier disruption and the repair rate were assessed by measuring the transepidermal water loss (TEWL) at regular time intervals using the TEWA-meter TM 210 (Courage & Khazaka, Cologne, Germany). The TEWL was measured by holding the probe lightly against the test area until a constant TEWL value was obtained. The pressure applied to the probe was just enough to prevent leakage of air between the lower rim of the Teflon cylinder and the skin.

The percentage of barrier recovery was calculated using the following equation: 1 – ((TEWL at indicated time point – TEWL of average control 'undamaged skin')/(TEWL immediately after stripping – TEWL of average control 'undamaged skin')) × 100%.

The AUC (area under the curve) of the recovery curve was calculated at the initial phase (1 day), at an intermediate period (3 days) and after full recovery (8 days). The different treatments were compared using a one-way ANOVA with a Bonferroni post-test; $P < 0.05$ was considered as statistically significant. All data analysis was performed using GraphPad Prism 4.0.

c. Histology

After the animals were sacrificed, biopsies were taken after 3 and 8 days using a pair of scissors in conjunction with metal tweezers, from the central part of the (treated) sites. The biopsies were immediately placed in a gelatine capsule, processed by fixation in Tissue-Tek®, frozen in liquid nitrogen and stored in liquid nitrogen prior to slicing. Samples (thickness 5 µm) were sliced perpendicular to the skin surface with a cryotome (Leica CM 3050S, Wetzlar, Germany). After fixation in cold acetone (4°C), contrast staining of the sections was performed for 1 min with a 1 % (w/v) aqueous safranin solution. Subsequently, the sections were washed with deionised water. To allow the corneocytes to swell, a 2 % (w/v) KOH solution was applied on the sections during 20 min [15, 16]. Visualization was performed with a light microscope combined with a digital camera (Carl Zeiss axioskop, Jena, Germany). The thickness of the viable epidermis was measured in at least 12 different locations of the stained cross-sections to obtain the mean.

Results & Discussion

Topical application of various synthetic biofilms on disrupted skin

The disruption of the skin was performed as described previously [11]. After sequential tape stripping (i.e. 12 tape strips) the skin was damaged in a controlled manner yielding a TEWL of 79 ± 6 g/m^2/h. In comparison, normal (undisrupted and untreated) skin has a TEWL of ~9 g/m^2/h. In our previous study we showed that the SC was completely removed after tape stripping while the remaining epidermis was intact. Moreover, a rather slow recovery (i.e. 200 h) was obtained, which makes it an excellent model to study the effect of formulations on both the initial and long-term barrier recovery. In a previous study, topical application of natural VC on this disrupted skin showed that complete recovery was enhanced, suggesting the potential use of VC treatment clinically [11]. Therefore, we studied the effect of VC mimicking formulations on skin barrier recovery in comparison to natural VC and commonly used oil-based ointments (Vas and Euc). The various synthetic formulations and commercially available creams used in this study are given in table 2. The base of our synthetic biofilms consisted of particles embedded in a lipid matrix [12]. The various components as well as the particle/lipid ratio, the water content in the particles and the absence or presence of a lipid coating on the particles (Table 1) were varied to select the best performing biofilm. The various formulations had different effects on redness and crust formation (Fig. 1; Table 3), which were rated concerning their severity.

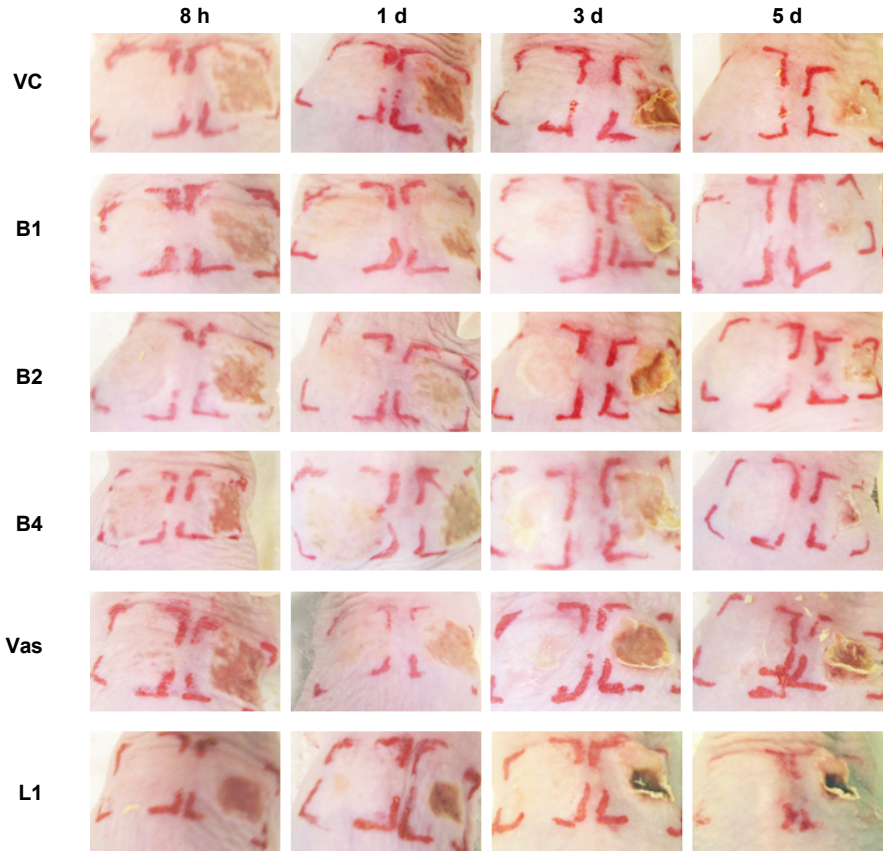

Figure 1. Representative macroscopic observations of the effect of topically applied native VC, the biofilms B1, B2 and B4, Vas and lipid mixture L1 on disrupted skin after 8 h, 1 day, 3 days and 5 days. Formulations were applied on the left side and the right side served as disrupted, untreated skin serving as control.

The disrupted, untreated site was clearly glistening and red following tape stripping, after which a crust was formed. Upon application of natural VC, redness disappeared in a few minutes and crust formation was prevented. Application of the various biofilms on the disrupted skin resulted in different observations (Table 3). The biofilms B1c and B2 (Fig. 1) showed predominantly intermediate crust formation (Table 3), whereas B2c showed both redness and crust formation up to 5 days post-disruption. However, it was clearly observed that application of B1 prevented largely both redness and crust formation (Fig. 1; Table 3): only after 1 and 3 days a slight crust formation was observed. Macroscopic observations (Fig. 1) showed once more that B1 improved skin conditions compared to the other biofilms, in which B2 was selected as representative.

Table 3. Rating of redness and crust formation of the disrupted sites followed in time. The average evaluation of digital pictures from three independent investigators is presented.

Treatment	Rating in time*			
	8 h	**1 d**	**3 d**	**5 d**
disr., untreated	++	++	++	+
VC	-	-	-	-
B1	-	+/-	+/-	-
B1c	+/-	+/-	+	-
B2	+/-	+/-	+	-
B2c	+/-	+/-	+/-	+/-
B3	+/-	+	+	-
B4	+/-	+/-	+	-
Vas	+/-	+/-	+/-	-
Euc	+/-	+/-	+/-	-
L1	-	+/-	+/-	-
L2	+/-	+/-	+/-	-

* Redness and crust formation on skin were assessed as obviously (++), intermediately (+), slightly (+/-) or absent (-)

The recovery of the skin was also monitored by TEWL measurements (Fig. 2). Initially the effect of the biofilm mimicking most closely the properties (in terms of water level in the particles, lipid composition and the presence of a lipid coating) of VC on barrier recovery was studied. This biofilm, referred to as biofilm B2c, with particle/lipid ratio of 2:1 using lipid coated particles with an initial water content of 80 % (w/w). It was observed that upon application of this biofilm B2c (5 mg/cm^2) on disrupted skin, complete recovery occurred already within ~100 h as compared to ~200 h for disrupted, untreated skin (Fig. 2A). The inset (Fig. 2A) shows the initial recovery period (phase 1) where a rapid barrier recovery was observed (TEWL decreased from 79 ± 6 g/m^2/h to 38 ± 6 g/m^2/h). Visually, the biofilm B2c disappeared within 3 to 4 h (phase 2, Fig. 2A). As the skin was not fully recovered a high TEWL was measured (56 ± 5 g/m^2/h; barrier recovery of 34 ± 6%). Further monitoring of the skin barrier (phase 3, Fig. 2A) showed complete recovery within 100 h. Upon application of biofilm B2c, the initial recovery was similar to VC treated skin, whereas the recovery period between 3 and 75 h was slower. Complete recovery, however, occurred within the same time span (i.e. 100 h) as VC treated skin.

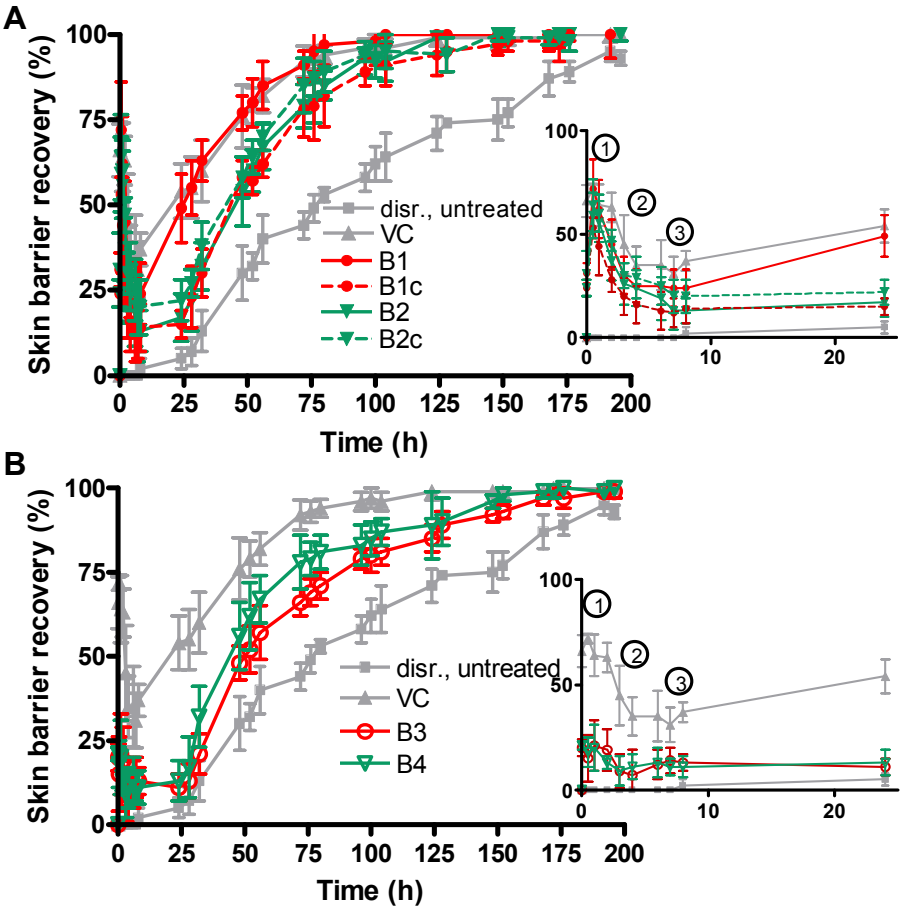

Figure 2. Skin barrier recovery after tape stripping as function of time and after application of various formulations. (A) Formulations with particle/lipid ratio 2:1, B1 (●; continuous line), B1c (●; dotted line), B2 (▼; continuous line) and B2c (▼; dotted line) are depicted and compared to VC (▲; 5 mg/cm²) and disrupted, untreated skin (■). (B) Formulations with particle/lipid ratio 5:1, B3 (o), B4 (▽), are depicted and compared to VC (▲; 5 mg/cm²) and disrupted, untreated skin (■). The inset shows the recovery during the first 24 h that can be divided into three distinct stages: in phase 1 the formulations covered the skin, in phase 2 the formulations disappeared and in phase 3 further monitoring of the skin barrier was performed. Data are shown as average ± SD (n = 6).

When decreasing the initial water content of the particles to 50% (w/w) and maintaining other components equal including the particle coating (biofilm B1c; Table 1), a similar barrier recovery profile as for biofilm B2c was obtained (Fig. 2A). A comparable skin barrier repair outcome was also obtained (Fig. 2A) when applying biofilm B2 on disrupted skin. B2 is very similar to B2c, except that uncoated particles were used. The particle/lipid ratio of this biofilm B2 was 2:1

and the initial water content of the microgels was 80 % (w/w; Table 1). A biofilm composed of uncoated particles with 50 % (w/w) initial water content and particle/lipid ratio of 2:1 (biofilm B1; Table 1), however, showed a different profile. Up to 10 h, the barrier recovery profile was still comparable to the biofilms B1c, B2 and B2c and VC (see inset Fig. 2A). However, from that moment (i.e. 10 h after stripping) until complete recovery the profile was similar to that of VC and, hence, more rapid compared to the other biofilms. Moreover, complete recovery was already obtained within 75 h, which is slightly faster than VC (i.e. 100 h). Overall, B1 mimics most closely the barrier recovery profile of VC (based on TEWL data).

Aforementioned, only the effect of various formulations with a particle/lipid ratio of 2:1 was shown. However, we observed previously that biofilms with a 5:1 particle/lipid ratio showed a more dense and random particle distribution and a higher water content more closely mimicking the corneocyte distribution and water level in VC [12]. Therefore, the topical application of biofilms with a particle/lipid ratio of 5:1 with an initial water content of 50 % (w/w) or 80 % (w/w) in the particles (biofilm B3 and B4, respectively; Table 1) was also evaluated. Lipid coating on the particles was omitted as this did not increase barrier recovery for the 2:1 particle/lipid formulations (Fig. 2A). Topical application of B3 and B4 on disrupted skin resulted predominantly in intermediate crust formation (Table 2; Fig 1). TEWL measurements showed a complete recovery within 150 h after application of both biofilms (Fig. 2B). Application of B3 and B4 (5 mg/cm^2) decreased only slightly the TEWL from 79 ± 6 g/m^2/h to 61 ± 8 g/m^2/h and 62 ± 7 g/m^2/h, respectively (barrier recovery increased to 20 ± 7% and 21 ± 8%, respectively, indicated as phase 1 in Fig. 2B). Within 1 to 2 h the biofilms B3 and B4 disappeared visually (phase 2, Fig. 2B). The skin was not fully recovered as indicated by a TEWL of 71 ± 6 and 69 ± 4 g/m^2/h, respectively. Further skin barrier repair was monitored (phase 3, Fig. 2B) until complete recovery was achieved (i.e. 150 h). Apparently, increasing the initial water content of the particles (i.e. B4 compared to B3; Fig. 2B) or increasing the amount of particles (e.g. B4 compared to B2; Fig. 2) did not improve further the skin barrier repair. This might also be due to the lower lipid content of the formulations B3 and B4 as these biofilms contain less lipids compared to the biofilms B1, B1c, B2 and B2c (i.e. 16.7 % vs. 33.3 %) as was shown previously [12]. Moreover, when comparing water release from the biofilms as described in the previous study [12], it is observed that initial high water release from the biofilms resulted in more enhanced barrier repair compared to the sustained water release from the pre-coated microgels (i.e. B1 vs. B1c, respectively)

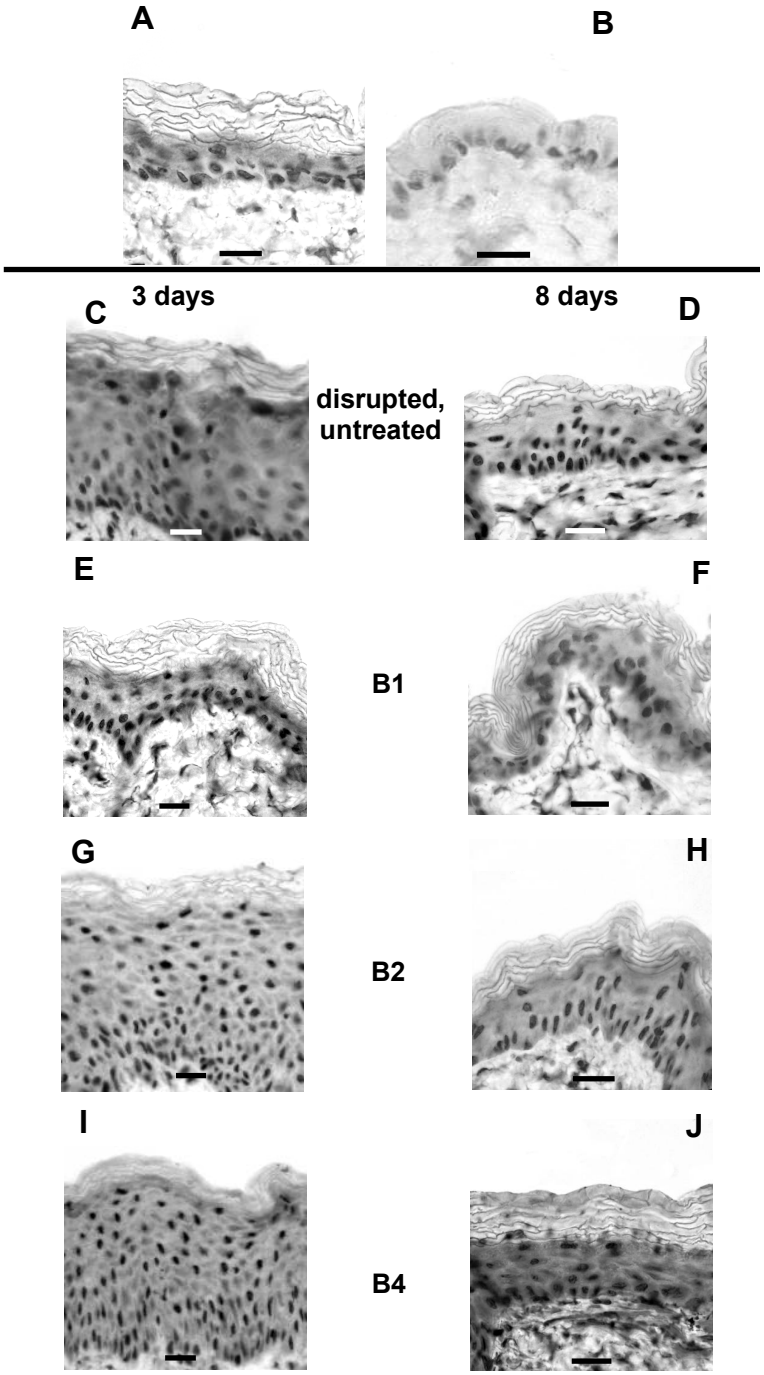

Figure 3. Cross-sections of hairless mouse skin prior to (A) and directly after tape stripping (B). Cross-sections of disrupted, untreated (C, D), B1 treated (E, F), B2 treated (G, H) or B4 treated (I, J) after 3 days and 8 days of recovery, respectively. *Scale bars* = 20 µm

The effect of the biofilms on the recovery of extensively disrupted skin was also histologically studied. Normal skin is characterized by viable epidermal cells and stratum corneum containing 4 to 6 corneocyte layers (Fig. 3A). After complete barrier disruption, using the tape stripping method applied in this study, the SC was removed (Fig. 3B) [11]. Three days after tape stripping, corneocytes gradually reappeared on disrupted, untreated skin (Fig. 3C) whereas only 8 days after recovery, similar to normal murine SC, untreated skin exhibited 3-5 corneocyte layers (Fig. 3D). Morphological features of biofilm-treated skin after 3 and 8 days are depicted in Fig. 3B: biofilms B1, B2 and B4 were chosen as representatives for this purpose. After 3 days, the presence of 3-5 corneocyte layers was observed (Fig. 3B) for all biofilms. However, the viable epidermis was largely thickened for B2 and B4, similar to disrupted, untreated skin (Fig. 3C). Epidermal thickening has been partly associated with hyperproliferation [17]. Application of B1 on the disrupted skin showed a normal thickness of the viable epidermis (Fig. 3G, 3H) indicating a further stage in the healing process. After 8 days, the various treatments (Fig. 3) showed similar results in SC and viable epidermis appearance compared to normal hairless mouse skin (Fig. 3A).

In addition, the average thickness of the viable epidermis was determined by measuring 12 random locations of the biopsies. The data obtained 3 and 8 days after treatment are presented in figure 4. After 3 days, the viable epidermis of B2 and B4 was up to 4 times thicker compared to the negative control (undisrupted, untreated skin). Upon treatment with B1, however, the thickness of the epidermis after 3 days was 31.0 ± 8.9 µm and comparable to VC treated skin (i.e. 25.2 ± 4.8 µm). After 8 days, the thickness of the epidermis of all biofilm-treated areas was similar to undisrupted, untreated skin. In comparison, the disrupted, untreated skin still showed a 2 times thicker epidermis compared to undisrupted skin.

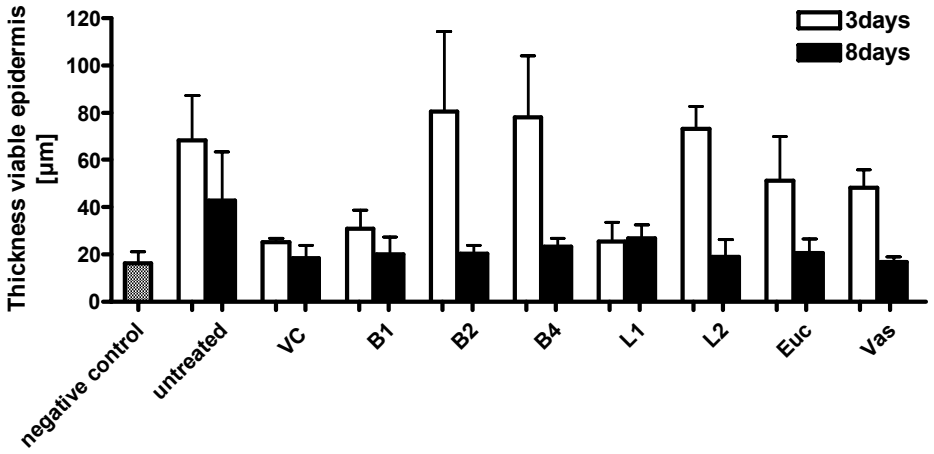

Figure 4. Thickness of the viable epidermis of undisrupted, untreated (negative control), disrupted, untreated or disrupted, treated hairless mouse skin after 3 (white) and 8 days (black) of recovery. At least 12 different random locations of the cross-sections were measured per treatment. Data is shown as average + SD.

Topical application of lipid formulations on disrupted skin

B1, the biofilm inducing the most accelerated barrier recovery, was also applied on the disrupted skin using 15 mg/cm^2. TEWL measurements showed a similar barrier recovery profile (data not shown) as was obtained for the biofilm B1 applied at 5 mg/cm^2 (Fig. 2A). To determine whether the lipids play a role in the recovery, the effect of lipids in the absence of particles (formulation L1, 5 mg/cm^2; Table 2) on barrier recovery was evaluated as well. It was observed that application of L1 showed similar effect as B1: redness and crust formation were largely prevented (Fig. 1; Table 2) and hence only slight curst formation was observed after 1 and 3 days. However, when omitting the barrier lipids (i.e. cholesterol, fatty acids, and ceramides [12, 14]) from the lipid matrix (lipid mixture L2), prevention of redness and crust formation was less effective compared to L1 and B1 (Table 2). Treatment with L2 resulted in occurrence of a crust and/or redness after 8 h to 3 days. TEWL measurements showed that application of L1 on the disrupted skin resulted in a similar barrier recovery as observed for the biofilms B1c, B2c and B2c (Fig. 5A). Application of L2, which has similar lipid composition as L1 but without the barrier lipids, on disrupted skin, decreased clearly the barrier recovery (Fig. 5A): complete barrier recovery

was obtained within 150 h compared to 100 h for L1. The histological assessment also indicates an improved recovery with L1 compared to L2 (Fig. 6).

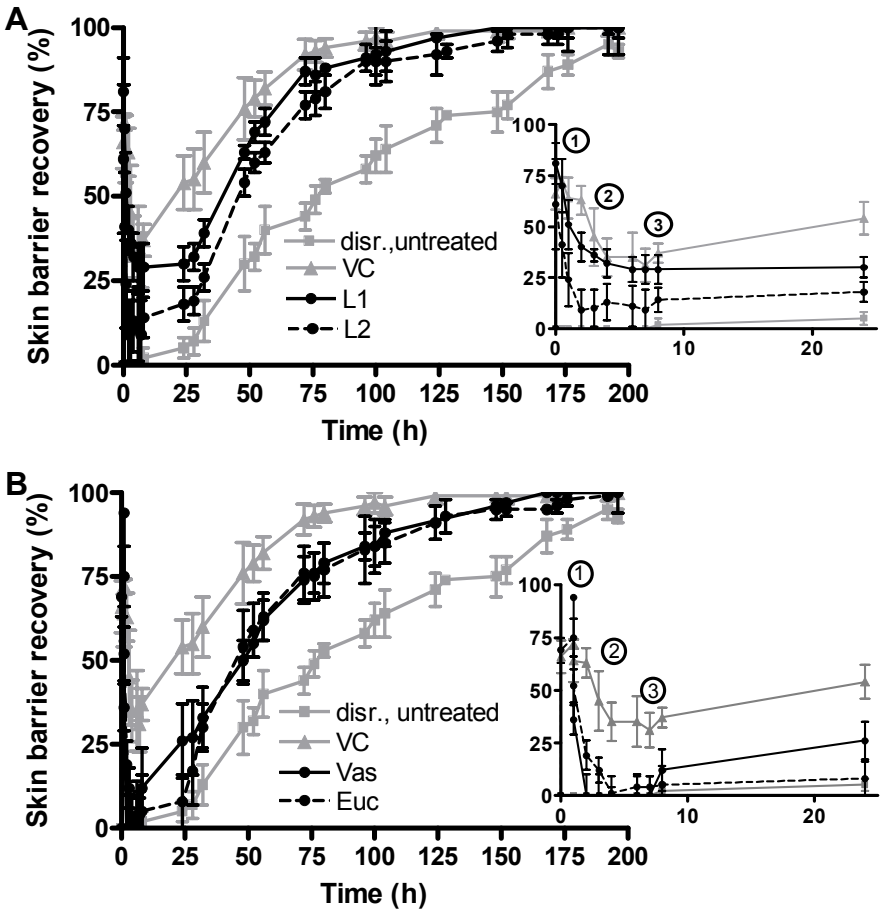

Figure 5. Skin barrier recovery after tape stripping as function of time and after application of various formulations. (A) synthetic lipid mixture L1 (●; continuous line) and synthetic lipid mixture without barrier lipids L2 (●; dotted line) are depicted and compared to VC (▲; 5 mg/cm²) and disrupted, untreated skin (■). (B) The commonly used oil-based ointments Vas (●; continuous line) and Euc (●; dotted line) are depicted and compared to VC (▲; 5 mg/cm²) and disrupted, untreated skin (■). The inset shows the recovery during the first 24 h. In the initial phase (phase 1) the formulations covered the skin and in phase 2 the formulations disappeared (phase 2). In phase 3 further monitoring of the skin barrier was performed. Data are shown as average ± SD (n = 6).

In addition, a very thick SC (~10 layers; Fig. 6A) was observed after 3 days of recovery for L1 whereas L2 only showed 3 layers (Fig. 6C). Despite of the thick SC, the TEWL is still increased which indicates a perturbed barrier. Upon application of L2 also the viable epidermis was thicker compared to L1-treatment after 3 days (Fig. 4), which in turn was comparable to undisrupted, untreated skin. After 8 days the thickness of epidermis with both treatments (Fig. 6B and 6D, respectively) are similar to normal skin (Fig. 3A). Hence, it was showed that microscopic observations and rating after topical application of L1 on disrupted skin were similar to the best performing biofilm B1, although the skin barrier recovery profile was not as effective (i.e. 100 h and 75 h, respectively, for complete barrier recovery). Moreover, the presence of barrier lipids (L1 vs. L2) is of major importance as they promoted barrier repair as was observed previously [18, 19]. The lipid formulation L1 contains about 10% of barrier lipids [12]. It was shown in literature that lipid mixtures containing ceramides (using acyl-ceramides isolated from mouse skin), fatty acids and cholesterol, increase the barrier recovery in acetone disrupted, hairless mouse skin [18].

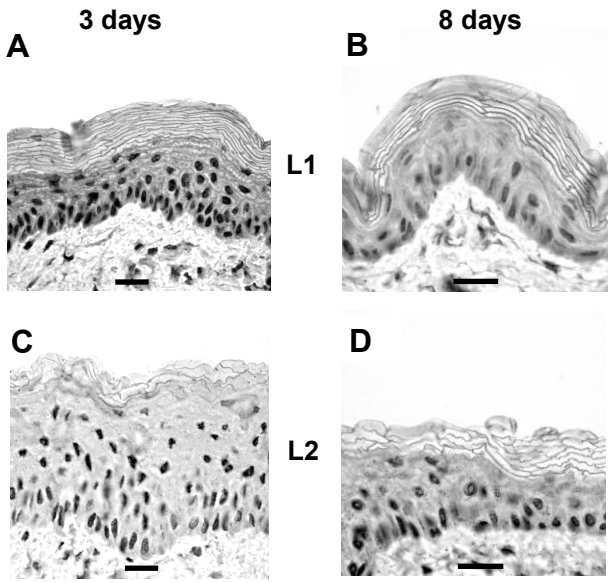

Figure 6. Cross-sections of tape stripped hairless mouse skin treated with L1 (A, B) and L2 (C, D) after 3 days or 8 days of recovery. *Scale bars* = 20 µm.

Although our lipid mixture contains synthetic (acyl-) ceramides, also a clearly improved barrier recovery rate was observed between these lipid mixtures and lipids without barrier lipids (Fig. 5A). Mechanistically, this effect can be attributed to the uptake of the barrier lipids by the viable epidermal cell layers where they can be incorporated in the lamellar bodies and at a latter stage may be involved in the formation of the intercellular lamellae [20, 21].

Topical application of Vas and Euc on disrupted skin

The effect of the aforementioned VC mimicking formulations on skin barrier recovery were also compared to the commonly used oil-based ointments Vas and Euc. Vas has been speculated to be occlusive and to increase barrier recovery [22, 23]. Euc is a water-in-oil emulsion that contains large amounts of water (i.e. 50%) [1], which is known to be of high importance in wound healing. It was observed that upon application of Vas on disrupted skin the redness did not disappear within the first hours as opposed to VC. Two hours after application, Vas was not visible anymore at the skin surface and the skin had a similar appearance as disrupted, untreated skin although crust formation was largely prevented (Fig. 2). Upon application of Euc, the emulsion was not visible anymore after 2 h and the redness almost completely disappeared (data not shown). Crust formation, however, was not completely prevented. The treatments showed a slight crust development, indicating an improved wound healing compared to untreated but clearly less effective than native VC (Table 2).

The recovery of the disrupted skin after application of Vas and Euc, was also monitored by TEWL measurements at various time points (Fig. 5B). Application of Vas (5 mg/cm^2) immediately restored the barrier function of the skin (indicated as phase 1 in Fig. 5B; TEWL decreased from 79 ± 6 g/m^2/h to 3 ± 0.5 g/m^2/h) demonstrating the occlusive properties of Vas. As mentioned, Vas disappeared visually within 2 h, which was associated with a higher TEWL (phase 2, Fig. 5B). Four hours after application the TEWL was 77 ± 7 g/m^2/h; for disrupted, untreated skin the TEWL was in the same range. Subsequent monitoring of the skin barrier showed that complete recovery occurred within 150 h (phase 3, Fig. 1). An immediate restored skin barrier function was observed as well upon application of Euc (TEWL decreased from 73 ± 5 g/m^2/h to 25 ± 8 g/m^2/h; phase 1 in Fig. 5B). Four hours after application Euc disappeared visually and TEWL increased to 74 ± 8 g/m^2/h (phase 2, Fig. 5B). The subsequent recovery profile (phase 3, Fig. 5B) was similar to Vas and complete recovery was observed within 150 h. Since the disrupted, untreated skin showed nearly complete recovery within 200 h, application of both Vas and Euc did enhance barrier recovery to some extent. However, when comparing the effect of Vas and

Euc to natural VC (Fig. 5B; [11]) or to our biofilms (Fig. 2), barrier recovery was slower (i.e. 100 h compared to 75-100 h, respectively).
Subsequently, the effect of Vas and Euc on the recovery of extensively disrupted skin was histologically studied. Vas and Euc treated skin showed the presence of 5-6 corneocyte layers 3 days after recovery and after 8 days no major differences in SC could be observed (data not shown). However, it was observed that both Vas and Euc showed thickened viable epidermis (i.e. 2.5 times thicker epidermis compared to undisrupted skin) after 3 days of recovery (Fig. 4). The average thickness of the viable epidermis was determined by measuring 12 random locations of the biopsies. After 8 days, the thickness of the epidermis of the Vas and Euc treated areas was similar to undisrupted, untreated skin (Fig. 3A). In comparison, the disrupted, untreated skin still showed a 2 times thickened epidermis compared to undisrupted skin.
In summary, macroscopic observations, TEWL measurements as well as histological analysis showed that barrier recovery was enhanced and that crust formation was partly prevented upon application of Vas or Euc on disrupted skin, however, not as effective as VC or the best performing biofilm B1. In agreement with the literature [21], our results indicate that the barrier lipid containing biofilms perform better than Vas (from 8 h onwards), whereas in the initial recovery phase Vas treatment shows lower TEWL values which can be attributed to the occlusive nature of Vas.

Comparison of the recovery after the various treatments
In order to evaluate the recovery curves of the various treatments in a statistic manner, the AUCs of the individual treatments were calculated and compared. The results after 1, 3 and 8 days of recovery are presented in figure 7. In general, the same trends can be observed for all 3 time points: disrupted, untreated skin (white bars) exhibits the lowest AUC compared to disrupted, treated (applied for all treatments) skin, indicating the lowest recovery rate. After 3 and 8 days, natural VC showed significantly better recovery than Vas and Euc, however, no significant difference to the best performing biofilm, i.e. B1. In turn, B1 demonstrated a significantly improved recovery versus B1c, B3, B4, L2, Vas and Euc after both 3 and 8 days. Moreover, B1 showed a significant improvement of barrier recovery after 3 days compared to L1, although after 8 days the difference between both formulations was not significant. It has been suggested that the water-handling properties are of major importance for the proper functioning of a VC substitute [24]. We therefore optimized the water release from the biofilms in our previous study [12], mimicking as closely as possible the water release rate from VC. When focusing on the water release rate from the various biofilms, no clear correlation was observed between the skin barrier repair rate and the water release rate from the biofilms. However, it was observed that the biofilm with the

fastest water release rate, biofilm B1, resulted in a better performance than the other biofilms concerning crust formation (Table 2), epidermal thickening (Fig. 4) and barrier recovery (Fig. 2A). When comparing the biofilms with a different particle/lipid ratio, it is obvious that the biofilms with a 2:1 particle/lipid ratio resulted in a better performance than the biofilms with a 5:1 particle lipid ratio. This indicates that the amount of lipids might play an important role for the biological effect.

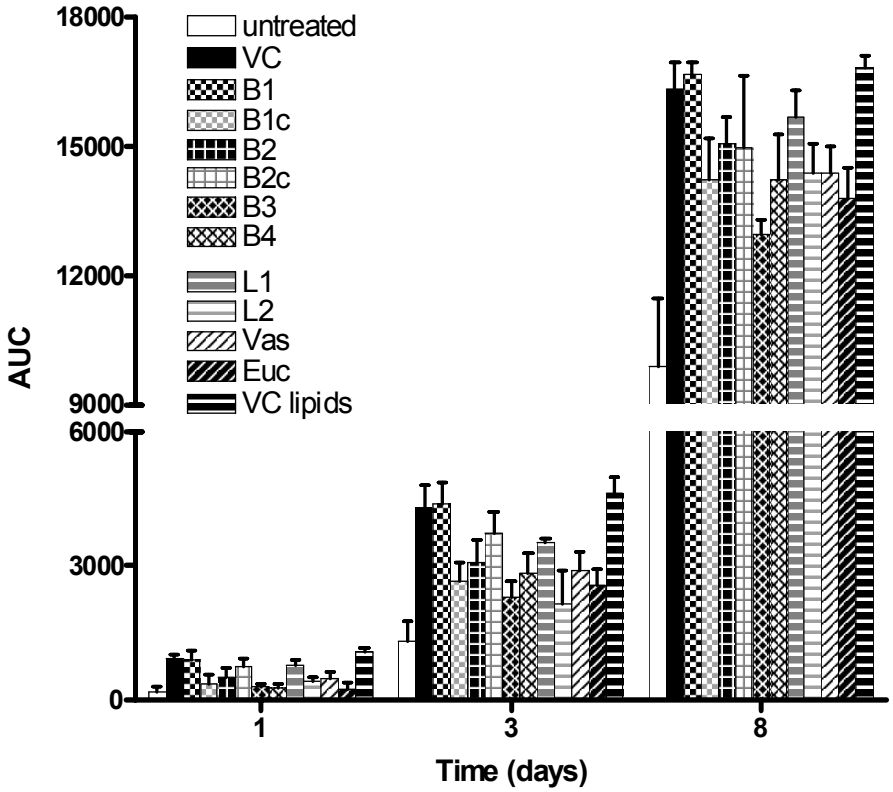

Figure 7. Area under the curve (AUC) of the recovery curves after tape stripping of hairless mouse skin after 1, 3 and 8 days of recovery. All treatments are depicted: untreated (white), VC treated (black), B1 (black; checked), B1c (grey; checked), B2 (black; crossed), B2c (white; crossed), B3 (black; crossed diagonally), B4 (white; crossed diagonally), L1-L2 (grey and white; striped horizontally, respectively), Vas-Euc (white and black; striped diagonally, respectively) and isolated VC lipids (black, striped horizontally). Data is represented as average + SD (n = 6).

Therefore, also with respect to natural VC, the question arises how important are the water-handling properties as well as the presence of corneocytes for barrier recovery? Therefore, we examined also the barrier recovery of VC lipids in the absence of water and corneocytes. These studies revealed that VC lipids resulted in a similar barrier recovery as was observed for natural VC (Fig. 7). This is very similar to the results that were obtained for the synthetic biofilms vs. the lipid formulation without water and particles (L1), but in the presence of barrier lipids. This demonstrates that the lipids, including barrier lipids, play a more prominent role in barrier recovery than the water content and the presence of corneocytes. However, the water-containing corneocytes may be very beneficial for increasing skin hydration, especially important for the treatment of dry and diseased skin. Importantly, the particles (synthetic corneocytes) can also be used as drug delivery matrix in our biofilm formulation. Incorporation of e.g. growth factors or natural moisturizing factor is an attractive approach and will be subject of future studies.
The obtained results clearly indicate that for an improved barrier recovery several aspects are important for the formulation. I) Besides the presence of barrier lipids, the initial high water release from the formulations as observed for B1 (likely due to a low particle/lipid ratio and particles without lipid coating) [12] appears to be beneficial. In contrast, native VC exhibited a fast barrier recovery rate, although a very slow water release and only little water in the external lipid matrix was reported for the natural biofilm [5]. This enhancement of barrier recovery might be explained by the fact that VC comprises a number of components (e.g. antioxidants such as alpha-tocopherol) that might stimulate the epidermal metabolism [8]. II) Occlusion of impaired skin (i.e. application of Vas) only enhances recovery to a small extent whereas a more permeable formulation (i.e. lipid mixtures) seems more suitable. III) The balanced ratio of particles, water and the lipids improve barrier recovery. This clearly indicates that the presence of both the barrier lipids and the highly hydrated particles (initial water content 50% (w/w)) in a specific ratio are necessary to obtain the best performing biofilm inducing a fast skin barrier repair similar to native VC.

In conclusion, a clear improvement of skin barrier recovery, reduced crust formation and epidermal hyperproliferation was demonstrated upon application of all tested formulations. However, the synthetic VC analogues showed stronger effects concerning the recovery rate than Vas and Euc, especially biofilm B1 mimicked the effects of native VC most closely. The importance of the presence of individual components, i.e. barrier lipids and water, as well as the ratio of these components was observed. In future, these biofilms will be tested in humans to demonstrate their beneficial effect with a potential use of the particles as drug delivery systems.

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