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

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Mimicking vernix caseosa - preparation and characterization of synthetic biofilms

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Abstract

The multiple protecting and barrier-supporting properties of the creamy, white biofilm vernix caseosa before and after birth suggest that a vernix caseosa biomimetic could be an innovative barrier cream for barrier-deficient skin. The aim of this study was the rational design and preparation of synthetic biofilms mimicking the unique composition and properties of natural vernix caseosa. Hexagonal, highly hydrated hyperbranched polyglycerol microgel particles (30 μm in diameter) were embedded in a synthetic lanolin-based lipid mixture using a micromixer. In these formulations, the water content of the particles (i.e. 50% and 80%), an additional lipid coating of the particles and different particle/lipid ratios were varied. Characterization with confocal laser scanning microscopy 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 vernix caseosa very 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 vernix caseosa most closely. Comparable results between native vernix caseosa and the synthetic formulations were obtained concerning water handling-properties while lower elasticity and lower viscosity were observed for the synthetic biofilms. The biofilm formulations were stable for at least one month at 4°C. In conclusion, our formulations mimic natural vernix caseosa very closely and are promising candidates for *in vivo* studies.

Introduction

Vernix caseosa (VC) is the creamy white skin-surface biofilm which covers the skin of the fetus and the newborn. It is suggested to feature multiple biological functions such as waterproofing and facilitation of the skin formation *in utero* [1, 2]. During delivery it acts as a lubricant, while exhibiting anti-infective[3], antioxidant[4] and skin cleansing properties postnatally [5]. Moreover, the dehydration profile of VC shows a strong temperature-dependence, which enables VC to hydrate the newborn's skin in a sustained manner [6, 7]. These multiple biological functions and unique properties of VC suggest that the generation of a synthetic VC equivalent could lead to a new generation of biofilms. The biofilms might be applicable for extremely low birth weight infants with deficient barrier function and absence of VC [5, 8] but may also be beneficial for adult skin to enhance wound healing [9].

VC consists of dead cells (corneocytes) that are embedded in lipids, thus very similar to the structure in stratum corneum (SC), the uppermost layer of the skin. VC is composed of 80% water, about 10% proteins and 10% lipids [1, 8]. The most abundant protein present in VC is keratin, which forms the scaffold of the corneocytes. These dead cells represent the main water reservoir of VC and are mostly polygonal and flat in shape with a diameter of approximately 30 μm .

Additionally, VC contains antibiotic peptides and polypeptides with innate immune functions [3, 10, 11]. VC lipids consist of a large variety of different lipid classes, including squalene, sterol esters/wax esters, triglycerides and free sterols [12]. Similar to SC, also barrier lipids -cholesterol, fatty acids, and ceramides- are present but they comprise only between 10% and 30% of the total amount of VC lipids [8, 13]. At the interface between the corneocytes and the free lipids, a monolayer of covalently linked lipids is present [13].

Because of the excellent properties of VC for the treatment of barrier-deficient skin [14], the aim of the present study is the generation of synthetic biofilms mimicking the unique composition and properties of natural VC. Previously, the thermal transitions and lipid organization of series of lanolin-derived, synthetic lipid mixtures were examined [15]. From these studies the lipid mixture mimicking most closely the physical properties of VC lipids was selected and used in the present study. Furthermore, it was reported that hydrated hydrogel microparticles can be prepared from hyperbranched polyglycerol [16, 17]. This biocompatible polymer [18] enabled us to reconstruct synthetic corneocytes (referred to as particle), similar to natural corneocytes in shape and size (i.e. hexagonal, Ø 30 µm) [17]. In the present study, these particles were embedded in a lipid mixture composed of the above mentioned (semi-) synthetic lipids using a specially designed micromixer. Compositions with various particle/lipid ratios as well as particles with different water content in the absence and presence of a surrounding lipid coating were studied to obtain the best performing biofilm. The homogeneity of these biofilms was characterized by confocal laser scanning microscopy (CLSM). Moreover, stability, water handling-properties, rheology and thermotropic behaviour of these VC mimetics were investigated.

Materials & Methods

Materials

HyPG (M_n 2000 g/mol, on the average 32 hydroxyl groups per molecule) was obtained from Hyperpolymers GmbH (Freiburg, Germany). Dimethyl sulfoxide (DMSO, $H_2O \leq 0.005\%$), glycidyl methacrylate (GMA) were purchased from Fluka (Buchs, Switzerland). 4-(*N,N*-dimethylamino)pyridine (DMAP) was purchased from Acros Chimica (Geel, Belgium). 2-hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone (Irgacure 2959, purity 98%), triolein, tripalmitolein, trinervonin, squalene, cholesterol, palmitic acid, palmitoleic acid, oleic acid, methyl sulfoxide- d_6 (99.9% atom D) and fluorescein isothiocyanate (FITC)-dextran ($M_w = 70$ kDa) were provided by Sigma-Aldrich (Zwijndrecht, The Netherlands). Ascorbic acid (vitamin C, purity 99%) was obtained from BUFA B.V. (Uitgeest, The Netherlands). Diethyl ether was purchased from Biosolve Ltd. (Valkenswaard, The Netherlands). Hydrous lanolin was purchased from Caesar & Loretz (Bonn, Germany). The synthetic ceramides were kindly provided by Cosmoform B.V. (Delft, The Netherlands). DOPC (dioleoyl phosphatidylcholine) and DOTAP (dioleoyl trimethylammonium-propane) were

obtained from Avanti Polar Lipids Inc (Alabaster, AL, USA). Texas Red and Nile Red were manufactured by Molecular Probes Europe BV (Leiden, The Netherlands). The commercial cream 'Zwitsal – protective cream with vernix protection' was purchased from Sara Lee (The Hague, The Netherlands).

Synthesis of hyperbranched polyglycerol derivatized with glycidyl methacrylate

Photocrosslinkable HyPG-MA was prepared as described previously [16]. The degree of substitution (DS, the percentage of derivatized hydroxyl groups) of the obtained product was 11 as determined by proton nuclear magnetic resonance spectroscopy (^1H NMR; Gemini 500 MHz spectrometer, Varian Associates Inc. NMR Instruments, Palo Alto, CA) in dimethyl sulfoxide- d_6 .

Preparation of the particles

Structured HyPG-MA hydrogel microparticles were prepared by photolithography [17]. In brief, HyPG-MA (50% or 20% (w/w)) was dissolved in phosphate buffer (10 mM, pH 7.2) also containing 1% (w/v) Irgacure 2959 and 2% (w/v) vitamin C and flushed with nitrogen. To obtain fluorescent labelled particles a few drops of 4.5% FITC-dextran solution in phosphate buffer were added to 1 ml of the HyPG-MA solution. This solution was applied between a glass plate (40x40x0.7 mm) and a patterned mask (Philips Research Laboratories, Micro Fabrication Center, Eindhoven, The Netherlands; hexagons of \varnothing 30 μm) using a spacer of 20 μm . Then, the HyPG-MA was polymerized via exposure to UV light (intensity of 11 mW/cm², UV light model Bluepoint 4 UVC, Dr. Hoenle AG, UV-Technology, Gräfelfing, Germany) for 120 seconds. Subsequently, the mask was removed and the microparticles were collected by rinsing the plate with water.

Coating of the particles using liposomes

Preparation of lipid vesicles and subsequent coating of the microparticles was performed as described previously [19]. In short, to prepare the lipid vesicles, DOPC (20 mM) and DOTAP (35 mM) were dissolved in 2 ml chloroform. Next, 4 μl Texas Red solution was added to 190 μl of the DOPC-DOTAP solution as fluorescent label. The chloroform was evaporated from the lipid matrix yielding a lipid film on a glass vial with cylindrical shape (\varnothing 3 cm). HEPES buffer (2 ml, 10 mM, pH 7) was added to yield a lipid concentration of 1 mg/ml and the mixture was sonicated (Braun Labsonic tip-sonicator; Braun Biotech, Melsungen, Germany) on ice for 10 minutes to obtain the liposomes. These liposomes (500 μl) were mixed with a suspension (100 μl) of HyPG-MA microgels and shaken (1000 rpm) for 1 h at room temperature to allow adsorption of the liposomes onto the surface of the microgels. The samples were centrifuged for 10 min at 2000 rpm, the supernatant removed and the particles were redispersed in 1 ml MilliQ-water. The centrifugation and resuspension was repeated three times. Finally, the microparticles were redispersed in 150 μl of MilliQ-water and

directly used for further studies. Visualization of the lipid coated HyPG-MA microparticles was performed by CLSM.

Preparation of the lipid matrix

The composition and preparation of the lipid mixture has been described previously [15]. Briefly, the nonpolar sterol ester and wax ester fractions of lanolin were isolated by column-chromatography. Subsequently, chloroform/methanol 2:1 lipid solutions were pipetted into a glass tube with following composition: combined sterolester/waxester/dihydroxywaxester 48.0%, trivervonin 11.9%, tripalmitolein 11.9%, triolein 11.9%, squalene 6.4%, cholesterol 3.5%, ceramide EOS (C30) 0.7%, ceramide NS (C24) 1.0%, ceramide NP (C24) 0.4%, ceramide NP (C16) 0.4%, ceramide AS (C24) 0.8%, ceramide AP (C24) 1.6%, palmitic acid 0.8%, palmitoleic acid 0.3%, stearic acid 0.1%, oleic acid 0.3% (w/w). Then, the lipid solution was dried under a flow of gaseous nitrogen at 40°C.

Preparation and visualisation of the biofilms mimicking vernix caseosa

a. Mixing of the particles with the lipids

In order to homogeneously distribute the particles within the lipid matrix, an automatic ointment-mixer Topitec® (WEPA, Germany) was modified in-house for small-scale purposes. A small amount of lipids (~10 mg) was first transferred into the small mixing tube (Fig. 1) in order to coat the surface of the mixing tube and prevented the breaking of the particles. Then, the suspension of fully hydrated microgels was added, centrifuged (5 min, 500 rpm) and the supernatant was removed. Subsequently, the microgels were dispersed in a minimal amount of water. The rest of the lipids were added and the mixing tube was mounted onto the stirrer of the mixing device. Various formulations were prepared by using two particle/lipid ratios (i.e. 2:1 and 5:1 (w/w)). Particles with an initial water content of either 50% (w/w) or 80% (w/w) were selected for the studies. The composition of the various formulations is given in table 1. In the manuscript, the biofilms are denoted by an acronym presented in the first column (Table 1). The particles were mixed with the lipids until a homogeneous mixture was obtained while particles were still intact. For this purpose rotating speed and mixing time could be adjusted.

The composition of the various formulations is given in table 1. The biofilms are denoted in the manuscript by the acronym of the first column of table 1. The biofilms B1 and B2 comprise a particle/lipid ratio of 2:1 (w/w) while B3 and B4 contain a particle/ lipid ratio of 5:1 (w/w). Particles with an initial water content of either 50% (B1, B3) or 80% (w/w) (B2, B4) were selected for the studies. When using lipid-coated particles, the biofilms are denoted with 'c', e.g. B1c, B2c, B3c and B4c.

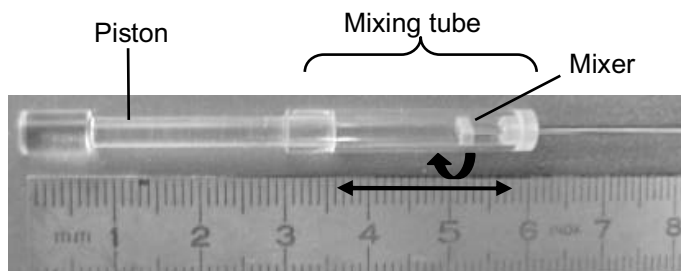


Figure 1. A custom-designed micromixer for sample sizes between 25 and 220 mg was designed to embed artificial corneocytes (particles) within the lipid matrix. During the mixing process, the mixer moves within the mixing tube (arrows). After mixing, the piston can be moved to eject the obtained biofilm.

Table 1. Composition of the various biofilms prepared in this study

Entry	Sample	Composition		
		Particle/lipid ratio	Initial water content particles (% w/w)	Lipid coated particles
B1	2:1_50	2:1	50	No
B1c	2:1_50_coated	2:1	50	Yes
B2	2:1_80	2:1	80	No
B2c	2:1_80_coated	2:1	80	Yes
B3*	5:1_50	5:1	50	No
B3c	5:1_50_coated	5:1	50	Yes
B4§	5:1_80	5:1	80	No
B4c	5:1_80_coated	5:1	80	Yes

*only for visualization study; §only for follow-up study

b. Visualization of the biofilms by means of confocal laser scanning microscopy

The homogeneity of the biofilms was characterized by CLSM. For this purpose, we labelled the lipids with Nile Red (0.1 µg/mg lipid) whereas the particles contained FITC-dextran as a label. Immediately after mixing, the biofilm was transferred onto a microscopic glass-slide, covered with a glass cover slip and visualized by CLSM. Images were processed using a Bio-Rad Radiance 2100 CLSM equipped with a Nikon Eclipse TE2000-U inverted microscope and a 40x S Fluor (NA 1.30; Nikon, Japan) oil-immersion objective. The images were captured using a helium neon laser with a wavelength of 543 nm. An emission filter excluded fluorescence with a wavelength below 570 nm. FITC-labelled

particles were visualized after excitation with an Argon laser (488 nm) combined with a 515/30 nm emission filter. At least 3 images of different sites in the biofilm were acquired with the Laser Sharp 2000 software (Bio-Rad, Hercules, USA). For stability studies two biofilms (i.e. B1 and B3; n=2) were re-evaluated by CLSM after storage for 1 month at 4°C in a closed eppendorf tube.

Thermotropic behaviour by differential scanning calorimetry

The thermotropic behaviour of various biofilms was examined by differential scanning calorimetry (DSC). The measurements were carried out on a Q-1000 calorimeter (TA Instruments, New Castle, Delaware, USA). An aluminium pan was filled with ~5 mg of biofilm and subsequently hermetically sealed. After 5 min equilibration at 5°C, DSC analysis was performed with a heating rate of 2°C/min and a modulation of $\pm 1^\circ\text{C}/\text{min}$ up to 50°C.

Water-handling properties

a. Water content by means of thermogravimetric analysis

The water content of the various biofilms was determined by thermogravimetric analysis (TGA; Q50 thermogravimetric analyzer, TA Instruments, New Castle, Delaware, USA). Approximately 30 mg of sample was placed into an open platinum crucible. Samples were then heated at a rate of 10°C/min from 20 to 200°C. The balance was purged with a constant flow of nitrogen (40 ml/min). The water content was calculated from the weight loss which occurred during the heating process.

b. Dehydration of synthetic biofilms

The water-holding capacity of the biofilms was assessed gravimetrically and compared to natural VC. For the dehydration study, custom-made weighing boats were filled with the formulation and dried over P_2O_5 in a desiccator at room temperature (21-24°C). During dehydration, the samples were weighed (Microbalance, Mettler TG50, Switzerland) at various intervals until a constant weight was achieved. The percentage of water release was calculated by using the following equation: $m(\text{evaporated water})/m(\text{total water}) \times 100\%$.

Rheology

Rheological properties of VC were studied on a rheometer (AR1000-N, TA instruments, Etten-Leur, The Netherlands) with a steel cone (1°, 20 mm diameter). In order to prevent evaporation of water, a solvent trap was installed above the cone. The shear storage modulus (G' , correlated to the elasticity) and the shear loss modulus (G'' , correlated to the viscosity) of the different biofilms were recorded in the oscillation mode with a controlled strain of 0.1% at a frequency of 1 Hz. Temperature-induced changes were studied between 10 and 40°C with a heating/cooling rate of 2°C/min.

Results & Discussion

Visualization of the lipid-coated particles

HyPG-MA microgels [17] (also referred to as particles) were selected as mimetic of the highly hydrated corneocytes in natural VC. These microgels were labelled with FITC-dextran to enable visualization by CLSM (Fig. 2A). The hexagonal shape, as well as the \varnothing 30 μ m particle size is apparent. The lipid coating of the microparticles was intended to facilitate embedding of the hydrophilic particles and to control water release from the biofilm. The liposomes were labelled with the lipophilic dye Texas Red which enables the distinction in another channel of the microscope (Fig. 2B). An uniform lipid coating surrounding the entire particle is observed. When merging both channels, the successful coating can be easily perceived (Fig. 2C).

Homogeneity of biofilms as observed by confocal laser scanning microscopy

A suspension of highly hydrated microparticles was embedded in the lipids using a modified Topitec® micromixer (Fig. 1). For convenience the different compositions of the obtained biofilms are listed in table 1 and will be referred to by the given abbreviations (first column). The captured CLSM pictures of the synthetic biofilms and natural VC are depicted in figure 3. VC is characterized by corneocytes that show only little auto-fluorescence (Fig. 3A). The Nile Red labelled lipids of VC are surrounding the corneocytes (black) and the structure of VC is clearly visible (Fig. 3B and 3C). The size of the corneocytes varies between 15 and 40 μ m and they are randomly distributed within the lipids. The synthetic biofilms are characterized by the FITC-dextran labelled particles (50% initial water content of the particles) embedded in the Nile Red labelled lipids (Fig. 3F and 3I). At the optimal mixing condition (i.e. 5 min and 400 rpm) the particles remained intact and were homogenously distributed within the lipid matrix. When mixing longer (e.g. 10 min at 400 rpm) or with higher mixing speeds (e.g. 5 min at 800 rpm) fragmented particles were visible, whereas shorter mixing times (e.g. 2 min at 400 rpm) resulted in a visually inhomogeneous biofilm (data not shown). In figure 3G the hexagonal top view of the particles (cross section of 30 μ m) and the side view of the particles with a thickness of 20 μ m and a rectangular shape is clearly visible. When comparing natural VC (Fig. 3A - 3C) and the synthetic biofilm B1 (Fig. 3D - 3F) it can be clearly observed that the particle density is higher in natural VC. Increasing the particle/lipid ratio to 5:1 (Fig. 3G - 3I) resulted in a biofilm (B3) with a particle distribution similar to the natural counterpart. VC consists of 80 % water, 10% lipid and 10% proteins [1, 8], whereas formulation B3 consists of 61% water, 22% HyPG-MA and 17% lipids. Although the water levels in the synthetic biofilms are less, an excellent resemblance in structure was obtained with VC.

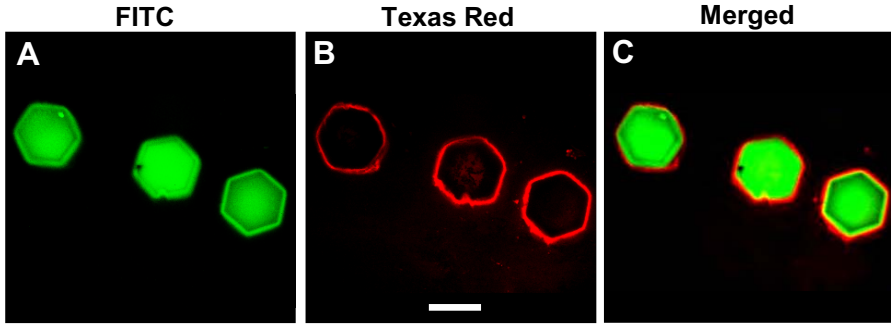


Figure 2. HyPG-MA microgels labelled with FITC-dextran (A) and the coating lipids (labelled with Texas Red) surrounding the microgels (B). Both images can be superposed (C) to visualize the labels of particles and lipids simultaneously. *Scale bar* = 25 μ m.

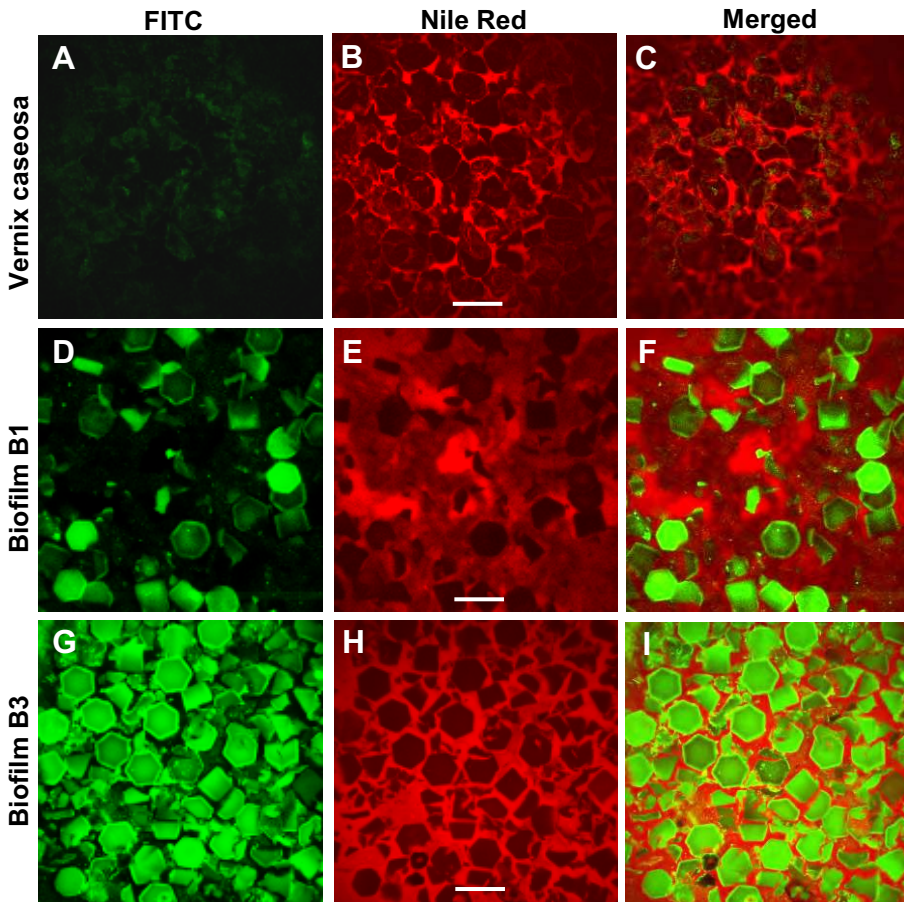


Figure 3. Photomicrographs of native VC and different biofilm formulations, obtained by CLSM, are depicted. VC is characterized by corneocytes (black) which are embedded in Nile Red-labelled lipids (A-C). Synthetic biofilms with particle/lipid ratios of 2:1 (B1) (D-F) and 5:1 (B3) (G-I), exhibit red labelled lipids and FITC-labelled synthetic particles. The particles are intact and are randomly distributed within the lipid mixture. *Scale bars* = 50 μ m.

Stability

After storage of various biofilms in a closed eppendorf tube for one month at 4°C, the obtained confocal images revealed a similar particle distribution in the lipid matrix (data not shown). Neither sedimentation nor separation occurred during storage, indicating that our biofilms have an excellent stability.

Thermotropic behaviour of the biofilms

The thermotropic behaviour of the various biofilms was evaluated by DSC (Fig. 4). The depicted biofilms (B1 and B2) are characterized by two overlapping lipid transitions with onset temperatures at 21°C and ~25°C and corresponding enthalpies of -0.17 J/g and 0.28 J/g (biofilm B2), respectively. The formulations with a higher particle/lipid ratio (5:1) and formulations containing the pre-coated particles showed similar onset temperatures of the transitions (data not shown). The enthalpies and temperatures of the observed lipid transitions are very similar to the thermotropic behaviour of natural VC (Fig. 4) and the synthetic lipid mixtures without particles in the formulation [15]. It has already been speculated that these thermotropic transitions, which coincide with a change in lipid disordering might result in the reduced dehydration rate of VC at lower temperatures. Therefore, the transitions may play an important role in minimizing the evaporative heat and water loss from the skin of the newborn [6]. Taken this into account, the presence of these transitions in the biofilms might also be of beneficial character for the skin.

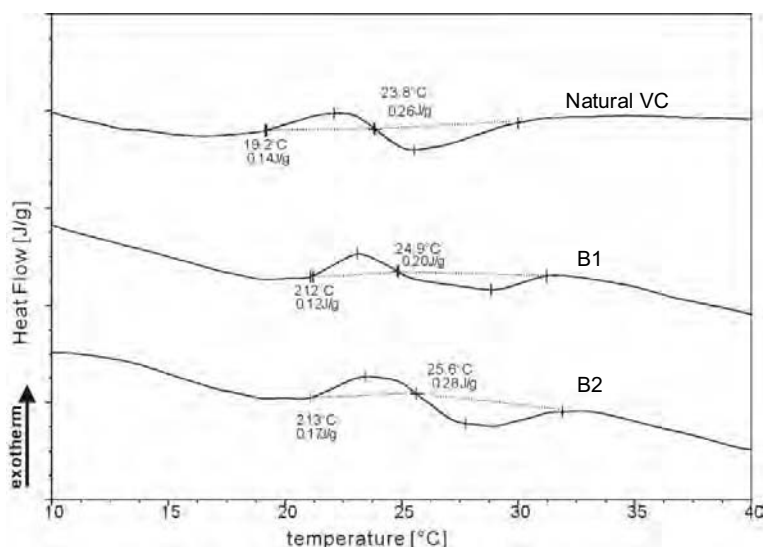


Figure 4. DSC of natural VC (upper trace) and synthetic VC biofilms (lower two traces).

Water handling properties - water content and dehydration studies

The water content of natural VC and different biofilm formulations is depicted in table 2. Natural VC is characterized by the highest amount of water ($77.8 \pm 0.6\%$) whereas the commercial formulation 'Zwitsal' contains a little less water ($73.4 \pm 0.5\%$). The biofilms with particle/lipid ratio 2:1 using lipid coated or uncoated particles (i.e. B1, B1c, B2, B2c) exhibited a water content ranging from $51.0 \pm 0.0\%$ up to $59.8 \pm 0.1\%$. A substantial difference between the theoretically calculated water level and experimental water was observed for biofilms containing particles with 50% water. The calculated water content was 33%, while the measured value was between 50 and 60%. In the biofilm preparation procedure, the microgels are dispersed in a minimal volume of 'free' water and subsequently mixed with the lipids using a micromixer. Using this suspension of concentrated microgels might result in an excess of water in the synthetic biofilm lipids and explains the difference between expected and experimentally obtained amounts of water in the formulations as shown in table 2. An increase in initial water content from 50% (w/w) to 80% (w/w) in the microgels did not show a considerable influence on total water content of the biofilms ($59.8 \pm 0.1\%$ and $55.7 \pm 3.5\%$ for B1 and B2, respectively) but showed a better correlation between experimental and calculated values (52.8%). This indicates that less excess of external water was present. When increasing the particle/lipid ratio to 5:1, maintaining the use of coated particles, the formulations showed $61.2 \pm 1.3\%$ and $68.0 \pm 0.0\%$ water content for B3c and B4c, respectively. The high water content of the latter biofilm is close to natural VC, however, also at higher particle levels, the 50% formulation resulted in higher water levels than the predicted values.

Table 2. Theoretical and experimental water content (w/w) of VC, a commercial formulation (Zwitsal) and biofilms by means of TGA. Data presented as mean \pm SD (n=3).

Samples*	Theoretical	Experimental
	water content (%)	water content (mean \pm SD %)
VC	N.A.	77.8 ± 0.6
Zwitsal	-	73.4 ± 0.5
B1	33.3	59.8 ± 0.1
B1c	33.3	51.0 ± 0.0
B2	52.8	55.7 ± 3.5
B2c	52.8	57.0 ± 1.4
B3c	41.7	61.2 ± 1.3
B4c	66.7	68.0 ± 0.0

*Acronyms according to Table 1.

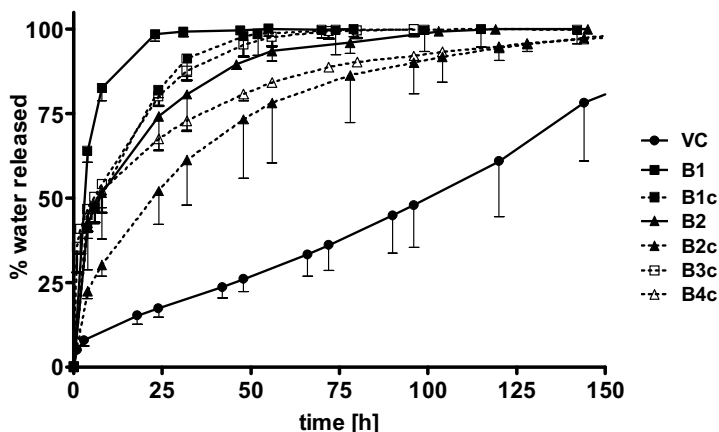


Figure 5. Water release profiles of native VC (●) and various biofilms were obtained by monitoring the weight loss of the specimen in a desiccator over P_2O_5 at room temperature. Various parameters were changed in the formulations: the initial water content of the particles was either 50% (■, □) or 80% (▲, △). The particles were coated with a lipid layer (dashed lines) or kept uncoated (solid lines) prior to embedding in the lipid matrix. The particle/lipid ratio was either 2:1 (■, ▲) or 5:1 (□, △). Data is presented as mean (w/w) \pm SD (n=3).

Water release profiles of VC and the biomimetics are presented in figure 5. As was already described previously [6], VC is characterized by an initial rapid water loss prior to a sustained, steady dehydration at room temperature (Fig. 5).

In contrast, a very fast release of water was observed with the biofilm B1 with non-coated particles and the dehydration process was completed within 24 h. Interestingly, a lipid coating of the microgels (B1c) prolonged the dehydration period to 48 h (Fig. 5). Furthermore, the increase of the initial water content of the microgels from 50% to 80% (w/w) (i.e. biofilm B2c) slowed down the complete water release to 140 h. This formulation was also mimicking the dehydration behaviour of the natural biofilm most closely. The increase in particle/lipid ratio to 5:1 did not result in a further retardation in water release kinetics.

As the postnatal water-handling properties of VC [6] are an important aspect, it should be considered to achieve a similar high skin hydration [7] with the synthetic VC. The water release from our synthetic biofilms could elegantly be controlled by the adjustment of the particle/lipid ratio and by the pre-coating of the particles. However, there is still a difference between the water-holding properties of synthetic and native VC. This might be due to I) the polymer used to prepare the microgel, i.e. HyPG-MA, might be less efficient in binding water than keratin, the most important biopolymer for water-binding present in the corneocytes of VC [20] III) the thick and highly cross-linked cornified envelope in native corneocytes [21] being absent in the biofilms and III) the presence of long

chain ceramides and fatty acids [13] chemically bound to the cornified envelope in natural VC, while in synthetic VC shorter chain DOTAP and DOPC were used to coat the hydrogels. Although some differences are observed between the water holding properties of VC and the biofilm, the water release could be extended from 24 to 140 h by adjusting the composition of the biofilms. This prolonged water-release from the optimized biofilm is also much longer than observed for the Zwitsal formulation which completed dehydration after 72 h (data not shown).

Rheological characterization

The biofilms were characterized for their viscoelastic properties by means of rheology. The shear storage modulus (G') and the shear loss modulus (G'') of the different biofilms were measured as function of temperature. A representative rheogram of a biofilm (i.e. B1) is shown in Fig. 6. Both G' and G'' decrease with increasing temperature from 10 to 28°C (closed symbols) where a plateau is reached. Upon cooling from 40°C to 10°C, G' and G'' returned to the original values (open symbols) demonstrating that the temperature induced changes in the biofilm were reversible. The $\tan \delta$ (i.e. the quotient of G'' and G') of this formulation varied between 0.11 and 0.18 during the heating/cooling cycle underlining the viscoelastic behaviour of this formulation. Other biofilm formulations showed a similar reversible profile (data not shown).

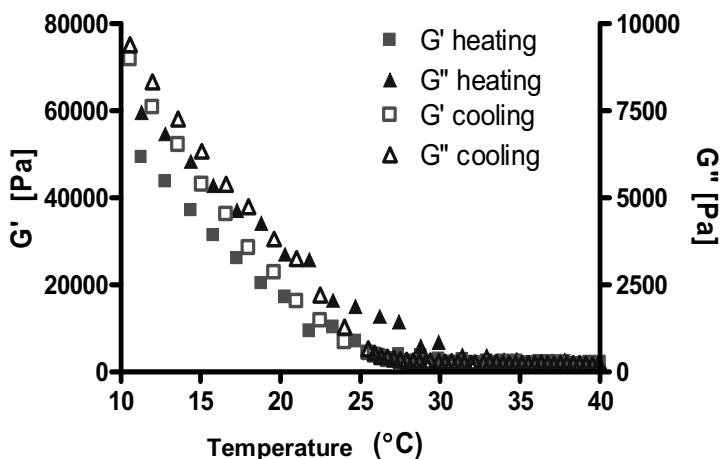


Figure 6. Rheological properties of one representative biofilm (B1) as a function of temperature. The shear storage modulus (G' , ■) is correlated to the elasticity whereas the shear loss modulus (G'' , ▲) is correlated to the viscosity. The heating values are depicted by closed symbols while the open symbols represent the cooling cycle.

Table 3. Rheological properties of VC, a commercial formulation (Zwitsal) and biofilms.

Samples*	G' (Pa) [§]	G'' (Pa) [§]	$\tan \delta$
VC	19,000 / 20,000	4,000 / 2,000	0.22 / 0.10
Zwitsal	80 / 1	60 / 6	0.70 / 8.20
B1	3,000 / 2,000	500 / 300	0.17 / 0.15
B1c	1,500 / 1,300	500 / 300	0.35 / 0.25
B2	2,000 / 2,000	300 / 300	0.15 / 0.14
B2c	2,000 / 1,600	500 / 300	0.27 / 0.20
B3c	4,000 / 4,000	600 / 600	0.22 / 0.43
B4c	2,000 / 1,600	500 / 300	0.27 / 0.20

*Acronyms according to Table 1. The mean of the experimental data is presented (n=3).

[§] G' , G'' and $\tan \delta$ at 32°C at heating / cooling step

For comparison, G' , G'' and $\tan \delta$ of the various biofilms and VC at 32°C (skin temperature) are listed in table 3. Natural VC shows a higher G' and G'' at skin temperature than all biofilm formulations. This is presumably due to the difference of water localization within the specimen, i.e. higher amount of external water in the biofilms. However, several similarities between natural VC and synthetic formulations are noticed: I) biofilms exhibit before and after the heating/cooling cycle G' and G'' values in the similar range indicating a reversible process as was also reported for VC [6]. II) the observed plateau of G' and G'' in the biofilms was also described for VC to occur approximately at (adult) skin temperature [6]. III) The $\tan \delta$ of native and synthetic VC is within the similar range indicating that both natural VC and synthetic counterpart exhibit viscoelastic properties. The Zwitsal cream was characterized by a much lower G' and G'' compared to natural VC and all biofilms, indicating a very different rheological behaviour. In conclusion, the rheological study showed that natural VC was mimicked very well by the synthetic formulations. Moreover, the lower G' and G'' of the biofilms might result in a better patient acceptance and compliance, since the biofilms are more tractable than the natural counterpart.

In conclusion, an excellent resemblance was achieved in composition, structure and properties between native VC and synthetic biofilms. Our in vitro optimization demonstrates that the biofilms with a particle/lipid ratio of 5:1 mimic VC most closely concerning the structural appearance whereas B2c exhibits water handling properties that mimics those of native VC most closely. In future, additional hydrophilic (into the microgels) and lipophilic (into the lipids) compounds could be added, e.g. drug substances, to apply our biofilms as drug delivery matrix.

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