Glycerol and urea can be used to increase skin permeability in reduced hydration conditions

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ABSTRACT

The natural moisturizing factor (NMF) is a group of hygroscopic molecules that is naturally present in skin and protects from severe drying. Glycerol and urea are two examples of NMF components that are also used in skin care applications. In the present study, we investigate the influence of glycerol and urea on the permeability of a model drug (metronidazole, Mz) across excised pig skin membranes at different hydrating conditions. The degree of skin hydration is regulated by the gradient in water activity across the membrane, which in turn depends on the water activity of the formulation in contact with the skin membrane. Here, we determine the water activity of all formulations employed using an isothermal calorimetric method. Thus, the gradient in water activity is controlled by a novel experimental set-up with well-defined boundary conditions on both sides of the skin membrane. The results demonstrate that glycerol and urea can retain high steady state flux of Mz across skin membranes at dehydrating conditions, which otherwise would decrease the permeability due to dehydration. X-ray diffraction measurements are performed to give insight into the effects of glycerol and urea on SC molecular organization. The novel steady state flux results can be related to the observation that water, glycerol, and urea all affect the structural features of the SC molecular components in a similar manner.

1. Introduction

Transdermal drug delivery is an attractive alternative to oral drug delivery because it avoids first pass metabolic degradation (Prausnitz and Langer, 2008). Optimization of transdermal drug delivery applications include considerations of interactions within the formulation as well as interactions between formulation ingredients and the molecular components of the skin barrier (Barry, 2001). After application of a transdermal or topical formulation onto the skin surface, several new gradients across the skin membrane are established, which may affect the properties of the skin barrier. The understanding of how the skin barrier is affected by changes in physical and chemical gradients is therefore highly relevant for the development of transdermal drug delivery systems. We have previously demonstrated that changes of a gradient in water activity across the skin membrane, which effectively determines the degree of skin hydration, can be used as a switch to regulate the skin permeability to model drugs with different lipophilic characteristics (Björklund et al., 2010). The proposed explanation for these observations is that changes in the water gradient can induce reversible structural alterations in SC lipid or protein components, which can lead to drastic changes in the transport characteristics (Björklund et al., 2010; Björklund et al. 2013a; Sparr and Wennerström, 2001). In the present study we explore the effect of glycerol and urea on the permeability of skin membranes, which are also exposed to a gradient in water activity.

The outermost layer of skin is called the stratum corneum (SC) and constitutes the main barrier towards both inward and outward diffusional transport (Scheuplein and Blank, 1971). The barrier properties of SC are assured by its organization of corneocytes embedded in a multilamellar lipid (Madison et al., 1987; Weerheim and Ponec, 2001). The corneocytes are packed with keratin filaments that are enclosed by the cornified cell envelope (Candi et al., 2005). Despite that SC normally experience low relative humidity (RH), the exposure to very dry environments can lead to defective skin conditions (e.g., winter xerosis). The continuous hydration of SC from the inside of the body is therefore crucial for maintaining healthy skin as water regulates, for example, SC flexibility (Blank, 1953) and enzymatic reactions in SC (Harding et al., 2000). Moisturizers are substances commonly used for

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treatment or prevention of defective dry skin conditions to make the SC more soft and pliable. Humectants comprise a subclass of moisturizers encompassing small polar molecules with hygroscopic properties. Humectants are also naturally present in SC, referred to as the natural moisturizing factor (NMF), which is a mixture of free amino acids and their derivatives, inorganic salts, lactic acid, urea, and glycerol (Choi et al., 2005; Harding et al., 2000). There is a well-regulated interplay between the water gradient in SC and the filaggrin-degradation into NMF components (Harding et al., 2000) and the importance of the NMF molecules is illustrated by the noticeable correlation between the absence of the NMF and conditions of SC abnormality (Marstein et al., 1973; Sybert et al., 1985).

Glycerol and urea are also used in commercial skin care lotions and creams where the beneficial function of these compounds is ascribed to their hygroscopic properties, as the suggested role for NMF. Still, it is clear that the barrier function as well as the mechanical properties of SC do not only depend on its water content, but more important, on the state and molecular organization of non-aqueous SC lipid and protein components. These properties can be affected by hydration (Alonso et al., 1996; Björklund et al., 2010; Björklund et al., 2013a; Blank et al., 1984; Nakazawa et al., 2012; Ohta et al., 2003), and also by the addition of other small polar molecules. For example, the presence of glycerol (10 wt%) in hydrated model skin lipids in a liquid crystalline state impede the transition into a crystalline state at dry conditions (6% RH), as compared to the same lipid mixture in the absence of glycerol (Froebe et al., 1990). In previous studies, we have shown that osmolytes like glycerol and urea can stabilize fluid structures in phospholipid bilayer systems at low RH where the lipids would form solid bilayer structures in the absence of these osmolytes (Costa-Balogh et al., 2006; Nowacka et al., 2012). These observations indicate that glycerol and urea can maintain the physical properties of hydrated lipid systems under dry conditions. It is also possible that a similar mechanism can act on the SC molecular components if these molecules are present inside SC under dehydrating conditions.

In this study, we explore the influence of glycerol and urea on the in vitro permeability of excised skin membranes and the molecular structure of SC at varying hydrating conditions. We use an experimental set-up of flow-through diffusion cells, where we have control of the boundary conditions and steady state conditions, to study the situation of opposite gradients in water and humectant across the skin membrane. In the absence of glycerol or urea, a decrease in water activity on the upper side of the skin membrane would lead to dehydration, a reduction of the fraction of fluid SC components (Björklund et al., 2013a), and ultimately a decrease in the skin permeability (Björklund et al., 2010). However, the addition of humectant to the same side of the membrane may prevent the transition from fluid to solid structures and thus retain the permeability of a hydrated skin membrane. To investigate this hypothesis, we study diffusional transport of a model drug (metronidazole, Mz) through pig skin membranes in vitro where we control both the gradient in water activity and the gradient in either glycerol or urea. Further, we correlate the effect of glycerol and urea on the skin permeability with their influence on the molecular organization of the SC lipid lamellar structures and the soft keratin proteins by performing small- and wide-angle X-ray diffraction measurements.

2. Materials and methods

2.1. Chemicals

Metronidazole (Mz) was purchased from Mediolast (Milan, Italy). Poly(ethylene glycol) 1500 Da (ultragrade) (PEG), glycerol,

urea, trypsin, and methanol were obtained from Sigma–Aldrich. NaCl, $Na_2HPO_4 \cdot 2H_2O$, KH_2PO_4 were obtained from Merck.

2.2. Preparation of skin and silicone membranes

Pig ears were obtained fresh from a local abattoir (Dalsjöfors slakteri, Sweden) and frozen at $-80\,°C$ until use. Split-thickness skin membranes (approx. 500 μm thick) were prepared from tissue of the inside of the outer ear by using a dermatome (TCM 3000 BL, Nouvag). Circular membranes (16 mm in diameter) were cut out to fit the diffusion cells (9 mm in diameter). Circular silicone membranes (Speciality Manufacturing, Michigan, USA) were used for reference purposes to confirm that all donor formulations had the same release rate of Mz.

2.3. Preparation of SC

Strips of dermatomed pig ear were placed, dermal side down, on filter paper soaked in 0.2% trypsin in PBS solution for 12 h at $4\,°C$. Next, the SC was removed with forceps and washed in PBS solution. The SC was rubbed with cotton tipped applicators to remove tissue not belonging to SC and further washed in PBS solution. The SC was dried in vacuum and stored in refrigerator until use.

2.4. Model drug formulations

The model drug used in this work was Mz, which is an antibiotic drug used in commercial formulations for e.g. treatment of the skin disease rosacea. It has low molecular weight (171 g mol^{-1}), is noncharged in the present experimental conditions, and partition approx. equally in octanol and water (log $P_{o/w}$ = 0 (Kasprzyk-Hordern et al., 2007)). All Mz formulations were prepared in phosphate buffered saline, PBS (130.9 mM NaCl, 5.1 mM $Na_2HPO_4 \cdot 2H_2O$, 1.5 mM KH_2PO_4, pH 7.4) and varying concentrations of glycerol or urea with or without PEG. The molecular weight of the polymer used in this work is $MW_{PEG} \sim 1500$ Da, which corresponds to roughly n = 34 where n is the number of ethylene oxide units according to $H(OCH_2CH_2)_nOH$. The reason for using this particular size is that it is small enough to allow for a considerable decrease in water activity, while at the same time being sufficiently large to assure that the polymer does not penetrate into the skin membrane (Albèr et al., Unpublished results; Tsai et al., 2001, 2003). Thus, the addition of this polymer only act as a dehydrating agent, in analogy with osmotic stress technique measurements (LeNeveu et al., 1976).

2.5. Uniform release rate of the model drug from all formulations

The release rate of Mz from the formulation depends on the chemical potential (activity) of the model drug in the formulation, which is strongly related to the formulation composition. We aim at an experimental set-up where the chemical potential of Mz is the same in all formulations. As we cannot get direct experimental data on the chemical potential of Mz, we use an approximate condition by adjusting the concentration in relation to the total solubility in each formulation. The solubility of Mz was determined for all formulations in three replicates following the procedures in (Björklund et al., 2010). The solubility data are summarized in Table 1. The drug concentration in each formulation was then adjusted by multiplying the total Mz solubility with an arbitrary factor so that the concentration in neat PBS solution was 0.75 wt% (7.5 mg ml^{-1}), which is the concentration used in several commercial topical formulations containing Mz (e.g. Rosex cream and Rosex gel, Galderma Nordic AB). This procedure, i.e. to adjust the Mz concentration to achieve similar chemical potential of Mz, is

Table 1
Compilation of experimental data (average ± S.E.M.) on steady state flux, J_{ss} (µg cm^{-2} h^{-1}) of Mz across skin and silicone membranes, water activity (a_w), and Mz solubility (mg ml^{-1}). Number of replicates is given by n.

Formulation[a] (no. in wt%)	J_{ss} skin	J_{ss} silicone	a_w	Solubility
PBS	10.4 ± 1.8 (n = 9)	2.0 ± 0.1 (n = 12)	0.992	11.8 ± 0.1 (n = 3)
G 7	15.1 ± 2.5 (n = 7)	2.0 ± 0.2 (n = 3)	0.985	11.8 ± 0.1 (n = 3)
G 15	15.0 ± 2.9 (n = 6)	2.1 ± 0.1 (n = 3)	0.959	12.1 ± 0.1 (n = 3)
G 20	9.6 ± 1.6 (n = 10)	2.1 ± 0.1 (n = 3)	0.943	12.0 ± 0.2 (n = 3)
G 30	9.1 ± 1.8 (n = 8)	2.1 ± 0.1 (n = 3)	0.912	12.2 ± 0.1 (n = 3)
U 5	10.5 ± 2.4 (n = 6)	1.9 ± 0.1 (n = 3)	0.978	13.6 ± 0.2 (n = 3)
U 10	13.7 ± 3.6 (n = 6)	2.1 ± 0.1 (n = 3)	0.962	16.3 ± 0.2 (n = 3)
U 15	17.5 ± 4.1 (n = 6)	2.3 ± 0.1 (n = 3)	0.945	19.5 ± 0.1 (n = 3)
U 20	15.1 ± 2.7 (n = 6)	2.2 ± 0.1 (n = 3)	0.925	21.3 ± 0.3 (n = 3)
G 20/PEG 10	6.8 ± 0.9 (n = 6)	1.8 ± 0.1 (n = 3)	0.930	12.4 ± 0.1 (n = 3)
G 20/PEG 20	4.5 ± 1.2 (n = 6)	1.9 ± 0.1 (n = 3)	0.917	13.5 ± 0.1 (n = 3)
G 20/PEG 30	3.3 ± 0.9 (n = 6)	1.9 ± 0.2 (n = 3)	0.867	15.4 ± 0.1 (n = 3)
G 20/PEG 40	2.5 ± 0.5 (n = 6)	2.0 ± 0.2 (n = 3)	0.810	16.8 ± 0.1 (n = 3)
U 20/PEG 10	11.3 ± 2.0 (n = 8)	2.0 ± 0.1 (n = 3)	0.916	19.7 ± 0.2 (n = 3)
U 20/PEG 20	7.5 ± 1.2 (n = 7)	2.1 ± 0.1 (n = 3)	0.898	19.0 ± 0.2 (n = 3)
U 20/PEG 30	3.1 ± 0.8 (n = 7)	2.0 ± 0.1 (n = 3)	0.870	17.5 ± 0.2 (n = 3)
U 20/PEG 40	2.7 ± 0.5 (n = 8)	1.9 ± 0.2 (n = 3)	0.817	16.3 ± 0.2 (n = 3)

[a] Abbreviations: G, glycerol; U, urea; PEG, poly(ethylene glycol) 1500 Da.
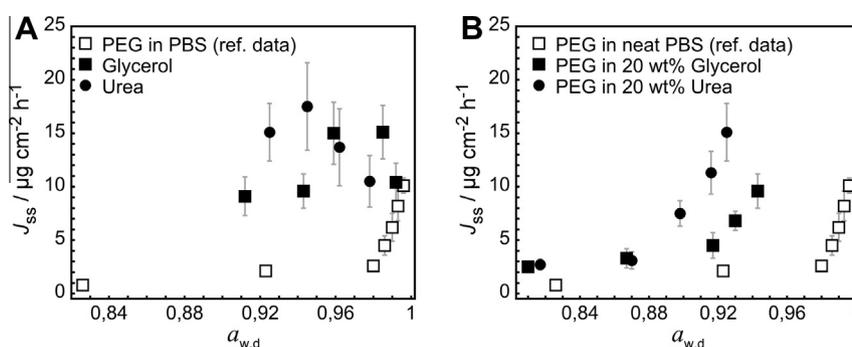
Fig. 1. Average steady state flux (J_{ss}) of Mz across skin membranes (n = 6–10) as a function of the water activity in the donor formulation ($a_{w,d}$). (A) Water activity regulated by glycerol or urea. (B) Water activity regulated by PEG 1500 Da together with a fixed amount of 20 wt% glycerol or urea. Error bars show ± S.E.M. (cf. Table 1). For comparison, ref. data of Mz flux across pig skin membranes from formulations containing no humectants and varying amounts of PEG 1500 Da in PBS solution are included in the figures. Ref. data are from (Björklund et al., 2010).
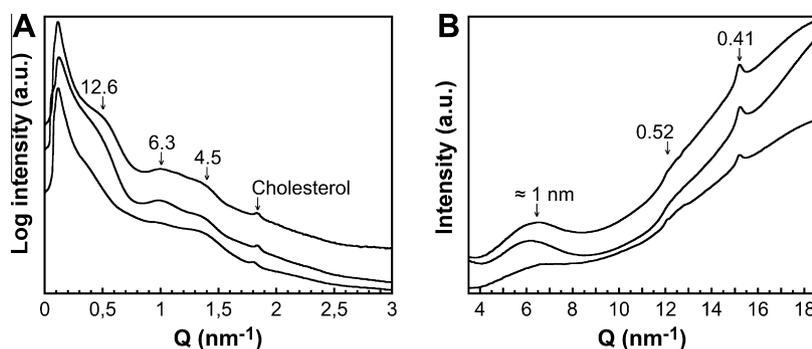
Fig. 2. X-ray diffraction data obtained at 32 °C of SC pretreated at 32 °C for 24 h in neat PBS solution (*middle curve*), 20 wt% glycerol in PBS (*top curve*), or 20 wt% urea in PBS (*bottom curve*). (A) SAXD data. (B) WAXD data. The numbers by the arrows give the *d*-spacing in nm. One replicate per SC sample was measured.

supported by diffusion measurements with silicone membranes showing that the release rate from all formulations is the same (see Figs. 1 and 2).

2.6. Water activity in the model drug formulations

In the steady state flux experiments, the water activity gradient is defined by the boundary conditions given by water activity in the donor formulation and the receptor solution. The water gradi-

ent can be expressed in terms of the water activity, a_w, or the chemical potential of water, $\Delta\mu_w$, by the relation $a_w = \exp(\Delta\mu_w/RT)$. The water activity (ranging from zero to unity) is defined as the ratio between the vapor pressure of water above a solution, p, and the vapor pressure above pure water, p_0, and related to the relative humidity, RH, by $a_w = p/p_0 = RH/100$. The water activity in the formulations used in this study was determined with an iso-thermal calorimetric method, developed in house, that allows for high precision measurements in the high range of water activities

(Björklund and Wadsö, 2011). Measured values for the water activities for all formulations studied are compiled in Table 1.

2.7. Steady state flux of Mz

The experimental method to determine the steady state flux (J_{ss}) of Mz was the same used as in previous studies (Björklund et al., 2010). In brief, the system consists of 15 flow-through cells (receptor phase flow-rate was 1.5 ml h^{-1}) with mixing from magnetic stirrers placed in both the donor and the receptor phase. The temperature in the diffusion cells was 32 ± 0.3 °C. To enable studies of steady state flux and constant boundary conditions in Mz, glycerol, urea, and water, we used large donor formulation volumes of 2 ml. In average, the decrease in Mz concentration in the donor phase after 24 h was less than 1%, taking all formulations into account. Similar changes can be predicted for glycerol and urea, based on previously published permeation studies in nude mouse skin under similar conditions (Ackermann and Flynn, 1987). As a control, we also determined the concentration of glycerol in the donor solution before and after a 24 h experiment on skin membranes. No detectable difference was observed from free glycerol assay kit measurements ($n = 15$, BioVision, California, USA). The PBS solution in the receptor phase was continuously renewed by the flow-through set-up, assuring minimal concentration build-up. With these precautions steady state conditions are satisfied reasonably well. Steady state flux values of Mz were calculated from the slope of curves of cumulative permeated mass per membrane area plotted against time. The data from individual skin or silicone membranes were treated separately to calculate the steady state flux, which then were used to determine the average value for the corresponding model drug formulation. In this calculation, five time points between 16 and 24 h was used for skin membranes, while eight time points between 4 and 18 h was used in the case of silicone membranes. The selection of the time intervals used for determining steady state is rationalized by the time required to reach steady state conditions, which is influenced by the water activity in the model drug formulation (Björklund et al., 2010). Representative curves of cumulative permeated mass of Mz across skin and silicone membranes as a function of time is given in Fig. S1 in the Supplementary material.

2.8. Analytical procedure

Mz concentration was determined at $\lambda = 319$ nm from calibration curves of standard solutions prepared in PBS solution (0.5–20 µg ml^{-1}). The concentration of Mz in the formulations and in the receptor phase from the diffusion study employing silicone membranes was determined by UV/visible spectrophotometry (Anthelie Advanced, Secoman). Receptor phase concentrations of Mz, from the skin membrane diffusion study, were analyzed by reversed phase HPLC-UV. Samples were injected using an automatic sample injector (Rainin Dynamax model AI-1A) with a 10 µl injection loop. The mobile phase consisted of filtered and degassed methanol:phosphate buffer (10 mM KH$_2$PO$_4$) (20:80 v/v). Flow rate was 2.0 ml min^{-1} (Varian 9012 solvent delivery system). A Phenomenex SecurityGuard (Gemini C$_{18}$, 4 × 3.0 mm) was used in series with a Phenomenex Gemini 5 µm C$_{18}$ column (110 Å, 100 × 4.6 mm) for chromatographic separation. The retention time for Mz detection (Thermo Separation Products, Spectra 100) was 1.9 min.

2.9. Small- and wide-angle X-ray diffraction (SAXD and WAXD)

Dry SC (approx. 30 mg) was placed in 2 ml formulations of PBS, 20 wt% glycerol in PBS, or 20 wt% urea in PBS, respectively, for 24 h at 32 °C. Next, the SC pieces were removed from the formulation and gently wiped with paper tissues to remove excess formulation and loaded into the SAXD sample holders by folding them several times. The diffraction measurements were performed at Maxlab in Lund (Sweden) at a new set-up in one of the five side stations of the Swedish–Danish beamline Cassiopeia (I911-4, $\lambda = 0.91$ Å) (Labrador et al., 2012). Diffraction intensities were corrected for air (empty cell) scattering and primary-beam intensity changes to enable comparison between different measurements. The corrected diffraction intensities are plotted as a function of the scattering vector Q defined as $Q = (4\pi \sin \theta)/\lambda$, where θ and λ are the diffraction angle and the wavelength, respectively. One measurement per SC sample was performed at 32 °C. To investigate if glycerol and urea affect the SC molecular organization differently than water at elevated temperatures, as previously shown (Bouwstra et al., 1995), we performed additional measurements on all samples at elevated temperatures. One measurement was performed per sample at following temperatures: 50 °C, 70 °C, 80 °C (WAXD) 90 °C (SAXD), and finally again at 32 °C after allowing the samples to cool down for approx. 1 h. In these experiments the SC samples were heated for approx. 30 min at each temperature. The results from the measurements at elevated temperatures are presented in Fig. S2 in the Supplementary material.

3. Results

3.1. Steady state flux of Mz

We study the steady state flux (J_{ss}) of the model drug Mz across skin membranes, focusing on the effect of a varying water gradient in the presence of glycerol and urea. Thus, the skin membrane is placed in several gradients; a gradient in water activity, a gradient in glycerol or urea activity, and a gradient in Mz activity. The water activity in the receptor solution (PBS solution) is held constant at physiological conditions, and the water activity in the donor formulation is regulated by the addition of glycerol or urea, or a combination of one of these molecules and the water-soluble polymer PEG (MW$_{PEG}$ ∼ 1500 Da, see Section 2.4.). Any addition of solute molecules to an aqueous solution leads to a reduction of the water activity, and it is therefore clear that all donor formulations investigated have water activities lower than one (Evans and Wennerström, 1999). The experiments presented here can be divided into two types; in the first type the concentration of glycerol or urea is adjusted, and in the second type the concentration of glycerol or urea is fixed at 20 wt% and the concentration of PEG is regulated. Glycerol and urea are small molecules that are likely to partition into the skin membrane, similar to what is expected for water. On the other hand, it is established that the relatively large size of the polymer used in this work assures that it does not penetrate into the skin membrane due to size exclusion (Albèr et al., Unpublished results; Tsai et al., 2001, 2003).

Table 1 summarizes experimental data on steady state fluxes of Mz across skin and silicone membranes for all formulations investigated. It is important to note that the data in Table 1 from the experiments employing silicone membranes show that all formulations have the same release rate of Mz, resulting in a steady state flux across silicone membranes of ∼2 µg cm^{-2} h^{-1} irrespective of formulation composition. This means that any variations in the Mz flux across skin membranes due to differences in the release pattern from the various formulations can be ruled out, which enable a quantitative comparison between Mz fluxes across skin membranes from the different formulations.

To be able to relate the data on steady state flux of Mz to the water activity in the formulations, we determined the water activity in all formulations studied. This was done using a calorimetric method previously developed in house, which allows for precision measurements at high water activities (Björklund and Wadsö,

2011). The results are compiled in Table 1. It is noted that the water activity in the formulations containing glycerol or urea in PBS solution is consistent with previous reported values on glycerol or urea in pure water, taking into account the small drop in water activity due to the PBS buffer salts (Scatchard et al., 1938).

3.1.1. Skin permeability at reduced water activity – influence of glycerol and urea

The average steady state flux of Mz across skin membranes as function of water activity in the donor formulation ($a_{w,d}$) is shown in Fig. 1. For comparison, previous flux data of Mz from formulation containing PEG in PBS solution are also included (Björklund et al., 2010). It is clear that the subsequent addition of glycerol, urea or polymer to the donor formulations leads to a reduced water activity (Table 1). Still, the addition of these compounds does not affect the permeability of the skin membrane in the same way (Fig. 1A and B). It is a striking observation that the flux of Mz remains high for all formulations that contain either glycerol or urea in PBS solution, irrespectively of the water activity (Fig. 1A). This is in clear contrast to the case when the water activity is regulated by the addition of the PEG polymer (ref. data in Fig. 1A), which does not partition into the skin membrane. In the latter case, there is a 6-fold decrease in Mz flux when the water activity goes below approx. 0.96. The data in Fig. 1A show that for some of the glycerol or urea formulations the average flux is increased compared to the neat PBS formulation, of which the latter corresponds to the data point at $a_{w,d}$ = 0.992. However, the variations is not statistically significant (treated by one-way ANOVA, p-level 0.18).

In the second set of experiments (Fig. 1B), the water activity in the formulations containing glycerol or urea is regulated by the addition of PEG in the same way as described for the reference samples with no humectant (Björklund et al., 2010). Again, the addition of PEG to the formulations leads to a sharp decrease in flux of Mz at reduced water activities. However, from the comparisons in Fig. 1B, it is clear that the onset of the sharp decrease in permeability is shifted towards lower water activities when glycerol or urea is present in formulations, as compared to the case when they are not. This effect is more pronounced in the case of urea, but the effect in the case of glycerol is also obvious as compared to the case without humectants present.

3.2. Molecular organization of the SC components – influence of glycerol and urea

The barrier properties of the skin membrane depend on the molecular organization of the SC components. Considering this, we employed SAXD and WAXD to investigate the effect of glycerol and urea on both the organization of the SC extracellular lipid lamellae and on the soft keratin structures. The results from the SAXD and WAXD measurements at 32 °C are presented in Fig. 2A and B, respectively. We start by concluding that the results obtained for the SC sample without glycerol or urea are in good agreement with previous SAXD and WAXD studies on hydrated pig SC (Bouwstra et al., 1995). Further, it is shown that the SC pretreated in glycerol or urea formulations give rise to similar diffraction curves as the SC pretreated in neat PBS solution.

All SAXD curves in Fig. 2A have one broad peak centered around Q = 1.0 nm^{-1} (6.3 nm in d-spacing). The strong diffraction at low Q is attributed to protein structures of the SC (Bouwstra et al., 1995; Garson et al., 1991), which obscures the diffraction pattern of any lipid structures in this region. However, centered around Q = 0.5 nm^{-1} (12.6 nm in d-spacing) a shoulder is present in the descending diffraction curves, which implies that the peak around 6.3 nm in d-spacing is a 2nd order peak of a lamellar phase with approx. 12.6 nm in d-spacing. When the SC sample has been pretreated in the formulation that contain urea (*bottom curve*), the

shoulder around Q = 0.5 nm^{-1} is nearly absent, and the intensity of the peak around Q = 1.0 nm^{-1} is weaker compared to the other samples. A weak shoulder centered around Q = 1.4 nm^{-1} (4.5 nm in d-spacing) is present in all diffraction curves in Fig. 2A. In the literature, the same peak at 4.5 nm has been interpreted as the 2nd order of a 9 nm periodicity lamellar phase (Bouwstra et al., 1995). However, no signs of a 1st order peak of this 9 nm lamellar phase was observed here. Considering that all reflections are diffuse and broad it cannot be ruled out that all of the above peaks/shoulders belong to the same lamellar phase with repeat distance of approx. 12.6 nm. Finally, a peak centered around roughly Q = 1.8 nm^{-1} (3.4 nm in d-spacing) is observed in all diffraction curves, which is attributed to phase separated crystalline cholesterol (Bouwstra et al., 1995).

Fig. 2B shows WAXD data for the corresponding conditions as in Fig. 2A. A distinct peak at approx. Q = 15.2 nm^{-1} (0.41 nm in d-spacing) is present in all diffraction curves, irrespective of pretreatment formulation. This peak corresponds to hexagonal packed lipid carbon chains. No signs of orthorhombic packing was observed under any conditions (i.e., no peak was present at approx. Q = 17 nm^{-1} or 0.37 nm in d-spacing), which is in agreement with previous studies on pig SC (Bouwstra et al., 1995; Caussin et al., 2008). The keratin filaments of SC are generally classified as soft keratin, associated with less order and irregular secondary structure, as compared to hard keratin in nail and hair (Kreplak et al., 2004). The broad diffraction peak with maxima around Q = 6.1–6.6 nm^{-1} (0.95–1.00 nm in d-spacing) is attributed to soft keratin (Bouwstra et al., 1995; Garson et al., 1991; Nakazawa et al., 2012). It is noted that the intensity of this broad peak is rather low for the SC sample pretreated in the urea formulation (*bottom curve*). Finally, a very weak shoulder is observed at approx. Q = 12 nm^{-1} (0.52 nm in d-spacing) in all diffraction curves, which may indicate that at least a minor portion of the SC proteins are associated with a secondary structure in the α-helical form (Kreplak et al., 2004).

We investigated the influence of glycerol or urea on the X-ray diffraction patterns from the SC samples at different temperatures. These experiments were performed in a similar manner as the procedure previously employed on pig SC without glycerol or urea (Bouwstra et al., 1995). The diffraction results obtained at elevated temperatures are presented in Fig. S2 in the Supplementary material. The data show that the SC sample pretreated in either glycerol or urea formulation in general give rise to similar diffraction pattern also at elevated temperatures as the SC sample pretreated in neat PBS formulation. The measurement obtained after the heating-cooling cycle show peaks representing a lamellar phase with a repeat distance around 13.2 nm, associated with hexagonally packed lipid carbon chains, and no signs of phase separated cholesterol. We note that diffraction data on SC are associated with natural variability (Garson et al., 1991). However, a comparison between the diffraction curves from the different SC samples at varying temperature conditions show little variability and are also in agreement with previous studies under similar temperature conditions (Bouwstra et al., 1995).

4. Discussion

We have previously shown *in vitro* that exposure of the SC side of the skin membrane to low water activity, regulated by non-penetrating polymers, leads to dehydration and decreased skin permeability of two model drugs (methyl salicylate and Mz) (Björklund et al., 2010). In this work we used a similar approach and investigated how the permeability of Mz across skin membranes is affected by the gradient in water activity when the NMF components glycerol or urea are present in the transdermal

formulation. This was performed by regulating the water activity in the model drug formulation in two ways: (i) by addition of glycerol or urea and (ii) by addition of the non-penetrating polymer PEG in the presence of glycerol or urea. To connect the effect of glycerol and urea on the skin permeability to SC structural properties we studied the influence of these molecules on the molecular organization of SC using X-ray diffraction.

4.1. Water, glycerol, and urea affect the skin permeability in a similar manner

The results of Mz steady state flux across skin membranes show that water-based formulations containing either glycerol or urea have similar effect on the skin permeability as a neat PBS formulation, and the transport of Mz remains virtually unchanged. We argue that this is a result of two opposing effects – dehydration from low water activity and retention of high skin permeability properties. When glycerol or urea is subsequently added to the formulations the water activity is lowered to approx. 0.9 (Table 1). This decrease in water activity does not lead to a decrease in the Mz flux, which is in contrast to what is observed when the water activity is lowered by addition of PEG in absence of glycerol or urea (Fig. 1A). By comparing flux values from either glycerol or urea formulations to flux values from PEG formulations at similar water activities in Fig. 1A it is clear that the difference in Mz flux is substantial. These results demonstrate that addition of either glycerol or urea to water-based formulations can act to retain the permeability properties associated with a fully hydrated skin membrane at dehydrating conditions.

In the second case, when the polymer PEG is added to the donor formulations that also contain glycerol or urea, the water activity is further decreased to approx. 0.8 (Table 1). In this case, the corresponding flux data show that the onset of the sharp decrease in Mz flux is shifted towards considerably lower water activities as compared to the case of PEG in neat PBS solution (Fig. 1B). Also, by comparing flux values at similar water activities from the different formulations it is clear that the formulations containing glycerol or urea results in increased Mz flux.

The variation in skin permeability of Mz with hydration observed in Fig. 1B should be considered in relation to previous in vitro studies on water diffusion across SC as a function of RH (Alonso et al., 1996; Blank et al., 1984), demonstrating an abrupt change of skin permeability to water at approx. 85–95% RH. In previous studies (Björklund et al., 2010), we demonstrated the same qualitative behavior for skin permeability of Mz at varying water activity (see the relation between a_w and RH in Section 2.6), although the position of the abrupt change was observed at higher values of water activity (RH) (ref. data in Fig. 1). In the present study we show that the onset of the abrupt increase can be shifted towards lower water activities (RHs) by adding glycerol or urea to the SC samples (Fig. 1B). This implies that the presence of glycerol or urea, as well as other small polar NMF compounds, may actually determine the position in terms of water activity for which there is an abrupt change in SC permeability towards water and other compounds. This could be of significance for the interplay between, TEWL, SC hydration, and biochemical processes (Harding et al., 2000).

4.2. Water, glycerol, and urea affect the molecular organization of the lipid lamellae and the protein components in a similar manner

Glycerol and urea can act to retain as high permeability of Mz as a fully hydrated skin membrane at reduced water activities (Fig. 1A). This effect on the skin permeability can be related to the influence of glycerol and urea on the SC structure under similar conditions. The lipid lamellae form the only continuous path across

the SC and are important for the barrier properties of SC (Boddé et al., 1989; Potts and Guy, 1992). However, depending on the diffusional transport path taken by the substance, one might also need to consider the barrier properties of the protein components, which indeed constitute the main fraction of the total SC material. It is clear that structural changes in the lipid or protein components in response to interactions with molecules present in the formulation in contact with the skin membrane can have important implications for the SC barrier properties. The SAXD and WAXD results (Fig. 2A and B, respectively) show that pretreatment of SC in formulations that contain either glycerol or urea (water activities around 0.93–0.94) has a similar effect on the organization of the lipid lamellae and the soft keratin proteins as pretreatment in neat PBS solution (water activity of 0.996). Considering these results it may be expected that the skin permeability is similar for these formulations, as observed in the present results (Fig. 1A). Thus, the steady state flux results in Fig. 1A may be related to that glycerol and urea penetrate into the SC and retain the structure of a fully hydrated SC membrane, which leads to similar transport characteristics of Mz across the skin membrane at reduced water activities. The effect of glycerol and urea is in contrast to the relatively larger polymer molecules, which do not partition into the skin membrane (Albèr et al., Unpublished results; Tsai et al., 2001, 2003) and thus only affect the skin membrane by dehydration irrespective of the presence of glycerol or urea. The abrupt decrease in permeability upon dehydration in Fig. 1B can thus be attributed to a larger fraction of less permeable solid SC components (lipids and proteins) (Alonso et al., 1996; Björklund et al., 2013a, 2013b).

In relation to the present diffraction data it has previously been demonstrated from SAXD and FTIR measurements that pretreatment of human SC in glycerol solution (35% w/v) for 24 h at 32 °C does not alter the organization of the lipid lamellar structures as compared to pretreatment in pure water (Caussin et al., 2008). Likewise, previous EPR spectroscopy studies, using spin labels to probe lipid dynamics, showed that treatment of SC with 8 M urea (approx. 43 wt%) only has a minor effect on the fluidity of the SC lipids (do Couto et al., 2005). These findings are in line with the present results (Fig. 2A and B). The position of the diffraction peak from soft keratin is slightly affected by the type of pretreatment as it is shifted from around 1.00 nm in the pure SC sample to approx. 0.95 nm when glycerol or urea are present in SC sample (Fig. 2B). We also note that the diffraction from this peak is weaker for the SC sample pretreated in urea formulation, which makes the determination of the peak position less certain. This peak has been attributed to the interchain packing of the soft keratin and a similar change in peak position has been observed in human SC (Nakazawa et al., 2012) and human callus (Hey et al., 1978) as a function of water content and RH, respectively. Considering that the swelling is regulated by the water activity (RH) the observed shift in peak position is in accordance with these previous studies as the water activity is higher in neat PBS compared to the glycerol or urea formulations. From previous EPR studies it has been shown that the protein mobility increases by urea treatment (Alonso et al., 2001; do Couto et al., 2005). This effect was demonstrated to be concentration dependent with an increase in protein mobility starting from 1 M (approx. 6 wt%) urea and further increasing at higher concentrations (Alonso et al., 2001; do Couto et al., 2005). An increased disorder of the soft keratin proteins when exposed to urea may explain the present weak diffraction peak around $Q = 6$ nm^{-1} from these structures (Fig. 2B).

4.3. Implications for transdermal drug delivery

The present results demonstrate the interplay between the water activity and the excipients/vehicle in a transdermal

formulation and stress the importance of defining and controlling the water activity. The results also show how either glycerol or urea can be used to regulate and control the skin permeability. An important implication of this study is that glycerol and urea may be used to substitute for water in transdermal formulations. Water has a relatively high vapor pressure compared to glycerol or urea, and the polar humectants can therefore possibly be used to retain the properties of a hydrated skin membrane also in dry conditions.

5. Conclusions

In this work we explore the effect of small polar molecules like glycerol and urea on the permeability of Mz across skin membranes, which are also exposed to a controlled gradient in water activity. We characterize the effect of glycerol and urea on the molecular organization of SC using small- and wide-angle X-ray diffraction. The main conclusions are:

i. Addition of glycerol or urea to water-based transdermal formulations lowers the water activity without decreasing the skin permeability of Mz. This effect is substantial in comparison to the effect from addition of PEG to the formulations, which results in an abrupt decrease of the skin permeability of Mz at a certain water activity (Björklund et al., 2010).

ii. In the presence of glycerol or urea, the abrupt decrease in skin permeability caused by addition of the polymer is shifted to considerably lower water activities.

iii. Conclusions i. and ii. imply that glycerol and urea penetrate the skin membrane and can retain the skin permeability characteristics associated with a hydrated skin membrane, even at situations of low water activities, which otherwise would dehydrate the skin membrane and decrease the permeability.

iv. Pretreatment of SC with water, glycerol, or urea formulations results in similar structural features of the SC lipid and protein components, which is in line with the observed similar steady state flux values of Mz across skin membranes from these formulations.

Acknowledgements

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ejps.2013.04.022.

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