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The skin barrier from a lipid perspective

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This contribution summarises the results from a number of investigations undertaken in the spirit of the Domain Mosaic Model proposed by Forslind in 1994. Atomic Force Microscopy (AFM) studies on the two-dimensional phase behaviour of some stratum corneum lipids revealed phase separation of the lipids in the typical case and the ability of cholesterol to reduce the line tension between phases. A theoretical model was developed describing the response of an oriented stack of polar lipid bilayers in the presence of a gradient in water chemical potential (water solution to humid air). The gradient gives rise to an inhomogeneous water swelling, and presumably to a liquid crystal-to-gel transition in the lamellar region closest to humid air. Skin penetration enhancers such as Azone and oleic acid cause phase transformations in lipid bilayer systems which may be relevant in the context of skin permeation. ***Key words: AFM; responsive membrane; penetration enhancer.***

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INTRODUCTION

The outermost layer of skin, the horny layer or *stratum corneum (SC),* is about 10 micrometer thick but functions as the major barrier towards inward and outward flux of compounds. It has therefore received considerable interest from research groups wishing to understand, learn from and perhaps manipulate its barrier function, including research activities in biology and medicine, chemistry (analytical chemistry, biochemistry and physical chemistry), pharmacy and membrane technology.

SC is often modelled as brick-and-mortar (1, 2), where the bricks are flattened keratin filled cells, the corneocytes, and the role of mortar is played by polar lipids arranged as stacked bilayers parallel to the skin surface. In 1994 Forslind proposed a Domain Mosaic Model (DMM) for the skin barrier where focus was on the lateral organisation of the polar lipids in the bilayers (3, 4). In the DMM the polar lipids are arranged laterally in a fashion similar to brick and mortar, but in this case the 'bricks' consist of polar lipids in a crystalline (gel) state as mosaic pieces surrounded by 'mortar' consisting of polar lipids in the fluid state.

Inspired by Forslind's model we have undertaken a number of separate studies on polar lipids with relevance for *SC*. These studies include: (i) monitoring the lateral arrangement of mixtures of polar lipids on a substrate by means of Atomic Force Microscopy (AFM) (5, 6), (ii) modelling the response of stacked bilayers subject to a gradient in water chemical potential (from an aqueous inner environment to a gaseous outer environment) (7), and (iii) the effect of some penetration enhancers such as Azone and oleic acid on bilayer forming polar lipids (8–10).

Invaluable in these studies have been the work by Bouwstra *et al.*11 and Kitson, Thewalt *et al.* (12) on stratum corneum lipid phase behaviour by means of X-ray and deuterium NMR, respectively. The analyses of the molecular composition of the polar lipids of *SC* by Wertz (13) and Norlén (14) are of course a necessity for a careful chemical modelling.

MATERIAL AND METHODS

AFM studies were performed on a Nanoscope® IIIa (Digital Instruments, Santa Barbara, CA). The lipid monolayers were prepared on a Langmuir–Blodgett trough type 611 from Nima Technology (Coventry, England). The fatty acids used were palmitic acid (C_{16}) and lignoceric acid (C_{24}) (Larodan Fine Chemicals, Malmö, Sweden). Cholesterol were purchased from Sigma Chemicals (St Louis, MO), and a synthetic ceramide (lignoceric acid amide-linked to a phytosphingosine base) from Cosmoferm b. v. (Delft, the Netherlands) was kindly provided by Dr Joke Bouwstra, Leiden University, NL. For a detailed description of the experimental procedures the reader is referred to refs. (5, 6).

The model used for calculation of lamellar water swelling is sketched in Fig. 1 The outer side of the stack of bilayers is supposed to be exposed to air with a specified relative humidity RH, and the inner side of the lamellar stack is exposed to a physiological saline solution. The variation in interlayer water separation along the gradient in water chemical potential can be calculated by knowing the surface forces between the lamellae. In the model the forces are given by an electrical double layer repulsion, calculated from the Poisson–Boltzmann equation, and an attractive dispersion force including a Hamaker constant. Besides the interlayer water separations within the stacked lamellae, it is also possible to calculate the water flux across the bilayer, as well as the flux of other small solute molecules. For a detailed description of the model the reader is referred to ref. 7

The studies of the effect of penetration enhancers were performed by adding the enhancer to lipid mixtures. The lipid mixtures were chosen from the literature on skin lipid models, and consisted of fatty acids and cholesterol. After equilibration, the mixtures were studied by X-ray diffraction in order to detect the phases present. For a detailed description of the experiments the reader is referred to refs. (8–10).

RESULTS AND DISCUSSION

Lateral arrangement of stratum corneum lipids

One major feature of the DMM is that of lipid segregation based on the fact that polar lipids tend to phase separate in the crystalline state. Even molecules of the same kind, for example fatty acids, phase separate if the difference in hydrocarbon chain length is typically more than four carbons, due to the packing constraints the chain length difference impose. Since the typical hydrocarbon chain length of a polar lipid in the horny layer exceeds twenty carbon atoms, we expect the lipids to prefer an ordered chain packing which in

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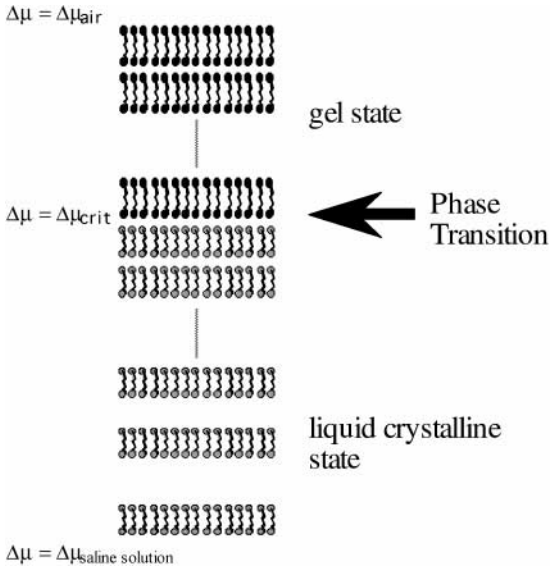

Fig. 1. Schematic representation of a liquid crystalline lamellar phase in the presence of a gradient in water chemical potential, Δμ. The thickness of every bilayer is constant while the interlamellar separation is calculated from the local value of Δμ. On the lower side of the system the chemical potential corresponds to a physiological saline solution, while the boundary condition on the upper side is determined from the relative humidity of air. Adapted from ref. 7.

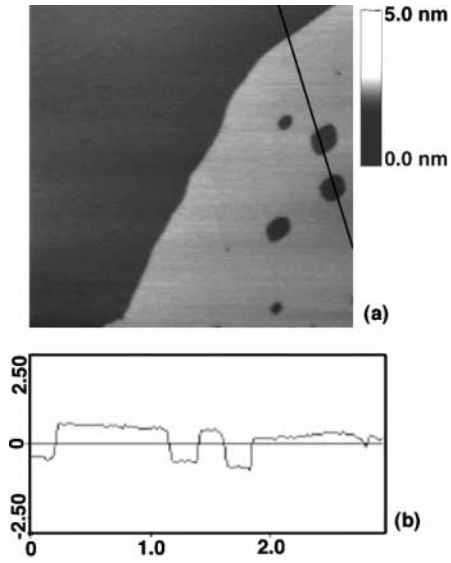

Fig. 2. (a) Topographic AFM image (3 × 3 μm) of a transferred monolayer of lignoceric acid:palmitic acid, molar ratio 1 : 1. Z-range: 5 nm. (b) A cross-section along the line indicated in (a).

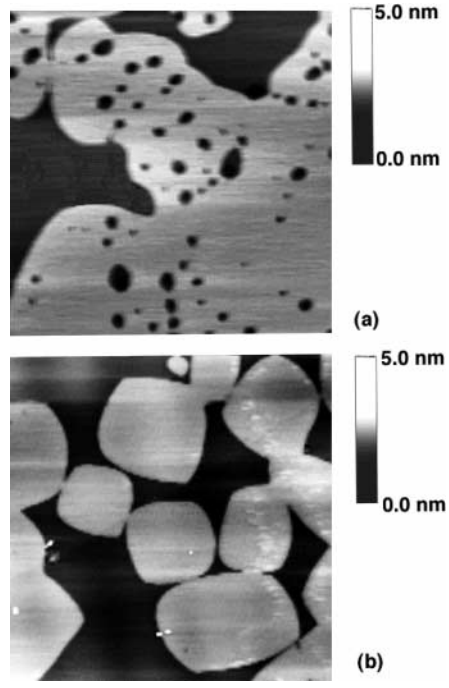

Fig. 3. Topographic AFM images (8 × 8 μm) of the transferred monolayers of (a) palmitic acid:lignoceric acid, molar ratio 1 : 1, and (b) palmitic acid:lignoceric acid:cholesterol, molar ratio 1 : 1 : 0.01. The films were relaxing at a surface pressure of 0 mN/m for 12 hours before deposited on mica at a surface pressure of 22 mN/m. Z-range: 5 nm.

turn should lead to phase separation. On the other hand, the high amount of cholesterol may have an opposing effect, known from other types of biological membranes. Cholesterol is said to have a 'line active' effect in the bilayer system, acting at the borders between different regions on the surface, in the same way as a surface active agent border regions in three-dimensional space. A series of AFM experiments illustrates this nicely.

Fig. 2a shows an AFM image (3 × 3 μm) of a transferred monolayer of lignoceric acid:palmitic acid, molar ratio 1 : 1. The cross-section along the line in Fig. 2a, given in Fig. 2b, shows the smoothness of each domain and also the height difference of 1.1 nm between the two domains. Since a difference of eight carbons in length between two fatty acids, both in all-trans conformation, is estimated to 1.02 nm, it is an plausible conclusion that the two fatty acids do not mix.

If cholesterol is added in small amounts to the fatty acid mixture above, the images change from large domains of lignoceric acid and palmitic acid in the cholesterol free case (Fig. 3a), to smaller domains as shown by Fig. 3b. Fig. 3 thus reveals that the presence of cholesterol reduces the driving force for minimising the interface between the lignoceric and palmitic domains. Cholesterol decreases the interfacial *line tension* and can therefore be regarded as a *line active agent (a lineactant)*.

In order to study a lipid model system more relevant for *stratum corneum* lipids, ceramides have to be included. The AFM images presented in Fig. 4 show a system with ceramide:lignoceric acid:cholesterol, molar ratio 1 : 1 : 1, which should be a good representative of the skin lipid composition. The image in Fig. 4 reveals a phase separation, with the lower phase essentially consisting of cholesterol and the higher of a mixture of fatty acid, ceramide and cholesterol. Without cholesterol the fatty acid and the ceramide mixing is very limited (not shown). The lineactive properties of cholesterol is thus manifested. Similar results have been obtained by ten Grotenhuis *et al.* (15).

We have previously suggested that the size of a mosaic piece could be given by the typical dimension of a lamellar body from which the lipids are extruded (4). This should

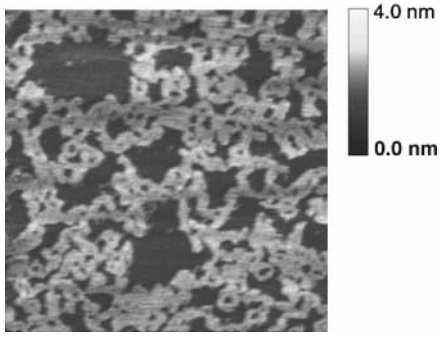

Fig. 4. Topographic AFM images (5×5 μm) of the transferred monolayers of lignoceric acid:ceramide:cholesterol, molar ratio 1 : 1 : 1. The film was deposited on mica at a surface pressure of 26 mN/m. Z-range: 4 nm.

imply an upper limit of about 0.1 micron, a size not detectable by light microscopic techniques. Our AFM studies on skin lipid model monolayers have revealed lipid domains of this size or less (Fig. 4) supporting the idea of phase separation proposed by the DMM. If the situation monitored in our systems exist in SC, with more lipid species present, remains of course an open question.

Modelling of water swelling and molecular transport across stratum corneum.

The assumption behind the model used here is that the stacked lipid bilayers are able to respond to a gradient in water chemical potential, since it causes a gradually increasing osmotic pressure as one moves from the inner aqueous region to the outer gaseous region. For polar lipids in the liquid crystalline state the changing osmotic pressure will give rise to an inhomogeneous swelling of the bilayer. This is typical for lipid bilayers in the liquid crystalline state, *i.e.* where the hydrocarbon chains are fluid. Since most of the lipids of SC have long hydrocarbon chains ($>C_{20}$) we expect them to adopt a more or less crystalline state, in which water swelling normally is not observed. The model may therefore be representative for the lower part of SC and/or more fluid regions within the SC lipids, *i.e.* in the grain borders assumed by the DMM.

A number of properties may be calculated within the model such as water swelling, water flux and solute transport. An important consequence of the gradient in swelling is that it is reasonable to extend the model to allow for phase separation (crystallisation) when the water layer between two bilayers becomes too thin. In the result presented below, such a possibility has been included.

Fig. 5 shows the swelling results from the model in comparison with experimental data by Blank *et al.* (16). The theoretical results are expressed as the relative swelling W/W_0, where W_0 is the swelling obtained for the case where the water chemical potentials are equal on both sides of the bilayer stack, *i.e.* RH = 99.5%. In this case the swelling is homogeneous. W is the inhomogeneous water swelling obtained for lower relative humidities. By using W/W_0 as the swelling parameter, the exact number of bilayers in the stack (1000 in this case) becomes irrelevant. If Blank's data for the swelling at RH = 99.5% is set equal to $W/W_0 = 1$, a comparison between theory and experiment is made possible.

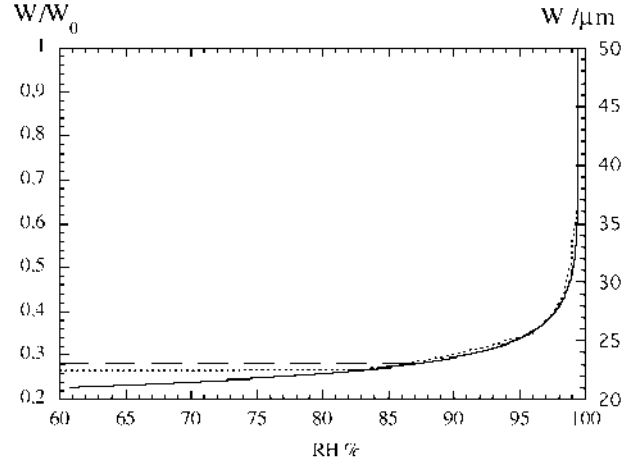

Fig. 5. The relative swelling of a liquid crystalline lamellar phase as a function of the relative humidity boundary conditions. Calculated profile W/W_0, where W_0 is the thickness of a homogeneously swollen phase (i. e. where the water chemical potential is equal on both sides, RH = 99.5%), for a liquid crystalline system (solid line) and a system where a phase transition from liquid crystal-to-gel is assumed to occur for too small interlamellar separations (dashed line). For comparison the experimental results (dotted line) from Blank *et al.*16 are also included (right hand axis).

The Figure shows that the higher the relative humidity of air, the higher the swelling capacity. It is also evident that the model is able to account for the experimental data at high RH values, whereas below about 87% RH, the model gives a continuous decrease in swelling in contrast to the experimental constant swelling. If the point where deviation occurs in Fig. 5 is taken as the point of crystallisation, a decreasing relative humidity will lead to a propagating liquid crystal-to-gel transition down the stack of bilayers (see Fig. 1). Since the water layer thickness between two bilayers in the gel state is assumed to be constant (but small) and independent of the relative humidity, the decrease in relative swelling is hindered resulting in a better agreement between theory and experiment.

The good quantitative agreement between theory and experiment in Fig. 5 is to some extent fortuitous, since the surface charge density of the polar lipid interface (1 e/nm^2) and other parameters are arbitrarily chosen. However, it turns out that the theoretical result is relatively insensitive to the choice of parameter set. On the other hand, one can argue that the experimental results obtained by Blank *et al.* reflect the water swelling of corneocytes rather than the lipids. An explanation for the good agreement may be that the water swelling of keratin-filled corneocytes follows the same electrostatic mechanism, showing a similar swelling behaviour as the liquid crystalline phase in respond to the gradient in water chemical potential.

Fig. 6 shows the qualitative behaviour of the water flux given by the theoretical model in comparison with experimental data(16). At high RH values the flux is low due to a low gradient in water chemical potential. When RH decreases the flux increases since the gradient increases. The model gives a monotonically increasing water flux whereas the experimental flux levels off at lower RH values. If the critical RH value from Fig. 5 is used to account for a phase transition, and that the diffusion coefficient of water through lipids in

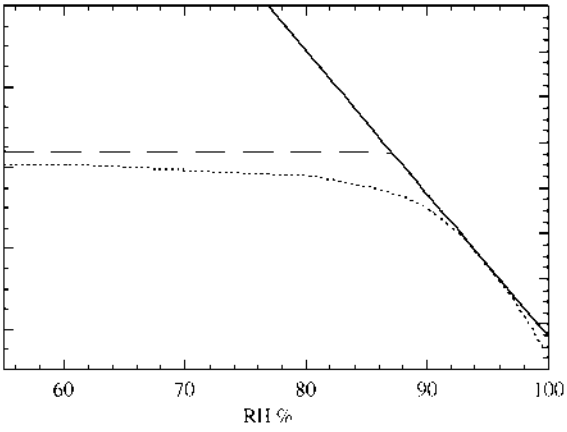

Fig. 6. Water flux as a function of the relative humidity RH. Calculated profile without (solid line) and with (dashed line) a liquid crystal-to-gel transition allowed. Experimental results from Blank *et al.* (16) (dotted line). The flux values has been left out in order to emphasise the qualitative agreement between theory and experiment. See text and ref. (7) for a discussion of quantitative comparisons.

the gel state is assumed to be one hundredth (it is probably less) of the corresponding value for the liquid crystalline state, then the qualitative agreement between experimental and model results are good. Quantitatively the model exaggerates the flux by about two orders of magnitude (7). This discrepancy is not surprising since the model only accounts for behaviour of those lipids which may be in a liquid crystalline state, i.e. lipids in grain borders.

In summary, modelling SC as a responsive membrane results in good qualitative agreement with experimental results with regard to water swelling, water flux and solute transport at high relative humidity. The deviation occurring at low relative humidity can be accounted for by adding the possibility of a phase transition from a liquid crystalline to a gel state within the membrane. An extended study is in progress focusing on the effect of lipid phase transitions on transport properties.

Skin penetration enhancers promote structural changes in lipid systems

From a barrier point of view, the organisation of the polar lipids of stratum corneum as stacked bilayers oriented parallel to the skin surface seems ideal. This organisation prevents pronounced hydrophilic and hydrophobic compounds from entering or leaving the body since they will be stopped at either the hydrophobic or hydrophilic domains. Compounds soluble in both water and oil, such as the solvents DMSO and propylene glycol, should be, and are, less hindered, making them potential skin penetration enhancers. In addition there is a group of amphiphilic molecules such as Azone (ε-caprolactam) and oleic acid which act as skin penetration enhancers as well. We have undertaken a number of phase studies in order to find out if these compounds may cause structural changes in skin lipid model systems which can explain their mechanism of action (8–10).

In our studies the enhancer molecules are added in relatively small amounts in order to find out their effect on the phase behaviour of our model systems. In the real situation, however, the penetration enhancer systems applied on skin typically contains hundred to thousand times more enhancer molecules than skin lipid molecules beneath the area of administration. This means that many mechanisms most probably are present at the same time which makes it difficult to sort out their relative importance. One such mechanism is the formation of solvent pools between the bilayers, which may alter (increase or decrease) the effective diffusional path lengths.

We have been particularly interested in the well-known penetration enhancer Azone. According to Small's classification scheme of polar lipids it belongs to the class of water insoluble polar lipids which form stable monolayers on the air-water surface (17). Being relatively hydrophobic Azone is expected to promote the formation of reversed types of phases (*i.e.* reversed cubic, reversed hexagonal and reversed micellar phases) in polar lipid water systems. This was clearly revealed in soy bean lecithin-water and monoolein-water systems (8), in fatty acid-soap-water system (9) and cholesterol-fatty acid-water systems (10).

Since transformation from lamellar structures to reversed types of structures in water implies the formation of an oil-continuous path in the system, one would expect Azone to promote the penetration hydrophobic substances through skin only. This is not the case, since Azone is known to promote the penetration of hydrophilic substances as well. In view of the phase studies undertaken and the complexity of the skin a more reasonable explanation of the effect of Azone is that it locally promotes the formation of reversed types of structures which in turn excludes water. This water is concentrated at adjacent places in the system thus possibly giving rise to a more efficient water path in skin than without Azone. The same type of reasoning can be applied to oleic acid and lidocaine base.

In summary, phase studies on penetration enhancers in skin lipid model systems reveal that molecules such as Azone create reversed types of phases in the test tubes, phases with less water content than the parent lamellar phase. In a situation with constant water this water is localised to other areas which may lead to increased swelling. Whether such a mechanism is relevant in a real situation where the typical number of enhancer molecules exceeds the number of lipid molecules by 2–3 orders of magnitude is an open question. On the other hand, small areas of continuous hydrophilic and/or hydrophobic paths across the horny layer may result in significant enhancer effects (4).

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We dedicate this paper to Prof Bo Forslind at the Karolinska Institute at the time of his retirement from academic duties. No doubt that Prof Forslind's enthusiasm and willingness to combine different scientific disciplines has brought the understanding of the barrier function of skin further. If his Domain Mosaic Model, questioned not least by his own PhD student Lars Norlén (18), is a relevant description of the polar lipid organisation of the horny layer remains an open question. Nevertheless, it has been and will be an inspiration to us in the search for a deeper knowledge of skin barrier structure and function.

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