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Molecular models of the intercellular lipid lamellae from epidermal stratum corneum

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Abstract

The purpose of the present study was to test the hypothesis that the 13 nm trilamellar repeat units within the intercellular spaces of epidermal stratum corneum are composed of lamellae with alternating 5–3–5 nm dimensions as presented in previous models [J. Invest. Dermatol. 92 (1989) 251, P.W. Wertz, Integral lipids in hair and stratum corneum, in: P. Jolles, H. Zahn, H. Hocker (Eds.), Hair: Biology And Structure, Birkhauser Verlag, Basel, 1996, pp. 227–238, Acta Derm.-Venereol., Suppl. 208 (2000) 23]. Electron density profiles were measured from transmission electron micrographs of porcine stratum corneum prepared using ruthenium tetroxide [J. Invest. Dermatol. 92 (1989) 251]. Center-to-center distances of adjacent electron-dense bands as well as adjacent lucent bands were measured. Dense band center-to-center measurements were consistent with a 5–3–5 nm arrangement. However, lucent band center-to-center measurements revealed uniform lamellar thickness. It is suggested that linoleate chains in the central lamella reduce more ruthenium than the predominantly saturated chains in the outer lamellae and that this additional reduced ruthenium accumulates under the polar head group regions. A similar phenomenon involving the sphingosine moieties of the covalently bound ω-hydroxyceramide molecules accounts for the three-band pattern seen between the ends of adjacent corneocytes. It is concluded that the component lamellae of the several types of 13 nm trilamellar units of the stratum corneum are all of equal thickness.

Keywords: Stratum corneum; Lamellar thickness; Intercellular lipid lamellae

1. Introduction

In 1973, it was discovered through use of the freeze fracture electron microscopic technique that the intercellular spaces contain extensive arrays of lipid lamellae [4]; however, these lipid lamellar structures were not evident in routine transmission electron micrographs. It was suggested that the failure to see lamellae by transmission electron microscopy reflected removal of lipids during dehydration of the samples [5]. In 1987, it was reported that substitution of the stronger oxidizing agent, ruthenium tetroxide, for the more commonly used osmium tetroxide in the preparation of samples for transmission electron microscopy revealed extensive, multiply stacked lamellae in the intercellular spaces of epidermal stratum corneum [6]. The lipid was not being removed during processing. Rather, the stratum corneum lipids have a paucity of functional groups capable of reacting with osmium tetroxide. Furthermore, the lamellae appeared to be organized into trilamellar repeat units with a broad–narrow–broad appearance and an overall dimension of 13 nm [6]. The most frequently seen lamellar patterns include three, six or nine lamellae [7]. The three-band pattern is seen between the edges of adjacent corneocytes, while the six- and nine-band patterns are seen between the broad flat surfaces of adjacent cells.

Subsequently, a 13 nm lamellar periodicity was reported on the basis of small angle X-ray diffraction analysis of stratum corneum [8–10]. Initially, it was suggested that this may represent two bilayers complexed with some protein structure [8]; however, it was subsequently demonstrated that the 13 nm repeat unit could be reconstituted in vitro from stratum corneum lipids in the absence of protein [11]. In addition, it was shown that a linoleate-containing acylceramide was required for formation of this periodicity [12,13]. This acylceramide consists of long ω-hydroxyacids amide-linked to sphingosine and bearing linoleate ester-linked to the ω-hydroxyl group [14]. In the nomenclature

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system of Motta et al. [15] this is designated CER EOS. In this system, E indicates the presence of an ester-linked fatty acid. The type of amide-linked fatty acid is indicated by N, A or O for normal, α-hydroxy or ω-hydroxy, respectively. The base component is indicated by S, P or H for sphingosine, phytosphingosine or 6-hydroxysphingosine, respectively.

Factors that determine the 13 nm periodicity would include the detailed structures of the lipid components, chain tilt relative to the plane of the polar regions and possible interdigitation [16]. Hydration does not appear to be a factor in that the there is no lamellar swelling with increasing hydration [9].

In 1989, molecular models were proposed to account for the 13 nm broad–narrow–broad lamellar structures based on the known structures of the stratum lipids [1]. It was proposed that the framework of the three-band patterns seen mainly between the edges of adjacent corneocytes was based on the covalently bound ω-hydroxyceramides. The ω-hydroxyacyl portions of the molecules were main components of the "broad" lamellae adjacent to the cornified envelopes, and it was suggested that the sphingosine tails interdigitated in the central "narrow" lamella. This interaction linked the edges of adjacent corneocytes at a molecular level. Free lipids filled in spaces to complete this model. A common six-band pattern consisted of covalently bound hydroxyceramides on either side of the intercellular space, a central pair of bilayers, and "narrow" lamellae between the central pair of bilayers and the covalently bound lipid layers. It was suggested that some ceramides assumed a splay configuration with one chain in one of the bilayers in the center of the intercellular space and the other in the "narrow" interdigitated layer. It was also suggested that ceramides in the central pair of bilayers could assume an extended configuration to effectively link the bilayers together. No special significance was attributed to any one ceramide over others.

Subsequently, a revision of this model was published in which the linoleate-containing acylceramide molecules had a unique role [2]. Basically, it was proposed that acylceramide molecules assumed an extended configuration with the ω-hydroxyacylsphingosine portion of the molecule in the "wide" lamellae and the linoleate tails inserted into the "narrow" interdigitated layers. In this revision, other ceramide molecules were not presented in splay configurations. A similar model has recently been arrived at independently [3,17]. The purpose of the present study was to use careful densitometric analyses to test the overall validity of these "broad–narrow–broad" lamellar models.

2. Methods

2.1. Densitometry

Digitized images were captured from micrographs using a PowerLook II flatbed scanner (UMAX, Fremont CA), and density profiles were obtained using TN-Image image analysis software (Thomas Nelson, Bethesda MD). Printouts of these electron density profiles were used to measure center-to-center distances of adjacent electron-dense bands as well as adjacent lucent bands.

2.2. Reconstitution and electron microscopy of stratum corneum lipids

Porcine stratum corneum lipids were prepared, and the composition was determined [18]. Lipids were reconstituted essentially as previously described [19]. The lipid (150 mg) was dissolved in *n*-hexane/isopropanol, 3:2, at a concentration of 2 mg per ml and was applied slowly to Millipore filter disks (1.5 cm diameter, 0.1 μm). The solvent was allowed to evaporate, after which the disks were suspended in a beaker of distilled water and heated to 80 °C for 1 h. After cooling to room temperature, disks were placed in a closed chamber over silica gel desiccant and stored for 2 days under house vacuum. After desiccation, disks were fixed for 2 h in Karnovsky's fixative at 4 °C. Disks were then post-fixed for 1 h in 0.2% ruthenium tetroxide in 0.1 M cacodylate buffer at pH 6.8 [6,18]. The specimens were dehydrated in a series of ethanol solutions (30%, 50%, 70%, 95% and 100%) before embedding in

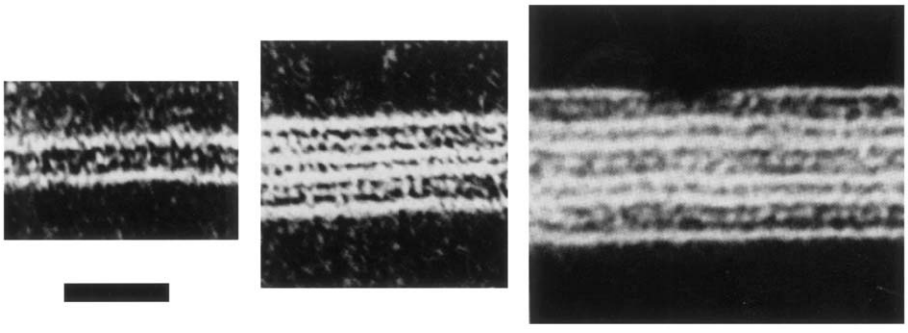

Fig. 1. Transmission electron micrographs displaying three, six or nine lamellae within the intercellular space of stratum corneum. Bar = 25 nm. Modified with permission from Swartzendruber et al. [1].

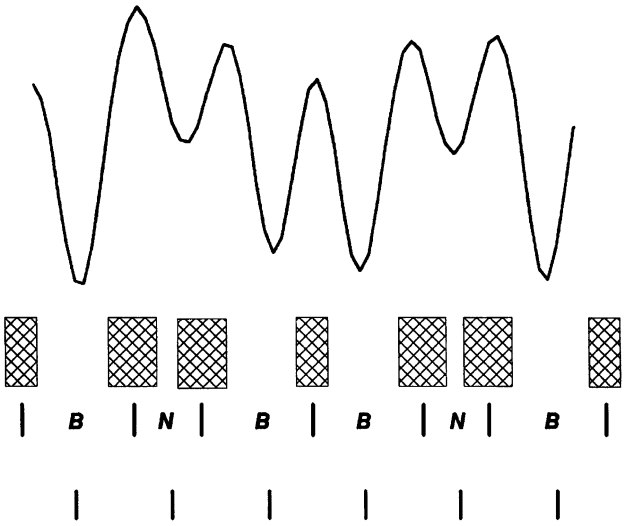

Fig. 2. Densitometer tracing of the six-band pattern of lamellae from the intercellular space of stratum corneum. Shaded boxes represent the pattern of reduced ruthenium on the micrograph. The first set of tick marks beneath the boxes indicate the centers of the electron dense bands, while the lower set of tick marks indicate the centers of the lucent lucent bands.

Spurr's resin. Ultrathin sections were cut and collected on carbon-stabilized Formvar-coated grids. Sections were stained with lead citrate or, in some cases, examined without staining in a Zeiss EM 10.

3. Results

Fig. 1 illustrates the most common lamellar patterns observed in the intercellular spaces of the stratum corneum [1], and Fig. 2 is a densitometer tracing of the six-band pattern from the middle of Fig. 1. The lamellar patterns shown in Fig. 1 have been reproduced from the manuscript in which the original molecular models were proposed. Note the uniform lamellar thickness as judged by the center-to-center distances of the lucent bands indicated by the bars beneath the tracing. Also, notice the alternating depths of the curve in Fig. 2 corresponding to the lucent band regions. A similar density profile has previously been reconstructed from optical diffractometry data [10].

Fig. 3 is a proposed model for the molecular arrangement of lipids within the three central lamellae in the nine-band pattern from Fig. 1. The key feature of this model is that the central lamella contains linoleate chains and the outer two lamellae of this trilaminar structure do not. As a result, the central lamella will reduce more ruthenium during post-fixation with ruthenium tetroxide. It is suggested that this additional ruthenium is not symmetrically deposited about the polar head groups of this lamella. It is suggested that the ruthenium accumulates beneath the plane of the polar head groups in this central lamella, as indicated by the shaded rectangles. Thus, the three-component lamellae of the 13 nm repeat units can be of equal thickness, but the uneven

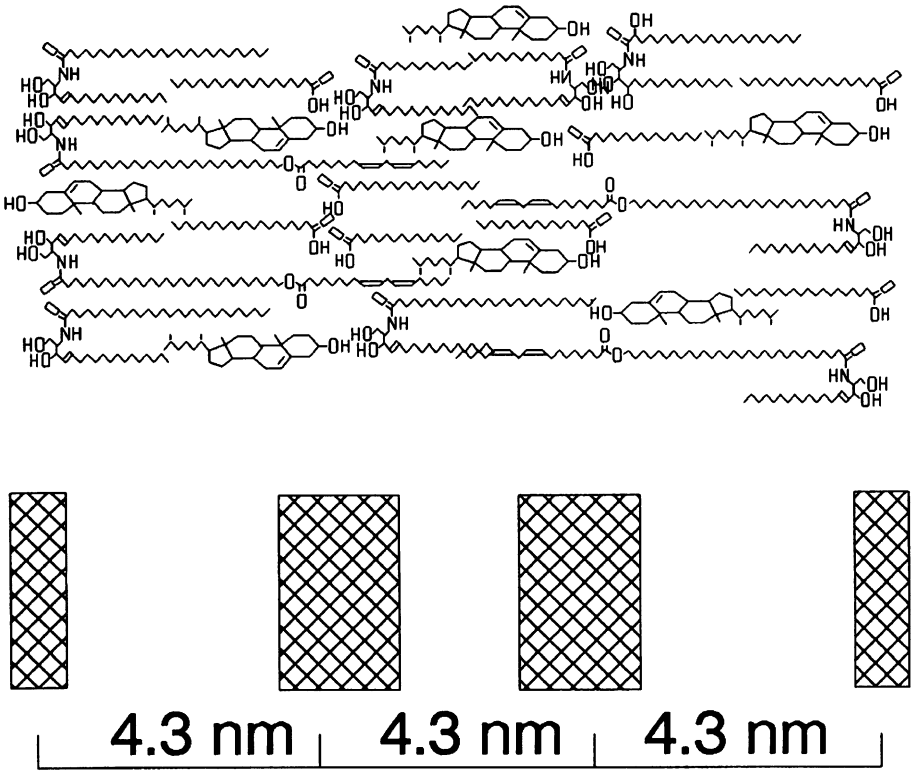

Fig. 3. Molecular model of the central three bands of the nine-band pattern. Note the linoleate tails of acylceramide molecules in the central lamella. Shaded boxes represent the pattern or reduced ruthenium on a corresponding portion of a transmission electron micrograph.

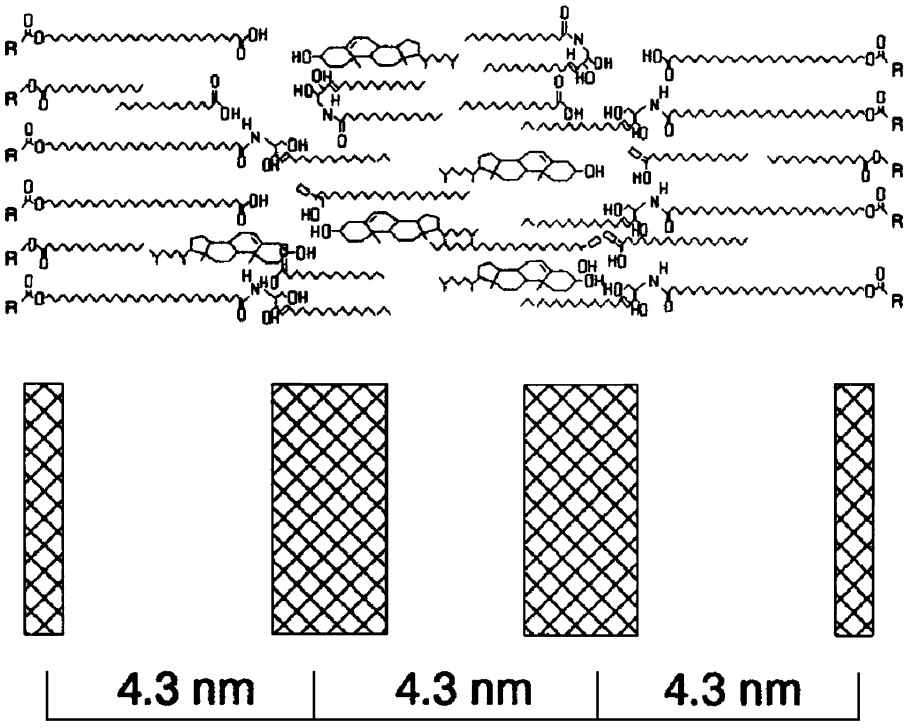

Fig. 4. Molecular model for the three-band pattern between the edges of adjacent corneocytes. As in Fig. 3, the shaded boxes represent the pattern of reduced ruthenium on a corresponding portion of a transmission electron micrograph.

distribution of ruthenium results in a broad–narrow–broad pattern of lucent bands on transmission electron micrographs. This is also consistent with the alternating depths of the densitometer tracing in the lucent band regions from Fig. 2.

Fig. 4 is a proposed molecular model for the three-band pattern seen mainly between the ends of adjacent corneocytes [7]. In this case, the primary interaction is between the covalently bound lipids on the outer surfaces of the cornified envelopes. It is proposed that the sphingosine tails of the ω-hydroxyceramide molecules insert into the central lamella without interdigitation. The double bonds from the sphingosine chains would, in this case, reduce additional ruthenium, and again, would lead to an asymmetric distribution of reduced ruthenium beneath the plane of the polar regions of the central lamella. This is indicated by shaded rectangles.

The lamellae containing the narrow lucent band shown in the center of Fig. 1 would be hybrids between their counterparts in Figs. 3 and 4. That is, they would contain sphingo-

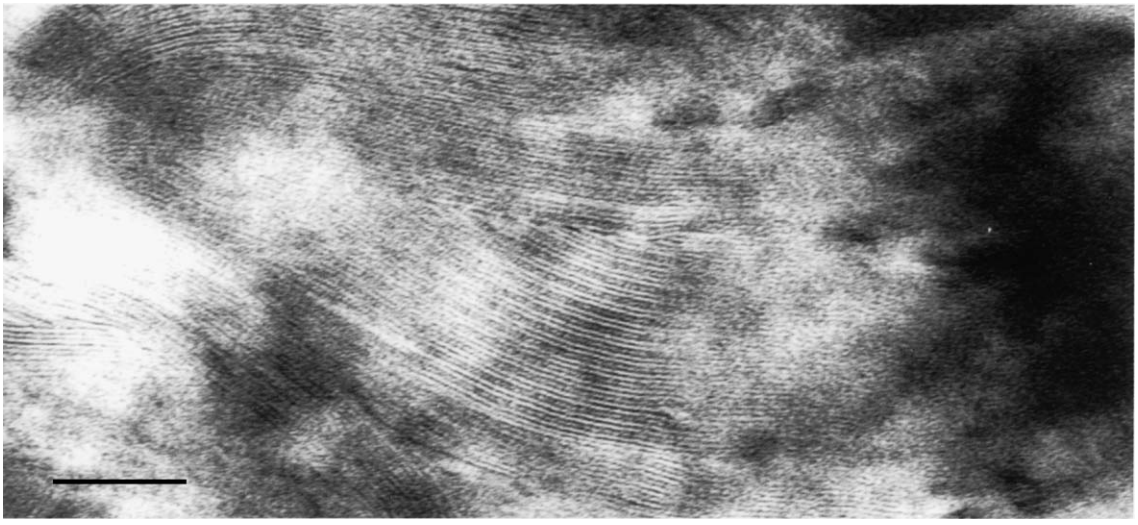

Fig. 5. Reconstituted porcine epidermal stratum corneum lipids. Bar = 100 nm. The composition (weight percent) of the lipid is 10.8% cholesterol esters, 13.8% fatty acids, 28.7% cholesterol, 6.5% CER EOS, 14.0% CER NS, 5.4% CER NP, 4.8% CER AS (long chain), 4.3% CER AS (short chain), 7.4% CER AP and 4.3% cholesterol sulfate.

sine chains from the covalently bound hydroxyceramides on the cornified envelopes and linoleate chains from acylceramide molecules from the pair of bilayers in the center of the intercellular space. Again, the chains would not interdigitate, and the thickness of all six lamellae would be equal.

Fig. 5 is a transmission electron micrograph of reconstituted porcine epidermal stratum corneum lipids within the pores of a Millipore filter disk. Although there is some variation in the lamellar pattern, there are extensive regions in which alternating broad–narrow–broad lucent bands are evident.

4. Discussion

The barrier function of the skin is fundamental to life on dry land [20]. Understanding the permeability barrier function of the skin is important for rational design of transdermal drug delivery systems as well as for our understanding the etiology, and possible treatment, of a range of skin diseases in which barrier function is compromised.

It must be acknowledged that the present study relies upon transmission electron micrographs, X-ray diffraction data and known chemical structures of the stratum corneum lipids. Transmission electron micrographs are artifacts, and depending upon the methods of sample preparation, X-ray diffraction may be subject to some of the same difficulties. It is an underlying assumption of the present work that both of these methods reflect reality with reasonable accuracy. This is an assumption that has attracted some controversy [21,22].

The present findings have enabled us to refine the molecular models for the structures of the intercellular lamellae. Although the lucent bands of the intercellular lamellae display an alternating repeating broad–narrow–broad pattern, the actual lamellae are of uniform 4.3 nm width. The false impression that the lamellae differ in thickness results from the reduction of ruthenium tetroxide by the linoleate chains in the central lamella and the asymmetric distribution of the reduced ruthenium beneath the polar head group regions.

The current model for the free lipid trilaminar unit differs from their predecessors in that the acyl chains in the trilamellar 13 nm repeat units are not interdigitated. In addition, in the previous models [1–3], only cholesterol and the shorter chained fatty acids and ceramides could fit into the central lamella of the trilaminar units. This restriction is removed in the present model. Except for the acylceramide molecules, there is no compelling reason to think that the other ceramide, cholesterol and fatty acid molecules are other than randomly distributed within the trilaminar units.

Although the present models are intended to address the lamellar spacing only, a few comments on lateral chain packing are in order. The central lamella of the trilamellar units would be fluidized by the linoleate from CER EOS, and possibly CERs EOP and EOH, and is probably in a liquid crystalline phase as proposed for the sandwich model [3,17]. In contrast, the outer lamellae of the trilamellar units would be rich in cholesterol and ceramides with long acyl chains. This composition makes these outer lamellae well-suited for formation of a liquid ordered phase [23]. In the liquid ordered phase acyl chain packing is tight, like in a gel or crystalline phase, but the lateral motion of the lipids is more rapid than in a gel phase. In fact, a model stratum corneum lipid mixture has been shown to form a liquid ordered phase [24].

The original molecular model included ceramide molecules in an extended conformation such that one aliphatic chain was inserted into each of a pair of adjacent lamellae [1]. This form of chain interdigitation was thought to increase the stability of the multilamellar arrays by linking all of the lamellae together. This sort of interdigitation is known to occur in ceramide crystals [25]. This feature is not depicted in the present models; however, it cannot be ruled out.

The present model for the trilaminar structures involving the covalently bound ω-hydroxyceramides is somewhat different than the trilaminar units formed from free lipids within the intercellular space. Although the double bond in the sphingosine chain is capable of reducing ruthenium tetroxide, it is a *trans* double bond so it will not have the same fluidizing effect as a *cis* double bond. In contrast, the trilaminar units formed from the free lipids would have a central lamella rich in linoleate chains containing two *cis* double bonds each. The acyl chains in the outer lamellae in these repeat units are relatively saturated. This would result in relatively rigid, possibly gel phase, outer lamellae with a relatively fluid, possible liquid crystalline, lamella in the center. This is consistent with the small angle X-ray diffraction studies, which have indicated co-existence of crystalline and liquid phases within stratum corneum lipids [3,26].

It should be noted that X-ray diffraction work reveals a 6 nm lamellar periodicity in addition to the dominant 13 nm periodicity [3]. The 6 nm periodicity has been assumed to represent a simple bilayer. In fact, although quantitatively relatively minor, uniformly spaced lamellae have been seen in regions of intercellular space by transmission electron microscopy [7]. These could represent stacked arrays of 6 nm bilayer. Neither the present models, nor those previously published [1–3] address the 6 nm periodicity.

One potential advantage of the ordered–fluid–ordered arrangement of the lamellae is that noxious substances that partitioned into the stratum corneum would diffuse laterally much faster through the fluid phase than across the ordered phase. This would have the effect of diluting a penetrant before it reached the living layers of the epidermis and, thereby, minimizing damage to the tissue.

It is somewhat surprising that the 13 nm trilaminar repeat units can be reconstituted from extracted lipids [11,12,19]. It would have been expected that the lipids would have been randomly oriented in the reconstituted bilayers, and this may

initially be the case. If this is correct, it would appear that the arrangement with the acylceramide linoleate chains in the central layer of the trilaminar units would represent a thermodynamically stable arrangement. Alternatively, it is possible that there is a kinetic advantage to formation of trilaminar structures with the linoleate chains in the central lamella, and there is a slow approach to equilibrium after formation of the initial structure. This could have implications for the biology of the stratum corneum. The broad–narrow–broad pattern of lucent bands is clearly present in the outer portion of the stratum corneum [6], and it has also been demonstrated between exfoliated cells [27]. However, in the later case, uniformly spaced lucent bands appear to predominate. This may be worthy of further study.

Previous studies have demonstrated that CER EOS containing sphingosine with an amide-linked ω-hydroxyacid and ester-linked linoleate is required for formation of the 13 nm repeat units [12,13], and the alternating broad–narrow–broad lucent band pattern [19]. In addition to CER EOS, two additional acylceramides have been identified among human stratum corneum lipids-CER EOH [28] and CER EOP [29]. Each of these contains linoleic acid as the principal ester-linked acid [29]. Future research will be directed at elucidating the roles of the several acylceramides in determining the physical properties of the stratum corneum lamellae.

Acknowledgements

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