The Characterization of Molecular Organization of Multilamellar Emulsions Containing Pseudoceramide and Type III Synthetic Ceramide

B. D. Park,* J. K. Youm,* S. K. Jeong,†‡ E. H. Choi,§ S. K. Ahn,§ and S. H. Lee†¶

*NeoPharm Co., Ltd, Taejeon, Korea; †BK21 Project for Medical Sciences, Yonsei University, Seoul, Korea; ‡Central Research Laboratories, Aekyung Industrial Co. Ltd, Taejeon, Korea; §Department of Dermatology, Yonsei University Wonju College of Medicine, Wonju, Korea; ¶Department of Dermatology, Yonsei University College of Medicine, Seoul, Korea

To investigate the molecular organization and phase behavior of physiologic lipid mixtures that contain either newly synthesized pseudoceramide or type III synthetic ceramide, various analytical techniques were used. The phase transition temperatures detected in differential scanning calorimetry analysis were 51.19 and 50.52 for the pseudoceramide-containing physiologic lipid mixture and synthetic type III ceramide-containing lipid mixture, respectively. From the small angle XRD patterns, the multilamellar emulsion-pseudoceramide showed 11.5 nm and 7.61 nm lamellar phases, while the multilamellar emulsion-synthetic ceramide showed only a 7.61 nm lamellar phase. The nonceramide containing lipid mixture did not show any distinct repeat pattern. Lateral packing distances of multilamellar emulsion-pseudoceramide and multilamellar emulsion-synthetic ceramide were measured as 0.4119 and 0.4110 nm at 30, respectively, which indicated the presence of hexagonal lattice. On the contrary, non-multilamellar emulsion did not show any definite repeat pattern. Transmission electron microscopy observation showed nearly comparable lamellar structures in all of the tested emulsions compared to the structure of human stratum corneum intercellular lipid. Decrease of water contents resulted in phase transition into liquid phase for all the tested emulsions, whereas phase transition into orthorhombic phase was observed only in multilamellar emulsion-pseudoceramide. From these results, we concluded that the molecular organization of multilamellar emulsion-pseudoceramide was characterized as the lateral hexagonal phase and both the long and short periodicity lamellar phases, which showed structural similarity with the native human stratum corneum intercellular lipid. ***Key words: epidermal permeability barrier/multilamellar emulsion/pseudoceramide/stratum corneum lipid/synthetic ceramide. J Invest Dermatol 121:794–801, 2003***

The epidermal permeability barrier is known to play an essential part in human physiology by not only preventing the loss of water from the body but also protecting the body from external physical, chemical, and microbial insults. The skin barrier function is closely related to the structure of the outermost layer of the epidermis, the stratum corneum (SC), which consists of two major components: corneocytes and intercellular lipids. By the "brick and mortar model", which is one of the most commonly employed analogies for describing SC organization, the hydrophilic corneocyte (brick) fills the major volume of the wall (SC) surrounded by the hydrophobic intercellular lipid (mortar); the most dominant part of the epidermal permeability barrier function seems to be presented by the intercellular lipids.

Diseased skin often shows an impaired skin barrier function, possibly due to the deviation of SC intercellular lipid composition and subsequent defects in molecular organization (Yamamoto *et al*, 1991; Lavrijsen *et al*, 1995; Bleck *et al*, 1999; Pilgram *et al*, 2001). In terms of the molecular organization of SC intercellular lipids, skin diseases such as atopic dermatitis and lamellar ichthyosis show increased hexagonal lattice, along with deficiencies in long chain fatty acids and certain type of ceramides (Bouwstra *et al*, 1996, 1998a; Pilgram *et al*, 1999). Thus detailed information about the relationship between the molecular organization of SC intercellular lipids and their composition is of great importance to understand the skin barrier function as well as the disorder of the SC itself (Elias *et al*, 1983).

Although the molecular organization of the intercellular lipids has not been completely elucidated, it is clear from many studies that the lipids are organized as highly ordered lamellar structures (Swartzendruber *et al*, 1989; Kitson *et al*, 1994; ten Grotenhuis *et al*, 1996; Kuempel *et al*, 1998; Bouwstra *et al*, 1999; Moore and Rerek, 2000). As for the lateral organization, it seems that the intercellular lipids exist *in vivo* as a balanced state between either orthorhombic and hexagonal phases or liquid crystalline phase depending on the various environmental and intrinsic factors, such as relative humidity, exposure to topical agents, skin temperature, and the depth within SC (Rhein *et al*, 1990; Frobe *et al*, 1990; Rawling *et al*, 1995; Pilgram *et al*, 1998).

In previous studies using pig, murine, and human skin models, a lamella phase with a periodicity of about 13 nm was shown. A second lamella phase with a periodicity of 6 nm was also present

Manuscript received June 14, 2002; revised September 10, 2002; accepted for publication April 16, 2003

Address correspondence and reprint requests to: Seung Hun Lee, Department of Dermatology, Yonsei University College of Medicine, Yongdong PO Box 1217, Seoul, 135-270, Korea. Email: ydshderm@yumc.yonsei.ac.kr

Abbreviations: SC, stratum corneum.

in human and pig SC (White *et al*, 1988; Bouwstra *et al*, 1991, 1994, 1995). Furthermore, in murine and human skin, the lipids were mainly organized in an orthorhombic lateral structure, whereas in pig skin, the lipids were in a hexagonal structure (White *et al*, 1988; Bouwstra *et al*, 1992, 1994). As the 13 nm phase existed in all examined species and was an unusual lipid arrangement, this phase has been considered to be important for the permeability barrier function (Bouwstra *et al*, 2002).

Recently, we have reported the synthesis of new pseudoceramide and the formulation of multilamellar emulsion using the newly synthesized pseudoceramide (MLE-PC) (Park *et al*, 1999, 2000). Also, the topical application of MLE–PC on barrier disrupted hairless mice skin, both acutely and chronically, was proved to be effective for barrier function recovery and maintaining normal skin homeostasis, compared with nontreated skin. Another emulsion was also prepared with nearly the same components as MLE-PC, except the pseudoceramide contents and some emulsifying agents. The emulsion without pseudoceramide (non-MLE) did not express the multilamellar structure and did not show a significant effect on the recovery rate (Park *et al*, 2001a).

According to the previous reports, topical application of physiologic lipid mixtures, which contain the main components of SC intercellular lipids such as ceramide, free fatty acids, and cholesterol, leads to the effective recovery of both acutely and chronically disrupted skin barrier function (Mao-Qiang *et al*, 1996). As the MLE-PC has the main physiologic lipid components, and non-MLE has only free fatty acids and cholesterol contents, it seems that the pseudoceramide content plays a major part for recovery of skin barrier function. In addition to the pseudoceramide contents, another significant difference between MLE-PC and non-MLE is the existence of a lamellar structure of the emulsion. Although the lamellar structure could play some part in barrier function recovery, little research has been done about the role of the molecular organization of topical formulation on skin barrier function.

In this study, we investigated the molecular organization of MLE-PC and non-MLE and tried to correlate the clinical effects of topically applied emulsion and its structure. In addition, we have formulated another emulsion with type III synthetic ceramide (MLE-SC) (**Table I**) and investigated its structure. In a previous report about the effects of specific ceramide on the molecular organization of lipid mixture, it was proposed that the presence of ceramide 1 is crucial for proper lipid phase behavior in the mixture (Bouwstra *et al*, 2002). According to this proposal, the MLE-SC with synthetic type III ceramide, could not express the proper phase behavior.

At first, we investigated the molecular organization and structure of MLE-PC, non-MLE, and MLE-SC using differential scanning calorimetry (DSC), X-ray diffraction (XRD), and transmission-electron microscopy (TEM) methods. Secondly, we observed whether any structural changes occurred under dry conditions, which could result in the phase transition. Finally, we investigated whether there is any relationship between the molecular organization of emulsion before and after topical application on the skin. This structural change could provide valuable information about the effects of the molecular organization of emulsions on skin barrier function when topically applied on the skin, as well as information about the structure of native intercellular lipids in SC.

Table I. The main components of MLE1, MLE2, and placebo

Components	MLE1	MLE2	Placebo
Pseudo-stratum corneum lipid	2.3	2.3	0.8
Pseudoceramide	0.6	–	–
Ceramide	–	0.6	–
Stearic acid	1.5	1.5	0.8
Cholesterol	0.2	0.2	–
Oils and common base	8.0	8.0	10.5
Cetanol	3.5	3.5	2.0
2-octyldodecanol			3.0
Squalane	2.0	2.0	2.0
Fatty acid triglyceride	3.5	3.5	3.5
Polyols	10.0	10.0	10.0
Glycerin	5.0	5.0	5.0
1,3-butylene glycol	5.0	5.0	5.0
Emulsifiers	4.5	4.5	4.5
Glyceryl monostearate	1.5	1.5	1.5
POE(15) Glyceryl monostearate	3.0	3.5	3.5
Thickener	0.2	0.2	0.2
Carbomer	0.2	0.2	0.2
Water	74.0	74.0	74.0

MATERIALS AND METHODS

Preparation of emulsion Stearic acid, cholesterol, and fatty acid triglyceride were purchased from Junsei Chemical Co. (Tokyo, Japan) and glyceryl monostearate and POE(15) glyceryl monostearate were from Nihon Emulsion Co (Tokyo, Japan). Cetanol and 2-octyldodecanol were from Kao Co. (Tokyo, Japan) and Henkel (Düsseldorf, Germany), respectively. Squalane (Kishimoto Co., Hyogo, Japan) and carboxyl vinyl polymer (B.F. Goodrich, Cleveland, Ohio) were also used for the preparation of emulsions. Synthesis of pseudoceramide (myristyl/palmityl-oxo-stearamide/arachamide MEA: $C_{34}H_{67}NO_3/C_{36}H_{71}NO_3/C_{38}H_{75}NO_3 =$ 11:42:47%; **Fig 1*A***) and its chemical characteristics were described in a previous report (Park *et al*, 2000). MLE-PC and non-MLE, which was composed of nearly the same components as the MLE-PC except the pseudoceramide, were prepared according to a previously reported method (Park *et al*, 2001b). In brief, the physiologic lipid components (pseudoceramide, stearic acid, and cholesterol), oils, common base (cetanol, 2-octyldodecanol, squalane, and fatty acid triglyceride), and emulsifiers (glyceryl monostearate, POE(15) glyceryl monostearate) were mixed and melted at 85 to 90°C. The water and water-soluble components (glycerine, 1,3-butylene glycol) were added slowly with vigorous agitation by homomixer (T.K. Homomixer Mark II f-model, Tokyo Co. Ltd, Tokyo, Japan) at 70°C. After emulsifying, the mixtures were cooled to room temperature. MLE-SC was prepared with the same components as MLE-PC except for the use of type III synthetic ceramide (ceramide IIIB type: $C_{36}H_{73}NO_4$; **Fig 1*B***) instead of pseudoceramide.

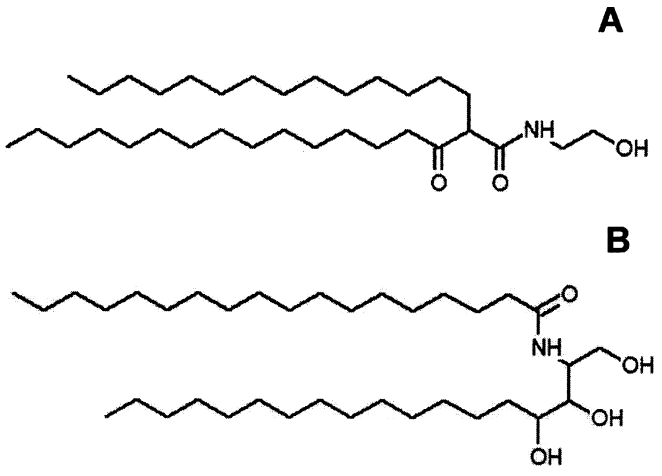

Figure 1. Structures of pseudoceramide and type IIIB synthetic ceramide. (*A*) Pseudoceramide (INCI name: myristyl/palmityl-oxo-stearamidel arachimide MEA, Neopharm, Taejeon, Korea), is a mixture of myristyl-oxostearamide MEA ($C_{34}H_{67}NO_3$, $11 \pm 2\%$), palmityl-oxostearamide MEA ($C_{36}H_{71}NO_3$, $21 \pm 3\%$), myristyl-oxoarachamide MEA ($C_{36}H_{71}NO_3$, $21 \pm 3\%$) and palmityl-oxoarachamide MEA ($C_{36}H_{75}NO_3$, $47 \pm 3\%$). All percentage values were based on the HPLC analysis. (*B*) Type IIIB synthetic ceramide ($C_{36}H_{73}NO_4$, $>90\%$ by HPLC).

Light and electron microscope A small amount of emulsions was spread on the slide glass and the optical anisotropy was observed under the cross-polarized light microscope (Optiphot-2, Nikon, Tokyo, Japan). For electron microscopic observation, all of the emulsions were embedded in 3% (w/v) agar solution. After complete dispersion of the agar solution, 10% (w/v) of emulsion was added and hardened at room temperature for 48 h. The embedded specimens were processed as per previously described methods (Choi *et al*, 1997; Ahn *et al*, 1999). Briefly, the agar-embedded emulsions were minced to 0.5 mm^3, fixed in modified Karnovsky's fixative solution overnight, washed with 0.1 M of cacodylate buffer, and postfixed in 0.2% ruthenium tetroxide (Polsciences, Warrington, Pennsylvania) in 0.1 M cacodylate buffer for 45 min in the dark at room temperature. After rinsing the buffer, the specimens were dehydrated in a graded ethanol series and substituted with propylene oxide, and finally embedded in Epon resin. Ultrathin sections (60–80 nm) were double-stained with uranyl acetate and lead citrate, and examined with a Philips CM10 electron microscope operating at 80 kV.

XRD All measurements were carried out at the Korea Research Institute for Chemical Technology (KRICT; Taejeon, Korea) using D8 Discover XRD (Bruker, Germany) with GADDS (General Area Detector Diffraction System). For small-angle X-ray scattering (SAXD), the samples were placed in a capillary (Mark-Röhrchem aus Glas no. 10, length 80 mm, Außen Ø 0.7 mm (external diameter), article no. 4007807, Hilgenberg, Germany). Cu-Kα radiation ($\lambda = 1.5405$Å, using monochromator) was used with power at 40 kV and 45 mA. The collimeter was fixed at 0.1 mm and the counting time was 30 min per step. The scattered beams from the samples were transmitted through 30 cm of helium gas tube. The scattered intensities were measured as a function of θ, the scattering angle.

For wide-angle X-ray scattering (WAXD), the samples were placed in a capillary (Mark-Röhrchem aus Glas no. 10, length 80 mm, Außen Ø 0.7 mm, article no. 4007807, Hilgenberg, Germany) and the temperatures of the samples were controlled by the D8 Discover XRD for high temperature WXRD. Cu-Kα radiation ($\lambda = 1.5405$Å, using monochromator) was used with power setting of 40 kV and 40 mA. The collimeter was fixed at 0.3 mm and the counting time was 150 s per step. The scattered beams from the samples were transmitted through 30 cm of helium gas tube.

Phase transition For the study of phase transition, 5 g of each emulsion was spread in the Petri dish and incubated at 45°C for 24 h. The phase-transited samples were placed in the general sample holder and observed with XRD.

DSC DSC (model 2910, TA Instruments, New Castle, Delaware) was used for the phase transition study of emulsions according to the temperature elevation. The heat of fusion was observed between −10°C and 90°C with nitrogen purge at the rate of 50 mL per min. The heating rate was 10°C per min.

RESULTS

MLE-PC and MLE-SC showed typical optical anisotropy under the cross-polarized microscope We have formulated the pseudoceramide, cholesterol, stearic acid, and other components to make an emulsion that mimics SC lipid composition (Park *et al*, 2000). To make lamellar structures in the emulsion, POE(15) glyceryl monostearate, and glyceryl monostearate were used as emulsifiers (Myers, 1988). Detailed explanation of the composition and formulation of the emulsions has been described in a previous report (Park *et al*, 2001a). MLE-SC was formulated by using type III synthetic ceramide, whereas other components and the procedure were the same as for MLE-PC. Non-MLE was prepared with the same emulsifying system, but the ceramide contents were excluded and the long chain fatty acid composition was slightly changed.

Both MLE-PC and MLE-SC showed a typical optical anisotropy by cross-polarized microscopy (**Fig 2*A,B***). This "Maltese cross" appearance is a characteristic configuration observed in concentric lamellar emulsion under cross-polarized microscopy. Several droplets of non-MLE also showed a faint "Maltese cross", but the optical anisotropy was not so distinct

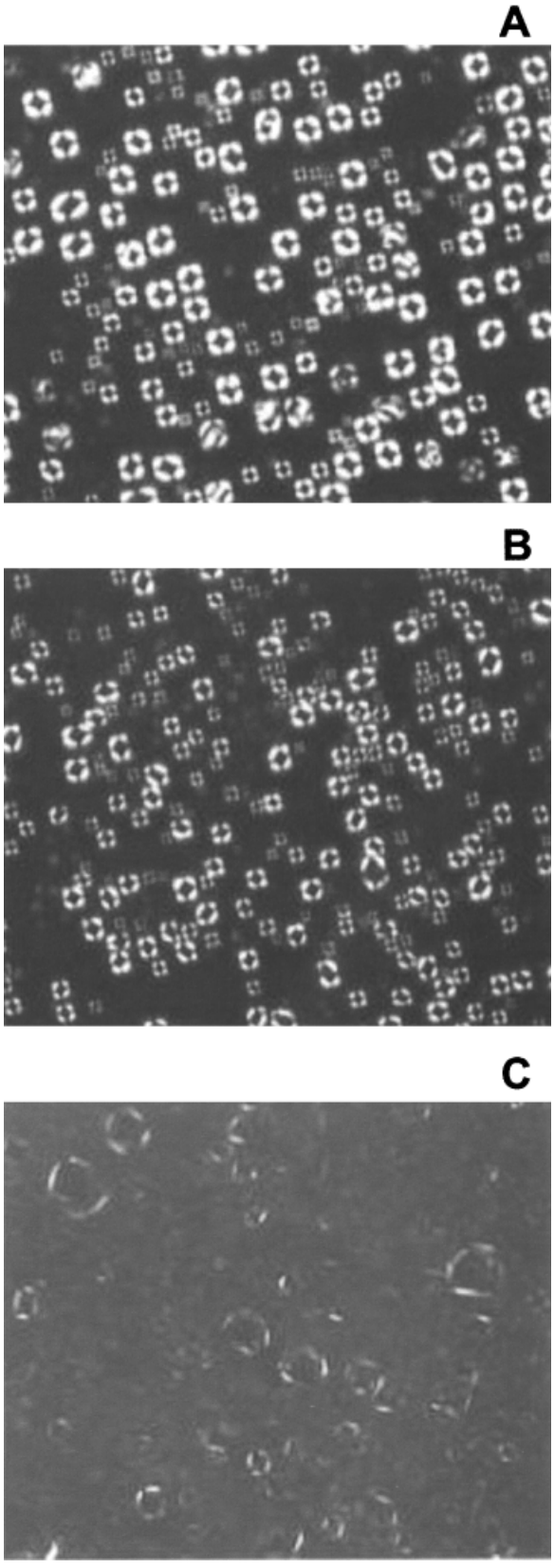

Figure 2. Cross-polarized microscopic observations of MLE-PC, MLE-SC and non-MLE. MLE-PC (*A*) and MLE-SC (*B*) show distinct optical anisotropy under the cross-polarized microscope. The "Maltese cross" is a characteristic configuration observed in concentric lamellar emulsions. Several droplets of non-MLE (*C*) also show faint optical anisotropy.

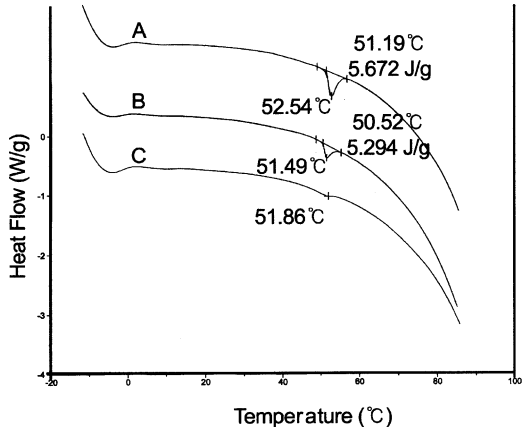

Figure 3. Endothermic heat adsorption profiles of MLE-PC, MLE-SC and non-MLE. The phase transition temperatures of each emulsion calculated from the DSC results are 51.19°C, 50.51°C and 51.86°C for MLE-PC (*A*), MLE-SC (*B*) and non-MLE (*C*), respectively. The heat of adsorptions for MLE-SC and MLE-SC are 5.6762 J/g and 5.294 J/g, respectively. The heat of adsorption for non-MLE was not calculated (*C*).

compared with the MLE-PC and MLE-SC and it might be due to the emulsifying system (**Fig 2*C***).

The heat of adsorption peaks from MLE-PC and MLE-SC represents the phase transition behavior of the tested emulsions In a previous report, the pseudoceramide/fatty acid/cholesterol mixture showed a phase transition at 48.4°C (Park *et al*, 2000). The phase transition temperature of MLE-PC, MLE-SC, and non-MLE emulsion were 51.19°C, 50.52°C, and 51.86°C, respectively, which were slightly higher than that of the three components system mentioned above (**Fig 3**). In addition, the heat of adsorptions for MLE-PC and MLE-SC were 5.6762 and 5.294 (J g), respectively, but not significant (<0.01 J g) in the non-MLE system. This presence of phase transition temperature in DSC was possibly due to the structural change of emulsion. The heat of adsorption of MLE-PC was slightly higher than that of MLE-SC, which, in part, might be due to the more rigid molecular organization of MLE-PC than that of MLE-SC.

Lateral packing: Only the hexagonal phase was observed in MLE-PC and MLE-SC The diffraction patterns of the tested emulsions are depicted in **Fig 4**. MLE-PC and MLE-SC showed a weak, but distinct diffraction peak at a spacing of 0.4119 nm and 0.4110 nm, respectively, at 30°C, which indicated the presence of hexagonal lateral packing (**Fig 4*A***). Although an increase of temperature could result in an increase of chain mobility of lipid components and the disturbance of the molecular organization of the emulsion, an identical series of diffraction patterns were observed for both emulsion at elevated temperature up to 60°C. A slight increase in packing space, however, was also seen with elevated temperature and no distinct diffraction pattern was observed at 80°C for either MLE-PC or MLE-SC (**Fig 4*B,C***).

Friberg *et al* (1987, 1999) suggested that glycerin and other polyols could slow down the phase transition under a dry environment and could exert some maintenance effects on the epidermal lipid as a fluid, hexagonal, or orthorhombic structure. Thus, glycerin, 1,3-butylene glycol, and water in MLE-PC could prevent crystallization. Moreover, the emulsifiers with high HLB (hydrophilic–lipophilic balance) also could exert a preventive role against the formation or transition into crystalline phase. Under room temperature, the hexagonal lateral structure of MLE-PC was not disturbed after 6 mo (data not shown).

Non-MLE emulsion, however, did not show any distinct peak in the WAXD analysis (**Fig 4*A***). As we attempted to make the

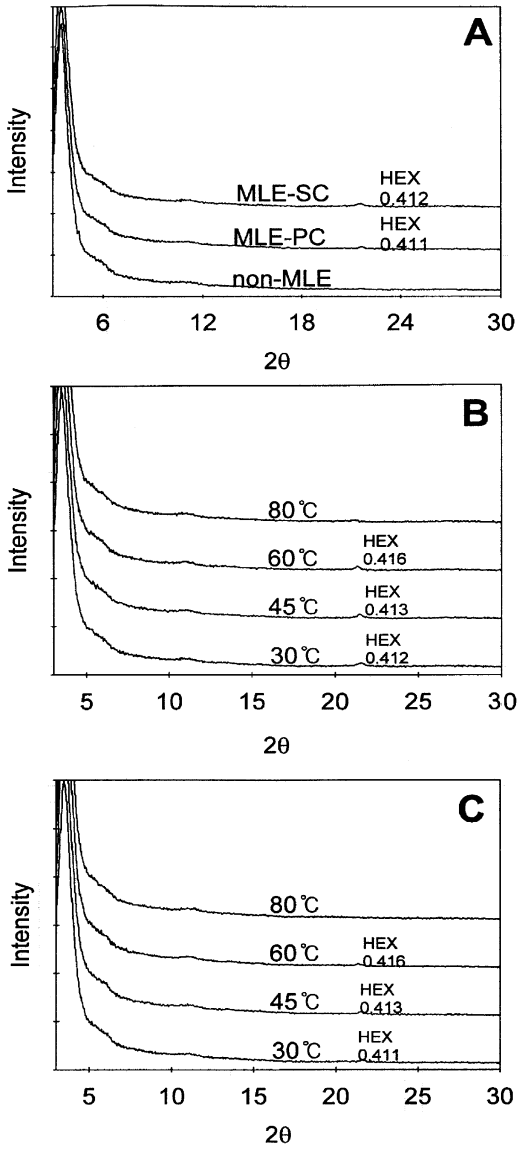

Figure 4. Lateral packing of MLE-PC, MLE-SC and non-MLE; HEX denotes diffractions attributed to hexagonal lateral packing. (*A*) While the WAXD peak of MLE-PC and MLE-SC reveals a weak but distinct peak at approximately 0.411 nm, which could be attributed to the hexagonal lateral packing, non-MLE does not show any distinct peak at 0.411 nm. An identical series of diffraction patterns are obtained for MLE-PC (*B*) and MLE-SC (*C*) at elevated temperatures up to 60°C, but no distinct pattern is observed at 80°C.

non-MLE emulsion not express lamellar structure, the absence of repeated peak pattern is not surprising.

In previous studies using WXRD, the human SC lipids showed lattice spacing of 0.415 nm and 0.375 nm at room temperature, which could be assigned to the orthorhombic, and one peak at 0.412 nm at 45°C, which could be interpreted as from the hexagonal phase (White *et al*, 1988). Both MLE-PC and MLE-SC contain the major components of human SC lipids such as cholesterol, free fatty acid, and (pseudo)ceramide. The peaks that could be assumed for orthorhombic packing, however, were not seen in all the observed emulsions, although a broad but distinct peak for the lateral hexagonal packing was observed. It might be due to the polyols and water components that could present the hexagonal phase at room temperature. The structural difference of pseudoceramide and synthetic ceramide from the ceramide 1 also might be a reason for this structural discrepancy between the MLC-PC and MLE-SC from native

human SC lipid, as the presence of ceramide 1 is considered crucial for proper lipid phase behavior in lipid mixtures (Bouwstra *et al*, 1998b, 1999).

SAXD: MLE-PC showed repeated distances of 7.61 nm and 11.5 nm but MLE-SC and non-MLE did not show a well-ordered repeated distance The SAXD diffraction patterns of MLE-PC, MLE-SC, and non-MLE are plotted in **Fig 5**. For MLE-PC, four peaks (second, third, fourth, and fifth order) were attributed to the long periodicity lamellar phase with a repeat distance of 11.5 nm, which is slightly lower than the long periodicity phase (LPP) shown in intact human SC. A short periodicity phase (SPP) with a repeat distance of 7.61 nm was also seen in MLE-PC, as could be deduced from the three peaks of the first, second, and third order (**Fig 5*A***).

The relatively short distance of the LPP in MLE-PC compared with that in native SC lipids might be due to the short fatty acid chain length of the pseudoceramide and free fatty acid used in MLE-PC. In pig SC, the fatty acid chain length linked to ceramide varies between C16 and C33, in which fatty acids of C24 and C26 chain lengths are most abundantly present, whereas the pseudoceramide used in MLE-PC has fatty acids of C16 and C18 chain lengths. The free fatty acid used for MLE-PC has a short chain of C18, compared with the free fatty acids in SC lipids with major factions being C24 and C26. The pseudoceramide and free fatty acids of relatively short chain length could decrease the long periodicity spacing, in accordance with the suggestion by Bouwstra *et al* (1999) that the match in chain length between free fatty acid and ceramides might be important for phase formation.

Whereas MLE-PC showed both LPP and SPP, MLE-SC showed only SPP with a repeat distance of 7.61 nm. It seemed that the second order peak of LPP existed at a spacing of 5.88 nm, but the apparent repeated pattern was absent, possibly due to the low intensity (**Fig 5*B***).

In non-MLE, a repeated peak pattern was not detected (**Fig 5*C***).

Electron microscopic observation showed the lamellar structure of emulsion TEM pictures of each emulsion are shown in **Fig 6**. Upon TEM observation using the ruthenium tetroxide postfixation method, the gross morphology of droplets in each emulsion was twisted and destroyed, compared with the round shapes in **Fig 2**. Although the emulsions were embedded in agar, the exposure to graded ethanol for dehydration and high temperatures, which are routine processes for TEM, might have twisted the shapes of the emulsion droplets.

From the cross-polarized microscopic observations, which showed a definite optical anisotropy, the existence of multilamellar structures in MLE-PC and MLE-SC were predicted; however, non-MLE also showed a typical lamellar structure, and we could not find a significant difference between MLE-PC, MLE-SC, and non-MLE. The appearance of the lamellar structure in the non-MLE might be due to the emulsifiers used to stabilize the emulsion. Even though non-MLE did not show a distinct optical anisotropy, some droplets were seen with faint optical anisotropy where the cetanol and emulsifier might locate the colloidal surface and stack with lamellar structure (Suzuki, 1986).

During the ruthenium tetroxide postfixation, hydrophilic groups showed an electron dense pattern, so the hydrophilic head group and hydrophobic tail domain were stained with different intensity. Compared with conventional SC lipid, the emulsions showed increased intensity, possibly due to the hydrophilic emulsifier systems. POE(15) glyceryl monostearate has many polyethylene groups, and in the emulsion system, a total 4.5% (w/w) ratio of emulsifier to total emulsion was used. At high temperature, the polyoxyethylene group lost its hydrophilic characteristics so it could stack with hydrophobic hydrocarbon, which produced the more intense staining compared with the human SC intercellular lipids.

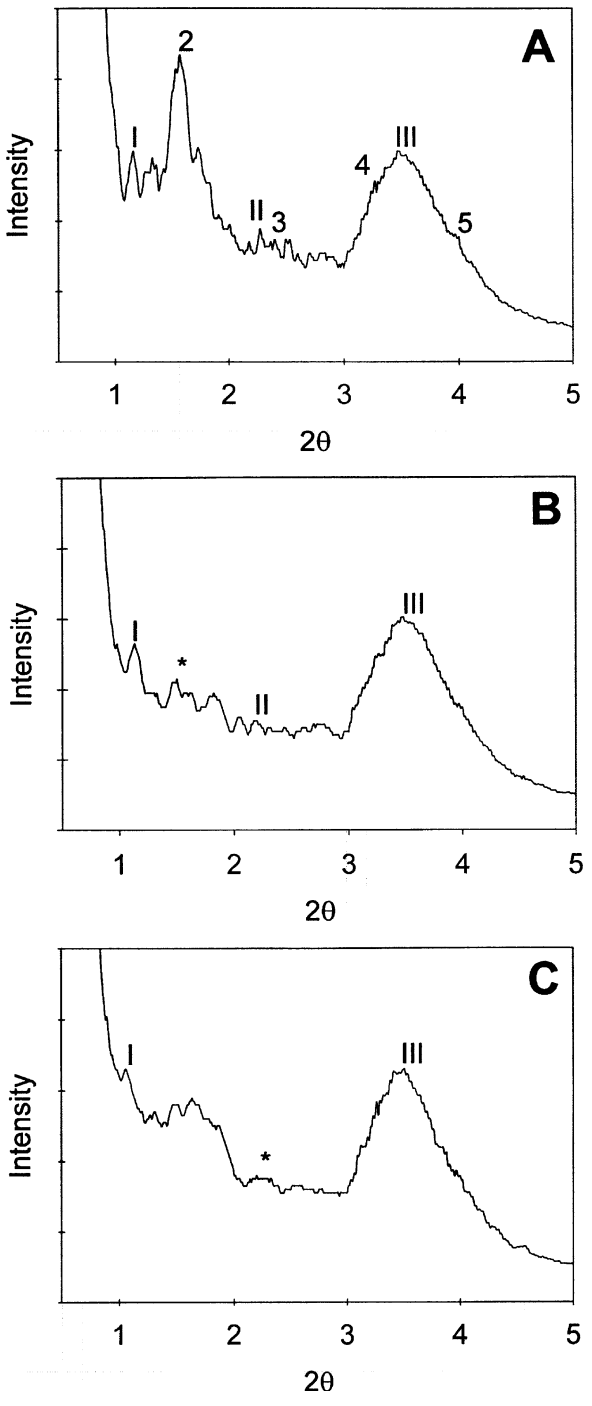

Figure 5. The diffraction pattern of MLE-PC (*A*), MLE-SC (*B*) and non-MLE (*C*) at 30°C. (*A*) The peaks indicated by the Arabic numbers in the figure are attributed to the long periodicity lamellar phase of 11.5 nm (second, third, fourth, and fifth order). The peaks, which are indicated by the roman numbers, located at a spacing of 7.61 nm ($2\theta = 1.16$), 3.88 nm ($2\theta = 2.27$) and 2.54 nm ($2\theta = 3.47$), are attributed to a short periodicity lamellar phase. (*B*) In MLE-SC, a lamellar phase with a periodicity of 7.61 nm is observed. It seems that the 2nd order peak of long periodicity lamellar phase is seen at a spacing of 5.88 nm ($2\theta = 1.50$), but the repeating pattern is absent (single asterisk). (*C*) The diffraction pattern of non-MLE shows peaks at a spacing of 8.32 nm ($2\theta = 1.06$) and 2.51 nm ($2\theta = 3.51$), but no repeating pattern is seen.

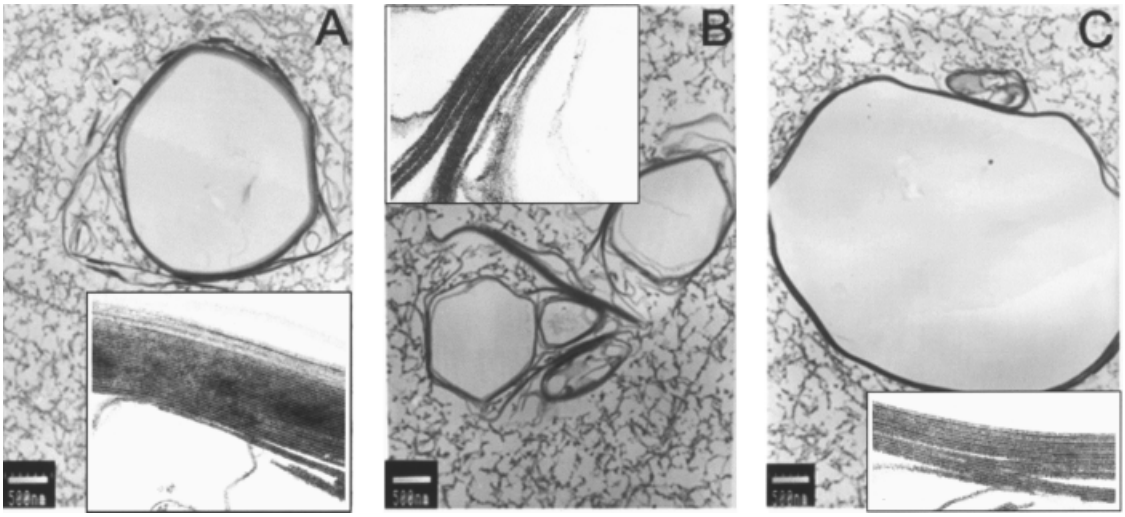

Figure 6. Electron microscopic observations of MLE-PC (*A*), MLE-SC (*B*) and non-MLE (*C*) show twisted and destroyed droplets in all of the emulsions. The structure of lipid layers is stacked as lamellar planes in all of the emulsions and there seem no significant differences among the structures of lipid (inlet).

After phase transition according to water loss, both orthorhombic and liquid sublattices, as well as hexagonal phase were shown in MLE-PC, whereas liquid and hexagonal phases were observed in MLE-SC and non-MLE.

To investigate the phase transition behavior according to the water loss in emulsion, a small amount of each emulsion was spread in the Petri dish, and incubated at 45°C under constant humidity. During the incubation, the weight loss was measured by analytical balance. After 24 h of incubation, the incubated samples were observed by XRD.

The WXRD peak patterns of the dried emulsions are shown in **Fig 7**. After drying, the non-MLE showed a distinct peak at a spacing of 0.4595 nm and a shoulder peak at a spacing of 0.4141 nm, which could be attributed to the liquid crystalline and hexagonal phases, respectively. MLE-SC showed a sharp peak at a spacing of 0.4119 nm and a broad peak at 0.4431 nm, which could be attributed to the hexagonal and liquid crystalline phases, respectively. Whereas MLE-PC also showed the two peaks, which could be attributed to the liquid crystalline lattice in addition to the hexagonal structure, it showed another weak, but distinct peak at a spacing of 0.3929 nm, which could be attributed to the orthorhombic lattice of MLE-PC after the phase transition by lowering the water content.

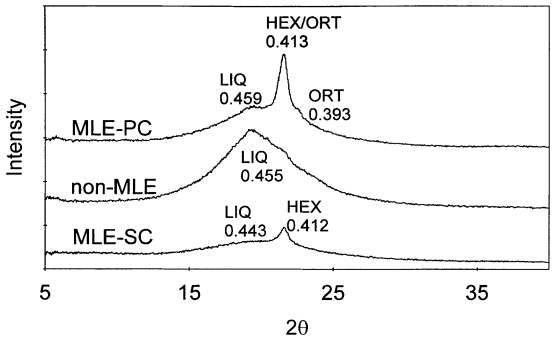

Figure 7. The diffraction patterns of MLE-PC, MLE-SC and non-emulsion after incubation; LIQ, ORT and HEX denotes diffractions attributed to liquid phase, orthorhombic phase and hexagonal phase, respectively. After incubation, all the observed emulsions show a phase transition to liquid phase due to the water loss. In MLE-PC, a distinct peak at a spacing of 0.413 nm and a weak peak at 0.393 nm suggest the phase transition to orthorhombic, while only the lateral packing of hexagonal and liquid phases are observed in MLE-SC and non-MLE.

DISCUSSION

In a previous paper, we reported that the topical application of a MLE-PC could accelerate the recovery rate of acutely or chronically barrier disrupted skin. Mao Qiang *et al* reported that topical application of physiologic lipid mixture accelerated the recovery rate of barrier function, whereas incomplete lipid mixture might inhibit the normal recovery process (Mao-Qiang *et al*, 1996). As non-MLE did not have any significant effect on the barrier recovery, we concluded that the pseudoceramide content could play a major part in the barrier recovery effects.

The use of pseudoceramide, however, resulted in the formation of a lamellar structure and it has not yet been reported whether the lamellar structure itself might play a part in the barrier recovery process. To investigate the relationship between the emulsion structure and its clinical efficacy, we tried to observe the structural difference of the emulsions according to their compositions. In addition to MLE-PC, we have formulated another emulsion that contains synthetic type III ceramide (MLE-SC). In a preliminary study, we compared the efficacy of both emulsions, MLE-PC and MLE-SC, on the skin barrier function recovery after acute disruption using a hairless mouse model. Although not significant ($p > 0.05$), MLE-PC treated skin showed a slightly higher recovery rate after acute disruption than MLE-SC treated skin (data not shown). In addition, MLE-PC treated skin showed a significantly ($p < 0.05$) higher recovery rate than non-MLE treated skin.

From the many previous reports, LPP of 13 nm is suggested as being crucial for the proper organization of lipid lamellar and important for the permeability barrier function of the skin. The presence of the LPP therefore could be an important factor to characterize the molecular organization of emulsions. From the SAXD results, MLE-PC showed two lipid lamellar phases with repeat distances of 11.5 nm and 7.61 nm. Considering the short carbon chain length of free fatty acid and fatty acid moiety in pseudoceramide used for MLE-PC, it is possible that the LPP of MLE-PC with a repeat distance of 11.5 nm could correspond with the LPP in intact human SC. According to the molecular organization model proposed by Bouwstra *et al* (2002) the hairpin conformation of ceramide 1 in the mixture of cholesterol, free fatty acid, and ceramide results in SPP, which is represented by the narrow low-electron density region of the characteristic

"broad-narrow-broad" band in TEM observation. In the electron microscopic observation of MLE-SC, however, the low-electron region was nearly identical in width (**Fig 7*A***, inset). Whether the conformation of pseudoceramide in MLE-PC might result in an identical bandwidth remains to be elucidated by further investigation and modeling.

When a lipid mixture is topically applied to the skin, it undergoes two different phase transition pathways. First, the skin surface lipid could be mixed with the topically applied lipid mixture, and it could exert some disturbing or enhancing effects for the structure of the lipid mixture, thereby resulting in phase transition. But, the significant difference in the quantity of the skin surface lipid and topically applied lipid makes this effect relatively negligible. The second, and possibly dominant pathway for phase transition is due to the decrease in water content of the lipid mixture according to the spontaneous evaporation or adsorption into the skin. When an oil-in-water emulsion is applied to the skin, it generally passes the liquid crystal or gel phase and finally stabilizes as a water-in-oil emulsion (Friberg *et al*, 1999).

To investigate the phase transition behavior of the emulsions after topical application on skin, we designed a simple model: spreading the emulsions on Petri dishes and incubating under a constant temperature, which resulted in decrease of water contents. After 24 h of incubation, three tested emulsions showed different phase transitions. In general, phase transition to the liquid crystalline phase was observed in all of the tested emulsions, although the initial hexagonal phase was also maintained. Only MLE-PC showed an orthorhombic phase, which predominantly exist throughout the human SC intercellular lipids (Pilgram *et al*, 1999). Pilgram *et al* (2001) reported that the lateral organization of intercellular lipid is predominantly a hexagonal phase in lamellar ichthyosis and atopic dermatitis skin, and suggested that orthorhombic packing is required for proper SC barrier function. Phase transition to orthorhombic phase was seen in MLE-PC, and this structural similarity to native human SC lipid could render some beneficial effects on barrier function recovery. In addition, in an open clinical crossover evaluation in childhood atopic dermatitis, the average SCORAD (SCORe Atopic Dermatitis) index improved in both the MLE-PC treated and commercial moisturizing cream (5% urea) treated subgroups, but the patients who received MLE-PC treatment had better results (31–35% decrease, $p < 0.05$) than the commercial moisturizing cream treated patients (13% increase to 14% decrease). The subjective satisfaction scores of the symptoms and signs of the patients, including itching, erythema, and dry skin, were higher in the MLE-treated patients than in the commercial moisturizing cream treated patients (Lee *et al*, in submission).

In conclusion, MLE-PC was shown to express a lateral hexagonal phase and lamellar structure with both the LPP and SPP, which corresponds to the molecular organization of intercellular lipid in human SC. Furthermore, phase transition of initial hexagonal phase into orthorhombic phase was observed in MLE-PC in an experimental model for topical application on skin, and this could be beneficial for the barrier function of the skin.

We wish to thank Bo Hyung Lee for help on the XRD study and Jong-Bae Lee for help on the DSC study in KRICT. A part of this study was presented at the 63rd Annual Meeting of the Society for Investigative Dermatology.

REFERENCES

Ahn SK, Hwang SM, Jiang SJ, Choi H, Lee SH: The changes of epidermal calcium gradient and transitional cells after prolonged occlusion following tape stripping in murine epidermis. *J Invest Dermatol* 113:189–195, 1999

Bleck O, Abeck D, Ring J: Two ceramide subfractions detectable in ceramide positions by HPTLC in skin surface lipids of non-lesional skin of atopic eczema. *J Invest Dermatol* 113:894–900, 1999

Bouwstra JA, Gooris GS, van der Speck JA, Bras W: Structural investigations of human stratum corneum by small angle X-ray scattering. *J Invest Dermatol* 97:1005–1012, 1991

Bouwstra JA, Gooris GS, Salommons-de Vries MA, VanderSpeck JA, Bras W: Structure of human stratum corneum as a function of temperature and hydration: A wide angle X-ray diffraction study. *Int J Pharm* 84:205–216, 1992

Bouwstra JA, Gooris GS, van der Speck JA, Lavrijsen S, Bras W: The lipid and protein structure of mouse stratum corneum: A wide and small angle diffraction study. *Biochim Biophys Acta* 1212:183–192, 1994

Bouwstra JA, Gooris GS, Bras W, Downing DT: Lipid organization in pig stratum corneum. *J Lipid Res* 36:685–695, 1995

Bouwstra JA, Gooris GS, Cheng K, Weerheim A, Bras W, Ponec M: Phase behavior of isolated skin lipids. *J Lipid Res* 37:999–1011, 1996

Bouwstra JA, Gooris GS, Weerheim A, Ponec M: pH, cholesterol sulfate and fatty acids affect the stratum corneum lipid organization. *J Invest Dermatol Symp Proc* 3:69–74, 1998a

Bouwstra JA, Gooris GS, Dubbelaar FER, Weerheim A, Ljzerman AP, Ponec M: The role of ceramide 1 in the molecular organization of the stratum corneum lipids. *J Lipid Res* 39:186–196, 1998b

Bouwstra JA, Dubbelaar FER, Gooris GS, Weerheim AM, Ponec M: The role of ceramide composition in the lipid organization of the skin barrier. *Biochim Biophys Acta* 1419:127–136, 1999

Bouwstra JA, Gooris GS, Dubbelaar FER, Ponec M: Phase behavior of stratum corneum lipid mixtures based on human ceramides: The role of natural and synthetic ceramide 1. *J Invest Dermatol* 118:606–617, 2002

Choi EH, Ahn SK, Lee SH: The changes of stratum corneum interstices and calcium distribution of follicular epithelium of experimentally induced comedones (EIC) by oleic acid. *Exp Dermatol* 6:29–35, 1997

Elias PM, Bonar L, Grayson S, Baden HP: X-ray diffraction analysis of stratum corneum membrane couplets. *J Invest Dermatol* 80:213–214, 1983

Friberg SE, Kayali I, Rhein L, Hill R: A model for stratum corneum lipids and some implications. *Cosmet Toiletries* 102:135–140, 1987

Friberg SE, Al-Bawab A, Sandburg JD, Barber JL, Yin Q, Aikens PA: Phase behavior of a fragrance compound system: Water/phenethyl alcohol/laureth 4/glycerol. *J Surfactants Detergents* 2:159–165, 1999

Frobe C, Simion FA, Ohlmeyer H, Rhein LD, Matti J, Cagan RH, Friberg S: Prevention of stratum corneum lipid phase transition *in-vitro* by glycerol: An alternative mechanism for skin moisturization. *J Soc Cosmet Chem* 41:51–65, 1990

ten Grotenhuis E, Demel RA, Ponec M, Boer DR, van Miltenburg JC, Bouwstra JA: Phase behavior of stratum corneum lipids in mixed Langmiur-Blodgett monolayers. *Biophys J* 71:1389–1399, 1996

Kitson N, Thewalt J, Mafleur M, Bloom M: A model membrane approach to the epidermal permeability barrier. *Biochemistry* 33:6707–6715, 1994

Kuempel D, Swartzendruber DC, Squier CA, Wertz PW: *In vitro* reconstitution of stratum corneum lipid lamellae. *Biochim Biophys Acta* 1372:135–140, 1998

Lavrijsen SPM, Bouwstra JA, Gooris GS, Weerheim A, Bodde HE, Ponec M: Reduced skin barrier function parallels abnormal stratum corneum lipid organization in patients with lamellar ichthyosis. *J Invest Dermatol* 105:619–624, 1995

Lee EJ, Shur KB, Lee JH, *et al*: The clinical efficacy of a multi-lamellar emulsion containing pseudoceramide in childhood atopic dermatitis: An open crossover study. *Br J Dermatol* (in submission)

Mao-Qiang M, Feingold KR, Thornfeldt CR, Elias PM: Optimization of physiological lipid mixtures for barrier repair. *J Invest Dermatol* 106:1096–1101, 1996

Moore DJ, Rerek ME: Insight into the molecular organization of lipids on the skin barrier from infrared spectroscopy studies of stratum corneum lipid models. *Acta Derm Venereol Suppl* 208:16–22, 2000

Myers D: Surfactants in solution. Micellization and related association phenomena. In: *Surfactant Science and Technology.* New York: VCH Publishers Inc., 1988; p 136–141

Park BD, Lee M, Kim Y, Lee JG: The synthesis of new pseudoceramides using alkylketene dimer and their physical properties. *J Ind Eng Chem* 5:228–234, 1999

Park BD, Lee M, Kim Y, Youm JK: Synthesis of N-ethanol-2-(myristyl/palmityl)-3-oxo (stearamide/arachamide) and its physical properties for a cosmetic raw materials. *J Cosmet Sci* 51:253–262, 2000

Park BD, Kim Y, Lee M, Youm JK, Jeong SK, Choi EH, Lee SH: Formulation of a multi-lamellar emulsion using a pseudoceramide and its properties *in vitro* and *in vivo* system. *Cosmet Toiletries* 116:65–75, 2001a

Park BD, Youm JK, Lee SH: The clinical efficacy of multi-lamellar emulsion (MLE) contained pseudoceramide (PC-9S) on atopic dermatitis. *JSBR* 3:81–89, 2001b

Pilgram GSK, Elgelsma-van pelt AM, Oostergetel GT, Koerten HK, Bouwstra JA: Study on the lipid organization of stratum corneum lipid models by cryoelectron diffraction. *J Lipid Res* 39:1669–1676, 1998

Pilgram GSK, Englesma-Van Pelt AM, Bouwstra JA, Koerten HK: Electron diffraction provides new information on human stratum corneum lipid organization in relation to depth and temperature. *J Invest Dermatol* 113:403–409, 1999

Pilgram GSK, Vissers DCJ, van der Meulen H, Pavel S, Lavrijsen SPM, Bouwstra JA, Koerten HK: Aberrant lipid organization in stratum corneum patients with atopic dermatitis and lamellar ichthyosis. *J Invest Dermatol* 117:710–717, 2001

Rawlings A, Harding C, Watkinson A: The effect of glycerol and humidity on desmosome degradation in stratum corneum. *Arch Dermatol Res* 287:457–464, 1995

Rhein LD, Simion FA, Froebe C, Mattai J, Cagan RH: Development of a stratum corneum lipid model to study the cutaneous moisture barrier properties. *Colloid Surfaces* 48:1–11, 1990

Suzuki T: Secondary droplets formed in O/W emulsions. *J Oil Chem Soc Jpn* 35: 113–119, 1986

Swartzendruber DC, Wertz PW, Kitko DJ, Madison KC, Downing DT: Molecular modeling of the intercellular lipid lamellae in mammalian stratum corneum. *J Invest Dermatol* 92:251–257, 1989

White SH, Mirejovski D, King GI: Structure of lamellar lipid domains and corneocyte envelopes of murine stratum corneum: An X-ray diffraction study. *Biochemistry* 27:3725–3732, 1988

Yamamoto A, Serizawa S, Ito M, Sato Y: Stratum corneum lipid abnormalities in atopic dermatitis. *Arch Dermatol Res* 283:219–223, 1991