Phase behaviors, lamellar structures, and physical properties of synthetic vitamin E ceramide (Tocomide) mixed with cholesterol and linoleic acid

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Summary

∏-A isotherms and phase behaviors of 'tocomide', a newly synthesized 1,3-bis(N-(2-hydroxyethyl)-tocopherol succinylamino)-2-hydroxypropane, mixed with cholesterol and linoleic acid, was studied for its monolayer miscibility and a stable delivery formulation for antioxidant applications. The monolayer of tocomide and cholesterol was formed in a homogeneously mixed state at air-water interface. The ternary mixtures with linoleic acid showed various bulk structures, including a stable and transparent solution of thermodynamically stable lamellar phase. The lamellar structure was confirmed by the X-ray diffraction (XRD) patterns and polarized microscopy such that pure tocomide formed a liquid crystal at room temperature with a lamellar periodicity of 36.7 Å (2θ=2.41°).
(Keywords: ceramide, tocomide, liquid crystal, lamellar structure)

Introduction

The skin protection methods against oxidative stressors such as ultraviolet radiation, ozone, and chemicals have been focused on the ability of stratum corneum to maintain water contents and oxidation resistance of bilayers because the layers at an outermost barrier of body are mainly exposed to the cutaneous oxidation target of atmospheric ozone, a major part of photochemical smog[1]. Here, the bilayer lamellar structure in the compartments of intercellular spaces as well as the stratum cornea, plays key role of protecting skins and mainly composed with ceramides, cholesterol, and fatty acids [2,3].

Among the components in bilayers ceramides play an important role in maintaining well-balanced water contents, which affect the elasticity, appearance, and barrier function of skin stratum corea [4]. The extracts of ceramides are available from diverse natural sources, but not feasible for supplying industrial demands. Alternatively, several pseudoceramides have been used in commercial products [5,6,7]. For example, Kim et al. synthesized pseudo-ceramides[8], named

Pacific pseudoceramide(PC) 102, 104[9], and 107. Meanwhile, the physical aging of a skin barrier by an UV irradiation has been studied with antioxidants, such as vitamin E (α- and γ-tocopherol forms), vitamin C, uric acid, and glutathione [10,11]. It has been known that vitamin E has a powerful anti-oxidant effect within a mammalian body, particularly for lipids [12,13]. As a model lipid, linoleic acid was carefully considered with two oxidation active sites of unsaturated double bond, which might change the physical and/or chemical properties of emulsion products [14]. Linoleic acid without any chemical modifications can be utilized for skin because of its presumed benefits on scaling phenomena caused by cosmetic defects [15]. Further, it is one of mandatory dietary elements to keep skin healthy, since mammals cannot synthesize this fatty acid [16].

By combining the two concepts above, lipid analogs of vitamin E ceramide(SVC) were synthesized, having the antioxidation ability of vitamin E (α-tocopherol) [17]. Tocomide, a synthetic vitamin E ceramide derivative has two conceptual functions; the ability of bilayer formation to mimic stratum cornea and the ability of antioxidation by tocopherol group. It is interesting to know if it can effectively form a stable internal structure and reduce the oxidation of membrane fatty acid. Therefore, we excluded any complicated oxidation mechanism such as cytochrom P450 in cellular metabolisms [18], but modeled a direct contact oxidation and its inhibitory mechanism involved in the outer most cellular bilayers.

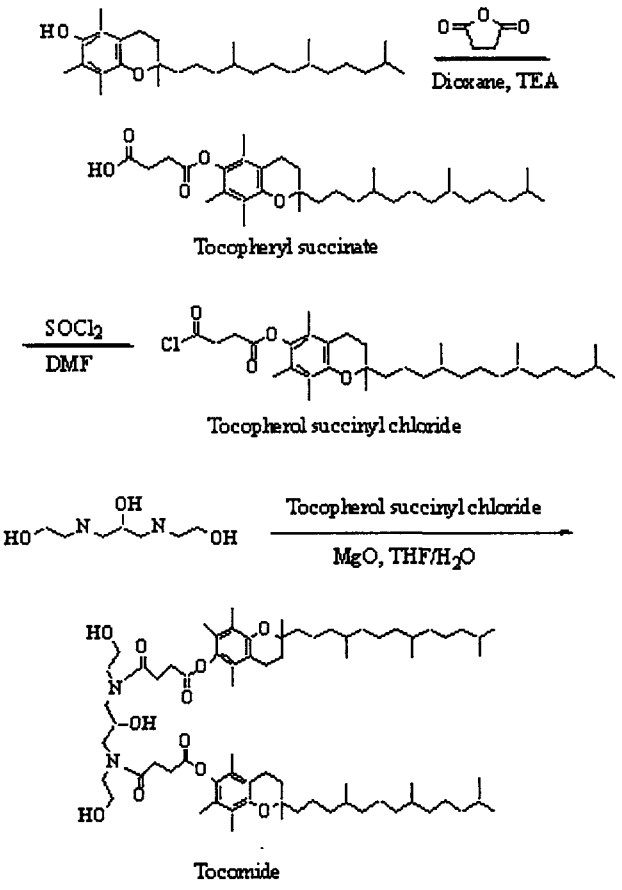
In this paper, we report the phase behaviors of ternary mixture of tocomide, cholesterol and linoleic acid for consumer practice and the monolayer formation of tocomide with cholesterol. A stable lamellar structure composed of cholesterol and fatty acids has been attracted for their simpilicity of membrane lipid structures, and for practical applications of drug carriers such as liposome and/or emulsion. The monolayers consisting of tocomide and cholesterol on air-water interface apparently simulate the half of bilayer membrane and the inclusion of linoleic acid into such mixed membrane provides significant variations of structures and phase behaviors

Materials and Method

Materials

A pseudo-ceramide, 1,3-bis(N-(2-hydroxyethyl)-tocopherol succinyl amino)-2-hydroxy-propane, was synthesized and purified[17], and linoleic acid and cholesterol are purchased from Sigma Chemical Co.(USA). The molecular structure of tocomide and its synthetic procedure are given in the scheme 1. The synthesized tocomide was identified by a spectral analysis of a ^{1}H NMR ($CDCl_3$, 300 MHz) as 0.85(m, 24H), 1.14~1.50(m, 48H), 1.85(m, 4H), 1.96(s, 6H), 2.04(s, 6H), 2.07(s, 6H), 2.57(t, 4H, J = 6.6 Hz), 2.85(t, 4H, J = 6.3 Hz), 2.95(t, 4H, J =6.3 Hz), 3.18(m, 4H), 3.48(m, 4H), 3.65(m, 4H), 4.20(m, 1H) and IR spectra (KBr) as of 3387, 2926, 1749, 1619 cm^{-1}. Tocomide has two tocopherol succinyl chains aligned to the same direction in the lamellar structure. The three hydroxyl groups form an array suitable to intermolecular hydrogen bonding or hydrogen bonding to water molecules. The mixture of linoleic acid, tocomide, and cholesterol was placed and weighed in glass vials

(10ml). The mixtures in leveled vials were melted to be clear, and then kept in an incubator(thermostated bath) at 30℃.



Scheme 1. Synthetic route to tocomide (1,3-bis(N-(2-hydroxyethyl) tocopherolsuccinyl- amino)-2-hydroxypropane)

Π-A isotherms

The pure components or mixtures of water-insoluble tocomide and cholesterol were spread out at the air/water interface of a Joyce Loebl type LB trough (Lauda KSV-5000). Binary lipid mixtures of tocomide and cholesterol were prepared in the mole ratios of 100/0, 75/25, 50/50, 25/75, and 0/100. HPLC-grade chloroform was used as a solvent, and deionized water was used for a subphase. The concentration of solutions was adjusted to 0.1 mmol/l. Each solution was spread out using with a Hamilton syringe.

After spreading, the monolayer was allowed to equilibrate until all chloroform was evaporated and then compression started. The spread monolayers on the water surface was compressed with a speed of 15mm/min equivalent to 2.5 $Å^2$ $molecule^{-1}$ min^{-1} and Π-A isotherms were determined at the maximum spreading area of 876.00cm^2. The Π-A isotherms were monitored on the computer interfaced to LB trough.

Phase behavior and textures

The mixtures of tocomide, cholesterol and linoleic acid prepared in a designed compositions were kept in an incubator at 30℃ and their textures were observed visually with and without crossed polarizers. The structures of a liquid crystalline phase in the samples were identified by a phase texture of optical microscopy under polarized light or by a X-ray diffraction[6,19].

X-ray diffraction

The lattice spacing and aggregation structure of lipids were identified by a powder X-ray diffraction(XRD) with a wide-angle diffractometer (Rigaku/USA, D/max-RB) of CuKα radiation, λ=1.54 Å. The scanning rate was 4°/min, and X-ray data were analyzed by the Bragg's equation.

Results and Discussion

Π–A isotherms and miscibility of tocomide and cholesterol

The Π–A isotherm and miscibility of tocomide with cholesterol were shown in Figure 1. The monolayers were conrstructed with pure tocomide and cholestrol and their mixtures of molar ratios of 25:75, 50:50, and 75:25, respectively. Since the cross-sectional area of tocomide is larger than that of cholesterol, Π–A isotherms were shifted upon an addition of cholesterol. The surface area per molecule in a condensed monolayer, A_0, was calculated as 39 $Å^2$ for cholesterol[20] and 83 $Å^2$ for tocomide. Also the surface area of tocomide, A_0 is larger than the limiting areas of other c eramides reported[7], approximately 50 $Å^2$/molecule, because the head group of tocomide is bigger and bulkier than the others are.

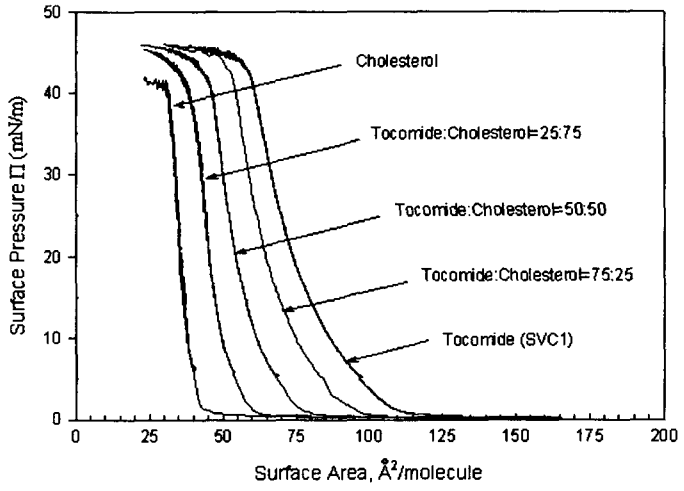


Figure 1. Π-A isotherms of mixtures of tocomide and cholesterol in various mole ratio at air-water interface

The collapse pressures decreased slightly at the mixed film as shown in Figure 1, but remained

unchanged to be 45 mN/m for pure tocomide. Brewster Angle Microscopy(BAM) showed that no separate domains of tocomide or cholesterol exist. The smooth transition of isotherms from low pressure to high pressure also indicates that the mixtures of these species form a homogeneous monolayer.

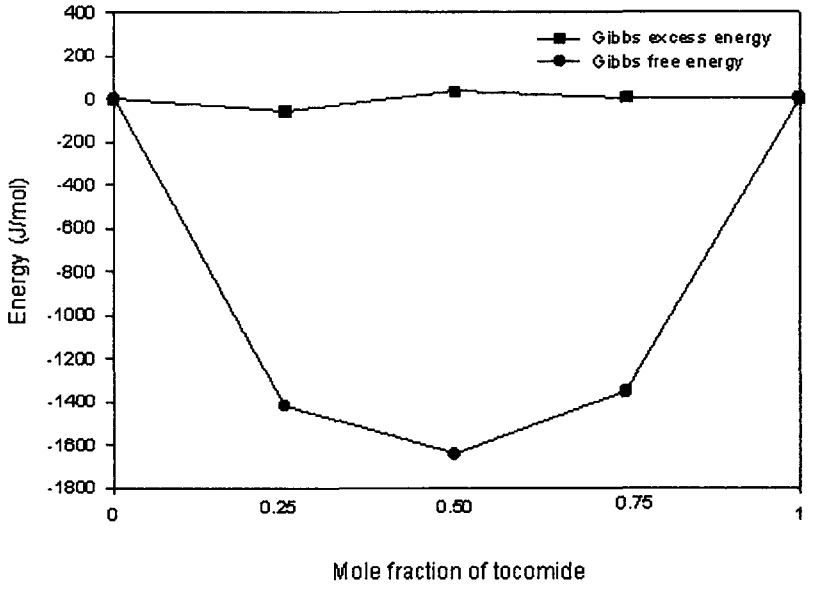


Figure 2. Gibbs excess energy (■) and Gibbs free energy of the mixed monolayer (●) determined from the isotherm of cholesterol/tocomide

Figure 2 shows the Gibbs free energy of mixtures obtained from the integration of isotherms at 25 mN/m [21,22]. If a monolayer contains the mole fractions N_1 and N_2 of components 1 and 2 , the free energy of mixing can be evaluated by a mixing process of two pure species. The molar free energy of mixing at surface pressure π, ΔG_{mix}^{π}, can be defined by

$$\Delta G_{mix}^{\pi} = G_{12}^{\pi} - N_1 G_1 - N_2 G_2 \qquad (1)$$

where ΔG_{mix}^{π} is the free energy difference between the Gibbs free energy (per mole) of a system of a mixed film and that of the same amounts of system composed of two pure films, which are separated from one another. By extending the system to low pressure ($\pi \rightarrow 0$), we have

$$\Delta G_{mix}^{\pi} = \int_0^{\pi} (A_{12} - N_1 A_1 - N_2 A_2) d\pi + RT(N_1 \ln N_1 + N_2 \ln N_2) \qquad (2)$$

Where A_1 and A_2 are the molar areas of two pure films and A_{12} is the mean molar area of the mixed film. The evaluation of the free energy of mixing can therefore be obtained directly from the π-A curves of the pure and mixed monolayers in Figure 1. Then we have the excess free energy of mixing written as [21].

$$\Delta G_{XS}^{\pi} = \Delta G_{mix}^{\pi} - \Delta G_{mix}^{I} = \int_0^{\pi} (A_{12} - N_1 A_1 - N_2 A_2) d\pi \qquad (3)$$

The Gibbs free energies of mixing shown in Figure 2 have a negative deviation as a function of added fraction of tocomide into cholesterol. The negativity of the Gibbs free energy of mixing indicates that the mixed monolayers of tocomide and cholesterol are miscible and stable at an air-water interface. However, it is indicated that the mixing process is entropic since the excess free energies are almost zero. The mean molar areas of a mixed film, $A_{12} = N_1 A_1 + N_2 A_2$, are plotted in Figure 3 at different surface pressure, and a little deviation of mean molecular areas indicates no excess areas of tocomide and cholesterol.

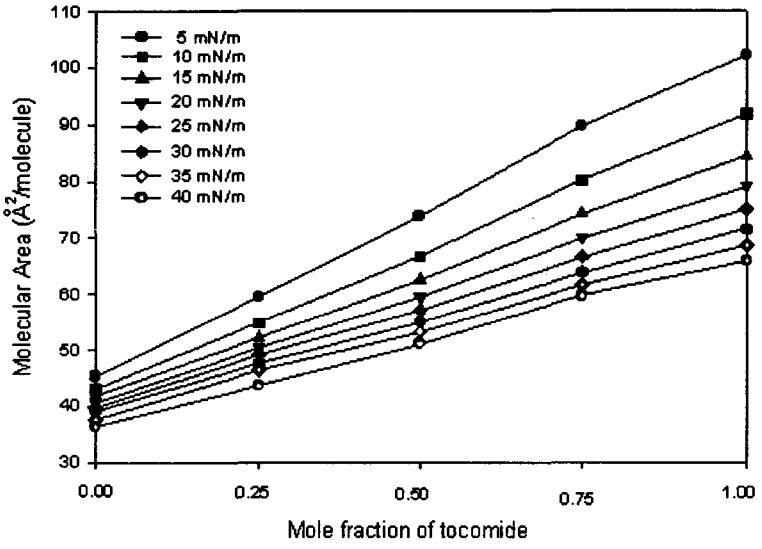


Figure 3. Variation of mean molecular area of mixed monolayers of tocomide and cholesterol

The surface compressional modulus, Cs^{-1} (mN/m) of mixed lipids (tocomide - and cholesterol) was obtained by the equation (4) [21].

$$Cs^{-1} = -A(\partial\Pi/\partial A)_T = -(\partial\Pi/\partial \ln A)_T \qquad (4)$$

The surface compressional moduli suggested that stable monolayer states were formed for all of tocomide (22.98, Liquid expanded), mixed 75%(39.84, Liquid expanded), mixed 50%(55.03, Liquid

expanded-Liquid condensed), mixed 25%(64.71, Liquid expanded-Liquid condensed), and cholesterol (451.87, Liquid condensed-Solid condensed) at 30 mN/m.

Therefore, the mixtures of tocomide and cholesterol form a stable and homogeneous monolayer at the air-water interface. It is concluded that two tocopherol succinyl chains are aligned straight toward air, and are not disturbed by cholesterol which is inserted into monolyers. This observation has some significances of forming monolayer and in fact bilayers, since it dissolves cholesterol as if it is an ideal solution. However, cholesterol has a profound effect on the polarization of lipid bilayers and interacts with lipid molecules merely by a hydrophobic force. The thermal motion or mobility of the disordered hydrocarbon chain of EPC lipid is significantly reduced by the presence of rigid sterol molecules. However, it does in fact depend on the molecular state of lipid membranes.

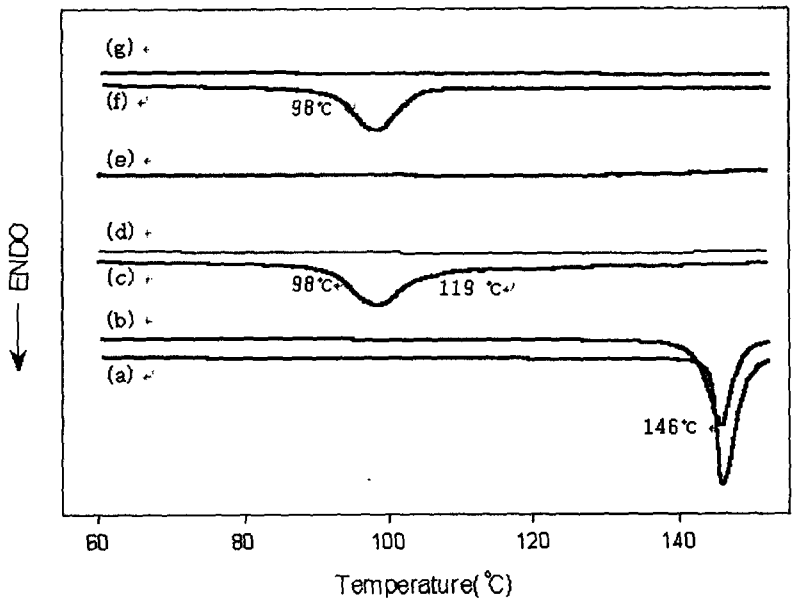


Figure 4. DSC thermograms of cholesterol, tocomide, and their mixtures (1mol/1mol), (a) cholestrol 1st heating, (b) 2nd heating with (a) sample, (c) cholestrol/tocomide powder mixture 1st heating, (d) 2nd heating with (c) sample, (e) cholestrol/tocomide mixture treated by evaporating after solving in CHCl3, (f) tocomide 1st heating, and (g) 2nd heating with (f) sample

Figure 4 shows DSC thermograms of cholesterol, tocomide, and their mixtures (1mol/1mol). During the first heating process, pure tocomide and cholesterol melted at 98℃(f), and 146℃(a) respectively, while during the second heating process, no change occurs with cholesterol(b), but with tocomide(g), melting disappears. With the mixture of tocomide and cholesterol powder(c), there was a sharp peak around 98 ℃ corresponding to the melting point of tocomides, while no peak of cholesterol was observed around 146 ℃. From the graph(c), an undetectably small broad peak was observed around 119 ℃. This suggests that tocomide would melt at temperatures above 98℃ and this solution could then affect the cholesterol to dissolve. As shown in the second melting process(d), tocomide and cholesterol could form amorphorous or homogeneous substances. The

same result was observed by the experiment (e) where tocomide and cholesterol powders are dissolved in cosolvent ($CHCl_3$) and then the resulting solvent is evaporated to obtain a desired blend. There was no sharp peak of the melting point, which was expected from theoretical data. As shown before, this is due to the property change caused by melting the two substances and forming a blend that is very likely to be mixed.

Therefore, it may be inferred that the mixture of tocomide and cholesterol forms a homogeneous monolayer or bilayer in biologically active skin compositions, where the mixture of these species could interact with other functional ingredients.

Phase behaviors and textures of tocomide, cholesterol and linoleic acid mixtures

Since linoleic acid (C18:2) was currently established as a fatty acid which is known to prevent EFA deficiency(causes skin defects), it is interesting whether this component can form a stable mixture with tocomide/cholesterol as a simulated stratum conium(SC) membrane. In the previous section, it is observed that the mixture of tocomide and cholesterol forms a stable and homogeneous monolayer. Therefore, the question we have is whether the addition of linoleic acid to the tocomide–cholesterol mixture can produce diverse phase behaviors. The set of experiments was d esigned to screen the possible appearance of new phases as shown in Figure 5. The phase characteristics of each sample were observed through physical and optical appearances during the incubation for at least 6 months.

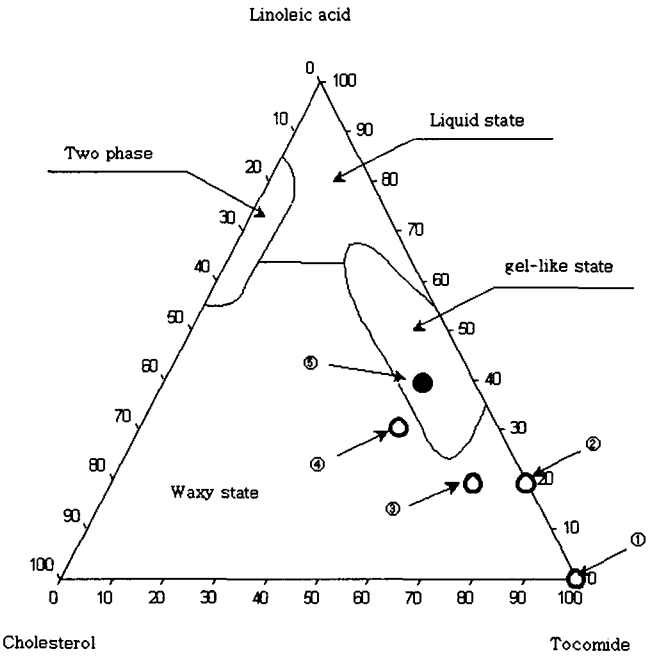


Figure 5. Ternary plot of linoleic acid/tocomide/cholesterol at 25 ℃; (group I => ①, ②, and ③ , group II => ①, ②, ③, ④, and ⑤)

Figure 5 shows the ternary plot of linoleic acid/tocomide/cholesterol at 30℃. Most samples showed

waxy states after cholesterol was added, and liquid states were seen at or near linoleic acid rich phases. The color and opacity changes can be recognized in the diagram. Interestingly, a transparent gel-like state is observed at low concentrations of cholesterol as shown in Figure 5. T he composition of the filled circle is clear and stable for more than 1 year.

In the group I (①, ②, and ③ in the Figure 5), the liquid crystalline phase of the artificial SC liquid mixtures was identified by the optical images through polarized microscopy. Figure 6 shows the optical micrographs of SC lipid mixtures under polarized light at room temperature (R.T.). The texture appeared in focal conic patterns, indicating the multi-lamella structure of lipids. At the immediate observation, the structures and/or the states could not be identified by polarized microscope. After 2 months, the focal conic shapes of lamellar structure appeared. Further, any transitions of multilamellar phases under temperature controlled polarized microscopy were not found during heating(up to 150℃ with 10℃/min) and cooling(down to 25℃ with 3℃/min). This means that it takes longer time for melted tocomide(α-gel) to change to other state(α- crystal)[5].

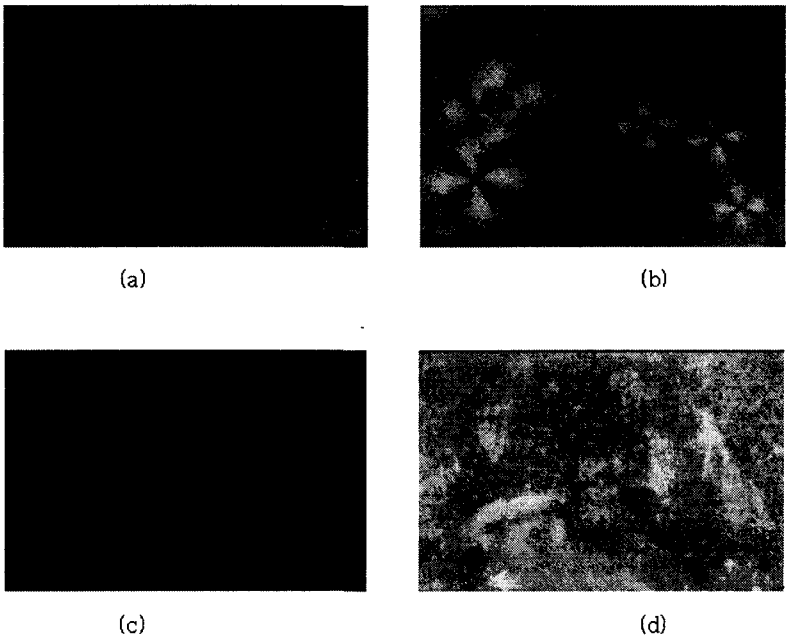


Figure 6. Polarizing optical micrographs at 25℃ of artificial stratum corneum lipids, (a) tocomide powder state, (b) metastable tocomide state (waxy phase), (c) linoleic/ tocomide/cholestrol (2/7/1), (d) linoleic/tocomide/cholestrol (2/8/0) ; (b), (c), and (d) matched with ①, ②, and ③ in Figure 5, respectively, were kept in R.T. for one month after heating and cooling treatment.

Lamellar structure by X-ray diffraction

The mixture of tocomide with cholesterol and linoleic acid was examined by a X-ray diffraction method and a stable lamellar structure was observed which was similar to intercellular stratum cornea.

Figure 7 shows the diffraction patterns of pure tocomide and a monomeric crystal, which formed a

liquid crystal at room temperature. The lamellar periodicity with 36.7Å(2θ=2.41°) and a broad reflection at around 4.7Å(2θ=18.8°) are attributed to the liquid packing of the hydrocarbon chains of tocomide in the lamellar liquid-crystal state. The spectral pattern of the broad reflection has a typical nematic shape, while a smectic(lamellar) liquid crystal phase shows a very broad peak in the range of 15-25° in 2θ (6-3.5 Å). This might be due to intermolecular distances [23]. After incubation of a prolonged period, the intensity of X-ray peaks decayed, but the pattern was similar as it was at the initial.

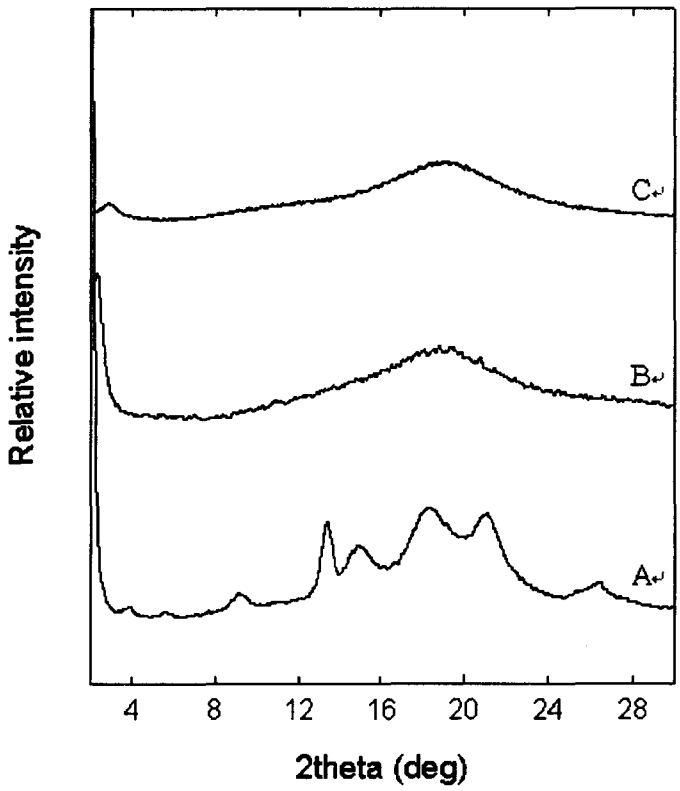


Figure 7. Changes in the molecular structure of tocomide measured by X-ray diffraction patterns at 25℃. (A) crystal (powder) state; (B) cooled after melting; (C) keep at 30℃ incubator for 1 month.

X-ray diffraction profiles of linoleic acid/tocomide/choleserol mixtures in the group II (①, ②, ③, ④, and ⑤ in the Figure 5) are shown in Figure 8, where lamellar periodicities of approximately 30-40 Å, and a single broad reflection at around 4.7Å were observed. The single broad reflection can be attributed to the liquid packing of the hydrocarbon chains of lipid complex in the lamellar liquid crystal state. There was only a diffusion halo in short-spacing region (at around 2θ=18.5), suggesting that lipids should still be in a liquid crystalline (LC) state[6].

Pure tocomide formed a liquid crystal at room temperature with a lamellar periodicity and the single broad reflection. In the whole group II region, there was a diffusion halo in a short-spacing region, suggesting that a lipid complex should still be in a liquid-crystalline state, although the focal conic patterns were not observed in the polarized microscopy, but all samples showed the lamellar XRD

pattern[23].

In the case of linoleic acid/tocomide/cholesterol=3/5/2 (weight fraction), sharp diffraction peaks appeared at 12.7Å(2θ=6.95) and 15.4Å(2θ=5.73). These diffraction peaks could be an evidence of the phase change from a liquid crystal structure to other structure of the lipid complex, although these peaks are not shown in Figure 8A~D.

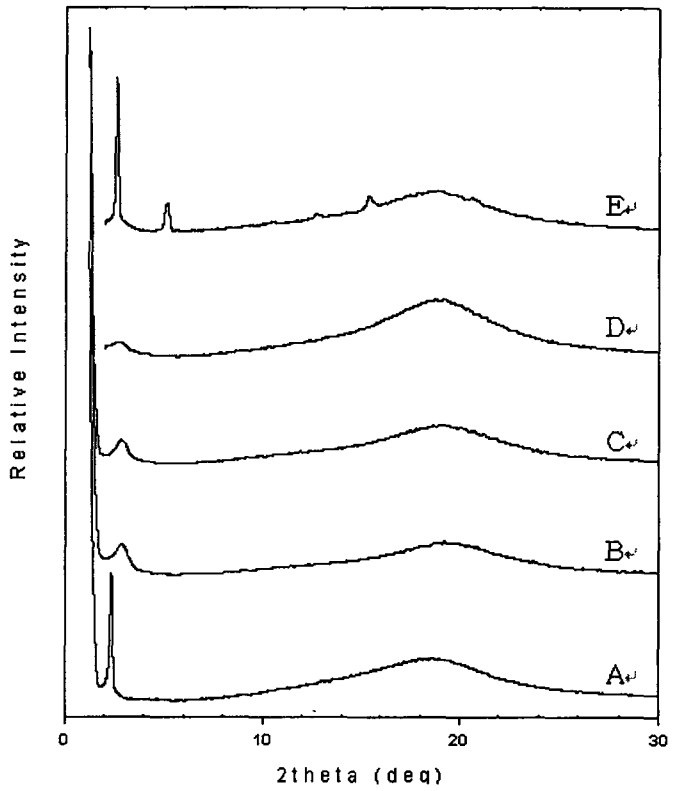


Figure 8. X-ray diffraction patterns of linoleic acid/tocomide/cholesterol (wt %); (A) 3/5/2, (B) 4/5/1, (C) 2/7/1, (D) 2/8/0, and (E) 0/10/0; (A), (B), (C), (D), and (E) are matched with ①, ②, ③, ④, and ⑤ in Figure 5, respectively.

Conclusions

In conclusion, this study describes that monolayer and phase behaviors of mixture of cholesterol and 'tocomide'(SVC1), a newly synthesized 1,3-bis(N-(2-hydroxyethyl)-tocopherol succinylamino)-2-hydroxypropane, was studied for its monolayer miscibility and a stable delivery formulation for antioxidant applications.

The mixture of tocomide and cholesterol forms a homogeneous monolayer at air-water interface and tocomide and cholesterol forms the homogeneous mixture in a bulk phase. There are focal conic patterns, which clearly indicates the existence of multi-lamella structure of lipids. The structures and/or the states of tocomide could not be identified by polarized microscopy at fresh

samples after preparation, but after several weeks, the focal conic shapes of lamellar structure are developed among the samples.

The lamellar structure was confirmed by the X-ray diffraction (XRD) and polarized microscopic images such that pure tocomide formed a liquid crystal at room temperature with lamellar periodicity of 36.7Å(2θ=2.41°). A mixture of linoleic acid and cholesterol formed a thermodynamically stable and transparent solution, which is attributed to the liquid packing of the hydrocarbon chains of tocomide in a lamellar liquid-crystal state.

References

[1] J. Cotovio, L. Onno, P. Justine, S. Lamure and P. Catroux, *Toxicology in Vitro*, **15** (2001) 357.

[2] M.Q. Man, K.R. Feingold, C.R. Thornfeldt and P.M. Elias, *J. Invest. Dermatol.*, **106** (1996) 1096.

[3] N.Y. Schurer and P.M. Elias, *Adv. Lipid Res.*, **24** (1991) 27.

[4] P. Elsner, E. Berardesca, H.I. Maibach, Bioenginnering of the skin: water and the stratum corneum, CRC Press, Florida, 1994

[5] H. Iwai, J. Fukasawa and T. Suzuki, *International Journal of Cosmetic Science*, **20** (1998) 87.

[6] H. Mizushima, J. Fukasawa, and T. Suzuki, *J. Colloid and Interface Sci.*, **195** (1997) 156.

[7] E. sparr, L. Eriksson, J. A. Bouwstra, and K. Ekelund, *Langmuir*, **17** (2001) 164.

[8] D.H. Kim, B.S. Lee, M.S. Koo, H.J. Kim, H.K. Lee, M.J. Choi, and O.S. Lee, 20th IFSCC congress, Cannes, France, September 1998, p. 87.

[9] M.W. Jeong, S.G. Oh, D.H. Kim, J.S. Ko and S.W. Oh, *Colloids and Surfaces A: Physicochem. Eng. Aspects*, **193** (2001) 197.

[10] S.U. Weber, J.J. Thiele, C.E. Cross and L. Packer, *J. Invest. Dermatol.*, **113** (1999) 1128.

[11] K. Kramerstickland and D.C. Liebler, *J. Invest. Dermatol.*, **111** (1998).

[12] J.J. Thiele, M.G. Traber, T.G. Polefka, C.E. Cross and L. Packer, *J. Invest. Dermatol*, **108** (1997).

[13] J.J. Thiele, S.U. Wever and L. Packer, *J. Invest. Dermatol.* **113** (1999) 1006.

[14] S.F. Mühlebach and Patrick J. K. Steger, *Nutrition* **14** (1998) 720.

[15] G. Brooks, *Cosm. & Toil.*, **99** (1984) 45.

16] M.R. Martin, *Cosmetics and Toiletries Magazine*, **109**(1994)57

[17] Korean Patent Application Number: 10-2000-0022835; filed on April 28, 2000.

[18] A.L. Lehninger, David L. Nelson and M.M. Cox, Principles of Biochemistry, Worth, New York, 1993.

[19] H. Mizushima, J. Fukasawa and T. Suzuki, *J. lipid Res.* **37** (1996) 361.

[20] W.A. Arthur, Physical Chemistry of Surfaces, Wiley, New York, 1990.

[21] J.T. Davies and E. K. Rideal, Interfacial Phenomena, Academic press, Inc., 1963.

[22] G.L. Gaines Jr., Insoluble Monolayers at Liquid-gas Interfaces, Interscience, 1966.

[23] M. Kijima, I. Kinoshita, T. Hattori and H. Shirakawa, *Synthetic Metals* **100** (1999) 61.