Preparation and Characteristics of Arginine Oleate Liquid Crystal Holding a Large Amount of Water

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Abstract: Solution properties of arginine oleate (ArgOA) were investigated by means of small angle X-ray scattering device, tensiometer, transmittance measuring device, transmission electron microscope, NMR, etc. Whereas arginine stearate which is different in hydrophobic moiety or sodium oleate which is different in counterion from ArgOA formed lamellar liquid crystals in a limited region, ArgOA formed over a wide ranges of temperature and concentration. The surface tension versus concentration curve of ArgOA had two bending points (C1 and C2). When the state of its aqueous solution was observed, transmittance of the aqueous solution decreased in the concentration range of C1 and more and, in the region until C2 where the concentration further increased, particles of several tens nm and several hundreds nm were observed. At concentrations above C2, the viscosity of the aqueous solution began to increase due to the formation of bigger molecular aggregates. The formation of micelles, vesicle-like highly associated aggregates, and lamellar liquid crystals with large amount of water were observed as a result of an increase in concentration of ArgOA.

Key words: arginine oleate, lamellar liquid crystal, surface tension, viscosity, micelle, vesicle, transmittance, NMR

1 Introduction

Unsaturated fatty acids play an important role *in vivo* not only as energy sources but also as main components of biomembranes (1-3). It is well known that fluidity of biomembrane is affected by its characteristic dynamic structure and is dependent on the unsaturated fatty acid, oleic acid (4-8). Oleic acid, which has a double bond at C9 position in its molecule and freely-rotating, adjacent methylene groups, is one of the representative *cis*-monoene unsaturated fatty acids which are able to bend comparatively freely. Until recently the purity of avail-

able oleic acid is low, and there has been restriction in its study for basic physical properties as well as its application for a speciality chemical field such as pharmaceuticals, cosmetics and biosciences. In recent years however, a product where purity is 99% or greater is constantly available and there have been many reports on the analysis of its basic physical properties (9-13) as well as the basic physical properties of nonionic surfactants which are its derivatives (14-16) and its use as an enhancer of drug penetration through the skin (17-20). These studies have shown that oleic acid has a big space-filling volume (21,22) based on *cis*-type double

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bond of its hydrophobic moiety and a fluidity near body temperature of constant-temperature animals. Incidentally, the melting point of oleic acid is 13.4°C for an α -type and is 16.3°C for a β -type, on the other hand, in the case of stearic acid having the same hydrocarbon chain length as oleic acid, it is 69.6°C (12).

L-Arginine is one of basic essential amino acids in living body and shows four ion dissociation states depending upon pH of its aqueous solution (Fig. 1) (23,24). For example, since the guanidyl group at ω -position shows a strong basic property, it neutralizes substances having counterion, whereas the carboxyl group and amino group at α -position have a characteristic of forming self dipolar zwitterions. It has been reported that many amino acids have melting points of 200°C or higher, because they form the zwitterion that has an ionic crystalline structure similar to inorganic salts (23). Amino acids have been very useful in the field of cosmetics as well and play an important role for governing a moisturizing ability of the skin being called a NMF (natural moisturizing factor) (25).

Combining the properties of big space-filling volume and fluidity of oleic acid with the high self dipolar zwitterion forming ability of L-arginine should give rise to a novel function, namely the formation of a biomimetic film.

In view of the above, in the present study, characteristic solution properties of an amphiphilic substance (arginine oleate) comprising highly purified (99.9%) oleic acid and L-arginine were investigated utilizing various surface chemical means.

Fig. 1 Four Different Dissociate Ion States of L-arginine Depending on pH.

2 Experimental

2.1 Materials

Arginine oleate (ArgOA) is a fatty acid soap where hydrophobic moiety is oleic acid (OA) and counterion is L-arginine (Arg). Oleic acid of 99.9% purity (Extra Oleic 99 manufactured by NOF Corporation) and L-arginine (of a pharmaceutical grade manufactured by Kyowa Hakko Kogyo) were used for the preparation. Preparation method was that L-arginine was added to distilled water of 75°C and dissolved with stirring, oleic acid was then gradually dropped to the solution and the mixture was subjected to natural cooling. Their adding amounts were in a molar ratio of Arg/OA = 1/1. After that, annealing was conducted (by three cycles where one cycle comprised allowing to stand at -5°C for 12 hours and at room temperature for 12 hours, followed by stirring) to prepare a homogeneous solution. The resulting 10 wt% aqueous solution of arginine oleate was freeze-dried to give powder of arginine oleate (ArgOA) crystals. It was confirmed by a Karl Fisher's method that amount of water in this ArgOA was 2 to 3%. Distilled water for injection (Otsuka Pharmaceutical) was used as the distilled water. Preparation of sodium oleate (NaOA; molar ratio = 1/1) and of arginine stearate (ArgSA; molar ratio = 1/1) was also carried out by the same method.

2.2 Preparation of Phase Diagram

Preparation of a phase diagram was conducted in such a manner that a sample having a predetermined composition was placed in a screwed test tube equipped with a Teflon seal and allowed to stand in a constant-temperature water bath and the resulting state was observed. For the measurement of phase transition temperature, a differential scanning calorimeter (DSC 6100 manufactured by Seiko Instruments) was used. Observation of the liquid crystalline structure was conducted in such a manner that a temperature rising stage (type THMS 600 manufactured by Linkam) was installed in an inverted optical microscope (type IMT-2 manufactured by Olympus) and changes in temperature were observed under a transmission Nomarski type differential interference contrast or under polarized light. Final confirmation of liquid crystalline structure was carried out by a small angle X-ray scattering (SAXS).

2.3 Measurement of Small Angle X-ray Scattering (SAXS)

Final confirmation of liquid crystalline structure and measurement of the interlayer spacing were conducted by means of small angle X-ray scattering (SAXS). As to a device, RU-200 manufactured by Rigaku was used and a sample was filled in stainless steel plates with a thickness of 1 mm having pores (5 mm $\times$ 40 mm), both surfaces were held by a polyimide film and measurement was conducted at a constant temperature of 25°C. However, for the measurement by SAXS of a sample in a region where concentration of water was 80% or more, ArgOA was diluted in such a manner that data with a good precision were not available by the SAXS used at this time and, therefore, a polarized microscope or a transmission electron microscope (TEM) was used for the observation of a diluted region.

2.4 Measurement of Surface Tension

Measurement of surface tension was conducted at 25°C by a Wilhelmy method using an automatic tensiometer of a Wilhelmy plate type (type CBVP-Z manufactured by Kyowa Kaimen Kagaku).

2.5 Measurement of Transmittance

Measurement of transmittance of a solution upon temperature was conducted at the wavelength of 700 nm using a UV/VIS Spectrophotometer (V-550 manufactured by Jasco).

2.6 TEM Observation by a Freeze Fracture Method

Preparation of a sample for observation of an aqueous solution of ArgOA under a transmission electron microscope (TEM) was carried out by a freeze fracture method. Thus, a frozen replica of the sample was prepared by a frozen replica manufacturing device (FR-7000A manufactured by Hitachi Science Systems) with using a quick freezing system (EM CPC manufactured by Leica). A method for replica preparation was that the sample solution was quickly frozen in liquid propane (-187°C) and platinum carbon was vapor-deposited on the cracked cross section to form shadows. After that, the above was washed with acetone and distilled water and transferred to a grid of 150 mesh made of copper to prepare a replica. The prepared metal replica membrane was observed under a TEM (JEM-1200EX manufactured by Nippon Denshi).

2.7 Measurement of Viscoelasticity

Measurement of viscoelasticity was conducted using a rheometer of a stress control type (Carri-Med CLS 2100 manufactured by TA-Instruments). Viscosity was calculated by an approximation of a curve for shearing stress versus shearing velocity by a Bingham's formula. Temperature of the sample was controlled at $25 \pm 0.1^{\circ}\text{C}$ by a Peltier element.

2.8 Measurement of pH

Measurement of pH was conducted using pH electrodes (GST-5311C manufactured by TOA Electric) and ion meter (1M-40S manufactured by TOA Electric).

2.9 Measurement of NMR

Measurement of chemical shifts for amino group proton based on ^1H -NMR was conducted by JNM AL-300 Type manufactured by JEOL.

3 Results and Discussion

3.1 Phase Diagram of Arginine Oleate/Water System

Figure 2 shows a phase diagram of ArgOA/water system, in which molar ratio of L-arginine to oleic acid is 1/1. As will be apparent from the region (A) of this diagram, a hydrated crystal of ArgOA was present in a very high concentration range of ArgOA, while transition temperature (T_c) to a lamellar liquid crystalline phase suddenly lowered as water increased and, when it

Fig. 2 Phase Diagram of ArgOA–water System. ArgOA (1/1, molar ratio).
LLC : Lamellar Liquid Crystal
W : Aqueous solution
 T_c : Phase transition temperature (LLC-Hydrated Crystal)

Fig. 3 Polarized Microscopic Observation.

(a) 15 wt%ArgOA aq.solu.

(b) 35 wt%ArgOA aq.solu.

exceeded 6 wt%, the temperature became 0°C or lower and most of the region was occupied by lamellar liquid crystals. That is because L-arginine as a counterion was apt to be hydrated, and similar phenomena have been observed in potassium stearate, dipalmitoylphosphatidylcholine, etc. (26).

Figure 3 shows pictures under a polarized microscope of aqueous solutions of (a) 15 wt% of ArgOA and (b) 35 wt% of ArgOA in **Fig. 2** at 25°C. In a 15 wt% aqueous solution of ArgOA in the LLC + W region (a) in the figure, concentric lamellar liquid crystals (multi-lamellar vesicles) dispersed in water were observed, while in a 35 wt% aqueous solution of ArgOA in the LLC region (b), lamellar liquid crystalline structure was formed.

In addition, as will be noted from the area of the lamellar liquid crystalline region (**Fig. 2**), it is expected that the lamellar liquid crystal by ArgOA is able to retain a large amount of water in its structural body. Therefore, in order to analyze the structure in more

detail, a small angle X-ray diffraction pattern (25°C), where the ratio by weight of ArgOA/water is 1/1 corresponding to **Fig. 2(c)**, is shown in **Fig. 4**. The scattering peak ratio of the primary peak to the secondary peak in the small angle diffraction was 1:1/2, by which the formation of lamellar liquid crystalline structure was confirmed. Then, the interlayer spacing of lamellar liquid crystals by small angle X-ray diffraction was measured (**Fig. 5**). Incidentally, the ordinate is the interlayer spacing (*d*) of the lamellar liquid crystals and the abscissa is the ratio (*C_w/C_{ao}*) of weight fraction of water (*C_w*) to that of ArgOA (*C_{ao}*). Here, spacing (*d*) of the lamellar liquid crystal was calculated by a Bragg's formula ($2d \sin \theta = n\lambda$ in which *d* is interlayer spacing, θ is X-ray scattering angle, *n* is integer and λ is wavelength of incoming X-ray) from the scattering peak of small angle X-ray diffraction. It will be considered that an increase in *d* means that water molecules are distributed into the hydrophilic parts of the lamellar liquid crystalline structure (27). As will be apparent from **Fig. 5**, when *C_w/C_{ao}* passed over 0.3, *d* increased almost linearly and became near a constant value when the ratio

Fig. 4 Small Angle X-ray Diffraction Pattern (25°C) : ArgOA/Water (1/1 by Weight). **Fig. 5** Interlayer Spacing of LLC Phase as a Function of the Water : ArgOA Weight Ratio (*C_w/C_{ao}*) at 25°C.

became more than about 2. This means that more water than the above is unable to be incorporated into the structure but is separated as a water phase being coexisted with the lamellar liquid crystalline phase (LLC + W). Thus, it shows that ArgOA forms lamellar liquid crystals containing about 70% of water and, at the same time, ArgOA has an ability of retaining more amount of water than lecithin which is a main component of biomembrane (28). Therefore, a multi-lamellar vesicle region of ArgOA shown by LLC + W of **Fig. 2** also retains about 70% of water in vesicles and a difference in specific gravity from water as the external phase also becomes small, whereby it was relatively stable causing no separation even after one month at room temperature. It has been found that, although ArgOA is a single-chained amphiphilic substance, it has a high ability to form lamellar liquid crystals like branched-chain type (29) or double-chained (30) amphiphilic substances and that much more water is able to be retained in its structural body.

Accordingly, in order to further understand the characteristics of solution properties of ArgOA, investigations were conducted for a phase diagram of sodium oleate/water system using sodium instead of arginine as a counterion and also for arginine stearate/water system using stearic acid instead of oleic acid as a hydrophobic moiety.

3.2 Phase Diagrams of a Sodium Oleate/Water System and an Arginine Stearate/Water System

Figure 6 shows a phase diagram of sodium oleate (NaOA)/water system. When it is compared with a hydrated crystal region (A) of **Fig. 2**, the hydrated crystal existed until about 32°C even when hydrated to an extent of 60% as shown in the point (e). It is presumed to be due to the fact that, since counterion for NaOA is sodium, there is no high hydrating ability of arginine, and changes in T_c as a result of an increase in water concentration do not take place quickly. In an NaOA/water system, both types of lamellar and hexagonal liquid crystals were present unlike the case of ArgOA. This is considered to be caused by a molecular structure (cone type) originated in the molecular balance between hydrophobic moiety (oleyl group) and hydrophilic moiety (sodium). Furthermore, compared the phase diagram of NaOA/water system in **Fig. 6** with that of ArgOA/water system in **Fig. 2**, NaOA showed a wide range of micellar phase (Wm), while in the case of ArgOA, it showed a wide range of dispersed phase of lamellar liquid crystal (LLC + W). It is interpreted that, since a lateral intermolecular repulsion in NaOA aggregate is higher than that in ArgOA aggregate with self dipolar zwitterionic interaction, NaOA forms smaller aggregate (in Wm phase) than the aggregate of ArgOA in LLC + W phase. As a result of it, it causes more hydrophilicity of NaOA than hydrophile-lipophile

Fig. 6 Phase Diagram of NaOA-water System.
NaOA (1/1, molar ratio)
LLC : Lamellar Liquid Crystal
HLC : Hexagonal Liquid Crystal
Wm : Micellar solution

Fig. 7 Phase Diagram of ArgSA-water System.
ArgSA (1/1, molar ratio)
LLC : Lamellar Liquid Crystal
R-HLC : Reversed Hexagonal phase
W : Aqueous solution

balance(HLB) of ArgOA.

Figure 7 shows a phase diagram of an arginine stearate (ArgSA)/water system where hydrophobic moiety of ArgOA was different. As will be clear from **Fig. 7**, hydrated crystal of ArgSA was present in a wide concentration range at about 60°C and lower. This is due to a high melting point of stearyl group. In the case of the hydrated crystal (I) in this region, water was stably incorporated into its structure and, even after one month, no separation of water took place, whereby it is regarded that an α -gel structure (26) is formed. When the attention is paid to the region of about 60°C and higher, a reversed hexagonal phase and a lamellar liquid crystalline phase appeared and, unlike the case of sodium oleate, a dispersed phase of lamellar liquid crystal (LLC + W) was present at the low concentration region.

From these facts, it is concluded that a molecular structure of ArgOA, in which oleic acid having a bulky structure as a hydrophobic moiety due to a cis-type double bond and arginine having a strong intermolecular interaction as a zwitterion are combined, is suitable for the formation of lamellar liquid crystals.

3.3 Solution Properties in a Diluted System of an ArgOA Aqueous Solution

It is very important to know the solution properties of ArgOA in a diluted solution system for understanding the surface chemical characteristic of ArgOA. In view of the above, a surface tension versus concentration curve of ArgOA aqueous solution was prepared (**Fig. 8**). As will be apparent from **Fig. 8**, there were two

Fig. 8 Surface Tension v.s. Concentration of ArgOA at 25°C.

bending points. Similar tendency was also reported for diluted aqueous solutions of a Pluronic type surfactant and of sodium salt of 1,2-dilauroylphosphatidylglycerol (DLPG) having an ability to form bilayer membrane and, at their bending points, it was analyzed that a phase change was resulted (31,32). Therefore, the bending points of **Fig. 8** were named the first cmc (C1: 3.0×10^{-4} M) and the second cmc (C2: 1.0×10^{-2} M). When an attention is paid to the concentration range near these two points, a change was resulted in the appearance of the solution from C1 and, therefore, transmittance of the solution near C1 and C2 were measured (**Fig. 9**). The transmittance began to lower from C1 and lowered down to near C2. This is interpreted to be due to the co-existence of micelle and highly associated aggregates from near C1. Therefore, an aqueous solution between C1 and C2 was subjected to an obser-

Fig. 9 Transmittance v.s. Concentration of ArgOA at 25°C.

Fig. 10 Transmission Electron Microscopic Observation (ArgOA 1.0×10^{-3} M) in the region from C1 to C2.

vation under TEM by a freeze fracture method (**Fig. 10**). In this picture, granular substances (highly associated aggregates) of 10 to 50 nm and 200 to 300 nm were observed. Taking a decrease in transmittance of the solution from C1 to C2 and a high ability of ArgOA to form lamellar liquid crystals as mentioned already into consideration, there may be a high possibility that those highly associated aggregates are vesicles. From these results, it is considered that the area between C1 and C2 is a region where micelle-vesicle like highly associated aggregates coexist. After that, changes in the viscosity of ArgOA aqueous solution were resulted from near C2 (**Fig. 11**). Viscosity at the concentration

range before C2 was similar to that of water but, after C2, it abruptly increased. The increase in the viscosity is judged to be due to a phase transition to a lamellar liquid crystalline phase region.

From these results, it has been cleared that ArgOA is able to form a highly associated aggregates from its diluted solution region.

3·4 Effect of L-arginine on the Formation of Highly Associated Aggregates

As mentioned above, it has been found that, although ArgOA is a single-chained amphiphilic substance, it shows a specific phase behavior and forms a highly

Fig. 11 Viscosity v.s. Concentration of ArgOA.

Fig. 12 pH v.s. Concentration of ArgOA.

associated aggregates from very low concentrations. Such a specificity of the ArgOA should be greatly affected by a structural characteristic of oleic acid as a hydrophobic moiety as well as L-arginine as a counterion. Therefore, we focused on the counterion and investigated the effect of arginine on the formation of highly associated aggregates in a diluted solution system.

ArgOA is a fatty acid soap and the dissociation states of counterion is able to be presumed from the changes in pH. Thus, the relation between pH and concentration of an ArgOA solution near C1 and C2 was investigated (Fig. 12). As will be apparent from Fig. 12, it was noted that the relation between pH and ArgOA concentration had a correlation to a surface tension versus concentration curve (transcribed from Fig. 8). Firstly, the pH began to increase from near C1 and, until C2, it increased almost in proportion to the concentration. The pH reached its maximum near C2 and, after that, a decrease in pH tended to become slow. If C2 is an initiation point for the phase transfer to the lamellar liquid crystalline phase, changes in pH should reflect the hydrated state around the bonded area of guanidyl group in strongly basic arginine for carboxyl group of oleic acid, whereby its relation with the associated state may be presumed as well. Accordingly, in order to investigate the state around the bonded area, chemical shift of amino group proton was measured by means of ^1H -NMR (Fig. 13). As noted in this figure, when ArgOA concentration increased from C2 (0.01 M) which is judged to be an initiation point for the phase

transfer as mentioned above to 0.1 M, chemical shift was shifted to the side of an up-magnetic field. Generally speaking, nucleus of atom having a relatively high electron density appears in an upfield while nucleus of atom having a relatively low electron density appears in a downfield. In accordance with such a qualitative chemical shift rule (33), it is meant that, as the ArgOA concentration increased, dissociation degree of amino group (a guanidyl group of arginine in the present case) lowered (lowering in deviation of electron density), whereby the electron density became high and amino group proton appeared in the area of an upfield. Therefore, it is able to be concluded that changes in pH reflect the hydrated state around the bonded area. From the above, with regard to the formation of lamellar liquid crystals of ArgOA, it is considered that, as the concentration increases, an interaction in the two-dimensional direction (in a lateral direction) between adjacent L-arginine molecules becomes strong and the hydrating ability of the bonded area lowers. Therefore, a dissociation degree of the bonded area of L-arginine guanidyl group lowers (lowering in pH), a hydrophobic interaction is further accelerated and a formation of the associated aggregates is promoted, as a result of it, lamellar liquid crystal which is an infinitely associated aggregates is formed.

4 Conclusion

In spite of the fact that ArgOA is a single-chained amphiphilic substance, it has been found to have a characteristic to form lamellar liquid crystals over a wide ranges of temperature and concentration. It has been concluded that a structural characteristic as a result of a cis-type double bond of oleic acid and a structural balance with L-arginine are inevitable in the formation of lamellar liquid crystals. It has been also confirmed that ArgOA is able to form a lamellar liquid crystalline structure having a large amount of water.

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Fig. 13 ^1H -NMR Chemical Shift in ppm in D_2O (Chain- NH_2).

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