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Monolayer Behavior of Cyclic and Linear Forms of Surfactins: Thermodynamic Analysis of Langmuir Monolayers and AFM Study of Langmuir-Blodgett Monolayers

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Abstract: The molecular interactions of monolayers composed of cyclic and linear forms of surfactins (SFs) were evaluated through atomic force microscopy (AFM) together with a Langmuir monolayer technique. The surface pressure (π)-area per molecule (A) isotherm of a pure cyclic surfactin (CSF) monolayer exhibited a liquid expanded (Le) monolayer, while that of a pure linear surfactin (LSF) monolayer exhibited a liquid condensed (Lc) monolayer, demonstrating that the CSFs are in a rather loose molecular packing state owing to its bulky heptapeptide ring. The plots of the mean area per molecule of the CSF/LSF monolayers were well fitted to the ideal curves, suggesting that ideal mixing occurs, or that the two components are immiscible in a monolayer. The AFM images of the CSF/LSF monolayers transferred at 25 mN/m gave phase-separated microdomain structures, indicating that the CSFs and LSFs are almost immiscible and separated into a CSF-rich and LSF-rich phases, as suggested from the analysis of the mean area per molecule of the monolayers. Our results clearly demonstrated that the cleavage of the cyclic heptapeptide headgroup of CSF drastically changes its molecular packing state in a monolayer and that AFM observation combined with the Langmuir monolayer technique is quite useful to explore the manner of self-assembly of a binary system of microbial products such as CSFs and LSFs.

Key words: biosurfactant, surfactin, cyclic peptide, atomic force microscopy, Langmuir monolayer

1 INTRODUCTION

Biosurfactants (BSs) are amphiphilic compounds that are produced abundantly from renewable resources by a variety of microorganisms[1-3]. Because of their structural diversity and unique properties, including high surface activity, low toxicity, and various biological functions, BSs have attracted recent attention for potential applications in environmental protection, contributing to the realization of a low carbon society.

Surfactin (CSF, Fig. 1 (a)) is a promising BS produced by various strains of *Bacillus subtilis*, and consists of a heptapeptide headgroup (*L*-Glu-*L*-Leu-*D*-Leu-*L*-Val-*L*-Asp-*D*-Leu-*L*-Leu) interlinked with a β-hydroxy fatty acid[4]. The cyclic peptide adopts a "horse-saddle" structure in solution with the two carboxylic acids as the hydrophilic parts[5, 6]. In spite of its bulky headgroup, CSFs are known to efficiently form versatile aggregates; CSFs have strong hydrogen bonding interactions, leading to the formation of β-sheet structures[7]. CSF also provides remarkable surface and biological activities derived from its unique cyclic structure. CSF reduces the surface tension of pure water from 72 to 26 mN/m[8]. CSF has quite a low critical micelle concentration (CMC) of 9.4×10^{-6} M in water. The p*K*a for the micelle of CSF is approximately 6[9]. CSF shows biological functions including hemolytic activity[10, 11] and antiviral[12, 13] and antimycoplasma properties[14]. These observations have attracted considerable interest because they could be related to the effect of the cyclic structure of CSF on the interaction with the biological membrane.

CSF also has a long alkyl chain of varying length as a hy-

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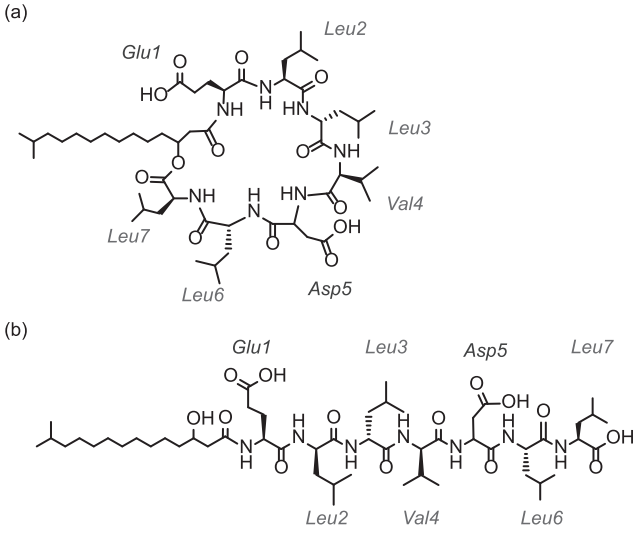

Fig. 1 Molecular structures of (a) cyclic surfactin (CSF) and (b) linear surfactin (LSF).

drophobic tail, so that hydrophobic interactions in water induce the spontaneous formation of a micelle or an extension into a lipid bilayer. CSF is mainly composed of a variety of mixtures, giving rise to homologues involving variations of the chain length, fatty acid branching, and amino acid substitutions in the peptide ring. Interestingly, CSF contains a trace amount of the linear form of surfactin (LSF), in which the lactone ring is cleaved to form a linear conformation, as shown in Fig. 1(b)[7, 15].

Microbial products generally show structural diversity as discussed above, and these compounds exhibit miscibility to maximize their synergy effects in biological environments. In order to understand the synergy effect of CSF in lipid membranes, the interfacial properties of CSFs in a monolayer have been studied[16–21]. Moreover, studies on the interactions between LSF and lipid membranes were reported by Deleu *et al.*[22]. These studies revealed that the cyclic heptapeptide moiety of CSF plays a crucial role in their remarkable interfacial properties in lipid membranes. However, the interfacial behavior of monolayers composed of CSF and LSF at the air/water interface has never been examined. These studies would provide a more detailed understanding of the mechanism of self-assembly of CSF both in solution and in biological environments.

Herein, we report the preparation of Langmuir monolayers composed of cyclic and linear surfactin at the air/water interface, and the molecular interactions were investigated from measurements of surface pressure (π)-area per molecule (A) isotherms. The miscibility between CSF and LSF was then evaluated by direct observation of the Langmuir monolayers transferred on mica substrates (LB films).

2 EXPERIMENTAL

Surfactin sodium salt was kindly supplied by Kaneka Co. Ltd., Japan. The primary structure and composition of the surfactin were ascertained by analytical RP-HPLC (Inertsil SIL-100A 5 mm C18 column, 0.46×25 cm, GL Sciences Inc.) using a mobile phase consisting of acetonitrile/water (80/20) containing 0.1% trifluoroacetic acid (TFA). It was found that the surfactin sodium salts are composed of mixtures with different chain lengths of C13 (17%), C14 (52%), and C15 (31%). MALDI-TOF mass spectrometry (autoflex speed TOF/TOF, Bruker Daltonics, Inc.) measurements of the mixture of surfactin sodium salts revealed that they are consists of a heptapeptide headgroup (*L*-Glu-*L*-Leu-*D*-Leu-*L*-Val-*L*-Asp-*D*-Leu-*L*-Leu) as a main compound. Very minor amounts of compounds with different peptide sequences were also observed in the spectrum. The CSFs were prepared by neutralization of the surfactin sodium salts with dilute HCl, and they were then used in this study without further purification. Sodium methoxide, methanol, and chloroform were purchased from Wako. Co. Ltd., Japan. Pure water was obtained from a Millipore (Milli Q) apparatus.

2.1 Synthesis of LSF

To a 1 mM solution of surfactin sodium salts (5.0 g, 4.8 mmol) in methanol was added sodium methoxide (0.78 g, 14.4 mmol). The mixture was stirred for 30 min at 60℃. After the reaction, the mixture was cooled to room temperature and the solvent was evaporated *in vacuo*, then dilute HCl was added until a precipitate was formed. The solid product was separated by centrifugation (13000 rpm, 1 h) and washed with a small amount of methanol and pure water to yield LSF as a white solid (97%). The structure of LSF was confirmed by ^{1}H-NMR, MALDI-TOFMS, and HPLC measurements.

2.2 Surface pressure (π)-area per molecule (A) isotherm measurements

Stock solutions (1 mM) were prepared by dissolving CSF/LSF in a mixed solvent system (chloroform/methanol = 2/1 (v/v)). The CSF/LSF monolayers were formed by spreading the stock solutions on distilled water. After 10 min of solvent evaporation, π-A isotherms were measured at 25℃ with a Langmuir-Blodgett trough type 611 (NIMA Ltd., UK). The surface pressure was obtained by the Wilhelmy plate method using a platinum plate. The barrier speed was 15 mm/min.

2.3 Atomic force microscopy (AFM)

AFM (Model SPI 4000, Seiko Instruments Co. Ltd., Japan) observations were performed in air at 25℃ in the dynamic force mode (tapping mode). A silicon cantilever (SII Nanotechnology, Japan, SI-DF 20, spring constant = 15 N/m) was used for observation. The Langmuir monolayers

on mica were prepared using a vertical dipping method[23]. The dipping speed was 10 mm/min. The produced LB films were imaged with scan rates ranging from 0.95 to 2.5 Hz. The applied force was maintained as low as possible during the imaging.

3 RESULTS AND DISCUSSION

3.1 Surface pressure (π)-area per molecule (*A*) isotherms of CSF/LSF monolayers

π-*A* isotherms of the CSF/LSF monolayers with various CSF mole fractions (X_{CSF}/X_{LSF} = 1.0/0, 0.7/0.3, 0.5/0.5, 0.3/0.7, and 0/1.0) were measured at the air/water interface at 25℃, and the results are shown in Fig. 2. From the figure, the pure CSF monolayer at the air/water interface presents a π-*A* isotherm with a mirrored sigmoidal shape and exhibits a liquid-expanded (Le) phase with a significantly large limiting molecular area value (A_0 = 191 Å^2/molecule). The value of A_0 gives an indication of the cross-sectional area of the cyclic peptide at the air/water interface and is in agreement with the values reported in previous studies[22]. At higher surface pressure, the CSF isotherm shows a plateau. On the other hand, the π-*A* isotherm of the pure LSF monolayer exhibited a higher collapse pressure and a steeper slope than the pure CSF monolayer, suggesting that LSF could form a liquid-condensed (Lc) phase and a less compressible monolayer than CSF (Fig. 2a). The pure LSF monolayer also shows a break point at 152 Å^2/molecule. The surface pressure of the break point increases with an increase in the temperature, indicating that the break point represents a phase transition from an Le to an Lc phase. A_0 of the LSF monolayer is 136 Å^2/molecule. We note that Deleu *et al*. also reported that LSF presented a π-*A* isotherm with a mirrored sigmoidal shape and a relatively large limiting molecular area value (A_0 = 202 Å^2/molecule; 10 mM Tris/150 mM NaCl; pH 7.2; 20℃)[22]. The difference between Deleu's results and ours could arise from the effect of the sodium salts or from the difference in the solvent used. The LSF monolayer was found to be expanded by the addition of CSF. The mean areas for the mixed monolayers are always between those of the pure monolayers. If CSF and LSF are miscible at the air/water interface, the collapse pressures of the monolayers will depend on the molar fraction[24]. The collapse pressures of the monolayers in Fig. 2 do not correlate well with the mole fraction, suggesting that CSF and LSF are not ideally miscible at the air/water interface (77.8 mN/m for X_{CSF} = 0, 81.0 mN/m for X_{CSF} = 0.3, 77.1 mN/m for X_{CSF} = 0.5, 80.5 mN/m for X_{CSF} = 0.7, and 76.5 mN/m for X_{CSF} = 1.0). In contrast, the transition pressures from the Le to the Lc phase of LSF varies with the mole fraction, suggesting partial miscibility of the two components (3.2 mN/m for X_{CSF} = 0, 4.6 mN/m for X_{CSF} = 0.3, 7.7 mN/m for X_{CSF} = 0.5, and 8.1 mN/m for X_{CSF} = 0.7).

In order to discuss the miscibility as well as the interaction between the CSF and LSF in a monolayer, the mean area per molecule of the monolayers at 10, 20, and 30 mN/m were plotted as a function of the mole fraction of CSF (X_{CSF}); the results are shown in Fig. 3. The dashed lines are ideal curves given by Equation (1).

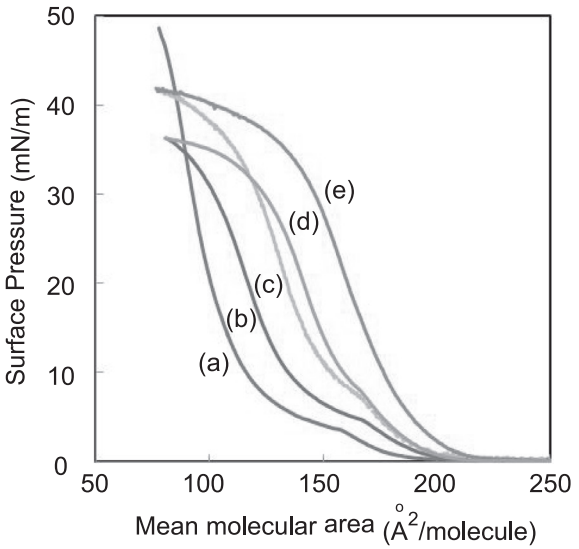

Fig. 2 Surface pressure (π) – area per molecule (*A*) isotherms of CSF/LSF monolayers with various compositions at the air/water interface at 25℃, (a) X_{CSF}/X_{LSF} = 0/1.0, (b) X_{CSF}/X_{LSF} = 0.3/0.7, (c) X_{CSF}/X_{LSF} = 0.5/0.5, (d) X_{CSF}/X_{LSF} = 0.7/0.3 and (e) X_{CSF}/X_{LSF} = 1.0/0.

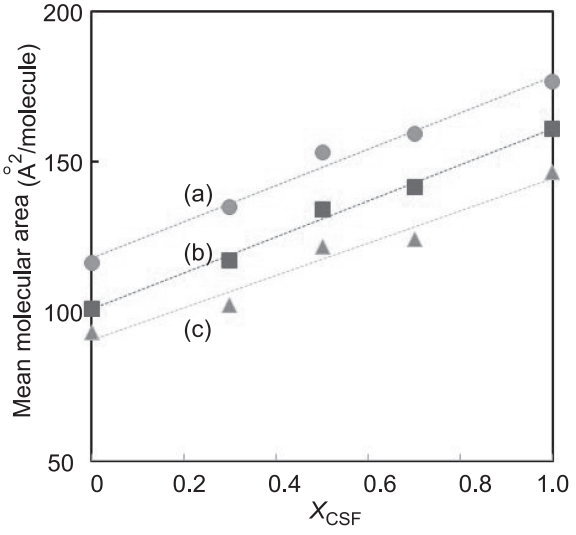

Fig. 3 Mean area per molecule as a function of composition for CSF/LSF monolayers at the air/water interface at various surface pressures (a) 10 mN/m, (b) 20 mN/m and (c) 30 mN/m.

$$A_{ideal} = X_1A_1 + X_2A_2 \quad (1)$$

Where A_{ideal} is the ideal area per molecule of the monolayer at a given surface pressure. A_1 and A_2 represent the areas per molecule of each pure monolayer at the same surface pressure, and X_1 and X_2 indicate the respective mole fractions in the monolayer. A_{ideal} denotes ideal mixing, or when the two components are completely immiscible in the monolayer. Otherwise, negative or positive values of the actual occupied area per molecule indicate that the two components are miscible and form non-ideal mixed monolayers. The plots of the mean area per molecule of the CSF/LSF monolayers show a good fit to the ideal curves, indicating that the CSF and LSF are ideally miscible or basically immiscible. Considering the independence of the collapse pressures on the mole fraction and the AFM observation of the monolayers (see below), the two components are basically immiscible, whereas partial miscibility of the two components was also indicated by the changing transition pressures.

We assume that CSFs form a β-sheet structure via hydrogen bonding interactions between bulky head groups at the air/water interface[7]. These bulky head groups at the interface provide spaces where the long alkyl chains could fold and move easily, leading to a loose molecular packing state. The π-*A* isotherm of the pure CSF monolayer indicates that CSF forms a stable Le phase at the air/water interface, probably due to the less effective van der Waals interactions between the long alkyl chains. Gallet *et al.* calculated that the hydrophobic chains of CSF folded back to interact mainly with the Leu2 and Val4 side chains at the air/water interface[25]. In the case of LSF, such a linear molecular could adopt extended conformations to minimize steric interactions and surface area, so that LSF could adopt a dense molecular packing to form an Lc phase. The β-sheet structure of CSFs may be more stable than that of LSFs because of its macrocyclic entropy effect, where CSF loses the freedom of motion of the head group.

3.2 AFM images of the CSF/LSF monolayers

When the CSF/LSF monolayers reached a steady-state surface pressure, they were transferred onto mica at 25 mN/m (Fig. 4), and the resulting monolayers were investigated by AFM. Figure 4 shows the top-view AFM images observed by the tapping mode on a scale of 5 μm × 5 μm. Generally, when two components are immiscible and the measured areas per molecule follow ideal behavior, phase-separated microdomain structures are observed at this scale. The AFM images of the pure CSF and LSF monolayers exhibit almost homogeneous contrast, as shown in Figs. 4(a) and (c). On the other hand, the CSF/LSF monolayer ($X_{CSF}/X_{LSF} = 0.5/0.5$), which was transferred onto mica at 25 mN/m, shows microdomain structures (Fig. 4(b)). We note that the higher domains are elongated to form line shape

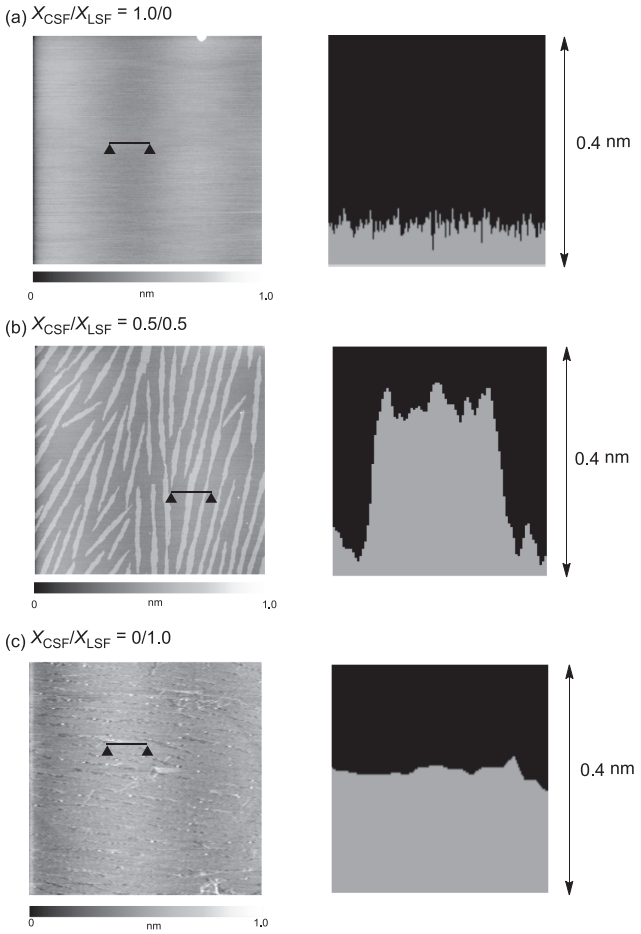

Fig. 4 Top-view AFM images of Langmuir-Blogget films (a) pure CSF Langmuir-Blogget films (5 μm × 5 μm in lateral, 1.0 nm in vertical), (b) X_{CSF}/X_{LSF} (= 0.5/0.5) Langmuir-Blogget films (5 μm × 5 μm in lateral, 1.0 nm in vertical), (c) pure LSF Langmuir-Blogget films (5 μm × 5 μm in lateral, 1.0 nm in vertical).

domains. This pattern is indicative of an immiscible morphology, which was predominantly induced by the driving force of the electrostatic dipole-dipole repulsion between the molecular dipoles[18]. The interfacial organization properties of the CSF/LSF monolayers are quite similar to those of CSF/DPPC[18, 22]. LSF and DPPC tend to form a liquid-condensed state with compact organization. They are thus more likely to form well-organized elongated domain structures with CSF. In this system, the difference of the height of the LSF in LSF/DPPC was about 0.3 nm higher than that of the CSF in the CSF/DPPC monolayers[22]. From these results, we attributed the higher matrix to the LSF-rich Lc domains and the lower level to the CSF-enriched Le phase (difference of height: Dh = ca. 0.3 nm). LSF is in a solid-like Lc state, while CSF adopts a fluid-like Le state. These two opposite states promote the immiscibility between the

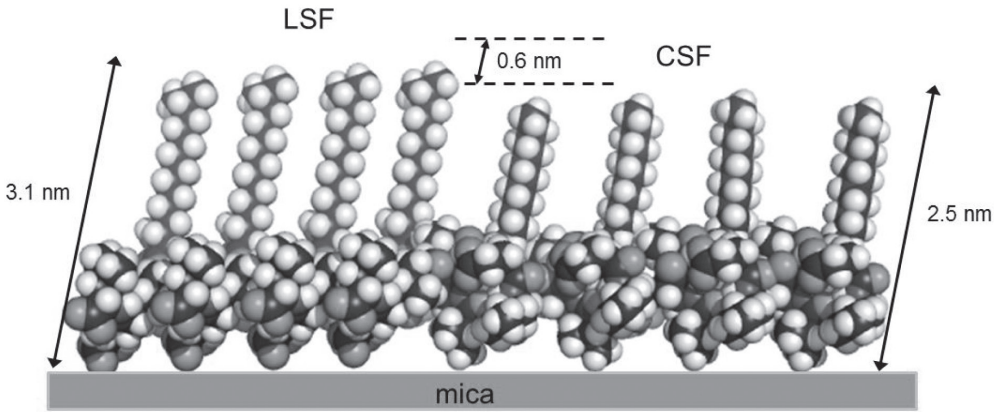

Fig. 5 Cartoon representation of a CSF/LSF Langmuir-Blogget film on mica.

two components at the interface.

The possible structures of CSF and LSF were calculated by a semi-empirical molecular orbital method (CAChe v. 6.1, PM5). A cartoon representation of a Langmuir monolayer of CSF/LSF is shown in Fig. 5. According to the energy-minimized structure, CSF, with a 15 carbon atom alkyl chain, forms a horse-saddle structure (2.5 nm in length). On the other hand, LSF shows a longer molecular conformation (3.1 nm in length). The difference in the molecular height in the line profiles was in good agreement with the calculation of the difference in the lengths of the CSF and LSF. These results also indicate that the protruding higher objects are mainly composed of LSF molecules.

To our knowledge, this is the first molecular interaction study of monolayers composed of CSF and LSF at the air/water interface. Our results clearly demonstrated that AFM together with the Langmuir monolayer technique is quite useful to understand the self-assembly mechanism of CSF in greater detail.

4 CONCLUSION

We report for the first time the preparation of Langmuir monolayers composed of CSF and LSF at the air/water interface. The π-*A* isotherm of the pure CSF monolayer exhibits a liquid-expanded monolayer (Le), while that of the LSF monolayer shows a phase transition from a liquid-expanded monolayer (Le) to a liquid-condensed monolayer (Lc). These results suggest that the cyclization of the heptapeptide moiety of surfactin leads to the formation of a stable Le phase at the air/water interface. The plots of the mean area per molecule of the CSF/LSF monolayers fit the ideal curves, suggesting that the CSF and LSF molecules are ideally miscible or basically immiscible. Considering the non-dependence of the collapse pressures on the mole fraction, the two components are basically immiscible; the partial miscibility of the two components was also indicated by the changing transition pressures.

The AFM images of the CSF/LSF monolayers transferred at 25 mN/m showed phase-separated microdomain structures, indicating that CSF and LSF are basically immiscible, as predicted from the plots of the mean area per molecule of the CSF/LSF monolayers.

Strains of *Bacillus subtilis* are known to secrete both CSF and LSF in a culture medium (mainly CSF). Our results clearly demonstrated that the cleavage of the cyclic heptapeptide headgroup of surfactin drastically changes its molecular behavior in a monolayer. AFM observation combined with the Langmuir monolayer technique was also shown to be quite useful to explore the mechanism of self-assembly of binary systems of microbial products such as CSFs and LSFs.

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