

Monolayer Behavior of Binary Systems of Lactonic and Acidic Forms of Sophorolipids: Thermodynamic Analyses of Langmuir Monolayers and AFM Study of Langmuir–Blodgett Monolayers

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Abstract: The synergic effect and miscibility of the lactonic and acidic forms of sophorolipids (SLs) produced by *Starmerella bombicola* NBRC 10243 were first evaluated through atomic force microscopy (AFM), together with the Langmuir monolayer technique. The π - A isotherm of a pure lactonic sophorolipid (LS) monolayer mostly exhibited a liquid expanded monolayer, while that of an acidic sophorolipid (AS) monolayer showed a liquid condensed monolayer, suggesting that the lactonization of SLs makes the molecules more bulky and prevents them from adopting a close-packed arrangement. Plots of the mean area per molecule of mixed LS/AS monolayers gave positive deviations from the ideal curves, implying that the LS and AS molecules are miscible. Interestingly, the positive deviation of excess area (A_{ex}) from ideality was most significant at a mole fraction (X_{AS}) of 0.3, which closely resembles the composition of the LS/AS mixture secreted by *S. bombicola* in culture. The AFM images of mixed LS/AS monolayers transferred at 20 mN/m revealed no phase-separated microdomain structures, but rather showed small protruding objects for all compositions, indicating that LS and AS are partially miscible, as predicted by the positive deviations from the ideal curves. Cross-section analysis of the AFM images indicated that the observed protruding objects are AS-rich monolayers formed on the LS/AS monolayer. Our results clearly demonstrate that AFM combined with the Langmuir technique is useful for the exploration of the miscibility and synergic effects of microbial products.

Key words: biosurfactant, sophorolipid, *Starmerella bombicola*, atomic force microscopy, Langmuir monolayer

1 INTRODUCTION

Biosurfactants (BSs) are amphiphilic molecules that are produced abundantly from renewable resources by a variety of microorganisms. They have received much attention owing to their unique properties including their high surface activity, low toxicity, and versatile biological functions compared with established petroleum-based surfactants¹⁻³. Of such BSs, sophorolipids (SLs), generally produced by yeasts (primarily *Starmerella bombicola*⁴), are among the most promising types because they exhibit a considerably high production yield of over 100 g/L and are recovered efficiently from culture broths, mainly by precipitation⁵. They are, therefore, of increasing commercial in-

terest in the cosmetic, food, and detergent industries.

In general, most microbial products such as BSs are known to consist initially of a mixture of slightly different structures; SLs produced by *S. bombicola* ATCC 22214 are classified into two main species: the lactonic and acidic forms, as shown in Fig. 1⁶. The lactonic form of SL (LS) was synthesized through an internal esterification between the terminal carboxylic acid and the sophorose head at the 4" position (Fig. 1 (a)), and the acidic form of SL (AS) was obtained with a free fatty acid tail (Fig. 1 (b)). The sophorose unit also contains acetyl groups at the 6' and/or 6" positions.

The lactonization of SLs is known to improve their

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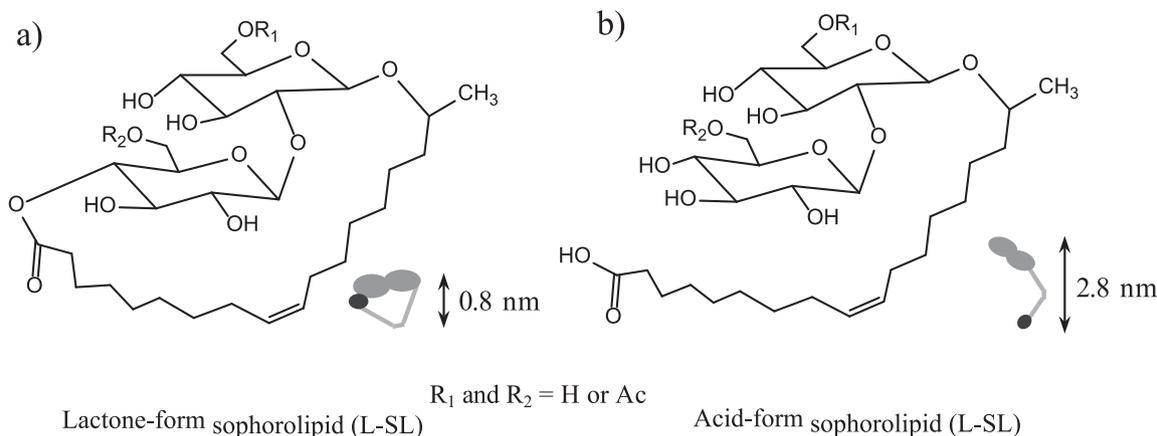


Fig. 1 Structure of the lactonic (LS) and the acidic (AS) sophorolipids (a) LS (b) AS.

various physicochemical and biological properties. LS is more surface active than AS⁷, and the critical micelle concentration (CMC) of LS is one order of magnitude lower than that of AS. LS, with reduced rotational freedom of the molecules, often forms crystals in aqueous solution, while AS is less crystalline. It has also been reported that LS shows a higher antimicrobial activity against gram-positive bacteria than AS⁸.

Recently, Hirata et al. found a synergic effect between LS and AS, and the CMC decreased upon mixing LS with AS⁹. Although *S. bombicola* secretes a mixture of both LS and AS in the culture, the miscibility or synergic effect of both compounds, especially at the molecular level, has not been elucidated.

During the past decade, atomic force microscopy (AFM), has emerged as a powerful tool for the visualization of the structures of biological systems^{10, 11}. AFM together with the Langmuir monolayer technique allows us to explore the miscibility or synergic effect of two different amphiphilic molecules at the molecular level^{12, 13}.

In this study, mixed Langmuir monolayers composed of lactonic and acidic forms of sophorolipids were first prepared at the air/water interface, and the molecular interactions between them were investigated through measurements of surface pressure–area per molecule (π - A) isotherms. The miscibility and synergic effect between the two SLs were also evaluated through direct observation of mixed Langmuir monolayers transferred on mica substrates (LB films).

2 EXPERIMENTAL

2.1 Yeast strains and growth medium

S. bombicola NBRC 10243 was obtained from the National Institute of Technology and Evaluation of Japan (NITE). Stock culture was cultivated for three days at 25°C on a YM plate medium containing 1% glucose, 0.5%

peptone, 0.3% yeast extract, 0.3% malt extract, 3.0% agar. It was stored at 4°C and renewed every 14 days.

2.2 Media preparation and culture conditions^{14, 15}

Seed cultures (1.5 × 5 mL) were prepared by inoculating cells grown on slants into test tubes containing a YM medium (1% glucose, 0.3% peptone, 0.3% yeast extract, 0.3% malt extract) at 25°C on a reciprocal shaker at 200 strokes/min for two days. The culture broths (30 × 5 mL) were inoculated in 2.3 L of the medium (10% jatropha oil, 5% glucose, 0.01% NaCl, 0.05% MgSO₄, 0.1% KH₂PO₄, 0.1% urea, 0.3% yeast extract) in a 5-L jar fermenter at 600 rpm at 25°C. Jatropha (*Jatropha curcas* L.) oil was kindly supplied by Allied Carbon Solutions Co., Ltd (Tokyo, Japan).

2.3 Isolation and structural analysis of SLs

The produced SLs were extracted from the culture with an equal amount of ethyl acetate. The extracts were first analyzed by thin-layer chromatography (TLC) on silica plates (Silica gel 60F; Wako) with a solvent system consisting of chloroform/methanol (80:20 v/v). The compounds on the plates were located by charring at 110°C for 5 min after spraying an anthrone/sulfuric acid reagent.

The ethyl acetate fraction was then separated and evaporated. The concentrated SLs were dissolved in chloroform and then purified through silica gel (Wako-gel C-200; Wako, Osaka, Japan) column chromatography using a gradient elution of chloroform/acetone (10:0 to 0:10 v/v) mixtures as solvent systems. The LS/AS composition was determined through high-performance liquid chromatography (HPLC) on an HPLC system (SSPC; Tosoh, Tokyo, Japan) equipped with a silica gel column (Inertsil SIL-100A 5 μm, 4.6 mm × 250 mm; GL science, Japan) with a low-temperature evaporative light-scattering detector (ELSD-LT; Shimadzu, Kyoto, Japan) using a gradient solvent program consisting of various proportions of chloroform and methanol (from 100:0 to 0:100) at a flow rate of 1 mL/min. The structure

was assigned through $^1\text{H-NMR}$ and MALDI-TOF-MS, as reported previously¹⁵⁾.

2.4 Surface pressure (π)–area per molecule (A) isotherm measurements

Stock solutions (1 mM) were prepared by dissolving LS/AS mixtures in chloroform. The mixed LS/AS monolayers were formed by spreading the stock solutions on distilled water. After 10 min for solvent evaporation, the surface pressure–area (π – A) isotherms were measured at 25°C with an LB trough model 611 (Nima Ltd., U.K.). The surface pressure was obtained through the Wilhelmy plate method using a filter paper provided by the trough manufacturer. The barrier speed was 10 mm/min.

2.5 Atomic force microscopy (AFM) measurements

AFM (Model SPI 4000, Seiko Instruments Co. Ltd., Japan) observations were performed in air at 25°C in dynamic mode (tapping mode). The silicon cantilever (SII Nanotechnology, Japan, SI-DF 20, spring constant = 15 N/m) was used for observation. The mixed Langmuir monolayers on mica were further prepared by using a vertical dipping method¹⁶⁾. 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 Composition of the LS/AS mixture in culture

After cultivation, the produced SL mixture was extracted from the culture with an equal amount of ethyl acetate. The extracts were firstly analyzed by TLC, and the ethyl acetate fractions were spotted on a plate. The SLs were detected with the anthrone reagent as shown in Fig. 2(a). Among the SL homologues, it was already found that spots 1 and 2 were lactonic forms and spots 3 and 4 were acidic forms: the spots were mainly diacetylated LS (spot 1), monoacetylated LS (spot 2), diacetylated AS (spot 3), and monoacetylated AS (spot 4)¹⁷⁾. Because the presence or absence of acetyl groups on the head had little effect on the interfacial properties, spots 1 and 2 were corrected as the LS sample and spots 3 and 4 were corrected as the AS sample for further experiments.

For the determination of the composition of the LS/AS mixture, the ethyl acetate fractions were also subjected to HPLC with a silica gel column; the results are shown in Fig. 2(b). The HPLC chart gave two peaks: the first at 6.9 min was ascribed to LS and the second at 8.1 min was attributed to AS.

The composition of the LS/AS mixture estimated by the standard curves using pure SL and AS as standards was 72:28, which corresponded well to the composition of the

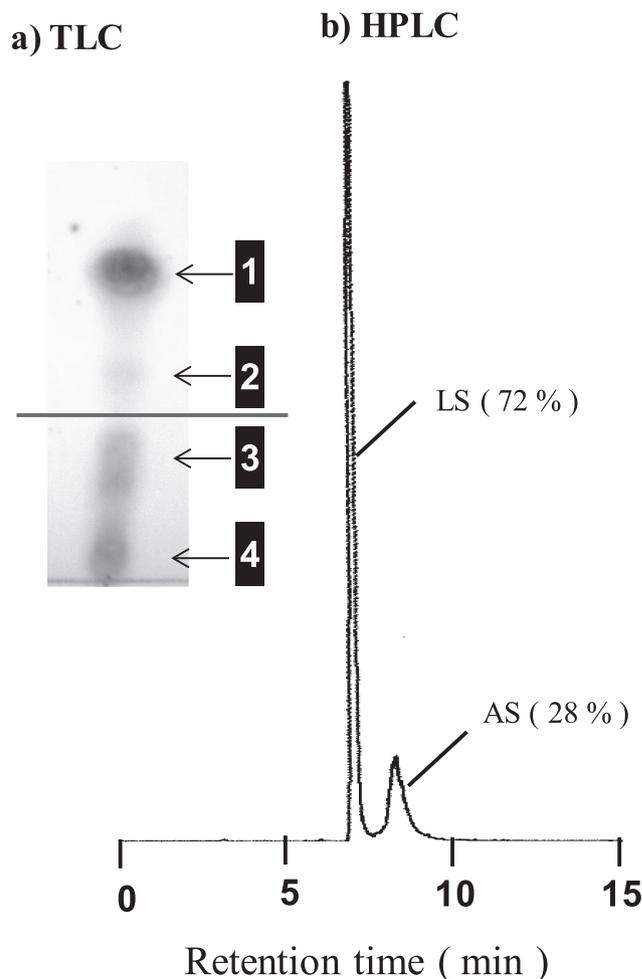


Fig. 2 (a) TLC analysis and (b) HPLC chart of a SL mixture.

mixture of SL produced from soybean oil by *S. bombicola* NBRC 10243⁹⁾. Through ELSD-LT detection, the peak intensity of LS was found to be rather low compared to that of AS, even at the same concentration.

3.2 Surface pressure (π)–area per molecule (A) isotherms of mixed LS/AS monolayers

Surface pressure–area per molecule (π – A) isotherms of mixed LS/AS monolayers with various AS mole fractions (X_{AS}) were measured at the air/water interface at 25°C, and are shown in Fig. 3. The π – A isotherm of the pure LS monolayer exhibited a phase transition from a liquid expanded monolayer to a liquid condensed monolayer, while that of the pure AS monolayer showed a liquid condensed monolayer. This indicates that the lactonization of SL makes the molecules more bulky and prevents them from adopting a close-packed arrangement, resulting in a larger area per molecule of LS. Moreover, the LS monolayer was found to be expanded by the addition of AS.

The molecular miscibility in a monolayer can be justified

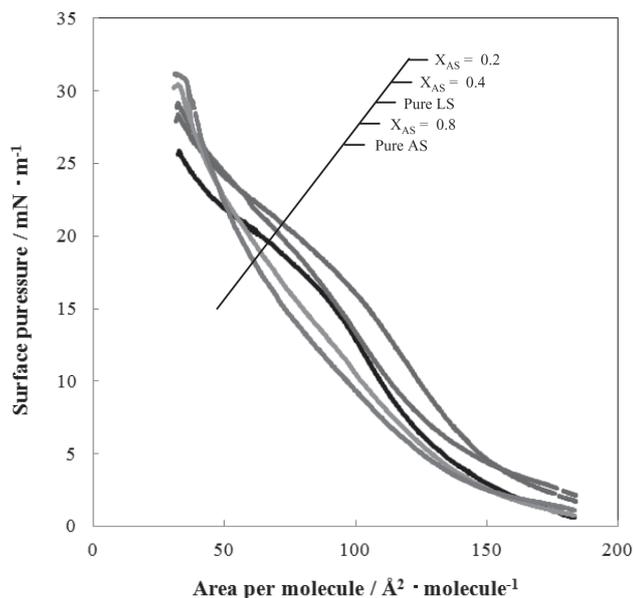


Fig. 3 Surface pressure (π) – area per molecule (A) isotherms of mixed AS/LS monolayers with various compositions at the air/water interface at 25°C.

by the use of the interface phase rule¹⁸). If the two components are miscible at the air/water interface, the collapse surface pressures of the mixed monolayer will vary with the composition. A monolayer composed of two miscible components generally exhibits two distinct collapse surface pressures corresponding to those of the pure components. The collapse surface pressure of the mixed monolayer observed in Fig. 3 did change with the composition, suggesting that LS and AS are miscible at the air/water interface.

For a further quantitative discussion of the miscibility and interaction between LS and AS in a monolayer, the mean area per molecule of the mixed monolayer at several different surface pressures is plotted as a function of mole fraction of AS (X_{AS}), with the results shown in Fig. 4(a). The dashed lines are the ideal curves given by equation (1):

$$A_{ideal} = X_1 A_2 + X_2 A_1 \tag{1}$$

where A_{ideal} is the ideal area per molecule of the mixed monolayer at a given surface pressure. A_1 and A_2 denote the area per molecule of each pure monolayer at the same surface pressure, and X_1 and X_2 indicate the respective mole fractions in the mixed monolayer. A_{ideal} means that ideal mixing occurs, or that the two components are completely immiscible in a 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 mean area per molecule of the mixed LS/AS monolayer showed positive deviations from the ideal curves, which means that LS and AS are not ideally miscible but partially miscible, as sug-

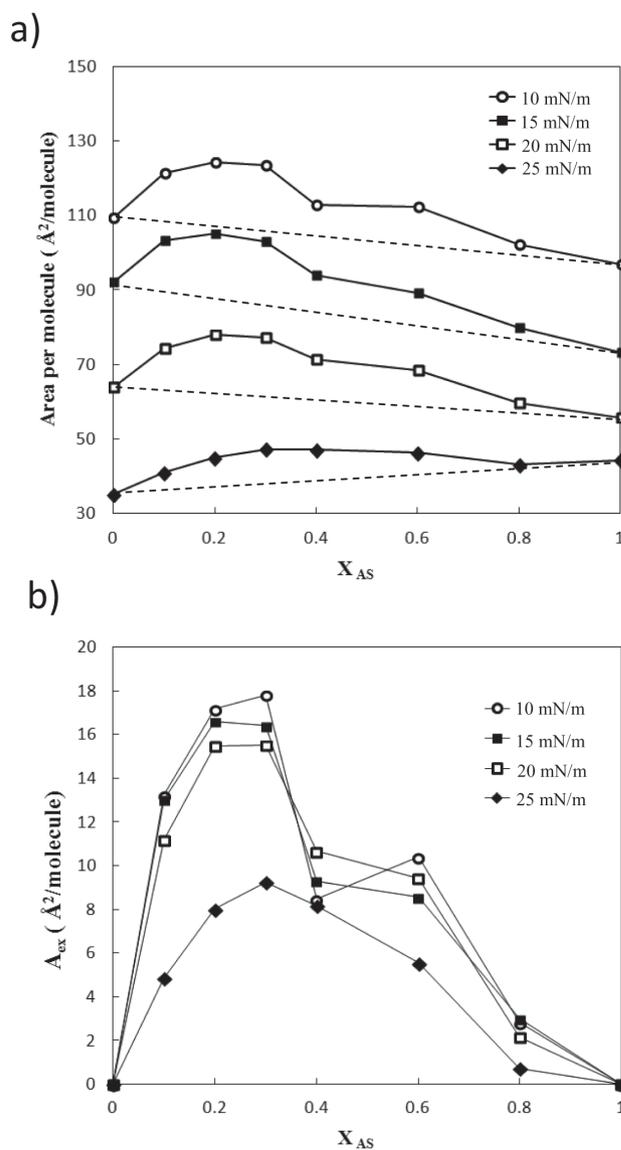


Fig. 4 (a) Mean area per molecule and (b) Excess area (A_{ex}) as a function of composition for mixed AS/LS monolayers at the air/water interface at various surface pressures.

gested from the analysis of the surface collapse pressures.

The excess area (A_{ex}) was then estimated from equation (2) at a given surface pressure¹²):

$$A_{ex} = A_{12} - A_{ideal} \tag{2}$$

where A_{12} is the actual occupied area per molecule of the mixed monolayer at a given surface pressure. Figure 4(b) shows A_{ex} versus X_{AS} for the mixed monolayers at several surface pressures. The plots of excess area exhibited positive deviations from ideality, with the most significant deviation observed at $X_{AS} = 0.3$. Interestingly, this rather resembles the composition of the LS/AS mixture produced from *S. bombicola* in culture⁹). The appropriate mixing of LS

and AL molecules effectively influences the molecular interactions and makes the molecular packing looser, especially at $X_{AS} = 0.3$. The largest mean area per molecule at this composition is likely to give the lowest CMC and γ_{CMC} values for an LS/AS mixture. Similar positive deviations are also found for the molecular interactions between Hepatitis G virus peptides with a bulky structure and phospholipids such as dimyristoyl phosphatidylcholine (DMPC), dimyristoyl phosphatidylglycerol (DMPG), and palmitoyl-oleoyl phosphatidylglycerol (POPG)¹³.

3.3 AFM images of mixed LS/AS monolayers

Mixed LS/AS monolayers at various compositions were then transferred onto mica at 20 mN/m, and visualized through AFM. Figure 5 shows top-view AFM images observed in tapping mode at a scale of $5 \times 5 \mu\text{m}$. In general, if two components are completely immiscible and the measured areas per molecule follow the ideal behavior, phase-separated microdomain structures are known to be observed at this scale in many systems^{16,19}. The AFM images in Fig. 5 show no microdomain structures, but rather, small protruding objects at all compositions, indicating that LS and AS are partially miscible, as predicted from the posi-

tive deviations from the ideal curves.

Figure 6 also shows top-view AFM images and cross-section profiles of mixed LS/AS monolayers at various compositions at the scale of $1 \times 1 \mu\text{m}$. The protruding objects were imaged clearly, and the number of such objects in the images was found to increase with increasing mole fraction of AS (X_{AS}). The cross-section images in Fig. 6 were almost the same, and the height of the protruding objects was estimated to be $2.9 \pm 0.5 \text{ nm}$, suggesting that they are composed mainly of AS molecules.

The possible structures of LS and AS were calculated by using the semiempirical molecular orbital method (CACH v. 6.1, PM5). According to the energy-minimized structure, LS forms a disk-like 3D structure, where the methylene moiety links the hydrophilic sugar groups together and provides a hydrophobic face at the opposite site (0.8 nm in height), as illustrated in Fig. 1(a). On the other hand, AS shows a linear structure having a hydrophobic tail and a hydrophilic head like general surfactants (2.8 nm in height), as illustrated in Fig. 1(b). The height of the protruding objects was in good agreement with the calculation of the height of the AS molecules, indicating that the protruding higher objects are composed mainly of AS mole-

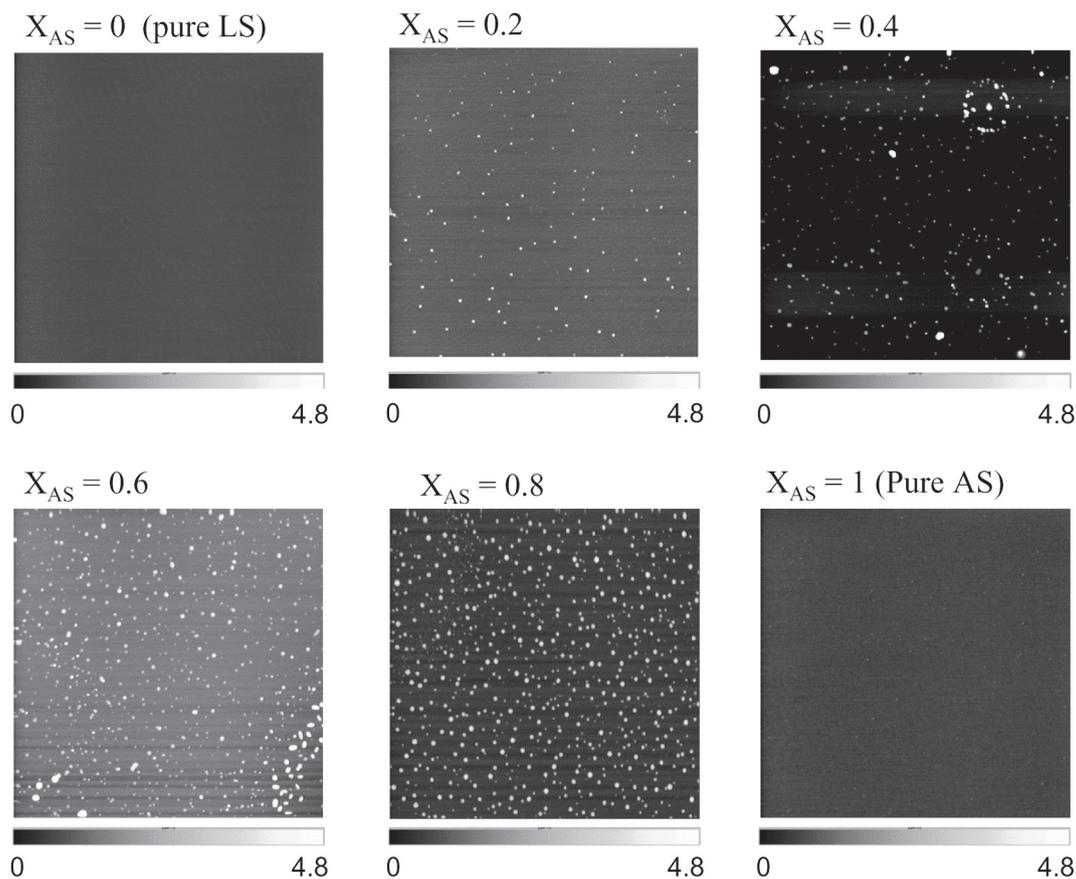


Fig. 5 Top-view AFM images of mixed AS/LS LB films deposited at 20 mN/m. The size of these images is $5 \mu\text{m} \times 5 \mu\text{m}$ in lateral, 4.8 nm in vertical.

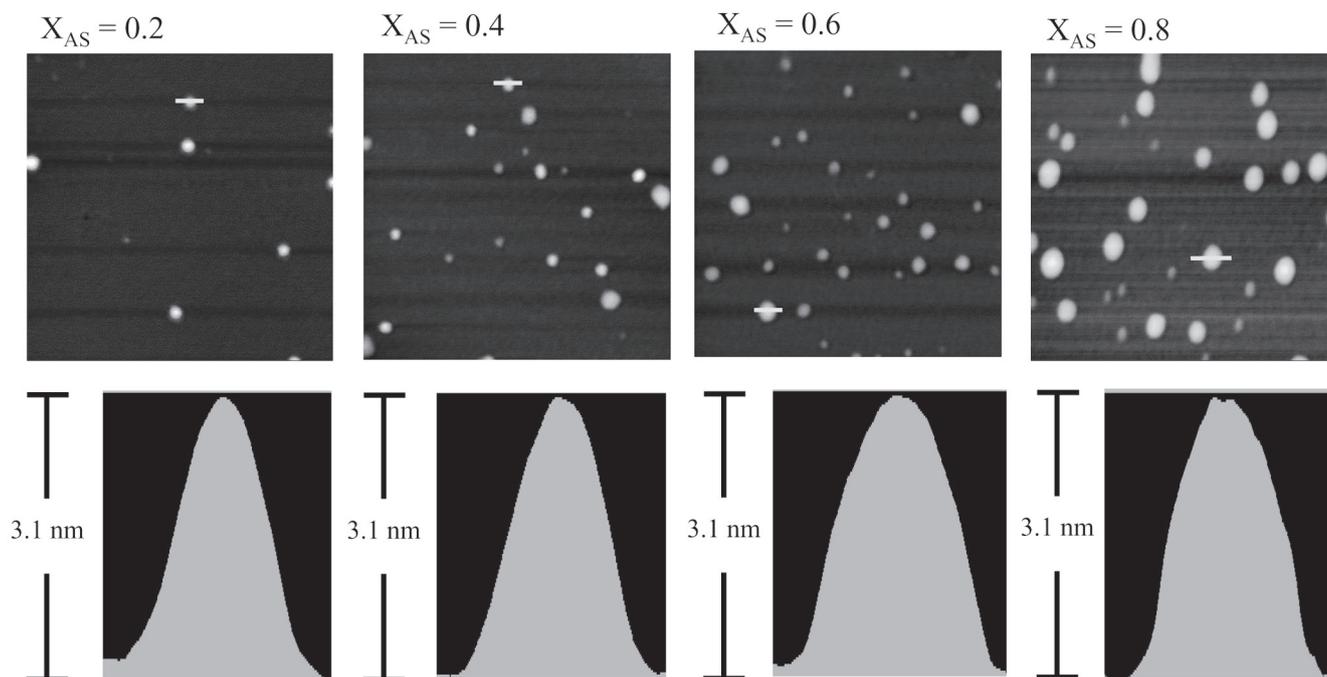


Fig. 6 Top-view AFM images and cross section profiles along the corresponding line in the images of mixed AS/LS LB films deposited at 20 N/m. The size of these images is $1 \mu\text{m} \times 1 \mu\text{m}$ in lateral, 4.8 nm in vertical.

cules. The distinctive bolaform structure of AS may give another AS-rich monolayer on the LS/AS monolayer.

To our knowledge, this is the first AFM observation of a naturally occurring mixture of microbial products. Our results clearly demonstrate that AFM together with the Langmuir technique is useful for exploration of the miscibility and synergic effects of microbial products.

4 CONCLUSION

The π - A isotherm of a pure LS monolayer showed a phase transition from a liquid expanded monolayer to a liquid condensed monolayer, while that of an AS monolayer gave a liquid condensed monolayer, suggesting that the lactonization of SL makes the molecules more bulky and prevents them from forming a close-packed arrangement.

The plots of mean area per molecule of mixed LS/AS monolayers gave positive deviations from the ideal curves, which means that LS and AS molecules are miscible. Interestingly, the positive deviation of excess area (A_{ex}) from ideality was most significant at the mole fraction (X_{AS}) of 0.3, which closely resembles the composition of the LS/AS mixture produced from *S. bombicola* in culture.

The AFM images of mixed LS/AS monolayers transferred at 20 mN/m showed no phase-separated microdomain structures, but rather, small protruding objects at all compositions, indicating that LS and AS are partially miscible, as predicted from the positive deviations from the ideal

curves. The cross-section analysis of the AFM images shows that the observed protruding objects are an AS-rich monolayer formed on the LS/AS monolayer.

Our results demonstrate that the appropriate mixing of LS and AS molecules influences their molecular interactions effectively and makes the molecular packing looser, especially at $X_{AS} = 0.3$. The largest mean area per molecule at this composition is likely to give the lowest CMC and γ_{CMC} values of naturally occurring microbial products of an LS/AS mixture.

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