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Deposition of Langmuir–Blodgett alternating bilayers of valinomycin and chemically modified bipolar lipid from archaea

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Abstract

Langmuir–Blodgett films of valinomycin appear to be unstable when kept in normal conditions. To prevent recrystallization of the valinomycin, alternating bilayers of this compound and barium salt of disuccinilated glycerol-dialkyl-glycerol tetraether (DS-GDGT) produced from lipid extract of thermophilic archaeon *Sulfolobus solfataricus* have been deposited. Optical and electron microscopy observations show that the film morphology does not deteriorate for at least three months after deposition. Study of the structure by the X-ray small-angle diffraction technique proves the suggested alternation of valinomycin and lipid bilayers. The period in the direction normal to the substrate plane equals 5.34 nm while the period of the DS-GDGT barium salt multilayer is equal to 3.97 nm. The thickness of the valinomycin monolayer of 0.68 nm is determined from these data. To clarify some structural peculiarities of the prepared films, alternating bilayers of valinomycin and barium behenate have been deposited and studied as well.

Keywords: Alternating bilayers; Bipolar lipids; Valinomycin; Langmuir–Blodgett

1. Introduction

Valinomycin (Fig.1) is a cyclic dodecadepsipeptide which has been widely investigated because it selectively transports potassium ions across biological membranes [1–3]. In principle, such a property may be used to produce potassium ion sensors [4,5]. Valinomycin forms an insoluble monolayer at the air–water interface [6,7] and can be deposited onto solid substrates by Langmuir–Blodgett (LB) technique [8,9], although valinomycin molecules possess a ring-shaped structure unlike that of normal surfactant molecules. The LB technique is a promising way of fabricating highly ordered thin films for sensor applications [9]. Monolayers of valinomycin equimolar mixtures with cholesterol and with stearic acid on the water surface and on the substrates have been studied as well [6], and a considerable decrease of the average area per molecule with respect to that calculated from the data for components of the mixtures was discovered. The authors propose different explanations, but "squeezing" the ionophore out of the monolayer seems to be highly probable at such high concentrations of the valinomycin molecules. Howarth et al. [10] report on the structure of mixed valinomycin/arachidic acid monolayers at the air–water interface and multilayers on the substrates at different mole fractions of acid in the mixture. Analyses of compressibility of the monolayers and small-angle X-ray diffraction curves for LB films indicate the possible miscibility of the two components at low concentrations of the ionophore. However, over most of the composition range the film probably consists of aggregates of each compound. Transfer of the mixtures of different phospholipids and fatty acids with valinomycin by LB technique onto platinum electrodes was accomplished by Fare et al., and the impedance response to potassium was measured [11].

Attempts to repeat the results of Ref. [9] in our laboratory were successful. Our conclusions coincide completely with those reported in that paper. However, films of valinomycin appear to be unstable when keeping the samples under normal room temperature conditions. In the best case the morphology

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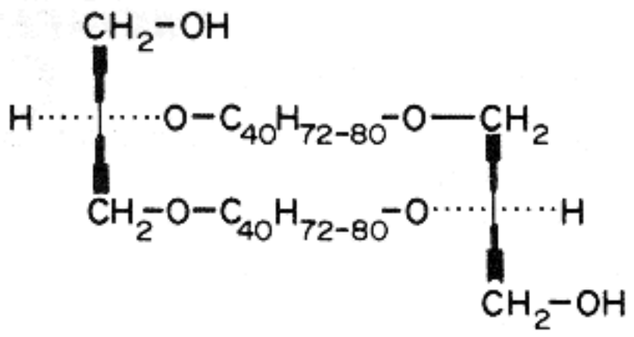
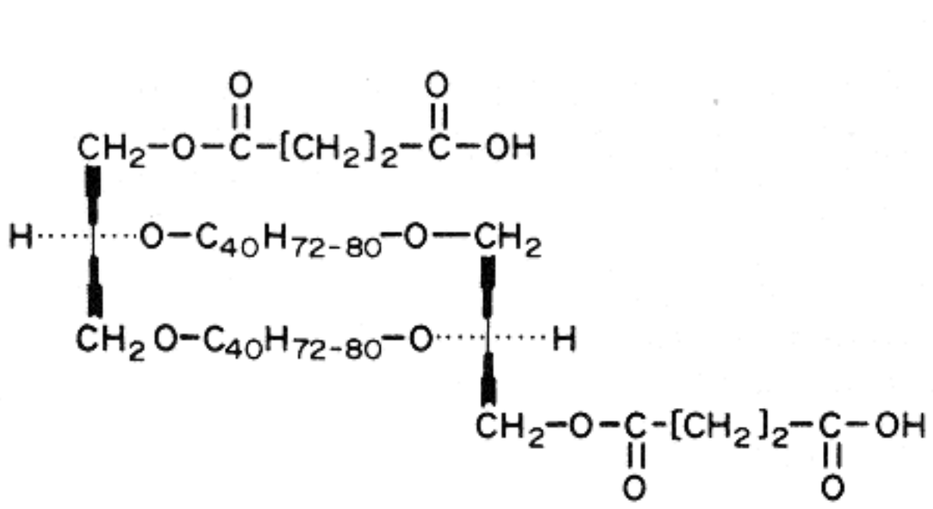
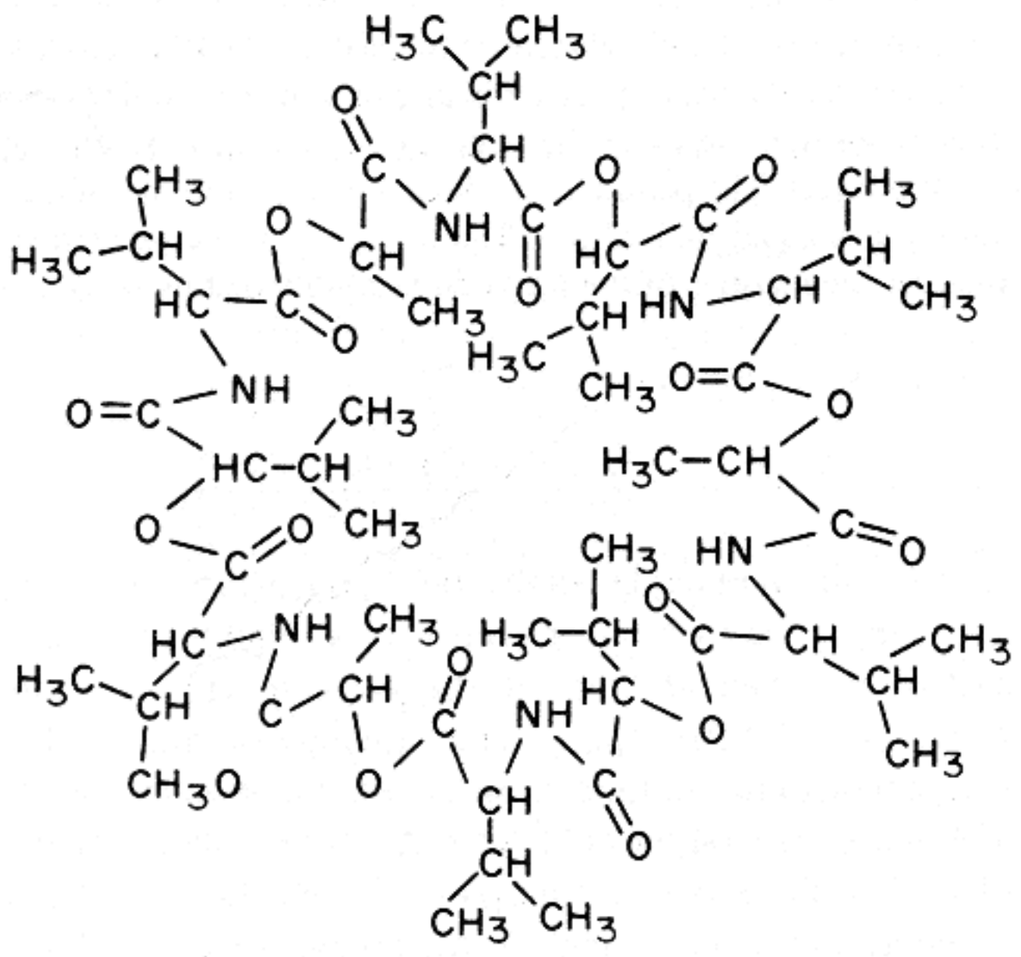


GDGT



DS–GDGT



valinomycin

Fig. 1. Hydrolized lipid GDGT, its synthetic disuccinilated derivative DS-GDGT and valinomycin used for deposition of alternating bilayers.

of the multilayer deteriorates in one week. Bulky aggregates arise, and the film becomes non-uniform and non-continuous. Although electrical measurements may be carried out, the application of such films when used in sensing devices is questionable. Then, following the strategy of Ref. [10], valinomycin mixtures with barium salts of behenic acid and disuccinilated glycerol-dialkyl-glycerol tetraether (DS-GDGT) (Fig. 1) produced from lipid extract of thermophilic archaeon *Sulfolobus solfataricus* were deposited to improve the film stability. But valinomycin bulk precipitates always arise if a considerable concentration of the latter is used in the mixture.

These experiments led us to the idea of alternating LB layer deposition [12,13] to prevent the recrystallization of valinomycin, i.e. we tried to insert some durable molecular layers between those of valinomycin to make the film more stable. Such an attempt has been realized for alternating bilayers of valinomycin and barium salt of DS-GDGT. On the one hand, valinomycin bilayers interacting with stable lipid bilayers lose the possibility of forming bulk aggregates, while on the other hand we achieve a considerable concentration of the ionophore molecules in the film, which can be important to increase the sensor effect. The motivation for using the derivative of bipolar lipid from archaea [14] as the matrix for valinomycin molecules is the unusual architecture of these molecules and the structure of the biological archaeol membranes, which play a key role in the survival of the extremophilic micro-organisms at high temperatures [15–18]. For example, the archaeon *S. solfataricus* grows at a temperature of about 87 °C and pH 3. Since the LB films of such compounds are expected to be stable up to high temperatures we may suppose also that our artificial systems created for sensor applications will possess increased thermal stability.

LB films of alternating bilayers mentioned above have been studied by X-ray small-angle diffraction, optical microscopy and electron microscopy techniques. Alternating bilayers of valinomycin and barium behenate have been deposited and studied as well for better understanding of the structural peculiarities of the films.

2. Experimental details

Non-substituted glycerol-dialkyl-glycerol tetraether (GDGT) (Fig. 1) was produced from total lipid extract as reported by De Rosa et al. [18]. GDGT was used for synthesis of the lipid DS-GDGT.

To obtain the DS-GDGT lipid, the succinic anhydride (654 mg) was added to the solution of GDGT in pyridine (170 mg in 5 ml). The mixture was stirred at room temperature for 12 h and the reaction was monitored by thin-layer chromatography (TLC) on a 0.25 mm layer of silica gel 60 F_{254}, Merck (R_F 0.7 for GDGT in chloroform). The mixture was acidified with 20 ml of aqueous 0.05 M HCl and extracted with three portions of ethyl ether. The extract was washed with water until it became neutral and was dried under reduced pressure. The product was purified by chromatography on a silica gel RP 18 column (Lobar, Merck 30×1.5 cm) with chloroform–methanol (1:1 v/v) as eluent and dried in vacuo yielding 68 mg (35%). It showed one spot with R_F 0.30 on TLC in the solvent system chloroform–methanol (9:1 v/v). The ^{13}C NMR spectrum shows one signal of methine carbons at 76.48 p.p.m. and one signal of C-1 *sn* carbons at 64.44 p.p.m.

Behenic acid and valinomycin were obtained from Sigma Chemical Co. Solutions of behenic acid in benzene (0.5 mg ml^{-1}), valinomycin in chloroform (0.5 mg ml^{-1}) and DS-GDGT in chloroform–hexane (1:2 v/v, 0.23 mg ml^{-1}) were prepared to spread the compounds at the air–water interface.

Deposition was carried out by using an LB-MDT system (Russia) with the trough consisting of two separated sections and with the possibility of automatic substrate translation from the first section to the second one. Water first distilled with an AQUATRON A4S distiller and then deionized with a UHQ-II ELGASTAT system was used for preparation of the subphases. Valinomycin was deposited from the surface of pure water. Barium salts of the DS-GDGT and of the behenic acid were both deposited using a buffered subphase of pH 7.0 and barium acetate concentration of 10^{-4} M. Monolayers of barium behenate, barium salt of the DS-GDGT and valinomycin were compressed at speeds of 0.8×10^{-4}, 4.2×10^{-4} and 6.9×10^{-4} nm^2 $molecule^{-1}$ s^{-1} up to the values of surface pressure of 29, 27 and 23 mN m^{-1} respectively. The deposition was carried out at a speed of 0.08 mm s^{-1}. The sequence of monolayer alternation was AAB-BAABB..., where B is a monolayer of barium behenate or DS-GDGT barium salt and A is a monolayer of valinomycin. The films composed of 40 monolayers, i.e. of 10 periods in the direction normal to the layer plane, were deposited on the hydrophobic silicon substrates covered by two monolayers of barium behenate for X-ray small-angle diffraction and optical microscopy studies. Twenty eight monolayers, i.e. 7 periods, were deposited onto collodion films 10–20 nm thick covered by three monolayers of barium behenate for electron microscopy study. Multilayers of valinomycin and barium salt of the DS-GDGT were also deposited separately under the same conditions to compare the morphology of different films by optical and electron microscopy techniques. Monolayers of valinomycin mixtures with DS-GDGT in the ratios of 1:2 and 1:3 were transferred from the surface of 10^{-4} M solution of barium acetate at pH 7.0 and at surface pressure of 23 mN m^{-1}. All other conditions were the same as described above.

The LB films were observed with a Carl Zeiss optical microscope AXIOSCOP at magnifications up to 1000. Transmission electron micrographs were recorded by a Philips CM-12 electron microscope at an accelerating voltage of 80 kV. X-ray small-angle diffraction patterns were measured by using an AMUR-K diffractometer with a position-sensitive detector.

3. Results and discussion

As stated above, valinomycin LB films are unstable and recrystallize in a rather short time. The electron micrograph in Fig. 2(a) illustrates the deterioration of film morphology, which has been recorded approximately one month after dep-

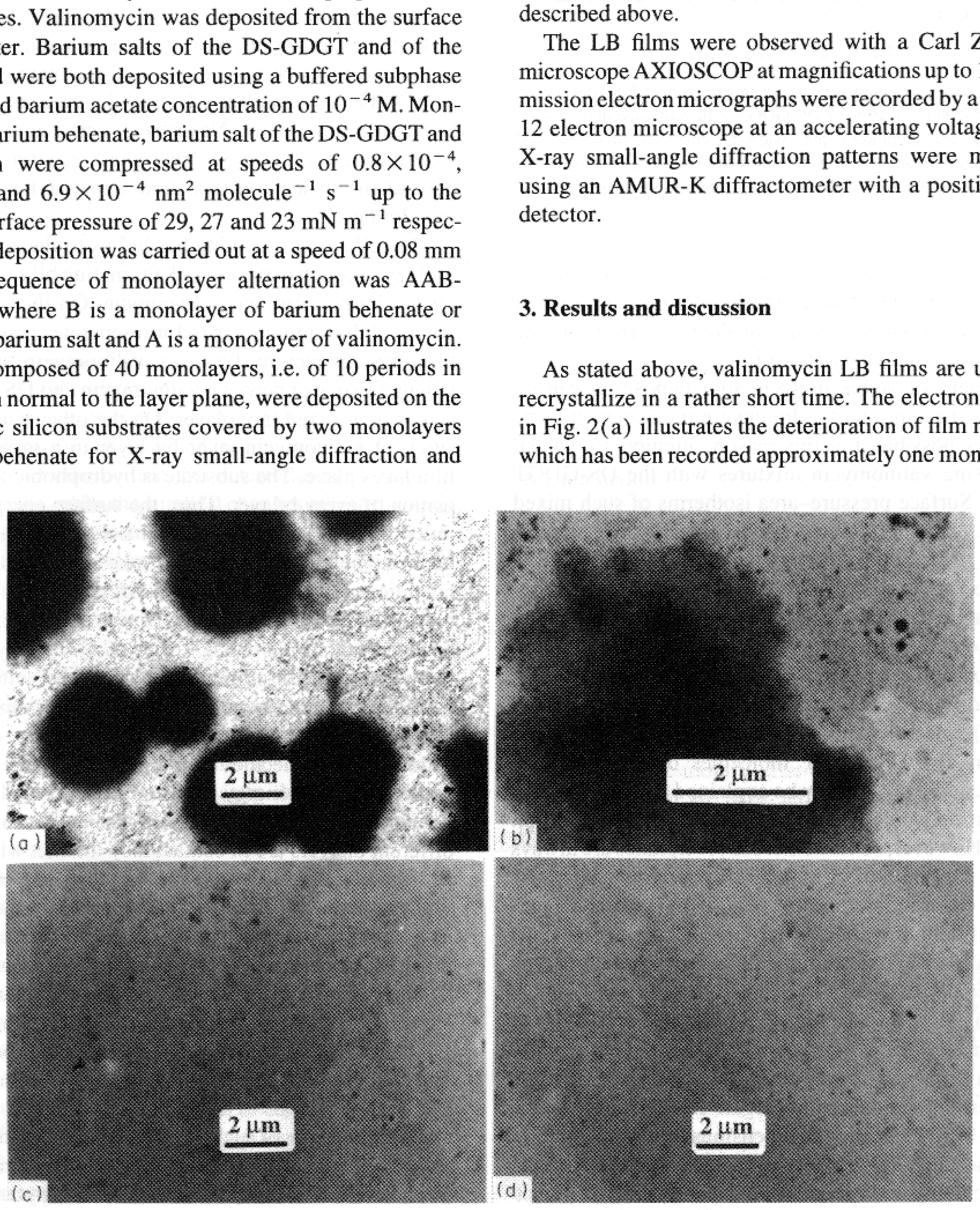


Fig. 2. Transmission electron micrographs of the LB films deposited on collodion substrates covered by three monolayers of barium behenate: (a) valinomycin after recrystallization; (b) valinomycin mixture with DS-GDGT in a molar ratio of 1 to 2; (c) barium salt of DS-GDGT; (d) alternating bilayers of valinomycin and barium salt of DS-GDGT.

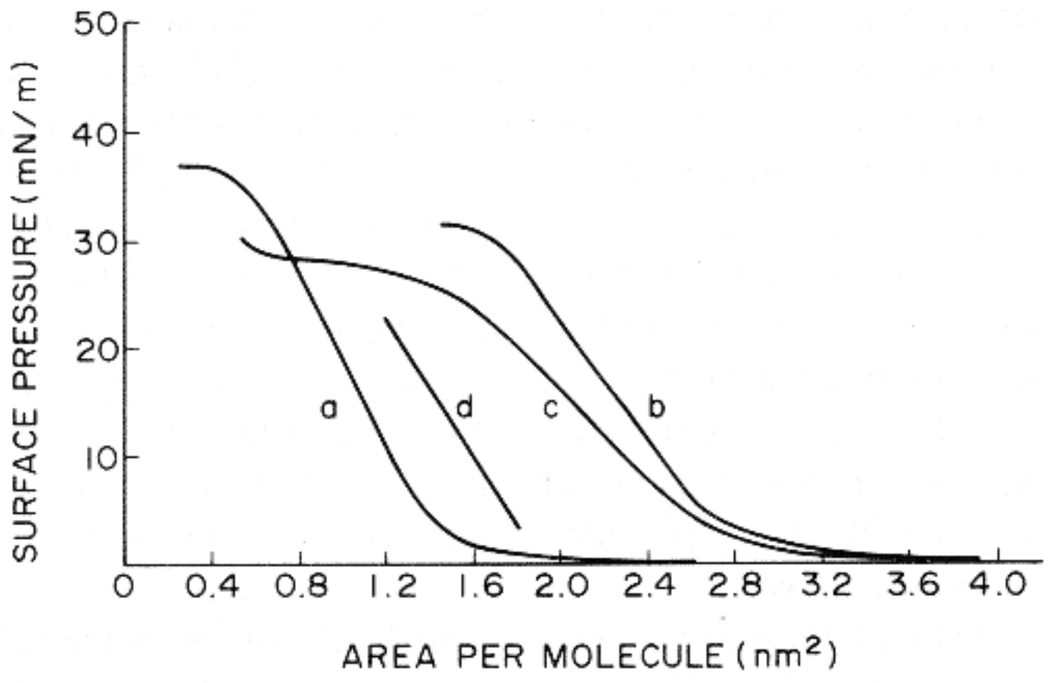


Fig. 3. Surface pressure–area isotherms of monolayers of DS-GDGT (a), valinomycin (b), and a mixture of DS-GDGT with valinomycin in a molar ratio of 2:1 (c). The subphase is pure water for curve (b) and a 10^{-4} M solution of barium acetate at pH 7.0 for curves (a) and (c). The solid line (d) is the calculated position of the isotherm of the mixed monolayer.

osition. A similar morphology was also observed by optical microscopy. At the beginning the film was quite uniform if we exclude the gradual decrease of thickness from the top end of the substrate to the bottom [9].

The problem of stable uniform film deposition can be solved if the dissolution of valinomycin molecules in some monolayer is possible. For this reason, attempts to deposit LB films using valinomycin mixtures with the DS-GDGT were made. Surface pressure–area isotherms of such mixed monolayers (Fig. 3) allowed us to hope for the complete dissolution of valinomycin molecules in the DS-GDGT matrix up to very high concentrations. Indeed, a strong non-additive change of the surface pressure–area isotherm of the mixed monolayer with respect to the isotherms of valinomycin and barium salt of the DS-GDGT could be caused just by dissolution. Curve (c) is shifted to the right from the ideal position (d) calculated on the assumption of fixed areas per valinomycin and DS-GDGT molecules determined from curves (a) and (b). This may be explained by the change of valinomycin molecule orientation caused by dissolution in the matrix. However, some numerous precipitates are always detected in the mixed film immediately after deposition by optical and electron microscopy (Fig. 2(b)) whereas monocomponent valinomycin and DS-GDGT barium salt films are practically ideal. An example of an electron micrograph of a monocomponent DS-GDGT barium salt film is shown in Fig. 2(c). Thus we conclude that these are precipitates of valinomycin. Further confirmation of valinomycin precipitation is an observed strong point-like electron diffraction pattern from the crystalline aggregate in Fig. 2(b), which looks like a big dark spot with dimensions of several microns. At the same time, LB films of DS-GDGT barium salt are always practically amorphous in the layer plane. The dimensions of crystalline aggregates of valinomycin vary approximately from 0.5 to 10 μm, but large aggregates consist of numerous arbitrarily oriented microcrystals. It seems that there is no phase separation of the two components in the areas of the film between these aggregates because electron diffraction patterns from such places show no Bragg reflections. However, that is not sufficient evidence, since the valinomycin phase may possess poor crystalline order as well. In spite of a contradiction between the data on surface pressure–area isotherms and those on optical and electron microscopy studies it became obvious after numerous repetitions of the experiments that uniform distribution of valinomycin at high concentration is impossible under such simple mixing of the compounds. This conclusion is in agreement with the results of Howarth et al. [10]. Quantitative data and especially the character of phase separation seem to be different in the two cases because the crystalline matrix of arachidic acid has been used in Ref. [10], while in our experiments the matrix was amorphous. Nevertheless, the same tendency for such different films shows that phase separation may be a general rule when trying to find a matrix for valinomycin dissolution.

The following reasoning has led us to the solution of the problem through deposition of alternating bilayers of the DS-GDGT barium salt and valinomycin. On the one hand, the ability of the valinomycin to form separate crystallites shows that the interaction between valinomycin molecules is stronger than that between valinomycin and DS-GDGT molecules in a mixed monolayer. On the other hand, the deposition of valinomycin layer by layer in a monocomponent film takes place. The substrate is hydrophobic after the deposition of every bilayer. Thus, the surface energy of valinomycin bilayer is rather low, and a rather strong interaction between the molecules inside it takes place. Under just such conditions is the formation of a uniform layer on the substrate by the LB technique possible. However, in reality this interaction is not strong enough to provide stability of thin valinomycin films for a long time. In contrast with the case of valinomycin, thin films of the DS-GDGT barium salt are quite stable, in particular, due to the salt formation, i.e. the interaction of the molecules inside each bilayer is very strong, while the surface energy is approximately equal to that of the valinomycin bilayer. When the hydrophobic surfaces of two different bilayers are in contact they interact with each other and the boundary between the bilayers will be the surface with approximately zero surface energy. Thus, after film deposition the reasons for recrystallization of each separate bilayer disappear and only the surface energy of the external boundary may cause a recrystallization of the film as a whole. However, to do that is difficult because strong bilayers of DS-GDGT barium salt are inserted into the multilayer. For this reason, the structure of alternating bilayers of the two compounds appears to be stable.

The realization of such an idea has resulted in uniform and stable films with a high content of valinomycin molecules. Studies of the films by electron microscopy and X-ray diffraction techniques have been carried out after film deposition and repeated after approximately one month and a month and a half by the first and the second techniques respectively.

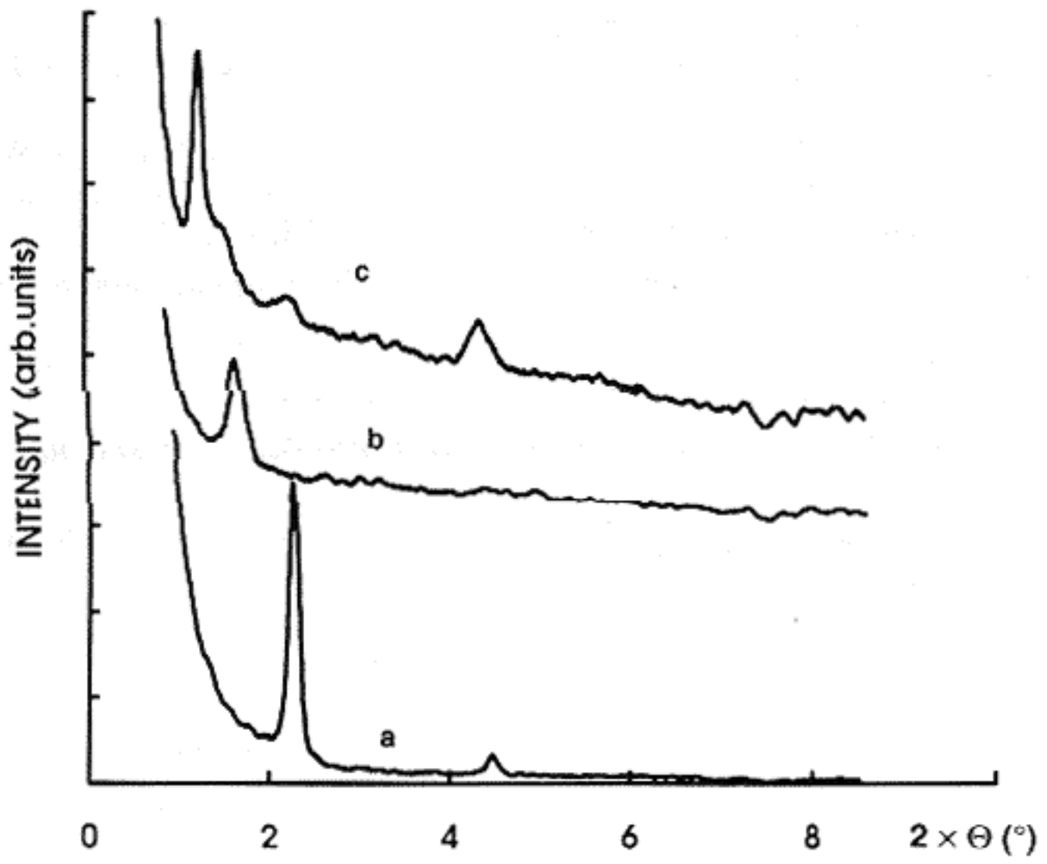


Fig. 4. X-ray small-angle diffraction curves of the LB films deposited on silicon substrates covered by two monolayers of barium behenate: (a) barium salt of DS-GDGT; (b) alternating bilayers of valinomycin and barium salt of DS-GDGT; (c) alternating bilayers of valinomycin and barium behenate.

These studies show no changes of either morphology or structure. The electron micrograph in Fig. 2(d) and the X-ray small-angle diffraction curve (b) in Fig. 4 demonstrate this fact. The films were also checked by optical microscopy for three months after deposition. No deterioration of morphology was detected, while the smallest changes of film thickness of about several nanometres could be observed due to the intensive interference colour of the film deposited on a silicon substrate at a magnification of up to 1000.

The diffraction curves in Fig. 4 show periods of 3.97 ± 0.03 nm and 5.34 ± 0.05 nm for the film of DS-GDGT barium salt and for the film of alternating bilayers respectively. No Bragg peaks corresponding to the separate phase of the DS-GDGT barium salt are detected in the second case. The thickness of the valinomycin monolayer calculated from these data is equal to 0.685 ± 0.040 nm, which is in the agreement with that reported by Ries and Swift [6] and by Howarth et al. [9].

To confirm the correctness of the valinomycin monolayer thickness determination, films composed of alternating bilayers of barium behenate and valinomycin were deposited and studied as well. The X-ray diffraction curve (c) in Fig. 4 shows a Bragg reflection with a period of 7.47 ± 0.05 nm corresponding to this structure. The period of the barium behenate multilayer is equal to 6.13 ± 0.05 nm. Thus, the thickness of the valinomycin monolayer equals 0.67 ± 0.05 nm, which coincides with the value presented above. However, the diffraction curve (c) in Fig. 4 shows also weak and broad reflections of a separate barium behenate phase. This can be understood easily because the diffraction pattern of the barium behenate multilayer displays very strong reflections at the 2θ values of 1.44, 2.88, 4.32, 5.76 and 7.20°. Detailed optical microscope observations of films composed of alternating bilayers of both types deposited on silicon substrates show small areas of defective deposition, which are detected due to slight variations of interference colour. The total area of such defects does not exceed 5% and the thickness decrease is not more than 5–6 nm, which can be roughly evaluated according to the changes of film interference colour. Taking into account the X-ray diffraction data and the thickness of the monolayer, one may reach the conclusion that these are the areas of defective deposition of valinomycin. Indeed, due to the absence of 2–3 valinomycin bilayers in some places a separate phase of another compound 6–8 monolayers thick will arise. Just such aggregates may result in broad and weak Bragg peaks in the diffraction pattern shown in Fig. 4. With very high probability the origin of defects in the films composed of alternating bilayers of valinomycin and DS-GDGT barium salt is the same. Bragg reflections corresponding to the DS-GDGT barium salt phase in the diffraction curve (b) seem to be absent because they are in principle considerably less intense than those of the barium behenate phase.

Finally, we would like to note that the LB films similar to those described above were used for the detection of sodium and potassium ions. Our preliminary measurements demonstrate a selective and reversible interaction with potassium ions. These results will be published separately.

4. Conclusion

We have deposited Langmuir–Blodgett alternating bilayers of valinomycin and barium salt of a derivative of lipid from archaea. The bilayers of the latter stabilize the film, preventing recrystallization of the valinomycin bilayers. The period of the structure in the direction normal to the substrate plane equals 5.34 nm. The thickness of the valinomycin monolayers included in the film is determined to be equal to 0.68 nm. No deterioration of the film morphology was revealed for at least three months after deposition. Films prepared in a similar manner are expected to be selectively sensitive to potassium ions.

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