Fibril-Like Aggregate Formation of Peptide Carboxylate Langmuir Films Analyzed by Surface Pressure, Surface Dipole Moment, and Infrared Spectroscopy

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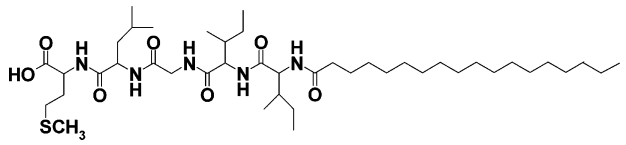
The fibril formation process of a synthetic peptidolipid compound in a Langmuir monolayer at the air−water interface has been analyzed by surface pressure and surface dipole moment-area isotherms, followed by infrared spectral analysis of related Langmuir−Blodgett films. Thus far, the analysis of randomly oriented molecular assemblies has been a difficult matter, especially for spectroscopic measurements. In the present study, the Langmuir film isotherms were discussed in detail, and they have readily been correlated to the infrared spectra. For the spectral analysis, infrared multiple-angle incidence resolution spectroscopy (MAIRS) was employed, which was compared to the results by conventional techniques. Since the peptide assemblies greatly responded to a metal surface, the reflection−absorption technique was not useful for our analysis. Instead, MAIRS was found to be powerful to reveal the anisotropic structure of the Langmuir films, and a disordered molecular architecture has been revealed via the molecular orientation analysis. As a result, the fibril-like aggregation formation process during the monolayer compression, which was suggested by previous topographical study, has been found to be due to the stiff domain formation in the Langmuir films.

Introduction

The architecture of molecular aggregation is an important chemical factor to understand material characteristics, which have a strong relation to the functions of the material. The cell membrane, for example, consists of a double layer of lipid molecular aggregates, in which the layer has a highly ordered fundamental architecture. The fluid mosaic model is based on this ordered molecular architecture, although the molecules have dynamic motions in the membrane.[1] Since the architecture can theoretically be discussed in relation to the material's functions, the ordered molecular structure attracts much interest.

The disordered structure in molecular assemblies is, on the other hand, of great importance, an example being the fibril formation of peptides. The peptide fibrils comprise peptide β-sheets.[2] Although the β-sheet itself has a highly ordered structure, a number of microdomains of the β-sheets randomly assemble to form the fibril, which has a disordered architecture as a whole. The role of the fibril in a biological system is one of the recent matters of bioscience, but very few have been revealed thus far. For example, abnormal disease-causing isoforms (prion peptides) are recognized to exhibit fibrillar molecular assemblies.[3] Although the biological functions and molecular roles of the isoforms are not known well enough, the precipitates of the molecular assemblies in the human body are believed to have strong relations with Alzheimer and BSE (Creutzfeldt−Jakob) diseases.

CHART 1: Chemical Structure of C_{18}−IIGLM−OH



To understand the biological function of the molecular assemblies, we have to reveal the molecular aggregation mechanisms for the fibril formation. The analysis of ordered molecular assemblies is a relatively easy task, while that of disordered systems is a difficult matter. In infrared spectral analysis, the ordered structure is reflected in the orientation angles of the molecules.[4−8] It is difficult, however, to prove the disordered molecular architecture by use of the conventional techniques of the orientation analysis since the discrimination between perfectly random molecules and the random aggregates of structured molecular domains is difficult.

In the present study, a synthesized peptide, C_{18}−IIGLM−OH (see Chart 1), which contains five amino acid residues that have a saturated hydrocarbon chain on the N-terminus of isoleucine and a carboxylic group on the C-terminus of methionine, has been used for the fibril formation analysis. In our previous paper,[9] the monolayer of this compound was analyzed in terms of topography using epi-fluorescence microscopy. The report revealed that fibril-like isoforms were generated by compression of the monolayer, but it was difficult to follow the process of the isoform formation. Therefore, in

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this study, the analysis in more detail of the fibril of C_{18}−IIGLM−OH that has disordered molecular architecture has been performed.

To do that, the Langmuir film has been analyzed by measuring the hysteresis isotherms[10] and the surface dipole moment against the molecular surface area. This analysis makes it possible to discriminate whether the monolayer is collapsing or forming fibril-like molecular aggregates. Following the interfacial analysis, the monolayer was transferred onto a solid support using the Langmuir−Blodgett (LB) film deposition technique,[11] and it was subjected to the infrared transmission, reflection−absorption (RA),[8] and multiple-angle incidence resolution spectrometry (MAIRS).[12−14] Among them, infrared MAIRS was found to be the most suitable for the analysis of the disordered molecular assemblies since the metal substrate for the RA analysis greatly influenced the monolayer architecture.

One of the benefits of the infrared MAIRS technique is that the disordered structure can be analyzed by calculation of the orientation angle. The analytical results by MAIRS were consistent with those of Langmuir films, which revealed a fibril formation process at the air−water interface.

Experimental Procedures

For the synthesis procedure of C_{18}−IIGLM−OH, the reader is referred to a previous paper.[9]

The pure water was obtained using a Millipore (Molsheim, France) Elix UV-3 pure-water generator, which was subjected to a Yamato (Tokyo, Japan) Autopure WT100U water purifier equipped with a microfilter (Millipak-40) having micropores of 0.22 μm to remove unexpected organic contaminants. The resistivity of the pure water was 18.2 MΩ cm or higher with a surface tension of 72.8 mN m^{-1} at 25 °C.

The peptidolipid compound (C_{18}−IIGLM−OH) was dissolved in a mixture solvent of chloroform and methanol (5:1 v/v) at a concentration of 0.55 mg mL^{-1}. A volume of 20 μL was spread on the water surface (18 °C) using a microsyringe. The monolayer formed by Langmuir adsorption on water was compressed by a barrier at a compression rate of 5×10^{-2} nm^2 molecule^{-1} min^{-1}. The surface pressure−area (π−A) isotherm was measured by a USI System (Fukuoka, Japan) FSD-220 Langmuir trough equipped with a paper Wilhelmy plate. The measurements of the surface dipole moment of the monolayer were performed by measuring the surface potential (ΔV)[15] using an ionizing electrode (^{241}Am) against a reference electrode (Ag/AgCl). The surface potential was converted to the perpendicular surface dipole moment[16] using eq 1.

$$\mu_{\perp} = \frac{A\Delta V}{12\pi} \tag{1}$$

where $\mu_{\perp}$ is the perpendicular dipole moment (calculated in Debye); A is the surface area, and ΔV is the measured surface potential (volts).

With the same Langmuir trough, the monolayer was transferred onto a germanium (Ge) substrate using the LB technique for the FT−IR transmission and MAIRS analyses. In a similar manner, the monolayer was deposited onto a gold-evaporated glass substrate for the FT−IR RA measurements. The surface pressure for the LB deposition was 33.0 ± 0.3 mN m^{-1}, and the withdrawing speed of the substrate for the transfer was 5.0 mm min^{-1}.

The FT−IR RA spectra were obtained with the use of a Harrick Scientific Co. (Ossining, NY) Versatile Reflection Attachment (VRA-NIC), and the angle of incidence was fixed at 80° from the surface normal with a wire-grid infrared polarizer to eliminate the *s*-polarization. The FT−IR transmission and MAIRS measurements were performed with the use of a Harrick Scientific Co. Brewster's Angle Sample Holder (BXH-S1G). For the MAIRS measurements, the angle of incidence was changed manually from 10 to 45° with an increment of 5°. This technique requires no polarizer.[12] The collection of infrared transmission single-beam spectra was performed on a Thermo-Electron Nicolet (Madison, WI) Magna 550 FT−IR spectrometer equipped with a mercury−cadmium−telluride (MCT) liquid N_2 cooled detector with the aperture fully opened.[13] The laser modulation frequency for the interferogram collection was 60 kHz. The interferogram was accumulated 2000 times to improve the signal-to-noise ratio.

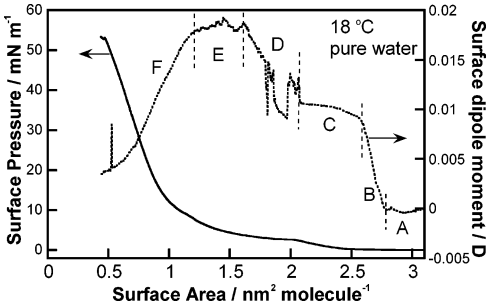

Figure 1. Surface pressure and surface dipole moment isotherms of the C_{18}−IIGLM−OH monolayer on pure water at 18 °C against the surface area of the monolayer.

Results and Discussion

The surface pressure and surface dipole moment-area isotherms of a C_{18}−IIGLM−OH monolayer were measured at 18 °C on pure water as presented in Figure 1 by the solid and dotted lines, respectively. When the monolayer was compressed from a surface area of 3.1 nm^2 molecule^{-1}, the surface dipole moment exhibited a constant value (region A) until the area was decreased to ca. 2.7 nm^2 molecule^{-1}. On further compression, the surface dipole moment sharply increased up to near 0.01 D (region B), and the slope of the change became small below 2.5 nm^2 molecule^{-1}. When the sharp increase of dipole moment stopped, the surface pressure began to increase gradually (region C). Note that this surface pressure increase is simultaneously observed with that of the gradual change of the surface dipole moment.

The change of the surface dipole moment reflects the change of orientation of a chemical group with a large dipole. In the present case, the C═O bond would correspond to the large dipole group. Therefore, the sharp increase of the surface dipole while the change of the surface pressure is negligible suggested that a drastic orientation change of the C═O groups occurred in the monolayer during compression. A large change of the orientation suggests that the molecules have room for the orientation change. Following the orientation change, therefore, the molecules would lose the room for any orientation change, in which pressure relaxation would not be possible against the compression. In this manner, the synchronization of the increase of the surface pressure and the stop of the surface dipole moment is understandable.

Below the surface area of 2.0 nm^2 molecule^{-1}, the increase of the surface dipole moment began again, and the surface pressure increase was suppressed at the same time (region D). It is of note that the surface dipole moment-area isotherm in this region showed instability. Because the position and intensity of the unstable changes were not reproduced, the change is

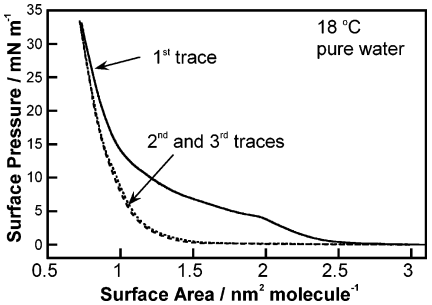

Figure 2. Surface pressure−area isotherms of an identical C_{18}−IIGLM−OH monolayer repeatedly measured 3 times (hysteresis isotherms).

interpreted as due to the formation of domains in the Langmuir films. This region was always accompanied a similar fluctuation for every measurement of the isotherm. These experimental results suggest that the monolayer formation process is not smooth and that the island−sea structure via domain formation appears on the water surface. During the island−domain formation process, the molecules are considered to be deeply associated with each other by hydrogen bonding. This association process would be encountered with the monitored increase of the surface dipole moment.

In a previous study,[9] the inhomogeneous surface structure was monitored for the same compound by epi-fluorescence microscopy as the monolayer was compressed. The inhomogeneous structure was explained by molecular aggregation that forms amyloid-like fibril structures. The fibril aggregates remind us of the collapse of the monolayer, but no further details have been given thus far.

The increase of the dipole moment finishes at about 1.6 nm^2 $molecule^{-1}$, and the slope of the surface pressure−area isotherm becomes larger (region E), which suggests that molecular association has already finished and that molecular packing is on the process.

In the final region (region F), the surface pressure increases sharply, while the surface dipole moment decreases monotonically. According to Li et al.,[9] the decrease is caused by molecular close packing since the surface dipole moment is a measure of the dipole−dipole interaction, which is a relatively long distance interaction in comparison to van der Waals force. Therefore, in region F, the molecules are highly packed by the compression, but no change in molecular association and orientation is expected.

According to Li et al.,[9] the fibril-like aggregates appear in a region that corresponds to the regions of E and F in the present study. This suggests that the linear change (solid-film like $\pi-A$ isotherm) in region F does not correspond to the solid film but to the compression of domains. If the domains are formed by fibril-like structures, compression−decompression cycle would result in a decrease of the surface area of the traces.

The results of the compression−decompression cycle of the monolayer are presented in Figure 2. When the surface pressure attained 33.0 mN m^{-1}, the monolayer was decompressed until the compression barrier went back to the starting position. Then, the second compression was performed as presented by the dotted line (second trace). It is found that the plateau region has disappeared and that the limiting molecular area is decreased. It is of note, however, that the second trace readily gets back to the returning point (33.0 mN m^{-1}) with no decrease of the surface area. This suggests that the domains are truly fibril-like structures that are stable after compression−decompression (hysteresis) of the monolayer. Since further cycles were performed and there is no change with regard to the second cycle, we can say that following the first cycle, the fibril-like structures formed were stable. In other words, the fibril-like structure in the monolayer is different from the collapsed film, and they have a specific structure that has been formed in the monolayer.

This discussion has been confirmed by performing the third compression of the identical monolayer. The trace of the third compression is plotted by the dashed line in Figure 2 (third trace). It is of surprise that the second and third traces are almost identical to each other and that the returning point has again been reproduced. Therefore, it can be concluded that the fibril-like structure has a stable topography and that the conformational changes occurring in the first compression yielded stiff molecular aggregates.

Since the molecule has a unique three-dimensional chemical structure, in which the directions of the amide groups and the residues of amino acid are perpendicular to each other,[17,18] the amide groups would readily form intermolecular hydrogen bonding. If the hydrogen bonding is formed, a stiff molecular aggregate would make the monolayer stable.

To investigate the hydrogen bonding formation, the monolayer was transferred onto a germanium substrate and a gold substrate at 33.0 mN m^{-1}, and the single-monolayer LB films were subjected to infrared transmission and RA spectrometries, respectively. It would be ideal that another LB film at a lower surface pressure would be subjected to the spectroscopic analysis. The molecular density of the LB film prepared at the lower surface pressure, however, is too low for the monolayer analysis by infrared spectroscopy, and only the LB film at 33.0 mN m^{-1} was studied.

In Figure 3, it is striking that the amide I band that is mainly due to the C═O stretching vibration appeared in the transmission spectrum at 1632 cm^{-1} and that it is largely split to give a high-

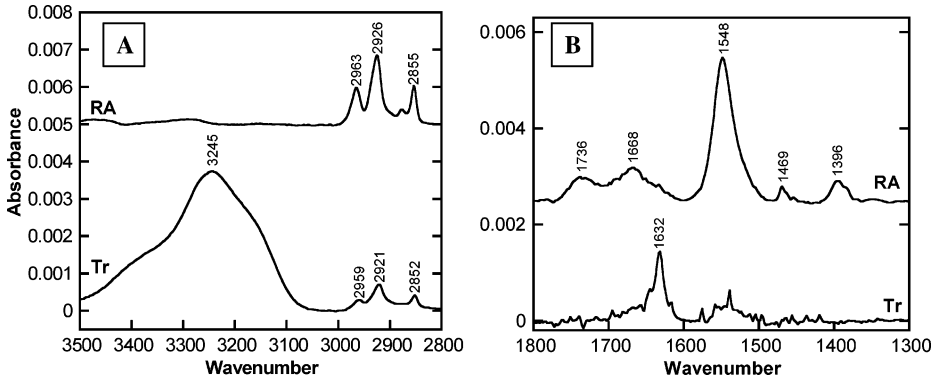

Figure 3. FT−IR transmission (Tr) and RA spectra of the C_{18}−IIGLM−OH monolayer LB film. Ge and gold surfaces were used for the Tr and RA measurements, respectively. (A) 2800−3500 cm^{-1} and (B) 1300−1800 cm^{-1}.

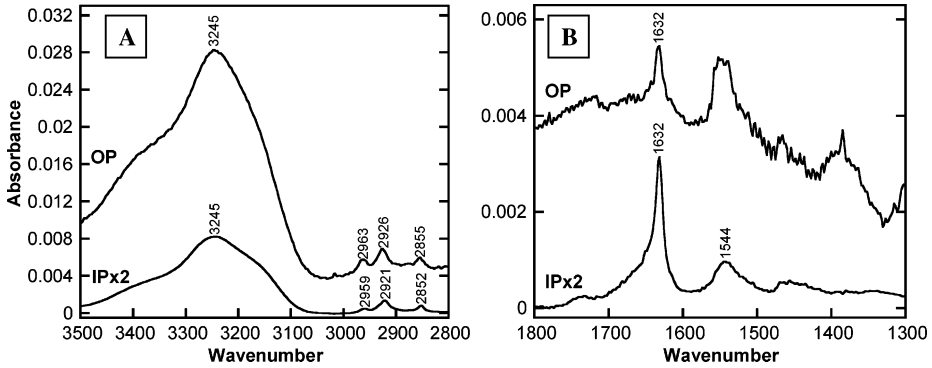

Figure 4. FT−IR MAIRS spectra of the C_{18}−IIGLM−OH monolayer LB film deposited on a Ge substrate. (A) 2800−3500 cm^{-1} and (B) 1300−1800 cm^{-1}.

wavenumber broad band in the RA spectrum at about 1700 cm^{-1}. The band location in the transmission spectrum suggests that the molecules form the parallel β-sheet structure,[17] which is reasonable.[10] The high wavenumber bands in the RA spectrum, on the other hand, suggest that the parallel β-sheet structure is largely damaged and that a portion of the amide groups are free from the hydrogen bonding. In conclusion, it is suggested that the monolayer structure greatly responds to the metallic surface that is used for the RA measurements. In other words, it is impossible to prepare the same monolayer on the two surfaces.

Another interest with the conventional technique is that the significantly strong amide A band at 3245 cm^{-1} in the transmission spectrum is silent in the RA spectrum. This is consistent with that the amide I band due to the parallel β-sheet is also silent in the RA spectrum. In addition, the band shifts are found for the C−H stretching vibration bands (2800−3000 cm^{-1}), which will be discussed with MAIRS spectra.

Since it has been found that the conventional transmission/RA technique has an analytical limitation,[10] the infrared MAIRS technique has been employed to obtain both in-plane (IP) and out-of-plane (OP) spectra for the single-monolayer LB film. The spectra are presented in Figure 4. It is found that the amide I band (mainly due to the C═O stretching vibration) at 1632 cm^{-1} is obtained as a singlet band in both IP and OP spectra, which means that the MAIRS technique is good for the analysis of the β-sheet structure without damaging the monolayer structure. With the MAIRS spectra, the orientation angle of the C═O groups is calculated by use of the following equation:[9]

$$\varphi = \tan^{-1}\sqrt{2A_{IP}/A_{OP}} \qquad (2)$$

The orientation angle has been calculated to be 54° from the surface normal. This angle corresponds to the magic angle, which has a perfectly unoriented state.[6] Therefore, the analytical angle suggests that the parallel β-sheets are randomly oriented in the monolayer. Since the β-sheet itself has an ordered molecular structure, the random orientation means that the β-sheet units are randomly oriented.

On the other hand, the orientation angle of the amide A band (mainly due to the N−H stretching vibration mode; 3245 cm^{-1}) is of interest. This band position is assigned to the hydrogen bonded N−H group, and it is reasonable that the N−H group plays a role of the counterpart of the hydrogen bonding with the C═O group in the parallel β-sheet. The orientation angle of this mode has been calculated by use of eq 2 to be 30°. This analytical angle is largely different from that of the amide I band, although the N−H and the C═O groups are hydrogen bonded with each other in the same random island aggregates. This discrepancy suggests that the band at 3245 cm^{-1} is not the pure amide A band, but another band is overlaid on the band. For example, the O−H stretching vibration band would appear near this position, and the absolute intensity would be inaccurately obtained.

If this discussion is true, the stronger intensity of the OP spectrum would indicate that the transition dipole of the O−H stretching vibration would prefer to be directly perpendicular to the surface since the IP spectrum is larger than the OP one for the amide I band. This seems reasonable because the hydroxyl group would interact with not only the adjacent molecule via hydrogen bonding but also with the substrate surface, given that the surface is hydrophilic.

Conclusion

It has been a difficult matter to experimentally prove a random orientation of a molecule by use of spectroscopy, and a topographical measurement technique has mainly been used for the purpose thus far. In the present study, the infrared MAIRS technique has been found to be a powerful tool to reveal the random orientation. Since the repeated traces of the $\pi-A$ isotherm exhibited a good reproducibility, it was found that the monolayer had not collapsed, but instead, very stiff molecular aggregates were formed in the monolayer, which played the role of island domain in the island−sea topography. The infrared MAIRS spectra supports this interrelation, and these spectra are consistent with the previous study with the use of the epi-fluorescence microscopy, in which fibril-like aggregates were monitored.

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