e-Polymers **2007**, no. 026

http://www.e-polymers.org

ISSN 1618-7229

Construction of an arranged nano-fibrous structure by self-organization of a designed amphiphilic peptide based on β-strand

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(Received: 13 November, 2006; published: 1 March, 2007)

Abstract: An amphiphilic peptide having alternate sequence of hydrophobic and charged amino acid residues, RFDF16 (CH_3CO-RFDFRFDFRFDFRFDF-NH_2), was designed to form a β-sheet monolayer at the air/water interface. The peptide monolayer was prepared by Langmuir-Blodgett (LB) method. The LB films were transferred onto mica substrates without the compressing process at various pH values in order to investigate a self-organized structure of peptide monolayer. Surface morphology of LB films showed arranged nano-fibers which have differences in interval between fibers and their orientation depending on pH of the subphase. On the other hand, the self-organized nano-fiber was also re-arranged by compressing process. The nano-fibers became elongated and aligned upon compression to higher surface pressures. The exploitation of an electrostatic interaction and compressing process will allow a larger two-dimensionally regulated nano-structure.

Introduction

The functional surface having a regulated morphology has a number of potential applications [1-5]. The ordered structure is explored to create architecture in submicro/nano-meter scale for the application of electro-devices, biointerfaces and super-hydrophilic/hydrophobic materials etc. Many methods are suggested in order to fashion a fine-structure on the material surface. For example, a top-down approach such as lithography and etching can construct a well-defined architecture at sub-micron scale [6, 7]. Similarly, a bottom-up approach would be able to construct various nano-architectures, such as nano-fibers, nano-tubes and vesicles etc [8, 9]. In recent years, a molecular self-assembly has been used to create fine-structure in nano-meter scale.

Peptides and proteins are useful as a building block for self-assembly to produce nano-structured materials, since the character of each amino acid and its sequence promote a favourable secondary structure such as α-helix, β-sheet and flexible loop. These structural units show inter/intra-molecular interaction, and thus resulting in specific higher-ordered nano-organizations [10]. Many researchers are making efforts to exploit the self-assembly of peptides and proteins. The formed nano-architectures such as micelle, gel and film, have versatile application in the field of drug-delivery, tissue-engineering and surface science [11, 12]. On the other hand, two-dimensional

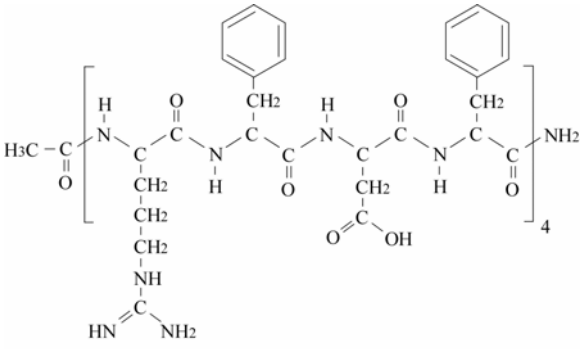
self-assembled structures are also attractive by virtue of the regulated structures such as stripe, lattice and honeycomb [13, 14]. These nano-architectures would be applied as a template for electro device and surface modification.

In this papar, we have focused on the construction of self-assembled structure of peptide at the air/water interface. The LB technique is an advanced method for preparing ordered organic monolayer, and it is expected to lead to designing of novel nano-device systems [15]. Furthermore, it would control the phase direction of the ordered structure by the use of amphiphilic molecule, and fix various external factors such as pH of aqueous subphase and compressing degree. Then, we will regulate the self-organized structure for understanding molecular character and exploit its potential. Previously, we have reported that the LB film constructed by RFDF16 showed an arranged nano-stripe [16]. RFDF16 having an alternate sequence of hydrophobic and charged amino acid residues has an equivalent number of cationic and anionic residues. Thus, we assume that peptide nano-fiber would respond to pH change of the subphase. In this study, we will discuss the construction of monolayer at various pH values and surface pressure.

Results and discussion

Characterization of RFDF16 monolayer

The amphiphilic peptide of RFDF16 is composed of Arginine (R) and Aspartic acid (D) as hydrophilic moieties and Phenylalanine (F) as hydrophobic moiety in alternate fashion (Scheme 1).



Scheme 1. Chemical structure of RFDF16.

Such peptides having alternate composition, RADA16 (CH_3CO-RADARADARADA RADA-NH_2) [17], or FEK16 (NH_2-FEFEFKFKFEFEFKFK-COOH) [18], tend to take a β-sheet conformation. When RFDF16 takes a β-sheet structure, the hydrophilic phase and hydrophobic phase should face opposite sides, and the peptide would turn the hydrophilic side to aqueous subphase at the air/water interface. Fig. 1 (isotherm (a)) shows a π-A isotherm of RFDF16 at air/water interface. The surface pressure was increased at a certain area by compressing the spread RFDF16. The obtained curve was typical as of a monolayer formation, thus, it was suggested that RFDF16 formed a monolayer. From the π-A isotherm, limiting area per molecule was estimated to be 2.2 nm^2, which was slightly smaller than the calculated value, 2.58 nm^2, on the basis of β-sheet formation.

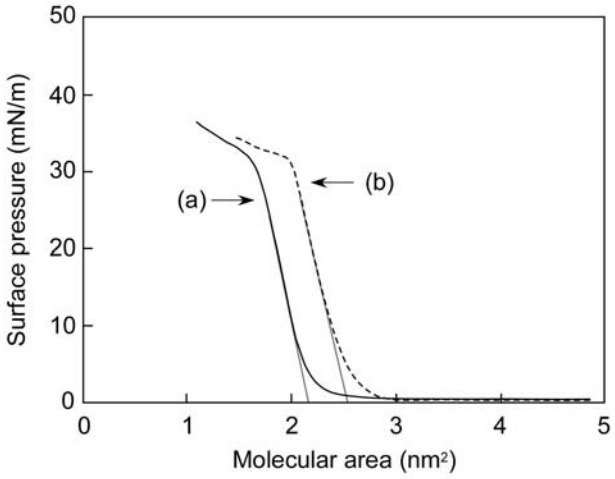


Fig. 1. Surface pressure-area (π-A) isotherms for monolayer of RFDF16 at the air/water interface (solid line (a): deionized water, dotted line (b): 0.3 M NaCl solution, gray lines: extrapolations to the x axis from the points of steepest slope on the isotherms)

Fourier transform infrared (FT-IR) spectrum of multilayer of RFDF16, which was prepared by repeatedly transferring of the monolayer, showed a strong peak at 1627 cm^{-1} and 1540 cm^{-1}, and shoulder including a peak at 1665 cm^{-1} (Fig. 2). The former peaks were attributed to anti-parallel β-sheet structure, and the latter peak was corresponding to random coil structure [19], indicating that RFDF16 formed β-sheet structure with a trace of random coil structure at the air/water interface. The observed limiting area would be attributed to partly dissolving of the spread peptide into aqueous subphase. The π-A isotherm of RFDF16 was measured on 0.3 M NaCl aqueous solution in order to prevent the spread molecule from dissolving into aqueous subphase (Fig. 1 isotherm (b)). The limiting area was estimated to be 2.5 nm^2, which almost agreed with the theoretical molecular area of RFDF16 on the basis of β-sheet formation.

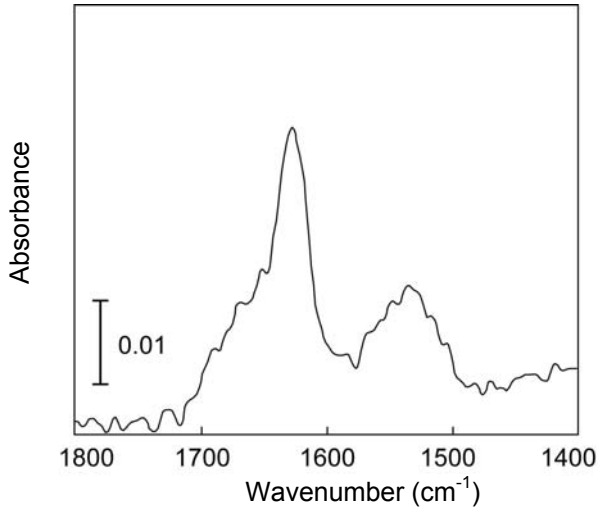


Fig. 2. FT-IR transmission spectrum of RFDF16 multilayer transferred onto a silicon substrate.

Characterization of RFDF16 monolayer at an air/water interface was investigated at various pH values (3, 4, 5.6 and 12). π-A isotherms at each pH were successfully obtained, and the limiting area per molecule were estimated to be 2.0~2.4 nm^2, which is slightly smaller than the calculated value of RFDF16. It was suggested that RFDF16 partly dissolved into aqueous subphase, since Arginine and Aspartic acid moieties transformed into cationic and anionic moieties according to the pH of aqueous subphase. Thus, π-A isotherms of RFDF16 were measured on 0.3 M NaCl additional subphase at various pH values (Fig. 3). From the π-A isotherms, the limiting area was estimated to be 2.6 nm^2 at pH 3. At pH 4 and pH 12, limiting areas were estimated to be 2.5 nm^2 and 2.3 nm^2, respectively. These values of limiting area showed that the peptide formed expanded monolayer at pH 3, and then it formed aggregated monolayer at pH 12 at the air/water interface. This behaviour suggests that RFDF16 monolayer influences the charge on the side chain.

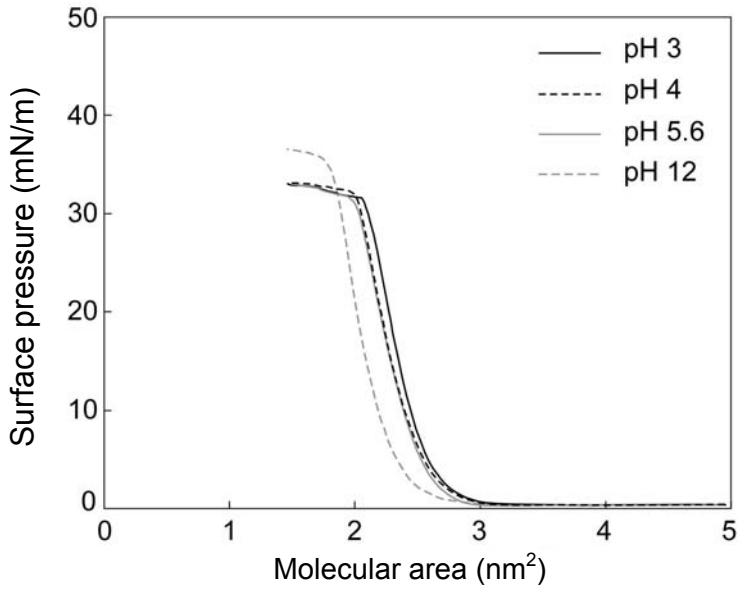


Fig. 3. π-A isotherms for monolayer of RFDF16 at the air/water interface with 0.3 M NaCl in subphase at pH 3, 4, 5.6, 12.

Morphology of RFDF16 LB film and its pH dependency

In order to verify the self-organization ability of RFDF16, LB films were constructed without compressing process, i.e. π=0 mN/m (A = 4.0 nm^2 in Fig. 1 isotherm (a)), at pH 3, 4, 5.6, and 12. The surface morphology of the LB films on a mica substrate was investigated by AFM (Fig. 4).

We considered that the LB films were successfully prepared at π=0 mN/m because the transfer ratios were approximately 1 when monolayers were transferred onto a mica at various pH values at the other surface pressure (ex. π=2 mN/m). AFM images at any pH showed an arranged nano-stripe structure, whose width and thickness were estimated to be 6~8 nm and 0.7~1.1 nm, respectively (Fig. 5).These values corresponded with the theoretical size of RFDF16 on the basis of β-sheet formation, suggesting that the observed stripe pattern was attributed to the self-organized construction of β-sheet nano-fibers. The stripe pattern formed by orientation of the nano-fibers was parallel to the lifting-up direction of the substrate.

This behavior suggested that the vertical lifting-up process provided the transferred nano-fibers with parallel aligning due to the surface flow during the deposition. This phenomenon is well-known and has been reported by Ijiro *et al.* and Wegner *et al.* [20, 21].

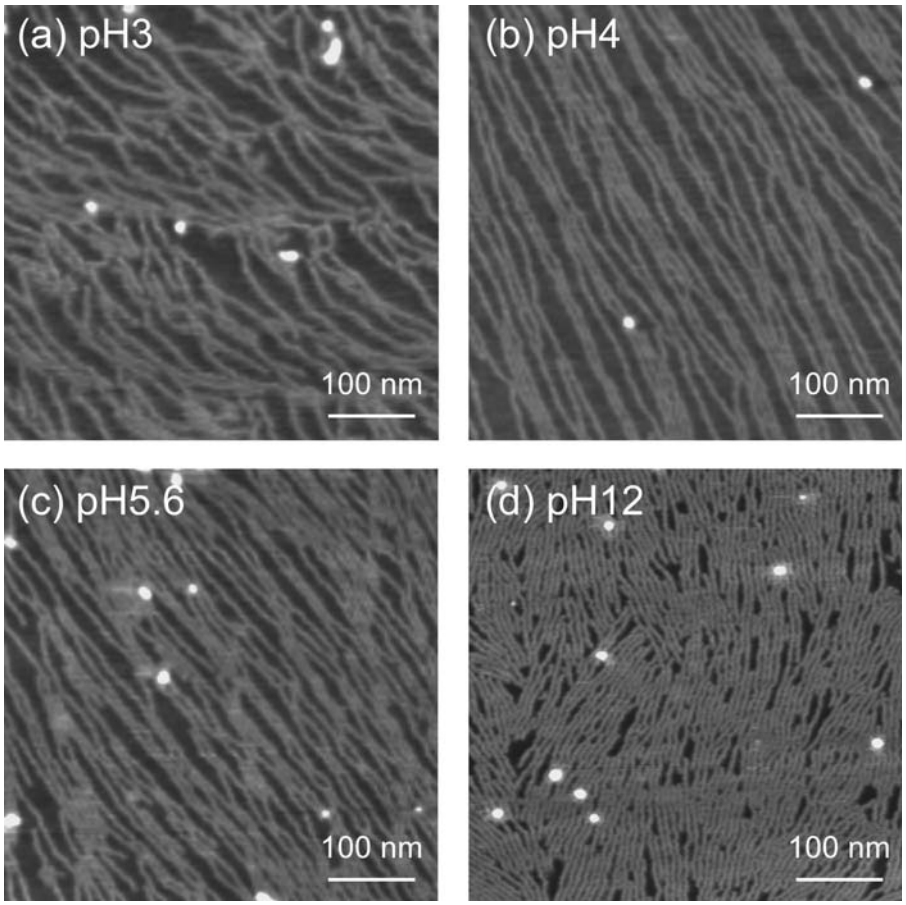


Fig. 4. (a) AFM images (500 nm × 500 nm) of RFDF16 LB film transferred onto a mica substrate without compressing process at pH 3, (b) pH 4, (c) pH 5.6 and (d) pH 12.

The morphology of nano-fiber at each pH seemed to be different in the interval between fibers and its orientation. Fibers constructed at pH 3 were bent and its interval has wide distribution (about 5~50nm), and its average interval was estimated to be ca. 20 nm (Fig. 4(a)). Fibers constructed at pH 4 and 5.6 were straight in micron order and some of fibers were packed in places (Fig. 4(b), (c)). The interval of fibers were estimated to be about 10~15 nm, respectively. And fibers constructed at pH 12 was straight in sub-micron order and its interval was relatively constant (ca. 8 nm) but orientation of fibers was not uniform in places (Fig. 4(d)). These behaviours can be explained by considering the charge balance between the fiber-constructed peptides. It was considered that the self-organized nano-fiber was constructed by hydrogen bonding, electrostatic interaction, and steric hindrance between phenyl groups of Phenylalanine moieties. Among them, the electrostatic interaction would be the most sensitive, when pH of the subphase was changed. Thus the RFDF16 was fully positive at pH 3, the peptide was amphoteric but relatively positive at pH 4, equivalent at pH 5.6, and amphoteric but relatively negative at pH 12, which are correlating with pKa values of Arginine and Aspartic acid. Then, the electrostatic

interaction acted at inter/intra-fibers as a repulsive force to give the fiber bent and make interval between fibers wider at pH 3. Fibers constructed at pH 4 and 5.6 have both negative and positive charge in places, resulting in an attractive force to promote propagation of the fiber. In the same way, fibers constructed at pH 12 also have both negative and positive charge in places to give an attractive force.

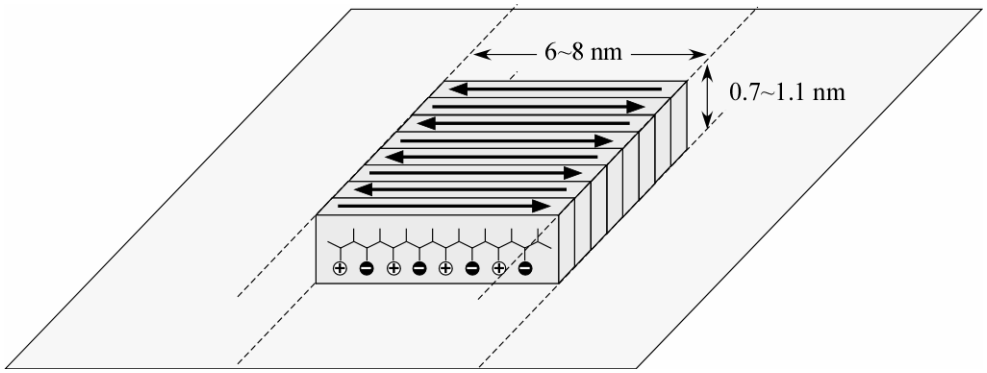


Fig. 5. Schematic illustration of RFDF16 formed nano-fiber on the basis of β-sheet structure on a mica substrate at various pH values. (The length of β-strand is estimated to be 6~8 nm and the thickness is to be 0.7~1.1 nm).

The difference of morphology between acidic and alkaline condition might be caused by kind of the excess charge on the side chain. This kind of behaviour was also discussed with the peptide sequence and the length of side chain of charged amino acid. The Arginine is located at terminus of the peptide, and has relatively long side chain attributed to the methylene group. Thus, cationic moieties would be exposed to outer side, when the peptide formed fibrous structure by anti-parallel β-sheet. Then, the positive repulsion efficiently affected to the intra/inter-fibrous interaction to give the fibrous structure bent and the interval wider in acidic condition. The Aspartic acid is located at inside of the peptide and has short side chain, so that anionic moieties would not be exposed to out side of peptide nano-fiber. Thus, fibres would not be sensitive to ionic repulsion in alkaline condition. It might be essential for constructing the oriented nano-fibers in secondary dimension to control the balance of the charged moieties.

We demonstrated that the various pH values of aqueous subphase influence the surface morphology of RFDF16 monolayer. But in this case, there is a possibility that peptide dissolved into aqueous subphase. Then, the RFDF16 LB film was observed which was transferred onto mica substrate from 0.3 M NaCl additional subphase at different pH values (pH 3 and 12) at 0 mN/m. Both AFM images also showed an arranged nano-stripe structure. The morphology of nano-fiber at pH 12 with 0.3 M NaCl seemed to be identical with Fig. 4(d) which is prepared from water subphase (pH 12). The fiber constructed at pH 3 with 0.3 M NaCl subphase had wide interval between nano-stripe patterning (about 5~40 nm), but it is compared with the film was prepared from water subphase (pH 3), the interval was narrowed and nano-fibers were partially packed. It is suggested that the salt was shielded the charges with peptide side chain. These results supported that the surface morphology of RFDF16 LB film was responded to pH change of aqueous subphase.

Dependence of surface pressure into RFDF16 LB film

The self-organized nano-fiber of RFDF16 was re-arranged by compressing the spread peptide. Fig. 6 shows surface structure of RFDF16 LB films, which was transferred onto a mica substrate at 1~2 mN/m at pH 3. On compressing further, the fibres became straight and highly oriented, and interval between fibres became narrower and constant. It is noteworthy that the short fibre and branched moieties, which can be observed in Fig. 4(a), disappeared on compressing at higher pressure (π=2 mN/m), to give longer and straight fibers. It suggested that the nano-fibers re-arranged its structure to avoid the steric hindrance and positive repulsion between Arginine moieties.

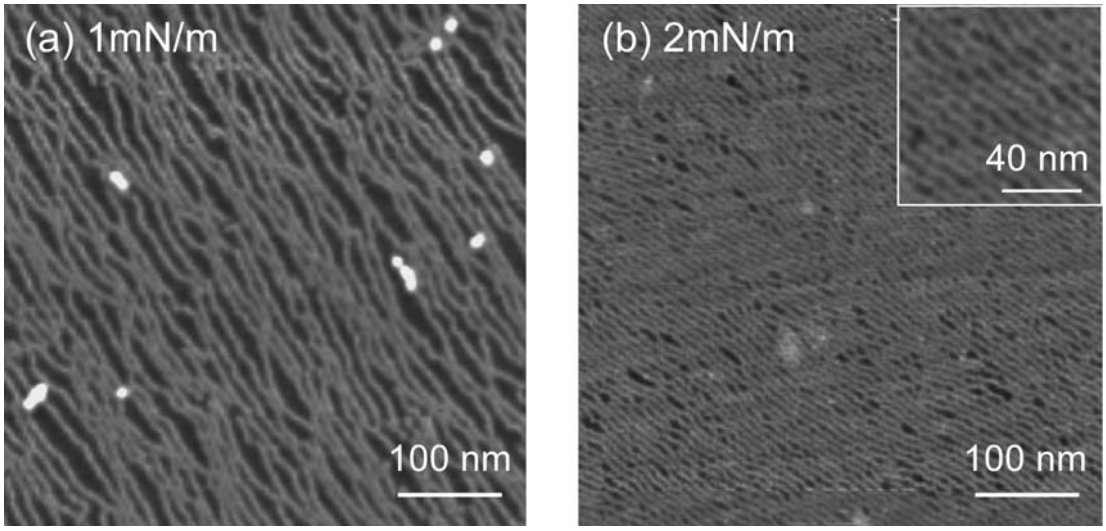


Fig. 6. (a) AFM images of (500 nm × 500 nm) RFDF16 LB film transferred onto a mica substrate at 1 mN/m, and (b) 2 mN/m (pH 3).

Taking account of the corresponding π-A isotherm, the pressure value, 1~2 mN/m, is according to which molecules interact weakly each other. These weak interactions might have promoted the re-arranging to give larger two-dimensionally regulated structures.

Conclusions

We had demonstrated that the LB films of amphiphilic peptide designed to form a β-sheet showed an arranged nano-stripe structure. The peptide having alternate sequence of hydrophobic and charged amino acid residues spontaneously formed a nano-fibrous structure at an air/water interface. Morphology of the monolayer transferred onto a mica substrate showed an arranged stripe pattern. Interval between fibers and their orientation were different depending on the pH of aqueous subphase, suggesting that the ionic interaction based on the charged residues at each pH affected the intra/inter-fibrous interaction. The self-organized nano-fiber of RFDF16 was re-arranged by compressing the spread peptide. The nano-fibers became elongated and aligned upon compression to higher surface pressure. Thus, the self-organized structure of LB films would be controlled by tuning of interaction between fibers. These results would be applied as a larger secondary regulated nano-structure and surface modification technique.

Experimental part

Materials

The amphiphilic peptide, RFDF16 was prepared by solid phase method using 9-fluorenylmethoxycarbonyl (Fmoc) strategy. The peptide chain was synthesized on a CLEAR-Amide Resin (cross-linked ethoxylate acrylate resin, Peptide Institute), by using Fmoc-amino acid derivatives (3 equiv.). The activation and coupling reactions have been conducted using 1-hydroxy-7-benzotriazole (HOAt, 3 equiv.), and 1,3-diisopropylcarbodiimide (DIPCDI, 3 equiv.) in N,N-dimethylformamide (DMF). And 20% of piperidine was used in DMF for Fmoc removal. The N-terminus of the peptide was acetylated by acetic anhydride (10 equiv.) in DMF. To cleave the peptide from the resin and the deprotection of the side chain were carried out with trifluoroacetic acid (TFA)/1,2-ethanedithiol/thioanisole/water (84:8:4:4). The resultant peptide was identified by matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry (PerSeptive Biosystems, VoyagerRP, m/z calc. for RFDF16 $[M+H]^+$: 2323.6; found: 2322.6) and reverse-phase high performance liquid chromatography (HPLC)(HITACHI High-Technologies, L-2000). The purity of the peptide was found to be ca. 80%.

Methods

-Surface pressure-area measurements

Surface pressure-area (π-A) isotherms of RFDF16 were measured using a NL-BIO40-MWCT (Nippon Laser & Electronics Lab.) at 25 °C. Monolayers were prepared by spreading a solution of the peptide in benzene/2,2,2-trifluoroethanol (6:4) at a concentration of approximately 0.1 mg/ml through a microsyringe onto deionized water surface. After evaporation of organic solvents at 20 min, the monolayer was compressed at a speed of 5 mm/min. Surface pressure was measured by Wilhelmy plate. The monolayer was transferred onto a freshly cleaved mica substrate in vertical method (up-stroke) at a speed of 5 mm/min after the surface pressure was reached at targeted pressure and then the monolayer was stabilized for 20 min. The subphase pH was controlled by addition of HCl for acidic condition and NaOH for alkaline condition.

-Atomic force microscope observations

Atomic force microscope (AFM) observation was carried out on the Nanoscope IV (Digital Instruments) using a silicon cantilever (NCH-10V, Veeco Instruments) operated in tapping mode. All images were obtained in air at room temperature. A 14 μm x 14 μm scanner was used for imaging. The scanning speed was at a line frequency of 1 Hz. Original images were sampled at a resolution of 512 x 512 points. Obtained images were filtered by plan-fit and flatten routine.

-Fourier transform infrared spectroscopy measurements

Fourier transform infrared (FT-IR) spectrum was collected on a Perkin Elmer spectrum 2000 instrument equipped with a mercury-cadmium-tellurium detector at 4 cm^{-1} resolution under nitrogen atmosphere. Multilayer of RFDF16 were prepared by repeat transferring of the monolayer with vertical method (up- and down-stroke) from

an air/water interface (π=20 mN/m) onto a silicon substrate modified with octadecyl triethoxy silane.

Acknowledgements

This research was supported partly by grants from the Japan Aerospace Exploration Agency (JAXA), and the NITECH 21st Century COE Program, "World Ceramics center for Environmental Harmony".

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