Tailoring a Tyrosine-Rich Peptide into Size- and Thickness-Controllable Nanofilms

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ABSTRACT: Self-assembled nanostructures of tyrosine-rich peptides have a number of potential applications such as biocatalysts, organic conducting films, and ion-selective membranes. In modulating a self-assembly process of peptides, the interfacial force is an important factor for kinetic control. Here, we present the formation of large-sized and thickness-controllable nanofilms from the YYACAYY peptide sequence (Tyr-C7mer peptide) using Langmuir–Blodgett and Langmuir–Schaefer deposition methods. The Tyr-C7mer peptide showed typical surfactant-like properties, which were demonstrated via the isotherm test (surface pressure–area) by spreading the Tyr-C7mer peptide solution onto an air/water interface. Uniform and flat peptide nanofilms were successfully fabricated and characterized. The redox activity of densely packed tyrosine moieties on the peptide nanofilm was also evaluated by assembling silver nanoparticles on the nanofilm without requiring any additives.

1. INTRODUCTION

In nature, specific peptide sequences encode a lot of information, drive structural assembly, and determine biological functions. To mimic these complex biological systems, which are based on the specific folding of peptides, considerable efforts have been made to fabricate hierarchical self-assembled architectures including tubes,1−3 fibers,4−6 planar structures,7 and 3D networks.8−10 In particular, peptide-based two-dimensional (2D) thin films are of great interest for a lot of applications such as biomaterials,11,12 surface coating,13,14 and electronics.15,16 However, various hurdles have to be overcome to fabricate thickness-controllable, homogeneous, and large-size 2D peptide films for practical applications.

Recently, we reported that a tyrosine-rich peptide (TyRP) (Tyr-Tyr-Ala-Cys-Ala-Tyr-Tyr, YYACAYY, Tyr-C7mer peptide) could be assembled into a closely-packed and flat nanosheet form at the air/water interface, which could be used as a template for the oxidative polymerization of pyrrole by generated tyrosyl radicals.17 The challenges here are (i) to transfer the nanofilm from the air/water interface onto a substrate and (ii) to increase its size. As an easy and facile transfer method of nanofilms from the air/water interface to a substrate, Langmuir–Blodgett (LB) assembly can be utilized, which enables the precise control of the monolayer thickness and packing density, inducing homogeneous deposition over a large surface area.18,19 While Langmuir and LB films have been studied with peptides,20−22 most of the cases have focused on the structural analysis of the peptide film rather than its function.

Herein, we demonstrate a new fabrication method of thickness-controllable 2D nanofilms of a Tyr-C7mer peptide at the air/water interface using Langmuir techniques. We further investigate the role of the Tyr moieties. Obtained nanostructures of assembled Tyr show electrochemical activities, similar to those of the photosystem II under physiological conditions.23−26 The LB or Langmuir–Schaefer (LS) technique can be used to induce intermolecular interactions on a densely packed assembled nanostructure. When the Tyr-C7mer peptide solution was spread on a metal ion containing an aqueous subphase, metal nanoparticles (NPs) were formed on the peptide nanofilm under neutral conditions. We demonstrate that TyRPs are a versatile tool kit for the assembly of ordered nanomaterials and metal NPs on them.

2. RESULTS AND DISCUSSION

Generally, peptides that have been used for LB films appeared to be amphiphilic. For example, surfactant-like peptide molecules,27,28 with a tail consisting of six consecutive hydrophobic amino acids and a head consisting of hydrophilic charged amino acids, or β-pleated sheet peptides29 comprising...
alternating hydrophilic and hydrophobic amino acid residues were used for LB films. All of them have adsorption properties onto the air/water interface. It is rather difficult to assign a hydrophobic tail and a hydrophilic head in the Tyr-C7mer peptide (Figure 1a). Nevertheless, the Tyr-C7mer peptide has surface-active properties in water, as demonstrated in our previous paper.20

2.1. Surface Pressure–Area Isotherm. Surface pressure versus area (π–A) isotherms of the Tyr-C7mer peptide monolayer on deionized (DI) water can provide important information about the behavior of the Tyr-C7mer peptide at the air/water interface. To get $A_0$ value correctly, surfactant molecules are commonly dissolved in a solvent that is immiscible with water. In our case, the Tyr-C7mer peptide is not soluble in a common water-immiscible solvent. Thus, we cannot but select dimethyl sulfoxide (DMSO) for peptide dissolving, which can lead to a partial loss of peptide molecules into the subphase. Therefore, $A_0$ values obtained from the π–A isotherms are not absolutely reliable. Despite these limitations, the π–A isotherm of the Tyr-C7mer peptide exhibited a behavior similar to that of amphiphilic β-hairpin peptide, which is slightly soluble in water26 (Figure 1b).

To check the phase change of the Tyr-C7mer peptide at the air/water interface during barrier pulling, the peptide film of each state was transferred onto a Si substrate and its morphology was analyzed using atomic force microscopy (AFM). For the deposition of Langmuir monolayers of liquid-condensed and solid phases, where the surface pressure is sufficiently high to ensure lateral cohesion of the interfacial film, classical LB technique [i.e., vertical deposition (VD)] is an acceptable method.31 The high-quality transfer of a monolayer at a low surface pressure (gaseous state) or of a collapsed one onto a solid substrate by the VD method is rather difficult to perform. In those cases, the horizontal precipitation (HP) method has been used.32 As shown in Figure 1b–d, Tyr-C7mer peptide LB films were successfully deposited from the aqueous subphase onto a Si surface at π ≈ 0 mN/m (lower pressure region), π ≈ 7 mN/m (higher pressure region), and π ≈ 13 mN/m (collapsed film region), which was verified through AFM analysis. According to the cross-sectional analysis, the thickness of the film in the lower pressure region and the higher pressure region were found to be 1.0 and 2.3 nm, respectively. Further increase of the surface pressure above π ≈ 13 mN/m resulted in film collapse.

It should be noted that the replacement of one amino acid in the middle position by Ala results in crucial changes of the peptide properties. The resulting Tyr-A7mer peptide did not show any surface-active properties (Figure S2). From the result, we can conclude that the Cys in the Tyr-C7mer plays an important role in the adsorption property onto air/water interface.

2.2. Characterization of YYACAYY LB Films. In our previous study of flattening water droplet surface (faceting) by the Tyr-C7mer peptide, we showed that a dimer formation via cysteine oxidation (Cys, C) played an important role in the stabilization of the peptide secondary structure.20 The dimer content had a direct effect on the facet formation. To confirm the effect of the disulfide bond on the Tyr-C7mer peptide film formation via the Langmuir method, Raman analysis was performed. As shown Figure 2a, the –SH stretching vibration (2571 cm$^{-1}$) was observed in the monomer but disappeared in the peptide film. Further, a new strong peak (489 cm$^{-1}$) appeared in the peptide film, which corresponds to the S–S stretching frequency, as a result of the formation of disulfide
bonds between cysteines. The value was shifted (−14 cm⁻¹) from the 503 cm⁻¹ band that was previously assigned to the S–S stretching frequency.30 The S–S stretching frequency and intensity are sensitive to a changing CS–SC dihedral angle, therefore providing detailed structural and conformational information about various disulfide-containing proteins such as oxytocin, vasopressin, glutathione, and malformin.33–35 Although various factors need to be considered, it is possible to estimate the approximate CS–SC dihedral angle of the Tyr-C7mer peptide dimer in Langmuir films based on Raman data. Thus, the 489 cm⁻¹ band corresponds to a dihedral angle of about 20°, and the 503 cm⁻¹ band corresponds to a dihedral angle of about 43°. As expected, the pressure applied to the peptide molecules by the LB barrier affects the peptide conformation and leads to a reduced CS–SC dihedral angle.

To investigate the molecular conformations of the 2D assembled peptide structure, solid circular dichroism (CD) spectra were obtained. As shown in the CD spectra of the peptide dimer sheets (Figure 3), strong positive and negative peaks at around 200 and 218 nm appeared, which can be assigned to n–π* and π–π* transitions, respectively. Those peaks correspond to the typical β-sheet conformation of the peptide. An additional weak peak at around 236 nm is known to be related to the π–π* transition of the disulfide bond.36,37 A deviation from 90° of the CS–SC dihedral angle causes this peak to appear,38 which matches well with the result obtained above via Raman spectroscopy.

2.3. Micropatterning of the Peptide LB Film. It is of great interest to fabricate patterns of functional soft materials such as peptides and proteins for biosensors,39,40 tissue engineering,41–43 and fundamental studies in biochemistry.44–46 Patterned mono- and multilayer Tyr-C7mer peptide films can be easily fabricated via stamping the peptide monolayer obtained by the LS method. The common method of stamp “inking” starts with the adsorption of “ink” molecules from solution. Previously, we have developed an alternative way based on LB technology that allowed the formation of patterned LB films of small- and large-size surfactants.47 In the present work, stamp inking was performed via the LS method. This allows us to obtain Tyr-C7mer peptide LB films with a flexible design as well as to control their thickness. The LB film thickness was determined via cross-sectional AFM analysis as the natural blemish depth. When the coating is highly uniform, it is necessary to make an artificial defect in its structure, for example, with the AFM tip while scanning a small area in the contact mode at a load of ~10 nN. The film material is supposed to be completely removed at that location. However, it can be difficult to estimate the extent to which the film material has been removed from the required location. This is critical in cases where the coating is sufficiently thick (tens of nanometers) or shows an increased wear resistance caused by either high adhesion to the surface or reorganization processes during scanning. In such cases, it becomes necessary to increase the scanning time at high loads, which may cause considerable probe damage and result in bad-quality AFM images.

Because the patterned LB mono- and multilayers are formed using the microcontact printing (μCP) technique, they consist of alternating regions with and without films, and the coating thickness can be easily and reliably controlled. Peptide film patterns of various thicknesses were evaluated by cross-sectional AFM analysis (Figure 4). The film thicknesses of patterned 1-layer, 3-layer, 5-layer, and 10-layer peptide LB films were ~3.1, ~9.4, ~15.7, and ~33.4 nm, respectively. The thickness could be easily tuned by simply controlling the number of μCPs by the PDMS stamp. The thickness of the peptide LB monolayer film obtained from the AFM data was compared with the geometric parameters of the Tyr-C7mer peptide molecule, estimated using semiempirical quantum chemistry method PM6 (length × width × height = 2.80 nm × 1.45 nm × 1.10 nm) (Figure S4). These parameters were obtained from the most stable conformation of YYACAYY in vacuum. The calculated peptide length (2.80 nm) is approximated to the measured peptide LB film thickness (3.1 nm). The slight difference between the two values might come from the adsorption of water molecules into the peptide in the film. In addition, the monolayer thickness of the LB peptide film is quite different from that of the faceted peptide film (1.4 nm),30 which might be attributed to the vertical arrangement of peptide molecules as a result of monolayer compression.

2.4. Application of Peptide Nanofilms for the Formation of Metal NPs. To characterize the redox-active behavior of the closely packed tyrosines in multilayered Tyr-C7mer peptide films, cyclic voltammetry (CV) was performed in 0.1 M NaCl solution (pH 6.5) with a Ag/AgCl electrode under the potential range of 0.4–1.6 V at 50 mV s⁻¹, as shown in Figure S3. The largest peak current was obtained at 0.9 V.
which indicates that tyrosyl radicals are generated in the peptide nanofilm (Tyr → Tyr••). To explore the possibility of using the densely packed tyrosines as a bioinspired scaffold for the formation of metal NPs, we attempted the reduction of silver ions as a model reaction. A Tyr-C7mer peptide solution in DMSO (5 mg/mL) was loaded onto the surface of an aqueous solution of silver nitrate (AgNO₃) (1 mM) (Figure 5a). From the π−A isotherm of the Tyr-C7mer peptide monolayer on the AgNO₃ aqueous solution (Figure S5), we have found that 6 mN/m is enough to form a liquid-condensed monolayer of the peptide. Interestingly enough, we noticed that Ag⁺ ions were reduced to NPs on the Tyr-C7mer peptide film without using any reducing agents such as sodium borohydride, hydrazine, or ascorbic acid. Through AFM and transmission electron microscopy (TEM) analyses of the resulting LB peptide film, we could confirm that the Tyr-C7mer peptide showed a redox-active property even under neutral condition. As shown in Figure 5b,c, silver nanoparticles (Ag NPs) covered the whole surface of the peptide film. It is known that tyrosine in its unstructured and solubilized form shows reducing properties only under basic conditions or at high temperatures,⁴⁸−⁵⁰ which has been demonstrated for gold and silver ions and graphene oxide.⁵⁰ On the basis of these findings, we consider that structured functional amino acids could play an important role for the metal reduction. The thickness of the peptide/Ag NP hybrid nanofilm was 3.2 nm, which is the same as the thickness of the peptide monolayer, indicating that the Ag⁺ ions did not affect the peptide conformation. In addition, the size of Ag NPs was in the range between 18 and 54 nm (mean and standard deviation: 34.3 ± 7.3 nm; Figure 5f).

The crystalline property of the Ag NPs was characterized through a high-resolution transmission electron microscopy (HR-TEM) image (Figure S5f) together with the electron diffraction data (Figure S6), wherein a plane distance of 0.21

Figure 4. LS micropatterned peptide nanofilms. (a) Schematic illustration of the fabrication of micropatterned peptide films using μCP. (b−e) Cross-sectional AFM images as a function of the number of μCPs.

Figure 5. Synthesis of the Ag NP-deposited peptide LB film. (a) Schematic illustration of the fabrication of Ag NP-deposited peptide LB films. (b) AFM image, (c) TEM image, and (d) size distribution histogram of Ag NPs with an average size of 34.3 nm and a standard deviation of 7.3 nm. The arrows in (b,c) point out the Ag NPs. (e) HR-TEM image obtained from Ag NPs on the peptide film transferred to the TEM grid. (f) Enlarged HR-TEM image recorded from a red square in (e).
nm, that is, the \{200\} lattice plane, appeared instead of the most stable \{111\} lattice plane of Ag(0) NPs in most cases. It is believed that the TyRP film stabilizes the high-index facet of the Ag(0) NPs.

3. CONCLUSIONS

Uniform, thickness-controllable, and large-scale 2D nanofilms of the Tyr-C7mer peptide were fabricated at the air/water interface by barrier pressure and successfully transferred onto Si substrates via LB and LS depositions. When an aqueous AgNO3 solution was used as a subphase, Ag NPs were formed on the peptide nanofilm via reduction of Ag by densely packed tyrosine moieties under neutral condition without requiring an additional reducing agent. The 2D peptide nanofilms have great potential for various fields such as biocatalysis, organic conducting films, or bioelectronics.

4. MATERIALS AND METHODS

4.1. Materials. 2-Chlorotriyl chloride (CTC) \((100\text{–}200\) mesh, 1.34 mmol/g) resin, fritted polypropylene tubes (Libra tube RT-20M, 20 mL), 2-{(H-benzotriazole-1-yl)-1,1,3,3-tetramethyllumuronium hexafluorophosphate (HBTU)}, hydroxy-benzotriazole (HOBt), and Fmoc-protected amino acids were purchased from BeadTech Inc. (Seoul, Korea). Dichloromethane (DCM), thiol (EDT) were purchased from Aldrich (St. Louis, MO, USA). Dichloromethane (DMC), N,N-Dimethylformamide (DMF), methanol, piperidine, diethyl ether, and trifluoroacetic acid (TFA) were obtained from Dae-Jung Chemicals (Korea). Hydrogen peroxide solution (30%), sulfuric acid (98%), and HCl were of analytical grade and used as purchased.

4.2. Synthesis and Purification of the YYACAYY Peptide. The YYACAYY peptide was synthesized on CTC resin using Fmoc/Bu-chemistry. Fmoc-amino acid (2 equiv), HBTU (2 equiv), HOBt (2 equiv), and DIPEA (4 equiv) were dissolved in DMF and used as a coupling solution. The coupling reaction was performed in a shaking incubator for 2 h at room temperature, and each coupling reaction was checked by the Kaiser test. After each coupling and deprotection reaction, the resin was washed with DCM, DMF, and methanol sequentially. To deprotect the Fmoc group, 20% piperidine/DMF was added to the resin and reacted for 30 min. The coupling and deprotection steps were repeated with Fmoc-Tyr(Bu)-OH, Fmoc-Tyr(Bu)-OH, Fmoc-Ala-OH, Fmoc-Cys(Trt)-OH, Fmoc-Ala-OH, Fmoc-Tyr(Bu)-OH, and Fmoc-Tyr(Bu)-OH. After all amino acids were coupled on the resin sequentially, the final peptide was cleaved from the resin for 90 min by using the cleavage cocktail \((\text{TFA/phenol/water/thioguanosine/EDT} = 82.5:5:5:5:2.5, \text{vol}-\text{ratio})\). The peptide-cleaved resin was filtered, and the peptide-containing filtrate was transferred to a round-bottom flask. The resin filtrate was washed with DCM and methanol to extract the cleaved peptides that remained in the resin, and then, all filtrate and washing solutions were combined and concentrated. To precipitate the peptide product, cold diethyl ether was added and centrifuged. The precipitate was dried in vacuum. The Tyr-C7mer was purified with reversed-phase HPLC using a linear gradient of 10–80% MeCN (containing 1% TFA) in water (containing 1% TFA) over 30 min and confirmed by electrospray ionization mass spectrometry (ESI-MS). The purity was above 99\% (Figure S1); ESI m/z: [M + H]+ calcld for C21H38N4O6S, 475.2591; found, 475.2593.

4.3. Surface Pressure–Area Measurements. The surface pressure–area (\(\pi\text{–}A\)) isotherms were measured with an LT-103 device (Microtestmachines Co). It is a one-barrier LB trough with an effective surface area of 253 \text{cm}^2. Aliquots of the Tyr-C7mer peptide solution in DMSO (1 mg/mL) were spread on the surface of DI water or an aqueous ionic subphase (aq AgNO3 solution) and allowed to stand for 20 min before \(\pi\text{–}A\) isotherm recording. The dimerized peptide through disulfide bond formation is less soluble in DMSO than the monomer peptide. Therefore, immediately after dissolving the peptide in DMSO, the Tyr-C7mer peptide solution was spread onto the surface.

4.4. Transfer of Peptide Films Using Langmuir Methods. VD and HP were used to transfer the LB films onto a Si surface. First, Si (100) wafers were cleaned in a mixture of H2SO4/H2O2 (7/3, v/v) by heating to ca. 70 \text{°C} for 15 min, followed by thorough rinsing with DI water and drying under a N2 stream. The precleared substrates were dipped into the subphase volume either vertically (in the case of VD) or horizontally (in the case of HP). Thus, an aliquot of the Tyr-C7mer peptide solution in DMSO (1 mg/mL) was spread onto the subphase surface. After a stabilization period of 20 min, the Langmuir monolayer was compressed up to the required surface pressure and transferred onto the Si substrate either through slow (0.012 mm/s) upstroke of the substrate (in the case of VD) or through lowering the substrate into the subphase level (in the case of HP).

4.5. Fabrication of Patterned LB Films by \(\mu\text{CP}\). On the basis of the well-known \(\mu\text{CP}\) technique, \(\mu\text{CP}\) were fabricated by replica molding using a TGAZ3 AFM calibration grating as a master and a SYLGARD 184 silicon elastomer kit (Dow Corning, Midland). Briefly, prepolymer and catalyst were thoroughly mixed as a 10–1 ratio (w/w). The mixture was degassed with a water-jet pump, spread as \(\sim 1\) mm thick layer onto the master, and then cured at 100 \text{°C} for 20 min. After cooling to room temperature, the elastomeric stamp was peeled off from the master and used for \(\mu\text{CP}\). The precleaned Si wafer was touched by the “inked” stamp to transfer the peptide LB film to those regions of the substrate that contact the stamp.

4.6. Characterization. 4.6.1. Electrospray Ionization Mass Spectrometry. The peptide film was dissolved in 0.5\% acetic acid/methanol. Mass data were confirmed by an analytical HPLC–ESI-mass spectrometer (MS; Thermo–Finnigan LCQ deca XP MS ESI-MS) and a Phenomenex C4 reverse-phase column (4 mm, 100 \times 3 mm\(^2\)). The gradient was 10–65\% acetonitrile in H2O with 0.5\% formic acid over 21 min.

4.6.2. Raman Spectroscopy. Raman spectra of the Tyr-C7mer peptide (monomer or peptide films) were analyzed with a HORIBA Jobin–Yvon/LabRAM Aramis Raman spectrometer (diode laser 785 nm, power = 1 mW). The film at the air/water interface was transferred onto a cleaned Si wafer. The single transfer of film did not give significant signals. Therefore, multiple transfers of the films were necessary on the substrate. The scanned wavenumbers ranged between 200 and 3000 cm\(^{-1}\) with 300 s scanning in average.

4.6.3. Solid CD Spectroscopy. Solid CD spectra of peptide sheets were measured with a J-815 spectropolarimeter (Jasco, Tokyo, Japan) at 25 \text{°C}. Spectra were collected from 260 to 180 nm by using a 0.1 cm path length quartz substrate and keeping the HT voltage < 500 V for reliability. The experimental
conditions were a 0.5 nm data pitch, a 20 nm/min scan speed, a 16 s response time, and a 1 nm bandwidth. The data were accumulated from three repeated runs for reproducibility. To acquire conformational information on peptide films, peptide sheets were transferred onto a quartz substrate by PDMS microstamping.

4.6.4. Cyclic Voltammetry. Current density of the Tyr-C7mer peptide film on FTO glass in 0.1 M NaCl electrolyte was measured, as the potential was sweeping from 0.4 to 1.6 V versus Ag/AgCl electrode at a scan rate of 50 mV s⁻¹.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.8b00395.

HPLC and ESI-MS analysis data of the Tyr-C7mer peptide, π-A isotherm of the Tyr-A7mer peptide on an aqueous subphase, CV polarization analysis of peptide nanofilm, most stable conformation of the Tyr-C7mer peptide, π-A isotherm of the Tyr-C7mer peptide on AgNO₃ aqueous solution, and electron diffraction image of Ag NPs (PDF)

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Notes

The authors declare no competing financial interest.

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