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Molecular Design of Helix-Turn-Helix Polypeptides and Their Aggregation Properties in Water

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In this study we designed and synthesized two types of helix-turn-helix polypeptides (**1** and **2**) which consist of poly(L-glutamic acid) (PLGA) as the helix moiety and *m*-xylylenediamine (Xyl) or 2,7-diaminofluorene (Flu) as the turn moiety. The secondary structure and self-assembling property in water were evaluated by CD, FTIR spectroscopies and AFM. The CD spectra showed that both of peptides formed α-helical structure at pH 4.0, and then as incubation, decrease of the $[\theta]$ value and spectral shift were observed based on the helix-aggregation in the case of Flu-$(PLGA)_2$ (**2**), whereas no spectral change was observed in the case of Xyl-$(PLGA)_2$ (**1**). In parallel with these changes in CD spectra, AFM and FTIR studies showed that the peptide **2** self-assembled into rectangular plate-shape nanostructures through helix-helix interactions. On the other hand, such controlled self-assembly did not occur for peptide **1**. These observations strongly suggest that the structure of turn moiety significantly affects the self-assembling properties of these peptide-architectures in water.

Key words: Helix-Turn-Helix, Self-assembly, Poly(L-glutamic acid), Secondary Structure, Nano-structure

1. INTRODUCTION.

The self-assembly of peptides, proteins and their derivatives into shape-specific nano-structures has attracted much attention as a powerful approach for the design of new functional nano-materials and because of their association with neurodegenerative diseases (β-sheet fibrils) like Alzheimer's in some case.[1] A number of artificial proteins have been prepared in aqueous solutions and investigated the interactions between peptides involved in protein folding problem. A recent approach to artificial protein design is to use template molecules such as metal ligands, rigid plane molecules, flexible polymer, and dendrimer.[2] The design process is simple; secondary structure modules are created and then assembled by using appropriate templates that direct the component peptide blocks into protein-like packing arrangements. For example, Kelly's group reported the artificial peptidemimetics, that consisted of two β-sheet formable peptides connected by rigid templates, self-assembled into filaments, ribbons, and fibers with well-defined nano-structure depending on the molecular structure and environmental conditions in aqueous solution.[3] These self-assembled polymeric architectures are expected to possess potential as novel biomaterials with a wide range of applications, such as use in nano-scaffold, nano-reactors, and nano-template.

We have developed a strategy in which α-helical polypeptides like poly(L-glutamic acid) (PLGA) are aligned on two-dimensional media such as water and Au surfaces.[4] In the course of this series of works, we have found that the helical PLGA assemblies can discriminate between *N*- and *C*-termini of guest helical polypeptides, owing to an antiparallel association property of helical rods.[5] By using this property of helical rods, functional groups, including redox active[6] and DNA-intercalatable[7] moieties that are incorporated at the chain end of PLGA, have been successfully immobilized at a desired position in the PLGA assemblies.

In this paper, we describe the preparation of novel water-soluble helix-turn-helix peptides (**1** and **2**) with protein-like stable tertiary structures and their self-assembly into nanostructure through the specific helix-helix interactions in water. Helix-turn-helix architectures were developed as a model for the study of protein folding intermediates and were designed to consist of two PLGA helices connected by rigid templates. A detailed analysis of conformation and morphology in water was performed in the view of molecular structures of templates (tertiary structure). These studies should provide simple and/or essential insight into the mechanism of peptide aggregation, and be useful for the design of novel peptide-based nano-biomaterials.

2. EXPERIMENTAL

2.1 Preparation of poly(L-glutamic acid)-based Helix-turn-helix peptides.

The triblock helix-turn-helix polypeptides (Fig. 1), that consist of two poly(L-glutamic acid)s (PLGAs) linked by *m*-xylylene (Xyl) or fluorene (Flu) derivatives, were prepared by ring-opening polymerization of γ-benzyl-L-glutamate *N*-carboxylic anhydride (BLG-NCA). BLG-NCA was synthesized

by reacting γ-benzyl L-glutamate with triphosgen in a THF solution. The peptide **1**, Xyl-(PLGA)$_2$, was obtained as follows. First, the polymerization of BLG-NCA was carried out from the primary amino groups of the *m*-xylylenediamine in $CHCl_3$ at room temperature. Then the reaction mixture was poured into a large excess of diethyl ether, and the precipitate was separated by centrifuge and then washed with diethyl ether repeatedly and dried. Subsequently, Xyl-(PBLG)$_2$ thus obtained was dissolved in 5% HBr in acetic acid, and the removal of benzyl groups was carried out by stirring for 4 h at 45 °C. The reaction mixture was then poured into a large excess of diethyl ether, and the precipitate was washed with diethyl ether repeatedly. As a result, the desired Xyl-(PLGA)$_2$(**1**), was obtained as a white powder. The degree of polymerization of the polypeptide segment (*n*) was estimated to be 15 by means of ^{1}H-NMR spectroscopy (400 MHz; JEOL JNM GX-400 spectrometer)). ^{1}H-NMR (DMSO-d_6, TMS); δ (ppm) 1.4-2.8 (-C*H*$_2$C*H*$_2$-CO-(side chain of PLGA), 3.6-4.5 (-NHC*H*$_2$Ph-(Xyl), -COC*H*NH-(main peptide chain)), 6.7-7.2 (Ph (Xyl)), 7.4-8.7 (-N*H*-). IR (cast film); 1700 cm^{-1} ($\nu_{C=O}$ Side chain), 1660 cm^{-1} ($\nu_{C=O}$ Amide), 1545 cm^{-1} (δ_{NH} Amide).

The peptide **2**, Flu-(PLGA)$_2$, was obtained by using 2,7-diaminofluorene as an initiator in a manner similar to that described above. The degree of polymerization of the polypeptide segment (*n*) was estimated to be 12 by means of ^{1}H-NMR spectroscopy. ^{1}H-NMR (DMSO-d_6, TMS); δ (ppm) 1.4-2.8 (-C*H*$_2$C*H*$_2$-CO-(side chain of PLGA), 3.6-4.5 (-COC*H*NH-(main peptide chain)), 6.7-7.2 (Ph (Flu)), 7.4-8.7 (-N*H*-).

2.2 Measurements.

CD spectra were recorded on a J-720 spectro-polarimeter (JASCO Ltd.) under a nitrogen atmosphere. Experiments were performed in a quartz cell with a 1 mm path length over the range of 190-250 nm at ambient temperature. Final peptide concentration was 1.6 x 10^{-3} Glu unit M in water. The pH of the sample solution was adjusted with 0.1 M HCl or 0.1 M NaOH. The helix content was calculated by using the following equation (1)[8]:

Helix content (%) = $([\theta]_{222} - [\theta]_c / [\theta]_h - [\theta]_c) \times 100$ (1)

where $[\theta]_{222}$, $[\theta]_h$ and $[\theta]_c$ are the molar ellipticity at 222 nm, –35000 and 5000 deg cm^2 dmol^{-1}, respectively.

Reflection absorption FTIR spectra (Thermo Nicolet Co. Nexus 470) were obtained on an Au-deposited glass plate using a MCT detector (resolution, 2 cm^{-1}; number of scans, 1024). The *p*-polarized light was introduced onto the sample at 80° to the surface normal. The sample and the detector chamber were purged with dried nitrogen before and during measurement.

The AFM images were collected at ambient temperature on a Nanoscope IIIa (Digital Instrument, Inc.) operated in a tapping mode using silicon cantilevers (125 μm, tip radius 10 nm). An aliquot of peptide **1** in water was placed on freshly cleaved mica. After adsorption for 1 min, the excess solution was removed by absorption onto filter paper and the samples were stored in a covered container to protect them from contamination until they were imaged (within 1 h). It should be noted that the AFM images obtained on other substrates (glass, cationic mica etc.) were similar to that obtained on mica substrate. A 10 μm x 10 μm scanner was used for imaging. The scanning speed was at a line frequency of 1 Hz, and the original images were sampled at a resolution of 512 x 512 points.

3. RESULTS AND DISCUSSION

3.1 Conformational Studies of Helix-Turn-Helix Polypeptides in Water

The conformational properties of the triblock peptides (**1** and **2**) in water were first investigated by means of circular dichroism (CD) spectroscopy. Figure 2 shows the time dependence of CD spectra for **1** and **2** at pH 4.0 (peptide concentration 1.6 x 10^{-3} Glu unit M). For peptide **1** (Fig. 2*a*), the spectra give a typical pattern of right-handed α-helical polypeptides with two negative maxima, one at 222 nm and one at 208 nm, and show no significant change with incubation time. The helicity of the PLGA units was calculated to be about 65 % from the observed molar ellipticity at 222 nm (see experimental section). This helicity is reasonable taking account of relatively short segment length as *n* = 15. By elevating the pH value to 10.0, the spectrum changes to that of random coil structure, similar to a normal PLGA, with a broad positive peak at 218 nm and negative peak at 197 nm (data not shown). These spectral changes with pH value are found to proceed through an isodichroic point at around 204 nm. On the other hand, for peptide **2** (Fig. 2*b*), the CD spectra showed the typical aggregated α-helix pattern,[9] as evidenced by the red shifting of 222 nm band toward

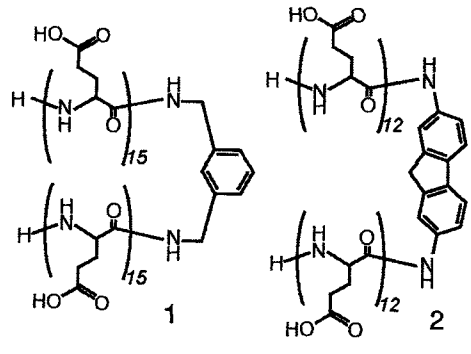

Fig. 1. Chemical structures of helix-turn-helix triblock polypeptides (**1** and **2**).

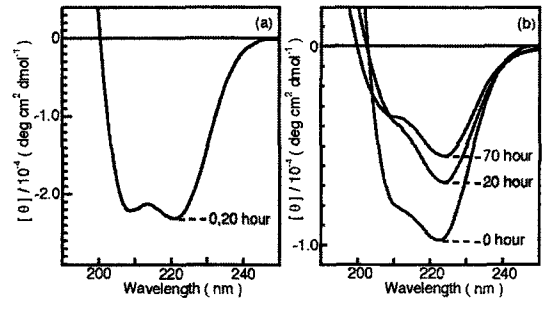

Fig. 2. CD spectral changes of peptide **1** (a) and **2** (b) in water at pH 4.0. The peptides were incubated at room temperature within the period indicated. [Glu]=1.6x10^{-3} unit M.

224 nm and the flattening of 208 nm band, even shortly after preparation of the sample solution. In addition, the molar ellipticity was reduced to half its initial value after incubation for 70 hours. This reduction in ellipticity was probably caused by the large aggregation[10] of **2** with the α-helical form. Such time-dependent spectral changes for **2** were not observed at pH 10.0, *i.e.* at a pH at which PLGA segments existed as random coil structures as well as the peptide **1**. It is therefore considered that the self-aggregation of **2** would be attributable to the helix-helix interaction, although the appropriate packing arrangement of two helices (tertiary structure) is required in causing such helix interaction.

3.2 Self-assembled Nanostructures from the Helix-Turn-Helix Polypeptides.

To gain insight into the aggregation properties of **2** (suggested by the CD study), we performed atomic force microscopy (AFM) measurements. In the field of structural biology, AFM is a useful technique to evaluate the three-dimensional structural features of proteins and their assemblies in nano-meter scale. Fig. 3 shows the time dependence of three-dimensional tapping-mode AFM images (1 x 1 μm^2) prepared from **2** in aqueous solutions ([Glu] = 1.6 x 10^{-3} M) of pH 4.0 and 10.0. AFM images obtained at pH 10.0 (Fig. 3C and D), revealed the absence of significant aggregates, indicating that the peptide **2** exists as a monomer (random coil structure) under this condition. In contrast, an AFM image obtained after incubation for 20 h at pH 4.0, in which the PLGA chain took mainly the α-helical form, revealed the presence of rectangular plate-shape aggregates (Fig. 3B). The average height of the plate aggregates was determined to be 1.7 or 5.0 nm, and the width and length were typically 40 nm and 70 nm, respectively (AFM level). It is important to note that AFM provides accurate measurements of a sample's height above the substrate, but the well-known convolution of the scanning tip leads to an overestimation of the sample width.[11] Thus, the

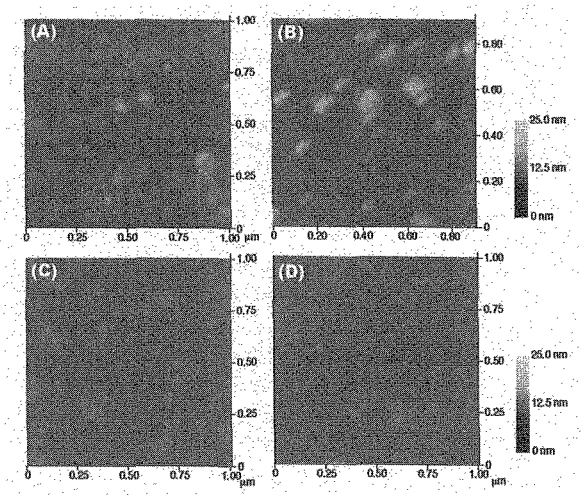

Fig. 3. Time dependence of tapping mode AFM images (1x1 μm^2) for peptide **2** at pH 4.0 (A, B) and 10.0 (C, D). The peptide **2** was incubated at room temperature for 0 h (A, C) and 20 h (B, D). [Glu]=1.6x10^{-3} unit M.

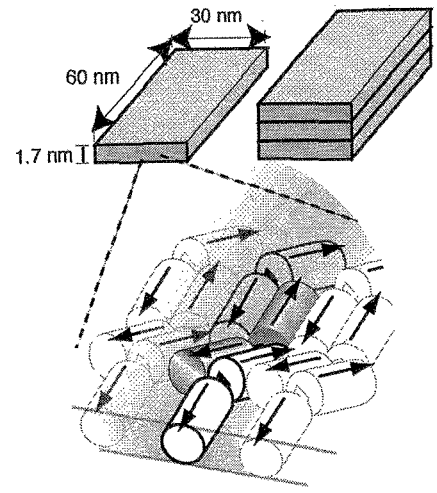

Fig. 4. Schematic illustration of the rectangular plate-shape aggregate observed in peptide **2** system.

observed dimensions have to be corrected. When the sample is assumed to be a plate, the real width (W) of the plate can be obtained by the following equation[12],

$$W = W_{obs} - 2(R_t H - H^2)^{1/2} \text{ ---- (2)}$$

where H is the observed height, and R_t is the radius of the AFM tip (10 nm). According to the above correction, the average width and length were calibrated to be 30 nm and 60 nm, respectively. The observed heights of the plate-shape aggregates (1.7 nm) are well consistent with the diameter of PLGA helix (*ca.* 1.3 nm).[13] Therefore, these plate-shape aggregates are supposed to consist of helix-monolayer, in which PLGA helices of **2** interact with each other in two-dimensionally, as shown in Fig. 4. The rectangular aggregates with 5.0 nm heights are probably due to the stacking of above thin plates (three plates (5.1 nm)) (Fig. 4). These structures of **2**-aggregates were also supported by FTIR measurements. Fig. 5 shows the reflection absorption (RA) FTIR spectrum of the **2**-aggregate on gold plate. Characteristic absorptions of amide I and II bands with the α-helix structure were

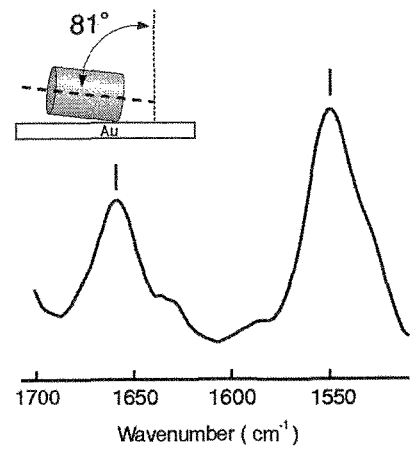

Fig. 5. RA-FTIR spectrum of the rectangular plate-shape aggregates composed of peptide **2** on Au plate. The plate-shape **2** aggregate was obtained after 20 h incubation in water at pH 4.0.

observed at 1660 and 1550 cm^{-1}. The average tilt angle of the α-helical axis of the PLGA from the surface normal was estimated to be 81° from the ratio of the individual intensities of amide I to amide II absorption bands, $D = A_I/A_{II}$.[14] In other words, the PLGA helices was oriented parallel in plate-like aggregates as suggested by AFM study.

The specific interaction between PLGA segments in the aggregates can be explained in view of a helix macrodipole. The dipole moments of amino acid in an α-helical peptide align in the same direction, nearly parallel to the helix axis, then the resulting macroscopic dipole generates an electrostatic potential, directed from the N-terminus to the C-terminus.[15] This electrostatic field plays an important role in the high-order structure and functions of proteins. We reported direct evidence for helix-helix macrodipole interaction by exploring an attractive interaction between the disulfide-modified PLGA self-assembled monolayer on gold and redox active PLGA derivatives as guest helices.[5,6] Thus, the helix-macrodipole interaction (namely, the head-to-tail antiparallel orientation of the PLGA chain, which is energetically more favorable) probably also contributes to the stability of the high-order structure of **2**-aggregate.

On the other hand, such self-assembly into shape-specific nanostructures was not observed in the case of peptide **1**. Even at pH 4.0, the peptide **1** was found to exist as a monomer, although the small portion of **1** formed poorly organized amorphous aggregates (data not shown). The peptide **1** has a flexible methylene parts between peptide segment and rigid template, and this part will probably provide sufficient mobility of PLGA chains, which prevents the controlled growth of plate-shape aggregates as observed in peptide **2** system. A similar situation was also observed for PLGA homo-polypeptide (n=15), namely the homo-polypeptide didn't show the spectral change in CD measurement and the nanostructure formation at pH 4.0 even after incubation for 24 h. From these results, it can be concluded that the self-assembling properties of the triblock peptide-architectures (**1** and **2**) was closely related to their secondary and tertiary structure, which can be controlled by manipulating the pH and/or appropriate design of template molecules.

4. CONCLUSION

In the present study we successfully synthesized two types of artificial helix-turn-helix triblock peptides (**1** and **2**), and described their conformational and self-assembling properties in water. Only under the condition of pH 4.0 for peptide **2** did the inter-peptide attractive force based on the PLGA helices and their stable tertiary structure (helix-turn-helix conformation) permit the controlled self-assembly, resulting in induction of highly-ordered nanostructure formation with rectangular plate-like form. We believe the ability to control the distribution of high-order structures (conformation and/or assembled nanostructure) of the artificial peptide provides important insights not only for understanding the protein folding mechanism, but also for developing novel peptide-based biomaterial with shape-specific nanostructure.

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[14] Calculation of the tilt angle has been carried out by using eqn. (3), proposed by Samulski et al.[16] where the angles of the transition moment (amide I and amide II) from the helix axis are supposed as 39° and 75°, respectively, and K (1.5) is a proportionality constant that is related to the intrinsic oscillator strengths of the amide I and amide II vibrational modes.

$$D = A_I/A_{II} = K \frac{0.5(\sin<\theta>\sin 39)^2+(\cos<\theta>\sin 39)^2}{0.5(\sin<\theta>\sin 75)^2+(\cos<\theta>\sin 75)^2} \quad (3)$$

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