Double Assembly Composed of Lectin Association with Columnar Molecular Assembly of Cyclic Tri-β-peptide Having Sugar Units

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A novel double assembly was prepared by association between a columnar molecular assembly of cyclic tri-β-peptides having sugar units and lectins. The NMR, FT-IR, and circular dichroism (CD) spectroscopy as well as computational calculations revealed that this compound took a flat and C_3 symmetrical conformation and that the amide N−H and C═O groups protruded vertically to the ring plane. This disk-shaped molecule stacked one by one to form a columnar structure via intermolecular hydrogen bonds between the amide groups. WGA lectin moderately bound to this columnar assembly to form a double assembly. Another lectin (Con A) disturbed the columnar structure upon strong binding, and RCA lectin showed no binding. Fluorescence spectroscopy revealed that the association between WGA lectin and columnar assembly of cyclic glycopeptide could be achieved due to the high density of the hydroxyl groups on the assembly surface (cluster effects). Interestingly, after cross-linking the lectins bound to the columnar assembly (the double assembly) by glutaraldehyde, the core column of cyclic tri-β-peptides could be washed away to leave the protein nanotube.

Introduction

Tobacco mosaic virus (TMV) attracts much attention due to the well-known self-assembled structure, which is composed of viral RNA in a helical structure and coating proteins around the helix.[1,2] Specific interactions between protein and RNA makes it possible to assemble them reversibly depending on pH and solvent due to the change of secondary interactions acting between them. Even though we have learned valuable points for the supramolecular assembly of two components from TMV, it is challenging and opens a new aspect that the one component of RNA as a template for the assembly is replaced with a molecular assembly, in total two assembling systems coexisting in the molecular system. One of the interesting points about such double assembling systems is that the core of the template and the shell of the aligned proteins are formed spontaneously without the concern of tedious covalent chemistry that usually requires many synthetic steps for a large molecular system. Further, the shell structure will be left behind, for example, as a nanotube of proteins after cross-linking the proteins and washing out the template assembly of the core in the double assembly of a cylindrical shape.

To construct such a double assembling system, we designed here a columnar molecular assembly with the exterior being covered by proteins due to the association of proteins specifically with the surface of the columnar assembly. The difficulty of this assembly formation is that the association of proteins with the columnar molecular assembly may destroy the columnar structure through steric effects when the interactions of the protein with the column are too strong. We therefore have to pay attention to the adjustment of two kinds of interactions, one for the association of proteins with the column and the other for the molecular stacking in the columnar structure, not to interfere with each other during their molecular assembling.

For the columnar molecular assembly, we chose a cyclic tripeptide of β-amino acid, which is a member of a peptide nanotube family.[3−12] Acyclic and cyclic β-peptides are known to form unique and interesting helical[13−22] and tubular structures,[5,23,24] respectively. To be endowed with affinity sites for proteins, a novel cyclic tri-β-peptide having sugar units is newly designed here as a component of the columnar assembly. Lectins are well-known to bind to specific sugars, and the affinity will become large when the sugars form clusters. Such a multivalent effect can be expected for the nanotube assembly of cyclic tri-β-peptide having sugar units at the exterior. On the basis of such molecular designs, the double assembly system was constructed from lectins as the shell and the tube assembly of the cyclic tri-β-peptides as the core template.

The double assembly formation was evaluated by fluorescence microscopy and TEM with using lectins labeled by fluorescein and gold nanoparticles, respectively. Further, the lectins were cross-linked by glutaraldehyde, and the tube assembly of the cyclic tri-β-peptides was washed away to leave the cross-linked lectins. The structure of cross-linked proteins left behind was studied by fluorescence spectroscopy on the construction of a novel protein assembly.

Experimental Procedures

General Procedure. Fluorescein isothiocyanate (FITC) labled wheat germ agglutinin, *Ricinus communis* agglutinin I, and *Concanavalin A* (FITC-WGA, FITC-RCA120, FITC-Con A) were purchased from

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Vector Laboratories, Inc. Au nanoparticle (15 nmϕ)-labeled WGA (Au-NP-WGA) was purchased from EY Laboratories, Inc. All other reagents were purchased from commercial sources and used as received. NMR spectra were recorded with a Bruker DPX-400 spectrometer. FAB-MS was performed on a JEOL XPS spectrometer using 2,4-dinitrobenzyl alcohol and thioglycerol as matrices. IR spectra were measured on a Nicolet 6700 FT-IR spectrometer. Molecular assembly formation was confirmed by dynamic light scattering (DLS) with a Photal DLS-8000. Circular dichroism (CD) spectra were measured at room temperature on a JASCO J-600 CD spectropolarimeter using an optical cell of 1 cm path length with concentration of 5×10^{-5} M.

Geometry Optimization. The initial geometry of the compound was generated by the Fujitsu CAChe software, and it was optimized by the semiempirical Austin Model 1 (AM1) method in the MOPAC 2002 package.[25] Using the obtained geometry as input, ab initio calculations were carried out by the Gaussian 03 program.[26] Finally, the geometry was further optimized at the Hartree−Fock (HF) level.

Peptide Synthesis. Compound 1′. Compound **1**[27] (25 mg, 26.4 μmol) was dissolved in DMSO (2.0 mL), and then a 1 N NaOH aqueous solution (285 μL) was added. After stirring at 80 °C for 12 h, the mixture was neutralized with a 1 N HCl aqueous solution. After that, MeOH was added to the solution until the insoluble solids appeared. The precipitate was washed with DMSO and hot MeOH to give **1′** (14 mg, 94%) as a white solid: ^{1}H NMR (400 MHz, DMF-d_7) δ 4.22 (t, 3H), 4.01−3.93 (m, 6H), 3.81 (m, 3H), 3.66 (m, 3H), 3.62−3.56 (m, 6H); IR (KBr) 3276, 3094, 2893, 1672, 1570, 1373, 1296, 1099, 1058 cm^{-1}; FAB-MS (matrix: thioglycerol): (*m*/*z*) calcd. for $C_{21}H_{34}N_3O_{15}$ [(M + H)$^+$]: 569.20. obsd 569.13.

Optical and Fluorescence Microscopy. Optical microscopy and fluorescence microscopy were performed using an Olympus IX70. The saturated solution of **1′** in water was dropped onto a glass substrate, and the water was evaporated at 40 °C for 3 h. Another thin glass plate was put on the sample surface, and the sample was observed in the crossed nicols with a sensitive tint plate positioned between the polarization filters. In the fluorescence microscopy, the crystal of **1′** prepared on a glass substrate was incubated in a HEPES buffer (pH 7.5) containing a fluorescein-labeled lectin (0.05 mM) at 4 °C for 1 h. Thereafter, the sample was rinsed with pure HEPES buffer three times. In the inhibition experiment, the buffer containing the fluorescein-labeled lectin and *N*,*N*′-diacetylchitobiose as an inhibitor (0.1 mM) was used.

Lectin tubes were prepared by cross-linking lectin attached to the columnar assembly as described next. The double assembly composed of the crystal of **1′** and FITC-WGA was incubated in HEPES buffer containing glutaraldehyde (2%) and further FITC-WGA (0.05 mM) at 4 °C for 1 h. After this period, the sample on the substrate was thoroughly rinsed with a pure HEPES buffer and water to remove nonspecifically absorbed lectin and the inner molecular column of **1′**.

Transmission Electron Microscopy and Electron Diffraction. The images and diffractions were taken by using a JEOL JEM-2000EXII at an accelerating voltage of 100 kV. For the observation of columnar assembly of **1′**, the dispersion of **1′** in water was mounted on a carbon coated Cu grid, and the sample was stained negatively with a 2% uranyl acetate and 1% trehalose solution. For observation of the double assembly, the sample prepared by the method described previously was incubated with an Au-NP-WGA solution (HEPES buffer, pH = 7.3, [protein] = 2.5×10^{-4} mM) at 4 °C for 10 min, and it was rinsed with the pure HEPES buffer.

The electron diffraction diagrams were obtained in the microdiffraction mode.[28,29] A small condenser with an aperture of 20 μm was inserted in the second condenser lens, and the first condenser lens was fully overfocused to achieve an electron probe of approximately 100 nm at the sample level. The samples were observed at 2500× under extremely low dosage conditions, at the limit of the dark current, with the help of a Fiber Optics Coupled TV image intensifier. After proper zone identification, the beam was manually blanked. The beam intensity was then adjusted at the desired value, and the electron microscope was switched to the diffraction mode. The beam was de-blanked, followed immediately by the opening of the mechanical shutter and the recording of the diffraction diagram on a preset film. The sample to camera length was calibrated with the (111) diffraction ring of evaporated Au particles.

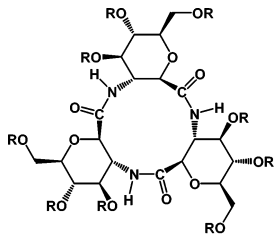

Figure 1. Chemical structures of cyclic tri-β-peptides.

Fluorescence Spectroscopy. Fluorescence spectra were recorded on a Hitachi MPF-4 fluorometer at an excitation wavelength of 494 nm. The excitation and emission band passes were set at 5 and 10 nm, respectively. The measurements were performed with a 10 mm quartz cell at 10 °C to avoid nonspecific absorption. The concentrations of FITC-WGA in a HEPES buffer (pH 7.3) were 0.5 μM (condition H) and 0.05 μM (condition L), respectively, and the total solution volume before addition of the glycopeptide solutions was 2 mL. The glycopeptide solutions with two kinds of concentrations were prepared according to the previous conditions: 2.0 mM (condition H) and 0.2 mM (condition L), respectively. In condition H, the glycopeptides formed the self-assembly, while in condition L, they were dispersed as one molecule. After the addition of a 10 μL aliquot of the glycopeptide solution to the FITC-WGA solution, the solution was left for a while until it reached equilibrium.

Results and Discussion

Conformational Analysis. Our designed cyclic glycopeptoid **1′** obtained from an acetylated compound **1** is shown in Figure 1. Our previous paper demonstrated that compound **1** formed the regular columnar assembly in an appropriate solvent.[27] If the ring structure of deacetylated compound **1′** is the same as that of **1**, **1′** is also expected to form the columnar structure via hydrogen bonds. We investigated the conformation of **1′** by various spectroscopic measurements and computational calculations. The trans-configuration of the amide groups was confirmed by FT-IR spectroscopy of the bulk sample, where amide I (C═O stretching mode) and amide II (N−H bending and C−N stretching mode) were observed at 1672 and 1570 cm^{-1}, respectively. Those wave numbers are characteristic of a parallel β-sheet structure.[30,31] Moreover, amide A (N−H stretching) was observed at 3276 cm^{-1} in overlapping peaks of multiple hydroxyl groups, suggesting that a hydrogen-bond network should be formed between the amide groups homogeneously throughout the crystal of **1′**. It is therefore concluded that the cyclic glycopeptides form nanotubes similar to those in the previous reports for cyclic tri-β-peptides.[4,5,24,27,32,33] The trans-configuration of the amide groups was also supported by circular dichroism (CD) experimentally and theoretically.[34] the CD spectrum of **1′** in water is constituted of a positive Cotton effect around 215 nm and a negative Cotton effect around 206 nm with zero interception around 206 nm as a typical pattern of a parallel β-sheet conformation.[33] CD analysis indicates that the ring structure of **1′** was the same as that of **1**. Moreover, the conformation of **1′** was studied further by computational geometry optimization (Figure 2a). Compound **1′** has a planar conformation with three *trans*-amide groups orienting perpen-

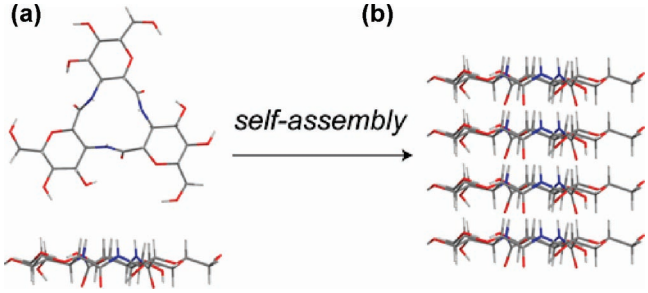

Figure 2. (a) Top view (top) and side view (bottom) of geometry-optimized structure of cyclo tri-β-peptide **1′** and (b) a schematic illustration of columnar assembly from **1′**.

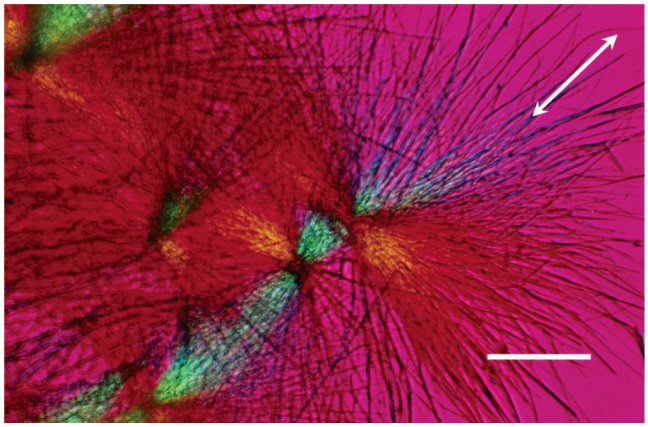

Figure 3. Spherulite-like assembly from **1′** prepared on a glass substrate. The double-headed arrow shows the orientation of the z'-axis of a sensitive tint plate. Scale bar = 100 μm.

dicularly to the ring plane and is expected to form the columnar structure via hydrogen bonds (Figure 2b). As shown in Figure 2b, hydroxyl groups at the side rings form a cluster, which should become the affinity site for sugar-recognition proteins.

Microscopic Observation. The molecular assembly of **1′** was successfully obtained by recrystallization from water as needle-like crystals (Figure 3). When the crystals were observed in the cross-nicol configuration with a sensitive tint plate inserted at 45°, the crystals show as blue (addition retardation) or yellow (subtraction retardation) depending on the orientation of the long axis of the crystal being parallel or perpendicular to the z'-axis of a sensitive tint plate. The observation indicates that the long axis of the crystal has a larger refractive index ($n_{\parallel}$) than that of the short axis ($n_{\perp}$). The crystal showed the same retardation type as cyclo(ACHC)$_3$, indicating that amide groups orient along the long axis of the crystal, which also leads to the interpretation that **1′** stack with each other along the long axis via an intermolecular hydrogen-bonding network to form a columnar assembly.[24]

We also prepared water or HEPES buffer dispersion of **1′** and investigated the molecular assembly formation of **1′** by an electron diffraction analysis (Figure 4). The electron crystallography of the molecular assembly revealed that **1′** stacked up with a spacing of 4.8 Å along the needle axis, which is consistent with the columnar structure as shown in Figure 2b.[24,27] On the other hand, we estimate a stacking distance from the N−H stretching frequency in FT-IR spectra according to Krimm's analysis. The calculated value is 4.8 Å, which agrees with the result of the ED analysis.[35] The diameter of the needle-like crystals is as large as 50−200 nm, indicating that the columnar assemblies of **1′** associate together to form the thick bundles. In the bundle, the columns should orient in an anti-parallel way

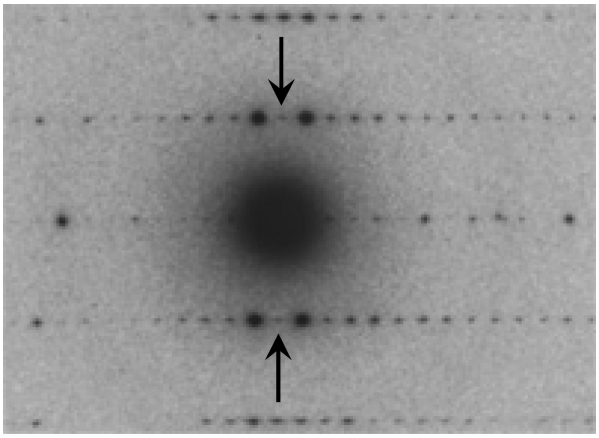

Figure 4. Electron diffraction pattern obtained with an incident electron beam perpendicular to the columnar axis of the assembly of **1′**. The spots indicated by arrows suggest that the columns in the bundle orient in an anti-parallel manner.

because diffraction appears at the intersections between a central meridian axis and the first layer lines.

Construction of the Double Assembly. The exterior of the needle-like crystals must be covered by many hydroxyl groups of the glucosamine units of **1′**, which will provide binding sites for lectins. The association of fluorescein-labeled lectins with the needle-like crystals of **1′** was investigated by fluorescence microscopy. When the crystals were incubated with FITC-Con A, the binding of FITC-Con A with the assembly of **1′** was identified, but the needle structure was destroyed (Figure 5a). On the other hand, FITC-WGA bound to the needles with keeping the original shape of the crystals (Figure 5b). The binding of WGA to the crystals of **1′** was inhibited by the presence of *N*,*N*′-diacethylchitobiose (Figure 6a). It is thus concluded that the WGA binding is due to the molecular recognition of WGA with the sugar unit of **1′** and not due to nonspecific interactions. Indeed, in the control experiment using RCA120, which is a galactose-specific lectin and binds neither to glucose nor *N*-acetylglucosamine (GlcNAc), no significant fluorescence from the needle-like crystals was observed upon incubation with fluorescein-labeled RCA120 (Figure 6b).

The double assembling by lectin association specifically with the columnar assembly of **1′** was thus successfully attained using WGA. WGA is known to bind specifically to a GlcNAc unit. **1′** possesses the glucosamine unit, but the amino group was used to connect the glycamino acid to form a cyclic skeleton. It has been reported that the *N*-acetyl group and the hydroxyl group at C-3 are involved in the binding of WGA to GlcNAc through the formation of hydrogen bonds.[36,37] Because **1′** has no *N*-acetyl group to form a hydrogen bond with WGA, which should make the binding strength of WGA with **1′** less than with GlcNAc, the association of WGA to the columnar assembly of **1′** should be compromised but not disrupt the columnar assembly. On the other hand, in the binding of Con A with a glucose unit, hydroxyl groups at C-3, C-4, and C-6 are reported to be essential.[38] Since the columnar assembly of **1′** exposes these three hydroxyl groups at the exterior, Con A could bind to them strongly (even though the binding of Con A to **1′** must be weaker than that to a glucose unit) and disturb the columnar structure of **1′**.

The association constant (K_a) between WGA and the molecular assembly was evaluated by measuring the changes in fluorescence intensity of fluorescein-labeled WGA upon the addition of the cyclic peptide solutions with changing the concentrations. When the concentration of cyclic tri-β-peptide

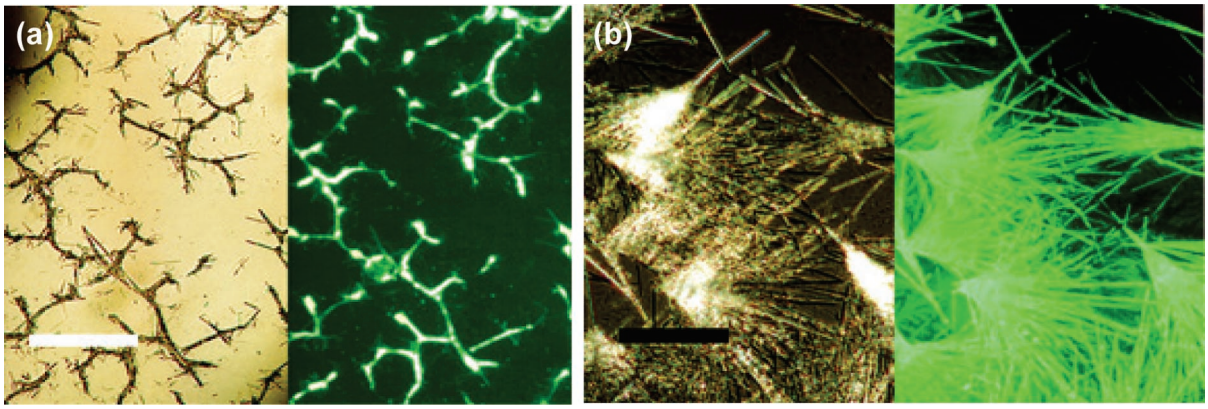

Figure 5. (a) Association of FITC-labeled Con A with the molecular assembly of **1′**: (left) optical microscope image and (right) fluorescence image, bar = 200 μm and (b) association of FITC-labeled WGA with **1′**: (left) optical microscope image under cross nicol and (right) fluorescence image. Scale bar = 100 μm.

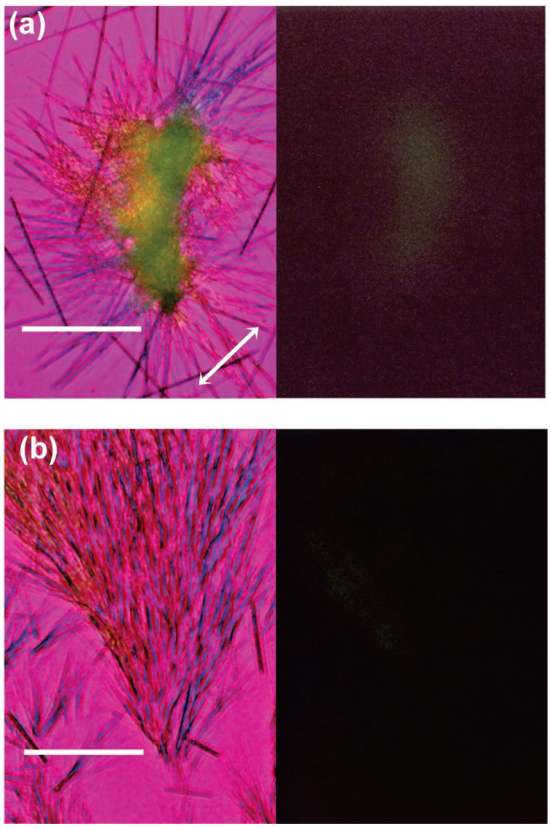

Figure 6. Cross-polarized optical (left) and fluorescence (right) microscopic images of **1′** + FITC-WGA + *N,N′*-diacetylchitobiose (a) and **1′** + FITC-RCA120 (b). The double-headed arrow shows the orientation of the *z′*-axis of a sensitive tint plate. Scale bar = 100 μm.

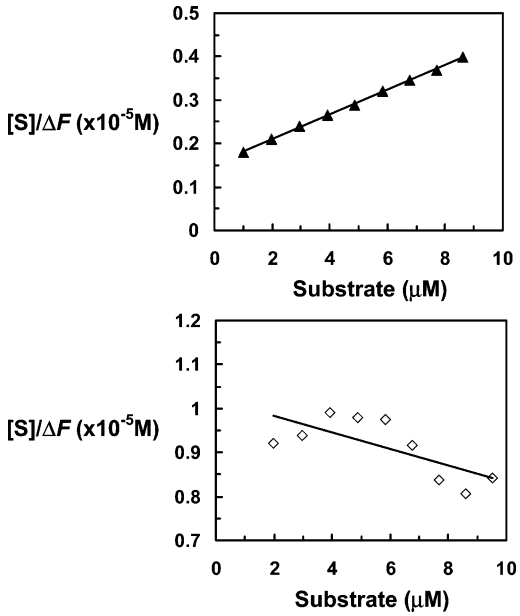

Figure 7. Plots from the fluorescence emission spectra of FITC-WGA upon the addition of the assembly solution (under the condition **H**; 2.0 mM, top panel) and monomer solution (under the condition **L**; 0.2 mM, bottom panel).

was around 10^{-6} M (condition L), the molecular assembly size was as small as the hydrodynamic diameter of 3 nm by dynamic light scattering (DLS) measurements (vide infra). Under this concentration range, no fluorescence intensity change was observed. On the other hand, under the concentration range of 10^{-5} M (condition H), large assemblies of 100 nm by DLS measurement were formed, which decreased the fluorescence intensity of WGA upon mixing in a buffer. This observation suggested that the fluorescein unit of WGA moved from a hydrophobic environment to relatively hydrophilic surroundings due to the binding of glycopeptide by WGA. It is well-known that a blue shift of the emission maximum is observed in a binding event between WGA and GlcNAc residue. However, we did not observe a significant shift, which is probably due to the fluorescein unit of WGA being apart from the binding sites.[39]

The change of the fluorescence intensity with the addition of the glycopeptide solution was recorded, and the result was analyzed on the basis of a Langmuir-type absorption (eq 1).[40,41] [S] and [substrate] are the concentration of the sugar ligand and glycopeptide concentration, respectively; [S] is equal to three times [substrate] because the glycopeptides have three sugar units. ΔF, ΔF_{max}, and K_a stand for the fluorescence change, the fluorescence change after the addition of an excess amount of the glycopeptide solution, and the association constant, respectively.

$$\frac{[S]}{\Delta F} = \frac{1}{\Delta F_{max}}[\text{substrate}] + \frac{1}{\Delta F_{max} K_a} \quad (1)$$

From the section of the plot of $[S]/\Delta F$ versus [substrate], the association constant in condition H was evaluated to be $K_a = 1.9 \times 10^4$ ($-\Delta G = 5.5$ kcal/mol^{-1}) (Figure 7, top). The exterior of the molecular assembly provides densely packed hydroxyl groups, which should promote WGA binding due to the cluster effect. The columnar assembly is stabilized by three hydrogen

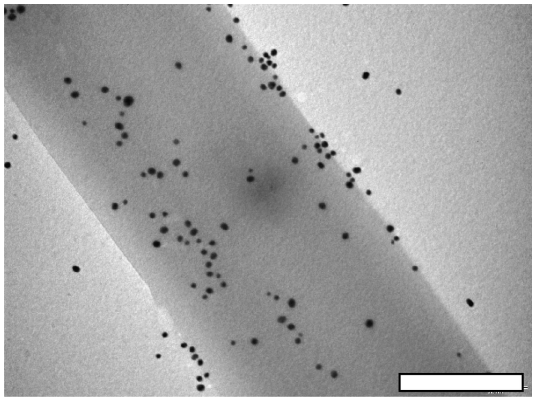

Figure 8. TEM image of the double assembly composed of Au-NP-WGA and columnar assembly of **1′**. Scale bar = 200 nm.

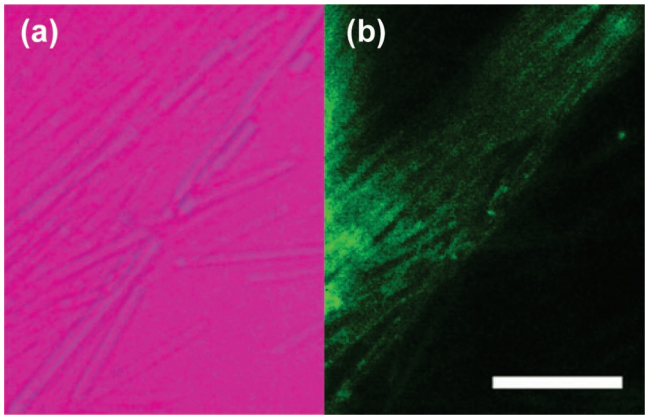

Figure 9. Observations of the FITC-WGA assemblies after the treatments of the double assembly system with the crosslinking reagent and buffer washing by (a) optical microscopy under cross nicol with a tint plate and (b) fluorescence microscopy. Scale bar = 50 μm.

bonds between two cyclic tri-β-peptides, which will need more than 7 kcal/mol^{-1} to break.[42] It is therefore considered that WGA and the columnar assembly of cyclic tri-β-peptide could moderately associate together without disruption of the columnar assembly. On the other hand, we could not obtain a reasonable fitting line under condition L and concluded that WGA did not bind to a monomeric **1′** specifically (Figure 7, bottom).

Moreover, to confirm that there is no change in the morphology of the molecular assembly of the glycopeptide during measurements, we measured DLS (10 °C) for three samples: (i) the FITC-WGA solution, (ii) the FITC-WGA solution with the addition of **1′** (100 μL of 2.0 mM), and (iii) the FITC-WGA solution with the addition of **1′** (100 μL of 2.0 mM). The preparation of samples ii and iii was carried out in the same manner as described previously. The results of DLS revealed that hydrodynamic diameters of i−iii were 3.6, 102, and 3.3 nm, respectively, suggesting that the molecular assembly of cyclic glycopeptides in the case of H did not change its morphology drastically upon an injection operation to the lectin solution and that the cyclic glycopeptide did not form a large molecular assembly in the case of L. It is therefore concluded that the different result between H condition and L condition in fluorescence measurements should be due to the density difference of the hydroxyl groups on the molecular or the assembly surface (cluster effects). The DLS data also support that we succeeded in the evaluation of binding between the columnar assembly and WGA under the H condition and between the monomeric unit of **1′** and the WGA under the L condition.

To confirm the association of lectin on the surface of the columnar assembly, we used WGA labeled with gold nanoparticles of 15 nm diameter (Au-NP-WGA).[43,44] The columnar assembly of **1′** was incubated with Au-NP-WGA, and the mixture was washed with a buffer solution on a TEM grid. As shown in Figure 8, specific staining of the molecular assembly of **1′** with gold nanoparticles was observed by TEM, indicating clearly that WGA should bind to the exterior of the columnar assembly of **1′**. Electron diffraction of the molecular assembly showed the same diffraction pattern as that of the original columnar assembly in the absence of WGA, supporting that the columnar assembly in the double assembly maintains the original structure. The low surface density of gold nanoparticles in Figure 8 is due to the limited concentration of Au-NP-WGA used for sample preparation. The exterior of the columnar assembly will be fully covered with WGA at high concentrations of WGA as shown in Figure 5b. The tight packing of the lectins on the surface is indeed supported by the following experiment.

The columnar assembly of **1′** was incubated with FITC-WGA at a high concentration to obtain the double assembly system (Figure 5b). Then, the assemblies were treated with glutaraldehyde to cross-link WGA proteins and were washed with a buffer solution and water. The fluorescence microscopic observation of the assembly shows the same staining in a rod-like shape as that before washing (Figure 9b). Because without the treatment of glutaraldehyde all the assemblies of FITC-WGA and **1′** disappeared after washing, FITC-WGA proteins stayed in a rod-like shape due to the cross-linking between the proteins. Interestingly, polarized optical microscopic observation of the assembly with a tint plate shows no crystalline structure of the columnar assembly of **1′**, indicating that **1′** was washed away. It is thus concluded that nanotubes of FITC-WGA proteins cross-linked by glutaraldehyde were prepared from the double assembly system.

Conclusion

We report here synthesis and conformation of the novel water-soluble cyclic tri-β-peptide having sugar units and the construction of the double assembly composed of lectin and the columnar assembly from the cyclic tri-β-peptide. WGA associates with the exterior of the columnar assembly of **1′** without disruption of the assembly. The key to construction of the double assembly is to adjust the association force in each assembly and not to interfere. Further, nanotubes made of cross-linked proteins were obtained from the double assembly with the columnar assembly of **1′** as a template. A double assembly of protein and thinner columnar assembly is challenging and leads to the production of protein nanotubes with a diameter of a few nanometers in length, which is now under investigation using a single columnar assembly of **1′**.

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Supporting Information Available. Theoretical simulation of CD spectrum. This material is available free of charge via the Internet at http://pubs.acs.org.

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