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Rod and Vesicular Structures of Cyclosophoraose-Based Ionic Self-assembly

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Cyclosophoraoses (Cys), a class of unbranched cyclic 1,2-β-D-glucans, are produced by many strains of *Rhizobium* and *Agrobacterium*.[1] They form a variety of large-ring molecules that comprise a variable number of glucose residues (17–23) per ring. Conformational analyses have indicated that Cys have flexible backbones and narrow cavities.[2] Based on their structure, their ability to form inclusion complexes with guest molecules has been studied to investigate their potential as solubility enhancers of poorly soluble guests.[3,4] Chemical modification of Cys could also expand its ability for complexation with various drugs.[5,6] To the best of our knowledge, here we show for the first time, the self-assembled supramolecular structure formed by Cys, and demonstrate that the secondary structure has the potential for application in development of drug delivery and releasing systems.[7,8]

Self-assembled materials with well-defined shapes and dimensions are currently attracting considerable interest, and are promising candidates for development of functional molecular devices and nanomaterials.[9] Among the various fabrication techniques, ionic self-assembly by coulombic interaction is considered to be a powerful tool for its ease of use, reliability, flexibility, and universality.[10] Thus, development of building blocks for ionic self-assembly is required. Negatively charged hyaluronan has been investigated for self-assembly with oppositely charged styrylpyridinium or chitosan.[11,12] Cationic chitosan has also reported to form nano-assembly with dextran sulfate using ionic interaction.[13] In the present study, we introduce a building block based on microbial carbohydrate.

Cys were isolated and purified from *Rhizobium trifolii TA-1* as described in the Experimental section. The native Cys was modified with quaternary ammonium (QA), and the synthesized QA–Cys was used as a cationic participant in ionic self-assembly. The modified structures were investigated using elemental analysis (EA) and nuclear magnetic resonance (NMR) spectroscopy (Figure 1). Nitrogen contents (N%) of QA–Cys were measured to be 4.28 using an elemental analyzer. The degree of substitution (DS) value was determined to be 0.92 by measurement of nitrogen contents and calculated according to the following equation:[14]

$$DS_{pract.} = \%N_{cation} \times 162.15/1401 - (\%N_{cation} \times 151.64)$$

In ^{13}C NMR distortionless enhancement polarization transfer (DEPT)-135 spectrum (Figure 1(b)), new peaks were observed and assigned to the methine (C4′), methylenes (C7–9/C7–9′), and quaternary ammonium N-methyl carbons (C10/C10′). In the ^{1}H–^{13}C heteronuclear single quantum coherence (HSQC) spectrum (Figure 1(c)), the C6′ carbons shifted downfield to 72.57 ppm compared to the C6 carbons with a free OH group. In addition, the signal of the H4′ proton was correlated with the corresponding carbon signal at 80.22 ppm. Three cross-peaks at 4.23/76.17, 4.47/67.85, and 3.50/71.00 were detected by the direct correlation between protons and carbons of the –C7,7′H_2–, –C8,8′H–, and –C9,9′H_2– groups, respectively. The C10,10′/H10,10′ correlations at 3.24 ppm for ^{1}H and at 57.06 ppm for ^{13}C were also assigned. These structural analyses indicated that the novel QA–Cys had been successfully synthesized.

To form the ionic counterpart, Cys was derivatized with carboxymethyl (CM) groups and the structure was confirmed, as described in our previous study.[6] The native Cys was substituted with CM groups at the OH-4 and OH-6 of the glucose residues with DS of 0.38.

The synthesized cationic and anionic Cys were used as building blocks for ionic self-assembly. Since DS of QA-Cys and CM-Cys are different from each other (DS 0.92 for QA-Cys and DS 0.38 for CM-Cys), the charge imbalance present in the assembled structure is valuable for charged guest molecules. In addition, a carbohydrate backbone can provide a mild amphiphilic character, because of the hydrophobic or apolar surfaces in its sugar chain, as well as its hydroxyl groups.[15,16] Actually, the organic assembled structure is achieved by a variety of forces, including hydrogen bonds, hydrophobic effects, and π-π interactions.[17] Therefore, QA-Cys and CM-Cys are considered to make the self-assembled supramolecular structure by electrostatic interactions, hydrogen bonding, and hydrophobic effects.

The self-assembled morphologies formed by QA–Cys and CM–Cys were analyzed using scanning electron microscopy (SEM). Figure 2(a) and 2(b) show SEM images of the original QA–Cys and CM–Cys. The surface is flattened and there is no network structure. However, ionic interactions between QA–Cys and CM–Cys, induced to a thickness of 1–5 μm, showed a branched rod-type morphology (Figure 2(c)); when the molecules were placed in pH 2-buffered solution, the morphology was no longer observed.

In the presence of hexane, self-assembly of the supramole-

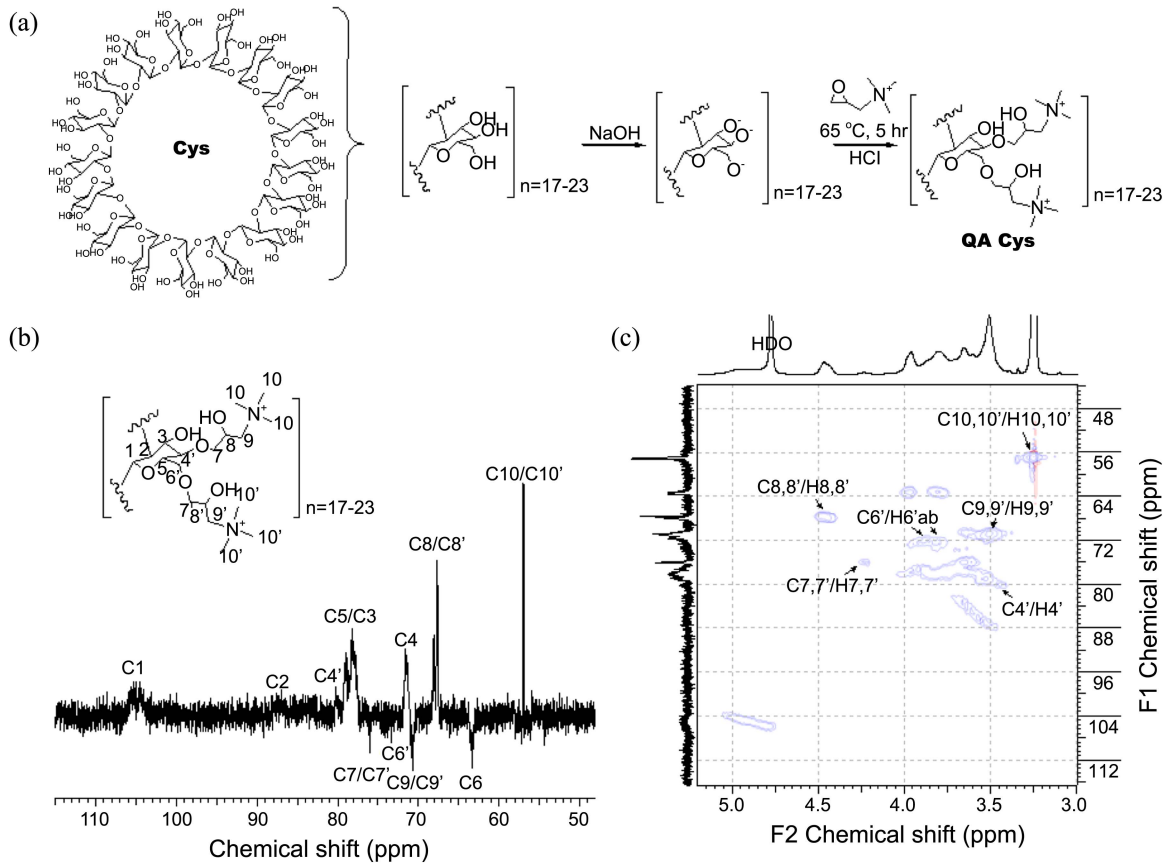

Figure 1. Synthetic protocol (a), DEPT-135 spectrum (b), and HSQC spectrum of QA-Cys.

cular structure was diversified. In the case of 1:20 ratio of hexane to buffer, fiber-type structure was made with 3-5 μm thickness (Figure 2(d)). As increasing the relative concentration of hexane, web-like structure of 1-5 μm thickness and bumpy coralliform were observed, respectively (Figure 2(e) and 2(f)). The organic solvent may have imposed structural distortions or alterations on the supramolecule shown in Figure 2(c).[18] These results indicate that novel supramolecular self-assembled structures were created by coupling of the oppositely charged building blocks (QA–Cys and CM–

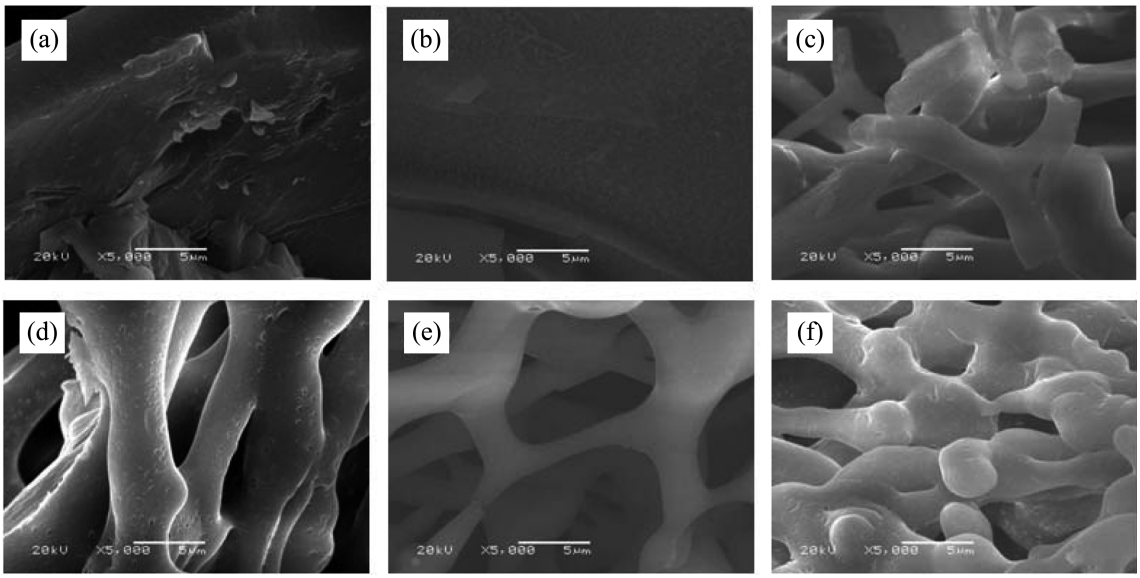

Figure 2. SEM images of QA-Cys (a), CM-Cys (b), and QA-Cys/CM-Cys ionic self-assembly in pH 7 phosphate buffer (c), in 1:20 (d), 1:10 (e), 1:5 (f) hexane : pH 7 phosphate buffer (v/v).

Cys) and changed by organic solvent. However, the above supramolecular structures have more surface area and interspace than unit molecule. Therefore, these self-assembled interwoven structures can provide favourable environment in addition to their respective inclusion complexes to encapsulate neutral or charged guest molecules.[7]

For comparison with an anionic counterpart, deoxycholic acid (DCA) was used for ionic self-assembly of QA–Cys. DCA is a bile acid composed of a steroid skeleton and an acid tail, and well known to possess many exciting biological properties.[19] Since QA-Cys could not make self-assembled aggregates with cholesterol, it is proved that the ionic interaction with DCA is critical step forming the supramolecule.

The self-assembly formed by QA–Cys and DCA in aqueous solution was revealed using transmission electron microscopy (TEM). Figure 3(a) and 3(b) show clear spherical micro-aggregates with vesicular shells, the morphology of which contrasts strongly with the morphology of QA–Cys/CM–Cys. The diameters ranged from approximately 100–200 nm in the TEM image; results of dynamic light scattering (DLS) showed diameters from 150–300 nm (Figure 3(c)). The discrepancy may be accounted for by noting that TEM and DLS show solid and swollen vesicles, respectively.[20] Anthraquinone modified β-cyclodextrin and DCA-modified chitosan have also been used for carrying drugs and DNA with the self assembled spherical structures of 200 nm and 130-300 nm in diameter, respectively.[7,21] Although the synthetic amphiphilic carbohydrates have been reported as the building block for self-assembled vesicle,[7,21,22] the vesicle based on electrostatic interactions has not been elucidated. The present self-assembled vesicular structure represents a novel phenomenon based on the ionic interactions between hydrophobic anion and cationic carbohydrate. Furthermore, these self-assembled vesicles can be used for biomimetic models, drugs or gene carriers, nanoreactors.[23-25]

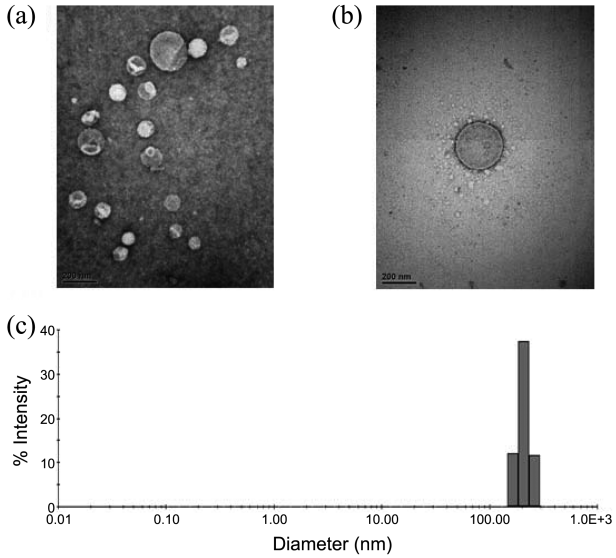

Figure 3. TEM image (a), enlarged image (b) (scale bars = 200 nm), and DLS profile of QA-Cys/DCA ionic self-assembly in water.

In conclusion, Cys isolated from *Rhizobium trifolii TA-1* were modified with quaternary ammonium groups and carboxymethyl moieties through chemical derivatization, respectively. Their modified structures were characterized with NMR, EA, and MALDI-TOF MS. The QA–Cys and CM–Cys were used as novel oppositely charged carbohydrate building blocks for self-assembled structures. Electrostatic attraction between the cationic and anionic portions produced ionic self-assembly behavior, and the resulting supramolecular structure and morphology were analyzed with electron microscopy (SEM and TEM). Furthermore, the self-assembled morphology could be controlled by changing the reaction conditions (pH, solvent, and addition of another anion). The present microbial carbohydrate-based supramolecule can be applied to tunable self-assembling materials for eco-friendly electronic devices or drug/gene delivery systems with its dual functionality as the macrocyclic host and supramolecular architecture. Further studies of applications using these supramolecules are in progress.

Experimental

Purification of Cys. The isolation and purification of Cys from *Rhizobium trifolii TA-1* were carried out as previously described.[26] Culture supernatants were concentrated fivefold by rotary evaporation, and the concentrated sample was precipitated by adding 3 volumes of ethanol. After centrifugation, the supernatant was concentrated by rotary evaporation and the product was collected by adding 7 volumes of ethanol. After decanting the supernatant, the precipitates were applied to Bio-Gel P-6 column. The fractions were assayed for carbohydrates using the phenol-sulfuric acid method. The fractions that contained Cys were pooled, concentrated, and desalted using Bio-Gel P-4 column. The desalted Cys were confirmed by NMR spectroscopy and MALDI-TOF MS.

Synthesis of QA–Cys. Cys (1 g) was dissolved in 7% NaOH aqueous solution for 30 min, and 2,3-epoxypropyltrimethylammonium chloride (3.1 g) was added. The mixture was reacted for 5 h at 65 °C, neutralized with HCl and evaporated to a viscous residue. After desalting on a Bio-gel P-2 column, the product was lyophilized, and the final structure was elucidated with EA (ThermoFinnigan, Flash2000) and NMR spectroscopy.

Synthesis of CM–Cys. CM–Cys was prepared as described in a previous report.[6] Cys (500 mg) and NaOH (2.8 g) were mixed in distilled water (7.4 mL). Monochloroacetic acid solution (20%) was added. After reacting for 4 h at 50 °C, the mixture was neutralized with 6 M HCl. The mixture was precipitated with 5 vol MeOH and left overnight at 4 °C. The precipitate was dissolved in water and desalted on a Sephadex G-10 column. The product was confirmed using MALDI-TOF MS (Voyager-DETM STR Biospectrometry Workstation) and NMR spectroscopy.

Nuclear Magnetic Resonance (NMR) Spectroscopy. For the NMR spectroscopic analysis, a Bruker Avance 500

spectrometer was used to record the ^{1}H–NMR, ^{1}C-NMR, DEPT and HSQC spectra. The HSQC spectrum was measured with a spectral width of 3401 Hz in both dimensions and 256/2048 complex data points in t_1 and t_2, respectively. NMR analyses were performed in D_2O at room temperature.

Scanning Electron Microscopy (SEM). QA–Cys (3 mM) and CM–Cys (3 mM) were mixed in 100 μL of 20 mM phosphate buffer (pH 7). Hexane was added to a QA–Cys/CM–Cys mixture in phosphate buffer (pH 7), and a turbid solution was obtained by vortexing for 5 min. After centrifugation, the precipitate was lyophilized. The lyophilized samples were mounted onto stubs using double-sided adhesive tape and then made electrically conductive by coating with a thin layer of gold. The surface morphologies of the materials were examined under a scanning electron microscope (Jeol, JSM 6380, Tokyo, Japan).

Transmission Electron Microscopy (TEM). QA–Cys (3 mM) and DCA (3 mM; Sigma Aldrich) were mixed in 800 μL of 20 mM phosphate buffer (pH 7). After sonication for 30 s, the aqueous suspension (10 μL) containing the supramolecular aggregates formed by the QA–Cys/DCA mixture was adsorbed onto a carbon-coated copper grid (300-mesh) and air-dried for 1 min. For clear negative staining, the supernatant of 2% uranyl acetate following centrifugation at 13,200 rpm for 2 min was used. The aggregates were examined using a transmission electron microscope (JEOL, JEM 1010, Tokyo, Japan).

Dynamic Light Scattering (DLS). DLS measurements were carried out with a Wyatt Technology DynaPro Plate Reader at constant room temperature.

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