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Supramolecular Nano-aggregates Directed by Phenyl Derivatives of Rhizobial Exopolysaccharides

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Supramolecular self-assembly is a spontaneous process in which a disordered system of pre-existing components forms an organized structure or pattern through local interactions among the components.[1] Proteins, DNA, and polysaccharides are examples of such supramolecular architectures in living systems. The self-assembled structure is formed by various non-covalent bonds, including hydrogen bonds, hydrophobic effects, and π-stacking interactions.[2-5] For example, the DNA double helix is stabilized by hydrogen bonds occurring between the bases attached to the two strands and π-stacking interactions between contiguous base pairs.[6] The DNA assembled architectures exhibit unique structural properties and functions that are not typical of single nucleotides. In this respect, researchers have structurally mimicked relevant natural phenomena, with a focus on self-assembly or aggregation of amphiphilic biopolymer, such as polysaccharides.[7-9]

Rhizobial exopolysaccharides (EPS) are secreted heteropolymers, which are crucial for the nitrogen-fixing symbiosis between *Sinorhizobium melioti* and its host plant *Medicago sativa* (alfalfa).[10] The polymer chain of EPS consists of linear octasaccharide subunits containing one galactose and seven glucoses substituted by acetyl, pyruvyl, and succinyl groups (Figure 1(a)).[11] The molecular weight ranges from 1.0×10^5 to 8.7×10^6 Da.[12] Thanks to their high viscosity and acid stability, EPS can be attached to surfaces, improve nutrient acquisition, or provide protection from environmental stresses and host defenses. In addition, EPS can be employed in various industries such as food additives and oil recovery.[13] Chemical modification of EPS will thus broaden their range of applications in many fields *e.g.*, new materials for chiral separation, organic catalysis, and metal complexation.[14-16] In this work, we functionalized the EPS with a hydrophobic pendant (phenyl group) *via* pentynylation, and studied their self-assembling behavior.

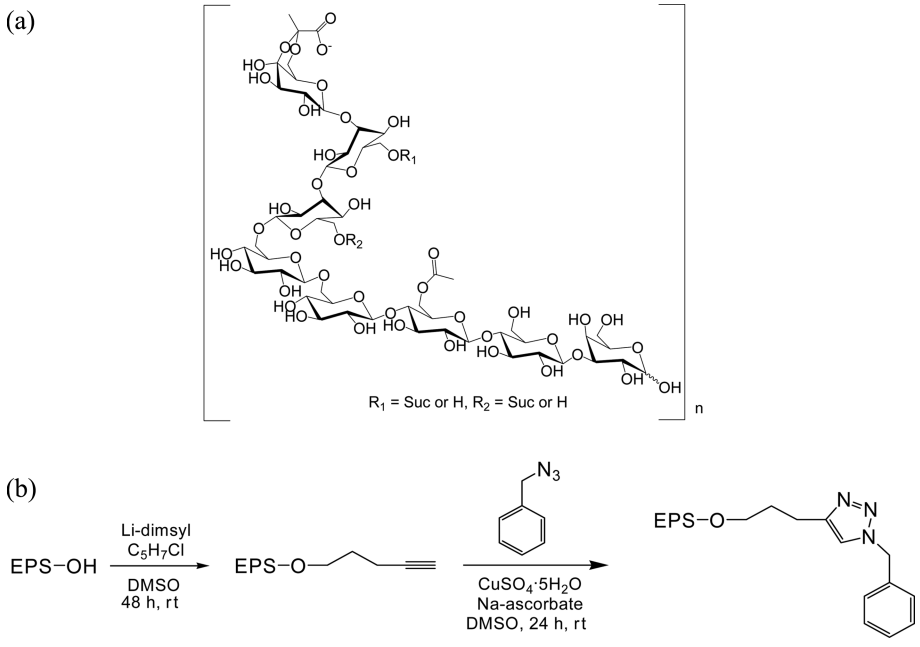

Figure 1. Chemical structure of EPS (a) and synthetic scheme of phenyl EPS (b).

Table 1. Elemental analysis of EPS, pentynyl EPS and phenyl EPS

	C[%]	H[%]	N[%]	$DS_{pentyne}$ [a]	$DS_{triazole}$ [b]
				EA	EA
EPS	38.56	6.35			
Pentynyl EPS	56.62	7.35		1.16	
Phenyl EPS	56.53	6.44	9.71		0.67, N[c] 0.76, C[d]

[a]Degree of substitution (DS) in pentynyl ether of EPS. [b]DS of phenyl group after click reaction. [c]DS calculated on the basis of N content. [d]DS calculated on the basis of C content

Pentynylation introduces a terminal acetylene group in the polymer backbone of EPS. This procedure allows tuning of the hydrophilic/hydrophobic balance of the EPS, and facilitates the functionalization by many other groups by copper-catalyzed azide-alkyne cycloaddition (CUAAC) click reactions.[16] To enhance hydrophobicity, phenyl groups were introduced on the polymer chain of EPS by combining pentynyl EPS with benzylazide (Figure 1(b)). The products of this reaction were then characterized by elemental analysis (EA), nuclear magnetic resonance (NMR) spectroscopy, and scanning electron microscopy (SEM).

As shown in the ^{1}H NMR spectrum of pentynyl EPS (Figure 2(b)), the protons of the two methylene groups at the 3- and 2-position of the pentynyl residues were observed at 1.67 and 2.21 ppm; the terminal proton at the 1-position appeared at 2.70 ppm. The degree of substitution (DS) values calculated from EA and NMR are very similar, 1.16 and 1.18, respectively; the latter value was obtained by comparing the intensity of the peak corresponding to H-1 of the glucose unit in EPS with that at the 1-position in the pentynyl group. In addition, the morphology changed from an amorphous lump, typical of natural EPS, to complex pieces (Figure 2(b) and 2(d)). The pentynyl- and hexynyl dextrans also showed a complex mixture in SEM image.[16] In the case of phenyl EPS, aromatic protons from the benzyl group and protons at the 1-position in the triazole group are designated at 7.29 and 7.82 ppm, respectively (Figure 2(c)). Glucose ring protons and unreacted acetylene protons appeared between 1.00 and 4.00 ppm, and protons at the 2-position at 5.49 ppm. The DS values of phenyl EPS, calculated from C and N content of EA, is equal to 0.76 and 0.67, respectively (Table 1). The DS of triazole, calculated from the NMR spectrum by comparing the intensity of the H-1 peak of glucose with that of the triazole proton (1-position), is equal to 0.76. Conversion from the pentynyl to the phenyl group is 64%. The SEM image of phenyl EPS showed laminar sheet-like structures, which differ from previously observed pentynyl EPS shapes (Figure 2(f)). These new structures may be related to the reduced ratio of surface to volume by phenyl substitution. These structural analyses indicate that phenyl EPS was successfully synthesized. Since polymer amphiphiles are also of interest to the design of new materials in biotechnology and biomedicine,[17,18] this novel functionalized biomaterial is important for further applications.

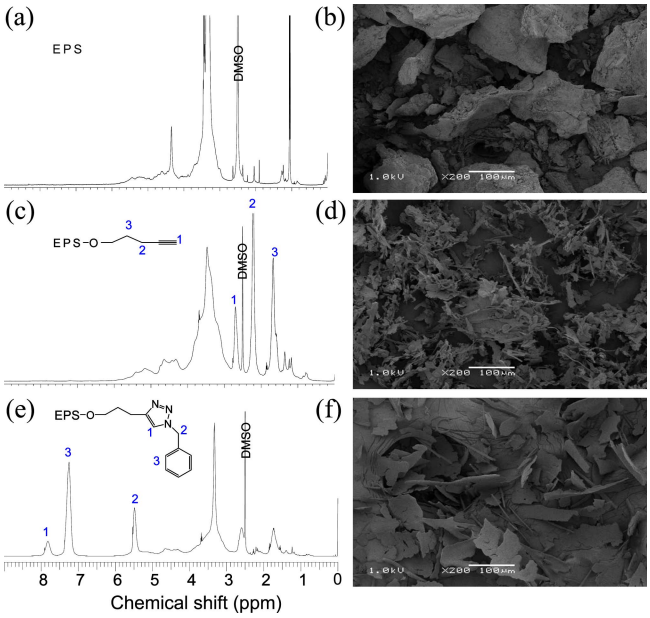

Figure 2. ^{1}H-NMR spectra and SEM images of EPS (a and b), pentynyl EPS (c and d), and phenyl EPS (e and f).

The use of transmission electron microscopy (TEM) revealed that phenyl EPS self-assembled in spherical nano-aggregates in aqueous solution (Figure 3(b)). The diameters of the spheres range between approximately 50 and 200 nm in the TEM image, whereas dynamic light scattering (DLS) showed a mean diameter of 78 nm (Figure 3(c)). The size of the nano-particle may be controlled by tuning the degree of substitution of phenyl EPS.[19,20] In a previous study, cholesterol-bearing pullulan and deoxycholic acid-modified chitosan have been reported to form a self-assembled hydrogel

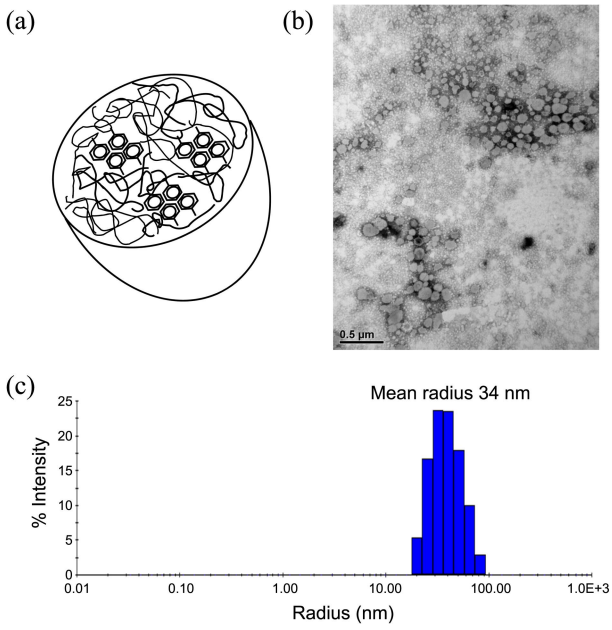

Figure 3. A schematic picture (a), TEM image (scale bar = 500 nm) (b), and DLS profile of self-aggregates formed by phenyl EPS in water.

spherical nano-particle and cylindrical bamboo-like structure in aqueous solution, respectively.[20,21] The rigidity of polysaccharide backbone may affect the final supramolecular structure. Furthermore, the cholesterol or the deoxycholic acid moieties are expected to provide sites where non-covalent cross-links may occur. In the present study, phenyl EPS containing linear octasaccharide backbone and less bulky hydrophobic substituent have been studied. In particular, π-stacking interactions occurring between the attached phenyl groups may be considered as the driving force for the self-assembly of the phenyl EPS.[22] In addition, hydrogen bonding and van der Waals interactions occur between sugar chains, and the possible supramolecular architecture is proposed in the scheme displayed in Figure 3(a). To the best of our knowledge, this is the first report on a self-assembled structure directed by polysaccharides substituted with phenyl groups. The major advantage of polysaccharides as nano-particle components is their natural molecular recognition ability,[23] which allows them to be potentially used for drug delivery and protein encapsulation.[19,24,25]

The self-aggregation of phenyl EPS in aqueous media was monitored by the fluorescence technique using the hydrophobic fluorescent probe, pyrene. Pyrene is preferentially solubilized into the interior of the hydrophobic regions in the presence of a hydrophobic region or microphase in aqueous media.[26] It exhibits five emission bands between 370 and 400 nm, and the intensity ratio of the first to the third bands (I_1/I_3) is considered as a polarity indicator of the micro-environment around the pyrene moiety.[27] For example, the I_1/I_3 value is 1.87 in water and 0.58 in hexane.[28] Thus, the critical aggregation concentration, lowest concentration of self-aggregation formation by intramolecular or intermolecular association, can be determined from the intersecting point of linear extension of the rapidly decreasing part and the horizontal part of the curve (Figure 4).[29] The obtained critical aggregation concentration of phenyl EPS is 0.1 mg/mL, and it can decrease with the increasing of the derivatized phenyl groups. The resulting self-aggregates are potent for making a stable complex with various hydrophobic substances.

In conclusion, EPS isolated from *S. meliloti* were modified with pentynyl and phenyl groups, and their final structures characterized with NMR, EA, and SEM. The synthesized phenyl EPS form self-assembled nano-spherical aggregates, and provide colloidally stable nanoparticles above the critical concentration. This process may be driven by π-stacking interactions between the substituted phenyl groups and by hydrogen bonding and van der Waals interactions occurring between sugar backbones. Further studies are in progress in order to explore possible applications and the structure-function relationship of the supramolecules proposed in this work.

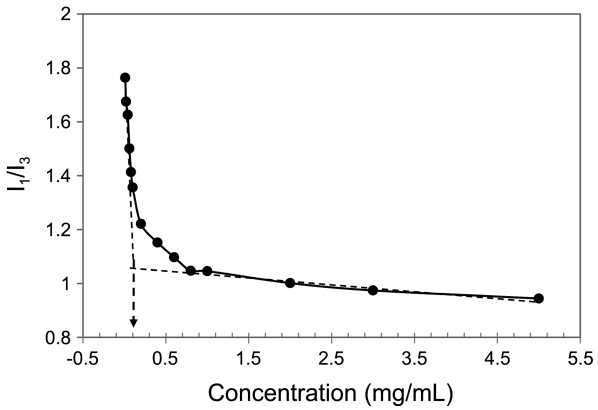

Figure 4. The relationship of pyrene fluorescence intensity ratios (I_1/I_3) with concentration of phenyl EPS.

Experimental

Purification of EPS. The isolation and purification of EPS from *Sinorhizobium meliloti* were carried out as previously described.[15,30] *S. meliloti* strain Rm 1021 was grown in a glutamate mannitol salt (GMS) medium for 5 d at 30 °C. Cells were removed by centrifugation, and the supernatant was concentrated to one-fifth of its original volume using a rotary evaporator. After adding 3 volumes of ethanol, the EPS turned into a gelatinous precipitate and was then collected by centrifugation. The EPS was dialyzed (MWCO > 10000) against distilled water and then lyophilized. EA: C 38.56%, H 6.35%.

Synthesis of Li-dimsyl. In a round bottom flask dried by heating under nitrogen, 1.6 M methyllithium (5% solution in diethyl ether) was added to an equal volume of DMSO through a septum. A needle was inserted into the septum to release methane and evaporate diethyl ether. The mixture was stirred under nitrogen for 90 min and freshly prepared Li-dimsyl was used immediately.

Synthesis of Pentynyl EPS. Pentynylation of the EPS was carried out according to the procedure described for dextran pentynylation.[16] Purified EPS (7.62 g, 47 mmol) was dissolved in 300 mL DMSO. After formation of a clear solution, Li-dimsyl (143 mL, 1.5 eq./OH) was added. After 1 h stirring at room temperature, 5-chloro-1-pentyne was slowly added under ice cooling and stirring was continued for 48 h. The product was purified by dialysis against demineralized water, during which time the water was changed 10 times for 5 d. The product was subsequently freeze-dried (8.80 g, whitish solid).

Click Reaction of Pentynyl EPS with Benzylazide. Py-EPS (5 g) was dissolved in DMSO/H_2O (4:1 v/v, 150 mL). After formation of a clear solution, benzylazide (2 Eq./alkynyl group) followed by freshly prepared 1 M aqueous solution of Na-L-ascorbate (20 mol % per alkynyl group), and $CuSO_4 \cdot 5H_2O$ (5 mol % per alkynyl group) was added. The reaction mixture was stirred at room temperature for 96 h. The product was purified by dialysis against deionized water and freeze-dried (6.98 g).

Preparation of Phenyl EPS Self Aggregates. Phenyl EPS (5 mg/mL) was suspended and swollen in 10 mM phosphate buffer under stirring for 2 d to produce a milky suspension.[16] The resulting suspension was probe sonicated for 10 min using a Sonics VC-505 instrument at 40% of its

maximum power. The procedure was repeated twice. The resulting solution was filtered through a 0.8 mm filter (Satorius, Minisart).

Nuclear Magnetic Resonance (NMR) Spectroscopy. A Bruker Avance 500 spectrometer was used to record the ^{1}H–NMR spectra. NMR analyses were performed in d_6-DMSO at room temperature.

Scanning Electron Microscopy (SEM). 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). The phenyl EPS aggregates were absorbed onto Formvar-coated copper grids (200 mesh) and air-dried for 5 min. For negative staining, 2% uranyl acetate solution was used. TEM images of the self-assembled structures were obtained using a JEM 1010 microscope (JEOL, Tokyo, Japan) operating at 80 kV.

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

Fluorometric Measurements. The self-aggregation property of phenyl EPS was determined using fluorescence spectroscopy with pyrene as a fluorescent probe. Fluorescence emission spectra of pyrene probe were recorded using a fluorescence spectrophotometer (SIMADZU, RF-5310PC) at room temperature. The probe was excited at 335 nm, and the emission spectra were obtained in the range of 350-550 nm. The excitation and emission slits had a width of 3.0 and 1.5 nm, respectively. A small amount of pyrene solution in acetone was added into each sample giving a final concentration of 1.0 mM in solution. Acetone was not removed, and its final content was 0.1%, v/v. All samples were sonicated for 15 min and left for 17 h at 25 °C before measurement.

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