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A Fructan, from Radix Ophiopogonis, Stimulates the Proliferation of Cultured Lymphocytes: Structural and Functional Analyses

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A fructan, Opaw-2, with molecular mass of about 14 kDa, was isolated from the tuberous roots of *Ophiopogon japonicus*. Opaw-2 comprises fructose and glucose with a molar ratio of 30:1. Linkage and NMR analyses indicated that Opaw-2 has a backbone structure of β-(1→2)-Fru*f* and β-(2→6)-Fru*f* residues that branches at O-6 of β-(1→2)-Fru*f* residues with α-1-linkage to the Glc*p* residues and terminates with Fru*f* residues. In cultured lymphocytes, the application of Opaw-2 significantly stimulated the proliferation of lymphocytes in a dose-dependent manner. By using atomic force microscopy, Opaw-2 showed a morphological change from globular to helical fibrous shape at increasing concentrations.

Fructans are widely distributed as carbohydrate storage polymers in the vegetative tissue of many families of plants, bacteria, and fungi. According to the type of linkage, fructans are classified into several families including inulin, which comprises (2→1)-linked β-D-fructofuranosyl units, levan, which comprises (2→6)-linked β-D-fructofuranosyl units, and graminan, which comprises both (2→1)-linked and (2→6)-linked β-D-fructofuranosyl units. Although there are many reports on the structural analysis of fructans, reports on the bioactivity of fructans are still scanty.

O. japonicus Ker-Gawler (Liliaceae) is an evergreen perennial. Its roots are widely used in traditional Chinese medicine (TCM) as Radix Ophiopogonis. In combination with the roots of *Panax ginseng* (Radix Ginseng) and flowers of *Schisandra chinensis* (Fructus Schisandra Chinensis), Radix Ophiopogonis has been used for treating disorders of the cardiovascular system.[1] In folk medicine, Radix Ophiopogonis serves as an expectorant and an anticough and tonic agent and to reduce blood sugar. From this root many steroidal glycosides and homoisoflavonoids as well as monoterpene glycosides have been isolated and characterized.[2] The major pharmacological effects of *O. japonicus*, such as reducing blood sugar and treating myocardial ischemia, are closely associated with its carbohydrate contents.[3] Oligosaccharides and polysaccharides are the main constituents of the tuberous roots of *O. japonicus.* The structure and function of these carbohydrates have never been well characterized. So far, only some oligosaccharides and one low molecular weight polysaccharide from Radix Ophiopogonis have been reported.[4]

In order to reveal the nature of active polysaccharides and their pharmacological mechanism, polysaccharides from Radix Ophiopogonis were analyzed. In this paper, a fructan, named as Opaw-2, was isolated from the roots of *O. japonicus*. The structure and function of Opaw-2 were characterized, and the results suggested that Opaw-2 could be responsible for the immune function of *O. japonicus*.

Results and Discussion

The water-boiled extract of *O. japonicus* roots was precipitated by four volumes of EtOH to obtain a crude polysaccharide fraction. After removal of protein, the fraction was dissolved in H_2O and further precipitated with 45% EtOH. The precipitate, dissolved in H_2O, was loaded onto DEAE and Sepharose CL-6B columns for purification. The profile of polysaccharides was followed, and a single peak of polysaccharide with the highest abundance was chosen for further analysis (data not shown). This polysaccharide showed a symmetrical narrow peak, as revealed by a sizing column

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Table 1. EIMS Fragmentation of the Derivatives of Opaw-2 Produced by Reductive Cleavage and Acetylation

1,5-anhydro-2,3,4,6-tetra-*O*-methyl-D-glucitol (5.87):[a] *m/z* 41 (32%), 43 (18), 45 (53), 55 (8), 58 (14), 59 (27), 71 (80), 75 (50), 85 (12), 88 (35), 99 (25), 101 (100), 111 (11), 115 (13), 125 (15), 143 (23), 175 (12), 188 (5)
2,5-anhydro-1,3,4,6-tetra-*O*-methyl-D-mannitol (5.99):[a] *m/z* 41 (24%), 43 (10), 45 (100), 55 (13), 59 (25), 71 (67), 74 (25), 75 (23), 83 (24), 85 (15), 87 (26), 89 (51), 99 (37), 101 (96), 111 (33), 115 (37), 125 (20), 126 (12), 143 (65), 156 (30), 175 (32), 188 (3), 221 (3)
2,5-anhydro-1,3,4,6-tetra-*O*-methyl-D-glucitol(6.33):[a] *m/z* 41 (12%), 45 (57), 55 (8), 59 (15), 71 (35), 74 (11), 83 (7), 85 (10), 87 (23), 89 (27),99 (20),101 (100), 111 (25), 115 (18), 125 (5), 143 (33), 221 (3)
1-*O*-acetyl-2,5-anhydro-3,4,6-tri-*O*-methyl-D-mannitol (8.39):[a] *m/z* 41 (24%), 43 (92), 45 (72), 55, 59 (20), 69 (20), 71 (100), 75 (18), 83 (28), 85 (22), 87 (50), 89 (21), 99 (22), 101 (95), 111 (73), 115 (53), 117 (30), 126 (33), 143 (67), 156 (10), 158 (12), 171 (9), 175 (5), 188 (7), 203 (10), 216 (3)
6-*O*-acetyl-2,5-anhydro-3,4,6-tri-*O*-methyl-D-glucitol (8.61):[a] *m/z* 41 (18%), 43 (73), 45 (55), 59 (17), 71 (72), 75, 83 (20), 87 (43), 89 (19), 98 (17), 101 (100), 111 (59), 114 (26), 115 (25), 117 (70), 126 (16), 143 (40), 156 (7), 171 (5), 188 (3), 203 (7), 216 (3)
1-*O*-acetyl-2,5-anhydro-3,4,6-tri-*O*-methyl-D-glucitol (8.78):[a] *m/z* 41 (24%), 43 (85), 45 (58), 55 (7), 59 (23), 71 (95), 75 (10), 83 (20), 87 (56), 89 (19), 99 (20), 101 (100), 111 (59), 114 (24), 115 (23), 117 (40), 126 (48), 143 (25), 158 (10), 171 (7), 188 (7), 203 (5)
1,6-di-*O*-acetyl-2,5-anhydro-3,4-di-*O*-methyl-D-mannitol (11.60):[a] *m/z* 41 (13%), 43 (100), 45 (15), 59 (10), 71 (33), 83 (13), 85 (8), 87 (38), 101 (17), 111 (10), 117 (20), 154 (6), 186 (5), 216 (7)
1,6-di-*O*-acetyl-2,5-anhydro-3,4-di-*O*-methyl-D-mannitol (12.09):[a] *m/z* 41 (13%), 43 (100), 71 (40), 87 (37), 101 (13), 111 (15), 115 (16), 117 (17), 124 (10), 125 (5), 143 (5), 156 (4), 216 (5)

[a] The retention time of the derivatives produced by reductive cleavage and acetylation.

on HPLC with an ELSD detector. Comparing to dextran standards, the calibrated molecular mass of this homogeneous polysaccharide was approximately 14 kDa. We named this polysaccharide Opaw-2.

Opaw-2 was free of protein and uronic acid according to the determination by the Lowry[5] and *m*-hydroxyldiphenyl methods.[6] Its specific rotation $[\alpha]_D^{20}$ was −56 (*c* 0.1, H_2O). The composition of sugars, as determined by acid hydrolysis and HPLC, gave fructose and glucose in a molar ratio of 30:1. The absolute configurations of the sugars after acid hydrolysis were determined by the analysis of TMSi(−)-2-butylglycosides as described.[7] Identification of trimethylsilylated (−)-2-butyl glycosides of the sugars was carried out from the retention time relative to trimethylsilylated methyl α-D-glucopyranoside and standard substance. To further determine the linkage of sugars in Opaw-2, the fructan was methylated and reductively cleaved, then the partially methylated fructan was analyzed by GC-MS to show seven peaks. According to the published literature and database,[8] the fragmentation patterns of these peaks were identified (Table 1). The peak area ratio of the methylated fragments was used as a parameter for comparison, and the integrated peak areas were corrected using the effective-carbon response method.[9] The fragmentation patterns of the methoxy derivatives were identical to the reported values.[8,10] The molar ratio of (2→1)-linked and (2→6)-linked fructose residues was calculated according to the reported method.[10] Table 2 shows the proportions of the several types of linked β-D-Fru*f* and α-D-Glc*p* residues. As shown in Table 2, the molar ratio of terminal D-fructosyl residues and (1,2→6)-linked D-fructose residues was about 1:1, indicating that there was a nonreducing fructosyl end linked at each of the branch points.

The summation of (2→1)-linked fructose residues corresponded to approximately 61% of the total applied sample, and the D-fructosyl residues having the (2→6) branching was approximately

Table 2. Assignment and Molar Ratio of β-D-Fru*f* and α-D-Glc*p* Residues in Each Opaw-2 Molecule[a]

	assignment[a]	molar ratio[b]
β-D-Fru*f*	terminal	2.8
	2,1-linked	11.2
	2,6-linked	2.5
	1,2,6-linked	2.3α-D-Glc*p*
terminal	0.6	

[a] Assignments were based on methylation analysis. [b] Molar ratio of monosaccharides was determined by GC-MS analysis.

10% of the sample. Reductive cleavage gave the only glucose-derived unit, suggesting that the α-linked-D-Glc*p* residue was in the nonreducing terminal position and was linked only at the 1-position.

The NMR spectra of Opaw-2 indicated that the above deduced sugar linkages coincided with the predicted structure of Opaw-2 (Figure 1). According to ^{13}C NMR and published values,[9,11,12] the anomeric carbon resonances at δ 103.9 and 103.6 were assigned to C-2 of the (2→1)-linked fructose residues, and the anomeric carbon signals at δ 104.3 and 104.5 to C-2 of the terminal and branched fructose residues. Those signals at δ 104.7 and 104.9 were assigned to (2→6)-linked fructose residues. The occurrence of (2→6)-linked fructose residues was also strongly supported by the signal at δ 80.9. The resonance at δ 81.7 indicated linkage at C-5. The broad signal at δ 63.7 was characteristic of 6-substitution from the (2→6)-linked fructose residues.[9] Table 3 summerizes the NMR data of Opaw-2.

The structure of Opaw-2 was further confirmed by its HMQC and HMBC spectra. In the HMBC spectra, two resonances, at δ 103.9 for (2→1)-linked fructose and δ 104.3 for (1,2→6)-linked fructose, correlated with the H-1 signal at δ 5.22 of the α-D-Glc*p* residues. No other correlative signal between the β-D-Fru*f* residue and the α-D-Glc*p* residue was identified. The α-D-Glc*p* residues were therefore linked at the 1-position by O-2 of the (2→1)-linked fructose residue or the (1,2→6)-linked fructose residue. The H-1 signals of (2→1)-linked β-D-Fru*f* residues correlated with the C-2 signal of the (26)-linked β-D-Fru*f* residue. The C-2 signal of the nonreducing β-D-Fru*f* residue showed cross-peaks with the H-6 signal of the (1,2→6)-linked β-D-Fru*f* residue. Opaw-2 is thus a fructan in which the O-1 of the (2→1)-linked β-D-Fru*f* residue is linked at C-2 of the (2→6)-linked β-D-Fru*f* residue, the O-6 of the (2→6)-linked β-D-Fru*f* residue is linked at C-2 of the (1,2→6)-linked β-D-Fru*f* residue, and the ends consist of α-1-linked D-Glc*p* residues and a terminal β-D-Fru*f* residue.

From the above data, a feasible structure of Opaw-2 is proposed in Figure 1, where *n* indicates the unit number of the polymer. Opaw-2 has a highly branched structure with predominantly (2→1)-D-linked and a small amount of (2→6)-linked D-fructosyl backbone branched at O-6 of (2→1) D-fructosyl residues that terminated with a fructosyl residue, while the terminal glucosyl residue was linked at O-2 of the fructosyl residue at the end of the polymer. Opaw-2 is therefore a typical fructan.

The biological function of Opaw-2 was elucidated. In cultured Balb/c mice lymphocytes, Opaw-2 was applied from 10 to 100 μg/mL for 3 days. In order to quantify the proliferation rate, the DNA synthesis as revealed by the incorporation of [^3H] thymidine was determined in the treated lymphocytes. The application of Opaw-2 induced lymphocyte proliferation in a dose-dependent manner (Figure 2). Compared with the basal proliferation, the treatment of Opaw-2 at 30 μg/mL for 3 days increased the incorporation of [^3H] thymidine by 2-fold, while the increase reached 3-fold when using 100 μg/mL Opaw-2.

In order to determine the relationship between the immunological activity and the conformation of Opaw-2, the microscopic morphologies of this fructan were determined using atomic force microscopy (AFM). A sample was dissolved in distilled H_2O at two concentrations: 10 and 30 μg/mL. The fructan showed a

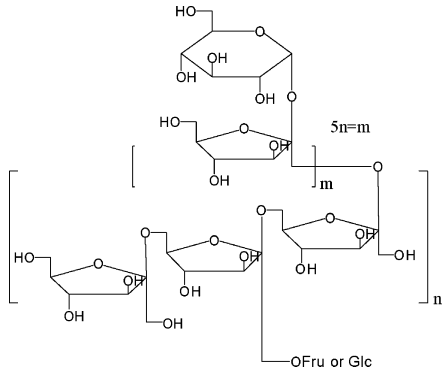

Figure 1. Deduced unit structure of Opaw-2, where *n* is the number of residues ($n = 1, 2, 3, 4$; $5n = m$).

Table 3. Chemical Shift Assignments for the ^{13}C NMR Spectra of Opaw-2 (125 MHz, in D_2O)[a]

	→6)-Fru*f*-(2←	→1)-Fru*f*-(2←	Fru*f*-(2←	→1,6)-Fru*f*-(2←	α-D-Glc*p*
C-1	60.7, 61.2	63.7, 63.2	62.9	62.9	92.9
C-2	104.7, 104.9	103.9, 103.6	104.2	104.5, 104.3	72.2
C-3	77.2	77.9,78.0	77.3[b]	77.4, 77.7	71.7
C-4	75.8	75.0, 75.2[a]	75.2	75.3	69.8
C-5	80.9	81.7	81.7	80.9	73.1
C-6	63.7	63.2	62.9, 61.5	63.7	60.4

[a] The linkage assignments were based on HMQC and HMBC experiments. [b] Unresolved from other signals.

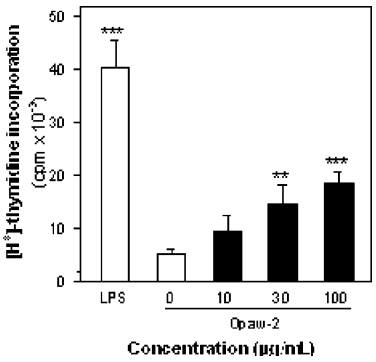

Figure 2. Opaw-2-induced proliferation of cultured lymphocytes. The mitogenic activity of Opaw-2 was examined at concentrations from 10 to 100 μg/mL. Cultured mouse lymphocytes (4×10^5 cells) were treated with Opaw-2 or LPS at 5 μg/mL for 3 days. [^{3}H]-thymidine ([^{3}H]-Thy) was added 6 h before cell collection. Each triplicate data point represents the mean ± SD, $n = 6$. LPS (5 μg/mL) served as a positive control. ** indicates $p < 0.01$ and *** indicates $p < 0.001$.

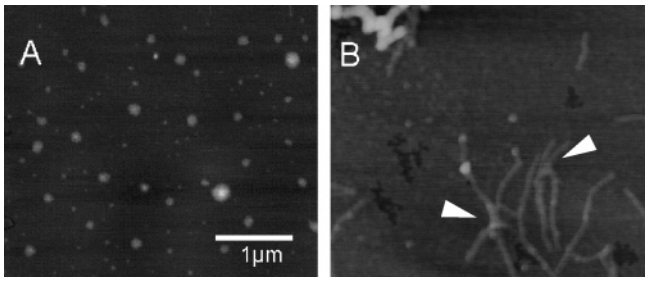

Figure 3. Images of Opaw-2 under AFM: 10 μg/mL (A) and 30 μg/mL (B). Opaw-2 samples were prepared and viewed under ATF as described in the Experimental Section. Arrowheads show the formation of fibrous structure at high concentration of Opaw-2.

globular shape at 10 μg/mL (Figure 3A). In contrast, it formed a fibrous structure at 30 μg/mL (Figure 3B). The fibrous structures had a helical conformation, some of which formed a knot in the linear length. The thickness of the individual molecules was less than those of the native molecules due to the reduction in probe broadening. Figure 3 shows that the width of the individual molecule, the tip shape, and the tip size could be broadened by several factors when changing the scanning speed during microscopy. Nevertheless, AFM imaging revealed the morphological change of Opaw-2 at high concentration.

Most of the reported fructans from plants have small molecular weights. However, Opaw-2 has a molecular mass of about 14 kDa. By comparing to previously reported fructans,[9−12] Opaw-2 belongs to the graminan-type fructans, which have a distinct structural feature with a special molar ratio of (2→1)-D-linked and (2→6)-linked D-fructosyl residues and O-linkage types.

In accord with the proposed biological functions of Opaw-2 isolated from Radix Ophiopogonis as described here, bioactivities of other fructans have also been reported. An inulin-type fructan from *Arctium lappa* L., var. *Herkules* was shown to have mitogenic activity.[13] In addition, several active graminan-type fructans were reported to have antitumor or antivirus action.[9,14] Comparing to these reported active fructans, Opaw-2 has a distinctly different chemical structure, which may explain their functional differences.

There are reports indicating that inulin was linear in solution, while others suggested that the favorable conformation for levan was a helix and inulin tended to form random coil structures.[15−17] A synthesized high molecular weight inulin (M_w 90 × 10^6) was described to have branches and be globular in shape in dilute aqueous and DMSO.[18] Except for these fructans, other polysaccharides showed different molecular morphologies. For example, under AFM, scleroglucan displays a linear triple-helical form at 10 μg/mL.[19] As a bacterial polysaccharide, gellan gum showed a linear chain image at less than 3 μg/mL and formed a network at more than 3 μg/mL with AFM.[20] In the present paper, the images obtained with AFM for our Opaw-2, a graminan-type fructan, revealed for the first time that this polymer was globular in shape in dilute aqueous solution (10 μg/mL), but formed a helical fibrous chain structure at 30 μg/mL. This was presumably due to molecular interactions at high concentration resulting in drastic conformation changes. This result is therefore different from those molecules described earlier. The relationship between the morphology and immunological activity of Opaw-2 is not very clear, and further studies will be carried out.

Experimental Section

General Experimental Procedures. Optical rotation of Opaw-2 was measured in distilled H_2O at 20 °C using a 1 cm light path length cell with a Perkin-Elmer 243B polarimeter. The infrared spectra (IR) of native polysaccharides and the methylated polysaccharides were recorded on a Perkin-Elmer 599B spectrometer. GC-MS analyses were performed on a Finnigan Trace GC-MS instrument. Carbohydrates were determined by the phenol/H_2SO_4 method. Uronic acid and protein contents were determined by the *m*-hydroxydiphenyl and the Lowry methods,[5,6] respectively. The hydrolyzates were analyzed using TLC on a silica gel plate containing 5% NaH_2PO_4 and developed with BuOH−EtOAc−isopropyl alcohol−HOAC−H_2O−pyridine (3.5:10:6:3.5:3:3). The plate was visualized with 1,3-naphthalenediol reagent, then heated at 110 °C for 10 min. Homogeneity and molecular weight were detected and determined by HPSEC, which was performed on an Agilent 1100 series apparatus with a Shodex KS-805 column (Shoko, Japan). The column was calibrated with T-series dextrans, T-2000, T-500, T-200, T-70, and T-40, and glucose (Amersham Pharmacia, Sweden). The sample concentration was 1% (w/v). Distilled H_2O was used as the solvent and eluent. The flow rate was kept at 1.0 mL/min. An 20 μL aliquot was injected for each run. The completeness of methylation was confirmed by the disappearance of the hydroxy absorption in the IR spectrum. Samples were dissolved in D_2O (99.96% D atom), deposited 24 h, freeze-dried, and redissolved in D_2O in 5 mm tubes. All NMR spectra were obtained with a Bruker AM 500 spectrometer with a dual probe in the FT mode at room temperature.

Plant Material. Roots of *O. japonicus* were collected from Sichuan Province of China in April 2002. The herbs were identified by our experts at the School of Pharmaceutical Sciences. A voucher specimen of this plant, [M-2-(6)], was deposited at the School of Pharmaceutical Sciences, Peking University.

Extraction and Isolation of Opaw-2 from *O. japonicus*. The tuberous roots of *O. japonicus* (1500 g) were extracted continually with 95% EtOH under reflux and hot distilled H_2O (3) (1.5 h). The hot H_2O extract was filtered and centrifuged. The supernatant (189.6 g) was precipitated with four volumes of EtOH, to give 50.6 g of precipitate. After protein removal, the resulting residue (42.5 g) was dissolved in distilled H_2O and further precipitated with 45% EtOH as the OPa fraction (10.2 g). The OPa (2 g) was dissolved in distilled H_2O (100 mL, 8%w/v) and further separated using a DEAE column (70 × 5 cm) and a gradient of 0 to 2 M NaCl in 1.5 L. The distilled H_2O fraction of Opaw was dialyzed and lyophilized (yield: 1.2 g). Opaw (300 mg) was separated by gel-permeation chromatography on a Sepharose CL-6B column (80 × 2.6 cm) eluted with 0.2 M NaCl (1500 mL). Polysaccharide fractions of 5 mL corresponding to Opaw-2 were pooled, dialyzed, and freeze-dried.

After desalting by Sephadex G-25 (60 × 1 cm column), the Opaw-2 fraction (110 mg) was further purified by gel-filtration chromatography on a Sephadex G-75 fine column (80 × 1 cm) eluted with distilled H_2O and was collected and lyophilized (85 mg). Opaw-2 was analyzed as a single peak by a sizing column on HPLC with an ELSD detector.

Chemical and Physical Analyses of Opaw-2. In order to determine the composition of monosaccharides, Opaw-2 (5.0 mg) was completely hydrolyzed with 50 mM H_2SO_4 (3 mL) at 60 °C for 1 h, neutralized with $BaCO_3$, then centrifuged, filtered, and concentrated. The hydrolyzate was analyzed using HPLC at 25 °C on a Prevail Carbohydrate ES 5μ (Alltech Associates, Inc., 250 mm × 4.6 mm) with 80:20 MeCN−H_2O as eluent at a flow rate of 0.6 mL/min, giving fructose and glucose in a molar ratio of 30:1. The absolute configurations of fructose and glucose were determined by the analysis of TMSi(−)-2-butylglycosides as described.[7] Identification of trimethylsilylated (−)-2-butylglycosides of the sugars was carried out from the retention time relative to trimethylsilylated methyl α-D-glucopyranoside and standard substance on GC using a HP-5 capillary column (30 m × 0.32 mm).

For methylation of the sugar, Opaw-2 (5.6 mg) was methylated three times using the Ciucanu method.[21] The sample was dissolved in DMSO (0.5 mL), and finely powered, dry NaOH (20 mg) was added under nitrogen. The mixture was sonicated under nitrogen for 10 min, then left for 90 min. Iodomethane (0.1 mL) was added at 0 °C during 20 min and sonicated for 10 min, then rested for 30 min at room temperature. The reaction mixture was extracted three times with $CHCl_3$. The combined $CHCl_3$ layers were extracted with water three times, and the solvent was evaporated. The completeness of methylation was confirmed by the disappearance of the hydroxy absorption in the IR spectrum.

The methylated polysaccharide was subjected to reductive cleavage as described previously.[22] The reducing agent was prepared from BF_3·OEt_2 (155 μL), Et_3SiH (200 μL), CF_3COOH (32 μL), and CH_2Cl_2 (130 μL). The reducing agent mixture was added to the methylated product and allowed to react for 24 h at 0 °C. Ac_2O was added, and the temperature was raised to 40 °C for 2 h. The solution was extracted with H_2O three times and dried under nitrogen. CH_2Cl_2 was added to obtain partially methylated alditol acetates. The product was analyzed on GC-MS using a DB-5 capillary column (30 m × 0.32 mm) at an oven temperature from 120 to 200 °C at 5 °C/min, then to 250 °C at 8 °C/min. Identification of partially methylated alditol acetates was carried out from the retention time relative to 1,5-anhydro-2,3,4,6-tetra-*O*-methyl-D-glucitol and its fragmentation pattern (Table 1). Optical rotation of Opaw-2: $[\alpha]_D^{20}$ −56 (*c* 0.1, H_2O); NMR, Table 3.

Lymphocyte Culture and its Proliferation Assay. Male Balb/c mice of age 6−8 weeks (weight 20 ± 2 g) were purchased from Peking University Health Science Center Experimental Animal Laboratory. Mice were sacrificed, and their spleens were removed and gently pressed through a sterilized iron sieve to obtain single cell suspensions. The cell suspension was washed with PBS, and red blood cells were lysed with lysis buffer (0.15 M NH_4Cl, 1.0 mM $KHCO_3$, 0.1 mM EDTA, pH 7.2) for 3 min. The spleen cells were washed before culturing in U-bottom well culture plates (10^5/well) in a volume of 200 μL per well. After 3 days of drug treatment, DNA synthesis was measured by [^{3}H]-thymidine (Amersham Pharmacia, Sweden) incorporation (1 μCi/well) in the final 6 h of the culture period. The data were tested for statistical differences using the Student's *t*-test.

Microscopy Morphologies of the Polymer. The morphologies of polymers were determined using an atomic force microscope manufactured by NanoDevices, Inc. (DI Digital Instruction, Veeco Metrology Group) as described in the literature.[23] Images were acquired in dc contact mode under constant force conditions. Polysaccharide solutions (~1.0 mg/mL) were diluted to 10 and 30 μg/mL. The sample (10 μL) was deposited onto freshly cleaved mica and then allowed to dry in air (~30 min). Imaging was carried out in air while the samples remained visibly moist.

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References and Notes

(1) Zhu, Y. X.; Yan, K. D.; Tu, G. S. *Yao-Xue-Xue-Bao* **1987**, *22*, 679−684.
(2) Kaneda, N.; Nakanishi, H.; Kuraishi, T.; Katori, T. *Yakugaku-Zasshi* **1983**, *103*, 1133−1139.
(3) Lin, X.; Xue, D. S.; Feng, Y.; Shen, L. *Anal. Chem.* **2005**, *342*, 179−185.
(4) Yakugaku, Z.; Sano, T.; Murayama, T.; Hirai, Y.; Shoji, J. *Chem. Pharm. Bull.* **1993**, *41*, 566−570.
(5) Hartree, E. F. *Anal. Biochem.* **1972**, *48*, 422−427.
(6) Blumenkrantz, N.; Asboe-Hansen, G. *Anal. Biochem.* **1973**, *54*, 484−489.
(7) Gerwig, G. J.; Kamerling, J. P.; Vliegenthart, J. F. G. *Carbohydr. Res.* **1978**, *62*, 349−357.
(8) Sweet, D. P.; Shapiro, R. H.; Albersheim, P. *Carbohydr. Res.* **1975**, *40*, 217−225.
(9) Chen, X. M.; Tian, G. *Carbohydr. Res.* **2003**, *338*, 1235−1241.
(10) Simms, P. J.; Boyko, W. J.; Edwards, J. R. *Carbohydr. Res.* **1990**, *208*, 193−198.
(11) Carpita, N. C. *Carbohydr. Res.* **1991**, *217*, 127−136.
(12) Seymour, F. R. *Carbohydr. Res.* **1979**, *72*, 57−69.
(13) Kardosova, A.; Ebringerova, A.; Alfoldi, J.; Nosal'ova, G.; Franova, S.; Hribalova, V. *Int. J. Biol. Macromol.* **2003**, *33*, 135−140.
(14) Jarrell, H. C.; Conway, T. F.; Moyna, P.; Smrith, C. P. *Carbohydr. Res.* **1979**, *76*, 45−57.
(15) Harada, T.; Suzuki, S.; Taniguchi, H.; Sasaki, T. *Food Hydrocoll.* **1993**, *7*, 23−38.
(16) Kawai, G.; Taniguchi, H.; Nakamura, M. *Agric. Biol. Chem.* **1973**, *37*, 2111−2119.
(17) Vereyken, I. J.; van Kulk, A. J.; Evers, T. H.; Rijken, P. J.; Kruijff, B. D. *J. Biophys.* **2003**, *84*, 3147−3154.
(18) Wolff, D.; Czapla, S.; Heyer, A. G.; Radosta, S.; Mischnick, P.; Springer, J. *Polymer* **2000**, *41*, 8009−8016.
(19) Mcintire, T. M.; Penner, R. M.; Brant, D. A. *Macromolecules* **1995**, *28*, 6375−6377.
(20) Gunning, A. P.; Kirby, A. R.; Ridout, M. J.; Brownsey, G. J.; Morris, V. J. *Macromolecules* **1996**, *29*, 6791−6796.
(21) Needs, P. W.; Selvendran, R. R. *Carbohydr. Res.* **1993**, *245*, 1−10.
(22) Rolf, D.; Gray, G. *J. Am. Chem. Soc.* **1982**, *104*, 3539−3541.
(23) Kirby, A. R.; Gunning, A. P.; Morris, V. J. *Biopolymers* **1996**, *38*, 355−366.

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