Macromolecules

Volume 41, Number 12 June 24, 2008

Communications to the Editor

Supergiant Ampholytic Sugar Chains with Imbalanced Charge Ratio Form Saline Ultra-absorbent Hydrogels

Maiko K. Okajima,[†] Takeshi Bamba,[‡] Yasuhiro Kaneso,[†] Kazumasa Hirata,[‡] Eiichiro Fukusaki,[§] Shin'ichiro Kajiyama,[§] and Tatsuo Kaneko*,[†]

School of Materials Science, Japan Advanced Institute of Science and Technology, 1-1 Asahidai, Nomi 923-1292, Japan; Department of Applied Environmental Biology, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita 565-0871, Japan; and Department of Biotechnology, Graduate School of Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan

Received February 10, 2008
Revised Manuscript Received May 8, 2008

Water-absorbing materials have been sought after in the fields of cell engineering, drug controlling, and planting materials.[1] A good absorption capacity for saline is very important, since various types of salts are contained in cell culture medium, body fluids, and nutritional solutions. However, the development of a high-performance saline absorber has been very difficult, even if an absorber showing ultrahigh absorption capacity for pure water was used initially. For instance, ionic polymers generally showed much better water absorption than nonionic ones and have been used in practice, but their saline absorption was so low that further applications were limited.[2] This is the reason why the ions in saline showed charge screening effects[3] on the polyelectrolyte chains. On the other hand, *Aphanothece sacrum*, which is a freshwater unicellular cyanobacterium (Figure 1a) mass-aquacultured in rivers with a high ionic concentration, has an abundance of a jelly-like extracellular matrix (ECM) (see inset of Figure 1a)[4] with a high water content (97.5%).[5] We hypothesized that this ECM must mainly contain hydrophilic materials such as sugar chains (SC) with a high capacity for saline absorption. Therefore, we extracted the SC and discovered that they consisted of polyampholytes with an ultrahigh molecular weight (over 10^7 Da) and an imbalanced charge ratio and that they showed a salt-induced chain extension for keeping a saline absorption capability extremely high. The unique structural character of *sacran* may serve as a guide to designing high-performance saline absorbers.

The SC were extracted by alkaline solution (0.1 N NaOH) as fibers (Figure 1b), and the yields were on the gram scale (50−80 wt %), which were high enough to investigate their structures and physical properties. The SC were soluble in hot water to give a homogeneous solution regardless of pH and formed a physical hydrogel after cooling. The IR absorption (Figure S1) and XPS spectra (Figure S2) data demonstrated the presence of sulfated groups in the SC. CHN S elemental analyses of the SC showed the following composition: C: 36.04%; H: 5.91%; N: 0.30%; S: 2.07%. If the average molecular weight for a sugar unit is assumed to be 162, then the molar composition of S to the total sugar units can be estimated at 10 mol %. The presence of an amino group in the SC was confirmed by a positive result on the ninhydrin test. On the other hand, an absence of peptide groups could be presumed, since the SC were negative on the Biuret test. These results showed the extracted SC contained amino sugars but did not contain any peptides or proteins. The uronic acid content was estimated at 22% by the carbazole−sulfuric acid method (525 nm). Gas chromatography/mass spectroscopy (GC/MS) and GC analyses of trimethylsilylated samples of methanolyzed SC indicated that the main monosaccharides were Glc, Gal, Man, Xyl, Rha, Fuc, GalA, and GlcA, with a composition of 25.9: 11.0: 10.0: 16.2: 10.2: 6.9: 4.0: and 4.2. We also confirmed the presence of trace amounts (ca. 1.0%) of Ara, GalN, and Mur. One can presume that the N element detected by elemental analysis was derived from GalN and Mur. Fourier transform ion cyclotron resonance mass spectroscopy (FT-ICR-MS) of methanolyzed SC (Figure S3) showed a milli-mass unit value of 358.0806, corresponding to a $[M - H]^- = 358.0808$ from sulfated dimethylmuramic acid (structure; Figure 1c) whose two methyl groups were considered to be attached to reducing terminal and carboxylic acid group in the process of the methanolysis. Furthermore, the MS/MS measurement showed 278.1244 to $[M - H]^- = 278.1240$ from dimethylmuramic acid, indicating the elimination

* Corresponding author: Tel +81-761-51-1631; fax +81-761-51-1635; e-mail kaneko@jaist.ac.jp.
[†] Japan Advanced Institute of Science and Technology.
[‡] Graduate School of Pharmaceutical Sciences, Osaka University.
[§] Graduate School of Engineering, Osaka University.

10.1021/ma800307w CCC: $40.75
Published on Web 05/30/2008

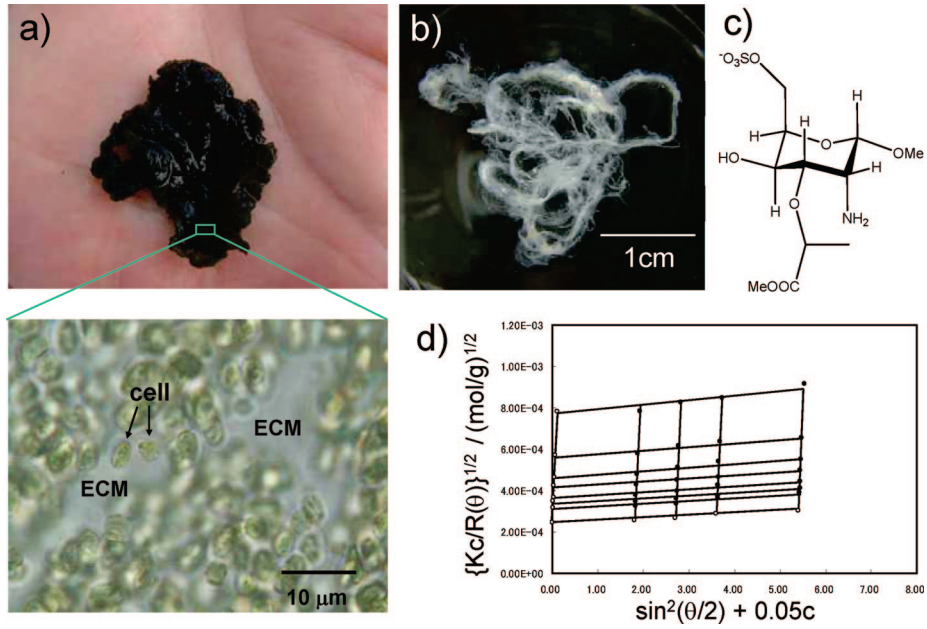

Figure 1. (a) Macroscopic view of *Aphanothece sacrum*. Inset: ECM is present between cells in microscopic image. (b) Macroscopic view of the fibers formed by precipitation of an aqueous eluate into isopropanol. (c) Chemical structure of sulfated muramic acid confirmed as a constituent of *sacran* from FT-ICR-MS analyses. (d) Zimm−Berry plot of the *sacran* solution in 0.1 M $NaNO_3$.

of a sulfate group. Sulfated muramic acid is a newly found sugar in nature, and thus one can state that the extracted SC was a novel one. Here, we named the SC "*sacran*". We also found sulfated muramic acid in the SC extracted using the following method: the ECM of *A. sacrum* was eluted by hot pure water using an autoclave and ultracentrifuged (at 50 000 rpm for 30 min) to obtain a clear supernatant which was poured into isopropanol to precipitate the SC fibers. FT-ICR-MS indicated that the presence of di- and trisaccharides composed of hexoses and muramic acid derivatives. The results of connection of muramic acid with hexoses demonstrated that the muramic acid derivative found as a SC sugar unit was not merely a contaminant from cell wall constituents but rather a constituent of the capsular SC of *A. sacrum* because muramic acid generally exists with *N*-acetylgulucosamine in the cell walls of bacteria.[6] It was demonstrated that *sacran* was an ampholytic SC with an imbalanced charge ratio, resulting in a high content of anionic sugars with sulfate and carboxylic acid groups and a low content of cationic amino sugars (anions/cations; ca. 30/1).

Gel permeation chromatography (GPC) of the SC solution in 0.1 M $NaNO_3$ indicated that *sacran* had a high weight-average M_w of 2.0×10^7 with a narrow polydispersity of 1.2 (Figure S4). Since this value is very high but just a relative molecular weight, we measured the absolute molecular weight using multiangle (from 15° to 40°) laser light scattering (MALLS). We successfully obtained a typical Zimm−Berry plot with a very small error of 1.4% (Figure 1d) by repeating the challenges under careful filtration (pore size: 5 μm) under a pressure. When the absolute M_w of the SC in pure water was measured, the error value was high due to a severe dependence on *sacran* concentration, and we did not obtain a valid lattice shape of the Zimm−Berry plot. The addition of $NaNO_3$ may be useful for adopting the not electrostatically restrained conformation of *sacran*. From an analysis of the Zimm−Berry plot, the absolute M_w and radius of gyration, $\langle s^2 \rangle^{1/2}$, for *sacran* were estimated at 1.6×10^7 Da and 402 nm, respectively. The absolute M_w was comparable with the relative M_w obtained by gel permeation. To the best of our knowledge, this is the first confirmation of an absolute M_w over 10^7 Da for a water-soluble bioderived polymer (e.g., hyaluronic acid and xanthan gum: $\sim 10^6$ Da)[7,8] although it has been reported that some associative SC had extremely high apparent M_w values ($\sim 10^9$ Da).[9] Atomic force microscopy (AFM; left image in Figure 2a) of specimens dried from a diluted aqueous solution (0.5 ppm) on a mica sheet showed the formation of network structures containing nanosized loops whose diameter ranged from 100 nm to 2 μm. AFM also showed the height of thread lines ranged 0.4−0.7 nm comparable with the diameter of sugar chains. Transmission electron microscopy (TEM; right image in Figure 2a) of specimens dried from a diluted MeOH/water (20/1 v/v) solution of *sacran* (ca. 10 ppm) on a carbon-coated Cu grid showed that almost all of the *sacran* chains appearing as black threads (which contain O and Na elements illustrated by EDX in Figure S5) formed nanoloops, supporting the AFM results. This form was often seen in microscopic images of RNA and specific proteins as a lariat structure, indicating that the associating points dispersed in the polymer chains. In *sacran*, the association can occur by electrostatic attraction of a small amount of amino cations with carboxylate and sulfate ones. The formation of the physical hydrogels and the variability of the MALLS measurement without $NaNO_3$ may be due to the dispersion of the electrostatically associating points.

In general, high-molecular-weight polymers are difficult to dissolve in water because of their own strong segregation effects.[10] On the other hand, *sacran* had both water solubility and an ultrahigh molecular weight, which can lead to some unique properties. Although physical hydrogels of *sacran* were stable in the still-standing state, they transited into the sol state by vigorous agitation. The rotation viscosity measurement of the *sacran* sol (1 wt %) showed a very high zero-share viscosity value (83 000 cP) whereas hyaluronic acid ($M_w \sim 150-180$ kDa) showed a value of 8900 cP. Such a large difference in the viscosity can be attributed to the difference in the M_w. Although the addition of NaCl (0.9%) reduced the viscosity of the hyaluronic acid solution to 4400 cP as a result of charge screening effects of salts,[3] it raised the viscosity of the *sacran* sol to 153 000 cP (Figure S6). This result indicated that the addition of NaCl had some crucial effects on the chain state of

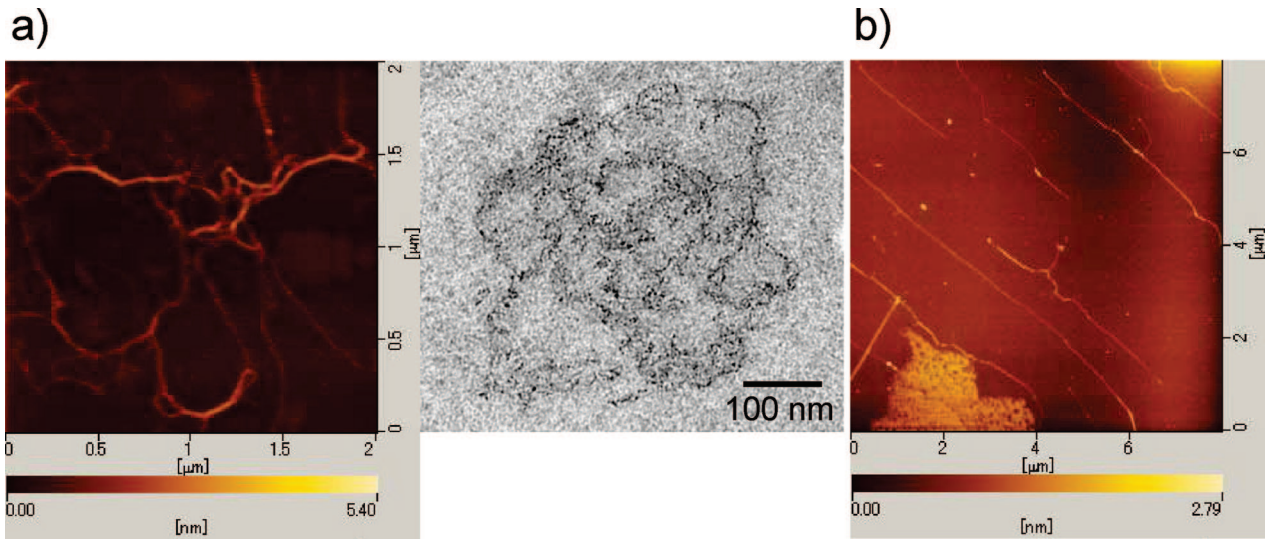

Figure 2. Microscopic images of *sacran*. (a) Atomic force microscopic (left) and transmission electron microscopic (right) images of specimens dried from a dilute solution of *sacran*. The images show representative networks containing nanosized loop structures. (b) Atomic force microscopic image of specimen dried from a dilute saline solution of *sacran*. The image shows representative rodlike *sacran* chains.

sacran. Then we took AFM images of *sacran* dried in the presence of NaCl and found that *sacran* chains drew almost straight lines (Figure 2b), indicating that cross-linking points of *sacran* networks were broken. The lines were 0.4−0.7 nm in thickness (Figure S7) and then were composed of the single *sacran* chain to suggest the adoption of the extended conformation. Since *sacran* may be semirigid rod as other natural polysaccharides,[7] one can discuss that they recovered extended conformation by the breakage of electrostatic association.

The *sacran* physical gel absorbed very large quantities of pure water (6100 mL g^{-1}) to its dry weight, as confirmed by the tea-bag method using a membrane with submicroscaled pores 0.8 μm smaller than most widely used filters with microscaled pores.[11] The water absorption of hyaluronic acid is 1200 mL g^{-1} to the dry weight using the submicro-pored filter, and λ-carrageenan showed only 700 mL g^{-1}. We showed that the water absorption efficiency of *sacran* was quite high as compared to these typical water-absorbable polysaccharides, which may be due to the extremely high M_w of *sacran*. Nanoloopes can hold water molecules efficiently as the chemically cross-linked hydrogels inside of their compartments. Subsequently, we showed that the value for saline absorption was as high as 2700 mL g^{-1} to dry weight. Although the saline absorption efficiency of *sacran* decreased to about one-half of the value for fresh water, the value was still very high. The saline absorption efficiency of hyaluronic acid was 240 mL g^{-1}, about one-sixth lower than its pure water absorption, which is a normal phenomenon due to the charge screening of polyelectrolytes by Na and Cl ions to reduce their hydration performance. One hypothesizes that the small decrease in *sacran* may be related to the salt-induced nanostructure transformation to extend chains to micrometer scale in length. Although the addition of NaCl could break the nanoloop structures to attenuating the water absorbability, it contributes to increasing the hydration performance by extending the chains, as shown in the schematic model of Figure 4. Furthermore, *sacran* still showed high absorption values for other salines (0.9%) containing multivalent metal ions such as Ca and Mg at 2000 and 2200 mL g^{-1}, respectively. The result that a lower saline absorption value was observed for the larger valence ions may be due to the stronger electrostatic forces with the polyanions to cross-link the sugar chains. In the case of an artificial urine containing $CaCl_2$ (0.02%), $MgSO_4$ (0.04%), NaCl (0.8%), and urea (2.0%), the saline absorption was also a high value at 2600 mL g^{-1}. In addition, we showed that *sacran* was successfully cross-linked by L-lysine to form hydrogels, and then water absorbency was controlled by changing the cross-linking density. The *sacran* physical gels was successfully wrapped in widely used nonwoven cloth even if no chemical cross-linker was used, and one can expect that *sacran* can be utilized as a high-performance urine absorber. All the results shown above suggest that *sacran* physical gels can be expected to have other various applications such as high moisturizing agents, good thickening agents, high-performance cell scaffolds, drug-release carriers in blood, and tree-planting materials for deserts.

Some reports pointed out the existence of supergiant sugar chains with a M_w over 10^7 Da in nature,[12] but their properties have never been confirmed due to the low extraction efficiency. For example, DNA is the highest M_w biopolymer,[13] but the amount of DNA that can be extracted with a M_w over 10^7 Da is too small to investigate its physical properties. Sugar sequence and bonding pattern of *sacran* have not been determined yet since cyanobacterial sugar chains generally had very complex structures composed of a great variety of constitutive monosaccharides without repeating unit.[14] The present paper is the first report showing the function of the cyanobacterial SC. We finally claim the usefulness of the SC which must have some unique structures because the cyanobacteria produced them to protect their own body from various external mechanical stressors in individual circumstances.

In conclusion, we extracted a novel sugar chain from the jelly-like extracellular matrix of a cyanobacterium, *Aphanothece sacrum*, living in rivers with a high ionic concentration. FT-ICR-MS analyses of the sugar chains demonstrated the presence of novel sugar, sulfated muramic acid as a chain constituent, which indicates that the sugar chain was novel. Then we named it *sacran*. GC-MS, GPC, and MALLS analyses of *sacran* demonstrated that it is an extremely high molecular weight polysaccharide (M_w: 1.6×10^7) composed of a high content of anionic sugars with a low content of cationic sugars. The supergiant glycoampholytes formed a physical hydrogels by cooling its homogeneous aqueous solution. Although the physical hydrogels of *sacran* were stable in the still-standing state, they transited into the sol state by vigorous agitation. The

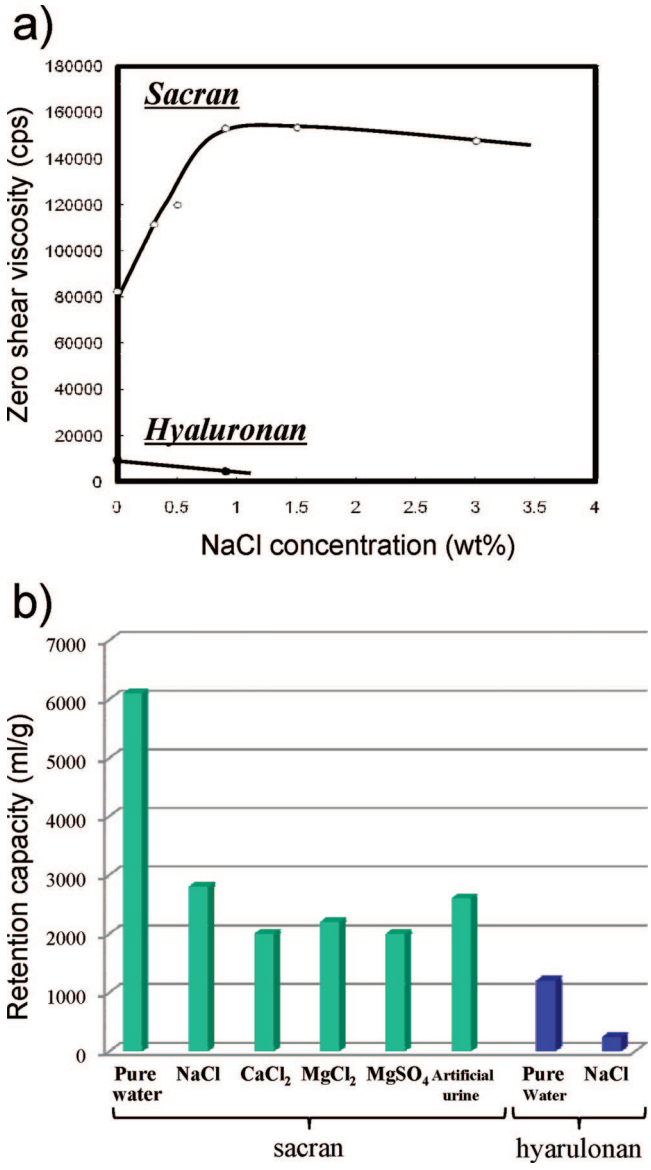

Figure 3. (a) Changes in zero-shear viscosity of NaCl solution of *sacran* and hyaluronan as a function of NaCl concentration. (b) Capacity of sugar chains for water, saline (0.9%), and artificial urine retention. *Sacran* showed ultrahigh retention capacities. Salt addition effects in *sacran* were weaker than those in hyarulonan.

rotation viscosity measurement of the *sacran* sol (1 wt %) showed a very high zero share viscosity value (83 000 cP) whereas hyaluronic acid ($M_w \sim 150-180$ kDa) showed a value of 8900 cP. While the addition of NaCl (0.9%) reduced the viscosity of the hyaluronic acid solution to 4400 cP as a result of reduction effects of salts on charge screening, it drastically raised the viscosity of the *sacran* sol to 153 000 cP. The hydrogels showed an ultrahigh absorption efficiency not only for water (6100 mL g^{-1}) but also for saline (2700 mL g^{-1}) and artificial urine (2600 mL g^{-1}). Atomic force microscopy of *sacran* illustrated that the *sacran* gels formed network structures containing nanolooped chains which showed a salt-induced transformation into extended rodlike chains several angstroms in diameter and several micrometers in length. Chain extension enhancing the hydration power countervailed the effects of salts.

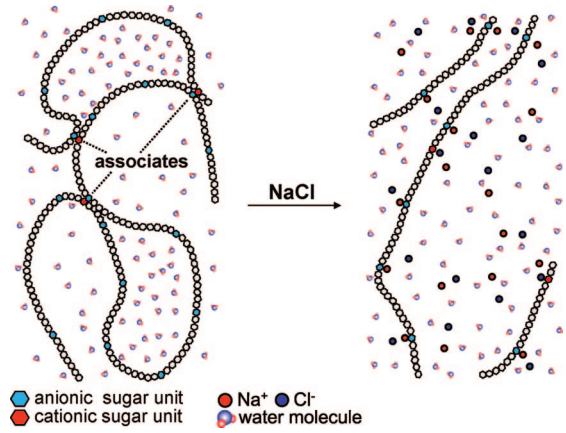

Figure 4. Schematic illustration of nanostructure transformation of *sacran* chains. *Sacran* formed networks containing nanoloops through electrostatic association of a small amount of cationic sugar units with anionic ones. Water molecules are efficiently retained in the nanolooped networks. Salt addition broke the association points and network structures to transform into rods several micrometers in length, keeping the retention capacity high.

Acknowledgment. This work was mainly financially supported by NEDO (08C46218d) and partially supported by Sekisui Integrated Research, Sumitomo Foundation, Yazaki Memorial Foundation for Science and Technology. We thank the technician Dr. K. Higashimine for taking the TEM image and Dr. K. Miyabayashi for recording the FT-ICR-MS.

Supporting Information Available: Procedures for extraction and purification of sugar chains, spectroscopic analysis, monosaccharide analysis, GPC, MALLS, TEM, AFM, zero-shear viscosity measurement, seven supporting figures, and detailed studies on retention capacity of pure water and saline. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) (a) Yeomans, K. *Chem. Rev.* **2000**, *10*, 2. (b) Osada, Y. *Adv. Polym. Sci.* **1987**, *82*, 1.
(2) Tanaka, T.; Fillmore, D.; Sun, S.-T.; Nishio, I.; Swislow, G.; Shah, A. *Phys. Rev. Lett.* **1980**, *45*, 1636.
(3) Zhang, Y.-Q.; Tanaka, T.; Shibayama, M. *Nature (London)* **1992**, *360*, 142.
(4) Hori, K.; Ueno, T.-M.; Okita, T. *Plant Foods Hum. Nutr.* **1992**, *42*, 36.
(5) Fujishiro, T.; Ogawa, T.; Matsuoka, M.; Nagahama, K.; Takeshima, Y.; Hagiwara, H. *Appl. Environ. Microbiol.* **2004**, *70*, 3338.
(6) Frank, H.; Lefort, M.; Martin, H. H. *Biochem. Biophys. Res. Commun.* **1962**, *7*, 322.
(7) Fraser, J. R. E.; Laurent, T. C.; Laurent, U. B. G. *J. Intern. Med.* **1997**, *242*, 27.
(8) Kim, J.-H.; Yoo, S.-J.; Oh, D.-K.; Kweon, Y.-G.; Park, D.-W.; Lee, C.-H.; Gil, G.-H. *Enzyme Microbiol. Technol.* **1996**, *19*, 440.
(9) Christensen, B. E.; Smidsroed, O.; Elgsaeter, A.; Stokke, B. T. *Macromolecules* **1993**, *26*, 6111.
(10) De Gennes, P.-G. *Scaling Concepts in Polymer Physics*; Cornell University Press: Ithaca, NY, 1979.
(11) Nohata, Y.; Azuma, J.; Kurane, R. *Carbohydr. Res.* **1996**, *293*, 213.
(12) For example: Smiley, K. L.; Boundy, J. A.; Hensley, D. E. *Carbohydr. Res.* **1982**, *104*, 319.
(13) Richter, J.; Seidel, R.; Kirsch, R.; Mertig, M.; Pompe, W.; Plaschke, J.; Schackert, H. K. *Adv. Mater.* **2000**, *12*, 507.
(14) De Philippis, R.; Vincenzini, M. *FEMS Microbiol. Rev.* **1998**, *22*, 151.

MA800307W