Chemical Reaction at Specific Sites and Reaction-Induced Self-Assembly as Observed by in Situ and Real Time SANS: Enzymatic Polymerization to Synthetic Cellulose

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Received May 31, 2006; Revised Manuscript Received July 25, 2006

We have investigated the self-assembling process of cellulose artificially synthesized via enzymatic polymerization by means of in situ and time-resolved SANS (small-angle neutron scattering). The results elucidated the following: (i) Cellulose molecules synthesized at a special reaction site of the enzyme (cellulase) located on or near the smooth surface of self-assembled enzymes formed in the reaction medium. (ii) The synthesized molecules associated themselves via DLA (diffusion-limited association) and crystallized into fibrils. (iii) The fibrils formed the aggregates, which had surface fractal dimension D_s increasing from 2 to 2.3 with the reaction time, on the smooth surface of the enzyme aggregates.

In this communication, we would like to present our recent experimental results concerning chemical-reaction-induced self-assembly of reaction products as observed by time-resolved small-angle neutron scattering (SANS) as a probe to unveil mesoscopic-scale structure formation. Full details of the experimental results will be described elsewhere.[1] Although the particular system to be reported here is a system comprising cellulose molecules artificially synthesized via enzymatic polymerization,[2] this type of research is considered to be one of the general problems in macromolecules which is interdisciplinary in nature, involving both chemistry (chemical reaction at a specific site of enzyme) and physics (reaction-induced self-assembly of reaction products). The problems will be found in various open nonequilibrium macromolecular systems that are open for various kinds of flows. The chemical reaction introduced to the system is considered to be a kind of external energy imposed on the system: we should note a strong interplay between molecular self-assembly and the chemical reaction.

In this work, we prepare separately two stable solutions A and B, as will be detailed below. Mixing the two solutions provides energy required for the enzymatic polymerization reaction and the reaction-induced self-assembly. In this sense, our system is considered to belong to an open nonequilibrium system. We focus on self-assembling processes, mechanisms, and structures particularly on the mesoscopic scale, because they have not been well-explored, despite its general importance for understanding pattern formation in nature, including biological systems.

An enormous number of studies concerning structures, biosynthesis methods, and chemical and physical properties of cellulose have accumulated so far, from both scientific and practical viewpoints.[3] We can find some successful reports leading to synthesis of cellulose with a biosynthetic pathway utilizing *Acetobacter xylinum* or *Phaseolus aureus* which extracts a nucleoside diphosphate sugar (ADP-, CDP-, or GDP-glucose) as substrate monomers.[4,5] In vitro synthesis of cellulose also has long been one of the most difficult and challenging research subjects. Actually, since 1941, a large number of studies tried to overcome the difficulties of attaining regio- and stereoselective synthesis of cellulose, i.e., construction of stereoregular polysaccharides having β-1,4-glycosidic linkage.[6−10]

In 1991, Kobayashi et al. reported the first successful nonbiosynthetic pathway of synthesizing cellulose via enzymatic polymerization.[2] The cellulose thus synthesized had a degree of polymerization (DP) with respect to the glucose unit as high as 22. Despite this advance in the cellulose chemistry, the self-assembling mechanism, processes, and structures of cellulose molecules created at a specific site on the enzyme have not yet been fully explored. Furthermore, physical factors controlling the self-assembly of the reaction product are not yet fully understood.

In this work, we aim to clarify those problems as described above. For this purpose, we employed exactly the same polymerization process as established previously by Kobayashi et al.:[2] To a solution of β-cellobiosyl fluoride (10.3 mg, 2.9×10^{-5} mol) in 170 μL of acetate buffer in D_2O (pD = 5.0, 0.05 M) was added acetonitrile (1000 μL) (solution A). To this solution A, 30 μL of acetate buffer in D_2O (pD = 5.0, 0.05 M) containing unpurified cellulase (originating from *Trichoderma viride*; 0.52 mg, 5 wt % per weight of substrate monomer; solution B) was added to give a total reaction volume of 1200 μL (acetonitrile/buffer = 5/1 v/v) and a monomer concentration of 0.025 M. The use of deuterated species for the reaction medium is to enhance the scattering contrast of the monomers and the polymers (cellulose). The mixed solution (A + B) was rapidly transferred into a quartz cell with 2 mm thickness, and the cell was set into a chamber controlled precisely at 30 °C.

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10.1021/bm0605255 CCC: $33.50
Published on Web 08/17/2006

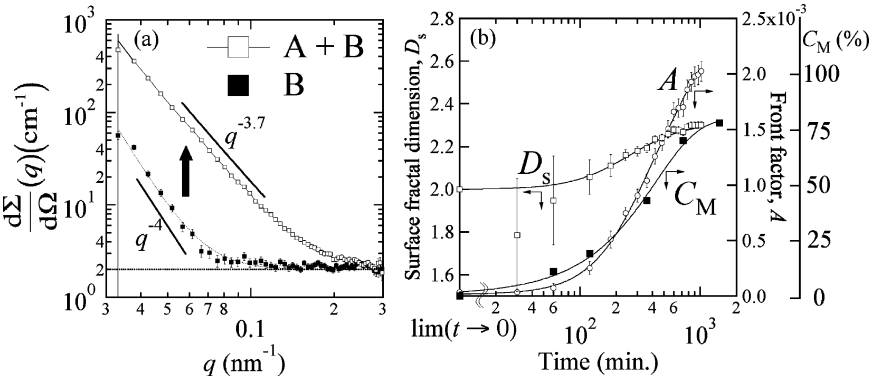

Figure 1. (a) SANS intensity distribution (differential scattering cross section in the absolute unit) for the enzyme solution B (filled square) and the reaction solution A + B at 18 h after the enzymatic polymerization (open square). The q-independent intensity level of 2 cm^{-1} arises from incoherent scattering from the solutions. (b) Time evolutions of aggregated cellulose molecules on the surface of the enzyme association as illuminated by time evolution of surface fractal dimension D_s, the front factor A related to an amount of the aggregated cellulose molecules, and monomer conversion C_M (%).

Then, the polymerization started, and time was set equal to zero for the time-resolved studies. The time-resolved SANS measurements were conducted with an SANS-J spectrometer installed at research reactor JRR-3 of Japan Atomic Energy Agency (JAEA) at Tokai. Circularly averaged scattered intensity was obtained as a function of q, where q is a magnitude of the scattering vector, defined by $q = (4\pi/\lambda)\sin(\theta/2)$ with λ and θ being the wavelength and the scattering angle, respectively. Further details of the SANS experiments should be documented elsewhere.[1]

It should be noteworthy that the SANS profile for the enzyme solution itself (solution B) shows an upturn toward the lower q region of $q < 0.1$ nm^{-1}, obeying Porod's law,[11] $d\Sigma/d\Omega = Aq^{-4}$, as indicated by the solid line in the Figure 1a where $d\Sigma/d\Omega$ is the differential scattering cross section in absolute intensity scale (cm^{-1}) and A is the front factor independent of q. This suggests the existence of superstructures in solution B having an average size of more than several hundreds of nanometers. Moreover, the superstructures must have a smooth interface and infinitesimally thin interface thickness (sharp interface), as revealed by the Porod's law type of scattering profile. Since the diameter of one cellulase molecule is estimated to be about 5 nm,[12] the superstructures are considered to be associations of large numbers of enzyme molecules. The association of enzymes is quite reasonable, because enzymes have both hydrophobic and hydrophilic parts, and the hydrophobic interactions of enzymes may outweigh their hydrophilic interactions with the reaction medium.

After the onset of polymerization, the scattered intensity drastically increased with time, as also shown by the scattering profile in Figure 1a obtained at 18 h after the polymerization. The profile is expressed by a power law, $d\Sigma/d\Omega = Pq^{-\alpha}$ with α being equal to 3.7, indicating a possibility that synthesized cellulose forms an aggregated structure with self-similarly rough surface having surface fractal dimension D_s given by $D_s = 6 - \alpha$.[13,14] The time-resolved SANS experiments revealed that as the polymerization proceeds the power-law exponent α tends to decrease from 4.0 to 3.7, and therefore, surface fractal dimension D_s increases from 2.0 to 2.3 with time, as shown in Figure 1b. This means that, as polymerization proceeds, the surface of the aggregates becomes rougher due to the successive formation of cellulose molecules as revealed by the increase of the front factor A in parallel to the increase of monomer conversion C_M, as also shown in Figure 1b.

Since the time-resolved SANS experiments would be able to cover only the narrow range of q as shown in Figure 1a, the power law for $d\Sigma/d\Omega$ elucidated extends over only about one decade with respect to q. To further check validity of the power law, we applied a combined small-angle scattering (SAS) method of USANS (ultra-small-angle neutron scattering), SANS, and USAXS (ultra-small-angle X-ray scattering) to the system in which the polymerization reaction was terminated at 18 h after onset of the polymerization. The details of the combined SAS method as well as those of confirmation of no change of the SAS before and after the termination should be referred to elsewhere.[1] The results are shown in Figure 2, which clearly elucidates the power law with $\alpha = 3.7$ extending over 3 orders of magnitude in the length scale (from ~30 nm to at least ~30 μm). The same system was explored also under field-emission electron microscopy (FE-SEM). The FE-SEM observation under varying magnification also elucidated self-similar rough surface with upper and lower cutoff lengths of ~30 μm and 30 nm, respectively.[1] The inset to Figure 2 shows a typical FE-SEM micrograph obtained under the highest magnification, elucidating the lower cutoff length of the fractal surface of ~30 nm. It remains as future challenging work to check whether the power law exists during the polymerization over such a wide q-range as observed above.

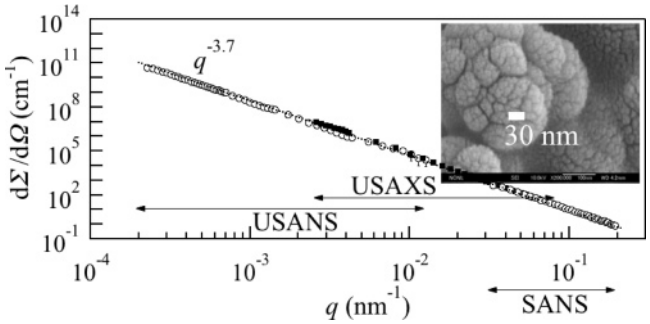

Figure 2. Combined SAS scattering profiles (USANS, USAXS, and SANS) for the self-assembly of the synthetic cellulose. SANS profile was corrected for incoherent scattering. The inset shows a typical FE-SEM micrograph.

On the basis of the results elucidated in Figures 1 and 2 together with previously established information on enzyme research,[2,15] we can now visualize a "specific reaction field" composed of enzyme associations in the reaction medium as schematically shown in Figure 3. In the length scale $r = 2\pi/q$ covered by the SANS ($7 < r < 200$ nm), the enzyme association appears to have effectively smooth and planar interface with a sharp interface boundary as shown in part (a). The enzyme association contains only a small number of active enzymes

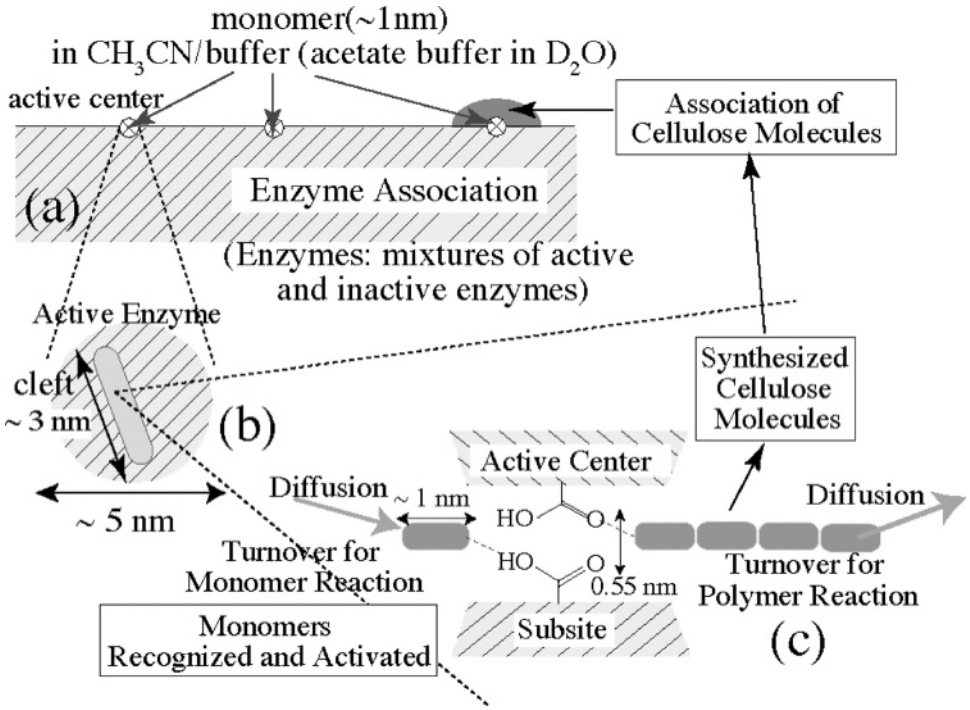

Figure 3. Schematic illustration of the reaction field of enzymes and the reaction-induced self-assembly of the reaction products.

(less than 1%), which can activate the enzymatic polymerization, because unpurified enzymes were used in these studies.[16] As a consequence, the active enzymes are statistically isolated and far apart among others in the enzyme association, which is a crucial point in this work.

An active enzyme shown in Figure 3b contains an active center as shown in Figure 3c, and the active center has one set of active subsites in a special place of the enzyme of the so-called cleft having ~3 nm in length and 0.55 nm along the cross-sectional directions.[2,15] Driven by osmotic pressure, a substrate monomer (~1 nm) diffuses into the active site, from the reaction medium outside the enzyme association, where it is recognized, activated, and reacted (chemically linked via condensation reaction) to a growing polymer chain recognized and anchored in another active subsite as shown in part (c). After a completion of this reaction cycle, another monomer diffuses into the same active subsite where it is recognized, activated, and reacted. As a consequence, the mass center of the growing polymer chain shifts. This cycle is repeated, which may be considered as a "turnover for the monomer reaction" as remarked in the left side of part (c). A polymer chain grows due to the successive turnovers for the monomer reaction. Then, the growing polymer chain end may be eventually brought away from the active subsite, which may be driven also by osmotic pressure, due to an increased local polymer concentration in the cleft and its surrounding space (a very narrow and confined space) as a consequence of increased DP. It should be noted that the polymer concentration outside the enzyme association is generally very small. The polymer chain brought away from the active subsite terminates polymerization. Another chain must start to grow at the same subsite, which may be considered as "turnover for polymer growth" as also remarked on the right-hand side of part (c). The polymer chains thus created diffuse out from the active center toward reaction medium outside the enzyme association, again driven by osmotic pressure, and associate themselves into aggregates via hydrogen bonding, because they are not solubilized in the medium, as schematically shown in the right edge of part (a) in Figure 3. The SANS will trace time

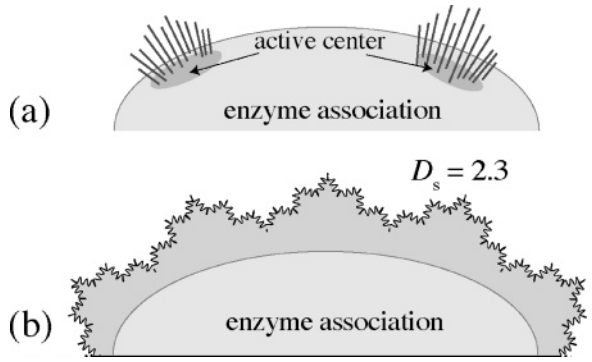

Figure 4. Schematic illustration of reaction-induced self-assembling process of cellulose molecules. (a) The unpurified enzymes self-assemble into associations of size of >200 nm where active sites are expected to locate far apart from each other. Each active center produces an enormous number of cellulose molecules as schematically shown by the lines via the turnover reactions.[17] (b) The cellulose molecules diffused out from the active center toward the reaction medium, driven by osmotic pressure, where they associated themselves via DLA and would eventually crystallize into fibrils. The illustration of part b represents a cellulose self-assembly developed in a relatively early stage of the polymerization. At the end of polymerization, the enzyme association will occupy only a minor fraction of the net assembly comprising celluloses and enzymes[17] and will be fully covered by the self-assembled cellulose fibrils in the average volume fraction which is expected to be very small in the assembly.[19]

evolution of the cellulose association formed in the periphery of the enzyme association.

The results obtained in Figures 1 and 2 suggest the following scenario for the reaction-induced self-assembly of cellulose molecules. A great number of cellulose molecules are created one by one in each active center in the enzyme association[17] as schematically depicted by a number of lines in Figure 4a, due to the two kinds of the successive turnovers as discussed above (Figure 3). Here, the length scale of observation was made larger than the case of Figure 2 (e.g., of the order of micrometers), so that the enzyme association shows a curvature. As a consequence, particular portions of surface of enzyme association are

enriched by reaction-terminated polymers, which should diffuse away from the surface along the direction (defined as *z*-direction) normal to the surface (longitudinal diffusion), driven by osmotic pressure. It should also be noted that the polymers created at active sites may be wet on and spread over the surface of the enzyme association through hydrophobic interactions of cellulose molecules and hydrophobic parts of enzymes (lateral diffusion). As a consequence of these lateral and longitudinal diffusion of cellulose molecules, a concentration gradient of cellulose molecules will be developed along the *z*-direction such that the concentration decays with z from the interface located at $z = 0$.

The synthesized cellulose having the low DP[18] is more or less rigid and has an extended or rodlike conformation with a length of ~5.5 nm and diameter of ~0.3 nm, thus having an aspect ratio of ~18. The polymers cannot be dissolved in the aqueous reaction medium and hence associate themselves into the aggregates, when they encounter each other, driven by diffusion, and eventually crystallized into fibrils. This reaction-induced self-assembling process driven by diffusion-limited aggregation (DLA)[13,14] results in the self-assembled structure having a fractal surface ($D_s = 2.3$) at the long time limit of the polymerization, as shown in Figure 1b and schematically depicted in Figure 4b.

It is important to note the the self-assembled cellulose molecules does not form a densely condensed phase, though the schematics shown in Figure 3(a) and 3(b) may give such an impression that they give rise to the densely condensed phase. The self-assembled structures are expected to still have enough free space for the monomers to diffuse through the assembly of cellulose formed in the periphery of the enzyme association into the active sites and for the reaction-terminated polymers to diffuse out from the active sites through the surface of the enzyme association to the reaction medium, both driven by osmotic pressure, because the average volume fraction of polymers in the assembly is still as low as $\sim 1 \times 10^{-3}$.[19] As a consequence, the enzymatic polymerization further proceeds, and the self-assembled structure of cellulose grows in the periphery of a round enzyme association, as shown in the inset to Figure 2, where the enzyme associations, which are surrounded by the cellulose aggregates, occupy only a minor volume fraction of the cellulose assembly. The growth is expected to result in radial expansion of the cellulose assembly as schematically shown in Figure 4b.

According to our scenario for the reaction-induced self-assembly of cellulose, both the diffusion of the monomers to the active site and the diffusion of reaction-terminated polymers from the active site toward the reaction medium are considered to be crucial parameters which control DP of the polymer synthesized via in vitro enzymatic polymerization. The pioneering works by Kobayashi et al.[2] already implicitly elucidated this fact by indicating that yield of the reaction products depends on composition of organic solvent and acetate buffer as well as the type of organic solvent used. Perhaps we need a much finer control of the thermodynamic properties of the reaction medium than the previously reported control[2] in order to attain a higher DP value. This is because the local concentration of polymer in the narrow space restricted around the specific site of the enzyme becomes very high as polymerization proceeds (Figure 3b,c). The studies along this line deserve future work.

References and Notes

(1) Tanaka, H.; Hashimoto, T.; Koizumi, S.; Kurosaki, K.; Kobayashi, S. In preparation.

(2) Kobayashi, S.; Kashiwa, K.; Kawasaki, T.; Shoda, S. *J. Am. Chem. Soc*. **1991**, *113*, 3079−3084.

(3) Mark, H. *Cellul. Chem. Technol*. **1980**, *14*, 569−581.

(4) Colvin, J. R. *Nature (London)* **1959**, *183*, 1135−1136.

(5) Elbein, A. D.; Barber, G. A.; Hassid, W. Z. *J. Am. Chem. Soc*. **1964**, *86*, 309−310.

(6) Schlubach, H. H.; Luehrs, L. *Liebigs Ann. Chem*. **1941**, *547*, 73−85.

(7) Klar, J. *Chem.-Ztg*. **1963**, *87*, 731.

(8) Hirao, S. *Agric. Biol. Chem*. **1973**, *37*, 187.

(9) Micheel, F.; Brodde, O.-E.; Reinking, K. *Liebigs Ann. Chem*. **1974**, 124−136.

(10) Uryu, T.; Yamauchi, J.; Kato, T.; Higuchi, S.; Matsuzaki, K. *J. Am. Chem. Soc*. **1983**, *105*, 6865−6871.

(11) Porod, G. *Kolloid-Z*. **1951**, *124*, 83−111.

(12) Davies, G. J.; Dauter, M.; Brzozowski, M.; Bjørnvad, H. E.; Anderssen, K. V.; Schülein, M. *Biochemistry* **1998**, *37*, 1926−1932.

(13) Hurd, A. J.; Schaefer, D. W.; Glines, A. M. *J. Appl. Cryst*. **1988**, *21*, 864−869.

(14) Martin, J. E. *J. Appl. Cryst*. **1986**, *19*, 25−27.

(15) Lee, J. E.; Brown, R. M., Jr.; Kuga, S.; Shoda, S.; Kobayashi, S. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 7425−7429.

(16) During the purification procedures of the crude enzyme to obtain the active enzyme with assaying the polymerization process,[15] the total weight of the starting enzyme was much reduced to less than 1 wt % (approximately 0.1 wt %) after isolation of the purified active enzyme fraction.

(17) We can roughly estimate a total number of cellulose molecules $N_{t,cellulose}$ created per active enzyme during the whole reaction process on the basis of information obtained from the experimental conditions: substrate monomer concentration [M], C_M, DP, concentration of total amount of enzyme $[E]_n$ (g/L) including both active and inactive ones, a number fraction of active enzyme f_a, and molecular weight of enzyme M_{enz}. The concentration of synthesized cellulose [C] is estimated as $[C] = [M]C_M/DP = 0.025 \times 0.75/5.5 = 3.41 \times 10^{-3}$ M. Concentration of active enzyme $[E]_a$ is estimated as $[E]_a = ([E]_n/M_{enz})\, f_a = [0.433/(3.7 \times 10^4)]0.1 \times 10^{-2} = 1.17 \times 10^{-8}$ M where f_a is assumed to be 0.1% and $[E]_n$ is estimated to be 0.52-(mg)/1.2(mL) = 0.433(g/L) from our experimental condition. Then, $N_{t,cellulose}$ is estimated as $N_{t,cellulose} = [C]/[E_a] = 3.41 \times 10^{-3}/1.17 \times 10^{-8} = 2.9 \times 10^5$. This corresponds to weight ratio r_w of amount of synthesized cellulose molecules to one active enzyme, $r_w = N_{t,cellulose} M_{cellulose}/M_{enz} = (2.9 \times 10^5) \times 1840/(3.7 \times 10^4) = 1.4 \times 10^4$ where $M_{cellulose}$ is average molecular weight of cellulose (1840) and $M_{enz} = 3.7 \times 10^4$. Thus, both $N_{t,cellulose}$ and r_w are estimated to be quite large!

(18) At 18 h after onset of the enzymatic polymerization where C_M reached a constant value of 75%, the number average molecular weight, M_n, of the reaction product was determined to be 1840 by size exclusion chromatography, corresponding to DP = 11 in the glucose unit, as will be detailed elsewhere.[1] Generally in carbohydrate chemistry, a "polysaccharide" refers to a substance having more than 10 monosaccharide units [Kennedy, J. F.; White, C. A. Polysaccharides. In *Comprehensive Organic Chemistry*; Barton, D., Ollis, W. D., Haslam, E., Eds.; Pergamon Press: Oxford, 1979; Vol. 5, pp 755−830]. According to this terminology, the present product belongs to the cellulose, a typical polysaccharide, family.

(19) The average volume fraction of synthesized cellulose molecules ϕ can be estimated as follows. The number density of the synthesized cellulose molecules ρ_n is estimated from total number of monomers converted into cellulose as $\rho_n = 0.025 \times 0.75 \times 6.0 \times 10^{23}/5.5 \cong 2 \times 10^{21}$ (cellulose molecules/L) where 0.025 is molar concentration of monomer (mol/L), 0.75 is C_M, and 5.5 is DP of β-cellobiosyl fluoride monomer. Now, the volume fraction ϕ is estimated to be still very low; $\phi = 2 \times 10^{21} \times \pi \times 0.15^2 \times 5.5$ (nm^3/L) $= 7.8 \times 10^{20}$ (nm^3/L) $\cong 1.0 \times 10^{-3}$, noting that 1 L $= 10^{24}$ nm^3. This value of ϕ was obtained by assuming that the synthesized cellulose molecules are uniformly dissolved in the reaction medium. Actually, the cellulose molecules are aggregated in the periphery of the enzyme association, so that the local concentration of cellulose in the assembly is much higher than 1×10^{-3}. Nevertheless, we anticipate there is enough free space in the assembly for diffusion of monomers and reaction-terminated polymers.

BM0605255