C.p./m.a.s. ^{13}C n.m.r. study on microbial cellulose–fluorescent brightener complexes

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The structures of microbial cellulose–fluorescent brightener complexes, produced from the *Acetobacter* culture in the presence of a fluorescent brightener, and the cellulose samples regenerated from them have been examined by cross-polarization/magic-angle spinning (c.p./m.a.s.) ^{13}C n.m.r. spectroscopy. C4 and C6 resonance lines for the cellulose components of the complexes appear at about 84.0 and 63.0 ppm, respectively, with the disappearance of their downfield crystalline components. Since the chemical shifts of the lines are in good accord with those of the non-crystalline component of native cellulose, it is concluded that the cellulose component of the complex is in the non-crystalline state. ^{13}C spin-lattice relaxation time (T_{1C}) measurements have also confirmed that the T_{1C} values of the dried complexes are of the same order as those for the non-crystalline component of native cellulose. In contrast, the T_{1C} values of the non-dried complex are much shorter than those of the dried complexes, indicating a much enhanced molecular mobility in the non-dried complex. On the other hand, when the non-dried complex is subjected to dye extraction, C4 and C6 resonance lines assignable to the crystalline component can be observed in the c.p./m.a.s. spectrum. Moreover, selective measurements of the spectrum of the crystalline component have revealed that cellulose Iβ is preferentially grown from the non-dried complex by dye extraction. In the case of the dried complexes, only very small amounts of cellulose I crystals are regenerated, possibly as a result of the formation of tight hydrogen bonds in the complex.

(Keywords: microbial cellulose; cellulose–brightener complexes; c.p./m.a.s. ^{13}C n.m.r.)

INTRODUCTION

As is well known, the cellulose produced from the *Acetobacter* culture will usually form a microfibril with about 40 Å of the diameter composed of cellulose I crystals[1–3]. Haigler *et al.*[4,5], however, found that the cellulose prepared in the presence of a fluorescent brightener does not form such a microfibril but forms a ribbon, and cellulose I is regenerated after dye extraction. This fact indicates that the product cultured in the presence of the brightener still has the ability to form cellulose I.

From the detailed X-ray examinations of the product, Kai[6,7] and Kai and Kitamura[8] reported that this is the crystalline complex of cellulose and brightener molecules in which a monolayer of the brightener is formed between the monolayer cellulose sheets corresponding to the $(1\bar{1}0)$ plane of cellulose I. In contrast, recent electron and X-ray diffraction studies of the products prepared in the presence of various direct dyes suggest that these dyes may adhere on the surface of the protofibril extruded from the cellulose synthesis site in the cell membrane, and that the cellulose component is in the non-crystalline state[9,10].

More recently, Kai *et al.*[11] found that cellulose products show characteristically different X-ray diffraction patterns when they are produced in the presence of several direct dyes; a structure similar to cellulose IV is regenerated in the case of congo red after dye extraction, while cellulose II is regenerated for direct red 80. Moreover, our cross-polarization/magic-angle spinning (c.p./m.a.s.) ^{13}C n.m.r. study revealed that the spectrum of the cellulose component in the product cultured in the presence of the brightener is similar to that of the non-crystalline component of native cellulose[12]. These results strongly suggest that the brightener and direct dyes may form complexes with cellulose as a result of their inclusion inside the nascent protofibril of cellulose.

In the work described in this paper, we have further studied the structure of the product cultured in the presence of the brightener, hereafter referred to as the cellulose–brightener complex for simplicity, in a dried or non-dried state, as well as the structure of the regenerated cellulose, with the use of c.p./m.a.s. ^{13}C n.m.r. spectroscopy.

EXPERIMENTAL

Preparation of cellulose–brightener complexes

The brightener complexes were prepared by the method described in previous papers[6,7,11]. Around 60 ml of cell suspension (*Acetobacter xylinum*, IFO 13693)[2,6,7]

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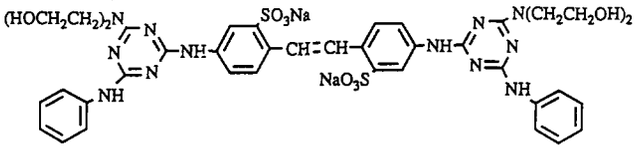
were added to 140 ml of Hestrin medium[13] (pH 7) containing the brightener 4,4′-bis[4-anilino-6-bis(2-hydroxyethyl)amino-1,3,5-triazin-2-ylamino]-2,2′-stilbene disulfonic acid



with concentrations ranging from 0.005 to 0.1 wt%, and the mixture was incubated at 28.0°C for 24 h. The product was washed thoroughly with 0.2 wt% aqueous NaOH solution for 48 h, rinsed with distilled water until alkali free, and then dried unless otherwise stated.

Preparation of dye-extracted samples

Non-dried and dried brightener complexes were subjected to dye extraction by boiling in 70 vol% aqueous ethanol for 18 h (fresh aqueous ethanol was used every 3 h). The residual samples were further boiled in a 1 wt% aqueous NaOH solution for 10 h under an N_2 atmosphere, rinsed with distilled water, and then dried.

N.m.r. measurements

C.p./m.a.s. ^{13}C n.m.r. experiments were performed at room temperature on a JEOL JNM-FX200 spectrometer operating at 4.7 T, as described elsewhere[14]. The m.a.s. rates were 3.2–3.4 kHz, and the contact time was 1.0 ms throughout this work. The chemical shifts relative to tetramethylsilane (Me_4Si) were determined by using the crystalline peak at 32.89 ppm of linear polyethylene as an internal standard. ^{13}C spin-lattice relaxation times (T_{1C}) were measured by the CPT1 pulse sequence with c.p.[15]. The dried or non-dried samples were packed in an m.a.s. rotor with an O-ring seal[16] to avoid the absorption of moisture or the loss of water during n.m.r. measurements.

RESULTS AND DISCUSSION

^{13}C n.m.r. spectra of microbial cellulose, cellulose–brightener complexes, and brightener powder

Figure 1 shows the c.p./m.a.s. ^{13}C n.m.r. spectra for dried microbial cellulose, dried complex and non-dried complex, obtained by incubation in the presence of the brightener in different concentrations, and the brightener powder. *Table 1* shows the ^{13}C chemical shifts of the respective carbons of microbial cellulose and the cellulose components of the brightener complexes shown in *Figure 1*. As can be clearly observed, no resonance line of the cellulose component in each complex overlaps the lines of the brightener, except for part of the upfield side of C6. It is therefore possible to discuss the structure of the cellulose component in the brightener complexes using these c.p./m.a.s. ^{13}C n.m.r. spectra.

All the resonance lines of the cellulose components in the dried complexes are broader than those of microbial cellulose. The C4 and C6 resonance lines for the complexes appear at 83.5–84.0 ppm and 62.8–63.0 ppm, respectively, which are very close to the corresponding chemical shifts of the non-crystalline component of microbial cellulose, as shown in *Table 1*. Furthermore, the C1 resonance line of each complex is shifted upfield by 1.0–1.3 ppm compared to that of microbial cellulose.

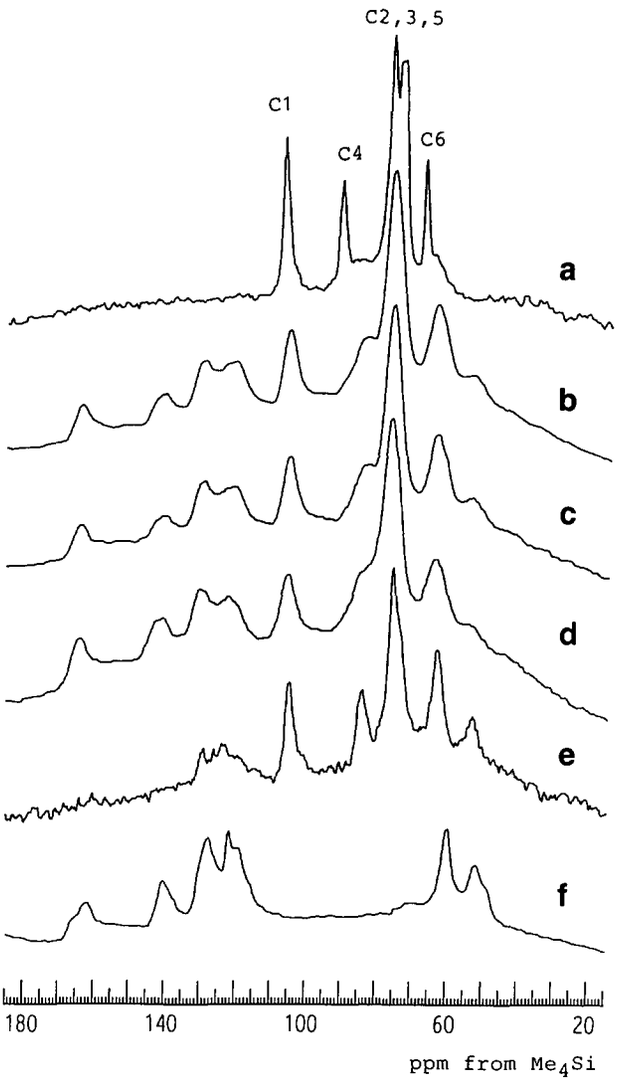


Figure 1 50 MHz c.p./m.a.s. ^{13}C n.m.r. spectra of (a) microbial cellulose, (b–d) dried cellulose–brightener complexes prepared in the presence of the brightener with concentrations of (b) 0.005 wt%, (c) 0.05 wt%, and (d) 0.1 wt%, (e) non-dried cellulose–brightener complex prepared in the presence of 0.1 wt% brightener, and (f) brightener powder

Table 1 ^{13}C chemical shifts of microbial cellulose, and cellulose–brightener complexes

	δ (ppm)			
Sample[a]	C1	C4	C2, 3, 5	C6
MC	105.7[b]	89.7[b], 84.1[c]	75.2, 73.1, 72.1	65.9[b], 63.0[c]
0.005D	104.5	83.5	75.0	62.8
0.05D	104.4	84.0	75.2	63.0
0.1D	104.7	83.9	75.5	63.3
0.1ND	104.4	83.9	74.7	62.5

[a] Naming for samples is as follows: MC, microbial cellulose; 0.1–0.005, concentration of the brightener in the medium (wt%); D, dried complex; ND, non-dried complex
[b] Crystalline component
[c] Non-crystalline component

The whole spectrum of the cellulose component of each complex seems to resemble that of the non-crystalline component of native cellulose[14,17] as well as that of non-crystalline cellulose[17,18]. No resonance lines corresponding to the C4 and C6 crystalline components

of microbial cellulose, which appear respectively at about 90 ppm and 66 ppm, can be recognized in the complex spectrum. These results indicate that the cellulose components in the brightener complexes are obviously in the non-crystalline state, in accord with the conclusion obtained by Haigler *et al.*[9,10].

In contrast to the dried complexes, the non-dried sample shown in *Figure 1e* has much narrower resonance lines, although the chemical shifts do not appreciably differ from those in the dried state. Such narrow lines may suggest a high mobility of cellulose chains and a more ordered structure with narrow distributions in conformation, chain packing, and hydrogen bonding.

Next we examine the structures and molecular mobilities for the different cellulose samples described above by analysing the ^{13}C spin-lattice relaxation process.

^{13}C spin-lattice relaxation times of microbial cellulose and brightener complexes

Table 2 shows the ^{13}C spin-lattice relaxation times (T_{1C}) measured by the CPT1 pulse sequence[15] for the dried and non-dried complexes. As previously reported[19,20], the T_{1C} values of microbial cellulose are 313–344 s for the crystalline component and 7–34 s for the non-crystalline component. Although the dried complexes also contain two components with different T_{1C} values, these values are as low as 24–39 s and 2–8 s. The longer T_{1C} values of the dried complexes are almost of the same order as those of the non-crystalline component of microbial cellulose. In contrast, the T_{1C} values for the non-dried complex are single values of 0.2–3.1 s, which are much smaller than the values of the dried complexes.

Horii *et al.*[19,20] have shown that water significantly reduces the T_{1C} values for the non-crystalline component of various celluloses, while there is almost no effect on the T_{1C} values for the crystalline component; T_{1C} values are 6.0–12 s for the non-crystalline component of wet cotton and 4.0–25 s for wet cupra rayon. The T_{1C} values of the non-dried complex are much smaller than these values. This fact indicates that the cellulose component of the complex is enhanced in its molecular mobility by water molecules. This may be due to a decrease in the level of hydrogen bonding in the cellulose component of the complex. In fact, a separate infra-red absorption study of the complex indicated that the hydrogen-bonding characteristic of cellulose I crystals does not exist in the cellulose component of the complex[21].

In the presence of water more stable complexes may be formed between cellulose and dyes, probably with the aid of hydrogen bonding associated with the water molecules. In such a complex, glucose residues undergo rapid fluctuations, resulting in very low T_{1C} values. However, when the complex is dried, some hydrogen bonds may be newly produced between cellulose molecules as well as between cellulose and dye molecules. This will lead to a reduction of the molecular mobility and thus to an increase in the T_{1C} values. Some disordering may also be introduced in the process of drying, as reflected by the lower T_{1C} components for the dried complexes. Nevertheless, in the region of more than 50% of the complex, as estimated from the higher T_{1C} component in the relaxation analysis, is assumed to be composed of somewhat ordered molecules. The ^{13}C chemical shifts suggest that such ordering may be similar to that in the non-crystalline regions of different native celluloses[14].

Table 2 ^{13}C spin-lattice relaxation times of the carbons of microbial cellulose–brightener complexes

Sample	T_{1C} (s)			
	C1	C4	C2, 3, 5	C6
0.005D	34, 4	26, 2	33, 6	31, 4
0.05D	34, 6	32, 5	39, 7	24, 4
0.1D	36, 6	29, 4	37, 8	27, 3
0.1ND	3.0	3.1	2.6	0.2

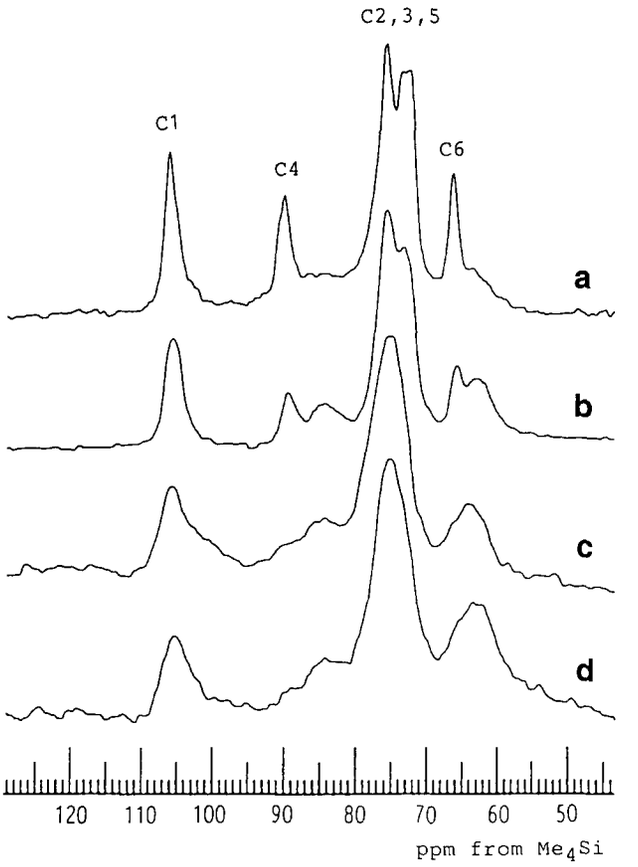


Figure 2 50 MHz c.p./m.a.s. ^{13}C n.m.r. spectra of (a) microbial cellulose, (b) the sample regenerated from the non-dried complex prepared in the presence of 0.1 wt% brightener, and samples regenerated from dried complexes prepared with (c) 0.005 wt% and (d) 0.1 wt% brightener

C.p./m.a.s. ^{13}C n.m.r. spectra and ^{13}C spin-lattice relaxation times of dye-extracted samples

Figure 2 shows the c.p./m.a.s. ^{13}C n.m.r. spectra of the cellulose samples regenerated from the dried and non-dried complexes by dye extraction. These spectra were measured in the dried state. There is no contribution from the brightener, indicating the satisfactory extraction of this material. *Table 3* shows the ^{13}C chemical shifts of the resonance lines of the spectra shown in *Figure 2*.

As can be clearly understood from the appearance of the downfield C4 and C6 lines in the c.p./m.a.s. ^{13}C n.m.r. spectrum (*Figure 2b*), cellulose I crystals are regenerated from the non-dried complex by dye extraction, although the degree of crystallinity is much lower than that in microbial cellulose. In contrast, only a very small amount of cellulose I is regenerated from the dried complexes.

Table 3 ^{13}C chemical shifts of different cellulose samples regenerated from dried and non-dried complexes

	δ (ppm)			
Sample[a]	C1	C4	C2, 3, 5	C6
MC	105.7	89.7, 84.1	75.2, 73.1, 72.1	69.5, 63.0
0.005DE	105.5	89.4, 83.9	75.0	65.6, 63.3
0.1DE	105.3	89.4, 83.9	75.3	65.1, 63.3
0.1NDE	105.4	89.5, 84.1	75.5	65.6, 62.8

[a] Naming for samples is as follows: DE, cellulose samples regenerated from the dried complex by dye extraction; NDE, cellulose sample regenerated from the non-dried complex; the numbers 0.005 and 0.1 indicate the concentrations (wt%) of the brightener

Table 4 ^{13}C spin-lattice relaxation times of different cellulose samples regenerated from dried and non-dried cellulose–brightener complexes

	T_{1C} (s)			
Sample	C1	C4	C2, 3, 5	C6
0.005DE	44, 9	50[a], 7[b]	–	52[c], 4[d]
0.1NDE	125, 16	125[a], 21[b]	–	115[c], 17[d]

[a] For the resonance at 105.5 ppm
[b] For the resonance at 83.9 or 84.1 ppm
[c] For the resonance at 65.6 ppm
[d] For the resonance at 63.3 or 62.8 ppm

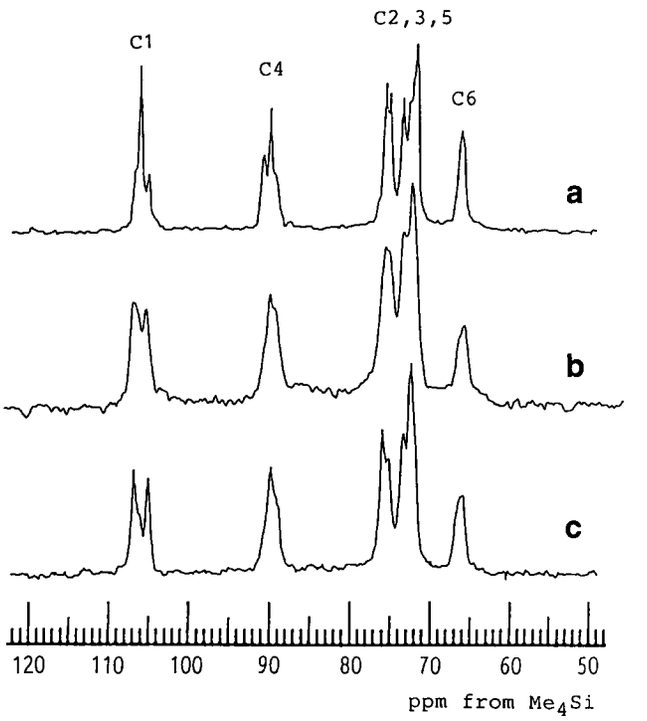


Figure 3 50 MHz c.p./m.a.s. ^{13}C n.m.r. spectra of the crystalline components of (a) microbial cellulose, (b) cellulose regenerated from the non-dried complex prepared in the presence of 0.1 wt% brightener, and (c) cotton cellulose

This suggests that some hydrogen bonds may be newly produced between cellulose molecules upon drying, and these will inhibit the rearrangement of cellulose chains for the regeneration of cellulose I crystals.

^{13}C spin-lattice relaxation times for some regenerated cellulose samples are given in *Table 4*. There are two components with different T_{1C} values: 4–9 s and 44–52 s for the sample (0.005DE) regenerated from the dried complex and 16–21 s and 115–125 s for the sample (0.1NDE) obtained from the non-dried complex. The higher T_{1C} values in the latter sample should be assigned to the crystalline component, because this component corresponds to the downfield C4 and C6 lines. However, the larger T_{1C} values for the dried complex are of a similar order to the T_{1C} values for the non-crystalline component of microbial cellulose. The crystalline region of the sample from the non-dried complex may be well formed compared to the case of the regeneration from the dried complex. Nevertheless, the T_{1C} values for the sample obtained from the non-dried complex are still lower than those for the crystalline component of microbial cellulose, implying that crystallite size and molecular orientation may be somewhat inferior in the regenerated cellulose.

As described above, in the case of the non-dried complex, the water molecules contained in the complex will be of use in the formation of the stable complex probably because of the suppression of direct hydrogen bonding among cellulose chains. The difficulty in regenerating cellulose I from the dried complex suggests that such hydrogen bonding, which may be formed upon drying, will inhibit the rearrangement of cellulose chains.

Crystal form of cellulose regenerated from the complex

There are two types of native cellulose, *Valonia*-microbial type and cotton-ramie type[22], which are rich in cellulose Iα and Iβ, respectively[23–25]. These are transformed almost entirely into cellulose Iβ by annealing at high temperatures[25–28] or by chemical treatments[29,30], as a result of the crystal transformation from cellulose Iα to Iβ. We have also examined the crystal forms of the cellulose samples regenerated by dye extraction.

Figure 3 shows the c.p./m.a.s. ^{13}C n.m.r. spectra of the crystalline components of microbial cellulose[22] and the sample regenerated from the non-dried complex, which was prepared in the presence of 0.1 wt% brightener. The crystalline spectrum of cotton cellulose is also shown for comparison[22]. Since the spectrum of the regenerated sample was measured in the dried state, the spectral resolution is somewhat lower than that for the spectra of microbial and cotton celluloses, which were measured in the hydrated state. Nevertheless, it is clear that the spectrum of the regenerated cellulose greatly differs from that of microbial cellulose, but resembles the spectrum of cotton cellulose. It is therefore concluded that cellulose Iβ is preferentially produced through the regeneration from the cellulose–brightener complex. This confirms that the complex has an ordered structure that will form cellulose Iβ crystals. Previous papers[6–8] have proposed that brightener molecules are included between the cellulose sheets corresponding to the $(1\bar{1}0)$ plane of the cellulose I unit cell. The preferential formation of cellulose Iβ may be associated with the transformation from Iα to Iβ by physical and chemical treatments[25–30]; cellulose Iβ is thermodynamically more stable than cellulose Iα.

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