

# X-ray diffraction and ultrastructural analyses of dye-altered celluloses support van der Waals forces as the initial step in cellulose crystallization

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Previous conflicting diffraction studies on dye altered cellulose have supported two opposing bonding schemes within a glucan minisheet: van der Waals forces and hydrogen bonds. Past molecular mechanics energy analysis has been used as evidence to support the van der Waals forces model. Additional evidence from electron microscopy and X-ray diffraction is presented here. Theoretical extrapolation supports a 3-ply sheet construction of a glucan dye sheets. A 9.4 Å reflection previously reported for glucan dye sheets as evidence for a cellulose I dye complex has been re-interpreted as a cellulose II-Tinopal composite structure where the dye molecules are intercalated between folded glucan chains. Glucan dye sheets and the cellulose II-Tinopal composite are thought to have arisen from glucan chains associated by van der Waals forces.

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## INTRODUCTION

The model for non-microfibrillar, dye-complexed cellulose (referred to as glucan dye sheets in this paper) has undergone several refinements since the first report of synthesis of tubes from extended glucan sheets and the inducement of microfibrils from them<sup>†</sup>. In 1984, Kai disputed the model of Brown *et al.*<sup>1</sup> in favour of a crystalline 'cellulose-brightener complex'. Based on an X-ray diffraction analysis, he proposed that dye molecules intercalate between the (110)<sup>†</sup> plane of the cellulose lattice to form a complex of many stacks of glucan sheets alternating with sheets of dye molecules. In 1985, Kai and Koseki<sup>2</sup> proposed that *in vivo* crystallization of cellulose I occurs when minisheets of glucan chains held together via van der Waals forces stack on top of each other and form hydrogen bonds between the sheets to form cellulose I microfibrils. This proposal was based on a combination of alkali swelling and electron diffraction studies.

Based on an electron diffraction analysis, Haigler and Chanzy in 1988<sup>3</sup> rejected Kai's cellulose-brightener complex model in favour of a modified tubular glucan dye sheet model. The helical orientation of microfibrils induced by acid washing and the ordering of a 3.99 Å reflection in four arcs suggested that the glucan sheet was helically coiled into a tube. The electron beam resistant, single 3.99 Å reflection was interpreted as the spacing between stacks of dye molecules associated with a single or a small packet of glucan sheets. The direction of dye

stacking was deduced to be oriented perpendicular to the glucan chain axis, and the researchers suggested that the long axes of the dye molecules probably were parallel to the glucan chain axes.

Using the conventional *a*, *b*, *c* three-dimensional coordinate system of crystallography, the glucan chain axis is often given as parallel to the *c* axis<sup>4,5</sup>. Both the *a* axis and the *b* axis are perpendicular to the *c* axis. Because two different planes may be perpendicular to a third plane in three-dimensional space, the Haigler–Chanzy description was unclear as to which direction the dye stacks were ordered. In recent work on the interruption of chitin synthesis with fluorescent brighteners<sup>6</sup>, this interaction, however, was diagrammatically illustrated with dye stacks associated via van der Waals forces with small, noncrystalline, packets of *n*-acetylglucosamine chains. By analogy, such a model would indicate that the predominant force holding the glucan chains together in a mini-sheet would be hydrogen bonds.

In 1991, Kai and Xu<sup>7</sup> presented new evidence for the original cellulose-brightener complex model<sup>8</sup>. Based on a modified X-ray diffraction analysis, a 9.4 Å reflection in addition to the previously reported 4.0 Å reflection was interpreted as evidence for multiple sheets in a highly crystalline cellulose-dye complex. They assigned the 9.4 Å reflection to the (110) plane<sup>8</sup>, and implied that the indexing was the same as for unaltered bacterial cellulose. Kai and Xu further suggested that the implicated spacings were modified due to the incorporation of the dye molecules in the spaces between the (110) plane. However, because the unit cell dimensions of the cellulose-dye complex could not be determined to verify this supposition, the indexing by Kai and Xu should still remain suspect.

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† While these colleagues indexed their cellulose I diffraction patterns according to Gardener and Blackwell<sup>4</sup>, the indexing has been translated according to Woodcock and Sarko<sup>5</sup> in order to maintain a constant system of indexing for this paper.

Thus, the Haigler/Chanzy model for glucan dye sheets<sup>3</sup> describes the interaction as a non-crystalline (but ordered) glucan sheet coated with dye molecules and helically coiled to form a tube. On the other hand, the Kai/Xu model<sup>7</sup> describes the interaction as a crystalline cellulose-dye complex. The differences in these interpretations may have arisen from differences in the synthesis conditions. These various structural interpretations can be evaluated by repeating the different synthesis conditions and observing the products with electron microscopy and X-ray diffraction. Thus, the goals of this study were: (1) to determine the structure of glucan dye sheets using the two different methods of synthesis, and (2) to determine the bonding between glucan chains with regard to the *in vivo* crystallization of cellulose I based on the structure of glucan dye sheets.

## EXPERIMENTAL

### Preparation of glucan dye sheets—Haigler/Chanzy protocol (HCP)

Glucan dye sheets were synthesized in static culture from resting cell suspensions incubated for 24 h in a volume of 100 ml at a final concentration of 1 mM Tinopal LPW<sup>TM</sup>, 40 mM glucose, and 50 mM sodium phosphate buffer (pH 7–8) in the dark at room temperature. This concentration of Tinopal LPW<sup>TM</sup> was chosen in order to maintain a high saturation level of brightener for the entire period of glucan dye sheet production. Resting cells of *A. xylinum* strain AY 201 (ATCC 23769) were prepared by the Celluclast<sup>TM</sup> method<sup>10</sup>. A single highly crenate colony (5–9 days) was used to inoculate 100 ml of Schramm and Hestrin (SH) medium<sup>11</sup> containing 150  $\mu$ l of filter sterilized Celluclast<sup>TM</sup>. The inoculated solution was incubated for 2 days at 28°C, in the dark on a rotary shaker at 120 rpm. After incubation, the solution was filtered through six layers of cheesecloth to remove undigested cellulose, then centrifuged at 1744  $\times$  *g* for 5 min at 4°C. The pellet was washed twice in excess 50 mM sodium phosphate buffer (pH 7–8) to remove any remaining Celluclast<sup>TM</sup>. The cells were resuspended in approximately 1 ml of 50 mM sodium phosphate buffer (pH 7–8).

### Preparation of glucan dye sheets—Kai/Xu protocol (KXP)

Another sample of glucan dye sheets was prepared according to the procedure of Kai and Xu<sup>7</sup>. In their study, Kai and Xu specified strain IFO 13693<sup>7</sup>, which is also distributed through the American Type Culture Collection as strain 23769 (AY 201). However, they did not describe the colony morphology used for the inoculum. Because the rough colony variant is the only one which has been identified which synthesizes glucan dye sheets<sup>1</sup>, strain AY 201 rc will be used. Ten Roux bottles (150 ml of SH medium<sup>11</sup> per bottle) each were inoculated with *A. xylinum* strain AY 201 rc and incubated for 4 days at 28°C in the dark in static culture. The cellulose pellicles were squeezed to release cells, and the resulting broth was filtered through cheese cloth. The filtrate was centrifuged for 10 min at 6160  $\times$  *g* at 4°C. The supernatant was removed, and the pellet was resuspended in excess 50 mM sodium phosphate buffer, pH 7.9. The resuspended solution was centrifuged again at 6160  $\times$  *g* at 4°C for 10 min. The pellet was resuspended in a final

volume of 30 ml of 50 mM sodium phosphate buffer, pH 7.9. The cell suspension was mixed with 50 ml of 80 mM glucose in 50 mM sodium phosphate buffer, pH 7.9 and 20 ml of 5 mM Tinopal LPW<sup>TM</sup> (in deionized water). This mixture was incubated in an Erlenmeyer flask wrapped with aluminium foil for 24 h at 28°C on an orbital shaker at 120 rpm. After 24 h, the cell suspension product was centrifuged for 10 min at 6160  $\times$  *g* at 4°C. The pellet was resuspended in excess 0.2% NaOH, incubated for 48 h in the dark on an orbital shaker at 90 rpm at room temperature, and washed thoroughly with deionized water to remove the alkali.

### Electron microscopy

Before preparation for X-ray diffraction, samples were contacted with formvar coated, 300 mesh copper grids and negatively stained with 2.5% uranyl acetate and observed using a Philips 420 transmission electron microscope (TEM) at 100 kV.

### X-ray diffraction analysis

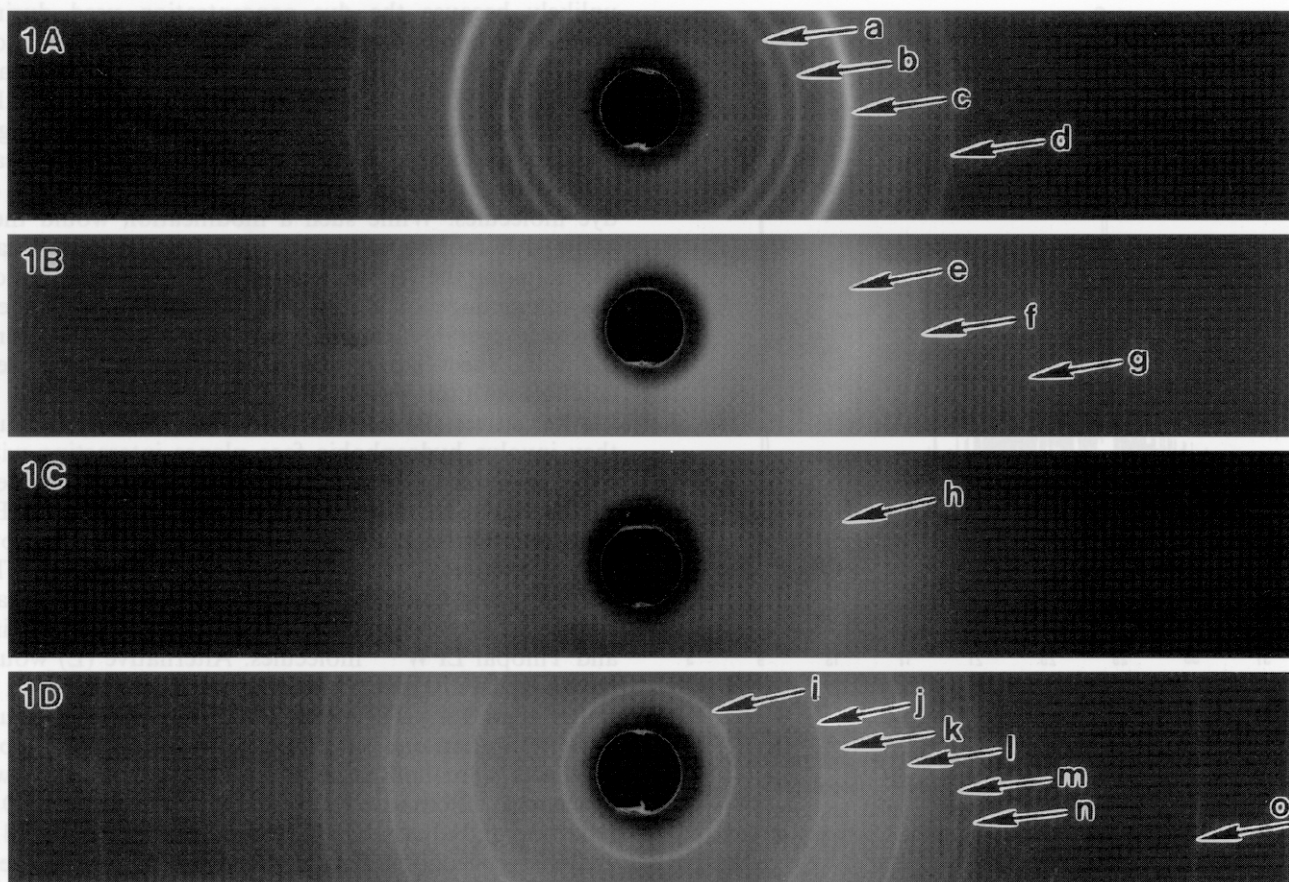
Powder patterns of the various samples were obtained with a Philips PW 1024/30 Debye Scherrer camera using Ni-filtered CuK $\alpha$  (1.542 Å) radiation at 35 kV and 25 mA. The reflections obtained were compared to those from a control sample of bacterial cellulose synthesized in the absence of Tinopal LPW<sup>TM</sup> and a control sample of Tinopal LPW<sup>TM</sup> recrystallized from a 5 mM stock solution by air drying under a black box.

For comparison with the data in Kai and Xu's study<sup>7</sup>, a sample of glucan dye sheets—(KXP) was analysed using an X-ray diffractometer with a diffracted beam graphite monochromator with CuK $\alpha$  radiation (1.5405 Å) over a  $2\theta$  range of 0–40°. This sample was air dried in layers on a glass slide, following the procedure of Kai and Xu<sup>7</sup>. After analysis, this sample was scraped from the glass slide and packed in a glass tube for powder diffraction analysis as described above.

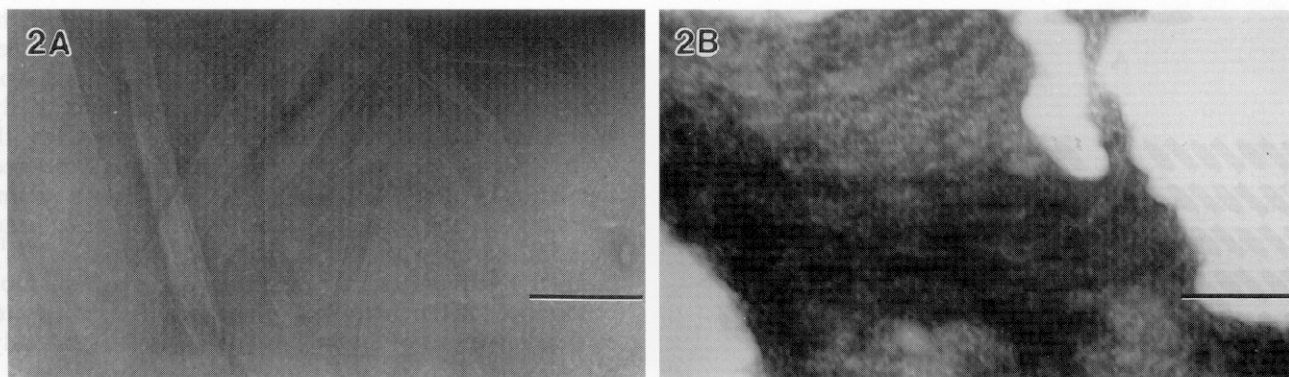
## RESULTS

X-ray powder diffraction for bacterial cellulose (Figure 1A) produced reflections at 6.1 Å, 5.3 Å, 3.9 Å, and 2.6 Å, characteristic of the expected cellulose I allomorph. The crystallized Tinopal LPW<sup>TM</sup> (Figure 1B) produced sharp reflections at 2.9 Å and 2.0 Å as well as a very line broadened reflection at 4.0 Å. The sample of glucan dye sheets—(HCP) gave a single 4.0 Å reflection (Figure 1C). The sample of glucan dye sheets—(KXP) gave several sharp reflections (Figure 1D) at 9.4 Å, 4.7 Å, 4.0 Å, 3.1 Å, 2.6 Å, 2.4 Å, and 1.5 Å as well as what appear to be several low intensity reflections at 3.7 Å, 2.2 Å, 2.1 Å, and 1.2 Å.

The quantity of reflections was not sufficient to determine a unit cell for the material synthesized with the Kai/Xu protocol because the several sharp reflections at 4.7 Å, 3.1 Å, and 2.4 Å are most likely multiple orders of the 9.4 Å spacing because the quotient of 9.4 Å and each of the other reflections yields an integer. The 4.0 Å reflection may be attributed to the stacking of the dye molecules and/or the ordering of glucan chains, and the 2.6 Å reflection probably arises from ordering of the glucan chains. The several low intensity reflections and background scatter indicate that the sample has a definite amorphous component in addition to the crystalline component determined from the several sharp reflections.



**Figure 1** X-ray diffraction analysis of glucan dye sheets. (A) Powder pattern of unaltered bacterial cellulose as a cellulose I control ( $a = 6.1 \text{ \AA}$ ;  $b = 5.3 \text{ \AA}$ ;  $c = 3.9 \text{ \AA}$ ;  $d = 2.6 \text{ \AA}$ ). (B) Powder pattern of Tinopal LPW<sup>TM</sup> crystallized from aqueous solution. Notice the line broadening of the  $4.0 \text{ \AA}$  reflection ( $e = 4.0 \text{ \AA}$ ;  $f = 2.9 \text{ \AA}$ ;  $g = 2.0 \text{ \AA}$ ). (C) Powder pattern of glucan dye sheets-(HCP). Notice the line sharpening of the  $4.0 \text{ \AA}$  reflection when compared to the same reflection in the crystallized Tinopal LPW<sup>TM</sup> ( $h = 4.0 \text{ \AA}$ ). (D) Powder pattern of glucan dye sheets-(KXP) ( $i = 9.4 \text{ \AA}$ ;  $j = 4.7 \text{ \AA}$ ;  $k = 4.0 \text{ \AA}$ ;  $l = 3.1 \text{ \AA}$ ;  $m = 2.6 \text{ \AA}$ ;  $n = 2.4 \text{ \AA}$ ;  $o = 1.5 \text{ \AA}$ ; several other low intensity reflections at  $3.7 \text{ \AA}$ ,  $2.2 \text{ \AA}$ ,  $2.1 \text{ \AA}$ , and  $1.2 \text{ \AA}$  are not labelled)



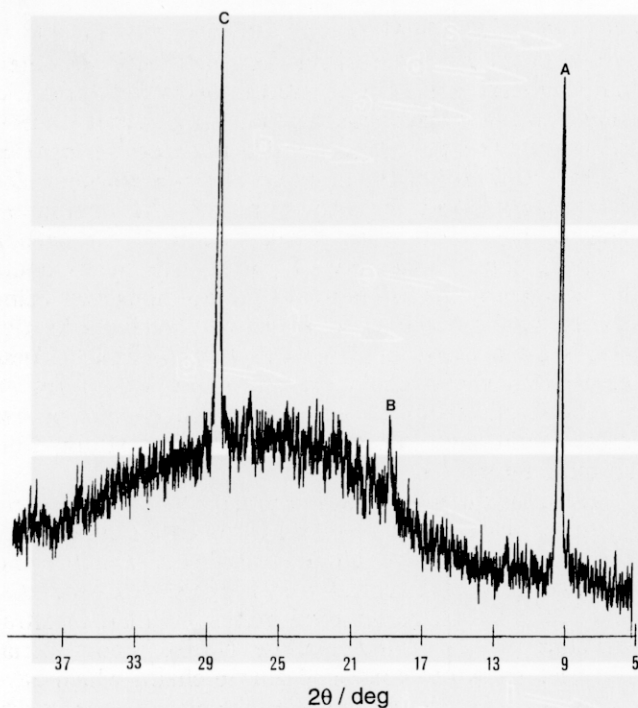
**Figure 2** Morphology of glucan dye sheet samples. (A) Electron micrograph of glucan dye sheets-(HCP); note the non-microfibrillar nature of the material. Compare morphology to the diffraction patterns in *Figure 1C*. (B) Electron micrograph of glucan dye sheets-(KXP); note the textured nature of the material. The image appears out of focus due to specimen thickness. Compare morphology to diffraction pattern in *Figure 4*. Bars =  $200 \text{ nm}$

Furthermore, the morphology of this sample was radically different from the samples which produced single  $4.0 \text{ \AA}$  reflections. While the sample of glucan dye sheets-(HCP) appeared as overlapping non-microfibrillar sheets (*Figure 2A*), the sample which produced the  $9.4 \text{ \AA}$  reflection appeared as a textured material extending perpendicularly from the longitudinal axis of the cells (*Figure 2B*). X-ray diffractometer analysis of the sample prepared with the Kai/Xu protocol also indicated a sharp reflection at  $9.4 \text{ \AA}$  with 1st, 2nd, and 3rd orders evident (*Figure 3*).

## DISCUSSION

### *Theoretical alternatives of glucan dye sheet structure*

Tinopal LPW<sup>TM</sup> molecules can interact with glucan chains by van der Waals forces or by hydrogen bonding. Five theoretical alternatives for these interactions (*Figure 4*) are: (A) alternating hydrogen bonded dye stacks with hydrogen glucan sheets; (B) alternating hydrogen bonded dye stacks with van der Waals associated glucan sheets; (C) intercalation of the dye molecules between glucan chains; (D) alternating dye



**Figure 3** X-ray diffraction profile of glucan dye sheets—(KXP). Notice the sharp reflection at  $d = 9.4 \text{ \AA}$  (A). The 2nd (B) and 3rd (C) orders were observed at  $d = 4.68 \text{ \AA}$  and  $d = 3.12 \text{ \AA}$ , respectively. (X-ray diffraction analysis courtesy of Dr Hugo Steinfink and co-workers in the Chemical Engineering Department at the University of Texas at Austin)

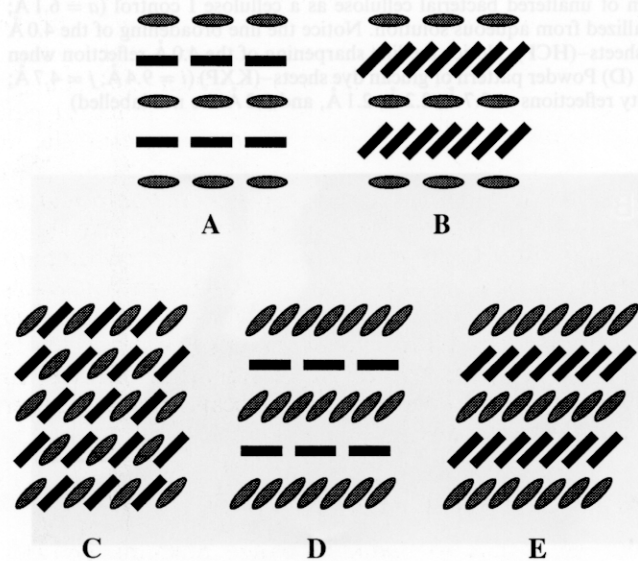
unlikely because the dye concentration used during synthesis is known to cause dye stacking, a phenomenon whereby dye molecules in an aqueous solution associate by van der Waals forces<sup>12</sup> into large stacks. Recently, Bartnicki-Garcia *et al.*<sup>6</sup> suggested a modified arrangement of alternative (A) in which a few layers of glucan chains are coated above and below many layers of dye molecules. While such a modification would then allow dye molecules to associate by van der Waals forces (along the vertical axis), this model is based upon the interactions of chitin with fluorescent brighteners in which several reflections were observed. Furthermore, the thin, sheet-like nature of the glucan dye sheets conflicts with this proposal.

Alternatives (B) and (D) also seem unlikely because they involve hydrophobic face planes interacting with hydrophilic face planes. Consequently, the various interactions can be narrowed down to alternatives (C) and (E). Both alternatives could produce a  $4.0 \text{ \AA}$  reflection, but only alternative (C) could produce a  $9.4 \text{ \AA}$  reflection. The  $9.4 \text{ \AA}$  spacing could be formed in the van der Waals associated sheet which alternates between glucan chains and Tinopal LPW<sup>TM</sup> molecules. Alternative (E) would have a repeat distance between layers of roughly  $17\text{--}21 \text{ \AA}$  rather than  $9.4 \text{ \AA}$ . Therefore, both alternatives should have more than one reflection. However, if the number of sheets were limited to three (one glucan and two dye stacks), alternative (E) would produce a single  $4.0 \text{ \AA}$  reflection and alternative (C) would produce both  $9.4 \text{ \AA}$  and  $4.0 \text{ \AA}$  reflections. This modified alternative (E), then, matches with the diffraction data obtained from glucan dye sheets with the Haigler/Chanzy protocol, and alternative (C) matches the data from sheets synthesized with Kai/Xu protocol.

*Structure of glucan dye sheets—Kai/Xu protocol*

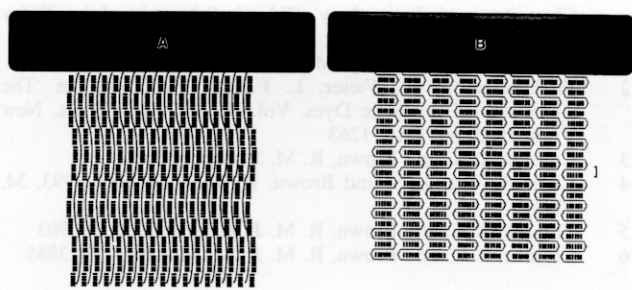
A  $9.4 \text{ \AA}$  reflection has been confirmed in the cellulosic material produced with the Kai/Xu protocol, but this reflection is *absent* in material produced by the Haigler/Chanzy protocol. Comparison of the reflections from these samples to the controls of crystallized dye and unaltered bacterial cellulose indicates that the  $9.4 \text{ \AA}$  spacing arises from the interactions between the Tinopal LPW<sup>TM</sup> molecules and the glucan chains. The  $4.0 \text{ \AA}$  spacing arises from the dye stacking, as indicated by comparison with the crystallized Tinopal LPW<sup>TM</sup> sample. Furthermore, the dye stacking becomes more pronounced in the presence of glucan chain, as illustrated by the sharpening of the  $4.0 \text{ \AA}$  reflection in the glucan dye complex—(KXP).

The morphology of the structure produced with the Kai/Xu protocol, however, is not that produced by the Haigler/Chanzy protocol. The difference in morphology could arise from washing with 0.2% NaOH, age of cells, or variation in temperature. While mechanical agitation is another variable between the two protocols, mechanical agitation following the Haigler—Chanzy protocol did not alter the morphology (data not shown). Glucan dye sheet synthesis has been shown to vary greatly between temperatures of  $25\text{--}28^\circ\text{C}$ <sup>13</sup>. While many smaller overlapping sheets are observed at  $25^\circ\text{C}$ , at  $28^\circ\text{C}$  glucan dye sheets are completely absent. The age of the cells may have been a factor as well. A milky layer was present on the surface of the pellicles used for production of the cell suspension, and such a milky layer has been implicated in the production of native band material<sup>14</sup>. The cellulosic

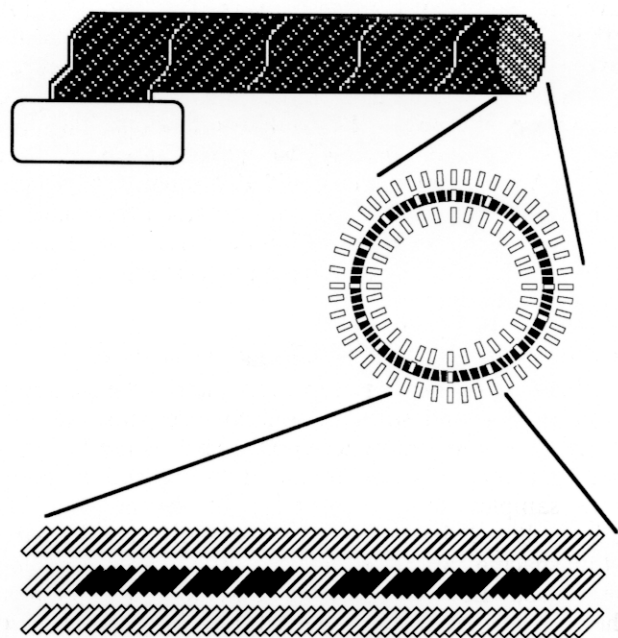


**Figure 4** The theoretical alternatives for interactions between glucan chains and Tinopal LPW<sup>TM</sup> molecules. A black rectangle represents a cross-section through the long axis of a glucan chain and a gray ellipse represents a cross-section through the long axis of a Tinopal LPW<sup>TM</sup> molecule. (A) Hydrogen bonded glucan sheets alternating with hydrogen bonded dye molecules, (B) van der Waals associated glucan sheets alternating with hydrogen bonded dye molecules, (C) alternating glucan chains and dye molecules within sheets, (D) hydrogen bonded glucan sheets alternating with van der Waals associated dye molecules, (E) van der Waals associated glucan sheets alternating with van der Waals associated dye molecules

stacks associated by van der Waals forces with hydrogen bonded glucan sheets; and (E) alternating dye stacks associated by van der Waals forces with van der Waals associated glucan sheets. Alternatives (A) and (D) appear



**Figure 5** Alternative schematic diagrams for the formation of the Kai/Xu complex. (A) If the complex forms from cells producing cellulose I, then the dye stacks must alternate with parallel glucan sheets and would be a cellulose I/dye complex allomorph. (B) If the complex forms from cells producing folded chains, then the dye molecules could intercalate between the folded sheets yields a cellulose II/dye complex allomorph. Note that the observed 9.4 Å spacing would occur between the stacks of sheets parallel to the long axis of the cells in the former case, but perpendicular to the long axis of the cell in the latter case (bracket = 9.4 Å spacing from Tinopal LPW<sup>TM</sup> stack to Tinopal LPW<sup>TM</sup> stack). This diagram is not to scale



**Figure 6** Schematic diagram of the glucan dye sheet model. Stacks of Tinopal LPW<sup>TM</sup> molecules associated by van der Waals forces (seen in long axis cross-section as rectangles) saturate the upper and lower surfaces of glucan minisheets as they are extruded from the TC subunits. The dye stacks also intercalate within minisheets which fuse into an extended sheet of glucans periodically interrupted by dye stacks. The glucan sheet is held together by van der Waals forces (groups of black rectangles), and the sheet binds to dye stacks on the upper and lower surfaces by hydrogen bonds. The codification scheme described in the text is represented by periodic dye intercalation within the plane of the glucan sheet. The exploded view (bottom) provides more details of the dye/cellulose interactions

material produced and referred to in this discussion as the Kai/Xu complex, is similar in ultrastructure to the cellulose II native band material reported by Kuga *et al.*<sup>14</sup>. Kai and Xu propose the complex is a composite of cellulose I and dye stacks (Figure 5A). A more likely model, based on the morphological similarity to native band cellulose, is the intercalation of dye stacks into the folds of cellulose II (Figure 5B).

### Structure of glucan dye sheets—Haigler/Chanzy protocol

Comparison of the diffraction data with the alternative interactions between glucan sheets and dye stacks supports a three-layer construction for glucan dye sheets (Figure 6). This three-layer construction also is supported by the flexibility of the material observed during video microscopy<sup>15</sup> and the very thin, sheet-like nature of glucan dye sheets even when measured on carbon support films<sup>1</sup>. Assembly of glucan dye sheets could be initiated by the formation of glucan minisheets being coated on both the upper and lower surfaces by dye stacks upon extrusion from the TC subunits<sup>15</sup>. These coated minisheets could interact laterally to form an extended glucan dye sheet. The extended glucan dye sheet could then helically coil to reduce strain; thus forming a tube.

Thus, the differences in interpretation of the structure of glucan dye sheets<sup>3,7</sup> has been based on the appearance of two different dye altered celluloses. Haigler and Chanzy<sup>3</sup> produced typical glucan dye sheets while Kai and Xu<sup>7</sup> synthesized textured material similar in ultrastructure to native band cellulose. Native band material has been shown to consist of glucan chains which have folded to form the antiparallel arrangement necessary for cellulose II<sup>14</sup>. Based on Kuga *et al.* and the data presented in this work, we propose that the textured material synthesized by the Kai–Xu protocol represents a new cellulose II/dye complex allomorph. Although Kai and Xu reported this material as a ‘crystalline complex’<sup>7</sup>, they assumed parallel glucan sheets, and this does not appear to be the case. In both dye altered celluloses, van der Waals associated glucan sheets play a major structural role. If the dye molecules contact the glucan chains during the folding process, then the cellulose II-composite results. If the dye molecules contact glucan chains which are not folding, then the nascent glucan chains (as a sheet) remain extended as they become coated with dye stacks. Such a process results in the formation of glucan dye sheets. The bonding in glucan dye sheets can be used to implicate van der Waals associations in native glucan mini-sheets. Although van der Waals forces are especially weak associations when compared to hydrogen bonding, energy analysis has shown that a van der Waals associated glucan mini-sheet is favoured over a hydrogen bond associated glucan mini-sheet under aqueous conditions<sup>16</sup>.

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### REFERENCES

- 1 Brown, R. M. Jr, Haigler, C. H. and Cooper, K. *Science* 1982, **218**, 1141

*Van der Waals forces in dye-altered celluloses: S. K. Cousins and R. M. Brown Jr*

- 2 Kai, K. and Koseki, T. *Makromol. Chem.* 1985, **186**, 2609
- 3 Haigler, C. H. and Chanzy, H. *J. Ultrastructure Mol. Structure* 1988, **98**, 299
- 4 Gardner, K. H. and Blackwell, J. *Biopolym.* 1974, **13**, 1975
- 5 Woodcock, C. and Sarko, A. *Macromolecules* 1980, **13**, 1183
- 6 Bartnicki-Garcia, S., Persson, J. and Chanzy, H. *Arch. Biochem. Biophys.* 1994, **310**, 6
- 7 Kai, A. and Xu, P. *Jpn. J. Polym. Sci. Technol.* 1991, **48**, 449
- 8 Kai, A. *Makromol. Chem. Rapid Commun.* 1984, **5**, 307
- 9 Distributed by Novo Nordisk Bioindustrials, Inc., Danbury, CT
- 10 Lin, F. C. and Brown, R. M. Jr in 'Cellulose and Wood: Chemistry and Technology' (Ed. C. Schuerch), John Wiley and Sons, New York, 1989, pp. 473-492
- 11 Hestrin, S. and Schramm, M. *Biochem. J.* 1954, **58**, 345
- 12 Venkataramon, K., Fieser, L. F. and Fieser, M. in 'The Chemistry of Synthetic Dyes, Vol. II', Academic Press, New York, 1952, pp. 1210-1263
- 13 Cousins, S. K. and Brown, R. M. Jr unpublished
- 14 Kuga, S., Takagi, S. and Brown, R. M. Jr *Polymer* 1993, **34**, 3293
- 15 Cousins, S. K. and Brown, R. M. Jr *Polymer* 1997, **38**, 903
- 16 Cousins, S. K. and Brown, R. M. Jr *Polymer* 1995, **36**, 3885