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Assessment of cellulose structural variety from different origins using near infrared spectroscopy

Yoshiki Horikawa

Abstract Near infrared (NIR) spectroscopy was tested as a rapid monitor of cellulose features by analyzing structurally distinguishable cellulose from different organisms ranging from Monera to Plantae as well as Animalia. The optimal spectral region was first identified using intra-crystalline deuteration, and then statistically analyzed based on second derivative spectra by principal component analysis. The score plots clearly distributed the samples according to crystalline structure such as relative crystallinity and allomorphism. These characteristics and the corresponding loading factors provided key NIR absorption criteria for identifying structural properties, especially in bands at 6527 and 6383 cm^{-1}, which correspond to I_α and I_β, respectively. In addition, calibration models were created for relative cellulose crystallinity using partial least square regression and for allomorph ratios using simple absorption band shifts at 6476–6446 cm^{-1}. NIR spectra of cellulose from various organisms combined with multivariate analysis can be used as a database for simple and rapid assessment of unknown cellulose materials.

Introduction

The plant cell wall has a central role in the mechanical function of the plant body, and its microstructure has evolved to adapt to environmental conditions (Sarkar et al. 2009). Therefore, survey of cell wall structure is a good approach to understand biological diversity as well as the progress of evolution. Cellulose is the main component of the plant cell wall, and is composed of β-1,4-homoglucan chains that are highly extended and packed to form microfibrils. It is synthesized by various organisms, including terrestrial plans, algae, bacteria, and tunicates (Tsekos 1999). However, the crystalline structure varies among biological organisms in terms of crystalline size, allomorphs, and uniplanar orientation. Therefore, the comparison of microfibril structures provides useful information for a better understanding of how the evolutionary process occurred.

Native cellulose has been characterized by different structural analyses such as X-ray diffraction, electron diffraction, nuclear magnetic resonance (NMR), and Fourier transform infrared spectroscopy (FTIR). Cross polarization/magic angle spinning (CP/MAS) ^{13}C-NMR spectra first demonstrated that cellulose microfibrils are composed of two crystalline units,

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namely I$_\alpha$ and I$_\beta$ (Attala and VanderHart 1984), which electron diffraction determined that correspond to unit cells as one chain triclinic and two chains monoclinic, respectively (Sugiyama et al. 1991a). Synchrotron X-ray and neutron diffraction further clarified the precise atomic coordinates and hydrogen bond formation (Nishiyama et al. 2002, 2003). The ratio of these allomorphs is different depending on the source of cellulose, and can be detected by NMR (VanderHart and Attala 1984) and some diffraction technique (Wada et al. 1995). FTIR is also a powerful tool to classify allomorphs, as demonstrated by infrared (IR) spectra combined with electron diffraction using crystalline phase transition of I$_\alpha$ to I$_\beta$ (Sugiyama et al. 1991b). The assignment of IR bands characteristic of the corresponding crystalline units has been widely applied to monitor the crystalline phase during chemical hydrolysis (Wada and Okano 2001), enzymatic degradation (Hayashi et al. 1998), and development of the chordate tunicate *Oikopleura dioica* (Nakashima et al. 2011).

Near infrared (NIR) spectroscopy is similar to IR spectroscopy in that the absorption of the electromagnetic wave is detected from chemical bond vibrations. However, in NIR spectroscopy, measurement is based on overtone and combinational vibration of functional groups, and NIR bands are less intense compared to those of IR. Therefore, NIR absorption is not saturated even in high crystalline material or wet samples, which allows monitoring of sugar content in fruit juice (Rodriguez-Saona et al. 2001) or biomass digestion in ethanol production (Horikawa et al. 2015). Cellulose structural properties have also been reported to successfully estimate relative crystallinities (Inagaki et al. 2010) and degree of polymerization (Horikawa et al. 2016). However, these reports are limited to cellulose from terrestrial plants, and few studies have explored the structural diversity of cellulose using NIR spectral analysis.

In the present work, I investigated structurally distinguishable cellulose from approximately 20 species of Monera, Plantae, and Animalia. NIR spectra were combined with multivariate analysis to evaluate correlation between sources and crystalline structure. First, I determined the NIR region corresponding to the crystalline structure using intra-crystalline deuteration. Second, principal component analysis (PCA) was carried out to study source distribution based on cellulose suprastructure. Third, I developed calibration models to predict cellulose crystalline features from NIR spectroscopy.

Experimental

Cellulose samples

Bacterial cellulose was obtained from *Acetobacter aceti* (Aj-12368) as described by Nge and Sugiyama (2007). Bacterial cells were statically cultured in Hestrin–Schramm medium at 27 °C for 2 weeks and then a thick gelatinous membrane (BC pellicle) was squeezed to collect the cells. The cell suspension was transferred to fresh medium and cultured statically at 27 °C for 9 days. The BC pellicles obtained were boiled in distilled water for 1 h, followed by 0.4% NaOH at 80 °C for 2 h, and then washed with distilled water several times.

Cell wall samples of *Glaucocystis nostochinearum*, *Oocystis apiculate, Valonia macrophysa*, *Cladophora* spp., *Boergesenia forbesii*, *Gelidium sesquipedale*, and *Erythrocladia subintegra* were boiled in 1% NaOH for 8–10 h followed by treatment in 0.05 N HCl at room temperature. The samples were then subjected to bleaching treatment in 0.3% NaClO$_2$ at pH 4.9 in acetate buffer at 70 °C for 3 h. The samples obtained were thoroughly rinsed with distilled water. The *G. sesquipedale* samples were boiled in water to remove agarose before the chemical treatment mentioned above.

Cellulose was obtained from *Halocynthia papillosa* as described by Sugiyama et al. (1991b). *Halocynthia roretzi* and *Ciona intestinalis* were treated with 5% KOH at room temperature overnight and then washed with distilled water. The samples were incubated in a bleaching solution at 70 °C for 2 h, which was repeated several times. After becoming colorless, cellulose samples were washed with distilled water.

Micrasterias crux-melitensis samples were boiled in 1% NaOH for 8 h, washed with distilled water, and then treated at 70 °C for 2 h in a bleaching solution. The bleaching treatment was followed by thorough washing and overnight dispersion into 5% KOH. The bleaching and KOH treatments were repeated several times before washing with water as described by Koyama et al. (1997).

Chara australis and *Egeria densa* were purchased from Kagaku Club (Shiga, Japan). To purify cellulose,

the specimens were treated with NaClO$_2$ solution at pH 4–5 at 70 °C, which is known as the Wise method (Wise et al. 1946). After repeating the process three times, the samples were boiled in 5% NaOH for 2 h and then washed with distilled water.

Cryptmeria japonica, *Eucalyptus globulus*, and sugarcane bagasse were subjected to cellulose extraction as described by Horikawa et al. (2013). After two-step milling using Orient mill VM-16 (Seishin, Tokyo, Japan) followed by Bantam mill AP-BL (Hosokawa Micron, Osaka, Japan), the products were subjected to the Wise method for lignin removal. The treatment was repeated and then samples were boiled in 5% NaOH for several hours. The cellulose samples obtained were washed with distilled water.

Cellulose was obtained from ramie as described by Sugiyama et al. (1991a).

Dewaxed cotton cellulose was purchased from Sogo Laboratory Glass Works (Kyoto, Japan).

Avicel PH-101, which is the commercial name of microcrystalline cellulose, was purchased from Asahi Kasei (Tokyo, Japan).

Nanocellulose was obtained from the purified cellulose from *C. japonica*. A cellulose suspension of approximately 0.8% was prepared and then passed through a grinder (Masuko, Saitama, Japan) at 1500 rpm as previously described (Abe et al. 2007).

Suspension-cultured cells induced from *Nicotiana tabacum*, known as BY-2 cells, were maintained in modified LS-medium (Nagata et al. 1981). Seven-day cultured cells were collected and subjected to the Wise method three times to remove proteins and lignin. The holocellulose obtained was treated with boiling 5% NaOH for 2 h and washed in distilled water for cellulose purification.

All purified cellulose samples were freeze-dried and molded to make 3 tablets from each source for measurement deviation. The tablets were made by collecting 10–40 mg of dehydrated cellulose and then hand-pressing it with a special die set (Kyoto Pastec, Kyoto, Japan) following a previously published protocol (Horikawa et al. 2011).

Intra-crystalline deuteration

Halocynthia roretzi cellulose was annealed in 0.1 N NaOD for 1 h at 210 °C, during which intra-crystalline deuteration reached completion (Nishiyama

et al. 1999). The deuterated samples were rinsed in distilled water several times.

IR spectroscopy

IR spectra were obtained using a PerkinElmer Frontier system equipped with microscopic accessory (PerkinElmer, Waltham, MA, USA). Cellulose suspension was placed on the BaF$_2$ window (13 mm diameter × 2 mm thickness) and dried completely. The spectra were recorded in the range of 4000–700 cm^{-1} using a low noise detector (HgCdTe) that was cooled to $-$ 196 °C with a spectral resolution of 4 cm^{-1} and acquisition of 128 scans.

NIR spectroscopy

NIR spectra were recorded under air-dry conditions using a PerkinElmer Spectrum 100 N system equipped with integrated sphere diffuse reflectance accessory (PerkinElmer, Waltham, MA, USA). Cellulose tablets were put directly on the sampling window where it was irradiated from below. The spectra were obtained in the range of 10,000–4000 cm^{-1} employing a triglycine sulfate detector with a spectral resolution of 16 cm^{-1} and acquisition of 32 scans.

Multivariate analysis

PCA and partial least square (PLS) regression analysis, which correspond to multivariate analyses, were performed using commercial software (Unscrambler v.10.3; CAMO Software, Woodbridge, NJ). To obtain more structural information, the original NIR spectral data with 2 cm^{-1} of interval, which were interpolated using spectral analysis software, were subjected to multiplicative scatter correction (MSC) and then converted with the Savitzky–Golay second derivative (1964) using a fifth-order polynomial for smoothing before multivariate analysis. Both algorithms for PCA and PLS regression analysis were executed on NIR second derivative spectra. PC loading was obtained from the model built for score plots. PLS regression model was created using optimal factors determined by cross-validation (leave-one-out): one sample was excluded from the model, and then its characteristics were predicted by making a model without the sample. The coefficient of determination for calibration (R_c^2)

and the root mean square error of calibration (RMSEC) were applied for evaluation of calibration performance. The models created were assessed using the coefficient of determination of prediction (R_P^2) and the root mean square error of prediction (RMSEP). A greater value of relative predictive determinant (RPD) reflects a more accurate fitting of data by calibration, and an RPD higher than 2.5 is considered satisfactory for screening (Williams and Sobering 1993).

Results and discussion

Optimal NIR region for cellulose crystal analysis suggested by intra-crystalline deuteration

When a cellulose sample is annealed in 0.1 N NaOD in D_2O, all OH bonds involved in microfibril formation are replaced by OD bonds (Fig. 1a i, ii). After cool down, the deuterated cellulose is washed with water, where surface OD bonds are accessible and revert to OH bonds whereas OD bonds in the crystalline core remain unaltered (Fig. 1a iii) (Horikawa and Sugiyama 2008). This behavior was detected by IR spectroscopy as shown in the cellulose spectra of *H. roretzi* before and after intra-crystalline deuteration (Fig. 1b). Intra-crystalline deuteration was reflected in a series of shaper bands around 3400 cm^{-1}, corresponding to OH stretching vibrations, shifting to 2400 cm^{-1}, where OD stretching vibrations are detected. However, broad and small IR absorption regions were observed around 3600 cm^{-1}, indicating that surface OD bonds reverted to OH bonds due to water access. This technique was applied to NIR spectra to determine OH stretching regions involved in cellulose crystal formation. Similar behavior can be seen in NIR spectra in which part of the overtones related to OH stretching vibrations in the range of 7000–6000 cm^{-1} disappeared and new bands appeared in 5200 cm^{-1} (Fig. 1c). A region with small absorption around 6900 cm^{-1} was observed after intra-crystalline deuteration, and should correspond to surface OH bonds by rehydration. In addition, combinational vibrations around 4800 cm^{-1} vanished after intra-crystalline deuteration, and these correspond to OH vibration during formation of the cellulose crystal. However, combinational vibrations include stretching and bending mode, which makes it difficult to assign the bands in light of the crystalline
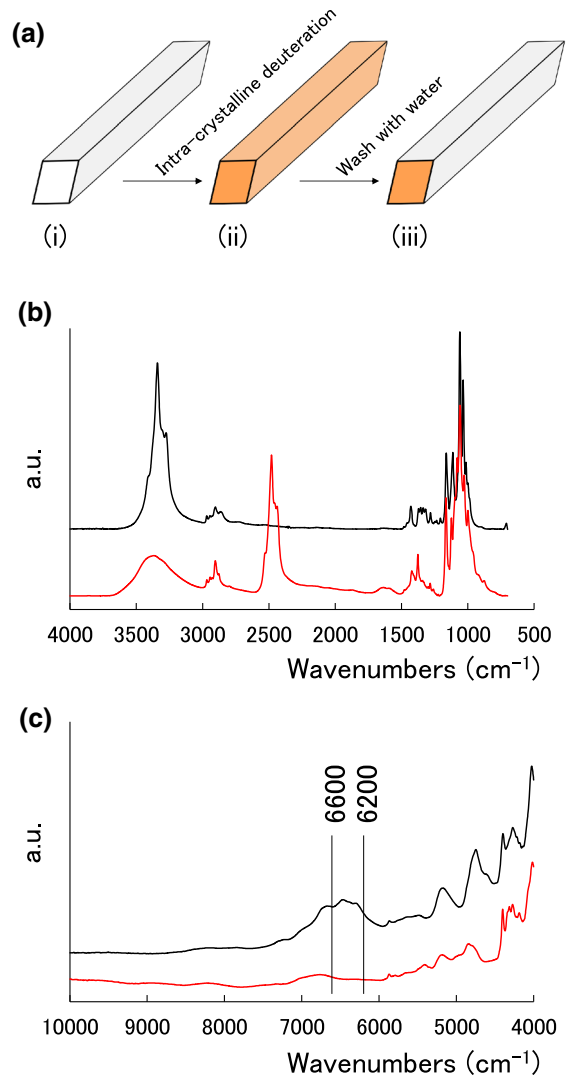

Fig. 1 a Schematic models of *H. roretzi* cellulose microfibrils (i) before and (ii) after intra-crystalline deuteration, and then (iii) washed with water where only surface OD bonds are replaced by OH bonds. b IR and c NIR spectra of *H. roretzi* cellulose before (black line) and after (red line) intra-crystalline deuteration. IR and NIR spectra after intra-crystalline deuteration correspond to model (iii) shown in a. (Color figure online)

cellulose structure. NIR bands in the range of 6600–6200 cm^{-1} were, therefore, used for the following multivariate analysis.

Determination of structural variety of cellulose from various sources using PCA

To better understand the structural relationships among cellulose produced by various organisms,

PCA score plots were computed based on NIR spectra of the relevant region. Original spectra were transformed to second derivatives before multivariate analysis to extract minor spectral features such as shoulder bands. Figure 2 exhibits score plots in PC1 (76%) and PC2 (19%) loading vectors, in which samples were clearly dispersed although some terrestrial plants and algae were clustered. Marine algae, tunicates, and bacterial cellulose localized in the left area, whereas higher plants, *Gelidium*, and *Chara* localized in right area. It is generally recognized that marine organisms generate large cellulose microfibrils, whereas higher plants produce small microfibrils of approximately 3–4 nm. Therefore, the PC1 vector might correspond to cellulose crystallinity. To verify this hypothesis, Fig. 3a shows the PC1 loading vector calculated with second derivative spectra of *Boergesenia* and *Egeria* (Fig. 3b). PC1 vector had three positive regions around 6527, 6454, and 6291 cm^{-1}, all of which coincided with the negative bands observed in the second derivative spectra of *Boergesenia*. Additionally, almost no or small negative band was observed at 6300 cm^{-1} in *Egeria* cellulose. The comparison of spectra obtained from different cellulose crystallinity samples further supported this hypothesis.

In the PCA score plots, terrestrial plants were ordered in left to right along PC 1 vector as follows: cotton, ramie, avicel, soft and hard wood, nanocellulose, grass, and cultured cells. Avicel can be prepared by removal of amorphous regions by chemical hydrolysis in microfibrils composing wood pulp, which shows a higher relative crystallinity. Grass is mainly constituted of parenchyma cells with the primary cell wall, and cultured cells also have only the thinner primary cell wall without lignification. Therefore, it is generally accepted that their cellulose crystallinities are lower than those of wood, which has a thick and lignified secondary cell wall. Interestingly, nanocellulose prepared from soft wood cellulose by grinder was clearly positioned to the right compared to its source. This distribution indicates that mechanical treatment decreases crystallinity, which is consistent with previously published reports (Hideno et al. 2016). To evaluate cellulose crystallinity, I performed X-ray diffractometry and estimated the full-width at half-maximum (FWHM) of the peak (200) presented in Table 1. FWHM is sometimes applied as an index of crystallinity and the values increase with lower crystalline cellulose. The FWHM values for terrestrial plants were mostly in accordance with their arrangement along the PC1 direction, suggesting that NIR spectroscopy successfully detected the molecular arrangements that form microfibrils.

In the PC2 direction, score plots show *Glaucocystis*, *Acetobacter*, *Oocystis*, *Cladophora*, *Valonia*, *Boergesenia*, and tunicates in order from top to bottom. This suggests that PC2 loading arranged cellulose samples based on allomorphs as shown in Table 1. Figure 4a shows PC2 loading vector with

Fig. 2 Principal component analysis plot of the first and second principal components of second derivative NIR spectra in the range of 6600–6200 cm^{-1} of cellulose from different organisms. (Color figure online)
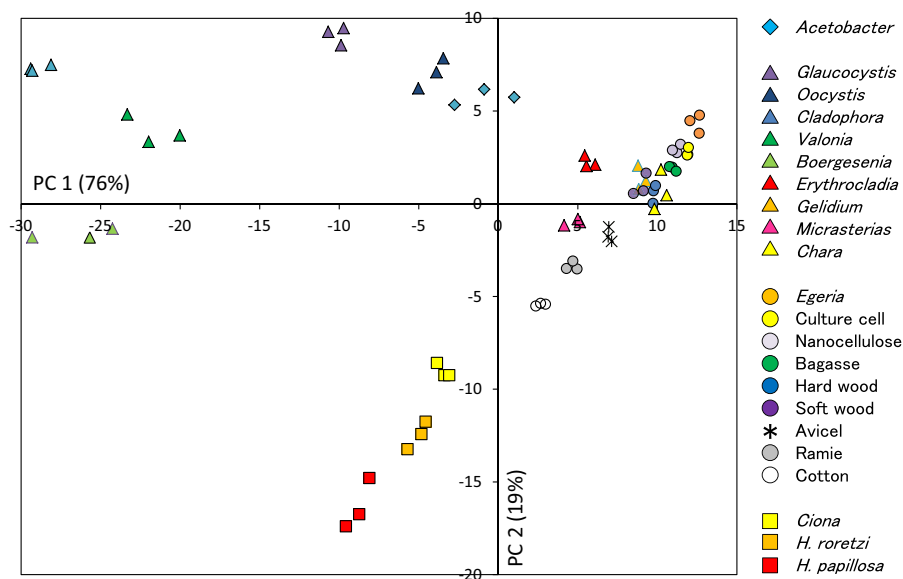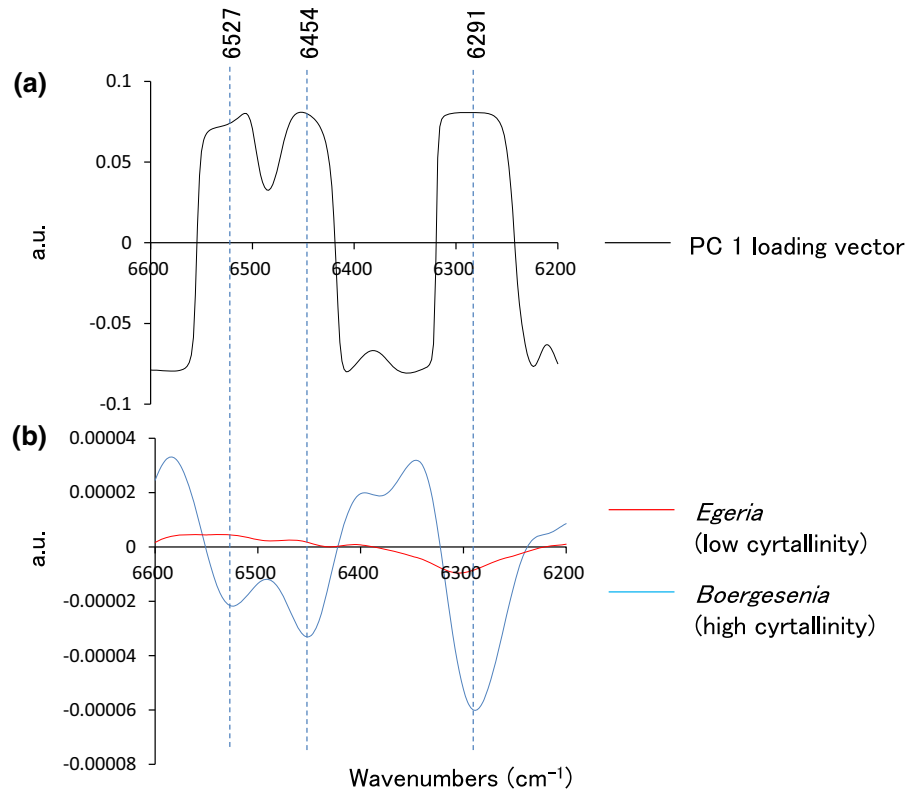
Fig. 3 a PC 1 loading vector for PCA in the range of 6600–6200 cm^{-1} and **b** second derivative spectra of *Egeria* (red line) and *Boergesenia* (blue line), which synthesize high and low crystalline cellulose microfibrils, respectively. (Color figure online)

Glaucocystis and *H. papillosa*, each representative of cellulose I$_\alpha$ and I$_\beta$, respectively (Fig. 4b). The bands in the PC2 loading vector at 6526 and 6446 cm^{-1} were negatively correlated, whereas shoulder bands at 6484 and 6383 cm^{-1} were positively correlated, with second derivative spectra. This can be interpreted as the former 2 bands being characteristic of I$_\alpha$ and the latter bands of I$_\beta$, which coincided with negative bands in the second derivative spectra of *Glaucocystis* and *H. papillosa*, respectively. Fackler and Schwanninger (2010) have tentatively assigned bands at 6270 and 6340 cm^{-1} to I$_\alpha$ and I$_\beta$, respectively, although these bands are different from those observed in this work. The characteristic bands in this study were determined using I$_\alpha$-rich and I$_\beta$-dominant cellulose samples, which is an improvement on previously published information.

Interestingly, terrestrial plants seemed to localize in score plots as an extension of *Ciona* and *Hallocynthia* samples, which consist mainly of I$_\beta$ crystal units. Though it is difficult to determine the allomorph type in lower crystalline cellulose microfibrils, Wada et al. (1997) showed by synchrotron X-ray diffraction analysis that wooden cellulose microfibrils are I$_\beta$ type. Algae such as *Micrasterias*, *Chara*, and *Glidium* are reported to be of type I$_\beta$ (Kim et al. 1996; Koyama et al. 1997), and they localized close to terrestrial plants. In contrast, *Erythrocladia* cellulose also has lower crystallinity and consists of I$_\alpha$ units (Koyama et al. 1997), and localized outside the line from tunicate to terrestrial plants. Therefore, score plots built using NIR spectra obtained from various sources further indicate that terrestrial plants synthesize cellulose microfibrils comprised of monoclinic units.

Calibration model for relative cellulose crystallinity

Based on the score plots in the PCA described above, I created calibration models for cellulose crystallinity by employing PLS regression. Statistics of the FWHM values is summarized in Table 2, where a wide variance is shown in both the calibration and prediction sets for randomly selected samples. To obtain a better model, second derivative spectra in the rage of 6600–6200 cm^{-1} as well as other regions were

Table 1 Full-width at half-maximum of peaks at (200) (°) of cellulose samples from different organisms calculated using X-ray diffractometry and compared to published I_α fractions

		Full-width at half-maximum (FWHM) at peak of (200) (°)	I_α fraction
Proteobacteria	*Acetobacter aceti* (AJ-12368)	1.44 ± 0.0284	
Glaucophyta	*Glaucocystis nostochinearum*	0.955 ± 0.0173	0.81[a]
Chlorophyta	*Oocystis apiculata*	1.07 ± 0.00244	0.83[a]
	Cladophora sp.	0.611 ± 0.00808	0.76[b]
	Valonia macrophysa	0.660 ± 0.0153	0.64[a]
	Boergesenia forbesii	0.663 ± 0.0127	0.55[b]
Rhodophyta	*Erythrocladia subintegra*	1.78 ± 0.0140	
	Gelidium sesquipedale	2.40 ± 0.128	
Charophyta	*Micrasterias crux-melitens*	2.57 ± 0.159	
	Chara australis	2.98 ± 0.260	
Spermatophyta	*Egeria densa*	3.30 ± 0.0958	
Pinophyta	*Cryptmeria japonica* (Soft wood)	2.55 ± 0.0118	
Magnoliophyta	*Eucalyptus globulus* (Hard wood)	2.40 ± 0.00533	
	Saccharum officinarum (Sugarcane bagasse)	2.60 ± 0.0311	
	Boehmeria nivea (Ramie)	1.87 ± 0.0175	
	Gossypium arboreum (Cotton)	1.62 ± 0.00395	
	BY2 culture cell induced from *Nicotiana tabacum*	3.51 ± 0.131	
	Nanocellulose from *Cryptmeria japonica*	3.17 ± 0.0998	
	Microcrystalline cellulose (Avicel)	1.94 ± 0.0151	
Chordata	*Ciona intestinalis*	1.45 ± 0.0670	
	Halocynthia roretzi	0.853 ± 0.00744	0.05[c]
	Halocynthia papillosa	0.793 ± 0.0196	0.05[c]

Standard deviation was calculated from three measurements

[a] Imai et al. (1999)

[b] Imai and Sugiyama (1998)

[c] Mean value of fractions reported by Belton et al. (1989) and Larrson et al. (1995)

obtained as follows: (1) 10,000–7500 cm^{-1}, in which the second and third overtones were present; (2) 7500–6000 cm^{-1}, where mainly OH overtone vibrations were detected; (3) 6000–5500 cm^{-1}, where the CH and aromatic framework vibrations were detected; and (4) 5500–4000 cm^{-1}, which corresponded to several combinational vibrations. Next, the optimum number of PLS factors to create the calibration model was determined to reduce the sum of squared residuals using the cross-validation algorithm. Although the sum of the residuals decreased as the number of PLS factors increased without any inflection point, too many numbers lead to overfitting, which results in

unreliable models. Therefore, the number of PLS factors used in this study was fewer than 6.

These selected regions, including the full-length NIR range of 10,000–4000 cm^{-1}, generated the PLS calibration models summarized in Table 3. The range of 6600–6200 cm^{-1} using 4 of PLS factor numbers produced a best model with an RMSEP of 0.273°, Rp^2 of 0.895, and RPD of 3.17 (Fig. 5). When using 5 PLS factors, regression performance became worse, with an RMSEP of 0.274°, Rp^2 of 0.860, and RPD of 2.74. The reduction in regression quality might be due to an overfitting problem. Table 3 also shows the regression performance of full length and other selected regions. With the exception of the model obtained for the NIR

Fig. 4 **a** PC 2 loading vector for PCA in the range of 6600–6200 cm^{-1} and **b** second derivative spectra of *Glaucocystis* and *H. papillosa*, which synthesize I$_\alpha$-rich and I$_\beta$-dominant cellulose microfibrils, respectively. (Color figure online)
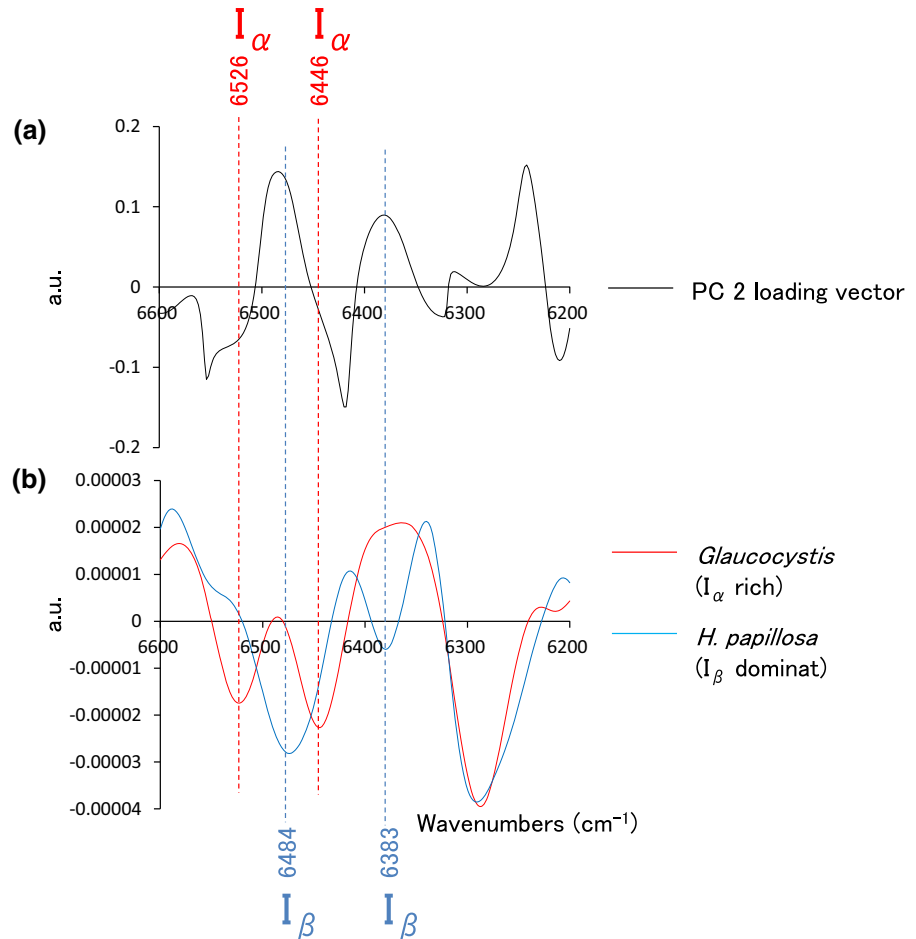

Table 2 Summary of the full-width at half-maximum of peaks at (200) (°) of cellulose samples estimated from X-ray diffractometry

	Calibration set					Prediction set				
	Spectra	Max.	Min.	Mean	SD	Spectra	Max.	Min.	Mean	SD
FWHM	45	3.66	0.602	1.87	0.957	21	3.27	0.643	1.87	0.864

Cellulose samples from 15 species were used in the calibration set and from seven species in the prediction set, which comprised 45 and 21 spectra, respectively

spectra ranging from 7500 to 6000 cm^{-1} and 5500–4000 cm^{-1}, PLS regression calibrations showed poor performance, as all Rp^2 were < 0.790 and all RMSEP were > 0.387°. Combinational vibrations in the range of 5500–4000 cm^{-1} gave an acceptable calibration model with an RMSEP of 0.377°, Rp^2 of 0.800, and RPD of 2.29. Considering this achievement, the range of combinational vibrations also involved crystalline structural information, as the

bands at 4800 cm^{-1} indicated in the intra-crystalline deuteration. The results in this study demonstrated that a broader range is not requisite to improve the model performance, and accordingly, the logical selection of the absorption range is an important process to create a more reliable regression model as a determination by the intra-crystalline deuteration.

Rambo and Ferreira (2015) recently showed that near infrared spectroscopy combined with

Table 3 Summary of statistical parameters of the calibration models for the full-width at half-maximum of peaks estimated from X-ray diffractometry of cellulose samples

	PLS factors	Calibration set		Prediction set		
		R_c^2	RMSEC(°)	R_p^2	RMSEP(°)	RPD
6600–6200 cm^{-1}	3	0.818	0.404	0.824	0.353	2.45
6600–6200 cm^{-1}	4	0.872	0.338	0.895	0.273	3.17
6600–6200 cm^{-1}	5	0.890	0.314	0.860	0.316	2.74
10,000–4000 cm^{-1}	5	0.990	0.095	0.789	0.388	2.23
10,000–7500 cm^{-1}	5	0.983	0.124	0.512	0.589	1.47
7500–6000 cm^{-1}	4	0.901	0.298	0.830	0.348	2.48
6000–5500 cm^{-1}	5	0.934	0.243	0.721	0.446	1.94
5500–4000 cm^{-1}	5	0.960	0.188	0.800	0.377	2.29

PLS partial least square, *RMSEC* root mean square error of calibration, *RMSEP* root mean square error of prediction, *RPD* relative predictive determinant
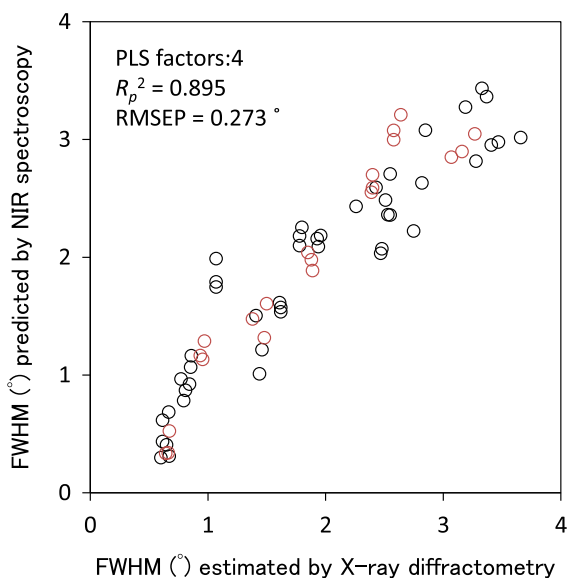

Fig. 5 Relationships between the full-width at half-maximum (FWHM) of peak at (200) (°) calculated by X-ray diffractometry and predicted by near infrared spectroscopy in the range of 6600–6200 cm^{-1}. Black and red plots correspond to calibration and prediction data sets, respectively. (Color figure online)

multivariate analysis allowed the evaluation of cellulose crystallinity of banana residues using almost the full-length NIR region. Inagaki et al. (2010) have successfully reported a calibration model for cellulose crystallinity using several specific NIR absorption regions assigned to amorphous, semi-crystalline, or crystalline structure in the range of 7200–6100 cm^{-1}. However, both published reports are limited to cellulose samples from terrestrial plants. This study demonstrates that short ranges such as 6600–6200 cm^{-1} allowed to estimate cellulose relative crystallinity from different organisms ranging from bacteria to higher plants as well as tunicates in Animalia.

Calibration model to determine the ratio of cellulose crystalline allomorph

I also designed a standard curve to determine the crystalline ratio of allomorphs in cellulose. The available allomorph data set is insufficient, as only six samples with NMR and FTIR data were reported (Imai and Sugiyama 1998; Imai et al. 1999). I initially surveyed the bands of second derivative spectra in the range of 6600–6200 cm^{-1}, which are summarized in Table 4. Bands around 6527 or 6383 cm^{-1} were characteristic of I$_\alpha$ and I$_\beta$, respectively, and both were independent of allomorph ratios in the various samples. In contrast, the band observed around 6450 cm^{-1} shifted position depending on the allomorph ratio. The cellulose I$_\alpha$ fraction was therefore plotted against the corresponding band position in each cellulose sample, clearly indicating a linear correlation (Fig. 6). The estimated cellulose I$_\alpha$ ratio is calculated as shown below,

$$\text{Cellulose I}_\alpha \text{ratio} = -0.0250 \times W_{6476-6446} + 162.08$$

Table 4 Wavenumbers from second derivative near infrared spectra in the range of 6600–6200 cm^{-1} of cellulose from different organisms

	Spectral bands (cm^{-1})			
	I$_\alpha$	I$_\alpha$ or I$_\beta$	I$_\beta$	
Glaucocystis	6526	6446	n. d.	6287
Oocystis	6527	6449	n. d.	6290
Cladophora	6527	6447	n. d.	6289
Valonia	6527	6449	6379	6290
Boergesenia	6527	6454	6380	6291
H. roretzi	n. d.	6475	6383	6291
H. papillosa	n. d.	6476	6383	6292
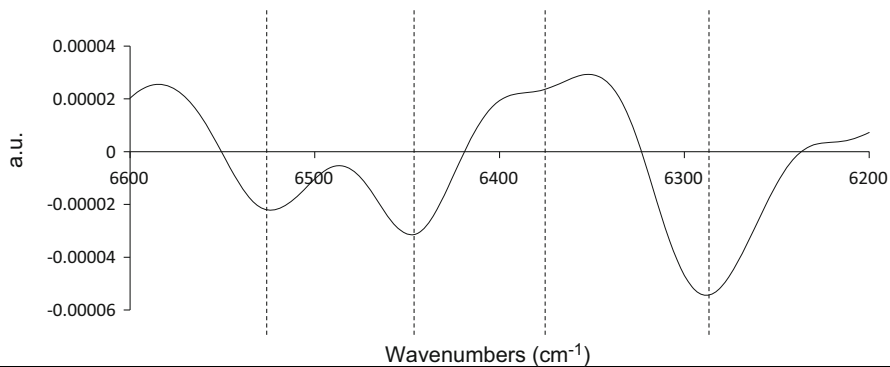
A second derivative spectra of *Valonia* with an I$_\alpha$ fraction of 0.64 has been included below the table to facilitate evaluation of each spectral band. Each mean value was determined from three measurements
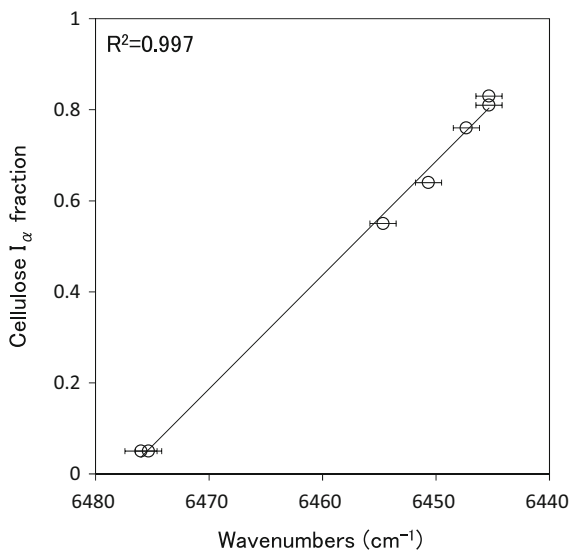
n. d. not detected

Fig. 6 Relationships between allomorph ratio and near infrared band shift ranging from 6476 to 6446 cm^{-1}. Cellulose I$_\alpha$ fractions of standard samples are applied as shown in Table 1

where $W_{6476 - 6446}$ is the wavenumber of the band position present in the range of 6476–6446 cm^{-1} in the second derivative spectra. Nakashima et al. (2011) have described a calibration model for the I$_\alpha$ fraction, which was created using IR band shifts at 3345 cm^{-1} and assigned to O3–H⋯O5. The authors describe a moving range in IR spectroscopy as narrow as 3350–3340 cm^{-1}, whereas in this study I describe a three times wider range of 6476–6446 cm^{-1}. The observed wide range of band shifts is promising to easily determine the cellulose crystalline allomorph faction, and allows a rapid evaluation of unknown cellulose samples even in small amounts. IR or NIR band shift is a result of changes in atomic distances. Therefore, a band shift can be observed, for example, when the measurement temperature is changed (Altaner et al., 2014). Given this background, a change in allomorph ratio should not result in a band shift but in a change in absorbance of specific bands. Therefore,

Table 5 Cellulose I_α fractions calculated using the calibration curve based on NIR band shift of samples from different organisms

		Spectral bands (cm^{-1})	I_α fraction
Proteobacteria	*Acetobacter aceti* (AJ-12368)	6449	0.86
Rhodophyta	*Erythrocladia subintegra*	6461	0.56
	Gelidium sesquipedale	6473	0.25
Charophyta	*Micrasterias crux-melitens*	6473[a]	0.25[a]
	Chara australis	6473	0.25
Spermatophyta	*Egeria densa*	6475[a]	0.20[a]
Pinophyta	*Cryptmeria japonica* (Soft wood)	6471	0.31
Magnoliophyta	*Eucalyptus globulus* (Hard wood)	6472	0.28
	Saccharum officinarum (Sugarcane bagasse)	6470	0.33
	Boehmeria nivea (Ramie)	6476	0.18
	Gossypium arboreum (Cotton)	6473	0.25
	BY2 culture cell induced from *Nicotiana tabacum*	6475	0.20
	Nanocellulose from *Cryptmeria japonica*	6476	0.18
Chordata	Microcrystalline cellulose (Avicel)	6475	0.20
	Ciona intestinalis	6474	0.23

The calibration curve was established using samples from different organisms. Each mean value was determined from three measurements

[a] Mean value was determined from two measurement because of difficulties to discriminate the band from each spectrum

specific bands of I_α and I_β are considered to be originally close to one another, and merging of these bands will appear as a shift with changed allomorph ratio.

The other cellulose samples were characterized in terms of crystalline allomorphism using the calibration curve created in this study (Table 5). Bacterial cellulose had a highly I_α type crystalline structure, which is consistent with a previous report (Attala and VanderHart 1984). The two samples from Rhodophyta (*Erythrocladia* and *Gelidium*) were rich in I_α and I_β, which is in agreement with a published report by Koyama et al. (1997). The authors have discussed that the I_α domain in the primitive red algae *Erythrocladia* (Bangiophyceae) may be replaced by I_β in a higher one such as *Gelidium* (Florideophyceae). In the case of Charophyta, it is well-known that *Micrasterias* produces flatter microfibrils (Kim et al. 1996), whereas *Chara* synthesizes smaller ones (Koyama et al. 1997). However, both crystalline types were reported to be of the I_β type (Koyama et al. 1997), which is similar to the results estimated by the standard curve in this study. For higher plants, the calibration model based on NIR spectra demonstrated that their microfibrils are fully composed of I_β allomorph, which is in agreement

with previous reports (Wada et al. 1997) and with the discussion of PCA score plots mentioned above. In addition, the calibration curve indicated that *Ciona* microfibrils are predominantly composed of I_β type crystalline units. Given that tunicates are generally recognized to synthesize I_β type cellulose microfibrils, the results obtained for *Ciona* are as expected.

In this study, using structurally distinguishable cellulose samples, I have designed a distribution map in terms of crystalline features. Though NIR spectra lack sharper bands compared to IR spectroscopy, the combination with multivariate analysis and monitoring of band shifts provided calibration models, which allowed the quantitative estimate of cellulose structural properties. The score plots and calibration models obtained will be useful for understanding the structural characteristics of unclear cellulose samples such as chemically treated or artificially synthesized cellulose microfibrils.

Conclusions

I have demonstrated that the short NIR range of 6600–6200 cm^{-1}, suggested by intra-crystalline

deuteration, involves structural information for relative cellulose crystallinity and allomorphism. PCA of this region revealed the crystalline structure characteristics in a linear arrangement from tunicates to cultured cells, which supports the hypothesis that cellulose microfibrils synthesized by trees are composed of I_β units. Furthermore, PLS regression allowed the quantitative evaluation of cellulose relative crystallinity in a wide range of organisms, from marine algae to cultured cells. Finally, I successfully designed a standard curve to estimate the ratio of I_α and I_β by a simple detection of NIR band shifts. Further investigation of spectral bands combined with its measurement may allow the detection of other crystalline features such as the uniplanar orientation or shape of the microfibril cross section. NIR spectroscopy therefore has the potential for accurate and quantitative assessment of unknown cellulose samples, and provides a better understanding of the relationships among cellulose-synthesizing organisms in terms of crystalline structure.

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