

Direct analysis of cellulose in poplar stem by matrix-assisted laser desorption/ionization imaging mass spectrometry

Seokwon Jung^{1,3}, Yanfeng Chen³, M. Cameron Sullards^{1,3} and Arthur J. Ragauskas^{1,2,3*}

¹BioEnergy Science Center, Georgia Institute of Technology, 500 10th St., Atlanta, GA 30332, USA

²Institute of Paper Science and Technology, Georgia Institute of Technology, 500 10th St., Atlanta, GA 30332, USA

³School of Chemistry and Biochemistry, Georgia Institute of Technology, 901 Atlantic Drive, Atlanta, GA 30332, USA

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Matrix-assisted laser desorption/ionization imaging mass spectrometry (MALDI-IMS) was applied to the analysis of the spatial distribution of cellulose on a cross-section of juvenile poplar (*Populus deltoids*) stems. Microcrystalline cellulose (MCC) was used to optimize matrix (2,5-dihydroxybenzoic acid) application and instrument parameters for the detection of low hexose oligomers, which originated from cellulose in the solid phase. A section of poplar cellulose isolated from juvenile poplar stem which consisted primarily of glucose (~95%) and minor components such as xylose and lignin was used for the MALDI-IMS studies. The mass spectrum of poplar cellulose consisted of a series of evenly spaced signals having a difference of 162 *m/z* units, which was similar to that of MCC in linear and reflectron positive ion modes. MS images of cellulose compounds with sodium ion adducts were generated and illustrated the distribution of cellulose on the surface of the poplar stem. Copyright © 2010 John Wiley & Sons, Ltd.

Cellulose is the most abundant biopolymer in nature and it is widely used due to its versatile application in many areas including the food, paper, and pharmaceutical industries. Recently, cellulose, one of the major components in lignocellulosic bioresources, has been further highlighted as a renewable energy resource since these materials can avoid the conflict over biofuel resources competing with food resources such as corn.¹ These renewable resources can be drawn from dedicated agro-energy crops and from biomass residues originating from both forest and agricultural ecosystems.^{2,3} Natural cellulose consists of glucose units linked by a β -(1 \rightarrow 4)-glycosidic bond and it exists as a linear polysaccharide embedded in hemicelluloses and lignin within the plant cell wall. Various analytical techniques have been used to characterize cellulose and its contribution to biomass recalcitrance, and therefore increase the overall efficiency of converting cellulose into bioethanol. Fourier transform (FT) Raman and infrared (IR) spectroscopy, nuclear magnetic resonance (NMR), and high-performance anion-exchange chromatography (HPAEC) have been widely used for cellulose characterization.^{4–6} However, the direct analysis of native biomass still remains a considerable challenge to the understanding of both the spatial and the temporal distribution of cellulose in plant cell walls during ethanol conversion processes.

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) is a powerful tool to analyze large molecules with high speed and sensitivity. Since MALDI-MS was successfully applied to the structural analysis of native carbohydrates by Harvey *et al.* and Stahl *et al.*, it has been frequently used to analyze carbohydrates.^{7,8} To increase the ionization efficiency of the carbohydrate moiety, many methodologies such as terminal derivatization and metal cationization can be applied.⁹ The versatility of MALDI-MS was extended by Caprioli *et al.* to direct image profiling of the surface of biological tissues.¹⁰ MALDI-imaging mass spectrometry (MALDI-IMS) can be used to determine the spatial distribution and relative abundance of specific molecules on many sample surfaces, and it has been successfully used to investigate sectioned tissue such as human brain and rat organs.^{11,12} Recent studies have reported the application of IMS in native plants as well. For example, Ng *et al.* demonstrated the spatial profiling of phytochemicals using direct analysis by MALDI-IMS in herbal tissue.¹³ MALDI-IMS has also been used to determine the distribution of agrochemicals in plants.^{14,15} Finally, the distribution of water-soluble carbohydrates and metabolites has been investigated in wheat plants by MALDI-IMS.^{16,17}

In this study, we utilized MALDI-IMS to obtain molecular images of native cellulose directly from a sectioned poplar stem. Microcrystalline cellulose (MCC) was used as a reference compound to optimize MALDI-IMS parameters such as matrix application, laser intensity, delay time, and accelerating voltage. The resulting analysis yields a series of intense signals having an inter-peak difference of 162 *m/z*

*Correspondence to: A. J. Ragauskas, BioEnergy Science Center, Georgia Institute of Technology, 500 10th St., Atlanta, GA 30332, USA.

E-mail: Art.Ragauskas@chemistry.gatech.edu

units for the solid-phase MCC in both reflectron and linear positive ion modes. A cross-section of poplar cellulose obtained by acid hydrolysis of poplar holocellulose was examined by bulk carbohydrate and Klason lignin analysis to determine the constituent monosaccharide contents. Subsequently, the cross-section of poplar cellulose was examined under optimal conditions in MALDI-MS, and molecular images of cellulose were directly acquired on the surface of the poplar sample.

EXPERIMENTAL

Materials

All chemicals used in this study, including Avicel PH-101, were purchased either from Aldrich Chemical Co. (Milwaukee, WI, USA) or VWR International (West Chester, PA, USA) and used as received. Juvenile poplar (*Populus deltoides*) stems were harvested between 2007 and 2008 by the National Renewable Energy Laboratory (NREL; Golden, CO, USA). *Populus deltoides* x *nigra* (DN34) clones were grown for 6 months in a greenhouse which maintained 16 h of light per day with 30–60% humidity. The poplar stems were cut and stored at -20°C .

Cryotome section of poplar stem

In a previous study, we reported the procedure for optimal sample preparation techniques using cryotome sections of poplar stems.¹⁸ In brief, a whole poplar stem was sectioned to 50 μm thickness using a Leica CM 3050 S cryostat (Leica Microsystems, Wetzlar, Germany) equipped with a disposable steel blade. To avoid chemical contamination from the embedding medium, the sample was directly attached onto the metal stage instead of being embedded and sectioned. The chamber temperature for the cryotome section was -8°C and the cutting speed was manually controlled. The cross-sections of poplar stem were stored at -20°C as stated previously.

Preparation of poplar cellulose

Extractives in the cross-section of poplar stems were removed by sequential Soxhlet extractions in order to obtain a section of extractive-free poplar based on the Laboratory Analytical Procedures (LAPs) provided by the NREL.¹⁹ Soxhlet extraction was performed for 5 h with the solvent being cycled five times per hour followed by air-drying overnight. The section of extractive-free poplar was then subjected to an oxidative treatment to prepare a section of poplar holocellulose (holocellulose: mixture of cellulose and hemicellulose) by removing lignin.^{20,21} In brief, the section of extractive-free poplar was dispersed into deionized (DI) water (100 mL/g sample) with sodium chlorite (40% by dry weight of sample) and glacial acetic acid (10% by dry weight of sample) at 70°C for 1 h in a sealed plastic pouch (Kapak Corporation, Minneapolis, MN, USA). The procedure was then repeated a further three times. Finally, the solid residue was filtered out and washed thoroughly with DI water. The cross-section of poplar cellulose was acquired from the poplar holocellulose by acid hydrolysis of the hemicellulose with 2.5 N HCl (100 mL/g sample) at 100°C for 4 h.²²

The solid residue (poplar cellulose) was then carefully filtered and washed with DI water. The section of each sample was subsequently stored between glass slides for subsequent MALDI-MS/IMS analysis.

Matrix application

Initially three matrices, 2,5-dihydroxybenzoic acid (DHB), α -cyano-4-hydroxycinnamic acid (CHCA) and sinapinic acid (SA), were prepared to determine which yielded the best signal response for cellulose. The concentrations of DHB, CHCA and SA were each 20 mg/mL in 1:1 acetonitrile/water containing 0.1% trifluoroacetic acid (TFA) (v/v). For MALDI-MS analysis, the section of each sample was ground for 10 min, while vibrating at 15 Hz using a MM200 mixer mill (Retsch Inc., Haan, Germany). After 2 min of vortexing the ground sample (100 mg/mL) in 1:1 acetonitrile/water containing 0.1% TFA (v/v) solution, the suspension was mixed with the matrix solution at a 1:100 (v/v suspension/matrix solution) ratio. The mixture ($\sim 2 \mu\text{L}$) was spotted onto a stainless steel MALDI plate and air-dried. For preparing samples of the sectioned poplar cellulose for MALDI-IMS, an oscillating capillary nebulizer (OCN) matrix application system, previously reported by Chen *et al.*,²³ was employed. The section of poplar cellulose was fixed onto a stainless steel MALDI plate using adhesive tape. The DHB matrix solution was delivered to the OCN sprayer using a syringe pump (KD Scientific, Holliston, MA, USA) at a flow rate of $\sim 60 \mu\text{L}/\text{min}$ and uniformly dispersed through an oscillating capillary (Polymicro Technologies, Phoenix, AZ, USA) with a nitrogen pressure of $\sim 345 \text{ kPa}$ for 20 min.

MALDI-MS and -IMS analysis

MALDI-MS was performed using a Voyager DE STR MALDI-TOF mass spectrometer (Applied Biosystems, Framingham, MA, USA) equipped with a 337 nm N_2 laser. The data was acquired under delayed extraction conditions in both reflectron and linear positive ion modes using external mass calibration. Positive ion MALDI-MS data was acquired in both linear and reflectron modes at an accelerating voltage of 12 and 20 kV, respectively, with an 85% grid ratio and a delay time of 400 ns. The laser power was set to a minimum for ionization (2900 arbitrary units (AU) for reflectron mode and 3300 AU for linear mode), and then increased by 100–200 units for medium and high laser intensity. The spectra were accumulated for 100 laser shots in a sample spot.

MALDI-IMS data were acquired at an accelerating voltage of 12 kV with optimal laser power (3100 AU) in linear positive ion mode. Raster scans were performed automatically on the sample surface with 12 shots for each spot. MALDI-IMS data was processed using the data acquisition software (MALDI MS Imaging Tool, MMSIT; Novartis Pharma AG, Basel, Switzerland; a free download program²⁴) over the sectioned poplar cellulose. Mass spectra were processed for baseline correction and normalization using Data Explorer 4.0 software, which was supplied with the mass spectrometer. The MS images were reconstituted using Biomap software (Novartis Pharma AG).

Carbohydrate and acid-insoluble lignin (Klason lignin) analysis

Samples for determining constituent monosaccharide and acid-insoluble lignin (Klason lignin) contents were prepared using a two-stage acid hydrolysis protocol based on the LAPs provided by the NREL.²⁵ In brief, primary hydrolysis of sample (175 mg) was performed with 72% (w/w) H₂SO₄ (1.5 mL) for 1 h at 30°C. The hydrolysates were diluted to 4% (w/w) H₂SO₄ with DI water and a second hydrolysis was performed at 121°C for 1 h in an autoclave (Tuttnauer Corp., Hauppauge, NY, USA). The resulting solution was cooled to room temperature and filtered using a G8 glass fiber filter (Fisher Scientific, Pittsburgh, PA, USA). The remaining residue, which is considered to be acid-insoluble (Klason) lignin, was oven-dried and weighed to obtain the Klason lignin content. The filtrate was analyzed for constituent monosaccharide content by anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) using a Dionex ICS-3000 system (Dionex Corp., Sunnyvale, CA, USA) and a CarboPac[®] PA-1 column (Dionex Corp.).⁶ Monosaccharides were eluted with DI water (0–20 min) with a gradient to 200 mM NaOH (20–55 min) and holding the concentration for 15 min at a flow rate of 0.3 mL/min. The column temperature was maintained at 23°C.

RESULTS AND DISCUSSION

Carbohydrates and Klason lignin analysis

Prior to MALDI-MS analysis, carbohydrate and Klason lignin analyses were performed on the sectioned samples; extractive-free poplar, poplar holocellulose, and poplar cellulose. The bulk chemical composition of each sample was determined using the relative monosaccharide and Klason lignin contents, as summarized in Fig. 1. Most of the Klason lignin in the section of the extractive-free poplar was removed by the holocellulose pulping treatment, whereas

the relative monosaccharide content of glucose and xylose in the section of poplar holocellulose was increased by 71% and 21%, respectively. The section of poplar cellulose obtained by removing most of the hemicellulose from the poplar holocellulose was composed of ~95% glucose and ~5% residual hemicelluloses and Klason lignin.

MALDI-MS analysis of cellulose

The ionization efficiency of insoluble polysaccharide (e.g. cellulose) in MALDI-MS is relatively low because of a lack of readily ionizable functional groups in the polysaccharide and the structural rigidity of the cellulose.²⁶ The experimental parameters such as matrix application, laser intensity, delay time, and accelerating voltage for MALDI-MS must therefore be optimized in order to detect insoluble polysaccharides. Microcrystalline cellulose (MCC), known as Avicel[®], was used as a model compound to evaluate the MALDI-MS conditions due to its structural similarity to natural cellulose. MCC is composed of glucose units connected by a β -(1 \rightarrow 4)-glycosidic bond with a high degree of crystallinity like natural cellulose. As a result, MCC and natural cellulose are both linear chain polymers containing large numbers of hydroxyl groups which make an internal hydrogen bonding network. The molecular size, in terms of degree of polymerization (DP) defined as the number of anhydroglucose units present in a single chain, is one difference between MCC and natural cellulose. The DP value of the MCC (150–300) is lower than that of wood plant fibers and other plant sources (800–10 000).²⁷

The resulting mass spectrum from solid-phase MCC with DHB matrix in reflectron positive ion mode reveals a series of intense ions (Fig. 2(a)). The mass intervals between adjacent signals were 162 *m/z* units, which are derived from the glycosyl unit (C₆H₁₀O₅) calculated to be 162.058 Da. The intense ions only appeared under certain instrumental parameters such as an accelerating voltage of 25 kV with a 85% grid ratio and laser power of 2900 AU when DHB matrix

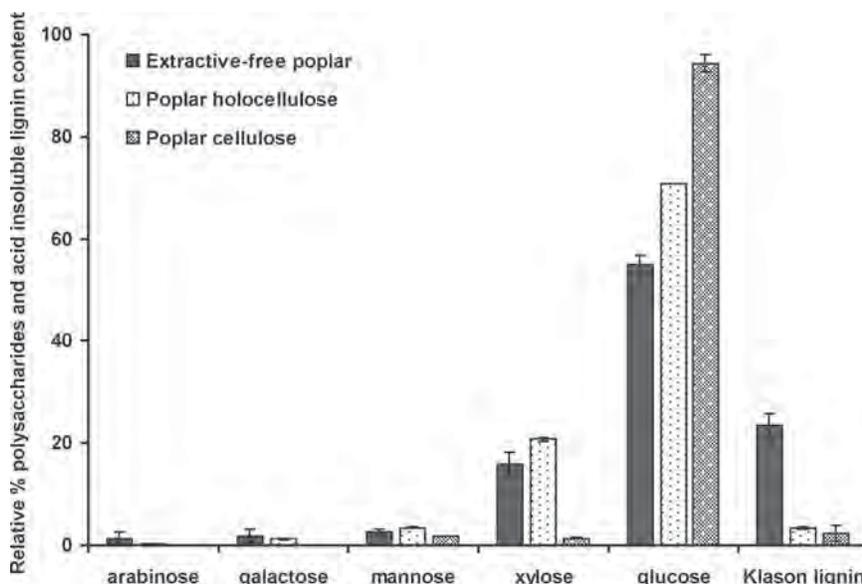


Figure 1. Monosaccharides and Klason lignin content in the treated sectioned poplar stems: extractive-free poplar, poplar holocellulose, and poplar cellulose.

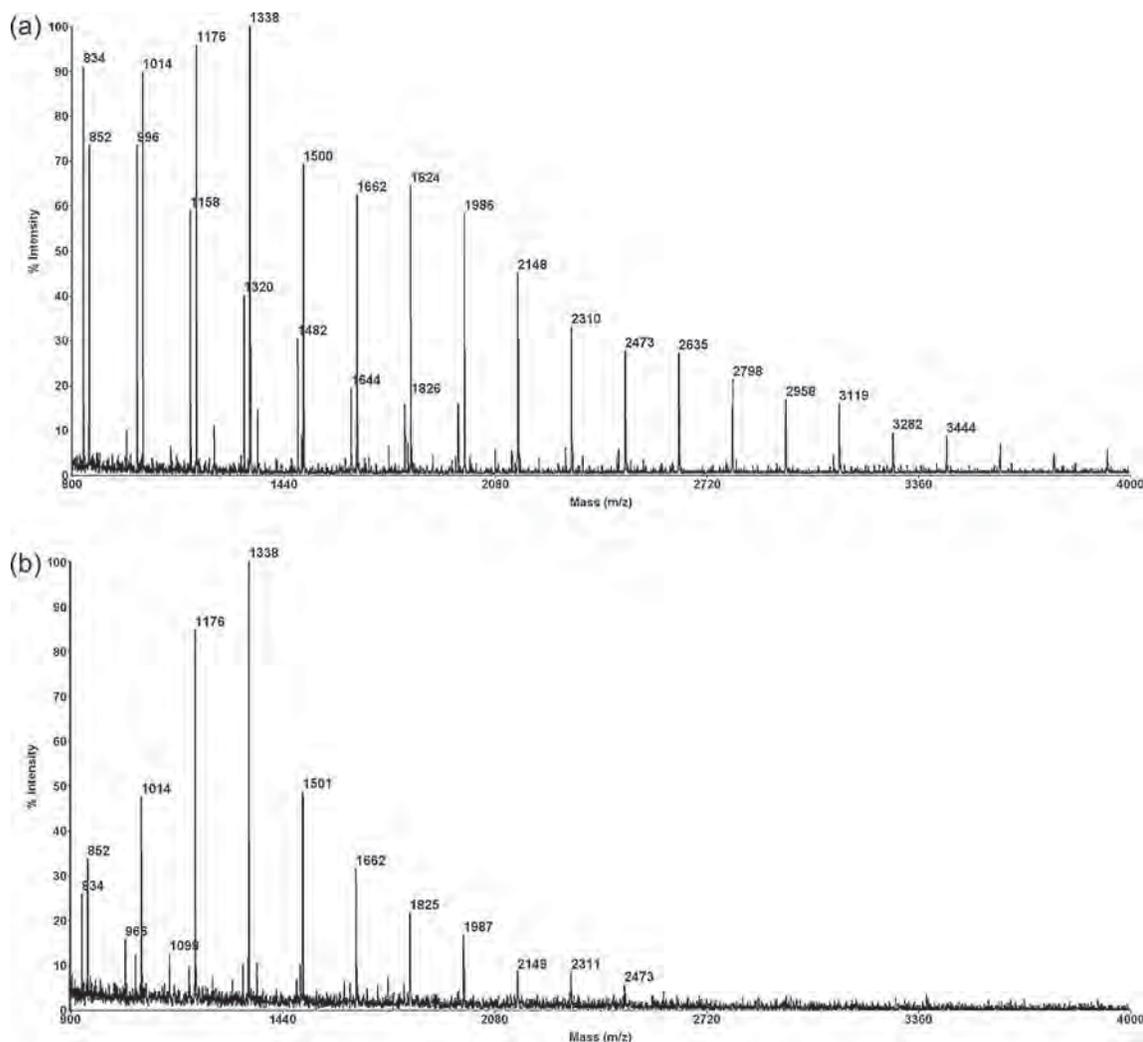


Figure 2. Positive ion MALDI mass spectra of (a) MCC and (b) the ground poplar cellulose from the sectioned poplar stems.

was used. In the evaluation of other common MALDI matrices (e.g. CHCA and SA) these ions were barely observed. The series of oligo- and poly-glucose ions in MCC were observed up to m/z 4000 although the intensities of the ions dropped off with increasing m/z (Fig. 2(a)). MCC produced the highest intensity of the oligo-glucose ion at m/z 1338, which corresponds to eight glucose units (DP 8) with sodium ion adducts in reflectron mode. The series of intense ions (e.g. m/z 1338) below m/z 2000 were accompanied by minor ions (e.g. m/z 1320) at 18 m/z units lower, probably corresponding to dehydration of the major ions. This is consistent with MALDI-MS results from potato and wheat starch debranched by isoamylase, which exhibited intense ions at intervals of 162 m/z units with sodium ion adducts and/or the loss of water.²⁸ The optimal MALDI conditions were also applied to the sectioned samples: extractive-free poplar, poplar holocellulose, and poplar cellulose. Prior to MALDI-MS analysis each sectioned sample was finely ground as small as the particle size of MCC in order to maintain the same level of experimental environment as for MCC. The mass spectrum of the ground poplar cellulose showed intense ions derived from oligo- and poly-glucoses

up to m/z 2500 (Fig. 2(b)). In terms of DP, intense ions up to DP 15 were detected in the ground poplar cellulose, whereas MCC exhibited ions up to DP 25 (Fig. 2). It was also observed that the intensities of ions in the ground poplar cellulose rapidly decreased above m/z 1338 (DP 8), whereas the signal intensities of the ions in MCC decreased more slowly up to m/z 4000. One possible explanation is that the small molecular size (\sim DP 300) of the cellulose in MCC relative to the large molecular size (\sim DP 3500) of the cellulose in ground poplar cellulose may affect the detection of intense ions. The mass spectra of the other ground samples (extractive-free poplar and poplar holocellulose) produced only very weak ion signals. It was assumed that the matrix structure of the biopolymers (e.g. cellulose/hemicellulose/lignins or cellulose/hemicellulose) in the cell wall may cause the low ionization efficiency observed in these MALDI-MS analyses. The linear positive ion mode with the same matrix system (2,5-DHB) was also evaluated for MCC and ground poplar cellulose. Here a lower accelerating voltage (12 kV) and higher laser power (3500 AU) proved optimal, resulting in higher intensity signals than in the reflectron mode. Intense ions corresponding to dehydration in MCC and

ground poplar cellulose were also detected in linear mode (Fig. S1, see Supporting Information).²⁹

Imaging MALDI-MS (IMS)

A section of poplar cellulose was coated with matrix using an oscillating capillary nebulizer (OCN) system, which can generate good matrix homogeneity on the sample surface.²³ The sample was evaluated in both linear and reflectron positive ion modes in order to find the best parameters for MALDI-MS (Fig. 3). The resulting mass spectra in reflection mode provided high-resolution data from the surface of the poplar cellulose. However, signals were only detected on a few spots across the sample surface due to low cellulose ionization yield (Fig. 3(a)). Interestingly, the sectioned poplar cellulose produced the highest intensity ion at m/z 1824, which was a higher mass than was obtained for the ground poplar cellulose at m/z 1338. This observation could result from a change in the cellulose structure by the mechanical milling process (e.g. ground poplar cellulose). The effect of mechanical milling on the cellulose structure was examined by Zhang *et al.*³⁰ and they reported a reduction in the

crystalline index along with thermal stability, indicating a disruption of inter/intra hydrogen bonding in cellulose. Compared with the reflectron mode, the linear positive mode provided higher intensity ions over the sample surface although the resolution was lower (Fig. 3(b)). In order to generate better ion images of the spatial distribution of cellulose compounds, we chose the linear positive ion mode. A low number of laser shots were employed to maintain the intense signals since higher laser power rapidly depleted analyte and matrix at a given spot. Consequently, a number of oligo- and poly-glucose ions were detected up to m/z 4000 over the sample surface (Fig. 3(b)).

The optical image of the sectioned poplar cellulose is shown in Fig. 4(a) for a cross-comparison with the MALDI-MS images. Numerous cell walls on the sectioned poplar cellulose exhibited inner cracks (arrowhead) generated during the drying process after acid hydrolysis. The positions of the inner cracks were used as standard points of alignment for spatial correlation between the optical image and the MALDI-MS images because the blanks could appear as a dark color in MALDI-IMS. MALDI-MS images were

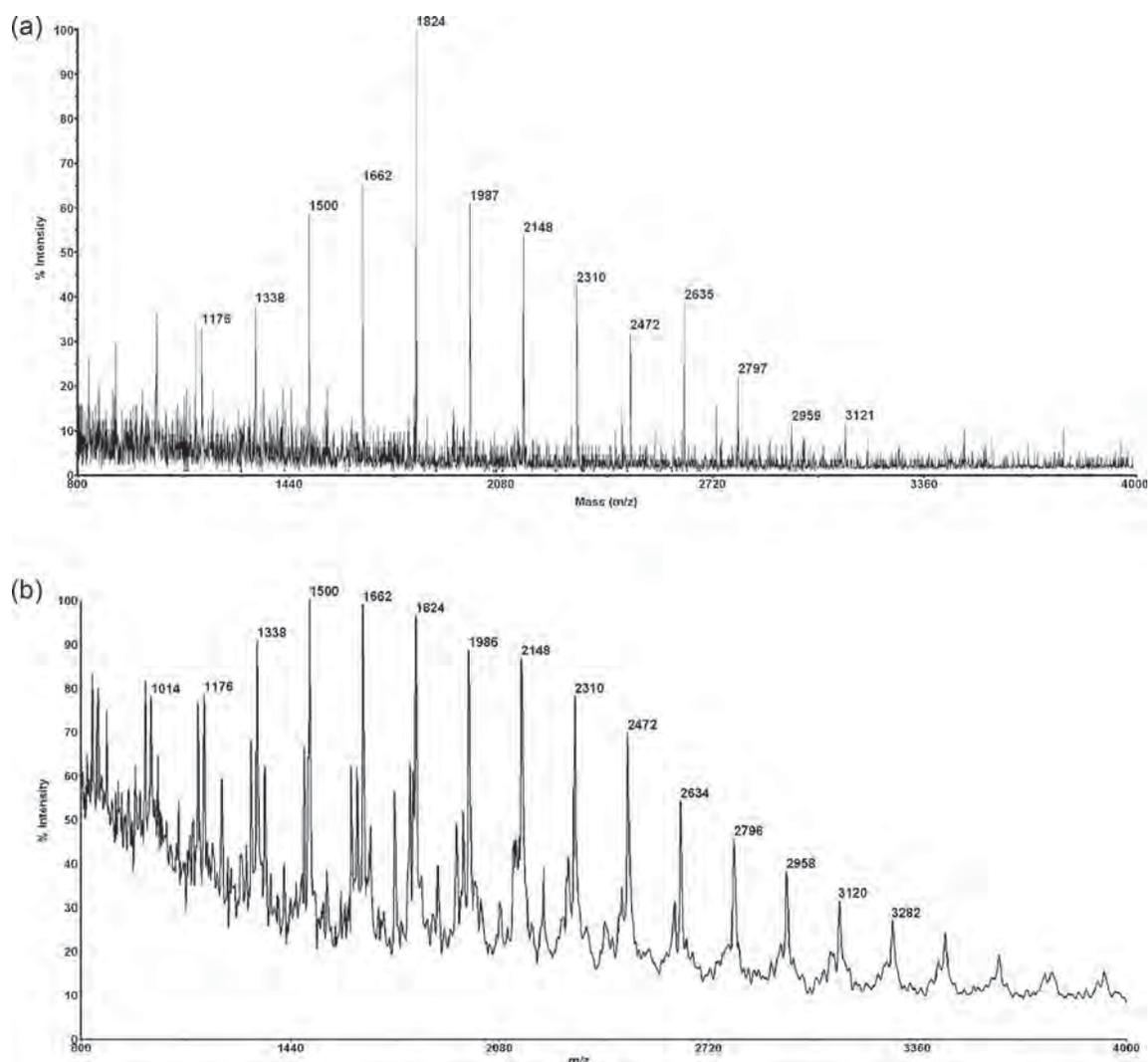


Figure 3. Positive ion MALDI mass spectra from the surface of sectioned poplar cellulose in: (a) reflectron mode and (b) linear mode.

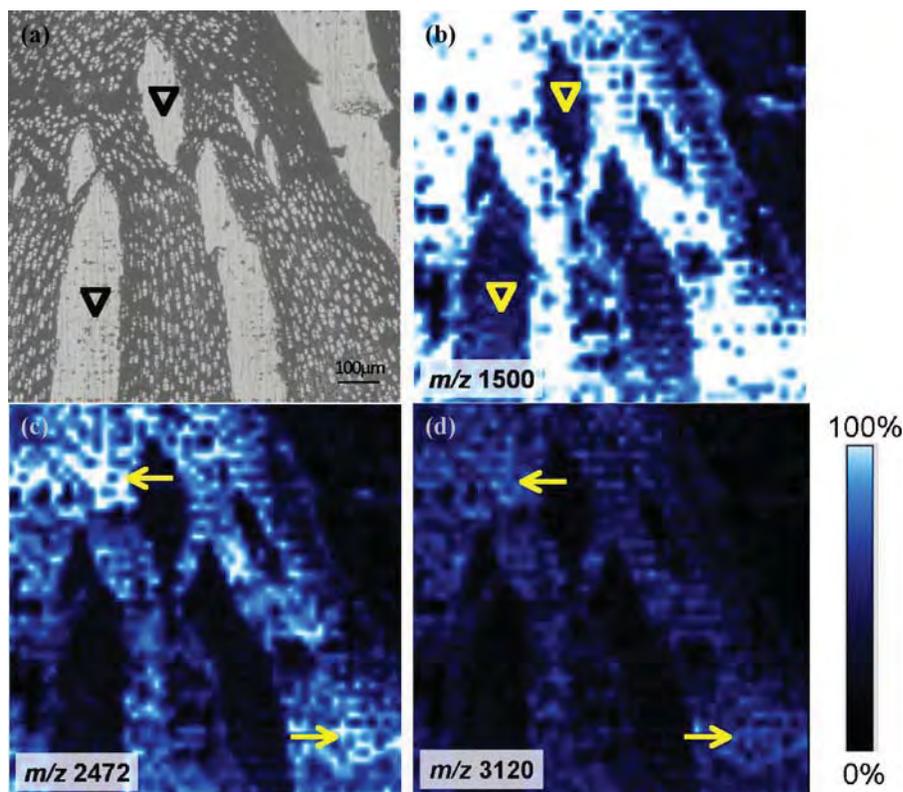


Figure 4. Images of the sectioned poplar cellulose: (a) optical image of sectioned sample coated with 2,5-DHB and MS images of (b) m/z 1500, (c) m/z 2472, and (d) m/z 3120. Scale bar = 100 μm . Color bar indicates the intensity levels. Typical metal surface is marked as an arrowhead; high signal intensity area is marked with an arrow.

generated from selected ions (m/z 1500, 2472 and 3120) corresponding to DP 9, 15 and 19 of oligo- and poly-glucose ions, respectively (Figs. 4(b)–4(d)). MALDI-MS images of the sectioned sample clearly showed the spatial distribution of cellulose compounds across the cell-wall surface while inner cracks were observed to be dark in color because of the lack of cellulose (arrowhead). The image generated from m/z 1500 (Fig. 4(b)) showed a considerably brighter color (higher intensity) along the cell walls. The border areas between cell wall and empty space were shown as a less intense color than the middle of the cell-wall areas. Decreasing brightness at m/z 2472 and 3120 in the MS images (Figs. 4(c) and 4(d)) clearly illustrates the reduction in the relative intensities of up to 30% and 70%, respectively, compared with that at m/z 1500 (Fig. 4(b)). Interestingly, the MS image of the cell walls at a particular m/z value represented different signal intensities over the surface. For example, more intense areas (brighter color) in the MS image at m/z 2472 were localized in the middle of the cell wall area (arrows) than for other cell wall areas. This same pattern was also observed in the MS image of m/z 3120. One possible explanation for this is that different molecular weight cellulose is distributed on the surface of cell walls and the relative DP of the cellulose affects its ionization efficiency in MALDI-MS, as observed in the MCC spectrum relative to the spectrum of the ground poplar cellulose (Fig. 2).

CONCLUSIONS

MCC and poplar cellulose were directly analyzed using MALDI-MS. Molecular ions generated from cellulose with various DP values were clearly observed in the resulting mass spectra. Lignin and hemicelluloses were observed to greatly reduce the detection of cellulose signals in MALDI-MS due to biological matrix effects. The optimization of experimental protocols for MALDI-MS was used to guide MALDI-IMS in order to generate glucose oligomer images in the section of poplar cellulose. The subsequently gathered molecular images revealed the spatial distribution of the different sizes of oligo- and poly-glucose ions which originated from the cellulose in the sectioned poplar stem.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article.

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