Appl Phys A (2013) 110:361–369
DOI 10.1007/s00339-012-7123-z

Applied Physics A
Materials Science & Processing

A novel sample preparation method to avoid influence of embedding medium during nano-indentation

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Received: 9 February 2012 / Accepted: 8 August 2012 / Published online: 25 August 2012

Abstract The effect of the embedding medium on the nano-indentation measurements of lignocellulosic materials was investigated experimentally using nano-indentation. Both the reduced elastic modulus and the hardness of non-embedded cell walls were found to be lower than those of the embedded samples, proving that the embedding medium used for specimen preparation on cellulosic material during nano-indentation can modify cell-wall properties. This leads to structural and chemical changes in the cell-wall constituents, changes that may significantly alter the material properties. Further investigation was carried out to detect the influence of different vacuum times on the cell-wall mechanical properties during the embedding procedure. Interpretation of the statistical analysis revealed no linear relationships between vacuum time and the mechanical properties of cell walls. The quantitative measurements confirm that low-viscosity resin has a rapid penetration rate early in the curing process. Finally, a novel sample preparation method aimed at preventing resin diffusion into lignocellulosic cell walls was developed using a plastic film to wrap the sample before embedding. This method proved to be accessible and straightforward for many kinds of lignocellulosic material, but is especially suitable for small, soft samples.

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1 Introduction

As a tool to explore the mechanical properties and microstructural features of small-scale or small-volume samples, nano-indentation is becoming popular in biological material research and has been successfully used to probe the small-scale mechanical properties of many biomaterials, among them bone [1, 2], tooth [3], soft tissue [4], crop stalks [5], wood [6], bamboo [7], Lyocell fiber [8], lignocelluloses [9] and others [10].

Performing nano-indentation on cell walls is obviously quite challenging. The high spatial resolution of nano-indentation means that every small roughness in the sample will be picked up; thus, the problem of rough surfaces causing false surface detection and inaccurate estimation of the contact area, yielding results with large and unacceptable errors, is a critical one to overcome. Therefore, a smooth sample surface with little roughness is crucial for nano-indentation, especially for shallow indentations [11]. In order to get a sample surface smooth enough to perform indentation, most biomass materials [1, 6, 8, 9, 11–14] need an embedding medium, typically Spurr's epoxy resin, for sample preparation; this preparation aims to support the cell wall during both microtoming [15] and performance of the indentation. However, the reliability and accuracy of the values obtained has been questioned due to the possible influence of the embedding medium. There are some caveats regarding this issue. First, previous research has not paid

sufficient attention to the embedding medium's penetration. Thus, any effects of the resin as an additive may have been missed [16]. Second, most biomaterials have complicated structures, especially for cellulose material, whose porosity, multiple layers and arrangement of microfibrils require further consideration in research on sample preparation [17].

Some previous studies have focused on the influence of adhesive penetration in wood [18]. As wood is a naturally porous and anisotropic material, considerable research efforts are being directed at studying what effects added adhesives have on the mechanical properties of wood cell walls [19]. Adhesive penetration has been agreed to affect cell-walls' mechanical properties at wood-bond lines [20–22], and it has been demonstrated that wood cell walls penetrated by phenol resorcinol–formaldehyde resin, urea–formaldehyde or melamine–urea–formaldehyde all had higher mechanical properties at the microscale [23]. Thus, based on previous studies, there is a suspicion that an embedding medium such as epoxy has the potential to modify the mechanical properties of cell walls. Even though few studies have explored the potential effects of sample preparation methods on nano-indentation results, some studies have already noticed the influence of the embedding medium when target samples were embedded to facilitate sample preparation and the nano-indentation test [13, 24]. Konnerth et al. mentioned that the epoxy resin for nano-indentation may affect measurements [24]. Jakes et al. adopted a surface preparation procedure without resin because of the concern about undesired chemical modifications being caused by the epoxy [25]. Studies of bone samples reported an increase of modulus values after the samples were embedded in polymethyl methacrylate (PMMA) [2]. Other research has shown that the penetration of epoxy resin into plant cell walls will increase the stiffness of fiber composites [26].

Although Jakes et al.'s method for preparing wood samples successfully avoids the effect of the embedding medium during nano-indentation, it is not useful for tiny or soft samples like fibers, wood chips and herb plant materials. Thus, the goal of this research is to develop a novel sample preparation method to avoid embedding resin penetration into the tested material. The new method should have wide application to all kinds of material of sizes ranging from single fibers to large wood blocks. Specifically, the effect of the embedding medium on the small scale mechanical property measurement of wood cell walls (as a representative woody biomass material) is investigated. In this research paper, we investigate in detail epoxy resin penetration as affected by the vacuum and curing time in order to explore its influence on the data when resin penetration is used. We propose a new sample preparation method that is reliable and efficient in avoiding embedding medium influence. This may result in the development of an efficient testing method for getting accurate measurements from nano-indentation.

2 Materials and methods

2.1 Material collection

Loblolly pine (*Pinus taeda L.*) and Red oak (*Quercus rubra*) were collected from a traditional plantation located in Crossett, Arkansas, USA. A Loblolly pine disk was cut 0.3 m above the ground. To make a wood block for testing without resin embedding, the 32nd annual ring was cut into a block with dimensions of 8 mm × 8 mm × 15 mm in the radial, tangential, and longitudinal directions, respectively [9]. For the wood sample embedded in epoxy, the latewood portion of the 32nd annual ring with a microfibril angle value of 31° was chosen and cut to a small block with dimensions of 1 mm × 1 mm × 5 mm radially, tangentially and longitudinally. Similarly, a Red oak disk was cut 0.5 m above the ground from a tree. The 38th annual ring was cut into blocks of the same two sets of dimensions as mentioned above. Late wood cells with a microfibril angle of 11.9° were chosen as the target location. The Sweetgum (*Liquidambar formosana*) with its 5th annual ring was taken from property at Oak Ridge National Laboratory, located in Oak Ridge, Tennessee, USA. The Silvergrass (*Miscanthus sacchariflorus*, family: Poaceae) was taken from Poyang Lake, Jiangsu, China. Samples were selected from the lower part of the stalk. Reed (*Phragmites australis*) samples were collected from Suqian, Jiangsu, China. Both Silvergrass and Reed are grass-like plants whose textures are soft and thin.

2.2 Conventional sample preparation methods

2.2.1 Small wood block embedded in epoxy resin

Latewood parts of the Loblolly pine, Red oak, Silvergrass, Sweetgum and Reed were cut into small pieces and then dehydrated at 40 °C for 30 min. The samples were successively embedded in Spurr's resin, which is a low-viscosity epoxy resin and one of the most easily prepared embedding materials for research on biomaterial microstructure [15]. The embedded specimens were treated in a vacuum-pressure system to remove air bubbles from the cell lumens, and the resin was then diffused into the specimens. Later, curing was conducted by putting the specimens in an oven for over eight hours at a consistent temperature of 70 °C. The cured specimens were mounted on holders ready for ultramicrotomy. Trapezoid-shaped samples from the epoxy-embedded specimens were cut, and tiny flat surfaces were prepared with a glass knife and were refined by means of a diamond knife. Figure 1a, b and c show the procedure of sample preparation in detail. Finally, a smooth surface with roughness less than 5 nm was obtained for nano-indentation.

Specimens with different vacuum treatment times were also investigated by nano-indentation. Loblolly pine specimens with the same growth ring and microfibril angles were

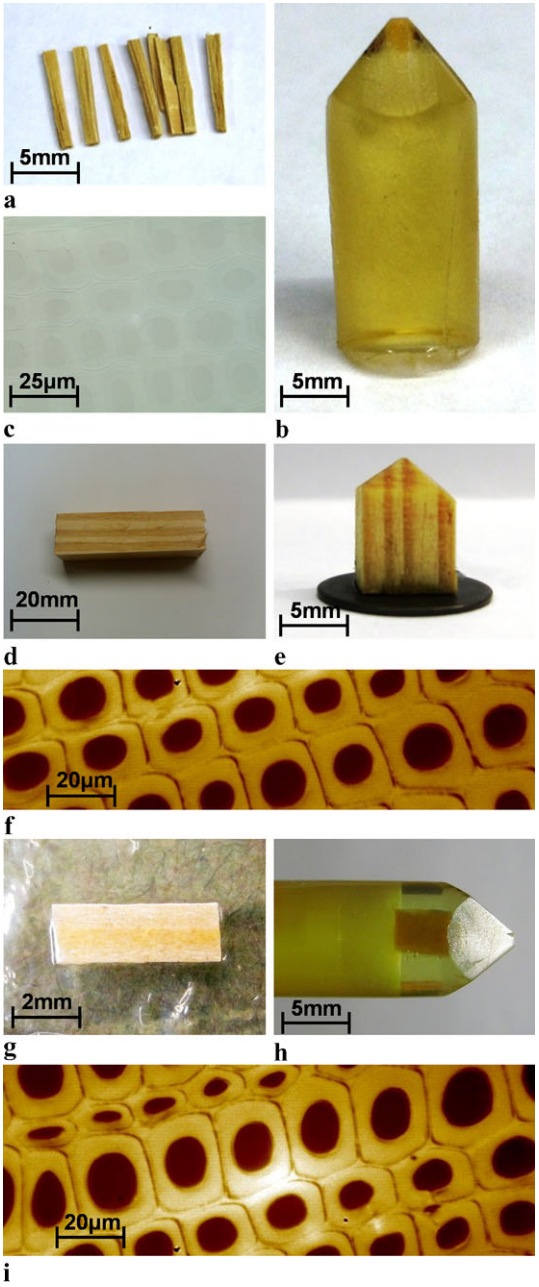

Fig. 1 Specimen preparation procedure for wood block embedded in epoxy resin, wood block without epoxy resin and wood block wrapped in thin film. (**a**) Loblolly pine small wood block; (**b**) Loblolly pine small wood block embedded in epoxy resin; (**c**) microscopic picture of embedded Loblolly pine cell wall; (**d**) Red oak block; (**e**) Red oak block with four sided pyramid shape; (**f**) interference microscopic picture of un-embedded Red oak cell wall; (**g**) Loblolly pine small wood block wrapped in plastic film; (**h**) Loblolly pine small wood block with wrapped film embedded in epoxy resin; (**i**) interference microscopic picture of Loblolly pine cell wall wrapped in thin film

treated at six different vacuum times (0 min, 10 min, 20 min, 30 min, 50 min and 120 min). All cell walls were tested in transverse section.

2.2.2 Wood blocks without embedding

Wood samples were cut into blocks with dimensions of 8 mm × 8 mm × 10 mm. A parallel cut was made to ensure that the bottom and top were parallel to each other and strictly conformed to the cell-wall's longitudinal axis. A gently sloping (30°) apex was created using a microtome on the transverse surface of the cube with the apex positioned in the latewood band. Figure 1d, e and f show the detailed preparation procedure. Additional details are given in Jakes et al.'s research [25]. This method provides a way to get rid of epoxy resin but requires the sample to be integral and strong so that it can support itself during the ultramicrotomy. The ultramicrotome equipped with a glass knife was chosen to cut the apex to make a small flat surface. Finally, a diamond knife was used to cut the apex, generating a smooth surface area of 1 mm^2. A wood block glued on the metal disk was ready for indentation or atomic force microscopy test when an intact cell-wall structure and a shiny surface were obtained when viewed under an optical microscope.

2.3 New sample preparation method for small wood blocks without resin penetration

We developed a novel method for sample preparation applicable for small wood chips or tiny fibers without epoxy resin diffusion. The procedure involves plastic sealing and embedding. Specimens were cut into small pieces and wrapped with high-density polyethylene (HDPE) film (FoodSaver®) as shown in Fig. 1g. A small wood block was placed between two films and quickly hot pressed by an electric iron at 160 °C. Then, the pre-sealed samples were embedded in epoxy resin using the aforementioned procedure; see Fig. 1h. As Konnerth et al.'s research mentioned [17], misalignment between the longitudinal cell axis and the indentation direction will introduce a test value bias caused by the artificial change of the microfibril angle. The sample obtained using the new preparation method with the pre-sealed thin film has the advantage of being easy to mount into an embedding mold in a direction strictly parallel to the longitudinal axis of the wood cell wall. The sample was mounted into a metal holder designed for ultramicrotomy. Figure 1i illustrates the interference microscopic image of the cell walls after ultramicrotomy. Smooth and intact cell walls with empty lumens were obtained.

2.4 Nano-indentation

Nano-indentation tests were performed using a TriboIndenter (Hysitron Inc. Minneapolis, MN) in conjunction with scanning probe microscopy, at a room temperature of 20 °C and a relative humidity of 25 ± 4 %. All the samples were

put into the TriboIndenter chamber at least 24 h before performing indentations to minimize the effect of thermal expansion or contraction of the samples during the indentation test [27].

On the basis of the theory of nano-indentation, the reduced modulus, E_r (the composite modulus for indenter and sample combination), can be evaluated from the nano-indentation measurements by employing the following Oliver and Pharr equation [28]:

$$E_r = \frac{\sqrt{\pi}(dP/dh)_{\text{unloading}}}{2\beta\sqrt{A_c}} \quad (1)$$

The Meyer hardness (H) can be obtained from the following equation:

$$H = \frac{P_{\max}}{A_c} = \frac{P_{\max}}{24.5h_c^2} \quad (2)$$

where P is the indentation load; h and h_c are the penetration and contact depths, respectively; β is a constant that depends on the geometry of the indenter ($\beta = 1.034$ for a Berkovich indenter) and A_c is the projected contact area, which is a function of the contact depth.

2.4.1 Morphological analysis

Images of all residual indents on the wood cell wall after indentation were acquired on an atomic force microscope (AFM, XE-100, PSIA Corp., Sang-Daewon-dong, Korea) operating in contact mode. With a rigid mounting in the AFM stage, images were collected at a scan rate of 0.5 Hz and a set point of 1.0 μN.

2.4.2 Measurements of hardness and reduced elastic modulus on lignocellulosic plant cell wall by nano-indentation

A TriboIndenter® system manufactured by Hysitron, Inc. was used for all the indentation tests. A Berkovich indenter, a three-sided pyramid with a known area-to-depth function, was loaded for all experiments [28]. All experiments were conducted with a closed-loop feedback control aimed at providing precise control of the nano-indentation probe in load-controlled modes. A drift monitor time of 40 s was set up for measuring the drift of the system before any tests. The single indentation procedure included four parts: first, there was a 2-μN set point force between the probe and the sample surface. The indentation test did not start until the 2-μN proload force was detected by the transducer. Second, the peak load was achieved at a loading rate of 30 μN/s. Third, at this peak load, the loading was held for 5 s to avoid the effect of creep occurring in viscous material during the unloading part [29]. Finally, the unloading was executed at the same loading rate as the loading segment. The scanning probe microscopy (SPM) assemblage in the TriboIndenter system is capable of accurately positioning the S_2 layer of wood cell walls. With the scanning size of 40 μm × 40 μm, interesting indent positions were marked on the SPM image and indentations were implemented and checked by rescanning. Only indentations in the middle of the cell-walls' S_2 layer were selected. Indentations performed in the embedding epoxy or at the border of cell walls were all expunged.

2.5 Scanning electron microscope and energy dispersive X-ray spectroscopy

Both embedded and non-embedded wood cell walls were characterized using a scanning electron microscope (SEM, S-3500, Hitachi Instruments Inc., Tokyo, Japan). Energy dispersive X-ray spectroscopy (EDS) was used for the investigation of surface chemical microanalysis. This technology is based on the interaction of the primary beam with atoms in the sample, which causes shell transitions and results in the emission of an X-ray [30].

3 Results and discussion

3.1 Influence of embedding medium on the reduced elastic modulus of lignocellulosic plant cell wall

3.1.1 Effect of epoxy resin on the reduced elastic modulus of lignocellulosic plant cell wall

Figure 2 shows the results of reduced elastic modulus in un-embedded and embedded Loblolly pine. The mean value of reduced elastic modulus of un-embedded cell walls was 13.4 ± 1.5 GPa, while it was 15.3 ± 1.9 GPa for embedded cell walls. The data obtained from Loblolly pine embedded in epoxy resin were significantly increased compared to the reference un-embedded cells (Wilcoxon rank sum test $p = 0.000$). The research supports the finding of Tze et al. [9], in which the reduced modulus was found to be 12.8 GPa with a 15 % coefficient of variation (note that the relative humidity was 25 % in this research and 60 % in Tze et al.'s experiment, leading to the increase of both hardness and elastic modulus). Clearly, the moisture content of wood cell walls is an important factor that dominates the mechanical properties. Differences between the parameters of the setup between the two facilities, such as upper fit and lower fit of the unloading curve, may be another explanation.

Similarly, the reduced elastic modulus of un-embedded Red oak was 17.9 ± 2.7 GPa, compared to that of embedded Red oak, which was 20.7 ± 1.4 GPa. The data from the epoxy resin-embedded sample lie in the same range as the findings of Wu et al. [12], 22.6 ± 1.5 GPa, considering the testing environment and individual difference among samples, such as microfibril angles and cell-wall density.

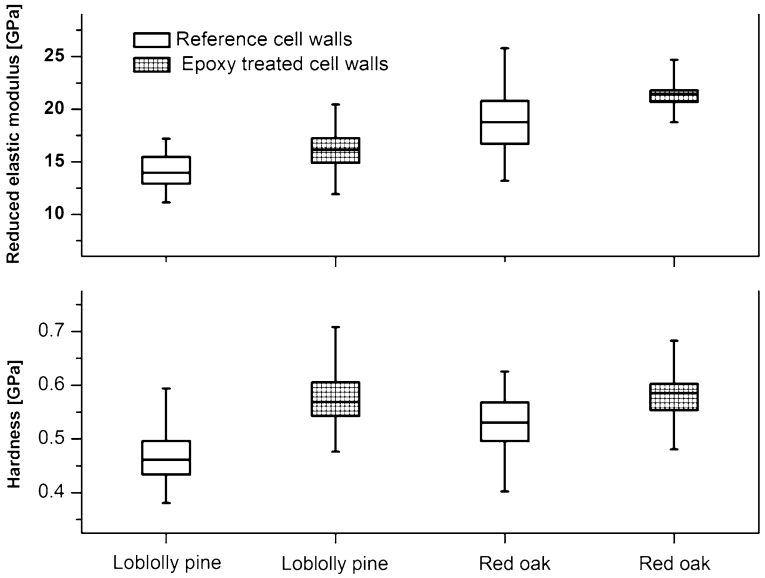

Fig. 2 Comparison between embedded and un-embedded samples of reduced elastic modulus and hardness for Loblolly pine and Red oak by nano-indentation

The effect of the embedding medium on wood cell-wall properties was highly significant (Wilcoxon rank sum test $p = 0.000$). Thus, we reject the null hypothesis and conclude that there is a significant difference between embedded and un-embedded Loblolly pine cell walls based on the measurement of the reduced elastic modulus. Specifically, the value of the reduced elastic modulus for Loblolly pine treated by epoxy resin increased by 14.2 % and the value of the reduced elastic modulus for Red oak treated by epoxy resin increased by 15.6 %. It is important to note that Red oak has higher microfibril angle and density than that of Loblolly pine. Previous research in our group has demonstrated that the orientation of stiff cellulose microfibrils in the cell walls and the density are two important factors that dominate the reduced elastic modulus [12]. Our results are in good agreement with the conclusion. The penetration of epoxy resin is believed to increase the value of the reduced elastic modulus, while the penetration amount is determined by the space in between microfibrils. The reduced elastic modulus of Red oak cell walls experienced a higher percentage increase than that of Loblolly pine. Thus, we may speculate that there is more space between the microfibrils inside Red oak cell walls than there is between those of Loblolly pine. Another hypothesis could be that it has to do with the support of epoxy resin in the cell lumen. The cell wall is less easily damaged or collapsed with the support of epoxy resin in the lumen.

3.1.2 Effect of vacuum time on the reduced elastic modulus of wood cell wall

To analyze all the data taken from indentations at different vacuum times, complete randomize design (CRD) with replication and repeated measurements were taken based on the data obtained. The definitions were based on the assumption that each sample is homogeneous, considering the individual differences among different cell walls. Previous experiments [9] performed on five adjacent tracheid walls and the analyses detected no significant differences in either hardness or modulus values among five tracheid walls of the same growth ring. Wimmer et al.'s results also confirmed this [14]. Linear regression was used to determine whether a relationship existed between the reduced elastic modulus and the vacuum time; data were collected and grouped by individual cell wall. All the indentations were performed under a relative humidity of 25 % and a temperature of 20 °C. Table 1 shows a series of mean values of the reduced elastic modulus and hardness of wood cell-walls' S_2 layers, obtained by single indentation with 150-μN peak load. Basically, the pooled data conform to a normal distribution.

The relationship between vacuum time and reduced elastic modulus was studied, revealing that the reduced elastic modulus of wood cell wall is nearly constant at different vacuum times ranging from 0 min to 120 min. Even though there was a negative regression coefficient value, there is no evidence that the vacuum time during the embedding procedure had a significant impact on the reduced elastic modulus ($p = 0.8878$, $R^2 = 0.035$). This implies that it is workable to make a successful infiltration in a short time by putting specimens directly into 100 % embedding medium [15]. The result also implies that the process of the infiltration of the embedding medium is quite rapid and is not affected by the vacuum time for small pieces, although the vacuum procedure will help and accelerate the diffusion. The low viscosity of Spurr's resin favored the fast infiltration into the tracheid cell wall.

Table 1 Reduced elastic modulus and hardness of Loblolly pine cell wall under different vacuum times

Vacuum time (min)	Number of valid data	Reduced elastic modulus (GPa)	Hardness (GPa)
0	47	17.9 (4.7)	0.7 (3.4)
10	46	16.8 (5.2)	0.6 (3.6)
20	39	16.5 (7.1)	0.6 (2.3)
30	38	17.5 (10)	0.7 (5.0)
50	35	17.3 (4.5)	0.6 (3.9)
120	40	17.2 (8.7)	0.6 (5.2)

Note: reduced elastic modulus and hardness represent the mean values of indents on five cell walls in each sample. The coefficient of variation (CV, in *brackets*; units in percentage) represents the variability of the reduced elastic modulus and hardness

3.2 Influence of embedding medium on the hardness of lignocellulosic plant cell wall

3.2.1 Effects of epoxy resin on the hardness of lignocellulosic plant cell wall

The hardness values of wood cell walls under the two different sample preparation methods were compared. The hardness measurement for the whole data set for each sample preparation method in the two different wood species is summarized in Fig. 2 as a box and whisker plot. The mean value of the hardness of un-embedded cell walls was 0.4 ± 0.1 GPa, while it was 0.6 ± 0.1 GPa for embedded samples. Statistical analysis showed there was a significant difference of measurement of hardness ($p = 0.000$) between the embedded Loblolly pine and the un-embedded one. There was a 32 % increase of hardness for epoxy-resin-treated Loblolly pine.

Following the definition of Meyer hardness, hardness was determined at peak load. Thus, the hardness value was dependent not only on the plastic but also the elastic deformation, which could be expressed by the ratio of elastic modulus and yield strength σ_y. Since the yield strength σ_y could not be measured directly by nano-indentation for most of the materials, the constraint factor given as the ratio H/σ_y, should be around 3. However, for cell corner middle lamellae (CCML), the constraint factor usually comes to 1.5, which has been proposed by Gindl et al. [31]. Thus, for wood cell-walls' S_2 layer in this case, when applying these values to the E/H ratio, E/σ_y should be between 45 and 90. Sink-in behavior was expected due to the small E/σ_y ratio and could be observed in topographic images obtained by AFM as shown in Fig. 3. Similarly, the hardness value for wood cell walls of Red oak was 0.5 ± 0.1 GPa for un-embedded samples and it was 0.6 ± 0.04 GPa for embedded ones. This is statistical evidence that the embedding medium has an influence on the hardness value of Red oak cell walls ($p = 0.000$). Analogously, there was an 11.3 % increase in hardness for Red oak cell walls with the treatment of epoxy resin.

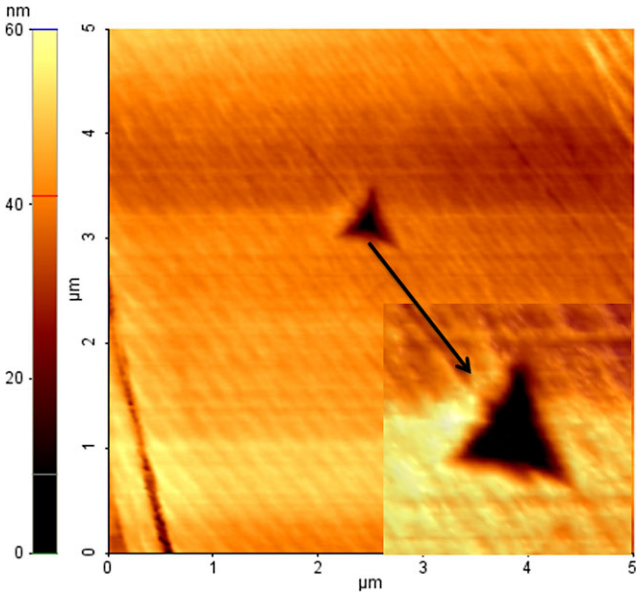

Fig. 3 AFM topographic image of single indent on Loblolly pine cell wall

3.2.2 Effect of vacuum time on the hardness of Loblolly pine cell wall

Hardness values obtained from Loblolly pine cell walls treated with different vacuum times are summarized in Table 2. Statistical analysis methods and linear regression were used to study the relationship between vacuum time and measured hardness values. As the vacuum time increases, the hardness reveals no significant changes. Our statistical analysis indicates that the vacuum time during the embedding procedure does not have a significant impact on hardness ($p = 0.235$, $R^2 = 0.016$).

3.2.3 Scanning electron microscopy (SEM) and energy dispersive spectroscopy (EDS) analysis

The possibility of resin penetration into cell walls and causal factors for the increase of reduced modulus and hardness

Table 2 Comparison of carbon/oxygen content between pure Spurr's resin, resin-embedded Loblolly pine cell wall and reference Loblolly pine cell wall

	Carbon content (%)	Oxygen content (%)
Epoxy resin	81.7 ± 1.8	18.3 ± 1.8
Reference cell wall A	72.2 ± 1.4	27.8 ± 1.4
Epoxy-resin-treated cell wall B	75.2 ± 1.7	24.8 ± 1.7

Note: values given are mean $\pm$ SD with units of percentage and were averaged from the four different areas in three samples each

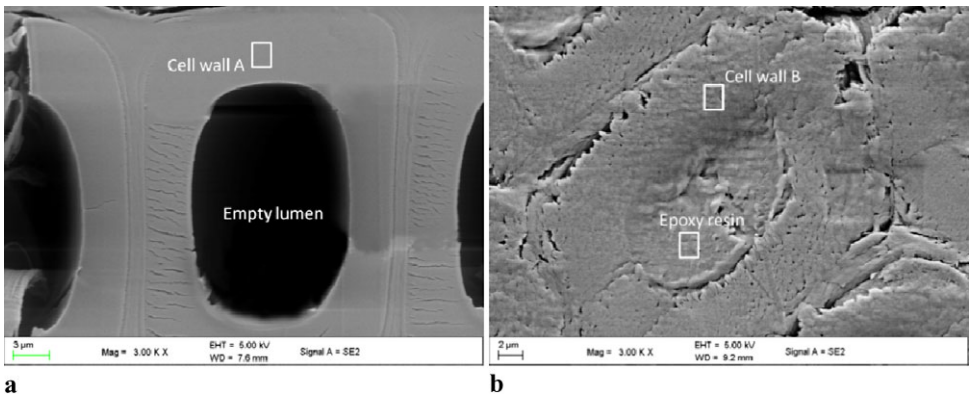

Fig. 4 (**a**) SEM image of Loblolly pine cell wall, (**b**) SEM image of Loblolly pine cell wall embedded in Spurr's resin

were investigated. Figure 4a and b are SEM images of wood cell walls. Several important characteristics of the SEM images are worthy of pointing out. First, the cell-wall lumen was fully diffused by epoxy resin in Fig. 4b compared to Fig. 4a. This observation suggests that epoxy resin had already occupied the cell-wall lumen after curing in the oven. However, it is insufficient to be a proof for resin infiltration into the cell wall. Hence, whether the resin penetrated into the cell wall or not could not be directly evaluated by the SEM images. Energy dispersive X-ray spectroscopy was used for further investigation. Interesting locations, such as cell wall A, cell wall B and epoxy resin, were picked up as shown in Fig. 4a and b. The major elements in Spurr's resin are carbon and oxygen, which are similar to the cell-wall's major elements. EDS element analysis was made for resin penetration by observing the carbon and oxygen contents. Table 2 shows the element analysis results; the carbon content of cell wall B, embedded by epoxy resin, was more than that of the epoxy resin and less than that of the reference cell wall. However, the oxygen content of cell wall B was lower than that of the reference but higher than that of the epoxy resin. The interpretation of content ratio changes could be an indication of resin penetration into the cell wall.

The penetration of embedding medium into cell walls increased both the values of reduced elastic modulus and the hardness. This is not surprising because of the wood cell-wall's texture and the properties of cell-wall components. Salmen and Burgert concluded that the interactions between constituents of cell walls occur on an extremely intermixed level [32]. The S_2 layer of wood cell wall can be considered as a natural, unidirectional fiber-reinforced composite, cellulose, made up of cellulose microfibrils playing the role of frame substance, where hemicellulose and carbohydrates serve as matrix substances to support the stiffness of the cell wall. Moreover, lignin diffused throughout is treated as an encrusting substance that will much increase the hardness of the cell wall. Although all indentation and EDS element measurements give indirect evidence of where and how exactly the epoxy resin is located within the cell wall, it can be assumed, with caution, that polymer chains of Spurr's resin penetrate into the cell wall through pit, lumen and nano-penetration due to its low-viscosity properties. Spaces in the cellulose–hemicellulose structures are filled with resin [33] and then the empty spaces among cellulose microfibrils are occupied, resulting in the modification of the cell-wall's chemical composition. The mainly hydrogen bonds between hemicellulose and cellulose enable a strong but relatively flexible connection, indicating a possible interaction between the C=O bond in the epoxy and the –OH bond in the cellulose. This procedure enables the effective bonding between Spurr's resin and microfibrils. Furthermore, the density of the cell wall was increased by the cell's being filled with resin. All the factors mentioned above could account for the change of both reduced elastic modulus and hardness of wood cell wall.

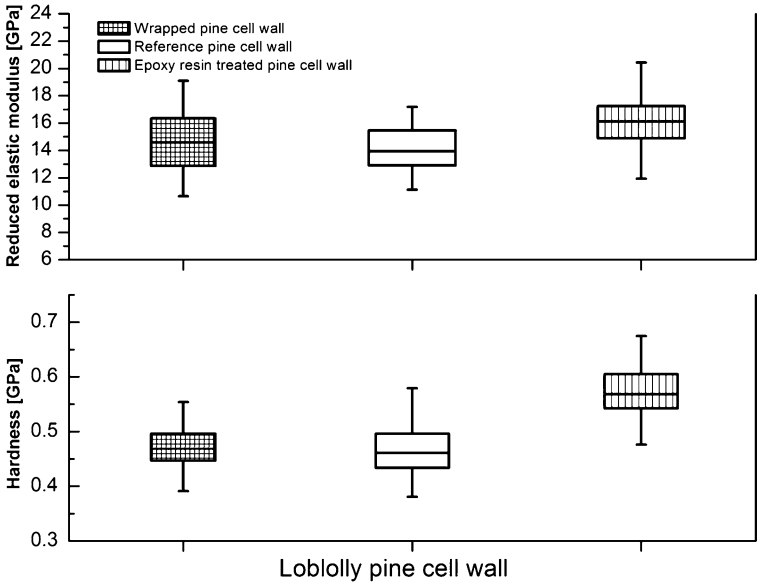

Fig. 5 Reduced elastic modulus and hardness of Loblolly pine by three sample preparation methods: wrapped, wood block and epoxy-resin-treated

Table 3 Comparison of nano-mechanical properties of cellulose materials under standard conventional sample preparation method and new method

Material	Embedded samples		Wrapped samples	
	Reduced elastic modulus (GPa)	Hardness (GPa)	Reduced elastic modulus (GPa)	Hardness (GPa)
Sweetgum	13.5 (1.9)	0.4 (0.05)	8.0 (1.4)	0.3 (0.03)
Reed	19.1 (0.9)	0.6 (0.04)	17.2 (4.3)	0.5 (0.02)
Silvergrass	22.5 (1.4)	0.6 (0.03)	20.1 (1.0)	0.5 (0.04)

Note: the coefficient of variation (CV, in *brackets*; units in percentage) represents the variability of the reduced elastic modulus and hardness

3.3 Test results and comparison of mechanical properties between embedded and wrapped cell walls

The influence of resin penetration and ways to avoid it are shown in Fig. 5, where the three sample preparation methods were compared using nano-indentation. To minimize the variability of microfibril angles and chemical composition due to anatomical differences among cell walls, indentations were performed on tracheid cell walls located in direct proximity. The mean value of the reduced modulus of wrapped pine cell wall is 14.6 ± 2.3 GPa. There is no significant difference with the value obtained from the reference cell wall, which is 14.1 ± 1.6 GPa ($p = 0.19$). An increase of cell-walls' reduced modulus was observed up to 16.1 ± 2.0 GPa after embedding with epoxy resin. Non-parametric analysis shows that there is a significant difference between wrapped samples and embedded samples ($p = 0.0001$). The mean value of the hardness of wrapped pine cell wall is 0.5 ± 0.04 GPa, while it is 0.6 ± 0.1 GPa after embedding into epoxy resin. The hardness value of the reference sample was 0.5 ± 0.1 GPa, which shows no significant difference from the wrapped sample ($p = 0.87$), while non-parametric analysis results show that there was a significant difference of hardness value between the embedded sample and the wrapped sample ($p = 0.02$). We interpret this to mean that the new sample preparation method achieves effective resin infiltration prevention and it strengthens the previous conclusion that Spurr's resin penetrating into tracheid cell walls has the potential of chemically modifying them, thus resulting in the modification of the mechanical properties of wood cell walls.

To provide further support for the new preparation methods, other lignocellulosic plant cell walls including Sweetgum, Reed and Silvergrass were tested under two sample preparation methods (Table 3 [34]). All the values of the reduced elastic moduli show a tendency to increase when epoxy resin penetrates into the cells. Clearly, the embedding medium for many lignocellulosic materials has a great influence on the reduced elastic modulus testing results during the nano-indentation test. Similarly, compared with those of the sample wrapped in plastic film, the hardness values of samples with epoxy resin directly penetrated were much higher. This table seems to be evidence that the embed-

ding medium can change the samples' properties and have a strong influence during the process of getting reliable data.

4 Conclusion

Nano-indentation has proven in recent years to be an effective tool in characterizing the mechanical properties of cell walls. However, this technique has still not been fully developed for biomaterial research due to the limitations of the testing capabilities at the facilities as well as in sample preparation. Many efforts have been made to improve the estimate of cell-wall properties obtained from nano-indentation. A better understanding of the effect of the embedding medium is crucial for insight into the interaction between epoxy resin and the cell-wall structure. Series contrast experiments were made to support the hypothesis that the embedding medium enhanced both the reduced modulus and the hardness of wood cell walls. Typically, for Loblolly pine, epoxy resin increased the values of the reduced elastic modulus by 14 % and the hardness by 32 %. Similarly, the value of the reduced elastic modulus of Red oak treated by epoxy resin increased by 15.5 % and the hardness value increased by 11.3 %. Other lignocellulosic materials, such as Reed, Silvergrass and Sweetgum, were tested as well and the results proved that the embedding medium had an influence on the cell-wall's mechanical properties. Cell-walls' porous and multiply layered structure would be considered as the major reason for resin penetration. This resinous penetration will essentially alter the chemical components inside the cell wall and the cell-wall density, leading to the observed change of mechanical properties under nano-indentation. Moreover, no significant differences of nano-indentation data were detected among different vacuum times. This outcome indicates that resin penetration is a fast procedure and happens at multiple access points, including pits, lumen and nano-penetration. Finally, a novel sample preparation method to prevent resin from penetrating into the lignocellulosic plant cell wall was developed and proved to be effective. This method overcomes the intrinsic limitations of conventional sample preparation and is appropriate for small, soft specimens like single fibers, wood chips, wood remains, herb plants etc, which, in turn, widens the potential application of nano-indentation.

Acknowledgements The authors gratefully acknowledge financial support by a USDA Wood Utilization Research Grant and the Forest Service Forest Products Laboratory and partial support by the Natural Science Foundation of China (No. 30928022).

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