Induction and quantification of fiber alignment in cellulose nanofibril films

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Abstract

Cellulose nanofibrils (CNF) have been explored as an emerging naturally sourced material for use in the preparation of new biomaterials. CNF fibrils have a high aspect ratio with fibril lengths of $\sim 1 \mu\text{m}$ and diameters of 20–40 nm. The assembly of CNF impacts both bulk mechanical properties as well as localized cellular interaction. The ability to reproducibly tune CNF fiber alignment is an active area of CNF-based biomaterial research. Here, we present a simple CNF fibril alignment strategy based on application of constant unilateral force on thin CNF films drying on a flexible substrate. CNF fibril alignment/orientation was characterized using both Polarized Light Microscopy (PLM) and conventional Scanning Electron Microscopy (SEM) approaches. CNF is optically birefringent; therefore, calculation of the birefringence orientation index (BOI) can infer the extent of CNF fibril alignment with a non-destructive, cost-effective technique. CNF fibril alignment is markedly increased with application of 10.2 N force as assessed by both SEM and PLM analysis. SEM imaging resolved individual CNF and the alignment was analyzed using OrientationJ, an ImageJ plugin, to extract fibril angle whereas PLM microscopy provided a BOI value. Both the fibril alignment and BOI score were in agreement; therefore, it is acceptable to infer fibril organization with PLM techniques. Furthermore, the addition of nanoparticle hydroxyapatite did not diminish the CNF fibril alignment as assessed by both PLM and SEM highlighting the utility of the CNF film fabrication technique. In summary, the application of unilateral force on thin CNF films adhered to latex, is an elegant, scalable, and cost-effective technique for generating CNF films with reproducible fibril alignment.

Introduction

Biopolymers are known to have superior biocompatibility compared to synthetic polymers and their nontoxic nature suits tissue engineering applications (Abdul Khalil et al. 2020). Cellulose is a primary component of every plant cell wall and is the most abundant polymer on earth. Cellulose nanofibrils (CNF) is a cellulose derivative which is produced using chemical and mechanical treatments on wood pulp. CNF offers a diverse range of properties including crystallinity, high specific area, alignment and orientation, barrier properties, and chemical reactivity. From a biological standpoint, it also benefits from critical features such as biocompatibility, biodegradability, and lack of toxicity as a biopolymer (Lin and Dufresne 2014). CNF is known for its outstanding mechanical properties and reinforcing characteristics (Zhang et al. 2019). The anisotropic character of CNF can lead to fiber orientation, impacting CNF's modulus of elasticity and tensile strength. In nature, cellulose fibrils are present in the outer cell wall layer of plants and are naturally aligned to create a strong durable structure (Ye et al. 2020). Controlling fiber alignment in engineered constructs has the ability to modulate mechanical properties which positively correlate with the degree of fiber alignment (Li et al. 2021). Beyond mechanical properties, cellular interactions such as cell polarity and cell elongation are directly influenced by fiber alignment of the contact surface (Kim et al. 2012). For instance, CNF based tissue scaffolds fabricated by electro-spinning have demonstrated enhanced cell alignment significantly affecting cell behavior including differentiation and adhesion (He et al. 2014).

Fiber alignment can be achieved by various techniques including vacuum filtration, (Ghasemi et al. 2020), electromagnetic (EM) fields (Kim et al. 2008), and hydrodynamic alignment (Håkansson et al. 2014). Although these methods are effective, the underpinning physics limit their applications. For example, vacuum filtration induces only a slight net orientation, and the extent of alignment is not readily tunable. Electromagnetic methods restrict the development of composites as the additional material must have similar dipole characteristics as CNF to avoid separation. This eliminates the possibility of adding mineral oxides with homogenous distribution for increased stiffness and biocompatibility. Similarly, hydrodynamic alignment methods require precise control of fluid viscosity and nozzle velocity and are generally not well suited for high concentration suspensions. In thin-film polymer science, it is common to dry films on an impermeable substrate. Furthermore, pinning opposite ends of an immobilized film on a substrate has been shown to result in CNF fiber orientation due to internal forces exerted on the individual fiber due to contraction caused during drying, but with limited success and no experimental control (Ghasemi et al. 2020).

The method described here is based on the application of a controllable unilateral elongational force applied via a deformable latex substrate during the CNF drying process to create orientated CNF films. Additionally, reducing the water content of the starting CNF suspension increases the adhesion of the CNF slurry to the latex substrate enabling even greater transmission of the alignment force. The utility of this method for fiber orientation in more complex CNF-composite films was also explored with up to 10% (dry basis) mineral additives (hydroxyapatite). Fiber orientation in this class of composite engineered materials are currently under exploration for bone-like biomaterials to spur bone regeneration. To aid in the development and characterization of these composite engineered materials, a non-destructive technique with sensitivity of fiber alignment is required. Here, we used Polarization Light Microscopy (PLM) and the innate birefringence of CNF to spatially map the degree of fiber orientation using the Birefringence Orientation Index (BOI) in a custom ImageJ macro. Scanning electron microscopy (SEM) images of the network of 20–50 nm nanocellulose fibrils were analyzed using OrientationJ, an ImageJ plugin to validate the BOI sensitivity to fiber orientation as PLM is not able to resolve individual fibers.

Experimental Section

All films were prepared using Cellulose Nanofibrils (CNF) at 3 wt% solids, in water, provided by the University of Maine Product Development Center (PDC). Hydroxyapatite (HA) paste (< 50 nm) was purchased from Sigma Aldrich and used without modification. Latex bands, used routinely for physical therapy (Thera-Band, 4.6 lbf @ 100% elongation), were implemented without modification as the deformable substrates for this study.

Thin film preparation

The stock 3wt% CNF suspension was diluted to 1.5 wt% solids in distilled water to decrease the viscosity and ease the spread of the CNF solution. Six grams of the resulting CNF suspension was spread using a standard laboratory spatula on a 2 x 5 inch latex band. The latex band was taped on all edges to a hard

flat rigid surface to ensure constant tension in the latex band and avoid dripping of the approximately 1 mm thick CNF films. These samples were left to dry in ambient room conditions overnight and were labeled as “unstretched”. The “stretched” pure CNF films were prepared following the same deposition process as above but were placed under a box (STP) to reduce air flow for 3 hours to decrease the sample moisture content by approximately 30% (by weight). The reduction in moisture content increases adhesion sufficiently to allow for both the latex substrate and the CNF film to be stretched during drying. At this stage, the latex band was stretched lengthwise by applying a controlled force to one end while the other end was fixed (Figure 1.b). The samples were left to dry at STP conditions overnight. The hydroxyapatite/CNF composite films were fabricated in the same way but with the addition of HA paste to the 1.5% CNF suspension (1:10), followed by magnetic stirring at 200 rpm for 4 hours, to ensure adequate dispersion of the HA into the CNF network. Dried films were carefully peeled off from the latex and stored in a dry place for characterization. In order to achieve the highest level of orientation and to determine the effect of an applied force on fiber alignment, a series of samples were prepared with applied forces ranging from 0 to 11.1 kN.

Polarized light microscopy

Polarized light microscopy (PLM) was used to map the fiber orientation of the prepared films. A standard Olympus IX73 inverted microscope, equipped with a 10X 0.4NA air objective and Amscope color (CMOS) camera, was used for imaging of all samples. Two broadband linear polarizers were crossed above and below the sample stage thereby eliminating all light transmission. A 530 nm full-wave retardation plate was fixed in a 360° rotation mount above the stage but below the first polarizer. Using this configuration, commonly used in mineralogy (Montana 2020), the 530 nm full wave plate introduces an optical path length difference of 530 nm for light of this same wavelength in the incident linearly polarized white light. These green photons (530 nm) therefore remain linearly polarized and are fully attenuated by the subsequent crossed polarizer, whereas all other wavelengths are elliptically polarized. As a result, all other wavelengths pass through the crossed polarizer combining to create magenta-red color using Newtonian color addition/subtraction. When a sample is introduced, the birefringence introduces a phase shift proportional to the interaction of the light relative to the optical axis of the birefringent material. This interaction results in either positive or negative interference when combined in the analyzer of the PLM microscope resulting in blue (additive) or yellow (subtractive) colors. To acquire images, one of the eye pieces was replaced with the Amscope camera and the multichannel (RGB) color information of the CMOS channel was recorded. Three samples of each type were tested, where 8 areas of each sample were imaged.

To obtain quantifiable isotropic information from the CNF films, control of the relative angles of orientation between the waveplate and the CNF sample orientation is required. Rectangular CNF films were fixed onto the microscope stage with the long axis (applied force axis, see Figure 1) parallel to the horizontal edge of the stage. RGB images are acquired at both +/- 45° rotation of the 530 nm full wave retardation plate with respect to the stage-mounted sample to be used in the BOI analysis.

BOI analysis

Birefringence imaging is routinely used to map inhomogeneities in lithographic samples (Burnett et al. 2001). More recently, this common technique has been adapted, where specific digital color channels are used to enhance and quantify birefringence contrast, referred to as the Birefringence Orientation Index (BOI) (Sørensen 2013). Specifically, the BOI index relates the relative intensity of the red and blue color channels to the positive or negative birefringence of the sample.

The BOI analysis was performed with a custom image processing algorithm using ImageJ (Schneider et al. 2012), an open source software package. A schematic depiction of the step-by-step image analysis process is shown in Figure 2. Here, The RBG images, at both $+45^\circ/-45^\circ$, were separated into their respective RGB color channels. The green channel images were used as a mask to reduce noise from the set-up due to the use of a 530 nm full wave retarder plate which extinguishes green wavelengths in the crossed polarizers in the absence of a sample. As a sample, CNF is a birefringent material, therefore when probed with linear polarization, the birefringence of CNF shifts the interfering wavefronts in the crossed analyzer resulting in either blue or yellow interference colors (Mashkour et al. 2014). All BOI calculations used the blue channel images. The blue channel images were filtered using the green channel as a mask to remove noisy pixels and the 8-bit images. The masked images were rescaled 1-256 to eliminate 0 as a pixel value. The BOI map was generated according to equation 1 where b is the pixel intensity of the blue channel at $-/+45^\circ$. A BOI value of ± 1 is only positively or negatively birefringent whereas a BOI of 0 has no preferred birefringence. Resulting BOI maps were smoothed using a median filter with a radius of 1. The histograms of each BOI image were recorded and normalized for direct comparison of BOI values.

See equation 1 in the supplementary files.

Fiber Orientation Analysis

Surface morphology and fiber directionality were investigated by Scanning Electron microscopy (SEM); using a Zeiss NVision 40 Microscope. Three SEM micrographs of each sample were obtained at 3 KV and 1000X magnification. The orientation of the fibers were quantified using a sliding gaussian window method to calculate a local gradient structure tensor to quantify fiber orientation. This analysis was performed using OrientationJ (Püspöki et al. 2016), an ImageJ plugin with a Gaussian window size of 3. A histogram of fiber orientation was created using OrientationJ which classifies the fibers based on pixel coherency (Rezakhaniha et al. 2012; Clemons et al. 2018).

Results

In this method, the alignment of fibers is directly affected by the longitudinal force applied to the deformable substrate. The force is applied to the sample once the solution is well adhered to the latex substrate. The amount of force applied to stretch the wet sample was quantified by measuring the maximum longitudinal force required to fully stretch the band without breaking the sample. For each weight applied, the amount of BOI was calculated accordingly. The results of the force optimization

experiments indicate that the average BOI increases with increasing applied force. However, when the longitudinal force exceeded 10.2 N, the sample failed to form a continuous film, fractured and delaminated from the latex support material. Table 1 indicates the amount of force applied to the samples before drying and the average BOI value for each force.

Figure 3 shows representative PLM images of unstretched and stretched samples. Unstretched samples had visual shifts in color due to the extrinsic birefringence of CNF; however, the shifts in colors are restricted to individual fibrillar structures. The apparent color shift of the stretched CNF samples were generally dominated by a single color and appeared more uniform across the sample area indicating that the fibrillar structure of the CNF is more uniformly arranged.

BOI maps, generated by method shown in Fig. 2, are shown in Fig. 4A. Here negative and positive birefringence are represented by black (-1) and white (+1), respectively. Both extremes indicate high fiber orientation whereas values closer to 0 indicate non-oriented samples. Highly oriented samples are expected to appear as dominantly black or white depending on the degree of positive or negative birefringence. Figure 3 shows that stretched samples have a black dominant BOI close to -1.

Representative normalized BOI histograms are shown in Fig. 4B. Comparison of the BOI histograms clearly illustrates that stretched CNF films have a BOI skewed towards -1; whereas, the unstretched CNF samples lack any dominant BOI feature. The average BOI value for stretched films was -0.80 whereas the average BOI of unstretched samples had a value of -0.16, suggesting a significant increase in birefringence, indicating greater fiber orientation. In order to demonstrate the robustness of this sample preparation method, HA was included in the initial slurry (10% by dry weight). The resulting PLM data suggest that the presence of HA did not significantly alter the BOI values relative to pure stretched CNF as demonstrated by both the BOI image Fig. 4A and histogram Fig. 4C. The average BOI achieved for the stretched CNF/HA sample was -0.82, comparable to the value of -0.80 obtained for the pure CNF sample.

Directionality Analysis

To further support that the BOI method is sensitive to CNF fiber orientation, SEM images were analyzed using the OrientationJ plugin in ImageJ. This canned algorithm makes use of the robustness of the structure tensor method to create a hue-saturation-brightness (HSB) image (Püspöki et al. 2016), from which the extent of feature orientation can be assessed. Resulting representative hue-saturation-brightness (HSB) color maps of analyzed SEM images are shown in Fig. 5A in which oriented fibers are apparent. To better quantify the individual image data, feature orientation polar diagrams were used. Figure 5D is a polar plot of the shifted dominant fiber angle normalized and centered at 0° of for the unstretched, stretched, and HA-doped stretched CNF films. These data clearly indicate that fiber alignment in both the CNF and CNF/HA composite films exhibit increased alignment compared to the unstretched films.

Conclusion

In this work, we developed and tested a novel method to induce fiber orientation in CNF and CNF/HA films. A BOI index method was adapted for our data using PLM results which showed that BOI was increased significantly for both pure CNF and hydroxyapatite incorporated CNF films. SEM data were used to validate the BOI results and provide insight into the narrow orientation distribution for stretched samples compared to unstretched samples. Both PLM and SEM results showed that incorporating HA into the CNF matrix did not interfere with the fiber orientation or film consistency. We found our method to be effective in generating oriented films for relatively low initial concentrations (1.5 wt%) of CNF. Higher initial concentrations were not explored in this study. Additionally, the use of other mineral, nanoparticulate, or polymer additives was not explored here, but will be the focus of future work. Highly oriented films, achieved by this method, could be beneficial for a broad range of tissue engineering applications.

Declarations

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Conflict of interests: The authors have no conflicts of interest to declare that are relevant to the content of this article.

Authors' contributions: SR, JH, MM, and KT conceived of CNF fabrication technique, experimental plan. SR, JH, and OA synthesized CNF films. SRH and JH characterized films using SEM and PLM respectively. SR, JH, MM, and KT wrote the manuscript. All authors have read and approved of the manuscript.

References

Abdul Khalil HPS, Jummaat F, Yahya EB, et al (2020) A review on micro- to nanocellulose biopolymer scaffold forming for tissue engineering applications. *Polymers* (Basel).

Burnett JH, Levine ZH, Shirley EL (2001) Intrinsic birefringence in calcium fluoride and barium fluoride. *Phys Rev B - Condens Matter Mater Phys* 64:1–4. <https://doi.org/10.1103/PhysRevB.64.241102>

Clemons TD, Bradshaw M, Toshniwal P, et al (2018) Coherency image analysis to quantify collagen architecture: Implications in scar assessment. *RSC Adv*. <https://doi.org/10.1039/c7ra12693j>

- Ghasemi S, Rahimzadeh-Bajgiran P, Tajvidi M, Shaler SM (2020) Birefringence-based orientation mapping of cellulose nanofibrils in thin films. *Cellulose*. <https://doi.org/10.1007/s10570-019-02821-2>
- He X, Xiao Q, Lu C, et al (2014) Uniaxially aligned electrospun all-cellulose nanocomposite nanofibers reinforced with cellulose nanocrystals: Scaffold for tissue engineering. *Biomacromolecules*. <https://doi.org/10.1021/bm401656a>
- Kim DH, Provenzano PP, Smith CL, Levchenko A (2012) Matrix nanotopography as a regulator of cell function. *J. Cell Biol.*
- Kim J, Chen Y, Kang KS, et al (2008) Magnetic field effect for cellulose nanofiber alignment. *J Appl Phys.* <https://doi.org/10.1063/1.3006140>
- Li K, Clarkson CM, Wang L, et al (2021) Alignment of Cellulose Nano fi bers: <https://doi.org/10.1021/acsnano.0c07613>
- Lin N, Dufresne A (2014) Nanocellulose in biomedicine: Current status and future prospect. *Eur Polym J.* <https://doi.org/10.1016/j.eurpolymj.2014.07.025>
- Mashkour M, Kimura T, Kimura F, et al (2014) Tunable self-assembly of cellulose nanowhiskers and polyvinyl alcohol chains induced by surface tension torque. *Biomacromolecules* 15:60–65. <https://doi.org/10.1021/bm401287s>
- Montana G (2020) Ceramic raw materials: how to recognize them and locate the supply basins—mineralogy, petrography. *Archaeol Anthropol Sci* 12:. <https://doi.org/10.1007/s12520-020-01130-1>
- Püspöki Z, Storath M, Sage D, Unser M (2016) Transforms and operators for directional bioimage analysis: A survey. *Adv Anat Embryol Cell Biol* 219:69–93. https://doi.org/10.1007/978-3-319-28549-8_3
- Rezakhaniha R, Agianniotis A, Schrauwen JTC, et al (2012) Experimental investigation of collagen waviness and orientation in the arterial adventitia using confocal laser scanning microscopy. *Biomech Model Mechanobiol.* <https://doi.org/10.1007/s10237-011-0325-z>
- Schneider CA, Rasband WS, Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis. *Nat Methods* 9:671–675. <https://doi.org/10.1038/nmeth.2089>
- Sørensen BE (2013) A revised Michel-Lévy interference colour chart based on first-principles calculations. *Eur J Mineral.* <https://doi.org/10.1127/0935-1221/2013/0025-2252>
- Ye D, Rongpipi S, Kiemle SN, et al (2020) Preferred crystallographic orientation of cellulose in plant primary cell walls. *Nat Commun.* <https://doi.org/10.1038/s41467-020-18449-x>
- Zhang K, Barhoum A, Xiaoqing C, et al (2019) Cellulose Nanofibers: Fabrication and Surface Functionalization Techniques. In: *Handbook of Nanofibers*

Table

Due to technical limitations, table 1 is only available as a download in the supplementary files section.

Figures

Figure 1

Schematic of the unilateral stretching force apparatus for aligning CNF samples using constant uniaxial force.

Figure 2

BOI calculation process flow chart.

Figure 3

Raw representative PLM image set. Images acquired at -45° (top) and $+45^{\circ}$ (bottom) for unstretched and stretched samples.

Figure 4

a) Representative BOI maps of stretched and unstretched samples. b) Comparison of normalized BOI frequency distribution of stretched and unstretched CNF films. c) Normalized BOI frequency distribution of stretched composite CNF/HA vs. stretched pure CNF films.

Figure 5

OrientationJ analysis results: a-c) Representative color-coded images of the surface of unstretched CNF film, stretched CNF film and stretched CNF/HA film. d) Polar plot of fibril distribution of angles normalized around 0° for stretched and unstretched CNF samples (n = 3).

Supplementary Files

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