Mistletoe as a Model Composite System: Self-assembling, Self-healing Natural Cellulosic Fibers

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Keywords: *Bio-inspiration; cellulosic fibers; natural composites; biomaterials; bio-adhesives*

Abstract:

Biological materials, optimized by nature over eons, offer among the highest specific mechanical properties of all known materials. Nacreous mollusk shells and trabecular bone, composites of protein and ceramic, have hierarchical structures offering high strength and toughness. Similarly, natural fibers like spider silk and the mussel byssus, have programmed nanostructures contributing to exceptional toughness, strength, and stiffness, even in extreme environments like the ocean's intertidal regions. Remarkably, such biological materials achieve these heights of performance through clever, yet inherently sustainable fabrication routes. Along these lines, European mistletoe (*Viscum album*) berries produce self-assembling composite fibers exhibiting stiffnesses of more than 14 GPa and tensile strengths of up to 170 MPa. Largely composed of cellulose nanocrystals (CNCs) as reinforcement, these fibers exhibit multi-material adhesion, self-healing, and water-responsive mechanics, attributed to the relationship between their high-aspect ratio CNCs and their polysaccharidic hemicellulose matrix phase. Stiffer than natural wood (yet without lignin) and stickier than urea-formaldehyde glues, the system accomplishes impressive mechanical performance with simple, natural building blocks. Aligned with the global initiative to phase out fossil fuel-derived materials, mistletoe fibers could inspire more sustainable composites after thorough elucidation of their self-assembly mechanisms using advanced materials characterization. Small and wide-angle X-Ray scattering, in addition to polarized light microscopy, were previously used to probe the mechanically induced re-alignment of the CNCs under tension along the tensile axis from a specialized tissue known as the mistletoe viscin cell bundle (VCB). Additionally, confocal Raman spectroscopy, atomic force microscopy and nano-spectroscopy (AFM-IR), rheology, and mechanical testing were utilized to further elucidate the structure-function relationships of the system. Here, we build on the current understanding of mistletoe fiber formation by exploring the possible role of proteins within this primarily polysaccharidic composite. Utilizing tandem mass spectrometry (MS/MS) based proteomic analysis, we identified the presence of several protein components with the VCB tissue – including lectins and viscotoxins – with possible roles in matrix cross-linking during fiber formation. Initial studies using AFM, TEM, and nano-IR spectroscopy support the hypothesis that these proteins are interacting with the hemicellulosic matrix of the VCB.

1. Introduction

Cellulose is the most abundant natural polymer on earth [1]. Its micro- to macro-structure varies depending on the source while maintaining the same biodegradable building blocks. Composed of crystalline and amorphous segments, cellulose nanofibrils are hierarchically assembled into a wide range of structures in natural systems. Given the biodegradability and mechanical properties of nanocellulose, (stiffness > 100 GPa, tensile strength > 5 GPa), it has been explored as composite reinforcement for decades [2]. While thermosets such as epoxies bond well to cellulose [3], more sustainable thermoplastic matrices suffer from poor fiber-matrix adhesion, leading to failure mechanisms like fiber pull-out and fiber-matrix debonding [4]. Furthermore, polymers like thermoplastic polyurethane (TPU), while recyclable, are fossil-fuel derived and not biodegradable, thus eliminating one of the major advantages of using cellulosic reinforcement.

Thus, we turn to nature, having the advantage of optimizing its natural cellulosic composite materials over hundreds of millions of years of evolution, as a source of bio-inspiration. European mistletoe (*Viscum album ssp. album*) – a hemi-parasitic plant prominent in Western Europe – is a remarkable, yet still understudied model system for processing of high-performance cellulosic composites. Propagated through bird droppings (Figure 1A), mistletoe berries possess a mucilaginous material around the seed that is capable of forming elongated sticky fibers whose stiffness exceeds 14 GPa [5]. Indeed, fibers of up to 2 meters in length can be pulled from a 5-millimeter-long tissue known as the viscin cell bundle (VCB) through simple tensile drawing. These fibers are composed of high aspect-ratio nanocellulose reinforcement with an adhesive matrix containing what we have coined Mistletoe Adhesive Extract (MAE) [6, 7]. While hygroscopicity is often the enemy of structural composites, study of MAE's moisture-responsive mechanical properties can offer insight into managing susceptibility to water uptake in other polysaccharidic systems. Through unravelling the self-assembly mechanisms of these fibers and characterizing the role of the matrix in mediating adhesion and mechanical properties, solutions towards durable biodegradable composites can be uncovered in the global initiative to phase-out fossil-fuel derived materials for more sustainable alternatives.

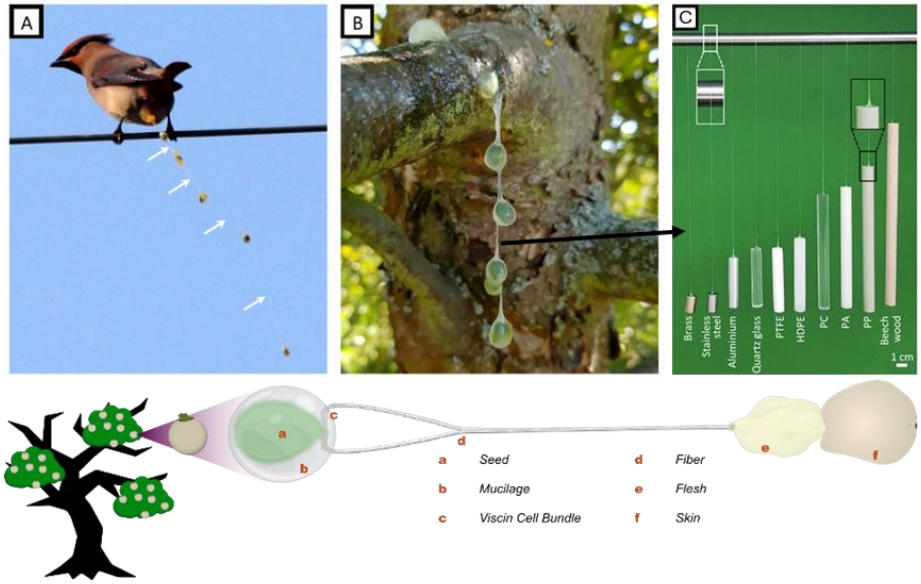


Figure 1: ***European Mistletoe Propagation Through Fiber Formation*** *A) Mistletoe thrush (Turdus viscivorus) propagating Viscum album seeds. B) Mistletoe fibers adhering to a tree branch. C) Multi-material adhesion of mistletoe fibers. D) Structure of Viscum album ssp. album berries, from feedstock to fiber. Includes images from reprinted from Horbelt (2021) [8] reprinted with permission.*

Previous investigation utilized polarized light microscopy (PLM) and X-ray diffraction to probe the changes in cellulose orientation during fiber formation from VCBs [5]. Cellulose demonstrates birefringence under polarized light, meaning that its orientation can be visualized in PLM. As shown in Figure 2A, the mistletoe berry's VCB starts with cellulose orientated perpendicular to the fiber drawing direction (appearing blue). During fiber formation, cellulose undergoes a near instantaneous 90-degree transformation, to colinear with the applied tension (appearing yellow). This overwhelmingly consistent orientation suggests the cellulose nanofibrils (CNFs) are synthesized and stored in the VCB in a manner important to their function. Wide-angle X-ray scattering (WAXS) was used to further probe cellulose orientation during fiber formation. Characteristic (200) peaks for cellulose nanocrystals (i.e. perpendicular to the long axis) appear in the scattering pattern, which shift to perpendicular orientation as the fiber is drawn (further illustrated in the azimuthal profiles, Figure 2B-E). Furthermore, the sharpening of these peaks from VCB to fiber suggests that the degree of cellulose alignment is much higher in the fibers than the VCB, thus strengthening and rigidifying the system under tension. The model for this transition is illustrated in Figure 2F, which includes small angle X-ray scattering (SAXS) scattering patterns to further illustrate how drying decreases the inter-fibrillar distance, further increasing mechanical properties.

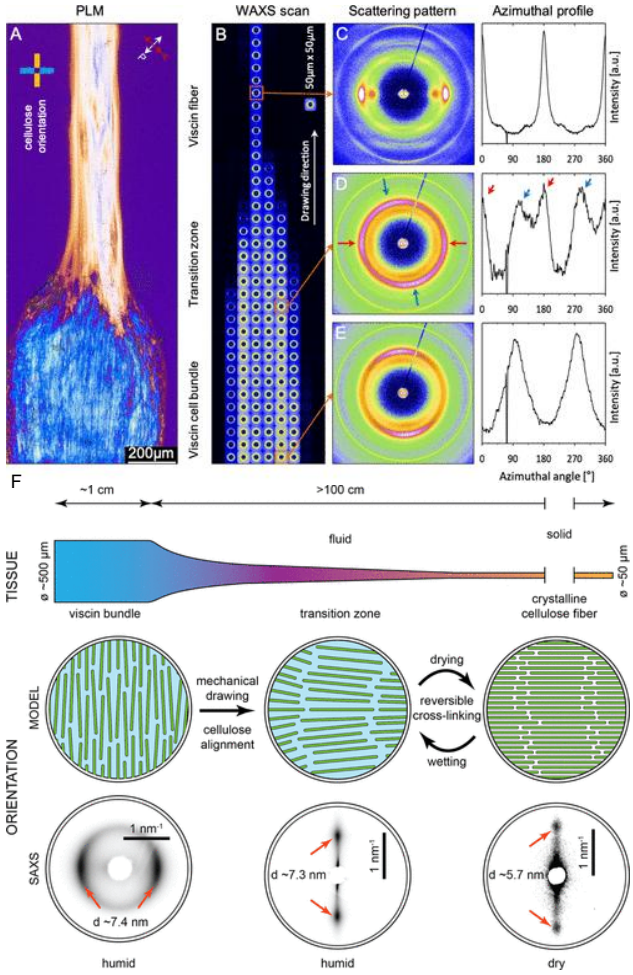


Figure 2: ***Investigation of cellulose orientation across the VCB transition zone****. A) PLM and B-E) WAXS from VCB to fiber. F) Model of nanocellulose reorganization and condensation under tension. VCBs offer immense hidden length, undergoing >200X length and ~35X stiffness increases during fiber drawing. Adapted from Horbelt et al. (2019) [5] with permission from the American Chemical Society.*

Key to the function of mistletoe viscin is its water-responsiveness. Owing to its transiently bonded hemicellulosic matrix, reversible, water-mediated hydrogen bonding enables the matrix to 'reset' and reorganize when water-vapour is applied. This enables self-healing, as illustrated in Figure 3. Previous results further show that the mere act of pre-failing the viscin in lap-shear prior to re-adhering it will increase its breaking strength [7]. The preferential orientation of the CNFs via shear forces is the underlying mechanism for this improvement, illustrated by quantitative PLM (Figure 3C-H). Thus, much like a traditional fiber-reinforced composite, reinforcement orientation is critical to function. Thus, this presents more evidence for the function of these self-assembling, reorientating CNF-hemicellulose fibers. This implies that strengthening a randomly oriented cellulosic composite via uniaxial stress aligns the reinforcement to the load axis thus providing stimuli-responsive strengthening and stiffening.

While much is now understood about the critical interplay between cellulose nanofibrils and hemicellulosic matrix components in dictating mistletoe fiber processing and adhesion [5, 7, 9], previous studies have also suggested the presence of protein components within mistletoe berries,

which could also contribute to the properties of this unusual biological composite. Indeed, other natural polysaccharidic adhesives, like gum arabic, rely on glycoproteins for their adhesive functionality [10], we hypothesized that mistletoe viscin, and its adhesive extract (MAE) rely on proteins for their adhesion. From spectroscopic data, we know that the matrix material in viscin is partially composed of highly branched arabinogalactans attached to a negatively charged backbone [6, 7, 11]. Research also suggests that mistletoe lectins (ML), highly abundant *Viscum album* extracts, bind to β-D-Galactose (β-Gal), a major building block of the hemi-cellulosic matrix [11, 12]. Recently, we developed a method to purify MAE, a water-soluble component of the viscin tissue known for its adhesive role, which also appears to function integrally in the malleable and hygroscopic matrix of the viscin composite. Here, we explore the presence of proteins in MAE using MS/MS proteomics and explore the structural organization of MAE and the associated proteins using TEM, AFM, and nano-IR spectroscopy. Our initial findings suggest that several key classes of proteins including lectins and viscotoxins are present in MAE and likely closely associated with the hemicellulosic components. Future studies will be necessary to untangle the contribution of these proteins to mechanics and processing of viscin fibers.

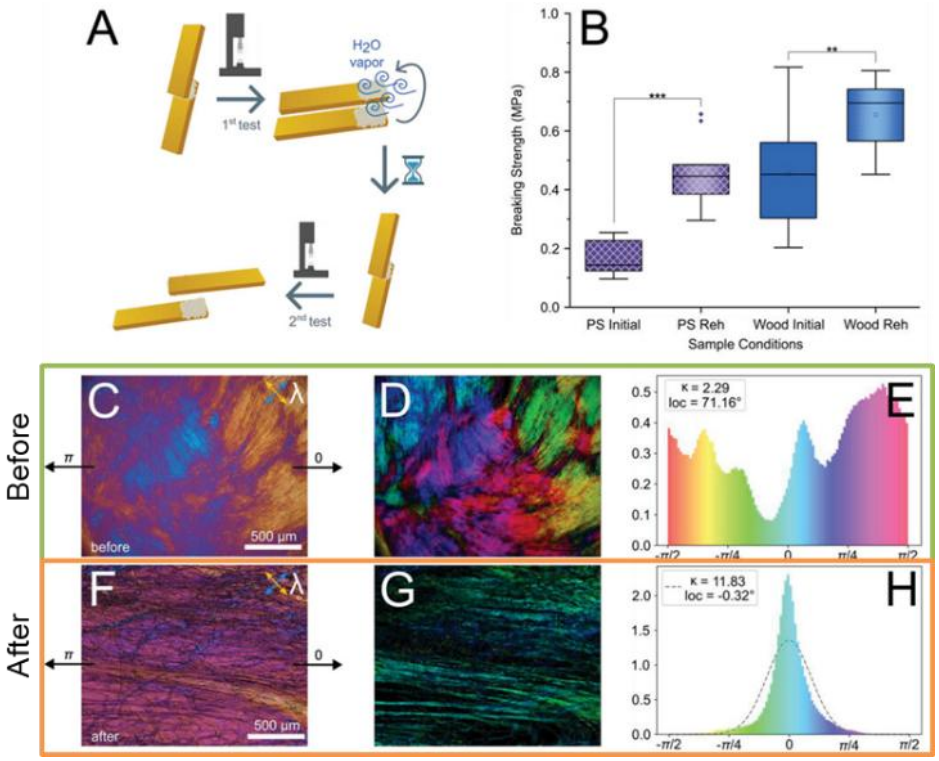


Figure 3: ***Self-healing of mistletoe viscin.*** *A) Schematic of re-adhesion process. B) Comparison of initial and rehydrated ("Reh") breaking strengths for two sample sets. Samples were tested between 41 and 47% RH and N ≥ 9 for all specimens. C, F) Raw PLM images, with full-wave retardance plate, of a single lap shear sample prepared between two glass slides. D, G) The orientation of CNFs within the viscin tissue relative to the horizontal axis. E, H) Von Mises distributions describing the orientations of cellulose nanofibrils orientations. Reprinted from George et al. (2024) [7] under CC BY-NC-ND 4.0.*

2. Materials and Methods

2.1. Materials

Mistletoe berries *(V. album* ssp. *album)* were collected from apple trees in Golm, Germany in winter. Berries were stored at −20 °C, and single berries were thawed for later use. The freeze–thaw process did not affect fiber-forming ability. The seed and viscin (containing VCBs and mucilage) were excised using razor blades and tweezers maintaining their integrity.

2.2. Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

Individual tissues (VCBs, Mucilage, Flesh) were excised from mistletoe berries using a scalpel and tweezers and vortexed in water for 1 minute. These samples, along with a bulk processing sample, were then centrifuged at 20,000 RPM for 10 minutes, with the pellet discarded. The supernatant was then filtered through 10 kDa centrifuge filters at 3000 rpm for 30 minutes. The residue was then washed with < 1mL of 8 M urea and re-filtered. The residue was then added to 1% formic acid and delivered to the McGill University Health Center Research Institute (RI-MUHC) where they were stacked on an SDS gel for ~15 minutes to form 1 band, which was cut out and prepared via in-gel digestion, reduction, alkylation, and trypsinization. The samples were then run through a uHPLC-MS/MS over a two hour gradient. Data was then searched against a database in MASCOT analyzed in Scaffold (Proteome Software).

2.3. Atomic Force Microscopy (AFM) and Nanospectroscopy (AFM-IR)

MAE (0.1-0.001 mg/mL) was ultrasonicated in an ice bath using a Branson ultrasonic homogenizer equipped with a 12.7 mm tip at 80% amplitude for 5 min at 30 s on/off intervals. The solution was drop-casted on freshly cleaved mica, silicon wafers (cleaned with RCA-1), or silicon wafers having a ~82 nm thick gold coating. AFM was performed in tapping mode on an Oxford Instruments ® MFP-3D, and AFM-IR performed in contact mode on a Bruker ® NanoIR. AFM-IR utilizes a specialized probe tip which, under the influence of an infrared laser, resonates at known frequency. Fluctuations in this frequency based on the chemical identity of the material which the probe tip is contacting (or oscillating over) correlate to infrared spectroscopic peaks. Both nanoscale point scans and frequency maps can be generated using this method.

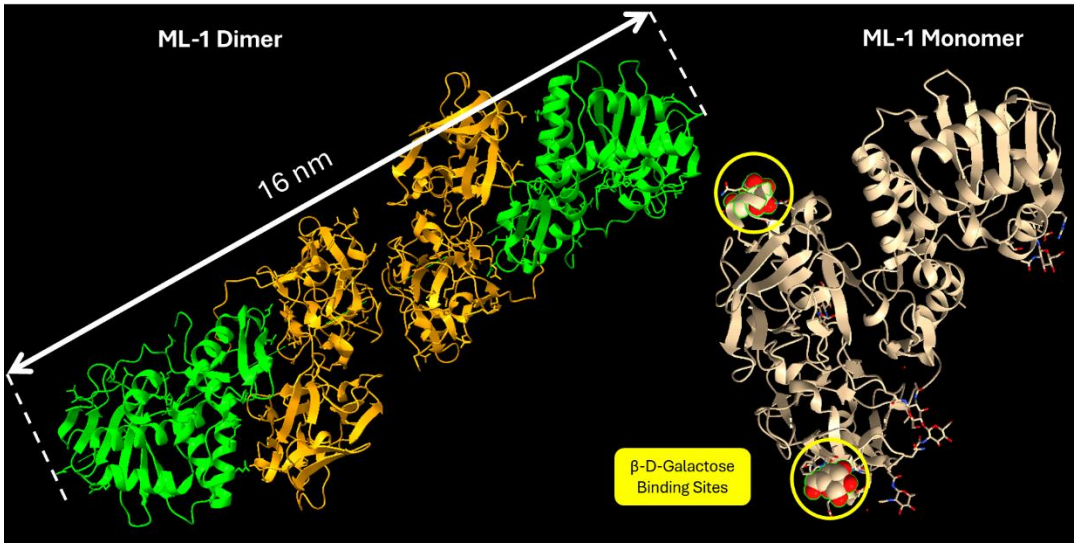
2.4. TEM

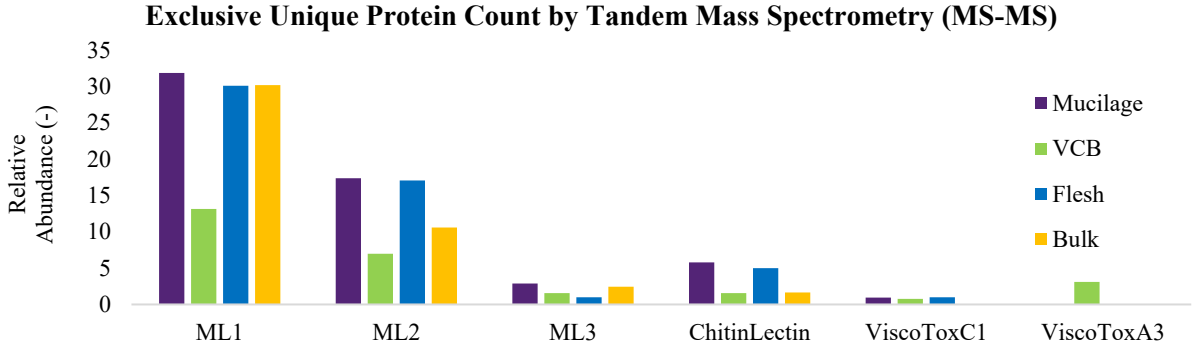
MAE (0.001 mg/mL) was ultrasonicated in an ice bath using a Branson ultrasonic homogenizer equipped with a 12.7 mm tip at 80% amplitude for 5 min at 30 s on/off intervals. The solution was drop-casted onto a carbon-backed copper grid (Electron Microscopy Sciences). A ThermoScientific Talos F200X S/TEM operated at 200 keV, with high brightness XFEG Schottky source was used for imaging along with Velox® software.

3. Results and Discussion

Reports in literature claim only 1-2% protein content in viscin [6], which were able to experimentally validate in MAE via Bradford microplate assay [13]. Initially, we performed SDS electrophoresis gels under fully reducing conditions, which showed evidence of individual chains of mistletoe lectins around the 30 kDa mark [14-16]. This prompted us to use a proteomics

approach to cross-reference tandem mass spectrometry (MS-MS) of mistletoe extracts from individual tissues (VCBs, Mucilage, Flesh, and Bulk) against the transcriptome (Figure 4). The proteins were broken down into smaller peptides via tryptic degradation, selectively lysing at the arginine and lysine residues. This ensures the proteins are of acceptable mass such that they can be processed and analyzed by the system and can be cross-referenced in the database of known *Viscum album* proteins. Results of these experiments provided strong evidence for the abundance of lectins, particularly the β-Gal binding ML-1. This glycoprotein is of particular interest given its ability to bind to four β-Gal termini concurrently in its dimerized form, meaning that it could hypothetically crosslink up to four polysaccharides [15-19].



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1   MNGHLASRRAWVWYFLMLGQVFGATVKAETKFSYERLRLRVTHQTTGEEYFRFITLLRDYVSSGSFSNEIPLLRQSTIPVSDAQRFVLVELTNEGGDSIT 100
101 AAIDVTNLYVVAYQAGDQSYFLRDAPRGAETHLFTGTTRSSLPFNGSYPDLERYAGHRDQIPLGIDQLIQSVTALRFPGGSTRTQARSILILIQMISEAA 200
201 RFNPILWRARQYINSGASFLPDVYMLELETSWGQQSTQVQQSTDGVFNNPIRLAIPPGNFVTLTNVRDVIASLAIMLFVCGERPSSSDVRYWPLVIRPVI 300
301 ADDVTCSASEPTVRIVGRNGMCVDVRDDDFHDGNQIQLWPSKSNNDPNQLWTIKRDGTIRSNGSCLTTYGYTAGVYVMIFDCNTAVREATLWEIWGNGTI 400
401 INPRSNLVLAASSGIKGTTLTVQTLDYTLGQGWLAGNDTAPREVTIYGFRDLCMESNGGSVWVETCVISQQNQRWALYGDGSIRPKQNQDQCLTCGRDSV 500
501 STVINIVSCSAGSSGQRWVFTNEGAILNLKNGLAMDVAQANPKLRRIIIYPATGKPNQMWLPVP 564
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*Figure 4: **MS-MS of extracts from various mistletoe berry tissues**. Top: Experimental X-Ray diffraction crystal structures of Mistletoe Lectin 1 visualized in Chimera X, via Protein Data Bank (1SZ6 and 1OQL). A and B chains are coloured green and orange, respectively. Middle: Semi-quantitative MS-MS data. Mistletoe lectins 1, 2 and 3 (ML-1, ML2, ML3) can be found in all tissues. Interestingly, Viscotoxin A3 was only detected in the VCBs, which may suggest a role in fiber-formation. Bottom: Matching ML-1 peptides detected in MS/MS, underlined. Amino acids responsible for β-Gal binding are coloured red.*

Having confirmed the presence of mistletoe lectins in our adhesive extract, we wanted to validate its co-localization with MAE hemicellulosic polymers. Firstly, using atomic force microscopy (AFM) and transmission electron microscopy (TEM), we were able to image the individual components of MAE, including the high MW polymers present, which appear as long branched fibrillar structures. (Figure 5A-B). Notably, with AFM, we observe raised nodules co-localized with the branched polymeric structure, and in TEM we see more-electron dense nodules co-localized with the polymer as well. We hypothesize that these nodular structure might be proteinaceous in nature, almost an inverse of gum arabic, whose glycoproteins have a protein backbone with arabinogalactan side chains [10]. In order to investigate this hypothesis, MAE was investigated using nano-IR spectroscopy – a cutting-edge method combining AFM mapping with vibrational spectroscopy (Figure 5C-D). The IR resolution limit requires higher concentrations of MAE (0.1 mg/mL vs. 0.001 mg/mL) for sufficient signal, thus individual polymers cannot be spectroscopically characterized. Thus, the bulk material was imaged targeting the amide I peak (~1650 cm^{-1}) – the primary infrared (IR)-active peak used for protein characterization [20]. In this imaging, there is clear signal above baseline in the MAE network, providing preliminary evidence for protein-arabinogalactan colocalization. The amphiphilic, variable charge surface of these proteins are hypothesized to contribute to the high adhesion offered by MAE by interacting with a large variety of chemistries and may be serving as crosslinks in the network, strengthening and stiffening the system (in a water-responsive manner). Protein crosslinks are a relatively unexplored mechanism in polysaccharidic materials and may prove useful in the design of future sustainable, biodegradable matrix materials for cellulosic composites.

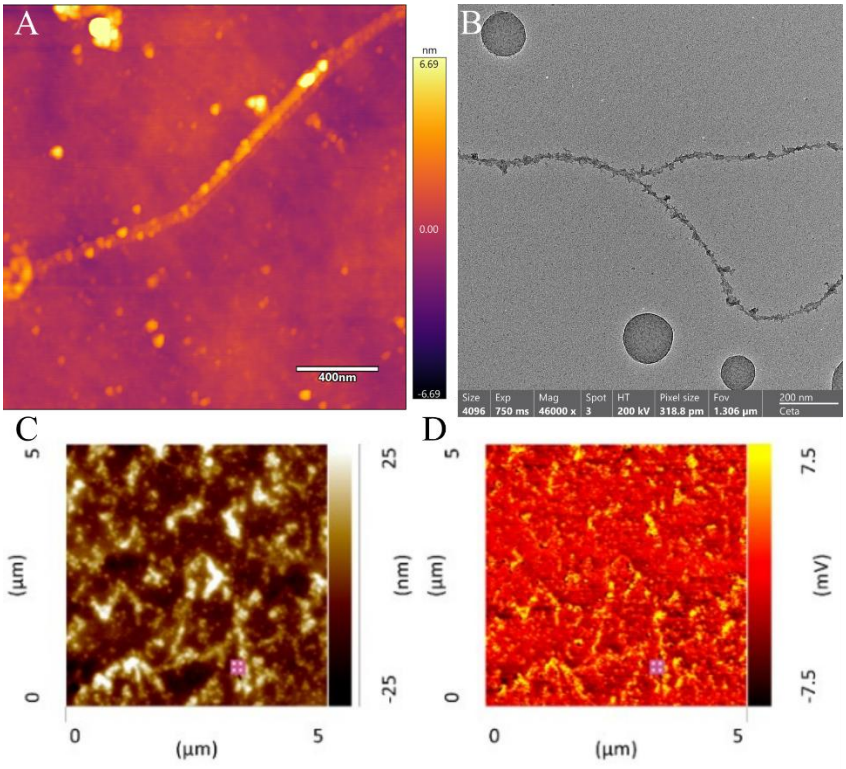

Figure 5: ***Nanoscale characterization of MAE.*** *A) AFM of MAE showing arabinogalactan polymer. B) Bright-field TEM of arabinogalactan polymer, with condensates of small molecules (largely free glucose). AFM-IR of MAE deposited on gold-coated silicon, with C) height map and D) IR intensity map for 1650 cm^{-1} amide I peak, an indicator of protein content.*

4. Conclusion

Mistletoe berry fibers transition from storage as a soft millimeter scale tissue into rigid, meter-long fibers, by promoting alignment and cross-linking of the cellulose nanofibrils, and acting as an in-situ deployable material. The preferential alignment of the reinforcement phase improves mechanical performance, enhancing function. Inspiration can be taken from this for processing stimuli-responsive materials, which stiffen and strengthen under load (which would uncoil, align, and densify the reinforcement phase).

As an intrinsically sustainable material, mistletoe viscin and its adhesive extracts offer potential unique alternatives to fossil fuel derived materials. While our current findings are still at the early stages, the protein components that we discovered to be present in MAE may be playing an important role in the material properties and processing of this amorphous, adhesive matrix. Future work will aim to further elucidate their role in the materials system, along with the production of bioinspired composites to validate the functionality of the individual components of the system.

Acknowledgement

This work was supported by the Natural Sciences and Engineering Research Council of Canada, and Fonds de recherche du Québec – Nature et technologies. Z.K acknowledges M. Rivard for his assistance with figure preparation.

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