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Tuning the water interactions of cellulose nanofibril hydrogels using willow bark extract

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ABSTRACT

Cellulose nanofibrils (CNFs) are increasingly used as precursors for foams, films and composites, where water interactions are of great importance. In this study, we used willow bark extract (WBE), an underrated natural source of bioactive phenolic compounds, as a plant-based modifier for CNF hydrogels, without compromising their mechanical properties. We found that the introduction of WBE into both native, mechanically fibrillated CNFs and TEMPO-oxidized CNFs increased considerably the storage modulus of the hydrogels and reduced their swelling ratio in water up to 5–7 times. A detailed chemical analysis revealed that WBE is composed of several phenolic compounds in addition to potassium salts. Whereas the salt ions reduced the repulsion between fibrils and created denser CNF networks, the phenolic compounds - which adsorbed readily on the cellulose surfaces - played an important role in assisting the flowability of the hydrogels at high shear strains by reducing the flocculation tendency, often observed in pure and salt-containing CNFs, and contributed to the structural integrity of the CNF network in aqueous environment. Surprisingly, the willow bark extract exhibited hemolysis activity, which highlights the importance of more thorough investigations of biocompatibility of natural materials. WBE shows great potential for managing the water interactions of CNF-based products.

1. Introduction

Cellulose is an important structural polysaccharide that has been used extensively during the long history of humankind as a robust and versatile material from renewable sources, e.g. plants and bacteria. However, its full potential as a nanomaterial was realized only in early 2000s with the development of pre-treatment strategies like chemical and enzymatic treatment of pulp fibres, which allowed for effective disintegration of cellulose bundles into nanofibrils at low energy consumption (Henriksson, Henriksson, Berglund, & Lindström, 2007; Isogai, Saito, & Fukuzumi, 2011; Pääkkö et al., 2007; Saito, Kimura, Nishiyama, & Isogai, 2007). Significant research work is now dedicated to expanding our understanding of nanocellulose materials and their behaviour so that they can be exploited for high-end functional materials, for example, in smart packaging and biomedical applications.

Nanocellulose is usually categorized into 3 groups: bacterial cellulose with ribbon-like morphology, cellulose nanofibrils (CNFs) and cellulose nanocrystals (CNCs). Nanocellulose can be modified chemically to manipulate its physical, chemical and optical properties. Most of its unique characteristics come from its chemical structure as well as high degree of crystallinity, which is inherently a result of the biosynthesis mechanism and the inter- and intramolecular hydrogen bonding network within and between cellulose chains in the CNFs (Klemm et al., 2018). In the plant cell wall the cellulose molecular chains are organized into microfibril bundles consisting of long crystalline fibrils with short-ranged defects or amorphous regions, embedded in the matrix of lignin and hemicellulose (Heise et al., 2021; Solhi et al., 2023). CNFs are the result of the defibrillation process of cellulose fibres, to create nanofibrils with very high aspect ratio. Therefore, chemical modification of CNFs is often only happening at the surface of the fibrils (Heise et al., 2021; Solhi et al., 2023).

A common knowledge in materials science is that surface chemistry governs the characteristics and behaviours of materials in different environments and this is especially true for nanomaterials. Native CNFs that are produced by mechanical disintegration methods from wood pulp exhibit low charge density, form opaque and weak hydrogels that

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are prone to collapse with time, e.g. after 3D printing. Saito and Isogai developed the method of 2,2,6,6-tetramethylpiperidine-1-oxyl radical (TEMPO) mediated oxidation to introduce carboxylic groups to the C6 primary hydroxyls of cellulose prior to mechanical disintegration, which significantly lowers the energy consumption of the process due to swelling induced by the electrostatic repulsion between the negatively charged moieties (Saito et al., 2007). The cellulose nanofibrils produced by this method are usually called TEMPO-oxidized cellulose nanofibrils or simply TEMPO-CNFs. Due to the extensive disintegration and the electrostatic repulsion, the TEMPO-CNFs are often of shorter diameter and length and more uniform in size than mechanically fibrillated CNFs, which render them to form more transparent and stronger gels than native CNFs, at the same concentration (Saito et al., 2007).

Although CNFs possess many appealing properties such as high strength-to-weight ratio, high aspect ratio, excellent gas barrier and optical clarity, they are very sensitive to water and moisture due to the extremely high density of -OH groups in their chemical structure. The hygroscopicity of CNFs is often perceived as an adverse characteristic since it can lead to the loss of mechanical and barrier properties of CNF-based products (Koivuniemi et al., 2021; Österberg et al., 2013; Peresin et al., 2017; Solhi et al., 2023). However, this is also the reason for many interesting characteristics of CNF, two of which are the tendency to form stable colloids with water at low concentration, i.e. hydrogels, and their shear thinning effect (Arola et al., 2022).

CNF-based hydrogels are found to be useful in many applications such as wound dressing, scaffolds for tissue engineering or rheology modifications for many industrial formulas (De France, Hoare, & Cranston, 2017; Heise et al., 2021; Klemm et al., 2018). The high affinity of CNFs to water was shown to be problematic in practice as it increases the duration and required energy of dewatering and drying process in CNF-based composites, films or coating for packaging (Amini, Tajvidi, Bousfield, Gardner, & Shaler, 2019; Collins & Tajvidi, 2022; Fall et al., 2022; Ringania, Harrison, Moon, & Bhamla, 2022). Uncontrollable water uptake and swelling when serving in moist and wet conditions can have a detrimental effect to the product performance. Wound dressing, for example, if poorly designed with too high water retention, can cause accumulation of wound exudates, which hinder the healing process. The volume expansion of an excessively swollen hydrogel-based wound dressing might compromise the wound site and risk bacterial infection (Holback, Yeo, & Park, 2011; Xu et al., 2016). The key issue is to understand the mechanism and find ways to control the interactions between cellulose nanofibrils and water.

The interaction between cellulose and water has been under investigation for roughly a century due to its significance in paper and packaging industry (Heise et al., 2021; Urquhart & Williams, 1924). However, nanocellulose has very different properties than its macroscopic pulp fibre counterpart (e.g. tendency to form gel at low concentration) due to its higher surface-to-volume ratio. It is utilized in a broad range of applications and in various forms, e.g. as ultrathin films, hydrogels or aerogels (De France et al., 2017; Kontturi & Spirk, 2019). When used alone, CNF hydrogels rely on weak physical interactions such as hydrogen bonding and chain entanglements to maintain their stability, and thus, the fibrous percolating network is prone to disintegration when immersed in water for too long (De France et al., 2017). Attempts have been made to improve the mechanical properties and manage the water affinity of CNF hydrogels, such as introduction of covalent crosslinkers, metal ions or using CNFs in combination with other polymers (Liang, Bhagia, Li, Huang, & Ragauskas, 2020; Markstedt et al., 2015; Markstedt, Escalante, Toriz, & Gatenholm, 2017; Narwade, Khairnar, & Kokol, 2018; Pfister et al., 2004; Wang, Wang, & Xu, 2020; Yang, Abe, Biswas, & Yano, 2018). However, the incorporation of crosslinking agents can greatly alter the interaction of CNFs and water. The water interactions of nanocellulose on their own and in multi-component complexes, thus, deserve some specific attention, especially at nanoscale.

To fight the depletion of non-renewable resources, extensive research activities are devoted to the identification of natural occurring substances that could substitute fossil-based counterparts and valorisation of waste stream. Utilizing biochemicals from wood bark waste stream is one of such efforts (Pásztory, Ronyecz Mohácsiné, Gorbacheva, & Börcsök, 2016), and in this study willow bark was used to extract useful phenolic compounds as an attempt to improve the properties of CNF hydrogels. Willow bark is a great source of bioactive phenolic compounds that serve as the plant's defence strategy against pathogens and other environmental attacks. The phenolic compounds present in willow barks – (+)-catechin, salicin, picein and triandrin – can be extracted easily via hot water extraction (Dou et al., 2018; Dou et al., 2022). Picein and catechin were found to possess antioxidant properties while willow bark extract as a whole was reported to offer protection against certain virus, bacteria and UV light, which can provide valuable functions to the hydrogels (Lohtander, Grande, Österberg, Laaksonen, & Arola, 2021; Luthria, Jones, Donovan, & Waterhouse, 1997; Tienaho et al., 2021).

Moreover, previous works show that (+)-catechin can be oxidized and polymerized using laccase enzymes. Lohtander et al. (2021) took advantage of this feature and attempted to make double network hydrogels by mixing willow bark extract (WBE) into TEMPO-oxidized CNF gels and polymerizing the bark extract in-situ using the *Trametes hirsuta* laccase (ThL) enzyme. Since there is very little data in current literature on the actual efficiency of the polymerization (e.g., degree of polymerization, molecular weight and structure of polymeric products), or on the spatial arrangement of WBE and TEMPO-CNFs, it is difficult to evaluate the success of earlier attempts, apart from speculations from indirect observations, such as rheological measurements and ATR-FTIR (Latos-Brozio & Masek, 2019; Lohtander et al., 2021).

It is desirable to control the water interaction of CNFs in many applications, for example to maintain hydrogel fidelity and structure in aqueous working environment, or to effectively dewater CNF-based thin films in a controlled manner. Hence, this work aims at providing a deeper understanding of the interactions between CNFs and WBE, and how those interactions affect the rheological properties and hydration of CNF hydrogels. Excess of electrolytes will have a decisive effect on swelling behaviour of hydrogels but to date the presence of salt in WBE or its possible effect on the hydrogel's water interactions have not been discussed. Both native, mechanically fibrillated CNFs and TEMPO-CNFs were selected for this study to analyse the effect of different CNF types (that is, with different surface charge and nanofibril size) on the interaction with WBE. We show that hot water extracted WBE contains electrolytes and both these electrolytes as well as the phenolic compounds of WBE affect rheology and swelling of CNF hydrogels. As WBE can offer protection against a number of biological pathogens and environmental factors, the hydrogel complexes from WBE and CNFs were also tested to explore their suitability for biomedical applications.

2. Experimental section

2.1. Materials

The phenolic compounds in willow bark were obtained using a hot water extraction method described by Lohtander, Arola, and Laaksonen (2020). Branches of two-year-old Klara hybrid (harvested in May 2019 from Kouvola, Finland) were removed from the freezer and debarked. The chopped willow bark was cooked with deionized water (bark-to-water ratio was kept at roughly 1:20) at 80 °C for 50 min with constant stirring at 250 rpm. The extract was then filtered with qualitative filter paper (particle retention 12–15 μm, VWR) and centrifuged (4500 *g*, 20 min, 21 °C). The supernatant was collected and freeze-dried into powder and stored at −20 °C. The enzyme *Trametes hirsuta* laccase (ThL—H345, P2) with the activity 5533 nkat/mL and protein concentration of 3.5 mg/mL was kindly provided by VTT Technical Research Center. Catechol (CAS 120–80-9, purity ≥95 %), (+)-catechin hydrate (CAS 225937–10-0, purity ≥98 %), and D-(−)-Salicin (CAS 138–52-3, purity

≥98.5 %) were purchased from Sigma-Aldrich as reference compounds for high performance liquid chromatography (HPLC), size-exclusion chromatography (SEC) analysis and hemolysis assay, and they were used as received.

Native CNFs were prepared by mechanical fibrillation of never-dried bleached hardwood kraft pulps from Finnish pulp mills following a procedure described by Österberg et al. (2013). Briefly, the pulp was first washed into sodium form, as described by Swerin, Odberg, & Lindström (1990) to control the counterion species and ionic strength, then with deionized water to remove excessive electrolyte. Next the washed pulp was refined with a Voith refiner (Heidenheim, Germany) and fibrillated using a M-110P fluidizer (Microfluidics, Newton, MA), with 6 passes. TEMPO-CNFs were also prepared using the same washed pulp, and the oxidation was carried out after the washing step following the procedure described previously by Saito et al. (2007), using 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO), sodium bromide and sodium hypochlorite, all chemicals were purchased from Sigma-Aldrich. The TEMPO-oxidized pulp was then fibrillated with 1 pass using the same fluidizer as for native CNF to obtain TEMPO-CNFs. The final dry matter content of native and TEMPO-CNFs was 2.08 wt% and 1.68 wt%, respectively. The hydrogels were stored at +4 °C until use.

2.2. Atomic force microscopy

The morphology of the CNFs was characterized with a Bruker MultiMode 8 atomic force microscope (AFM) connected to a NanoScope V controller (Bruker, Santa Barbara, CA). A thin layer of CNFs was deposited on Ti/Au-covered quartz crystal sensors by spin coating, as described in the Section 2.7. The topographical images were obtained using ScanAsyst in air, at room temperature, with a J scanner and ScanAsyst-Air probes (Bruker AFM probes, Camarillo, CA). The images were analysed with NanoScope Analysis 1.5 software (Bruker). First order flattening was the only image processing step.

2.3. Conductometric titration

Conductometric titration was performed to determine the carboxylate contents of the two CNFs with a Metrohm 712 conductometric titrator (Metrohm, Brazil). In brief, 500 mL of CNF suspensions were prepared with at least 0.3 g dry matter content and then protonated with 0.1 M HCl to reach pH 3–3.5. The suspensions were titrated with 0.1 M NaOH by adding 0.05 mL in 30 s intervals. The conductivity was monitored during the titration and the carboxylate contents were determined as weak acid groups on the titration curves.

2.4. Chemical analysis of willow bark extract

The elemental composition of WBE was determined by X-ray photoelectron spectroscopy (XPS) with a Kratos AXIS Ultra DLD X-ray photoelectron spectrometer using a monochromated Al K_α X-ray source (1486.7 eV) run at 100 W. Willow bark extract was deposited on clean silicon wafers for XPS by adsorption. Survey spectra were collected using a pass energy of 80 eV, at 1.0 eV step size while high resolution spectra were obtained using 20 eV pass energy and 0.1 eV step size. The X-ray beam spot is 1 mm in diameter and photoelectrons were collected at a 90° take-off angle under ultra-high vacuum conditions, with a base pressure typically below 1×10^{-9} Torr. Both survey and high-resolution spectra were collected from three different spots with a scan size of 300 μm × 700 μm to examine the homogeneity and surface charge effects. The average elemental composition from 3 spots is presented.

The phenolic content of willow bark powder was determined with a Shimadzu preparative high-performance liquid chromatography (HPLC) equipped with a SPD-M20A diode array detector and a coupled column system: Semi-preparative Luna® Omega 5 μm PS C18 100 Å (250 × 10 mm) and Kinetex® 5 μm Biphenyl 100 Å (250 × 10 mm). The samples were dissolved in a water-acetonitrile mixture (92:8) at the concentration of 5 mg/mL. An ultrapure water – acetonitrile mixture (90:10) was used as eluent with a flow rate of 3 mL/min. The injection volume was 200 μL, and the phenolic compounds were detected at the wavelength 210 nm. As catechin and salicin were the main compounds of interest in terms of possibility to polymerize and therapeutic properties, quantitative analysis was performed for these two compounds. Due to difficulties in obtaining picein and triandrin of high purity as standards, we skipped the quantitative analysis of these two phenolic compounds and used previous works as reference (Dou et al., 2016, 2018; Dou et al., 2022).

The molar mass of the enzymatically polymerized WBE was characterized with size-exclusion chromatography using the procedure previously described by Lohtander et al. (2021). Briefly, WBE solutions with increasing concentrations were polymerized with ThL for 4 h at 37 °C, then freeze-dried into powder. The polymerized WBE were then dissolved in 0.1 M NaOH to a final concentration of 2 mg/mL and filtered with a 0.45 μm syringe filter. The dissolved samples were analysed with HPLC using an Agilent 1100 series system (Agilent Technologies, US) equipped with a refractive index detector and three Polymer Standards Service MCX 300 × 8 mm columns (pore size of 100, 500 and 1000 Å). The calibration curve was obtained with polystyrene sulfonate standard (1000–64,000 g/mol), ascorbic acid (176 g/mol) and NaCl (58 g/mol). The test was performed at a flow rate of 0.7 mL/min, and the injection volume was 50 μL.

2.5. Preparation of CNFs and WBE hydrogels

As mentioned previously, there is a possibility to polymerize the catechin in WBE with laccase enzyme and create a percolating network of phenolic compounds that is intertwined with CNF network. Gong (2010) suggested that the ratio between the two networks should be very high, where the content of the second network is roughly tens of times of the first network. Therefore, high ratios of WBE to CNFs were used to evaluate the prospect of this theory where polymerized catechin was expected to act as the second network.

A WBE stock solution was prepared by dissolving freeze-dried WBE powder in deionized water. The CNF hydrogels were mixed with the WBE stock solution to achieve samples with the different final CNFs: WBE ratio by weight (1:2, 1:3, 1:5 and 1:10), keeping the CNF concentration fixed at 1.2 wt% for all samples. The homogenization was done with an electric handheld mixer for roughly 2 min until the mixture was uniform. The samples were then divided into two parts – one was kept untreated and the other was enzymatically treated with ThL (20 nkat/mL) at 37 °C for 4 h to promote in situ polymerization. All the samples were then refrigerated at +4 °C and used within 3 days.

The XPS results revealed that WBE contains a small amount of potassium salts, probably potassium sulfate, which can greatly affect the behaviour of CNF hydrogels. Therefore, hydrogels of native and TEMPO-CNFs with the presence of K_2SO_4 (analytical grade, Sigma Aldrich) at increasing concentration, from 5 to 40 mM, were also prepared and used for rheological measurements and swelling tests to determine the effect of inorganic salts.

2.6. Zeta potential measurements

The changes in zeta potential of cellulose nanofibrils with the presence of willow bark extract were also studied with a Zetasizer Nano ZS90 instrument (Malvern Instruments, UK) using the dip cell. A very diluted concentration of cellulose nanofibrils of around 0.01 wt% was chosen for studying the effect of increasing content of willow bark extract on the colloidal stability of the CNF suspensions. The zeta potential measurements were conducted in triplicate at 20 °C in 10 mM NaCl, at pH 8, using Smoluchowski equation.

2.7. Quartz crystal microbalance with dissipation monitoring

The affinity of WBE to CNF thin films was characterized by quartz crystal microbalance with dissipation monitoring (QCM-D) using a Q-Sense E4 instrument (Q-Sense, Gothenburg, Sweden). Ti/Au QCM-D sensors were purchased from AWSensors (Valencia, Spain) and cleaned with a UV ozone cleaner (BioForce Nanosciences, Virginia, US) for 15 min. A volume of roughly 200 μL of 2.5 mg/mL polyethyleneimine solution (PEI, branched, average $M_w \sim 25{,}000$ by LS, Sigma-Aldrich) was deposited on the cleaned surface by adsorption for 10 min, followed by rinsing with water and drying with nitrogen. Then, the PEI-coated sensors were spin coated with a CNF suspension (4000 rpm, 1 min) using a Laurell spin-coater WS-650SX-6NPP-Lite (Laurell Technologies Corp., North Wales, PA). The fine fraction of the suspensions of native CNFs and TEMPO-CNFs (ca. 0.03 wt% and 0.07 wt%, respectively) to be used for QCM-D experiments were prepared beforehand according to the procedure described by Valle-Delgado, Johansson, and Österberg (2016). Briefly, CNF hydrogels were diluted with deionized water to achieve a suspension of roughly 0.1 wt% that was then dispersed with an ultrasonication tip (Branson sonifier S-450 D) at 25 % amplitude for 1 min. The CNF suspensions were centrifuged using Eppendorf centrifuge 5804R (Germany) at 8000 *g* for 30 min at room temperature. The supernatants were considered as the fine fraction of CNFs, which were collected and stored at +4 °C before use for QCM-D experiments, while the CNF suspensions before centrifugation were called unfractionated CNFs and used for bulk characterizations.

QCM-D was used to track the adsorption and possible desorption of WBE on/from the prepared nanocellulose thin film. The corresponding changes in film viscoelasticity were studied to understand how the adsorbed WBE affected water interactions of different types of CNFs. Native and TEMPO CNF thin films were first stabilized with deionized water until a stable baseline was obtained. Then the WBE solution (10 mg/mL) was injected until the change in the resonance frequency value of the sensor became stable. The CNF thin films were finally rinsed with deionized water to evaluate the desorption of previously adsorbed WBE compounds. For comparison, the adsorption of catechin (10 mg/mL) on CNF thin films was also monitored following a similar procedure. The flow rate was kept constant at 100 μL/min during the experiments. The changes in resonance frequency Δf and dissipation factor ΔD of the fifth overtone were recorded and the corresponding sensed mass Δm were calculated by the Sauerbrey equation, where C is the mass sensitivity constant – a property of quartz, and n is the number of the harmonic (Sauerbrey, 1959).

$$\Delta m = -C \cdot \frac{\Delta f}{n}$$

For the 5 MHz Ti/Au coated quartz sensors used in these QCM-D experiments, the C equals 17.7 ng/(cm^2.Hz). The measurements were done in triplicate. One representative set of data and the average sensed mass after 120 min and after final rinsing with corresponding standard deviations are presented in this study.

2.8. Rheological measurements

The rheological measurements were performed on an Anton Paar MCR302 rheometer with a smooth 25 mm plate-to-plate geometry to observe the effect of WBE (untreated and ThL treated) on the viscosity and strength of native and TEMPO-CNF hydrogels. Amplitude sweeps (shear strain between 0.01 % - 100 %) were carried out at 25 °C and a constant frequency of 1 rad/s to determine the linear viscoelastic region (LVER). Measurements were done in duplicate.

2.9. Hydrogel swelling tests

The hydrogels were cast into a 1.5 × 1.5 × 0.5 cm (V_{before}) mould placed in a Petri dish. Deionized water was added to immerse completely the hydrogels, and they were left to swell overnight at room temperature with a second mould on top of the first one. Then the leaching solution was removed, and the swelling degree was measured using the method of Zu et al. (2012). Briefly, the swollen volume of the hydrogels was determined as the difference between the total volume of the container ($V_{container}$, that is the volume of 2 moulds) and the volume of an inert liquid (vegetable oil) added to fill this container. The volume of the added oil was obtained from the measured mass (m_{oil}) and density (ρ_{oil}). Thus, the swelling ratio of the hydrogels was calculated according to the following equation:

$$Swelling\ ratio\ (\%) = \frac{\left(V_{container} - \frac{m_{oil}}{\rho_{oil}} - V_{before}\right)}{V_{before}} \times 100$$

2.10. Hemolysis assays

The hemocompatibility of the hydrogels from WBE and CNFs were tested using human red blood cell (RBC) suspensions obtained from Finnish Red Cross – Blood Service under the permission No. 58/2021. The red blood cell suspension, stored at +4 °C before the test, was washed 3 times with sterile phosphate-buffered saline, centrifuged with a centrifuge 5804 (Eppendorf, Germany) at 2000 rpm for 5 min, and then the supernatant was discarded. Hydrogel samples of CNFs and WBE, pure WBE solutions, as well as reference compounds such as catechin and salicin, with increasing concentration (from 50 to 200 μg/mL) were distributed to 1.5 mL Eppendorf tubes. An amount of 20 μL of suspensions or 200 mg of hydrogels were used. A Triton X-100 was used as the assay positive control and pure phosphate-buffered saline as a negative control. Then, 1 mL of cell suspension was added to Eppendorf tubes and incubated for 1 h at 37 °C. After incubation, the samples were centrifuged with a microcentrifuge 5425 (Eppendorf, Germany) at 2000 rpm for 5 min, and the supernatants were transferred to a 96 well-plate. The absorbance of the supernatant of the samples at 540 nm was recorded with a BioTek Eon Microplate Spectrophometer (BioTek, Winooski, VT, USA) and Gen5 2.09 software (BioTek). The hemolytic activity was determined according to this equation:

$$Hemolysis\ (\%) = \frac{\left(Abs_{Sample} - Abs_{negative}\right)}{\left(Abs_{positive} - Abs_{negative}\right)} \times 100$$

where Abs_{sample} is the absorbance of samples, $Abs_{negative}$ is the absorbance of the negative control (PBS, 0 % hemolysis) and the $Abs_{positive}$ is the absorbance of the positive control (Triton X-100, 100 % hemolysis). The hemolysis assay was performed in triplicate.

3. Results and discussion

3.1. Analysis of raw materials

The water binding of CNFs depends on the charge density and accessible surface area of the fibrils. Both TEMPO-CNFs and native CNFs are often used in the literature but differ both in surface charge and fibril dimensions. Hence, we compared these 2 materials.

The morphology of both types of CNFs was characterized by AFM (Fig. 1). As expected, the diameter of TEMPO-CNF fibrils was smaller and more uniform in both unfractionated suspension (Fig. 1b) and the fine fraction (Fig. 1d), compared to that of mechanically fibrillated CNFs, due to the higher repulsion between the charged fibrils. The centrifugation step removed larger and coarser fibril fractions from the native CNFs (Fig. 1a), reducing the population of large fibril aggregates observed in the AFM images of the fine fraction (Fig. 1c). Conductometric titrations confirmed the higher surface charge of TEMPO-CNFs (amount of carboxylic groups 943 μmol/g), compared to that of native CNFs (40 μmol/g).

We used WBE to control the water binding and rheology of CNFs. It has been reported that the main components in hot water extracts of

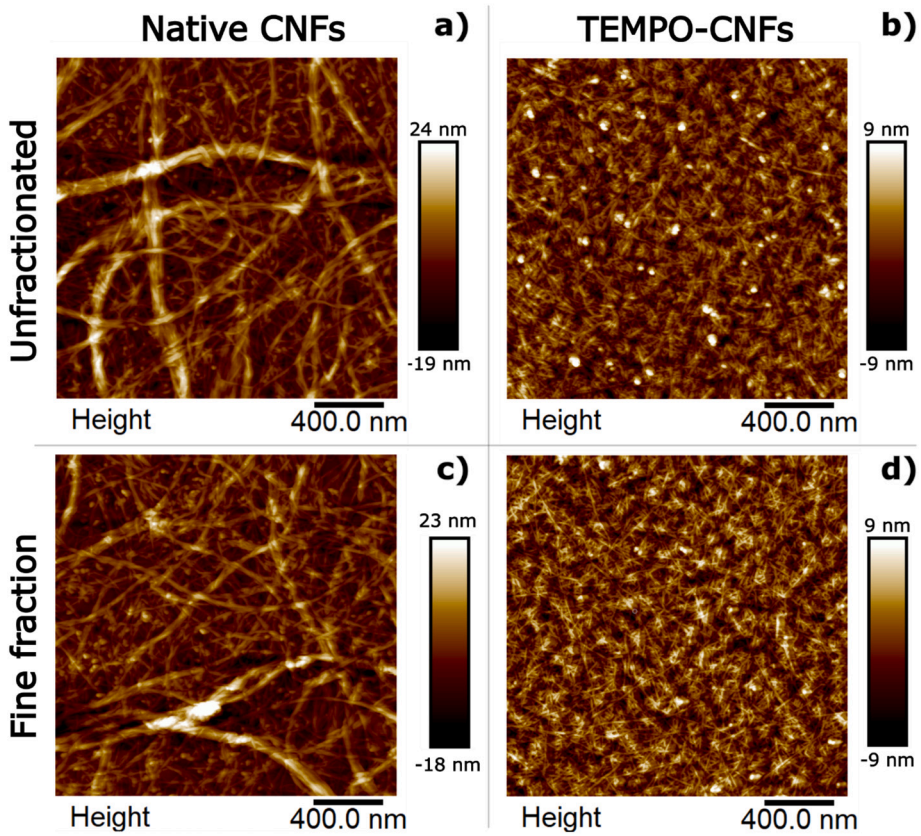

Fig. 1. Topography (height) images of unfractionated and the fine fraction of native, mechanically fibrillated CNFs (a and c) and TEMPO-CNFs (b and d). Scale bars represent 400 nm.

willow bark are (+)-catechin, salicin, picein and triandrin (Fig. 2), as well as other simple sugars (Dou et al., 2018). The content of phenolic compounds in our willow bark samples was analysed with HPLC.

Quantitative analysis of the HPLC was performed only for (+)-catechin and D-(−)-salicin due to the difficulties in obtaining picein and triandrin standards of high purity. The results show that (+)-catechin and D-(−)-salicin were present in the WBE at very low concentrations (approximately 2 wt% and $\leq$ 1 wt%, respectively) while triandrin might be present at higher quantity (Fig. 3). Dou et al. (2022) used GC-FID to quantify the content of these phenolic compounds and their results shows that WBE from Klara hybrid contains around 2 wt% of (+)-catechin and <1 wt% of D-(−)-salicin, and the triandrin accounts for roughly

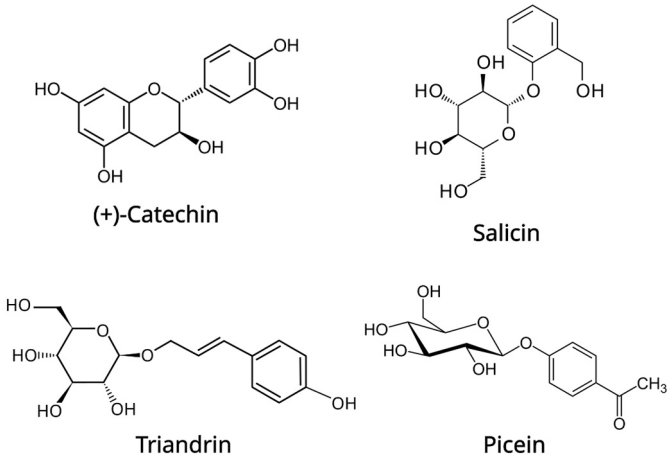

Fig. 2. Phenolic compounds present in willow bark extract.

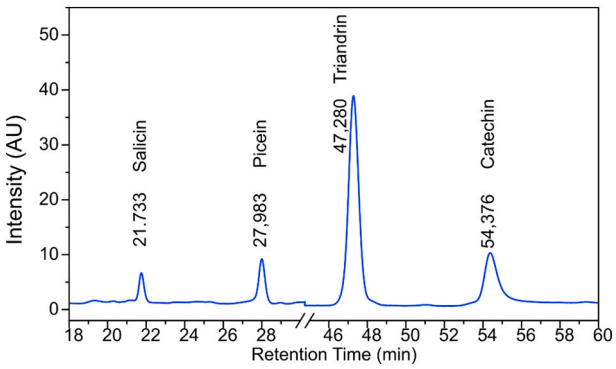

Fig. 3. HPLC chromatogram (at 210 nm) of willow bark extract.
The main detected peaks were of catechin, salicin, picein and triandrin.

12 wt% of WBE. Our results agree with their study (2022) but they were very far from another report of the same author group where catechin was quantified at 11 wt% in WBE from Karin hybrid (Dou et al., 2018). This discrepancy suggests that the age and species of the willow are important factors in the composition of WBE as found in a recent study (Dou et al., 2022).

As part of our study, we wanted to examine if the polymerization of WBE compounds affected their interaction with CNFs and the rheological properties of the hydrogels. Thus, WBE was treated with ThL enzyme to provoke the polymerization of catechin via the condensation of catechol moiety. Size-exclusion chromatography (SEC) results showed that the enzymatically-induced polymerization of WBE was actually quite limited. The molar mass of polymeric materials in WBE, regardless of the dry matter content, was roughly around 2000 g/mol,

indicating that the product was mostly oligomers of around 6 or 7 catechin monomers. This result was significantly lower than the value reported in a previous work, where the average molecular weight of enzymatic polymerized materials of willow bark extract was found to be 30,000 g/mol (Lohtander et al., 2021), even though a higher concentration of willow bark extract was used in this study. We hypothesize that the low amount of catechin in our samples that serves as the most prominent substrate for the laccase enzyme could be the reason for the limited polymerization observed.

Another important component of willow bark extracts is ash. Ash content of willow bark has been studied using muffle-oven, and found to range from 5 to 10 wt% of the extract (Dou et al., 2016). However, no exact elemental analysis was conducted in previous studies. Here we used XPS and discovered that WBE also contained a small amount of K (0.81 atomic %) and trace amount of Mg (0.02 atomic %), mostly in the form of sulfate and nitrite salts, which might be corresponding to the ash content (Fig. S1). When dissolved in aqueous solution and used together with CNFs, the salts could be an important source of counterions that affects the stability and behaviour of CNF network.

Due to the difference in both surface charge and fibril dimensions of CNFs and TEMPO-CNFs we expected them to interact differently with WBE and hence we utilized QCM-D to get a deeper understanding of their interactions as discussed in the next section.

3.2. Affinity of willow bark extract to CNF networks

A successful combination of WBE and CNF hydrogels requires a good affinity between them. To evaluate that affinity, the adsorption and desorption of WBE phenolic compounds to model CNF films (prepared from fine fractions as the ones presented in Fig. 1c and d) was investigated by QCM-D (Fig. 4). We used a higher WBE concentration than in a previous work (Lohtander et al., 2021) (10 mg/mL versus 0.1 mg/mL, respectively) to better visualize the affinity of willow bark components to CNF ultra-thin films.

The fast and significant increase in Δm shows that adsorption of WBE and catechin occurred on both native and TEMPO-CNFs (Fig. 4a and b). This indicates that the phenolic compounds in WBE have high affinity to both types of CNFs. Considering that catechin and other phenolic WBE compounds are non-charged molecules, their adsorption on CNF films is probably mainly driven by an increase in entropy upon the release of water molecules structured around hydrophobic moieties of the CNFs and the phenolic molecules (Österberg, Henn, Farooq, & Valle-Delgado, 2023; Wohlert et al., 2022). Once adsorbed on the cellulose surfaces, van der Waals forces, hydrogen bonds between hydroxyl groups, and carbohydrate-aromatic stacking interactions could contribute to strengthen the attachment of WBE phenolic compounds on CNFs and prevent their desorption upon rinsing (Asensio, Ardá, Cañada, & Jiménez-Barbero, 2013). Nevertheless, quite many WBE molecules seem to be weakly attached to the nanocellulose films and they were washed away during rinsing with water, as can be observed by the decrease in Δm in Fig. 4a and d. Still, around half of the adsorbed material remained on the substrates after rinsing. A similar trend was observed with a single-component solution of catechin, where desorption also occurred but only to a limited extent (Fig. 4b and d). It is noteworthy that the

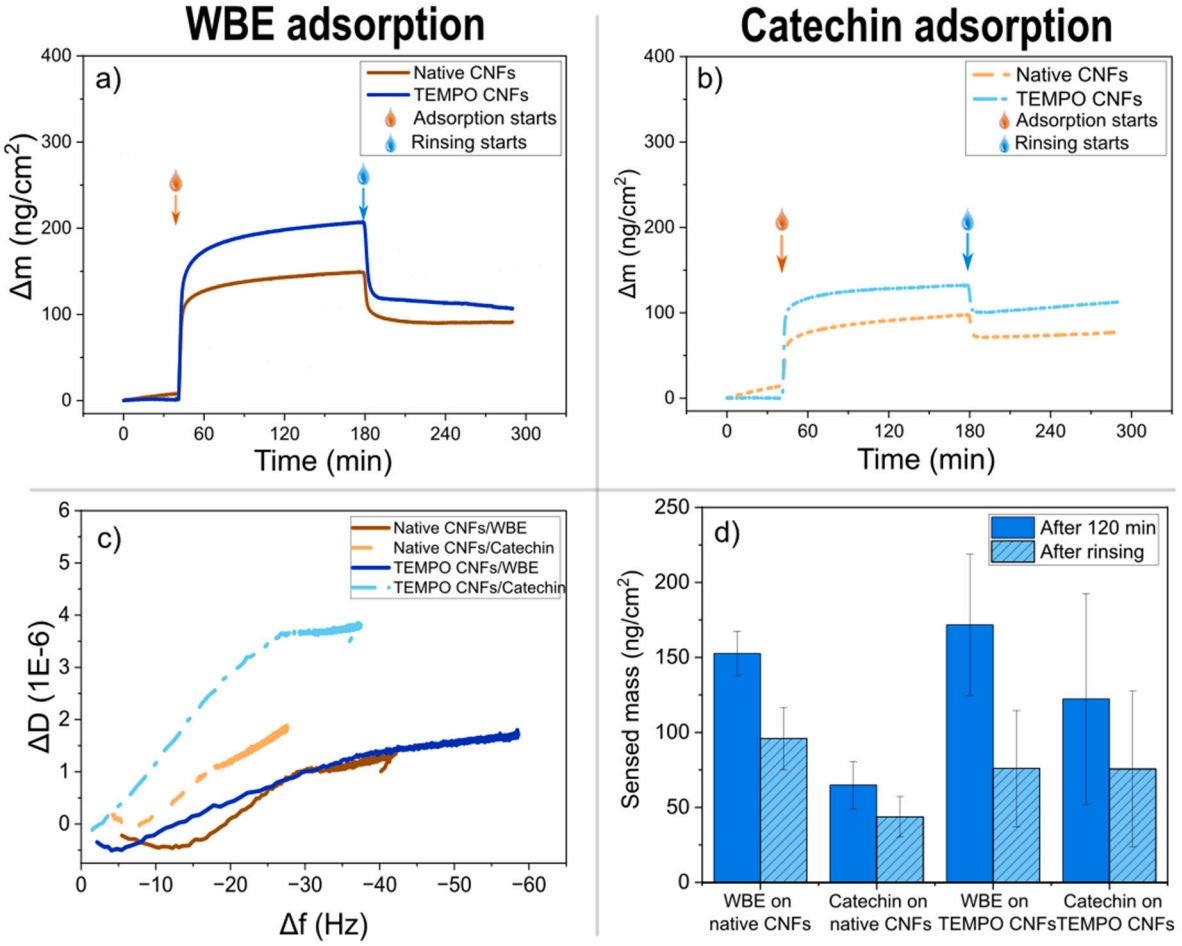

Fig. 4. The adsorption/desorption of WBE and catechin monitored with the QCM-D. Changes in adsorbed mass of WBE (a) and catechin (b) on native CNFs and TEMPO CNFs and the relation between Δf and ΔD during adsorption (c) were recorded. The sensed mass after 120 min and after rinsing (d) were calculated for all systems using eq. (1) and the mean value of three separate measurements is shown.

adsorption of catechin alone resulted in more viscoelastic layers than WBE, especially on TEMPO-CNFs, as indicated by the higher increase in the dissipation factor and the steeper slope of the ΔD-versus-Δ*f* curve (Fig. 4c).

It can be seen in Fig. 4a and b that TEMPO-CNFs attract more WBE and catechin than native CNFs. TEMPO-CNFs feature finer fibrils thanks to the high density of carboxylate groups on its surface, as observed in the AFM images (Fig. 1). This leads to a higher surface area, compared to thin films from native, mechanically fibrillated CNFs, which is probably the reason for greater adsorbed mass of both WBE and catechin.

Studying the Δ*D*-versus-Δ*f* curve (Fig. 4c) in more detail we observed that there was an initial decrease in Δ*D* while the sensed mass was still increasing (that is, Δ*f* was decreasing). That decrease in Δ*D* may suggest the formation of more compact (less swollen) CNF films due to the reduction of the electrostatic double layer repulsion between fibrils by the salt ions present in WBE. However, that deswelling of the CNF films should be accompanied by an increase in Δ*f* (loss of sensed mass) due to the released of water molecules. The fact that Δ*f* did not increase but steadily decreased seems to indicate that the mass gained by the adsorption of (+)-catechin and other phenolic compounds in WBE overcompensated the possible mass lost by the water release.

3.3. Effect of willow bark extract on the colloidal stability of CNFs

The effect of WBE on the colloidal stability of CNFs is an important issue that deserves attention. In this study, the colloidal stability of CNFs in the presence of WBE was investigated with zeta potential measurements (Fig. S2) and visual observation of aggregate formation. The negative zeta potential values confirm the negative surface charge of both native CNFs and TEMPO-CNFs. Although cellulose is a neutral molecule, native CNFs usually exhibit a low, negative surface charge attributed to remaining hemicellulose molecules attached to the nanofibrils (Li, Wu, Moon, Hubbe, & Bortner, 2021). The absolute value of zeta potential for TEMPO-CNFs is considerably larger than for CNFs, in agreement with the conductometric titrations showing a higher negative charge of the TEMPO-oxidized nanofibrils. No visible aggregates were observed in the diluted dispersions of the two CNFs in the absence of WBE, indicating that the electrical double layer repulsion between the fibrils induced by their surface charge was strong enough to make the CNF dispersions stable. However, the introduction of WBE caused a decline in the absolute value of the zeta potential of cellulose fibrils, resulting in less stable CNF dispersions as the WBE concentration increased. CNF aggregates were especially visible at the highest WBE concentration, although those aggregates could be reversibly dispersed by simply shaking. Since the phenolic compounds in WBE are non-charged molecules, the effect of WBE on CNF zeta potential and colloidal stability was only due to the ions (especially from potassium salts) present in WBE, which made the electrical double layer around the fibrils thinner and, consequently, reduced the double layer repulsion between fibrils more efficiently as the WBE concentration increased. The adsorption of phenolic compounds on CNFs and the effect of salt on CNF colloidal stability suggest that WBE may have a profound effect on the rheological properties and the swelling of CNF hydrogels, as discussed in the next section.

3.4. Effect of willow bark extract on rheological and swelling properties of CNF hydrogels

As QCM-D is a surface sensitive method using ultrathin film models, it gives information about surface interactions and from the results, we can assume that the willow bark extract interacts differently with the two examined types of CNFs. Rheological measurements, therefore, were done to reveal how the interfacial interactions between WBE and CNFs discussed above affect bulk properties of the hydrogels.

The influence of WBE on the viscoelastic properties of both native and TEMPO-CNF hydrogels was investigated using oscillatory shear rheology measurements (Figs. 5 and 6). The concentration of native CNFs and TEMPO-CNFs was kept at 1.2 wt% in all the samples. For both native and TEMPO-CNFs at a concentration of 1.2 wt%, storage modulus G' and loss modulus G" were found to be independent of the shear strain γ at low shear strains, indicating visco-elastic behaviour of the starting material. This is well in accordance with previous findings. CNFs are well known for their tendency to gel even at very low concentration, ranging from 0.05 wt% to 0.125 wt% depending on the charge density of the fibrils (Geng et al., 2018; Pääkkö et al., 2007).

The addition of WBE to the native CNF and TEMPO-CNF hydrogels significantly changed the microstructure and viscoelastic properties of the hydrogels. The amplitude sweeps showed that for native CNFs (Fig. 5), the linear viscoelastic region (LVER) of pure CNF sample extended beyond 3 % strain and the addition of WBE reduced the LVER to approximately 1 % strain, yet the storage modulus (G') and loss modulus (G") increased at least 10 folds. These observations indicate the formation of a denser, but less stable network compared to the original pure CNFs. As potassium salt was found in the WBE and the addition of WBE caused a reduction in zeta potential of CNF suspensions, the effect of potassium salt was further investigated by performing an amplitude sweep on CNF hydrogels with supposedly equivalent salt concentrations (Fig. S3). The moduli of CNF hydrogels in the presence of potassium sulfate alone followed an ascending trend as the ionic strength increased, while a significant increase was already observed in samples with low WBE content, followed by a plateau at higher WBE concentrations.

According to the Derjaguin-Landau-Verwey-Overbeek (DLVO) theory, an increase in electrolyte concentration shrinks the electrical double layers and reduces the repulsion between cellulose fibrils (Derjaguin & Landau, 1993; Verwey, 1947). Therefore, the addition of ions to the hydrogels provoked a reduction in interfibrillar distance and more contact points between fibrils. Arola et al. (2022) have also proposed that salt ions can increase the contact points between cellulose nanofibrils by affecting the arrangement of water molecules around cellulose surfaces. When comparing Figs. 5 and S3 it can be observed that the values of G' and G" are significantly higher for the hydrogels with WBE than for the hydrogels containing potassium sulfate but not phenolic compounds. That fact could be due to the higher dry matter content of WBE-CNF hydrogels, but it could also indicate that the adsorbed phenolic compounds might also contribute as bridging points between cellulose fibrils which, in addition to the reduction of interfibrillar distances induced by the salt ions, reinforce the entanglement of the cellulose nanofibrils. In any case, the introduction of WBE in the system resulted in a denser and more entangled fibril network that offered more resistance to deformation (increase in G' and G") but it also started losing its elasticity at lower strains (decrease in LVER).

An interesting feature that differentiates the effect of potassium salt from the whole WBE (including phenolic compounds) can be observed from the loss modulus. In pure CNF hydrogels, there was a small peak in the loss modulus G" right after the LVER, which is sometimes referred to as "overshooting phenomenon". This phenomenon of G" has also been reported for various soft materials and colloids (Asai, Masuda, & Kawaguchi, 2008; Donley, Singh, Shetty, Rogers, & Weitz, 2020; Hyun, Kim, Ahn, & Lee, 2002). It is typically associated with the interparticle interactions and amplitude dependent microstructure formation/breakdown, although alternative explanations have also been proposed (Donley, Singh, Shetty, Rogers, & Weitz, 2020; Hyun, Kim, Ahn, & Lee, 2002). CNF hydrogels, characterized by their high aspect ratio and strong interfibrillar interactions, exhibit a high tendency to form flocculated microstructures under shear, especially in concentrated regimes (Hubbe et al., 2017; Saarikoski, Saarinen, Salmela, & Seppälä, 2012). These fibre flocs exerted a resistant force before they were eventually broken down and the system started to flow (Arola et al., 2022). Remarkably, the addition of WBE to native CNFs eliminated the overshooting of G" (Fig. 5). The overshooting of G" was still observed in native CNFs in the presence of potassium sulfate, indicating that the

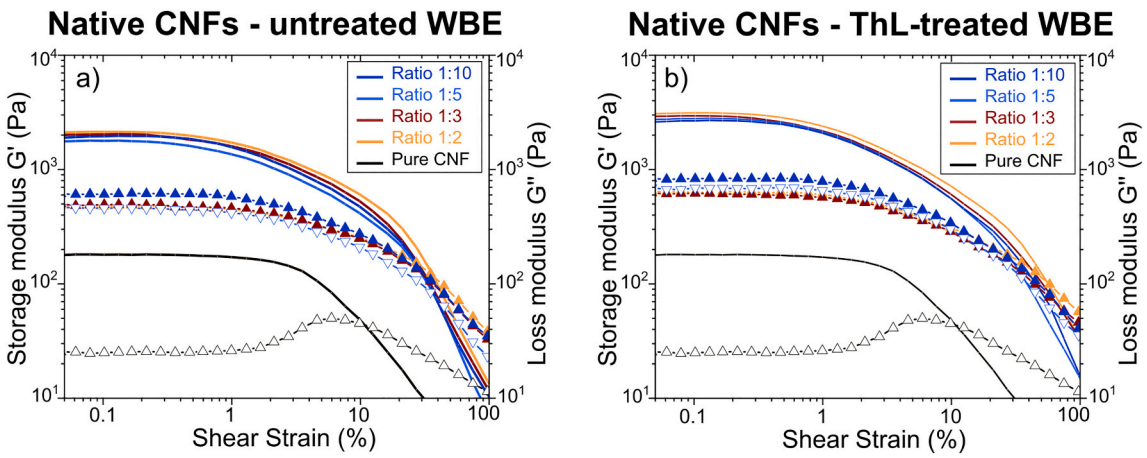

Fig. 5. Amplitude sweeps of hydrogel complexes from WBE (untreated (a) and ThL-treated (b)) and native, mechanically fibrillated CNFs for different CNFs:WBE weight ratios. Solid lines represent samples' storage modulus while the dash lines with triangle symbol represent loss modulus.

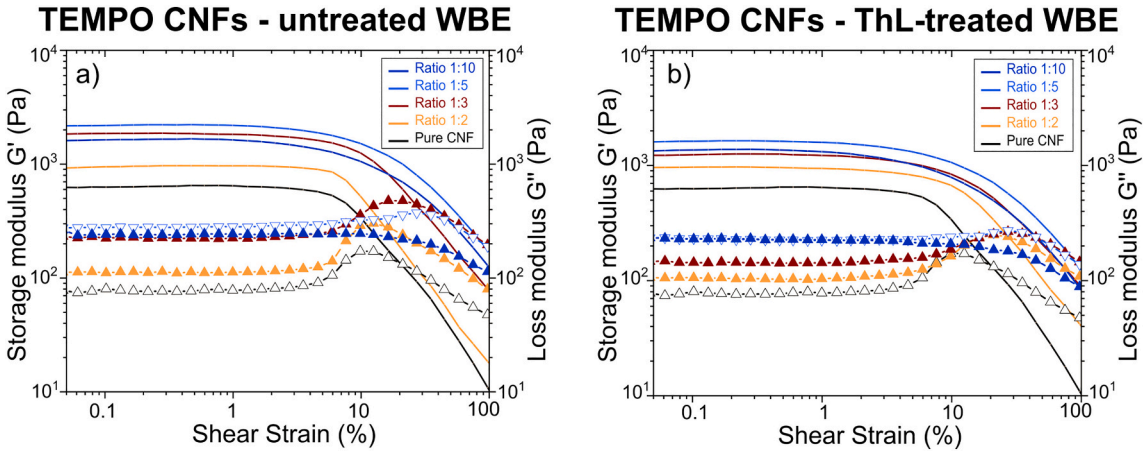

Fig. 6. Amplitude sweeps of hydrogel complexes from WBE (untreated (a) and ThL-treated (b)) and TEMPO-CNFs for different TEMPO CNFs:WBE weight ratios. Solid lines represent samples' storage modulus while the dash lines with triangle symbol represent loss modulus.

phenolic compounds of WBE were responsible for the elimination of the G" overshooting in WBE-CNFs hydrogels. In the light of these results, we hyphothesized that the phenolic compounds of WBE adsorbed on the CNF fibrils may enhance the lubrication between fibrils to some extent at high shear strains. That interfibrillar lubrication induced by the phenolic compounds could also explain the reduction in the LVER of the CNF hydrogels in the presence of WBE.

TEMPO-CNFs have a larger surface area as well as a higher surface charge compared to native CNFs, leading to stronger electrostatic double layer repulsions between fibrils and, consequently, a larger water binding capacity, which influences the viscoelastic properties of the hydrogels (Guccini et al., 2022; Österberg & Valle-Delgado, 2017; Salmi, Österberg, Stenius, & Laine, 2007). As shown in Fig. 6, pure TEMPO-CNFs had a higher storage modulus, indicating higher resistance to shear deformation compared to native, mechanically fibrillated CNFs. When WBE was incorporated into TEMPO-CNFs, the storage modulus gradually increased with the concentration of WBE until reaching a maximum value at CNF:WBE ratio 1:5. Furthermore, the G" overshoot remained in the hydrogels with low WBE concentrations but it was gradually diminished as the WBE content increased.

The TEMPO-CNFs exhibit a similar behaviour towards the presence of ions and phenolic compounds as CNF hydrogels. An increase in ionic strength decreased the electrostatic double layer repulsion between the fibrils, which resulted in a reduction in the separation distance between fibrils and the formation of a denser hydrogel with higher G'. On the other hand, phenolic compounds of WBE reduced to some extent the friction between cellulose nanofibrils, affecting the overshooting in G". Due to the higher surface charge of TEMPO-CNFs compared to native CNFs, higher concentrations of WBE (and higher concentration of potassium salt) were needed to screen effectively the repulsion between fibrils. Thus, the storage modulus showed a stepwise increase with increasing WBE content. In line with the QCM-D results (Fig. 4a), the larger surface area of TEMPO-CNFs in comparison to native CNFs required a higher concentration of WBE for the phenolic compounds to fully cover the nanofibril surface. As a consequence, the G" overshoot in TEMPO-CNFs gradually decreased and eventually disappeared at the highest WBE concentrations tested (ratios 1:5 and 1:10, Fig. 6), when the adsorbed phenolic compounds reached a sufficient surface coverage to facilitate the sliding of fibrils and prevent the formation of fibre flocs or facilitate their breakdown. Control rheological tests with TEMPO-CNFs in the presence of potassium sulfate showed an intensification of the G" overshooting as the salt concentration increased (Fig. S3), confirming that the phenolic compounds were primarily responsible for the vanishing of the G" overshooting.

Curiously, a slight decrease in G' was observed in TEMPO-CNFs for the highest WBE ratio 1:10 compared to other ratios. That could indicate that an excess of phenolic compounds saturating the cellulose surfaces could increase the lubrication between fibrils to the point that affects the elasticity of the CNF network. Indeed, the elasticity of CNF networks depends on interfibril contacts, and facilitating the sliding between

fibrils too much would decrease the elastic response of the hydrogels. This speculation correlates well with the slightly shorter LVER observed for 1:10 ratio compared to 1:5.

It was expected that the enzymatic treatment of willow bark extract would help to improve the strength of the hydrogels, thanks to the condensation of catechin under oxidation effects of ThL enzyme. However, the effect of the enzymatic treatment of WBE to the strength of the hydrogel complexes was relatively limited, and the hydrogel complex still exhibited strong liquid-like and shear thinning behaviour. The enzymatic polymerization only slightly improved the storage modulus of the native CNFs while the effect was inconclusive for TEMPO CNFs (Figs. 5b, 6b and 7). Although the condensed catechin was expected to act as the reinforcement for the percolating fibril network, the increase in molecular weight was not sufficient to create a remarkable effect. The smaller than expected increase in molecular weight of the condensed catechin was most probably due to the low amount of catechin found in the WBE used in this study. However, the G" overshooting receded faster in the presence of enzyme-treated WBE (Fig. 6), which could mean that the polymerized catechin could enhance the lubrication between cellulose fibrils more than individual, unpolymerized molecules, facilitating the flowing of CNF hydrogels at high strain. Nevertheless, we cannot discard a possible effect of the enzyme present in the system, which could also adsorb on the cellulose nanofibrils and reduce the friction between them.

To further strengthen the conclusions from surface sensitive QCM-D and rheological analyses and to indicate the practical implications of the WBE-CNFs interactions, the swelling of CNF hydrogels with different WBE concentrations was explored with water immersion tests (Figs. 8 and 9). Control swelling tests of CNF hydrogels in the presence of potassium sulfate were also carried out to discriminate the effect of salt from the effect of phenolic compounds of WBE (Fig. S4).

Both native and TEMPO-CNF hydrogel experienced a large volume expansion upon water immersion and the hydrogels were even partly disintegrated in the presence of excess water, especially in the case of TEMPO-CNFs. This swelling was immediately diminished in the presence of WBE. In the case of native CNF, the lowest concentration of WBE (2.4 %) was enough to decrease the swelling ratio from 42 % to 8 %. After this there was no change in the swelling ratio upon increasing the WBE content. Due to the higher water uptake of TEMPO CNFs the deswelling effect increased gradually with increasing the WBE content. This reduction in swelling was most likely due to the presence of ions from a potassium salt since a similar effect was observed in hydrogels

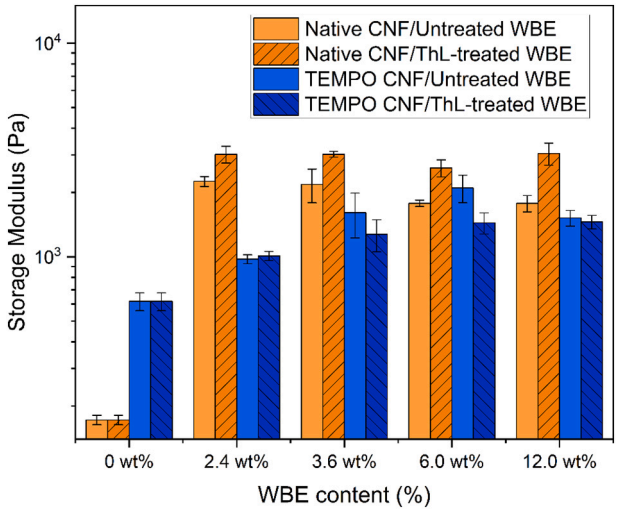

Fig. 7. Storage modulus at shear strain 0.1 % from the amplitude sweep for native CNFs and TEMPO CNFs as a function of WBE content and enzymatic polymerization.

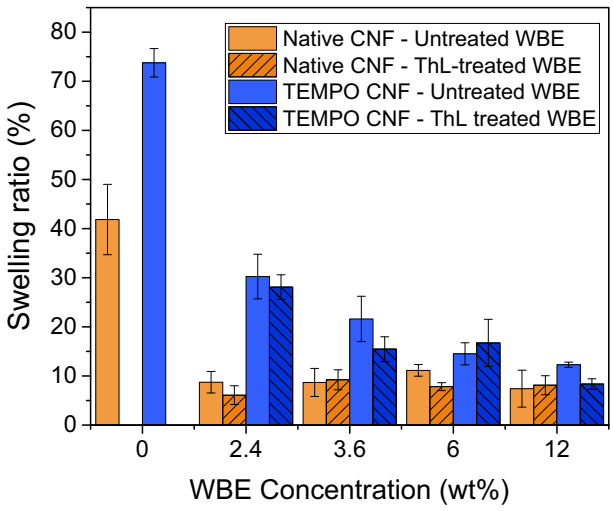

Fig. 8. Swelling ratio of CNF and WBE hydrogels from the water immersion tests.

with only K_2SO_4 (Fig. S4).

However, all samples containing WBE retained their shape with no disintegration observed. When only salts were incorporated in the CNF hydrogels, the casted structures were seen to disintegrate gradually if shaken. The phenolic compounds of WBE seem to act as cross-linking points that hold the fibril network together and prevent disintegration when an outside force is applied. Although the enzymatic treatment of WBE had an insignificant effect on further decreasing the swelling, all-in-all, the incorporation of WBE (either untreated or enzymatically treated) showed an excellent capability of controlling the swelling ratio, up to 5–7 times compared to the pristine CNFs, while maintaining the integrity of the CNF hydrogels without the use of any covalent cross-linking agents.

From the results of rheological measurements and water immersion tests, we speculate that the WBE modified the CNF networks via two main mechanisms. First, the introduction of salts from WBE shrinks the electrical double layer of CNFs, thereby reducing the electrostatic double layer repulsion between fibrils and favouring fibril aggregation. That decreases the number of water molecules entrapped between the fibrils and, consequently, the swelling of the hydrogels is hindered in the presence of WBE. This is consistent with the findings from previous works on the salt effect on CNF hydrogels (Arola et al., 2022; Fukuzumi, Tanaka, Saito, & Isogai, 2014; Hubbe et al., 2017; Mendoza, Batchelor, Tabor, & Garnier, 2018). Based on rheological data and molecular dynamics simulations, Arola et al. (2022) have also proposed that salt ions may affect the ordering of the hydration layers surrounding the cellulose fibrils, resulting in the formation of more and stronger contacts between the fibrils. This mechanism could also contribute to reducing the swelling of the CNF hydrogels.

Second, the phenolic compounds in WBE readily adsorb on CNF surface and assist the flowing of the hydrogel by facilitating the sliding of cellulose fibrils, preventing the formation of fibre flocs at high shear strain. In that way, they prevent the G" overshooting observed in pure CNFs or CNFs with salts (Arola et al., 2022). The adsorption of phenolic compounds of WBE on cellulose nanofibrils is probably an entropically-driven process related to the release of water molecules. Upon the initial attachment, the binding of WBE phenolic compounds to cellulose surfaces could be strengthened by van der Waals forces, hydrogen bonds, and carbohydrate-aromatic stacking interactions. In the presence of salt ions, the fibrils are closer to each other and the adsorbed phenolic compounds may also contribute as crosslinking points between fibrils, thereby, stabilizing the whole fibril network and preventing the disintegration upon water submergence. The role of salt in the WBE has to date not gained much attention and these results, showing both the role

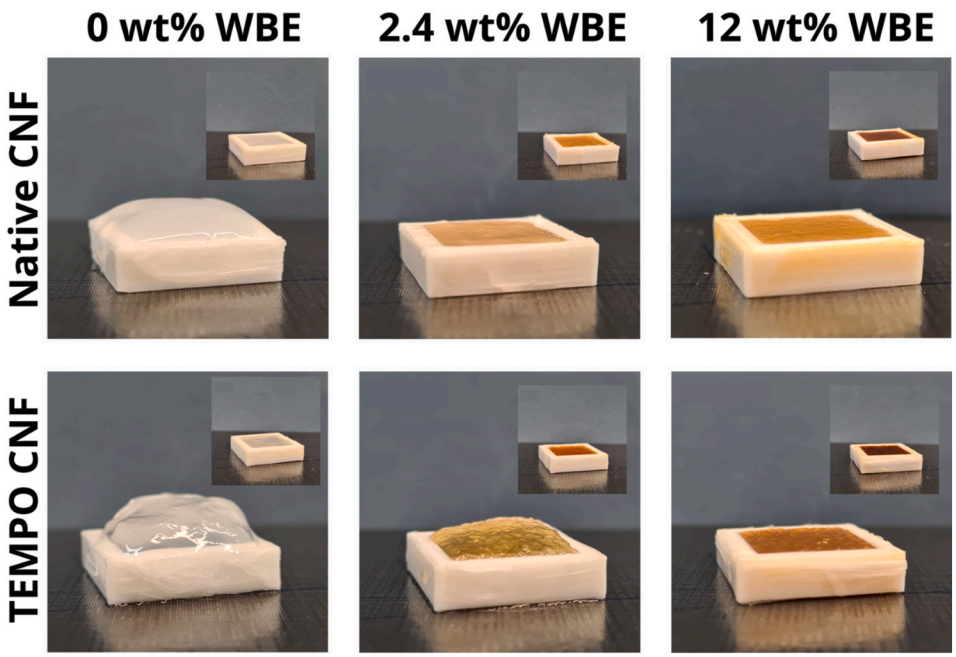

Fig. 9. CNF hydrogels after swelling test with varying WBE content.
Small images in the top right corner are of the samples before water immersion.
A reduction in water uptake can be clearly observed upon increasing the WBE content.

of the salt and the phenolic compounds of WBE, provide valuable understanding on the effects of willow bark extract to the nanofibrillar cellulose networks and its properties, in terms of water management and structural stabilization in aqueous environment. This could be useful in CNF-based products development since it offers a pathway to regulate the water affinity of CNF hydrogels where improvement in moduli and dimensional stability are achieved while retaining the beneficial aspects like shear thinning and relatively good water retention.

3.5. *Biocompatibility of WBE and CNF hydrogels*

Hydrogels are used extensively in biomedical applications, such as wound dressing and scaffolds for tissue engineering. Therefore, it is important to study the biocompatibility of newly developed materials. In this study, the hemolysis assay, i.e., the tendency of a material as a factor to cause red blood cell death, was used as an indication of the material's biocompatibility.

Willow bark extract, with its bioactive phenolic compounds, have been suggested to bring beneficial effects like anti-oxidant, anti-inflammatory, and anti-bacterial properties which are highly appealing for biomedical applications (Lohtander et al., 2021; Tienaho et al., 2021). These effects are mainly attributed to catechin and salicin. Hence, we expected that the CNFs/WBE hydrogel complexes would also have good bio and hemo-compatibility or even help to prevent red blood cell death. Surprisingly, on the contrary, the WBE exhibited some hemolytic activity, although it was only about 10–20 % at low WBE concentrations. This means that the studied WBE can kill red blood cells upon contact, and this effect intensifies with increased concentration of WBE (Fig. 10). For comparison (+)-Catechin and D-(−)-Salicin were tested with varying concentration, and no hemolytic activities were observed for these compounds, leading to the conclusion that the hemolytic activity was caused by other component of the WBE than catechin and salicin. We speculate that either triandrin or picein, as the main components of the WBE studied here, could be the reason for the observed hemolytic effect and the effect might depend on the composition, species, year and age of the willow. However, due to difficulties in obtaining high purity triandrin and picein, we could not go further to confirm the origin of the hemolytic activity of WBE.

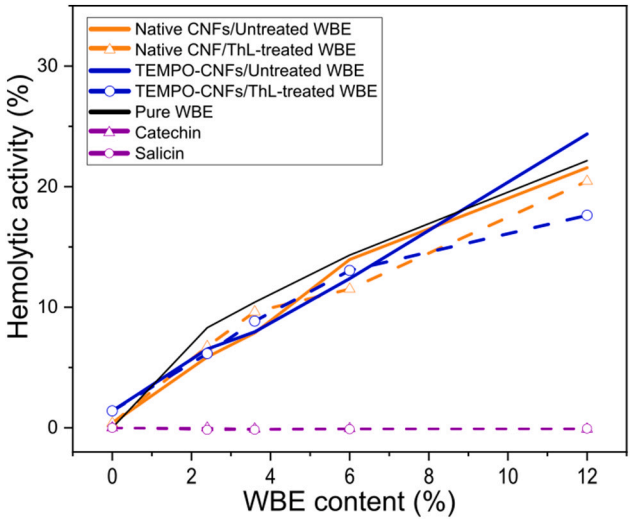

Fig. 10. Hemolysis assays of samples. Salicin and (+)-catechin solutions were used as reference compounds.

The hemolytic activity of WBE might pose a risk in using this specific WBE in those applications in which they are in direct contact with blood; and other aspects of safety and biocompatibility have to be tested carefully before using them for biomedical applications. WBE still possess high potential to be used as a bioactive ingredient in varies applications and the control of water binding is an interesting phenomenon to further explore. However, this surprising finding shows that the variability in chemical content and consequently bioactivity and biocompatibility of natural compounds like WBEs must be taken into account and detailed specifications of origin and history of samples should be reported.

4. Conclusions

CNFs have great potential in several high-value and functional applications like barrier coatings or wound dressing. However, CNF-based products and CNF hydrogels are still facing certain challenges, one of which is their high affinity to water, which can either increase the energy required for dewatering or drying, or adversely affect their performance in high humidity or wet conditions. Different strategies have been studied before and the incorporation of bioactive compounds from wood bark waste stream into CNF hydrogels is one interesting strategy to improve their performance and bring valuable functionalities to the end products. Willow bark is an underrated phytochemical source that can offer several bioactive phenolic species. In this study, we introduced these phytochemicals into CNF hydrogels and observed their interactions with the CNF surface and how they alter the water interactions and stability of CNF networks in aqueous environment.

We found that the willow bark extract has double effects on the rheological behaviour and water uptake of CNF hydrogels. On the one hand, willow bark extract introduced salt ions into the medium, decreasing the electrostatic double layer repulsion and, consequently, shortening the interfibrillar distance and increasing the contact points between fibrils. This resulted in an increase in the storage and loss moduli of the cellulose hydrogels upon shearing. On the other hand, the phenolic compounds in WBE readily adsorb on CNFs (more on TEMPO-CNFs than on native CNFs due to the larger surface area of the former), probably following an entropically-driven process (release of bound water molecules) reinforced with attractive van der Waals forces, carbohydrate-aromatic stacking interactions, and hydrogen bonds formed upon the initial attachment of the molecules. The adsorbed phenolic compounds on cellulose fibrils can reduce the friction between fibrils and assist the flowing of the hydrogels at high shear strain, preventing the formation of fibre flocs and the overshooting in G" usually observed in pure CNFs or CNF with salts. The introduction of WBE to the CNF network also reduced the penetration of water and swelling ratio in aqueous environment, increasing dimensional stability of the hydrogel without chemical crosslinking. We suggest that as the CNF network become more compact due to the presence of ions, the adsorbed phenolic compounds might also act as bridging points between fibrils, contributing to the increase in storage modulus and the reduction of the swelling of the hydrogels. The findings in this study are useful for the design and application of CNF-based materials, where water adsorption is undesirable, and dewatering is a challenge.

A surprising finding from the study was that willow bark extract showed hemolytic activity, and the effect increased with WBE concentration. Therefore, the use of this specific WBE might not be safe for biomedical applications where direct contact with blood is likely. Further screening tests are needed to determine the exact compound that induced the hemolysis.

CRediT authorship contribution statement

Ngoc Huynh: Methodology, Investigation, Data curation, Formal analysis, Software, Visualization, Writing – original draft. **Juan José Valle-Delgado:** Supervision, Formal analysis, Writing – review & editing. **Wenwen Fang:** Methodology, Writing – review & editing. **Suvi Arola:** Conceptualization, Funding acquisition, Methodology, Supervision, Writing – review & editing. **Monika Österberg:** Conceptualization, Funding acquisition, Methodology, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.carbpol.2023.121095.

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